Characterization of Metabolic Gene Targets in Response to Chromosomal Instability

Submitted in total fulfilment of the requirements of the degree of Doctor of Philosophy

By

Mahwish Khan

July 2018

Discipline of Genetics
School of Molecular and Biomedical Science
The University of Adelaide

Table of Contents

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.

I give consent to this copy of my thesis when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

The author acknowledges that copyright of published works contained within this thesis resides with the copyright holder(s) of those works. I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

17/ 07/2018

Mahwish Khan   Dated

Acknowledgments

First and foremost I would like to thank Almighty Allah for his immense blessing and for making my dream come true.

I would like to thank all the people who helped me in some way during my PhD study. Foremost, I would like to express my sincere gratitude to my supervisor and my mentor Dr. Stephen Gregory for his tremendous support, great patience, immerse knowledge and persistent encouragement throughout my study. I am extremely grateful for what he has offered me, without his support this thesis would not have been completed. I would also like to thank my co-supervisor Prof. Robert Saint for his insightful ideas, comments, encouragement and continuous support.

Beside my supervisors, I would like to thank Louise O'Keefe and Robert Richards for their great support during my PhD candidature. I would also like to acknowledge the Beacon of Enlightenment scholarship, University of Adelaide for the financial support which has allowed me to undertake my studies. I am very grateful to my lab mates Zeeshan Shaukat, Rashid Hussain, Dawei Liu, Sandeep Basenet,

I would also like to express my extremely gratitude to my family. Words cannot express how grateful I am to my mother (Munawar Begum) and father (Moeen-ul Abedien Khan) for all of the sacrifices that they've made on my behalf. Your prayer for me was what sustained me thus far. I would like to thank my loving sister (Nazish Khan) and brothers (Umair-ul-Abedien Khan and Zubair-ul-Abedien Khan). Last but not least, I would like to express appreciation to my beloved daughters (Lubaba and Hamda) and my husband Muhammad Tayyab who always my support during the entire period of carrying out this study. Finally, a special thanks to my close friends, Sara Altaf, Anila Ali Khan, Sana Ishaque, Sadia Fiaz, Naila Asif, Saira Ali, Manahil Badar for always being there when I needed you the most.

List of Publications

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

- Zeeshan Shaukat, Dawei Liu, Rashid Hussain, <u>Mahwish Khan</u> 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, <u>Mahwish Khan</u>, Robert Saint and Stephen L. Gregory, 2017. Phosphoenolpyruvate Carboxykinase Maintains Glycolysis-driven Growth in Drosophila Tumors. *Scientific reports*, 7(1), pp.11531.

- <u>Mahwish Khan</u>, Zeeshan Shaukat, Robert Saint and Stephen L. Gregory, 2018. Chromosomal instability causes sensitivity to protein folding stress and ATP depletion. (submitted for publication)

Abbreviations

17-AAG	17-N-allylamino-17-demethoxygeldanamycin
5-FU	5-Flurourocil
ADA	Adenosine deaminase
ADSS	Adenosine phosphoribosyltransferase
AICAR	5-amino-1-β-D-ribofuranosyl-imidazole-4-carboxamide
Ambra1	Activating molecule in BECN1-regulated autophagy protein 1
AMPK	AMP kinase
APC	Adenomatous *polyposis coli*
APC/C	Anaphase promoting complex/cyclosome
APRT	Adenosine phosphoribosyltransferase
Atg1	Autophagy-related 1
Atg4c	Autophagy-related 4c
Atg5	Autophagy-related 5
Atg7	Autophagy-related 7
ATM	*Ataxia telangiectasia mutated*
Aurora A	Aurora kinase A
Aurora B	Aurora kinase B
BRCA1	Breast cancer 1
BUB	Budding uninhibited by benzimidazoles
BubR1	Bub1-related protein kinase
Cdc20	Cell division cycle 20 homologue
CENPE	Centromere linked motor protein E
CIN	Chromosomal instability
CRC	Colorectal cancer
DNA	Deoxyribonucleic acid
dNTP	Deoxy nucleotide triphosphate
ER	Endoplasmic reticulum
ETC	Electron transport chain
G6PD	Glucose-6-phosphate dehydrogenase
GPO1	Glycerophosphate oxidase
HGPRT	Hypoxanthine-guanine phosphoribosyltransferase
HIF	*Hypoxia-inducible factor*
HK2	Hexokinase 2
HSP83	Heat shock protein 83
HSP90	Heat shock protein 90
IMP	Inosine monophosphate
JNK	*c-Jun N-terminal kinases*
KSP/Eg5	Kinesin spindle protein

LDHA	Lactate dehydrogenase
Mad1	Mitotic arrest deficient 1
Mad2	Mitotic arrest deficient 2
MCAK	Mitotic centromere-associated kinesin
mTOR	Mammalian target of rapamycin
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
NDC	Kinetochore protein NDC
Nek2	NIMA-related kinase 2
NRF2	Nuclear factor erythroid-derived related factor 2
NSCLC	Non-small cell lung cancer
p53	Tumor protein (SDS-PAGE: 53 kDa) p53
p62	Sequestosome 1
PARP	Poly ADP-ribose Polymerase
PASK	Pas domain-containing serine/threonine protein kinase
PEPCK	Phosphoenol-pyruvate carboxykinase
Pfrx	Phosphofructokinase
PI3K	Phosphoinositide 3-kinases
PRPP	Phosphoribosyl pyrophosphate
PRPS2	Phosphoribosyl-pyrophosphate synthetase
Rad21	RAD21 homolog (S. pombe), kleisin subunits of Cohesin Rad21
Ras	Rat sarcoma
Rb	Retinoblastoma
RNA	Ribonucleic acid
RNAi	RNA interference
RNR	Ribonucleotide reductase
ROS	Reactive oxygen species
SAC	Spindle assembly checkpoint
SAMHD	SAM And HD Domain Containing Deoxynucleoside Triphosphate
S-CIN	Structural chromosomal instability
STAGE	Stromal antigen
TCA	Tricarboxylic acid cycle
TKL	ribonucleotide reductase
TNFα	Tumour necrosis factor alpha
UPR	Unfolded proteins response
USFDA	U S Food and Drug Administration
XBP1	X-box binding protein 1

Abstract

CIN is frequently present in advanced tumours and associated with tumorigenesis and poor clinical outcomes. CIN is thought to contribute to the development of resistance against anti-cancer drugs. CIN is specific to cancer cells, so our lab is exploiting the idea of targeting CIN itself in order to develop novel therapies that kill CIN cells. We have developed *Drosophila melanogaster* induced-CIN models for *in vivo* screening and characterization and used RNAi lines to knock down candidates in a CIN background **(Chapter 2).** From our screening of phosphatase and kinase knockdowns that kill CIN cells, we identified some interesting metabolic candidates that caused lethality via apoptosis in CIN background. These metabolic changes generate elevated ROS levels, DNA damage, mitochondrial hyperactivity in CIN background, showing CIN cells are sensitive to redox stress. Subsequently, we investigated the effect of these metabolic candidates in our *Drosophila* tumour model and found a gluconeogenic protein, PEPCK, needed for proliferation of CIN tumours. PEPCK deficiency supressed the tumour growth by increasing the cytoplasmic NADH and ROS levels. Our data demonstrated that in PEPCK deficient tumours, the glycerol-3-phosphate shuttle produced high levels of ROS. High NADH and ROS production stopped the tumour growth **(Chapter 3)**. We also found that the purine biosynthesis pathway is needed to tolerate CIN. Depletion of nucleotide synthesis candidates altered the level of adenine nucleotides which led to DNA damage which in turn activated PARP for DNA repair, further depleting ATP levels. We found increased numbers of lysosomes in nucleotide deficient CIN cells and if their formation was blocked, CIN cells died, which suggested that autophagy is activated and required for CIN cell survival **(Chapter 4)**. In conclusion, our findings reveal that CIN is sensitive to metabolic aberrations. Several metabolic pathways including glycolysis, gluconeogenesis and nucleotide biosynthesis pathways are activated in response to CIN. Increasing understanding of these pathways that make CIN cell survive or die may ultimately allow the design of cancer-specific drug targets for cancer therapy.

This page intentionally left blank

INTRODUCTION

1.1-Cancer

Abnormal cell proliferation results in cancer development and cancer is one of the leading causes of early deaths worldwide. Cancer progression is associated with multistep changes in the genome such as gene mutations, deletions or insertions, and epigenetic aberrations, initiating less apoptosis, enhanced cell proliferation, angiogenesis, invasion and metastasis (Hanahan and Weinberg, 2011). In Australia, more than 14,000 new cases are diagnosed with colorectal cancer every year, with a mortality of around 30% and an annual healthcare cost of over $200m (Cancer Council of Australia). The median survival of metastatic colorectal cancer patients is still under 2 years (Sorbye et al., 2013). More than 80% of such tumours and many lung cancers exhibit CIN and become resistant to multiple chemotherapeutics (Lee et al., 2011, Coldren and Miller, 2011a). However, there are different next generation drugs such as anti-mitotic and anti-microtubule therapies that are available to eradicate the disease and prolong survival but they come with drastic side effects and may generate drug resistance (Coldren and Miller, 2011b). Chromosomal instability (CIN) is a common feature of advanced tumours, in which cells fail to maintain a stable chromosome number as they grow (Figure 1.1). Acquisition of drug resistance is often seen in cells with CIN in advanced tumours like lung, breast and colorectal carcinomas. 80% of these cancers exhibit CIN and develop resistance against multiple chemotherapeutics (Gerlinger and Swanton, 2010). Thus, there is a pressing need for novel therapies against cancer.

1.2-Chromosomal Instability (CIN)

CIN is often seen in solid tumours and many haematological malignancies (Mertens et al., 1994) and refers to the phenomena in which whole or large parts of chromosomes are added or lost by the tumour cells (Figure 1.1) at a higher frequency (10-100 times) than normal or chromosomally stable diploid tumour cells (Lengauer et al., 1997). CIN is a main cause of aneuploidy (Rajagopalan and Lengauer, 2004) and has major implications in carcinogenesis, cancer cell evolution, metastatic potential and drug resistance (Rajagopalan and Lengauer, 2004, Gao et al., 2007). The transition to a metastatic tumour requires genomic rearrangements that can be produced by CIN (Rao and Yamada, 2013). CIN induction in mouse models results in a two-fold increase in the frequency of spontaneous tumour growth (Duijf and Benezra, 2013), and CIN in human cancer is associated with significantly poorer

prognosis (McGranahan et al., 2012). It has been suggested that CIN promotes drug resistance and relapses upon chemotherapy because it is responsible for the formation of genetic diversity in tumours (Swanton et al., 2006).
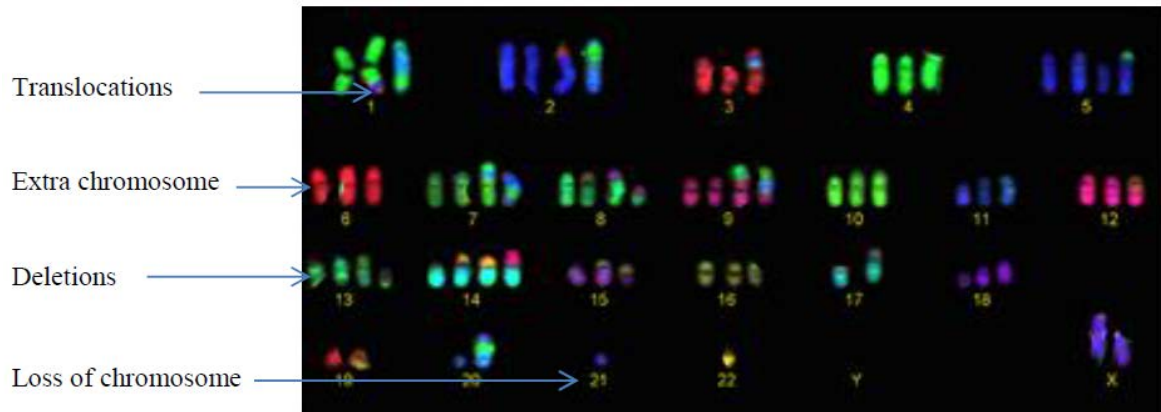
Figure 1.1: *Karyotype of a typical cancer cell (Janssen & Medema, 2013)*

Although several mechanisms have been identified that can generate CIN, a comprehensive understanding of CIN at a molecular level in most cancers is still lacking. Considering other phenotypic features of tumours, it is assumed that mutations in genes required for chromosome stability contribute to CIN (Pfau and Amon, 2012). However, few human cancers with gene mutations specifically known to cause CIN have been identified. CIN can be classified into either whole chromosome instability (W-CIN) which arises due to defective segregation of whole chromosomes or structural chromosome instability (S-CIN), where alteration in parts of chromosomes such as deletions, duplications and translocations arise (Kamata and Pritchard, 2011).

## 1.3-Mechanisms of CIN Development:

There are a wide range of possible mechanisms that cause CIN, including defects in the spindle assembly checkpoint (SAC), centrosome amplification, merotelic kinetochore attachment and DNA damage and chromosome bridges. (Jallepalli et al., 2001, Fukasawa and Woude, 1997, Pihan et al., 2001, Weaver and Cleveland, 2006, Thompson and Compton, 2008).

1.3.1- SAC Component Defects and CIN

Defects in chromosome segregation contribute to CIN phenotypes and tumorigenesis (Thompson and Compton, 2008). The SAC plays a crucial role in accurate chromosome

segregation by preventing cells from progressing into anaphase if kinetochores are either unattached to spindle microtubules or attached in a way that fails to generate normal tension between sister kinetochores (Musacchio and Salmon, 2007). This ensures proper alignment of all pairs of duplicated chromatids in metaphase (Musacchio and Salmon, 2007). Both heterozygous and hypomorphic mutations in SAC genes develop missegregation of chromosomes, detected by aneuploidy in mutant progeny (Foijer, 2010, Ricke et al., 2011). These SAC defects have been reported in colon, lung and breast cancer (Michel et al., 2001). SAC genes, including checkpoint sensors, mitotic arrest–deficient (*Mad*) and budding uninhibited by benzimidazoles (*BUB*) gene products, act to control sister chromatid separation and also work as signal transducers. Their activation inhibits a ubiquitin-protein ligase, the anaphase-promoting complex/cyclosome (*APC/C*), and causes cell cycle arrest (Cahill et al., 1998, Thompson et al., 2010). Mutation in other genes such as *P53*, *BRCA1* and *ATM, Rb, cyclins or cyclin dependent kinases,* which are involved in checkpoint control of the cell cycle, DNA repair and recombination and transcription (Yarden et al., 2002) can also induce CIN and aneuploidy. Ubiquitylation of *cyclin B*, activation of separase (caspase-related proteases) and reduction of securin (an inhibitor of the protease separase) are normally initiated by APC/C–associated Cdc20 at chromosome segregation (Herzog et al., 2009). Securin binding prevents the proteolytic activity of separase, which destroys sister chromatid association by dissolving the chromatid cohesin factor Scc1. Securin degradation is essential for the segregation of sister chromatids during mitosis. It is reported that core components including budding uninhibited by benzimidazoles–related 1(*BubR1*), *Mad2*, and *Bub3* form the mitotic check point complex with Cdc20 which causes conformational alteration in the APC/C that stops substrate binding (Herzog et al., 2009). Before anaphase and after condensation of chromosomes the Mad2 protein is localized at kinetochores, it associates with the APC/C complex and arrests cells at metaphase (Lopez-Girona et al., 1999) and plays a vital role in mediating the spindle checkpoint (Hardwick, 2005). A previous study showed that deletion of one allele of *Mad2* in both human cancer cells and murine primary embryonic fibroblasts is sufficient to cause a spindle checkpoint defect that results in early separation of sister chromatids and an elevated rate of unequal chromosome segregation (Michel et al., 2001). Early segregation of sister chromatids is typical of spindle checkpoint defects that have been found in *Drosophila Bub1* mutants as well as in yeast *Mad2* mutants (Basu et al., 1999, Minshull et al., 1996). In mice, *Mad2* null cells are non-viable and quickly undergo apoptosis owing to defects in the SAC (Dobles et al., 2000). Therefore, heterozygous mice *Mad2*+/- were examined for tumour development. *Mad2*+/-

mice were prone to develop lung tumour at an elevated rate (27%) compared to wild-type (Michel et al., 2001). In *Drosophila melanogaster*, CIN can be generated by knocking down the Mad2 protein, which shortens metaphase giving cells less time to align their chromosomes before the onset of anaphase (Figure 1.2), resulting in chromosomal bridges and lagging chromosomes (Buffin et al., 2007). Several studies also demonstrated that the overexpression of *Mad2* in B-cell lymphoma, lung adenocarcinoma and hepatocellular carcinoma causes genomic instability by delaying mitosis, increasing mitotic inaccuracy and the production of polyploid cells (Alizadeh et al., 2000, Chen et al., 2002, Garber et al., 2001, Sotillo et al., 2007).
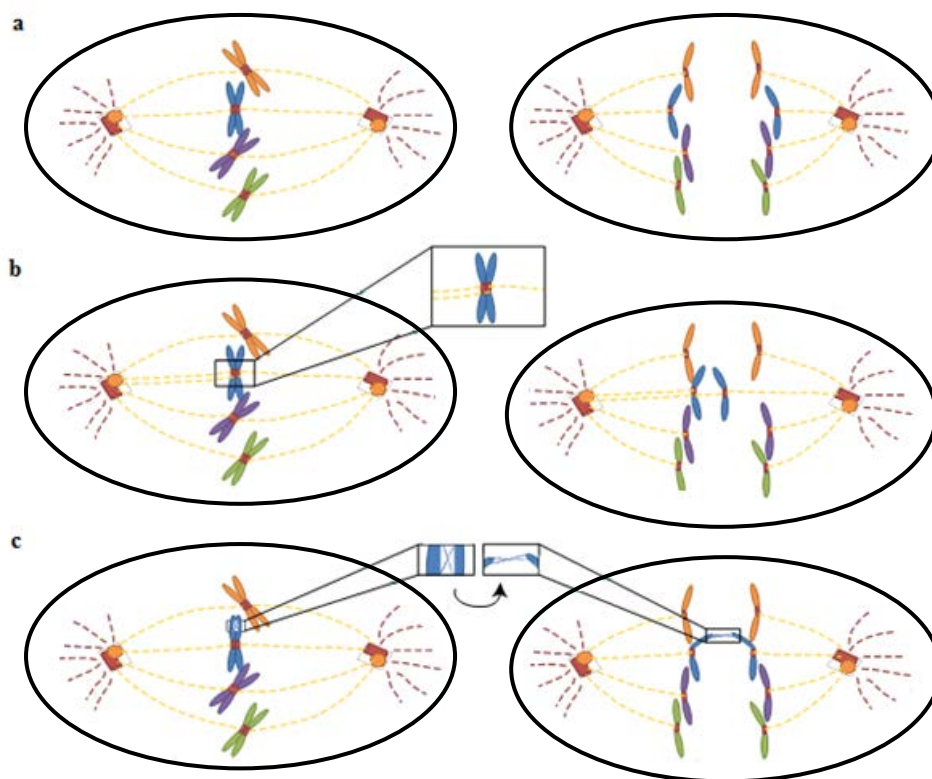

Figure1.2: *Aberrant spindle-chromosome attachments and chromosomal bridges lead to CIN. a) Normal chromatid separation during Anaphase, b) an induced spindle checkpoint defect, kinetochores attached to both poles may cause lagging chromosomes, c) ends of separating chromatids are unresolved, resulting in the formation of a chromosomal bridge.*

1.3.2- Centrosome Amplification and CIN

Centrosome abnormality both in number and function is considered to be another possible cause of CIN. Cells may acquire centrosome amplification by various mechanisms such as cell fusion, defects in cytokinesis and perturbation in centrosome biogenesis. It has been

demonstrated that supernumerary centrosomes result in multiple spindle poles during mitosis (Figure 1.3), which can lead to missegregation of chromosomes and CIN (Ganem et al., 2009). Work from Basto et al. (2008) found that centrosome amplification has a role in inducing metastatic tumours in flies (Basto et al., 2008). Several mechanisms like viral based cell fusion, high expressions of Eg5 and cytokinesis failure that generates tetraploidy, also result in the occurrence of centrosome amplification (Thompson et al., 2010). Thus, it is expected that tetraploid cells are more susceptible to CIN.
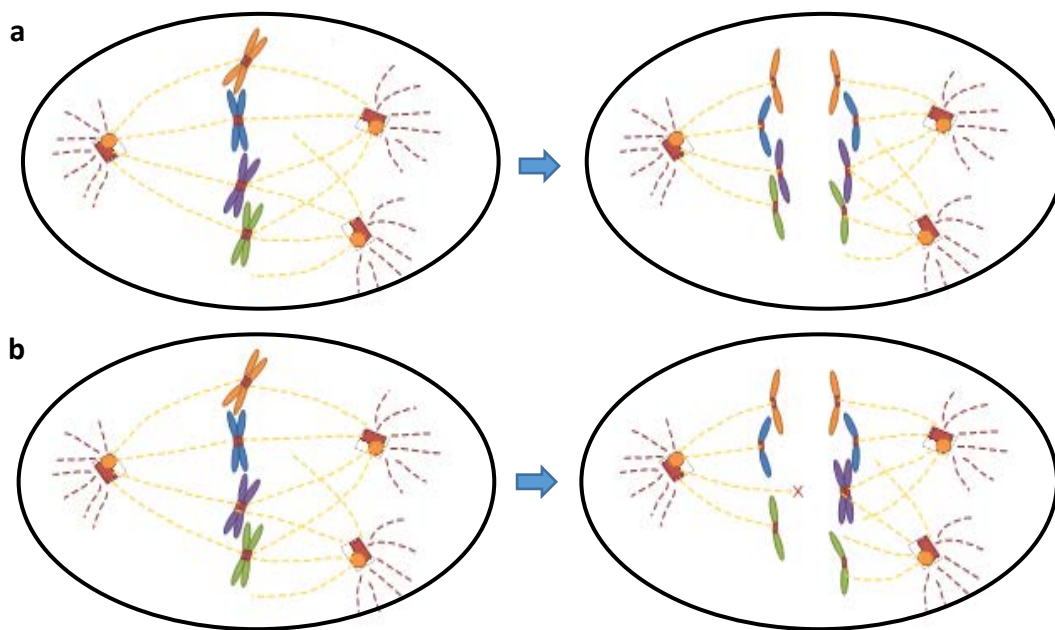

Figure 1.3: Multi-polar spindle and merotelic attachments lead to CIN. a) an induced defect in centrosomes that causes multiple attachments of sister chromatids to two or more centrosomes may delay mitosis but resolve normally, b) Multiple centrosome attachments, chromosomes fails to segregate: may cause CIN.

1.3.3- Merotelic Kinetochore Attachment and CIN

At least in some situations, centrosome amplification significantly increases merotelic binding at the kinetochore (Cimini et al., 2006). Merotely defects arise when a kinetochore simultaneously gets attached to microtubules originating from different spindle poles (Cimini, 2008) as shown in (Figure 1.4) and is commonly found in CIN cells (Thompson and Compton, 2008). Mutations in centromere and kinetochore structural proteins may cause kinetochore merotelic defects, and mutations in centromeric heterochromatin contribute to

6

kinetochore–microtubule attachment defects (Cimini, 2008). For example, mutations in histone deacetylases and retinoblastoma protein causes poorly arranged centromeric chromatin, leading to lagging chromosomes (Cimini et al., 2003, Manning et al., 2010). Additionally, alteration in microtubule-depolymerizing enzymes including the Ndc complex, CENP-E, CENP-F, MCAK and Kif2b cause merotely and lagging chromosomes at an elevated rate in anaphase, showing that these proteins are important for correcting errors in kinetochore-microtubule binding (DeLuca et al., 2002, Thompson et al., 2010). Specifically, perturbation of these proteins highly stabilizes kinetochore–microtubule binding, reducing the capacity to correct merotelic attachments, resulting in CIN.
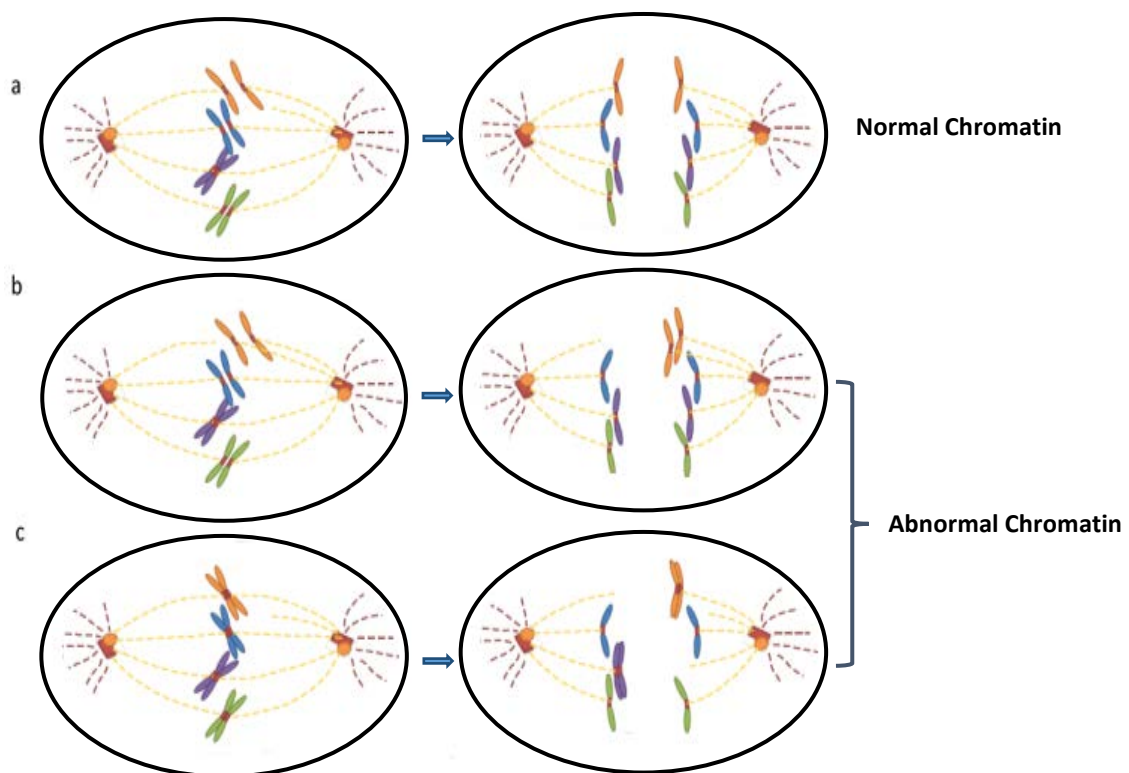
Figure 1.4: *Cohesion defects lead to CIN; a) normal sister chromatid separation b) a pair of sister chromatids wrongly moved to one spindle pole, causing aneuploidy c) two pairs of chromosomes erroneously moving towards one spindle pole, causing aneuploidy for the respective chromosomes.*

1.3.4- DNA Damage and Chromosome Bridges

Structural chromosomal instability (S-CIN) refers to a situation in which the rate of sub-chromosomal changes increase, which results in loss or alteration of, small regions of chromosomes in the form of translocations, insertions, deletion and DNA amplification.

Mutations in the double-strand DNA damage repair machinery can result in translocation and S-CIN (Duker, 2002, Natarajan and Palitti, 2008). Non homologous end joining is an error-prone DNA damage repair pathway, which leads to S-CIN by joining two non-specific broken ends of DNA (Natarajan and Palitti, 2008). Double strand breaks can therefore, generate non-specific chromosomal fusions particularly at dysfunctional telomeres (Hastie and Allshire, 1989). In eukaryotes, telomeres (DNA-protein complexes) contain short stretches of nucleotide repeats (TTAGGG) that prevent the chromosome ends from breaking and joining during segregation. Due to the end-replication problem, which arises because DNA polymerase does not completely synthesize the 3' end of chromosomes, telomere length shortens at each replication. Fusion at telomeres results in formation of di-centric chromosomes or ring chromosomes, which cause the formation of chromatin bridges at anaphase due to improper binding of microtubules to these di-centric chromosomes. These chromosomal bridges cause breakage at cytokinesis and fusion, a process that is repeated in the subsequent mitosis (Gisselsson, 2011). This breakage-fusion-bridge cycle is often found in multiple cell generations and can cause remarkable genome reorganization. S-CIN aberrations can lead to an increased rate of tumorigenesis (Pino and Chung, 2010).

1.4-The Consequences of Chromosomal Instability:

CIN is a common feature of cancer cells that plays a critical role in tumour progression and is associated with worse prognosis (Bakhoum and Compton, 2012). Approximately 85% of solid tumours, and 90% of advanced tumours exhibit the CIN phenotype (Mertens et al., 1994). The accepted outcome of a moderate level of CIN is tumorigenesis but its role in initiating tumorigenesis and cancer progression is still uncertain (Giam and Rancati, 2015).

Several studies revealed the involvement of CIN in tumour evolution and development. The overexpression of spindle assembly checkpoint proteins such as Mad2 in transgenic mice model resulted in CIN. A mouse model with increased or reduced expression of Mad2 has shown an elevated rate of tumourigenesis (Birkbak et al., 2011). In some mouse CIN models, CIN induced by Bub1, Bub3, BubR1 and Rad21 has developed tumourigenesis (Schliekelman et al., 2009, Solomon et al., 2014). Moreover, in another mouse model, over-expression of hec1 (a kinetochore-microtubule stabilizing protein) also developed tumours in lung, liver, and pancreas (Diaz-Rodríguez et al., 2008). However, numerous studies revealed that loss of tumour suppressor genes such as p53, p21 are required for tolerance to aneuploidy

in CIN cells (Bakhoum and Compton, 2012). This evidence supports a role for CIN in tumourigenesis, however, the role of CIN in tumor evolution is still unclear.

One possible mechanism is that chromosome missegregation can directly result in DNA damage and translocation which enables fractions of CIN cells to gain a selective advantage, for instance, losing cancer suppression genes through loss of certain chromosomes in which the genes reside, therefore, promoting tumourigenesis (Janssen et al., 2011). Disruption of the p53/p21 tumour suppressor pathways are common in CIN cells which leads to tumorigenesis (Janssen et al., 2011).

Another consequence of CIN is drug resistance (Giam and Rancati, 2015). Lee et al. (2011) have studied the panel of colorectal cancer (CRC) cell lines, classified as CIN+ or CIN− for their drug resistance. They reported that CIN+ CRC lines displayed multi-drug resistance compared to the CIN- CRC lines. Similarly, CIN+ CRC was associated with worse progression free survival relative to CIN- CRC disease (Lee et al., 2011). One mechanism of developing resistance in CIN cells could be due to the high expression of Nek2 in those cells. Nek2 over-expression induced drug resistance in cancer cells mostly via activation of the efflux pumps (Zhou et al., 2013). Thus, targeting CIN genes such as *Nek2* and the signalling pathways that activates these genes could be an important target for cancer therapy.

CIN can also promote metastasis, e.g., through the copy number gain of pro-metastatic genes, the copy number loss of anti-metastatic genes or a cytosolic DNA response. Bakhoum, et al. (2018) recently reported that CIN can promote metastasis via a cyclic GMP–AMP synthase (cGAS)–stimulator of interferon genes (STING) pathway-dependent response to cytosolic DNA. To examine causality, the authors transplanted CIN suppressed metastatic cancer cell lines (human breast cancer, mouse breast cancer and human lung cancer cells) into mice. To retain the aneuploid karyotypes during cell proliferation, the kinesin-like proteins KIF2B or KIF2C were overexpressed in these cancer cells (CIN-low cells). Intracardiac injection (CIN-low cells) showed that these cells are less able to colonize metastatic sites compared with cells overexpressing a (KIF2A) that did not affect CIN (CIN-high cells). They further examined the role of CIN in spontaneous metastasis and observed that suppression of CIN did not reduce the efficacy of primary tumour implantation but did reduce metastasis and prolong survival of the mice (Bakhoum et al., 2018).

1.5-Targeting Chromosomal Instability

Therapeutic interventions to target CIN is based on levels of CIN in the specific tumour (Bakhoum and Compton, 2012). It was found that depletion of kinetochore proteins such as APC or kinesin-13 protein MCAK, which are responsible for destabilization of attached microtubules could significantly decrease the level of CIN in cancer cell lines (Bakhoum et al., 2009). Conversely, another study reported that increased CIN levels were induced by suppressing mitotic checkpoint proteins such as BubR1 or Mad2, resulting in apoptosis in human cancer cells (Kops et al., 2005). Therefore, an elevated CIN phenotype could be used as a strategy to target CIN mediated tumorigenesis. However, targeting CIN mediated tumorigenesis by manipulating the CIN level has been constrained by the adverse effect of the drugs used, such as haematological and neurological dysfunction (Caley and Jones, 2012).

Another strategy to target cancer cells that exhibit CIN could be to target the pathways that are needed to maintain the CIN level or the adaptations which cancer cells acquire to tolerate CIN related stress. CIN results in an unbalanced genome which further evokes a proteotoxic stress response in aneuploid cells (Oromendia et al., 2012). Many cancers display overexpression of genes that are involved in protein translation and protein folding. It has been reported that protein aggregation (polyglutamine aggregate formation) was increased by aneuploidy, and overloading of chaperones such as HSP90 was observed in human aneuploid cells (Oromendia et al., 2012, Donnelly et al., 2014). Aneuploidy is also linked with increased autophagy and recent findings of our lab demonstrated that autophagy is activated in CIN cells and modulation of the autophagy pathway could affect the fate of CIN cells (Liu et al., 2016, Santaguida et al., 2015). CIN and autophagy protects cancer cells from apoptosis and promotes resistance to chemotherapy treatment (Li et al., 2017). Thus, targeting autophagy and its related pathways could improve the efficacy of anti-cancer drugs by reducing the chance of developing drug resistance caused by either CIN or autophagy.

CIN is also linked to metabolic alteration and oxidative stress; cancer cells exhibiting CIN adapt to tolerate these stresses (Shaukat et al., 2015). Targeting these metabolic adaptations could lead to cancer specific cell death (Shaukat et al., 2015). For instance, G6PD, a rate limiting enzyme of the Pentose Phosphate (PP) pathway that regulates the antioxidant and nucleotide levels in cells, is mostly high in cancer cells (Wang et al., 2012). G6PD overexpression increases tumorigenesis and its depletion makes the cells sensitive to

radiotherapy and chemical oxidants (Zhang et al., 2014). G6PD depletion in CIN cells result in an increase in oxidative stress and DNA damage which further cause cell death (Shaukat et al., 2015).

In order to kill CIN cells, Pas domain-containing serine/threonine protein kinase (PASK) and Phosphofructokinase (Pfrx) have also been identified as metabolic targets. PASK promotes glycolysis and loss of PASK led to elevated mitochondrial activity, ROS generation and apoptosis in CIN cells (Shaukat et al., 2012). Other specific knockdowns affecting the TCA cycle, fat metabolism, gluconeogenesis and oxidative stress response enzymes could all promote CIN-specific apoptosis. Thus, metabolic interventions are potentially a highly effective way of killing CIN cells without affecting normal dividing cells. Identification of metabolic targets that are either required for CIN-specific cell death or CIN tolerance could serve as potential therapeutic targets to specifically kill advanced tumours that exhibit CIN.

1.6-Cancer Metabolism

Otto Warburg observed the metabolic alterations that are specific for tumours nearly a century ago (Warburg, 1956). This discovery paved the way for understanding cancer metabolism and has become an emergent field of interest in the past decade. Multiple molecular mechanisms contribute to altering cellular metabolism in cancer, however, their main purpose is to provide support for the three basic requirements: rapid ATP production, enhanced biosynthesis of macromolecules and redox balance for the maintenance of tumorigenic state (Pavlova and Thompson, 2016).

1.6.1- Altered Bioenergetics

Warburg's observation that tumours utilise glucose and generate ample amounts of lactate despite the presence of oxygen (Warburg effect) due to impaired mitochondrial function has led to the hypothesis that cancer cells rely on aerobic glycolysis instead of oxidative phosphorylation (DeBerardinis and Chandel, 2016). The emerging evidence favours that the Warburg effect is vital for anabolic pathways, not just for energy production, and a high rate of glucose consumption is associated with activation of oncogenes or loss of tumour suppressors and up-regulation of the PI3K pathway (Pavlova and Thompson, 2016). Several studies revealed the importance of mitochondrial metabolism in cancer cells to conduct oxidative phosphorylation for ATP production (Weinberg et al., 2010, Martínez-Reyes et al., 2016). Moreover, depletion of mitochondrial DNA reduces the tumorigenic potential of

cancer cells (Tran et al., 2016). However, slowing down glycolysis in tumour cells has not been very useful to prevent tumorigenesis (Joshi et al., 2015). Furthermore, fatty acids and amino acids provide substrates to the TCA cycle to maintain the mitochondrial ATP supply in cancer cells. In mitochondria, breakdown of fatty acids produces acetyl-CoA, NADH and FADH$_2$, which are supplied to the ETC (electron transport chain) for mitochondrial ATP production. The amino acids isoleucine, valine, and leucine are elevated in some cancers and are converted to acetyl-CoA and other organic molecules that are used to fuel the TCA cycle for ATP production (Mayers et al., 2014). Under hypoxic and nutrient-deprived condition, tumour cells decrease the demand for ATP in order to adapt to the microenvironment. Subsequently, tumour cells maintain their ATP/ADP ratio to sustain viability in unfavourable conditions. If the ATP/ADP ratio is difficult to maintain, the increase in ADP can activate AMP kinase (AMPK) via a rise in AMP, which stimulates catabolic pathways in tumour cells to increase ATP production for survival. Certain cancer cells in nutrient deprived conditions employ fatty acids to fuel the TCA cycle for ATP production. Thus, in metabolic stress conditions cancer cells maintain their ATP/ADP ratio via multiple mechanism to sustain their viability (Hardie et al., 2016). Metabolic reprogramming in cancer cells makes it hard to target a specific metabolic dysregulation for cancer therapy. However, malignant cells use conventional metabolic pathways to produce more glucose, pyruvate, lactate, glutamine, fatty acids and nucleotide than the normal proliferating cells. Therefore, common metabolic features of malignant cells can potentially be targeted for cancer therapy.

1.6.2- Enhanced Biosynthesis

Enhanced biosynthesis is a vital characteristic of cancer metabolism because it enables cancer cells to produce the macromolecules needed for replicative cell division, DNA repair, angiogenesis, hypoxic response and tumour growth (DeBerardinis and Chandel, 2016). Biosynthetic pathways are involved in the acquisition of simple nutrients such as sugars and essential amino acids, taken from the extracellular space, that are converted into biosynthetic intermediates via core metabolic pathways such as glycolysis, the PPP, the TCA cycle, and nonessential amino acid synthesis. Glucose and glutamine are two principal nutrients that are needed for cell survival. It has been observed that glucose consumption is remarkably high in tumour cells as compared to normal non-proliferating cells (Warburg et al., 1927). An increase in aerobic glycolysis is used for lipogenesis, proteiogenesis and nucleogenesis to fulfil the demands of proliferating cells (Lunt and Vander Heiden, 2011).

Nucleotide biosynthesis is required for DNA replication, RNA production and a variety of cellular processes. De novo biosynthesis of nucleotides is an energy intensive process coordinating several metabolic pathways into one fine-tuned metabolic network. Glucose is converted to ribose-5-phosphate during the PP pathway, which is used for both purine and pyrimidine synthesis and increased glycolytic flux in cancer cells can enhance the nucleotide synthesis by upregulating the PP pathway. Another pool of nucleotides comes from the TCA cycle via oxaloacetate, which is transaminated to aspartate, and converted to nucleotides (Stanton, 2012). Various nonessential amino acids and methyl groups from the one-carbon/folate pool are used for the production of purine and pyrimidine bases. As well as these intermediates and pathways, NADH is also employed for nucleotide synthesis. For instance, conversion of ribonucleotides to deoxyribonucleotides via ribonucleotide reductase is NADPH dependent.

Balanced levels of the intracellular deoxynucleoside triphosphate (dNTP) pool is critical in maintaining genomic stability and preventing cancer development. In many types of cancer cells, reduction or imbalance of dNTP pools enhances the tumour progression and causes dysregulation of cell cycle (Aird and Zhang, 2015). In eukaryotes, nucleotide levels are maintained by two distinct pathways: the *de novo* synthesis in the cytoplasm, and the salvage pathway that takes place both in cytoplasm and mitochondria. In *de novo* synthesis, purine ribonucleotides are synthesized from basic components including phosphoribosyl pyrophosphate (PRPP), glutamine, and glycine and pyrimidine biosynthesis is accomplished by carbamoyl phosphate, aspartate and PRPP (Figure 1.5). The enzyme ribonucleotide reductase (RNR) catalyses the rate-limiting step of *de novo* pathway that converts ribonucleotide diphosphates to deoxyribonucleotides (Aird and Zhang, 2015).

Salvage pathways also supply nucleotides to organisms. In salvage reactions, the free purine bases, adenine, guanine, and hypoxanthine, can be reconverted to their corresponding nucleotides by phosphoribosylation after phosphoribosyl pyrophosphate (PRPP) has been made by the activation of ribose-5-phosphate. Two key transferase enzymes including adenosine phosphoribosyltransferase (APRT) and hypoxanthine-guanine phosphoribosyltransferase (HGPRT) are involved in the salvage of purines (Figure 1.5). APRT mediates AMP formation and HGPRT can act on either hypoxanthine to form IMP or guanine to form GMP. A critically important enzyme of purine salvage in rapidly dividing cells is adenosine deaminase (ADA) which catalyses the deamination of adenosine to inosine.

In the salvage pathway, dNTPs are synthesized by deaminases and phosphorylases, along with mammalian triphosphohydrolase, histidine/aspartate (HD)-domain containing protein 1 SAMHD1 (Goldstone et al., 2011). Maintaining dNTP concentrations at optimum levels is achieved by cell cycle-dependent activity and allosteric regulation of RNR and SAMHD1 (Ji et al., 2013). Elevated RNR activity in yeast cells increases the dNTP level that can lead to an increased mutation rate and can also affect its function in DNA repair but does not arrest cell cycle progression (Kumar et al., 2010). In mammalian cells, depletion of the dNTP pool decreases PARP-1 activity and impairs Chk1 activation, resulting in inaccurate DNA replication leading to ultrafine anaphase bridge formation (Gemble et al., 2015). Both excess and deficiency of one dNTP may be detrimental. Therefore, balanced dNTP levels are required for DNA damage repair, which is necessary to prevent cancer development.

Targeting nucleotide metabolism has long been considered a therapeutic strategy for cancer. Nucleotide enzyme inhibitors and nucleoside analogs aim to disrupt the synthetic pathways which result in imbalance of dNTPs in cancer cells; this is a common therapeutic target used in the treatment of many types of cancer. However, still very little is known about the effect of cancer metabolism on nucleotide biosynthesis (Shuvalov et al., 2017). Combination therapies that target both the *de novo* and salvage pathways of nucleotides would likely result in better outcomes.
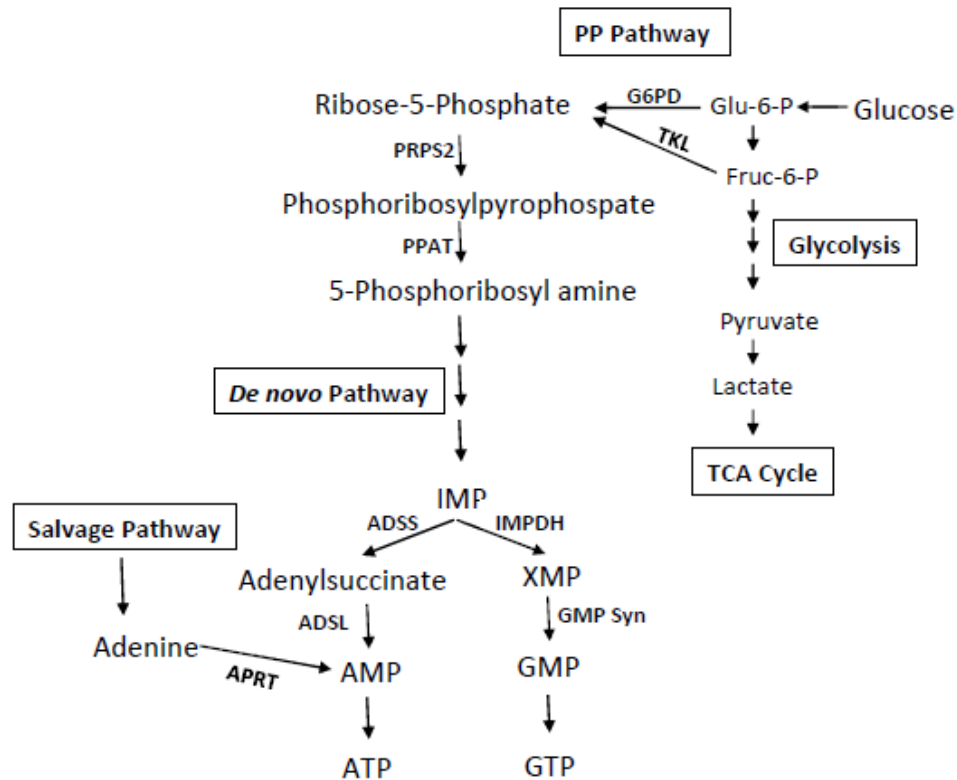
Figure 1.5*: Nucleotide synthesis from metabolic pathways. Enzymes known to be involved in nucleotide production in cells are G6PD = Glycerol Phosphate dehydrogenase and TKL= Transketolase from Pentose phosphate pathway (PPP), PRPS2= Phosphoribosyl pyrophosphate synthetase2, PPAT= Phosphoribosyl pyrophosphate amidotransferase ADSS= Adenylosuccinate synthase, IMPDH= Inosine monophosphate dehydrogenase, ADSL= Adenylosuccinate lyase, GMP SYN = Guanosine monophosphate synthetase, from de novo pathway, APRT= Adenine phosphoribosyltransferase from salvage pathway.*

1.6.3- Redox Balance

In addition to genetic alterations, abnormal tumour microenvironments play a major role in determining the metabolic phenotype of tumour cells. The redox status of tumour cells is also an important metabolic issue because it not only changes the microenvironment of a cell but also alters the signalling pathways for re-wiring of its metabolism (Dang, 2012). Reactive oxygen species (ROS) are radicals, ions or molecules such as superoxide (O_2^-), hydroxyl radical (OH), and hydrogen peroxide (H_2O_2). These molecules are synthesized within the cells via oxygen metabolism and at low levels they are considered important signalling molecules (Gough and Cotter, 2011). ROS are the most common outcome of redox alterations and have been known as a lethal by-product of cellular metabolism. Altered metabolism is expected to generate oxidative stress, which may affect the ability of cancer

15

cells to maintain ROS levels. Under normal physiological conditions, an increased ROS level can induce cancer by creating greater oxidative stress, oncogenic stimulation, increased metabolic activity, mitochondrial malfunctioning, damage of macromolecules such as DNA, lipid and protein (DeBerardinis and Chandel, 2016). It is also notable that different levels of ROS have different effects on normal and cancer cells; therefore, the role of ROS depends upon their levels (Majewski et al., 2004, Cairns et al., 2011). At low levels, ROS provide a beneficial effect, supporting cell proliferation and survival via post-translational modification of kinases and phosphatases (Finkel, 2012). However, at high level, ROS cause detrimental oxidative stress that can lead to apoptosis (Garrido et al., 2006, Han et al., 2003). To counter excessive oxidative stress, a cell produces antioxidants that prevent ROS inducing cellular damage. In cancer cells, the high level of ROS not only induces macromolecule damage but also it induces the production of anti-oxidant enzymes to escape from cell death. One of the important mechanisms to increase anti-oxidants in cancer cells is by activating nuclear factor (erythroid-derived 2)–related factor 2 (NRF2) (Jaramillo and Zhang, 2013). NRF2 activation induces the transcription of several antioxidant proteins as well as activating the enzymes that increase cytosolic NADPH (anti-oxidant) levels, which is needed for redox balance to evade cell death (Ye et al., 2014).

1.7-Cancer Metabolism: A Therapeutic Opportunity for Cancer Therapy

Cancer treatment is constrained by the high genetic diversity and adaptability of tumour cells, making it hard to develop treatments that discriminate between normal and cancer cells. Abnormal cell proliferation is a common feature in cancer cells. Anti-cancer drugs such as cyclophosphamide, mGluR1 inhibitors (riluzole and BAY36-7620), sorafenib or vemurafenib with riluzole have been used for targeting cancer. In addition to this, our current understanding of the role of glucose and glutamine in tumour metabolism has led to the idea that a novel class of anti-cancer drugs that altered metabolism could be developed. Several glycolytic enzymes that are required to maintain a high glucose metabolism have been targeted in cancer cells (DeBerardinis and Chandel, 2016). Lactate dehydrogenase (LDHA) is associated with pyruvate conversion to lactate and its over-expression has been linked to cancer metabolism. LDHA inhibitors were reported to cause regression in tumour growth (Xie et al., 2014). Similarly, fatty acid synthase, the enzyme which catalyses the synthesis of palmitate, was found to be increased in many human cancers and has been targeted in a clinical setting (Röhrig and Schulze, 2016). The mitochondrial metabolic inhibitor metformin

impedes mitochondrial complex I activity and has been observed to have an effect on reducing cancer incidence in clinical trials to test whether it could have an anti-cancer effect (Wheaton et al., 2014). Alternatively, autophagy can be targeted to inhibit mitochondrial metabolism in certain cancer cells. Cancer cells are more reliant on autophagy than normal cells or tissue. Autophagy affects cancer chemotherapy associated cell death and it is related to development of drug resistance by activating pro-survival pathways in cancer cells. Numerous studies show that autophagy has a dual role during cancer progression, it has both cancer-promoting and cancer suppressing functions (Galluzzi et al., 2015, Guo and White, 2013, Yang et al., 2014). For instance, monoallelic deletion of *Beclin1* (autophagy gene) or its regulator *Ambra1* causes increased cell proliferation and spontaneous tumour progression (Qu et al., 2003, Cianfanelli et al., 2015). Furthermore, in mice, targeted deletion of autophagy genes such as *Atg4c*, *Atg5* and *Atg7* induces a high level of tumorigenesis as compared to the wild type controls (Takamura et al., 2011). Moreover, autophagy deficiency is also associated with high level of ROS, DNA damage and CIN which are responsible for causing tumour progression (Poillet-Perez et al., 2015). Conversely, upregulation of autophagy is needed to promote cancer survival, growth and metastasis. In a mouse model of breast cancer, deficiency of the autophagy gene *ATG17/ FIP200* inhibits mammary tumour initiation and progression (Wei et al., 2011), suggesting the role of autophagy in promoting tumorigenesis. Because autophagy has a dual role in tumorigenesis, interventions to either stimulate or impede autophagy have been proposed as anticancer therapy. However, targeting autophagy as a cancer therapy is still controversial. Currently, chloroquine and hydroxychloroquine have been used in clinical trials to inhibit autophagy. These drugs deacidify the lysosome and inhibit the fusion of autophagosomes with lysosomes (Yang et al., 2013). Other autophagy-specific inhibitors including lysosomal inhibitors such as Lys05 and drugs that target earlier steps in the autophagy pathway are also in pre-clinical stages (McAfee et al., 2012, Egan et al., 2015).

As discussed earlier, altered metabolism may change the ability of cancer cells to maintain ROS levels. ROS depleting strategies depend upon antioxidants to suppress ROS signalling and tumour growth (Chandel and Tuveson, 2014). Different anticancer agents based on this strategy are approved by USFDA, for example, procarbazine, motexafin and gadolinium are used to increase ROS content and minodronate and histamine are used to eliminate ROS. However, ROS resistance under continuous ROS stress is a major drawback of these ROS strategies (DeBerardinis and Chandel, 2016).

Metabolic reprogramming in cancer cells contributes to tumorigenesis but also leads to metabolic liabilities that can be exploited for effective cancer therapy. Recent advancements in understanding the differential metabolic dependencies of tumours has developed new therapeutic plans to exploit altered metabolism (Luengo et al., 2017). However, targeting general proliferative metabolism may not produce an appropriate therapeutic option since many normal cells, such as those in the bone marrow, intestinal crypts, and hair follicles, are highly proliferating. Likewise, the proliferation rates of non-malignant cells are often greater than those of malignant cells (Vander Heiden and DeBerardinis, 2017) so adverse effects of antimetabolite chemotherapy arise due to destruction of normal rapidly proliferating cells. In addition, metabolic reprogramming in cancer cells at different stages of cancer development leads to drug-resistance, metastasis and tumorigenesis. These attributes make it hard to target a specific metabolic pathway in malignant cells, however, it may be more effective to address multiple metabolic targets in cancer cells.

Glycolysis has been targeted in several studies as a cancer therapy due to the high uptake of glucose in many tumour cells as compared to normal tissue (DeBerardinis and Chandel, 2016). Hexokinase 2 (HK2) catalyses the first step of glycolysis, and is overexpressed in many cancers. HK2 inhibition, in cancer models like NSCLC and breast cancer tissue, delays tumour progression. Additionally, systemic inhibition of HK2 in mice did not show adverse effects on normal tissues. Lactate dehydrogenase (LDH), which converts pyruvate to lactate has been another target from glycolysis. Increased glycolysis tends to generate high lactate levels for either conversion to other metabolites or to be secreted out of the tumour cells (Vander Heiden, 2011). LHD-A inhibition in MYC driven tumours including 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 (Vander Heiden, 2011).

Targeting nucleotide biosynthesis pathways for cancer therapy has been used for more than 60 years. Cancer cells are highly dependent on the *de novo* synthesis of nucleotides for DNA replication and RNA production. Inhibition of 5-phosphoribosyl-1-pyrophosphatase (PRPP) amidotransferase, the enzyme that catalyses the first step in *de novo* purine biosynthesis, by

purine analogs 6-mercaptopurine and 6-thioguanine has been successful in treating many cancers, including childhood leukemia (Elion, 1989). 5-fluorouracil (5-FU), a pyrimidine analog that inhibits thymidylate synthase, and other 5-FU-prodrugs such as capecitabine are commonly used for gastrointestinal cancer treatment (Wagner et al., 2005). Nucleotide biosynthesis is linked with other metabolic pathways such as the PP pathway, and aspartate and glutamine. Thus, targeting the PP pathway and amino acid synthesis can affect nucleotide production. Combinatorial treatment that targets several metabolic pathways seems to be an attractive strategy for cancer treatment.

1.8-Targeting Metabolism in CIN Cells

CIN inevitably generates aneuploidy in cancers, causing changes in chromosome copy number. This may generate proportional alterations in the transcript level of genes located on aneuploid chromosomes, which may result in protein dosage changes in the encoded protein, which can then lead to malfunctioning of biological processes (Potapova et al., 2013). Defects during mitosis can cause various abnormalities in cytokinesis, mitotic spindle formation and mitotic checkpoints. These aberrations can cause missegregation of chromosomes and tetraploidy, therefore increasing the rate of CIN. Through aneuploidy, CIN may enable tumours to develop resistance against stressful environments, the host immune system, and cytotoxic effects of chemotherapies by further generating genetic variations in tumour cells (Potapova et al., 2013). Continuous genetic variations in cancers make tumours a 'moving target' for anti-cancer therapies. In cancer research, the idea of targeting CIN itself has received much attention because this mechanism is tumour-specific and anti-CIN therapy can potentially inhibit the ability of tumours to develop resistance against drugs.

CIN is a hallmark of cancer cells and causes proteotoxic stress and metabolic aberrations (Oromendia et al., 2012). Recent studies to selectively kill CIN and aneuploid cancer cells revealed that an energy and proteotoxic stress inducer AICAR, a protein folding inhibitor (Hsp190 inhibitor 17-AAG) and chloroquine were found to be promising interventions to inhibit the growth of trisomic yeast cells and high-grade aneuploid colorectal cancer cell lines by increasing aneuploid-induced stress (Tang et al., 2011, García Martínez et al., 2015).

Our lab has carried out a genome wide screen for kinases and phosphatases that when depleted, cause CIN-dependent apoptosis. This screen identified a class of genes that has effects on cell metabolism. We have found that knockdown of metabolic candidates such as

PASK, Pfrx (a glycolysis regulator) or WW domain containing oxidoreductase (Wwox) (a tumour suppressor) alone has no effect on normal cells. However, inhibiting the function of these candidates or similar candidates that affect the TCA cycle, fat metabolism and oxidative stress response enzymes in a CIN model, generates elevated ROS levels, DNA damage and cell death (Shaukat et al., 2014). Autophagy also is activated in CIN cells which is necessary for their survival (Liu et al., 2016). This finding is crucial because both CIN and autophagy are linked with the development of drug resistance (Tan et al., 2017). Thus, we can target the autophagy pathway in order to reduce the development of drug resistance initiated by either CIN or autophagy.

1.9-Existing Models for Investigating CIN

1.9.1- Culture Model

Cell cultures that exhibit CIN are not ideal models for screening for targets that when depleted can induce death in CIN specific cells but avoid affecting the normal cells. Genomic diversity in cell lines makes them problematic for investigating CIN in general and there is also an issue of reproducibility, since cells have different genomes each time they are cultured (Roschke et al., 2005a, Wallqvist et al., 2005). However, this approach can be employed for the screening of chemical libraries in CIN lines (Roschke and Kirsch, 2005). NCI-60 human cell lines have been used to find drugs that show significant effects on these karyotypically diverse cell lines (Roschke et al., 2005b, Wallqvist et al., 2005). None of the tested current cancer drugs were found to have any effect specifically on unstable cell lines, but a few novel drugs were identified that could affect the growth of these cell lines. However, this model was not considered a suitable tool for drug discovery and additional procedures are needed in order to understand the mechanisms (Wallqvist et al., 2005). Therefore, animal CIN tumour models would be preferable if we are to identify and characterize the possible mechanisms of killing CIN cells.

1.9.2- *Drosophila Melanogaster* Model for CIN

Drosophila has been used as a model system for cancer research for decades (Gonzalez, 2013). It is considered to be an effective model for investigating aneuploidy and CIN due to several reasons such as the short generation time, and it is a simple and less expensive compared to vertebrate models. In addition, CIN can be induced in a specific tissue or the entire organism by tissue specific knockdown or increased expression of genes that can

generate CIN, which would be difficult in vertebrate CIN models. For the *Drosophila* system, temperatures of 18°C and 25°C can be used to control the effectiveness of the altered gene expression and hence the level of instability. Moreover, it is an excellent model for analysing the role of human genes in diseases such as neurological defects, metabolic and storage diseases, cardiovascular diseases and cancer (Pandey and Nichols, 2011)

We have used *Drosophila* induced-CIN models for *in vivo* screening and characterization. We induced chromosomal instability by knocking down the *mad2* gene or *Rad21*, giving CIN models with different CIN levels. Depletion of *mad2* provides less time for the cell to align kinetochores properly by reducing metaphase duration (Buffin et al., 2007). Reduced amounts of Mad2 have been shown to generate CIN, aneuploidy and tumorigenesis in human and mouse models (Michel et al., 2001). It has been observed that *mad2* knockdown in *Drosophila* causes lagging chromosomes and chromosome bridges during cell division in > 25% of cells without affecting the overall viability. Therefore, we employed this as a CIN model, knocking down target genes in normal or CIN background cells and screening for lethality specifically in CIN cells (Shaukat et al., 2012).

Drosophila Rad21 is a chromatid cohesion protein and its depletion is also known to cause CIN. Cohesin binds the sister chromatids together during cell division until the SAC is satisfied (Nasmyth, 2011). Cohesion is required for faithful segregation of replicated chromosome during mitosis. A cohesion defect leads to loosely attached or unattached sister chromatids and induces difficulty in the equal distribution of chromosomes between the two daughter cells. Premature chromatid separation or partial failure of segregation results in numerical CIN (Thompson et al., 2010). Genes including *Smc1*, *Smc3*, *Rad21*, *STAG1*, *STAG2*, *STAG3* and *separase* are involved in the cohesion of sister chromatids and frequently mutated in CIN cancers. We have found that knocking down of *Rad21* by RNA interference together with UAS-Dicer2, which enhances the processing of *Rad21*[RNAi] in *Drosophila*, results in anaphase errors in about 35% of brain mitoses, and aneuploidy in more than 50% of wing disc cells (Liu et al., 2016). The resulting lethality makes *Rad21* knockdown flies an ideal model organism to screen candidate genes whose depletion could give suppression of lethality in CIN cells. This CIN model is also used to validate that candidates show suppression or lethality as a result of the presence of CIN, not just as a result of *mad2* knockdown because CIN induced by *Rad21* knockdown caused a high level of CIN and aneuploidy compared to *mad2* knockdown.

1.10- Key Points

Key points of existing CIN research related to this study are as follows:

1. CIN and aneuploidy are essential hallmarks of cancer.
2. CIN and aneuploidy are associated with tumorigenesis, drug resistance, relapse and worse prognosis
3. CIN could be induced by several mechanisms such as cohesion defects, defects in kinetochore-microtubule attachment, defects in spindle assembly check point, DNA damage etc.
4. CIN is common in cancer cells as compared to normal cells. Therefore CIN itself can be employed as a therapeutic target.
5. CIN cells are sensitive to metabolic stress. Thus, specific metabolic interventions can be targeted for cancer therapy.
6. *Drosophila* CIN models are an ideal tool for characterization of metabolic gene targets responding to CIN.

1.11- Aims of the Study

The main objective of this study is an investigation of the role of metabolic pathways in response to CIN and the identification of the potential mechanism in CIN cells that amplifies small metabolic interventions to the point where they affect the survival of CIN cells.

Aim 1: Screening for metabolic candidates whose knockdown can trigger cell death in CIN cells.

Aim 2: Translating the CIN specific cell metabolic targeting into CIN tumours, to evaluate the effects of metabolism on cancer therapy.

Aim 3: Screening and characterization of metabolic candidates whose knockdown can give tolerance to CIN.

Aim 4: To investigate the effects of metabolic stress on mitosis in normal and CIN cells.

Aim 5: To characterize the effects of the unfolded protein response and oxidative stress in CIN cells.

Aims 1 and **2** are described in **Chapter 3**. **Aim 3**, **4** and **5** are described in **Chapter 4**.

Drosophila as a model for chromosomal instability

A common feature of advanced tumours is Chromosomal Instability (CIN), in which cells fail to maintain a stable chromosome number as they proliferate (Thompson et al., 2010). CIN is extremely common in tumours, being associated with tumour progression and poor clinical outcomes in a wide range of cancer types (Carter et al., 2006). Existing cell lines with CIN are not ideal as a CIN model research due to their high aberrant genome content, making it hard to distinguish whether any effect is due to the CIN or the disrupted genome.

Drosophila melanogaster as a model organism for cancer research is well validated, and several key pathways driving proliferation and metastasis were discovered and/or elucidated in this model organism, including signaling pathways (Brumby and Richardson, 2005). *Drosophila* CIN models are considered to be an effective model for investigating aneuploidy and CIN due to its short generation time and ease of maintaining large numbers of fly stocks. It is a simple and less expensive model; more than 70% of human disease causing genes have functional homology in *Drosophila* (Pandey and Nichols, 2011). Moreover, using this model, CIN can be induced in a specific tissue or the entire organism by tissue specific knockdowns or high expression of genes, allowing us to regulate the expression of multiple genes and screen for novel mutations in ways not feasible in vertebrate models. This makes it a good model for cancer research and targeted drug discovery studies. Several mechanisms have been used to induce CIN in *Drosophila* including aberrations in mitotic processes, such as disruption in spindle assembly checkpoint, cytokinesis defects, and DNA damage elevation to develop different CIN models in order to understand the cause of CIN (Bakhoum and Compton, 2012a). However, constant genome shuffling in CIN tumours make it hard to understand the specific changes that induced CIN, and various possible mechanisms to induce CIN may explain why it has been difficult to understand the individual causal mutations in specific tumours.

Nonetheless, it is clear that CIN is a hall mark of cancer cell. Therefore, we can exploit the CIN phenotype in order to target cancer cells exhibiting CIN. We can identify the targets that can be disrupted to specifically kill CIN cells without affecting normal proliferating cells and also understand the causes and cellular responses to CIN.

Drosophila CIN-inducible *in vivo* models are a powerful genetic tool for understanding and characterizing signalling pathways that significantly affect the fate of CIN cells.

Statement of Authorship

Title of Paper	Drosophila as a model for chromosomal instability
Publication Status	Published in AIMS Genetics
Publication Details	Review article Dawei Liu, Zeeshan Shaukat, Rashid Hussain, <u>Mahwish Khan</u>, Stephen L. Gregory Corresponding E-mail: stephen.gregory@adelaide.edu.au

Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author	Dawei Liu		
Contribution to the paper	Conceived and designed the review article Contributed in writing		
Signature		Date	

Name of Co-Author	Zeeshan Shaukat		
Contribution to the paper	Contributed in writing		
Signature		Date	

Name of Co-Author	Rashid Hussain		
Contribution to the paper	Contributed in writing	20 % contribution	
Signature		Date	

Name of Co-Author	Mahwish Khan	
Contribution to the paper	Contributed in writing	20% Contribution
Signature		Date	

Name of Co-Author	Stephen Gregory	
Contribution to the paper	Conceived and designed the Article Wrote the paper Correspondence	
Signature		Date	

Review

Drosophila as a model for chromosomal instability

Dawei Liu, Zeeshan Shaukat, Rashid Hussain, Mahwish Khan and Stephen L. Gregory *

School of Molecular and Biomedical Science, University of Adelaide, North Terrace, Adelaide S.A. 5006, Australia

* **Correspondence:** Email: stephen.gregory@adelaide.edu.au; Tel: +61 8 83137536; Fax: +61 8 83137534.

Abstract: Chromosomal instability (CIN) is a common feature of tumours that leads to increased genetic diversity in the tumour and poor clinical outcomes. There is considerable interest in understanding how CIN comes about and how its contribution to drug resistance and metastasis might be counteracted. In the last decade a number of CIN model systems have been developed in *Drosophila* that offer unique benefits both in understanding the development of CIN in a live animal as well as giving the potential to do genome wide screens for therapeutic candidate genes. This review outlines the mechanisms used in several *Drosophila* CIN model systems and summarizes some significant outcomes and opportunities that they have produced.

1. Introduction

The acquisition of mutations is a driving force in the formation of any tumour, as these mutations represent the genetic diversity from which aberrantly proliferative cells can emerge. Genomic instability, or an increased mutation rate, can be generated by numerous cellular defects, such as lack of DNA repair, and these typically give a strong predisposition to tumorigenesis [1]. Chromosomal instability, or CIN, refers to an increased rate of DNA changes on the largest scale: gain or loss of whole chromosomes or chromosome sections. As a common form of genomic instability, CIN is linked to tumorigenesis, particularly in solid tumours, where the frequency of chromosomal aberrations can be as high as 90% [2]. There are several reasons why CIN might be found so often in tumours: some common oncogenic mutations tend to promote CIN by disrupting mitosis, some tumours typically only progress with the gain or loss of specific chromosome arms, and for a tumour to go on to metastasize seems to require the kind of dramatic rearrangements that

CIN generates [3]. Inducing CIN in otherwise normal mouse models is able to double the rate of spontaneous tumour formation [4], and in human cancers CIN is associated with significantly worse clinical outcomes [5]. Because CIN generates genetic diversity in tumours it is thought to promote drug resistance and relapses following chemotherapy. For these reasons there has been considerable research into the causes and possible therapies for chromosomal instability.

One of the problems in trying to understand the onset of CIN in tumours is that by the time they are detected, they have divided unstably many times and become so genetically diverse that it is hard to identify the specific changes that induced the CIN. In theory, by examining many clinical CIN tumour samples we should be able to find the common changes, but the limited studies available have not clearly identified causal mutations [6]. An alternative approach, testing specific mitotic defects in culture or animal models, has shown that CIN can be caused by a range of defects in the attachment of chromosomes to the spindle as well as by loss of checkpoints, elevated DNA damage or replication stress [7]. This wide range of possible mechanisms may explain why it has been difficult to identify individual causal mutations in specific tumours, and also raises clinical challenges. It may be obvious that CIN is a dangerous cellular phenotype that we would like to prevent, but CIN prevention is problematic even in theory, with so many possible defects that can cause instability. An alternative is to accept that CIN will arise in tumours, and to look instead for therapies that can target such cells. From this perspective, the CIN phenotype is a therapeutic advantage, because CIN represents a significant difference from normal proliferating cells that may allow targeting of therapy to avoid damaging the patient. The question then is: can we find targets that can be disrupted to specifically induce death in CIN cells without affecting normal dividing cells?

2. CIN models and their limitations

An obvious approach to this problem is to get cultures of CIN cells, and screen them with chemical libraries to find anything that kills CIN, but not normal cells. To some extent this has been attempted [8], but there are several challenges with this approach. CIN lines are inherently varied—potentially every cell has a different genome, and each cell varies as it is cultured. This means that it is problematic to do reproducible experiments. However, the objective is to find generalizable therapies that affect any CIN cells, so in theory the approach can still work if enough chemicals are tested enough times. Using karyotypic heterogeneity as a proxy for CIN, the well characterized panel of 60 human cancer cell lines from the NCI has been used to identify drugs that preferentially affect karyotypically diverse cell lines [9,10]. Perhaps unsurprisingly, none of the current cancer therapeutics tested were particularly effective against the unstable cell lines. Some novel drug classes were found that could inhibit the growth of unstable lines with some specificity, however, as the authors comment, these correlations are a "blunt tool for drug discovery requiring secondary experimental confirmation" [10]. In addition, the cell lines used have inevitably arisen with constraints very different to those faced by tumours *in vivo*, and the most effective chemicals identified gave no clear idea of how they might be working. For these reasons it is valuable to have animal CIN tumour models in which specific and reproducible mechanisms for killing CIN cells can be identified and characterized. This review will focus on the development of CIN models in *Drosophila*, discussing the relative merits of this system and the progress that has been achieved so far.

## 3. Advantages of using *Drosophila*

As an animal model for studying CIN, *Drosophila* has some significant advantages. It is now straightforward to manipulate the expression of any gene in *Drosophila*, at any stage of development in any tissue of interest. In the context of CIN, this means that animals can be grown that are genetically normal, with CIN induced by gene mis-expression in the proliferating tissue of choice when required. For example, the larval wing disc can be used to provide a testbed of cells that start out identical each time the experiment is done, while allowing the generation of a diverse set of CIN cells to study [11,12,13]. This avoids the issues of reproducibility faced when using CIN cell lines, as well as providing an *in vivo* epithelium that better reflects the environment of CIN tumours when they arise. This could theoretically be done in mice [14,15], but large scale screening in mice is impracticably time consuming and expensive. To identify and characterize novel mechanisms by which CIN cells can be specifically killed, it is useful to have an inducible *in vivo* system that allows genome-wide screening. Using *Drosophila* provides such a system as well as giving access to unparalleled resources for genetic analysis of any candidates identified [16]. This includes easy screening for genetic interactions, pathway dissection by epistasis and modelling of most of the hallmarks of cancer [17-21].

4. *Drosophila* CIN models

As mentioned above, there are numerous mitotic processes that are known to give CIN when disrupted in cell culture or mouse models, and several of these have been exploited in *Drosophila* to create inducible CIN models. They can be broadly classified as disruption of the mitotic spindle, checkpoints, cytokinesis or DNA repair.

4.1. Mitotic spindle disruption

Defects in spindle structure or kinetochore dynamics represent straightforward mechanisms for generating CIN. For example, if the spindle is incorrectly formed with too many poles, then chromosome segregation is affected (Figure 1A). Centrosome number is frequently aberrant in cancers, so this form of CIN has been modelled by altering the levels of centrosome regulatory genes such as *polo* or *sak*, and showing that neural cells with aberrant numbers of centrosomes go on to form malignant and metastatic tumours in *Drosophila* [22,23]. It is interesting that although very little CIN was initially detected in the mutant brains, when they had been transplanted and grown in a host they became strikingly aneuploid. Exactly when and how the CIN arises in these models is not well characterized, but they appear to recapitulate human tumorigenesis in which CIN is generally not an early trigger, but arises in an already hyper-proliferative tissue. Another striking observation from cells with elevated centrosome numbers was that spindles are surprisingly effective at generating a bipolar array even with many extra centrosomes present, as long as the spindle checkpoint is working to allow enough time to cluster the centrosomes [23,24]. This checkpoint control is not completely effective in neuroblasts, though, which can lose the correct spindle alignment and cell polarity, leading to a failure to differentiate and consequent overproliferation. Interestingly, CIN tumours can also be made without altering the centrosomes simply by disrupting neuroblast polarity. In this case, again there is a failure to differentiate, and an expansion of
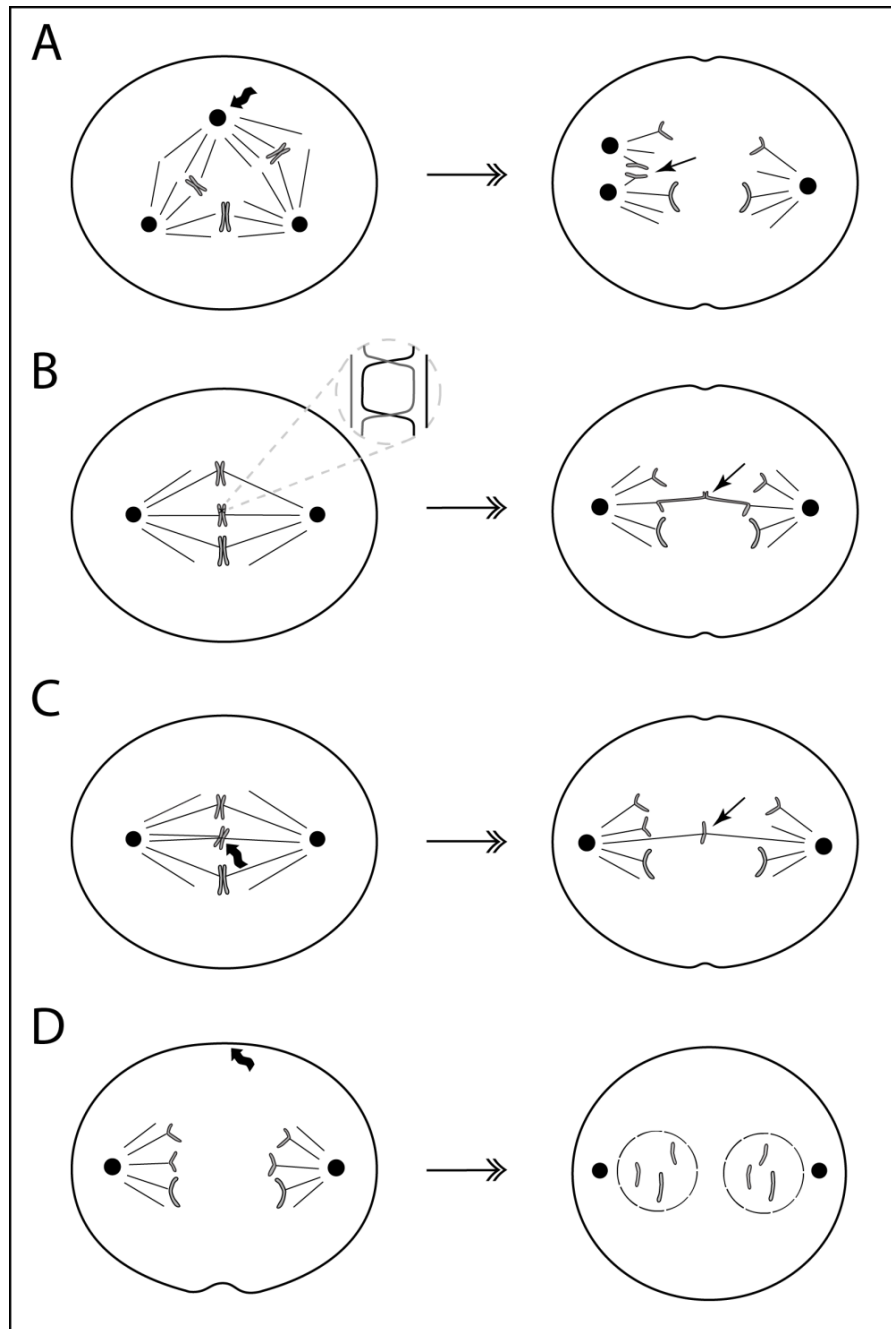
Figure 1. *Drosophila* **CIN models: (A) An induced defect in centrosome number (wavy arrow) leads to a tripolar spindle. This is resolved into a bipolar array before anaphase, but one chromosome fails to segregate (arrow). (B) An induced defect in DNA damage repair or replication leads to still catenated chromatids (enlarged). At anaphase this leads to a chromosome bridge (arrow) that will break, leading to loss or gain of chromosome segments. (C) An induced spindle checkpoint defect means that a kinetochore attached to both poles (wavy arrow) may not be resolved before anaphase, leading to failure to segregate a lagging chromosome (arrow) that is attached to both poles.（D）An induced cytokinesis defect (wavy arrow) leads to no separation of daughter cells, and results in a binucleate cell with a tetraploid genome and an extra centrosome.**

proliferative neuroblasts that can be transplanted to form malignant and metastatic tumours [25]. The initial neuroblasts are karyotypically normal, but become aneuploid and acquire centrosome defects as the tumour develops. From these studies and others [26] it seems that most tissues in *Drosophila* resist tumorigenesis from centrosome alterations by dying, but neuroblasts are an exception. In these cells, anything that expands the 'stem-like' population of transit amplifying lineage neuroblasts will tend to not only cause overgrowth, but also the acquisition of additional defects that cause CIN and allow metastatic tumour growth. It will be interesting to find out what changes arise so rapidly and frequently to transform overgrowing neuroblasts; recent work in this direction has implicated DNA damage [27].

4.2. Elevated DNA damage

Mutations that either increase the rate of DNA damage or that reduce the cell's ability to repair DNA damage are another type of CIN model available in *Drosophila* (Figure 1B). For example, high levels of instability can be induced by blocking *tefu,* the *Drosophila* homolog of *ATM*, which is needed for DNA damage repair and telomere maintenance [28]. Interestingly, ATM also acts as a redox sensor protein, so it is activated either by DNA damage itself or by oxidative stress that threatens the DNA [29]. Balancing the levels of cellular pro- and anti-oxidants is clearly important for cells, as too much of either is able to generate CIN [30]. Furthermore, the aneuploidy generated by CIN is known to cause oxidative stress [31], which can damage DNA to cause further aneuploidy, so there is potential for feedback reinforcement of initially minor oxidative insults. Sophisticated tools are now available in *Drosophila* to monitor the levels and locations of redox stress in live tissues [32]. DNA damage itself is harder to monitor live, but some markers are available [33,34]. It would be particularly useful to be able to monitor the persistence of DNA damage into mitosis, as there are many unresolved questions about chromosome bridges and the resolution of double strand breaks in mitosis. For example, replication stress can induce CIN [35], but it is not clear whether this mechanism is often found in CIN tumours [36]. Ultrafine anaphase bridges, which result from late decatenation of chromatids [37], represent another potential source of CIN that has not yet been explored in *Drosophila*. These bridges indicate an unexpected level of DNA processing during mitosis even after the DNA damage and spindle checkpoints have been passed. This may explain why mitotic stability is so dependent on DNA damage repair being completed in time. The need for efficient DNA repair has been highlighted by our work showing that CIN cells are particularly dependent on JNK signalling in G2 to prevent DNA damage accumulation and apoptosis [38]. Even just altering G2 duration strongly affects the survival of CIN cells; we suggest this is because CIN cells are particularly sensitive to entering mitosis with unrepaired DNA damage. If DNA damage or aneuploidy levels get too high, the JNK pathway is also used to drive apoptosis [13,26,39]. How the JNK pathway integrates multiple stress signals to decide between repair versus death is an area of active research [40,41,42].

4.3. Spindle assembly checkpoint mutations

The best characterized cause of CIN is a failure in the spindle assembly checkpoint that allows segregation of chromosomes that are not correctly bioriented on the spindle (Figure 1C). There is abundant evidence that a defect in the spindle checkpoint in cell culture [43], animal models [44] or

humans [45], leads to CIN. Analysis of mutations found in tumours suggests that complete loss of spindle checkpoint proteins is rare [46], presumably because that would generate an intolerably high level of instability. However there are numerous examples of tumours in which the checkpoint is aberrant, either in protein levels or localization [44,47,48].

Using a defective spindle checkpoint as a CIN model has some advantages: the previously mentioned centrosomal and DNA damage models will tend to trigger checkpoints that promote cell cycle arrest and apoptosis rather than the desired unstable proliferation. In vertebrate systems, the level of spindle checkpoint disruption must be carefully controlled to avoid lethal levels of aneuploidy, but in *Drosophila*, the process of capturing the four chromosomes is sufficiently robust that even complete loss of the checkpoint can give viable animals [49]. This CIN model provides a perfect sensitized background for genetic screening in which even minor disruptions to genes needed for CIN cell survival can push these checkpoint compromised animals over the threshold of viability [11]. This screen tested the set of kinases and phosphatases and identified the JNK pathway and centrosomal signaling as key areas of CIN cell sensitivity. Although this type of viability screening has the benefit of being high-throughput, it has the drawback of a relatively non-specific phenotype: the death could be due to a range of developmental defects that would not be relevant to CIN tumours. However, further analysis allows confirmation that depleting the candidates by RNAi in proliferating CIN cells, such as the wing imaginal disc, can cause cell death [11]. This screen discarded mutations that were lethal in wild type as well as CIN animals, potentially missing some effective ways to kill CIN cells, however this approach should tend to identify more clinically useful approaches with fewer side effects on normal cells. Subsequent testing of good candidates in *Drosophila* tumour models [18] may allow confirmation that the genes in question are needed for the growth of *bona fide* CIN tumours. Our viability screen induced CIN by using RNAi to deplete the spindle checkpoint protein Mad2, but numerous alternative CIN models are available to confirm the generalizable effectiveness of candidates, including models depleting the checkpoint protein BubR1, or the cohesin Rad21 [38,50].

Another use of spindle checkpoint CIN models in *Drosophila* has been to explore the fate of CIN cells if apoptosis is prevented [12,13]. Although the spindle checkpoint is not strictly needed for *Drosophila* survival, checkpoint mutants lose many cells to apoptosis as they grow, so preventing apoptosis allows analysis of the behaviour of the most aneuploid and aberrant cells, which might also be found in apoptosis-resistant cancers. These were found to activate the JNK pathway and drop out of the epithelium, a metastatic behaviour seen in other similarly "undead" cells [39,51].

4.4. Cytokinesis defects

It has been noticed in human tumours that although they frequently show CIN and grossly aberrant karyotypes, in many cases the chromosome number seems to vary around a tetraploid rather than a diploid complement [52,53]. In some cases tetraploidy strongly predicts human tumour progression [54], and elegant studies in mice have shown that simply blocking one round of cytokinesis to give tetraploid cells can trigger tumorigenesis [55]. *Drosophila* is an excellent model system for studying cytokinesis—many of the genes and interactions that drive the process were discovered in flies [56-59]. Although there are numerous *Drosophila* RNAi lines that allow induction of cytokinesis failure and the generation of tetraploid cells, the relationship of tetraploidy to CIN development and tumorigenesis has not been intensively studied in flies [60]. Recent work showing

the involvement of the Hippo pathway in tetraploid cell survival [61] may prompt further investigation in this area.

All these methods for generating CIN involve genetic disruption, but it is also possible to induce CIN chemically, for example by inhibiting kinesins with monastrol to cause monopolar spindle formation [62]. This approach has been used in vertebrate cell culture for some time, and has recently been developed in *Drosophila* by replacing the fly kinesin with the human version, which is sensitive to inhibition by small molecules [63]. Chemical induction of CIN has the advantage that it is convenient for developing high-throughput screening in culture, and by using *Drosophila* cells, it is convenient to then rapidly screen candidate hits in flies for *in vivo* phenotypes.

5. Conclusion

At the beginning of this review we posed a question of particular clinical interest: can we find targets that can be disrupted to specifically induce death in CIN cells without affecting normal dividing cells? Use of induced CIN models in *Drosophila* has allowed some significant progress to be made in this direction. It has been known for over a century that centrosomes are often aberrant in cancers [64], but studies in *Drosophila* have made it clear that just having the wrong number of centrosomes is not in itself enough to result in tumorigenesis [18], because either the spindle checkpoint can delay anaphase until a bipolar spindle has formed, or because the grossly aneuploid progeny will die by apoptosis. The striking exception to this generalization is also significant—in brains, neither the spindle checkpoint nor apoptosis are able to prevent tumorigenesis from neuroblasts that have lost their polarity cues. In these cells, too many or too few centrosomes or disruption of cortical polarity markers can all trigger failure to differentiate and hyperplasia that often progresses to metastatic tumours. It is not yet clear why neuroblasts are insensitive to genomic disruption that triggers apoptosis in other cells, but since human CIN tumours share this cell death resistance, either neuroblasts or disc cells with blocked apoptosis are appealing CIN models for looking at aneuploid cell behaviour [65].

The significance of centrosomes has been emphasized: in CIN cells even slight perturbation of the centrosomes tends to give cell death [11]. This is consistent with reports showing that the spindle checkpoint is needed to survive extra centrosomes [66]. The JNK pathway is also clearly implicated in CIN cell survival and proliferation. JNK is typically activated in response to cell stress, but it is important to recognize that it gives two possible outcomes: if the stress is low level it promotes repair, whereas if the stress is acute, JNK promotes apoptosis [40]. Consequently, blocking JNK signalling in CIN cells can either cause their death through failed DNA repair [11,38] or prevent their death in response to gross aneuploidy [13,26]. Although targeting either centrosomes or JNK signalling may allow effective manipulation of CIN cell fates, neither is ideal as a clinical target, due to their critical functions in normal cells. An alternative that may offer better clinical promise is to genetically or chemically target the metabolism of CIN cells. We have found that CIN cells are highly sensitive to RNAi knockdowns that give a range of mild metabolic perturbations that do not affect normal cells [50]. Some of these (e.g. Pas Kinase) may be amenable to chemical inhibition. It is known that aneuploid cells experience redox stress [67], so one possible hypothesis is that CIN cells, with their ongoing and varied aneuploidy, are close to the limits of their ability to buffer redox stress, and hence are vulnerable to metabolic intervention. Because tumours frequently display an aberrant metabolism as well as chromosomal instability, there are reasons to hope that metabolic therapy may be effective at

generating tumour-specific apoptosis with minimal side effects [68].

There are a number of remaining challenges in understanding the causes and cellular responses to CIN. For example we still have no clear explanation for why the many varied genotypes generated in CIN populations so frequently seem to give the same stereotypical cell phenotype that includes overactive mitochondria, reactive oxygen species production, activation of the JNK pathway etc. It may be simply that any gene dosage variation gives protein folding stress [69], but we suspect that there may be other mechanisms that contribute to the response to aneuploidy. For example, many organisms can partially buffer gene dosage changes by down- or up-regulating the expression of genes in trisomic or monosomic DNA segments [70,71]. Several dosage compensation mechanisms exist for ensuring equal gene expression from sex chromosomes in males and females [72], and there are suggestions that extra somatic chromosomes can be shut down by nuclear compartmentalization [73], so it will be interesting to see whether CIN cells use similar processes to allow tolerance of gross aneuploidy. Because CIN develops fairly rapidly in *Drosophila* tumour models [25], they may be ideal for characterizing the acquisition of the changes that result in cells not only tolerating aberrant karyotypes, but also going on to proliferate and metastasize.

Conflict of Interest

All authors declare no conflicts of interest in this paper.

References

1. Lengauer C, Kinzler KW, Vogelstein B (1998) Genetic instabilities in human cancers. *Nature* 396: 643-649.
2. Weaver BA, Cleveland DW (2006) Does aneuploidy cause cancer? *Curr Opin Cell Biol* 18: 658-667.
3. Rao CV, Yamada HY (2013) Genomic instability and colon carcinogenesis: from the perspective of genes. *Front Oncol* 3: 130.
4. Duijf PHG, Benezra R (2013) The cancer biology of whole-chromosome instability. *Oncogene* 32: 4727-4736.
5. McGranahan N, Burrell RA, Endesfelder D, et al. (2012) Cancer chromosomal instability: therapeutic and diagnostic challenges. *EMBO Rep* 13: 528-538.
6. Carter SL, Eklund AC, Kohane IS, et al. (2006) A signature of chromosomal instability inferred from gene expression profiles predicts clinical outcome in multiple human cancers. *Nat Genet* 38: 1043-1048.
7. Bakhoum SF, Swanton C (2014) Chromosomal instability, aneuploidy, and cancer. *Front Oncol* 4: 161.
8. Roschke A, Kirsch I (2010) Targeting karyotypic complexity and chromosomal instability of cancer cells. *Curr Drug Targets* 11: 1341-1350.
9. Roschke AV, Lababidi S, Tonon G, et al. (2005) Karyotypic "state" as a potential determinant for anticancer drug discovery. *Proc Natl Acad Sci U S A* 102: 2964-2969.
10. Wallqvist A, Huang R, Covell DG, et al. (2005) Drugs aimed at targeting characteristic karyotypic phenotypes of cancer cells. *Mol Cancer Ther* 4: 1559-1568.

11. Shaukat Z, Wong HWS, Nicolson S, et al. (2012) A Screen for Selective Killing of Cells with Chromosomal Instability Induced by a Spindle Checkpoint Defect. *PLoS One* 7: e47447.

12. Morais da Silva S, Moutinho-Santos T, Sunkel CE (2013) A tumor suppressor role of the Bub3 spindle checkpoint protein after apoptosis inhibition. *J Cell Biol* 201: 385-393.

13. Dekanty A, Barrio L, Muzzopappa M, et al. (2012) Aneuploidy-induced delaminating cells drive tumorigenesis in Drosophila epithelia. *Proc Natl Acad Sci U S A* 109: 20549-20554.

14. Silk AD, Zasadil LM, Holland AJ, et al. (2013) Chromosome missegregation rate predicts whether aneuploidy will promote or suppress tumors. *Proc Natl Acad Sci U S A* 110: E4134-E4141.

15. Foijer F, DiTommaso T, Donati G, et al. (2013) Spindle checkpoint deficiency is tolerated by murine epidermal cells but not hair follicle stem cells. *Proc Natl Acad Sci U S A* 110: 2928-2933.

16. St Johnston D (2002) The art and design of genetic screens: Drosophila melanogaster. *Nat Rev Genet* 3: 176-188.

17. Brumby AM, Richardson HE (2005) Using Drosophila melanogaster to map human cancer pathways. *Nat Rev Cancer* 5: 626-639.

18. Gonzalez C (2013) Drosophila melanogaster: a model and a tool to investigate malignancy and identify new therapeutics. *Nat Rev Cancer* 13: 172-183.

19. Tipping M, Perrimon N (2014) Drosophila as a model for context-dependent tumorigenesis. *J Cell Physiol* 229: 27-33.

20. Gladstone M, Su TT (2011) Chemical genetics and drug screening in Drosophila cancer models. *J Genet Genomics* 38: 497-504.

21. Pastor-Pareja JC, Xu T (2013) Dissecting social cell biology and tumors using Drosophila genetics. *Annu Rev Genet* 47: 51-74.

22. Castellanos E, Dominguez P, Gonzalez C (2008) Centrosome dysfunction in Drosophila neural stem cells causes tumors that are not due to genome instability. *Curr Biol* 18: 1209-1214.

23. Basto R, Brunk K, Vinadogrova T, et al. (2008) Centrosome amplification can initiate tumorigenesis in flies. *Cell* 133: 1032-1042.

24. Kwon M, Godinho SA, Chandhok NS, et al. (2008) Mechanisms to suppress multipolar divisions in cancer cells with extra centrosomes. *Genes Dev* 22: 2189-2203.

25. Caussinus E, Gonzalez C (2005) Induction of tumor growth by altered stem-cell asymmetric division in Drosophila melanogaster. *Nat Genet* 37: 1125-1129.

26. Poulton JS, Cuningham JC, Peifer M (2014) Acentrosomal Drosophila Epithelial Cells Exhibit Abnormal Cell Division, Leading to Cell Death and Compensatory Proliferation. *Dev Cell* 30: 731-745.

27. Jüschke C, Dohnal I, Pichler P, et al. (2013) Transcriptome and proteome quantification of a tumor model provides novel insights into post-transcriptional gene regulation. *Genome Biol* 14: r133.

28. Purdy A, Su TT (2004) Telomeres: not all breaks are equal. *Curr Biol* 14: R613-R614.

29. Krüger A, Ralser M (2011) ATM Is a Redox Sensor Linking Genome Stability and Carbon Metabolism. *Sci Signal* 4: 4-6.

30. Li TS, Marbán E (2010) Physiological levels of reactive oxygen species are required to maintain genomic stability in stem cells. *Stem Cells* 28: 1178-1185.

31. Pfau SJ, Amon A (2012) Chromosomal instability and aneuploidy in cancer: from yeast to man. *EMBO Rep* 13: 515-527.
32. Albrecht SC, Barata AG, Grosshans J, et al. (2011) In vivo mapping of hydrogen peroxide and oxidized glutathione reveals chemical and regional specificity of redox homeostasis. *Cell Metab* 14: 819-829.
33. Dronamraju R, Mason JM (2011) MU2 and HP1a regulate the recognition of double strand breaks in Drosophila melanogaster. *PLoS One* 6: e25439.
34. Chiolo I, Minoda A, Colmenares SU, et al. (2011) Double-strand breaks in heterochromatin move outside of a dynamic HP1a domain to complete recombinational repair. *Cell* 144: 732-744.
35. Burrell RA, McClelland SE, Endesfelder D, et al. (2013) Replication stress links structural and numerical cancer chromosomal instability. *Nature* 494: 492-496.
36. Bakhoum SF, Silkworth WT, Nardi IK, et al. (2014) The mitotic origin of chromosomal instability. *Curr Biol* 24: R148-R149.
37. Liu Y, Nielsen CF, Yao Q, et al. (2014) The origins and processing of ultra fine anaphase DNA bridges. *Curr Opin Genet Dev* 26C: 1-5.
38. Wong HW, Shaukat Z, Wang J, et al. (2014) JNK signaling is needed to tolerate chromosomal instability. *Cell Cycle* 13: 622-631.
39. McNamee LM, Brodsky MH (2009) p53-independent apoptosis limits DNA damage-induced aneuploidy. *Genetics* 182: 423-435.
40. Shaukat Z, Liu D, Hussain R, et al. (2014) The Role of JNK Signaling in Responses to Oxidative DNA damage. *Curr Drug Targets* [in press].
41. Karpac J, Biteau B, Jasper H (2013) Misregulation of an adaptive metabolic response contributes to the age-related disruption of lipid homeostasis in Drosophila. *Cell Rep* 4: 1250-1261.
42. Christmann M, Kaina B (2013) Transcriptional regulation of human DNA repair genes following genotoxic stress: trigger mechanisms, inducible responses and genotoxic adaptation. *Nucleic Acids Res* 41: 8403-8420.
43. Thompson SL, Compton DA (2008) Examining the link between chromosomal instability and aneuploidy in human cells. *J Cell Biol* 180: 665-672.
44. Perez de Castro I, de Carcer G, Malumbres M (2007) A census of mitotic cancer genes: new insights into tumor cell biology and cancer therapy. *Carcinogenesis* 28: 899-912.
45. Hanks S, Coleman K, Reid S, et al. (2004) Constitutional aneuploidy and cancer predisposition caused by biallelic mutations in BUB1B. *Nat Genet* 36: 1159-1161.
46. Wood LD, Parsons DW, Jones S, et al. (2007) The genomic landscapes of human breast and colorectal cancers. *Science* 318: 1108-1113.
47. Privette LM, Weier JF, Nguyen HN, et al. (2008) Loss of CHFR in human mammary epithelial cells causes genomic instability by disrupting the mitotic spindle assembly checkpoint. *Neoplasia* 10: 643-652.
48. Schvartzman JM, Sotillo R, Benezra R (2010) Mitotic chromosomal instability and cancer: mouse modelling of the human disease. *Nat Rev Cancer* 10: 102-115.
49. Buffin E, Emre D, Karess RE (2007) Flies without a spindle checkpoint. *Nat Cell Biol* 9: 565-572.

50. Shaukat Z, Liu D, Choo A, et al. (2014) Chromosomal instability causes sensitivity to metabolic stress. *Oncogene* [Epub ahead of print].

51. Rudrapatna VA, Bangi E, Cagan RL (2013) Caspase signalling in the absence of apoptosis drives Jnk-dependent invasion. *EMBO Rep* 14: 172-177.

52. Olaharski AJ, Sotelo R, Solorza-Luna G, et al. (2006) Tetraploidy and chromosomal instability are early events during cervical carcinogenesis. *Carcinogenesis* 27: 337-343.

53. Margolis RL (2005) Tetraploidy and tumor development. *Cancer Cell* 8: 353-354.

54. Galipeau PC, Cowan DS, Sanchez CA, et al. (1996) 17p (p53) allelic losses, 4N (G2/tetraploid) populations, and progression to aneuploidy in Barrett's esophagus. *Proc Natl Acad Sci U S A* 93: 7081-7084.

55. Fujiwara T, Bandi M, Nitta M, et al. (2005) Cytokinesis failure generating tetraploids promotes tumorigenesis in p53-null cells. *Nature* 437: 1043-1047.

56. Ebrahimi S, Gregory SL (2011) Dissecting protein interactions during cytokinesis. *Commun Integr Biol* 4: 243-244.

57. D'Avino PP, Savoian MS, Glover DM (2005) Cleavage furrow formation and ingression during animal cytokinesis: a microtubule legacy. *J Cell Sci* 118: 1549-1558.

58. Somma MP, Ceprani F, Bucciarelli E, et al. (2008) Identification of Drosophila mitotic genes by combining co-expression analysis and RNA interference. *PLoS Genet* 4: e1000126.

59. Eggert US, Mitchison TJ, Field CM (2006) Animal cytokinesis: from parts list to mechanisms. *Annu Rev Biochem* 75: 543-566.

60. Fox DT, Duronio RJ (2013) Endoreplication and polyploidy: insights into development and disease. *Development* 140: 3-12.

61. Ganem NJ, Cornils H, Chiu SY, et al. (2014) Cytokinesis Failure Triggers Hippo Tumor Suppressor Pathway Activation. *Cell* 158: 833-848.

62. Lampson MA, Renduchitala K, Khodjakov A, et al. (2004) Correcting improper chromosome-spindle attachments during cell division. *Nat Cell Biol* 6: 232-237.

63. Salemi JD, McGilvray PT, Maresca TJ (2013) Development of a Drosophila cell-based error correction assay. *Front Oncol* 3: 187.

64. Holland AJ, Cleveland DW (2009) Boveri revisited: chromosomal instability, aneuploidy and tumorigenesis. *Nat Rev Mol Cell Biol* 10: 478-487.

65. Milán M, Clemente-Ruiz M, Dekanty A, et al. (2014) Aneuploidy and tumorigenesis in Drosophila. *Semin Cell Dev Biol* 28: 110-115.

66. Marthiens V, Piel M, Basto R (2012) Never tear us apart--the importance of centrosome clustering. *J Cell Sci* 125: 3281-3292.

67. Sheltzer JM, Torres EM, Dunham MJ, et al. (2012) Transcriptional consequences of aneuploidy. *Proc Natl Acad Sci U S A* 109: 12644-12649.

68. Gorrini C, Harris IS, Mak TW (2013) Modulation of oxidative stress as an anticancer strategy. *Nat Rev Drug Discov* 12: 931-947.

69. Oromendia AB, Dodgson SE, Amon A (2012) Aneuploidy causes proteotoxic stress in yeast. *Genes Dev* 26: 2696-2708.

70. Lundberg LE, Figueiredo MLA, Stenberg P, et al. (2012) Buffering and proteolysis are induced by segmental monosomy in Drosophila melanogaster. *Nucleic Acids Res* 40: 5926-5937.

71. Siegel JJ, Amon A (2012) New insights into the troubles of aneuploidy. *Annu Rev Cell Dev Biol* 28: 189-214.

72. Ferrari F, Alekseyenko AA, Park PJ, et al. (2014) Transcriptional control of a whole chromosome: emerging models for dosage compensation. *Nat Struct Mol Biol* 21: 118-125.
73. Davidsson J, Veerla S, Johansson B (2013) Constitutional trisomy 8 mosaicism as a model for epigenetic studies of aneuploidy. *Epigenetics Chromatin* 6: 18.

Phosphoenolpyruvate carboxykinase maintains glycolysis-driven growth in Drosophila tumors

Our initial broad screening identified some metabolic regulators, such as phospho-fructokinase (Pfrx) and PAS kinase. These glycolysis regulators were not previously known to affect mitotic events, but we found that their knockdown causes strong CIN-specific apoptosis (Shaukat et al., 2015). Pfrx and PAS kinase both function to promote glycolysis so we tested whether a range of other metabolic disruptions could also affect CIN cells. Consistent with this findings, we observed that specific knockdowns affecting the TCA cycle, fat metabolism, gluconeogenesis and oxidative stress response enzymes could all promote CIN-specific apoptosis (Shaukat et al., 2015). These processes all affect the maintenance of cellular anti-oxidant levels, so we tested whether depletion of these candidates caused oxidative stress in CIN cells. We observed disruption of mitochondria and glutathione levels, with high levels of reactive oxygen species (ROS) and oxidative damage to DNA in these cells. The oxidative damage was responsible for the cell death, because overexpression of antioxidant enzymes could block apoptosis in these cells. Significantly, oxidative damage was not seen when the metabolic candidates were depleted in normal cells.

Having found that metabolic candidates when depleted cause apoptosis in CIN cells, we wished to understand their mechanism in a CIN tumor model. Like many animals, *Drosophila* are prone to cancer as they age (Gonzalez, 2013), but the process can be reliably induced by depleting tumour suppressors. We use RNAi to knock down *brat*, which produces aggressive, metastatic brain tumours that can be explanted into normal *Drosophila* hosts and passaged to follow their development (Rossi and Gonzalez, 2015). We observed that these tumours rapidly acquire CIN and their ROS levels are elevated. This represents a genetically manipulable CIN tumour model that is ideal for testing the effect of metabolic interventions on the tumour and host animal.

We tested our best RNAi candidates from earlier screening to identify those that could block the growth of CIN tumours. Some *G6PD*, and *JNK* knockdowns gave elevated cell death and smaller primary tumours, but were not able to prevent the growth of explants and their metastases. Of note, we found that depletion of Phosphoenol-pyruvate Carboxykinase (PEPCK) as well as feeding the host a PEPCK inhibitor, was able to completely block the growth of CIN tumours. PEPCK catalyzes the formation of phosphoenolpyruvate from oxaloacetate, and is an early step in gluconeogenesis in the liver. PEPCK also drives the formation of Glycerol-3-Phosphate (Beale et al., 2004). This glyceroneogenesis is used to provide the backbones for lipid synthesis, which is vital for proliferating cells, as they need to

double their membrane content with every cell division. Glucose can be used for this purpose, but glyceroneogenesis appears to be the preferred source, even in fed animals (Nye et al., 2008).

We wished to understand why PEPCK depletion blocks the growth of tumours, while it has so little effect on normal cell proliferation. We checked for ROS because induced-CIN cells and tumours that acquire CIN have elevated ROS levels and depend on antioxidants for survival (Shaukat et al., 2015). We found that feeding antioxidant to the host can restore growth to PEPCK depleted CIN tumours. This suggests that PEPCK does something to lower redox stress. In this study we proposed that the critical role of PEPCK in these tumours is to lower cytoplasmic NADH levels.

We concluded that CIN tumours need glycolysis to grow, so they must generate NADH, but as aneuploid cells they suffer redox stress that limits their capacity to tolerate any further ROS. PEPCK represents a way to lower NADH without making ROS, but if we block this, we have shown that CIN tumours have a major problem

Statement of Authorship

Title of Paper	Phosphoenolpyruvate carboxykinase maintains glycolysis-driven growth in *Drosophila* tumors.
Publication Status	Published
Publication Details	Rashid Hussain, Zeeshan Shaukat, <u>Mahwish Khan</u>, Robert Saint and Stephen Gregory Affiliations: School of Genetics and Evolution, University of Adelaide, Adelaide, SA, Australia Corresponding E-mail: stephen.gregory@adelaide.edu.au

Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author	Rashid Hussain
Contribution to the paper	Conceived and designed the experiments Performed the experiments Analysed the data Contributed reagents/materials/analysis tools Wrote the paper
Signature	Date

Name of Co-Author	Zeeshan Shaukat
Contribution to the paper	Performed the experiments Draft writing Supervision
Signature	Date

Name of Co-Author	Mahwish Khan
Contribution to the paper	Performed the experiments　　　　20% Contribution
Signature	Date

Name of Co-Author	Robert Saint
Contribution to the paper	Conceived and designed the experiments Contributed reagents/materials/analysis tools Funding
Signature		Date	

Name of Co-Author	Stephen Gregory
Contribution to the paper	Conceived and designed the experiments Analysed the data Contributed reagents/materials/analysis tools Wrote the paper Funding Correspondence
Signature		Date	

SCIENTIFIC REP⚙RTS

OPEN

Phosphoenolpyruvate Carboxykinase Maintains Glycolysis-driven Growth in Drosophila Tumors

Rashid Hussain[1], Zeeshan Shaukat[1], Mahwish Khan[1], Robert Saint[2] & Stephen L. Gregory[1]

Tumors frequently fail to pass on all their chromosomes correctly during cell division, and this chromosomal instability (CIN) causes irregular aneuploidy and oxidative stress in cancer cells. Our objective was to test knockdowns of metabolic enzymes in *Drosophila* to find interventions that could exploit the differences between normal and CIN cells to block CIN tumor growth without harming the host animal. We found that depleting by RNAi or feeding the host inhibitors against phosphoenolpyruvate carboxykinase (PEPCK) was able to block the growth of CIN tissue in a *brat* tumor explant model. Increasing NAD+ or oxidising cytoplasmic NADH was able to rescue the growth of PEPCK depleted tumors, suggesting a problem in clearing cytoplasmic NADH. Consistent with this, blocking the glycerol-3-phosphate shuttle blocked tumor growth, as well as lowering ROS levels. This work suggests that proliferating CIN cells are particularly vulnerable to inhibition of PEPCK, or its metabolic network, because of their compromised redox status.

Chromosomal instability (CIN) refers to cell divisions that cannot maintain chromosomal integrity or number. This can be caused by defects including elevated DNA damage, weakened cell cycle checkpoints or an aberrant mitotic spindle[1]. CIN is a common phenotype of human tumours and generates genetic variation that has been associated with tumour evolution, the development of drug resistance and the consequent poor prognosis of CIN cancer patients[2]. We and others have proposed that CIN itself could be an attractive target for chemotherapy, as it is a relatively cancer-specific phenotype [3–6]. However, as CIN cells are necessarily genetically diverse, it is challenging to identify conserved features of CIN cells as potential targets. Our approach has been to induce CIN in a genetically uniform population of cells *in vivo* in *Drosophila* and to screen for genes that can be knocked down to kill CIN cells without affecting normal proliferating cells[3, 7, 8]. We hypothesize that the candidates giving widespread cell death in CIN cell populations are targeting vulnerabilities common to a wide range of aneuploidies.

This approach identified plausible targets such as JNK signalling and centrosomal regulators that could be depleted to give CIN-specific lethality[3]. In addition, we found metabolic targets such as Phosphoenol pyruvate carboxykinase (PEPCK), and Glucose-6-phosphate dehydrogenase (G6PD)[7]. Knockdown of these genes gave increased mitochondrial output, reactive oxygen species (ROS), DNA damage and cell death in CIN cells without affecting normal proliferating cells. Tumours are often metabolically unlike their surroundings, with elevated glycolysis for anabolism rather than ATP generation[9]. This metabolic demand is shared to some extent by all proliferating cells, as they must generate cellular building blocks before they can replicate their DNA and divide. CIN tumours carry an additional burden, however, as it has been observed that aneuploid cells suffer redox stress in proportion to their aneuploidy[10]. Though we lack a detailed understanding of how aneuploidy causes redox stress, the evidence implicates a combination of elevated ROS levels and protein turnover problems[11]. The combination of this redox stress and a Warburg metabolism makes CIN tumours potentially vulnerable to metabolic intervention that does not affect normal cells.

Having found metabolic targets that were able to kill proliferating cells with induced CIN, we wished to understand their mechanism of action in the context of a growing CIN tumour. In this paper we describe the CIN status of *brat* explant tumours and their use as a fly CIN tumour model. Chemical as well as genetic inhibition of

[1]Department of Genetics and Evolution, University of Adelaide, Adelaide, 5006, Australia. [2]Flinders University, Adelaide, 5042, Australia. Correspondence and requests for materials should be addressed to S.L.G. (email: stephen. gregory@adelaide.edu.au)

1
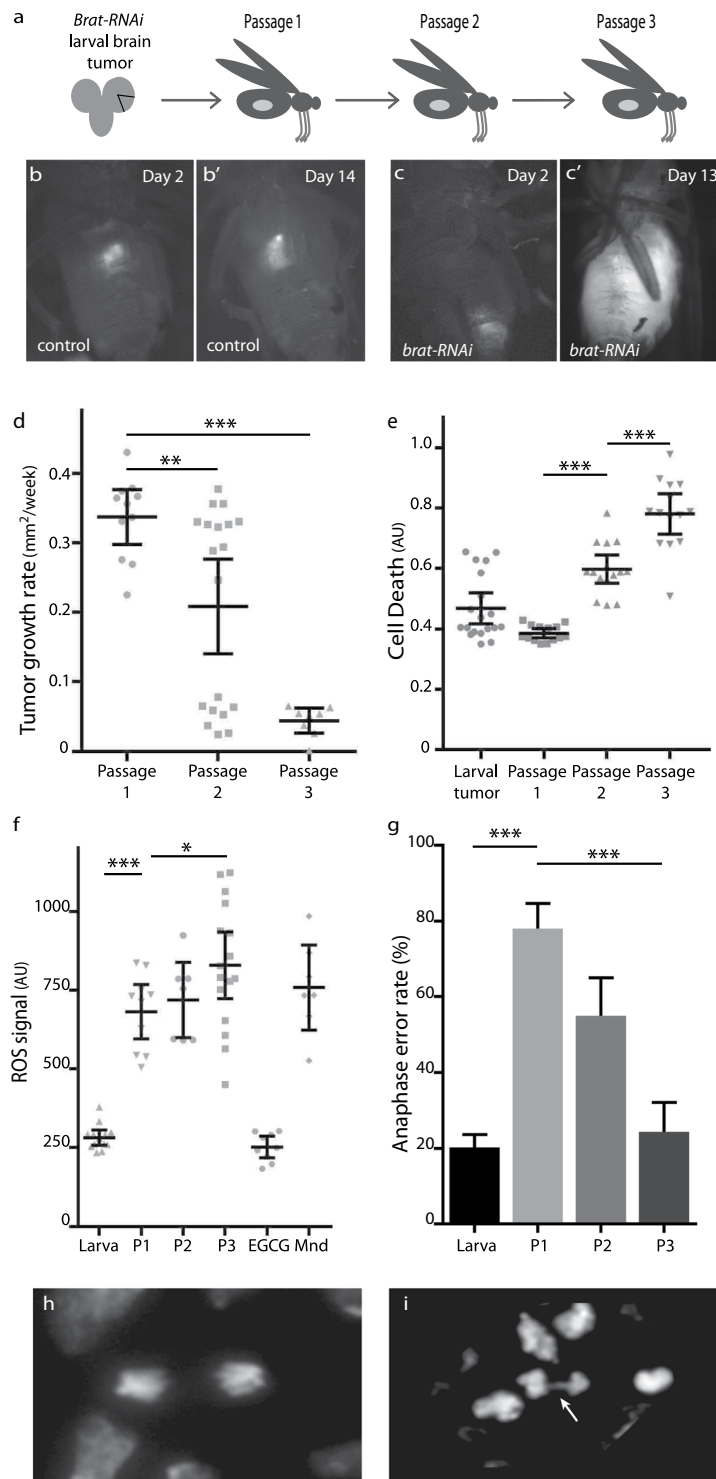
Figure 1. Characterization of *brat-RNAi* as a CIN tumor model. (**a**) RFP-labelled 3rd instar larval brain tissue depleted for Brat (*da > Gal4*; UAS-RFP; UAS-*brat*-RNAi) was dissected and transplanted into the abdomen of wild type adult hosts. Serial passages of the tumor explant were carried out into a new host within a fortnight to allow the tumor growth to be continued. (**b**) Control 3rd instar larval brain tissue (*da > Gal4*; UAS-RFP) was transplanted and showed no growth after 2 (**b**) or 14 days (b'). Labelled 3rd instar larval brain tissue explants depleted for Brat (*da > Gal4*; UAS-RFP; UAS-*brat*-RNAi) showed considerable growth on day 13 (**c**) compared to day 2 (**c**) after transplantation. (**d**) The average growth rate of *brat* tumor explants (*da > Gal4*; UAS-RFP; UAS-*brat*-RNAi). A significant decrease in the mean growth rate was observed in Passage 2 (p < 0.01, n = 19) and Passage 3 (p < 0.001, n = 8) relative to the first transplantation. (**e**) Cell death was measured in 3rd instar larval brains depleted for Brat (*da > Gal4*; UAS-RFP; UAS-*brat*-RNAi) and in serial passages of this tissue. The rate of cell death significantly increased in the second and third passage (p < 0.001, n > 13 for each), but was not significantly different from the larval tissue to the first explant (p > 0.06, n > 13). (**f**) The level of reactive

oxygen species (ROS), was measured in labelled 3rd instar larval brains depleted for Brat (*da* > *Gal4*; UAS-RFP; UAS-*brat*-RNAi), and compared with subsequent serial passages of the explanted tissue (P1 to P3) or with tissue treated with an antioxidant (EGCG) or pro-oxidant (Mnd; Menadione) as controls. A strong increase in ROS was observed in the first passage (p < 0.001, n = 10), with a modest increase from passage 1 to passage 3 (p < 0.05, n = 16). (**g**) The rate of chromosomal instability was measured in larval brains (*da* > *Gal4*; UAS-RFP; UAS-*brat*-RNAi) and subsequent passages of this explanted tissue. The proportion of visibly aberrant anaphase figures was significantly higher in the first passage (78 ± 3%, n = 141 compared to 20 ± 3%, n = 622, p < 0.001), but then decreased over the next two passages (p < 0.001, n > 100 for each). (**h,i**) Representative images of normal and defective anaphases from Brat depleted brain tissue (*da* > *Gal4*; UAS-RFP; UAS-*brat*-RNAi), quantitated in (**g**). The arrow indicates an anaphase bridge. In all graphs, error bars show the 95% confidence intervals. Variation in means were tested for significance using Dunnett's multiple comparisons test. Variation in proportions were tested for significance using Fisher's exact tests.

metabolic candidates in this model identified targets such as PEPCK that could effectively block tumour growth. Elevated levels of ROS were observed in the targeted tissue, and adding antioxidants could rescue growth. Experiments to identify the source of the ROS suggested that pressure to clear cytoplasmic NADH generated in glycolysis was leading to ROS generation by the glycerol phosphate shuttle. Our results suggest that metabolic interventions that constrain clearance of NADH can generate toxic ROS levels in CIN tumours without harming the host.

Results

Characterization of *brat^RNAi* as a CIN tumor model.

Mutations in the gene *brain tumour (brat)* cause tumours in *Drosophila* larval brains due to a failure of neuroblast differentiation[12], and this tissue can be grown indefinitely as explant tumors if serially transplanted into the abdomen of host adult flies[13] (Fig. 1a). We initially tested whether depletion of Brat by RNAi gave effective tumour growth comparable to mutant alleles. Dissection of third instar larval brains from control animals marked with RFP and transplanted in to a wild type adult host showed no growth (Fig. 1b), but depletion of Brat by RNAi resulted in strong growth of the RFP-tagged transplanted tissue (Fig. 1c) that would typically kill the host within two weeks. Serial passaging of the tumor tissue after ten days' growth allowed the development of the tumors to be followed. We observed a significant decrease in the rate of growth (Fig. 1d) and increased cell death (Fig. 1e) in these tumors over the first three passages. Levels of reactive oxygen species (ROS) were high in the explants, with a significant increase relative to the larval brain tumour and from the first to third passage (Fig. 1f). We also observed metastasis, which is a common feature of these explant tumors[14]. Measuring the frequency of aberrant anaphases showed that the CIN rate was 20.2% (±3.2%) in *brat^RNAi* larval brains (n = 15), comparable to our previous CIN model, *mad2^RNAi*, which showed defects in 24% of anaphases[3]. We further analysed the CIN rate in *brat^RNAi* tumor explants up to three passages and found the highest CIN rate in passage 1 which decreased over time in the subsequent passages, similar to the explant growth rate (Fig. 1g). These initial studies confirmed that depletion of *brat* by RNAi was a viable method for generating aggressive CIN tumors that shared the high-ROS phenotype that we and others identified when CIN was induced in normal proliferating tissue[7, 15, 16].

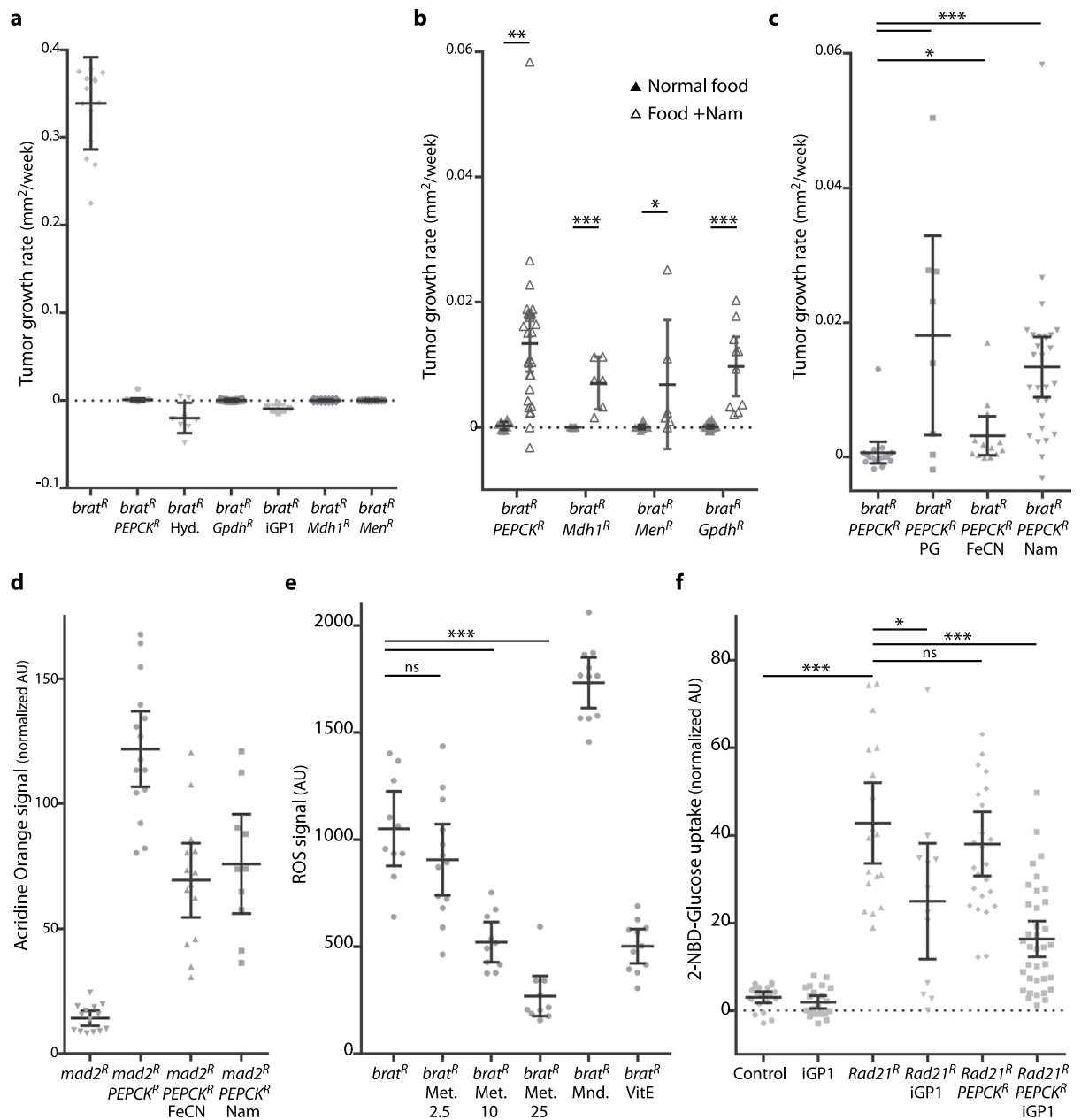### Effect of metabolic interventions on CIN tumors.

Having previously shown that cells with induced CIN are sensitive to several metabolic interventions[7], we now wished to test the effect of such knockdowns on CIN tumors. We tested a range of genes affecting glucose usage (G6PD, PEPCK, Wwox), lipid metabolism (Mfe2) and antioxidant responses (JNK). We initially measured their effect on the size of overgrowth observed in *brat^RNAi* larval brains (Fig. 2a). All of the candidate knockdowns tested gave significantly reduced overgrowth at this stage, with some no larger than non-tumorous controls (e.g. *brat^RNAi JNK^RNAi*). Reduced overgrowth could have been caused by less proliferation or more cell death; our data from wing discs suggested that cell death was likely to be occurring[7]. Surprisingly, we did not observe a significant increase in cell death relative to *brat* alone for any of the candidate knockdowns except Glucose-6-phosphate dehydrogenase (G6PD; Fig. 2b). Elevated CIN rates can also impact proliferation, so we tested whether the knockdown of the candidates was affecting the incidence of CIN in *brat* larval brains. Depletion of Jun N-terminal kinase (JNK) gave a significant increase in the CIN rate, while the others had little (*Mfe2*) or no effect (Fig. 2c). These data indicated that all of the candidates were able to impact the growth of *brat* larval brains, and suggested that varied mechanisms were responsible, with G6PD depletion giving cell death and JNK depletion causing more CIN. When these candidates were tested for their effect on ongoing tumor proliferation in explants, we were surprised to find that neither G6PD nor JNK depletion were able to effectively inhibit *brat* tumor growth (Fig. 2d). However, depletion of either Phosphoenolpyruvate Carboxykinase (PEPCK) or Multifunctional enzyme type 2 (Mfe2) did block the growth of explanted *brat* tumors (Figs 2d and 3).

The role of NADH in *brat* tumour growth inhibition by PEPCK depletion.

As an enzyme with a relatively well characterized function in glucose metabolism, PEPCK became the focus of further investigation aimed at explaining how CIN tumor growth can be blocked. We found that feeding the hosts an inhibitor to PEPCK (hydrazine; Hyd) was able to block *brat* explant growth, consistent with our depletion of PEPCK in the tumor by RNAi (Fig. 3). PEPCK catalyses the inter-conversion of oxaloacetate and phosphoenolpyruvate (Fig. 4a) and is rate limiting for gluco- and glyceroneogenesis[17, 18]. Proliferating cells must generate lipids and nucleotides, and to do so typically drive glycolysis at a high rate. This leads to a build-up of NADH in the cytoplasm which must be cleared for glycolysis to continue. The glycerol-3-phosphate shuttle is an important sink for NADH that could be significant for the growth of *brat* tumors[19]. PEPCK is required for glyceroneogenesis (Fig. 4a), which is
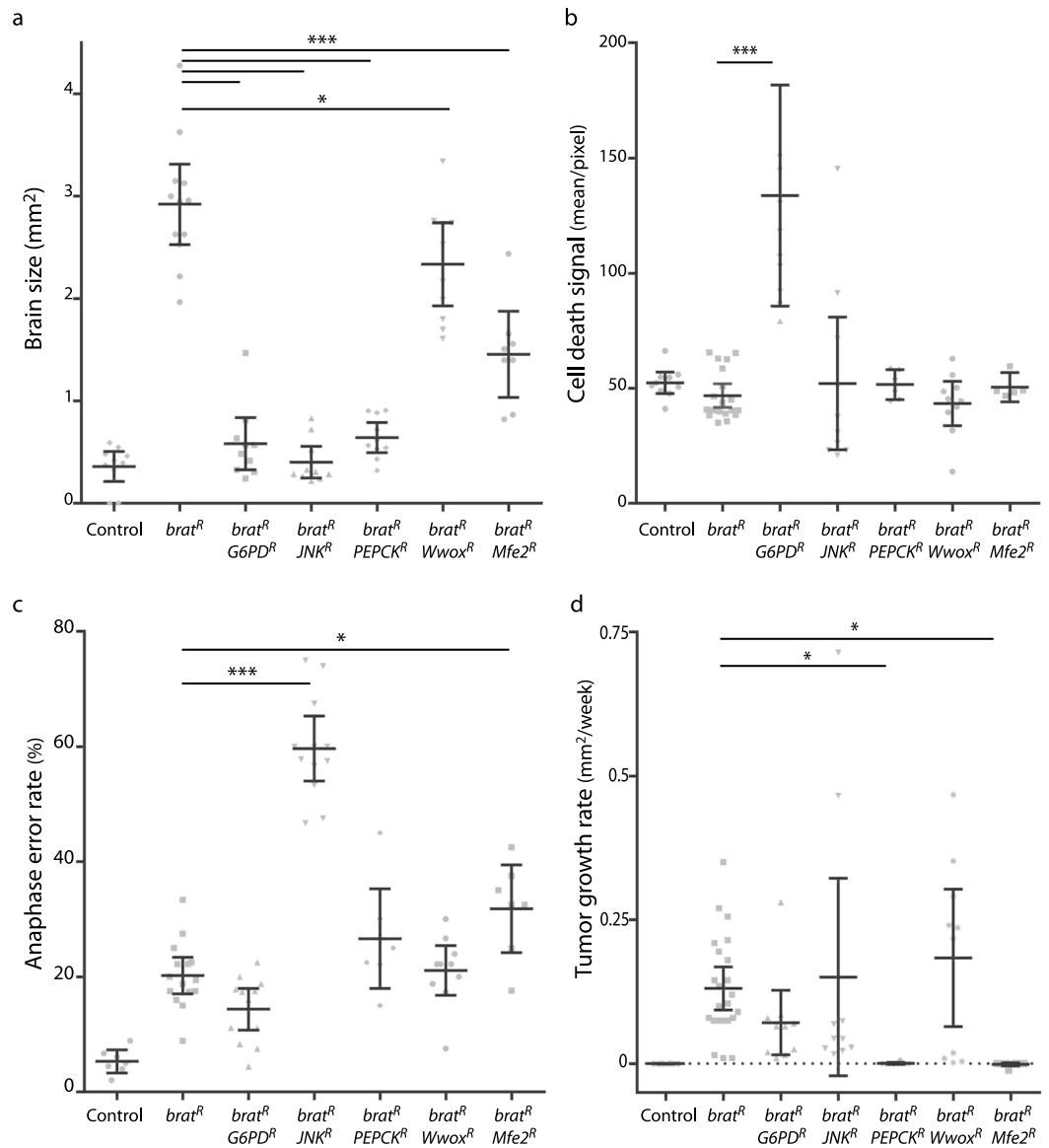
Figure 2. Identifying gene knockdowns that affect CIN tumor growth. (**a**). Comparison of the size of 3rd instar larval brains depleted for Brat (*da > Gal4*; UAS-RFP; UAS-*brat*-RNAi) or *brat* and one of five genes known to increase apoptosis in CIN cells. Control brains were *da > Gal4*; UAS-RFP. Error bars indicate 95% confidence intervals, n > 10 in all cases. All candidate gene knockdowns gave a significant reduction in larval brain size relative to *brat*-RNAi alone (p < 0.001 for all except *Wwox*-RNAi, p < 0.05). (**b**) Apoptosis in *brat*-RNAi larval brains was compared with *brat* plus candidate knockdowns. Depletion of G6PD showed significantly elevated apoptosis compared to the *brat* alone control (p < 0.001, n ≥ 10). All other comparisons with the control showed no significant variation (p > 0.05). (**c**) The rate of chromosomal instability was measured in larval brains depleted for Brat (*da > Gal4*; UAS-RFP; UAS-*brat*-RNAi) and compared with brains lacking Brat and a candidate. The proportion of aberrant anaphases was elevated relative to the *brat* control (20%, n = 622 anaphases) when JNK (56%, p < 0.001, n = 495) or Mfe2 (32%, p < 0.05, n = 257) were also depleted. All other comparisons with the *brat* control showed no significant variation (p > 0.05 using Fisher's exact test, n > 200 for each). (**d**) Growth rate of tumor explants. RFP labelled brain tissue from 3rd instar larvae was transplanted into a wild type adult host and growth was measured over two weeks. No growth was observed in control tissue (*da > Gal4*; UAS-RFP). Depletion of Brat led to rapid explant growth, which was not significantly affected by co-depleting G6PD, JNK or Wwox (p > 0.05, n ≥ 10). Co-depletion of PEPCK gave a strongly reduced growth rate relative to *brat* alone (p < 0.05, n = 10), as did co-depleting Mfe2 (p < 0.05, n = 10). All error bars show the 95% confidence interval, p values are from Dunnett's multiple comparisons test except for proportions, for which Fisher's exact test was used.

a significant contributor to the generation of glycerol-3-phosphate, even in the presence of glucose[20]. We tested the requirement for this shuttle by either depleting cytoplasmic Glycerol-3-Phosphate Dehydrogenase (Gpdh) in the tumour or by feeding the host a specific inhibitor (iGP1) of GPO1, the mitochondrial Glycerophosphate

Figure 3. Effect of metabolic intervention on the growth of CIN tumors. (**a**) Labelled 3rd instar brain tissue depleted for Brat (*actin > Gal4*; UAS-RFP; UAS-*brat*-RNAi) was grown in wild type hosts for up to two weeks. Feeding the host with the PEPCK inhibitor Hydrazine (Hyd.) blocked tumor growth, as did depleting PEPCK by RNAi. Co-depletion of the cytoplasmic Glycerol-3-Phosphate Dehydrogenase (Gpdh), Malate dehydrogenase (Mdh1) or Malic Enzyme (Men) with Brat was able to block growth, as did feeding the host iGP1 to inhibit the mitochondrial Glycerophosphate Oxidase. All treatments gave a statistically significant decrease in growth relative to the *brat* control (p < 0.001 for each, n ≥ 5) (**b**) The effect of supplementing food with nicotinamide (Nam) was tested on host adults carrying explanted tumors with the indicated genotypes. All genotypes showed significantly increased growth when given nicotinamide (multiple t-tests using the Holm-Sidak method, *p < 0.05, **p < 0.01, ***p < 0.001). (**c**) Explants depleted for Brat and PEPCK (*actin > Gal4*; UAS-RFP; UAS-*brat*-RNAi; UAS-*PEPCK*-RNAi) did not grow, but could be induced to grow by feeding their adult hosts the antioxidant propyl gallate (PG). Increasing the NAD+/NADH ratio by feeding the hosts ferricyanide (FeCN) or nicotinamide (Nam) could also rescue the explant growth. Significant increases over the *brat PEPCK* control (n = 18) were seen for PG and Nam (p < 0.001, n = 8 and 28), with a modest increase seen for FeCN (p < 0.05 by one-tailed t-test, n = 13). (**d**) The effect of NADH on cell death seen in non-tumorous CIN cells depleted for PEPCK. Cells in the posterior half of 3rd instar larval wing discs depleted for mad2 and PEPCK (*en > Gal4*; UAS-CD8-GFP; UAS-*mad2*-RNAi; UAS-*PEPCK*-RNAi) show a high rate of cell death, as measured by Acridine Orange incorporation[7], relative to the wild type cells in the anterior half of each disc. The mad2 alone control (en > Gal4; UAS-CD8-GFP; UAS-*mad2*-RNAi; UAS-*LacZ*-RNAi) shows little signal. Increasing the NAD+/NADH ratio by feeding with either ferricyanide (FeCN) or nicotinamide (Nam) significantly decreased the level of cell death (p < 0.001 for both, n ≥ 10). (**e**) The effect of inhibiting the

glycerol-3-phosphate shuttle on reactive oxygen species produced in Brat depleted 3rd instar larval brain tissue (*actin* > *Gal4*; UAS-RFP; UAS-*brat*-RNAi). Adding Metformin at 10 mM or 25 mM significantly decreased the level of ROS observed in *brat* tissue (***p < 0.001, n ≥ 10; ns: p > 0.05). Menadione (Mnd.) and vitamin E (VitE) were used as control pro- and anti-oxidant treatments respectively. (**f**) The uptake of labelled glucose in CIN cells was decreased by blocking both PEPCK and the glycerol-3-phosphate shuttle. The posterior halves of 3rd instar larval wing discs were depleted for Rad21 (*en* > Gal4; UAS-CD8-GFP; UAS-*Rad21*-RNAi; UAS-Dicer2) to induce CIN. This strongly increased the uptake of fluorescently labelled 2-NBD-Glucose. Depletion of PEPCK in CIN cells did not significantly decrease the rate of glucose uptake (p > 0.05, n = 25) relative to CIN alone (n = 18). Blocking the glycerol-3-phosphate shuttle by inhibiting GPO1 with iGP1 caused some decrease (p < 0.05, n = 12), while adding iGP to wings depleted for PEPCK strongly decreased the rate of glucose uptake (p < 0.001, n = 36). In all graphs, error bars show the 95% confidence interval. P values given are from Dunnett's multiple comparisons tests unless otherwise noted.

Oxidase[19]. In either case, the growth of *brat* explants was blocked (Fig. 3a). We were not able to block growth by inhibiting hexokinase or uncoupling mitochondrial respiration (data not shown). The inhibition of tumor growth by blocking the glycerol phosphate shuttle suggested that these tumors may be sensitive to cytoplasmic NADH build-up.

Replacing NAD+ can rescue *PEPCK brat* explant growth.   This model for the role of NADH in mediating the effect of PEPCK on *brat* tumor explants would predict that providing the tumor with NAD+ should bypass the need for PEPCK, allowing glycolysis to proceed and *PEPCK brat* explants to grow. We tested this model by feeding the host adult nicotinamide (Nam), a precursor in the synthesis of NAD+[21]. While *brat* tumors depleted for PEPCK did not grow, we found that the same tumors in hosts fed nicotinamide were able to grow, albeit slowly (Fig. 3b). This strongly suggested that lack of NAD+ was a limiting factor when PEPCK was depleted. We also found that nicotinamide rescued the growth of *brat* tumors lacking Gpdh (Fig. 3b), confirming the importance of the glycerol-3-phosphate shuttle in oxidising NADH in these tumors. Ideally we would have confirmed this by measuring cytoplasmic NAD+/NADH ratios in the explants, but the low NADH concentration combined with tiny tissue size made this technically problematic. As an alternative approach, we confirmed that cytoplasmic NADH was growth limiting in PEPCK depleted tumors by feeding the hosts ferricyanide (FeCN)[22] which externally drives NADH oxidation, and this was also able to increase growth (Fig. 3c). To test whether this rescue of *PEPCK* phenotypes by NADH oxidation was a feature only of *brat* tumors, we tested non-tumorous proliferating cells in which CIN had been induced by Mad2 depletion. The level of cell death and ROS induced by PEPCK depletion in this CIN tissue was significantly rescued by feeding the larvae either ferricyanide or nicotinamide (Figs 3d and 5). Proliferating wing disc tissue in which CIN was induced by BubR1 depletion showed similar sensitivity to PEPCK depletion (Supplementary Figure 2).

An important side-effect of using the glycerol-3-phosphate shuttle is the production of ROS by mitochondrial Glycerophosphate Oxidase (GPO1)[23, 24]. Given that we observed high levels of ROS in *brat* tumor explants (Fig. 1f), we wished to test whether ROS levels were limiting the usefulness of the glycerol-3-phosphate shuttle when PEPCK was depleted. When adult hosts were fed with an antioxidant (propyl gallate; PG), we observed a significant rescue of the growth of PEPCK depleted *brat* tumor explants (Fig. 3c). We tested whether the antioxidant rescue seen with propyl gallate was affecting ROS generated by the glycerol phosphate shuttle, by feeding Brat depleted larvae metformin, which inhibits mitochondrial Glycerol-3-phosphate Dehydrogenase/GPO1[25] (Fig. 3e). Increasing levels of metformin gave decreasing levels of ROS, consistent with the ROS in *brat*-RNAi brain tissue being generated by the glycerol-3-phosphate shuttle. Similarly, adding a specific inhibitor to the glycerol phosphate shuttle in cells depleted for PEPCK and Mad2 also lowered ROS levels (Fig. 5f).

CIN tissues can have an elevated metabolic rate, consuming energy stores faster and producing more mitochondrial output than normal proliferating cells[7]. This was reflected in an increased rate of uptake of labelled glucose in proliferating CIN tissue (*Rad21*-RNAi) relative to controls (Fig. 3f). Feeding the animals a Gpo1 inhibitor to block the glycerol-3-phosphate shuttle significantly decreased the uptake of glucose (Fig. 3f), consistent with this shuttle being an important sink for NADH that allows glycolysis to continue at a high rate. Depletion of PEPCK did not strongly block glucose uptake (Supplementary Figure 3), however in *brat* tumors, depletion of PEPCK had a stronger effect (Fig. 4b), and could be rescued by feeding ferricyanide, confirming the importance of NADH oxidation for continued glycolysis in these tumors.

Discussion

CIN induced in *Drosophila* wing discs has reproducible phenotypic effects[3–5, 15, 26], even though the genetic instability is effectively random. CIN cells typically generate reactive oxygen species, activate the p38 and JNK/Upd pathways, drop out of the epithelial layer and trigger an innate immune response. Because of this stereotypical response to induced aneuploidy, we reasoned that there should be ways to effectively target the response that should be specific to CIN cells. By screening for such CIN-killing interventions, we found several, such as targeting JNK or centrosomes, that were effective but not ideal due to their important roles in normal proliferating cells[3]. Metabolic intervention, on the other hand, has better potential as a therapeutic tool because normal cells can tolerate large changes in metabolite concentrations, which they experience during feeding and fasting. On that basis we wished to further investigate the effect of metabolic intervention not just on epithelial cells with induced CIN, but on a CIN tumour growing *in vivo*.

Explanted *Drosophila* brain tumours have been used for over a decade[13] and allow the development of a tumour to be followed for months by regular passaging. These tumours have been shown to accumulate additional
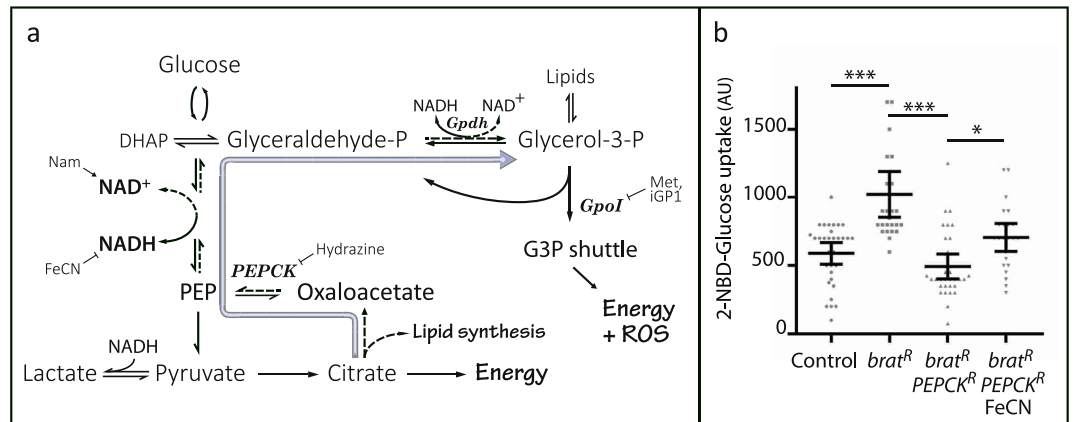
Figure 4. Model for the effect of PEPCK and NADH on glucose metabolism. (**a**) Proliferating cells require glucose for processes including the synthesis of nucleotides (via the pentose-phosphate pathway), membrane lipids (via pyruvate and citrate) and energy (via pyruvate or the glycerol-3-phosphate shuttle). Cytoplasmic NADH is produced in making phosphoenolpyruvate, which must be oxidised for glycolysis to continue. NADH can be oxidised by making lactate, but this prevents the use of pyruvate for lipid synthesis or energy. The glycerol-3-phosphate shuttle can oxidise NADH, but does so at the cost of generating elevated ROS levels. PEPCK mediates glyceroneogenesis (broad arrow), which uses citrate exported from mitochondria to generate glycerol-3-phosphate while oxidising NADH. Our model is that depletion of PEPCK decreases the levels of cytoplasmic NAD+, which inhibits glycolysis unless the NAD+ can be regenerated by the glycerol-3-phosphate shuttle. This shuttle generates reactive oxygen species, so its use is limited in CIN cells which are already redox stressed. The inhibitors used to test this model are shown with their targets. Abbreviations: DHAP: dihydroxyacetone phosphate, Gpdh: Glycerol-3-phosphate Dehydrogenase, Gpo1: mitochondrial Glycerol-3-Phosphate Dehydrogenase/Glycero-phosphate oxidase, iGP1: inhibitor of Glycerophosphate oxidase 1, Met: Metformin, Nam: nicotinamide, FeCN: ferricyanide, PEPCK: Phosphoenolpyruvate Carboxykinase. (**b**) The effect of PEPCK depletion on glucose uptake in *brat* depleted brain tissue. The uptake of fluorescently labelled 2-NBD-glucose in 3rd instar larval brains was increased by the depletion of Brat (*actin > Gal4*; UAS-RFP; UAS-*brat*-RNAi; $p < 0.001$, $n \geq 25$). Depleting PEPCK in these *brat* tumors (*actin > Gal4*; UAS-RFP; UAS-*brat*-RNAi; UAS-*PEPCK*-RNAi) led to significantly lower uptake of labelled glucose ($p < 0.001$, $n \geq 25$), which was rescued by feeding the larvae with ferricyanide (FeCN, $p < 0.05$, $n \geq 23$). Error bars show 95% confidence intervals. Comparisons were done by multiple t-tests using Tukey's method.

centrosomes and become aneuploid, so we were not surprised to find that the CIN rate in transplanted *brat* tissue was relatively high. We also observed an elevated level of reactive oxygen species, consistent with previous findings from *Drosophila* cells with induced CIN[7, 15] and from stable aneuploidy in a range of organisms[27]. Of course, *brat* tumours have other changes in addition to CIN[28], but the phenotype of the *brat* tumours was sufficiently similar to our induced-CIN model to encourage us to examine the best metabolic candidates we had identified in the wing disc CIN model. Surprisingly, depleting proteins like G6PD or JNK had little effect on tumour explant growth (Fig. 2d), though they both strongly induced cell death in our wing disc model[3]. We considered whether the number of unstable divisions during the extended proliferation period available to the explants might have generated sufficient variation to allow the appearance of mutant clones resistant to the effect of our knockdowns. We think this unlikely, as other similar interventions, such as *PEPCK*-RNAi never acquired resistance and for *G6PD* or *JNK* we did not see a period of limited growth followed by rapid expansion, which would be expected if tumours were acquiring resistance. Instead we saw immediate growth upon transplantation, suggesting that in this environment, those knockdowns were not able to effectively block cell proliferation. Prior to transplantation, knockdown of *G6PD* did give reduced brain size and increased ROS and cell death in *brat* larval brains, suggesting that some feature of the transplantation environment was able to rescue their growth. Further investigation will be needed to account for this growth difference.

Following our initial tests, we focused our attention on *PEPCK*, which consistently blocked the growth of explanted tumours. PEPCK is best known for its role in the liver, where it mediates gluconeogenesis during fasting[29]. However, PEPCK is expressed widely in non-gluconeogenic tissue in most organisms, notably in muscle and gut as well as cancer cells, where its role is more complex, facilitating the catabolism of either glucose or glutamine[30]. PEPCK is regulated transcriptionally by p38 via ATF-2[31] and post-translationally by acetylation[32]. PEPCK is rate limiting for glyceroneogenesis, the synthesis of glycerol-3-phosphate from TCA cycle intermediates[33]. This is particularly important for the re-esterification of free fatty acids, which is needed to prevent the depletion of fats that we and others have noted when PEPCK is removed[7, 34]. Cell proliferation requires the doubling of membrane lipids at each division, so this demand for lipid synthesis could be an important role for PEPCK in non-gluconeogenic tissues. However, *PEPCK* null mutants and RNAi knockdowns remain viable[3, 34], so this is insufficient to explain why *PEPCK* depleted *brat* explants cannot grow and why *PEPCK* depleted CIN wing disc cells apoptose. A possible explanation is suggested by our observation that providing antioxidants or increasing NAD+ could rescue the growth of *PEPCK* depleted *brat* explants. Our data suggest that these interventions
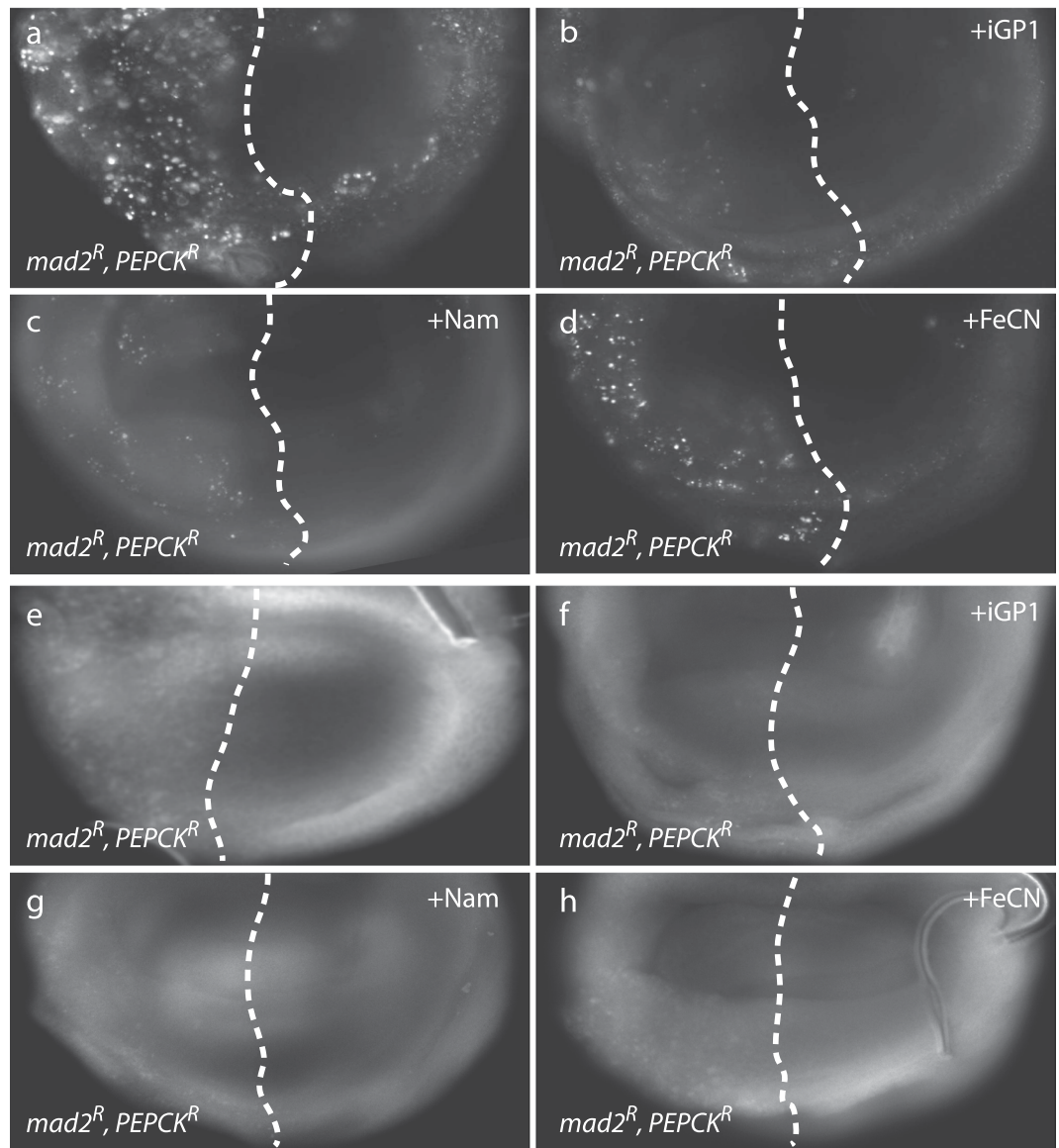
Figure 5. The effect of cytoplasmic NADH levels on the survival and metabolism of CIN cells depleted for PEPCK. (**a**–**d**) Cell death caused by depletion of Mad2 and PEPCK could be rescued by feeding larvae chemicals to alter cytoplasmic NADH levels. (**a**) Cells in the posterior half of 3rd instar larval wing discs (left of the dotted lines) were depleted for Mad2 and PEPCK (*en* > Gal4; UAS-CD8-GFP; UAS-*mad2*-RNAi; UAS-*PEPCK*-RNAi) and showed an elevated level of cell death, as visualized by Acridine Orange[7], relative to the wild type cells in the anterior half of each disc. Increasing the cytoplasmic NAD+/NADH ratio by feeding with either ferricyanide (**d**, FeCN) or nicotinamide (**c**, Nam) significantly decreased the level of Acridine staining, as did blocking the glycerol phosphate shuttle by feeding iGP1 (**b**). Quantitation of the rescue is shown in Fig. 3d. (**e**–**h**) ROS caused by depletion of Mad2 and PEPCK could also be rescued by changing NADH availability. (**e**) Cells in the posterior half of 3rd instar larval wing discs (left of the dotted lines) were depleted for Mad2 and PEPCK (*en* > Gal4; UAS-CD8-GFP; UAS-*mad2*-RNAi; UAS-*PEPCK*-RNAi) and showed an elevated level of ROS, as visualized by CellRox, relative to the wild type cells in the anterior half of each disc. Increasing the cytoplasmic NAD+/NADH ratio by feeding with either 5 mM ferricyanide (h, FeCN) or 5 mM nicotinamide (g, Nam) decreased the level of CellRox staining, as did blocking the glycerol phosphate shuttle by feeding with 2 μM iGP1 (**f**).

affected the glycerol phosphate shuttle, which may be an important sink for the NADH generated during glycolysis, but which also generates ROS[23].

NADH is made from NAD+ in the cytoplasm during glycolysis, and must be oxidised for glycolysis to continue. Pyruvate can be converted to lactate to regenerate NAD+, however this option is limited in proliferative cells as pyruvate is in demand for energy and lipid synthesis (Fig. 4a). In some organisms the Malate/Aspartate shuttle is used to lower levels of cytoplasmic NADH, however in *Drosophila*, the Mdh1 and Got1 enzymes that would catalyse the cytoplasmic half of the shuttle are found in peroxisomes[35]. Nonetheless, our data suggest Mdh1

is still needed to regulate NADH in CIN tumors (Fig. 3a,b). An effective alternative for oxidising cytoplasmic NADH is via the glycerol-phosphate shuttle. This shuttle uses Glycerol Phosphate Dehydrogenase to divert from glycolysis as much glyceraldehyde phosphate as required to oxidise NADH. This makes glycerol-3-phosphate, which can be removed by dephosphorylation to glycerol, or by acylation to make fats, however the shuttle uses mitochondrial Glycerophosphate Oxidase to generate energy in the mitochondria while recovering the glyceraldehyde phosphate for glycolysis. This pathway is heavily used in muscle, but it can also generate significant levels of ROS[23]. We found that the shuttle is important in proliferating CIN cells, as inhibiting or depleting Glycerophosphate Oxidase caused a significant decrease in glucose uptake, and blocked the growth of *brat* explants.

We found that increasing the availability of NAD+ by feeding the host nicotinamide rescued the growth of PEPCK depleted *brat* explants, suggesting that in these tumours, the glycerol phosphate shuttle has not been able to sufficiently oxidise NADH, despite the availability of glucose to fuel this pathway. One possible contributor to this limitation was the ROS generated by mitochondrial Glycerophosphate Oxidase (GPO1)[23], because we know that cells with CIN are already redox stressed[7]. Consistent with this model (Fig. 4a), we found that inhibiting Glycerophosphate Oxidase lowered ROS levels but did not allow growth of *brat* explants (as the shuttle was then unavailable for NADH oxidation). However, lowering ROS by feeding the host antioxidants was able to rescue growth: in this case the shuttle is available without the toxic consequences of high ROS. Lowering endogenous antioxidants by depleting Malic enzyme (hence NADPH)[36] was able to block the growth of *brat* explants. This effect of ROS levels on PEPCK depleted explants suggests that the glycerol-3-phophate formed in glyceroneogenesis is not just used for lipids, but is also used to clear cytoplasmic NADH via the mitochondria. It is still poorly understood how PEPCK mediates retrograde carbon flow in the presence of glucose, but high rates of PEPCK-mediated glyceroneogenesis are observed in numerous cell types and diets, including cancer cells[18, 20, 37, 38].

Our model, then, for effectively blocking the growth of *brat* tumours is that treatments that decrease the use of the glycerol-3-phosphate shuttle (*PEPCK*-RNAi, hydrazine, *GPDH*-RNAi, iGP1) are inhibiting growth by preventing the oxidation of cytoplasmic NADH. If the shuttle is available, its activity is significantly limited by the production of ROS in redox stressed CIN cells, so treatments that lower ROS levels or that provide an alternative source of NAD+, will tend to rescue tumour growth. By using the appropriate level of PEPCK and GPO1 inhibitors we could completely block the growth of these aggressive CIN tumours.

These results emphasize the benefit of starting with an unbiased genetic approach to identifying the key sensitivities of CIN cells. We would not have predicted that PEPCK had a significant role in managing NADH, nor that this would be enough to block tumour growth *in vivo*. It is not surprising that ROS levels can limit tumour growth, as they provide a mutagenic advantage to tumours only at the cost of widespread cellular damage. We had not previously considered mitochondrial Glycerophosphate Oxidase as a significant source of ROS, however we find that in *PEPCK brat* tumors, the glycerol-3-phosphate shuttle generates growth limiting ROS levels. Blocking this pathway lowers ROS levels, but it also prevents PEPCK-inhibited tumours from oxidising NADH, which then becomes growth limiting. This trade-off between NADH and ROS suggests that the relationship between the glycerol-phosphate shuttle and NADH sources may be a fruitful area of research for combination therapy directed at CIN tumors.

Experimental procedures. *Oxidative stress analysis.* Oxidative damage was analysed in tumor tissue using CellRox Green (Invitrogen) according to the manufacturers' recommendations. Menadione (10 mM), and Vitamin E (5 mM) in D22 media were used as a pro-oxidant positive control and anti-oxidant control. Another anti-oxidant epigallocatechin gallate-EGCG (10 μM) was used in some experiments. Tissue was dissected in D22 media then stained in the ROS dye for 20 minutes. Photographs were taken on a Zeiss Axioplan 2 using a fixed exposure time for each experiment determined by the positive control (Menadione) treated tissue.

Passaged *brat*^{RNAi} tissue was dissected and incubated for ROS staining in fly extract media. Fly extract was prepared using Schneider's *Drosophila* media (0.93 ml), whole fly extract of 200 fly (50 μl), insulin 0.5 mg/1 ml (5 μl), penicillin/streptomycin 10,000U/ml (5 μl)[39].

Glucose assay. A labelled fluorescent glucose, 2-NBDG (Sigma) was used to detect the uptake of glucose as recommended by the supplier. Wing discs were taken from 3rd instar larvae that were still feeding to ensure glycolytic metabolism and immediately incubated in 2 μM 2-NBDG for 1.5 h in wing disc culture media[39] before imaging. Images were taken at 20X with a fixed exposure time for all genotypes and fluorescent intensity was measured by using ImageJ software.

Explant and measurement techniques. An injection system was developed using 1.0mm O.D × 0.78mm I.D. borosilicate glass capillaries, a capillary holder, a suction tube and a suction apparatus. All the explants were done under a dissection microscope, into the ventral side of the fly abdomen[13, 40]. RFP was used to detect the explant presence and its growth. All explants were photographed at 3X under a fluorescence dissecting microscope (Nikon SMZ1500). The number of hosts that survived the transplant and hence contributed to the results for each experiment is shown by the number of points on its respective graph.

CIN analysis. CIN was analysed by measuring the rate of anaphase errors. Whole larval brains were fixed using 3.7% formaldehyde in PBS for 20 minutes, put in 45% and 60% acetic acid in ddH$_2$O for 2 minutes and 45 seconds respectively, then squashed onto a cover slip and frozen in liquid nitrogen. Hoechst 33342 (Sigma) at 2 μg/ml in PBS was used to stain chromosomes. 50 anaphases were identified per brain and anaphase aberrations were scored. CIN analysis for passaged tissue was treated similarly, except all the available anaphases of each explant were scored, due to the smaller amount of tissue.

Cell death analysis. Brain: whole brains were incubated in CellEvent Caspase 3/7 Green (Thermo Fisher) according to the recommended protocol. After the treatment, brains were mounted and photographed at 10X on a Zeiss Axioplan 2. GFP signals were counted using the Analyse Particle plugin in ImageJ. Passaged tissues were treated in a similar way, except the signal was manually counted using the Cell Counter plugin.

Wing discs: wing discs were treated with 1 mM Acridine Orange/PBS stain for three minutes, then briefly washed. The discs were mounted in PBS and photographed at 10X on a Zeiss Axoplan 2. The intensity of RFP signal was measured in ImageJ and normalized relative to the control half of the disc as described[3]. For ROS analysis of wing discs we used CellRox Deep Red (Invitrogen) as described [7].

Drug treatments. Drugs were obtained from Sigma except where noted. For adult fly feeding, drugs were mixed in 20% sucrose solution. For larvae, drugs were mixed in standard fly food (water, molasses, yeast, glucose, acid-mix, agar, semolina, Tegosept) and were given to the host fly when solidified. Drugs used were as follows unless otherwise noted in figure legends: iGP1 (Vitas-M lab, 1 mM), hydrazine sulphate-HS (10 mM), Metformin-Met (25 mM), Ferricyanide-FeCN (0.05 mM), Nicotinamide-Nam (5 mM), Propyl gallate-PG (1 mM). Because hydrazine and metformin are relatively non-specific in their targets, they were only used in cases where we could verify the relevance of the phenotype by comparison with the RNAi phenotype of the relevant enzyme (PEPCK or GPO1).

Drosophila stocks. Fly stocks were from either the Vienna *Drosophila* Resource Centre or Bloomington Stock Centre, raised at 25 °C, and all treatments were done at room temperature. Gal4 drivers included *daughterless*-gal4 and *actin*-gal4 for brain expression and *engrailed*-gal4 for wing expression of UAS transgenes. UAS-*RFP* was used as a reporter gene in *actin*-Gal4 and *da*-Gal4 driven UAS-*brat*^*RNAi* tumours. Canton S female flies were used as hosts for the CIN tumor explants. Stocks used were as follows; UAS-*brat*^*RNAi* (34646), UAS-RFP (27391), UAS-*Mfe2*^*RNAi* (v108880), UAS-*PEPCK*^*RNAi* (v20529), UAS-*G6PD*^*RNAi* (v101507), UAS-*JNK*^*RNAi* (v34138), UAS-*Wwox*^*RNAi* (v108307), UAS-*Men*^*RNAi* (41652), UAS-*Gpdh*^*RNAi* (v29013/GD), UAS-*Mdh1*^*RNAi* (v41437/GD), UAS-*mad2*^*RNAi* (v47918), UAS-*rad21*^*RNAi* (v13669), UAS-*dicer2*. Standard crosses were used to generate the genotypes tested, using segregation away from *Bl/CyO;TM2/TM6b* to combine markers on 2nd and 3rd chromosomes. Where recombination was necessary (*en > Gal4 UAS-mad2*), the stock was tested for each locus and taken through single pair matings to ensure a consistent genotype. The level of depletion by RNAi shown in Supplementary Table 1 was measured by qPCR as described (7) using the following primers:

Pepck: (f) CCGTGTGCTGGAATGGATC (r) TTGGGCAGCGAGAAGATCT;
brat: (f) AACCACAACAACTTCAACCTGAC (r) GCGATATATGTAGAGCCGATAGTC;
Gpdh: (f) TCACGACGTGTTACGGTGG (r) CCTCAATGGTTTTTCCAGAAGT;
rp49 control (f) GACGCTTCAAGGGACAGTATCTG (r) AAACGCGGTTCTGCATGAG.

Statistical analysis. In most cases, Dunnett's multiple comparisons test was used to assess the significance of variation in the means of each genotype of test tumour from that observed in the control. A single-tailed t-test with Welch's correction was also used for testing growth relative to a non-growing control. This was only used in one case of doubtful significance (the effect of FeCN on *brat PEPCK* tumor growth), which was also confirmed by other tests (e.g. cell death rescue and the effect of nicotinamide). Fisher's exact tests were used to compare proportions (CIN rates). In all graphs, error bars show 95% confidence intervals.

Data availability. All data generated or analysed during this study are included in this published article (and its Supplementary Information files).

References

1. Nicholson, J. M. & Cimini, D. Link between aneuploidy and chromosome instability. *Int. Rev. Cell Mol. Biol.* **315**, 299–317 (2015).
2. de Bruin, E. C. *et al.* Spatial and temporal diversity in genomic instability processes defines lung cancer evolution. *Science* **346**, 251–256 (2014).
3. Shaukat, Z., Wong, H. W., Nicolson, S., Saint, R. B. & Gregory, S. L. A screen for selective killing of cells with chromosomal instability induced by a spindle checkpoint defect. *PLoS One* **7**, e47447 (2012).
4. Liu, D., Shaukat, Z., Hussain, R., Khan, M. & Gregory, L. S. Drosophila as a model for chromosomal instability. *AIMS Genet.* **2**, 1–12 (2014).
5. Liu, D., Shaukat, Z., Saint, R. B. & Gregory, S. L. Chromosomal instability triggers cell death via local signalling through the innate immune receptor Toll. *Oncotarget* **6**, 38552–38565 (2015).
6. Janssen, A., Kops, G. J. & Medema, R. H. Targeting the mitotic checkpoint to kill tumor cells. *Horm Cancer* **2**, 113–116 (2011).
7. Shaukat, Z. *et al.* Chromosomal instability causes sensitivity to metabolic stress. *Oncogene* **34**, 4044–55 (2014).
8. Wong, H. W.-S., Shaukat, Z., Wang, J., Saint, R. & Gregory, S. L. JNK signaling is needed to tolerate chromosomal instability. *Cell Cycle* **13**, 622–31 (2014).
9. Cairns, R., Harris, I. S. & Mak, T. W. Regulation of cancer cell metabolism. *Nat. Rev. Cancer* **11**, 85–95 (2011).
10. Sheltzer, J. M., Torres, E. M., Dunham, M. J. & Amon, A. Transcriptional consequences of aneuploidy. *Proc Natl Acad Sci USA* **109**, 12644–12649 (2012).
11. Dephoure, N. *et al.* Quantitative proteomic analysis reveals posttranslational responses to aneuploidy in yeast. *Elife* **3**, e03023 (2014).
12. Bowman, S. K. *et al.* The tumor suppressors Brat and Numb regulate transit-amplifying neuroblast lineages in Drosophila. *Dev. Cell* **14**, 535–46 (2008).
13. Caussinus, E. & Gonzalez, C. Induction of tumor growth by altered stem-cell asymmetric division in Drosophila melanogaster. *Nat Genet* **37**, 1125–1129 (2005).
14. Beaucher, M., Hersperger, E., Page-McCaw, A. & Shearn, A. Metastatic ability of Drosophila tumors depends on MMP activity. *Dev Biol* **303**, 625–634 (2007).
15. Clemente-Ruiz, M. *et al.* Gene Dosage Imbalance Contributes to Chromosomal Instability-Induced Tumorigenesis. *Dev. Cell* **36**, 290–302 (2016).
16. Shaukat, Z., Liu, D., Hussain, R., Khan, M. & Gregory, S. L. The Role of JNK Signaling in Responses to Oxidative DNA damage. *Curr. Drug Targets* **17**, 154–63 (2015).

17. Wagner, A. E. *et al*. Epigallocatechin gallate affects glucose metabolism and increases fitness and lifespan in Drosophila melanogaster. *Oncotarget* **6**, 30568–30578 (2015).
18. Li, Y. *et al*. Upregulation of cytosolic phosphoenolpyruvate carboxykinase is a critical metabolic event in melanoma cells that repopulate tumors. *Cancer Res.* **75**, 1191–6 (2015).
19. Orr, A. L. *et al*. Novel inhibitors of mitochondrial sn-glycerol 3-phosphate dehydrogenase. *PLoS One* **9**, e89938 (2014).
20. Nye, C. K., Hanson, R. W. & Kalhan, S. C. Glyceroneogenesis Is the Dominant Pathway for Triglyceride Glycerol Synthesis *in Vivo* in the Rat. *J. Biol. Chem.* **283**, 27565–27574 (2008).
21. Kirkland, J. B. Niacin requirements for genomic stability. *Mutat. Res.* **733**, 14–20 (2012).
22. Baker, M. A., Lane, D. J. R., Ly, J. D., De Pinto, V. & Lawen, A. VDAC1 is a transplasma membrane NADH-ferricyanide reductase. *J. Biol. Chem.* **279**, 4811–9 (2004).
23. Miwa, S., St-Pierre, J., Partridge, L. & Brand, M. D. Superoxide and hydrogen peroxide production by Drosophila mitochondria. *Free Radic. Biol. Med.* **35**, 938–948 (2003).
24. Orr, A. L., Quinlan, C. L., Perevoshchikova, I. V. & Brand, M. D. A refined analysis of superoxide production by mitochondrial sn-glycerol 3-phosphate dehydrogenase. *J. Biol. Chem.* **287**, 42921–35 (2012).
25. Madiraju, A. K. *et al*. Metformin suppresses gluconeogenesis by inhibiting mitochondrial glycerophosphate dehydrogenase. *Nature* **510**, 542–6 (2014).
26. Dekanty, A., Barrio, L., Muzzopappa, M., Auer, H. & Milán, M. Aneuploidy-induced delaminating cells drive tumorigenesis in Drosophila epithelia. *Proc. Natl. Acad. Sci. USA.* **109**, 20549–54 (2012).
27. Pfau, S. J. & Amon, A. Chromosomal instability and aneuploidy in cancer: from yeast to man. *EMBO Rep* **13**, 515–527 (2012).
28. Jüschke, C. *et al*. Transcriptome and proteome quantification of a tumor model provides novel insights into post-transcriptional gene regulation. *Genome Biol.* **14**, r133 (2013).
29. Burgess, S. C. *et al*. Impaired tricarboxylic acid cycle activity in mouse livers lacking cytosolic phosphoenolpyruvate carboxykinase. *J. Biol. Chem.* **279**, 48941–9 (2004).
30. Montal, E. D. *et al*. PEPCK Coordinates the Regulation of Central Carbon Metabolism to Promote Cancer Cell Growth. *Mol. Cell* **60**, 571–583 (2015).
31. Okamura, T., Shimizu, H., Nagao, T., Ueda, R. & Ishii, S. ATF-2 regulates fat metabolism in Drosophila. *Mol. Biol. Cell* **18**, 1519–29 (2007).
32. Jiang, W. *et al*. Acetylation Regulates Gluconeogenesis by Promoting PEPCK1 Degradation via Recruiting the UBR5 Ubiquitin Ligase. *Mol. Cell* **43**, 33–44 (2011).
33. Beale, E. G., Hammer, R. E., Antoine, B. & Forest, C. Disregulated glyceroneogenesis: PCK1 as a candidate diabetes and obesity gene. *Trends Endocrinol. Metab.* **15**, 129–135 (2004).
34. Bartok, O. *et al*. The transcription factor Cabut coordinates energy metabolism and the circadian clock in response to sugar sensing. *EMBO J.* **34**, 1538–1553 (2015).
35. Baron, M. N., Klinger, C. M., Rachubinski, R. A. & Simmonds, A. J. A Systematic Cell-Based Analysis of Localization of Predicted Drosophila Peroxisomal Proteins. *Traffic* **17**, 536–553 (2016).
36. Geer, B. W., Krochko, D. & Williamson, J. H. Ontogeny, cell distribution, and the physiological role of NADP-malic enzyme in Drosophila melanogaster. *Biochem. Genet.* **17**, 867–79 (1979).
37. Chaves, V. E. *et al*. Increased Glyceride–Glycerol Synthesis in Liver and Brown Adipose Tissue of Rat: *In-Vivo* Contribution of Glycolysis and Glyceroneogenesis. *Lipids* **47**, 773–780 (2012).
38. Moura, M. A. F. *et al*. Brown adipose tissue glyceroneogenesis is activated in rats exposed to cold. *Pflugers Arch. - Eur. J. Physiol.* **449**, 463–469 (2005).
39. Handke, B., Szabad, J., Lidsky, P. V., Hafen, E. & Lehner, C. F. Towards long term cultivation of Drosophila wing imaginal discs *in vitro*. *PLoS One* **9**, e107333 (2014).
40. Rossi, F. & Gonzalez, C. Studying tumor growth in Drosophila using the tissue allograft method. *Nat. Protoc.* **10**, 1525–34 (2015).

Acknowledgements

Author Contributions

Conceptualisation: R.H., R.S., S.G.; Investigation: R.H., Z.S., M.K.; Initial draft R.H., S.G.; Writing, reviewing and editing: R.H., Z.S., R.S., S.G.; Supervision: Z.S., R.S., S.G.; Funding acquisition: R.S., S.G.

Additional Information

Supplementary information accompanies this paper at doi:10.1038/s41598-017-11613-2

Competing Interests: The authors declare that they have no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Chromosomal instability causes sensitivity to protein folding stress and ATP depletion

A plausible contributor to the effect of CIN on cells under oxidative stress is aneuploidy. Trisomy is known to give inappropriate gene expression levels and stress responses, in diverse organisms, consistent with perturbation in the endoplasmic reticulum (ER) resulting in protein folding problems (Sheltzer et al., 2012, Oromendia et al., 2012a). The ER is the main site for protein folding, translocation and post-translation modification. Perturbation in the ER environment by nutrient deprivation, altered glycosylation, calcium depletion, ROS, DNA damage and energy disturbance/fluctuation lead to ER stress with subsequently causing unfolded protein response (UPR) activation (Malhotra and Kaufman, 2007). During tumorigenesis, cancer cells require increased activities of ER protein folding, assembly and transport, which induce ER stress (Lee, 2007). Robust ER stress responses have been documented in variety of human cancer, including breast, pancreatic, lung, skin, prostate, brain, and even liquid malignancies (Wang and Kaufman, 2014). The ER stress response is considered cytoprotective and is involved in advanced-stage disease and drug resistance (Healy et al., 2009, Ma and Hendershot, 2004). The UPR represents to primarily a pro-survival process, sustained and/or prolonged stress may result in cell death induction. UPR activation shows a vital process in tumour development, stage progression, and resistance to therapies.

In yeast, cells with an extra chromosome suffer proteotoxic stress and protein aggregation (Oromendia et al., 2012a) and vertebrate CIN lines are particularly sensitive to Hsp inhibition (Santagata et al., 2013), suggesting protein folding stress. In this study we assessed whether CIN cells, with their abnormal gene dosage, struggle to maintain chaperone function by testing the toxicity of aggregation-prone Poly-Q proteins with and without CIN or the protein folding response proteins such as hsp90. Increased protein-folding load in the ER may result in the accumulation of ROS (Wang and Kaufman, 2014).

Aneuploid cells also show elevated ROS levels (Shaukat et al., 2015, Sheltzer et al., 2012). In ER-stressed cells, Ca^{2+} released from the ER is taken up by mitochondria and release cytochrome c that inhibits complex III of the ETC and boosts ROS production. Moreover, increased Ca^{2+} in the mitochondria stimulates Krebs cycle dehydrogenases, thus increased oxygen consumption and ROS production. Mitochondrial Ca^{2+} also activates nitric oxide synthase, whose product disturbs the ETC and enhances ROS generation (Brand, 2010, St-Pierre et al., 2002). However it is not clear what is the primary cause as the accumulation of a misfolded protein within the ER leads to prolonged UPR activation, which in turn causes accumulation of ROS via the UPR-regulated oxidative protein folding machinery in the ER and the mitochondria (Haynes et al., 2004). In addition, aneuploid cells typically have an

aberrant metabolism that generates ROS and redox stress.

We wished to understand whether the stressed ER generates the ROS (by Ca^{++} release) or whether the ROS generates the stressed ER (by damaging proteins), or both. In this study we addressed this question by adding antioxidants (Catalase, propyl gallate) to CIN cells and measuring the level of ROS (by CellRox staining) and ER stress (by XBP1-GFP) expression (Sone et al., 2013). We tested the effect of protein folding stress by expressing hard-to-fold poly-glutamine (van Eyk et al., 2011) or chaperones such as Hsp90 (Vos et al., 2016) and measured ROS and Ca^{++} release (GCaMP3) in CIN versus normal tissue (Tian et al., 2009).

Nucleotide metabolism has a crucial role in senescence and autophagy in cancer cell (Kohnken et al., 2015). Thus, nucleotide metabolism has implications in genomic instability as part of tumour initiation and resistance to apoptosis during tumorigenesis. To determine how nucleotide metabolism affects CIN cells, we have examined the effect of the nucleotide inhibition in CIN cells by depleting the nucleotide synthesis enzymes and see their effect on DNA damage, ROS, cell death, cell-cycle progression and autophagy. In addition, alteration in adenine nucleotide level activates Poly (ADP-ribose) Polymerase (PARP), causing depletion of its substrate, NAD. Depletion of NAD lead to loss of ATP. Thus, in order to understand why ATP might be limited in CIN cells we test for PARP upregulation in CIN cells and examined whether adding ATP and Nicotinamide (a precursor in the synthesis of NAD+) rescue the CIN phenotype.

We know that tumours adapt to therapy, so it is important to identify ways to block the available stress response pathways that potentially mitigate metabolic therapy we wished to understand the association between aneuploidy, oxidative stress, nucleotide stress and protein folding because CIN and aneuploidy are common, tumour-specific phenotypes that offer the prospect of similarly tumour-specific therapies.

Statement of Authorship

Title of Paper	Chromosomal instability causes sensitivity to protein folding stress and ATP depletion**.**
Publication Status	Submitted for Publication
Publication Details	Mahwish Khan, Zeeshan Shaukat, Robert Saint and Stephen Gregory Affiliations: School of Genetics and Evolution, University of Adelaide, Adelaide, SA, Australia Corresponding E-mail: stephen.gregory@adelaide.edu.au

Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author	Mahwish Khan		
Contribution to the paper	Conceived and designed the experiments Performed the experiments Analysed the data Contributed reagents/materials/analysis tools Wrote the paper	80% contribution	
Signature		Date	

Name of Co-Author	Zeeshan Shaukat		
Contribution to the paper	Performed the experiments		
Signature		Date	

Name of Co-Author	Robert Saint
Contribution to the paper	Conceived and designed the experiments Contributed reagents/materials/analysis tools Funding
Signature		Date	

Name of Co-Author	Stephen Gregory
Contribution to the paper	Conceived and designed the experiments Analysed the data Contributed reagents/materials/analysis tools Wrote the paper Funding Correspondence
Signature		Date	

Chromosomal instability causes sensitivity to protein folding stress and ATP depletion.

Mahwish Khan*[1], Zeeshan Shaukat*[1], Robert Saint[2] and Stephen L. Gregory[§][1,2].

* Equal first author.

[1] Department of Genetics, University of Adelaide, Adelaide, Australia 5006.

[2] College of Medicine and Public Health, Flinders University, Adelaide, Australia 5042.

[§] Corresponding author: Stephen Gregory, College of Medicine and Public Health, Flinders University, Adelaide, Australia. Ph. +618 82067146 email: stephen.gregory@flinders.edu.au

Keywords: endoplasmic reticulum, protein aggregation, *Drosophila*, genomic instability, reactive oxygen species (ROS).

Summary statement: Cells that gain or lose chromosomes during cell division are shown to be sensitive to ATP levels and protein folding stress.

1   **ABSTRACT**

2   Aneuploidy, having an unbalanced genome, is poorly tolerated at the cellular and organismal level. It

3   gives rise to proteotoxic stress as well as a stereotypical oxidative shift which makes these cells

4   sensitive to internal and environmental stresses. Using *Drosophila* as a model, we found that protein

5   folding stress is exacerbated by redox stress that occurs in response to ongoing changes to ploidy

6   (chromosomal instability, CIN). We also found that if *de novo* nucleotide synthesis is blocked, CIN

7   cells are dependent on a high level of lysosome function to survive. Depletion of AMP synthesis

8   enzymes led to DNA damage in CIN cells, which showed elevated activity of the DNA repair

9   enzyme PARP. PARP activation causes depletion of its substrate, NAD+ and subsequent loss of

10   ATP, and we found that adding ATP or nicotinamide (a precursor in the synthesis of NAD+) could

11   rescue the observed phenotypes. These findings provide ways to interpret, target and exploit

12   aneuploidy, which has the potential to offer tumour-specific therapies.

13   **INTRODUCTION**

14   Aneuploidy is known to have detrimental effects on most cells, including reduced proliferation and

15   elevated redox stress (Sheltzer et al., 2017; Siegel and Amon, 2012). The concept of "unbalanced"

16   mutations giving a phenotype when single chromosomes are gained or lost is now almost a century

17   old (Blakeslee et al., 1920). In yeast, changes in gene dosage brought about by aneuploidy have been

18   shown to generate an excess of some proteins, and increase the burden on the protein degradation

19   apparatus (Dephoure et al., 2014). The additional demand for ATP to synthesize, fold, and turn over

20   these excess proteins has been proposed to cause increased mitochondrial activity and the production

21   of reactive oxygen species (ROS). ROS could also be produced from a stressed endoplasmic

22   reticulum if there were protein folding problems (Malhotra and Kaufman, 2007), but at least in

23   disomal yeast, activation of the unfolded protein response was not detected (Oromendia et al., 2012).

24   Nonetheless, protein aggregation was increased by aneuploidy, and in vertebrate cells, aneuploidy

25   has been linked with increased autophagy and overloading of chaperones such as HSP90 (Donnelly

26   et al., 2014; Santaguida et al., 2015). Consequently, it seems likely that aneuploidy imposes a

27   chronic strain on protein homeostasis, with the exact phenotype depending partly on the particular

28   gene dosage changes in each aneuploid cell, but also on the type of cell. With the whole protein

29   production and turnover system under strain, cell types are likely to differ in what breaks first –

30   limiting levels of antioxidants, chaperones, ATP, or lysosome function have all been implicated in

31   different cell types.

32   The unfolded protein response is typically activated in situations where the endoplasmic reticulum

33   (ER) is unable to manage the load of protein folding that is required, and there is some evidence, at

34   least in vertebrate cells, that it can be induced by aneuploidy. Studies differ as to whether simple

35   tetraploidy is (Senovilla et al., 2012) or is not (Ohashi et al., 2015) enough to trigger robust

36   activation of the unfolded protein response. Furthermore, it is not clear whether aberrant protein

37   levels are the primary cause, because aneuploid cells typically also have an aberrant metabolism that

38   generates ROS and redox stress. Protein folding is a highly redox sensitive process, so aneuploidy

39   may both increase the load of proteins to be folded and impair the ability to process them.

40   Nucleotide depletion is another homeostatic imbalance that affects aneuploid cells (Bester et al.,

41   2011). Insufficient nucleotides during S-phase lead to replication stress, which has been strongly

42   linked to the induction of chromosomal instability (CIN) that produces highly aneuploid cells.

43   Several mechanisms linking replication stress and CIN have been demonstrated, including increased

44   double stranded DNA breaks, increased anaphase bridges, segregation errors and production of ROS

45   (Burrell et al., 2013; Chan et al., 2009; Marchetti et al., 2006; Minocherhomji et al., 2015).

46   Consequently, we expect that aneuploid cells will be sensitive to perturbation of nucleotide levels,

47   which may increase aneuploidy beyond the cell's threshold of tolerance.

48   We wished to understand the causal relationships between aneuploidy and these failures of

49   homeostasis because chromosomal instability and its consequent aneuploidy is a common, tumour-

50   specific phenotype that offers the prospect of similarly tumour-specific therapies. This will require

51   the identification of features of aneuploid cells that are invariant, regardless of which DNA has been

52   gained or lost. In this paper we describe a common phenotype of protein folding stress in genetically

53   diverse aneuploid cells using a *Drosophila* model for chromosomal instability. We find that the

54   protein folding defects are caused by redox stress and contribute to that stress. We have tested

55   nucleotide depletion and find that it can be tolerated by aneuploid cells without causing redox stress,

56   but this tolerance requires high levels of lysosome function. Finally, we find that CIN cells are

57   sensitive to decreased ATP synthesis and have activated poly(ADP ribose) polymerase (PARP),

58   which leaves these cells deficient in NAD+ and ATP.

59   **RESULTS**

60   **CIN causes ER stress and oxidative stress in *Drosophila***

61   Up-regulation of protein chaperones has been observed in aneuploid cells from budding yeast, mouse

62   and humans (Pfau and Amon, 2012; Sheltzer et al., 2012). To determine whether cells in which

63   aneuploidy was induced by chromosomal instability (CIN) were under protein folding stress, we

64 tested levels of the HSP70 family. We observed an increased in chaperone levels (HSP 83) in cells in

65 which CIN had been induced by Rad21 depletion, relative to wildtype controls (Fig 1a). In this paper

66 we use two models for induced CIN: depletion of the cohesin Rad21, which makes 46% of

67 metaphases aneuploid, typically with gain or loss of a whole chromosome, and causes ROS and cell

68 death (Liu et al., 2015). Alternatively we use depletion of the spindle checkpoint protein Mad2,

69 which causes bridges or lagging chromosomes in approximately 25% of anaphases and gives very

70 little cell death (Shaukat et al., 2012). We typically use high CIN (Rad21 depletion) where we are

71 measuring strong CIN phenotypes and low CIN (Mad2 depletion) where we are testing for genetic

72 enhancement of mild CIN effects. The transcription factor X-box-binding protein 1 (XBP1) is

73 required for the activation of several unfolded protein response genes that control ER protein folding,

74 intracellular trafficking, and ER membrane expansion in response to ER stress (Bravo et al., 2013).

75 To observe ER stress in CIN cells, we used recombinant UAS-XBP1-GFP which only produces GFP

76 when its mRNA is appropriately spliced in response to ER stress (Ryoo, 2015; Samali et al., 2010).

77 We found that GFP was increased, indicating the presence of ER stress in CIN cells (Fig 1b'). We

78 have previously observed that CIN cells have elevated levels of reactive oxygen species (ROS) and

79 mitochondrial dysfunction (Shaukat et al., 2015), so we wished to test whether that might be caused

80 by release of Ca^{++} ions from the stressed ER. The ER is a principal cytoplasmic store for Ca^{++}, which

81 can be released in response to protein folding stress, potentially causing loss of mitochondrial

82 integrity and ROS generation (Pinton et al., 2008). We observed an increase in GCaMP3 signal (Tian

83 et al., 2009), which indicates increased Ca^{++} release in CIN cells relative to controls (Fig 1c).

84 **CIN affects protein aggregation in *Drosophila***

85 Having observed activation of the unfolded protein response in CIN cells, we wished to test whether

86 adding proteins that were difficult to fold would lead to aggregate formation and cell damage under

87 these conditions (Eenjes et al., 2016). To observe protein aggregation in CIN cells, we expressed

88 proteins containing poly-Glutamine (polyQ) repeats of different lengths (CAG55 and CAG91) that

89 can form aggregates (van Eyk et al., 2012; Landrum and Wetzel, 2014). We observed greatly

90 increased aggregation of myc-tagged CAG91 in CIN cells compared to the wild-type control (Fig

91 2b), consistent with CIN cells having difficulty managing this additional protein folding burden. To

92 test the effect of this burden on CIN cell survival, we measured Acridine Orange staining (Shaukat et

93 al., 2012). Increased Acridine staining was observed in CIN cells expressing either CAG55 or

94 CAG91 compared to their controls (Fig 2c, d). Our data suggests that CIN cells suffer protein folding

95 stress so that chaperones are incapable of resolving additional stress from aggregation-prone poly-

96 Glutamine proteins.

97 **Overexpression of antioxidants rescues the effect of protein aggregation in CIN cells.**

98   Reactive oxygen species (ROS) production has been associated with ER stress and the unfolded

99   protein response (Cao and Kaufman, 2014). Oxidative stress causes the formation of oxidatively

100   modified proteins that tend to aggregate in cytosol and trigger tumour progression (Kim et al., 2010).

101   In order to test our hypothesis that the protein aggregation in CIN cells is ROS-dependent, we

102   measured the effect of overexpression or depletion of the antioxidant enzyme Catalase together with

103   polyQ repeats. Catalase only directly removes hydrogen peroxide, not other reactive oxygen species,

104   but we have previously noted that this can significantly affect CIN cell fates (Shaukat et al., 2015).

105   We observed a significant decrease in polyQ protein aggregation in CIN wing discs of larvae co-

106   expressing UAS-catalase and polyQ repeats compared to CIN discs expressing polyQ repeats only

107   (Fig 2e). We also observed an increase in aggregation when Catalase was depleted by RNAi in CIN

108   cells expressing poly-Glutamine protein. These results suggest that ROS contributes to protein

109   folding stress in CIN cells which makes them vulnerable to the addition of hard-to-fold proteins.

110   **Effect of nucleotide interventions on CIN cells**

111   Like protein folding stress, nucleotide stress has been linked to CIN and cancer development (Bester

112   et al., 2011). Nucleotide pool disequilibrium can result in genetic abnormalities and oncogenic

113   transformation (Aird and Zhang, 2015). To identify the genes from nucleotide synthesis pathways

114   that could be involved in regulating the fate of CIN cells, we tested a range of genes affecting

115   purine biosynthesis (ADSS, PRPS2, GMP Synthetase, IMP dehydrogenase, PRAT), pyrimidine

116   biosynthesis (CTP Synthetase, Carbamoyl Phosphate Synthetase) and the Pentose Phosphate (PP)

117   pathway (TKL, Transaldolase, PP Epimerase). We initially measured their effect on CIN cells

118   using RNAi to deplete the nucleotide pathway candidates in third instar larval wing discs with

119   CIN induced by Mad2 depletion (Fig 3a). We selected the candidates based on their Acridine

120   Orange (AO) phenotype, a cell viability assay that we have previously used to identify genes such

121   as the positive control *G6PD*, that are required for CIN cell survival (Shaukat et al., 2012). The

122   knockdown of ADSS, PRPS2 and TKL gave significantly elevated AO staining in the CIN region,

123   while candidate depletion in non-CIN cells gave little or no AO, similar to the negative control, in

124   this case RNAi to a gene not expressed in *Drosophila* (mCherry) (Fig 3c-g). Quantification of AO

125   signals from nucleotide pathway candidates with or without CIN showed elevated AO staining in

126   CIN cells compared to non-CIN cells (Fig. 3b). These results suggest that CIN cells are sensitive

127   to defects in nucleotide synthesis.

128   **Effect of depletion of nucleotide synthesis enzymes on oxidative stress.**

129   We have previously found that disruptions to glucose metabolism in CIN cells not only increase

130   Acridine staining, but also generate ROS and cause apoptosis (Shaukat et al., 2015). We carried

131 out ROS assays to examine whether depletion of nucleotide synthesis enzymes may also elevate

132 oxidative stress in CIN cells. We found that that knockdown of ADSS, PRPS2 and TKL gave no

133 detectable increase ROS in CIN cells, in contrast to the positive control G6PD (Fig 4a-d). These

134 results suggest that depletion of nucleotide synthesis enzymes does not affect CIN cells by

135 increasing ROS production.

136 **DNA damage in response to nucleotide synthesis enzyme depletion in CIN cells.**

137 Low nucleotide pools are known to cause replication stress and DNA damage in dividing cells

138 (Kohnken et al., 2015). We next examined DNA damage by using anti-P-H2avD (γH2aX)

139 antibody staining of larval wing discs with CIN in which nucleotide candidates had been knocked

140 down (Fig 4e-i). Quantification showed that depletion of ADSS, PRPS2 and TKL in CIN cells

141 gave an elevated level of DNA damage, compared to the *mad2*-RNAi, *mCherry*-RNAi negative

142 control (Fig 4j). As expected, these data confirm that depletion of nucleotide synthesis enzymes

143 caused DNA damage in CIN cells, consistent with decreased nucleotide availability.

144

145 **Depletion of nucleotide synthesis enzymes does not result in cell death in CIN cells.**

146 Having observed elevated Acridine staining and DNA damage, we expected that apoptosis might

147 be elevated in CIN cells depleted for nucleotide synthesis enzymes (Abrams et al., 1993). To

148 visualize apoptosis, we used anti-cleaved-Caspase 3 and anti-Dcp-1 antibody staining of wing

149 discs. The levels of these antibody stainings were not elevated in double knockdowns of

150 nucleotide candidates with Mad2 (Fig S1, Fig 5b-e) unlike the positive control (*mad2*-RNAi,

151 *PASK*-RNAi) which generates ROS, DNA damage and apoptosis in CIN cells (Shaukat et al.,

152 2015). Quantification of Dcp-1 signals from nucleotide candidates with CIN showed no

153 significant increase in cell death compared to the *mad2*-RNAi, *mCherry*-RNAi negative control

154 (Fig 5f). However, cell death could have been caused by a caspase independent mechanism such

155 as necrosis. We used Propidium Iodide (PI) staining to test for necrosis when our candidates were

156 depleted in CIN cells. Consistent with the apoptosis data, these knockdowns in CIN cells were

157 found to be negative for PI staining compared to the positive control (*JNK*[RNAi], Fig S2). Together,

158 these results suggest that that depletion of nucleotide synthesis enzymes does not cause cell death

159 either by apoptosis or necrosis in CIN cells. This surprising finding contrasts with the high levels

160 of apoptosis induced by disrupting glucose metabolism in CIN cells (Shaukat et al., 2015).

161

162 **Effect of depletion of nucleotide synthesis enzymes on the cell cycle.**

163 We observed a significant increase in DNA damage when our nucleotide candidates were

164 depleted in CIN cells, however we did not see cell death. Based on this finding, we hypothesized

165   that this DNA damage could have led to cell cycle arrest in CIN cells. To test this hypothesis, we

166   used Phospho-Histone 3 (PH3) antibody staining as a marker for scoring mitotic cells. We tested

167   whether loss of our nucleotide candidates in CIN cells could prevent cell cycle progression by

168   scoring the mitotic cells from larval wing discs of nucleotide candidates knocked down in cells

169   with and without CIN. We did not observe any significant increase or decrease in PH3 staining in

170   knockdowns of ADSS, PRPS2 and TKL in CIN or non-CIN cells (Fig S3), suggesting that

171   depletion of nucleotide synthesis enzymes does not arrest the cell cycle even in CIN cells showing

172   DNA damage. Consistent with this, we did not see any change in S phase duration as measured by

173   EdU incorporation (Figure S3).

174

175   **Effect of depletion of nucleotide synthesis on activation of autophagy in CIN cells**

176   It is known that autophagy is activated in CIN cells, and is needed for their survival (Liu et al.,

177   2016). We wished to examine the effect of depleting nucleotide candidates on the activation of

178   autophagy in CIN cells, to see if this could explain the Acridine staining, which normally stains

179   the lysosomes in apoptotic cells (Abrams et al., 1993). We used lysotracker staining and found

180   that, like Acridine, it was elevated when any of the nucleotide candidates were depleted in CIN

181   cells, relative to non-CIN controls (Fig S4, 6A). We used a tagged form of Atg8a to confirm

182   whether the high lysotracker staining observed was due to activation of autophagy in knockdowns

183   of ADSS, PRPS2 and TKL in CIN cells. Unlike the lysotracker staining, only knockdown of TKL

184   gave robust Atg8a puncta formation in CIN cells (Fig S4, 6B). However, we did not observe

185   Atg8a puncta in knockdown of ADSS or PRPS2 in CIN cells (Fig S4, 6B). We expect that

186   knockdowns of ADSS and PRPS2 decrease the rate of AMP synthesis and AMP is used to maintain

187   phosphorylated AMPK to activate autophagy (Choi and Lee, 2011). However, increased lysotracker

188   staining in these candidates suggest that in absence of autophagy, lysosomes work hard to

189   compensate.

190

191   **Feeding rATP and dATP rescue some AO phenotypes in CIN cells:**

192   Depletion of ADSS and PRPS2 should decrease the level of AMP synthesis, which will further lead

193   to depletion of ATP. So, we examined whether feeding rATP or dATP to larvae would have an effect

194   on the AO phenotype. First, we fed rATP to larvae knocked down for ADSS, PRPS2 and TKL in

195   cells with CIN induced by Mad2 depletion. We observed that feeding rATP significantly rescued the

196   AO phenotype when ADSS or PRPS2 were depleted in CIN cells. (Fig 7A, S5). The same rescue of

197   ADSS and PRPS2 was seen when larvae were fed dATP (Fig 7B, S5). However, no rescue was seen

198   when TKL depleted larvae were fed either nucleotide (Fig 7, S5). This was consistent with the

199   autophagy seen in the TKL knockdowns: these cells do not seem to be short of adenosine. We also

200   tested a high CIN model (Rad21 depletion) to see if CIN might generate sensitivity to ATP levels in

201   otherwise normal cells. Depletion of Rad21 leads to high levels of aneuploidy, Acridine Orange,

202   ROS and cell death (Liu et al., 2016). Surprisingly, we found that feeding Rad21 depleted larvae

203   rATP strongly rescued the Acridine phenotype, but only in males (Fig 7A, S5). dATP had no effect

204   and females were unaffected by either nucleotide (Fig 7B, S5). We conclude that elevated CIN can

205   cause a loss of rATP, but do not know why females are less sensitive to this (c.f. Clemente-Ruiz et

206   al., 2016). When AMP synthesis is compromised, our data suggest that CIN cells increase lysosome

207   formation to compensate for the lack of autophagy.

208

209   **Lysosomal ATP levels in CIN cells.**

210   Effective lysosomal function is known to play a vital role in cancer progression and metastasis

211   (Appelqvist et al., 2013). To examine the induction of lysosomes as a result of depletion of

212   nucleotide synthesis enzymes in CIN cells, we used Quinacrine (QA), a marker for lysosomal ATP

213   (Cao et al., 2014). Robust QA staining was observed in knock downs of ADSS, PRPS2 and TKL in

214   CIN cells relative to the inert *mCherry*-RNAi control (Fig 8A, S6). This suggests that there is no lack

215   of ATP inside lysosomes, although our feeding experiments argue that these cells lack ATP overall.

216   In fact, we observed more QA staining in CIN cells than non-CIN controls (Fig S6). A multi-subunit

217   lysosomal membrane protein, v-ATPase, maintains lysosomal acidification by importing protons

218   from the cytoplasm to the lysosomal lumen (Collaco et al., 2013), and is required for ATP transport

219   into the lysosome (Mauvezin et al., 2015). Blocking v-ATPase with bafilomycin significantly

220   reduced the QA signal (Fig 8A) as expected. By blocking lysosome acidification, this inhibition of v-

221   ATPase also strongly rescued the Acridine Orange phenotype seen in CIN cells depleted for

222   nucleotide synthesis enzymes (Fig 8B, S6).

223

224   **Lysosomes are needed for CIN cell survival**

225   It has been reported that advanced cancers exploit autophagy-lysosomal pathways to avoid cell

226   death. Recent studies indicate that compromised lysosomal function in mammalian cell lines resulted

227   in cell death (Cao et al., 2014). Moreover, having previously found that autophagy is needed for

228   CIN cell survival (Liu et al., 2016), we wished to investigate the role of lysosomes in determining

229   the fate of CIN cells, particularly in absence of autophagy. We treated CIN wings discs knocked

230   down for our candidates with bafilomycin to block v-ATPase, and then stained the wings discs

231   with Dcp-1 to measure the level of cell death. The positive control was depletion of PEPCK,

232   which gives elevated apoptosis in CIN cells and tumours (Hussain et al., 2017). We found that

233   bafilomycin treatment caused some cell death in CIN cells knocked down for ADSS or PRPS2

234   and perhaps even TKL, but not in CIN cells alone (Fig 8C, S7). These results suggest that in CIN

235   cells in the absence of autophagy, lysosomes were able to compensate, but if we also blocked

236   lysosomes, the CIN cells died.

237

238   **Replacing NAD+ rescues the AO phenotype**

239   We have shown that that depletion of ADSS and PRPS2 caused DNA damage in CIN cells and

240   both rATP and dATP significantly rescued the AO phenotype. This led us to hypothesize that

241   knockdowns of ADSS and PRPS2 depleted the level of adenosine, causing DNA damage during

242   replication, which we would expect to then activate poly(ADP ribose) polymerase (PARP) as part of

243   the repair process (Weaver and Yang, 2013). In normal cells, the depletion of nucleotide synthesis

244   had no effect, so we wondered whether CIN cells were particularly vulnerable to PARP activation,

245   which can further decrease ATP levels (Weaver and Yang, 2013). We found that cells with high CIN

246   levels activated PARP to some degree even without nucleotide depletion (Fig S8). In these high CIN

247   cells, we know that AO marks cell death (Liu et al, 2015), and we found that blocking PARP

248   activation rescued a significant amount of that cell death (Fig 8D). Because PARP consumes NAD+

249   and leads to ATP depletion (Zong et al., 2004), we predicted that providing extra NAD+ to high CIN

250   cells, might rescue the AO phenotype in these cells. To test our model, we fed larvae with

251   nicotinamide (NAM), a precursor in the synthesis of NAD+ (Kirkland, 2012). We observed a

252   significant reduction in the AO phenotype in wing discs when NAM was fed to larvae with

253   decreased nucleotide synthesis (ADSS, PRPS2 or TKL) or with high CIN levels (Rad21) relative to

254   controls (Fig 8E, S8). We conclude that CIN cells tend to activate PARP, which decreases their

255   levels of NAD+ and ATP, making them particularly vulnerable to interventions that further limit

256   ATP availability. At moderate CIN levels, depleted adenosine does not kill the cell, but does limit

257   autophagy, leading to an increased dependence on lysosomes, which then become critical for CIN

258   cell survival.

259

260   **DISCUSSION**:

261   Aneuploidy is a state in which cells carry unbalanced genome. Errors in chromosomal segregation

262   lead to whole chromosome aneuploidy while segmental aneuploidy mainly originates due to defects

263   and delays in the DNA replication and repair mechanism (Gordon et al., 2012; Janssen and Medema,

264   2013). Aneuploidy is a hallmark of cancer and has been shown to be poorly tolerated at the cellular

265   and organismal level (Weaver and Cleveland, 2006). Aneuploidy can have a range of effects

266   including aberrant cell growth, proliferation, proteotoxic and oxidative stress (Pfau and Amon,

267   2012). We have found that the induction of aneuploidy makes cells highly glycolytic and vulnerable

268   to oxidative stress and they show DNA damage and apoptosis in response to metabolic interventions

269   that do not damage normal cells (Shaukat et al., 2015). The mechanism that regulates the change in

270   metabolism in response to aneuploidy is unclear, therefore, we wished to identify the stereotypical

271   metabolic signature of aneuploidy. Aneuploidy is known to cause ER stress and oxidative stress in

272   most organisms such as yeast, plants, mouse and humans (Pfau and Amon, 2012; Sheltzer et al.,

273   2012). Aneuploidy related impaired proliferation, altered protein folding and ER stress is thought to

274   be due to the changes in protein stoichiometry from the aberrant chromosomes. In this study,

275   increases in chaperone levels (Hsc70, Hsp83) and ER stress markers (XBP1) were observed in

276   aneuploid cells, we also observed that ER stress can cause ROS by Calcium release in aneuploid

277   cells. Our data confirms that aneuploid cells suffer protein folding stress so that chaperones are

278   incapable of resolving additional stress (i.e. aggregation prone Poly-Glutamine (polyQ) proteins).

279   Successful aneuploidy tolerant cells must be able to enhance repair and reduce oxidative stress and

280   cell death, so we wished to identify the regulation of pathways required for aneuploidy tolerance.

281   Autophagy is known to be induced by ER stress and ROS, and is a plausible mechanism for

282   aneuploidy tolerance (Sheltzer, 2013). Aneuploid cells have three related defects that autophagy

283   could help to moderate: proteotoxic stress, defective mitochondria and oxidative damage (Li et al.,

284   2017; Shaukat et al., 2015). We have shown that aneuploid cells are dependent on autophagy of

285   defective mitochondria to tolerate their aneuploidy (Liu et al., 2016). We further analysed the

286   production of ROS and ER stress in aneuploid cells. Previous studies revealed that in ER-stressed

287   cells, Ca^{2+} released from the ER is taken up by mitochondria and releases cytochrome c that inhibits

288   complex III of the electron transport chain and increases ROS production. Moreover, increased

289   Ca^{2+} in the mitochondria stimulates Krebs cycle dehydrogenases, thus increasing the oxygen

290   consumption and ROS production. Mitochondrial Ca^{2+} also activates nitric oxide synthase, whose

291   product disturbs the ETC and enhances ROS generation (Brand, 2010; St-Pierre et al., 2002).

292   However it is not clear whether the ER stress generates the ROS or ROS generates the stressed ER or

293   both. In this study, we addressed this question and showed that ROS contributes to protein folding

294   defects in aneuploid cells, which could be significantly rescued by adding antioxidants.

295   We have previously observed that aneuploid cells have overactive mitochondria, which produce

296   ROS and hence oxidative damage to proteins, lipids and DNA and damage to macromolecules

297   (Shaukat et al., 2015), which we now find causing protein folding defects and ER stress. Moreover,

298   effective repair mechanisms, autophagy and antioxidant levels are required to tolerate the deleterious

299   effects of aneuploidy.

300   Further we tested the effect of nucleotide stress on CIN cells. We carried out preliminary screening

301   based on AO staining and tested that range of genes affecting purine biosynthesis, pyrimidine

302   biosynthesis and the PP pathway. We found that the knockdown of ADSS, PRPS2 and TKL gave

303   significantly higher AO staining in a CIN background. These nucleotide candidates play a central

304   metabolic role in the synthesis of nucleotides. The PRPS enzyme adds a pyrophosphate group from

305   ATP to ribose-5-phosphate generated from the PP pathway to produce a nucleotide precursor called

306   5-phosphoribosyl-1-pyrophosphate (PRPP). PRPP is a substrate for all nucleotide salvage pathway

307   enzymes, as well as for the rate-limiting enzymes of purine and pyridine biosynthesis. Modulation of

308   PRPS levels by either knockdown or overexpression respectively inhibit or potentiate nucleotide

309   production, suggesting that levels of PRPP may be sufficient to govern the overall rate of purine

310   metabolism (Cunningham et al., 2014). ADSS is downstream of the de novo pathway and it is a vital

311   component of the de novo pathway as well as the salvage pathway. Similarly, ADSS is a highly

312   conserved enzyme among all living organisms, it converts inosine monophosphate (IMP) to

313   adenosine monophosphate (AMP) as part of ATP biosynthesis.

314   The high AO phenotypes in nucleotide candidate knockdowns in CIN cells suggest that CIN cells

315   are sensitive to changes in nucleotide levels. In addition, increased lysotracker and DNA damage

316   phenotypes were observed when nucleotide synthesis enzymes ADSS, PRPS2 and TKL were

317   depleted in a CIN background. However, we did not see any involvement of ROS or cell cycle arrest

318   in this case. Cell death either by apoptosis or necrosis was also not observed when these candidates

319   were depleted in CIN cells. Therefore, we expected that the autophagy pathway was being activated.

320   Autophagy is known to be activated in response to various stresses including nutrient starvation

321   (Jiang and Mizushima, 2014; White, 2015). We have shown that CIN cells are dependent on the

322   autophagy of mitochondria to tolerate their aneuploidy and avoid triggering innate immune

323   signalling (Liu et al., 2016). For example, blocking the autophagy pathway in CIN cells led to an

324   increased number of dysfunctional mitochondria, increased levels of oxidative stress, DNA damage

325   and apoptosis, while enhancing autophagy could reduce the level of ROS and apoptosis. So we

326   hypothesized that autophagy might be triggered in response to nucleotide depletion. Surprisingly,

327   autophagy was not detected in ADSS and PRPS2 deficient CIN cells. However, elevated puncta of

328   tagged Atg8a were observed in TKL deficient CIN cells, suggesting that autophagy is activated in

329   TKL deficient CIN cells. TKL is a pivotal enzyme of the non-oxidative arm of the PP pathway. It

330   catalyses the common two-substrate reactions in order to generate glyceraldehyde-3-phosphate and

331   fructose-6-phosphate, which re-enter glycolysis. When cells are in need of nucleotides, the PPP

332   produces ribose via the oxidative as well as the nonoxidative arm from fructose-6-phosphate and

333   glyceraldehyde-3-phosphate. TKL1 also enhances the PPP flux for biosynthetic reactions, as the PPP

334   generates ribose-5-phosphate for nucleic acid synthesis and NADPH for fatty acid synthesis and

335   maintaining redox homeostasis to protect cells against oxidative stress and apoptosis (Diaz-Moralli et

336   al., 2016; Vaughn and Deshmukh, 2008). Moreover, inhibition of TKL will suppress the PP pathway

337   and interrupt the synthesis of important coenzymes such as ATP, CoA, NAD(P)+, FAD, and genetic

338   material, RNA and DNA. We found that depletion of TKL in CIN cells triggered autophagy, which

339   we interpret as a CIN cell survival response, based on previous work by ourselves and others

340   (Santaguida et al, 2015, Liu et al, 2016).

341   Increased lysosome staining in ADSS and PRPS2 deficient CIN cells suggests lysosomal

342   involvement in CIN cells. Lysosomes play a crucial role in cancer progression by regulating complex

343   processes involving protein secretion, endocytic receptor recycling, energy metabolism, and cell

344   signalling (Appelqvist et al., 2013, Stingele and Storchova, 2013). Moreover, the autophagy–

345   lysosome pathway is closely linked with the hallmarks of cancer including escaping cell death

346   pathways, evading immune surveillance, and deregulating metabolism (Hanahan and Weinberg,

347   2011). Advanced cancer cells are extremely reliant on effective lysosomal function. As a

348   consequence, cancer progression and metastasis are related with unusual changes in lysosomal

349   compartments, such as lysosome volume, composition, cellular distribution, and lysosomal enzyme

350   activity, as compared with normal cells (Gocheva et al., 2006; Nishimura et al., 1998). Furthermore,

351   when we blocked lysosomes, ADSS or PRPS2 depleted CIN cells showed increased cell death.

352   These results suggest that in the absence of autophagy, lysosomes worked hard in these cells to

353   compensate for the lack of autophagy and when we blocked lysosomes the CIN cells died. So, our

354   model is that CIN cells use lysosomes to compensate for loss of autophagy when AMP is depleted.

355   Depletion of ADSS and PRPS2 decrease the synthesis of AMP which further leads to depletion of

356   ATP, and we observed that in larvae knocked down for ADSS or PRPS2, feeding rATP and dATP

357   significantly rescued the AO phenotype in CIN cells relative to their controls. These results suggest

358   that CIN cells are sensitive to ATP changes. To further understand why ATP might be limited in CIN

359   cells we tested for the Poly (ADP-ribose) Polymerase (PARP) and found that it is upregulated in CIN

360   cells. We also observed rescue of oxidative stress and cell death when we knocked down PARP in

361   CIN cells. The PARP enzyme PARylates a variety of protein substrates and alters their interaction

362   with DNA and other proteins. PARP is currently in clinical trials for DNA damage sensitive cancers

363   because of its role in the DNA damage response (Weaver and Yang, 2013). PARP activation causes

364   depletion of its substrate NAD+, which further leads to loss of ATP (Zong et al., 2004) . We found

365   that adding ATP or nicotinamide (a precursor in the synthesis of NAD+) rescued the AO phenotype

366 in CIN cells. These results suggest that ATP and NAD+ are consumed for PARP activation and CIN

367 cell survival.

368 Our model is that knockdowns of ADSS and PRPS2 in CIN cells deplete the nucleotide level which

369 result in DNA damage. DNA damage activates PARP that causes a reduction in both the NAD^+ and

370 the ATP pool. CIN cells already lack ATP due to activation of PARP, and less ATP in CIN cells is

371 paralleled by increasing AMP levels. High AMP levels, sensed by AMPK, should lead to activation

372 of autophagy, which is required for CIN cell adaptation in a nutrient starved condition. However,

373 loss of AMP by ADSS knockdown in CIN cells seems to prevent activation of autophagy. An

374 elevated lysosomal phenotype in ADSS deficient CIN cells shows that in the absence of autophagy,

375 lysosomes work hard to compensate for the autophagy defect, and this is required for the cells to

376 survive. Overall, our results suggest that cellular homeostasis is significantly disrupted by

377 aneuploidy, with defects originating from elevated ROS levels that exacerbate protein folding stress

378 and damage DNA. Because DNA damage activates PARP, aneuploid cells are sensitive to levels of

379 NAD+ and ATP, making this metabolic pathway a promising therapeutic option for the treatment of

380 aneuploid tumours.

381

Material and Methods

383 **Drosophila Stocks**

384 The fly stocks used in this paper are as follows:

385 *mad2*-RNAi (VDRC 47918) , *Rad21*-RNAi (Bloomington #36786), *ADSS*-RNAi (Bloomington

386 #33993), *PRPS2*-RNAi (Bloomington #35619), *TKL*-RNAi (Bloomington #32884), Ribulose-

387 *Phosphate3-Epimerase*-RNAi (Bloomington #42816), *Carbamoyl Phosphate Synthetase*-RNAi

388 (Bloomington #38332), *PRAT*-RNAi (Bloomington #51492), *Transaldolase*-RNAi (Bloomington

389 #51709), *GMP Synthetase*-RNAi (Bloomington #31055), *CTP Synthetase*-RNAi (Bloomington

390 #31752), UAS-*IMP dehydrogenase* (Bloomington #11284), *JNK*-RNAi (VDRC 34138), *mCherry-*

391 *Atg8a* (Bloomington #37750), *PEPCK*-RNAi (Bloomington #17725), *PASK*-RNAi (Bloomington

392 #3105), *G6PD*-RNAi (Bloomington #12529), UAS-XBP1-GFP (Bloomington #39719), Hsp83-GFP

393 (DGRC #109-697), UAS-CAG55 and UAS-CAG91 (a gift from Prof. R. Richards), UAS-Catalase

394 (Bloomington #24621), PARP-RNAi (Bloomington #35792), Parg-RNAi (Bloomington #61333),

395 UAS-GCaMP (Bloomington #32236). The driver stock *engrailed* (*en-Gal4*) for gene expression in

396 the posterior region of wing discs is Bloomington #30564.

397 **Acridine Orange (AO) Staining:**

398   Third instar larvae were dissected in PBS then discs were incubated in 1mM AO for 2 mins then

399   transferred to a slide after a brief wash. The treated imaginal discs were immediately mounted under

400   a bridged cover slip and imaged in PBS. For quantification, the AO stain was normalized by

401   subtracting the wild type region value from the test region value (*engrailed*-driven region). The

402   background noise of all images was subtracted in ImageJ using a rolling ball radius of 10 pixels.

403   **Propidium Iodide (PI) Staining:**

404   Propidium Iodide staining was used to measure caspase-independent cell death. Third instar larvae

405   were dissected in PBS for imaginal discs, the collected imaginal discs were incubated in 3uM PI for

406   5 mins then transferred to a slide after a brief wash. Mounting, imaging and quantification was done

407   similar to AO staining as described above.

408   **Lysotracker and Hoechst staining**

409   Lysotracker staining was used to detect lysosomes in larval wing imaginal discs. The dissected

410   imaginal discs were transferred from PBS and incubated in 1uM lysotracker (Lysotracker red DND-

411   99, Invitrogen) and 6ug/ml Hoechst (Hoechst 33342, Sigma) for 5 mins and then mounted to a slide

412   with PBS for microscopy after a quick wash in PBS.

413   **Quinacrine (QA) Staining:**

414   To identify lysosomal ATP level, we used QA staining on the wings disc of third instar larvae.

415   Candidates were knocked down using *en-gal4* driver, approximately equal aged larvae were selected

416   and wing discs were dissected out in PBS. Then dissected discs were incubated in 50uM Quinacrine

417   for 2 min. Mounting and imaging was done as for the Acridine Orange staining described above.

418   **Oxidative stress assay**

419   The level of reactive oxygen species (ROS) in CIN cells was measured by using the fluorogenic

420   probe CellROX Deep Red from Life Technologies according to manufacturer's recommendations.

421   The third instar larvae were dissected in D22 media with pH 6.8. Then the dissected imaginal wing

422   discs were transferred into 5μM CellRox (in D22 media) for 20 mins. The wing discs were quickly

423   washed in PBS and fixed in 3.7% formaldehyde for 5 min then mounted in 80% glycerol for

424   imaging.

425   **Calcium imaging**

426   Intracellular calcium was detected by imaging of GCaMP3. Third instar wing discs were dissected in

427   PBS, briefly fixed in 4% formaldehyde and mounted in 80% glycerol, then imaged as for GFP.

428   **Immunostaining:**

429   Immunostaining was used on dissected wing imaginal discs for different purposes. Third instar

430   larvae were dissected in PBS for imaginal discs then the collected imaginal discs were fixed in 3.7%

431   formaldehyde for 20 mins and then wash for 30 mins in 0.2% PBST (1xPBS+0.2% Tween). The

432   fixed imaginal wing discs were then blocked in PBSTF (1xPBS+0.2% Tween+5% fetal calf serum)

433   for 30 mins and stained with the primary antibody for overnight at 4℃. After staining with the

434   primary antibody, the wing discs were washed in 0.2% PBST for 30 mins then transferred to a

435   secondary antibody solution for 2.5 hrs at room temperature in the dark. After 30 mins washing in

436   PBST, the wing discs were mounted in 80% glycerol for imaging. For detecting Parylation, discs

437   mutant for the deParylating enzyme Parg were used to improve the sensitivity of PAR detection (Fig.

438   S8).

439   The source and concentration of antibodies used are as follows:

440   Rabbit anti-cleaved caspase 3 (D175, 1: 100) from (Cell Signalling #9661S), Rabbit anti-cleaved

441   Drosophila Dcp1 (Asp216, 1:100) from (Cell Signalling #9578), Mouse anti-P-Histone3 (Ser10,

442   1:500) from (Cell Signalling #9706S), Rabbit anti-P-H2avD (1:500) from (Rockland, Lot # 30352),

443   Mouse anti-pADPr (PAR, Clone 10H; 1:100) from Tulip Biolabs, #1020/N).

444   The secondary antibodies used were goat anti-rabbit CY3 (1: 100, Life Technologies), rhodamine

445   anti-mouse (1: 200) and rhodamine anti-rabbit (1:200).

446   **Drug Treatments:**

447   For wing disc culture experiments, drugs were mixed with fly extract media, fly extract media was

448   prepared by using Schneider's media (Invitrogen) 94%, whole fly extract 5% (Currie *et al.*, 1988),

449   Bovine Insulin (Sigma) (1mg/ml) 0.5%, penicillin/streptomycin 1000U/ml 0.5%.

450   For drug feeding experiments, drugs were mixed in standard fly food (semolina, yeast, molasses,

451   agar, glucose, water, Tegosept and acid-mix) and were given to larvae when solidified. Drugs used in

452   this paper are as follows:

453   6-Aminonicotinamide (6-ANA) 500 uM, Bafilomycin 150 nM, Adenosine 5′-triphosphate disodium

454   salt hydrate (rATP) 1mM, dATP PCR Grade, sodium salt 1mM, Nicotinamide (NAM) 1mM. All

455   drugs were obtained from SIGMA.

456   **Data analysis**

457   Statistical analysis was done in Prism (Graph Pad) using Dunnett's multiple comparisons and two-

458   tailed t-test with Welch's correction. In all graphs, error bars show 95% confidence intervals. All

459   microscopy was done on a Zeiss Axioplan2 microscope. Axiovision software (Carl Zeiss), Adobe

460   Photoshop, Adobe Illustrator and ImageJ (https://fiji.sc/) were used for image processing and

461   quantification.

462

464
465   **REFERENCES**:
466

Aird, K. M. and Zhang, R. (2015). Nucleotide metabolism, oncogene-induced senescence and cancer. *Cancer letters* **356**, 204-210.

Appelqvist, H., Wäster, P., Kågedal, K. and Öllinger, K. (2013). The lysosome: from waste bag to potential therapeutic target. *Journal of molecular cell biology* **5**, 214-226.

Bester, A. C., Roniger, M., Oren, Y. S., Im, M. M., Sarni, D., Chaoat, M., Bensimon, A., Zamir, G., Shewach, D. S. and Kerem, B. (2011). Nucleotide deficiency promotes genomic instability in early stages of cancer development. *Cell* **145**, 435-446.

Blakeslee, A. F., Belling, J. and Farnham, M. (1920). Chromosomal duplication and Mendelian phenomena in Datura mutants. *Science* **52**, 388-390.

Brand, M. D. (2010). The sites and topology of mitochondrial superoxide production. *Experimental gerontology* **45**, 466-472.

Bravo, R., Parra, V., Gatica, D., Rodriguez, A. E., Torrealba, N., Paredes, F., Wang, Z. V., Zorzano, A., Hill, J. A. and Jaimovich, E. (2013). Endoplasmic reticulum and the unfolded protein response: dynamics and metabolic integration. *International review of cell and molecular biology*, vol. **301**, 215-290

Burrell, R. A., McClelland, S. E., Endesfelder, D., Groth, P., Weller, M.-C., Shaikh, N., Domingo, E., Kanu, N., Dewhurst, S. M. and Gronroos, E. (2013). Replication stress links structural and numerical cancer chromosomal instability. *Nature* **494**, 492.

Cao, Q., Zhao, K., Zhong, X. Z., Zou, Y., Yu, H., Huang, P., Xu, T.-L. and Dong, X.-P. (2014). SLC17A9 protein functions as a lysosomal ATP transporter and regulates cell viability. *Journal of Biological Chemistry* **289**, 23189-23199.

Cao, S. S. and Kaufman, R. J. (2014). Endoplasmic reticulum stress and oxidative stress in cell fate decision and human disease. *Antioxidants & redox signaling* **21**, 396-413.

Chan, K. L., Palmai-Pallag, T., Ying, S. and Hickson, I. D. (2009). Replication stress induces sister-chromatid bridging at fragile site loci in mitosis. *Nature Cell Biology* **11**, 753.

Choi, Y. and Lee, J.-H. (2011). The combination of tephrosin with 2-deoxy-D-glucose enhances the cytotoxicity via accelerating ATP depletion and blunting autophagy in human cancer cells. *Cancer biology & therapy* **12**, 989-996.

Collaco, A. M., Geibel, P., Lee, B. S., Geibel, J. P. and Ameen, N. A. (2013). Functional vacuolar ATPase (V-ATPase) proton pumps traffic to the enterocyte brush border membrane and require CFTR. *American Journal of Physiology-Cell Physiology* **305**, C981-C996.

Cunningham, J. T., Moreno, M. V., Lodi, A., Ronen, S. M. and Ruggero, D. (2014). Protein and nucleotide biosynthesis are coupled by a single rate-limiting enzyme, PRPS2, to drive cancer. *Cell* **157**, 1088-1103.

467   **Dephoure, N., Hwang, S., O'Sullivan, C., Dodgson, S. E., Gygi, S. P., Amon, A. and
468   Torres, E. M.** (2014). Quantitative proteomic analysis reveals posttranslational responses to
469   aneuploidy in yeast. *Elife* **3**, e03023

Diaz-Moralli, S., Aguilar, E., Marin, S., Coy, J. F., Dewerchin, M., Antoniewicz, M. R., Meca-Cortés, O., Notebaert, L., Ghesquière, B. and Eelen, G. (2016). A key role for transketolase-like 1 in tumor metabolic reprogramming. *Oncotarget* **7**, 51875.

Donnelly, N., Passerini, V., Dürrbaum, M., Stingele, S. and Storchová, Z. (2014). HSF1 deficiency and impaired HSP90-dependent protein folding are hallmarks of aneuploid human cells. *The EMBO journal* **33**, 2374-2387.

Eenjes, E., Dragich, J. M., Kampinga, H. H. and Yamamoto, A. (2016). Distinguishing aggregate formation and aggregate clearance using cell-based assays. *J Cell Sci* **129**, 1260-1270.

Gocheva, V., Zeng, W., Ke, D., Klimstra, D., Reinheckel, T., Peters, C., Hanahan, D. and Joyce, J. A. (2006). Distinct roles for cysteine cathepsin genes in multistage tumorigenesis. *Genes & development* **20**, 543-556.

Gordon, D. J., Resio, B. and Pellman, D. (2012). Causes and consequences of aneuploidy in cancer. *Nature Reviews Genetics* **13**, 189.

Hanahan, D. and Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell* **144**, 646-674.

Janssen, A. and Medema, R. (2013). Genetic instability: tipping the balance. *Oncogene* **32**, 4459.

Jiang, P. and Mizushima, N. (2014). Autophagy and human diseases. *Cell Res* **24**, 69-79.

Kim, C. H., Jeon, H. M., Lee, S. Y., Jeong, E. K., Ju, M. K., Park, B. J., Park, H. G., Lim, S.-C., Han, S. I. and Kang, H. S. (2010). Role of reactive oxygen species-dependent protein aggregation in metabolic stress-induced necrosis. *International journal of oncology* **37**, 97-102.

Kirkland, J. B. (2012). Niacin requirements for genomic stability. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* **733**, 14-20.

Kohnken, R., Kodigepalli, K. M. and Wu, L. (2015). Regulation of deoxynucleotide metabolism in cancer: novel mechanisms and therapeutic implications. *Molecular cancer* **14**, 176.

Landrum, E. and Wetzel, R. (2014). Biophysical underpinnings of the repeat length dependence of polyglutamine amyloid formation. *Journal of Biological Chemistry* **289**, 10254-10260.

Li, Y.-J., Lei, Y.-H., Yao, N., Wang, C.-R., Hu, N., Ye, W.-C., Zhang, D.-M. and Chen, Z.-S. (2017). Autophagy and multidrug resistance in cancer. *Chinese journal of cancer* **36**, 52.

Liu, D., Shaukat, Z., Xu, T., Denton, D., Saint, R. and Gregory, S. (2016). Autophagy regulates the survival of cells with chromosomal instability. *Oncotarget* **7**, 63913.

Malhotra, J. D. and Kaufman, R. J. (2007). Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword? *Antioxidants & redox signaling* **9**, 2277-2294.

Marchetti, M. A., Weinberger, M., Murakami, Y., Burhans, W. C. and Huberman, J. A. (2006). Production of reactive oxygen species in response to replication stress and inappropriate mitosis in fission yeast. *Journal of cell science* **119**, 124-131.

Mauvezin, C., Nagy, P., Juhász, G. and Neufeld, T. P. (2015). Autophagosome–lysosome fusion is independent of V-ATPase-mediated acidification. *Nature communications* **6**, 7007.

Minocherhomji, S., Ying, S., Bjerregaard, V. A., Bursomanno, S., Aleliunaite, A., Wu, W., Mankouri, H. W., Shen, H., Liu, Y. and Hickson, I. D. (2015). Replication stress activates DNA repair synthesis in mitosis. *Nature* **528**, 286.

Nishimura, Y., Sameni, M. and Sloane, B. F. (1998). Malignant transformation alters intracellular trafficking of lysosomal cathepsin D in human breast epithelial cells. *Pathology & Oncology Research* **4**, 283.

Ohashi, A., Ohori, M., Iwai, K., Nakayama, Y., Nambu, T., Morishita, D., Kawamoto, T., Miyamoto, M., Hirayama, T. and Okaniwa, M. (2015). Aneuploidy generates proteotoxic stress and DNA damage concurrently with p53-mediated post-mitotic apoptosis in SAC-impaired cells. *Nature communications* **6**, 7668.

Oromendia, A. B., Dodgson, S. E. and Amon, A. (2012). Aneuploidy causes proteotoxic stress in yeast. *Genes & development* **26**, 2696-2708.

Pfau, S. J. and Amon, A. (2012). Chromosomal instability and aneuploidy in cancer: from yeast to man. *EMBO reports* **13**, 515-527.

Pinton, P., Giorgi, C., Siviero, R., Zecchini, E. and Rizzuto, R. (2008). Calcium and apoptosis: ER-mitochondria Ca 2+ transfer in the control of apoptosis. *Oncogene* **27**, 6407.

Ryoo, H. D. (2015). Drosophila as a model for unfolded protein response research. *BMB reports* **48**, 445.

Samali, A., FitzGerald, U., Deegan, S. and Gupta, S. (2010). Methods for monitoring endoplasmic reticulum stress and the unfolded protein response. *International journal of cell biology* **2010**. doi:10.1155/2010/830307

Santaguida, S., Vasile, E., White, E. and Amon, A. (2015). Aneuploidy-induced cellular stresses limit autophagic degradation. *Genes & development* **29**, 2010-2021.

Senovilla, L., Vitale, I., Martins, I., Tailler, M., Pailleret, C., Michaud, M., Galluzzi, L., Adjemian, S., Kepp, O. and Niso-Santano, M. (2012). An immunosurveillance mechanism controls cancer cell ploidy. *Science* **337**, 1678-1684.

Shaukat, Z., Liu, D., Choo, A., Hussain, R., O'keefe, L., Richards, R., Saint, R. and Gregory, S. (2015). Chromosomal instability causes sensitivity to metabolic stress. *Oncogene* **34**, 4044-4055.

Shaukat, Z., Wong, H. W., Nicolson, S., Saint, R. B. and Gregory, S. L. (2012). A screen for selective killing of cells with chromosomal instability induced by a spindle checkpoint defect. *PloS one* **7**, e47447.

Sheltzer, J. M. (2013). A transcriptional and metabolic signature of primary aneuploidy is present in chromosomally unstable cancer cells and informs clinical prognosis. *Cancer research* **73**, 6401-6412.

Sheltzer, J. M., Ko, J. H., Replogle, J. M., Burgos, N. C. H., Chung, E. S., Meehl, C. M., Sayles, N. M., Passerini, V., Storchova, Z. and Amon, A. (2017). Single-chromosome gains commonly function as tumor suppressors. *Cancer cell* **31**, 240-255.

Sheltzer, J. M., Torres, E. M., Dunham, M. J. and Amon, A. (2012). Transcriptional consequences of aneuploidy. *Proceedings of the National Academy of Sciences* **109**, 12644-12649.

Siegel, J. J. and Amon, A. (2012). New insights into the troubles of aneuploidy. *Annual review of cell and developmental biology* **28**, 189-214.

Stingele, S. and Storchova, Z. (2013). Activation of autophagy in cells with abnormal karyotype. *Autophagy* **9**, 246-248.

St-Pierre, J., Buckingham, J. A., Roebuck, S. J. and Brand, M. D. (2002). Topology of superoxide production from different sites in the mitochondrial electron transport chain. *Journal of Biological Chemistry* **277**, 44784-44790.

van Eyk, C., McLeod, C.J., O' Keefe, L.V., Richards, R.I. (2012). Comparative toxicity of polyglutamine, polyalanine and polyleucine tracts in Drosophila models of expanded repeat disease. *Human Molecular Genetics* **21**,536-47.

Vaughn, A. E. and Deshmukh, M. (2008). Glucose metabolism inhibits apoptosis in neurons and cancer cells by redox inactivation of cytochrome c. *Nature Cell Biology* **10**, 1477.

Weaver, A. N. and Yang, E. S. (2013). Beyond DNA repair: additional functions of PARP-1 in cancer. *Frontiers in oncology* **3**, 290.

Weaver, B. A. and Cleveland, D. W. (2006). Does aneuploidy cause cancer? *Current opinion in cell biology* **18**, 658-667.

White, E. (2015). The role for autophagy in cancer. *Journal of Clinical Investigation* **125**, 42-6.

Zong, W.-X., Ditsworth, D., Bauer, D. E., Wang, Z.-Q. and Thompson, C. B. (2004). Alkylating DNA damage stimulates a regulated form of necrotic cell death. *Genes & development* **18**, 1272-1282.

Figure Legends

Figure 1: CIN cells are under ER stress.

ER stress was detected by the levels of chaperones (HSP83-GFP expression, **A**, **A'**), the activation of XBP1 by alternative splicing (XBP1-GFP expression, **B**, **B'**) and measuring intracellular Ca^{++} release (GCaMP3 signal, **C**, **C'**) in CIN (**A'**, **B'**, **C'**) versus normal (**A**, **B**, **C**) wing imaginal tissue. The dotted lines show the posterior compartment of a representative wing disc pouch region from each genotype. (**A**, **B**, **C**) control wing discs show background expression of HSP-83-GFP, XBP1-GFP and GCaMP3 in non-CIN cells. (**A'**, **B'**) Increased GFP levels which indicate elevated levels of chaperone (Hsp83-GFP) and XBP1-GFP were observed when CIN was induced by depletion of Rad21 (UAS-Rad21^{RNAi}, UAS-Dicer2). (**C'**) increased GCaMP signal was observed in CIN cells relative to wild type controls (**C**).

Figure 2: CIN increases protein folding stress via reactive oxygen species.

Increased aggregation of hard-to-fold poly-glutamine protein (myc-tagged CAG91 or CAG55) was observed in CIN cells. (**A**, **B**) Images show the anterior/posterior boundary of a representative wing disc from each genotype. Right of the dotted line is the *engrailed*-Gal4 expressing region in which myc-tagged poly-CAG peptides were expressed. (**A**) In cells expressing a shorter polyglutamine peptide (CAG55), aggregation was not observed, and mild CIN induction by Mad2 depletion had little, if any, effect on this (**A'**). (**B**) Some aggregation was observed of the hard-to-fold longer polyglutamine peptide CAG91 in otherwise wild type cells, which was greatly increased by the induction of CIN (*mad2*^{RNAi}, **B'**). (**C**, **C'**) Third instar imaginal discs were stained for Acridine Orange (AO) to indicate vesicle acidification, a common precursor to cell death. The dashed line indicates the posterior compartment expressing CAG55 or CAG91 peptides, while the unmarked anterior compartment was wild type in every disc. (**D**, **D'**) Imaginal discs in which CAG55 and CAG91 peptides were expressed in CIN cells (*mad2*^{RNAi}), gave rise to high levels of AO staining compared to the non-CIN controls. (**E**) Quantification of polyglutamine peptide aggregation shows that ROS significantly contribute to the proteotoxic stress generated in CIN cells. Aggregation of myc-tagged CAG55 increased when co-expressed with Catalase-RNAi, while aggregation decreased when catalase was overexpressed (UAS-Catalase). Overexpression of Catalase was able to strongly rescue the aggregation seen in CIN cells. The signal was normalized by subtracting the signal from the wild type anterior compartment of each disc. Error bars indicate the 95% CI. $n \geq 11$ in all cases. P-values were calculated by two-tailed t-tests with Welch's correction: **** $p < 0.0001$, *** $p < 0.001$.

Figure 3: **Nucleotide synthesis gene knockdowns affect CIN cells**.

(**A**) A quantitative analysis of Acridine Orange (AO) staining in third instar larvae wing discs knocked down for nucleotide synthesis enzymes. The y axis shows the normalized AO signals obtained by the subtracting the mean value of the control region from the affected region for each wing disc. Error bars represent 95% Confidence Intervals (CIs), n≥8 in all cases. P-values were calculated by two-tailed t-tests with Welch's correction: *** p<0.001. All tests compare candidate^RNAi *mad2*^RNAi with the negative control *mCherry*^RNAi *mad2*^RNAi. (**B**) Quantification of AO staining in candidate^RNAi imaginal wing discs with and without CIN (see C. below). The y axis shows the normalized AO signals obtained by the subtracting the mean value of the control region for each wing disc. Knockdown of the candidate in non-CIN cells is represented by grey bars. Candidate knockdowns in CIN cells are represented by black bars. Error bars represent 95% CIs, n≥8 in all cases. P-values were calculated by two-tailed t-tests with Welch's correction: p<0.0001= ****, p<0.01=**. (**C - G**) Acridine Orange staining (AO) of the third instar larvae wing discs. The dotted regions (*engrailed>Gal4*, *UAS-CD8-GFP, with or without UAS-mad2*^RNAi^) show the posterior compartment of wing disc in which the candidates were knocked down while the remainder of each wing disc is a wild type internal control (C) Negative control *mCherry mad2*^RNAi (D) Positive Control *G6PD mad2*^RNAi showing high levels of AO staining (E) *ADSS*^RNAi (E') *ADSS*^RNAi *mad2*^RNAi (F) *PRPS2*^RNAi (F') *PRPS2*^RNAi *mad2*^RNAi (G) *TKL*^RNAi (G') *TKL*^RNAi *mad2*^RNAi. Knock down of *ADSS*, *PRPS2* and *TKL* in CIN cells show high AO staining as compared to their knockdown in wild-type cells.

Figure 4: **The effect of depletion of nucleotide synthesis enzymes on oxidative stress and DNA damage in CIN cells**.

Reactive oxygen species (ROS) levels were visualized by CellRox staining in third instar larval wing discs. Images show the anterior/posterior boundary of a representative wing disc pouch region from each genotype. The dotted line shows the *engrailed*-Gal4 expressing knockdown area in each image and left of the dotted line is wild type (**A**) *G6PD* ^RNAi *mad2*^RNAi positive control showing elevated ROS levels (**B, C, D**) We did not detect elevated ROS when nucleotide synthesis candidates were depleted in CIN cells. The experiment was repeated twice. (**E-I**) Third instar larvae wing discs were stained with anti-phosphorylated H2AvD (γH2AX) antibody staining to measure the level of DNA damage. The dashed line indicates the posterior compartment expressing (**G**) *ADSS*^RNAi, (**H**) *PRPS2*^RNAi, (**I**) *TKL*^RNAi in CIN cells, (**E**) negative control *mCherry*^RNAi and (**F**) positive control *PASK*^RNAi. The unmarked anterior compartment is wild type in each disc. (**J**) A quantitative analysis of P-H2AvD staining in third instar larval wing disc knockdowns of nucleotide synthesis enzymes in

CIN cells. The y axis shows the number of P-H2AvD positive puncta cells in the engrailed-driven knockdown region, normalized by subtracting the number of stained cells in the control region for each disc. Elevated rates of P-H2AvD staining were seen when nucleotide synthesis enzymes were depleted in CIN cells. Error bars represent 95% CIs, n≥8 in all cases. P-values were calculated by two-tailed t-tests with Welch's correction: **** p<0.0001. All tests compare candidate^{RNAi} $mad2^{RNAi}$ with $mCherry^{RNAi}$ $mad2^{RNAi}$.

Figure 5: **Depletion of nucleotide synthesis enzymes did not cause apoptosis in CIN cells**.

Death caspase-1 antibody was used to test for apoptosis in wing discs in which candidates were knocked down in CIN cells (dashed region). (**A**) Positive Control *PASK mad2*^{RNAi} showed elevated apoptosis; (**B**) negative control *mCherry mad2*^{RNAi} , (**C**) *ADSS*^{RNAi} *mad2*^{RNAi}, (**D**) *PRPS2*^{RNAi} *mad2*^{RNAi}, (**E**) *TKL*^{RNAi} *mad2*^{RNAi} did not. (**F**) Quantification of Dcp-1 staining of positive control and candidate-RNAi imaginal wing discs. The y-axis shows the Dcp-1 stained cells in the engrailed driven region normalized by subtracting the number of stained cells in control region of each wing disc. The error bar indicates the 95% CIs, n≥10 in all cases. The p values were calculated by two-tailed t-tests with Welch's correction. ns=non-significant. No significant elevation in apoptosis was seen when nucleotide synthesis enzymes were depleted in CIN cells. The experiment was repeated twice and confirmed by using Caspase3 antibody staining (Fig. S1).

Figure 6: Nucleotide candidate knockdowns caused the accumulation of lysosomes, but not always autophagy, in CIN cells.

Lysotracker staining was used to detect lysosomes. Quantification of lysotracker staining is shown in (**A**). The y axis shows the number of lysotracker puncta in the *en*-driven knockdown region, normalized by the subtracting the number of puncta in the control region for each wing disc. The black bars represent the candidate knockdowns in CIN cells (*en>mad2*^{RNAi}) and grey bars represent the candidate knockdowns in wild type cells. Error bars indicate 95% CIs, n≥8 in all cases. The P values were calculated by two-tailed t-test with Welch's correction: p<0.0001 ****, p<0.001 ***. Representative images are shown in Figure S4. (**B**) Quantification of the level of mCherry-Atg8a puncta, measured as for lysotracker. In all cases n≥10 and error bars show 95% CIs. The p values were calculated using two-tailed t-tests with Welch's correction: p<0.001 ***, p<0.05 *, n.s.=non-significant. Representative images for this data are shown in Figure S4.

Figure 7: **Feeding ATP to larvae depleted for some nucleotide synthesis enzymes rescued their AO phenotype**.

(**A**) Graph showing that depletion of *ADSS*, *PRPS2* or *JNK* in CIN cells (induced by *mad2* ^{RNAi}) showed high AO staining, which was rescued by feeding the larvae with rATP (1mM). No rescue was observed in ATP fed larvae of genotype *TKL*^{RNAi} *mad2*^{RNAi}. Male larvae with high CIN induced
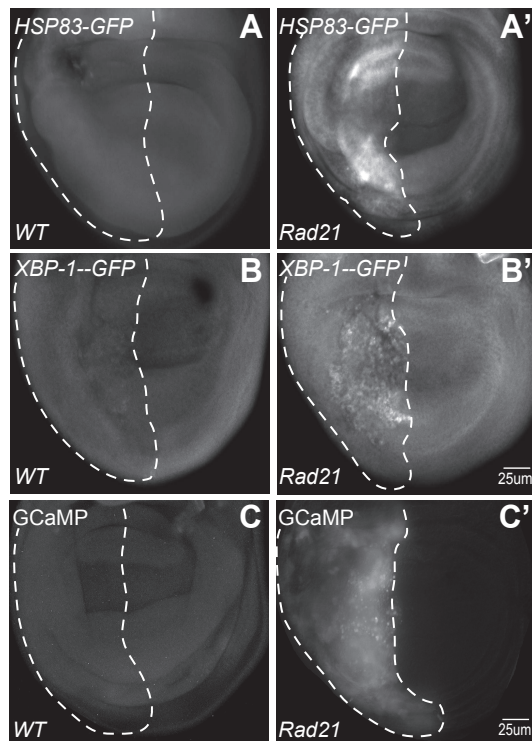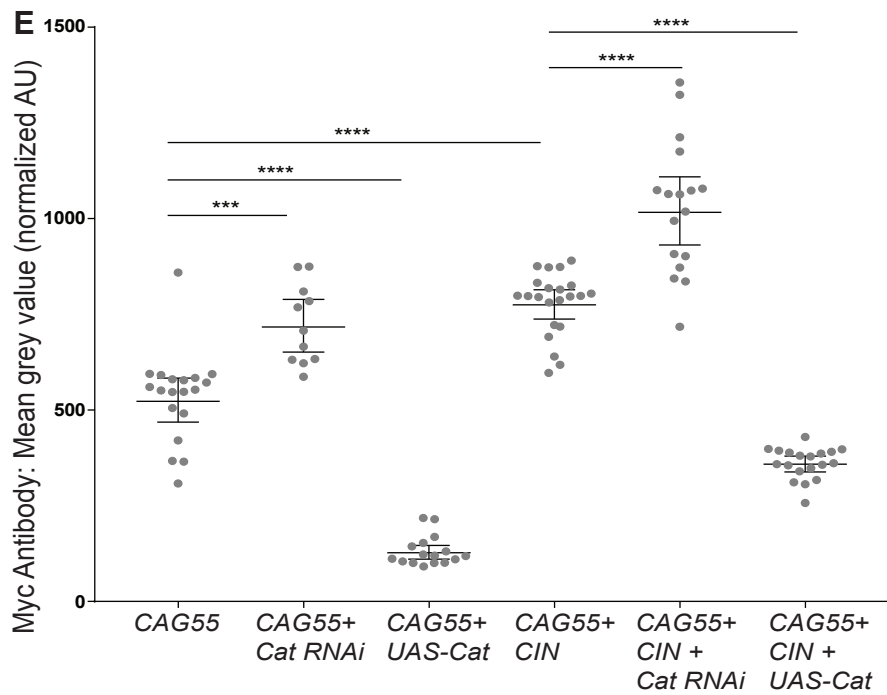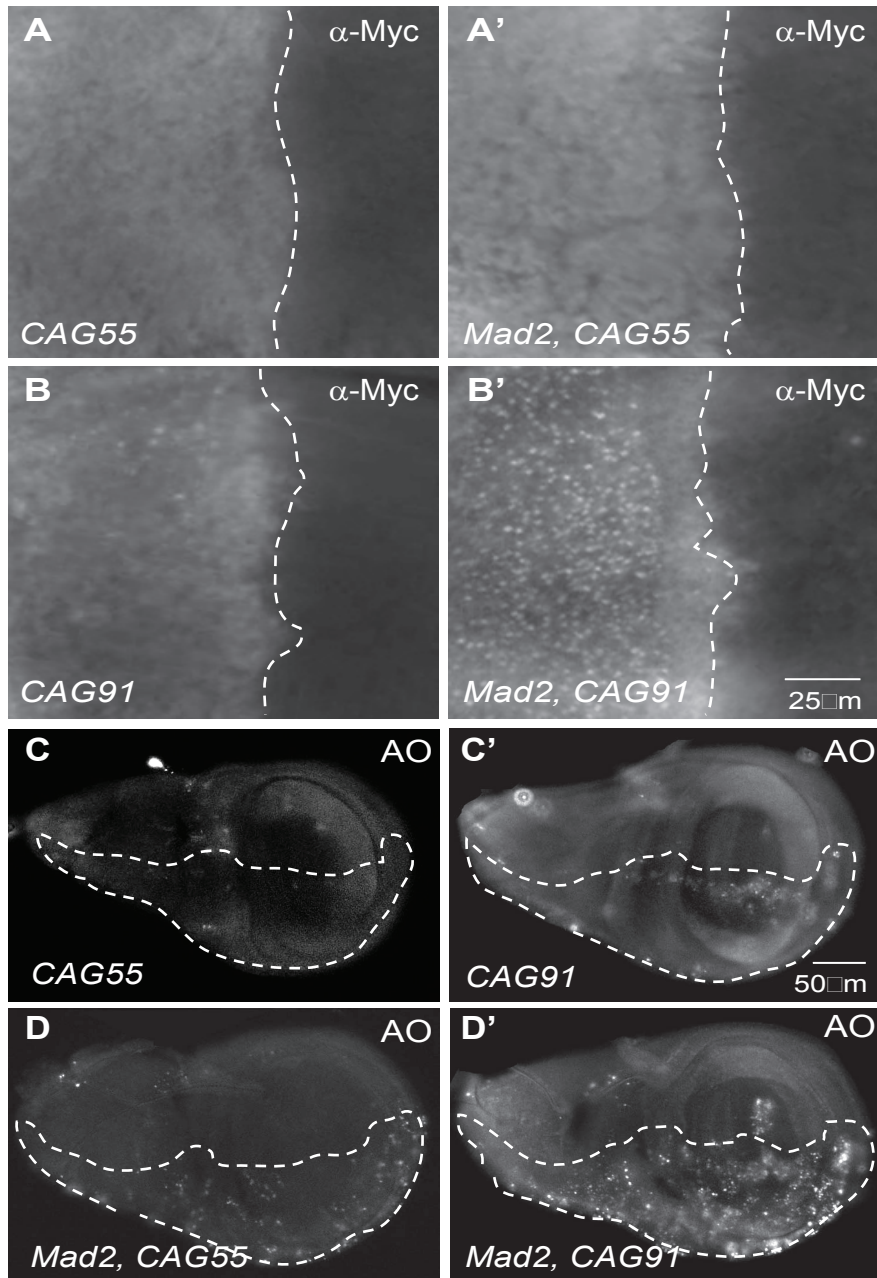
by Rad21 depletion (UAS-*rad21*[RNAi], UAS-*Dicer2*) also showed high AO staining that could be rescued by rATP feeding, while feeding ATP to female larvae of the same genotype did not show reduction in their AO phenotype. Candidate knockdowns in CIN cells are represented by black bars and rATP fed candidate knockdowns in CIN cells are represented by grey bars. The error bars represent the 95% CIs, n ≥10 in all cases. The experiment was repeated three times. Representative images are shown in Figure S6. (**B**) Feeding the larvae with dATP (1mM) rescued the AO phenotype in ADSS and PRPS2 knockdowns in CIN cells (grey bars) as compared to their controls (black bars). No rescue was observed in dATP fed larvae of genotypes *TKL*[RNAi]*mad2*[RNAi] and in high-CIN larvae (UAS-*rad21*[RNAi], UAS-*Dicer2*). The error bars represent the 95% CIs, n ≥10 in all cases. The p values were calculated by two-tailed t-tests with Welch's correction: $p < 0.0001$ ****, $p < 0.01$ **, $p < 0.05$ *, ns=non-significant. This experiment was repeated twice. Representative images are shown in Figure S7. (**C**) Graph showing Quinacrine (QA, a marker for lysosomal ATP) staining on larval wing discs. High QA staining was observed, when nucleotide synthesis candidates were depleted in CIN cells (dark grey bar) as compared to candidates knocked down in non-CIN cells (light grey bar). Wing discs were incubated with bafilomycin (150nM) for 30 min and then stained with QA (15uM). Candidates' knockdown in CIN cells (black bar) show significantly lower QA staining after bafilomycin treatment. Quantifications show the normalized grey value of staining, which is obtained by subtracting the mean grey value of wild type from the affected region of each disc. Error bar indicate 95% CIs, n ≥10 in all cases. The p values were calculated by two-tailed t-test with Welch's correction: $p < 0.0001$ ****, $p < 0.001$ ***, $p < 0.01$ **, ns=non-significant. Representative images are shown in Figure S8. (**D**) Graph showing that blocking v-ATPase by bafilomycin (75nM) inhibits the AO phenotype in nucleotide candidate knockdowns in CIN cells. When wing discs of candidate knockdowns in CIN cells were incubated with bafilomycin for 30 min it significantly reduced the AO staining in these discs compared to controls. Quantifications show the normalized grey value of staining, obtained by subtracting the mean grey value of wild type from the mean of the affected region of each disc. The error bars represent 95% CIs, n ≥12 in all cases. The p values were calculated by two-tailed t-tests with Welch's correction. This experiment was repeated twice. Representative images are shown in Figure S9.
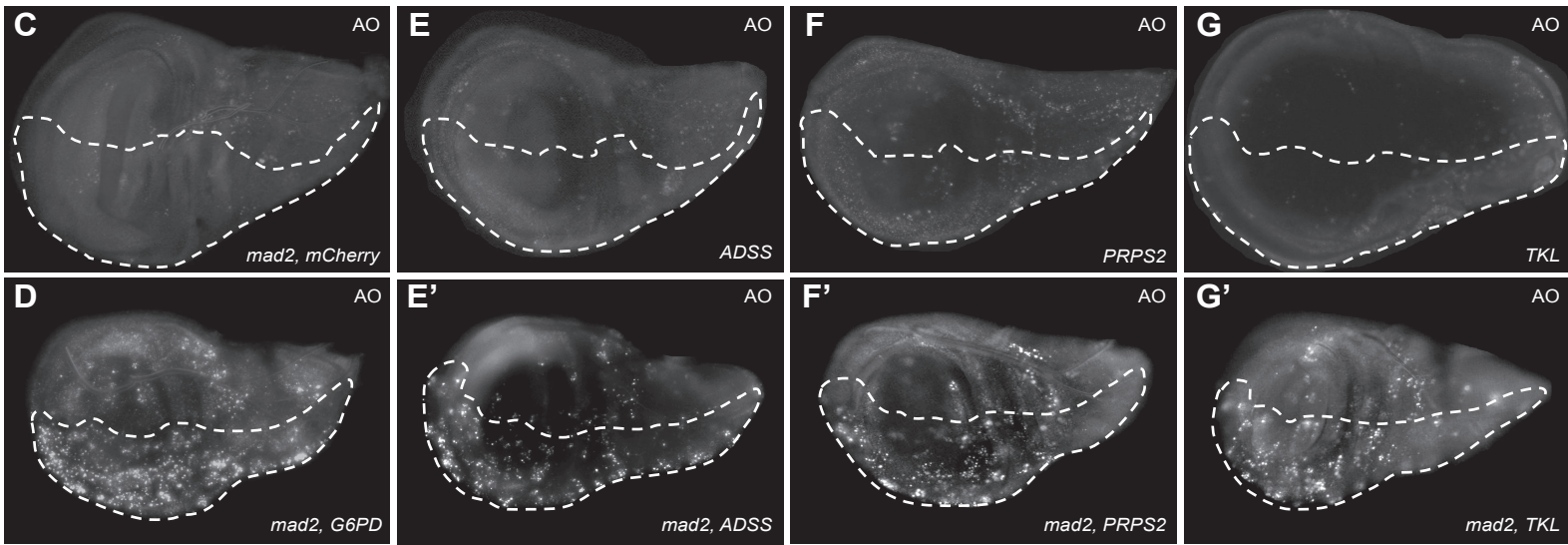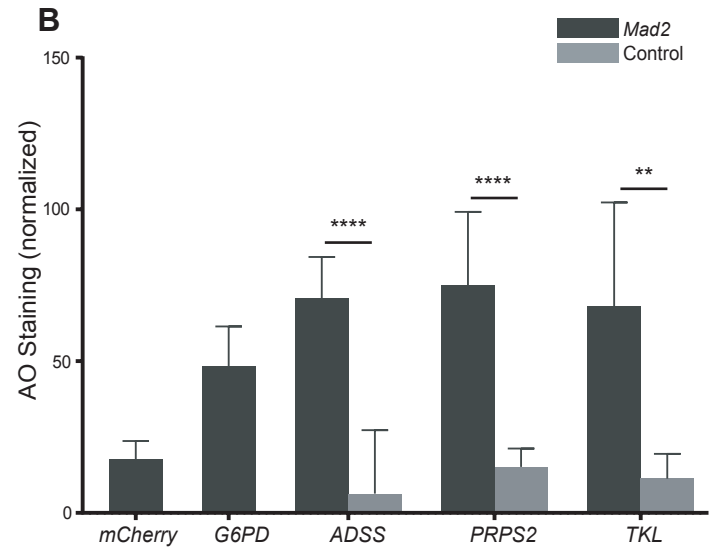
Figure 8: Blocking v-ATPase causes cell death in nucleotide depleted CIN cells

470   (**A**) A model for the effect of depletion of nucleotide synthesis enzymes on CIN cells. Loss of ADSS,

471   PRPS2 or TKL decrease ATP synthesis and contribute to DNA damage in CIN cells, which are

472   already ATP depleted by PARP activation. (**B**) Dcp-1 antibody staining was used to detect the level

473   of apoptosis in bafilomycin treated wing discs of candidate knockdowns in CIN cells. The graph

474   shows that depletion of ADSS and PRPS2 significantly increased the level of apoptosis in CIN cells

475   after treatment with bafilomycin. Depletion of TKL caused a non-significant increase in apoptosis in

476   bafilomycin treated CIN cells. In all cases n≥10 and error bars show 95% CIs around the mean. The

477   p values were calculated by two-tailed t-tests with Welch's correction. Representative images are

478   shown in Figure S10. (**C**) Graph showing that PARP knockdown in CIN cells (induced by Rad21

479   depletion) significantly rescued the AO phenotype in these cells, which in this case we know

480   represents apoptosis (Liu et al., 2015). Quantifications show the normalized grey value of staining

481   obtained by subtracting the mean grey value of wild type from the affected half of each disc. In all

482   cases n≥23 and error bars show 95% CIs around the mean. The p values were calculated by two-

483   tailed t-tests with Welch's correction. Representative images are shown in Figure S11. (**D**)

484   Nicotinamide feeding rescued the AO phenotype caused by nucleotide synthesis enzyme depletion in

485   CIN cells. Quantification showing that the AO phenotype of ADSS, PRPS2 and TKL depletion in

486   CIN cells was significantly rescued by feeding the larvae with Nicotinamide (1mM). A similar

487   reduction was observed in high CIN cells (induced by Rad21 depletion). In all cases n≥12 the error

488   bar show 95% CIs around the mean. The p values were calculated by two-tailed t-tests with Welch's

489   correction. This experiment was repeated twice. Representative images are shown in Figure S12.
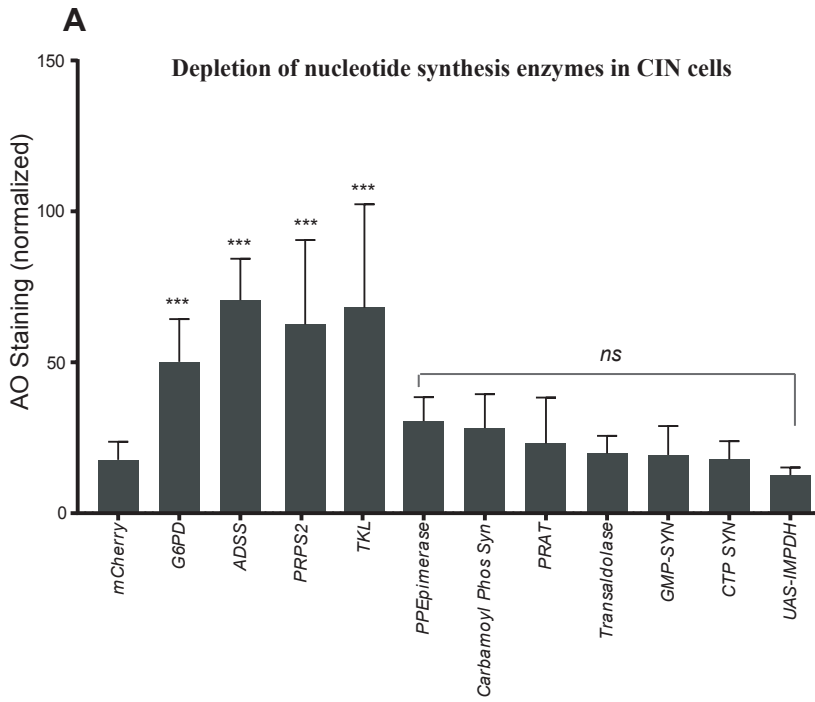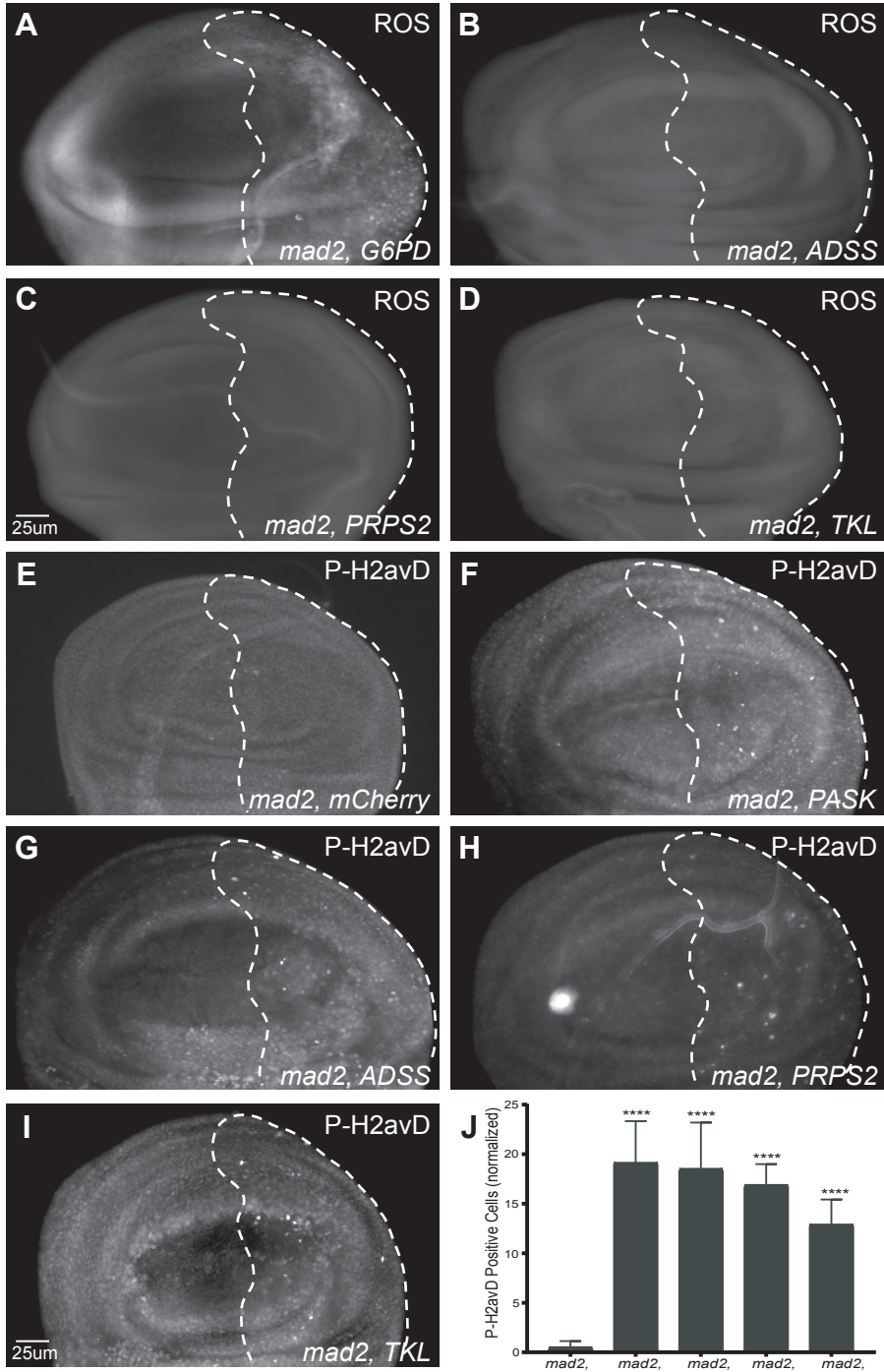
HSP83-GFP — A — WT

HSP83-GFP — A' — Rad21

XBP-1--GFP — B — WT

XBP-1--GFP — B' — Rad21 — 25um

GCaMP — C — WT

GCaMP — C' — Rad21 — 25um

A α-Myc   CAG55

A' α-Myc   Mad2, CAG55

B α-Myc   CAG91

B' α-Myc   Mad2, CAG91   25 μm

C AO   CAG55

C' AO   CAG91   50 μm

D AO   Mad2, CAG55

D' AO   Mad2, CAG91

E

Myc Antibody: Mean grey value (normalized AU)

CAG55 | CAG55+ Cat RNAi | CAG55+ UAS-Cat | CAG55+ CIN | CAG55+ CIN + Cat RNAi | CAG55+ CIN + UAS-Cat

A

Depletion of nucleotide synthesis enzymes in CIN cells

AO Staining (normalized)

*** G6PD
*** ADSS
*** PRPS2
*** TKL

ns

mCherry, G6PD, ADSS, PRPS2, TKL, PPEpimerase, Carbamoyl Phos Syn, PRAT, Transaldolase, GMP-SYN, CTP SYN, UAS-IMPDH

B

AO Staining (normalized)

Mad2
Control

**** ADSS
**** PRPS2
** TKL

mCherry, G6PD, ADSS, PRPS2, TKL

C AO
mad2, mCherry

D AO
mad2, G6PD

E AO
ADSS

E' AO
mad2, ADSS

F AO
PRPS2

F' AO
mad2, PRPS2

G AO
TKL

G' AO
mad2, TKL

A — Dcp-1 — *mad2, PASK*

B — Dcp-1 — *mad2, mCherry*

C — Dcp-1 — *mad2, ADSS*

D — Dcp-1 — *mad2, PRPS2*

E — Dcp-1 — 50um — *mad2, TKL*

F — Death Caspase-1 (normalized) — **** — ns — ns — ns — *mad2, PASK* — *mad2, mCherry* — *mad2, ADSS* — *mad2, PRPS2* — *mad2, TKL*

A

Purine biosynthesis

Glucose → G6P

Ribulose-5-P ← **PP Pathway** / **G6PD** ← G6P

Ribulose-5-P → Ribose-5-P

Ribose-5-P ← **TKL** ← F6P

Ribose-5-P → Phospho Ribosylpyro P (**PRPS2**)

Phospho Ribosylpyro P → 5-Phosphoribosyl amine (**PPAT**)

5-Phosphoribosyl amine ⇢ IMP

IMP → Adenylsuccinate

Adenylsuccinate → ATP (**ADSS**)

G6P → F6P → Glyceraldehyde-P ⇢ Glycolysis

TKL → Glyceraldehyde-P

CIN → **PARP** (↓NAD, ↓ATP) → DNA Damage

DNA Damage ⇢ ATP

B

Dcp-1 signals (normalized)

- *Mad2*
- *Mad2*+bafilo

Pepck, mCherry, ADSS (**), PRPS2 (***), TKL (*ns*)

C

Acridine Orange (normalized)

CIN, CIN+*PARP* (****)

D

Acridine Orange (normalized)

- *Ctrl*
- NAM

mad2 mCherry, mad2 ADSS (****), mad2 PRPS2 (***), mad2 TKL (**), Rad21 (****)

Figure S1: (**A**) Quantification of XBP1-GFP expression. Increased GFP levels which indicate XBP1-GFP were observed when CIN was induced by depletion of *en*>Rad21 (UAS-Rad21^RNAi, UAS-Dicer2) compared to the control *en*>UAS-XBP1-GFP. The y axis shows the normalized anti-GFP staining obtained by the subtracting the mean value of the control region from the mean of the affected region for each wing disc. The error bars indicate the 95% confidence 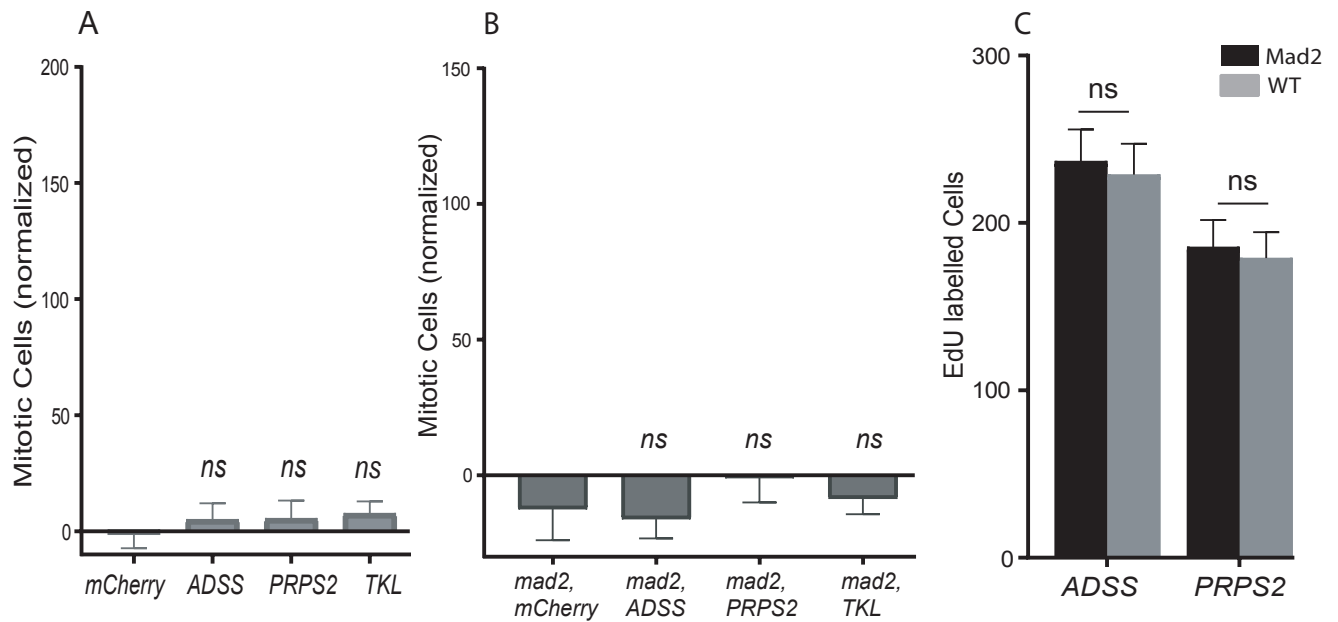interval, n≥9 in all cases. The p values were calculated by two-tailed t-tests with Welch's correction. (**B**) Quantification of GCaMP3 signals. Increased GCaMP signal was observed in *en*>Rad21 (UAS-Rad21^RNAi, UAS-Dicer2 relative to wild type controls. The y axis shows the normalized GCaMP3 signals obtained by the subtracting the mean value of the control region from the mean of the affected region for each wing disc. The error bars indicate the 95% confidence interval, n=8 in all cases. (**C**) Quantification of ROS staining. The y axis shows the normalized CellROX staining obtained by the subtracting the mean value of the control region from the mean of the affected region for each wing disc. The error bars indicate the 95% confidence interval, n≥8 in all cases. Genotypes tested were (*engrailed* driven) positive control *G6PD mad2*^RNAi, negative control *mCherry mad2*^RNAi, *ADSS*^RNAi *mad2*^RNAi, *PRPS2*^RNAi *mad2*^RNAi, *TKL*^RNAi *mad2*^RNAi (**d, e, f, g, h**) Caspase3-driven apoptosis was not detected when candidates were depleted in CIN cells. Anti-cleaved caspase 3 antibody was used to measure the apoptosis in third instar larval wing discs. In every disc, the unmarked region does not express RNAi constructs, while the dashed line shows the area affected by CIN (*mad2-RNAi*) and depletion of candidates. Genotypes tested were (d) positive control *PASK mad2*^RNAi (e) negative control *mCherry mad2*^RNAi (f) *ADSS*^RNAi *mad2*^RNAi (g) *PRPS2*^RNAi *mad2*^RNAi (h) *TKL*^RNAi *mad2*^RNAi.
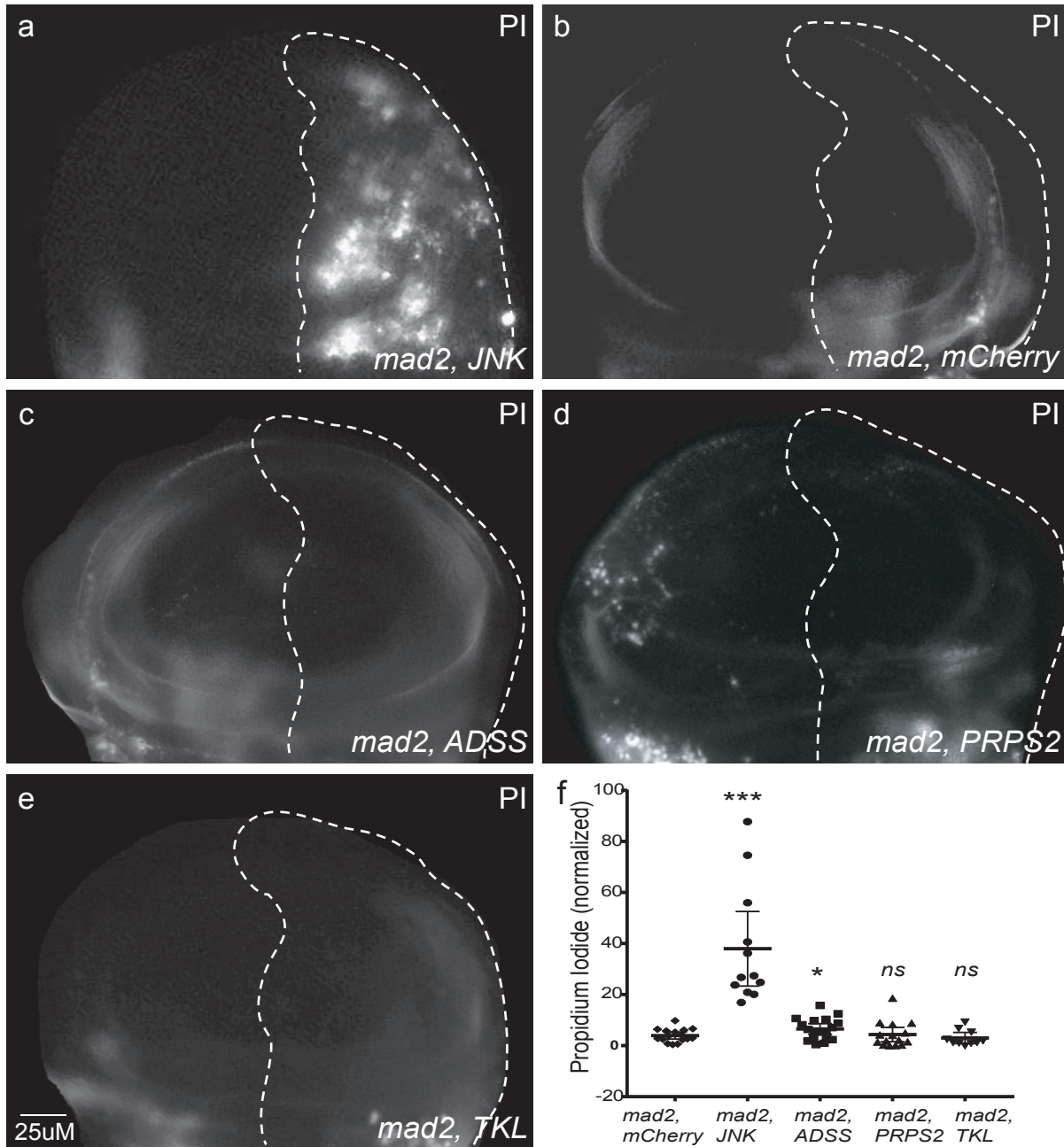
Figure S2: Propidium Iodide (PI) staining was used to measure the level of necrosis in nucleotide depleted CIN cells. The candidate knockdowns in the posterior half of each wing disc are indicated by the dotted line; the remainder of each disc was wild type. PI staining was high in positive control (a) $JNK^{RNAi}mad2^{RNAi}$, while (b) negative control $mCherry\ mad2^{RNAi}$, and the other genotypes (c) $ADSS^{RNAi}mad2^{RNAi}$, (d) $PRPS2^{RNAi}mad2^{RNAi}$, (e) $TKL^{RNAi}mad2^{RNAi}$ were negative for PI staining. (f) Quantification of PI staining. The y axis shows the normalized PI staining obtained by the subtracting the mean value of the control region from the mean of the affected region for each wing disc. The error bars indicate the 95% confidence interval, n≥10 in all cases. The p values were calculated by two-tailed t-tests with Welch's correction. p <0.001= ***.

Figure S3: The effect of nucleotide depletion on the cell cycle in CIN cells. Phospho-Histone 3 antibody staining was used to detect the mitotic cells in CIN larval wing discs with nucleotide candidate knockdowns. (**A**) no significant difference was observed in the frequency of mitotic cells in wing discs of control and genotype ADSS[RNAi], PRPS2[RNAi] and TKL[RNAi] when these candidates depleted with or without CIN as shown in graph (**B**). The error bar represents the 95% CIs, n≥8 in all cases. The p values were calculated by two-tailed t-tests with Welch's correction. (**C**) Quantification of EdU labelling on nucleotide synthesis deficient CIN cells. EdU labelling was used to detect the S-Phase cells in CIN larval wing discs with nucleotide candidate knockdowns. No significant difference was observed in the frequency of S-Phase cells in wing discs of control and genotype *ADSS*[RNAi] and *PRPS2*[RNAi] when these candidates depleted with or without CIN as shown in graph.
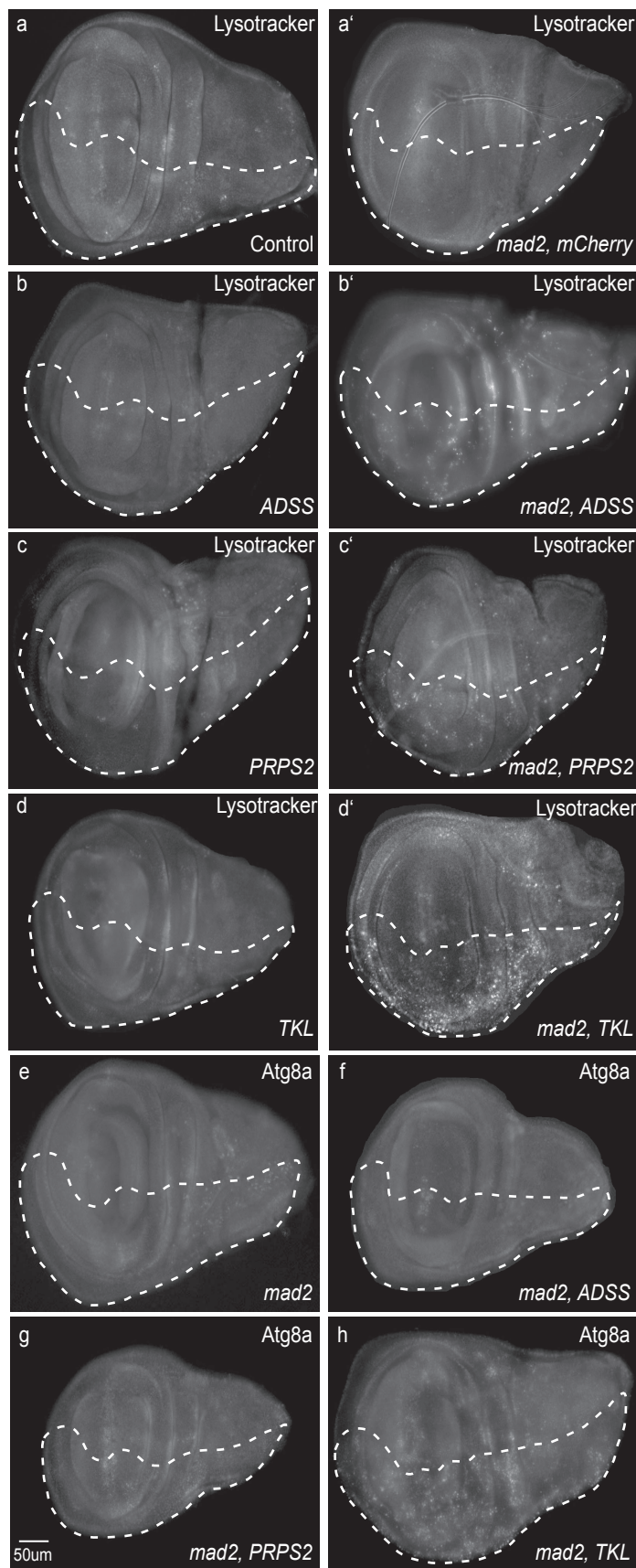
Figure S4: Third instar larvae wing discs were stained with lysotracker and mCherry-Atg8a puncta level detection to test for effects on lysosomes. In every disc, the unmarked region does not express RNAi constructs, while the dashed line shows the area affected by CIN (*mad2-RNAi*) and depletion of nucleotide synthesis enzymes. Control wings (a, a') show no lysotracker staining when CIN is induced. (b, c, d) Imaginal discs in which candidates have been depleted giving rise to no lysotracker staining. (b', c', d') Imaginal discs showing that when candidates were depleted in CIN cells high lysotracker was observed compared to controls. (e) negative control *UAS-mCherry-Atg8 UAS-mad2*[RNAi] (f, g) Wing discs of *ADSS*[RNAi] and *PRPS2*[RNAi] with CIN induced by mad2 depletion (*engrailed > Gal4, UAS-CD8-GFP, UAS-mCherry-Atg8 UAS-mad2*[RNAi]) show no induction of autophagy (mCherry-Atg8a puncta). (h) Depletion of TKL in CIN background shows a high level of mCherry-Atg8a puncta, indicating the activation of autophagy
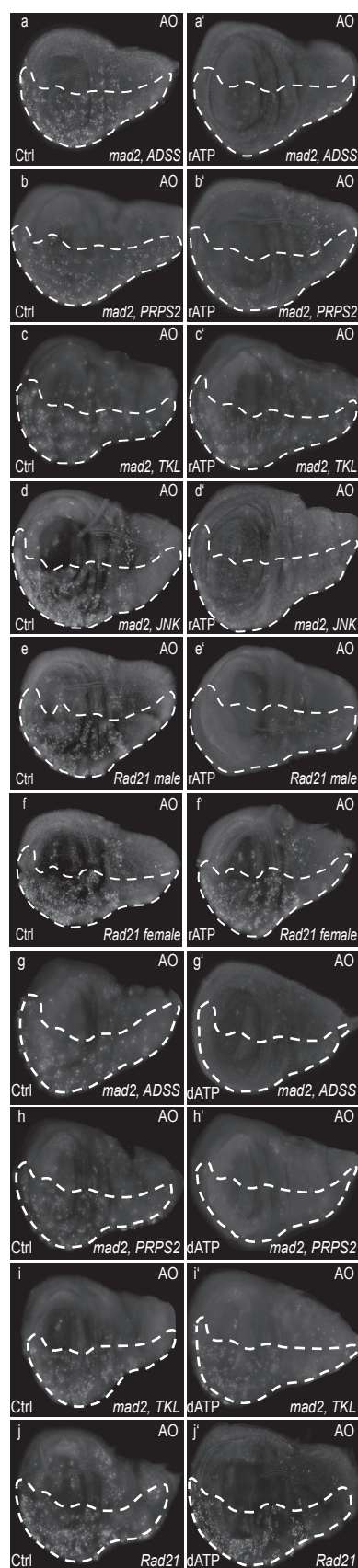
Figure S5: Depletion of nucleotide synthesis enzymes in CIN cells showed high AO staining which was rescued by feeding the larvae with rATP and dATP (1mM). (a, b, c, d) controls wing disc of candidates $ADSS^{RNAi}$, $PRPS2^{RNAi}$, TKL^{RNAi} and JNK^{RNAi} depleted in CIN cells induced by $mad2^{RNAi}$. (a', b', d') wing discs of rATP fed larvae of $ADSS^{RNAi}mad2^{RNAi}$, $PRPS2^{RNAi}mad2^{RNAi}$ and $JNK^{RNAi}mad2^{RNAi}$ which show reduction in the AO phenotype. (c') no rescue was observed in ATP fed larvae of genotype $TKL^{RNAi}mad2^{RNAi}$. (e, f) control wing discs of male and female larvae with high CIN induced by Rad21 depletion (UAS-$Rad21^{RNAi}$, UAS-$Dicer2$) show a high AO phenotype but (e') feeding rATP to male larvae of genotype (UAS-$Rad21^{RNAi}$, UAS-$Dicer2$) rescues the AO phenotype in wing discs while (f') female larvae of same genotype did not show a reduction in the AO phenotype. Feeding larvae with dATP (1mM) rescued the AO phenotype in ADSS and PRPS2 knockdowns in CIN cells (g', h') as compared to their controls (g, h). No rescue was observed in dATP fed larvae of genotypes $TKL^{RNAi}mad2^{RNAi}$ and (UAS-$Rad21^{RNAi}$, UAS-$Dicer2$) (i', j') as compared to their controls (i, j).
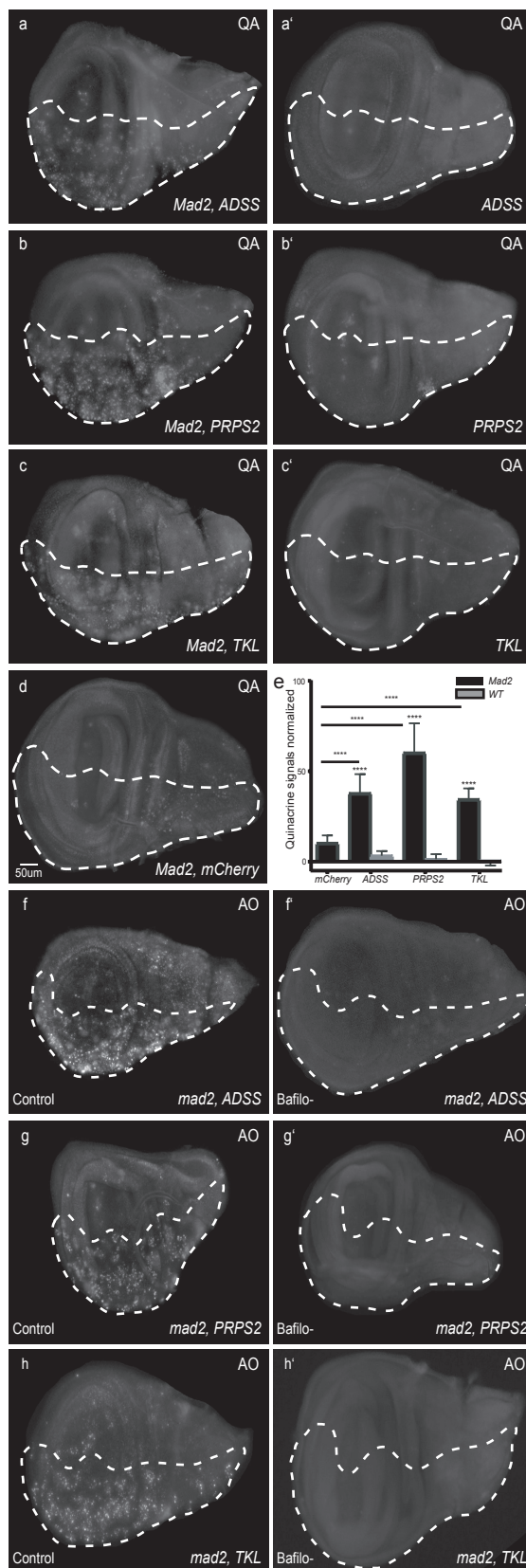
Figure S6: Effect of quinacrine (QA) staining on larval wing discs with depletion of nucleotide synthesis enzymes in CIN cells. All wing discs were stained with QA (50uM). The dotted line shows the *en*>CD8GFP marked posterior compartment in which the genes were depleted. The other half of each disc serves as a wild type internal control. (a, b, c,) high QA staining was observed, when nucleotide candidates ADSS, PRPS2 and TKL were depleted in CIN cells as compared to candidates knocked down in non-CIN cells (a', b', c'). (d) Control disc of *mCherry mad2*[RNAi.] (e) Quantification of QA stainings show the normalized grey value of staining, obtained by subtracting the mean grey value of the wild type from the affected region of each disc. The error bars represent the 95% CIs, n ≥10 in all cases. The p values were calculated by two-tailed t-tests with Welch's correction. (f, g, h) control wing discs of nucleotide synthesis enzymes ADSS, PRPS2 and TKL depleted in CIN cells show high AO phenotypes. (f', g', h') wing discs of same genotype treated with bafilomycin (75nM) treatment for 30 min significantly reduced the AO staining.
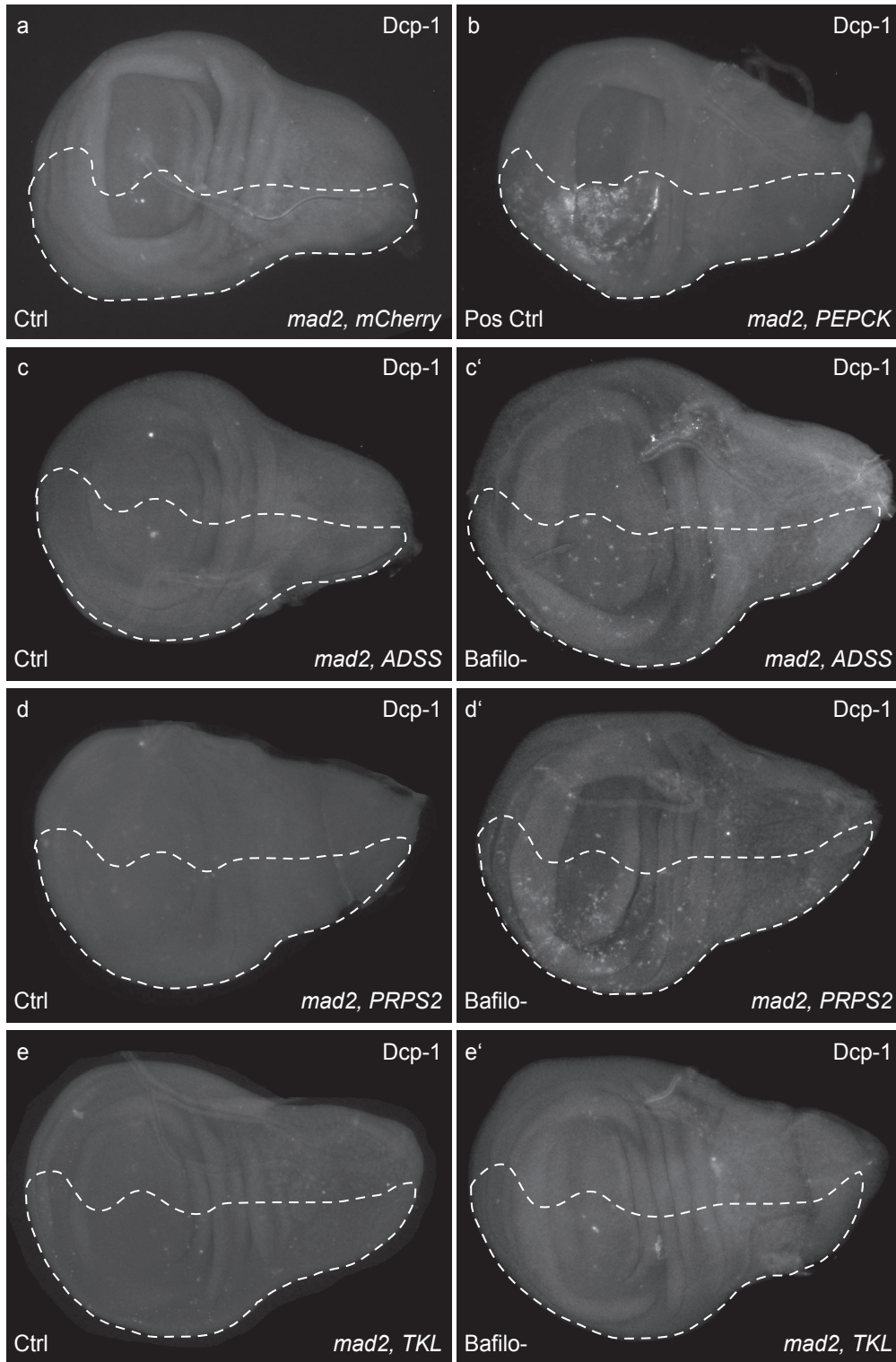
Figure S7: Blocking v-ATPase activity causes some apoptosis in nucleotide depleted CIN cells. Dcp-1 antibody staining was used to detect the level of apoptosis in bafilomycin treated wing discs of candidates' knockdowns in CIN cells. (a) Negative control (b) positive control *PEPCK*[RNAi] *mad2*[RNAi]. (c, d, e) wing discs of knockdowns of ADSS, PRPS2 and TKL in CIN cells without bafilomycin treatment show no apoptosis. (c', d') Knockdown of ADSS and PRPS2 significantly increased the level of apoptosis in CIN cells after treatment with bafilomycin. (e') knockdown of TKL in CIN cells did not show significant apoptosis in these proliferating cells.
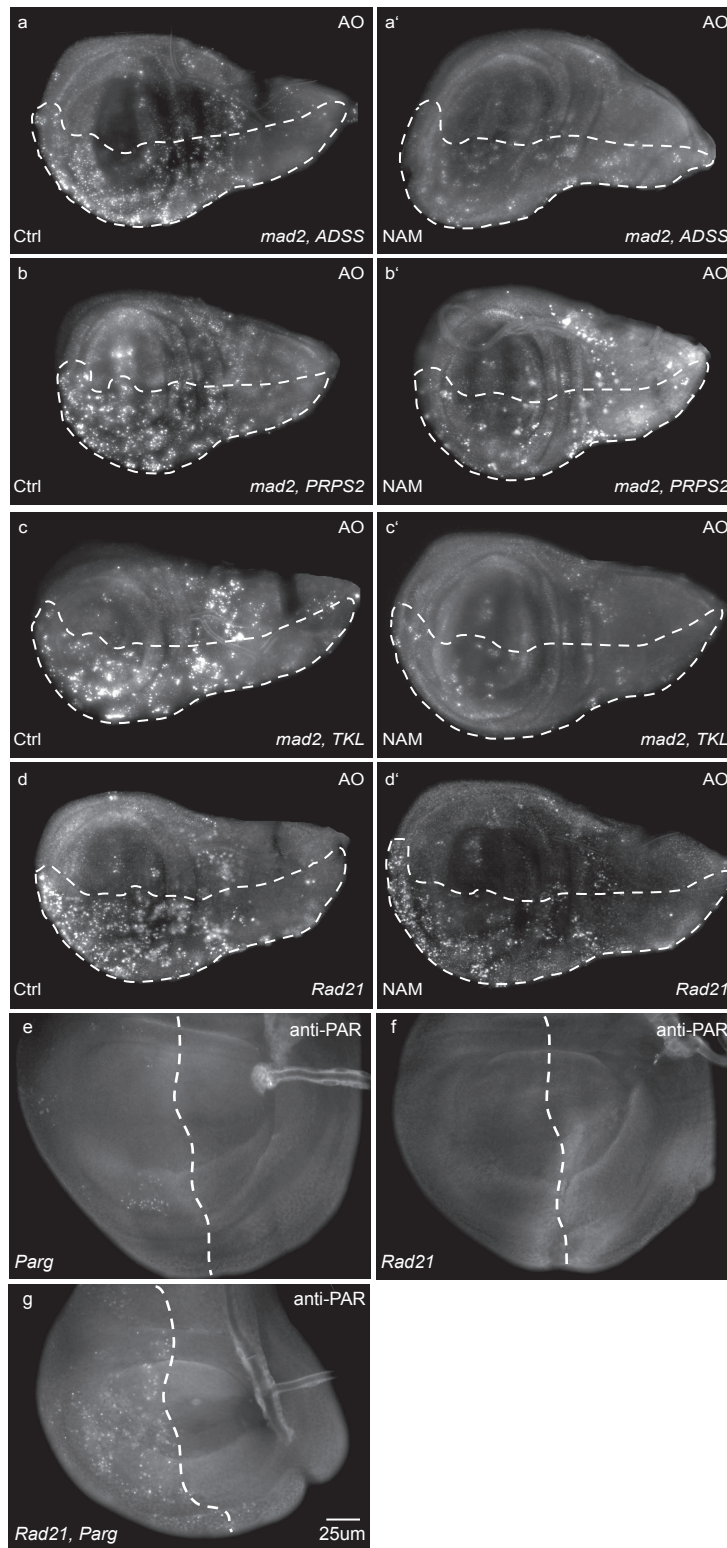
Figure S8: Nicotinamide rescues the AO phenotype in CIN cells. (a, b, c) represent the AO staining in control wing discs without NAM treatment of candidates *ADSS*[RNAi], *PRPS2*[RNAi] and *TKL*[RNAi] in non-CIN cells. (a', b', c',) feeding the larvae with NAM (1mM) significantly rescued the AO in wing disc of genotype *ADSS*[RNAi], *PRPS2*[RNAi] and *TKL*[RNAi] in CIN cells. (d) Wing discs with high CIN induced by Rad21 depletion showed high AO staining. (d') feeding NAM to the larvae with high CIN caused reduced AO staining. (g) increased in anti-PAR antibody staining in Parg with high-CIN cells (UAS-*Rad21*[RNAi], UAS-*Dicer2*) was observed compared to the controls alone (e, f).

DISCUSSION

Chromosomal instability (CIN) is a hallmark of cancer cells in which they fail to maintain their stable chromosomal number and/or integrity. CIN is frequent in most human solid tumours and is linked to tumorigenesis, metastasis, worse prognosis, relapse and drug resistance (Bakhoum and Compton, 2012, Hanahan and Weinberg, 2011a). CIN is common in cancer cells but not in normal cells, thus, it has been hypothesized that targeting CIN provides an opportunity to specifically target cancer cells that exhibit CIN without affecting normal cells as well as limiting the ability of tumours to develop drug resistance in late stage cancers. In our lab we have used *Drosophila melanogaster* as a model organism to study CIN. We generated different levels of CIN by either weakening spindle assemble checkpoint function or depleting *cohesin* genes. *mad2* is an essential spindle checkpoint gene and its knockdown in *Drosophila* causes lagging chromosomes and chromosome bridges during cell division in > 25% of brain cells without affecting overall viability (Shaukat et al., 2012). *Rad21* is involved in chromatid cohesion during mitosis and its co-expressing with dicer2 (to enhance the level of RNAi knockdown) in proliferating wing discs cells led to aneuploidy in 46% of metaphase cells, indicating a higher rate of CIN with this approach (Liu et al., 2015)

Our lab has carried out a genome-wide screen for kinases and phosphatases that when depleted, cause CIN-dependent apoptosis. This screen identified candidate genes that belonged to various pathways such as the JNK pathway, the DNA damage response pathway, centrosomal function, and metabolic pathways that are required to maintain the survival of CIN cells (Shaukat et al., 2012, Wong et al., 2014). Identification of clinically significant cancer targets such as Nek2 from our screen confirms that the screen has the potential to select promising therapeutic targets for cancer treatment (Kokuryo et al., 2007).

 CIN and aneuploidy are known to cause proteotoxic and oxidative stress (Oromendia et al., 2012b). CIN cells adapt to high stress conditions by increasing their glucose consumption, which could also lead to increased mitochondrial output. We have observed that CIN cells are sensitive to metabolic disruption. Depletion of metabolic candidates such as PEPCK (a key enzyme of gluconeogenesis) or G6PD gave increased mitochondrial output, oxidative stress, DNA damage and apoptosis in CIN cells without affecting normal cells. Having found metabolic targets that were able to induce CIN-specific cell death, we further tested the effect of these candidates on CIN tumours in order to understand their mechanism of action in the context of a growing CIN tumour. We observed that knockdown of candidates such as *G6PD* and *JNK* gave elevated cell death and smaller primary tumours, however they were not able to prevent the growth of CIN tumours and their metastases. Interestingly, depletion of

PEPCK, however, was able to completely hamper the growth of these tumours. PEPCK depletion was consistent with PEPCK inhibitors and their ability to stop tumour growth (Chapter 3). These result were encouraging, 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., 2007), but it is also known to be expressed in non-gluconeogenic tissues as well as in cancer cells, suggesting PEPCK may have other roles (Previs et al., 2009). Many cancers such as neuroendocrine prostate cancer and uterine carcinoma express elevated levels of PEPCK despite having high hexokinase activity for glycolysis. In addition, whether PEPCK is a rate-limiting factor in gluconeogenesis is still unclear. Rognstad (1979) found that inhibiting PEPCK with mercaptopicolinic acid stops gluconeogenesis, but later studies found a limited effect of PEPCK inhibition on gluconeogenic rate (Rognstad, 1979, Argaud et al., 1991). Some recent studies found that PEPCK is involved in glucose metabolism in tumour growth and proliferation (Montal et al., 2015, Leithner et al., 2014, Leithner, 2015). These studies showed that PEPCK is involved in catabolism of glutamine in the absence of glucose. It was observed that PEPCK converts oxaloacetate to phosphoenolpyruvate which can support the TCA cycle and biosynthetic intermediates for tumour growth and survival in glucose limited conditions (Leithner, 2015, Vincent et al., 2015), and enhance glycolysis when glucose is available. Major metabolic intermediates like pyruvate, glutamate, citrate, fumarate, phosphoenolpyruvate, and malate were decreased with PEPCK deficiency (Vincent et al., 2015, Leithner, 2015, Montal et al., 2015). However, these studies did not address the glycerogenic role of PEPCK. PEPCK is one of the rate limiting steps in glyceroneogenesis (Hanson and Reshef, 2003, Bartok et al., 2015). 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 (Shaukat et al., 2015). Bartok et al., (2015) also found PEPCK inhibition lowered glucose, glycerol and TAG levels in the animal suggesting an effect of PEPCK on lipid metabolism (Bartok et al., 2015). Our data suggest that the glyceroneogenic role of PEPCK is an important factor to restrict tumour growth by affecting ROS production and manipulating the rate of glycolysis by affecting levels of cytoplasmic NADH.
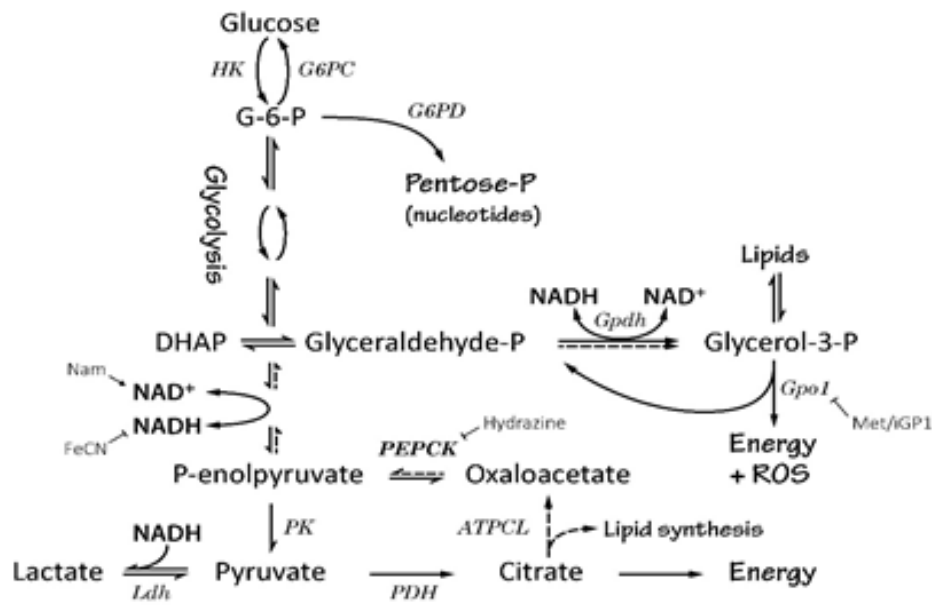
Figure 5.1 Proposed model for the role of PEPCK in CIN cancer cells. *CIN cells are vulnerable to metabolic interventions that increase ROS. Depletion of PEPCK increases cytoplasmic NADH, which drives ROS via mitochondrial GPDH. High ROS and high NADH blocks glycolysis, so PEPCK depletion is effective in CIN cells.*

NADH is made from NAD+ during glycolysis in proliferating cells, and must be oxidized for glycolysis to continue. NADH can also be oxidized by converting pyruvate to lactate, but this option is limited in proliferating cells because pyruvate is needed for energy and lipid synthesis. Our data reveals that the glycerol-3-phosphate shuttle is the primary pool for NADH production: Glycerol phosphate dehydrogenase is utilised by this shuttle in order to oxidize cytoplasmic NADH through mitochondria. This process permits glycolysis to continue, however, by doing so it produces ROS in the mitochondria via glycerophosphate oxidase (GPO1) (Miwa et al., 2003, Orr et al., 2012). We know that CIN cells have elevated ROS levels and depend on antioxidants for survival (Shaukat et al., 2015). Consistent with this finding, we observed that feeding hosts inhibitors to the shuttle (hydrazine or iGP1) or RNAi mediated knockdown (*GPDH*-RNAi or *PEPCK*-RNAi) lowers ROS levels. Inhibition of GPO1 reduced ROS levels as well as limiting the growth of *brat* tumours by preventing the oxidation of cytoplasmic NADH because in this case the shuttle was unavailable for NADH oxidation (Hussain et al., 2017). In contrast, we observed that lowering ROS by feeding the host antioxidants (propyl gallate) was able to rescue tumour growth: in this scenario the shuttle is available without the toxic consequences of high ROS. These findings suggest that

the mechanism of PEPCK depletion to block CIN tumour growth is through inhibition of the oxidation of cytoplasmic NADH by glyceroneogenesis, and that elevated NADH generates more redox stress than the CIN cells are able to cope with. Further, feeding nicotinamide (added NAD+) to the host animal, rescued the growth in PEPCK depleted *brat* tumours, suggesting that in these tumours, the glycerol phosphate shuttle has not been able to adequately oxidise NADH, regardless of the availability of glucose to fuel this pathway. Oxidizing cytoplasmic NADH by feeding with ferricyanide had the same effect. This suggests that growth was being limited by the accumulation of NADH. By using the appropriate level of PEPCK and GPO1 inhibitors we could completely inhibit the growth of these aggressive CIN tumours.

A plausible contributor to the effect of CIN on cells under oxidative stress is aneuploidy. Trisomy is known to give inappropriate gene expression levels and stress responses in diverse organisms, consistent with perturbation in the endoplasmic reticulum (ER) resulting in protein folding problems (Sheltzer et al., 2012, Oromendia et al., 2012a). We are also interested to understand the causal relationships between aneuploidy, oxidative stress and protein folding because chromosomal instability and its consequent aneuploidy are common, tumour-specific phenotypes that offer the prospect of similar tumour-specific therapies. We have observed increased chaperone levels (Hsc70, Hsp83) and ER stress markers (XBP1) in aneuploid cells and we hypothesize that aneuploidy causes oxidative stress due to altered metabolism and this oxidative stress enhances the proteotoxic effect of aneuploidy. Our data also confirms that aneuploid cells suffer protein folding stress such that chaperones are incapable of resolving additional stress (i.e. aggregation-prone poly-glutamine (polyQ) proteins).

Published evidence demonstrated that in ER-stressed cells, Ca^{2+} released from the ER is taken up by mitochondria and releases cytochrome c which then inhibits complex III of the ETC and increases ROS production. Moreover, increased Ca^{2+} in the mitochondria stimulates Krebs cycle dehydrogenases, thus increasing the oxygen consumption and ROS production. Mitochondrial Ca^{2+} also activates nitric oxide synthase, whose product disturbs the ETC and enhances ROS generation (Brand, 2010, St-Pierre et al., 2002). However it is not clear whether ER stress generates ROS or ROS generates stressed ER or both. We wished to address this question and observe whether the stressed ER generates ROS (by Ca^{2+} release) and whether ROS generates stressed ER (by damaging proteins). We added antioxidants to CIN cells and measured the level of ROS and ER stress expression (Chapter 4). We found that antioxidants rescued protein folding stress in CIN cells, demonstrating that ROS is responsible for protein folding stress in CIN cells, which could be rescued by antioxidants.

Nucleotide stress has been linked to CIN and cancer development. Nucleotide pool disequilibrium results in genetic abnormalities and oncogenic transformation. In order to identify the genes of nucleotides pathways that could be involved in regulating the fate of CIN cells, we tested a range of genes affecting purine biosynthesis (Adenylosuccinate synthetase (ADSS), phosphoribosyl-pyrophosphate synthetase2 (PRPS2), GMP Synthetase, IMP dehydrogenase, PRAT), pyrimidine biosynthesis (CTP Synthetase, Carbamoyl Phosphate Synthetase) and the PP pathway (Transketolase (TKL), Transaldolase, PP Epimerase). We selected the candidates on the basis of their acridine orange (AO) phenotype. AO, a metachromatic dye, has long been known as a convenient non-specific tracer of acidic vesicles. This dye readily crosses plasma membranes as well as membranes of cytoplasmic vesicles and becomes trapped in vesicles with low internal pH (Pierzyńska-Mach et al., 2014). Its mechanism of accumulation in acidic vesicles involves molecule protonation in the interior of an acidic vesicle. The positive charge impedes the its ability to cross the vesicle membrane. Hence, the protonated dye molecules are trapped inside the acidic vesicles and reach significantly higher local concentrations than in the cytoplasm. Such brightly stained vesicles can be readily detected and traced by fluorescence microscopy. The fluorescence spectra of AO make it a sensitive marker to measures the changes in the cells. The change in AO staining was reliable indicator of cell injury or death (Plemel et al., 2017).

The knockdown of ADSS, phosphoribosyl-pyrophosphate synthetase (PRPS2) and TKL gave significantly higher AO staining in CIN cells. The nucleotide candidates such as PRPS2 and ADSS play a central metabolic role in the synthesis of nucleotides. The PRPS enzyme adds a pyrophosphate group from ATP to ribose-5-phosphate generated from the PP pathway to produce a nucleotide precursor called 5-phosphoribosyl-1-pyrophosphate (PRPP). PRPP is a substrate for all nucleotide salvage pathway enzymes, as well as for the rate-limiting enzymes of purine and pyrimidine biosynthesis. Modulation of PRPS levels by either knockdown or overexpression respectively inhibited or potentiated nucleotide production, suggesting that levels of PRPP may be sufficient to govern the overall rate of purine metabolism. Notably, *PRPS1 and PRPS2* are two isoforms of PRPP synthetase expressed in somatic tissues. Interestingly, the PRPS2 isoform is largely resistant to the allosteric feedback inhibition by the nucleotide biosynthetic pathway products ADP and GDP (Nosal et al., 1993). This enzymatic property of PRPS2 may facilitate the PRPS2-expressing cells to continue production of nucleotides when their intracellular concentrations are elevated. PRPS2 activity is more sensitive to fluctuations in ATP concentration, elevated in Myc-overexpressing B cells and less

sensitive to feedback inhibition by purine nucleotides biosynthesis products than PRS1 which explains why the levels of PRPS2, but not PRPS1, are increased in cancer cells (Nosal et al., 1993). Genetic knockout of PRPS2 gave a normal physiology in mice. However, is essential for Myc-driven tumorigenesis (Cunningham et al., 2014). ADSS is downstream of the de novo pathway and it is a vital component of the de novo pathway as well as the salvage pathway. ADSS is a highly conserved enzyme among all living organisms, it converts inosine monophosphate (IMP) to adenosine monophosphate (AMP) as part of ATP biosynthesis.

These high AO phenotypes in nucleotide candidate knockdowns in CIN cells suggest that CIN cells are sensitive to changes in nucleotide levels. In addition, the increased Lysotracker and DNA damage phenotype was also observed in nucleotide candidate knockdowns of ADSS, PRPS2 and TKL in a CIN background. However, the involvement of ROS and cell cycle arrest were not observed when these candidates were depleted in CIN cells. Cell death either by apoptosis or necrosis was also not observed when these candidates were depleted in CIN cells. Therefore, we suspected that the autophagy pathway could be activated in CIN cells. Autophagy is activated in response to various stresses including nutrient starvation (White, 2015, Jiang and Mizushima, 2014). We found that CIN cells are dependent on the autophagy of mitochondria to tolerate their aneuploidy and avoid triggering innate immune signalling (Liu et al., 2016). For example, blocking the autophagy pathway in CIN cells led to an increased number of dysfunctional mitochondria, increased levels of oxidative stress, DNA damage and apoptosis, while enhancing autophagy could reduce the level of ROS and apoptosis (Chapter 4). So we hypothesized that autophagy might be triggered in response to nucleotide depletion. Surprisingly, autophagy was not detected in ADSS and PRPS2 deficient CIN cells. However, elevated puncta of mCherry-tagged Atg8a were observed in TKL deficient CIN cells, suggesting that autophagy is activated in that case.

TKL is a pivotal enzyme of the PP pathway. The PP pathway is connected with glycolysis and mainly involved in the production of ribose-5-phosphate and NADPH via the non-oxidative and oxidative arms, respectively (Figure 5.2). TKT is a reversible enzyme of the non-oxidative arm of the PP pathway which catalyses the common two-substrate reactions. First it transfers two-carbon groups from xylulose-5-phosphate to ribose-5-phosphate to generate sedoheptulose-7-phosphate to glyceraldehyde-3-phosphate. Second, TKL transfers two-carbon groups from xylulose-5-phosphate to erythrose-4-phosphate to generate glyceraldehyde-3-phosphate and fructose-6-phosphate, which re-enter glycolysis. When cells are in need of nucleotides, the PP pathway produces ribose through the oxidative as well as the nonoxidative

arm from fructose-6-phosphate and glyceraldehyde-3-phosphate (Figure 5.2). Increased expression of TKL was reported in various cancers such as cervical, lung, gastric, colorectal, and endometrial cancers (Krockenberger et al., 2010, Schultz et al., 2008, Staiger et al., 2006). Induced expression of TKL1 in advanced colorectal and urothelial cancer is associated with malignancy, invasiveness, therapeutic resistance and poor prognosis (Schwaab et al., 2011). Published evidence suggested that TKL1 also enhances the PPP flux for biosynthetic reactions, as the PPP generates ribose-5-phosphate for nucleic acid synthesis and NADPH for fatty acid synthesis and maintaining redox homeostasis to protect cells against oxidative stress and apoptosis (Vaughn and Deshmukh, 2008, Diaz-Moralli et al., 2016).

Other studies also showed that the non-oxidative arm of PPP to be the major provider of PRPP, with more than 70% of nucleic acid ribose in several human cancer cell lines derived through the TKL, transaldolase, and triose phosphate isomerase reactions and inhibition of TKL suppress the PP pathway and interrupt the synthesis of important coenzymes such as ATP, CoA, NAD(P)+, FAD, and genetic material, RNA and DNA in cancer cells (Cascante et al., 2000, Comín-Anduix et al., 2001, Saha et al., 2014). Though these findings suggest TKL may control purine synthesis and represent a potential target for cancer therapy.
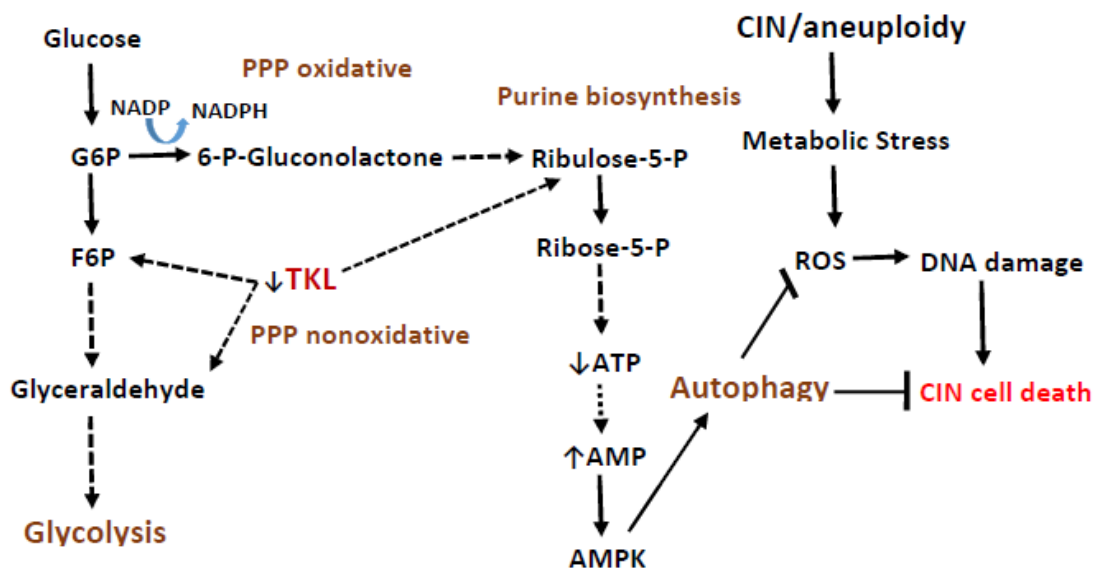

Figure 5.2: A model for triggering autophagy in CIN cells by TKL. CIN cells are sensitive to nucleotide stress. Transketolase (TKL, depletes the level of ATP which leads to disequilibrium in ATP and AMP ratio that activates AMPK and triggers autophagy, which required for CIN cell survival.

Besides the importance of TKT in the nucleotide production, a recent study showed that TKL has been implicated in the regulation of autophagy via interacting with Atg1 kinase protein and changes in the endogenous TKT activity in *Dictyostelium discoideum* strains lacking or overexpressing Atg1, suggesting that additional metabolic pathways between autophagy and the pentose phosphate pathway influence the regulation of autophagy in *D. discoideum* (Mesquita et al., 2015). In this study, we found that depletion of TKL in CIN cells triggered autophagy, so we proposed that depletion of TKL decreased the level of ATP (Figure 5.2). Low ATP and high AMP levels activate AMPK and trigger autophagy which is needed for CIN cell survival (Chapter 4).

We observed high lysotracker staining in ADSS and PRPS2 deficient CIN cells. Lysosomes play a crucial role in cancer progression by regulating complex processes involving protein secretion, endocytic receptor recycling, energy metabolism, and cell signalling (Appelqvist et al., 2013). Moreover, the autophagy–lysosome pathway is closely linked with the hallmarks of cancer including escaping cell death pathways, evading immune surveillance, and deregulating metabolism (Hanahan and Weinberg, 2011b). Advanced cancer cells are extremely reliant on effective lysosomal function. As a consequence, cancer progression and metastasis are related with unusual changes in lysosomal compartments, such as lysosome volume, composition, cellular distribution, and lysosomal enzyme activity, as compared with normal cells (Nishimura et al., 1998, Gocheva et al., 2006). Thus, targeting lysosomes in cancer has great therapeutic potential because it not only triggers apoptotic and lysosomal cell death pathways but also inhibits autophagy. Furthermore, lysosomes contribute to the development of drug resistance by sequestering cancer drugs in their acidic environment which reduces the effect of the drug (Gotink et al., 2011). Therefore, the lysosomes are thought of as an Achilles' heel of cancer cells. Furthermore, consistent with the lysotracker staining, high quinacrine (a lysosomal ATP marker) was observed in knockdown of nucleotide synthesis enzyme in CIN cells, suggesting lysosomal induction in these cells (chapter 4). However, as expected, when we blocked lysosomes by inhibiting v-ATPase, the CIN cells died. These results suggest that in the absence of autophagy, lysosomes worked hard to compensate for the lack of autophagy and when we blocked lysosomes the CIN cells died. So, we concluded that CIN cells use lysosomes to compensate for AMP-blocked autophagy loss. However it remains unclear how loss of AMP blocks autophagy in CIN cells.

Moreover, depletion of ADSS and PRPS2 decreased the level of AMP which further led to depletion of ATP in CIN cells (Figure 5.3). So, we examined whether feeding both rATP and dATP to ADSS and PRPS2 deficient CIN larvae would have an effect on the AO phenotype. First, we fed rATP to larvae with double knockdown of ADSS and PRPS2. We observed that in larvae knocked down for ADSS or PRPS2 feeding rATP significantly rescued the AO phenotype in CIN cells relative to their controls. Similarly, we fed dATP to larvae knocked down for ADSS or PRPS2. We observed a significant reduction in the AO phenotype in knockdown of ADSS and PRPS2 in CIN cells. These results suggest that CIN cells are sensitive to ATP changes. To further understand why ATP might be limited in CIN cells we tested for the Poly (ADP-ribose) Polymerase (PARP) and found that it is upregulated in CIN cells.

In addition, our preliminary screen for candidates that are needed for death in response to high aneuploidy levels has identified several members of the dosage compensation complex (e.g. Unr, Msl1, Mof, roX1, CLAMP, JIL-1). Significantly, we have observed that loss of Unr, CLAMP and JIL-1 can lower ROS levels and rescue cell death in aneuploid cells (unpublished data). The chromatin modifiers such as CLAMP, JIL-1 are associated with aneuploidy sensing and regulation of redox response through PARP. CLAMP depletion in highly aneuploid cells rescues oxidative stress and lethality by reducing the level of PARP, suggesting CLAMP uses PARP to induce aneuploidy specific ROS and cell death. Similarly we also observed rescue of oxidative stress and cell death when we knockdown PARP in CIN cells. The PARP enzyme PARylates a variety of protein substrates and alters their interaction with DNA and other proteins. PARP is currently in clinical trials for DNA damage sensitive cancers because of its role in the DNA damage response (Weaver and Yang, 2013). PARP activation causes depletion of its substrate, which further leads to loss of ATP (Zong et al., 2004) . We found that adding ATP or nicotinamide (a precursor in the synthesis of NAD+) rescued the AO phenotype in CIN cells. These results suggest that ATP and NAD+ are consumed for PARP activation and CIN cell proliferation (Figure 5.3).
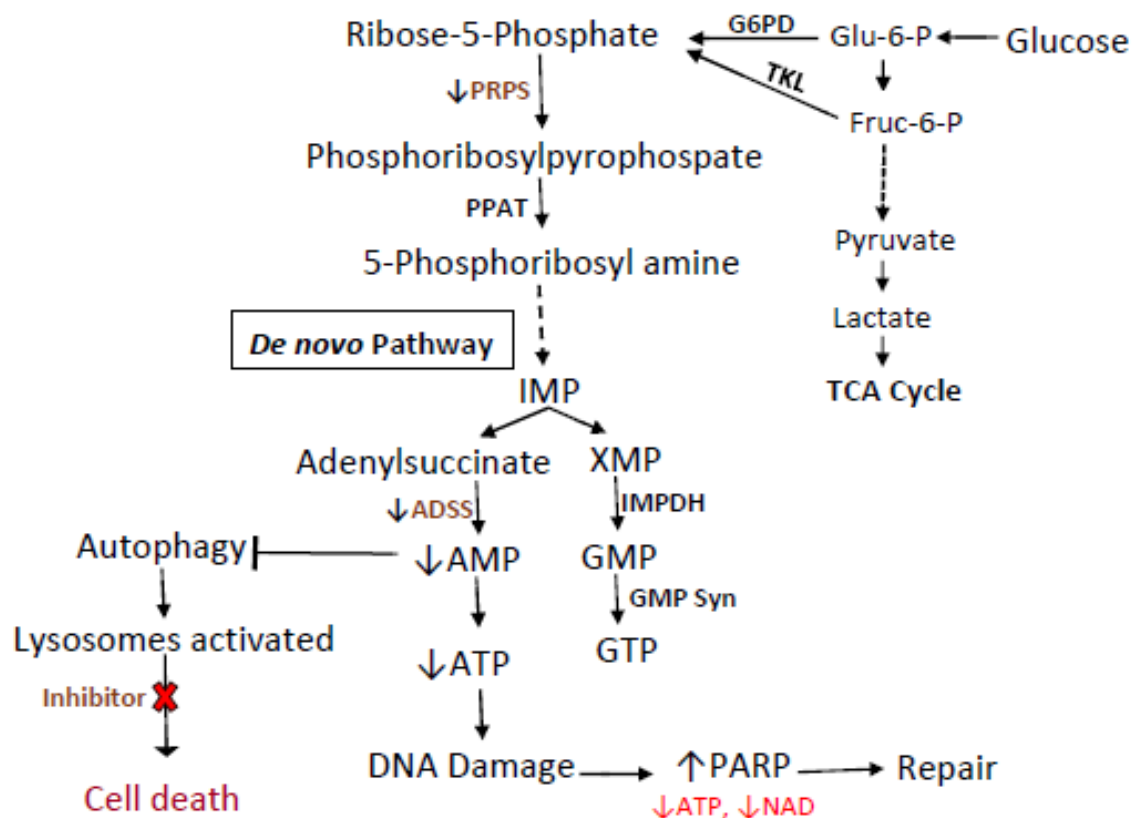
Figure 5.3: CIN cells are sensitive to nucleotide stress. PRPS2 and ADSS, deplete the nucleotide level and cause DNA damage as a result PARP is activated for repair. ADSS depletion in CIN cells decreases the AMP level which could not activates AMPK and trigger autophagy, so in absence of autophagy lysosomes work hard to compensate autophagy in CIN cell.

We proposed that knockdown of ADSS and PRPS2 in CIN cells depletes the nucleotide level which results in DNA damage. DNA damage activates PARP and causes a reduction in both the NAD^+ and the ATP pool. CIN cells already lack ATP due to activation of PARP and lower levels of ATP in CIN cells is normally paralleled by increasing AMP levels. High AMP levels, sensed by AMPK, lead to activation of autophagy, which is required for CIN cell adaptation in a nutrient starved condition. However, blocking AMP synthesis by ADSS knockdown in CIN cells prevented activation of autophagy (Figure 5.3). An elevated lysosomal phenotype in ADSS deficient CIN cells suggests that in the absence of autophagy, lysosomes worked hard to compensate for the autophagy defect. When we blocked lysosomes by inhibitor, the CIN cells died, which suggest the importance of lysosomes in CIN progression (Figure 5.3).

Targeting lysosomes in cancer by exploring lysosomal biogenesis and its role could emerge as a new therapeutic strategy to overcome drug resistance in cancer.

Future Directions

Our studies demonstrate the importance of metabolism in CIN tumour therapy. Further exploration would provide a mechanistic understanding for these complex metabolic interactions in the cell to be able to target cancer. However, it is ineffective to depend on any single therapeutic agent due to the emergence of drug resistance during tumorigenesis. Thus, targeting additional features that CIN cells have in common, along with our metabolic approach could develop a strategy that robustly clears the tumour. There are still some open questions that need to be addressed.

- We have shown that CIN tumours depend on PEPCK to decrease cytoplasmic NADH but the level of NADH has not been measured. By measuring the effect of removing PEPCK on NADH metabolism in normal and induced-CIN tissue as well as in CIN tumours, this data could help to confirm our hypotheses about the inhibitory role of NADH in PEPCK deficient CIN cells or tumours.

- Our study suggests that elevated NADH in CIN cells leads to increased ROS production from mitochondria, however this still needs to be validated. Identification of the most effective targets for perturbing NADH levels in a range of tissue types will show what effect this has on CIN cells

- We know that tumours adapt to therapy, therefore it is important to identify ways to block the available stress response pathways that potentially mitigate metabolic therapy. We found that ER stress responses, mitophagy and innate immune signalling pathways are regulated in CIN cells to avoid CIN-specific cell death. However, we did not test their ability to block NADH-induced ROS and growth arrest in CIN cells and tumours to identify effective inhibitors.

- In this project, we found that the CIN cells are sensitive to nucleotide changes but depletion of purine nucleotide synthesis enzymes did not trigger apoptosis in CIN cells. This strongly suggested that other pathways might be activated in CIN cells to compensate for the demand for nucleotides. The role of the salvage pathway in CIN cells is not clear. Therefore, manipulation of nucleotide recycling pathway candidates in CIN cells by either RNAi or inhibitor is necessary to investigate its function in CIN cell progression.

- Why is ATP limited in CIN cells? We suggest that is due to activation of PARP in CIN cells and showed that feeding ATP rescued the AO phenotype, but we did not measure the ATP level in CIN cells. ATP measurement in nucleotide depleted CIN cells and high CIN (*Rad21 with Dicer2*) will provide valuable confirmation of the effect of ATP in CIN cells.

- In this study, induction of lysosomes was observed in response to nucleotide stress in CIN cells and when we blocked the lysosomes, CIN cells died. Consequently, this study shows the importance of lysosomes in CIN cell progression under nucleotide stress. Thus, further investigations to determine the interaction between the lysosomal pathway and nucleotide pathway in response to CIN will be significant.

- The effect of ADDS, PRPS2 and TKL in tumours has not been tested. We still do not know whether these candidates that are directly involved in nucleotide synthesis can affect CIN tumours, or if they only affect normal cells with induced CIN.

Chapter 6

References

AIRD, K. M. & ZHANG, R. 2015. Nucleotide metabolism, oncogene-induced senescence and cancer. *Cancer letters,* 356**,** 204-210.

ALIZADEH, A. A., EISEN, M. B., DAVIS, R. E., MA, C., LOSSOS, I. S., ROSENWALD, A., BOLDRICK, J. C., SABET, H., TRAN, T. & YU, X. 2000. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature,* 403**,** 503-511.

APPELQVIST, H., WÄSTER, P., KÅGEDAL, K. & ÖLLINGER, K. 2013. The lysosome: from waste bag to potential therapeutic target. *Journal of molecular cell biology,* 5**,** 214-226.

ARGAUD, D., HALIMI, S., CATELLONI, F. & LEVERVE, X. 1991. Inhibition of gluconeogenesis in isolated rat hepatocytes after chronic treatment with phenobarbital. *Biochemical Journal,* 280**,** 663-669.

BAKHOUM, S. F. & COMPTON, D. A. 2012a. Chromosomal instability and cancer: a complex relationship with therapeutic potential. *The Journal of clinical investigation,* 122**,** 1138.

BAKHOUM, S. F. & COMPTON, D. A. 2012b. Chromosomal instability and cancer: a complex relationship with therapeutic potential. *J Clin Invest,* 122**,** 1138-43.

BAKHOUM, S. F., GENOVESE, G. & COMPTON, D. A. 2009. Deviant kinetochore microtubule dynamics underlie chromosomal instability. *Current Biology,* 19**,** 1937-1942.

BAKHOUM, S. F., NGO, B., LAUGHNEY, A. M., CAVALLO, J.-A., MURPHY, C. J., LY, P., SHAH, P., SRIRAM, R. K., WATKINS, T. B. & TAUNK, N. K. 2018. Chromosomal instability drives metastasis through a cytosolic DNA response. *Nature,* 553**,** 467.

BARTOK, O., TEESALU, M., ASHWALL-FLUSS, R., PANDEY, V., HANAN, M., ROVENKO, B. M., POUKKULA, M., HAVULA, E., MOUSSAIEFF, A. & VODALA, S. 2015. The transcription factor Cabut coordinates energy metabolism and the circadian clock in response to sugar sensing. *The EMBO journal,* 34**,** 1538-1553.

BASTO, R., BRUNK, K., VINADOGROVA, T., PEEL, N., FRANZ, A., KHODJAKOV, A. & RAFF, J. W. 2008. Centrosome amplification can initiate tumorigenesis in flies. *Cell,* 133**,** 1032-1042.

BASU, J., BOUSBAA, H., LOGARINHO, E., LI, Z., WILLIAMS, B. C., LOPES, C., SUNKEL, C. E. & GOLDBERG, M. L. 1999. Mutations in the essential spindle

checkpoint gene bub1 cause chromosome missegregation and fail to block apoptosis in Drosophila. *The Journal of cell biology,* 146**,** 13-28.

BEALE, E. G., HAMMER, R. E., ANTOINE, B. & FOREST, C. 2004. Disregulated glyceroneogenesis: PCK1 as a candidate diabetes and obesity gene. *Trends in Endocrinology & Metabolism,* 15**,** 129-135.

BIRKBAK, N. J., EKLUND, A. C., LI, Q., MCCLELLAND, S. E., ENDESFELDER, D., TAN, P., TAN, I. B., RICHARDSON, A. L., SZALLASI, Z. & SWANTON, C. 2011. Paradoxical relationship between chromosomal instability and survival outcome in cancer. *Cancer research,* 71**,** 3447-3452.

BRAND, M. D. 2010. The sites and topology of mitochondrial superoxide production. *Experimental gerontology,* 45**,** 466-472.

BRUMBY, A. M. & RICHARDSON, H. E. 2005. Using Drosophila melanogaster to map human cancer pathways. *Nature Reviews Cancer,* 5**,** 626-639.

BUFFIN, E., EMRE, D. & KARESS, R. E. 2007. Flies without a spindle checkpoint. *Nature Cell Biology,* 9**,** 565-572.

BURGESS, S. C., HE, T., YAN, Z., LINDNER, J., SHERRY, A. D., MALLOY, C. R., BROWNING, J. D. & MAGNUSON, M. A. 2007. Cytosolic phosphoenolpyruvate carboxykinase does not solely control the rate of hepatic gluconeogenesis in the intact mouse liver. *Cell metabolism,* 5**,** 313-320.

CAHILL, D. P., LENGAUER, C., YU, J., RIGGINS, G. J., WILLSON, J. K., MARKOWITZ, S. D., KINZLER, K. W. & VOGELSTEIN, B. 1998. Mutations of mitotic checkpoint genes in human cancers. *Nature,* 392**,** 300-303.

CAIRNS, R. A., HARRIS, I. S. & MAK, T. W. 2011. Regulation of cancer cell metabolism. *Nature Reviews Cancer,* 11**,** 85-95.

CALEY, A. & JONES, R. 2012. The principles of cancer treatment by chemotherapy. *Surgery (Oxford),* 30**,** 186-190.

CARTER, S. L., EKLUND, A. C., KOHANE, I. S., HARRIS, L. N. & SZALLASI, Z. 2006. A signature of chromosomal instability inferred from gene expression profiles predicts clinical outcome in multiple human cancers. *Nature genetics,* 38**,** 1043-1048.

CASCANTE, M., CENTELLES, J. J., VEECH, R. L., LEE, W.-N. P. & BOROS, L. G. 2000. Role of thiamin (vitamin B-1) and transketolase in tumor cell proliferation. *Nutrition and cancer,* 36**,** 150-154.

CHANDEL, N. S. & TUVESON, D. A. 2014. The promise and perils of antioxidants for cancer patients. *New England Journal of Medicine,* 371**,** 177-178.

CHEN, X., CHEUNG, S. T., SO, S., FAN, S. T., BARRY, C., HIGGINS, J., LAI, K.-M., JI, J., DUDOIT, S. & NG, I. O. 2002. Gene expression patterns in human liver cancers. *Molecular biology of the cell,* 13**,** 1929-1939.

CIANFANELLI, V., D'ORAZIO, M. & CECCONI, F. 2015. AMBRA1 and BECLIN 1 interplay in the crosstalk between autophagy and cell proliferation. *Cell cycle,* 14**,** 959-963.

CIMINI, D. 2008. Merotelic kinetochore orientation, aneuploidy, and cancer. *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer,* 1786**,** 32-40.

CIMINI, D., MATTIUZZO, M., TOROSANTUCCI, L. & DEGRASSI, F. 2003. Histone hyperacetylation in mitosis prevents sister chromatid separation and produces chromosome segregation defects. *Molecular biology of the cell,* 14**,** 3821-3833.

CIMINI, D., WAN, X., HIREL, C. B. & SALMON, E. 2006. Aurora kinase promotes turnover of kinetochore microtubules to reduce chromosome segregation errors. *Current Biology,* 16**,** 1711-1718.

COLDREN, C. D. & MILLER, Y. E. 2011. Progressive Endobronchial Premalignancy: Marked by Original CIN. *American journal of respiratory and critical care medicine,* 184**,** 869-870.

COMÍN-ANDUIX, B., BOREN, J., MARTINEZ, S., MORO, C., CENTELLES, J. J., TREBUKHINA, R., PETUSHOK, N., LEE, W. N. P., BOROS, L. G. & CASCANTE, M. 2001. The effect of thiamine supplementation on tumour proliferation. *The FEBS Journal,* 268**,** 4177-4182.

CUNNINGHAM, J. T., MORENO, M. V., LODI, A., RONEN, S. M. & RUGGERO, D. 2014. Protein and nucleotide biosynthesis are coupled by a single rate-limiting enzyme, PRPS2, to drive cancer. *Cell,* 157**,** 1088-1103.

DANG, C. V. 2012. Links between metabolism and cancer. *Genes & development,* 26**,** 877-890.

DEBERARDINIS, R. J. & CHANDEL, N. S. 2016. Fundamentals of cancer metabolism. *Science advances,* 2**,** e1600200.

DELUCA, J. G., MOREE, B., HICKEY, J. M., KILMARTIN, J. V. & SALMON, E. 2002. hNuf2 inhibition blocks stable kinetochore–microtubule attachment and induces mitotic cell death in HeLa cells. *The Journal of cell biology,* 159**,** 549-555.

DIAZ-MORALLI, S., AGUILAR, E., MARIN, S., COY, J. F., DEWERCHIN, M., ANTONIEWICZ, M. R., MECA-CORTÉS, O., NOTEBAERT, L., GHESQUIÈRE, B. & EELEN, G. 2016. A key role for transketolase-like 1 in tumor metabolic reprogramming. *Oncotarget,* 7**,** 51875.

DIAZ-RODRÍGUEZ, E., SOTILLO, R., SCHVARTZMAN, J.-M. & BENEZRA, R. 2008. Hec1 overexpression hyperactivates the mitotic checkpoint and induces tumor formation in vivo. *Proceedings of the National Academy of Sciences,* 105**,** 16719-16724.

DOBLES, M., LIBERAL, V., SCOTT, M. L., BENEZRA, R. & SORGER, P. K. 2000. Chromosome missegregation and apoptosis in mice lacking the mitotic checkpoint protein Mad2. *Cell,* 101**,** 635-645.

DONNELLY, N., PASSERINI, V., DÜRRBAUM, M., STINGELE, S. & STORCHOVÁ, Z. 2014. HSF1 deficiency and impaired HSP90-dependent protein folding are hallmarks of aneuploid human cells. *The EMBO journal,* 33**,** 2374-2387.

DUIJF, P. & BENEZRA, R. 2013. The cancer biology of whole-chromosome instability. *Oncogene,* 32**,** 4727-4736.

DUKER, N. J. 2002. Chromosome breakage syndromes and cancer. *American journal of medical genetics,* 115**,** 125-129.

EGAN, D. F., CHUN, M. G., VAMOS, M., ZOU, H., RONG, J., MILLER, C. J., LOU, H. J., RAVEENDRA-PANICKAR, D., YANG, C.-C. & SHEFFLER, D. J. 2015. Small molecule inhibition of the autophagy kinase ULK1 and identification of ULK1 substrates. *Molecular cell,* 59**,** 285-297.

ELION, G. B. 1989. The purine path to chemotherapy. Portland Press Limited.

FINKEL, T. 2012. From sulfenylation to sulfhydration: what a thiolate needs to tolerate. *Sci. Signal.,* 5**,** pe10-pe10.

FOIJER, F. 2010. CINister thoughts. *Biochemical Society transactions,* 38**,** 1715.

FUKASAWA, K. & WOUDE, G. V. 1997. Synergy between the Mos/mitogen-activated protein kinase pathway and loss of p53 function in transformation and chromosome instability. *Molecular and cellular biology,* 17**,** 506-518.

GALLUZZI, L., PIETROCOLA, F., BRAVO-SAN PEDRO, J. M., AMARAVADI, R. K., BAEHRECKE, E. H., CECCONI, F., CODOGNO, P., DEBNATH, J., GEWIRTZ, D. A. & KARANTZA, V. 2015. Autophagy in malignant transformation and cancer progression. *The EMBO journal,* 34**,** 856-880.

GANEM, N. J., GODINHO, S. A. & PELLMAN, D. 2009. A mechanism linking extra centrosomes to chromosomal instability. *Nature,* 460**,** 278-282.

GAO, C., FURGE, K., KOEMAN, J., DYKEMA, K., SU, Y., CUTLER, M. L., WERTS, A., HAAK, P. & WOUDE, G. F. V. 2007. Chromosome instability, chromosome transcriptome, and clonal evolution of tumor cell populations. *Proceedings of the National Academy of Sciences,* 104**,** 8995-9000.

GARBER, M. E., TROYANSKAYA, O. G., SCHLUENS, K., PETERSEN, S., THAESLER, Z., PACYNA-GENGELBACH, M., VAN DE RIJN, M., ROSEN, G. D., PEROU, C. M. & WHYTE, R. I. 2001. Diversity of gene expression in adenocarcinoma of the lung. *Proceedings of the National Academy of Sciences,* 98**,** 13784-13789.

GARCÍA MARTÍNEZ, J., GARCÍA–INCLÁN, C., SUÁREZ, C., LLORENTE, J. L. & HERMSEN, M. A. 2015. DNA aneuploidy-specific therapy for head and neck squamous cell carcinoma. *Head & neck,* 37**,** 884-888.

GARRIDO, C., GALLUZZI, L., BRUNET, M., PUIG, P., DIDELOT, C. & KROEMER, G. 2006. Mechanisms of cytochrome c release from mitochondria. *Cell Death & Differentiation,* 13**,** 1423-1433.

GEMBLE, S., AHUJA, A., BUHAGIAR-LABARCHÈDE, G., ONCLERCQ-DELIC, R., DAIROU, J., BIARD, D. S., LAMBERT, S., LOPES, M. & AMOR-GUÉRET, M. 2015. Pyrimidine pool disequilibrium induced by a cytidine deaminase deficiency inhibits PARP-1 activity, leading to the under replication of DNA. *PLoS genetics,* 11**,** e1005384.

GERLINGER, M. & SWANTON, C. 2010. How Darwinian models inform therapeutic failure initiated by clonal heterogeneity in cancer medicine. *British journal of cancer,* 103**,** 1139-1143.

GIAM, M. & RANCATI, G. 2015. Aneuploidy and chromosomal instability in cancer: a jackpot to chaos. *Cell division,* 10**,** 3.

GISSELSSON, D. 2011. Aneuploidy in cancer. *Cell Cycle,* 10**,** 1-3.

GOCHEVA, V., ZENG, W., KE, D., KLIMSTRA, D., REINHECKEL, T., PETERS, C., HANAHAN, D. & JOYCE, J. A. 2006. Distinct roles for cysteine cathepsin genes in multistage tumorigenesis. *Genes & development,* 20**,** 543-556.

GOLDSTONE, D. C., ENNIS-ADENIRAN, V., HEDDEN, J. J., GROOM, H. C., RICE, G. I., CHRISTODOULOU, E., WALKER, P. A., KELLY, G., HAIRE, L. F. &

YAP, M. W. 2011. HIV-1 restriction factor SAMHD1 is a deoxynucleoside triphosphate triphosphohydrolase. *Nature,* 480**,** 379-382.

GONZALEZ, C. 2013. Drosophila melanogaster: a model and a tool to investigate malignancy and identify new therapeutics. *Nature Reviews Cancer,* 13**,** 172-183.

GOTINK, K. J., BROXTERMAN, H. J., LABOTS, M., DE HAAS, R. R., DEKKER, H., HONEYWELL, R. J., RUDEK, M. A., BEEREPOOT, L. V., MUSTERS, R. J. & JANSEN, G. 2011. Lysosomal sequestration of sunitinib: a novel mechanism of drug resistance. *Clinical Cancer Research,* 17**,** 7337-7346.

GOUGH, D. & COTTER, T. 2011. Hydrogen peroxide: a Jekyll and Hyde signalling molecule. *Cell death & disease,* 2**,** e213.

GUO, J. Y. & WHITE, E. 2013. Autophagy is required for mitochondrial function, lipid metabolism, growth, and fate of KRASG12D-driven lung tumors. *Autophagy,* 9**,** 1636-1638.

HAN, D., ANTUNES, F., CANALI, R., RETTORI, D. & CADENAS, E. 2003. Voltage-dependent anion channels control the release of the superoxide anion from mitochondria to cytosol. *Journal of Biological Chemistry,* 278**,** 5557-5563.

HANAHAN, D. & WEINBERG, R. A. 2011a. Hallmarks of cancer: the next generation. *Cell,* 144**,** 646-74.

HANAHAN, D. & WEINBERG, R. A. 2011b. Hallmarks of cancer: the next generation. *cell,* 144**,** 646-674.

HANSON, R. W. 2005. Metabolism in the era of molecular biology. *Journal of Biological Chemistry,* 280**,** 1705-1715.

HANSON, R. W. & RESHEF, L. 2003. Glyceroneogenesis revisited. *Biochimie,* 85**,** 1199-1205.

HARDIE, D. G., SCHAFFER, B. E. & BRUNET, A. 2016. AMPK: an energy-sensing pathway with multiple inputs and outputs. *Trends in cell biology,* 26**,** 190-201.

HARDWICK, K. G. 2005. Checkpoint signalling: Mad2 conformers and signal propagation. *Current Biology,* 15**,** R122-R124.

HASTIE, N. D. & ALLSHIRE, R. C. 1989. Human telomeres: fusion and interstitial sites. *Trends in Genetics,* 5**,** 326-331.

HAYNES, C. M., TITUS, E. A. & COOPER, A. A. 2004. Degradation of misfolded proteins prevents ER-derived oxidative stress and cell death. *Molecular cell,* 15**,** 767-776.

HEALY, S. J., GORMAN, A. M., MOUSAVI-SHAFAEI, P., GUPTA, S. & SAMALI, A. 2009. Targeting the endoplasmic reticulum-stress response as an anticancer strategy. *European journal of pharmacology,* 625**,** 234-246.

HERZOG, F., PRIMORAC, I., DUBE, P., LENART, P., SANDER, B., MECHTLER, K., STARK, H. & PETERS, J.-M. 2009. Structure of the anaphase-promoting complex/cyclosome interacting with a mitotic checkpoint complex. *Science,* 323**,** 1477-1481.

HUSSAIN, R., SHAUKAT, Z., KHAN, M., SAINT, R. & GREGORY, S. L. 2017. Phosphoenolpyruvate Carboxykinase Maintains Glycolysis-driven Growth in Drosophila Tumors. *Scientific reports,* 7**,** 11531.

JALLEPALLI, P. V., WAIZENEGGER, I. C., BUNZ, F., LANGER, S., SPEICHER, M. R., PETERS, J.-M., KINZLER, K. W., VOGELSTEIN, B. & LENGAUER, C. 2001. Securin is required for chromosomal stability in human cells. *Cell,* 105**,** 445-457.

JANSSEN, A., VAN DER BURG, M., SZUHAI, K., KOPS, G. J. & MEDEMA, R. H. 2011. Chromosome segregation errors as a cause of DNA damage and structural chromosome aberrations. *Science,* 333**,** 1895-1898.

JARAMILLO, M. C. & ZHANG, D. D. 2013. The emerging role of the Nrf2–Keap1 signaling pathway in cancer. *Genes & development,* 27**,** 2179-2191.

JI, X., WU, Y., YAN, J., MEHRENS, J., YANG, H., DELUCIA, M., HAO, C., GRONENBORN, A. M., SKOWRONSKI, J. & AHN, J. 2013. Mechanism of allosteric activation of SAMHD1 by dGTP. *Nature structural & molecular biology,* 20**,** 1304-1309.

JIANG, P. & MIZUSHIMA, N. 2014. Autophagy and human diseases. *Cell Res,* 24**,** 69-79.

JOSHI, S., TOLKUNOV, D., AVIV, H., HAKIMI, A. A., YAO, M., HSIEH, J. J., GANESAN, S., CHAN, C. S. & WHITE, E. 2015. The genomic landscape of renal oncocytoma identifies a metabolic barrier to tumorigenesis. *Cell reports,* 13**,** 1895-1908.

KAMATA, T. & PRITCHARD, C. 2011. Mechanisms of aneuploidy induction by RAS and RAF oncogenes. *American journal of cancer research,* 1**,** 955.

KOHNKEN, R., KODIGEPALLI, K. M. & WU, L. 2015. Regulation of deoxynucleotide metabolism in cancer: novel mechanisms and therapeutic implications. *Molecular cancer,* 14**,** 176.

KOKURYO, T., SENGA, T., YOKOYAMA, Y., NAGINO, M., NIMURA, Y. & HAMAGUCHI, M. 2007. Nek2 as an effective target for inhibition of tumorigenic growth and peritoneal dissemination of cholangiocarcinoma. *Cancer research,* 67**,** 9637-9642.

KOPS, G. J., WEAVER, B. A. & CLEVELAND, D. W. 2005. On the road to cancer: aneuploidy and the mitotic checkpoint. *Nature Reviews Cancer,* 5**,** 773-785.

KROCKENBERGER, M., ENGEL, J., SCHMIDT, M., KOHRENHAGEN, N., HÄUSLER, S., DOMBROWSKI, Y., KAPP, M., DIETL, J. & HONIG, A. 2010. Expression of transketolase-like 1 protein (TKTL1) in human endometrial cancer. *Anticancer research,* 30**,** 1653-1659.

KUMAR, D., ABDULOVIC, A. L., VIBERG, J., NILSSON, A. K., KUNKEL, T. A. & CHABES, A. 2010. Mechanisms of mutagenesis in vivo due to imbalanced dNTP pools. *Nucleic acids research,* 39**,** 1360-1371.

LEE, A. J., ENDESFELDER, D., ROWAN, A. J., WALTHER, A., BIRKBAK, N. J., FUTREAL, P. A., DOWNWARD, J., SZALLASI, Z., TOMLINSON, I. P. & HOWELL, M. 2011. Chromosomal instability confers intrinsic multidrug resistance. *Cancer research,* 71**,** 1858-1870.

LEE, A. S. 2007. GRP78 induction in cancer: therapeutic and prognostic implications. *Cancer research,* 67**,** 3496-3499.

LEITHNER, K. 2015. PEPCK in cancer cell starvation. *Oncoscience,* 2**,** 805.

LEITHNER, K., HRZENJAK, A. & OLSCHEWSKI, H. 2014. Gluconeogenesis in cancer: door wide open. *Proceedings of the National Academy of Sciences***,** 201415680.

LENGAUER, C., KINZLER, K. & VOGELSTEIN, B. 1997. Genetic instability in colorectal cancers. *Nature,* 386**,** 623-627.

LI, Y.-J., LEI, Y.-H., YAO, N., WANG, C.-R., HU, N., YE, W.-C., ZHANG, D.-M. & CHEN, Z.-S. 2017. Autophagy and multidrug resistance in cancer. *Chinese journal of cancer,* 36**,** 52.

LIU, D., SHAUKAT, Z., SAINT, R. B. & GREGORY, S. L. 2015. Chromosomal instability triggers cell death via local signalling through the innate immune receptor Toll. *Oncotarget,* 6**,** 38552.

LIU, D., SHAUKAT, Z., XU, T., DENTON, D., SAINT, R. & GREGORY, S. 2016. Autophagy regulates the survival of cells with chromosomal instability. *Oncotarget,* 7**,** 63913.

LOPEZ-GIRONA, A., FURNARI, B., MONDESERT, O. & RUSSELL, P. 1999. Nuclear localization of Cdc25 is regulated by DNA damage and a 14-3-3 protein. *Nature,* 397**,** 172-175.

LUENGO, A., GUI, D. Y. & VANDER HEIDEN, M. G. 2017. Targeting Metabolism for Cancer Therapy. *Cell chemical biology,* 24**,** 1161-1180.

LUNT, S. Y. & VANDER HEIDEN, M. G. 2011. Aerobic glycolysis: meeting the metabolic requirements of cell proliferation. *Annual review of cell and developmental biology,* 27**,** 441-464.

MA, Y. & HENDERSHOT, L. M. 2004. The role of the unfolded protein response in tumour development: friend or foe? *Nature Reviews Cancer,* 4**,** 966-977.

MAJEWSKI, N., NOGUEIRA, V., BHASKAR, P., COY, P. E., SKEEN, J. E., GOTTLOB, K., CHANDEL, N. S., THOMPSON, C. B., ROBEY, R. B. & HAY, N. 2004. Hexokinase-mitochondria interaction mediated by Akt is required to inhibit apoptosis in the presence or absence of Bax and Bak. *Molecular cell,* 16**,** 819-830.

MALHOTRA, J. D. & KAUFMAN, R. J. 2007. Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword? *Antioxidants & redox signaling,* 9**,** 2277-2294.

MANNING, A. L., LONGWORTH, M. S. & DYSON, N. J. 2010. Loss of pRB causes centromere dysfunction and chromosomal instability. *Genes & development,* 24**,** 1364-1376.

MARTÍNEZ-REYES, I., DIEBOLD, L. P., KONG, H., SCHIEBER, M., HUANG, H., HENSLEY, C. T., MEHTA, M. M., WANG, T., SANTOS, J. H. & WOYCHIK, R. 2016. TCA cycle and mitochondrial membrane potential are necessary for diverse biological functions. *Molecular cell,* 61**,** 199-209.

MAYERS, J. R., WU, C., CLISH, C. B., KRAFT, P., TORRENCE, M. E., FISKE, B. P., YUAN, C., BAO, Y., TOWNSEND, M. K. & TWOROGER, S. S. 2014. Elevation of circulating branched-chain amino acids is an early event in human pancreatic adenocarcinoma development. *Nature medicine,* 20**,** 1193-1198.

MCAFEE, Q., ZHANG, Z., SAMANTA, A., LEVI, S. M., MA, X.-H., PIAO, S., LYNCH, J. P., UEHARA, T., SEPULVEDA, A. R. & DAVIS, L. E. 2012. Autophagy inhibitor Lys05 has single-agent antitumor activity and reproduces the phenotype of a genetic autophagy deficiency. *Proceedings of the National Academy of Sciences,* 109**,** 8253-8258.

MCGRANAHAN, N., BURRELL, R. A., ENDESFELDER, D., NOVELLI, M. R. & SWANTON, C. 2012. Cancer chromosomal instability: therapeutic and diagnostic challenges. *EMBO reports,* 13**,** 528-538.

MERTENS, F., JOHANSSON, B. & MITELMAN, F. 1994. Isochromosomes in neoplasia. *Genes, Chromosomes and Cancer,* 10**,** 221-230.

MESQUITA, A., TÁBARA, L. C., MARTINEZ-COSTA, O., SANTOS-RODRIGO, N., VINCENT, O. & ESCALANTE, R. 2015. Dissecting the function of Atg1 complex in Dictyostelium autophagy reveals a connection with the pentose phosphate pathway enzyme transketolase. *Open biology,* 5**,** 150088.

MICHEL, L. S., LIBERAL, V., CHATTERJEE, A., KIRCHWEGGER, R., PASCHE, B., GERALD, W., DOBLES, M., SORGER, P. K., MURTY, V. & BENEZRA, R. 2001. MAD2 haplo-insufficiency causes premature anaphase and chromosome instability in mammalian cells. *Nature,* 409**,** 355-359.

MINSHULL, J., STRAIGHT, A., RUDNER, A. D., DERNBURG, A. F., BELMONT, A. & MURRAY, A. W. 1996. Protein phosphatase 2A regulates MPF activity and sister chromatid cohesion in budding yeast. *Current Biology,* 6**,** 1609-1620.

MIWA, S., ST-PIERRE, J., PARTRIDGE, L. & BRAND, M. D. 2003. Superoxide and hydrogen peroxide production by Drosophila mitochondria. *Free Radical Biology and Medicine,* 35**,** 938-948.

MONTAL, E. D., DEWI, R., BHALLA, K., OU, L., HWANG, B. J., ROPELL, A. E., GORDON, C., LIU, W.-J., DEBERARDINIS, R. J. & SUDDERTH, J. 2015. PEPCK coordinates the regulation of central carbon metabolism to promote cancer cell growth. *Molecular cell,* 60**,** 571-583.

MUSACCHIO, A. & SALMON, E. D. 2007. The spindle-assembly checkpoint in space and time. *Nature reviews Molecular cell biology,* 8**,** 379-393.

NASMYTH, K. 2011. Cohesin: a catenase with separate entry and exit gates? *Nature cell biology,* 13**,** 1170-1177.

NATARAJAN, A. T. & PALITTI, F. 2008. DNA repair and chromosomal alterations. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis,* 657**,** 3-7.

NISHIMURA, Y., SAMENI, M. & SLOANE, B. F. 1998. Malignant transformation alters intracellular trafficking of lysosomal cathepsin D in human breast epithelial cells. *Pathology & Oncology Research,* 4**,** 283.

NOSAL, J. M., SWITZER, R. L. & BECKER, M. A. 1993. Overexpression, purification, and characterization of recombinant human 5-phosphoribosyl-1-pyrophosphate synthetase isozymes I and II. *Journal of Biological Chemistry,* 268**,** 10168-10175.

NYE, C. K., HANSON, R. W. & KALHAN, S. C. 2008. Glyceroneogenesis is the dominant pathway for triglyceride glycerol synthesis in vivo in the rat. *Journal of Biological Chemistry,* 283**,** 27565-27574.

OROMENDIA, A. B., DODGSON, S. E. & AMON, A. 2012a. Aneuploidy causes proteotoxic stress in yeast. *Genes & development,* 26**,** 2696-2708.

OROMENDIA, A. B., DODGSON, S. E. & AMON, A. 2012b. Aneuploidy causes proteotoxic stress in yeast. *Genes Dev,* 26**,** 2696-708.

ORR, A. L., QUINLAN, C. L., PEREVOSHCHIKOVA, I. V. & BRAND, M. D. 2012. A refined analysis of superoxide production by mitochondrial sn-glycerol 3-phosphate dehydrogenase. *Journal of Biological Chemistry,* 287**,** 42921-42935.

PANDEY, U. B. & NICHOLS, C. D. 2011. Human disease models in Drosophila melanogaster and the role of the fly in therapeutic drug discovery. *Pharmacological reviews,* 63**,** 411-436.

PAVLOVA, N. N. & THOMPSON, C. B. 2016. The emerging hallmarks of cancer metabolism. *Cell metabolism,* 23**,** 27-47.

PFAU, S. J. & AMON, A. 2012. Chromosomal instability and aneuploidy in cancer: from yeast to man. *EMBO reports,* 13**,** 515-527.

PIERZYŃSKA-MACH, A., JANOWSKI, P. A. & DOBRUCKI, J. W. 2014. Evaluation of acridine orange, LysoTracker Red, and quinacrine as fluorescent probes for long-term tracking of acidic vesicles. *Cytometry Part A,* 85**,** 729-737.

PIHAN, G. A., PUROHIT, A., WALLACE, J., MALHOTRA, R., LIOTTA, L. & DOXSEY, S. J. 2001. Centrosome defects can account for cellular and genetic changes that characterize prostate cancer progression. *Cancer research,* 61**,** 2212-2219.

PINO, M. S. & CHUNG, D. C. 2010. The chromosomal instability pathway in colon cancer. *Gastroenterology,* 138**,** 2059-2072.

PLEMEL, J. R., CAPRARIELLO, A. V., KEOUGH, M. B., HENRY, T. J., TSUTSUI, S., CHU, T. H., SCHENK, G. J., KLAVER, R., YONG, V. W. & STYS, P. K. 2017. Unique spectral signatures of the nucleic acid dye acridine orange can distinguish cell death by apoptosis and necroptosis. *J Cell Biol***,** jcb. 201602028.

POILLET-PEREZ, L., DESPOUY, G., DELAGE-MOURROUX, R. & BOYER-GUITTAUT, M. 2015. Interplay between ROS and autophagy in cancer cells, from tumor initiation to cancer therapy. *Redox biology,* 4**,** 184-192.

POTAPOVA, T. A., ZHU, J. & LI, R. 2013. Aneuploidy and chromosomal instability: a vicious cycle driving cellular evolution and cancer genome chaos. *Cancer and Metastasis Reviews,* 32**,** 377-389.

PREVIS, S. F., BRUNENGRABER, D. Z. & BRUNENGRABER, H. 2009. Is there glucose production outside of the liver and kidney? *Annual review of nutrition,* 29**,** 43-57.

QU, X., YU, J., BHAGAT, G., FURUYA, N., HIBSHOOSH, H., TROXEL, A., ROSEN, J., ESKELINEN, E.-L., MIZUSHIMA, N. & OHSUMI, Y. 2003. Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. *Journal of Clinical Investigation,* 112**,** 1809.

RAJAGOPALAN, H. & LENGAUER, C. 2004. Aneuploidy and cancer. *Nature,* 432**,** 338-341.

RAO, C. V. & YAMADA, H. Y. 2013. Genomic instability and colon carcinogenesis: from the perspective of genes. *Frontiers in oncology,* 3.

RICKE, R. M., JEGANATHAN, K. B. & VAN DEURSEN, J. M. 2011. Bub1 overexpression induces aneuploidy and tumor formation through Aurora B kinase hyperactivation. *The Journal of cell biology,* 193**,** 1049-1064.

ROGNSTAD, R. 1979. Rate-limiting steps in metabolic pathways. *Journal of Biological Chemistry,* 254**,** 1875-1878.

RÖHRIG, F. & SCHULZE, A. 2016. The multifaceted roles of fatty acid synthesis in cancer. *Nature Reviews Cancer,* 16**,** 732-749.

ROSCHKE, A. V. & KIRSCH, I. R. 2005. Targeting cancer cells by exploiting karyotypic complexity and chromosomal instability. *Cell Cycle,* 4**,** 679-682.

ROSCHKE, A. V., LABABIDI, S., TONON, G., GEHLHAUS, K. S., BUSSEY, K., WEINSTEIN, J. N. & KIRSCH, I. R. 2005a. Karyotypic "state" as a potential determinant for anticancer drug discovery. *Proceedings of the National Academy of Sciences of the United States of America,* 102**,** 2964-2969.

ROSSI, F. & GONZALEZ, C. 2015. Studying tumor growth in Drosophila using the tissue allograft method. *Nature protocols,* 10**,** 1525-1534.

SAHA, A., CONNELLY, S., JIANG, J., ZHUANG, S., AMADOR, D. T., PHAN, T., PILZ, R. B. & BOSS, G. R. 2014. Akt phosphorylation and regulation of

transketolase is a nodal point for amino acid control of purine synthesis. *Molecular cell,* 55**,** 264-276.

SANTAGATA, S., MENDILLO, M. L., TANG, Y.-C., SUBRAMANIAN, A., PERLEY, C. C., ROCHE, S. P., WONG, B., NARAYAN, R., KWON, H. & KOEVA, M. 2013. Tight coordination of protein translation and HSF1 activation supports the anabolic malignant state. *Science,* 341**,** 1238303.

SANTAGUIDA, S., VASILE, E., WHITE, E. & AMON, A. 2015. Aneuploidy-induced cellular stresses limit autophagic degradation. *Genes & development,* 29**,** 2010-2021.

SCHLIEKELMAN, M., COWLEY, D. O., O'QUINN, R., OLIVER, T. G., LU, L., SALMON, E. & VAN DYKE, T. 2009. Impaired Bub1 function in vivo compromises tension-dependent checkpoint function leading to aneuploidy and tumorigenesis. *Cancer research,* 69**,** 45-54.

SCHULTZ, H., KÄHLER, D., BRANSCHEID, D., VOLLMER, E., ZABEL, P. & GOLDMANN, T. 2008. TKTL1 is overexpressed in a large portion of non-small cell lung cancer specimens. *Diagnostic pathology,* 3**,** 35.

SCHWAAB, J., HORISBERGER, K., STRÖBEL, P., BOHN, B., GENCER, D., KÄHLER, G., KIENLE, P., POST, S., WENZ, F. & HOFMANN, W.-K. 2011. Expression of Transketolase like gene 1 (TKTL1) predicts disease-free survival in patients with locally advanced rectal cancer receiving neoadjuvant chemoradiotherapy. *BMC cancer,* 11**,** 363.

SHAUKAT, Z., LIU, D., CHOO, A., HUSSAIN, R., O'KEEFE, L., RICHARDS, R., SAINT, R. & GREGORY, S. 2014. Chromosomal instability causes sensitivity to metabolic stress. *Oncogene*.

SHAUKAT, Z., LIU, D., CHOO, A., HUSSAIN, R., O'KEEFE, L., RICHARDS, R., SAINT, R. & GREGORY, S. 2015. Chromosomal instability causes sensitivity to metabolic stress. *Oncogene,* 34**,** 4044-4055.

SHAUKAT, Z., WONG, H. W., NICOLSON, S., SAINT, R. B. & GREGORY, S. L. 2012. A screen for selective killing of cells with chromosomal instability induced by a spindle checkpoint defect. *PLoS One,* 7**,** e47447.

SHELTZER, J. M., TORRES, E. M., DUNHAM, M. J. & AMON, A. 2012. Transcriptional consequences of aneuploidy. *Proceedings of the National Academy of Sciences,* 109**,** 12644-12649.

SHUVALOV, O., PETUKHOV, A., DAKS, A., FEDOROVA, O., VASILEVA, E. & BARLEV, N. A. 2017. One-carbon metabolism and nucleotide biosynthesis as attractive targets for anticancer therapy. *Oncotarget,* 8**,** 23955.

SOLOMON, D. A., KIM, J.-S. & WALDMAN, T. 2014. Cohesin gene mutations in tumorigenesis: from discovery to clinical significance. *BMB reports,* 47**,** 299.

SONE, M., ZENG, X., LARESE, J. & RYOO, H. D. 2013. A modified UPR stress sensing system reveals a novel tissue distribution of IRE1/XBP1 activity during normal Drosophila development. *Cell Stress and Chaperones,* 18**,** 307-319.

SORBYE, H., CVANCAROVA, M., QVORTRUP, C., PFEIFFER, P. & GLIMELIUS, B. 2013. Age-dependent improvement in median and long-term survival in unselected population-based Nordic registries of patients with synchronous metastatic colorectal cancer. *Annals of oncology,* 24**,** 2354-2360.

SOTILLO, R., HERNANDO, E., DÍAZ-RODRÍGUEZ, E., TERUYA-FELDSTEIN, J., CORDÓN-CARDO, C., LOWE, S. W. & BENEZRA, R. 2007. Mad2 overexpression promotes aneuploidy and tumorigenesis in mice. *Cancer cell,* 11**,** 9-23.

ST-PIERRE, J., BUCKINGHAM, J. A., ROEBUCK, S. J. & BRAND, M. D. 2002. Topology of superoxide production from different sites in the mitochondrial electron transport chain. *Journal of Biological Chemistry,* 277**,** 44784-44790.

STAIGER, W. I., COY, J. F., GROBHOLZ, R., HOFHEINZ, R.-D., LUKAN, N., POST, S., SCHWARZBACH, M. H. & WILLEKE, F. 2006. Expression of the mutated transketolase TKTL1, a molecular marker in gastric cancer. *Oncology reports,* 16**,** 657-661.

STANTON, R. C. 2012. Glucose-6-phosphate dehydrogenase, NADPH, and cell survival. *IUBMB life,* 64**,** 362-369.

SWANTON, C., TOMLINSON, I. & DOWNWARD, J. 2006. Chromosomal instability, colorectal cancer and taxane resistance. *Cell cycle,* 5**,** 818-823.

TAKAMURA, A., KOMATSU, M., HARA, T., SAKAMOTO, A., KISHI, C., WAGURI, S., EISHI, Y., HINO, O., TANAKA, K. & MIZUSHIMA, N. 2011. Autophagy-deficient mice develop multiple liver tumors. *Genes & development,* 25**,** 795-800.

TAN, Q., JOSHUA, A., WANG, M., BRISTOW, R., WOUTERS, B., ALLEN, C. & TANNOCK, I. F. 2017. Up-regulation of autophagy is a mechanism of resistance

to chemotherapy and can be inhibited by pantoprazole to increase drug sensitivity. *Cancer chemotherapy and pharmacology,* 79**,** 959-969.

TANG, Y.-C., WILLIAMS, B. R., SIEGEL, J. J. & AMON, A. 2011. Identification of aneuploidy-selective antiproliferation compounds. *Cell,* 144**,** 499-512.

THOMPSON, S. L., BAKHOUM, S. F. & COMPTON, D. A. 2010. Mechanisms of chromosomal instability. *Current biology,* 20**,** R285-R295.

THOMPSON, S. L. & COMPTON, D. A. 2008. Examining the link between chromosomal instability and aneuploidy in human cells. *The Journal of cell biology,* 180**,** 665-672.

TIAN, L., HIRES, S. A., MAO, T., HUBER, D., CHIAPPE, M. E., CHALASANI, S. H., PETREANU, L., AKERBOOM, J., MCKINNEY, S. A. & SCHREITER, E. R. 2009. Imaging neural activity in worms, flies and mice with improved GCaMP calcium indicators. *Nature methods,* 6**,** 875-881.

TRAN, Q., LEE, H., PARK, J., KIM, S.-H. & PARK, J. 2016. Targeting cancer metabolism-revisiting the Warburg effects. *Toxicological research,* 32**,** 177.

VAN EYK, C. L., MCLEOD, C. J., O'KEEFE, L. V. & RICHARDS, R. I. 2011. Comparative toxicity of polyglutamine, polyalanine and polyleucine tracts in Drosophila models of expanded repeat disease. *Human molecular genetics,* 21**,** 536-547.

VANDER HEIDEN, M. G. 2011. Targeting cancer metabolism: a therapeutic window opens. *Nature reviews Drug discovery,* 10**,** 671-684.

VANDER HEIDEN, M. G. & DEBERARDINIS, R. J. 2017. Understanding the intersections between metabolism and cancer biology. *Cell,* 168**,** 657-669.

VAUGHN, A. E. & DESHMUKH, M. 2008. Glucose metabolism inhibits apoptosis in neurons and cancer cells by redox inactivation of cytochrome c. *Nature cell biology,* 10**,** 1477.

VINCENT, E. E., SERGUSHICHEV, A., GRISS, T., GINGRAS, M.-C., SAMBORSKA, B., NTIMBANE, T., COELHO, P. P., BLAGIH, J., RAISSI, T. C. & CHOINIÈRE, L. 2015. Mitochondrial phosphoenolpyruvate carboxykinase regulates metabolic adaptation and enables glucose-independent tumor growth. *Molecular cell,* 60**,** 195-207.

VOS, M. J., CARRA, S., KANON, B., BOSVELD, F., KLAUKE, K., SIBON, O. & KAMPINGA, H. H. 2016. Specific protein homeostatic functions of small heat-shock proteins increase lifespan. *Aging cell,* 15**,** 217-226.

WAGNER, A., GROTHE, W., BEHL, S., KLEBER, G., GROTHEY, A., HAERTING, J. & FLEIG, W. 2005. Chemotherapy for advanced gastric cancer.

WALLQVIST, A., HUANG, R., COVELL, D. G., ROSCHKE, A. V., GELHAUS, K. S. & KIRSCH, I. R. 2005. Drugs aimed at targeting characteristic karyotypic phenotypes of cancer cells. *Molecular cancer therapeutics,* 4**,** 1559-1568.

WANG, J., YUAN, W., CHEN, Z., WU, S., CHEN, J., GE, J., HOU, F. & CHEN, Z. 2012. Overexpression of G6PD is associated with poor clinical outcome in gastric cancer. *Tumor Biology,* 33**,** 95-101.

WANG, M. & KAUFMAN, R. J. 2014. The impact of the endoplasmic reticulum protein-folding environment on cancer development. *Nature Reviews Cancer,* 14**,** 581-597.

WARBURG, O. 1956. On the origin of cancer cells. *Science,* 123**,** 309-314.

WARBURG, O., WIND, F. & NEGELEIN, E. 1927. The metabolism of tumors in the body. *The Journal of general physiology,* 8**,** 519.

WEAVER, A. N. & YANG, E. S. 2013. Beyond DNA repair: additional functions of PARP-1 in cancer. *Frontiers in oncology,* 3**,** 290.

WEAVER, B. A. & CLEVELAND, D. W. 2006. Does aneuploidy cause cancer? *Current opinion in cell biology,* 18**,** 658-667.

WEI, H., WEI, S., GAN, B., PENG, X., ZOU, W. & GUAN, J.-L. 2011. Suppression of autophagy by FIP200 deletion inhibits mammary tumorigenesis. *Genes & development,* 25**,** 1510-1527.

WEINBERG, F., HAMANAKA, R., WHEATON, W. W., WEINBERG, S., JOSEPH, J., LOPEZ, M., KALYANARAMAN, B., MUTLU, G. M., BUDINGER, G. S. & CHANDEL, N. S. 2010. Mitochondrial metabolism and ROS generation are essential for Kras-mediated tumorigenicity. *Proceedings of the National Academy of Sciences,* 107**,** 8788-8793.

WHEATON, W. W., WEINBERG, S. E., HAMANAKA, R. B., SOBERANES, S., SULLIVAN, L. B., ANSO, E., GLASAUER, A., DUFOUR, E., MUTLU, G. M. & BUDIGNER, G. S. 2014. Metformin inhibits mitochondrial complex I of cancer cells to reduce tumorigenesis. *Elife,* 3**,** e02242.

WHITE, E. 2015. The role for autophagy in cancer. *J Clin Invest,* 125**,** 42-6.

WONG, H. W., SHAUKAT, Z., WANG, J., SAINT, R. & GREGORY, S. L. 2014. JNK signaling is needed to tolerate chromosomal instability. *Cell Cycle,* 13**,** 622-31.

XIE, H., HANAI, J.-I., REN, J.-G., KATS, L., BURGESS, K., BHARGAVA, P., SIGNORETTI, S., BILLIARD, J., DUFFY, K. J. & GRANT, A. 2014. Targeting lactate dehydrogenase-a inhibits tumorigenesis and tumor progression in mouse models of lung cancer and impacts tumor-initiating cells. *Cell metabolism,* 19**,** 795-809.

YANG, A., RAJESHKUMAR, N., WANG, X., YABUUCHI, S., ALEXANDER, B. M., CHU, G. C., VON HOFF, D. D., MAITRA, A. & KIMMELMAN, A. C. 2014. Autophagy is critical for pancreatic tumor growth and progression in tumors with p53 alterations. *Cancer discovery,* 4**,** 905-913.

YANG, Y.-P., HU, L.-F., ZHENG, H.-F., MAO, C.-J., HU, W.-D., XIONG, K.-P., WANG, F. & LIU, C.-F. 2013. Application and interpretation of current autophagy inhibitors and activators. *Acta Pharmacologica Sinica,* 34**,** 625-635.

YARDEN, R. I., PARDO-REOYO, S., SGAGIAS, M., COWAN, K. H. & BRODY, L. C. 2002. BRCA1 regulates the G2/M checkpoint by activating Chk1 kinase upon DNA damage. *Nature genetics,* 30**,** 285-289.

YE, J., FAN, J., VENNETI, S., WAN, Y.-W., PAWEL, B. R., ZHANG, J., FINLEY, L. W., LU, C., LINDSTEN, T. & CROSS, J. R. 2014. Serine catabolism regulates mitochondrial redox control during hypoxia. *Cancer discovery,* 4**,** 1406-1417.

ZHANG, C., ZHANG, Z., ZHU, Y. & QIN, S. 2014. Glucose-6-phosphate dehydrogenase: a biomarker and potential therapeutic target for cancer. *Anti-Cancer Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Cancer Agents),* 14**,** 280-289.

ZHOU, W., YANG, Y., XIA, J., WANG, H., SALAMA, M. E., XIONG, W., XU, H., SHETTY, S., CHEN, T. & ZENG, Z. 2013. NEK2 induces drug resistance mainly through activation of efflux drug pumps and is associated with poor prognosis in myeloma and other cancers. *Cancer cell,* 23**,** 48-62.

ZONG, W.-X., DITSWORTH, D., BAUER, D. E., WANG, Z.-Q. & THOMPSON, C. B. 2004. Alkylating DNA damage stimulates a regulated form of necrotic cell death. *Genes & development,* 18**,** 1272-1282.

Appendix

The role of JNK signalling in response to oxidative DNA damage

Statement of Authorship

Title of Paper	The role of JNK signalling in response to DNA damage
Publication Status	Published in Current drug targets
Publication Details	Review article Zeeshan Shaukat, Dawei Liu, Rashid Hussain, <u>Mahwish Khan</u>, Stephen L. Gregory Corresponding E-mail: stephen.gregory@adelaide.edu.au

Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author	Zeeshan Shaukat		
Contribution to the paper	Conceived and designed the article Contributed in writing		
Signature		Date	

Name of Co-Author	Dawei Liu		
Contribution to the paper	Contributed in writing		
Signature		Date	

Name of Co-Author	Rashid Hussain		
Contribution to the paper	Contributed in writing		
Signature		Date	

Name of Co-Author	Mahwish Khan	
Contribution to the paper	Contributed in writing	20% contribution
Signature		Date	

Name of Co-Author	Stephen L. Gregory	
Contribution to the paper	Conceived and designed the article Wrote the article Correspondence	
Signature		Date	

This page intentionally left blank

The Role of JNK Signalling in Responses to Oxidative DNA Damage

Zeeshan Shaukat, Dawei Liu, Rashid Hussain, Mahwish Khan and Stephen L. Gregory*

School of Molecular and Biomedical Science, University of Adelaide, North Terrace, Adelaide S.A. 5006, Australia

Stephen L. Gregory

Abstract: The production of reactive oxygen species is a normal part of cell physiology, but many internal and external stimuli are able to trigger the production of excess levels of oxidants that are potentially damaging. The threat of oxidative damage is particularly significant to DNA, as damaged bases can interfere with replication to generate lasting mutations. Signalling through the JNK pathway is a key cellular response to oxidative damage. Depending on the intensity and duration of the damage signal, JNK signalling can lead to distinct alternative responses including DNA repair, anti-oxidant production or cell death. These responses are highly relevant to cancer therapy, as tumours are often under oxidative stress that produces elevated JNK levels and therapy often involves inducing DNA damage with the intention of driving cell death. In this review we examine the causes and consequences of JNK activation that relate to oxidative DNA damage, with a focus on the potential therapeutic implications.

Keywords: Aneuploidy, cell cycle, chromosomal instability, DNA damage, *Drosophila*, JNK, oxidative stress, ROS, therapeutic target.

INTRODUCTION

The family of Jun N-terminal kinases (JNKs) was originally identified as UV responsive proteins that activate Jun to give transcription of a range of stress response target genes [1]. There is now a huge literature on JNK that documents its role in processes as diverse as cell migration, proliferation, apoptosis, insulin signalling and neuronal survival [2]. In this review we will focus on how JNK signalling is triggered by oxidative damage to DNA, and the downstream responses that determine cell fates after oxidative damage. This topic is relevant to cancer treatment for several reasons. Firstly, tumours often have an altered metabolism that favours glycolysis but generates elevated levels of reactive oxygen species (ROS) from the mitochondrial electron transport chain [3]. Because high ROS levels can damage DNA, tumours can potentially generate heightened genetic diversity that promotes drug resistance and cancer progression [4]. The JNK pathway is frequently activated in tumours, as part of the oxidative stress response [5], so inhibiting JNK signalling may be an effective way to prevent cancers from being able to tolerate a high ROS metabolism. Oxidative damage to DNA can also be incurred by the cancer therapy, such as radiotherapy or metabolic interventions that rely on the production of ROS to kill tumours. However, the activation of JNK in response to DNA damage can have widely divergent outcomes including proliferation, cellular repair or apoptosis [6]. Consequently, an understanding of what drives cellular responses to JNK is essential to allow effective therapeutic intervention.

ACTIVATION OF JNK SIGNALLING BY OXIDATIVE DAMAGE

A wide range of cellular insults are known to result in activation of JNK, primarily by causing phosphorylation of conserved residues in the JNK kinase domain [7]. These stresses include UV light, genotoxic damage, oxidative stress, hypoxia, wounding, nearby apoptosis, growth factor signalling, and immune cell activation. In each case, a sensor protein detects changes in the cellular environment, triggering the activation of a phosphorylation cascade that culminates in the phosphorylation of JNK. The process may be better described as a network response than a pathway, as numerous inputs can contribute to JNK activation and these signals are integrated with negative feedback from JNK targets to generate a dynamic response (Fig. **1**) [8].

There is evidence that the strength and duration of JNK activation can significantly alter the cellular response to JNK signalling. For example, sustained JNK activation is observed following treatment with cisplatin [9]. Cisplatin may initially trigger JNK activation by oxidative stress, but cisplatin also activates JNK later *via* the DNA damage sensing pathway to give the sustained response. Whereas ionising radiation can trigger JNK to drive rapid apoptosis of damaged cells [10], sustained weak activation of JNK by low concentrations of cisplatin may improve cell survival by upregulating DNA damage repair [9]. However, a sustained high level of activation of JNK can lead to robust apoptosis, as seen in cells lacking feedback inhibition of JNK [11, 12]. Consequently, interpretation of JNK activation requires careful consideration of dosage and timing as well as other potential variables such as cell type [13], cell cycle stage [14] and metabolic status [15].

*Address correspondence to this author at the School of Molecular and Biomedical Science, University of Adelaide, North Terrace, Adelaide S.A. 5006, Australia; Tel: +61 8 83137536; Fax: +61 8 83137534;
E-mail: stephen.gregory@adelaide.edu.au
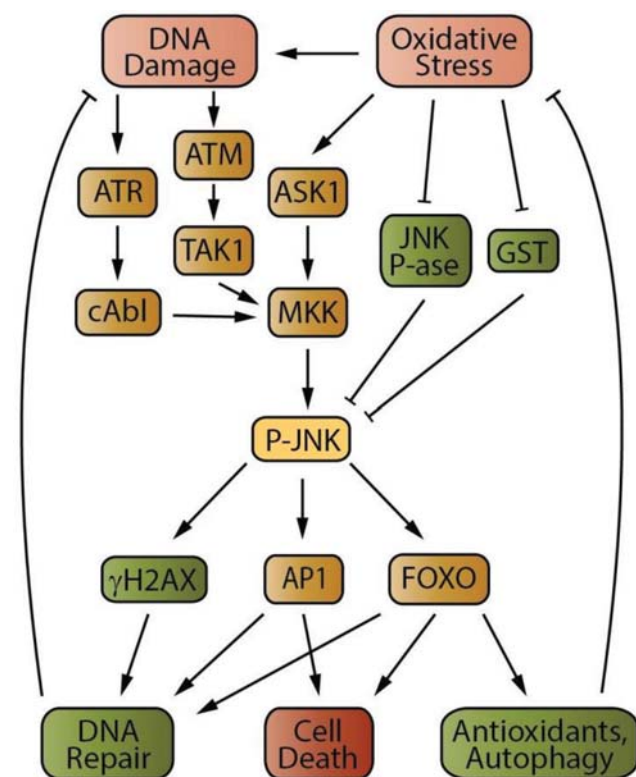
Fig. (1). Cell stresses that generate oxidative damage activate JNK signalling via multiple redox sensors including JNK Phosphatase (JNK P-ase), Glutathione-S-Transferase pi (GST) and ASK1 (via Thioredoxin). Oxidative DNA damage also activates JNK via several routes including the damage sensors ATM and ATR. The result of JNK activation generally includes either DNA repair, autophagy and antioxidant production to promote stress tolerance, or the induction of apoptosis.

Two features frequently seen in stressors that activate JNK signalling are that they cause elevated levels of reactive oxygen species (ROS) and that they lead to DNA damage. High ROS levels are thought to increase JNK activity primarily through loss of JNK inhibition, as negative regulators of JNK, such as Glutathione-S-Transferase and JNK phosphatase, are ROS sensitive [16]. ROS can be generated by external stimuli including ionising radiation, pro-oxidant chemicals, hypoxia, toxins like alcohol that activate cytochrome p450 enzymes, and drugs like cisplatin that affect mitochondria [17]. Internal sources of ROS include the mitochondrial electron transport chain, NADPH oxidase (Nox), and peroxisomes. The levels of ROS from these sources are normally balanced by appropriate levels of antioxidants such as reduced Glutathione or Thioredoxin and antioxidant enzymes including Superoxide Dismutase, Catalase and Peroxiredoxins. JNK activity can thus be stimulated either by increasing ROS generation or by depleting the cell of antioxidants. It seems likely that a strong upregulation of JNK by elevated ROS levels contributes to the apoptosis generated by ROS-producing cancer therapies such as fenretinide and cisplatin [18, 19].

High levels of ROS lead to oxidative damage. In the case of lipids and proteins they may be repaired or replaced, but oxidative DNA damage can lead to lasting mutations. This is

particularly significant for mitochondrial DNA, which is exposed to superoxide and peroxide from the electron transport chain in an organelle that lacks catalase [20]. Ideally, oxidatively damaged nucleotides are removed before DNA replication to avoid fork stalling and the resulting double strand breaks that contribute to genomic instability [21]. Because mitochondrial superoxide has a short half-life [22], the nuclear DNA is usually less exposed to oxidative stress, and in some tissues is better able to replace damaged nucleotides [23]. An exception to this may be in mitosis, when the DNA is no longer shielded by the nuclear membrane and DNA repair is limited [24].

Whether generated by ROS or other means, DNA damage can activate JNK signalling. In particular, double stranded breaks in DNA have been shown to lead to the activation of JNK [6, 25]. Possible mechanisms include the DNA damage sensors ATM and ATR triggering the JNK signalling pathway via Tak1 and c-Abl, or DNA damage effects on translation reducing the amount of the JNK phosphatase MKP-1. Replication stress can also lead to JNK activation, in this case via MKK4 and independent of ATM/ATR damage sensing [26]. This defective replication mechanism can explain how simply adding DNA that carries intra-strand crosslinks can activate JNK [9]. In this context it is interesting that inter-strand crosslinks do not produce the same effect on JNK [27], partly because the mismatch repair system does not detect inter-strand crosslinks. There are significant clinical differences between drugs that predominantly generate one or other type of crosslink [28], so it is tempting to speculate that JNK activity contributes to these differences.

There are a number of features of tumourigenesis that relate to oxidative induction of JNK signalling. Prior to tumour formation, chemicals that promote ROS, such as cigarette smoke and alcohol, are implicated in the accumulation of oncogenic mutations. JNK is typically activated by these kinds of cellular stresses, either to repair or, if necessary, to kill damaged cells [7]. It is intuitively obvious that oxidative DNA damage can generate deleterious mutations, but the opposite hypothesis, hormesis, should also be considered. Hormesis suggests that an ongoing low level of oxidative stress encourages the production of cellular antioxidants, repair and immune surveillance that results in a lower rate of surviving deleterious mutations [29]. The role of JNK in mediating hormesis is still poorly characterised, though there is evidence that hormetic radiation resistance may require JNK [30].

Once a tumour has formed, it is common to observe high levels of ROS [31, 32] and JNK activation [7]. ROS production is known to result from growth factor stimulation of Nox or from mitochondrial disruption that accompanies a metabolic shift to oxidative glycolysis [33]. In these cases, the demands of proliferation result in higher levels of ROS generation that must be counteracted for the tumour to avoid self-destruction. Another feature of tumours that induces oxidative stress is chromosomal instability (CIN). CIN is a common feature of tumours that gives ongoing variation in karyotype due to impaired mitotic fidelity [34]. This means that most tumours are aneuploid, and aneuploidy has been shown to cause oxidative stress [35], though the mechanisms

are still unclear. Nonetheless, we have found that the induction of CIN causes an increase in ROS and activation of JNK (our unpublished results). Because JNK signalling is a primary oxidative stress response, it is thought to be critical for tolerating high ROS production as it can enhance DNA repair, autophagy and antioxidant levels (see below). It is in this context, to block the ability of tumours to tolerate high ROS levels, that JNK inhibitors are being considered for cancer therapy [7, 36].

Finally, treatment of tumours also often results in further elevated levels of ROS, in this case with the objective of generating JNK-driven apoptosis [12]. For example, cisplatin is a front-line therapy that damages mitochondrial DNA leading to loss of mitochondrial gene products and hence a defective electron transport chain that produces ROS [37]. There is evidence that activation of JNK is necessary for cisplatin to be able to induce apoptosis [12] and to effectively treat tumours in animal models [38]. Radiation therapy also induces high levels of ROS, and similarly relies on JNK induction to give effective apoptosis in tumours [39].

In each of these cases it can be seen that JNK activation can be beneficial or detrimental depending on the signalling outcomes. For example, JNK activation can lead to the induction of DNA damage repair enzymes and the production of antioxidants [40]. This is a highly adaptive response for cells that have been exposed to low levels of an environmental toxin, but is the opposite of the desired effect for cisplatin chemotherapy. Conversely, JNK signalling can lead to apoptosis, which might suggest that upregulating JNK would be an effective cancer therapy, but JNK can also promote autophagy , which can be counterproductive for therapy [41]. Clearly, a more detailed understanding is needed of how stimulus and response are coupled in JNK signalling.

JNK SIGNALLING OUTCOMES

In any multicellular organism, the cellular response to genotoxic damage has to be delicately balanced. On one hand, a decision to keep damaged cells and attempt repair, risks the build-up of mutations and the threat of diseases such as cancer. On the other hand, a too robust apoptotic response to damage risks self-destruction or too much reliance on compensatory proliferation from stem cells that may also have been damaged. Consequently, organisms have developed stress sensing mechanisms such as the JNK pathway, which to some extent assess the threat level in order to produce the appropriate response: to maximise the chance that surviving cells are effectively repaired and to ensure that where cell death is needed, apoptotic cells are efficiently removed and replaced. These responses will be discussed in more detail in the following sections.

Survival: DNA Damage Repair and Protection

Cells are continually exposed to oxidative stresses from both internal sources such as active mitochondria, and external threats such as radiation or UV light. Cells generate antioxidants to counter these threats, but even so, a certain amount of ongoing oxidative DNA damage appears to be an inevitable feature of metazoan metabolism. It has been estimated that mammalian cells suffer 20,000 DNA lesions per cell per day, mostly from reactive oxygen species [42].

These lesions are repaired, typically by the constitutively active base excision repair process in G1 or G2 [43]. This results in "silent" DNA repair that is completed before the lesion has any impact on the cell [44]. Oxidative damage during replication, however, is more problematic: damaged bases can cause C:G to A:T transversion mutations [42], or stall the replication fork [45], potentially generating double strand breaks. To avoid breaks, cells can employ alternative polymerases such as Pol ι that can tolerate abnormal DNA structures and carry out trans-lesional synthesis [21]. Consistent with the role of JNK in oxidative stress responses, JNK signalling can upregulate Pol ι [46, 47], and this polymerase has recently been shown to be essential to avoid tumorigenesis in a mouse model [48]. JNK can also phosphorylate Histone 2AX to form part of the DNA double strand break repair complex [49]. There is now evidence for the activation of a wide range of DNA damage recognition and repair enzymes in response to JNK signalling, mostly via the JNK activated transcription factors FOXO or AP1.

The JNK-activated heterodimer AP1 is unusual in that its structure is directly redox sensitive: both Fos and Jun have ROS sensitive Cysteines that inactivate AP1 if oxidised [50]. Consequently, although lower levels of ROS can lead to AP1 activation by JNK-dependent phosphorylation, strong oxidative stress directly inhibits AP1 function. This threshold effect may contribute to generating dose dependent alternative outputs from JNK signalling. AP1 activity stimulated by DNA damage increases the transcription of a wide range of genes that are known to mediate DNA damage repair including MSH2, MLH1, XPA, RAD21, RAD50, GADD45 and ERCC1 [51]. It is interesting to note that AP1 activity also inhibits p53 transcription [52], which seems counterintuitive given that p53 can activate many of the same DNA repair genes. On the other hand, JNK has also been seen to directly stabilize p53 by phosphorylation [53]. These varied effects on p53 may reflect the difference between the steady state role of AP1 in cell cycle progression versus differing stress-activated levels of JNK signalling, in which the priority may be either to avoid or to ensure p53-dependent apoptosis depending on the damage level.

Activation of FOXO-dependant transcription is another JNK signalling output that has been connected with the stimulation of DNA repair. JNK pathway signalling leads to the nuclear localization of FOXO, preventing the retention and degradation of FOXO in the cytoplasm [54]. It is interesting to note that although FOXO can be negatively regulated by growth factors via the PI3K/Akt pathway, activation by stress through JNK appears to be a dominant effect, allowing FOXO activation even in proliferating cells [55]. Active nuclear FOXO has two effects that are relevant to oxidative DNA damage. Firstly, like AP1, FOXO activates the transcription of GADD45α, which has been shown to mediate the repair of either UV [56] or nitric oxide damaged DNA [57].

The second effect of FOXO activation is to drive the expression of a range of antioxidants. This is distinct from the role of AP1 which downregulates some antioxidants [58]. FOXO induces targets that include Superoxide Dismutase, Catalase and Peroxiredoxins [40, 54, 59] to remove superoxide and peroxide radicals, and Sestrin, which regenerates

Peroxiredoxins as well as increasing levels of autophagy and the antioxidant-regulating transcription factor Nrf2 [60]. As might be expected, loss of FOXO confers sensitivity to oxidative stress in a range of organisms [47, 61, 62]. Given that FOXO contributes to the survival of oxidatively stressed cells by both repairing damaged DNA and by boosting antioxidant defences, it might seem that depletion of FOXO would be a logical strategy for chemotherapy. Unfortunately, activation of FOXO is known to be needed for the effectiveness of several frontline cancer therapies including cisplatin and microtubule poisons [63]. This underlines the need to understand the role of JNK targets in detail to allow separation of their pro- and anti-apoptotic roles.

Cellular Repair: Autophagy

As well as repairing damaged DNA, cells that have suffered oxidative stress must react to the accumulation of oxidised protein and lipids. Autophagy is a common process cells employ to degrade unnecessary protein aggregates and dysfunctional organelles via the lysosomes. Autophagy is initiated with the formation of double-membraned vesicles referred to as autophagosomes to isolate targeted cytoplasmic components, then the autophagosomes are delivered and fused with lysosomes for the degradation of the sequestered components [64]. The degradation of those cytoplasmic components allows cells to eliminate harmful organelles, recycle nutrients and maintain energy homeostasis. It is a self-protection process which can be activated in response to a variety of cellular stresses including nutrient starvation, oxidative stress, DNA damage and damaged organelles [65].

JNK signalling in response to oxidative stress (Fig. **2**) is known to increase the levels of sestrins [60], which can activate AMPK to increase autophagy by blocking the effect of mTOR [66]. Consistent with this model, JNK signalling has been seen to activate autophagy through up-regulating essential autophagy genes such as *atg1*, *atg8a* and *atg18* in response to chemical induced oxidative stress [67]. An important result of this stress-induced autophagy is the removal of damaged mitochondria, which would otherwise produce ROS and increase the level of oxidative stress [60]. Consistent with this, JNK signalling is required for the survival of cells with induced chromosomal instability (CIN), which causes oxidative stress and DNA damage [68]. The shutdown of either JNK signalling or the autophagy pathway leads to an increased number of dysfunctional mitochondria, increased levels of ROS, and DNA damage as well as apoptosis in CIN cells, while inducing autophagy is able to significantly rescue these cells (our unpublished data). Autophagy has also been implicated in regulating the timing of mitosis in the presence of DNA damage [69], which may also contribute to the need for JNK signalling in CIN cells.

Aberrant autophagy is associated with tumorigenesis; however, its actual role in cancer development is highly controversial. The current view is that functional autophagy can suppress early stage tumorigenesis whereas it can facilitate advanced tumours to develop resistance against chemotherapy [70]. Autophagy exerts its protective function and suppresses tumorigenesis in many ways: autophagy increases tolerance of metabolic stress and limits chromosomal insta-

bility in cells, two hallmarks of cancer [64, 71]; autophagy could remove dysfunctional mitochondria which are the main source of endogenous ROS generation that contributes to tumorigenesis [72]; autophagy could also suppress tumorigenesis by eliminating the accumulation of certain oncoproteins, such as p62 which can alter the NF-kB pathway and promote tumorigenesis [73]. Therefore, by activating autophagy, ROS-mediated JNK activation could effectively protect cells from endogenous and external cellular stresses and prevent tumorigenesis.

On the other hand, in tumours being treated with chemotherapy, autophagy has been reported to exert either a pro-survival or pro-death role [74]. Pro-survival autophagy is seen in P53 deficient colorectal cancer cells that activate autophagy through JNK signalling to develop chemoresistance against the widely used chemotherapeutic 5-fluorouracil (5-FU). Inhibition of either JNK signalling or autophagy can effectively kill the tumour cells after 5-FU treatment [41]. Similarly, treating human glioblastoma cells with evodiamine gave autophagy activation via JNK signalling, and shutting down either autophagy or JNK signalling increased apoptosis and reduced tumour cell viability [75].

However, other research showed the pro-death function of autophagy: in chalcone-24 treated lung and bladder tumour cells, the sequential activation of JNK signalling induce autophagy-dependent necroptosis which effectively killed the cancer cells [76]. Similarly, human hepatoblastoma (HepG2) cells activate autophagy through the ROS-JNK signalling pathway to facilitate the efficacy of anticancer drug isoorientin (ISO), and inhibition of autophagy reduces the apoptosis after isoorientin treatment [77]. It appears that JNK-mediated autophagy can have both tumour suppressing and tumour promoting roles during the process of tumorigenesis which is highly dependent on the cell type and anticancer drug properties. Clearly we need to know more about the interactions between autophagy and chemotherapy to be able to avoid chemo-resistance and maximize drug efficacy.

Arrest: Cell Cycle Effects

Oxidative stress signals are sensed by FOXO, forkhead transcription factors that act as effectors of the subsequent cellular response i.e cell proliferation, apoptosis, or regulation of oxidative stress and DNA damage. As a key integrator of pro- and anti-proliferative signals, it is not surprising that aberrant regulation of FOXO leads to tumour development and progression [78].

Oxidative stress and ROS signalling can affect FOXO not only through JNK, but also via activation of the PI3K/Akt pathway (Fig. **2**) [79]. Akt phosphorylates FOXO and inhibits its activity by ensuring its cytoplasmic localization and degradation [55, 80]. This allows progression through G1, which is otherwise inhibited by FOXO activity [81]. Activated FOXO has nuclear localization and acts as a transcription factor to block cell cycle entry via p27^{kip1}. FOXO activity also decreases the transcription of genes such as CyclinB and Cdc2 that are needed for G2/M progression [82]. Overall, the activity of FOXO is anti-proliferative and acts at several stages to prevent cell cycle progression unless it is inhibited by growth factor signalling through Akt.
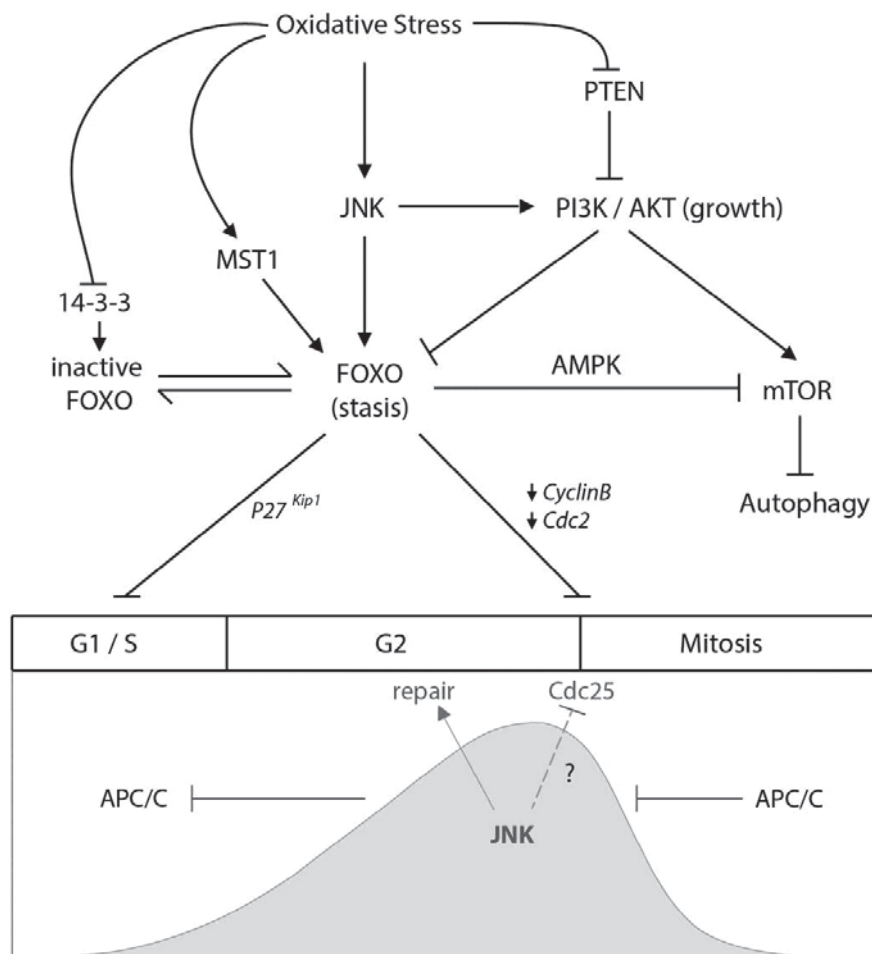
Fig. (2). Oxidative stress typically activates FOXO by several mechanisms including direct phosphorylation, blocking 14-3-3 binding and blocking the PI3K/Akt pathway that otherwise inhibits FOXO during growth factor signalling. Activation of FOXO typically leads to cell cycle arrest in G1 or G2 as well as activation of autophagy via AMPK. JNK activity normally peaks during G2, and may ensure effective DNA repair before mitotic entry, when it is degraded by the APC/C.

The oxidative stress-activated JNK pathway is known to activate FOXO. This can be by direct phosphorylation/activation of FOXO4 at Thr447 and Thr451 [83], and by blocking the inhibitory effect of PKB/Akt on FOXO [84]. Oxidative stress can also activate FOXO by directly activating MST1, which phosphorylates FOXO1 and FOXO3a [85], or by disrupting inhibitory 14-3-3 binding [86]. However, ROS can also activate PI3K/Akt signalling by inhibiting the PI3K negative regulator (PTEN) phosphatase [87], so it has become clear that, like AP1, FOXO is either up- or down-regulated by ROS through multiple mechanisms to coordinate an appropriate response [88].

In a normal cell cycle, nuclear JNK is degraded by the APC/C in mitosis [14], resulting in low levels of JNK during mitosis and G1. However, activation of JNK in response to oxidative stress leads to inhibition of the APC/C which results in persistent cyclinB, Plk and cdc20, giving mitotic arrest [14]. Oxidative stress mediated activation of JNK also correlates with inhibitory phosphorylation of cdc25 at Ser-168 [89], again resulting in G2/M arrest. It would seem plausible that JNK might contribute to checkpoint function in G2 to prevent mitotic progression in the presence of DNA damage, however the evidence for this is limited. Although JNK

can phosphorylate cdc25c [90], there is inconclusive evidence that it does so *in vivo*, primarily because the JNK inhibitors used (SP600125 or TAT-JIP) efficiently inhibit other cell cycle regulators such as CDK1, MPS1 and CDK2 [91, 92]. This lack of a specific inhibitor has meant that some cell cycle roles of JNK are still poorly characterised.

We and others have directly tested whether JNK function is needed for the DNA damage checkpoint in G1 [93] or G2 [68], and have found that cells lacking JNK can still effectively arrest in response to irradiation. JNK is activated in response to DNA damage, but it seems that its role is not to induce arrest so much as to drive either repair or apoptosis. We found that JNK depletion increased both the level of DNA damage and apoptosis in stressed cells, but that increasing the duration of G2 could rescue these defects [68], suggesting that if repair can be completed before mitotic entry, there is no lasting damage done. DNA damage persisting into mitosis, on the other hand, is likely to be perpetuated by mitotic errors [94]. In addition, we and others have observed activated JNK on centrosomes during early mitosis which is then lost during anaphase [68, 95]. This is consistent with degradation of JNK by APC/C cdh1 [14]. Several DNA damage response genes and cell cycle regulators are

also localized on centrosomes. Their functional interactions are still poorly understood, but consistent with vertebrate data showing evidence of JNK-regulated targets in mitosis [90, 96-98].

Although oxidative stress can activate JNK and FOXO to give cell cycle arrest or apoptosis, JNK is also implicated in triggering compensatory proliferation to replace dying cells [99, 100]. It is superficially paradoxical that a cellular response to genotoxic damage should encourage proliferation, but this tension has been noted for decades [101], and several explanations exist, including the need for multicellular animals to maintain enough cells to support organ function, despite high levels of damage. Because chemotherapy can generate high levels of apoptosis, JNK-mediated compensatory proliferation is of significant clinical interest. Apoptosis can trigger proliferation in the neighbouring cells by activating Wnt and JNK signalling pathways via Dronc/Caspase 9 [99]. It is not yet clear how Caspase 9 activates JNK, nor why JNK stimulates proliferation in this setting, though it may relate to a known developmental requirement for JNK in EGF signalling [102]. JNK is also activated in the cells near to wounds, possibly by localized ROS production [103], and is required for the onset of regeneration in the absence of cell death [100], consistent with the known roles of JNK in migration and differentiation during development [104, 105].

Death: Induction of Apoptosis

Experiments using JNK mutants have clearly shown that JNK is needed for normal apoptotic responses [93, 106]. Lack of JNK prevented apoptosis in response to irradiation and DNA damage, and was accompanied by a failure to release cytochrome c and activate caspases. In vertebrates, a range of mechanisms seem to be involved downstream of active JNK, centring on the activation of Bax and the inactivation of Bcl at the mitochondria [107, 108]. Bax activation can come from JNK triggering cleavage of Bid [109] or stabilization of p53 [110]. Similarly, JNK can also stabilize p73, which is needed for the appropriate apoptotic response to the DNA damaging pro-oxidant cisplatin [111]. Elevated levels of JNK have been shown to sensitize cells to cisplatin induced apoptosis [38].

Activated JNK is known to move to the mitochondria to facilitate the release of pro-apoptotic factors [112-114], as well as its nuclear role in transcriptional upregulation through AP1 and FOXO [63, 115]. The transcriptional role in apoptosis is thought to be particularly significant in organisms such as *Drosophila* where JNK signalling gives increased transcription of genes that lead to the degradation of DIAP and the release of apoptosis inhibition [116]. Interestingly, this pathway can give p53 independent apoptosis in response to DNA damage [10], which may be helpful in the context of tumour treatment, which often involves DNA damage to p53 mutant cells.

As described earlier, activation of JNK that leads to apoptosis is typically protracted and intense, and can come from either external or internal sources. For example, JNK1 is activated by the pro-oxidant and pro-apoptotic factor TNF-α. This occurs via the JNK activator ASK1 (Apoptosis signal regulating kinase 1), which responds to a broad spectrum of stimuli including TNF-α, ROS, lipopolysaccharide (LPS)

and endoplasmic reticulum (ER) stress [107]. For activation by oxidative stress, an inhibitory interaction between ASK1 and thioredoxin is removed when ROS oxidises thioredoxin [117]. This promotes ASK1 dimerization, allowing the recruitment of TNF-receptor associated factors (TRAF2 and TRAF6) and the activation of JNK to drive apoptosis. JNK can be transiently activated by TNFα/ROS through other pathways (such as DNA damage), but ASK1 is needed for persistent activation that gives apoptosis [118]. ROS can activate JNK to activate AP1, which increases transcription of TNF-α to produce more ROS, so feedback amplification of cell death signals may occur, though as noted earlier, this may be limited by oxidative inactivation of AP1. Furthermore, TNF-α can be secreted, so in some cases the induction of cell death in one cell can lead to the JNK-dependent death of nearby cells [119]. What prevents this mechanism from spreading indefinitely is not known, but it may relate to evidence that cell death can also generate protection from apoptosis in surrounding cells [120, 121]. The balance between these processes is currently poorly understood, but JNK signalling is likely to be a critical component.

THE JNK PATHWAY AS A CANCER THERAPY TARGET

Sequencing of human tumours has identified mutations that inactivate either JNK or its upstream regulators [122-125], suggesting that mutations that disable the JNK pathway can promote cancer development. However, such mutations are relatively rare (<1% of COSMIC database samples have mutated JNK1) and studies on both rodent model systems and human cancers, have suggested that JNKs can have either oncogenic [126] or tumour suppressive effects [127]. For example, in hepatocellular carcinomas (HCC), JNK1 can be oncogenic, as increased kinase activity is associated with tumour formation [128]. Similarly, JNK activity is needed for cellular transformation by common oncogenic mutations in RAS, Bcr-Abl [129], but conversely, JNK is also needed for the apoptotic effectiveness of several chemotherapies [19, 63]. Since JNK activity can either promote or prevent carcinogenesis, therapeutic intervention in the JNK pathway must consider the cellular environments that trigger each response. For example, oxidative stress plays a vital role in liver and colorectal cancers, at both the initiation and progression steps [130], so it is essential to understand the relationship between oxidative stress-triggered JNK signalling and cellular outcomes.

Chemicals that produce high levels of ROS in the liver normally trigger a JNK-dependent stress response, and defects in this response can, for example, lead to increased HCC tumorigenesis [131, 132]. In this case, lack of JNK-dependent antioxidant production may speed up the accumulation of mutations needed for transformation. Once a tumour has formed, however, the same lack of antioxidants may restrict the growth of chemical induced HCC tumours [128]. Similarly, mice lacking JNK1 show a marked decrease in the growth of gastric tumours caused by the ROS generating drug N-methy-N-nitrosurea, as compared to wild-type controls [5]. This case is interesting because the mutant mice apparently showed less DNA damage and less apoptosis than wild types, but still developed fewer tumours. In this case it may be that in wild type animals the chemical-

induced high ROS levels gave JNK-mediated apoptosis triggering undesirable compensatory proliferation that promoted tumorigenesis as well as being able to effectively increase antioxidant levels to promote tumour survival. The problem here is evident: if a genotoxin generates more tumours in JNK mutants we can explain it by saying that JNK is needed for cell death, but if we see fewer tumours in JNK mutants we can instead explain the result by saying that JNK is needed for compensatory proliferation or for anti-oxidant production in stressed tumour cells. Presumably both processes are at work, and the outcome may depend on the levels and timing of JNK activation, though this has not been examined in detail in animal cancer models. Because we lack a detailed time-course of tumour induction, it is not clear where in the process JNK has its effect - it could be during the accumulation of oncogenic mutations, in the stress-response adaptation to those mutations, in the ability to apoptose in response to the mutations or in the ability to replace apoptosed cells, as well as the later processes of metastasis and therapy resistance.

Manipulating JNK signalling is of interest to drug companies due to its involvement in so many disease processes. The knock-down of JNK genes alone or in combination has been an important step in understanding JNK function and was significant in detecting the specific JNK pathway genes that are relevant. However, alternative approaches including antisense or RNAi techniques, and chemical and peptide inhibitors have now been developed that can inhibit JNK function. Mounting evidence has shown that these inhibitors can be effective *in vivo*, indicating that the JNK pathway may be accessible for therapeutic manipulation [36]. Three different kinds of inhibitors have been thoroughly studied to date. The first are the ATP-competitive inhibitors of the JNK pathway such as CEP-1347 and SP600125. CEP-1347 can inhibit the activation of the JNK pathway to prevent β - amyloid, NGF-withdrawal, oxidative-stress, and UV irradiation-induced cell death in PC12 cells and rat sympathetic neurons [133, 134]. However, high cellular levels of ATP can reduce the efficacy of ATP-competitive inhibitors, and this has been observed for CEP-1347 [135]. SP600125 also has the significant drawback of being poorly specific for JNK over other significant kinases such as MPS1 [92, 136]. The second type of inhibitor targets the substrate-binding site. For example, JNK-interacting protein-1 (JIP1) functions as a scaffold with other JNK pathway proteins to build a functional signalling complex. Overexpression of JIP1-derived peptides can effectively block JNK activity, though again there are concerns with specificity [91]. A third kind of inhibitor targets allosteric regulatory sites using peptides or chemicals to bind and block their phosphorylation. While all these types of inhibitor can effectively decrease JNK activity, their use in therapy remains problematic, perhaps due to the need for JNK in normal cell maintenance as well as for tumour cell apoptosis. Consequently, no JNK inhibitors are in clinical use, though several companies such as Celgene have been pursuing trials for some time (CC401, CC930). However, new inhibitors are being developed, and our understanding of the multiple roles of JNK is being refined [137], so there is reason to expect more specific and better targeted manipulation of the JNK pathway as a clinical approach in the near future.

CONFLICT OF INTEREST

ACKNOWLEDGEMENTS

REFERENCES

[1]   Hibi M, Lin A, Smeal T, Minden A, Karin M. Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. Genes Dev 1993; 7: 2135-48.

[2]   Karin M, Gallagher E. From JNK to pay dirt: jun kinases, their biochemistry, physiology and clinical importance. IUBMB Life 2005; 57: 283-95.

[3]   Cairns R, Harris IS, Mak TW. Regulation of cancer cell metabolism. Nat Rev Cancer 2011; 11: 85-95.

[4]   Gerlinger M, Swanton C. How Darwinian models inform therapeutic failure initiated by clonal heterogeneity in cancer medicine. Br J Cancer 2010; 103: 1139-43.

[5]   Shibata W, Maeda S, Hikiba Y, Yanai A, Sakamoto K, *et al.* c-Jun NH2-terminal kinase 1 is a critical regulator for the development of gastric cancer in mice. Cancer Res 2008; 68: 5031-9.

[6]   Picco V, Pages G. Linking JNK Activity to the DNA Damage Response. Genes Cancer 2013; 4: 360-8.

[7]   Wagner EF, Nebreda AR. Signal integration by JNK and p38 MAPK pathways in cancer development. Nat Rev Cancer 2009; 9: 537-49.

[8]   Wagner EF, Nebreda AR. Signal integration by JNK and p38 MAPK pathways in cancer development. Nat Rev Cancer 2009; 9: 537-49.

[9]   Helbig L, Damrot J, Hülsenbeck J, *et al.* Late activation of stress-activated protein kinases/c-Jun N-terminal kinases triggered by cisplatin-induced DNA damage in repair-defective cells. J Biol Chem 2011; 286: 12991-13001.

[10]   McNamee LM, Brodsky MH. p53-independent apoptosis limits DNA damage-induced aneuploidy. Genetics 2009; 182: 423-5.

[11]   Christmann M, Tomicic MT, Aasland D, Kaina B. A role for UV-light-induced c-Fos: Stimulation of nucleotide excision repair and protection against sustained JNK activation and apoptosis. Carcinogenesis 2007; 28: 183-90.

[12]   Sánchez-Perez I, Murguía JR, Perona R. Cisplatin induces a persistent activation of JNK that is related to cell death. Oncogene 1998; 16: 533-40.

[13]   Lang W, Wang H, Ding L, Xiao L. Cooperation between PKC-alpha and PKC-epsilon in the regulation of JNK activation in human lung cancer cells. Cell Signal 2004; 16: 457-67.

[14]   Gutierrez GJ, Tsuji T, Chen M, Jiang W, Ronai ZA. Interplay between Cdh1 and JNK activity during the cell cycle. Nat Cell Biol 2010; 12: 686-95.

[15]   Biteau B, Hochmuth C, Jasper H. JNK Activity in Somatic Stem Cells Causes Loss of Tissue Homeostasis in the Aging Drosophila Gut. Cell Stem Cell 2008; 3: 442-55.

[16]   Martindale JL, Holbrook NJ. Cellular response to oxidative stress: signalling for suicide and survival. J Cell Physiol 2002; 192: 1-15.

[17]   Bae YS, Oh H, Rhee SG, Yoo Y Do. Regulation of reactive oxygen species generation in cell signalling. Mol Cells 2011; 32: 491-509.

[18]   Osone S, Hosoi H, Kuwahara Y, *et al.* Fenretinide induces sustained-activation of JNK/p38 MAPK and apoptosis in a reactive oxygen species-dependent manner in neuroblastoma cells. Int J Cancer 2004; 112: 219-24.

[19]   Chiu W-H, Luo S-J, Chen C-L, *et al.* Vinca alkaloids cause aberrant ROS-mediated JNK activation, Mcl-1 downregulation, DNA damage, mitochondrial dysfunction, and apoptosis in lung adenocarcinoma cells. Biochem Pharmacol 2012; 83: 1159-71.

[20]   Kwong LK, Mockett RJ, Bayne AC, Orr WC, Sohal RS. Decreased mitochondrial hydrogen peroxide release in transgenic Drosophila melanogaster expressing intramitochondrial catalase. Arch Biochem Biophys 2000; 383: 303-8.

[21]   Jones RM, Petermann E. Replication fork dynamics and the DNA damage response. Biochem J 2012 443: 13-26.

[22]   Fisher AB. Redox signalling across cell membranes. Antioxid Redox Signal 2009; 11: 1349-56.

[23]   Szczesny B, Olah G, Walker DK, *et al*. Deficiency in repair of the mitochondrial genome sensitizes proliferating myoblasts to oxidative damage. PLoS One 2013; 8: e75201.

[24]   Janssen A, van der Burg M, Szuhai K, Kops GJ, Medema RH. Chromosome segregation errors as a cause of DNA damage and structural chromosome aberrations. Science 2011; (80)333: 1895-8.

[25]   Basu A, Krishnamurthy S. Cellular responses to Cisplatin-induced DNA damage. J Nucleic Acids 2010.

[26]   Llopis A, Salvador N, Ercilla A, *et al*. The stress-activated protein kinases p38α/β and JNK1/2 cooperate with Chk1 to inhibit mitotic entry upon DNA replication arrest. Cell Cycle 2012; 11: 3627-37.

[27]   Nehmé A, Baskaran R, Nebel S, *et al*. Induction of JNK and c-Abl signalling by cisplatin and oxaliplatin in mismatch repair-proficient and -deficient cells. Br J Cancer 1999; 79: 1104-10.

[28]   Rabik CA, Dolan ME. Molecular mechanisms of resistance and toxicity associated with platinating agents. Cancer Treat Rev 2007; 33: 9-23.

[29]   Tang FR, Loke WK. Molecular mechanisms of low dose ionizing radiation induced hormesis, adaptive responses, radioresistance, bystander effects, and genomic instability. Int J Radiat Biol 2014: 1-68.

[30]   Moskalev AA, Plyusnina EN, Shaposhnikov M V. Radiation hormesis and radioadaptive response in Drosophila melanogaster flies with different genetic backgrounds: the role of cellular stress-resistance mechanisms. Biogerontology 2011; 12: 253-63.

[31]   Glorieux C, Dejeans N, Sid B, *et al*. Catalase overexpression in mammary cancer cells leads to a less aggressive phenotype and an altered response to chemotherapy. Biochem Pharmacol 2011; 82: 1384-90.

[32]   Kamp DW, Shacter E, Weitzman SA. Chronic inflammation and cancer: the role of the mitochondria. Oncology (Williston Park) 2011; 25: 400-410, 413.

[33]   Gorrini C, Harris IS, Mak TW. Modulation of oxidative stress as an anticancer strategy. Nat Rev Drug Discov 2013; 12: 931-47.

[34]   Bakhoum SF, Silkworth WT, Nardi IK, *et al*. The mitotic origin of chromosomal instability. Curr Biol 2014; 24: R148-9.

[35]   Oromendia AB, Dodgson SE, Amon A. Aneuploidy causes proteotoxic stress in yeast. Genes Dev 2012; 26: 2696-708.

[36]   Bogoyevitch MA, Ngoei KR, Zhao TT, Yeap YY, Ng DC. c-Jun N-terminal kinase (JNK) signalling: recent advances and challenges. Biochim Biophys Acta 2010; 1804: 463-75.

[37]   Marullo R, Werner E, Degtyareva N, *et al*. Cisplatin induces a mitochondrial-ROS response that contributes to cytotoxicity depending on mitochondrial redox status and bioenergetic functions. PLoS One 2013; 8: e81162.

[38]   Pereira L, Igea A, Canovas B, Dolado I, Nebreda AR. Inhibition of p38 MAPK sensitizes tumour cells to cisplatin-induced apoptosis mediated by reactive oxygen species and JNK. EMBO Mol Med 2013; 5: 1759-74.

[39]   Kim Y-J, Lee W-S, Ip C, *et al*. Prx1 suppresses radiation-induced c-Jun NH2-terminal kinase signalling in lung cancer cells through interaction with the glutathione S-transferase Pi/c-Jun NH2-terminal kinase complex. Cancer Res 2006; 66: 7136-42.

[40]   Lee KW, Lee DJ, Lee JY, *et al*. (2011) Peroxiredoxin II restrains DNA damage-induced death in cancer cells by positively regulating JNK-dependent DNA repair. J Biol Chem 286: 8394-404.

[41]   Sui X, Kong N, Wang X, *et al*. JNK confers 5-fluorouracil resistance in p53-deficient and mutant p53-expressing colon cancer cells by inducing survival autophagy. Sci Rep 2014; 4: 4694.

[42]   Markkanen E, Hubscher U, van Loon B. Regulation of oxidative DNA damage repair. The adenine : 8-oxo-guanine problem. Cell Cycle 2012; 11: 1070-5.

[43]   Maynard S, Schurman SH, Harboe C, de Souza-Pinto NC, Bohr V. Base excision repair of oxidative DNA damage and association with cancer and aging. Carcinogenesis 2009; 30: 2-10.

[44]   Leroy C, Mann C, Marsolier MC. Silent repair accounts for cell cycle specificity in the signalling of oxidative DNA lesions. EMBO J 2001; 20: 2896-906.

[45]   Branzei D, Foiani M. Maintaining genome stability at the replication fork. Nat Rev Mol Cell Biol 2010; 11: 208-19.

[46]   Yuan F, Xu Z, Yang M, *et al*. Overexpressed DNA polymerase iota regulated by JNK/c-Jun contributes to hypermutagenesis in bladder cancer. PLoS One 2013; 8: e69317.

[47]   Jünger M, Rintelen F. The Drosophila forkhead transcription factor FOXO mediates the reduction in cell number associated with reduced insulin signalling. J Biol 2003; 2: 20.

[48]   Iguchi M, Osanai M, Hayashi Y, Koentgen F, Lee G-H. The error-prone DNA polymerase ι provides quantitative resistance to lung tumorigenesis and mutagenesis in mice. Oncogene 2014; 33: 3612-7.

[49]   Lu C, Zhu F, Cho YY, *et al*. Cell apoptosis: requirement of H2AX in DNA ladder formation, but not for the activation of caspase-3. Mol Cell 2006; 23: 121-32.

[50]   Go Y-M, Jones DP. Redox control systems in the nucleus: mechanisms and functions. Antioxid Redox Signal 2010; 13: 489-509.

[51]   Hayakawa J, Mittal S, Wang Y, *et al*. Identification of promoters bound by c-Jun/ATF2 during rapid large-scale gene activation following genotoxic stress. Mol Cell 2004; 16: 521-35.

[52]   Schreiber M, Kolbus A, Piu F, *et al*. Control of cell cycle progression by c-Jun is p53 dependent Control of cell cycle progression by c-Jun is p53 dependent. Genes Dev 1999; 13: 607-19.

[53]   Buschmann T, Potapova O, Bar-Shira A, *et al*. Jun NH2-terminal kinase phosphorylation of p53 on Thr-81 is important for p53 stabilization and transcriptional activities in response to stress. Mol Cell Biol 2001; 21: 2743-54.

[54]   Van der Horst A, Burgering BMT. Stressing the role of FoxO proteins in lifespan and disease. Nat Rev Mol Cell Biol 2007; 8: 440-50.

[55]   Puig O, Mattila J. Understanding Forkhead box class O function: lessons from Drosophila melanogaster. Antioxid Redox Signal 2011; 14: 635-47.

[56]   Tran H, Brunet A, Grenier JM, *et al*. DNA repair pathway stimulated by the forkhead transcription factor FOXO3a through the Gadd45 protein. Science 2002; 296: 530-4.

[57]   Hughes KJ, Meares GP, Chambers KT, Corbett J. Repair of nitric oxide-damaged DNA in beta-cells requires JNK-dependent GADD45alpha expression. J Biol Chem 2009 284: 27402-8.

[58]   Wilkinson J, Radjendirane V, Pfeiffer GR, Jaiswal AK, Clapper ML. Disruption of c-Fos leads to increased expression of NAD(P)H:quinone oxidoreductase1 and glutathione S-transferase. Biochem Biophys Res Commun 1998; 253: 855-8.

[59]   Ahn H-M, Lee K-S, Lee D-S, Yu K. JNK/FOXO mediated PeroxiredoxinV expression regulates redox homeostasis during Drosophila melanogaster gut infection. Dev Comp Immunol 2012; 38: 466-73.

[60]   Lee JH, Budanov AV, Park EJ, *et al*. Sestrin as a feedback inhibitor of TOR that prevents age-related pathologies. Science 2010; 327: 23-28.

[61]   Kops GJPL, Dansen TB, Polderman PE, *et al*. Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress. Nature 2002; 419: 316-21.

[62]   Yanase S, Yasuda K, Ishii N. Adaptive responses to oxidative damage in three mutants of Caenorhabditis elegans (age-1, mev-1 and daf-16) that affect life span. Mech Ageing Dev 2002; 123: 1579-87.

[63]   Gomes AR, Brosens JJ, Lam EW-F. Resist or die: FOXO transcription factors determine the cellular response to chemotherapy. Cell Cycle 2008; 7: 3133-6.

[64]   Mathew R, Kongara S, Beaudoin B, *et al*. Autophagy suppresses tumour progression by limiting chromosomal instability. Genes Dev 2007; 21: 1367-81.

[65]   Kroemer G, Mariño G, Levine B. Autophagy and the integrated stress response. Mol Cell 2010; 40: 280-93.

[66]   Budanov AV, Karin M. p53 target genes sestrin1 and sestrin2 connect genotoxic stress and mTOR signalling. Cell 2008; 134: 451-60.

[67]   Wu H, Wang MC, Bohmann D. JNK protects Drosophila from oxidative stress by trancriptionally activating autophagy. Mech Dev 2009; 126: 624-37.

[68]   Wong HW, Shaukat Z, Wang J, Saint R, Gregory SL. JNK signalling is needed to tolerate chromosomal instability. Cell Cycle 2013;13.

[69]   Dotiwala F, Eapen VV, Harrison JC, *et al*. DNA damage checkpoint triggers autophagy to regulate the initiation of anaphase. Proc Natl Acad Sci USA 2013; 110: E41-9.

[70]   Guo JY, Xia B, White E. Autophagy-mediated tumour promotion. Cell 2013; 155: 1216-9.

[71]   Karantza-Wadsworth V, Patel S, Kravchuk O, *et al*. Autophagy mitigates metabolic stress and genome damage in mammary tumorigenesis. Genes Dev 2007; 21: 21-35.

[72]   Deffieu M, Bhatia-Kiššová I, Salin B, *et al*. Increased levels of reduced cytochrome b and mitophagy components are required to trigger nonspecific autophagy following induced mitochondrial dysfunction. J Cell Sci 2013; 126: 415-26.

[73]   Mathew R, Karp CM, Beaudoin B, *et al*. Autophagy suppresses tumorigenesis through elimination of p62. Cell 2009; 137: 1062-75.

[74]   Sui X, Chen R, Wang Z, *et al*. Autophagy and chemotherapy resistance: a promising therapeutic target for cancer treatment. Cell Death Dis 2013; 4: e838.

[75]   Liu A-J, Wang S-H, Chen K-C, *et al*. Evodiamine, a plant alkaloid, induces calcium/JNK-mediated autophagy and calcium/mitochondria-mediated apoptosis in human glioblastoma cells. Chem Biol Interact 2013; 205: 20-28.

[76]   He W, Wang Q, Srinivasan B, *et al*. A JNK-mediated autophagy pathway that triggers c-IAP degradation and necroptosis for anticancer chemotherapy. Oncogene 2014; 33: 3004-13.

[77]   Yuan L, Wei S, Wang J, Liu X. Isoorientin induces apoptosis and autophagy simultaneously by reactive oxygen species (ROS)-related p53, PI3K/Akt, JNK, and p38 signalling pathways in HepG2 cancer cells. J Agric Food Chem 62: 2014; 5390-400.

[78]   Myatt SS, Brosens JJ, Lam EW-F. Sense and sensitivity: FOXO and ROS in cancer development and treatment. Antioxid Redox Signal 2011; 14: 675-87.

[79]   Yuan X, Zhou Y, Wang W, *et al*. Activation of TLR4 signalling promotes gastric cancer progression by inducing mitochondrial ROS production. Cell Death Dis 2013; 4: e794.

[80]   Burgering BMT. Decisions on life and death: FOXO Forkhead transcription factors are in command when PKB/Akt is off duty. J Leukoc Biol 2003; 73: 689-701.

[81]   Medema RH, Kops GJ, Bos JL, Burgering BM. AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1. Nature 2000; 404: 782-7.

[82]   Ho KK, Myatt SS, Lam EW-F. Many forks in the path: cycling with FoxO. Oncogene 2008; 27: 2300-11.

[83]   Essers MAG, Weijzen S, de Vries-Smits AMM, *et al*. FOXO transcription factor activation by oxidative stress mediated by the small GTPase Ral and JNK. EMBO J 2004; 23: 4802-12.

[84]   Sunters A, Madureira PA, Pomeranz KM, *et al*. Paclitaxel-induced nuclear translocation of FOXO3a in breast cancer cells is mediated by c-Jun NH2-terminal kinase and Akt. Cancer Res 2006; 66: 212-20.

[85]   Brent MM, Anand R, Marmorstein R. Structural basis for DNA recognition by FoxO1 and its regulation by posttranslational modification. Structure 2008; 16: 1407-16.

[86]   Yuan Z, Lehtinen MK, Merlo P, *et al*. Regulation of neuronal cell death by MST1-FOXO1 signalling. J Biol Chem 2009; 284: 11285-92.

[87]   Seo JH, Ahn Y, Lee S-R, Yeol Yeo C, Chung Hur K. The major target of the endogenously generated reactive oxygen species in response to insulin stimulation is phosphatase and tensin homolog and not phosphoinositide-3 kinase (PI-3 kinase) in the PI-3 kinase/Akt pathway. Mol Biol Cell 2005; 16: 348-57.

[88]   De Keizer PLJ, Burgering BMT, Dansen TB. Forkhead box o as a sensor, mediator, and regulator of redox signalling. Antioxid Redox Signal 2011; 14: 1093-106.

[89]   Goss VL, Cross J V., Ma K, Qian Y, Mola PW, *et al*. SAPK/JNK regulates cdc2/cyclin B kinase through phosphorylation and inhibition of cdc25c. Cell Signal 2003; 15: 709-718.

[90]   Gutierrez GJ, Tsuji T, Cross JV, *et al*. JNK-mediated phosphorylation of Cdc25C regulates cell cycle entry and G(2)/M DNA damage checkpoint. J Biol Chem 2010; 285: 14217-28.

[91]   Melino M, Hii CS, McColl SR, Ferrante A. The effect of the JNK inhibitor, JIP peptide, on human T lymphocyte proliferation and cytokine production. J Immunol 2008; 181: 7300-6.

[92]   Schmidt M, Budirahardja Y, Klompmaker R, Medema RH. Ablation of the spindle assembly checkpoint by a compound targeting Mps1. EMBO Rep 2005; 6: 866-72.

[93]   Tournier C, Hess P, Yang DD, *et al*. Requirement of JNK for stress-induced activation of the cytochrome c-mediated death pathway. Science 2000; 288: 870-4.

[94]   Ganem NJ, Pellman D. Linking abnormal mitosis to the acquisition of DNA damage. J Cell Biol 2012; 199: 871-81.

[95]   Xu D, Zhang F, Wang Y, Sun Y, Xu Z. Microcephaly-associated protein WDR62 regulates neurogenesis through JNK1 in the developing neocortex. Cell Rep 2014; 6: 104-16.

[96]   Lee K, Song K. Basal c-Jun N-terminal kinases promote mitotic progression through histone H3 phosphorylation. Cell Cycle 2008; 7: 216-21.

[97]   MacCorkle-Chosnek RA, VanHooser A, Goodrich DW, Brinkley BR, Tan TH. Cell cycle regulation of c-Jun N-terminal kinase activity at the centrosomes. Biochem Biophys Res Commun 2001; 289: 173-80.

[98]   Bogoyevitch MA, Yeap YY, Qu Z, *et al*. WD40-repeat protein 62 is a JNK-phosphorylated spindle pole protein required for spindle maintenance and timely mitotic progression. J Cell Sci 2012; 125: 5096-109.

[99]   Fan Y, Wang S, Hernandez J, *et al*. Genetic models of apoptosis-induced proliferation decipher activation of JNK and identify a requirement of EGFR signalling for tissue regenerative responses in Drosophila. PLoS Genet 2014; 10: e1004131.

[100]   Bergantiños C, Corominas M, Serras F. Cell death-induced regeneration in wing imaginal discs requires JNK signalling. Development 2010; 137: 1169-79.

[101]   Devary Y, Gottlieb RA, Smeal T, Karin M. The mammalian ultraviolet response is triggered by activation of src tyrosine kinases. Cell 1992; 71: 1081-91.

[102]   Weston CR, Wong A, Hall JP, *et al*. The c-Jun NH2-terminal kinase is essential for epidermal growth factor expression during epidermal morphogenesis. Proc Natl Acad Sci USA 2004; 101: 14114-9.

[103]   Razzell W, Evans IR, Martin P, Wood W. Calcium flashes orchestrate the wound inflammatory response through DUOX activation and hydrogen peroxide release. Curr Biol 2013; 23: 424-9.

[104]   Owusu-Ansah E, Banerjee U. Reactive oxygen species prime Drosophila haematopoietic progenitors for differentiation. Nature 2009; 461: 537-41.

[105]   Ishimaru S, Ueda R, Hinohara Y, Ohtani M, Hanafusa H. PVR plays a critical role via JNK activation in thorax closure during Drosophila metamorphosis. EMBO J 2004; 23: 3984-94.

[106]   McEwen DG, Peifer M. Puckered, a Drosophila MAPK phosphatase, ensures cell viability by antagonizing JNK-induced apoptosis. Development 2005; 132: 3935-3946.

[107]   Dhanasekaran DN, Reddy EP. JNK signalling in apoptosis. Oncogene 2008; 27: 6245-51.

[108]   Weston CR, Davis RJ. The JNK signal transduction pathway. Curr Opin Cell Biol 2007; 19: 142-9.

[109]   Deng Y, Ren X, Yang L, Lin Y, Wu X. A JNK-Dependent Pathway Is Required for TNFα-Induced Apoptosis. Cell 2003; 115: 61-70.

[110]   Oleinik NV, Krupenko NI, Krupenko SA. Cooperation between JNK1 and JNK2 in activation of p53 apoptotic pathway. Oncogene 2007; 26: 7222-30.

[111]   Jones EV, Dickman MJ, Whitmarsh AJ. Regulation of p73-mediated apoptosis by c-Jun N-terminal kinase. Biochem J 2007; 405: 617-23.

[112]   Chauhan D, Li G, Hideshima T, *et al*. JNK-dependent release of mitochondrial protein, Smac, during apoptosis in multiple myeloma (MM) cells. J Biol Chem 2003; 278: 17593-6.

[113]   Sinha K, Das J, Pal PB, Sil PC. Oxidative stress: the mitochondria-dependent and mitochondria-independent pathways of apoptosis. Arch Toxicol 2013; 87: 1157-80.

[114]   Chambers JW, LoGrasso PV. Mitochondrial c-Jun N-terminal kinase (JNK) signalling initiates physiological changes resulting in amplification of reactive oxygen species generation. J Biol Chem 2011; 286: 16052-62.

[115]   Yi CH, Sogah DK, Boyce M, *et al*. A genome-wide RNAi screen reveals multiple regulators of caspase activation. J Cell Biol 2007; 179: 619-26.

[116]   Martín F a, Peréz-Garijo A, Morata G. Apoptosis in Drosophila: compensatory proliferation and undead cells. Int J Dev Biol 2009; 53: 1341-7.

[117]   Soga M, Matsuzawa A, Ichijo H. Oxidative Stress-Induced Diseases via the ASK1 Signalling Pathway. Int J Cell Biol 2012; 439587.

[118]   Tobiume K, Matsuzawa A, Takahashi T, *et al*. ASK1 is required for sustained activations of JNK/p38 MAP kinases and apoptosis. EMBO Rep 2001; 2: 222-8.

[119] Pérez-Garijo A, Fuchs Y, Steller H. Apoptotic cells can induce non-autonomous apoptosis through the TNF pathway. Elife 2013; 2: e01004.

[120] Bilak A, Uyetake L, Su TT. Dying cells protect survivors from radiation-induced cell death in Drosophila. PLoS Genet 2014; 10: e1004220.

[121] Grusche FA, Degoutin JL, Richardson HE, Harvey KF. The Salvador/Warts/Hippo pathway controls regenerative tissue growth in Drosophila melanogaster. Dev Biol 2011; 350: 255-66.

[122] Greenman C, Stephens P, Smith R, *et al*. Patterns of somatic mutation in human cancer genomes. Nature 2007; 446: 153-8.

[123] Kan Z, Jaiswal BS, Stinson J, *et al*. Diverse somatic mutation patterns and pathway alterations in human cancers. Nature 2010; 466: 869-73.

[124] Parsons DW, Wang T-L, Samuels Y, *et al*. Colorectal cancer: mutations in a signalling pathway. Nature 2005; 436: 792.

[125] Pham TT, Angus SP, Johnson GL. MAP3K1: Genomic Alterations in Cancer and Function in Promoting Cell Survival or Apoptosis. Genes Cancer 2013; 4: 419-26.

[126] Sancho R, Nateri AS, de Vinuesa AG, *et al*. JNK signalling modulates intestinal homeostasis and tumorigenesis in mice. Embo J 2009; 28: 1843-54.

[127] She Q-B, Chen N, Bode AM, Flavell RA, Dong Z. Deficiency of c-Jun-NH(2)-terminal kinase-1 in mice enhances skin tumour development by 12-O-tetradecanoylphorbol-13-acetate. Cancer Res 2002; 62: 1343-8.

[128] Hui L, Zatloukal K, Scheuch H, Stepniak E, Wagner EF. Proliferation of human HCC cells and chemically induced mouse liver cancers requires JNK1-dependent p21 downregulation. J Clin Invest 2008; 118: 3943-53.

[129] Manning AM, Davis RJ. Targeting JNK for therapeutic benefit: from junk to gold? Nat Rev Drug Discov 2003; 2: 554-65.

[130] Klaunig JE, Kamendulis LM, Hocevar BA. Oxidative stress and oxidative damage in carcinogenesis. Toxicol Pathol 2010; 38: 96-109.

[131] Tasaki M, Kuroiwa Y, Inoue T, *et al*. Lack of nrf2 results in progression of proliferative lesions to neoplasms induced by long-term exposure to non-genotoxic hepatocarcinogens involving oxidative stress. Exp Toxicol Pathol 2014; 66: 19-26.

[132] Marra M, Sordelli IM, Lombardi A, *et al*. Molecular targets and oxidative stress biomarkers in hepatocellular carcinoma: an overview. J Transl Med 2011; 9: 171.

[133] Trotter L, Panton W, Hajimohamadreza I, *et al*. Mitogen-activated protein kinase kinase 7 is activated during low potassium-induced apoptosis in rat cerebellar granule neurons. Neurosci Lett 2002; 320: 29-32.

[134] Maroney AC, Finn JP, Bozyczko-Coyne D, *et al*. CEP-1347 (KT7515), an inhibitor of JNK activation, rescues sympathetic neurons and neuronally differentiated PC12 cells from death evoked by three distinct insults. J Neurochem 1999; 73: 1901-12.

[135] Kaneko M, Saito Y, Saito H, *et al*. Neurotrophic 3,9-bis[(alkylthio)methyl]-and-bis(alkoxymethyl)-K-252a derivatives. J Med Chem 1997; 40: 1863-9.

[136] Bain J, Plater L, Elliott M, *et al*. The selectivity of protein kinase inhibitors: a further update. Biochem J 2007; 408: 297-315.

[137] Bubici C, Papa S. JNK signalling in cancer: in need of new, smarter therapeutic targets. Br J Pharmacol 2014; 171: 24-37.