The Investigation of Signalling Pathways in Response to Chromosomal Instability

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By

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I would like to dedicate my thesis to my beloved father

Shiyong Liu

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List of Publications

- Dawei Liu, Zeeshan Shaukat, Tianqi Xu, Donna Denton, Robert Saint and Stephen L. Gregory*. Autophagy regulates the survival of cells with chromosomal instability. (Manuscript submitted).

- Dawei Liu, Zeeshan Shaukat, Robert Saint and Stephen L. Gregory* (2015). Chromosomal instability triggers cell death via local signalling through the innate immnue receptor Toll. ***Oncotarget***, doi: 10.18632/oncotarget.6035.

- Zeeshan Shaukat, Dawei Liu and Stephen Gregory* (2015). Sterile Inflammation in *Drosophila*. ***Mediators of Inflammation***, doi: 10.1155/2015/369286.

- Zeeshan Shaukat, Dawei Liu, Rashid Hussain, Mahwish Khan and Stephen Gregory* (2015). "The Role of JNK Signalling in Responses to Oxidative DNA Damage." ***Curr Drug Targets***, Volume 16 (E-pub ahead of print).

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

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

Abbreviations

5-FU	5-Flurourocil
ACT	Adoptive cell transfer
Ambra1	Activating molecule in BECN1-regulated autophagy protein 1
APC/C	Anaphase promoting complex/cyclosome
Atg1	Autophagy-related 1
Atg4c	Autophagy-related 4c
Atg5	Autophagy-related 5
Atg7	Autophagy-related 7
Atg18	Autophagy-related 18
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
CTLA4	Cytotoxic T-lymphocyte-associated antigen 4
DAMPs	Danger-associated molecular patterns
DNA	Deoxyribonucleic acid
FDA	Food and Drug Administration
GIN	Genomic instability
HIF	Hypoxia-inducible factor
HMGB1	High mobility group box 1
IL-2	Interleukin 2
JNK	c-Jun N-terminal kinases
KSP/Eg5	Kinesin spindle protein
Mad1	Mitotic arrest deficient 1
Mad2	Mitotic arrest deficient 2
MCAK	Mitotic centromere-associated kinesin
MCC	Mitotic checkpoint complex
Mmp1	Matrix metalloproteinase 1
mTor	Mechanistic target of rapamycin
NDC80	Kinetochore protein NDC80 homolog
Nek2	NIMA-related kinase 2

NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
p53	Tumour protein (SDS-PAGE: 53 kDa) p53
p62	Sequestosome 1
PCM	Pericentriolar material
PCS	Premature chromatid separation
PI3K	Phosphoinositide 3-kinases
PLKs	Polo-like kinases
Rad21	RAD21 homolog (S. pombe), kleisin subunits of Cohesin Rad21
Ras	Rat sarcoma
Rb	Retinoblastoma
RNA	Ribonucleic acid
RNAi	RNA interference
ROS	Reactive oxygen species
SAC	Spindle assembly checkpoint
TCA	Tricarboxylic acid cycle
TLRs	Toll-like receptors
TNFα	Tumour necrosis factor alpha

Abstract

Most human solid cancers show Chromosomal Instability (CIN) in which cancer cells show a higher rate of gain or loss of whole chromosomes or large chromosomal fragments. CIN is associated with the progression of tumorigenesis, the development of cancer drug resistance and the poor prognosis. Since CIN is a hallmark of cancers and not common in normal cells, it has been proposed that CIN is targetable for cancer therapy. In order to target CIN for cancer treatment, there is a need to determine the signalling pathways which enable cells to tolerate CIN.

The aim of this study is to identify signalling pathways activated in response to CIN which could potentially be targeted to specifically kill CIN cells. Using *Drosophila* as the model organism to study CIN **(Chapter 2)**, we found that CIN cells are specifically sensitive to metabolic disruption as the depletion of metabolic genes involved in glycolysis, tricarboxylic acid cycle and oxidative stress response led to high levels of oxidative stress, DNA damage and apoptosis only in CIN cells **(Chapter 3)**. Consistent with its role in stress responses, in the subsequent study, we found that the autophagy pathway is robustly activated in CIN cells and autophagy inhibition can specifically kill CIN cells. We also found that autophagy activation removes defective mitochondria in CIN cell which gives tolerance to CIN in proliferating cells **(Chapter 4)**.

We also found a systemic immune signalling activation in *Drosophila* larvae when CIN was induced in the engrailed region of wing discs. Moreover, we found that the immune signalling Toll pathway is also activated within CIN cells and manipulation of Toll pathway could affect the survival of CIN cells. We proposed that signals released from CIN cells such as reactive oxygen species (ROS) could trigger a local Toll pathway activation in CIN tissue which in turn recruits *Drosophila* blood cells (hemocytes) to the surface of the CIN tissues. These recruited hemocytes then initiate apoptosis in the CIN cell through the TNFα/JNK pathway **(Chapter 5)**.

In conclusion, our studies demonstrated that CIN leads to a variety of consequences in cells: several signalling pathways including metabolic pathways, autophagy and Toll signalling are activated in response to CIN stresses. Understanding the mechanisms of these pathways responding to CIN will provide insights into designing cancer specific drug targets and ultimately contribute to cancer treatment.

Chapter 1

INTRODUCTION

1.1- Cancer and Cancer therapy

Cancer or malignant tumour is a group of diseases defined by aberrant cell growth and division with the potential to invade to other organs in the body. Cancer is the leading cause of death worldwide: in 2012, 14 million new cancer cases were diagnosed and 8.2 million people died from cancer related diseases and the incidence of cancer is expected to increase by approximately 70% by 2034 (World cancer report 2014). In Australia, cancer related death accounts for about 30% of the overall death. It has been estimated that 128,000 new cancer patients will be diagnosed in 2015 and more than 4.5 billion dollars spent on cancer in the direct health system every year. The number of new cases is expected to increase to about 150,000 by 2020 (Cancer Council Australia).

There are different therapies to treat cancer depending on the location and stage of the cancer including surgery, chemotherapy, radiation therapy, hormonal therapy and immunotherapy (Caley and Jones 2012). Surgery is effective to remove solid tumours especially at their early stage, however, when cancer cells metastasize to other sites in the body, complete removal of cancer cells become impossible. Therefore, when treating advanced or metastasized tumours, chemotherapy is often applied after surgery (Schmidt and Bastians 2007; Caley and Jones 2012). Of note, cancer immunotherapy has become a mainstream cancer therapy due to dramatic progress achieved in the field (Rosenberg 2014).

1.1.1- Cancer chemotherapies

Cancer chemotherapies were initially identified using compounds which can effectively kill dividing cells (Chabner and Roberts 2005; Vanneman and Dranoff 2012). Currently, cancer chemotherapies are still the mainstream cancer treatment and most of the clinical anti-cancer drugs belong to three classes: drugs that affect DNA synthesis or enhance DNA damage or anti-mitotic drugs that target microtubules (Schmidt and Bastians 2007; Vanneman and Dranoff 2012).

DNA damaging agents like alkylating agents have been used in treating different type of cancers, they can kill cancer cells at all phases of the cell cycle by damaging their DNA (Hosoya and Miyagawa 2014; Tian et al. 2015). However, those drugs can also damage normal cells and cause long term damage in the marrow. In the worst scenario they can cause acute leukaemia (Tucker et al. 1987). The application of those drugs requires a functional DNA damage response pathway to initiate apoptosis which is often

compromised in cancer cells (Bouwman and Jonkers 2012; Hosoya and Miyagawa 2014). For example, p53, which has been described as the guardian of the genome is mutant or inactive in tumours of more than half of cancer patients (Lane 1992; Vazquez et al. 2008; Perot et al. 2010). In addition, the development of drug resistance and the lack of biomarkers for drug response or resistance further limit the application of the DNA damaging agents (Hosoya and Miyagawa 2014).

Antimetabolite drugs like 5-flurouracil (5-FU) have been widely and effectively used in clinic to treat various cancer including leukemia, breast, ovarian cancers for decades (Longley et al. 2003; Tiwari 2012). These drugs mimic the structure of natural molecules which interfere with nucleic acid (DNA and RNA) synthesis inducing cell death at S phase (Tiwari 2012). However, Antimetabolite drugs also inhibit DNA or RNA synthesis in normal somatic cells which could cause severe side effects such as acute cardiac and renal failure in cancer patients (Lukenbill and Kalaycio 2013; Polk et al. 2014).

Anti-mitotic drugs, such as taxanes and vinca alkaloids, are extraordinarily effective in clinic and they can bind to microtubules and disrupt their function, leading to cell arrest in mitosis and finally induce cancer cell death (Schmidt and Bastians 2007). However, because microtubules are also involved in many other important processes such as intracellular transportation and maintenance of cell shape and integrity in normal non-dividing cells, anti-microtubule drugs can kill not only cancer cells but also normal resting cells, thus resulting in many severe side-effects such as peripheral neuropathies and myelosuppression in patients (Jackson et al. 2007). Currently, the development of the "new generation" anti-cancer drugs is mainly focused on the inhibitors of mitotic proteins such as kinesin (KSP/Eg5), polo-like kinases (PLKs), aurora kinases, and centromeric protein E (CENPE) (Jackson et al. 2007; Schmidt and Bastians 2007; Hosoya and Miyagawa 2014). These proteins all have special functions in mitosis and are only expressed in dividing cells. Thus, the inhibitors of these proteins should have no effects on normal resting cells and therefore can potentially improve therapeutic efficacy compared to the existing anti-microtubule drugs (Mitchison 2012).

1.1.2-Targeting Autophagy as a novel chemotherapy

Autophagy is a key cellular homeostatic process which degrades and recycles unnecessary or dysfunctional macromolecules and organelles in the cell (Mizushima 2007; Glick et al. 2010). The current view about autophagy is that it mediates cancer

chemotherapy related cell death and is related to the development of drug resistance by activating pro-survival pathways in cancer cells (Notte et al. 2011; Rubinsztein et al. 2012; Sui et al. 2013). The process of autophagy involves the progressive formation of a double-membraned autophagosome which sequesters the cytoplasmic materials. Then the autophagosomes fuse with lysosomes to form autolysomes in which the sealed cytoplasmic materials will be degraded (Mizushima 2007; Galluzzi et al. 2015). Autophagy removes dysfunctional organelles and prevents the buildup of toxic materials in cells. Therefore, a basal level of autophagy is continuously functioning under normal condition. However, autophagy can be upregulated in response to various stimulus including starvation, metabolic stress, genotoxic and pathogenic stresses (Galluzzi et al. 2015; White 2015).

Autophagy is tightly controlled and its dysfunction is often associated with tumorigenesis (He and Klionsky 2009; Galluzzi et al. 2015). Numerous studies showed that the deficiency of essential autophagy genes could promote tumorigenesis. For example, heterozygous deficiency of the autophagy gene *Beclin1* or its regulator *Ambra1* leads to increased cell proliferation and spontaneous tumorigenesis (Qu et al. 2003; Cianfanelli et al. 2015). Similarly, mice carrying a deficiency for other essential autophagy genes, such as *atg4c*, *atg5* and *atg7*, all have higher rates of tumorigenesis than their wild-type littermates (Marino et al. 2007; Takamura et al. 2011). In addition, a deficiency in autophagy is commonly associated with increased levels of reactive oxygen species (ROS), DNA damage and chromosomal instability which are known to induce tumorigenesis (Mathew et al. 2007). On the other hand, upregulated autophagy is required to promote cancer cell survival, proliferation and metastasis. This is likely due to increased metabolic demands in cancer cells for biosynthesis due to excessive cell proliferation (Galluzzi et al. 2015; White 2015). For example, Ras-driven cancer cells heavily rely on autophagy which is known as “autophagy addiction” (Guo et al. 2011; Guo and White 2013). Therefore, suppression of cancer growth by autophagy inhibition has been shown in different studies (Guo and White 2013; Strohecker et al. 2013; Karsli-Uzunbas et al. 2014; Yang et al. 2014).

As discussed above, autophagy plays a dual role during tumorigenesis: it has both cancer-promoting and cancer-supressing functions. Normal tissues could tolerate absence of autophagy for at least a short term, as systemic *atg7* disruption in mice specifically killed established lung cancer cells but not normal tissue cells (Karsli-Uzunbas et al. 2014). This opened up a window for autophagy inhibition as cancer

therapy. However, the inhibition of autophagy as an anticancer therapy is still at an early stage. Currently, the antimalarial drugs chloroquine and hydroxychloroquine which could inhibit lysosomal degradation have been used in preclinical trials in combination with other anticancer drugs (Amaravadi et al. 2011). Other autophagy inhibitor drugs such as 3-methyladenine, which inhibit autophagosome formation, and quinacrine which inactivates lysosomes are also in the preclinical study stage (Milano et al. 2009; Gupta et al. 2010; Janku et al. 2011).

1.1.3- Cancer immunotherapy

The genetic and epigenetic changes in cancer cells provide various cancer specific antigens that could be recognised by human immune system. The concept of cancer immunotherapy refers to a type of cancer treatment by activating the immune system to attack cancer cells (Rosenberg 2014). Cancer immunotherapy has been under investigation for more than one century: in the late 1800s, Dr William Coley showed that the injection of certain kind of bacteria into cancer patients could provoke their immune system and kill cancer cells (Coley 1891). However, the first FDA approved immunotherapy was IL-2 administration in effectively treating renal cancer and melanoma in the 1990s (Atkins et al. 1999). Currently, the most common clinical immunotherapies are adoptive cell transfer (ACT) and immune checkpoint inhibition (Rosenberg et al. 2008; Pardoll 2012; Rosenberg 2014).

The adoptive cell transfer (ACT) immunotherapy refers to the treatment that uses autologous anti-tumour T cells to kill cancer cells. The normal procedure is to obtain anti-tumour cells either from tumour infiltrating lymphocytes or peripheral lymphocytes genetically modified to recognise tumour antigens. After expanding them *in vitro*, these anti-tumour T cells are then reinfused into patients after lymphodepletion (Rosenberg et al. 2008). The ACT immunotherapy has been effective especially in treating metastatic melanoma and lymphoma. Long term research showed that ACT mediates cancer regression in around 50% of the metastatic melanoma patients (Rosenberg et al. 2011; Bollard et al. 2014). However, the major obstacle for ACT application is that it is a labour-intensive and highly personalized treatment which is difficult to commercialize and to fit into oncological practice (Rosenberg et al. 2008).

In the past decade, the advance in the field of T cell inhibitory signalling pathways has led to increased use of immunotherapy using antibodies to block the inhibitory receptors on the T cell surface (Hodi et al. 2010; Pardoll 2012; Yao et al. 2013; Rosenberg 2014).

In the research published by Hodi *et al*. in 2010, the administration of Cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) antibody ipilimumab improved overall survival of cancer patients with metastatic melanoma from 6.5 months to 10 months (Hodi et al. 2010); This research led to the approval of ipilimumab by FDA as the first CTLA4 antibody in immunotherapy (Pardoll 2012). The second FDA approved lymphocyte receptor inhibitory antibody is programmed cell death protein 1 (PD1) (Pardoll 2012). The PD1 antibody was applied to patients with melanoma, non-small-cell lung cancer, prostate cancer, renal-cell or colorectal cancer. Among the 236 patients, objective responses were observed in 18% of the non-small-cell lung cancer patients, 28% of the melanoma patients, 27% of the renal-cell cancer patients (Topalian et al. 2012). These clinical successes are encouraging and open a new avenue for immunotherapy and there are more of these immune-modulatory cancer drugs currently under clinical trial (Yao et al. 2013; Sharma and Allison 2015).

1.1.4- Current therapies and drug resistance

One critical problem for all these cancer therapies is the development of drug resistance after drug administration and their side effects on cancer patients (Caley and Jones 2012; Holohan et al. 2013). For example, to those "new generation" anti-mitotic drugs, changes in microtubule composition and dynamics by deregulating expression of microtubule associated proteins are one cause of anti-microtubule drug resistance. Acquired mutations in cancer cells are considered to be the molecular basis of intrinsic multidrug resistance including mutations of the drug target, dysfunctions of the DNA damage response pathway, activation of pro-survival pathways to avoid cell death etc. (Lee et al. 2011; Holohan et al. 2013).

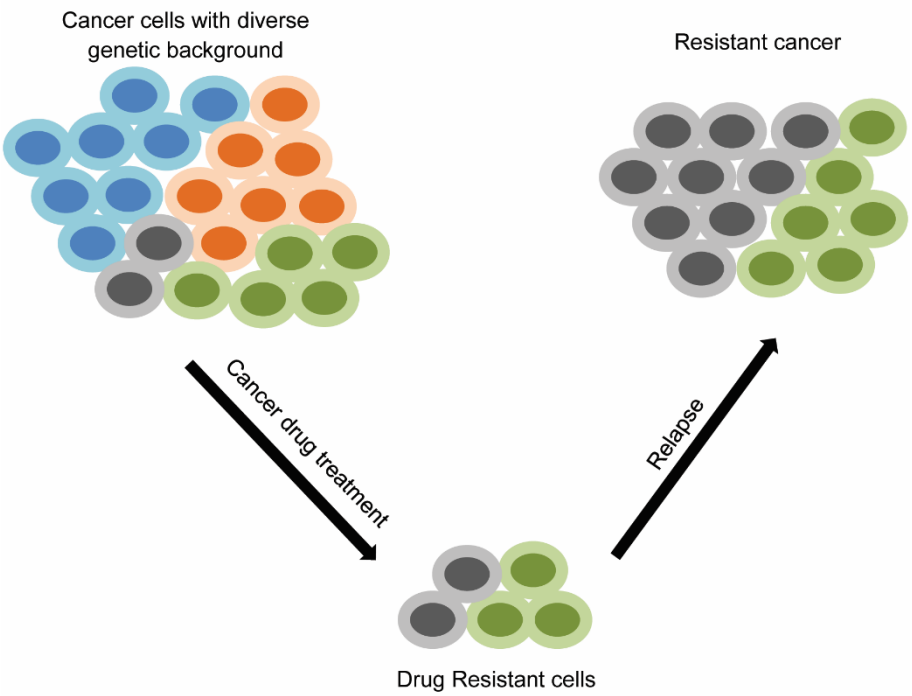

Figure 1.1: Darwinian evolution model of cancer cells. CIN could generate cancer cells with different genetic background, cancer drug treatment will kill most of the cancer cells at a time but cancer cells with resistance will survive and relapse.

Behind these, chromosomal instability (CIN) has been proposed to be the hidden master which allows cancer cells to acquire drug resistance (Swanton et al. 2009; Lee et al. 2011; Bakhoum and Compton 2012; Turner and Reis-Filho 2012). Chromosomal instability is a common phenomenon in almost all tumours in which cells have a higher rate of gain or loss of chromosomes or large chromosomal fragments (Thompson et al. 2010; Heng et al. 2013). Therefore, CIN can generate numerous cancer cell clones with massive genetic diversity while drug treatment selects those with drug resistance. Thus, by Darwinian evolution within cancer cells, CIN could change the cancer genomic landscape and enable cancer cells to develop multidrug resistance with time (Gerlinger and Swanton 2010) (Fig. 1.1). Consequently, it is not surprising that high levels of CIN correlate with poor clinical outcomes in multiple human cancers (Carter et al. 2006).

As most cancer cells show CIN, in order to minimize side-effects, increase therapeutic efficacy and reduce drug resistance among cancer cells, chromosomal instability itself could be an attractive therapeutic target (Gerlinger and Swanton 2010; Bakhoum and Compton 2012; Shaukat et al. 2012; Shaukat et al. 2014; Wong et al. 2014). In this thesis, in order to specifically kill CIN cells, using *Drosophila* as the model organism, we investigate pro-survival signalling pathways in response to CIN. The overall aim of this project is to investigate signalling pathways that involved in CIN tolerance, thus

targeting those pathways will have minimal side-effects on normal cells and can theoretically prevent cancer cells from developing drug resistance.

1.2. Chromosomal Instability

During the progression of tumorigenesis, there are six hallmarks of cancer cells which enable them to proliferate and metastasize. Among them, Genome Instability (GIN) is the most essential hallmark of cancer cells which confers selective advantage on neoplastic cells and enables them to acquire other cancer hallmarks required for the tumorigenesis (Hanahan and Weinberg 2011).

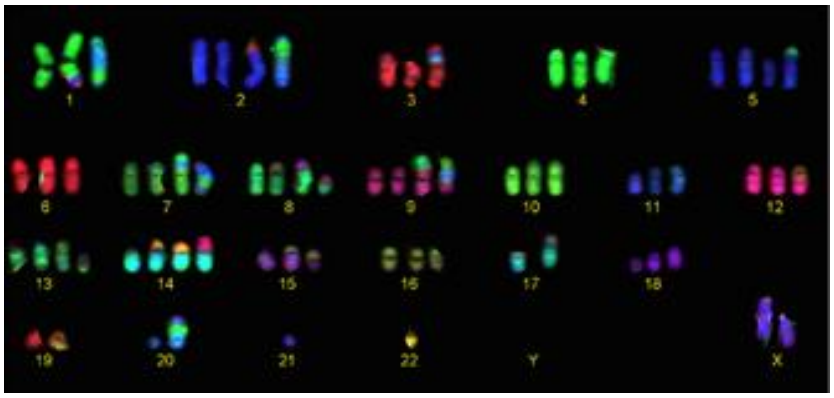

Figure 1.2: Karyotype of an osteosarcoma cancer cell showing the effect of CIN. [From (Jansson & Medema, 2013)]

As a common form of GIN, Chromosomal Instability (CIN) is defined as an elevated rate of gain and loss of chromosomes or large chromosomal fragments which has been commonly seen in most human solid cancers (Thompson et al. 2010) (FIG 1.2).

1.2.1- The mechanism of CIN

Mitosis is a process of cell reproduction by which one parent cell equally separates its duplicated chromosomes into two daughter cells. The process of mitosis could be further divided into five sequential stages including prophase, prometaphase, metaphase, anaphase and telophase. In a normal metaphase, sister chromatids are aligned at the cell equator with their kinetochores captured by microtubule fibres radiating from the centrosomes at the two cell poles. After all kinetochores are properly captured, the Cohesins which bind the sister chromatids together are cleaved, thus allowing the segregation of sister chromatids and the onset of anaphase (Foley and Kapoor 2013). Many regulators have been involved in the control of mitosis; any defects of these regulators could lead to CIN. These could include defects in cohesion

and its regulators (Cucco et al. 2014), defects in the dynamics of kinetochore-microtubule attachment (Foley and Kapoor 2013), defects in centrosomes and defects in the spindle assembly checkpoint (Pihan 2013).

1.2.1.1- Cohesion defects

Sister chromatids are physically bound together by chromatin-associated cohesin protein during mitosis (Nasmyth 2011). Defects in cohesin or cohesion regulators could induce CIN in human cancers (Jallepalli et al. 2001; Barber et al. 2008; Sajesh et al. 2013) (Fig. 1.3). Somatic mutations in genes regulating chromatid cohesion have been identified in human colorectal cancers, most of which show CIN and the subsequent depletion of these genes by RNA interference induced chromatid cohesion defects and CIN in a human chromosomally stable cell line (Barber et al. 2008). Elevated levels of separase, a cysteine protease which triggers the onset of anaphase by cleaving sister chromatid cohesion, could result in premature loss of sister chromatid cohesion which in turn leads to chromosomal mis-segregation probably by impairing the normal back-to-back orientation of sister kinetochores (Zhang et al. 2008). Consistent with this, abnormalities in securin, a protein which deactivates separase, could lead to a high frequency of chromosome loss in human cells (Jallepalli et al. 2001). In *Drosophila*, depletion of cohesin by RNAi leads to a high level of CIN and aneuploidy in around 40% of cells (Liu et al. 2015).

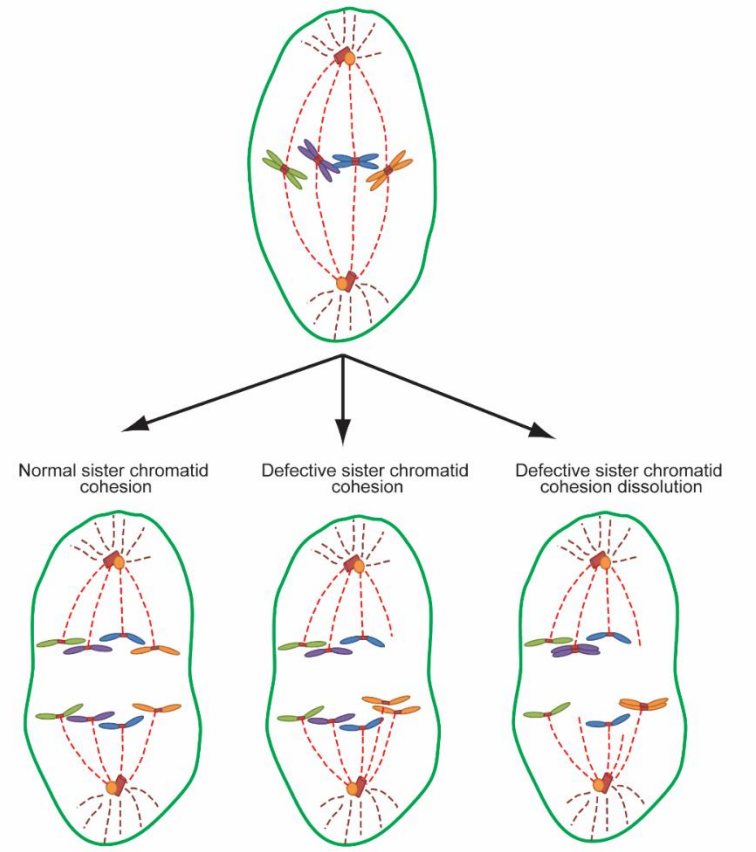

Figure 1.3: Cohesion defects lead to chromosomal instability. Cohesin defects leads to defective chromatid cohesion or dissolution which in turn case chromosomal mis-segregation.

1.2.1.2- Defects in the dynamic of kinetochore-microtubule attachment

The kinetochore is a protein complex to link the chromosomes to microtubules during mitosis. Microtubule attachment to kinetochores is a stochastic and dynamic process during prophase and metaphase, allowing incorrect attachments to be corrected (Cheeseman and Desai 2008).

Defects in the dynamics of kinetochore-microtubule attachment are commonly seen in CIN cancers (Thompson et al. 2010; Compton 2011) (Fig. 1.4). For example, increased levels of Mad2 lead to hyper-stability of kinetochore-microtubule attachments and consequently CIN and tumorigenesis (Kato et al. 2011; Kabeche and Compton 2012).

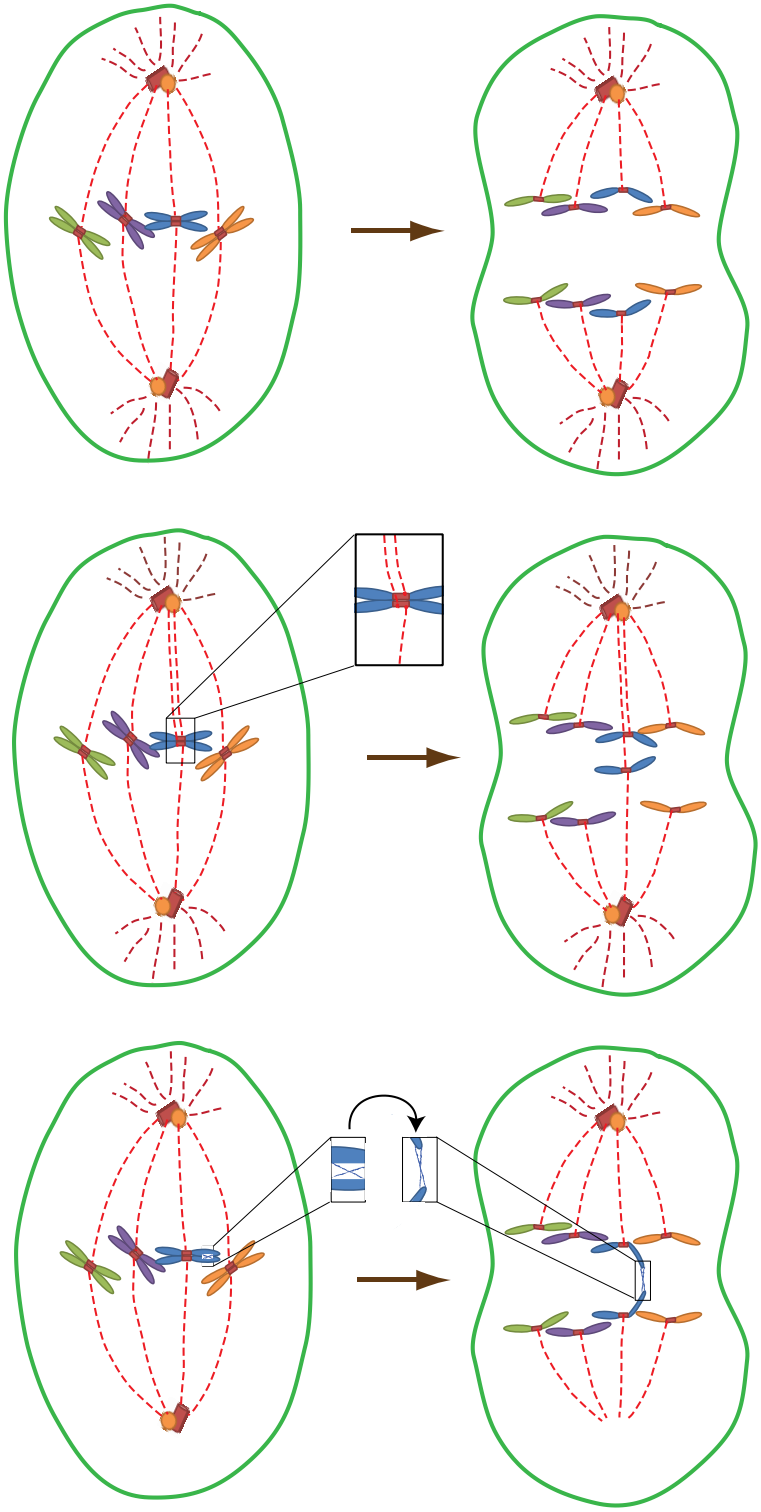

Figure 1.4: Defects in the dynamic of kinetochore-microtubule attachment lead to chromosomal instability. Defects in the dynamics of kinetochore-microtubule attachment could cause lagging chromosomes or chromosomal bridges during mitosis.

On the other hand, Mad2 depletion can cause kinetochore-microtubule attachment instability and CIN (Michel et al. 2001). Furthermore, it has been reported that a number of proteins such as NDC80 complex, Aurora B and APC/C are involved in this process and

their deregulation is associated with an increased frequency of lagging chromosomes in anaphase and subsequently CIN (Thompson et al. 2010; Bakhoum and Compton 2012).

1.2.1.3- Centrosome defects

The centrosome is a non-membranous organelle in animal cells which consists of a pair of linked centrioles surrounded by an amorphous matrix of proteins called the pericentriolar material (PCM) (Azimzadeh and Bornens 2007). Each centriole is a symmetrical barrel-shaped array composed of nine sets of triplet microtubules while the PCM contains hundreds of proteins which are involved in microtubule nucleation and other functions (Azimzadeh and Bornens 2007; Nigg and Raff 2009). The centrosome is the centre of spindle microtubule nucleation and anchoring in most animal cells, thus playing a significant role in microtubule-associated processes such as cell division (Silkworth et al. 2012). Centrosome abnormality in number, structure and size has been observed in almost all human tumours and is considered a hallmark of tumour aggressiveness and malignancy (Pihan 2013).

Ganem et al. found that although cells with extra centrosomes could undergo bipolar cell divisions, they show a significantly increased level of CIN in terms of lagging chromosomes (Ganem et al. 2009). Furthermore, CIN could also lead to DNA damage during cytokinesis (Janssen et al. 2011), while DNA damage has also been reported to induce centrosome amplification in human cells (Bourke et al. 2007). Thus, it seems that centrosome abnormality and DNA damage could form a positive feedback loop which drives the development of CIN in cancers.

Another mechanism is that the aberrant expression of some centrosome genes could lead to centrosome or microtubule defects which then lead to CIN. For example, the overexpression of Nek2, a serine/threonine-protein kinase which is involved in centrosome separation (Mardin and Schiebel 2012), leads to premature centrosomes generation while depletion of Nek2 causes defects in centrosome separation (Hayward and Fry 2006).

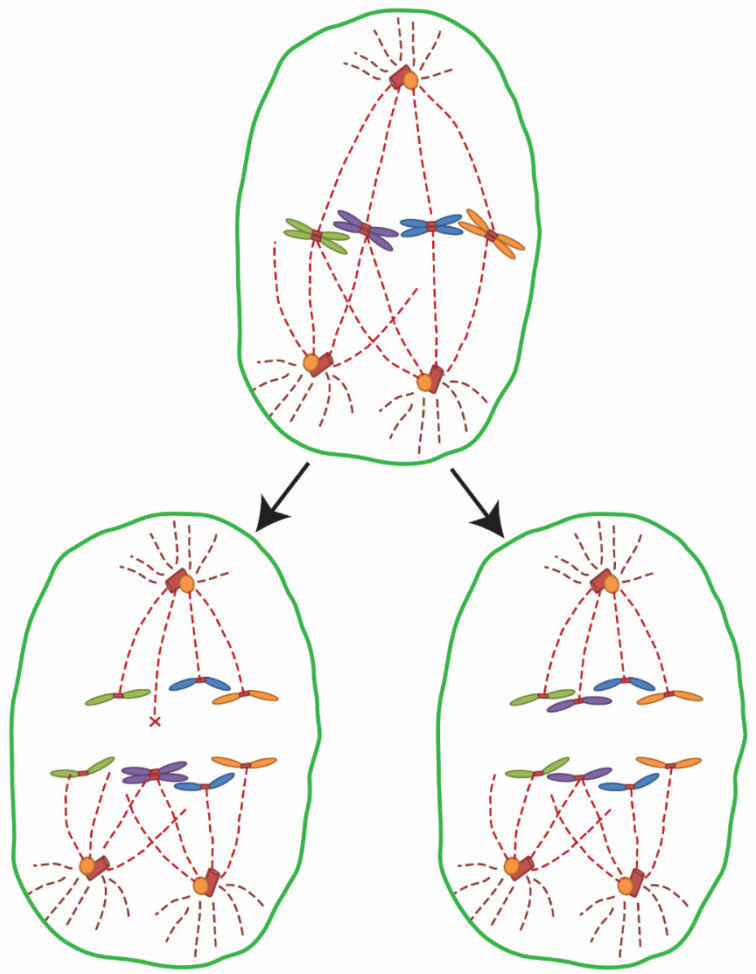

Figure 1.5: Centrosome defects lead to chromosomal instability. Centrosome abnormality in number, structure and size lead to multi-polar cell division. The daughter cells would have abnormal number of chromosome and DNA damage.

Another example is Aurora-A, a mitotic serine/threonine kinase which is involved in centrosome maturation and separation. It has been shown that overexpression of Aurora-A in mouse epithelium could induce centrosome amplification, CIN and subsequently tumorigenesis in a mouse model (Wang et al. 2006).

1.2.1.4- Defects in the spindle assemble checkpoint

In order to ensure the fidelity of chromosome segregation during the process of cell division, a mechanism named the spindle assemble checkpoint (SAC) has been developed by eukaryotes to prevent chromosome mis-segregation and aneuploidy (Musacchio and Salmon 2007; Lara-Gonzalez et al. 2012). The SAC machinery is highly conserved among eukaryotes and its exact mechanism is still under investigation. The current model for the SAC is that the SAC proteins mitotic-arrest deficient (MAD) protein MAD2, CDC20, budding uninhibited by benzimidazole (BUB) BUB1 and

BUBR1 (also called MAD3) could form the mitotic checkpoint complex (MCC) at unattached kinetochores, generating inhibitory signals to prevent the onset of anaphase (Musacchio and Salmon 2007; Lara-Gonzalez et al. 2012). Once all the kinetochores are properly captured by microtubules, CDC20 molecules are released from the MCC. The released CDC20 molecules then activate its target anaphase-promoting complex/cyclosome (APC/C) which subsequently mediates the degradation of Cyclin B and protease inhibitor Securin by the 26S proteasome. The released protease named Separase is required for the cleavage of Cohesin and the onset of anaphase while the degradation of Cyclin B is required for the exit from mitosis (Foley and Kapoor 2013). Taken together, by sequestering CDC20 in the MCC, the SAC prevents early onset of anaphase until all kinetochores are properly captured by microtubules, thus preventing chromosome mis-segregation during mitosis. Due to the function of the SAC, defects in the SAC machinery are considered as contributors to chromosomal instability (CIN) and aneuploidy, thereby promoting tumorigenesis in human cells (Musacchio and Salmon 2007; Li and Zhang 2009). For example, mutations in BUB1B (encoding BUBR1 protein) were found to be linked with a rare cancer-prone disorder called premature chromatid separation (PCS syndrome) which has CIN as one of its characteristics (Matsuura et al. 2006). Mice with heterozygous mutations in SAC genes such as Mad1 and Cdc20 showed an elevated incidence of chromosome mis-segregation and tumorigenesis (Iwanaga et al. 2007; Li et al. 2009). However, mutations in the SAC are rare in cancer cell lines (Musacchio and Salmon 2007). Normally, instead of complete loss, partial inactivation of SAC genes is more frequently observed in cancers, probably because complete loss of SAC genes can lead to embryonic lethality in most metazoans (Li and Zhang 2009). Consistent with this point, loss of one allele of the essential spindle checkpoint gene Mad2 leads to an impaired SAC and increased incidence of chromosome mis-segregation due to premature sister-chromatid separation in both human and murine cells (Michel et al. 2001). On the other hand, an increased level of Mad2 also leads to a compromised SAC, CIN and a wide spectrum of cancers including fibrosarcomas, lymphomas, lung adenomas and hepatoma carcinoma (Sotillo et al. 2007). A compromised spindle assembly checkpoint caused by the aberrant expression of other SAC genes has also been found to cause CIN in a variety of scenarios (Mondal et al. 2007; Gao et al. 2011; Ryan et al. 2012; Jahn et al. 2013).

The SAC machinery is well characterized and highly conserved In *Drosophila* (Conde et al. 2013; Foley and Kapoor 2013); Consistent with mammalian cells, the deletion of

SAC genes in *Drosophila* leads to CIN (Shaukat et al. 2012; Shaukat et al. 2014; Wong et al. 2014). For example, Mad2 depletion leads to short metaphase and CIN in terms of chromosomal bridges and lagging chromosomes (Buffin et al. 2007; Shaukat et al. 2012). Similarly, depletion of DNA damage response genes such as ATM, Rb or BRCA1 could also give rise to increased incidence of chromosomal mis-segregation (Thompson et al. 2010). In this study, we used depletion of SAC or cohesin genes in *Drosophila* to induce CIN.

1.2.2- The outcomes of Chromosomal instability

CIN is a hallmark of most human solid tumours and is required for the acquisition of multiple characteristics essential for the progress of tumorigenesis (Hanahan and Weinberg 2011; Bakhoum and Compton 2012). A moderate level of CIN is believed to play an initiating role in tumorigenesis and the level of CIN seen in tumours positively correlates with poor prognosis and drug resistance (Michor et al. 2005; Carter et al. 2006; Bakhoum and Compton 2012).

One outcome of CIN is that it creates diverse genetic background in daughter cells by which it confers selective advantages to neoplastic cells during the progress of tumorigenesis (Gerlinger and Swanton 2010; Hanahan and Weinberg 2011). CIN could be an underlying mechanism for the acquisitions of different cancer hallmarks including resistance to cell death and replicative immortality, sustaining cell growth and evading growth suppressors, angiogenesis, metastasis, escape immune surveillance and cellular metabolism reprogramming (Hanahan and Weinberg 2011).

For example, Sotillo et al. (2007) found that overexpression of Mad2 resulted in a wide range of tumours accompanied by both structural and numerical CIN in Mad2-inducible transgenic mice. When Mad2 expression was turned off, they found the tumour growth was not affected, suggesting that Mad2 is not essential for its growth and that the high incidence of tumorigenesis in Mad2 overexpression mice is probably due to the elevated CIN (Sotillo et al. 2007). One specific outcome of CIN is aneuploidy which refers to a state of having an abnormal number of chromosomes within cells and was found in cancer cells more than a century ago (Holland and Cleveland 2009). Numerous studies showed that deregulation of mitotic genes could lead to aneuploidy and consequently an increase the incidence of both spontaneous and carcinogen-induced tumorigenesis in mice (Holland and Cleveland 2009; Gordon et al. 2012). One plausible mechanism is that CIN and aneuploidy could lead to increased oxidative stress, DNA damage and

tumorigenesis (Williams et al. 2008; Shaukat et al. 2014). CIN cells have an elevated rate of gain and loss of chromosomes which directly causes DNA damage and translocations which enables a fraction of these cells to gain a selective advantage, such as losing cancer suppressor genes by the loss of certain chromosomes in which those genes lie, thus promoting tumorigenesis (Rajagopalan and Lengauer 2004; Janssen et al. 2011). For example, P53 acts as the "guardian of genome" which could suppress CIN and prevent tumorigenesis (Lane 1992; Dalton et al. 2010). Consequently, inactivation of p53 is common in CIN cancers (Sigal and Rotter 2000; Vazquez et al. 2008).

Another consequence of CIN is its association with anticancer therapy resistance, poor prognosis and relapses (Carter et al. 2006; McClelland et al. 2009; Swanton et al. 2009; Sotillo et al. 2010; Lee et al. 2011; Gordon et al. 2012).It has been reported that high levels of CIN positively correlated to taxane resistance in ovarian cancer cells in a clinical trial (Swanton et al. 2009).CIN cancer cells demonstrated a significant intrinsic multidrug resistance when compared to cancer cell lines without CIN (Duesberg et al. 2000; Lee et al. 2011; Zhou et al. 2013). One mechanism for the drug resistance could be due to the activation of efflux pumps in those CIN cells (Zhou et al. 2013). Furthermore, CIN cancer cells even demonstrated a significant intraline and interline variation of cell fate in response to antimitotic drugs, which makes those cancer cells a moving target for therapy (Gascoigne and Taylor 2008). Due to the drug resistance, cancer patients with CIN showed increased relapse and poor clinical outcomes (Carter et al. 2006; Sotillo et al. 2010).

1.3. Targeting Chromosomal instability

As most cancer cells show CIN, in order to minimize side-effects, increase therapeutic efficacy and reduce drug resistance among cancer cells, chromosomal instability itself could be an attractive therapeutic target (Zhang et al. 2008; Gerlinger and Swanton 2010).

In order to target CIN, one feasible solution is to reduce the level of CIN (Bakhoum and Compton 2012). The balance between the kinetochore-microtubule attachment stabilization and destabilization is critical for chromosomal stability maintenance. It has been reported that overexpression of kinetochore proteins such as MCAK could significantly reduce the level of CIN in cancer cell lines by correcting mal-attachments by increasing kinetochore-microtubule turnover (Bakhoum et al. 2009b). This can prevent cancer cell lines developing further drug resistance caused by massive

chromosome reshuffle. This research opened an avenue for targeting a moderate level of CIN in cancer cells (Bakhoum et al. 2009a).

Conversely, it has also been reported that a high level of CIN caused by disturbing the SAC proteins BUBR1 or MAD2 could lead to apoptosis in human cancer cells within six divisions (Kops et al. 2004). Thus, elevating CIN levels can be used as a strategy to target CIN affected cells. This was confirmed by a study which shows that enhancing CIN by either a low dose of taxol treatment or further reduction of SAC proteins leads to massive apoptosis in human cancer cell lines originally showing mild CIN but no lethality (Janssen et al. 2009). In addition, increasing the level of CIN by other mechanisms could also be used as an effective strategy to kill CIN cancer cells. For example, manipulation of kinetochore-microtubule dynamics could also be an effective way to kill CIN cancer cell by increasing the level of CIN. Increasing the kinetochore-microtubule stability by inhibiting its regulator Aurora B could effectively kill CIN cancer cells and could be used as a therapeutic strategy (Liu et al. 2009; Payton et al. 2010; Birkbak et al. 2011). Another example is that the inhibition of telomerase could enhance the level of CIN, leading to cancer cell death and increasing the efficacy of traditional cancer therapies in aggressive tumours (Stewenius et al. 2007). Similarly, supernumerary centrosomes lead to chromosomal mis-segregation and CIN. Therefore, targeting centrosomal pathways has been proposed as a therapeutic strategy against cancer cells with centrosome-related chromosomal instability (Kwon et al. 2008; Mazzorana et al. 2011; Kawamura et al. 2013; Korzeniewski et al. 2013).

However, targeting CIN cancer cells by manipulating the level of CIN could be limited by side effects of cancer drugs such as haematological and neurological dysfunction (Caley and Jones 2012). Furthermore, CIN induction could potentially lead to tumorigenesis in normal cells and drug resistance, and relapse as discussed above (Gisselsson 2011; Holohan et al. 2013). Therefore, as normal cells do not tolerate CIN and CIN is a hallmark of most solid tumours (Rajagopalan and Lengauer 2004), a third strategy to kill cancer cells showing CIN would be to investigate pathways necessary to maintain CIN, and then develop agents that can disrupt those pathways (Rajagopalan and Lengauer 2004). Therefore, the aim of this project is to investigate the potential signalling pathways that decide the fate of CIN cancer cells.

In this project, we found that autophagy is activated in CIN cells and manipulating autophagy could affect the fate of these CIN cells (Chapter 4). This is important because both CIN and autophagy are related to development of chemotherapy drug resistance

(Sui et al. 2013). By targeting the autophagy pathway, we could improve chemotherapy efficacy by reducing the possibility of developing drug resistance caused by either CIN or autophagy activation. We also found that the Toll pathway is also activated in response to CIN (Chapter 5). The main difficulty for the application of cancer immunotherapy is the characterisation of molecules from certain immune checkpoint pathway(s) that are targetable in different type of cancers (Pardoll 2012; Yao et al. 2013), however, CIN cells are thought to generate specific molecules that could be recognised by the immune system (Gasser and Raulet 2006; Yang et al. 2013).

In conclusion, the advantage of targeting CIN as a cancer therapy is obvious: CIN only exists in cancer cells and is positively correlated with poor prognosis. Therefore, CIN-specific cancer drugs would have minimal side-effects on normal cells and could reduce the frequency of metastasis. Moreover, CIN-specific drugs could prevent cancer cells from remodelling their genomic landscape by killing those CIN cells, and consequently limit the cancer cells' ability to develop drug resistance and relapse. The overall aim of this project is to develop drugs that can specifically kill cells with CIN, thus the drug will have minimal side-effects on normal cells and can theoretically prevent cancer cells from developing drug resistance.

1.4. Using *Drosophila* as the model organism to investigate chromosomal instability

Drosophila melanogaster has been used as a model organism for a century and is one of the most effective tools for analysing the function of human disease genes, including those are responsible for developmental and neurological disorders, cancer, cardiovascular disease, metabolic and storage diseases (Fortini et al. 2000; Bier 2005). 72% of cancer related genes have counterparts in *Drosophila*, making it an ideal model organism for cancer research (Fortini et al. 2000). Another important advantage of *Drosophila* in cancer research is that flies typically have only one copy of these genes whereas vertebrate cells often have several related paralogs. This characteristic simplifies the analysis of protein interactions within tumorigenesis (Reiter et al. 2001). In this study, *Drosophila* has been used as the model organism to investigate signalling pathways responding to CIN *in vivo*. The recent advances and progress of using *Drosophila* in CIN research has been reviewed in Chapter 2.

1.5. Key points

The key points of the CIN research related to this study are as follows:

1. Genomic instability is an essential hallmark of cancer.

2. Chromosomal instability is required for the acquisition of other hallmarks in cancer.

3. Chromosomal instability could be induced by many mechanisms including cohesion defects, kinetochore-microtubule attachment defects, spindle assembly checkpoint defects, centrosomal defects, etc.

4. Chromosomal instability could lead to the initiation of tumorigenesis, the development of multi-drug resistance, relapse and poor clinical outcomes.

5. Chromosomal instability is common in cancer cells but not in normal cells, and cancer cells tolerating CIN are under stress. Therefore, CIN itself could be a target for cancer therapy.

6. *Drosophila* CIN models are available to investigate signalling pathways responding to CIN.

1.6. Aims of the study

The main objectives of this project are to broaden the search for genes that are involved in aneuploidy and CIN response and to investigate the signalling pathways in controlling the apoptotic or survival signals in proliferating CIN cells.

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

Aim 2: To characterize the potential apoptotic pathways by which our selected candidates induce CIN specific apoptosis.

Aim 3: To screen and characterize candidates and pathways whose knockdown can give tolerance to CIN in *Drosophila*.

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

Aim 3 is described in **Chapter 5**.

Chapter 2

Drosophila as a Model for Chromosomal Instability

Chromosomal instability (CIN) is a common feature of almost all solid cancers. However, CIN is not common in normal cells, therefore, there is considerable significance in understanding how cancer cells respond to CIN and what signalling pathways are required for CIN tolerance. Due to their high variability in genomic content and poor reproducibility, cancer cell lines are not idea as models for CIN research.

Drosophila melanogaster has been widely used as a model organism for a century to investigate the function of human genes and identify signalling pathways underlying human genetic diseases. Apart from its advantages such as short life span, small size, fast generation time, ease of generating and maintaining large numbers of fly lines as well as cost effectiveness, more than 70% of human disease genes have their orthologue in *Drosophila*, making it a good model for cancer research and therapeutic drug discovery studies (Pandey and Nichols, 2011, Reiter et al 2001).

There are several advantages to use *Drosophila* as the model organism specifically to study CIN. For example, it is easy to manipulate gene expression to induce CIN in any tissue at certain stage of development. Several mechanisms have been used to induce CIN in *Drosophila* including mitotic spindle disruption, DNA damage elevation, spindle assembly checkpoint mutations and cytokinesis defects. To characterize signaling pathways that are critical for maintaining the survival of CIN cells, a *Drosophila* CIN-inducible *in vivo* system could be a powerful tool for gene screening for genetic interaction and pathway dissection. Moreover, due to the relatively rapid development of CIN in proliferating cells, *Drosophila* CIN models might also be good tools for studying the initiation of tumorigenesis.

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Review

Drosophila as a model for chromosomal instability

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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.

Keywords: aneuploidy; cell cycle; checkpoint; chromosomal instability; DNA damage; *Drosophila*; JNK; metabolism; ROS

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

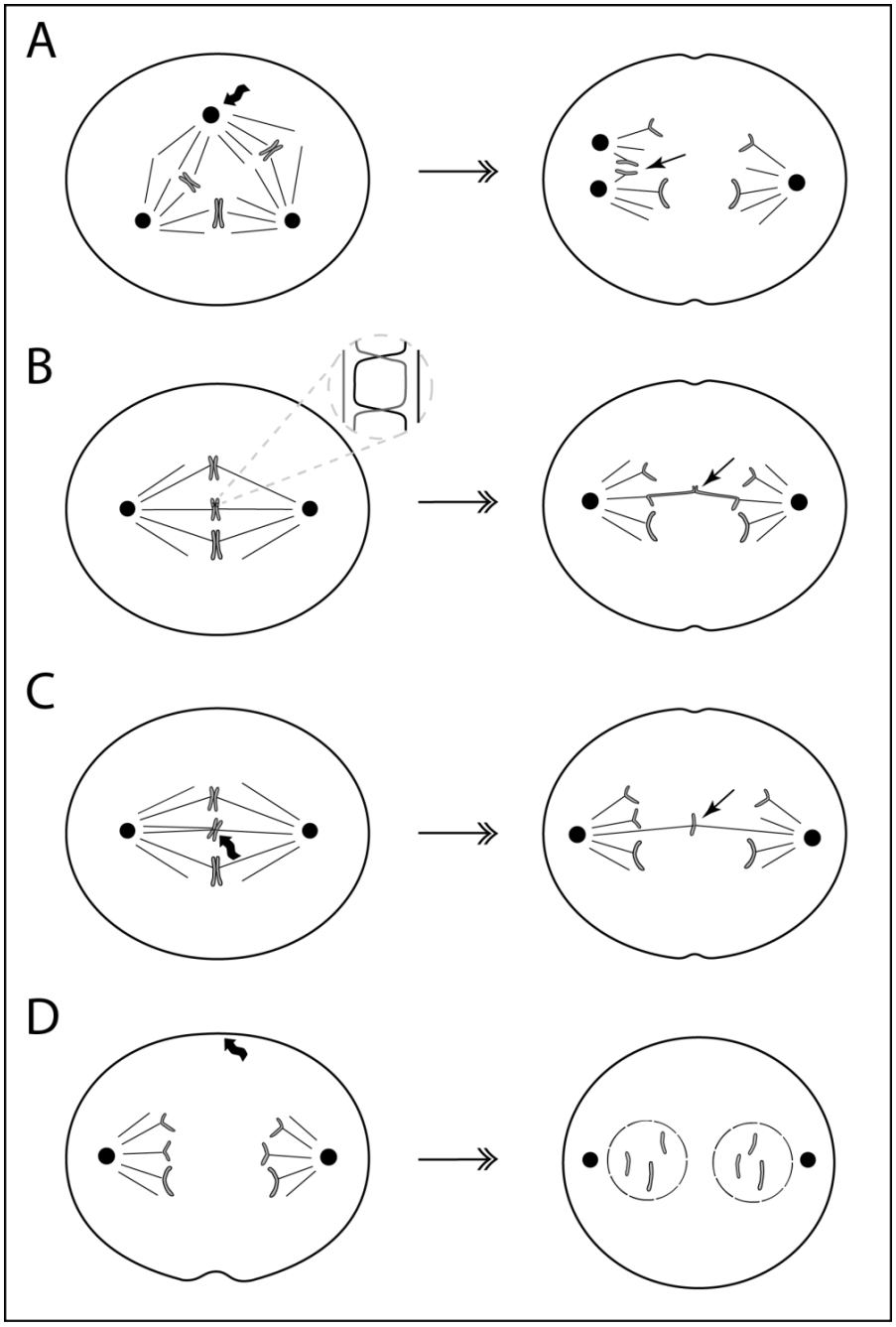

Figure 1. *Drosophila* CIN models: (A) An induced defect in centrosome number (wavy arrow) leads to a tripolar spindle. This is resolved into a bipolar array before anaphase, but one chromosome fails to segregate (arrow). (B) An induced defect in DNA damage repair or replication leads to still catenated 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.

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Chapter 3

Chromosomal Instability Causes Sensitivity to Metabolic Stress

Chromosomal instability or aneuploidy is common in cancer cells and also linked to selective metabolic adaptations in response to cellular and environmental stresses (Komarova, 2006; Yuneva et al, 2007; Pavelka et al, 2010). Continuous reshuffling of the genome and high mutation rates result in highly adaptive cells with a growth advantage and resistance to chemotherapies (Swanton et al, 2009; Sotillo et al, 2010).

Moreover, adjustment of metabolic pathways in order to support cell growth and division under stressed conditions is a hallmark of cancer cells. Most cancer cells reprogram their metabolic pathways to a high rate of glycolysis and lactic acid fermentation to fulfil their high energy and macromolecule demands (Warburg, 1956) and also to avoid the excessive use of mitochondria which are involved in initiation of apoptosis (Lopez-Lazaro, 2008). This metabolic shift was first reported by Otto Warburg in 1925, now it is an important hallmark of cancer (Warburg, 1925; Warburg et al, 1927; Warburg, 1956; Delbeke, 1999) and is widely considered as a potential anti-cancer target. (Kaplan et al, 1990; Clem et al, 2008; Holen et al, 2008; Jiralerspong et al, 2009; Gross et al, 2010; Le et al, 2010; Michelakis et al, 2010; Tennant et al, 2010). Currently, a few promising metabolic drugs are in clinical trials including inhibitors of lactate, fatty acid and nucleotides biosynthesis, glycolysis, HIF signalling and PI3K signaling (Tennant et al, 2010).

As CIN and these metabolic alterations are not present in normal cells, they can be a potential target for anti-cancer therapies (Raj et al, 2011). In this study we showed that the knockdown of certain metabolic genes results in high levels of mitochondrial dysfunction, oxidative stress, DNA damage and cell death only in CIN cells but not normal cells. Induction of CIN in otherwise normal cells makes them vulnerable to certain metabolic changes and can potentially be used as a target. We demonstrate that this is because the CIN and aneuploidy cause redox stress that pushes these cells close to their tolerance limits. Further studies can explore the possible mechanisms by which cells recognize and respond to CIN.

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Chapter 4

Autophagy Regulates the Survival of Cells with Chromosomal Instability

Autophagy is a pro-survival pathway that responds to various cellular stresses by degrading and recycling dysfunctional molecules and organelles in cells (Mizushima 2007; Glick et al. 2010). Defects in the autophagy pathway are associated with tumorigenesis. However, autophagy is also required to maintain the survival of advanced cancer cells. In addition, cancer cells can activate autophagy pathways after cancer chemotherapy in response to cellular damage and metabolic stress. Therefore, autophagy can maintain cancer cell survival which causes cancer growth and development of drug resistance (Notte et al. 2011; Rubinsztein et al. 2012; Sui et al. 2013).

In this study, consistent with the results that CIN cells showed metabolic stress, we found that the autophagy pathway is robustly activated in CIN cells and is critical for their survival. Blocking autophagy by depleting essential autophagy genes *atg1* or *atg18* increased the level of ROS, DNA damage and apoptosis in CIN cells. On the other hand, enhancing autophagy by either depletion of autophagy upstream negative regulator *mTor* or over-expressing mitophagy gene *parkin* reduced the level of ROS and rescued the apoptosis. Furthermore, we found a strong co-localization of mitochondria and lysosomes indicating these defective mitochondria are degraded by autophagy/mitophagy. We propose that autophagy reduces the level of oxidative stress and apoptosis by removal of dysfunctional mitochondria in CIN cells. In conclusion, our study demonstrated that the autophagy pathway is vital for maintaining the survival of CIN cells.

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Autophagy regulates the survival of cells with chromosomal instability.

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Key words: Chromosomal instability; autophagy; mitophagy; Parkin; Drosophila

ABSTRACT

Chromosomal instability (CIN) refers to genomic instability in which cells have duplicated or deleted chromosomes or chromosomal fragments. A high level of CIN is common in solid tumours and is associated with cancer drug resistance and poor prognosis. The impact of CIN-induced stress and the resulting cellular responses are only just beginning to emerge. Using proliferating tissue in *Drosophila* as a model, we found that autophagy signalling is activated in CIN cells and is necessary for their survival. Specifically, increasing the removal of defective mitochondria by mitophagy is able to lower levels of reactive oxygen species and the resultant cellular damage that is normally seen in CIN cells. In response to DNA damage CIN is increased in a positive feedback loop, and we found that increasing autophagy signalling could decrease the level of CIN in proliferating cells. These findings underline the importance of autophagy control in the development of CIN tumours.

INTRODUCTION

Chromosomal instability (CIN) refers to a state which cells are unable to maintain chromosomal integrity or number [1]. Chromosomal instability (CIN) or genomic instability (GIN) has been suggested as a pivot hallmark of cancer which facilitates the acquisition of other cancer hallmarks required for tumorigenesis [2]. CIN is seen in most human solid tumours and the genetic variation it generates can account for the development of drug resistance and the poor prognosis of CIN cancer patients [3, 4]. It has been proposed that CIN itself could be an attractive target for chemotherapy, as it is a relatively cancer-specific phenotype [5-7]. However, little is known about which properties of CIN cells might allow CIN tumours to be efficiently killed.

Autophagy is a normal cellular pathway for the degradation and recycling of unnecessary or dysfunctional cellular components [8-10]. The process of autophagy involves the sequestration of cytoplasmic material by double-membrane phagophores to form autophagosomes that then fuse with lysosomes to enable degradation of their cargo [8]. Autophagy is induced in response to various stresses to maintain metabolic homeostasis and prevent the build-up of dysfunctional cellular components [9]. The aberrant regulation of autophagy has been seen in several diseases, especially in neurodegenerative disease and cancer [10-12]. However, whether autophagy is protective or deleterious in the development of cancer has been widely debated [13]. The information currently available from clinical trials and mouse models suggests that a lack of autophagy predisposes tissue to develop tumours, possibly because autophagy normally moderates oxidative stress and DNA damage by removing defective mitochondria. However, in some model systems, autophagy is essential for the growth of the tumour [14, 15]. Consequently there are now ongoing clinical trials evaluating the combination of inhibition of autophagy with chemotherapeutics [16, 17] (ADD DONNA'S REFS). The expectation is that tumours may need autophagy to tolerate the metabolic demands of proliferation, to avoid excessive oxidative stress and consequently an unmanageable level of genome instability. Thus reduced autophagy may promote tumorigenesis by increasing DNA damage rates, but for tumours to thrive they may need to increase their autophagic flux to prevent deleterious levels of oxidative damage.

In characterizing pathways which facilitate the survival of CIN cells, we have previously reported that CIN cells are sensitive to metabolic stress and generate elevated levels of reactive oxygen species [18]. Based on that study, we carried out further screening for candidates whose depletion can specifically kill CIN cells. In this process,

we found that when CIN is induced in otherwise normal cells, they become sensitive to the depletion of autophagy. Here we show that CIN leads to an increase in autophagy, and that autophagy is needed to limit reactive oxygen species, DNA damage and cell death in CIN cells. Furthermore, elevated levels of autophagy promote the survival of CIN cells.

Altogether, our research highlights the significance of understanding autophagy pathways as a potential therapeutic target for the treatment of CIN tumours.

RESULTS

Autophagy is activated when CIN is induced in proliferating cells

We have previously used RNA interference knockdown of the spindle assembly check point gene *mad2* or cohesin gene *rad21* to generate inducible CIN models with different CIN levels [19]. From this work, and that of others [20] it has become clear that aneuploidy is associated with elevated levels of reactive oxygen species (ROS). We speculated that in response, CIN cells might induce autophagy to recycle damaged macromolecules. To test autophagy levels in cells with induced CIN, we used lysotracker staining, was elevated in both *mad2* and *rad21* CIN cells relative to normally proliferating cells (Fig. 1b, 1c). To confirm this result we examined the levels of a tagged form of Atg8a [21]. In line with the lysotracker staining, we found robust Atg8a puncta formation in CIN cells indicating autophagy activation (Fig. 1e, 1f). Stronger induction of autophagy was seen in *rad21* CIN cells than in *mad2* CIN cells, consistent with the higher level of CIN generated in the *rad21* model [19].

Depletion of Autophagy pathway leads to increased oxidative stress and apoptosis in CIN cells

Having found that the autophagy pathway is activated in CIN cells, we hypothesized that robust autophagy activation might be particularly needed for the survival of CIN cells. In order to address this hypothesis, we depleted essential autophagy genes *Atg1* or *Atg18* [22] by RNA interference in CIN cells. We found that knocking down either *Atg1* or *Atg18* led to dramatically increased levels of oxidative stress and DNA damage in CIN cells (Fig. 2, Fig.S1). Furthermore, ablation of *Atg1* or *Atg18* in CIN cells resulted in a significant increase in apoptosis as detected by active caspase staining (Fig. 3). Elevated levels of cell death were seen when autophagy was blocked in either CIN model (Fig S2). However, depleting *Atg1* or *Atg18* in normal proliferating cells had no detectable effect on ROS levels, DNA damage or apoptosis. These results are consistent with a protective role for autophagy in response to cellular stresses [23], and showed that that autophagy activation was required for the survival of CIN cells.

Enhancing autophagic flux rescues oxidative stress levels and apoptosis in CIN cells

Having observed that CIN cells required autophagy to avoid cell death, we wished to see whether enhancing autophagic flux could improve the survival of CIN cells. Autophagy induction is regulated by conserved upstream signalling pathways that converge on the target of rapamycin (TOR) kinase, which prevents autophagy by inhibiting Atg1 [24, 25]. By the removal of the autophagy inhibitor *Tor* using RNAi, we found that enhancing autophagic flux could rescue the oxidative stress and apoptosis phenotype in CIN cells (Fig. 4, Fig.S3). This suggested that autophagy is not normally

induced enough to protect cells with high levels of CIN, and that elevated autophagy, which is often seen in cancer [7, 8], can improve the survival of these cells.

Autophagy activation is responsible for the degradation of dysfunctional mitochondria in CIN cells

A selective function of autophagy pathway activation is the removal of defective mitochondria through *pink1*/*parkin*-mediated mitophagy [26]. CIN is known to cause defective mitochondria and increased oxidative stress in cells [18, 19], therefore, we checked whether mitophagy is involved in the response to CIN. We found that overexpression of the essential mitophagy gene *parkin* reduced the level of ROS and apoptosis in CIN cells even more effectively than increasing general autophagy by *Tor* depletion (Fig. 4). If removal of defective mitochondria is an essential function in CIN cells, we would expect to see elevated levels of mitochondria being processed in lysosomes in CIN cells. To test this we visualized lysosomes with lysotracker and mitochondria with mito-GFP (Fig. 5). In CIN cells we observed large cytoplasmic accumulations of lysotracker, and they co-localized with high levels of mito-GFP. Control cells did not have large lysosomes or any striking co-localization with mito-GFP. These results suggest that mitochondria in CIN cells are transported to lysosomes for degradation and that this process is necessary for the survival of CIN cells.

Enhancing autophagic flux reduces the level of CIN in proliferating cells

It has been reported that defective autophagy increases the level of CIN in cancer cells due to increased DNA damage and gene amplification [27]. Conversely, we would expect treatments that decrease DNA damage to lower CIN levels. As enhancing autophagic flux reduced the level of ROS (Fig. 4), and we have previously shown that

DNA damage in CIN cells is caused by elevated ROS [18] , we wished to test whether increasing autophagy could moderate the CIN level. In order to address this hypothesis, we checked the frequency of aneuploid metaphases after autophagy enhancement. We found that enhanced autophagic flux could significantly reduce the CIN level in a proliferating tissue (Fig. S4).

Discussion

Autophagy can function as a pro-survival protective pathway in cancer cells to fulfil their metabolic demands for rapid cell proliferation and to respond to cellular stresses that may include genomic instability and metabolic stress [27-30]. Therefore, we assessed the level of autophagy in cells with induced CIN and found a robust activation of autophagy (Fig.1). We found that this activation was vital for the survival of CIN cells as inhibiting autophagy led to increased oxidative stress, DNA damage and massive apoptosis in CIN cells (Fig. 2, Fig. 3, Fig.S1 and Fig.S2). On the other hand, we found that enhancing autophagic flux could significantly reduce the level of reactive oxygen species (ROS) and apoptosis in CIN cells (Fig. 4 and Fig.S3). Our findings are consistent with a protective role for autophagy in response to aneuploidy and the redox stress that comes with aneuploidy [23, 31]. It is interesting that the CIN should invoke a protective response as well as the cell lethal immune responses that remove defective cells [19]. Our interpretation is that autophagy is a buffering process that can manage stresses within the normal range and prevent any auto-immune responses, but this has a limit beyond which damaged mitochondria accumulate, the redox stress signals are produced and the immune response is triggered.

Autophagy has been reported to suppress CIN in tumour cells, however, the detailed mechanism is not clear [27]. In this study, we found that enhancing autophagy flux

could reduce the level of CIN in *Drosophila* proliferating cells (Fig.S4). We examined the possibility of chromatid removal by autophagy [32], but failed to observe any co-localization of DNA with lysosomes (Fig. 5 and data not shown), suggesting that autophagy does not directly degrade lagging chromosomes in our CIN models. However, we found a strong co-localization of mitochondria and lysosomes suggesting that defective mitochondria are degraded by autophagy (mitophagy) (Fig.5). Furthermore, we found that overexpression of the mitophagy regulator *parkin* could significantly rescue the level of ROS and apoptosis in CIN cells. Although mitochondria are built to tolerate ROS by producing localized antioxidants such as superoxide dismutase, it is not surprising that the high levels of ROS produced by mitochondria in CIN cells should damage them to the point where they require mitophagy [33]. In the absence of this quality control system, we observed high rates of DNA damage. While decreasing autophagy might be an effective mechanism for pre-tumourous tissue to increase its mutation rate, tumours need to balance their level of CIN to avoid intolerable genotoxic stress [34]. Modulating mitophagy is likely to play a key part in fine tuning the rate of CIN to an adaptive level.

In conclusion, our data suggests that autophagy effectively removes defective mitochondria in CIN cells thus reducing the level of ROS, DNA damage and apoptosis in CIN cells. Moreover, the reduced level of ROS and DNA damage further mitigate the level of CIN (Fig.6). Our study reveals a mechanism by which autophagy limits CIN in cells, which underscores the importance of understanding autophagy pathways in CIN tumour treatment.

MATERIALS AND METHODS

Drosophila Stocks

The fly stocks used in this paper are as follows: *mad2*-RNAi (VDRC 47918), *Rad21*-RNAi (Bloomington #36786), *mcherry-Atg8a* [21], *Atg1*-RNAi (VDRC 16133), *Atg18*-RNAi (VDRC 22643), *Tor*-RNAi (VDRC 35578), UAS-*park* (Bloomington #34746), *UAS-mito-GFP* (Bloomington #8442).

Lysotracker and Hoechst staining

Lysotracker staining was used to detect autophagy in larval wing imaginal discs. The dissected imaginal discs were transferred from PBS and incubated in 1uM lysotracker (Lysotracker red DND-99, Invitrogen) and 6ug/ml Hoechst (Hoechst 33342, Sigma) for 5 mins and then mounted to a slide with PBS for microscopy after a quick wash in PBS.

Oxidative stress assay

Fluorogenic probe CellROX (Life Technologies) was used to measure the level of reactive oxygen species (ROS) in CIN cells as detailed in [18].

Immunostaining

The standard method for immunostaining in our lab has been used in this study [18]. The details of used antibody in this study are listed here: The primary antibodies are Rabbit anti-caspase3 (D175, 1: 100) (Cell Signalling); Rabbit anti-H2AVD (Rockland, Lot# 30352, 1:700). The secondary antibody is CY3 anti-rabbit (1: 200).

Data analysis

The microscopy of CellROX staining, Immuno-staining was done on a Zeiss Axioplan2 microscope. The microscopy of lysotracker and mitoGFP co-localization was done on a confocal microscope. The details of data analysis are described in [18] and [19].

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Figure Legends

Figure 1 Autophagy is activated in tissues with Chromosomal Instability (CIN).

CIN was induced in the posterior half of each wing disc as indicated by the dotted line and the rest of each disc was wild type.

(a, b, c) Lysotracker staining of third instar larval wing discs. Wing discs with CIN induced by either Mad2 depletion (b, *engrailed>Gal4*, *UAS-mad2*^{RNAi}) or Rad21 depletion (c, *engrailed>Gal4*, *UAS-rad21*^{RNAi} *UAS-Dicer2*) showed increased lysosome staining.

(d, e, f) The level of mCherry-Atg8a in third instar larval wing discs. Wing discs with CIN induced by either Mad2 depletion (e) or Rad21 depletion (f) showed increased induction of autophagy as indicated by the level of mCherry-Atg8a puncta.

Figure 2 Blocking autophagy causes redox stress in CIN cells.

CellRox staining was used to detect the level of oxidative stress. The indicated genes were knocked down in the posterior half of each wing disc as indicated by the dotted line and the rest of each disc was wild type. Knocking down either Atg1 (*engrailed>Gal4*, UAS-*atg1*^{RNAi}) (c) or Atg18 (*engrailed>Gal4*, UAS-*atg18*^{RNAi}) (e) did not give oxidative stress, and the CellRox signal was low or absent in *mad2*^{RNAi} CIN

cells (b). However, when Atg1 (d) or Atg18 (f) were depleted in CIN cells, an elevated level of oxidative stress was observed.

Figure 3 Blocking autophagy increases cell death in CIN cells.

Anti-cleaved caspase3 antibody staining was used to show the level of apoptosis. The indicated genes were knocked down in the posterior half of each wing disc as indicated by the dotted line and the rest of each disc was wild type. Knocking down either Atg1 (*engrailed>Gal4*, UAS-$Atg1^{RNAi}$) (c) or Atg18 (*engrailed>Gal4*, UAS-$Atg18^{RNAi}$) (e) did not cause apoptosis in these proliferating cells. However, knocking down Atg1 (d) or Atg18 (f) in CIN cells, significantly increased the level of apoptosis in these cells relative to the CIN alone control (b). Quantification of the cleaved caspase3 staining is shown in (g). In all cases n≥9 and the error bars show 95% confidence intervals around the mean. The p values were calculated using two-tailed t-tests with Welch's correction.

Figure 4 Enhancing autophagy or mitophagy decreases redox stress and cell death in CIN cells.

Anti-cleaved caspase3 antibody was used to stain the level of apoptosis in cells while CellRox staining was used to detect the level of oxidative stress. The indicated genes were knocked down in the posterior half of each wing disc as indicated by the dotted line and the rest of each disc was wild type. Enhancing autophagy signalling by mTor knockdown (*engrailed>Gal4*, UAS-Tor^{RNAi}, UAS-$rad21^{RNAi}$, UAS-*Dicer2*) (b, e) reduced the level of oxidative stress (b) and apoptosis (e) observed in CIN cells relative to the CIN alone controls (a, d). A similar reduction in oxidative stress (c) and apoptosis (f) was observed in CIN cells when mitophagy was induced by the overexpression of Parkin (*engrailed>Gal4*, UAS-*parkin*, UAS-$rad21^{RNAi}$, UAS-*Dicer2*). Quantification of

the cleaved caspase3 staining is shown in (g). In all cases n≥12 and the error bars show 95% confidence intervals around the mean. The p values were calculated using two-tailed t-tests with Welch's correction.

Figure 5 Mitochondria accumulate in lysosomes in CIN cells.

Mito-GFP (b, d, f, h) was used to mark mitochondria and Lysotracker staining (c, d, g, h) was used to detect lysosomes in third instar larval wing discs. CIN cells induced by Rad21 depletion (*engrailed>Gal4*, UAS-*rad21*^{RNAi}, UAS-*Dicer2*) showed a strong co-localization (one example arrowed) of mitochondrial aggregates (f) and large lysosomes (g) while this co-localization was not seen in wild type cells (a-d).

Figure 6 A model for the effect of autophagy on the survival of CIN cells.

Chromosomal Instability leads to metabolic stress and the production of reactive oxygen species, which in turn cause defective mitochondria and further oxidative stress. Autophagy can be activated to effectively remove the defective mitochondria and thus reduce the level of oxidative stress, DNA damage and apoptosis in CIN cells. Moreover, autophagy signalling could reduce the level of CIN by reducing DNA damage in CIN cells.

Figure S1 The effect of knocking down autophagy signalling on the level of DNA damage in CIN cells.

Anti-phosphorylated H2AvD antibody was used to detect the level of DNA damage. Knocking down either Atg1 (c) or Atg18 (e) did not cause DNA damage in proliferating cells. However, knocking down Atg1 (d) or Atg18 (f) significantly increased the level of DNA damage in CIN cells compared to the CIN alone control (b).

Figure S2 The effect of knocking down autophagy signalling on cell death in CIN cells.

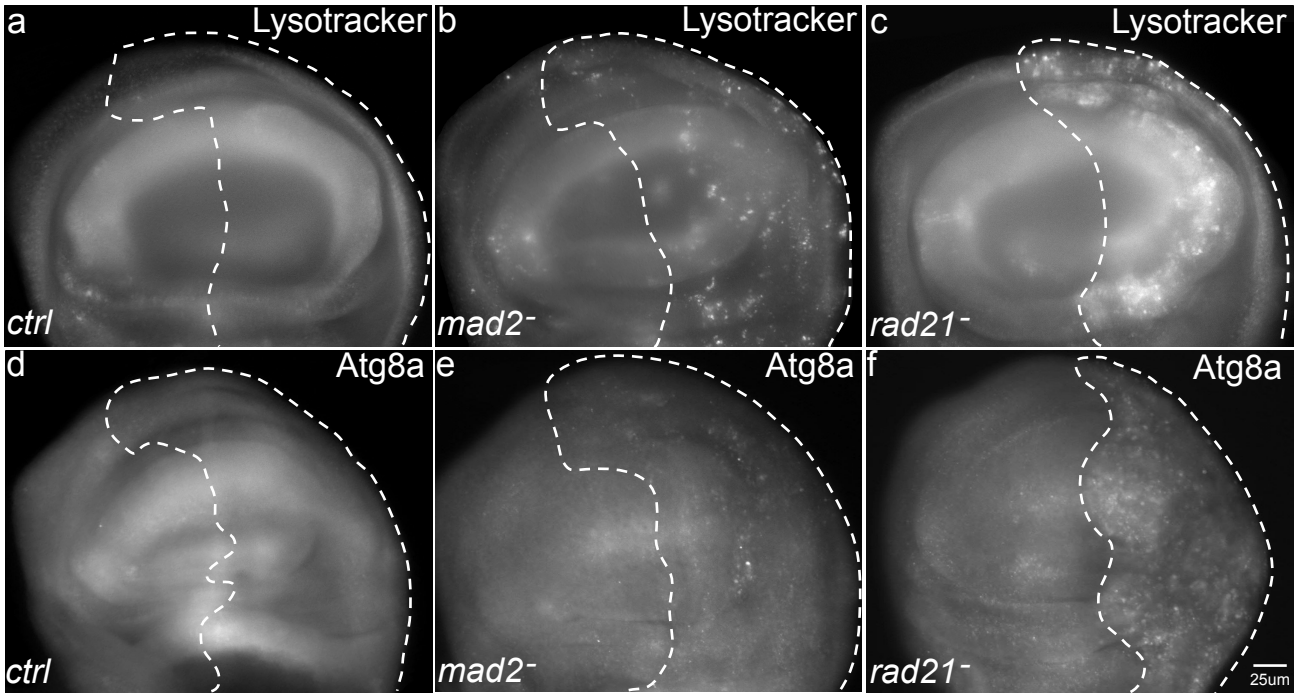
Anti-cleaved caspase3 antibody was used to detect the level of apoptosis. Knocking down either Atg1 (b) or Atg18 (c) significantly increased the level of apoptosis in CIN cells induced by Rad21 depletion compared to the CIN alone control (a). Quantification of the cleaved caspase3 staining is shown in (d). For all genotypes n>12 and the error bars show 95% confidence intervals around the mean. The p values were calculated using two-tailed t tests with Welch's correction.

Figure S3 The effect of enhancing autophagy signalling on the cell death in CIN cells.

Acridine orange staining was used to detect the level of cell death. Enhancing autophagy signalling by mTor knock down (b) significantly reduced the level of cell death in CIN cells compared to the Cin alone control (a). Quantification of the Acridine Orange staining is shown in (c).

Figure S4 Enhancing autophagy signalling reduces the level of CIN in proliferating third instar larval wing disc cells.

The level of CIN was evaluated by the frequency of aneuploid metaphases. Rad21 depletion gave aneuploidy in 39% of metaphase cells and the level of CIN could be significantly reduced to 22% by mTor knock down. The p value was calculated by the Fisher's exact test.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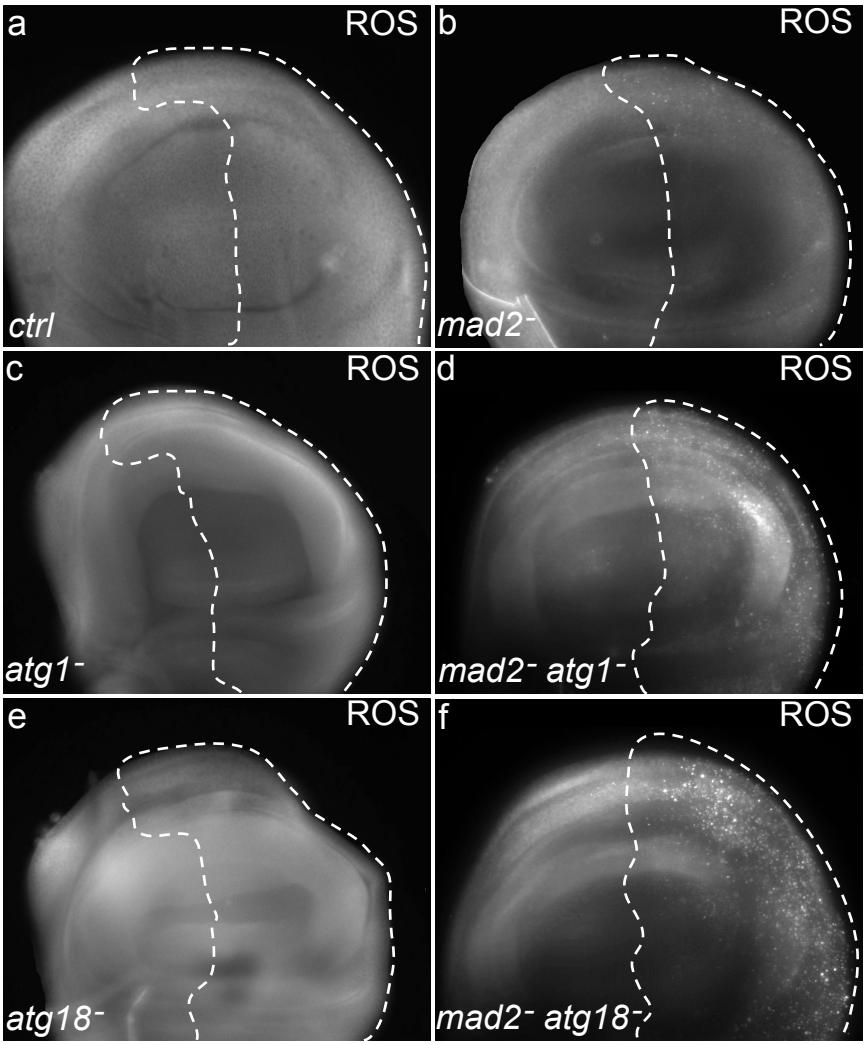
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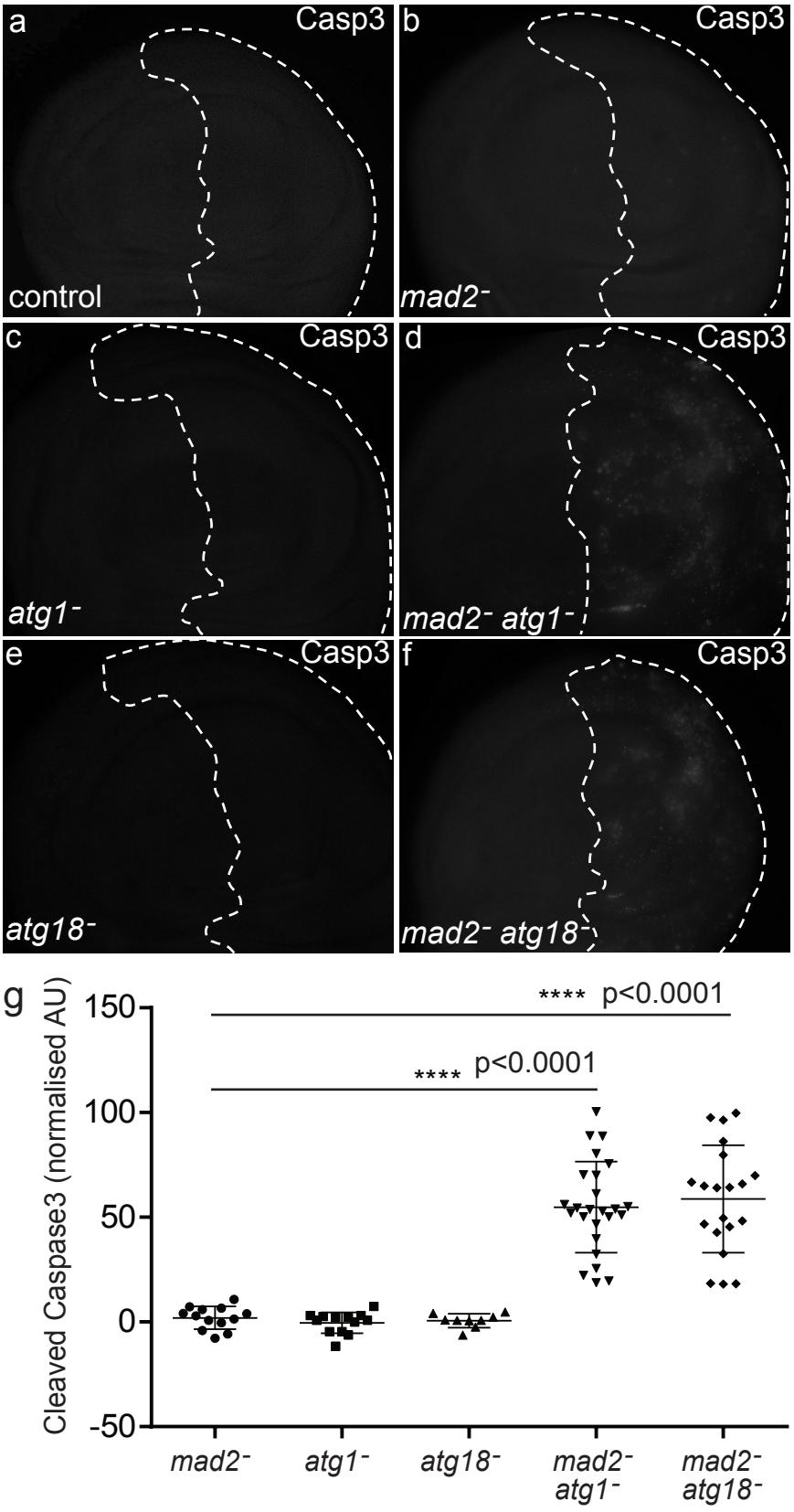
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mad2-
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e
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f
Casp3
mad2- atg18-
g
Cleaved Caspase3 (normalised AU)
150
100
50
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atg18-
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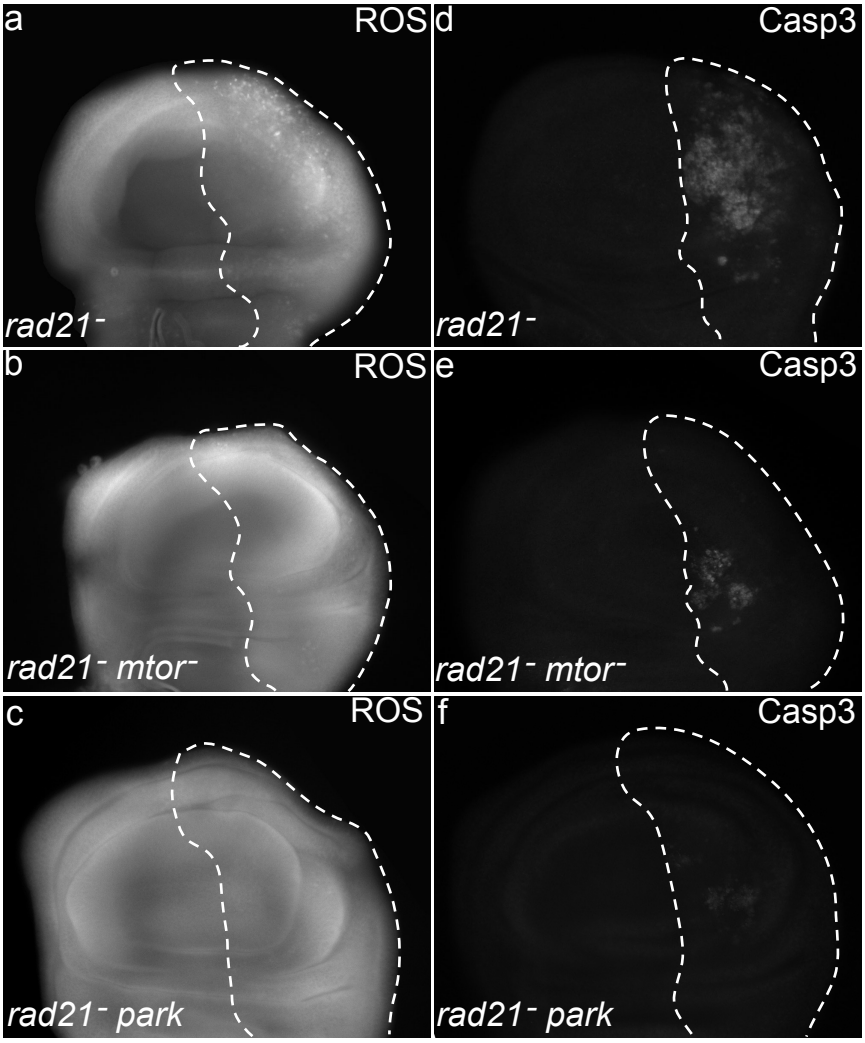
mad2- atg18-


a
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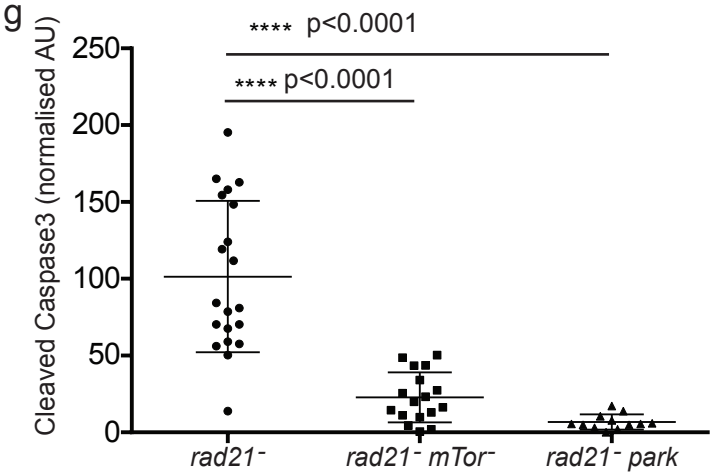
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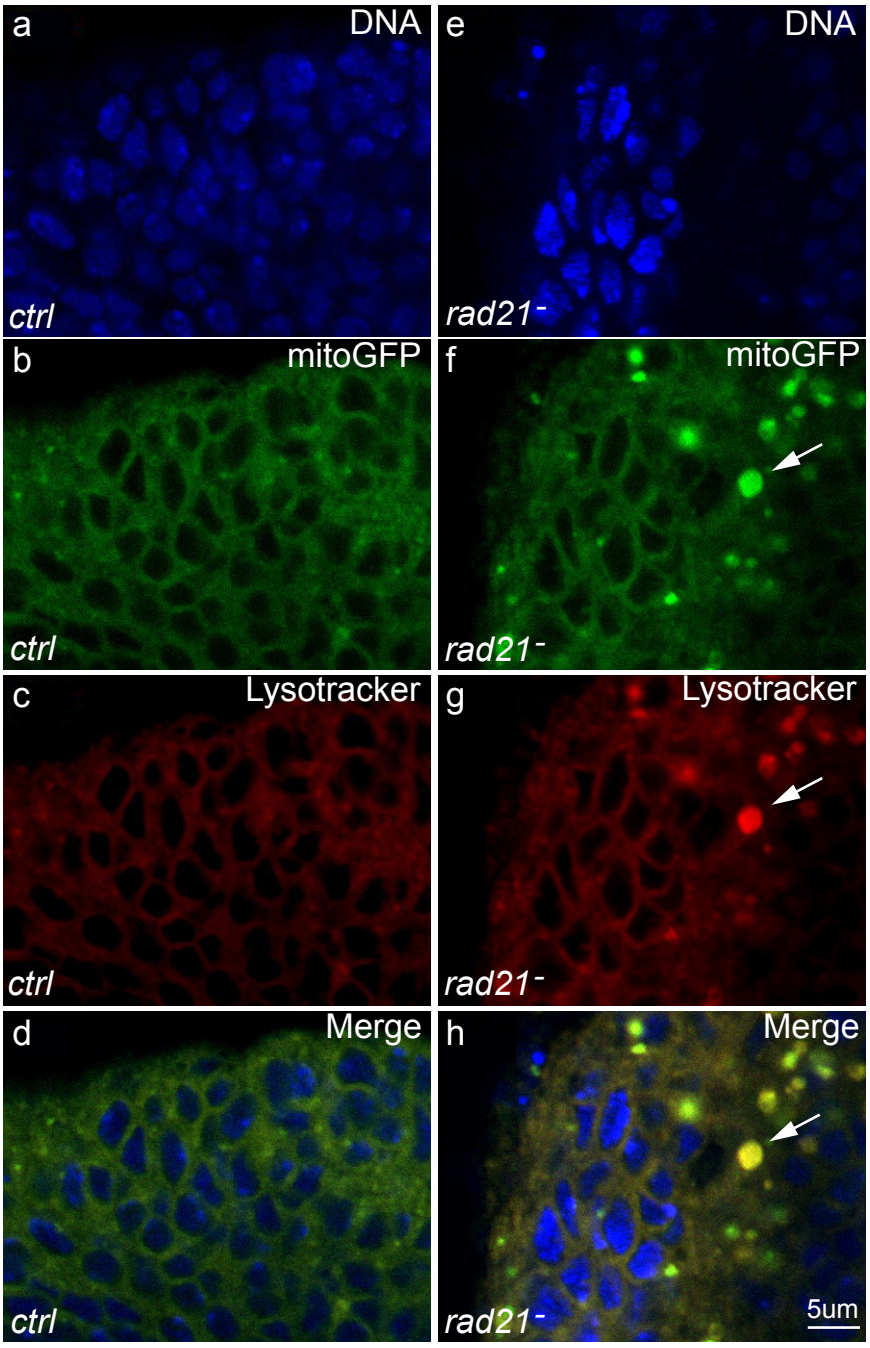
rad21- park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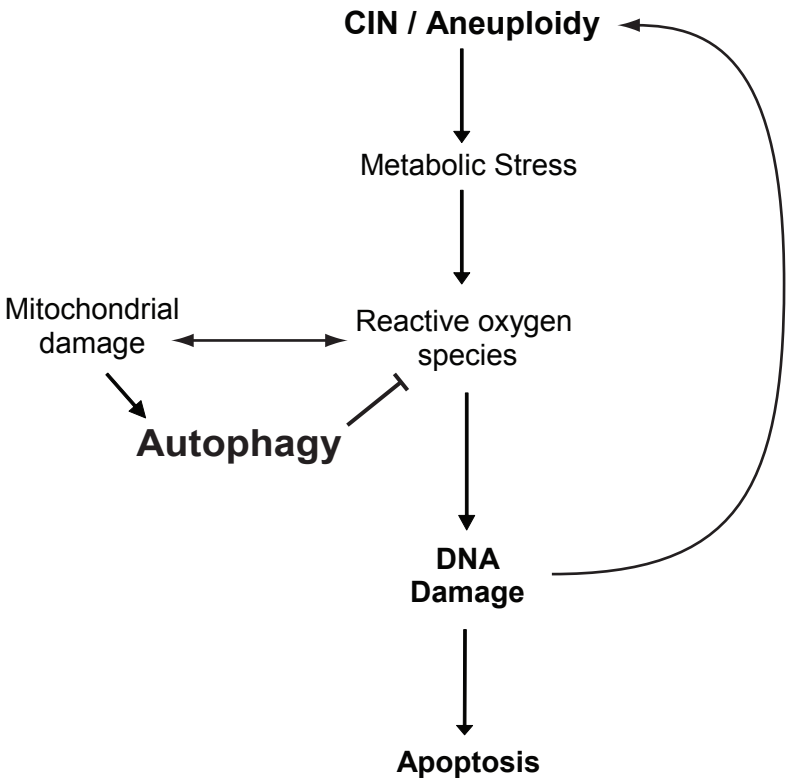
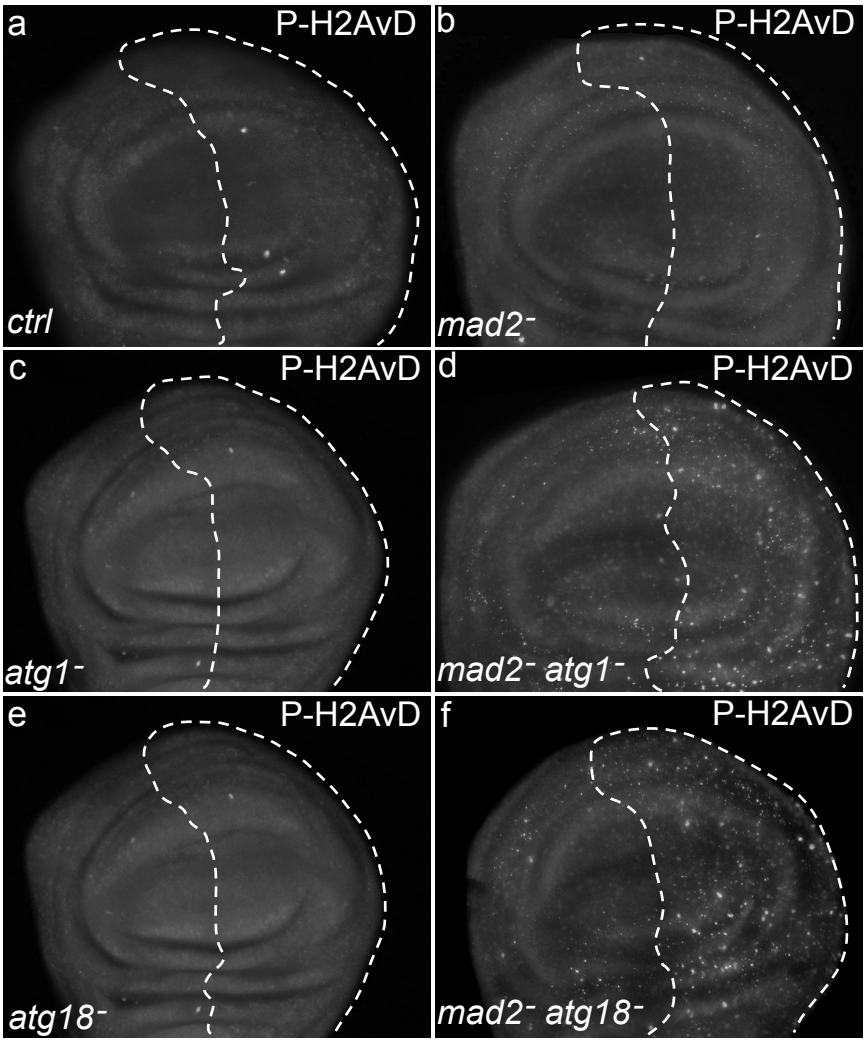

CIN / Aneuploidy
Metabolic Stress
Mitochondrial damage
Reactive oxygen species
Autophagy
DNA Damage
Apoptosis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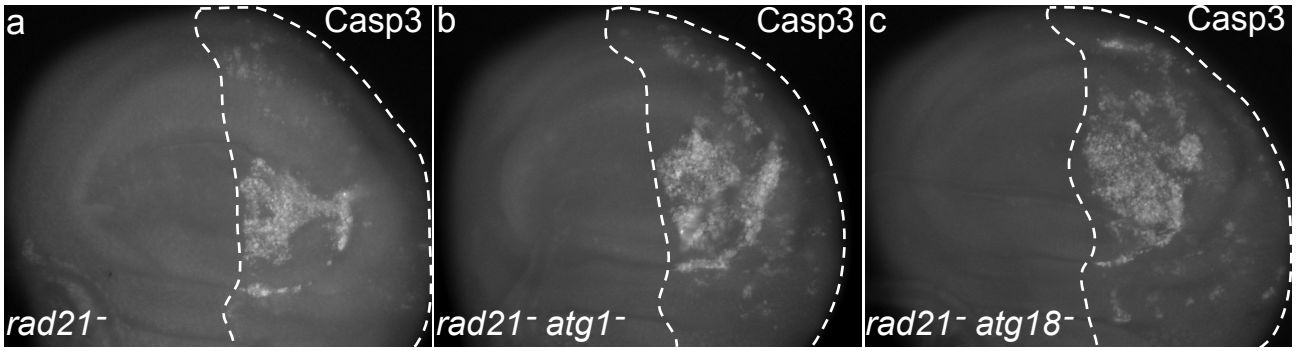
P-H2AvD
mad2- atg18-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c
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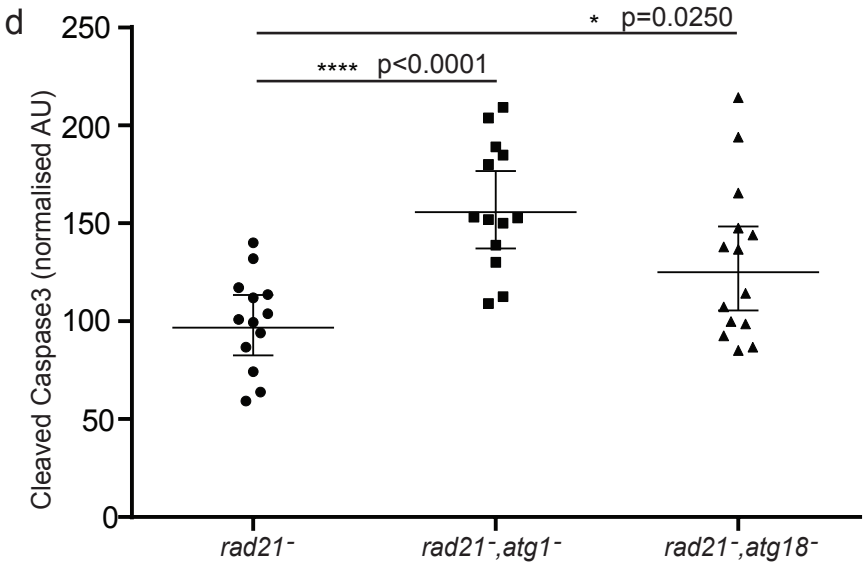
rad21- atg18-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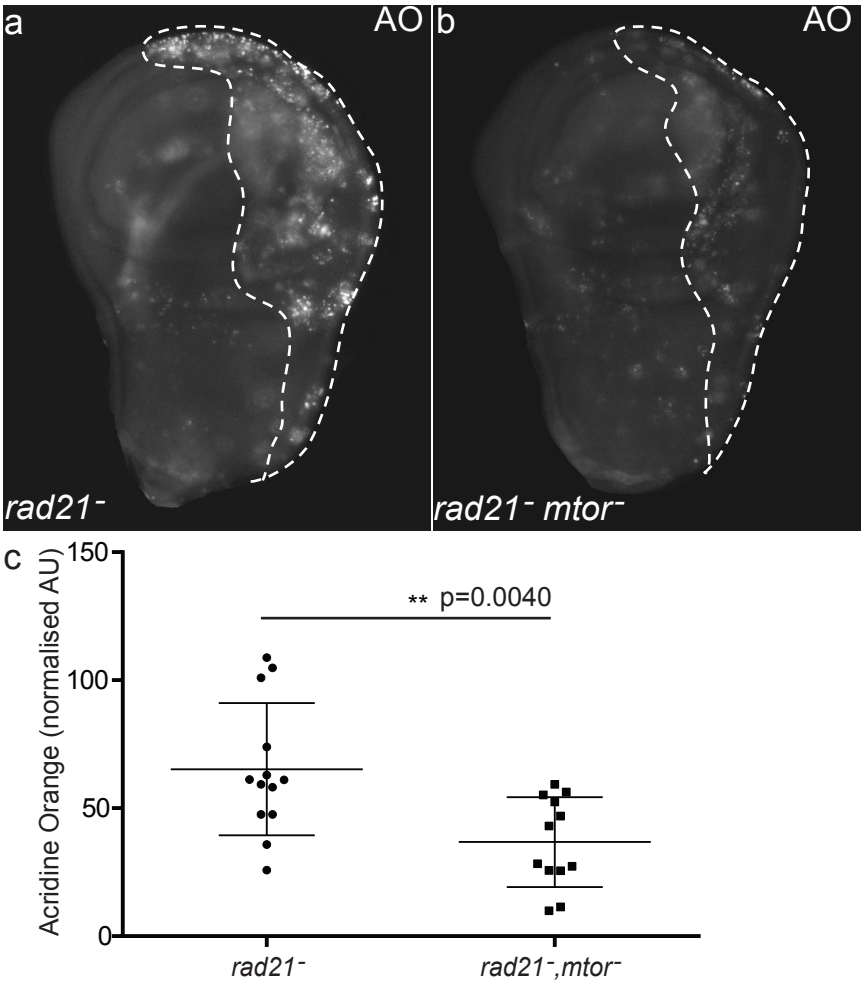
rad21-,atg18-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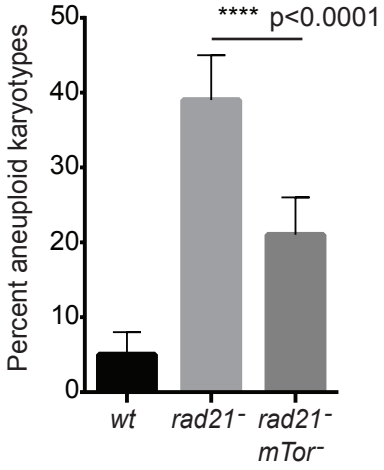
rad21-
rad21-,mtor-


Percent aneuploid karyotypes
50
40
30
20
10
0
**** p<0.0001
wt
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rad21-
mTor-

Chapter 5

Chromosomal Instability Triggers Cell Death via Local Signalling Through the Innate Immune Receptor Toll

A high level of chromosomal instability (CIN) is specifically found in cancer cells not in normal cells. (Thompson et al. 2010). As high level of CIN leads to metabolic stress in cells and is not tolerated by normal cells, one question is how CIN can be tolerated by cancer cells? In order to address this question, we tested genes from different signaling pathways for their effects on CIN tolerance. In order to investigate signaling pathways which give rise to CIN tolerance in cells, we tested 295 candidate genes involved in the cytoskeleton, centrosome, DNA replication and repair, apoptosis and immune response pathways (see Appendix 1). In this study, we found *Drosophila* Toll pathway genes are associated with the lethality of CIN flies as depletion of five Toll pathway genes could rescue the *Drosophila* lethality caused by CIN. In line with this, we found systemic innate immune activation in flies carrying CIN cells suggesting that CIN cells can be detected by the *Drosophila* immune system. This is consistent with the role of the immune system in response to induced tissue damage and overgrowth in *Drosophila* (Carvalho et al. 2014; Hauling et al. 2014; Parisi et al. 2014).

We also found Toll pathway is activated within CIN cells as shown by increased levels of Dorsal staining in those cells. Manipulation of the Toll pathway in CIN cells can significantly affect the fate of the CIN cells. Our results suggest that CIN cells recruit hemocytes to the surface of cells by activating the local immune system and its downstream JNK-Mmp1 pathway. Blocking signalling through Toll and JNK in CIN cells reduced the number of recruited hemocytes and consequently reduced the level of apoptosis in the CIN cells. In addition, we found that the apoptosis in CIN is mediated through the TNFα-JNK pathway in response to hemocytes. Knockdown of either TNFα or JNK could reduce the level of apoptosis in CIN cells which is consistent with its well characterized role in initiating apoptosis in *Drosophila* (Igaki et al. 2009).

Taken together, our research demonstrated that CIN cells can be detected and executed by the innate immune system which highlights the significance of the innate immune system in cancer immunotherapy.

Statement of Authorship

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Chromosomal instability triggers cell death via local signalling through the innate immune receptor Toll

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ABSTRACT

Chromosomal instability (CIN) is a hallmark of cancer and has been implicated in cancer initiation, progression and the development of resistance to traditional cancer therapy. Here we identify a new property of CIN cells, showing that inducing CIN in proliferating *Drosophila* larval tissue leads to the activation of innate immune signalling in CIN cells. Manipulation of this immune pathway strongly affects the survival of CIN cells, primarily via JNK, which responds to both Toll and TNFα/Eiger. This pathway also activates Mmp1, which recruits hemocytes to the CIN tissue to provide local amplification of the immune response that is needed for effective elimination of CIN cells.

INTRODUCTION

Chromosomal Instability (CIN) refers to a state in which cells have an increased rate of gain or loss of whole chromosomes or chromosomal pieces [1]. Several causes of CIN have been identified, and include dysfunction of the spindle assembly checkpoint, centrosomes, DNA replication or cohesion, leading to defects in chromosome segregation during mitosis [1, 2]. CIN is a defining feature of most human solid cancers (e.g. 85% of sporadic colorectal cancers) and is positively correlated with both drug resistance and poor prognosis [3]. Somatic cells with CIN can initiate the process of tumorigenesis [4], and CIN is responsible for the generation of cells with varied genetic backgrounds, out of which drug resistance can develop. This is thought to contribute to relapses following traditional cancer therapies that appear to initially succeed: the therapy creates selection pressure on cancer cells which drives rapid genetic evolution as CIN generates diverse cells from which those with a selective advantage and drug resistance can arise [5].

In this context, the role of the immune system is a double-edged sword during tumorigenesis [6]. On one hand, tumor-related inflammation is thought to foster tumorigenesis by supplying molecules into the tumour micro-environment that promote angiogenesis, resistance to apoptosis, and metastasis of malignant cells [7]. On the other hand, the immune system can detect and eliminate incipient cancer cells: there is good evidence for cancer immune surveillance. For example, immunocompromised mice that lack mature lymphocytes show a higher frequency of spontaneous tumorigenesis by the age of 14-16 months [8]. The frequency of carcinogen-induced tumorigenesis is also much higher in immunocompromised mice than in immunocompetent controls [9]. Furthermore, clinical evidence shows that at least for some kinds of tumours, increased infiltration with activated T cells is correlated with a better prognosis [10, 11]. Overall, the capability of cancer cells to circumvent attack by the immune system has been recognized as a hallmark of cancer [12].

Chromosomal instability represents a striking difference between the tumour and stromal cells, which do not normally have CIN. Consequently, CIN represents an excellent immune target if it can be recognized. Although the immune system has been reported to be activated by DNA damage [13] and tissue dysplasia [14], little is known about *in vivo* responses to CIN. While screening for genes that are required for the death of CIN cells *in vivo*, we identified several immune signalling genes. We found that the induction of CIN not only activates a systemic response from immune tissues, but also triggers a local immune reaction in proliferating epithelial cells. Manipulation of immune signalling strongly affects the

fate of these CIN cells. Altogether, our results showed that the immune system can detect and respond to CIN, and represents a critical feedback loop that is necessary to ensure the removal of defective cells that are a threat to the organism.

RESULTS

CIN leads to mitochondrial dysfunction, oxidative stress and cell death

We have previously reported that knockdown of the spindle assembly checkpoint gene *mad2* by RNA interference can be used to induce chromosomal instability (CIN) in *Drosophila* cells *in vivo*, which then show lagging chromosomes or chromosome bridges [15]. CIN caused by *mad2* knockdown leads to oxidative stress and

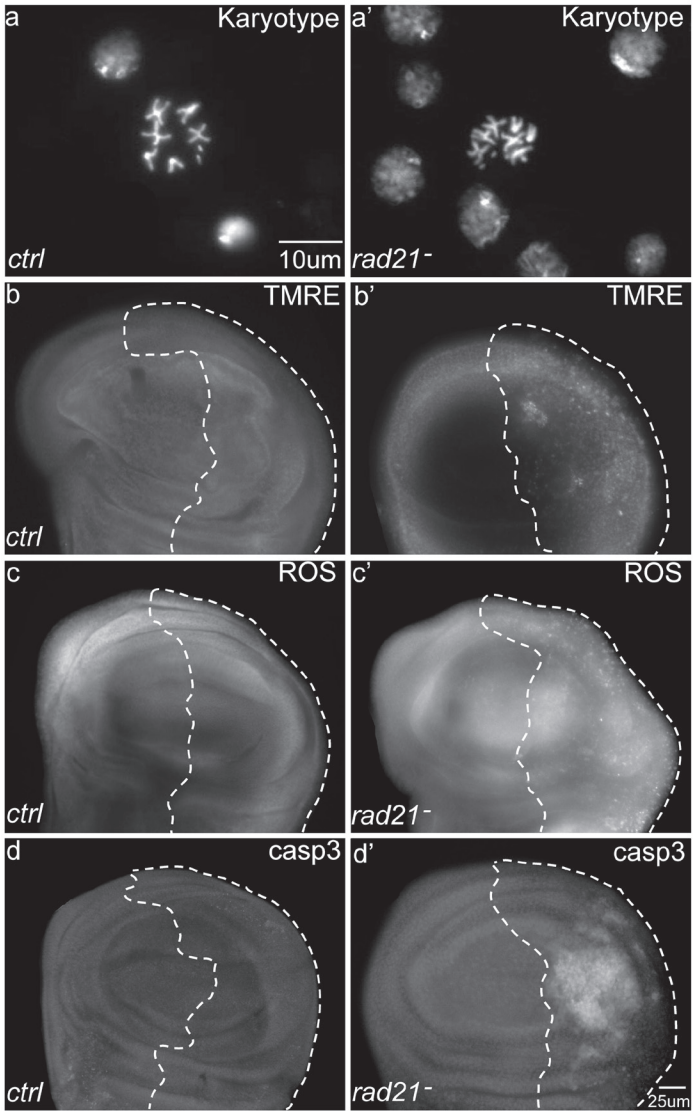

Figure 1: The effects of Chromosomal Instability (CIN) induced by Rad21 depletion on larval wing discs. CIN was induced in the *engrailed* expressing posterior region of the wing discs as shown by the dotted line; the rest of each disc was wild type. (**a**, **a'**) DNA stains of metaphase cells to show the karyotype. (**a**) A normal karyotype. (**a'**) Karyotype from a wing disc cell with induced CIN (*engrailed>Gal4*, *UAS-rad21*^{RNAi} *UAS-Dicer2*) showing a chromosome gain. Aneuploidy rates were quantified in Fig S1. (**b**, **b'**) TMRE staining of third instar larval wing discs. Tissue with induced CIN (*engrailed>Gal4*, *UAS-rad21*^{RNAi} *UAS-Dicer2*) showed increased mitochondrial membrane potential (**b'**) compared to the negative control (**b**). (**c**, **c'**) CellRox staining of third instar larval wing discs. CIN cells showed increased oxidative stress (**c'**) compared to the negative control (**c**). (**d**, **d'**) Anti-cleaved caspase3 antibody staining of third instar larval wing discs. CIN tissue showed an increased level of apoptosis (**d'**) compared to the negative control (**d**).

a repair response from the JNK pathway [16, 17]. In order to generate higher levels of CIN and to confirm that these CIN phenotypes were not specific to *mad2* knockdown, we created another inducible-CIN model. We knocked down *rad21*, a cohesin that regulates sister chromatid separation during cell division [18, 19]. While Rad21 mutation is not common in advanced cancers, its depletion results in CIN in vertebrates [20]. Co-expressing Dicer2 to enhance the RNAi knockdown of *rad21* in proliferating wing imaginal disc cells resulted in aneuploidy in 46% of metaphase cells, indicating a relatively high rate of CIN (Figure 1a' and Figure S1). To avoid missing cells that may have died from aneuploidy and been cleared, we blocked apoptosis by overexpression of p35 and in this case saw that around 70% of metaphase cells were aneuploid (Figure S1b). CIN induced by *rad21* depletion led to an increase in the level of TMRE staining, indicating elevated mitochondrial activity (Figure 1b'). As expected, this was accompanied by an increased level of oxidative stress (Figure 1c') and widespread cell death (Figure 1d', Figure S1). These effects were consistent with, but stronger than the effects of *mad2* knockdown [16, 21]. We found that we could similarly increase the rate of aneuploidy and cell death in the *mad2* model by using temperature to increase the RNAi expression level or by blocking apoptosis (Figure S1b). These results indicated that chromosomal instability generated by disparate means resulted in mitochondrial dysfunction and oxidative stress. Using strong depletion

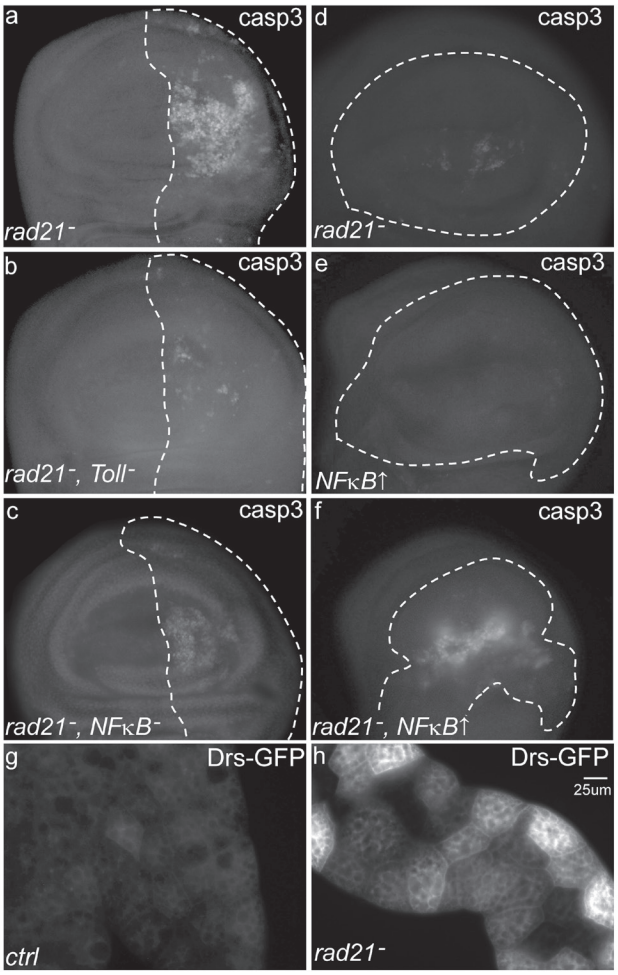

Figure 2 The effect of Toll pathway knock down or activation on CIN cell apoptosis. Anti-cleaved caspase3 antibody staining shows apoptosis in third instar larval wing discs with CIN induced by depletion of Rad21 (*UAS-rad21*^{RNAi} *UAS-Dicer2*). (**a**, **b** and **c**) CIN and Toll pathway gene double knockdowns were induced in the *engrailed* region (driven by *engrailed>Gal4*) of the wing discs as shown by the dotted line; the remainder of each disc was wild type. Knocking down Toll (**b**) or NFκB (*dorsal*) (**c**) significantly reduced the level of apoptosis in CIN cells (**a**). (**d**, **e** and **f**) CIN and gene overexpression were induced in the dotted-lined region (driven by *MS1096>Gal4*) while the rest of each disc was wild type. Activation of the Toll pathway by NFκB (*dorsal*) over-expression caused little apoptosis in normal cells (**e**), but greatly increased the level of apoptosis in CIN cells (**f**). Note that the level of cell death induced by *Rad21*-RNAi was lower using the *MS1096* driver (**d-f**) than with the *engrailed* driver (**a-c**), allowing detection of CIN enhancement without killing the animal. Quantitation for these results is shown in Fig S2. The innate immune response from the primary immune tissue, the fat body, was detected by visualizing levels of a GFP-tagged antimicrobial peptide (Drosomycin-GFP) in the larval fat body (**g**, **h**). Wild type larvae expressed low levels of Drosomycin-GFP (**g**), but this level was increased in animals in which CIN had been induced in a range of tissues including the wing, epidermis, gut and fat body (h, *engrailed>Gal4*, *UAS-rad21*^{RNAi} *UAS-Dicer2*).

of *rad21* or *mad2* we were able to generate high levels of instability making many cells inviable.

Depletion of the toll pathway rescues lethality and apoptosis caused by chromosomal instability

Having generated models in which high levels of CIN caused cell death, we were in a position to identify mechanisms that might be mutated in CIN cells (such as tumours) to improve their tolerance of this detrimental phenotype. Ubiquitous knockdown of *mad2* in *Drosophila* resulted in no adult survivors at 30°C, so we tested candidate gene knockdowns to identify any that could rescue this CIN lethality. While testing candidates involved in a variety of cellular processes, we found that knockdown of five *Drosophila* innate immune response genes from the Toll pathway could rescue the lethality in CIN flies (*Toll*, *dorsal*, *spatzle*, *cactus*, and *pelle*). These genes are part of a conserved signalling pathway that regulates patterning during early development and subsequently is used to mediate innate immune responses [22]. We next carried out cell death assays to examine whether the increased viability observed was due to a reduction in cell death when the Toll pathway was depleted in CIN cells. Knockdown of *Toll* or the NFκB homolog *dorsal* in CIN cells significantly reduced the rate of cell death as detected by Acridine Orange incorporation and anti-cleaved-caspase3 staining for apoptosis (Figure 2a-2c and Figure S2). We confirmed that the level of knockdown of *Mad2* was not decreased when we also knocked down *Toll* (Figure S1), excluding the possibility of Gal4 titration. Furthermore, we found that simulating Toll pathway activation by NFκB/*dorsal* overexpression greatly increased the level of apoptosis in CIN cells (Figure 2f) but had a limited effect on normal cells (Figure 2e). These results suggested that local activation of Toll pathway in CIN tissue is needed for the appropriate cell death response to high levels of CIN.

CIN leads to an immune response

The Toll transmembrane receptor has been implicated in the induction of innate immune responses

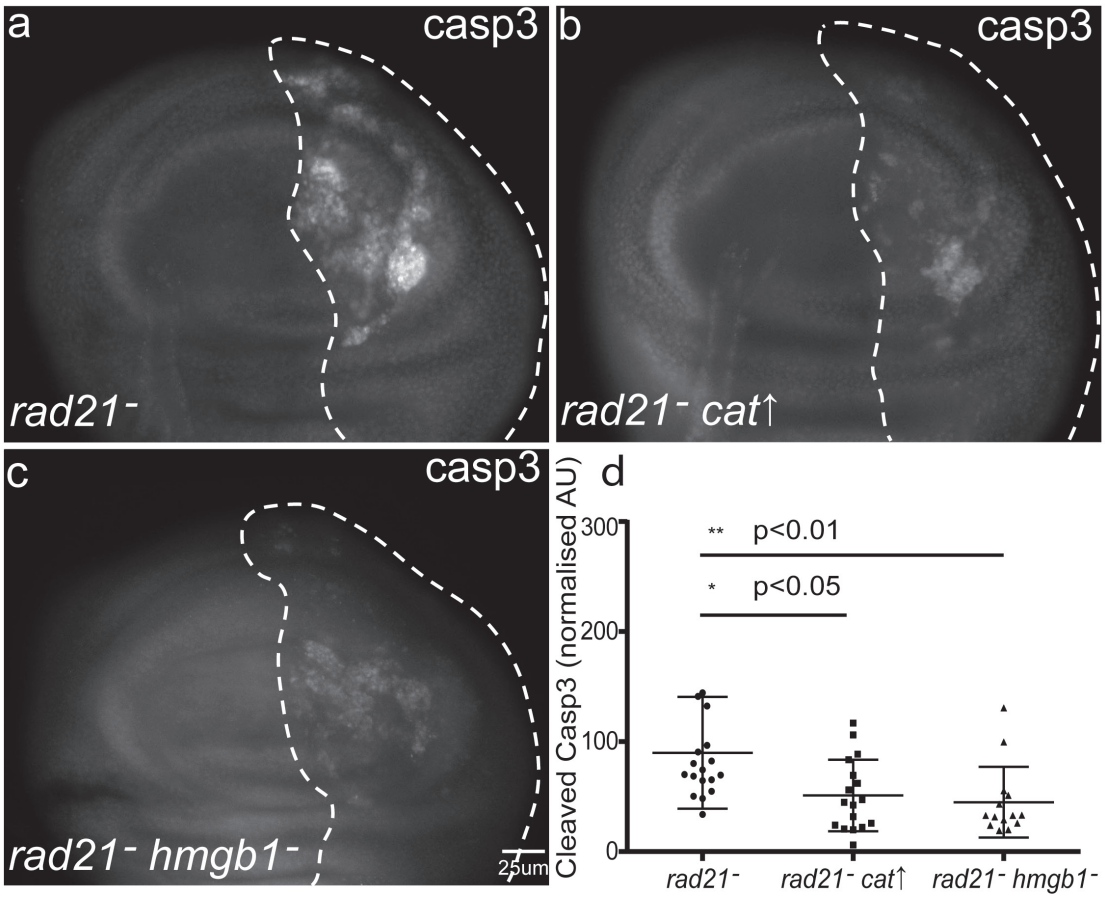

Figure 3: The role of reactive oxygen species (ROS) in determining the fate of CIN cells. Anti-cleaved caspase3 antibody was used to detect apoptosis in third instar larval wing discs with CIN induced in the posterior (dotted) region (*engrailed>Gal4*, *UAS-rad21*^{RNAi} *UAS-Dicer2*) (**a**). Overexpression of Catalase to reduce oxidative stress (**b**) or knocking down the redox-sensitive damage marker HMGB1 (**c**) significantly reduced the level of apoptosis in CIN cells. (**d**) Quantification of the cleaved caspase3 staining. In all cases n≥10 and the error bars show 95% confidence intervals. The *p* values were calculated using two-tailed t-tests with Welch's correction.

[23], so we hypothesized that the immune system could be activated in response to CIN. *Drosomycin* is an antimicrobial peptide gene that is a direct transcriptional target of Toll/NFκB signalling [24], so we used a Drosomycin-GFP reporter to detect its expression in the primary immune secretory tissue (fat body) of CIN larvae (Figure 2g, 2h). We observed a strong up-regulation of Drosomycin-GFP signal in 0 out of 12 control larvae and 11 out of 12 larvae with induced CIN. Together, these results suggest that induction of CIN leads to activation of the larval innate immune response. We also performed immunostaining against Dorsal and Relish, NFκB mediators of the *Drosophila* innate immune system downstream of Toll [25]. We found elevated levels of Dorsal in the cytoplasm of CIN wing disc cells (Figure S3). We observed a barely detectable increase in Relish (downstream of IMD) in CIN cells, even when using p35 to block apoptosis [26] and retain highly aneuploid cells (Figure S3).

The immune system responds to reactive oxygen species

Having found that the induction of CIN triggers an immune response we wished to understand what aspect of CIN cell biology is detected by the immune system. Reactive Oxygen Species (ROS) are known to activate both sterile and infectious inflammatory responses [27]. We have previously shown that CIN cells generate elevated levels of ROS [16], so we hypothesized that ROS might be a trigger. We found that over-expression of Catalase, which decreases ROS levels by converting H_2O_2 into H_2O, significantly rescues the apoptosis observed in CIN cells (Figure 3b and Figure S4a-b). Knocking down the *Drosophila* ortholog of HMGB1 (Dsp1), a ROS-responsive effector of immune activation in vertebrates

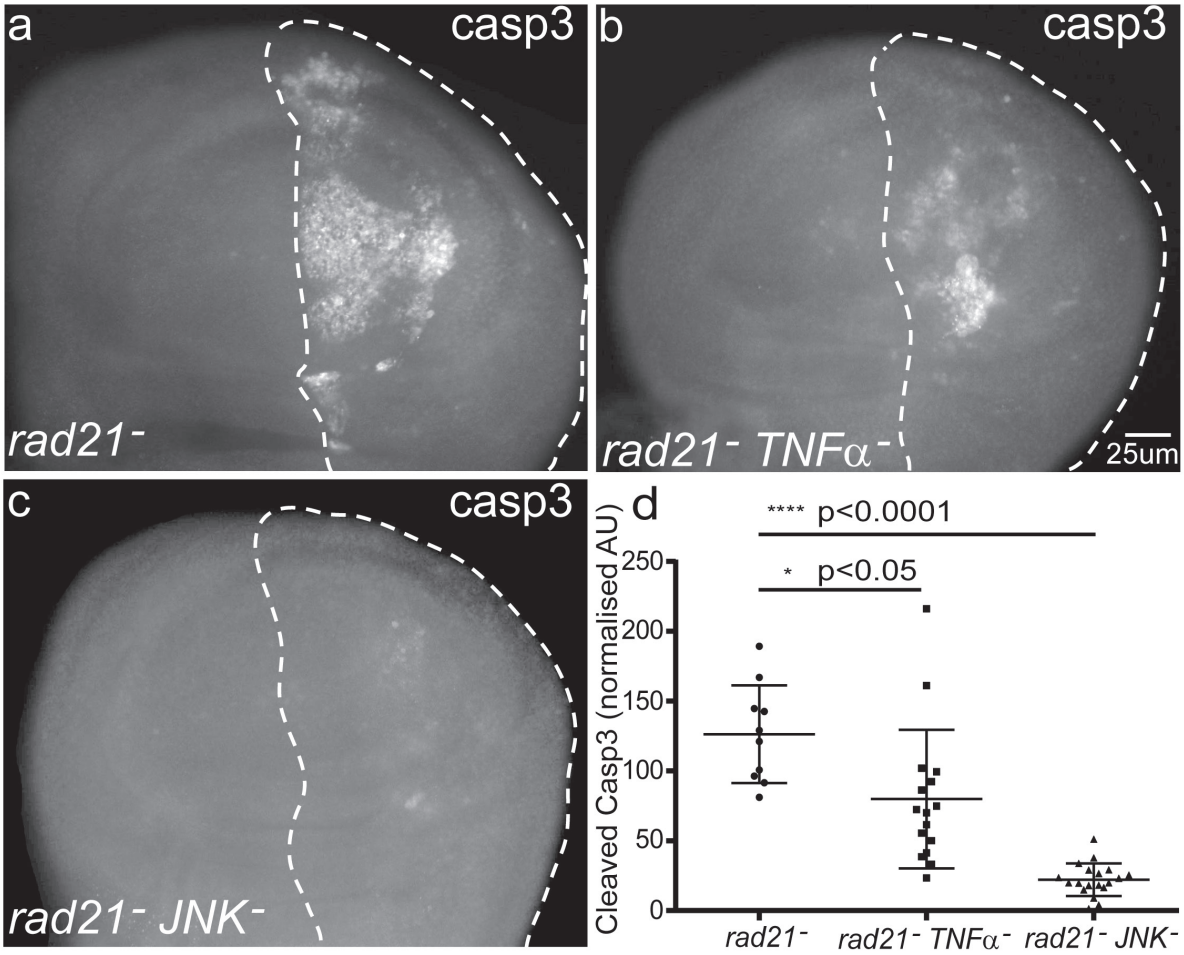

Figure 4: The effect of blocking TNFα signalling by depletion of Eiger or JNK, on the fate of CIN cells. (**a-d**) Anti-cleaved caspase3 antibody was used to detect apoptosis in third instar larval wing discs with CIN induced in the posterior (dotted) region (*engrailed>Gal4*, *UAS-rad21*^{RNAi} *UAS-Dicer2*). Knocking down either TNFα (UAS-*eiger*^{RNAi}) (**b**) or JNK (UAS-*bsk*^{RNAi}) (**c**) significantly reduced the rate of apoptosis in CIN cells. In these experiments TNFα production by immune cells such as circulating hemocytes was not altered; the knockdown was restricted to *engrailed*-expressing tissues such as the imaginal discs. Panel (**d**) shows quantification of the cleaved caspase3 staining. In all cases n≥10 and the error bars show 95% confidence intervals. The *p* values were calculated using two-tailed t-tests with Welch's correction.

[27, 28] also rescued the apoptosis phenotype in CIN cells (Figure 3c). These results suggest a model in which the ROS generated by CIN cells is responsible for triggering an immune response. While there are likely to be many substrates affected by a ROS signal, the response may be mediated by the release of redox sensitive substrates like HMGB1 that are known ligands for the Toll pathway in vertebrates; the inflammatory response driven by Toll activation then significantly contributes to CIN cell death.

Cell death caused by CIN is TNFα and JNK dependent

Toll signaling in *Drosophila* is known to generate a humoral response through antimicrobial peptides and to activate hemocytes that contribute to tumour clearance by TNF signalling [14, 29]. We tested whether Eiger, the *Drosophila* homolog of TNFα was also involved in mediating the apoptosis of CIN cells. Knockdown of TNFα/*eiger* by dsRNA in wing discs significantly reduced the apoptosis in CIN cells (Figure 4b). TNFα has been shown to cause cell death via the JNK pathway [30], so we tested the role of JNK in mediating the response to CIN. Knockdown of JNK strongly rescued the apoptosis of these CIN cells (Figure 4c). Looking downstream of JNK, we found that the JNK effector Mmp1 [31] was elevated in CIN cells (Figure 5b) but was lost if Toll signalling was reduced (Figure 5c). Overexpression of either TNFα or the Toll effector NFκB/Dorsal was sufficient to give elevated Mmp1 levels in normal wing discs (Figure 5e and Figure 5f), consistent with JNK and Mmp1 activation being downstream of Toll signaling. Our results show that Toll/NFκB signalling is needed in the CIN tissue itself for the TNFα-JNK mediated cell death usually seen when CIN is induced by *rad21* knockdown.

A local immune response is critical for hemocyte recruitment

One effect of activating the *Drosophila* innate immune response is the production and recruitment of hemocytes to sites of damage [32, 33]. Dysplastic or pre-tumorous tissue in flies can trigger this response, leading to increased numbers of hemocytes and recruitment of hemocytes to the surface of the abnormal tissue [14, 34]. We found that induction of CIN in otherwise normal, non-dysplastic tissue also increased the number of hemocytes recruited to the wing discs (Figure 6a-6b, 6d). Simulating local immune activation by NFκB overexpression was often sufficient to trigger the JNK-Mmp1 pathway and to recruit hemocytes (Figure 6e-6g). On the other hand, blocking local immune activation by Toll knockdown

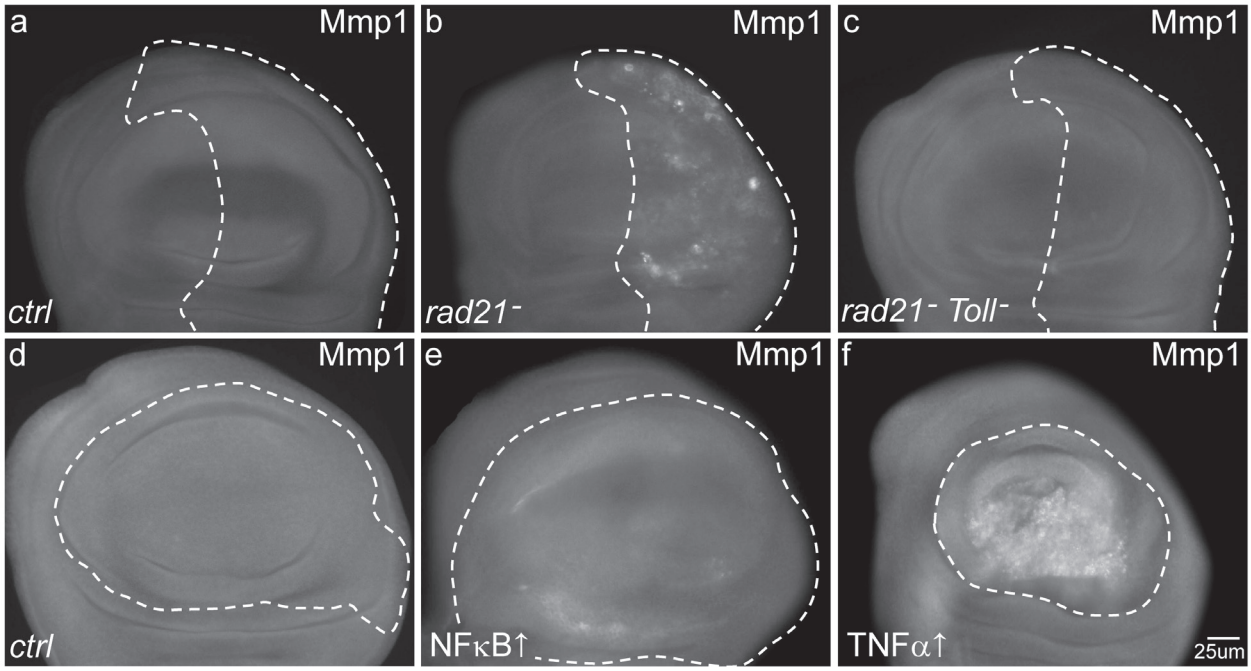

Figure 5: The effects of CIN and local immune signalling on the activation of matrix metalloprotease 1. Anti-Mmp1 antibodies were used to detect the levels of matrix metalloprotease 1 in third instar larval wing discs in which genes were knocked down (dotted regions) using *engrailed* (**a-c**) or *MS1096* (**d-f**) drivers. Normal wing discs (**a**, **d**) show low levels of Mmp1 staining. (**b**) When CIN was induced in the posterior region (dotted) of the disc (*engrailed>Gal4*, *UAS-rad21*^{RNAi} *UAS-Dicer2*), cells showed increased Mmp1 staining. Local signalling via Toll was needed for this effect, as Toll knockdown in these cells (**c**) reduced the level of Mmp1 staining in CIN cells. Overexpression of NFκB (*dorsal*) in the wing pouch (**e**, dotted region) led to a slightly increased level of Mmp1 staining. Overexpression of TNFα (*eiger*) in the same region (**f**) gave very high levels of Mmp1. *MS1096>Gal4* was used in these overexpression experiments to avoid lethality.

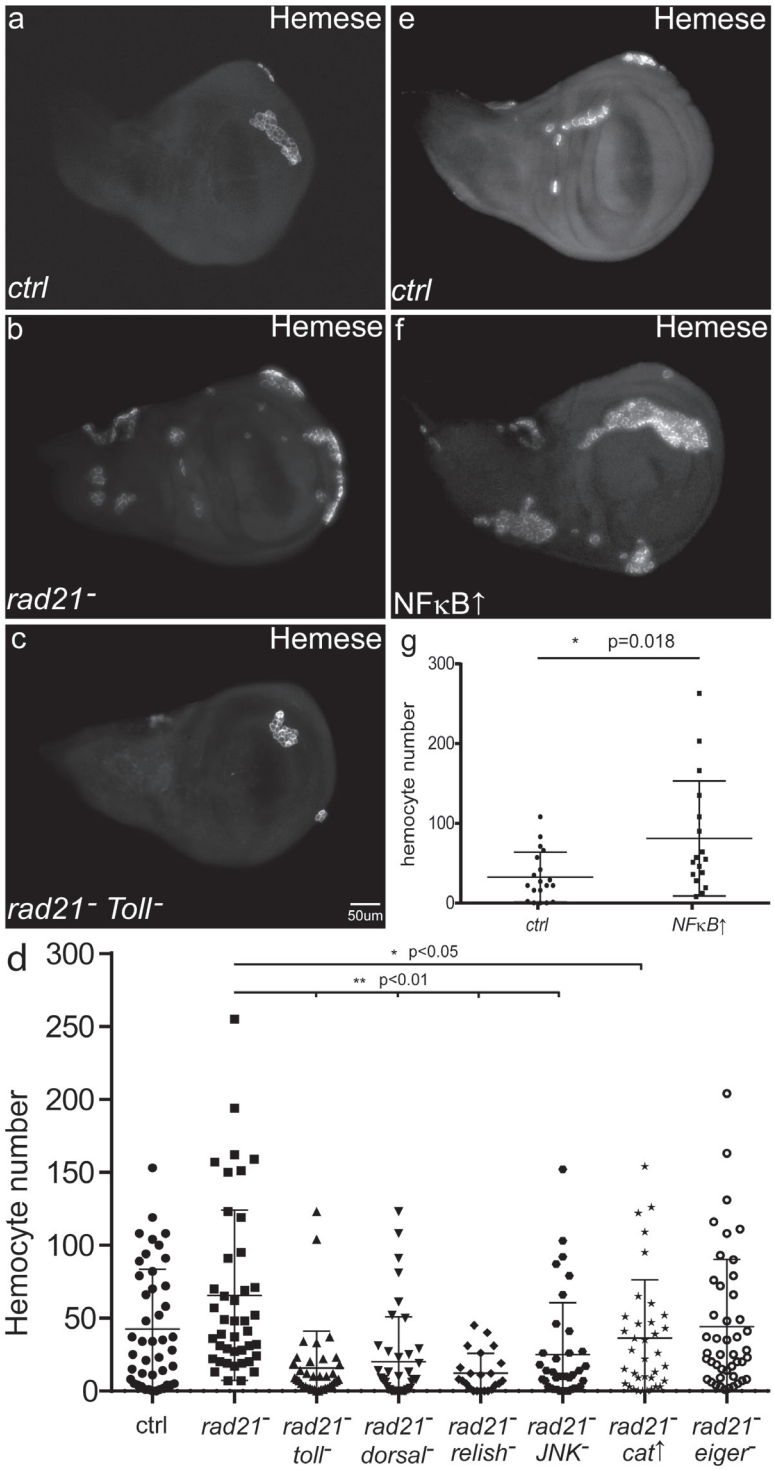

Figure 6: The effects of CIN and immune signalling on the recruitment of hemocytes. Anti-Hemese staining was used to visualize the recruitment of macrophage-like hemocytes to the surface of third instar larval wing discs. When CIN was induced in the wing discs (*engrailed>Gal4*, *UAS-rad21*^{RNAi} *UAS-Dicer2*), significantly more hemocytes were recruited (**b**, **d**, $p < 0.05$) than to wild type wing discs (**a**, **d**). Blocking immune signalling in the CIN cells by Toll knockdown (**c**, **d**) greatly reduced the number of hemocytes recruited. (**d**, **g**) The quantifications show the number of hemocytes recruited to the wing discs, n≥20 in all cases, the error bars show 95% confidence intervals around the mean. (d) Knockdown of Toll, NFκB homologs (*dorsal* or *relish*), or JNK (*bsk*) strongly reduced the number of hemocytes recruited to CIN wing discs ($p < 0.01$for each). Overexpression of Catalase to reduce the level of oxidative stress generated by CIN cells also significantly reduced the number of hemocytes recruited to CIN wing discs ($p < 0.05$). Knockdown of *eiger* (TNFα) did not have a strong effect on the number of hemocytes recruited to CIN wing discs ($p = 0.06$). Simulation of local immune signalling by overexpression of NFκB (*MS1096>Gal4*, UAS-*dorsal*) in wing discs (**f**, **g**) was sufficient to significantly increase the number of hemocytes recruited compared to control discs (**e**, **g**). All p values were calculated by two-tailed t-tests with Welch's correction.

reduced the level of Mmp1 activation within CIN cells and lowered the number of hemocytes recruited to the wing discs (Figure 5c and 6c-6d). The same loss of hemocyte recruitment was produced by blocking JNK or either NFkB homolog, or by decreasing ROS levels (Figure 6d). These results suggest that a ROS-triggered local immune response in the wing disc is critical for hemocyte recruitment and the effective killing of CIN cells (Figure 7).

DISCUSSION

As a feature of most human solid tumours, chromosomal instability (CIN) has been associated with the initiation of tumorigenesis [4], the development of drug resistance, and the poor prognosis of cancer patients after chemotherapy [35-38]. However, the induction of CIN in proliferating cells is usually detrimental or lethal, and the mechanisms by which cancer cells can tolerate CIN are poorly understood [39]. In order to investigate the signalling pathways that allow CIN tolerance, we carried out viability screening in *Drosophila* to select genes whose depletion could rescue lethality caused by CIN. Interestingly, five of the candidate genes obtained were from the *Drosophila* immune system. Further experiments showed that the depletion of several key genes in *Drosophila* immune pathways, such as *Toll*, *dorsal* and *relish*, could rescue the apoptosis phenotype caused by CIN in a proliferating tissue (Figure 2). These results strongly suggest that the innate immune system is normally induced to kill CIN cells.

Immune systems have long been thought to be involved in tumorigenesis [7, 40]. Chronic inflammation is thought to contribute to the initiation, promotion and progression of tumours [40]. However, the innate and adaptive immune systems may be able to eliminate transformed cells, so evasion of this immunosurveillance has been recognised as a hallmark of cancer [12, 41]. In this study, we showed that the induction of CIN in *Drosophila* larvae could trigger the production of antimicrobial peptides from the fat body, the main organ that drives humoral and cellular responses to damage and infection [24, 42]. This result is consistent with recent studies showing that induced tissue overgrowth in flies activates a systemic immune response [14, 42]. They also saw activation of the Toll pathway in the fat body, however they did not test the role of local immune signalling within the induced tumour.

In this study, we found that induction of CIN not only activated the immune organs, but also triggered an immune response within the proliferating CIN tissue (Figure S3). The Toll and Imd pathways are activated in CIN wing discs and depletion of either Toll or Dorsal just in the wing cells reduced the amount of apoptosis in response to CIN (Figure 2). Our data suggests a model in which the local immune response activates JNK and Mmp1 to recruit hemocytes which in turn trigger apoptosis in those cells (Figure 5 and 6). Consistent with this model, we have seen that enhancing the local immune response by *dorsal* overexpression greatly increased the level of apoptosis in CIN cells (Figure 2). On the other hand,

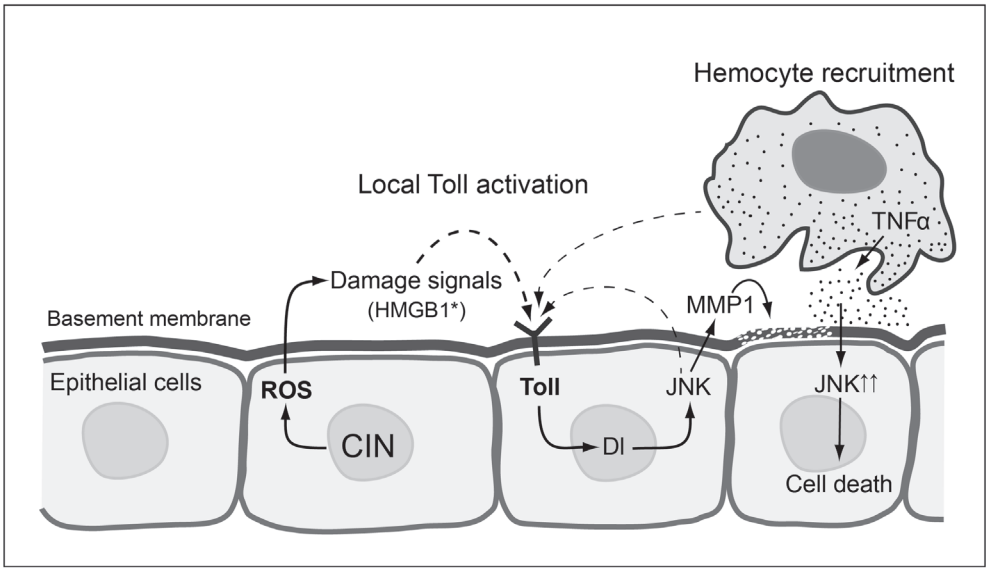

Figure 7: Proposed model for local Toll pathway activation giving apoptosis in response to CIN. CIN cells generate ROS and DAMPs like oxidised HMGB1, which trigger the local immune response in nearby cells through the Toll/NFκB pathway which in turn activates JNK and Mmp1. The activation of Mmp1 leads to basement membrane digestion and hemocyte recruitment. The recruited hemocytes subsequently trigger the apoptosis of underlying cells through secretion of TNFα and further activation of JNK. Note that the signalling processes are drawn in adjacent cells for clarity, but may all occur in the same cell. Dashed lines indicate the production of activated Toll ligand by a process that is not well characterized. This model is informed by data from [34] and [14] on hemocyte responses and [65] on JNK activation of Toll.

blocking the local immune response and its downstream effectors by knocking down Toll, Dorsal, Relish or JNK could significantly reduce the number of hemocytes recruited (Figure 6d). Hemocytes have been shown to secrete TNFα onto the underlying tissue as well as being a source of the Toll ligand Spz [14], so hemocyte recruitment appears to be a positive feedback loop by which damaged cells attract a source of signals to ensure their own demise (Figure 7).

The activation of local immune signaling in proliferating tissue has been shown to remove slow growing cells [43]. In that case the trigger(s) and targeting are not known, and the Toll receptor itself and JNK had little effect. Thus, although cell competition presents an interesting parallel, it appears to induce a response different to that seen in CIN cells. Local as well as systemic immune responses are also seen in response to infection by bacterial pathogens [44], suggesting that the systemic response alone is either insufficient or requires targeting. For example, bacterial pathogen elimination in infection requires local IMD/Relish pathway activation [45-47]. At least in the gut, it appears that tissue damage and ROS production are used as an additional trigger to improve immune responses to pathogens [44]. In the case of CIN, we think that ROS are made in the mitochondria [16] rather than at the plasma membrane by Duox [48], but the immune effects may be similar.

Toll and Toll-like receptors (TLRs) are the critical mediators of innate immune responses in *Drosophila* and mammals [23, 49]. Apart from external pathogens, many endogenous molecules released from damaged cells, referred to as DAMPs (damage-associated molecular patterns), can also activate Toll-like receptors [6]. Toll has recently been implicated in the response to tissue dysplasia and damage [14, 50], suggesting a similarity to vertebrate DAMP receptor TLRs. However it should be noted that TLRs frequently detect the DAMP directly, while activation of the Toll ligand Spz is likely to require several intermediate steps [51]. Our results showed that CIN cells exhibit dysfunctional mitochondria and oxidative stress, both of which are known to activate innate immune responses through TLRs [27, 52]. Reducing oxidative stress by over-expressing the antioxidant Catalase significantly rescued the apoptosis phenotype of CIN cells. Consistent with this model in which ROS triggers an immune response, Catalase overexpression also significantly reduced the number of hemocytes recruited (Figure 6d). In addition, removing dysfunctional mitochondria by over-expressing the mitophagy gene *park1*, which mediates the clearance of abnormal mitochondria [53], also rescues the apoptosis phenotype of CIN cells (our unpublished data). It is not known how CIN generates stressed mitochondria, but current models for stable aneuploidy suggest that altered stoichiometry of proteins can lead to saturation of the protein folding and degradation machinery, leading to ER stress and subsequent mitochondrial stress [54]. We are confident that ROS are an upstream trigger rather than a downstream consequence of apoptosis, because decreasing ROS levels reduced apoptosis in CIN tissue (Figure 3b) and we were able to almost completely block apoptosis without reducing ROS levels (Figure S4).

In order to identify potential mediators of the ROS signal, we tested HMGB1 (high mobility group box 1), which is one of the most intensively studied DAMP molecules. HMGB1 is redox state sensitive and can be released from oxidatively damaged cells, triggering immune responses by binding to Toll-like receptors [27]. We found that HMGB1 depletion could rescue the apoptosis phenotype of CIN cells, suggesting a model in which ROS triggers a local immune response by releasing oxidised HMGB1, leading eventually to CIN cell apoptosis. Consistent with this model, research in mammals has shown that HMGB1 released from dying cells triggers a TLR4 dependent immune response that affects the outcome of traditional cancer therapy [55].

We expected that apoptosis in response to CIN would be mediated by the TNFα/Eiger-JNK pathway, which has been well documented to trigger apoptosis in flies in response to a number of stimuli [30, 56, 57]. Eiger is the Drosophila ortholog of Tumour Necrosis Factor alpha (TNFα) which acts as tumour suppressor and typically drives apoptosis by activation of the intrinsic death pathway though JNK [30, 56]. JNK signalling has been shown to be dysregulated in several fly "pre-tumour" models with varying effects including apoptosis, migration, proliferation and DNA repair. [14, 17, 31, 57, 58]. In CIN cells, we detected increased JNK signalling (Figure 5), and depletion of either *eiger* or *JNK* by RNAi could significantly rescue the apoptosis phenotype (Figure 4). These results are consistent with the role of the TNFα-JNK pathway as a tumour suppressor signal to eliminate CIN cells by triggering apoptosis. JNK activation would also be expected to promote hemocyte proliferation to increase the local TNFα signal, as observed in response to tissue overgrowth [14, 34]. JNK signalling through Mmp1 can also lead to invasive cell migration [31, 59] typically when apoptosis has been blocked by strong growth factor signalling. Despite the activation of Mmp1 in CIN cells, we have not observed any invasion or metastasis. We speculate that this could be due to apoptotic clearance and the local immune response restraining the invasiveness of CIN cells.

Based on our results, we have hypothesised that CIN cells produce dysfunctional mitochondria and oxidative stress; the generation of ROS and DAMPs such as HMGB1 then triggers a local immune response. This involves signalling through Toll to give JNK activation, which is known to generate signals that attract [34] and expand [14] the hemocyte population. The recruited hemocytes then promote the death of CIN cells through TNFα-JNK signalling as well as secreting Spz to increase

Toll signaling (Figure 7). We also saw some reduction in cell death when TNFα was depleted just in the CIN cells, so we speculate that ROS can generate some local production of TNFα, as has been reported for eye discs [60]. However this more immediate route to cell death (ROS-TNFα-JNK) does not appear to be very effective in CIN cells, because without Toll and the involvement of an immune response to amplify the JNK signal, we saw very little CIN cell death (Figure 2).

Our results raise the question of whether such an anti-CIN immune response has clinical implications. Investigation of the innate immune system as a cancer treatment has been going on for more than a century. In the 1890s, Coley injected live bacterial cultures into cancer patients as a treatment to provoke the immune system with some success in treating certain cancer types such as soft tissue sarcoma and lymphoma [61]. Since the 1950s, bacteria-derived materials like polysaccharide have been investigated for cancer immunotherapy. Although the detailed mechanism of their anti-cancer effect is unclear, some of them have been approved for clinical use [6]. DNA damage or DNA repair mutations are known to provoke an innate immune response [41, 62], a response that is likely to also be seen in CIN cells, as they generate ongoing DNA stress [2, 63]. Our results have suggested that CIN cells are aberrant in a number of significant ways including glucose metabolism, mitochondrial output, ROS levels, JNK signalling, and DNA damage [15-17], and that some or all of these contribute to a signal that generates the local and systemic immune responses needed to eliminate the damaged cells. It remains to be seen to what extent this response can be exploited therapeutically.

MATERIALS AND METHODS

Drosophila stocks

The fly stocks used in this paper are as follows: *mad2*-RNAi (VDRC 47918), *Rad21*-RNAi (Bloomington #36786), *Eiger* (TNFα)-RNAi (VDRC108814), UAS-*catalase* (Bloomington #24621), *HMGB1*-RNAi (Bloomington #31960), *Drosomycin*-GFP [24], UAS-*p35* (Bloomington #5073).

Driver stocks: *daughterless* (*da*)-Gal4 for ubiquitous expression, *engrailed* (*en*)-Gal4 for gene expression in the posterior region of wing discs and *MS1096*-Gal4 for wing pouch expression, all from Bloomington *Drosophila* stock centre.

Viability screening

Candidate genes were knocked down in the CIN background (*mad2* knockdown) to see their effect on the viability of CIN flies: UAS>*mad2* RNAi/CyO; *da*>Gal4/TM6 *tubulin*>Gal80ts × UAS>candidate-RNAi. The crosses were set at 30°C which was lethal for CIN flies crossed to negative controls.

RNA purification and quantitative real-time PCR (qPCR) assays

Five third instar larvae from each genotype (in triplicate for each genotype) were chosen and washed in PBS and were quickly transferred and homogenised in cold Trizol reagent on ice and then stored at -80 °C before processing as described [15]. Primers pairs used in this paper:

mad2 F/R:GGCGACCAAAAACTGCATCA/GGTAAATTCCGCGTTGGAAGA

rp49 F/R:ATCGATATGCTAAGCTGTCGCAC/TGTCGATACCCTTGGGCTTG

Karyotype analysis

For measuring the level of aneuploidy, wing discs from third instar larvae were dissected out in PBS, and were incubated for 10 min in 0.5% sodium citrate solution. Then these discs were treated with 45% acetic acid for 2 min and 60% acetic acid for 1 min on a cover slip. Treated wing discs were squashed quickly between a coverslip and a slide and placed into liquid nitrogen. The cover slip was removed and the squashed discs were stained with Hoechst 33342 for 10 min and washed with PBST for 20 min before mounted in 80% glycerol. The karyotypes of different genotypes were compared using χ^2 analysis to detect significant variation from the expected proportions of euploid and aneuploid cells.

Cell death

Acridine Orange (AO) staining was used to measure the cell death in larval wing imaginal discs [15]. Third instar larvae were dissected in PBS for imaginal discs; the collected imaginal discs were incubated in 1mM AO for 2 mins then transferred to a slide after a brief wash. Then the treated imaginal discs were immediately mounted in PBS with a cover slip on before microscopy. The results of AO were normalized by subtracting the wild type region value from the test region value (eg. *engrailed*-driven region) as identified by UAS>*CD8-GFP* expression. The background noise of all images was subtracted in ImageJ using a rolling ball radius of 10 pixels.

Oxidative stress assay

The level of reactive oxygen species (ROS) in CIN cells was measured by using the fluorogenic probe CellROX from Life Technologies. The third instar larvae

were dissected in D22 media pH 6.8. Then the dissected imaginal wing discs were transferred into 5μM CellRox (in D22 media) for 15 mins; after this, the wing discs were quickly washed in PBS and fixed in 3.7% formaldehyde for 5 min then mounted in 80% glycerol for imaging.

Mitochondrial stress

The level of mitochondrial stress in CIN cells was measured by using the fluorogenic probe TMRE from Life Technologies. Third instar larvae were dissected in PBS and transferred into 0.05 μM TMRE solution for 10 mins incubation and then washed in PBS for 10 mins. Then the treated imaginal discs were immediately mounted in PBS for imaging.

Immunostaining

Immunostaining was used on dissected wing imaginal discs for different purposes. Third instar larvae were dissected in PBS for imaginal discs; the collected imaginal discs were fixed in 3.7% formaldehyde for 20 mins and then wash for 30 mins in 0.2% PBST (1xPBS+0.2% Tween). For anti-hemese staining, the fixation time was 4°C overnight, with no shaking through all the process. The fixed imaginal wing discs were then blocked in PBSTF (1xPBS+0.2% Tween+5% fetal calf serum) for 30 mins and stained with the primary antibody for 2.5 hrs (at room temperature) or overnight (at 4°C). After staining with the primary antibody, the wing discs were washed in PBSTF for 30 mins then transferred to a secondary antibody solution for 2.5 hrs at room temperature in the dark. After 30 mins washing in PBST, the wing discs were mounted in 80% glycerol-PBS.

The source and concentration of antibodies used in this paper are as follows: Rabbit anti-caspase3 (D175, 1: 100) from Cell Signalling; mouse anti-dorsal (7A4, 22 μg/ml) and mouse anti-MMP1 (14A3D2, 5.3μg/ml) from the Developmental Studies Hybridoma Bank; mouse anti-hemese (1.5μg/ml) [64].

The secondary antibodies used were CY3 anti-rabbit (1: 100), rhodamine anti-mouse (1: 200).

Data analysis

All microscopy was done on a Zeiss Axioplan2 microscope. Axiovision software (Carl Zeiss), Adobe Photoshop, Adobe Illustrator and ImageJ were used for image processing and quantification. Statistical analysis was carried out in GraphPad Prism using either t-tests or χ^2 tests as indicated.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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Chromosomal instability triggers cell death *via* local signalling through the innate immune receptor Toll

Supplementary Material

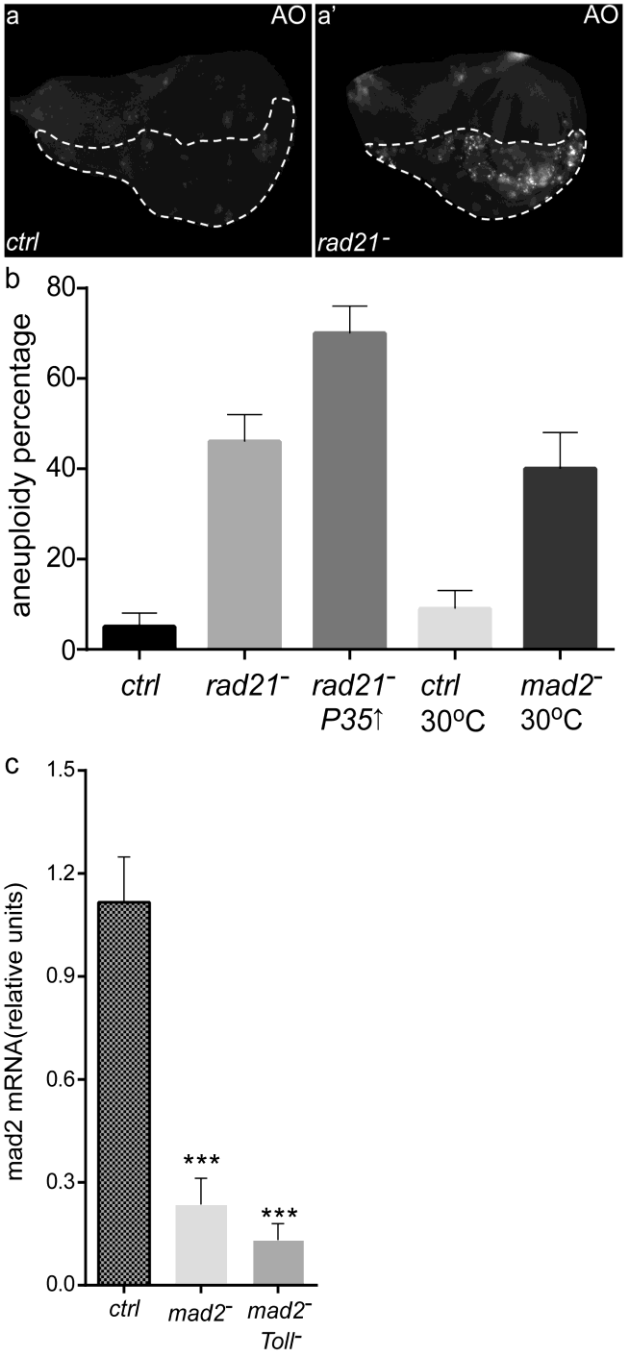

Fig S1 The effect of Chromosomal Instability (CIN) induced by Rad21 depletion on cell death in larval wing discs. (a, a') Acridine Orange staining of third instar larval wing discs. CIN induced in the posterior (dotted) region of wing discs showed increased cell death (a') compared to the negative control (a). (b) The level of aneuploidy in different CIN models. Metaphase karyotypes were used to evaluate aneuploidy as a proxy for the rate of CIN. *Rad21* knockdown in wing discs gave aneuploidy in 46% of metaphase cells while the level of aneuploidy increased to 70% if clearance of CIN cells by apoptosis was blocked by P35 over-expression. *Mad2* knockdown at 30℃ gave 40% metaphase aneuploidy. (c) The level of mad2 mRNA. qPCR shows that the ubiquitous expression of mad2 RNAi construct leads to mad2 mRNA reduction by 80%, and this knockdown effects is not affected by simultaneously toll knockdown indicating that more RNAi constructs do not affect RNAi machinery efficiency in UAS-Gal4 system.

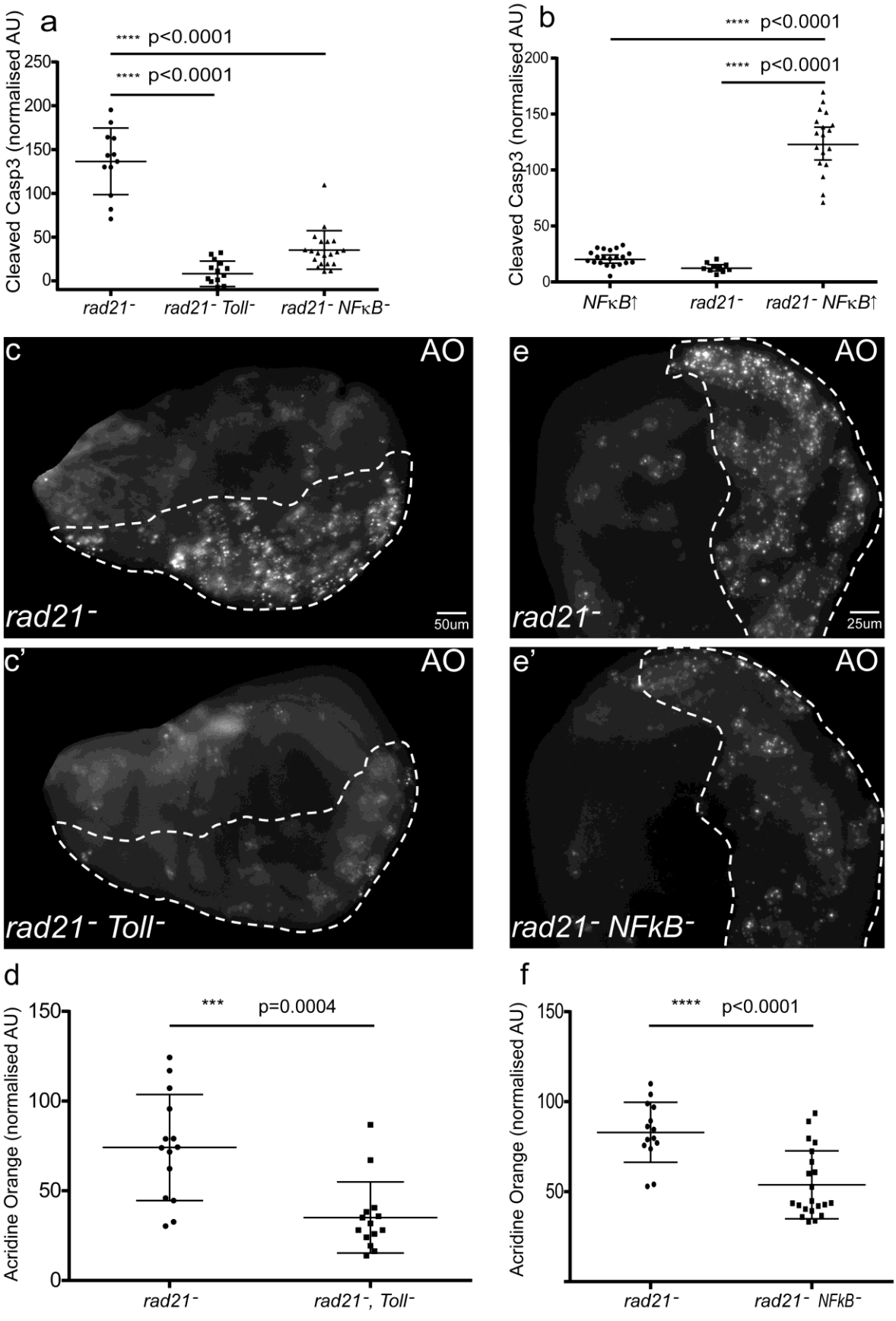
a
Cleaved Casp3 (normalised AU)
**** p<0.0001
**** p<0.0001
250
200
150
100
50
0
rad21-
rad21- Toll-
rad21- NFκB-
b
Cleaved Casp3 (normalised AU)
**** p<0.0001
**** p<0.0001
200
150
100
50
0
NFκB↑
rad21-
rad21- NFκB↑
c
AO
rad21-
50um
e
AO
rad21-
25um
c'
AO
rad21- Toll-
e'
AO
rad21- NFkB-
d
Acridine Orange (normalised AU)
*** p=0.0004
150
100
50
0
rad21-
rad21-, Toll-
f
Acridine Orange (normalised AU)
**** p<0.0001
150
100
50
rad21-
rad21- NFkB-

Fig S2 The effect of Toll pathway knockdown on CIN cell death.

(a and b) Quantification of the cleaved caspase3 staining in (Fig 2 a, b and c) and (d, e and f) respectively. The quantifications show the normalized grey value of staining that was obtained by subtracting the mean grey value of the wild type from that of the affected (dotted) region of each wing disc. n≥10 in all cases, the error bars show 95% confidence intervals. The p values were calculated using two-tailed t-tests with Welch's correction.

(c, c', e and e') Acridine Orange staining of third instar larval wing discs. Knocking down Toll (c') or NFκB (*dorsal*) (e') significantly reduced the level of cell death in CIN cells. (d, f) Quantification of Acridine Orange staining. The quantifications show the normalized grey value of staining obtained by subtracting the mean grey value of the wild type from that of the affected region of each wing disc. n≥10 in all cases, the error bars show 95% confidence intervals around the mean. The p values were calculated by two-tailed t-tests with Welch's correction.

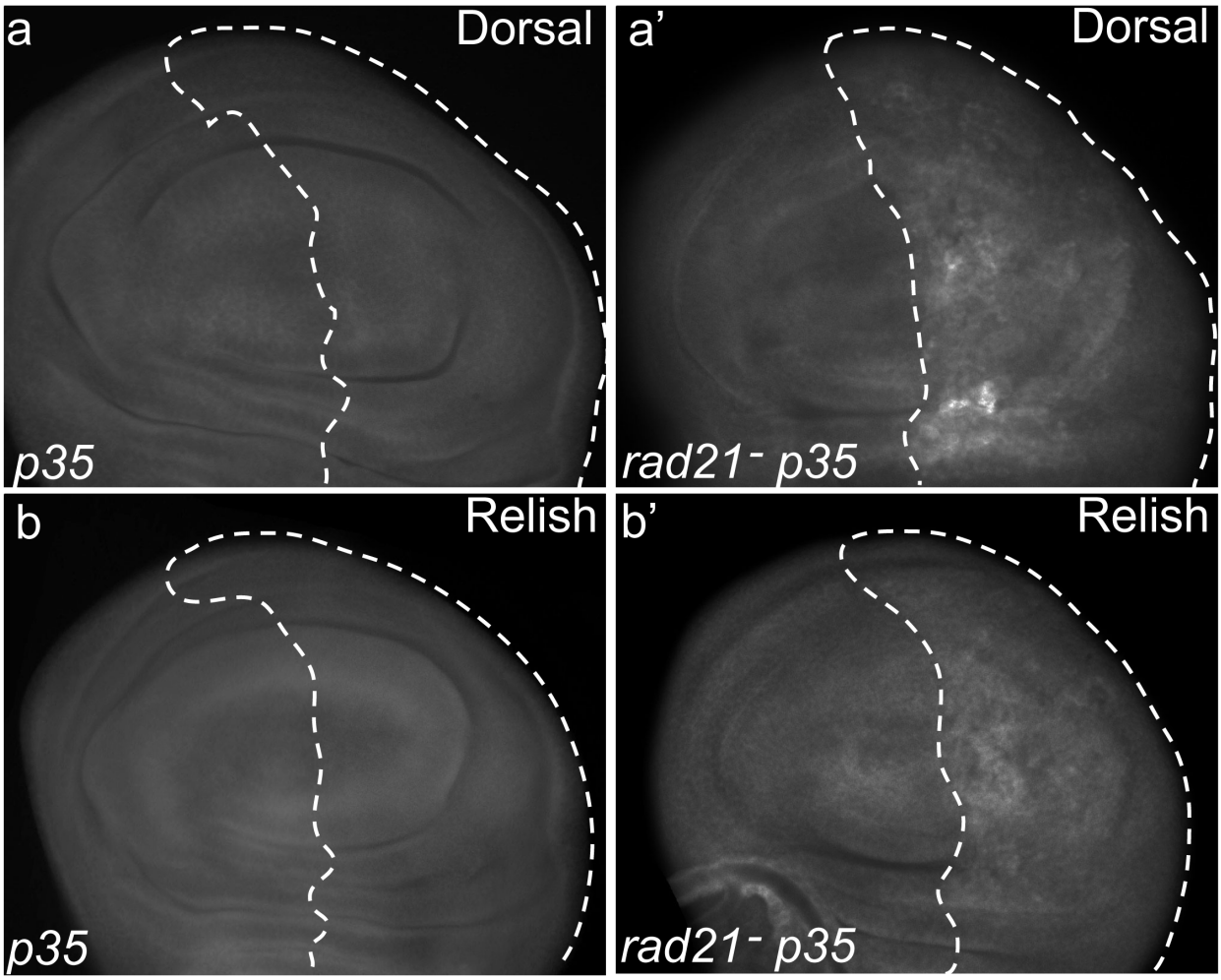

Fig S3 Visualizing activation of immune signaling by CIN.

(a, a', b, b') CIN was induced by depletion of Rad21 in the posterior half of third instar wing discs (*engrailed>Gal4, UAS-rad21*^{RNAi} *UAS-Dicer2 UAS-P35*), indicated by the dotted region. In this case, apoptosis was blocked by the expression of P35 to increase the retention of CIN cells that are otherwise cleared by apoptosis (see Fig 1). Activation of local immune signalling was detected by staining for the two NFkB homologs Dorsal (a, a') and Relish (b, b'). Levels of Dorsal were clearly elevated in CIN cells, with a barely detectable change in Relish. We did not observe any significant change in Dorsal subcellular localization; the Relish epitope detected would be expected to remain in the cytoplasm.

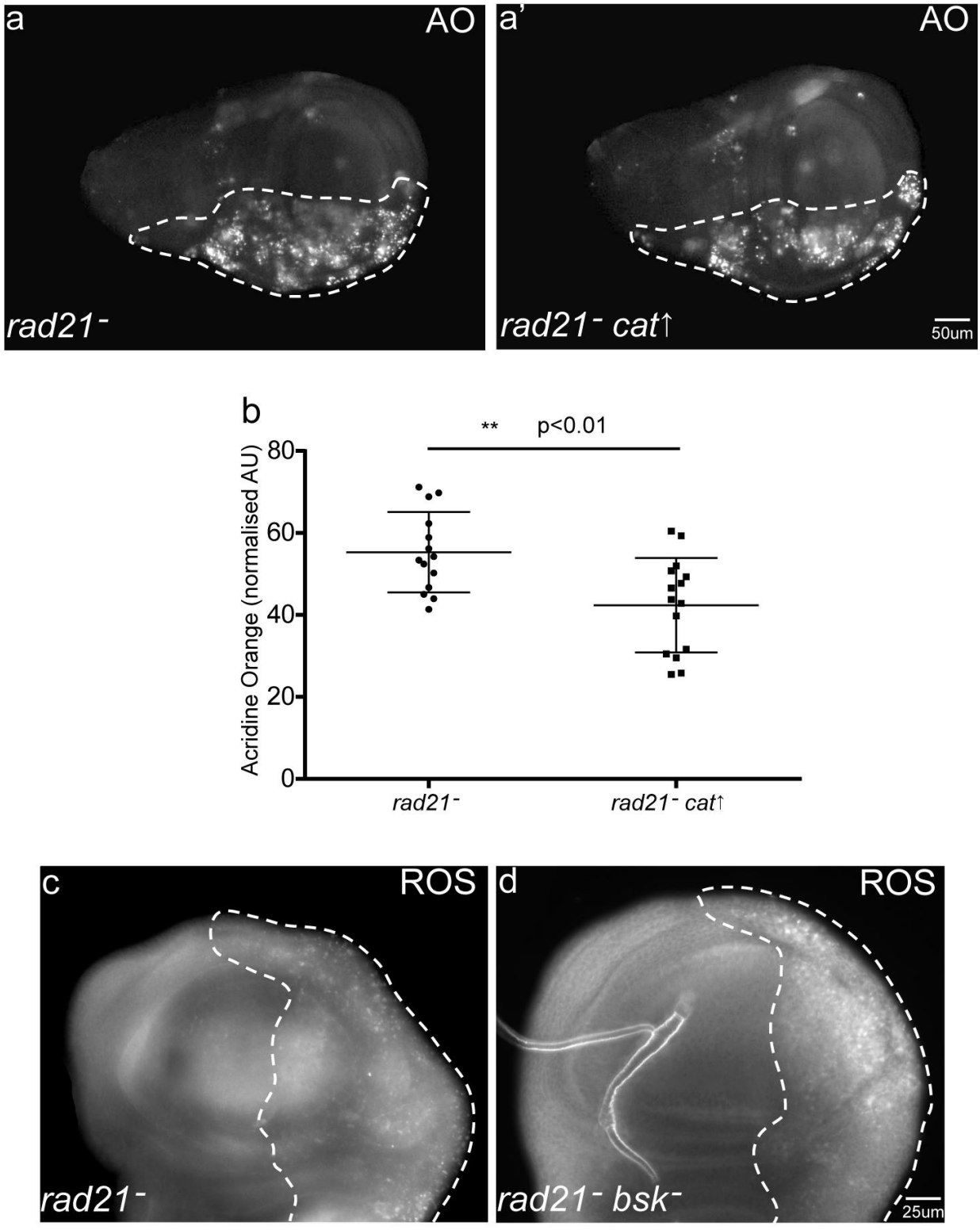

Fig S4 The role of reactive oxygen species (ROS) in determining the fate of CIN cells.

(a, a') Acridine Orange staining of third instar larval wing discs. Overexpression of Catalase to deplete reactive oxygen species (a') significantly reduced the level of cell death seen in CIN cells (a, b). (b) Quantification of the Acridine Orange staining with n>10 for each genotype, error bars showing 95% CIs and the p value calculated by a two-tailed t-test with Welch's correction. (c) Reactive oxygen species, visualized here by CellRox staining, were elevated in CIN cells (dotted region) relative to the adjacent wild type tissue. (d) Blocking signalling by JNK (bsk^{RNAi}) did not reduce the level of reactive oxygen species in CIN cells, though it almost completely eliminated cell death (Fig 4). We concluded that ROS can cause cell death and is not being generated by the process of apoptosis.

Table S1

List of immune candidates showing rescue of *mad2* knockdown CIN induced lethality at 30°C. Columns show the ID of the RNAi construct used; its chromosomal location; the ID of the affected gene; the gene name; the number of surviving adults obtained when the candidate gene was knocked down in a wild type background and the number survivors when the candidate gene was knocked down in the CIN background (*da>mad2*). Obtaining surviving progeny indicated that the candidate gene knockdown rescued the *mad2* depletion lethality phenotype (marked in grey). Candidate lines that are unable to rescue the lethality are shown for comparison.

Stock #	Chr	CG	SYMBOL	Candidate RNAi survivors	Candidate + CIN survivors
BL27650	3	CG6667	dorsal	30	2
BL34699	3	CG6134	Spz	2	2
B34733	3	CG5974	Pll	9	1
B34775	3	CG5848	Cact	0	2
B28538	3	CG6134	Spz	16	1
B35628	3	CG5490	Toll	34	1
B28543	3	CG18241	Toll-4	17	0
B35577	3	CG5974	Pll	21	0
B28519	3	CG6890	Tollo	3	0
B28526	3	CG1149	Mstprox	11	0
B30488	3	CG8595	Toll-7	7	0

Chapter 6

DISCUSSION

Chromosomal instability (CIN) is a common phenomenon in cancer cells in which cells fail to maintain their chromosomal number and/or integrity. CIN is common in most human solid tumours and is required for the acquisition of multiple characteristics essential for the progress of tumorigenesis (Hanahan and Weinberg 2011; Bakhoum and Compton 2012). A moderate level of CIN is believed to play an initiating role in tumorigenesis and the level of CIN seen in tumours positively correlates with poor prognosis and drug resistance (Michor et al. 2005; Carter et al. 2006; Bakhoum and Compton 2012).

CIN is not common in normal cells but in cancer cells and it has been hypothesized that CIN could be targeted for cancer therapy. Therefore, the overall aim of this project is to characterize pathways which respond to CIN or are related to CIN tolerance and can be targeted to specifically kill CIN cells. In our lab, using *Drosophila* as the model organism, we induce CIN of different levels by weakening spindle assemble checkpoint or knocking down *cohesin* genes. For example, *mad2* is an essential spindle checkpoint gene involved in metaphase-anaphase transition and its knock down gave rise to CIN in 25% of brain cells. *Rad21* is involved in chromatid cohesion during mitosis and its depletion led to aneuploidy 46% of metaphase cells (Shaukat et al. 2012). Initially, our lab performed a genome wide screen of kinases and phosphatases in *Drosophila* which aimed to select candidates whose knockdown can specifically kill CIN cells (Shaukat et al. 2012). We found that those candidate genes which specifically kill CIN cells are involved in various pathways including the JNK pathway, the DNA damage response pathway, centrosomal functioning, and metabolism were necessary in maintaining the survival of CIN cells(Shaukat et al. 2012; Wong et al. 2014). Of note, identification of Nek2 as one of the candidates indicates that the screen had the potential to pick up real cancer treatment targets as Nek2 is being targeted clinically for cancer therapy (Henise and Taunton 2011).

Although CIN cells give the advantage of resisting cancer treatment, CIN and aneuploidy are known to cause proteotoxic and oxidative stress (Oromendia et al. 2012). In order to tolerate these high-stress conditions, CIN cells have been shown to increase glucose usage which could increase mitochondrial output. Consistent with this, we found that CIN cells are sensitive to metabolic disruption. For example, depletion of genes such as PEPCK (a key enzyme of gluconeogenesis) which is expected to increase glucose usage imposes further stress on mitochondria and finally causes oxidative stress and apoptosis. Similarly, I found that genes whose depletion could induce CIN specific

apoptosis affect larval lipid storage (Shaukat et al. 2014). This could because deletion of these genes could elevate the glycolytic flux which in turn put a further burden on mitochondria. Thus, the depletion of these genes could commonly increase the level of oxidative stress and cause apoptosis in CIN cells. Moreover, we propose that the depletion of those genes could also affect the production of NADPH and GSH, the main antioxidant in cells (Shaukat et al. 2014). Our hypothesis is supported by the fact that the manipulation of antioxidant capacity could significantly affect the fate of CIN cells suggesting that CIN cells are close to their maximum capacity for buffering oxidative stress, so the depletion of the metabolic genes leads to a further increase of mitochondrial stress, oxidative damage and consequently apoptosis in those CIN cells (Shaukat et al. 2014).

Conventional cancer therapy such as DNA damaging agents has side effects on normal cells which might trigger subsequent cancer (Tucker et al. 1987). Cancer cells normally show high levels of oxidative stress due to their high metabolic rate, therefore, a pro-oxidant approach has been proposed as a new cancer therapy (Martin-Cordero et al. 2012). We have shown that metabolic disruption specifically increases oxidative stress and DNA damage in CIN cells. Therefore, CIN cancer cells should be more sensitive to metabolic disruption and that metabolic intervention could potentially be used to specifically kill CIN cancer cells without damaging normal cells.

Autophagy is known to be activated in response to various stresses including nutrient starvation (Jiang and Mizushima 2014; White 2015). As CIN cells show metabolic stress, we suspected that the autophagy pathway could be activated in CIN cells. As expected, we found that autophagy pathway flux is highly elevated in CIN cells and manipulation of the autophagy pathway could affect the fate of CIN cells (Chapter 3). For example, blocking the autophagy pathway in CIN cells leads 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 3). Furthermore, our study shows that the defective mitochondria in CIN cells are transported into lysosomes for degradation, the process is known as mitophagy. Moreover, we found that increased expression of the essential mitophagy gene *parkin* (Narendra et al. 2008; Jin and Youle 2012) decreases the level of ROS and consequently apoptosis in CIN cells. Taken together, these results suggest that the activated autophagy/mitophagy pathway is responsible for the selective degradation of dysfunctional mitochondria which determine the fate of CIN cells.

These results are consistent with the protective role of autophagy in response to oxidative stress in *Drosophila* (Wu et al. 2009). Autophagy is commonly activated in established cancer cells to cope with the intracellular and environmental stress and thus facilitate cancer proliferation and aggressiveness (Galluzzi et al. 2015; White 2015). For example, the autophagy pathway is often unregulated in Ras-transformed cancer cells and promotes cancer transformation and proliferation through facilitating glycolysis, maintaining oxidative stress levels and mitigating chromosomal instability (Guo et al. 2011; Lock et al. 2011; White 2015). Therefore, the autophagy pathway has been proposed as a promising target for treatment of advanced tumours (Gupta et al. 2010; Nagy et al. 2013).

In order to meet the high energy requirements for rapid growth, the majority of cancers show much higher oxidative stress and mitochondrial defects than normal cells (Gogvadze et al. 2010). We found that mitophagy signalling is critical for removing defective mitochondria. The inhibition of autophagy or mitophagy signalling has minor effects on normal cells probably they have relatively lower metabolic rate than cancer cells. Therefore, our studies suggest that targeting mitophagy could be an effective therapy to specifically kill CIN cancer cells as a pro-oxidant cancer therapy.

In order to investigate pathways which give CIN tolerance, we completed a screen at 30 degrees to select candidate genes whose knockdown could rescue the *Drosophila* lethality caused by CIN. Surprisingly, we found that depletion of five of the *Drosophila* Toll pathway genes including *Toll* and *dorsal* could rescue the lethality and apoptosis caused by CIN. In line with this, we found that the *Drosophila* systemic innate immune pathway is activated in response to induced CIN. This auto-immune effect has been observed in cancer cells which generate antigens that could be recognised by immune system and immune escape have been considered as a critical step during tumorigenesis (Apetoh et al. 2007). Our results are consistent with the recent studies which show that Toll pathway is activated in the fat body in response to tissue overgrowth and damage in *Drosophila* (Carvalho et al. 2014; Hauling et al. 2014; Parisi et al. 2014). However, in their studies, the trigger molecules for localizing the immune response are not clear.

As CIN cells show dysfunctional mitochondria and increased levels of oxidative stress, we think ROS might be a key trigger of the immune response to CIN in *Drosophila*, similar to the ROS-driven immune response to pathogens (Ha et al. 2005; Ha et al. 2009). As expected, enforced expression of the antioxidant enzyme *catalase* or deletion of the ROS downstream effector gene *hmgb1* (Apetoh et al. 2007) could reduce the

number of recruited hemocytes and consequently the level of apoptosis in CIN cells. On the other hand, we are able to block apoptosis without affecting the level of ROS which confirms that ROS generation is upstream of apoptosis. However, as ROS could cause DNA damage which is known to trigger an immune response (Gasser and Raulet 2006), we cannot rule out the possibility that DNA damage might be the trigger of immune activation seen in CIN flies. Furthermore, it is also possible that CIN could generate other antigens that can be recognised by the immune system.

Apart from a systemic immune activation which has been observed responding to various cancer models (Hauling et al. 2014; Parisi et al. 2014), we also observed that the level of Dorsal and Relish staining is increased in CIN cells, suggesting that a local immune pathway is activated. The local and systemic immune responses have been observed to be activated against bacterial infection (Buchon et al. 2014). Although the systemic immune activation is required for responding induced overgrowth in *Drosophila* (Parisi et al. 2014), its function in targeting CIN cells is not clear. As a consequence of the local immune signalling, we found that increased numbers of *Drosophila* blood cells (hemocytes) are recruited to the surface of CIN tissue. *Drosophila* larval hemocytes are known to attach to the surface of damaged or overgrown tissue in order to heal the wound or suppress cancer growth (Pastor-Pareja et al. 2008; Parisi et al. 2014). Consistent with this, we found that depletion of Toll pathway genes *Toll* or *NFkB* could significantly reduce the number of hemocytes recruited to CIN wing discs and consequently the level of apoptosis in CIN cells. These results suggest that a local immune activation and hemocyte recruitment is critical to trigger CIN specific apoptosis.

This microenvironment of cancer cells is critical for tumorigenesis and the immune homeostasis between cancer cells and host could potentially affect the fate of these cancer cells (Sounni and Noel 2013; Guo et al. 2014). The current cancer immune therapies are focused on the adaptive immune response. However, our research shows that CIN cells could release DAMPs such as HMGB1 that can be recognised by TLRs allowing us to consider promoting innate immunity as a novel strategy for cancer therapy (Lotfi et al. 2009). Therefore, in cancer immune therapy, it will be significant if we could promote such innate immune responses and attract human immune cells (eg, Natural Killer cells) to specifically target CIN cancer cells (Qiu et al. 2014).

Our data suggests that the JNK-Mmp1 pathway is activated downstream of the Toll pathway to recruit hemocytes through basement membrane disruption (Pastor-Pareja et

al. 2008). Knockdown of JNK significantly reduces the number of hemocytes recruited to the wing discs and the level of apoptosis in CIN cells.

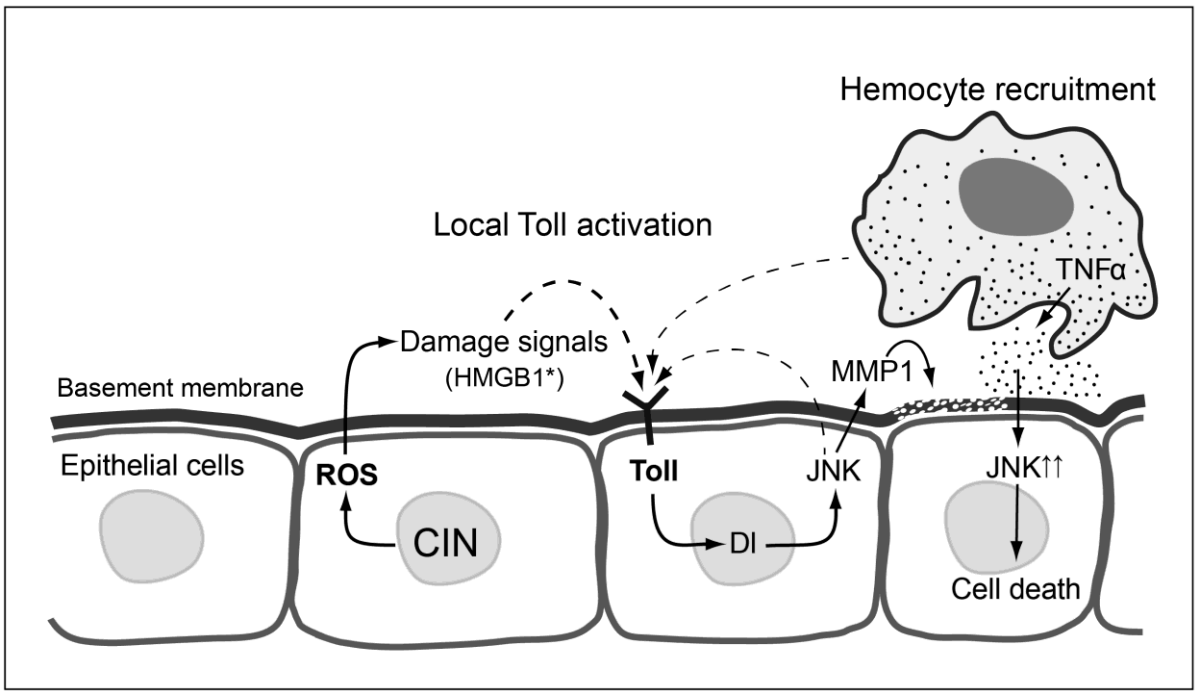

Figure 6.1: Activation of the Toll pathway in response to CIN triggers CIN-specific apoptosis. The increased level of ROS might oxidize DAMPs such as HMGB1 which in turn triggers a local Toll/NFkB signal in nearby cells. The activated Toll signaling activates JNK-Mmp1 signalling which recruits hemocytes to CIN tissue by basement membrane remodling. These recruited hemocytes then initiate CIN-specific apoptosis through TNFα/JNK signaling. This model is informed by studies from (Pastor-Pareja et al. 2008; Parisi et al. 2014) and (Wu et al. 2015).

However, a recent study shows depletion of the Toll pathway is also found to rescue the apoptosis caused by either TNFα/Eiger overexpression or JNK activation in the eye, suggesting that Toll signalling can be downstream of JNK (Wu et al. 2015). In addition, although they found that the local immune system is activated within the tissue, they failed to observe increased numbers of recruited hemocytes (Wu et al. 2015). These disparities could be due to different tissue used in their studies as we are able to show that local immune activation by *NFkB* overexpression is enough to activate the JNK pathway to recruit hemocytes in wing disc, while they observe no effects of *NFkB* overexpression on eye discs (Wu et al. 2015). Therefore, further work needs to be done to test whether TNFα/Eiger overexpression or JNK activation could give feedback activation of local Toll signalling in our model. However, we found that the apoptosis in CIN cells is dependent on the Eiger-JNK pathway which is consistent with their results

and its well characterized role in initiating apoptosis in *Drosophila* (Igaki et al. 2009; Wu et al. 2015) (Fig. 6.1).

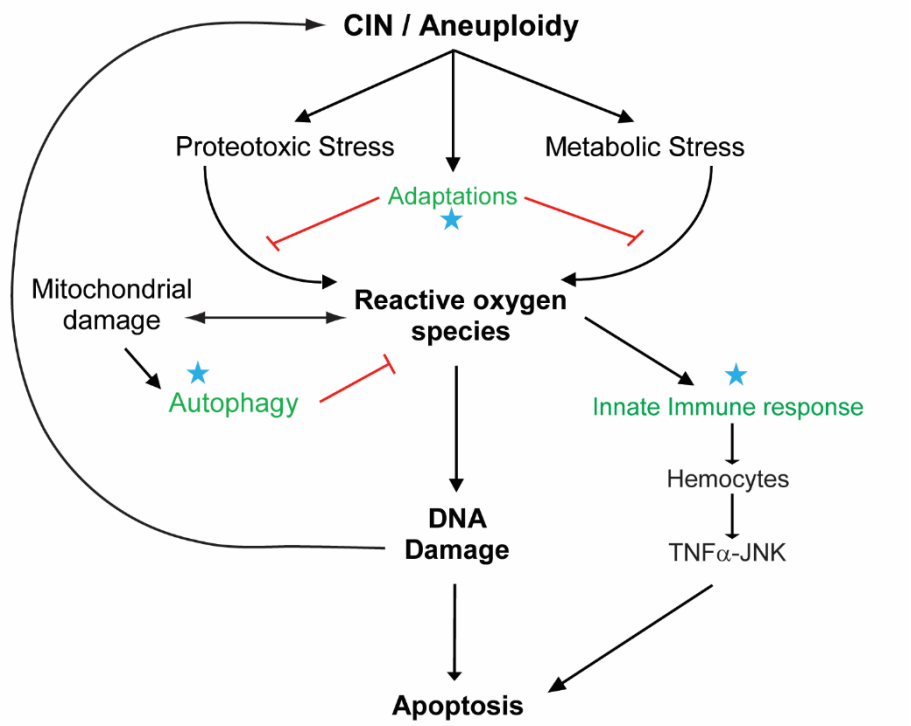

Figure 6.2: The current model for the signaling pathways activated in response to chromosomal instability. Chromosomal instability causes proteotoxic and oxidative stress in proliferating cells, these stresses could further cause DNA damage and apoptosis. In response to these stresses, autophagy signalling is activated to remove defective mitochondria thus reducing the level of oxidative stress. On the other hand, the innate immune signaling is activated to recruit hemocytes which in turn trigger CIN specific apoptosis through TNFα/JNK signaling (Stars indicate the pathways which may be targetable to specifically kill CIN cells).

The major signalling pathways are highly conserved between human and *Drosophila* which has made *Drosophila* a powerful model organism for human diseases research and therapeutic drug discovery (Bier 2005; Miles et al. 2011; Pandey and Nichols 2011). Given the main pathways and factors involved in this project such as the autophagy pathway (Galluzzi et al. 2015; White 2015), mitochondrial dysfunction and oxidative stress (Modica-Napolitano et al. 2007; Yang et al. 2013), Toll signalling (Apetoh et al. 2007), JNK-MMP signalling (Li et al. 2004) and TNFα induced apoptosis (Prins et al. 1997) are all well conserved in humans, we believe that our findings will provide further understanding of cancer biology and contributions to cancer treatment.

6.1 Future Directions

Although a certain level of Chromosomal instability (CIN) gives tumours diversity which facilitates tumorigenesis, it also causes significant stress such as oxidative stress and DNA damage to cancer cells. Our studies show that in order to cope with these stress, several signalling pathways are altered in response to CIN, including metabolism, autophagy and innate immune signalling (Fig. 6.2). Our research highlights the importance of understanding chromosomal instability as a target for cancer treatment. However, it also raised new questions that need to be addressed:

1. Although CIN suppressor genes have previously been identified (Burrell et al. 2013), the mechanism by which cells sense CIN or aneuploidy is not clear. In order to address this question, a screen could be carried out to investigate genes involved in CIN/aneuploidy sensing pathways. Understanding of the mechanism might provide insight into how CIN leads to metabolic stress, dysfunctional mitochondria and oxidative stress.
2. How mitochondria could respond to CIN is not clear. In this project, we have shown that CIN leads to dysfunctional mitochondria and oxidative stress. These defective mitochondria are transported to lysosomes for degradation. It has been reported that the accumulation of p62 in mitochondria could lead to tumorigenesis (Mathew et al. 2009). Therefore, a systematic analysis of mitochondria could be performed to verify the hypothesis that CIN could lead to tumorigenesis through the accumulation of dysfunctional mitochondria.
3. The nature of the signal(s) released from the CIN cells to trigger the immune signalling is not clear. We have proposed that ROS can oxidise HMGB1 which in turn triggered the innate immune response, however, the detailed pathway is not clear. As HMGB1 is a ubiquitous nuclear protein which interacts with transcriptional factors and histones (Tang et al. 2010), studies to rule out the possibility that knock down HMGB1 may affect gene transcription by which reducing CIN cell death are necessary. Moreover, a further investigation of the protein structure of HMGB1 including functional domains and sites required for the immune response to CIN is important.
4. Spaetzle (spz) is a toll receptor ligand and can be cleaved by proteolytic enzymes which in turn activates Toll pathway after immune challenge in *Drosophila* (Valanne et al. 2011). The function of spz in hemocytes is not clear in this project.

Therefore, the manipulation of spz in hemocytes by RNAi or mis-expression to investigate its function on the CIN cells is necessary.

5. We have shown that increased numbers of hemocytes are recruited to the surface of CIN tissue. However, the exact function of these hemocytes is not clear. Moreover, these hemocytes do not seem to attach precisely on the CIN cells. Therefore, an investigation of hemocyte function to pinpoint the pathways required to initiate CIN specific apoptosis is necessary.
6. In this project, the pro-survival autophagy pathway is activated in response to the stresses to remove dysfunctional mitochondria thus reducing the level of oxidative stress and apoptosis. Our studies also show that CIN cells can be detected and removed by the immune system. Since autophagy is involved in immune response regulation (Deretic et al. 2013), further investigations to determine the interaction between the autophagy pathway and the innate immune pathway in response to CIN will be significant.
7. In the project, *Drosophila* has been used as the model organism to carry out all the research. From the perspective of clinical cancer treatment, verifying these research results in mammalian cells including mouse models and human cancer cell lines are necessary.

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Appendix

Stock # B=Bloomington V=VDRC	Chr	CG	SYMBOL	# crosses	Candidate RNAi survivors	Candidate + CIN survivors	Avg candidate + CIN survivors
B10989	2	CG9325	Hts	5	64	50	10
V103631	2	CG9325	Hts	9	28	26	2.9
V108203	2	CG14979	Gr63a	2	46	4	2
V3785	2	CG9206	Gl	1	10	2	2
V16108	3	CG10939	Sip1	1	11	2	2
V102160	2	CG1480	bnk	1	0	2	2
BL27650	3	CG6667	dorsal	1	30	2	2
V109398	2	CG9699	Septin 4	3	29	5	1.7
V3152	3	CG42247	DCX-EMAP	2	24	3	1.5
V104566	2	CG2987	alpha-cater	4	38	4	1
BL34699	3	CG6134	Spz	2	2	2	1
V20518	3	CG1768	dia	1	0	1	1
V39116	3	CG31618	His2A	1	0	1	1
V101651	2	CG11152	fd102C	1	13	1	1
V106404	2	CG2368	psq	1	0	1	1
V100212	2	CG10694		2	0	2	1
V105385	2	CG6932	CSN6	1	2	1	1
V16138	3	CG10971	Hip1	1	6	1	1
V32964	3	CG40444	kl-5	1	17	1	1
V43777	3	CG3571	KLHL18	1	0	1	1
V48150	3	CG1763	nod	1	8	1	1
V49345	3	CG9155	Myo61F	1	0	1	1
V49385	3	CG5784	Mapmodul	1	13	1	1
V51526	1	CG5657	Scgβ	1	20	1	1
V100917	2	CG17237		1	29	1	1
V102504	2	CG42840	d	1	0	1	1
V104089	2	CG7438	Myo31DF	1	0	1	1
V105137	2	CG3085		1	7	1	1
V105707	2	CG14838		1	0	1	1

V29259	3	CG1900	Rab40	1	13	1	1
B34733	3	CG5974	Pll	1	9	1	1
B34775	3	CG5848	Cact	2	0	2	1
B28538	3	CG6134	Spz	1	16	1	1
B24621	2	CG6871	UAS-Catala	1	18	1	1
V106355	2	CG5023		6	56	5	0.83
V100726	2	CG8156	Arf51F	6	83	5	0.83
V44885	3	CG9623	if	2	6	1	0.5
B24754	2	CG11793	UAS-Sod1	2	34	1	0.5
V33615	1	CG5939	Prm	2	2	1	0.5
V42113	2	CG5450	Cdlc2	2	27	1	0.5
V104576	2	CG16944	sesB	3	0	1	0.33
B35628	3	CG5490	Toll	3	34	1	0.33
V101439	2	CG9765	tacc	1	0	0	0
V24083	3	CG9660	toc	1	10	0	0
V22007	3	CG9484	hyd	1	0	0	0
V6216	3	CG8440	Lis-1	1	0	0	0
V46607	3	CG16983	skpA	1	0	0	0
V14194	3	CG17081	Cep135	1	0	0	0
V108401	2	CG3738	Cks30A	1	0	0	0
V29788	3	CG8104	nudE	1	0	0	0
V43950	2	CG2684	lds	1	0	0	0
V40138	3	CG18214	trio	1	0	0	0
V36627	3	CG32434	siz	1	26	0	0
V19130	3	CG3157	γTub23C	1	1	0	0
V29073	2	CG9201	Grip128	1	0	0	0
V103202	2	CG9476	αTub85E	1	0	0	0
V27482	3	CG5688	Grip163	1	4	0	0
V100830	2	CG10346	Grip71	1	0	0	0
V107750	2	CG4453	Nup153	1	0	0	0
V37819	3	CG4217	TFAM	1	0	0	0
V40442	3	CG3403	Mob4	1	0	0	0
V110290	2	CG1451	Apc	1	0	0	0

V15884	3	CG31196	14-3-3ε	1	0	0	0
V43077	3	CG17697	fz	1	0	0	0
V51805	3	CG14781	mei-38	1	0	0	0
V10967	3	CG7538	Mcm2	1	0	0	0
V106648	2	CG4978	Mcm7	1	0	0	0
V10881	3	CG8142		1	0	0	0
V23702	2	CG9790	Cks85A	1	0	0	0
V34597	3	CG3333	Nop60B	1	0	0	0
V24704	2	CG6546	Bap55	1	3	0	0
V24258	3	CG8251	Pgi	1	0	0	0
V37412	3	CG9723		1	0	0	0
V107807	2	CG3945	Rad9	2	0	0	0
V12676	3	CG3240	Rad1	1	8	0	0
V109642	2	CG6768	DNApol-ε	1	0	0	0
V105478	2	CG30420	Atf-2	1	0	0	0
V100974	2	CG6064	TORC	1	4	0	0
V48691	3	CG5748	Hsf	1	0	0	0
V12752	3	CG4143	mbf1	1	0	0	0
V109255	2	CG6673		1	0	0	0
V36297	2	CG31884	Trx-2	2	4	0	0
V30892	3	CG11015	CoVb	1	0	0	0
V100587	2	CG1007	emc	1	0	0	0
V101511	2	CG8376	ap	1	0	0	0
V104313	2	CG14938	crol	1	8	0	0
V100514	2	CG31193	TotX	1	0	0	0
V5322	2	CG5582	cln3	1	3	0	0
V109637	2	CG8318	Nf1	1	3	0	0
V11381	2	CG9762	l(3)neo18	1	0	0	0
V35825	2	CG6770		1	0	0	0
V50381	2	CG31449	Hsp70Ba	1	0	0	0
V106219	2	CG10964	sni	1	0	0	0
V14374	2	CG5873		1	0	0	0
V108665	2	CG17753	CCS	1	18	0	0

V19819	2	CG3178	Rrp1	1	11	0	0
V30505	2	CG4208	XRCC1	1	2	0	0
V12580	2	CG8151	Tfb1	1	0	0	0
V105937	2	CG1163	RpII18	1	0	0	0
V31240	3	CG10387	tos	1	0	0	0
V35222	3	CG7376		1	0	0	0
V32267	3	CG10640	Uev1A	1	0	0	0
V104207	2	CG3473		1	0	0	0
V105408	2	CG4003	pont	1	2	0	0
V44027	3	CG7957	MED17	1	0	0	0
V34727	3	CG3889	CSN1b	1	0	0	0
V103803	2	CG8725	CSN4	1	0	0	0
V105248	2	CG11979	Rpb5	1	5	0	0
V27775	3	CG6987	SF2	1	0	0	0
V110498	2	CG9797		1	0	0	0
V7916	2	CG3595	sqh	1	0	0	0
V8058	3	CG10846	dyn-p25	1	0	0	0
V8141	3	CG8397		1	14	0	0
V8262	3	CG5022		1	0	0	0
V9265	3	CG7595	ck	2	2	0	0
V9788	3	CG11949	cora	1	0	0	0
V11670	3	CG32528	parvin	1	12	0	0
V11791	1	CG8705	pnut	1	1	0	0
V15817	3	CG1363	blow	1	0	0	0
V17344	3	CG1403	Septin 1	1	5	0	0
V17537	3	CG11259	MICAL-like	1	4	0	0
V17563	3	CG33484	zormin	1	0	0	0
V21549	3	CG4944	cib	1	1	0	0
V21908	2	CG4636	SCAR	1	0	0	0
V21930	3	CG4719	Tnks	1	5	0	0
V22125	2	CG5433	Klc	1	0	0	0
V22476	3	CG6224	dbo	1	0	0	0
V22823	3	CG9379	by	1	21	0	0

V22851	3	CG10724	flr	1	0	0	0
V23888	3	CG13503	Vrp1	1	1	0	0
V23954	3	CG17957	Sry-α	1	12	0	0
V24068	2	CG9595	osm-6	1	0	0	0
V24354	2	CG2331	TER94	1	1	0	0
V24795	3	CG13809	osm-1	1	0	0	0
V25024	3	CG31057	tau	1	5	0	0
V25044	3	CG31363	Jupiter	1	0	0	0
V25712	2	CG3121	CG3121	1	22	0	0
V25833	3	CG8683	mon2	1	0	0	0
V25906	3	CG42768	Msp-300	1	26	0	0
V26121	3	CG42734	Ank2	1	0	0	0
V26548	3	CG4560	Arpc3A	1	0	0	0
V27082	3	CG3722	shg	1	0	0	0
V27307	3	CG10695	Pat1	1	7	0	0
V27322	3	CG10859		2	50	0	0
V27837	3	CG7092	Dhc16F	1	0	0	0
V27853	2	CG7107	up	2	0	0	0
V28141	3	CG9881	Arpc5	1	0	0	0
V28471	2	CG30092	jbug	1	0	0	0
V28582	3	CG15831		1	14	0	0
V30035	3	CG3201	Mlc-c	1	0	0	0
V31319	3	CG10686	tral	1	0	0	0
V31488	2	CG11312	insc	1	14	0	0
V31623	2	CG12042		1	0	0	0
V31750	3	CG12363	Dlc90F	1	0	0	0
V31894	2	CG12770	Vps28	1	0	0	0
V32370	2	CG31641	stai	1	1	0	0
V32601	3	CG1539	tmod	1	0	0	0
V32751	3	CG16837		1	14	0	0
V32836	3	CG17046	klar	1	0	0	0
V32971	2	CG17629	kl-3	2	8	0	0
V33486	3	CG2174	Myo10A	2	2	0	0

V33595	3	CG2955		1	14	0	0
V34019	3	CG31907		1	10	0	0
V34098	3	CG34417		1	7	0	0
V34331	2	CG5596	Mlc1	1	0	0	0
V34908	3	CG4931	Sra-1	1	0	0	0
V35273	3	CG7794		1	1	0	0
V36107	2	CG9579	AnxB10	1	16	0	0
V37074	2	CG12008	kst	1	2	0	0
V37865	3	CG1106	Gel	1	6	0	0
V38330	3	CG10083	Abp1	1	2	0	0
V38854	3	CG31012	cindr	1	0	0	0
V39177	2	CG6383	crb	2	0	0	0
V40554	1	CG4696	Mp20	1	20	0	0
V40601	3	CG5629	Ppcs	1	5	0	0
V40603	2	CG5658	Klp98A	1	10	0	0
V41579	3	CG15171	robl37BC	1	28	0	0
V41918	3	CG3339		1	19	0	0
V42003	3	CG4824	BicC	1	3	0	0
V42053	3	CG5870	β-Spec	1	10	0	0
V42118	3	CG8800		1	17	0	0
V43641	2	CG17461	Kif3C	1	11	0	0
V44337	1	CG7765	Khc	1	8	0	0
V45594	2	CG33556	form3	1	4	0	0
V45981	3	CG42236	RanBPM	1	8	0	0
V46029	3	CG8649	Fim	1	7	0	0
V47207	3	CG8936	Arpc3B	1	0	0	0
V47301	2	CG1915	sls	1	0	0	0
V49776	3	CG33694	cana	1	5	0	0
V49957	2	CG31275		1	4	0	0
V51247	3	CG2671	l(2)gl	1	0	0	0
V52343	2	CG1873	Ef1α100E	1	6	0	0
V100094	2	CG10541	Tektin-C	1	0	0	0
V100573	2	CG8978	Arpc1	1	0	0	0

V100714	2	CG9749	Abi	1	0	0	0
V100773	2	CG10540	cpa	1	1	0	0
V100794	2	CG12530	Cdc42	1	0	0	0
V100856	2	CG6433	qua	1	12	0	0
V101016	2	CG6976	Myo28B1	1	0	0	0
V101058	2	CG7293	Klp68D	1	0	0	0
V101111	2	CG31802		1	27	0	0
V101222	2	CG18109		1	12	0	0
V101248	2	CG7051	Dic61B	1	0	0	0
V101340	2	CG1938	Dlic	1	0	0	0
V101704	2	CG1842	Dhc98D	2	1	0	0
V101818	2	CG10834		1	14	0	0
V101993	2	CG11063	jub	1	0	0	0
V102031	2	CG12408	TpnC4	1	21	0	0
V102052	2	CG3401	βTub60D	1	2	0	0
V102493	2	CG4463	Hsp23	1	13	0	0
V102759	2	CG9553	chic	1	19	0	0
V103358	2	CG10642	Klp64D	1	0	0	0
V103380	2	CG5837	Hem	2	0	0	0
V103746	2	CG9426		1	0	0	0
V103870	2	CG32397	jv	1	9	0	0
V103917	2	CG32296	Mrtf	2	1	0	0
V103977	2	CG7230	rib	1	13	0	0
V104043	2	CG14763		1	14	0	0
V104396	2	CG10954	Arpc2	1	0	0	0
V104425	2	CG7846	Arp8	2	19	0	0
V104438	2	CG31536	Cdep	1	2	0	0
V104485	2	CG8529	Dyb	1	0	0	0
V104502	2	CG8280	Ef1α48D	1	0	0	0
V104621	2	CG2184	Mlc2	2	1	0	0
V104759	2	CG1014	robl62A	1	0	0	0
V104894	2	CG2981	TpnC41C	1	7	0	0
V104992	2	CG7152	Syn1	1	0	0	0

V105397	2	CG7210	kel	1	1	0	0
V105579	2	CG11020	nompC	1	0	0	0
V105723	2	CG9028	ssp2	1	0	0	0
V105760	2	CG10751	robl	1	0	0	0
V105812	2	CG34347		1	0	0	0
V105822	2	CG13956	kat80	1	0	0	0
V105843	2	CG10838	robl22E	1	7	0	0
V105898	2	CG9492		1	0	0	0
V105984	2	CG8183	Khc-73	2	9	0	0
V106162	2	CG8953	Actn3	1	18	0	0
V106167	2	CG9313	CG9313	1	6	0	0
V106346	2	CG2916	Septin 5	1	3	0	0
V106401	2	CG34157	Dys	1	8	0	0
V106455	2	CG14996	Chd64	1	16	0	0
V106484	2	CG15112	ena	1	3	0	0
V106645	2	CG5753	stau	1	1	0	0
V106683	2	CG5410	Miro	1	0	0	0
V106867	2	CG5730	AnxB9	1	0	0	0
V107029	2	CG18250	Dg	1	8	0	0
V107176	2	CG5020	CLIP-190	1	13	0	0
V107344	2	CG11579	arm	1	0	0	0
V107789	2	CG31794	Pax	1	8	0	0
V107970	2	CG4843	Tm2	1	0	0	0
V108168	2	CG1193	kat-60L1	1	8	0	0
V108221	2	CG5695	jar	1	0	0	0
V108580	2	CG7483	eIF4AIII	1	0	0	0
V108601	2	CG6053		1	1	0	0
V108658	2	CG3723	Dhc93AB	1	0	0	0
V108886	2	CG4532	pod1	1	0	0	0
V108920	2	CG13221	Vhl	1	0	0	0
V108982	2	CG10839		1	4	0	0
V109084	2	CG6998	ctp	1	0	0	0
V109281	2	CG4114	ex	1	0	0	0

V109413	2	CG15609	Ehbp1	1	0	0	0
V109416	2	CG3849	Lasp	1	4	0	0
V109428	2	CG15097		1	5	0	0
V109442	2	CG33141	sns	1	0	0	0
V109562	2	CG17150		1	0	0	0
V109590	2	CG9359	βTub85D	1	22	0	0
V109644	2	CG9446	coro	1	8	0	0
V110200	2	CG5972	Arpc4	1	0	0	0
V110251	2	CG9121	CG9121	1	3	0	0
V110422	2	CG1424	mst	1	0	0	0
V110599	2	CG4254	tsr	1	2	0	0
V110602	2	CG4905	Syn2	1	0	0	0
V110694	2	CG17347	l(2)37Ce	1	1	0	0
V110719	2	CG4376	Actn	1	0	0	0
V18002	2	CG10105	Sin1	1	0	0	0
V39857	2	CG14992	Ack	2	0	0	0
V49961	3	CG10002	fkh	1	16	0	0
V100748	2	CG8357	Drep-1	1	15	0	0
V109437	2	CG10491	vn	1	1	0	0
V105390	2	CG17077	pnt	1	0	0	0
V107030	2	CG5535		1	0	0	0
V108833	2	CG4016	Spt-I	1	0	0	0
V102000	2	CG3208	RhoGAP5A	1	0	0	0
V51495	3	CG4163	Cyp303a1	1	0	0	0
V102812	2	CG18193		1	1	0	0
V21083	3	CG17136	Rbp1	1	0	0	0
V100790	2	CG12013	PHGPx	1	5	0	0
V103338	2	CG2135		1	7	0	0
B28543	3	CG18241	Toll-4	2	17	0	0
B35577	3	CG5974	Pll	1	21	0	0
B28519	3	CG6890	Tollo	1	3	0	0
B28526	3	CG1149	Mstprox	2	11	0	0
B30488	3	CG8595	Toll-7	1	7	0	0

B14150	2	CG9325	Hts	3	21	0	0
V101353	2	CG6878		1	0	0	0