Chromosomal instability-induced senescence potentiates cell non-autonomous tumourigenic effects

He, Qianqian

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**NANYANG
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SINGAPORE

Chromosomal instability-induced senescence potentiates cell non-autonomous tumourigenic effects

He Qianqian

SCHOOL OF BIOLOGICAL SCIENCES

2019

Chromosomal instability-induced senescence potentiates cell non-autonomous tumourigenic effects

He Qianqian

SCHOOL OF BIOLOGICAL SCIENCES

A thesis submitted to the Nanyang Technological University
in partial fulfilment of the requirement for the degree of
Doctor of Philosophy

2019

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This thesis contains material from one paper published in the following peer-reviewed journal where I was the first author.

Chapter 3 is published as He Q, Au B, Kulkarni M, Shen Y, Lim KJ, Maimaiti J, Wong CK, Luijten MNH, Chong HC, Lim EH, Rancati G, Sinha I, Fu Z, Wang X, Connolly JE, Crasta KC. Chromosomal instability-induced senescence potentiates cell non-autonomous tumourigenic effects. *Oncogenesis*. 2018 Aug 15;7(8):62. doi: 10.1038/s41389-018-0072-4.

The contributions of the co-authors are as follows:

- A/Prof Karen Crasta conceived the project in this thesis. I co-designed the study with A/Prof Karen Crasta and I performed the experiments at Lee Kong Chian School of Medicine (Experimental Medicine Building) and the Institute of Molecular and Cell Biology, Agency for Science, Technology and Research. I analysed the results with A/Prof Karen Crasta.
- I prepared the manuscript draft. The manuscript was revised by A/Prof Karen Crasta and read critically by the Crasta Lab.
- Bijin Au performed the Luminex cytokine assay and analysed the data with Prof John E. Connolly.
- Dr Madhura Kulkarni performed the choroid angiogenesis assay and analysed the data with Prof Xiaomeng Wang.
- Dr Yang Shen and I analysed RNA-seq data with Dr Zhiyan Fu.
- Kah J. Lim and Jiamila Maimaiti performed the zebrafish experiments and analysed them with Dr Indrajit Sinha.
- Cheng Kit Wong performed interphase FISH experiments on cell lines and analysed them with Prof Giulia Rancati.

- Dr Han C. Chong performed immunohistochemistry staining on patient breast cancer tissues and analysed the data with me and A/Prof Karen Crasta. Dr Elaine H. Lim provided breast cancer tissues.

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Dear Sir/Madam,

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Acknowledgements

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Summary

Chromosomal instability (CIN), a higher propensity for loss or gain of whole chromosomes, is a hallmark of cancer and is associated with poor prognosis and drug resistance in multiple types of cancers. CIN often generates aneuploid, including polyploid, cells which contain abnormal number of chromosomes. Despite the prevalence of aneuploidy in tumours, the cell fates following different degrees of chromosome mis-segregation and aneuploidy and how aneuploidy contributes to tumourigenesis remain unclear. Here in this study, we generated relatively mild and severe forms of random aneuploidy in chromosomally stable cells using nocodazole (a microtubule depolymerisation drug) and reversine (spindle assembly checkpoint (SAC) kinase Mps1 inhibitor). We found that severe forms of aneuploid, including polyploid cells, led to senescence, which is classically defined as an irreversible cell cycle arrest. Conversely, mild forms of aneuploidy continued proliferation. Additionally, aneuploidy induced by genetic intervention mechanisms through knockdown of SAC component BUB1 and cohesion subunit SMC1A also culminated in senescence, further substantiating the notion that aneuploidy is sufficient to trigger senescence. These senescent cells were observed to exhibit persistent DNA damage and robust p53 activation. We further found that depletion of p53 significantly reduced the number of senescent cells with concomitant increase in cells undergoing DNA synthesis, suggesting the senescence growth arrest is p53-dependent. Importantly, further characterisation revealed that these aneuploidy-induced senescent cells acquired the senescence-associated secretory phenotype (SASP) which is characterised by increased secretion of a variety of pro-inflammatory factors including cytokines, chemokines, growth factors and matrix remodelling factors and so on. Subsequent phenotypic analysis demonstrated that these SASP factors conferred paracrine pro-tumourigenic effects such as cell migration, invasion and angiogenesis both *in vitro* and *in vivo*. Finally, we observed a correlation between high levels of aneuploidy and senescence at the invasive front in invasive ductal breast carcinomas. Collectively, our findings demonstrate functional non-equivalence of discernable random aneuploidies on tumourigenesis and

suggest a cell non-autonomous mechanism by which aneuploidy-induced senescence and SASP can affect the tumour microenvironment to promote tumour progression.

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Abbreviations

17-AAG	17-N-Allylamino-17-demethoxygeldanamycin
53BP1	p53-binding protein 1
ABC	Avidin:Biotinylated enzyme Complex
AICAR	5-Aminoimidazole-4-carboxamide ribotide
AMP	adenosine monophosphate
ANGPTL4	angiopoietin-like 4
APC/C	anaphase-promoting complex/cyclosome
APS	aneuploidy-associated protein signature
AREG	amphiregulin
ATCC	American Type Culture Collection
ATF3	activating transcription factor 3
ATM	ataxia-telangiectasia mutated
BrdU	5-Bromo-2'-deoxy-uridine
BSA	bovine serum albumin
BTG2	B-cell translocation gene 2
CDC20	cell division cycle 20
CDK1	cyclin-dependent kinase 1
cDNA	complementary DNA
CENPE	centromere protein E
cGAMP	cyclic GMP-AMP
cGAS	cGAMP synthase
CHK2	checkpoint kinase 2
CIN	chromosomal instability
CM	conditioned media
COX-2	cyclooxygenase-2
DAB	3,3'-diaminobenzidine
DAPI	4',6-Diamidino-2-Phenylindole
DCF	2', 7'-dichlorofluorescein

DCFDA	2',7'-dichlorofluorescein diacetate
DDIT3	DNA damage inducible transcript 3
DDR	DNA damage response
DL-PDMP	N-[2-hydroxy-1-(4-morpholinylmethyl)-2-phenylethyl]-decanamide monohydrochloride
DMSO	dimethyl sulfoxide
DNMT1	DNA methyltransferase 1
DSBs	double-strand breaks
dsDNA	double-stranded DNA
ER	endoplasmic reticulum
ERS	environmental-stress response
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FGF2	fibroblast growth factor 2
FISH	fluorescent <i>in situ</i> hybridization
FITC	fluorescein isothiocyanate
GDF15	growth differentiation factor 15
GFP	green fluorescent protein
GMP	guanosine monophosphate
GO	Gene Ontology
h	hour
H&E	hematoxylin and eosin
HMGB1	high mobility group box 1
hpf	hours post-fertilization
HRP	horseradish peroxidase
IF	immunofluorescence
IL-1 α	interleukin 1 alpha
IL-1 β	interleukin 1 beta
IL-6	interleukin 6
IL-8	interleukin 8

ITGA2	integrin subunit alpha 2
JAG 1	jagged 1
L1CAM	L1 cell adhesion molecule
LRG1	leucine-rich alpha-2-glycoprotein 1
MAD1	mitotic arrest deficient 1
MAD2	mitotic arrest deficient 2
MAPK	mitotic centromere-associated kinesin
MAPKAPK2	mitogen-activated protein kinase-activated protein kinase 2
MCC	mitotic checkpoint complex
MEFs	mouse embryonic fibroblasts
Mieap	mitochondria eating protein
MIF	macrophage migration inhibition factor
min	minute
MMP1	matrix metalloproteinases-1
MMP9	matrix metalloproteinases-9
Mps1	monopolar spindle 1
MTOC	microtubule organizing center
MVA	mosaic variegated aneuploidy
NF- κ B	nuclear factor kappa B
N.A.	not available
NGS	normal goat serum
Noc	nocodazole
Nup153	Nucleoporin 153
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline with 0.1% v/v Tween 20
PCA	principle component analysis
PcG	Polycomb group
PCM	pericentriolar material
PFA	paraformaldehyde

PHA	phytohaemagglutinin
PI	propidium iodide
PTC	papillary thyroid carcinomas
PTHrH	parathyroid hormone-like hormone
PTTG	pituitary tumour-transforming gene
qRT-PCR	quantitative real-time PCR
Rb	retinoblastoma protein
Rev	reversine
RIN	RNA integrity number
RNA-seq	RNA-sequencing
ROS	reactive oxygen species
SA- β -gal	senescence-associated β -galactosidase
SAC	spindle assembly checkpoint
SASP	senescence-associated secretory phenotype
SB	SB203580
SCC	sister chromatid cohesion
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
sec	second
SEM	standard error of the mean
SESN2	Sestrin 2
shRNA	short hairpin RNA
SKY	spectral karyotyping
SMC	structural maintenance of chromosomes
SPP1	secreted phosphoprotein 1
STING	stimulator of interferon genes
TCGA	The Cancer Genome Atlas
VEGF-A	vascular endothelial growth factor A
WT	wild-type

YAC yeast artificial chromosome

1. Introduction

The overarching goals of this study were to investigate the effects of chromosome mis-segregation and aneuploidy on cell physiology and the mechanisms by which aneuploidy affects cancer progression. In this section I begin with a literature review with a focus on mechanisms of aneuploidy induction, cellular and physiological consequences of aneuploidy as well as the role of aneuploidy in tumourigenesis.

1.1. Chromosomal instability and aneuploidy

Chromosomal instability (CIN) and aneuploidy are both hallmarks of cancer and are closely related. Yet, they describe distinct concepts. CIN refers to the condition where cells mis-segregate their chromosomes at a high rate (1). In other words, the karyotypes in cells with CIN are constantly changing. CIN can be divided into numerical CIN (whole chromosome mis-segregation) and structural CIN (sub-chromosomal losses, gains, inversions or translocations) (2). Numerical CIN (referred to as CIN hereafter) often results in aneuploidy, a term describing the state of abnormal chromosome numbers (i.e. losses or gains of whole chromosomes). Aneuploid cells generated from CIN are referred to as "random aneuploidy" since chromosome mis-segregation is random and the resultant daughter cells display karyotypic heterogeneity (3). For organisms carrying multiple chromosomes, the possible combinations of chromosomes are limitless. Hence, aneuploidy can have a wide range of unbalanced karyotypes in a given organism. Another type of aneuploidy that is commonly used to study the effects of aneuploidy on cell physiology is "defined aneuploidy" or "model aneuploidy" usually derived from euploid, chromosomally stable cells through single-chromosome transfer or other methods (3). Since the aneuploid chromosomes are known, hence the name.

There are two types of aneuploidy in humans: constitutional aneuploidy and mosaic aneuploidy. Constitutional aneuploidy is caused by aneuploid gametes due to chromosome segregation errors during meiosis, which results in offspring with aneuploid cells throughout the body (4). For example, individuals with Down syndrome carry an extra copy of chromosome 21 (5). Chromosome mis-segregation in somatic cells during normal human development leads to mosaic aneuploidy with aneuploid cells

occurring in part of the human body. Although the rate of chromosome mis-segregation in normal tissues is very low and most cells are euploid (6), aneuploidy has been shown to be present in certain healthy tissues in adults such as in the human brain and liver (7, 8). However, cancers display the most frequent aneuploidy, with over 90% of all solid human tumours and around 75% of blood cancers being aneuploid (9).

CIN and aneuploidy have been observed in many types of cancers as early as in the late 1800s. Over 100 years ago, the German biologist Theodor Boveri described the detrimental effects of aneuploidy on cellular and organismal physiology by studying sea urchin embryos undergoing abnormal mitotic divisions (10). Based on his own findings as well as the observation of abnormal mitotic events in tumour cells described by others, Boveri proposed that aneuploidy might promote malignant transformation, which is known as the aneuploidy hypothesis (11). Tremendous efforts have been made towards studying the role of aneuploidy in tumourigenesis since then. To date, although it is crystal clear that aneuploidy is a striking feature of solid tumours, whether aneuploidy is a driving force or just a side-effect of malignant transformation remains a subject of debate.

To study the cellular consequences of chromosomal mis-segregation and aneuploidy, one fundamental question to understand is how aneuploidy arises. Before we delve into the mechanisms that cause CIN and aneuploidy, it is important to have a general understanding of cell cycle and its checkpoints, as discussed in the next section.

1.2. The cell cycle

1.2.1 Overview of the cell cycle and its checkpoints

Cell cycle (or cell division) is fundamental to all multicellular organisms. Cell cycle is a highly regulated process by various cell cycle checkpoints. Deregulation of cell cycle leads to diseases, including cancer which is characterised by uncontrolled cell proliferation. The cell cycle in eukaryotes consists of four stages: G1 (gap 1) phase, S (synthesis) phase, G2 (gap 2) phase and M (mitotic) phase (Fig. 1). G1, S and G2 phases are collectively known as interphase. In G1 phase, cells grow and prepare for DNA

synthesis after checking that DNA is damage-free by the G1/S checkpoint. The G1/S checkpoint, also known as "Start" in yeast and "Restriction Point" in mammalian cells (12), checks cell size, nutrients, DNA integrity and molecular signals to determine whether or not to move forward to S phase. Cells that do not meet these requirements are not allowed to progress to S phase. Cells can either halt the cell cycle to remedy the problems or enter a resting G0 phase and wait for further signals. In S phase, genetic material inside the cell is duplicated and each chromosome consists of two sister chromatids. Before proceeding to mitosis, cells double-check the duplicated chromosomes for any error and repair any damaged DNA in G2 phase. If the damage is irreparable, cells may undergo cell cycle arrest or apoptosis to make sure that no damaged DNA is passed on to daughter cells (13). In M phase, cells partition its content equally into two daughter cells through mitosis (nuclear division) by a surveillance mechanism called the spindle assembly checkpoint (SAC, also known as mitotic checkpoint) (14), followed by cytokinesis (cell division), which will be discussed in the next section.

Figure 1. A graphical illustration of the cell cycle. Please see text for further details.

1.2.2. Mitosis

Mitosis can be further broken down into five stages: prophase, prometaphase, metaphase, anaphase and telophase (Fig. 2A-E) (13). The details of each stage are discussed as below.

Prophase is marked by nuclear envelope breakdown and chromosome condensation. Two sister chromatids of each chromosome are held together by cohesin protein complex which is established in S phase during DNA replication. The two centrosomes duplicated in S phase start to move to the opposite ends of the cell and begin nucleating microtubules that will form the bipolar mitotic spindle (Fig. 2A) (15).

During prometaphase, chromosomes continue to condense and a large protein structure called kinetochore assembles on each sister chromatid for attachment to microtubules (16). Usually, one kinetochore will bind to microtubules first which is called monopolar attachment, and the other kinetochore establishes attachment with microtubules from opposing pole later to form bipolar attachment (17). Once a bipolar attachment is achieved, the chromosomes start to move to the spindle equator, a process called chromosome congression (Fig. 2B).

During metaphase, all the chromosomes have congressed and aligned in a plane known as the metaphase plate in the middle of two poles of the cell (Fig. 2C) (18). At this point, the sister chromatids are still held together tightly by sister chromatid cohesin, and the chromosomes display maximum condensation. The mitotic checkpoint ensures that the two kinetochores are correctly attached to microtubules from opposite poles and chromosomes align at the metaphase plate (19). Cells that satisfy this checkpoint will proceed to anaphase.

Degradation of cohesin proteins marks the onset of anaphase, which enables the rapid movement of sister chromatids to opposite poles pulled by the microtubules (Fig. 2D) (13). Once separated, each sister chromatid is called a chromosome. Non-kinetochore spindle fibers lengthen and the cell becomes elongated.

In telophase, the final stage of mitosis, segregated chromosomes arrive at opposite spindle poles and start to decondense into chromatin. Meanwhile, nuclear envelope reforms around each set of chromosomes, generating two daughter nuclei, and the mitotic spindle breaks down (Fig. 2E).

Following the completion of mitosis, a contractile ring structure composed of actin filament assembles at the metaphase plate just inside the plasma membrane (20). The actin ring contracts, forming a cleavage furrow, which eventually cleaves the cell into two, a process called cytokinesis.

The whole process of cell division must be tightly regulated to ensure faithful segregation of the genetic material, as any error can result in chromosome mis-segregation and aneuploidy, which will be discussed in the following section.

Figure 2. Stages of mitosis. (A) Prophase, (B) prometaphase, (C) metaphase, (D) anaphase and (E) telophase. Please see text for details.

1.3. Roads to aneuploidy

Aneuploidy is often caused by chromosome mis-segregation in mitosis. A large cohort of molecules and signalling pathways function to prevent chromosome mis-segregation and aneuploidy. Here, I describe various processes/pathways that can lead to cell division errors and aneuploidy when

deregulated: (1) defects in spindle assembly checkpoint (SAC), (2) defects in sister chromatid cohesion, (3) improper kinetochore-microtubule attachments, and (4) centrosome amplification (Fig. 3A-D).

1.3.1. Mitotic checkpoint defects

Under normal conditions, the SAC is a finely-regulated signalling cascade which is required to maintain fidelity of chromosome segregation in all eukaryotic organisms (14). The SAC functions by monitoring the proper attachment of mitotic microtubules to both kinetochores of each chromosome, as well as the tension between the sister kinetochores (21). One single unattached kinetochore or lack of tension in the kinetochore area is sufficient to activate the SAC and generate a molecular "wait" signal to anaphase onset (22). SAC activation delays the onset of anaphase until all chromosomes are perfectly aligned and attached to mitotic spindle microtubules in a bipolar fashion, allowing for satisfaction of the SAC and subsequent anaphase onset (14). A weakened SAC will permit anaphase entry in the presence of misaligned or unattached kinetochores, causing chromosome mis-segregation and aneuploidy (Fig. 3A). The SAC is not an "all-or-none" mechanism as it can be compromised to a certain extent. For instance, yeast and flies with a compromised SAC are viable (23-25). Loss of heterozygosity for SAC components is also tolerated in mice despite defects in SAC activity and chromosomal segregation fidelity (26). However, complete abrogation of SAC is reported to cause mitotic catastrophe, cell death and embryonic lethality (27-29).

The SAC components were initially identified in budding yeast by isolating mutant strains that failed to be arrested in mitosis in response to perturbation of microtubules (24), hence the name "spindle assembly checkpoint". Homologues of these SAC proteins were later identified in mammals, and the main components include MAD1 (mitotic arrest deficient 1) or MAD1L1, MAD2 (mitotic arrest deficient 2) or MAD2L1, BUBR1 (MAD3 in yeast), BUB1, BUB3, Mps1 (monopolar spindle 1) and CENPE (centromere protein E) (30). Upon SAC activation, a number of SAC proteins will be recruited to unattached kinetochores and participate in the assembly of the mitotic checkpoint complex (MCC), which functions as an anaphase inhibitor by sequestering the anaphase-promoting complex/cyclosome (APC/C)

activator cell division cycle 20 (CDC20) (14). APC/C is an E3 ubiquitin ligase that is responsible for targeting cell cycle proteins cyclin B1 and securin for degradation. Cyclin B1 degradation inactivates cyclin-dependent kinase 1 (CDK1), thereby causing mitotic exit. Destruction of securin releases its binding partner separase. As a result, separase will be activated to cleave the cohesin complex, thereby initiating anaphase onset.

Given the fundamental and indispensable role of SAC in maintaining genomic integrity, extensive studies have been conducted towards understanding whether malfunction of SAC, specifically loss or gain or mutation in SAC genes, is associated with tumour development. Mice haploinsufficient or heterozygous for SAC proteins such as MAD1 (31), MAD2 (32), BUBR1 (33), BUB1 (34) and CENPE (35) displayed increased rate of mitotic errors, aneuploidy and tumour formation. Over-expression of MAD2 in mice also promoted aneuploidy and tumourigenesis (36). In humans, altered expression of the SAC components such as BUBR1 (encoded by BUB1B) and BUB1 has been observed in a variety of cancer types including colon adenocarcinomas (37), bladder cancer (38), hepatocellular carcinoma (39), clear cell kidney carcinomas (40), gastric cancer (41) and oral squamous cell carcinoma (42). Over-expression of the kinetochore component Hec 1 (43) and MAD2 (44, 45) is also observed in various human cancers and associated with poor prognosis. Moreover, mosaic variegated aneuploidy (MVA), a rare disorder in humans caused by mutations in BUB1B gene (46) and the centrosomal protein CEP57 (47), causes around 25% or more aneuploid cells in many tissues (48). MVA is also associated with increased risk of developing childhood cancer (49). However, despite the prevalence of aneuploidy in human tumours, extensive studies have shown that mutations in SAC genes are rare and the SAC activity is rarely compromised but hyperactivated in human tumours (50), suggesting the link between overactivation of SAC and CIN in tumours.

1.3.2. Cohesion defects

The multisubunit cohesin ring complex holds sister chromatids together from S phase after their synthesis until anaphase onset. The cohesion complex consists of two SMC (structural maintenance of

chromosomes) (SMC1 and SMC3) and two SCC (sister chromatid cohesion) (SCC1 and SCC3) proteins (51). Sister chromatid cohesion is believed to be required for the formation of bipolar (amphitelic) attachment (52) through resisting microtubule forces and promoting the normal back-to-back configuration of sister kinetochores which promotes chromosome bi-orientation (53), thus promoting the equal distribution of chromosomes into daughter cells. Sister chromatid cohesion is removed in two steps. Most of the cohesin is dissociated from chromosome arms following polo-like kinase and Aurora B-mediated phosphorylation of SCC3 in prophase and prometaphase (54, 55), since phosphorylation of cohesin reduces its ability to bind to chromosomes (55). The remaining cohesin in the centromeric region is removed at anaphase onset by separase-mediated proteolytic cleavage of SCC1 following separase activation (56).

Dissociation of cohesion from sister chromatids is a finely regulated process, disruption of which may lead to either precocious loss of cohesion or persistent cohesion at anaphase, eventually resulting in chromosome mis-segregation (Fig. 3B). A systematic study in aneuploid colorectal cancers revealed that 10 mutations out of 11 were in genes involved in the sister chromatid cohesion machinery (57). Down-regulation or genetic disruption of these genes caused sister chromatid cohesion defects and CIN. Over-expression of separase induced premature loss of sister chromatid cohesion and aneuploidy in mouse mammary epithelial cells (58). Importantly, these cells caused aneuploid tumour formation in the mammary gland in an *in vivo* transplant model. Furthermore, separase is found to be significantly over-expressed in multiple human tumours of breast, prostate, brain and bone (58-60). Securin (also known as pituitary tumour-transforming gene (PTTG)) is also found to be over-expressed in multiple tumours and is able to induce aneuploidy and cellular transformation both *in vitro* and *in vivo* (61). These studies indicate the importance of cohesion in the fidelity of chromosome segregation.

A Mitotic checkpoint defects

B Cohesion defects

C Merotelic attachment

D Centrosome amplification

Figure 3. Roads to aneuploidy. (A) Mitotic checkpoint defects, (B) cohesion defects, (C) aberrant kinetochore-microtubule attachments and (D) centrosome amplification. Detailed description can be found in the text.

1.3.3. Improper kinetochore-microtubule attachments

Accurate kinetochore attachment to spindle microtubules is key to high-fidelity chromosome partitioning. Under normal circumstances, one kinetochore is firstly captured by spindle microtubules from one pole and transported poleward by the microtubules (17). Then, the other sister kinetochore also comes into contact with microtubules extending from the other spindle pole. Eventually, both sister kinetochores are attached to microtubules emanating from opposing spindle poles to form bi-orientation (amphitelic attachment) (Fig. 4A). As a consequence, sister chromatids move to opposite spindle poles and both daughter cells inherit an exact copy of the duplicated chromosomes. However, the initial

attachment of kinetochores to mitotic spindles is a random, dynamic and error-prone process in early stages of mitosis (62, 63), improper kinetochore-microtubule attachments can arise spontaneously, including monotelic, syntelic and merotelic (Fig. 4B-D) (64). In normal conditions, microtubule fibers undergo frequent attachment to and detachment from kinetochores to eliminate malattachments efficiently (62, 63), and eventually achieve amphitelic attachment. Disruption of the kinetochore-microtubule dynamics can result in erroneous attachments and subsequent chromosome mis-segregation.

Monotelic attachment describes the condition where only one kinetochore is captured by microtubules with the other one unattached (Fig. 4B). This type of attachment occurs frequently in early mitosis. Due to lack of tension between sister kinetochores, monotelic attachment can be sensed by mitotic checkpoint and trigger mitotic arrest until a correct form of attachment is formed. If the error is uncorrected, both copies of the sister chromosomes will be deposited into one daughter cell and the other daughter cell will lose the chromosome. However, cells with mitotic checkpoint defects may proceed to anaphase even in the presence of monotelic attachment, giving rise to aneuploid progeny.

If both sister kinetochores are attached to microtubules extending from the same spindle pole, this is called syntelic attachment (Fig. 4C). Failure to correct syntelic attachment will cause mis-segregation of sister chromatids. However, syntelic attachment is not stable (65) and is usually corrected before the onset of anaphase. Nicklas proposed that syntelic attachment is not common because of the back-to-back configuration of kinetochores (66). Since sister kinetochores are embedded in a pit, one kinetochore attached to the microtubules from one spindle pole will therefore make its sister kinetochore inaccessible to microtubules from the same pole. In support of this, cancer cells are not reported to show increased frequency of such attachment (67).

Merotelic attachment occurs when one sister kinetochore is attached to microtubules emanating from both spindle poles (Fig. 4D). This condition occurs frequently and transiently in early phases of normal mitosis (i.e. late prophase or prometaphase) and is corrected in most cases (68). Because of the tension generated across sister kinetochores and the absence of unattached kinetochores, merotelic attachment does not induce a SAC-dependent mitotic arrest (69) and can therefore persist until anaphase,

leading to lagging chromosomes (chromosomes lagged behind in anaphase at the spindle equator) and chromosome mis-segregation. Merotelic attachment is proposed to be a major mechanism contributing to aneuploidy in somatic cells (69) and CIN in cancer cells (70, 71).

Figure 4. Types of kinetochore-microtubule attachments. (A) Amphitelic attachment, (B) monotelic attachment, (C) syntelic attachment and (D) merotelic attachment.

Under normal circumstances, improper kinetochore-microtubule attachments are corrected through different signalling pathways. Disruption or inhibition of these pathways can disturb the dynamics of kinetochore attachment to microtubules and thus cause errors in mitosis and reduce the fidelity of chromosome segregation. Bakhoun *et al* reported that the kinetochore-microtubule attachments in cancer cells with CIN are more stable than those in normal diploid RPE-1 cells (62) and malattachments could persist into anaphase, which may underlie the high frequency of chromosome segregation errors. They further showed that increasing the stability of kinetochore-microtubule attachments in RPE-1 cells promoted chromosome segregation errors to a level similar to that in cancer cells, supporting the importance of kinetochore-microtubule dynamics in preserving genomic stability. Inhibition of Aurora B, a kinase required to ensure amphitelic attachment (72), reduced the turnover of kinetochore-microtubule in prometaphase, increased merotelic attachments, and therefore elevated the rate of chromosome segregation errors (73). Suppression of Hec 1, which is a substrate of Aurora B

kinase and plays an important role in regulating the dynamic behavior of kinetochore microtubules, has been shown to increase errors in kinetochore-microtubule attachment and lead to severe chromosome mis-segregation (74).

1.3.4. Centrosome amplification

Centrosomes are the microtubule organizing center (MTOC) and play a pivotal role in the formation of bipolar microtubule spindles in mitosis. A centrosome is composed of two barrel-shaped centrioles perpendicular to each other and is surrounded by a proteinaceous structure (also known as the pericentriolar material, PCM) that helps to nucleate microtubules (75). The centrosome duplication cycle in a cell cycle is tightly regulated to ensure that, upon cytokinesis, each daughter cell inherits one centrosome, which is duplicated only once in S phase and consequently cells enter mitosis with two centrosomes. Defects in centrosome duplication that cause centriole over-duplication in a single cell cycle will lead to centrosome amplification with more than two centrosomes. Centrosome amplification may also arise through cell fusion (76), cytokinesis failure and mitotic slippage (cells exit mitotic arrest and "slip" into interphase without chromosome segregation and cytokinesis) (77). Supernumerary centrosomes can lead to the formation of multipolar spindles and subsequent multipolar divisions (78), which will generate highly aneuploid but often inviable cells (Fig. 3D) (79). However, in most cases, multipolar spindles can cluster into two groups and form a bipolar spindle (80). Such clustering of centrosomes in mitosis comes with a price as this will greatly increase the formation of erroneous kinetochore-microtubule attachments such as merotelic attachments, finally resulting in lagging chromosomes and chromosome mis-segregation (Fig. 3D) (70, 79). In fact, centrosome amplification is one of the major contributing factors in promoting CIN and aneuploidy in tumours and is observed in almost all types of human cancers (80, 81). Recently, it has been shown that supernumerary centrosomes in a mouse model are sufficient to induce aneuploidy and promote spontaneous tumours in multiple tissues (82).

Apart from the numerical defects as described above, structural defects are another type of centrosomal abnormality (81). Defects in centriole structure (83) and the amount of PCM (81) can cause structural abnormalities in centrosomes. Both numerical and structural centrosomal abnormalities are associated with aneuploidy and CIN in cancers (84).

1.4. Consequences of aneuploidy

Intensive efforts have been invested towards understanding the consequences of aneuploidy during the last decade. Gain or loss of one or more whole chromosomes will immediately change the copy number of hundreds, if not thousands, of genes on the unbalanced chromosomes, which dramatically alters the genomic landscape inside a cell as well as cell behaviors. A large body of evidence has shown the detrimental effects of aneuploidy at the cellular and organismal levels. Nonetheless, aneuploidy is also observed to endow cells with beneficial effects such as growth advantages under selective circumstances. In this section, I'll first elaborate on the physiological consequences of aneuploidy, followed by the cellular response to aneuploidy.

1.4.1. Physiological consequences of aneuploidy

Aneuploidy is associated with abnormal development and diseases. Over one hundred years ago, Theodor Boveri observed that sea urchin eggs fertilized by two sperms, which underwent tripolar or tetrapolar division during the first mitosis and generated massive aneuploidy, displayed developmental defects and lethality, leading to Boveri's hypothesis that aneuploidy interferes with organismal growth and development. Since Boveri's discovery, aneuploidy is reported to be detrimental to most organisms analysed (85), and the severity of defects caused by aneuploidy correlates with the size of the additional chromosomes (86). In worms and flies, most whole-chromosome monosomies and trisomies are lethal (87). In mouse, all autosomal monosomies cause embryonic lethality and all trisomies except trisomy 19, the smallest autosome in mouse, result in death during embryogenesis (88). However, trisomy 19 mice die soon after birth. In humans, aneuploidy is the leading cause of miscarriage (89). All human autosomal monosomies and all autosomal trisomies but trisomy 13, 18 and 21 result in embryonic lethality (86).

Chromosomes 13, 18 and 21 are the smallest human chromosomes with regards to the number of transcripts encoded by them (86). Trisomy 13 (known as Patau's syndrome) and trisomy 18 (known as Edward's syndrome) usually die within a few months after birth (90). Trisomy for chromosome 21, which is the smallest human chromosome, causes Down syndrome and is the only autosomal trisomy that can survive to adulthood (86). These observations support the idea that the amount of chromosomal imbalances correlates with the severity of the defects. Individuals with Down syndrome exhibit varying levels of physical and mental defects, such as heart, vision and hearing abnormalities, mental retardation and reduced life expectancy (91, 92). Patients with MVA also display growth deficiency, mental retardation, microcephaly and childhood cancers. Interestingly, plants appear to be more resistant to aneuploidy than animals. Nonetheless, aneuploid plants were observed to grow more slowly than euploid counterparts (93, 94).

Despite the adverse effects of aneuploidy on normally euploid organisms, aneuploid cells are surprisingly found in certain normal tissues in mammals, especially in the liver and brain. Up to 50% of hepatocytes in adult human liver are reported to be aneuploid using fluorescence *in situ* hybridization (FISH) (95, 96), and around 20% of brain cells in adult mice and humans are found to be aneuploid by spectral karyotyping (SKY) and FISH (7, 97-100). However, subsequent single-cell sequencing revealed lower levels of aneuploidy in these tissues, with approximately 4% of human hepatocytes (101) and about 2-5% of human neurons to be aneuploid (101-103). Nevertheless, it is speculated that such karyotypic heterogeneity in hepatocytes confers phenotypic variability and adaptive potential under a variety of stressed conditions (104). High level of aneuploidy in the brain was proposed to endow neurons with beneficial phenotypic plasticity while predisposing the brain to neurodegeneration (105, 106). It remains to be determined whether these organs are more tolerant to the adverse effects of aneuploidy or instead use them to their advantage.

1.4.2. Cellular response to aneuploidy

Aneuploidy has dramatic effects on cell physiology. In the following section, the gene expression pattern of aneuploidy as well as aneuploidy-associated stresses will be discussed.

1.4.2.1. Effects of aneuploidy on transcriptome and proteome

Understanding how cells respond to aneuploidy at both the mRNA and protein levels is key to studying the impact of aneuploidy on cell physiology. Previous studies on aneuploid yeast (107-110), mice (111) and human cell lines (112-114) revealed that the majority of the genes carried on the aneuploid chromosomes are expressed in proportion to their DNA copy number. In addition, aneuploidy not only affects the expression of genes encoded on the aneuploid chromosomes directly, but also indirectly influences genes located on other chromosomes in two ways. Firstly, the transcriptional regulators located on the aneuploid chromosomes can affect the expression of their downstream targets on other chromosomes (115). Secondly, a conserved gene expression signature of aneuploidy has been observed in various organisms including yeast, plants, mice and humans (107, 116, 117), regardless of the identity of the aneuploid chromosomes. Transcripts associated with cell cycle, cell proliferation, nucleic-acid metabolism are found to be down-regulated, whereas transcripts involved in stress and membrane metabolisms are up-regulated. These transcriptional changes are reminiscent of the environmental-stress response (ERS) in yeast that is triggered by stresses or slow growth.

Similar to the transcriptomic changes, the protein levels of genes on the aneuploid chromosomes are observed to largely scale with the DNA copy number in various organisms. For instance, analyses of aneuploid yeast strains with distinct karyotypes showed that the protein expression of genes on the aneuploid chromosomes is proportional to the chromosome copy number changes (109, 118). Although aneuploid human cell lines displayed similar changes in protein expression, such correlation is, however, less pronounced compared to RNA expression (112). Subsequent study suggests that the observed dosage compensation was predominantly due to protein degradation and the translation efficiency was not decreased (119). These proteins that are prone to dosage compensation are enriched in components of macromolecular complexes (107, 112, 118, 119). This is because many subunits of protein complexes

rely on their binding partners or chaperones to remain soluble. Certain subunit encoded on the unbalanced chromosomes that lacks its binding partner may eventually succumb to degradation.

Besides the conserved transcriptional response to aneuploidy, another gene expression signature termed aneuploidy-associated protein signature (APS) was identified in aneuploid yeast strains (119), where proteins but not transcripts involved in the oxidative stress response, amino acid biosynthesis and cellular bioenergetics were up-regulated. The intensity of APS correlates with the level of aneuploidy, suggesting that higher level of aneuploidy and karyotype imbalances lead to increased ROS (reactive oxygen species) production, although the underlying mechanisms remain to be elucidated.

1.4.2.2. Proteotoxic stress

Under normal conditions, the protein abundance and composition in the cell are controlled at appropriate levels for optimal activity (120). Such protein homeostasis is achieved mainly through protein folding pathway and protein degradation pathway. Protein folding pathway is mediated by the chaperones to ensure proper folding of proteins, while protein degradation pathway makes sure misfolded proteins are eliminated through autophagy-mediated degradation of protein aggregates and ubiquitin proteasome-mediated removal of short-lived proteins (121, 122). When these protein quality control pathways are defective, the resulting accumulation of unfolded or misfolded proteins causes proteotoxic stress (85).

As discussed above, gain or loss of whole chromosomes has tremendous impact on the protein composition in the cell. Chromosome copy number changes in aneuploid cells usually result in a corresponding change in protein expression on the aneuploid chromosomes with the exception of subunits of multimolecular complexes as abovementioned. When protein complex subunits are produced continuously at the incorrect ratio in aneuploid cells, this can cause an elevated burden on the cell's protein quality control pathways to maintain protein homeostasis, eventually contributing to proteotoxic stress. In aneuploid yeast and human cells, Hsp90-mediated protein folding is reduced and cells harbour protein aggregates (123, 124), suggesting a link between aneuploidy and proteotoxic stress. It was further shown that aneuploid human cell lines and MEFs (mouse embryonic fibroblasts) were more sensitive to

the Hsp90 inhibitor 17-AAG (17-N-Allylamino-17-demethoxygeldanamycin) compared to euploid cells (123).

On the other hand, aneuploidy is also demonstrated to affect protein degradation pathways. Trisomic MEFs and aneuploid human cells display enhanced autophagy (112, 125) and are more sensitive to autophagy inhibitor chloroquine (126). Neuronal cells from patients of Down syndrome showed up-regulation in the ubiquitin-mediated proteosomal pathway (127). Furthermore, in line with the notion of increased protein degradation in aneuploid cells, some aneuploid budding yeast strains exhibited increased sensitivity to the proteasome inhibitor MG132 than wild-type (WT) cells (107). Deletion of the deubiquitinating enzyme Ubp6, which accelerates proteosomal degradation, was able to improve growth and cellular fitness in some aneuploid yeast strains by attenuating aneuploidy-induced changes in intracellular protein composition (118, 119). These findings imply that identification of compounds that interfere with pathways that are essential for aneuploid, but not euploid cells, could provide new therapies for aneuploid cancers.

1.4.2.3. Proliferation defects

Aneuploidy has been shown to interfere with cell proliferation in yeast and mammalian cells. In budding and fission yeast, cells carrying extra chromosomes exhibit decreased proliferation (107, 128, 129). Mutations in MAD2-interacting motif in CDC20, which lead to failure of inhibition by MAD2 and a dysfunctional SAC, caused proliferation defects in MEF cells (26), and these CDC20 mutant MEFs showed p53 activation (130). MEFs trisomies 1, 13, 16 and 19 and human fibroblasts trisomy for 21 also proliferate more slowly than their normal counterparts (111, 131). Human trisomic and tetrasomic cell lines derived from HCT116 all exhibited growth impairment (112). Chromosome mis-segregation and subsequent aneuploidy induced by both monastrol (kinesin Eg5 inhibitor) washout and depletion of MCAK (mitotic centromere-associated kinesin) were also reported to induce p53 activation and cell cycle delay in human cells (132). The anti-proliferative effect of aneuploidy is thought to be partly owing to the proteotoxic stress (107, 124). Aneuploid cancer cells in tumours, however, are characterized by

uncontrolled cell proliferative potential, a situation known as the "aneuploidy paradox"—the strong association of aneuploidy with tumours despite its anti-proliferative potential. It is conceivable that aneuploid cancer cells have evolved mechanisms to override the aneuploidy-associated proliferation defects, although the underlying molecular mechanisms remain to be unraveled.

1.4.2.4. Metabolic stress

Aneuploidy has been linked to altered metabolism. Aneuploid yeast strains showed elevated glucose uptake and, when grown under phosphate-limited conditions to achieve the same growth rate with the WT strains, displayed increased expression of genes related to nucleic acid metabolism and reduced expression of genes associated with carbohydrate metabolism (107). Proteome analysis in aneuploid yeast strains revealed enrichment of genes involved in oxidative stress response and metabolic processes (e.g. amino acid biosynthesis and cellular bioenergetics) (119). These aneuploid cells also harboured increased ROS that are a product of oxygen metabolism. It remains to be determined how aneuploidy regulates redox metabolism.

Altered metabolism is also observed in mammalian cells. Aneuploid MEFs (130) and human fibroblasts (133) contained enhanced ROS levels than diploid control cells. Four MEFs trisomies (1, 13, 16 and 19) all exhibited increased glutamine consumption and trisomy 13 also displayed increased use in glucose (111). All four trisomies produced more ammonium and lactate. Analyses of model aneuploid cell lines derived from HCT116 revealed down-regulation in pathways linked to DNA and RNA metabolism, as well as up-regulation in proteins involved in carbohydrate and membrane and oxidative metabolic processes (112). Furthermore, similar to aneuploid yeast and mammalian cells in culture, aneuploid lymphomas in mice induced by knockout of SAC kinase Mps1 also exhibited metabolic burden, as evidenced by up-regulation of pathways involved in cellular metabolism such as RNA metabolic pathway, nucleotide synthesis and so forth (134). Aneuploidy was recently found to cause increased intracellular ceramide levels, suggestive of dysregulation in sphingolipid metabolism (135). Remarkably, DL-PDMP (N-[2-hydroxy-1-(4-morpholinylmethyl)-2-phenylethyl]-decanamide

monohydrochloride), an antagonist of ceramide glucosyltransferase, could selectively inhibit proliferation of aneuploid colorectal cancer cells and trisomic MEFs through further increasing the levels of ceramide, leading to cell apoptosis (135). Consistent with this study, aneuploid yeast strains also exhibited increased ceramide levels, and further increasing ceramide levels genetically or pharmacologically decreased cellular fitness and interfered with cell proliferation (136). These findings indicate that identification of metabolic pathways that are essential for the survival of aneuploid cells poses an opportunity to specifically target aneuploid cancer cells.

1.4.2.5. Replication stress and DNA damage

Aneuploidy also causes replication stress, an event that is defined as stalling or slowing replication fork progression (137). Stalled replication fork can be repaired or collapse, leading to DNA double-strand breaks (DBSs). Aneuploid human RPE-1 cells induced by a Mps1 inhibitor has been reported to display decreased fork rates and increased fork stalling as well as DNA damage in comparison to diploid counterparts (138). Trisomic and tetrasomic HCT116 and RPE-1 cells also showed increased DNA damage and were more sensitive to replication stress which could be partly explained by the reduced levels of replicative helicase MCM2-7 (139). It was suggested that DNA replication defects underlie the genomic rearrangement that was observed in these model aneuploid cell lines. Consistent with the replication stress induced by aneuploidy, the same group previously reported that these aneuploid cells showed major cell cycle delays in S phase and G1 phase (112). Aneuploidy-associated replication stress has also been reported to be located at the telomeres which could be sufficiently abrogated by telomerase expression (140). Moreover, replication defects were also observed in disomic yeast strains, leading to DNA damage that persisted into mitosis (141). Although DNA damage was low in these aneuploid yeast strains, such DNA breaks act as a potent driver for genomic instability such as deletions and translocations that could fuel malignant transformation in cancer. Consistently, aneuploidy yeast strains exhibited genomic instability as demonstrated by increased chromosome loss, DNA damage repair defects and mitotic recombination (142).

1.4.2.6. Mitotic stress

Unbalanced chromosomes can disturb the normal stoichiometry of subunits of protein complexes that regulate various cellular pathways, including chromosome segregation, DNA replication and the SAC (143). Therefore, aneuploidy could cause mitotic stress, leading to errors in chromosome segregation and therefore drive CIN and karyotype diversity. Disomic yeast strains carrying yeast artificial chromosome (YAC) harbouring human DNA displayed an increased rate of YAC mis-segregation relative to the haploid counterparts (142). This phenomenon has been observed almost four decades ago by Campbell *et al.* who found multiple disomic yeast strains displayed repeated and nonrandom mitotic loss of chromosomes (144). Mitotic stress was observed in aneuploid mammalian cells as well. Trisomies 7 and 13 derived from colorectal cancer cell line DLD1 were found to exhibit higher frequency of lagging chromosomes than their euploid counterparts (145). Random aneuploid RPE-1 cells induced by a Mps1 inhibitor displayed higher degree of mitotic aberrations including lagging chromosomes and micronuclei formation (138). Furthermore, PHA (phytohaemagglutinin)-stimulated lymphocytes from patients with Turner's syndrome (X chromosome monosomy) showed significant higher levels of sporadic autosomal aneuploidy than those from healthy controls (146). The same group later reported that like X chromosome monosomy, patient-derived lymphocytes trisomic for chromosome 13, 18 and 21 were also coupled with random gain and loss of other chromosomes (147). However, findings from another group did not show increased rate in chromosome mis-segregation in constitutional aneuploid fibroblasts (trisomy 13, 18 and 21) derived from patients (148). Such discrepancy could be due to the cell types and detection methods used in these studies. Taken together, these findings suggest that aneuploidy *per se* could cause mitotic stress, leading to a vicious cycle of CIN and aneuploidy.

1.4.2.7. "Beneficial" effects of aneuploidy

Despite the tremendous deleterious effects of aneuploidy on cell physiology under normal optimal growth conditions as discussed above, the effects of aneuploidy on cellular fitness seem to be context dependent and may sometimes confer growth advantages. For instance, gain of an additional copy

of chromosome VI which carries the β -tubulin-encoding gene TUB2 was lethal in haploid budding yeast (149). However, such lethality was suppressed when an extra copy of α -tubulin-encoding gene TUB1 was present by introducing another copy of chromosome VIII (149). Moreover, aneuploid cells can promote survival and growth especially under challenging circumstances that are suboptimal for euploid counterparts. For example, aneuploidy is reported to confer drug resistance in *Candida albicans* (150), which is the most prevalent fungal pathogen in humans. Aneuploid yeast strains also show improved survival and growth under glucose-limited chemostats (151). Under various stress conditions and in the presence of chemotherapeutic or antifungal drugs, aneuploid yeast strains were found to grow significantly better than their euploid controls (109). Trisomy 7 in immortalized human colonic epithelial cells was also shown to outgrow their diploid controls under serum-free condition (152). Likewise, aneuploid human colorectal cancer cells also conferred a proliferative advantage under non-standard conditions, increased anchorage-independent growth and invasiveness in both standard and non-standard conditions (153). Li *et al.* also reported aneuploidy to be the primary cause of multidrug resistance in human breast and colon cancer cell lines (154).

Aneuploidy can also generate phenotypic variability and potentiate rapid adaptation and drug resistance. This has been extensively studied in yeast. Haploid yeast cells exposed to lethal-level Hsp90 inhibitor led to the formation of drug-resistant colonies through gain of chromosome XV (155) which carries two genes: STI1 and PDR5, encoding a Hsp90 co-chaperone and a drug pump respectively, demonstrating that aneuploidy is capable of inducing rapid phenotypic evolution under stress. The adaptive trait of aneuploidy is also exemplified in yeast by Rancati *et al.* (115). They showed that cells that evolved and adapted best to depletion of MYO1, a motor protein required for cytokinesis and its depletion caused lethality in most cases, displayed aneuploidy and polyploidy. A recent study by Beaupere and colleagues identified gain of chromosome II allowed yeast cells to adapt to ER (endoplasmic reticulum) stress (156). Similarly, human cancer cells with CIN displayed adaptive resistance to a compound UMK57 which potentiates the activity of MCAK (the kinesin-13 protein) and

suppresses chromosome mis-segregation, as the chromosome mis-segregation rate in drug-treated cells rebounded within a few days (157). Together, these findings highlight the strong association of aneuploidy with resistance to stress. This raises the interesting question as to whether aneuploidy underlies the rapid evolution of cancer cells, which will be discussed in the next section.

1.5. Aneuploidy and tumourigenesis

Despite the extensive association between aneuploidy and cancer, the role of aneuploidy in tumourigenesis remains a subject of hot debate. Some suggest that aneuploidy is not a driving force during tumourigenesis (158), while others argue that aneuploidy promotes tumour progression but not initiation (159). Others also argue that aneuploidy is only a side-effect during transformation and is inconsequential in tumour development (160). This is in stark contrast with structural chromosomal aberrations including deletions, amplifications, translocations and inversions, which are well-established causes of malignant transformation due to activation of oncogenic or tumour suppression signalling pathways. Although loss and/or gain of specific chromosomes or particular combination of certain chromosomes can be linked with gain of oncogenes or loss of tumour suppressors, it seems a clear link is lacking. In fact, cancer cells within a tumour are often genetically heterogeneous, a situation known as intratumour heterogeneity (161). Tumour development appears to correlate with increased aneuploidy and genome complexity, with neoplasms becoming more karyotypically complex as cancer progresses, followed by karyotypic stabilisation in advanced cancers (162). However, it remains controversial whether aneuploidy is a cause or consequence of tumourigenesis.

To understand the role of aneuploidy in tumour initiation and progression, various genetically-modified mouse models with CIN gene mutations, particularly within SAC genes, have been generated by many research groups. These mouse models displayed tremendous variability with regards to how aneuploidy impacts tumour development, and aneuploidy has been documented to act as both a tumour suppressor and an instigator. Mice that were haploinsufficient for both BUB3 and Rae1, two mitotic checkpoint genes, did not accelerate spontaneous tumour formation despite severe aneuploidy induction

(163). Mice heterozygous for CENPE, a kinesin-like motor protein required for stable spindle microtubule capture at the kinetochores, displayed a modest increase of spontaneous lung adenomas and lymphomas, albeit with incomplete penetrance and late onset (35). On the other hand, however, reduced levels of CENPE acted as a tumour inhibitor in spontaneous liver tumours, carcinogen-induced tumours and tumours caused by homozygous loss of the p19/ARF tumour suppressor. Similarly, either MAD1 (31) or MAD2 (32) haploinsufficiency caused a mild increase in the incidence of spontaneous tumours. However, over-expression of MAD2 in mice induced CIN and a wide spectrum of tumours, including lung adenomas, hepatoma and hepatocellular carcinoma, fibrosarcomas as well as lymphomas (164). BUB1 hypomorphic mice (BUB1^{-H} and BUB1^{H/H}) were observed to display high rates of chromosome mis-segregation and were highly susceptible to spontaneous tumourigenesis (34). Mutation in CDC20 protein (CDC^{+ /AAA}) that renders SAC dysfunctional also accelerated formation of spontaneous tumours in mice (26). Such variance in the tumourigenic phenotypes in mice carrying mutations in different SAC genes may be attributed to the levels of aneuploidy induced, other unknown functions of the SAC genes or even the genetic background of different mouse strains. Taken together, the observations that mouse models of CIN usually developed tumours at late onset and did not reach 100% penetrance suggest that aneuploidy *per se* may promote tumour development rather than initiate tumourigenesis.

The above studies strongly suggest the cancer-promoting capacity of aneuploidy, then how does aneuploidy benefit tumours? Two main hypotheses have been proposed in the literature. The first one is that aneuploidy could drive tumourigenesis through its quick adaptive potential under extreme pressure as discussed above. Tumours displaying CIN can give rise to heterogeneous aneuploid cancer cell populations that allow for enhanced adaptive potential under challenging environments (e.g. the tumour microenvironment), metastasis, resistance to therapy and poor prognosis (154, 165-169). In a mutant KRAS-driven mouse model of lung cancers, tumours in mice with combined over-expression of MAD2 and KRAS relapsed more quickly than those over-expressing KRAS only after KRAS withdrawal (170). Importantly, these relapsed tumours displayed highly aneuploid karyotypes, suggesting that MAD2-induced CIN and aneuploidy could facilitate the emergence of KRAS-independent tumours by generating

aneuploid karyotypes that confer a growth or proliferative advantage. This work suggests that CIN and aneuploidy may account for tumour relapse in patients after initial effective chemotherapy.

The second hypothesis is that aneuploidy promotes structural aberrations (142, 171), which are a well-known cancer-promoting factor. Consistent with this notion, gain of a single extra chromosome is shown to trigger replication stress and genomic rearrangement (139), which potentially leads to genomic instability and promotes tumour progression. Whole chromosome mis-segregation and aneuploidy have also been reported to cause structural lesions via micronuclei which can generate genomic instability (172). The resultant genomic instability may predispose cells to further mutations that favour malignant transformation and eventually cause cancer. However, these hypotheses remain to be tested in animal models.

Delineating the relationship between aneuploidy and tumourigenesis has been an active area of research, and the effects of aneuploidy on cell physiology and tumourigenesis and the underlying mechanisms are only beginning to be understood. In order to better understand the effects of aneuploidy on tumourigenesis, it is critical to identify both pro- and anti-tumourigenic effects of aneuploidy on cell physiology. Identification of compounds that interfere with the survival of aneuploid cells but not euploid cells could be exploited for specifically targeting aneuploid cancer cells. As reported, the autophagy inhibitor chloroquine, the protein folding inhibitor 17-AAG (Hsp90 inhibitor) and energy stress inducer AICAR (5-Aminoimidazole-4-carboxamide ribotide) displayed increased selectivity against MEFs trisomies 1, 13, 16 and 19 (126). Importantly, combination of 17-AAG and AICAR inhibited proliferation of aneuploid human cancer cells *in vitro* and in xenografts. DL-PDMP, an antagonist of ceramide glucosyltransferase was demonstrated to inhibit proliferation of aneuploid colorectal cancer cells by further increasing the cellular levels of ceramide, which is a characteristic of aneuploid cells (135). Therefore, identification and characterisation of novel pathways that promote or interfere with the growth of aneuploid cells would provide new therapies against aneuploid cancers.

1.6. Hypotheses and objectives of study

Aneuploidy compromises cellular fitness and impairs organismal development, yet it is closely associated with cancer, a disease of uncontrolled cell proliferation. It is of crucial importance to investigate the cellular consequences of aneuploidy and the mechanisms by which aneuploidy influences tumour development. Cancer cells in the majority of tumours are quite genetically heterogeneous and display a wide spectrum of aneuploid karyotypes, which are classified into "low grade" and "high grade" aneuploidies by Amon and colleagues (3). They refer cells with loss or gain of a few chromosomes from the euploidy as low-grade aneuploidy, whereas those with deviation of many chromosomes from the euploid chromosome number as high-grade aneuploidy. High-grade aneuploidy displays complex karyotypes and is often a combination of losses and gains of different chromosomes. Interestingly, it is proposed that low levels of genetic instability that leads to mis-segregation of one or a few chromosomes (low aneuploidy) promote cell proliferation and tumourigenesis, and conversely, losses or gains of many chromosomes (high aneuploidy) as a result of high levels of genetic instability cause tumour suppression owing to cell death (173). However, until now it is still unclear how losses or gains of chromosomes act oncogenically or as a tumour suppressor. We hypothesize that like defined aneuploidy, random aneuploidy with complex karyotypes also has negative impacts on cell fitness and proliferation. Based on the observations that an extra copy of the smallest chromosome is more tolerable than other chromosomes in mouse and human (86, 88), we hypothesize that random aneuploidy with many chromosome mis-segregation causes severe defects compared to aneuploid cells with one or a few chromosomes mis-segregated. In other words, the severity of stresses caused by random aneuploidy also correlates with the degree of aneuploidy.

Aneuploid cells usually grow slowly than their euploid counterparts in both normal and cancer cells as a result of the aneuploidy-associated stresses. In some cases, acute aneuploidy even succumbs to death or G1 cell cycle arrest depending on the cell type or specific combinations of chromosomes. We were also interested in the long-term cell fate of the G1-arrested cells in response to chromosome mis-segregation. Will they eventually undergo cell apoptosis, resume proliferation or enter an irreversible cell cycle arrest?

Based on the strong association of aneuploidy with tumourigenesis, it is plausible that a rare population of aneuploid cancer cells in the tumours may confer growth advantages, drug resistance and tumour relapse in a cell-autonomous manner by activation of oncogenes or loss of tumour suppressors on the aneuploid chromosomes. Moreover, aneuploidy could also fuel tumour development through giving rise to genomic instability, including structural chromosomal aberrations. However, since the tissue microenvironment plays a critical role in the survival and proliferation of cancerous cells, and vice versa cancer cells can also affect tissue microenvironment through altered expression or secretion of factors (174), we hypothesize that chromosome mis-segregation and aneuploidy might impact tumour progression in a cell non-autonomous fashion as well.

To address these hypotheses, the objectives of this study are to: (1) identify the possible mechanistic differences between low and high random aneuploidies; (2) characterize the role of aneuploidy in tumourigenesis and the underlying mechanisms.

2. Materials and Methods

2.1. Materials

2.1.1. Chemicals and reagents

Table 1. List of chemicals and reagents used in this study.

Chemical	Source
30% Acrylamide/Bis Solution	Bio-Rad
0.5M Tris-HCl, pH6.8	Bio-Rad
1.5M Tris-HCl, pH8.8	Bio-Rad
16% paraformaldehyde	Electron Microscopy Sciences
10× Phosphate-Buffered Saline (PBS)	1 st BASE
10× Tris/Glycine Buffer	Bio-Rad
10× Tris/Glycine/SDS Buffer	Bio-Rad
10% (w/v) Sodium Dodecyl Sulfate (SDS) Solution	1 st BASE
Acetic acid	Merck Millipore
Ammonium persulfate (APS)	Merck Millipore
Ampicillin	Sigma-Aldrich
Bio-Rad Protein Assay Dye reagent Concentrate	Bio-Rad
Bovine serum albumin	Sigma-Aldrich
DMEM/F12 Medium	Gibco
Dulbecco's Modified Eagle Medium (DMEM)	Gibco
Ethanol	Merck Millipore
Eosin	Merck Millipore
Fetal bovine serum	Hyclone, GE Healthcare
Fluka Eukitt® quick-hardening mounting medium	Sigma-Aldrich
FluorSave™ reagent	Calbiochem
Halt Protease & Phosphatase Inhibitor Cocktail (100×)	ThermoFisher Scientific
Hematoxylin	Merck Millipore
Hoechst 33342	Invitrogen
LB broth	BD Biosciences
Lipofectamine 2000	Life Technologies
Matrigel	BD Biosciences
McCoy's 5A Medium	Gibco
Methanol	Merck Millipore
Methyl green	Vector Laboratories
Nocodazole	US Biological
Opti-MEM reduced serum medium	Gibco
Penicillin and streptomycin	Invitrogen
Polybrene	Sigma-Aldrich
Ponceau S solution	Sigma-Aldrich
Prestained protein ladder	ThermoFisher Scientific
Propidium iodide	Sigma-Aldrich
Protease and Phosphatase Inhibitor Cocktail (100×)	ThermoFisher Scientific
Reversine	Cayman Chemicals,
SB203580	Calbiochem
Skim milk	Bio-Rad
TEMED	Bio-Rad

Thymidine	Sigma-Aldrich
Triton-X 100	Merck Millipore
Trypan blue stain (0.4%)	Gibco
Tween 20	Calbiochem
VECTASHIELD Antifade Mounting Medium with DAPI	Vector Laboratories

2.1.2. Antibodies

Antibodies used for Western blotting, immunofluorescence, flow cytometry and immunohistochemistry staining are listed in Table 2-5 as follows.

Table 2. List of antibodies used in Western blotting.

Antibody	Company	Catalog number	Dilution
Bub1	Bethyl Laboratories	A300-373A	1:1000
Donkey anti-Rabbit IgG-HRP	GE Healthcare Life Sciences	NA934	1:10000
FoxM1	Cell Signaling Technology	#5436	1:1000
GAPDH	Merck Millipore	CB1001	1:5000
HSP27	Cell Signaling Technology	#2402	1:1000
I κ B α	Cell Signaling Technology	#9242	1:1000
lamin B1	Abcam	ab16048	1:1000
MAPKAPK-2	Cell Signaling Technology	#3042	1:1000
Nup153	Abcam	Ab24700	1:1000
p21	Cell Signaling Technology	#2947	1:1000
p38	Cell Signaling Technology	#9212	1:1000
p53	Santa Cruz Biotechnology	sc-126	1:1000
p65	Cell Signaling Technology	#8242	1:1000
phospho-Histone H2AX (Ser139)	Cell Signaling Technology	#2577	1:1000
Phospho-HSP27(Ser82)	Cell Signaling Technology	#2401	1:1000
Phospho- I κ B α (Ser32/36)	Cell Signaling Technology	#9246	1:1000
Phospho-MAPKAPK-2 (Thr222)	Cell Signaling Technology	#3316	1:1000
phospho-p38 (Thr180/Tyr182)	Cell Signaling Technology	#9211	1:1000
phospho-p53 (Ser15)	Cell Signaling Technology	#9284	1:1000
phospho-Rb (Ser780)	Cell Signaling Technology	#9307	1:1000
p-p65(Ser536)	Cell Signaling Technology	#3031	1:1000
Rb	Cell Signaling Technology	#9309	1:1000
Sheep anti-Mouse IgG-HRP	GE Healthcare Life Sciences	NA931	1:10000
SMC1A	Bethyl Laboratories	A300-055A	1:1000
β -actin	Cell Signaling Technology	#4967	1:5000

Table 3. List of antibodies used in immunofluorescence.

Antibody	Company	Catalog number	Dilution
53BP1	Cell Signaling Technology	#4937	1:500

Goat anti-Mouse IgG-Alexa Fluor 488	Invitrogen	A-11001	1:1000
Goat anti-Rabbit IgG-Alexa Fluor 594	Invitrogen	A-11012	1:1000
phospho-Histone H2AX (Ser139)	Merck Millipore	#05-636	1:500

Table 4. List of antibodies used in flow cytometry.

Antibody	Company	Catalog number	Dilution
phospho-Histone H3 (Ser10) Rabbit mAb (Alexa Fluor®488 Conjugate)	Cell Signaling Technology	#2947	1:50

Table 5. List of antibodies used in immunohistochemistry staining.

Antibody	Company	Catalog number	Dilution
Biotinylated Goat Anti-Mouse IgG Antibody	Vector Laboratories	BA-9200	1:1000
Biotinylated Goat Anti-Rabbit IgG Antibody	Vector Laboratories	PK-4001	1:1000
p21	Cell Signaling Technology	#2947	1:100
p27	Abcam	Ab193379	1:200

2.1.3. Primers

Primers used for quantitative real-time PCR (qRT-PCR) in this study are listed in Table 6-7 as follows.

Table 6. List of primers for qRT-PCR in cell lines.

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
ANGPTL4	GGGTCTGGAGAAGGTGCATA	GTGGAGAAGGGTACGGAGAG
AREG	GTGGTGCTGTCGCTCTTGATA	ACTCACAGGGGAAATCTCACT
ARL6IP1	TGCTTGGCTGACTACCTTGT	TGAGAAGCAGGTTGTGGACT
ATF3	GGGTCACTGGTGTTTGAGGA	CCTCGGCTTTTGTGATGGAC
BMP2	CAAGCCAAACACAAACAGCG	CCAACGTCTGAACAATGGCA
BTG2	AGCACTACAAACACCACTGG	GTACAAGACGCAGATGGAGC
CCL20	CTGGCTGCTTTGATGTCAGT	CGTGTGAAGCCCAATAAAA
COX-2	CCTGAGCATCTACGGTTTGC	GAACAACCTGCTCATCACCCC
DDIT3	CATTGCCTTTCTCCTTCGGG	CCAGAGAAGCAGGGTCAAGA
DEK	TGTTAAGAAAGCAGATAGCAGCACC	ATTAAAGGTTTCATCATCTGAACATCCTC
DNMT1	CCATCAGGCATTCTACCA	CGTTCTCCTTGTCTTCTCT
EZH2	GGGACAGTAAAAATGTGTCCTGC	TGCCAGCAATAGATGCTTTTTTG
Fas	TGCCCCAAGTGACTGACATCA	CATCCCCATTGACTGTGCAG
FGF2	GGCTTCTTCCTGCGCATCCA	GCTCTTAGCAGACATTGGAAGA

FoxM1	GGAGGAAATGCCACACTTAGCG	TAGGACTTCTTGGGTCTTGGGGTG
GAPDH	GGTCGTATTGGGCGCCTGGTCACC	CACACCCATGACGAACATGGGGGC
GDF15	TTGCACTCCGAAGACTCCAG	CGCACTTCTGGCGTGAGTAT
HMGB1	TTGTGCAAACCTTGTCGGGAG	CTCTTGGGTGCATTGGGATC
HMOX1	CCCACGCCTACACCCGCTAC	GGTGGCACTGGCAATGTTGG
IL-1 α	GCCCAAGATGAAGACCAACC	AGTCAGTGATAGAGGGTGGC
IL-1 β	ACAGATGAAGTGCTCCTTCCA	GTCGGAGATTTCGTAGCTGGAT
IL-6	CCAGGAGCCCAGCTATGAAC	CCCAGGGAGAAGGCAACTG
IL-8	GAGTGGACCACACTGCGCCA	TCCACAACCCTCTGCACCCAGT
INHBA	TCGCACAGACCTTTCCTCAT	ATCCAGTCATTCCAGCCGAT
ITGA2	CAGCTTCTCACCTGCAACTC	TGTGTCTTTGTGGGGCCTAT
JAG 1	CATCGATGAATGTGCCAGCA	GCAGTGGTCTTTCAGGTGTG
Klf4	TCTCCACATGAAGCGACTT	ATGGGTCAGCGAATTGGAGA
Klf5	TCCACAACAGGCCACTTACT	TGTGAGTTAACTGGCAGGGT
L1CAM	CATCTACGTTGTCCAGCTGC	CGTCCTGAAGCACTGTTGTC
lamin B1	CACTGGCGAAGATGTGAAGG	CCCTGCTGGTGGAAAAGTTC
LRG1	GCTGAGGGTGCTGGATCTAA	TGGTTCTCCCCAAGGTCAAG
Mieap	AGAGAATAGGCGGTCAGAGC	ACCTTCCTTGGAGTGCATT
MMP1	CTACCCGGAAGTTGAGCTCA	CTAGGGAAGCCAAAGGAGCT
Nectin 4	ACATTGGCAGAGAAGGAGCT	TGCTTCCCAGAGTCTTCCTG
NOXA	GCAAGAACGCTCAACCGA	TGCCGGAAGTTCAGTTTGTC
P8	GTCGCACCAAGAGAGAAGCT	CCCCTCGCTTCTTCCTCTC
PDGFA	GTCCGCCAACTTCCTGATCT	CCGTGTCTCTTCCCGATAA
PGF	CAGAGGTGGAAGTGGTACCCTTCC	CGGATCTTTAGGAGCTGCATGGTGAC
PIM1	AAGAAGGTGAGCTCGGGTTT	TTTTCGTCCTTGATGTCGCG
PMEPA1	GAGAAGATGCCCTGTCTCA	CTGCAGGTACGGATAGGTGG
PTHLH	GCGACGATTCTTCCTTCACC	TCTTCCCAGGTGTCTTGAGC
S100A7	AAAGCAAAGATGAGCAACAC	AAGTTCTCCTTCATCATCGTC
Sestrin 2	GGCTCATCACCAAGGAACAC	CAGAGTTGTTCAACGGGTCC
SPP1	GGACAGCCAGGACTCCATTG	TGTGGGGACAACCTGGAGTGAA
TGF- α	CCCAGATTCCCACACTCAGT	ACCTGGCAGCAGTGTATCAG
TGF- β	CCCAGCATCTGCAAAGCTC	GTCAATGTACAGCTGCCGCA
TNFRSF10A	AAGCTGAAGGGTCTCAGAGG	CTGTACCAGCTCTGACCACA
TNFRSF10B	ACCCAACAAGACCTAGCTCC	CTGTGTTTCTGGTCGTGGTG
VEGF-A	CTTGCCTTGCTGCTCTACC	CACACAGGATGGCTTGAAG
WHSC1	AATATGACTCCTTGCTGGAGCAGG	ATTTCAACAGGTGGTCTTTGTCTC

WNT5A	CAGGAGTTGCTTTGGGGATG	TGGTCCTGATACAAGTGGCA
β -actin	AGAGCTACGAGCTGCCTGAC	AGCACTGTGTTGGCGTACAG

Table 7. List of primers for qRT-PCR in human breast tumours.

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
CCL2	CAGCCAGATGCAATCAATGCC	TGGAATCCTGAACCCACTTCT
CCL5	ATCCTCATTGCTACTGCCCTC	GCCACTGGTGTAGAAATACTCC
IL-6	GGTACATCCTCGACGGCATCT	GTGCCTCTTTGCTGCTTTTAC
IL-8	ATGACTTCCAAGCTGGCCGTGGCT	TCTCAGCCCTCTTCAAACTTCTC
MMP2	CTCAGATCCGTGGTGAGATCT	CTTTGGTTCTCCAGCTTCAGG
MMP9	ATCCAGTTTGGTGTGCGGAGC	GAAGGGGAAGACGCACAGCT
β -actin	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCACGCACGAT

2.1.4. Plasmids

The plasmids used in this study as listed below were either purchased or gifts. pMDLg/pRRE, pRSV-Rev and VSV.G plasmids were kindly gifted by Vinay Tergaonkar (Institute of Molecular and Cell Biology, Agency for Science, Technology and Research, Singapore). pBABE-H2B-GFP vector was a kind gift from Phillip Kaldis (Institute of Molecular and Cell Biology, Agency for Science, Technology and Research, Singapore).

Table 8. List of plasmids used in this study.

Plasmid	Source	Catalog number
pBABE-H2B-GFP	Gift	N.A.
pLKO.1-shBUB1-1	Dharmacon	TRCN0000040157
pLKO.1-shBUB1-2	Dharmacon	TRCN0000010307
pLKO.1-shp53	Addgene	#19119
pLKO.1-shSMC1A-1	Dharmacon	TRCN0000062555
pLKO.1-shSMC1A-2	Dharmacon	TRCN0000062557
pMDLg/pRRE	Gift	N.A.
pRSV-Rev	Gift	N.A.
VSV.G	Gift	N.A.

N.A. denotes not available.

2.1.5. Mammalian cell lines

hTERT RPE-1 (referred to as RPE-1), HCT116, U2OS and HEK293T cells were obtained from American Type Culture Collection (ATCC). HCT116 p53^{-/-} cell line was a kind gift from Duane A. Compton (Department of Biochemistry and Cell Biology, Geisel School of Medicine at Dartmouth, Hanover, USA). hTERT RPE-1 H2B-GFP (referred to as RPE-1 H2B-GFP) was generated through transduction with pBABE retrovirus encoding histone H2B C-terminally fused to GFP (green fluorescent protein). Cells were positively selected with puromycin and subsequent fluorescence-activated cell sorting (FACS).

2.2. Methods

2.2.1. Transformation of competent cells

Plasmids were transformed into ABLE K competent cells (Agilent Technologies, #200172) according to the manufacturer's instructions. 50 ng plasmid DNA was added into 100 µl of competent cells and incubated on ice for 30 min. Cells were heat-shocked at 42°C for 45 sec, followed by incubation on ice for 2 min. 900 µl of LB broth was then added to the cells and cells were allowed to grow at 37°C with shaking at 225-250 rpm in a shaking incubator. 200 µl of the transformation mixture was spread on LB agar plates containing 100 µg/ml ampicillin and incubated overnight at 37°C.

2.2.2. Plasmid DNA purification

A single colony on the LB agar plate was inoculated into 5 ml of LB broth containing 100 µg/ml ampicillin and cultured at 37°C with shaking at 225-250 rpm overnight. The following day, plasmid DNA was purified using QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions. To make glycerol stocks of bacterial, 800 µl of overnight culture was mixed with 800 µl of 30% sterilized glycerol (diluted in water) in a cryovial tube and stored at -80°C. To purify plasmid DNA, bacterial culture was centrifuged at 3500 rpm for 10 min at room temperature and cell pellet was re-suspended in 250 µl of Buffer P1 and transferred into a clean eppendorf tube. Equal amount of Buffer P2 (250 µl) was added to the cell suspension to lyse the cells. After mixing thoroughly by inversion 4-6 times, the samples were incubated at room temperature for less than 5 min until the solution turns clear. Samples were then

added with 350 µl of Buffer N3 (neutralization buffer) and mixed immediately and thoroughly by inversion 4-6 times, followed by centrifugation at 13000 rpm for 10 min at room temperature. The supernatant containing plasmid DNA was transferred to a spin column and spun down for 30 sec at 13000 rpm, leaving plasmid DNA bound to the membrane inside the spin column. After washing with 750 µl of Buffer PE by centrifugation, plasmids were eluted with 50 µl of Buffer EB or water. DNA concentration was measured by NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). Purified plasmid DNA was stored at -20°C.

2.2.3. Cell culture and synchronisation

Cells were maintained in DMEM/F12 (Gibco) (RPE-1), McCoy's 5A (Gibco) (HCT116) or DMEM (Gibco) (HEK293T and U2OS) supplemented with 10% fetal bovine serum (FBS) (Hyclone, GE Healthcare) and 1% penicillin/streptomycin (Invitrogen). All cells were cultured at 37°C with 5% CO₂ in a humidified incubator.

To synchronise cells at G1/S phase transition, cells were first treated with serum starvation for 24 h (G0/G1 phase), followed by wash-out and treatment with 3 mM thymidine (Sigma-Aldrich, #T1895) for 24 h (G1/S phase). Cells were then released in fresh media for 3 h and treated with either 100 ng/ml nocodazole (Noc) (US Biological, #N3000), dissolved in Dimethyl sulfoxide (DMSO), or 0.2 µM and 1 µM reversine (Rev) (Cayman Chemicals, #10004412), dissolved in DMSO, to induce aneuploidy.

2.2.4. shRNA lentiviral production and infection

To produce lentivirus, HEK293T cells were seeded in 6 cm or 10 cm tissue culture dishes and incubated overnight. On the following day, HEK293T cells at around 80% confluency were co-transfected with transfer plasmid (pLKO.1 (vector control), pLKO.1-shp53, pLKO.1-shBUB1 or pLKO.1-shSMC1A) and lentiviral packaging plasmids (pMDLg/pRRE and pRSV-Rev) and envelope plasmid (VSV.G) using LipofectamineTM 2000 Transfection Reagent (Life Technologies, ThermoFisher Scientific #11668030) according to the manufacturer's instructions. The ratio of these plasmids for transfection is: transfer plasmid (pLKO.1):pMDLg/pRRE:pRSV-Rev:VSV.G: 4:2:1:1. 6 µg (6 cm dish) or 15 µg (10 cm dish) of

DNA plasmids were diluted in 200 μ l (6 cm dish) or 500 μ l (10 cm dish) of Opti-MEM reduced serum medium in one sterilized eppendorf tube. In another tube, 12 μ l (6 cm dish) or 30 μ l (10 cm dish) of Lipofectamine 2000 was diluted in the same amount of Opti-MEM reduced serum medium as DNA. After incubation for 5 min, the DNA mixture and Lipofectamine mixture were mixed and incubated for 20 min at room temperature to allow the formation of DNA-lipid complex. The DNA-lipid complex was then added dropwise to HEK293T cells. The media were changed 6-8 h post-transfection. 36-48 h later, lentiviral supernatant was collected and centrifuged at $500 \times g$ for 5 min, filtered through a 0.45 μ m filter (Sartorius) and stored at -80°C . RPE-1 cells were infected with lentivirus in the presence of 8 $\mu\text{g/ml}$ of polybrene (Sigma-Aldrich #H9268) for 48 h. Infected cells were then selected with 5 $\mu\text{g/ml}$ puromycin for 10 days before used for further analysis.

2.2.5. Live-cell imaging for visualization of lagging chromosomes

RPE-1 H2B-GFP cells were cultured on glass-bottom 6-well tissue culture plates (MatTek) and filmed with an inverted fluorescence microscope (Nikon) equipped with EMCCD camera (Photometrics Evolve 512), a precision motorized stage and Nikon Perfect Focus. Microscope was equipped with an incubation chamber which maintained a temperature- and CO_2 -controlled atmosphere of 37°C and 5% humidified CO_2 . For Noc treatment, after incubation with Noc for 8 h and 16 h respectively, cells were washed with fresh media twice and replaced with fresh media, then filmed immediately on the microscope with a $60\times$ objective. GFP images were acquired every 3.5-5 min for 1.5-3 h at multiple points. Z-stacks with a 0.5 μ m step size were collected. Control cells (synchronised cells without drug treatment) were imaged 9 h after release from thymidine treatment. For Rev treatment, 3 h after release from thymidine treatment, cells were treated with DMSO, 0.2 μM or 1 μM Rev and filmed immediately with a $40\times$ objective at multiple sites every 5 min for 24 h. Z-stacks were captured with a 0.5 μ m step size. Images captured from each condition were analysed using MetaMorph software (Molecular Devices).

2.2.6. Chromosome spreads

Cells were treated with 100 ng/ml colcemid (Invitrogen) for 4-6 h, trypsinized, re-suspended in a hypotonic solution of 75 mM KCl and incubated at 37°C for 20 min. After incubation, freshly prepared ice-cold Carnoy's fixative (3:1 methanol:glacial acetic acid) was added dropwise while gently mixing, followed by centrifugation at 1000 rpm for 5 min. The supernatant was removed and cells were washed three times with Carnoy's fixative. Cells were then re-suspended in 1 ml of Carnoy's fixative and stored at -20°C. To prepare slides, cells in fixative were dropped onto pre-cleaned slides held up to 45 degree angle to the horizontal. Cells were allowed to dry, and stained with either Giemsa or 4',6-Diamidino-2-Phenylindole (DAPI) to visualize chromosomes. For Giemsa staining, cells were stained with Giemsa stain in 1× Gurr's buffer (Gibco) for 5 min, rinsed with Gurr's buffer and allowed to air dry. For DAPI staining, VECTASHIELD Antifade Mounting Medium with DAPI (#H-1200) was mounted on slides and covered by coverslips and sealed with nail polish. Slides were imaged with a 60× objective and chromosomes were counted manually. Minimum of 50 spreads were analysed per experimental condition.

2.2.7. Fluorescent *in situ* hybridization

For interphase fluorescent *in situ* hybridization (FISH) in cell lines, cells were trypsinized and collected by centrifugation, then fixed with Carnoy's fixative and dropped onto slides as described previously. FISH was performed following the manufacturer's instructions using either two centromere-specific probes for chromosome 6 (green, Cytocell Aquarius #LPE006G) and chromosome 8 (red, Cytocell Aquarius #LPE008R) or a locus-specific FISH probe set for XA13(green)/18(aqua)/21(red) (MetaSystems Probes #D-5607-100-TC). Briefly, cell samples were spotted onto slides and allowed to air dry. Probes were added to slides and both sample and probes were denatured simultaneously on a hotplate at 75°C for 2 min. Hybridization was then carried out by overnight incubation at 37°C in a humidified and lightproof chamber. After hybridization, slides were washed and counterstained with DAPI for imaging with either a Nikon Eclipse-Ti microscope or the automated slide scanner system (MetaSystems) using classifier Metacyte spotcount XA131821.

To perform interphase FISH on breast cancer tissues, 2 µm-thick cryosections from human breast tissues were obtained and probed for chromosome 13 and 21 using the same FISH probe set XA13(green)/18(aqua)/21(red) by Advanced Molecular Pathology Laboratory at SingHealth.

2.2.8. RNA-sequencing analyses

24 h after mitotic shake-off from Noc treatment for 8 h and 16 h, aneuploid and euploid control RPE-1 cells were collected. Total RNA was isolated using RNeasy plus Mini Kit (Qiagen) and two biological replicates were subjected to RNA-sequencing (RNA-seq). RNA-seq reads were aligned to the human genome by Tophat 2.1.0 (175). Quality control of single-ended reads was performed by RNA-SeQC (1.1.7) (176). The quality control results were all within acceptable ranges. Cuffdiff (2.2.1) (177) was used to quantify expression and detect differentially expressed genes with gene annotation GTF file downloaded from Ensembl (version GRCh37.75) (178). For each comparison between two conditions, genes with fold change at least two folds and *q* value less than 0.05 were selected as significantly differentially expressed genes.

Principal component analysis (PCA) was performed on all of two biological replicates using function PCAplot from R package CummeRbund (v2.8.2). Gene Ontology (GO) analysis for differentially expressed genes was conducted using online database DAVID (Version 6.7) (179). GO terms with Benjamini *p* value < 0.05 were considered to indicate significant differences.

2.2.9. RNA extraction and quantitative real-time PCR

Total RNA was extracted using RNeasy plus Mini Kit (Qiagen). The purity and concentration of total RNA were measured using NanoDrop 2000 spectrophotometer. 1 µg of RNA was reverse-transcribed into cDNA (complementary DNA) in 20 µl reactions using iScriptTM RT supermix (Bio-Rad) according to the manufacturer's instructions. 0.5-1 µl of cDNA synthesized was utilised in subsequent quantitative real-time PCR (qRT-PCR) reactions in 20 µl reaction volumes using SYBR® Select Master Mix for CFX (Life Technologies, #4472942) by StepOnePlusTM Real-Time PCR System (Applied Biosystem). The PCR reactions comprise of denaturation at 95°C for 10 min, followed by 40 cycles of amplification which

consists of 95°C for 15 sec for template denaturation, and 60°C for 1 min for extension. The relative abundance of mRNAs for target genes were normalised to β -actin or GAPDH as indicated in figure legends.

2.2.10. Western blotting

Cells were washed with PBS and collected by trypsinization and centrifugation. Cell pellets were lysed using RIPA Lysis and Extraction Buffer (Thermo Scientific) with protease and phosphatase inhibitor cocktail (ThermoFisher Scientific). The protein concentrations of cell lysates were determined by Bradford assay (Bio-Rad). Protein samples were diluted with 3 $\times$ loading buffer (Cell Signaling Technology #7722) and heated at 95°C for 5 min and cooled on ice. Approximately 30 μ g of protein samples per lane were resolved on 12% or 10% denaturing SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) gels and electrotransferred onto a nitrocellulose membrane (Bio-Rad) using wet transfer at 100V for 90 min using Bio-Rad electrophoresis and blotting apparatus. The prestained protein ladder (Thermo Fisher Scientific #26620) was used to determine the size of separated proteins and confirm transfer. Membranes were stained with Ponceau S solution (0.1% (w/v) in 5% acetic acid) (Sigma-Aldrich, #P7170) to further confirm transfer. Membranes were then blocked with PBST (phosphate-buffered saline with 0.1% (v/v) Tween 20) containing 5% (w/v) skim milk to prevent non-specific binding. After blocking for 1 h at room temperature, membranes were incubated with primary antibodies diluted in PBST containing 3% skim milk overnight at 4°C. After incubation, membranes were washed thrice with PBST for 10 min each time on a shaker, followed by incubation with horseradish peroxidase (HRP)-conjugated sheep anti-mouse or donkey anti-rabbit secondary antibodies (GE Healthcare) diluted in 3% skim milk (1:10000, GE Healthcare) for 1 h at room temperature. After washing with PBST for three times, a chemiluminescent substrate (GE Healthcare) was added onto the membrane and proteins of interest were detected immediately using chemiluminescence (Bio-Rad).

2.2.11. Senescence-associated β -galactosidase staining

Senescence-associated β -galactosidase (SA- β -gal) staining was performed using the Senescence β -Galactosidase Staining Kit (Cell Signaling Technology #9860) following the manufacturer's instructions. Briefly, cells grown on 6-well plates were fixed with 1 $\times$ fixative for 15 min at room temperature. After washing with PBS twice, fixed cells were incubated with freshly prepared SA- β -gal staining solution at 37°C in a dry incubator without CO₂. Plates were sealed with parafilm to prevent evaporation. The next day, cells were checked under the microscope for blue colour development. For long-term storage, cells were kept in 70% glycerol and stored at 4°C. Images were taken using bright-field microscopy with a 20 $\times$ objective. At least 100 cells were analysed per condition from three independent experiments.

2.2.12. Immunofluorescence

Cells grown on sterilized glass coverslips in 6-well tissue culture plates were washed with PBS and fixed with PBS containing 4% paraformaldehyde (PFA) for 15 min at room temperature. After fixation, cells were rinsed with PBS twice and kept at 4°C until all the samples at different timepoints were collected for the assay. Cells were then permeabilized with PBS containing 0.25% Triton X-100 for 10 min at room temperature. After washing with PBS twice, cells were blocked with blocking buffer (PBS containing 2% BSA (bovine serum albumin), 5% FBS and 0.1% Triton X-100) for 30 min at room temperature and subsequently incubated with mouse anti-phospho-Histone H2AX (Ser139) (1:500, Merck Millipore #05-636) and rabbit anti-53BP1 (1:500, Cell Signaling Technology #4937) antibodies diluted in blocking buffer overnight at 4°C. The next day, cells were washed thrice with PBS containing 0.1% Triton X-100 for 5-10 min each time and then incubated with fluorescence-conjugated secondary antibody (1:1000, Invitrogen) diluted in blocking buffer for 1 h at room temperature in the dark. Coverslips were then rinsed three times with PBS containing 0.1% Triton X-100. DNA was counterstained with Hoechst 33342 (1:5000, Invitrogen) diluted in PBS for 5 min. Coverslips were washed with PBS twice and mounted onto slides with FluorSaveTM Reagent (Calbiochem) for imaging. Slides were imaged with an inverted fluorescence microscope at a 60 $\times$ objective with identical acquisition parameters for all the samples tested. At least 100 cells were analysed per condition from three independent experiments.

2.2.13. BrdU incorporation assay

Cells grown on coverslips were labelled with 10 μ M BrdU for 24 h and fixed according to the manufacturer's instructions (5-Bromo-2'-deoxy-uridine Labeling and Detection Kit I, Roche #11296736001). Cells were then incubated with anti-BrdU (1:200) antibody overnight at 4°C and processed for immunofluorescence (IF) as described previously.

2.2.14. Flow cytometry

Cells collected after trypsinization were fixed with ice-cold 70% ethanol and stored at -20°C until staining. For phospho-Histone H3 (Ser10)/propidium iodide (PI) staining, fixed cells were centrifuged at 3500 rpm for 5 min at 4°C, and the supernatant was removed. Cells were washed once with PBS containing 0.5% BSA and then blocked in PBS containing 0.5% BSA for 10 min at room temperature. Cells were then incubated with phospho-Histone H3 (Ser10) antibody conjugated with Alexa Fluor® 488 (1:50, Cell Signaling Technology #3465) diluted in PBS with 0.5% BSA in the dark for 1 h at room temperature. After incubation, cells were washed with PBS containing 0.5% BSA, followed by incubation with PBS containing 250 μ g/ml RNase A and 10 μ g/ml PI at 37°C for 30 min in the dark before analysis. FACS analyses of stained cells were performed using a BD Acurri C6 cytometer (BD Biosciences) using FL2-A (PI)/FL1-H (FITC, fluorescein isothiocyanate) parameters, and data were analysed using FlowJo® (FlowJo, LLC).

2.2.15. Measure of ROS production by FACS

Cellular ROS levels were evaluated using DCFDA/H2DCFDA Cellular ROS Detection Assay Kit (Abcam #ab113851) according to the manufacturer's instructions. Briefly, cells were harvested by trypsinization, washed with PBS and then re-suspended in culture media to ensure a single-cell suspension ($< 1 \times 10^6$ cells/ml) by gently pipetting up and down. Cells were stained with 20 μ M of DCFDA and incubated for 30 min at 37°C, followed by flow cytometry analysis (FITC/FL1-A channel). Unstained cells were used as negative control for detection of autofluorescence. The mean DCF intensity

for each condition was calculated by subtracting the mean DCF intensity of unstained cells from the stained counterparts.

2.2.16. Preparation of conditioned media

To prepare conditioned media (CM) from euploid and 16 h Noc-induced highly aneuploid RPE-1 cells, 24 h after mitotic shake-off from Noc 16 h treatment, euploid and highly aneuploid cells were washed with PBS twice and incubated with DMEM/F12 supplemented with 0.5% FBS and 1% penicillin/streptomycin. After incubation for five days, CM were collected and filtered with 0.22 μ m pore filters (Sartorius).

To harvest CM from euploid and Rev-induced aneuploid cells, RPE-1 and HCT116 cells were treated with DMSO (control) or 1 μ M Rev for 24 h. After drug wash-out, cells were grown in fresh media for two more days, washed and replaced with DMEM/F12 (RPE-1) or McCoy's 5A (HCT116) supplemented with 0.5% FBS and 1% penicillin/streptomycin. Three days later, CM were collected and filtered as described previously.

For use in various phenotypic assays, CM containing different concentrations of FBS were prepared. CM containing 0.5% FBS were used for scratch wound healing assay. For use in transwell matrigel invasion assay and cellular migration and invasion in zebrafish assay, CM containing 10% FBS were prepared by mixing CM containing 0.5% FBS with culture media containing 40% FBS at a ratio of 3:1.

2.2.17. Multiplex cytokine assay

CM were generated from Noc 16 h-induced aneuploid and diploid control RPE-1 cells as described previously. The protein concentrations of 41 cytokines were determined using Human Cytokine Panel 1 (Merck Millipore) according to the manufacturer's protocols. The 41 cytokines consisted of EGF, Eotaxin/CCL11, FGF2, Flt-3 ligand, Fractalkine, sCD40L, G-CSF, GM-CSF, GRO (CXCL1), IFN- α 2, IFN- γ , IL-1 α , IL-1 β , IL-1RA, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17A, IP-10, MCP-1 (CCL2), MCP-3 (CCL7), MDC (CCL22), MIP-1 α (CCL3),

MIP-1 β (CCL4), PDGF-AB/BB, RANTES (CCL5), TGF- α , TNF- α , TNF- β , VEGF and PDGF-AA. Plates were washed using Tecan Hydrospeed Washer (Tecan) and read with Flexmap 3D system (Luminex Corp). The concentrations of target cytokines were determined according to the standard curves which were generated using Bio-Plex manager 6.0 (Bio-Rad) with a 5-parameter curve-fitting algorithm. For proteins out of range below the detection level, we assigned a value that is half of the lowest standard values to avoid zero values for statistical analysis. Three biological replicates were analysed.

2.2.18. Growth proliferation assay

To examine the proliferative capacity of U2OS and HCT116 cells upon treatment with indicted CM over time, cells were seeded in 6-well tissue culture plates at 5×10^4 cells per well (U2OS) or 1.0×10^5 cells per well (HCT116) and grown in standard complete media for 24 h. 24 h later, cells were washed twice with pre-warmed PBS and incubated with indicated CM containing 10% FBS for up to four days. For the Day 4 samples, media were replenished once through the experiment. Cells were collected each day by trypsinization and re-suspended in culture media. 10 μ l of cell suspension was mixed with equal amount of 0.4% trypan blue solution (Gibco) and 10 μ l of cell mixture was used for cell counting.

2.2.19. Cell viability assay

U2OS cells were plated at 2×10^4 per well in 96-well tissue culture plates in standard culture media. The next day, cells were washed with PBS twice and incubated with indicated CM in triplicate wells. Three days later, cells were incubated with CellTiter-Blue[®] Reagent for 1.5 h using the CellTiter 96[®] Aqueous One Solution Cell Proliferation Assay Kit (Promega #G3580). Cell viability was determined by measuring absorbance at 490 nm using a microplate reader (Biotek). Triplicate wells with CM but no cells were used to determine the background absorbance values. The average of absorbance values of the background were subtracted from the experimental wells with cells treated with respective CM. Relative cell viability was determined by comparing with the mean absorbance of cells treated with control CM.

2.2.20. Scratch wound healing assay

U2OS cells were seeded in 6-well plates at 4×10^5 cells per well in standard culture media. The following day, cells were washed with PBS twice and incubated with indicated CM containing 0.5% FBS for 24 h. Confluent monolayer was scratched with a 200 μ l sterile pipette tip to generate an even wound. Cells were washed with PBS twice gently, replaced into fresh CM with 0.5% FBS and subjected to time-lapse microscopy to track the closure of wound. Phase-contrast images were captured with a 10 $\times$ objective at multiple points every 1 h for 24-48 h as described above. The area of the wound was measured using ImageJ. The percentage of wound area = Area of actual wound/Area of original wound $\times$ 100%.

2.2.21. Transwell invasion assay

U2OS cells were seeded at 1.0×10^5 cells per well in 6-well plates and incubated with CM containing 10% FBS for two days. Cells were trypsinized and re-suspended in fresh CM. 4×10^4 cells in 500 μ l of CM were plated onto the transwell inserts (8 μ m pore size, BD Biosciences) uncoated or pre-coated with 200 μ g/ml matrigel (BD Biosciences) with 20% FBS added to the lower chamber as a chemoattractant. After incubation for 24 h, non-invasive cells on the upper surface of the inserts were gently scraped off using a cotton swab, and invasive cells on the lower surface were washed with PBS and fixed with 4% PFA for 15 min at room temperature. After fixation, cells were washed with PBS and stained with 0.05% (w/v) crystal violet for 20 min at room temperature. After staining, crystal violet staining solution was discarded and stained cells were washed with water gently to remove excess staining solution inside the insert. Inserts were allowed to air dry and invasive cells were observed under a 20 $\times$ objective by bright-field microscopy. The percentage of invasive cells were calculated using the formula: Invasive cells (%) = mean number of cells invading through matrigel matrix insert membrane/mean number of cells migrating through uncoated insert membrane $\times$ 100%. Quantification was performed by counting the total number of cells from 6-9 random fields per condition.

2.2.22. Preparation of nuclear protein lysates

The nuclear extract was isolated using the Nuclear and Cytoplasmic Extraction Reagents kit (ThermoFisher Scientific #78833) according to the manufacturer's instructions. Cells cultured in 6 cm

(HCT116) or 10 cm (RPE-1) dishes were harvested and washed with PBS. Cell pellet was suspended in 100 μ l of ice-cold CER I (Cytoplasmic Extraction Reagent I) with protease and phosphatase inhibitor and mixed well by vortexing vigorously for 15 sec on the highest setting, followed by incubation on ice for 10 min. 5.5 μ l of CER II (Cytoplasmic Extraction Reagent II) was then added to the suspensions and incubate the tube on ice for 1 min after vortexing for 5 sec. The suspensions were centrifuged at 16000 $\times g$ for 5 min at 4°C. The supernatant (cytoplasmic extract) was removed, and the insoluble pellet was re-suspended in 100 μ l of NER (Nuclear Extraction Reagent) containing protease and phosphatase inhibitor by vortexing, followed by incubation on ice for 40 min with vortexing every 10 min. After incubation, the suspensions were centrifuged at 16000 $\times g$ for 10 min at 4°C and the supernatant (nuclear extract) was transferred to a clean tube and stored at -80°C until use. The protein concentrations were measured by Bradford assay (Bio-Rad).

2.2.23. Choroid sprouting assay

The choroid tissues from eyes of P3 mice were separated in PBS containing 10% FBS. The peripheral choroid layer was separated from the retina and cut into approximately 1 mm $\times$ 1 mm segments. Each segment was placed in a 20 μ l drop of matrigel and incubated with indicated CM containing 0.5% FBS in 1:3 diluted with EGM2 media (Lonza) for four days. Media were changed every other day. On Day 4, phase-contrast images of individual explants were taken using a Nikon Eclipse-Ti microscope. The area of vascular sprouting and area of the explant were quantified using ImageJ. Minimum of 6 explants were analysed per experimental condition. This experiment was in collaboration with Wang Xiaomeng, Lee Kong Chian School of Medicine, Nanyang Technological University.

2.2.24. Cellular migration and invasion in zebrafish (Zgraft™)

The Zebrafish Research Facility and the Animal Care Committee in Acenzia Inc. are certified by Ontario Ministry of Agriculture, Food and Rural Affairs (OMAFRA). All protocols used in the laboratories were approved by the Animal Health and Welfare Branch, OMAFRA. Zebrafish were maintained using Tecniplast's ZebTec system with controlled light-dark cycles (14 h light/10 h dark). Embryos were

obtained from natural spawnings and developmental stages were defined as hours post-fertilization (hpf) at 28.5°C.

CM were generated from RPE-1 or HCT116 cells treated with 1 μ M Rev or DMSO as described above. Two days after drug treatment, cells were cultured with media supplemented with 0.5% FBS and 1% penicillin/streptomycin for three days. CM were collected, filtered and mixed with culture media containing 40% FBS in a 3:1 ratio to generate CM containing 10% FBS.

5×10^5 - 6×10^5 U2OS cells and 1.5×10^5 HCT116 cells were seeded in 10 cm dishes and 6 cm dishes, respectively. Both cell lines were incubated with indicated CM for two days, and then incubated for 45 min at 37°C in culture media containing DiI (Vybrant, Life Technologies) for visualization of cells with red fluorescence. After incubation, cells were washed twice with PBS, trypsinized using Trypsin-EDTA solution (Sigma) and re-suspended in serum-free media for injection. Zebrafish embryos at approximately 48 hpf were dechorionated (if required) and anesthetized with tricaine (Sigma). 100-200 cells were injected into the yolk sac of each embryo using Nanoject II injector (Drummond). After injection, embryos were further incubated at 35°C in fresh fish tank system water containing methylene blue. Approximately 2 h post-injection, embryos with fluorescent cells outside the yolk sac were excluded from further experimentation. The rest were incubated for up to four days post-injection. Each larva was anesthetized by immersion in system water containing tricaine and placed on a 1% agarose bed under an inverted fluorescence microscope for imaging (Leica M165 FC). Images were gathered, aligned to a specific orientation and analysed using ImageJ software to determine metastatic tumour foci position relative to injection site (0,0 on graph). For consistent quantitative comparison of the differentially treated cells, invasion index and migration index which measure the ability of cells to invade and migrate respectively, were calculated: Invasion index = Number of metastases formed per 100 cells injected; Migration index = Distance travelled by metastases as percent of the maximum distance cells can migrate within the fish.

2.2.25. Patient breast cancer tissues

Biopsies were harvested using mastectomy procedure and were snap-frozen in liquid nitrogen immediately after surgery. Frozen biopsies were thawed slowly, fixed and processed accordingly for analytical study. All human breast tissue samples were collected from patients undergoing surgery at the National Cancer Centre, Singapore. None of the patients underwent prior chemotherapy. The selected samples were de-identified, stored and analysed according to procedures described in protocols #CIRB 2015/2976 and #IRB-2018-04-009, approved by SingHealth CIRB and NTU IRB. Informed consents were obtained from all patients.

2.2.26. H&E staining and immunohistochemistry

Breast cancer tissue of 4 µm-thickness was sectioned. For cryosectioned tissue, the sections were fixed in 4% PFA and washed in PBS. For hematoxylin and eosin (H&E) staining, tissue sections were first stained with hematoxylin for 5 min, and dipped in 0.1% HCl for 2-3 sec. Next, sections were washed in running tap water for 5 min, followed by counterstain with eosin for 2 min. After rinse with tap water briefly, sections were mounted with aqueous mounting medium containing 90% glycerol and 10% PBS.

For immunohistochemistry, tissue endogenous peroxidase activity was quenched using 3% hydrogen peroxide for 15 min at room temperature to block non-specific staining. Tissue sections were then blocked in PBS containing 10% normal goat serum (NGS), 1% BSA and 0.1% Triton X-100 for 1 h at room temperature, followed by incubation with primary antibodies as follows: anti-human p21 Waf1/Cip1 (12D1) (1:100, Cell Signaling Technology #2947S) and anti-human p27 KIP1 (1:200, Abcam ab193379) in PBS containing 3% NGS overnight at 4 °C. After washing in PBS, sections were incubated with biotinylated goat anti-mouse or anti-rabbit secondary antibodies (Vector laboratories, USA) depending on the source of the primary antibodies, followed by incubation in Avidin:Biotinylated enzyme Complex (ABC; Vector Laboratories #PK-4001, USA) for 30 min. Sections were then washed with PBS and developed with 3,3'-diaminobenzidine (DAB) substrate (Vector Laboratories #SK-4100, USA) as a chromogen to produce a brown colour stain and counterstained with methyl green for nuclear staining. Slides were mounted with Fluka Eukitt® quick-hardening mounting medium (Sigma-Aldrich, USA).

Staining without primary antibodies served as negative controls, which resulted in the absence of staining. Images were obtained using Carl Zeiss slide scanner Axio Scan.Z1 with a Plan-Apochromat 40x/0.95 NA objective and Hitachi HV F202, and ZEN 2 slidescan software. The intensity of the staining was presented as negative=0, weak=1, moderate=2 or strong=3. The percentage of positively stained cells at several representative regions was recorded as numerical score in every 10% (Table 9). H-score was calculated by two observers using the formula: $1 \times \text{percentage of weakly stained nuclei} + 2 \times \text{percentage of moderately stained nuclei} + 3 \times \text{percentage of strongly stained nuclei}$. The range of H score is 0 to 300. The difference between two observers was less than 5%.

Table 9. Quantitative H scoring method.

Intensity of immunostaining (I)	0			1			2			3		
	negative			weak			moderate			Strong		
Percentage of stained cells in each intensity (P)	0	10	20	30	40	50	60	70	80	90	100	
	0%	0-10%	11-20%	21-30%	31-40%	41-50%	51-60%	61-70%	71-80%	81-90%	91-100%	

2.2.27. Statistical analysis

All experiments were repeated at least three times unless otherwise stated in figure legends. Data were blindly obtained and analysed. All the statistical analyses were performed with GraphPad Prism 6. Student's *t*-test (two-tailed, unpaired) was used to determine the statistical differences between different groups (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ and ns, not significant). Data are expressed as mean $\pm$ SD (standard deviation) or mean $\pm$ SEM (standard error of the mean) as indicated.

2.2.28. Data availability

RNA-seq data have been deposited in the NCBI Gene Expression Omnibus repository (<http://www.ncbi.nlm.nih.gov/geo/>) under the accession number GSE109519, which is scheduled to be publicly available on Feb 26 2019. Private link (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE109519>) with the key "ctulcymuhxorjgp_" before the release date.

3. Results

3.1. Induction of relatively low- and high-grade aneuploidies

As a first step towards studying the effects of different degrees of aneuploidy on cell physiology and tumourigenesis, we selected a stable, diploid, hTERT-immortalized non-transformed human cells RPE-1 (retinal pigment epithelial cells with a modal chromosome number of 46) and another chromosomally stable near-diploid human colon cancer cell line HCT116 (modal chromosome number of 45). To generate aneuploid cells, we treated cells with nocodazole (Noc) or reversine (Rev) which induces chromosome mis-segregation and subsequent aneuploidy with distinct mechanisms. Noc depolymerizes microtubules by binding to free tubulin dimers and therefore preventing their incorporation into microtubules (180), which ultimately disrupts the formation of mitotic spindles and kinetochore attachment to microtubules. This will activate the spindle assembly checkpoint (SAC) and arrest cells in prometaphase. After Noc wash-out, mitotically arrested cells induce merotelic attachment and lagging chromosomes (69). Contrary to a mitotic delay, the essential SAC kinase Mps1 (monopolar spindle 1) inhibitor Rev inactivates SAC and accelerates progression through mitosis even in the presence of unattached kinetochores or incorrect kinetochore-microtubule attachments (181), causing aneuploid progeny.

To induce chromosome mis-segregation by Noc treatment, we used Noc at a concentration of 100 ng/ml or 330 nM, which is sufficient to significantly reduce microtubule polymerization (182). Cells were treated with Noc for 8 h and 16 h to induce relatively low and high levels of chromosome mis-segregation and aneuploidy. To capture mitotic events after Noc treatment and wash-off, we performed live-cell imaging using RPE-1 cells stably expressing H2B-GFP. After cell synchronisation, RPE-1 H2B-GFP cells treated with Noc for 8 h and 16 h, respectively, were washed with drug-free media twice and subjected to time-lapse microscopy (Fig. 5A). Synchronised cells without drug treatment served as control. Noc wash-off was observed to result in chromosome segregation defects, including lagging chromosomes, broken chromatids and chromatin bridges (Fig. 5B). Notably, Noc 16 h treatment not only

caused an overall increase of cells with chromosome mis-segregation, but also higher number of mis-segregated chromosomes per cell and cytokinetic failure compared to Noc 8 h treatment, suggesting that Noc 16 treatment induced a severe form of aneuploidy compared to 8 h treatment. To generate aneuploid cell populations, mitotically arrested cells were isolated by shake-off, followed by incubation in fresh media for another 24 h to allow cells to complete cell division (Fig. 5C). Since mis-segregated chromosomes are often encapsulated in micronuclei (usually containing 1-2 chromosomes), and cytokinetic failure can produce multinucleated cells (containing several chromosomes per nucleus), we observed increased micro- and multinucleation after Noc 16 h treatment compared to 8 h treatment (Fig. 5D). Consistent with a previous report showing p53 activation following chromosome mis-segregation and aneuploidy (132), we observed a significant increase in p53 protein levels in RPE-1 cells 24 h after mitotic shake-off following Noc treatment, especially in Noc 16 h-treated populations (Fig. 5E). The protein levels of p21, which is a direct transcriptional target of p53, also increased in aneuploid cells (Fig. 5E). Quantitation of mitotic cells by FACS analyses of phosphorylated Histone H3 (Ser10), which is a mitotic marker, revealed a sharp decrease of mitotic cells in both low and high aneuploid cell populations in comparison to the control (control cells=1.01%, Noc 8 h (low aneuploidy)=0.16%, Noc 16 h (high aneuploidy)=0.10%) (Fig. 5F). In line with the live-cell imaging result, Noc 16 h treatment caused an increase in G1-arrested polyploid cell populations that were negative for phospho-Histone H3 staining (24%), indicative of failed cytokinesis, as compared to Noc 8 h treatment (2.87%) (Fig. 5F). These results demonstrate p53 activation and cell cycle arrest upon Noc-induced chromosome mis-segregation and aneuploidy.

Figure 5. Noc induces chromosome mis-segregation and cell cycle arrest in a duration-dependent manner in RPE-1 cells. (A) Schematic representation of time-lapse microscopy after Noc treatment and wash-off in RPE-1 H2B-GFP cells. (B) (Left) Representative anaphase images illustrating lagging chromosomes in RPE-1 H2B-GFP cells treated as per (A). Yellow arrows indicate lagging chromosomes.

Scale bar, 5 μ m. (Right) Plot showing the percentage of abnormal mitosis from the time-lapse microscopy after indicated treatments. Abnormal mitosis includes lagging chromosomes, chromosome bridges, broken chromatids, misaligned chromosomes and cytokinetic failure. A minimum of 20 cells were analysed per experimental condition. (C) Scheme for inducing low- and high-grade aneuploid cells by Noc treatment for 8 h and 16 h, respectively. (D) DAPI staining of nuclei showing micronucleated and multinucleated cells 24 h after mitotic shake-off from Noc treatment in RPE-1 cells. (Top) Representative images and (bottom) plot showing percentages of micronucleated and multinucleated cells after indicated treatments. Yellow and white arrows indicate micronucleated and multinucleated cells, respectively. Scale bar, 10 μ m. D1 represents Day 1. A minimum of 100 cells were analysed per condition. (E) Protein levels of p53 and p21 were determined in RPE-1 cells treated as per (C). Actin served as loading control. (F) RPE-1 cells treated as per (C) were co-stained with Alexa Fluor[®] 488-conjugated anti-phosphorylated Histone H3 (Ser10) antibody and propidium iodide (PI) and subjected to flow cytometry analysis. Numbers in each quarter indicate the percentage of different cell populations. FACS profile of PI staining on the right shows DNA content. Plots in (B) and (D) are expressed as mean $\pm$ SD from three independent experiments. * P <0.05, ** P <0.01, *** P <0.001 and **** P <0.0001 by Student's t -test.

To further determine the level of aneuploidy in post-mitotic cells, we set out to perform the conventional metaphase spreads for karyotyping. To this end, we first depleted p53 by short hairpin RNA (shRNA) in RPE-1 cells to allow cells to proceed to mitosis after chromosome mis-segregation (Fig. 6A). RPE-1 shp53 cells after Noc treatment as in Fig. 5C were incubated with 100 ng/ml colcemid for 4-6 h to arrest cells in mitosis. Metaphase spreads analyses revealed karyotypic heterogeneity between these conditions, with Noc 16 h-induced high aneuploidy displaying a greater extent of chromosome number loss or gain, especially increased near-tetraploid cell populations, than their Noc 8 h-treated counterparts (Fig. 6B). To further verify this variation in aneuploidy, two-chromosomes interphase Fluorescence *In Situ* Hybridization (FISH) was conducted in cells 24 h after release from Noc-induced arrest. Similar to the metaphase spreads result, Noc 16 h treatment led to a minor increase in the total percentage of aneuploid cells but a greater deviation from the diploid chromosome number compared to cells treated with Noc for 8 h (Fig. 6C). Together, these results demonstrate Noc treatment for 8 h and 16 h induced comparatively low and high levels of aneuploid populations in RPE-1 cells, respectively.

Figure 6. Noc induces mild and severe forms of aneuploid karyotypes in RPE-1. (A) Western blotting analysis of shRNA-mediated knockdown of p53 in RPE-1 cells. shCtrl denotes RPE-1 cells infected with lentivirus carrying empty vector. GAPDH was used as loading control. (B) (Left) Representative images of metaphase spreads from RPE-1 shp53 cells treated with Noc as per Fig. 5C. (Right) Plot shows percentage of cells with indicated number of chromosomes gained or lost. Data are expressed as mean $\pm$ SD from two independent experiments. At least 50 spreads were manually counted per condition. Near-tetraploid/Tetraploid: 81-110 chromosomes. Chr represents chromosome. (C) (Left) Representative interphase FISH images of RPE-1 cells treated as per Fig. 5C with centromere-specific probes for chromosome 6 (green) and chromosome 8 (red). (Right) Plot shows distribution of cell ploidy. "An" denotes nuclei with different number of signals for chromosome 6 and 8. Scale bar, 10 μ m. n=50 cells.

In contrast to Noc, Rev has been shown to accelerate progression through mitosis rather than enforce a mitotic arrest (138). As Rev is known to induce chromosome mis-segregation in a dose-dependent manner (125, 181), we used lower and higher concentrations (0.2 μ M and 1 μ M) of Rev to induce comparatively mild and severe chromosome segregation defects. Examination of the mitotic events in RPE-1 H2B-GFP cells in the presence of 0.2 μ M and 1 μ M Rev using time-lapse microscopy

revealed severe chromosome mis-segregation upon 1 μ M Rev treatment as compared to 0.2 μ M Rev (Fig. 7A and 7B). 1 μ M Rev was observed to induce chromosome segregation errors in virtually all the mitotic cells analysed, while 0.2 μ M Rev affected only around half of the cells entering mitosis (Fig. 7B). After treatment with Rev for 24 h (Fig. 7C), we observed 1 μ M Rev led to a significant increase in micronucleated and multinucleated cells compared to 0.2 μ M Rev treatment (Fig. 7D). Similar to Noc treatment, Rev was also found to induce a p53-dependent cell cycle arrest as shown by increased p53 protein expression (Fig. 7E) and decreased phospho-Histone H3 (Ser10) staining (Fig. 7F), therefore RPE-1 shp53 cells were used for karyotyping. Metaphase spreads of cells collected immediately following 1 μ M Rev treatment confirmed an overall increase in aneuploidy and a preferential increase in severe forms of aneuploidy (≥ 5 chromosomes lost or gained, including near-tetraploidy/tetraploidy (81-110 chromosomes)) compared to control cells (Fig. 7G). In contrast, cells treated with 0.2 μ M Rev displayed an increase in cells with less than 5 chromosomes lost or gained (Fig. 7G). Together, these data showed that 1 μ M Rev induced higher degree of aneuploidy in RPE-1 cells than 0.2 μ M Rev.

G

Figure 7. Rev causes chromosome mis-segregation, cell cycle arrest and aneuploidy in a dose-dependent manner in RPE-1. (A) Scheme for time-lapse microscopy performed on RPE-1 H2B-GFP cells upon Rev treatment. (B) (Left) Representative anaphase images of Ctrl (DMSO) and Rev-treated RPE-1 H2B-GFP cells. (Right) Percentage of abnormal mitosis is shown. Scale bar, 5 μ m. At least 30 cells were quantified per experimental condition. (C) Schematic representation of aneuploidy induction using 0.2 μ M and 1 μ M Rev. (D) (Top) RPE-1 cells treated as per (C) were stained with DAPI and representative images are shown. Yellow and white arrows indicate micronucleated and multinucleated cells, respectively. Scale bar, 10 μ m. (Bottom) Plot shows percentage of micronucleated and multinucleated cells. A minimum of 100 cells were quantified per condition. (E) Protein levels of p53 and p21 were determined by Western blotting analysis in RPE-1 cells treated as per (C). GAPDH was used as loading control. (F) RPE-1 cells treated as per (C) were subjected to flow cytometry analysis after double staining of phospho-Histone H3 (Ser10) and PI. FACS profile on the right shows PI staining. (G) (Left) Representative metaphase spreads from RPE-1 shp53 cells treated as per (C). Scale bar, 20 μ m. (Right) Plot depicting percentage of cells with indicated number of chromosomes lost or gained. At least 50 spreads were manually counted. Near-tetraploid/Tetraploid: 81-110 chromosomes. Chr denotes chromosome. Plots in (B) and (D) are expressed as mean $\pm$ SD from three independent experiments. * P <0.05, ** P <0.01, **** P <0.0001 and ns, not significant by Student's t -test.

To test whether Rev has a similar effect on chromosomally stable HCT116 colon cancer cells, we conducted metaphase spreads in HCT116 p53^{-/-} cells due to p53 activation in HCT116 wild-type (WT) cells (Fig. 8A and 8B). 1 μ M Rev was found to increase polyploid cells, including near-triploidy (60-80 chromosomes), near-tetraploidy/tetraploidy (81-110 chromosomes) and near-octoploidy/octoploidy (170-182 chromosomes), whereas 0.2 μ M Rev induced relatively mild forms of aneuploidy (Fig. 8C). Hereafter, we refer to Noc 16 h- and 1 μ M Rev-treated cells as severely aneuploid cell populations, and their respective counterparts treated with Noc 8 h and 0.2 μ M Rev as mildly aneuploid cell populations.

Figure 8. Rev induces low and high amount of chromosome imbalances in HCT116. (A) Western blotting analysis of indicated proteins in HCT116 cells treated as per Fig. 7C. (B) Western blotting analysis of p53 in HCT116 WT and p53^{-/-} cells. GAPDH was used as loading control. (C) HCT116 p53^{-/-} cells treated as Fig. 7C were subjected to metaphases spreads. (Left) Representative images and (right) plot shows percentage of cells with indicated number of chromosomes lost or gained. Chr means chromosome. Near-triploid/triploid: 60-80 chromosomes; near-tetraploid/tetraploid: 81-110 chromosomes; near-octoploid/octoploid: 170-182 chromosomes. Scale bar, 20 μ m. n $\geq$ 50 spreads.

3.2. Aneuploid cells display cell cycle- and stress-related changes in gene expression

To study the immediate transcriptional response to aneuploidy, aneuploid populations generated from RPE-1 cells collected 24 h after mitotic shake-off from Noc 8 h- and Noc 16 h-induced mitotic arrest were subjected to RNA-sequencing (RNA-seq) for differential gene expression analyses. Two sets of RNA samples showing good quality (RIN (RNA integrity number) $\geq$ 8) (Fig. 9A) were used for RNA-seq analysis. Principle component analysis (PCA) demonstrated that gene expression patterns of the two biological replicates were highly correlative (Fig. 9B). Genes with a minimum of two-fold changes

between samples were considered differentially expressed genes. Full lists of up- and down-regulated genes from the RNA-seq data can be found in Table 9-11 at the end of this thesis. Gene Ontology (GO) analyses using online database DAVID (179) revealed down-regulation of genes associated with cell cycle, DNA metabolic processes and DNA repair in both Noc 8 h- and Noc 16 h-treated cells compared to control (Noc 8 h and 16 h vs Ctrl), with 16 h-treatment-induced highly aneuploid cells showing greater down-regulation in these genes than the 8 h-treatment (Noc 16 h vs Noc 8 h) (Fig. 9C). 16 h-treatment also exhibited up-regulation of genes involved in extracellular matrix, response to stimulus and stress, as well as response to wounding and angiogenesis compared to control (Noc 16 h vs Ctrl) (Fig. 9C). It is noteworthy that such changes in gene expression observed in Noc-induced random aneuploidy shared high similarities with defined aneuploidy reported for both mouse and human cells (116, 117).

We then performed quantitative real-time PCR (qRT-PCR) of genes of interest to validate the RNA-seq data. In line with our RNA-seq data, qRT-PCR results displayed increased expression of genes involved in response to stress such as Sestrin 2 (SESN2), Mieap (mitochondria eating protein), BTG2 (B-cell translocation gene 2), DDIT3 (DNA damage inducible transcript 3, also known as GADD153 or CHOP) and Fas (Fig. 9D); inflammatory response including IL-1 α (interleukin 1 alpha), IL-1 β (interleukin 1 beta), SPP1 (secreted phosphoprotein 1), AREG (amphiregulin), PTHLH (parathyroid hormone-like hormone), COX-2 (cyclooxygenase-2, also known as PTGS2), CCL20 (C-C motif chemokine ligand 20) and GDF15 (growth differentiation factor 15) (Fig. 9E); angiogenesis including ANGPTL4 (angiopoietin-like 4), VEGF-A (vascular endothelial growth factor A), FGF2 (fibroblast growth factor 2), JAG 1 (jagged 1) and LRG1 (leucine-rich alpha-2-glycoprotein 1) (Fig. 9E) as well as cell migration such as MMP1 (matrix metalloproteinases-1), ITGA2 (integrin subunit alpha 2), L1CAM (L1 cell adhesion molecule) and Nectin 4 (Fig. 9F) in severely-aneuploid cells compared to control cells. We also observed decreased expression of transcription and chromatin-remodeling genes in highly aneuploid populations such as the transcription factor FoxM1 (forkhead box M1), EZH2 (a polycomb group histone H3 lysine-27 methyltransferase), lamin B1, DNMT1 (DNA methyltransferase 1), WHSC1

(known as MMSET, histone methyltransferase) and HMGB1 (high mobility group box 1) (Fig. 9G). Importantly, Noc 8 h-induced low aneuploidy in RPE-1 cells displayed up- or down-regulation of these genes to a lesser extent compared with Noc 16 h treatment, suggesting that the transcriptional changes in response to immediate random aneuploidy correlate with the degree of aneuploidy, which is consistent with the RNA-seq data.

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Figure 9. RNA-seq analyses reveal cell cycle- and stress-associated transcriptional changes upon chromosome mis-segregation. (A) Total RNA was isolated from euploid control cells and Noc-induced aneuploid RPE-1 cells as per Fig. 5C. RNA quality was measured by Agilent Bioanalyzer and a representative example is shown. The first peak represents 18s rRNA and the second peak denotes 28s rRNA. RNA with RIN not less than 8 is considered good quality. (B) PCA plot of the RNA-seq data from two biological replicates. (C) GO analyses of the RNA-seq data. (Top) Down-regulated GO terms depict enrichment of genes in both Noc 8 h- and Noc 16 h-induced low and high aneuploid cells compared to control cells. (Bottom) Up-regulated GO terms indicate enrichment of genes in high aneuploid cells induced by Noc 16 h treatment in comparison to euploid control. The X-axis indicate $-\log$ (Benjamini P value). GO terms with Benjamini P value <0.05 were considered significant. (D-G) Plots showing fold changes of mRNA expression of (D) cell stress genes, (E) pro-inflammatory factors, (F) migration-associated genes and (G) chromatin remodeling and transcriptional factors. mRNA levels were normalized to GAPDH. All data are shown as mean $\pm$ SD ($n=3$). * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$ and ns, not significant by Student's t -test.

3.3. Elevated chromosome mis-segregation and aneuploidy induce senescence and DNA damage

It is clear from the above data that cells with highly aberrant karyotypes trigger a cellular stress response (Fig. 9). The stress response usually results in either apoptosis or cell cycle arrest (183) that may lead to cellular senescence, a stable cell cycle arrest. To determine cell fates following chromosome mis-segregation, we collected RPE-1 cells at 1, 3 and 5 days after mitotic shake-off following Noc treatment for 8 h and 16 h respectively. Intriguingly, instead of induction of apoptotic cell death, highly aneuploid cells displayed enlarged and flattened morphological changes from Day 3, reminiscent of senescence (Fig. 10A). Indeed, SA- β -gal staining, the "gold standard" marker for cellular senescence (184), displayed significantly increased SA- β -gal activity 3 days following Noc 16 h treatment (Fig. 10A). Conversely, Noc 8 h treatment only caused a negligible increase in SA- β -gal-positive cells. To further confirm the senescent state, BrdU incorporation assay was conducted on cells collected on Day 5. As expected, we observed a dramatic decrease in DNA synthesis in highly aneuploid cell populations (Noc 16 h), whereas low aneuploid counterparts (Noc 8 h) exhibited a minor reduction in BrdU-positive cells compared to control cells (Fig. 10B). These BrdU-negative cells after Noc 8 h treatment could be attributed to the presence of a small percentage of senescent cells, as well as the proliferation defect that is a characteristic

of aneuploid cells. Similar results were observed in Rev-induced aneuploid cells where 1 μM Rev treatment culminated in a significant increase in SA- β -gal-positive cells, while the majority of 0.2 μM Rev-induced mildly aneuploid cells were negative for SA- β -gal staining (Fig. 10C). These findings were also found to be true in identically-treated HCT116 cells via both Noc and Rev (Fig. 10D and 10E). Together, these data demonstrate that severe chromosome mis-segregation induces senescence in both normal and cancer cells, while cells in response to mild chromosome segregation errors continue to proliferate.

Figure 10. Noc- and Rev-induced severe chromosome mis-segregation leads to senescence. (A) RPE-1 cells were treated with Noc for 8 h and 16 h as per Fig. 5C, followed by mitotic shake-off and culture in drug-free media for indicated number of days. (Left) Representative images of cells stained for SA- β -gal using bright-field microscopy. (Right) Plot showing percentage of SA- β -gal-positive cells. (B) RPE-1 cells were treated as per (A). BrdU-incorporation assay was performed 5 days after mitotic shake-off. (Left) Representative images and (right) percentage of BrdU-positive cells were shown. (C) RPE-1 cells were treated with 0.2 μ M and 1 μ M Rev as described in Fig. 7C. SA- β -gal staining was conducted on cells collected at 3 and 5 days after Rev wash-off. Plot showing percentage of SA- β -gal-positive cells. (D-E) HCT116 cells were treated identically as RPE-1 cells as in (A) and (C). SA- β -gal staining was performed on cells collected at 1, 3 and 5 days post drug wash-off. Plots show percentage of SA- β -gal-positive cells. At least 100 cells were quantified for each condition and data are expressed as mean $\pm$ SD from three independent experiments. * P <0.05, ** P <0.01, *** P <0.001, **** P <0.0001 and ns, not significant by Student's t -test.

However, as with many other synthetic drugs being used in biological studies that may exert side-effects on cells' fitness, Noc and Rev *per se* may cause cellular stress and render cells susceptible to senescence. To rule out the possibility of senescence induction due to "heavy" drug treatment, we generated aneuploid cells using genetic intervention mechanisms by lentiviral shRNA-mediated knockdown of the SAC component BUB1 and cohesin subunit SMC1A which were shown to promote chromosome segregation defects and aneuploidy (34, 133). Two shRNAs against both BUB1 and SMC1A were used to control for off-target effects. Empty vector (shCtrl) was used as negative control. Western blotting analyses confirmed the efficient knockdown of BUB1 and SMC1A in RPE-1 cells (Fig. 11A). Cells harvested 10 days after lentiviral transduction were assessed for ploidy status by three-chromosomes (chromosome 13, 18 and 21) interphase FISH. FISH results showed increased aneuploidy induction upon suppression of BUB1 and SMC1A in RPE-1 cells (Fig. 11B). Importantly, these aneuploid cells displayed a significant increase in SA- β -gal-positive cells (Fig. 11C). Hence, these results confirmed that chromosome mis-segregation indeed could induce cellular senescence.

Figure 11. Knockdown of BUB1 and SMC1A induces senescence. (A) Western blotting analyses of the knockdown efficiency of Bub1 and SMC1A in RPE-1 cells with GAPDH as loading control. (B) RPE-1 cells were cultured for 10 days after infection with lentiviral shRNA targeting BUB1 and SMC1A. Three-chromosome interphase FISH was performed with locus-specific probes for chromosomes 13 (green), 18 (aqua) and 21 (red). (Left) Representative images were shown. Numbers at the corners of each image indicate the number of signals for chromosomes with the same colour. (Right) Plot shows percentage of aneuploid cells based on FISH signals for chromosome 13, 18 and 21. $n=500$ cells. $**P<0.01$, $***P<0.001$ and ns, not significant by Fisher's exact test. (C) RPE-1 cells treated as in (B) were subjected to SA-β-gal staining. (Left) Representative images and (right) plot depicting percentage of SA-β-gal-positive cells treated with indicated shRNA. A minimum of 100 cells were quantified, and data are expressed as mean $\pm$ SD of three independent experiments. $***P<0.001$ and $****P<0.0001$ by student's t -test.

Since chromosome mis-segregation has been reported to be associated with DNA damage (139, 171), which is a well-known senescence stimulus (185), we then sought to investigate whether DNA damage accompanied aneuploidy-induced senescence in our context. To address this question, we first performed IF analysis of the well-characterised DNA damage marker γ H2AX (186) as well as the DNA damage repair protein 53BP1 (p53-binding protein 1) (187). We observed significantly higher amount of cells containing one or more γ H2AX foci in Noc 16 h-induced severely aneuploid cells in RPE-1, indicating double-strand breaks (DSBs), than Noc 8 h treatment and control cells (Fig. 12A and 12B). Interestingly, the γ H2AX signals were much stronger in the micronuclei than the primary nuclei (Fig. 12A). Contrary to γ H2AX foci, we observed the opposite for 53BP1 staining which was more prevalent in the primary nuclei rather than the micronuclei. More specifically, the γ H2AX foci with strong intensity in the micronuclei displayed none or weak 53BP1 signal, whereas the strong 53BP1 foci in the primary nuclei showed weak or even negative staining for γ H2AX. In other words, we did not observe a clear co-localization of 53BP1 with γ H2AX in these severely aneuploid cells. Such discrepancy could be explained by previous reports demonstrating that DNA damage response factors such as 53BP1 were not able to be efficiently and properly recruited to DSBs in the micronuclei owing to the trafficking defects through the micronuclear envelope membrane (172, 188). Micronuclei also had incomplete nuclear envelope with strongly reduced density of nuclear pores (189-191) and inhibited nuclear import (172). We therefore hypothesized that the majority of γ H2AX foci were already resolved in primary nuclei by the time of detection because of efficient DNA damage repair, whereas γ H2AX foci persisted in the micronuclei partly as a result of failure of recruitment of components of the DNA repair machinery.

Consistent with the IF data, immunoblots also demonstrated greater DNA damage accumulation in severely aneuploid RPE-1 cells upon Noc 16 h treatment compared to Noc 8 h-induced mildly aneuploid cells and control cells, as was evident from the increased γ H2AX protein levels (Fig. 12C). Similar to what was observed in Noc-treated cells, 1 μ M Rev treatment also displayed stronger

accumulation of γ H2AX in RPE-1 cells than their mildly aneuploid counterparts (0.2 μ M Rev) and control (Fig. 12D). Notably, we did not observe significant difference in DNA damage between mildly aneuploid cells (Noc 8 h and 0.2 μ M Rev) and control cells. The increase in DNA damage correlated with elevated levels of senescence-associated markers such as p53 activation (phosphorylation at serine 15 residue of p53), p21 expression and loss of lamin B1 (192). Like RPE-1 cells, HCT116 cells treated with 1 μ M Rev also exhibited elevated DNA damage and senescence-associated markers (Fig. 12E). Furthermore, depletion of BUB1 and SMC1A also caused DNA damage and increased p21 expression in RPE-1 cells with the exception of shBUB1-1 (Fig. 12F). These results imply that aneuploidy-associated stresses leading to DNA damage correlate with severity of chromosome mis-segregation and degree of aneuploidy.

In addition to DNA damage, increased ROS levels have been shown as another critical stimulus for senescence induction (193). Thus, we asked if increased ROS was involved in aneuploidy-induced senescence in our study. To test ROS levels, we used the fluorogenic dye DCFDA (2',7'-dichlorofluorescein diacetate), which will be eventually converted into highly fluorescent DCF (2', 7'-dichlorofluorescein) by intracellular ROS, and intracellular ROS levels were measured by FACS. We found that Noc 16 h-induced senescent RPE-1 cells induced higher DCF intensity (~2-fold) as compared to euploid control, while mildly-aneuploid cells displayed a mild increase (~1.3-fold) when compared to control (Fig. 12G). This result suggests increased ROS production in aneuploid cells which correlates with the degree of aneuploidy as observed for DNA damage.

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Figure 12. Severe aneuploidy is associated with DNA damage and increased ROS levels. (A-B) RPE-1 cells were treated with Noc for 8 h and 16 h, followed by mitotic shake-off and culture in drug-free media for 1, 3 and 5 days. Cells were stained for DNA damage marker γ H2AX (green) and 53BP1 (red). (A) Representative IF images were shown. Scale bar, 10 μ m. (B) Plot shows percentage of cells containing one or more γ H2AX foci (mean $\pm$ SD). At least 100 cells were quantified per experimental condition. $n=3$. (C-F) Western blotting analyses of DNA damage marker γ H2AX and other senescence-associated markers in (B) Noc- and (C) Rev-treated RPE-1 cells, (D) Rev-treated HCT116 cells and (E) RPE-1 cells depleted of Bub1 or SMC1A. GAPDH was used as loading control. Synchronised cells treated with 100 ng/ml doxorubicin for 24 h were used as positive control for DNA damage in (B). Dox stands for doxorubicin. (G) RPE-1 cells treated as (A) were cultured in drug-free media for 5 days, followed by measure of intracellular ROS levels using DCFDA by FACS. (Left) Representative histogram of DCF intensity and (right) quantification of the relative mean DCF intensity ($n=3$). Data are expressed as mean $\pm$ SD. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ and ns, not significant by student's t -test.

3.4. Aneuploidy-induced senescence is dependent on p53

Next, we went on to examine the signalling pathways that underlie senescence induction upon severe chromosome mis-segregation. As shown earlier, greater p53 activation was observed in severely aneuploid cells than mildly aneuploid and control cells (Fig. 12C and 12D). P53 is a canonical senescence inducer (194, 195), and its activation is reported to be required for replicative senescence (196), DNA damage- (197) and oncogene-induced senescence (198). Moreover, previous reports have described the biphasic functioning of p53 during senescence (195, 199): in early stage of senescence, p53 is essential for senescence induction, whereas down-regulation of p53 is necessary for senescence evolution at late stage. Consistent with this notion, we also observed a decrease in p53 protein levels following induction

of senescence (Fig. 12C and 12D). Therefore, we hypothesized that p53 may contribute to senescence induction in our context. To test this hypothesis, we depleted p53 through shRNA in RPE-1 cells and determined the cell fates of severely aneuploid cells generated via Noc 16 h and 1 μ M Rev treatments. Cells harvested at Day 5 post-drug treatment were subjected to SA- β -gal staining. A sharp decrease in SA- β -gal-positive cells was observed in RPE-1 cells depleted of p53 compared to control cells carrying empty vector (Fig. 13A). We next performed BrdU incorporation assay for further substantiation. As expected, we observed significantly increased amounts of BrdU-stained cells in p53-depleted cultures compared to control (Fig. 13B). Furthermore, we observed increased lamin B1 and decreased p21 at the protein levels upon Noc 16 h and 1 μ M Rev treatments in p53-depleted cells, as determined by Western blotting analyses (Fig. 13C and 13D). These results demonstrate that p53 contributes to senescence induction in RPE-1 cells following severe chromosome mis-segregation.

Figure 13. Aneuploidy-induced senescence is dependent on p53 in RPE-1 cells. (A-B) RPE-1 shCtrl and shp53 cells treated with Noc for 16 h and 1 μ M Rev for 24 h were cultured in drug-free media for 5 days, and then subjected to (A) SA- β -gal staining and (B) BrdU-incorporation assay. (Left) Representative images and (right) plots showing percentages of (A) SA- β -gal-positive and (B) BrdU-positive cells. Scale bar, 50 μ m. Minimum of 100 cells were quantified per sample from three

independent experiments. Data are expressed as mean $\pm$ SD. *P* values were derived from Student's *t*-test (**P*<0.05, ****P*<0.001 and *****P*<0.0001). (C-D) RPE-1 shCtrl and shp53 cells were treated with (C) Noc and (D) Rev as in (A) and (B) and subjected to Western blotting analyses of indicated proteins. GAPDH was used as loading control.

Similarly, we compared the amount of senescent cells in HCT116 p53^{-/-} cells with WT cells after the same treatment as done in RPE-1 shCtrl and shp53 cells. SA- β -gal staining exhibited significantly reduced SA- β -gal activity in HCT116 p53^{-/-} cultures compared to WT cells after Noc 16 h and 1 μ M Rev treatments (Fig. 14A). Additionally, we also tested other senescence-associated markers by immunoblotting, including p21 expression, loss of lamin B1, decreased total Rb (retinoblastoma protein) and phospho-Rb (200, 201). We found that loss of p53 reduced p21 and increased lamin B1 expression, elevated Rb and phosphorylated Rb protein levels in HCT116 p53^{-/-} cells compared WT cultures (Fig. 14B and 14C). Taken together, these results indicate that severe aneuploidy-induced senescence is dependent on p53 in HCT116 cancer cells as well.

Figure 14. Aneuploidy-induced senescence is dependent on p53 in HCT116 cells. (A) HCT116 WT and p53^{-/-} cells were treated with Noc for 16 h and 1 μ M Rev for 24 h, followed by culture in drug-free media for 5 days and were subjected to SA- β -gal staining. Minimum of 100 cells were quantified per sample. Data are expressed as mean $\pm$ SD (n=3). * P <0.05 derived from Student's t -test. **(B-C)** HCT116 WT and p53^{-/-} cells were treated with (B) Noc and (C) Rev as per (A), and the expression of indicated proteins was evaluated by Western blotting analyses. GAPDH was used as loading control.

3.5. Aneuploidy-induced senescence displays pro-tumourigenic effects via its secretory phenotype

Senescence triggered by telomere exhaustion, DNA damage or oncogene activation is accompanied by a complex secretome termed senescence-associated secretory phenotype (SASP). The SASP factors consist of a variety of biologically-active soluble signalling factors, including interleukins,

chemokines, growth factors, proteases and regulators, as well as insoluble factors such as extracellular matrix components collagens, fibronectin and laminins, that can affect senescent cells themselves and the microenvironment by activating or inhibiting signalling transduction pathways (202). To determine whether aneuploidy-induced senescence also develops a SASP, we first assessed the mRNA expression of some well-established SASP factors such as IL-1 α , IL-1 β , IL-6 (interleukin 6), IL-8 (interleukin 8), FGF2, MMP1 and CCL20. qRT-PCR analyses showed increased mRNA expression of these SASP-associated genes in severely aneuploid RPE-1 cells generated via both Noc and Rev treatments compared to control cells at Day 5, whereas the majority of these genes were not significantly affected in mildly aneuploid counterparts compared to untreated control (Fig. 15A and 15B). Moreover, highly aneuploid HCT116 cells induced by 1 μ M Rev treatment also displayed enhanced mRNA expression of IL-1 β , IL-8 and MMP1 (Fig. 15C). In order to quantitatively measure the secreted SASP proteins, we analysed the conditioned media (CM) of control and Noc 16 h-induced severely aneuploid senescent RPE-1 cells for the secretion of 41 interleukins, chemokines, cytokines and growth factors by Luminex cytokine assay (Fig. 15D). It was found that 15 of these tested proteins such as IL-1 α , IL-6, IL-8, VEGF, FGF2, CXCL1, CCL3, CCL4, CCL5, and CCL7 displayed statistically significant up-regulation in CM from aneuploidy-induced senescent RPE-1 cells relative to control cells (Fig. 15E). These results suggest that aneuploidy-induced senescence provokes a SASP.

Figure 15. Aneuploidy-induced senescence promotes SASP. (A) qRT-PCR analyses of the mRNA expression of SASP genes and pro-inflammatory factors in RPE-1 cells 5 days (D5) after mitotic shake-off from Noc treatment for 8 h and 16 h. mRNA levels were normalized to β -actin. (B-C) qRT-PCR analyses of SASP components in (B) RPE-1 or (C) HCT116 cells 5 days (D5) after Rev wash-off. mRNA levels were normalized to β -actin. (D) Experimental setup for preparation of CM from control and Noc 16 h-treated RPE-1 cells for Luminex cytokine assay. Cells were synchronised as in Fig. 5C. (E) Concentrations of various cytokines and chemokines from CM derived from euploid (Ctrl) and severely aneuploid RPE-1 cells by Luminex cytokine assay. All data are expressed as mean $\pm$ SD from three independent experiments. * P <0.05, ** P <0.01, *** P <0.001, **** P <0.0001 and ns, not significant by Student's t -test.

Intriguingly, although loss of p53 suppressed senescence induction upon severe chromosome mis-segregation as shown previously, we found that p53 depletion enhanced the mRNA expression of SASP factors such as IL-1 α , IL-1 β , IL-6, IL-8 and MMP1 in both RPE-1 and HCT116 cells treated with Noc 16 h or 1 μ M Rev, compared to cells with intact p53 (Fig. 16A and 16B). Interestingly, as can be seen from the data, loss of p53 in both cell lines even without any drug treatment showed increased expression of these genes at the mRNA level. These findings are in agreement with a previous study that demonstrated functional loss of p53 amplified the development of SASP (203).

Figure 16. Loss of p53 promotes mRNA expression of SAPS components. (A-B) Fold changes of mRNA expression of SASP factors in (A) RPE-1 shCtrl and shp53 cells or (B) HCT116 WT and p53 $^{-/-}$ cells 5 days (D5) after Noc 16 or 1 μ M Rev treatment by qRT-PCR analyses. mRNA levels were normalized to β -actin. Plots are shown as mean $\pm$ SEM of four independent experiments.

Cellular senescence is regarded as a potent tumour-suppressor mechanism that irreversibly restrains the proliferation of cells at risk for neoplastic transformation (204). However, increasing evidence has shown that senescent cells can impact tumour progression through modulating the tissue microenvironment through SASP. Depending on the context, the SASP has pro- or anti-tumourigenic potentials. On one hand, the SASP factors suppress tumour development by reinforcing senescence and inducing senescence in an autocrine or paracrine manner (205, 206). In addition, inflammatory and immune-modulatory SASP factors can activate the immune response against the tumour, thereby promoting tumour cell clearance. On the other hand, SASP can also be immunosuppressive and pro-tumourigenic on neighbouring premalignant cells by promoting cell proliferation, angiogenesis, cell migration and invasion in a paracrine fashion (203, 207-210). To determine whether aneuploidy-induced SASP factors have pro-tumourigenic capabilities, CM from RPE-1 and HCT116 cells were used in various phenotypic assays. To evaluate migration potential *in vitro*, we performed scratch wound healing assay on human osteosarcoma U2OS cells due to their metastatic potential. To limit cell proliferation during cell migration, we used CM containing 0.5% FBS. Our results showed that U2OS cells exposed to CM from severely aneuploid RPE-1 cells induced by both Noc 16 h and 1 μ M Rev treatments migrated much faster than those treated with control CM (Fig. 17A and 17B), suggesting enhanced cell migration. Moreover, 1 μ M Rev-induced senescent CM from HCT116 cells also promoted wound closer at a faster rate than control CM (Fig. 17C).

It has been shown that one of the most prominent pro-tumourigenic effects of SASP is to promote cell proliferation (202, 207-209). We then asked whether the observed pro-migratory effect as shown earlier was due to enhanced cell proliferation rate or cell viability. To address this, we examined the cell number and viability of U2OS cells after incubation with respective CM. It was found that Noc- or Rev-induced senescent CM from RPE-1 cells did not significantly affect the proliferation and cell viability of U2OS cells compared to control CM (Fig. 17D and 17E), which ruled out any possible effect of cell proliferation and viability on cell migration.

A**U2OS + CM RPE-1** **B****U2OS + CM RPE-1** **C****U2OS + CM HCT116**

Figure 17. Aneuploidy-associated SASP promotes migration of U2OS cells *in vitro* without significantly affecting cell proliferation and viability. (A-C) U2OS cells were incubated for 24 h with CM derived from (A) control or Noc 16 h-treated RPE-1 cells, (B) control or 1 μ M Rev-treated RPE-1 cells, or (C) control or 1 μ M Rev-treated HCT116 cells. U2OS cells treated with respective CM were subsequently subjected to scratch wound healing assay. (Left) Representative phase-contrast images and (right) plots showing the percentage of wound area relative to the initial wound area at time 0 h (immediately after the wound scratch). Scale bar, 200 μ m. (D) U2OS cells seeded identically as in (A) and (B) were incubated with CM from (left) control or Noc 16 h-treated RPE-1 cells, or (right) control or 1 μ M Rev-treated RPE-1 cells for indicated duration. To be consistent with the wound healing assay in (A) and (B), 0 h represents pre-incubation with CM for 24 h. Plots show cell number at indicated time points. (E) Plots showing relative cell viability of U2OS cells treated with indicated CM for 3 days. All data reflect three independent experiments (mean $\pm$ SD). * P <0.05, ** P <0.01, **** P <0.0001 and ns, not significant by Student's *t*-test.

To further eliminate the possibility that the above-mentioned pro-migratory effects were due to the side-effects (if any) of "heavy" drug treatment, we tested the mRNA expression of some SASP factors and associated migratory potential following senescence induction in RPE-1 cells via knockdown of

SMC1A. Our qRT-PCR data revealed increased mRNA levels of IL-8, MMP1, CCL20 and FGF2 in RPE-1 shSMC1A-2 cells compared to control cells (Fig. 18A). Importantly, CM derived from shSMC1A-2 cells resulted in faster wound closure on U2OS cells (Fig. 18B), demonstrating that factors secreted from shSMC1A-induced senescent cells could also afford migratory potential. These results further confirmed that the secreted SASP factors from aneuploidy-induced senescent cells indeed could induce migratory changes in cancer cells.

Figure 18. shSMC1A-induced senescence increases the expression of SASP factors that promote U2OS cell migration *in vitro*. (A) RPE-1 cells were infected with lentivirus containing empty vector pLKO.1 (shCtrl) or shSMC1A-2 for 48 h, followed by selection with puromycin for 10 days. mRNA expression of indicated SASP genes was determined by qRT-PCR analyses. Plot shows fold change of mRNA levels. β -actin was used as normalisation control. (B) RPE-1 cells were treated as in (A). 10 days after puromycin selection, RPE-1 shCtrl and shSMC1A-2 cells were cultured with media supplemented

with 0.5% FBS for 3 days. CM were then collected and used in scratch wound healing assay on U2OS cells. U2OS cells pre-incubated with CM for 24 h were subjected to scratch wound healing assay. (Left) Representative images are shown. (Right) Plot showing the percentage of wound area relative to the initial wound area at 0 h (immediately after the scratch). All data are expressed as mean $\pm$ SD (n=3). *P* values were derived from Student's *t*-test (***P*<0.01, ****P*<0.001 and *****P*<0.0001).

To assess whether CM from aneuploidy-induced senescent cells could enhance cell invasion, we carried out the matrigel transwell invasion assay using CM derived from HCT116 cells. CM containing 0.5% FBS were mixed with culture media containing 40% FBS at a ratio of 3:1 to generate CM with 10% FBS. U2OS cells were incubated with CM with 10% FBS for 48 h and subjected to transwell matrigel assay. Results demonstrated that a higher number of U2OS cells treated with senescent CM (1 μ M Rev CM) invaded the bottom of the transwell inserts than cells exposed to control CM (Fig. 19), indicating the pro-invasive ability of aneuploidy-induced SASP factors.

Figure 19. Aneuploidy-associated SASP enhances U2OS cell invasion *in vitro*. U2OS were treated for 2 days with CM from control (DMSO) or 1 μ M Rev-treated HCT116 cells. The invasive ability of U2OS cells was assessed by matrigel transwell invasion assay. (Left) Representative crystal violet stain images were shown. (Right) Plot shows percentage of invasive cells. Data are expressed as mean $\pm$ SD (n=3). ***P*<0.01 by Student's *t*-test.

As shown previously, we observed increased expression and secretion of pro-angiogenic factors such as VEGF, PGF, S100A7 (psoriasin), LRG1, FGF2 and ANGPTL4, along with other interleukins and chemokines from RNA-seq (Fig. 9C), qRT-PCR (Fig. 9E and 15A) and Luminex cytokine assay (Fig. 15E). To examine the pro-angiogenic capabilities of these SASP factors, the *ex vivo* choroid sprouting assay (211) was utilised. Here, choroid explants from mice were treated with Noc 16 h-induced senescent CM or control CM diluted with culture media for four days. Choroid explants treated with senescent CM were observed to display increased vascular sprouting than those treated with control CM (Fig. 20), suggesting enhanced pro-angiogenic abilities of secretory factors from aneuploidy-induced senescent cells.

Figure 20. Aneuploidy-associated SASP enhances *ex vivo* choroid angiogenesis. Choroid explants from mice were incubated with CM from control- or Noc 16-treated RPE-1 cells for four days. (Left) Representative images of choroid sprouts formed and (right) plot showing the ratio of choroid sprouting area to explant area. Scale bar, 200 μ m. Data are expressed as mean $\pm$ SD (n=3). ****P*<0.001 derived from Student's *t*-test.

Next, we sought to investigate if the migratory phenotype observed *in vitro* could be recapitulated *in vivo*, we employed the zebrafish embryo model system as a means to monitor cellular migration and

invasion. The zebrafish embryo represents a tractable model for studying the metastatic potential of tumour cells owing to its transparent characteristic, making *in vivo* imaging of metastatic cells straightforward using conventional microscopy (212). U2OS cells were treated with CM (10% FBS) derived from either 1 μ M Rev-induced senescent or control HCT116 cells for two days, followed by labelling with a red-fluorescent tracking dye Dil and injection into 48 h post-fertilization (hpf) zebrafish embryos. Four days post-injection, embryos were imaged and the invasion index and migration index were determined for consistent quantitative analysis of the metastatic behaviour of differentially-treated cells in zebrafish embryos. Invasion index is defined as the number of metastases formed per one hundred cells injected, and migration index describes the distance travelled by metastases as percentage of the maximum distance cells can migrate within the zebrafish. U2OS cells incubated with CM from severely aneuploid senescent HCT116 cells were observed to migrate and invade farther from the injection site into the zebrafish body than DMSO-treated control cells (Fig. 21A), indicating enhanced invasive and migratory abilities. To investigate whether HCT116-derived senescent CM promote the invasiveness of their parental cells, the migratory and invasive capabilities of identically-treated HCT116 cells were measured. Similarly, senescent CM-treated HCT116 cells displayed greater migration index and invasion index compared to those exposed to control CM (Fig. 21B), further confirming that aneuploidy-induced SASP factors exert pro-tumourigenic metastatic effects on cancer cells. To further test whether senescent CM from non-transformed RPE-1 cells could also induce the same phenotypic effect on cancer cells, we treated U2OS cells with RPE-1-derived senescent CM or control CM, and found that 1 μ M Rev-induced senescent CM promoted the invasion of U2OS cells from the injection site through the zebrafish body (i.e. invasion index) (Fig. 21C). However, these invasive cells exhibited non-significant difference in the distance travelled (i.e. migration index) compared to the control group. These results led us to postulate that aneuploidy-induced senescent CM from cancer cells like HCT116 confer more invasiveness than normal cells like RPE-1.

For the same reason for scratch wound healing assay, we also examined whether the pro-invasive phenotype of senescent CM (10% FBS) observed in zebrafish model was affected by cell proliferation rate. The cell number of U2OS and HCT116 cells upon incubation with indicated CM was determined during the course of four days. Although senescent CM from HCT116 cells were found to slightly promote the proliferation of U2OS cells from Day 3(Fig. 21D), senescent CM from both RPE-1 and HCT116 cells had no significant effect on the cell number of U2OS and HCT116 cells compared to control CM (Fig. 21E and 21F). These results exclude the possibility that the enhanced metastasis of U2OS and HCT116 cells in zebrafish embryos after treatment with senescent CM was due to the increased cell proliferation rate. Taken together, these data demonstrate the pro-tumourigenic paracrine effects of aneuploidy-induced SASP *in vivo*.

A

U2OS + CM HCT116

B

HCT116 + CM HCT116

C

U2OS + CM RPE-1

Figure 21. Aneuploidy-associated SASP promotes cancer cell migration and invasion *in vivo*. (A-C) U2OS or HCT116 cells were incubated with indicated CM (10% FBS) from control or 1 μ M Rev-treated HCT116 or RPE-1 cells for 48 h, and stained with red-fluorescent dye Dil before injection into zebrafish embryos. White arrows in the zebrafish images indicate cells that invaded the zebrafish body. Scatter plots shown below represent all tumour foci observed in all tested larvae that belong to indicated treatment group. Tumour foci in one larva are shown in one colour. "n" represents number of injected embryos of three independent experiments. The metastatic ability was measured by invasion index and migration index as shown in the bar plots on the right (mean $\pm$ SEM, n=3). ** P <0.01, *** P <0.001, **** P <0.0001 and ns, not significant by Student's *t*-test. (D-E) Cell number of U2OS cells incubated with CM (10% FBS) from control or 1 μ M Rev-treated (D) HCT116 or (E) RPE-1 cells for indicated number of days. (F) Cell number of HCT116 cells incubated with CM (10% FBS) from control or 1 μ M Rev-treated HCT116 cells for indicated number of days. Line graphs in (D-F) are expressed as mean $\pm$ SD (n=3). ns, not significant by Student's *t*-test.

3.6. Senescent and aneuploid cells are frequently present at the invasive front in breast tumours

Based on our findings as described above, we set out to investigate whether the *in vitro* correlation of high levels of aneuploidy and senescence could be extended *in vivo* by analysing invasive ductal breast carcinomas from patients. We categorised breast tissue sections into "nipple" and "centre" regions as graphically represented in Fig. 22A. As illustrated, the nipple region was composed of mainly peri-tumoural normal area of mostly stroma, epithelial glands and capillaries, while the centre region mostly consisted of cancer cells. We first examined senescence by SA- β -gal staining on snap-frozen breast tissue sections from 5 patients, and found that senescent cells were identified in the tumour region at a higher incidence compared to normal nipple region (Fig. 22A and 22B). Interestingly, these senescent cells were observed to be frequently located at the invasive fronts instead of random distribution throughout the tumours. To confirm senescence, we performed immunohistochemical staining for

senescence-associated markers p21 and p27 (213) using serial sections of the corresponding breast tissues. We found that the tissue sections that showed strong staining for SA- β -gal also showed stronger intensity for both p21 and p27 (Fig. 22A and 22B), suggesting that expression of p21 and p27 correlated with SA- β -gal staining. These findings were supported by a recent study in papillary thyroid carcinomas (PTC) that demonstrated senescent tumour cells were frequently present at the invasive borders (214). Notably, they found that these senescent tumour cells exhibited high invasive ability compared with non-senescent tumour cells via SASP, and contributed to the collective invasion and metastasis of PTC. This prompted us to evaluate the SASP expression at the invasive front where senescent tumour cells were predominantly located versus the tumour centre region. Regions of interest from the same tumour samples analysed previously were microscopically dissected by an experienced pathologist. We determined the mRNA expression of various SASP factors by qRT-PCR analysis, and observed statistically significant up-regulation of MMP2, CCL2, CCL5, IL-6 and IL-8 at the invasive front compared with the corresponding centre area, with MMP9 displaying non-significant difference between these regions (Fig. 22C). These results suggest that the SASP may promote tumour cell invasion in breast cancers in a paracrine manner, as reported in PTC.

Figure 22. Prevalence of senescent cells and SASP at the invasive front in human primary breast tumours. (A) Tissue sections of 5 pairs of breast tumours and corresponding normal nipple regions were stained with HE (hematoxylin and eosin), SA- β -gal, p21 and p27, or counterstained with methyl green.

Images depicting the nipple region, tumour centre and invasive front are shown on the top, and images below show the representative staining for HE, SA- β -gal, p21 and p27. Red arrows denote positive staining. Scale bar, 500 μ m. **(B)** Plots showing the percentage of positively-stained cells for SA- β -gal, p21 and p27 in the normal nipple region, cancer centre and invasive front as in (A). The intensity for SA- β -gal was analysed using Image J, and p21 and p27 analysed using H score as described in materials and methods. **(C)** Plot shows relative mRNA expression of indicated SASP genes at the invasive front compared to the corresponding centre area. Actin is used as normalisation control. Data are expressed as mean $\pm$ SD (n=3). * P <0.05, ** P <0.01, *** P <0.001, and ns, not significant by Student's t -test.

To examine whether the level of aneuploidy correlated with the incidence of senescence in the normal nipple region, the tumour centre area as well as the invasive fronts in breast tumour tissues, we assessed the levels of aneuploidy using two-chromosomes locus-specific FISH probes for chromosome 13 (green) and chromosome 21 (red) in the same tumour tissues analysed previously. Representative FISH images and quantitation of signals for Chromosome 13 and 21 from one tumour sample were shown in Fig. 23A, and the results of three other tumour samples were displayed in Fig. 23B. We found that cells in the normal nipple area displayed the lowest levels of aneuploidy for these two chromosomes tested, while the invasive fronts exhibited the highest deviation from the diploid chromosome number (2 signals) (Fig. 23A and 23B). Notably, we observed an enrichment of cells displaying 3 or more signals for these two chromosomes at the invasive fronts compared to cells in the centre region and the normal area, suggesting the existence of near-triploid and near-tetraploid cells at the invasive front. This finding is in line with a recent study that revealed the presence of near-tetraploid cancer cells at the invasive front in primary colorectal tumours, which led to their speculation that tetraploidy could promote the invasiveness and aggressiveness of cancer cells (215). Importantly, our study revealed a correlation of greater deviation from the diploidy with increased incidence of senescence and SASP at the invasion front, indicating that the pro-tumourgenic paracrine effects of SASP from senescent tumour cells might plausibly promote the aggressive behaviour of tumour cells at the invasive front, as described previously *in vitro*.

A

Chr 13/Chr 21

B

Figure 23. Enrichment of aneuploidy at the invasive front in breast tumours. (A) (Top) Representative interphase FISH images using probes for chromosome 13 (green) and 21 (red) at cancer invasive front, corresponding cancer centre area and normal nipple region obtained from one human invasive ductal breast tumour. (Bottom) Plots showing distribution of FISH signals for chromosome 13 and 21 of 30 cells in each condition. **(B)** Distribution of FISH signals for (top panel) chromosome 13 and (bottom panel) chromosome 21 at the invasive fronts, the corresponding centre area and normal regions obtained from three other human breast tumours. n=30 cells.

To sum up our results so far, we propose a model where cells in response to mild chromosome segregation errors continue proliferation, whereas severely aneuploid cells as a result of higher degree of chromosome mis-segregation entered senescence in a p53-dependent manner (Fig. 24). Importantly, aneuploidy-induced senescence represents a mechanism by which aneuploidy may contribute to tumourigenesis, as revealed by the paracrine pro-tumourigenic effects of the SASP produced.

Figure 24. Proposed model of aneuploidy-induced senescence and potential role in tumourigenesis.

Relatively mild and severe forms of chromosome mis-segregation were induced through anti-microtubule drug (Noc), SAC suppression (Rev and shBUB1) and cohesion defects (shSMC1A). Mildly aneuploid cells generated as a result of mild chromosome segregation errors tend to continue proliferation. In contrast, severe chromosome mis-segregation leads to severely aneuploid offspring that undergo senescence in a p53-dependent manner. DNA damage and other aneuploidy-associated stresses may also contribute to senescence induction. Importantly, these senescent cells elicit a SASP that confers pro-tumourigenic paracrine effects such as enhanced cell migration, invasion and angiogenesis.

3.7. Activation of NF- κ B and p38MAPK and potential candidate genes in aneuploidy-induced senescent cells

Given the pro-tumourigenic effects of aneuploidy-associated SASP, it is of crucial importance to identify factors and signalling pathways that lead to senescence and/or SASP in response to severe chromosome mis-segregation. NF- κ B (nuclear factor kappa B) pathway has long been considered as a prototypical inflammatory signalling pathway (216) and it has been reported to be the master regulator of SASP in senescent cells triggered by oncogenic Ras (217). This promoted us to investigate whether NF- κ B pathway is required for the development of SASP in aneuploidy-induced senescent cells. In mammals, the NF- κ B complex involves homo- or heterodimerization of the five transcription factors: p105/p50 (NF- κ B1), p100/p52 (NF- κ B2), p65 (RelA), RelB and c-Rel (218), and the most common complex is a dimer formed by p50 and p65 (219). In unstimulated cells, NF- κ B is inhibited by the inhibitor of κ B (I κ B) family in the cytoplasm. Upon various stress signals, phosphorylation of the I κ B and p65 subunits results in release of p65 and nuclear translocation of the p65/p50 complex, causing transcriptional activation of a wide range of proinflammatory genes (220). To assess NF- κ B activation, we prepared whole cell lysates from Noc 16 h- and 1 μ M Rev-treated RPE-1 cells and analysed total and p-p65 (Ser536) by Western blotting. Indeed, a dramatic increase in the protein levels of p-p65 was observed in highly-aneuploid cells, while total p65 protein levels remained unchanged, indicating p65 activation (Fig. 25A). In support of this, we also observed enhanced expression of p-I κ B and a concomitant decrease in total I κ B (Fig. 25A).

Likewise, we also observed increased p-p65, decreased I κ B and increased p-I κ B (Day 3) levels in HCT116 cells upon treatment with 1 μ M Rev (Fig. 25B). Furthermore, immunoblotting of the nuclear fraction of senescent cells confirmed accumulation of p65 on the chromatin (Fig. 25C). However, since these methods are indirect readouts for NF- κ B transcriptional activity, using the direct methods such as the chromatin immunoprecipitation assay and luciferase reporter assay to measure the promoter binding and actual transcriptional activity of NF- κ B is preferred in future study. Nonetheless, it would be interesting to examine if p65 suppression by either shRNA or small molecule inhibitors (e.g. BAY 11-7082) would modulate the expression of SASP in response to Noc- and Rev-induced severe chromosome mis-segregation.

Moreover, the Campisi team has described p38 α MAPK (mitogen-activated protein kinase) (MAPK14) as a key regulator of the SASP by enhancing NF- κ B activity in the context of genotoxic stress (221). Interestingly, Noc-induced senescent RPE-1 cells in our context indicated a sharp increase in p-p38 (Thr180/Tyr182) from Day 3 by Western blotting, with total p38 protein levels unchanged (Fig. 25A). However, 1 μ M Rev treatment only slightly increased the phosphorylation of p38 in both RPE-1 and HCT116 cells (Fig. 25A and 25B). Although the extent of p38 activation varies in these conditions, the general activation of p38 implies that the p38MAPK response potentially modulates the expression of SASP by enhancing the transcriptional activity of NF- κ B in aneuploidy-induced senescent cells.

To test whether p38 is indeed involved in SASP expression, we compared the mRNA expression of various cytokines and chemokines in the presence and absence of the well-characterised p38MAPK inhibitor SB203580 (SB). SB inhibits the catalytic activity of p38 by binding to its ATP binding pocket without inhibiting the phosphorylation of p38 itself by upstream kinases (222). We used SB at a concentration of 10 μ M which is reported to have minimal off-target effects (223). Western blotting analysis of MAPKAPK2 (mitogen-activated protein kinase-activated protein kinase 2) which is the direct target of p38, and its target HSP27, confirmed that daily treatment with SB suppressed phosphorylation of

both MAPKAPK2 and HSP27 (Fig. 25D), suggesting inhibition of the p38MAPK signalling cascade. Interestingly, qRT-PCR analyses revealed that severely aneuploid cells treated with SB blunted the mRNA expression of IL6 and CCL7 but increased the mRNA levels of other SASP factors such as IL-1 β , IL-8 and CCL3 (Fig. 25E). These preliminary data suggest that p38 activation may be responsible for, either by itself or in cooperation with other factors or signalling pathways, the expression of a subset of SASP genes in Noc-induced senescent cells. To further understand to what extent p38MAPK regulates the SASP, analysis of the secretome of senescent cells by either cytokine array or mass spectrometry could be done in future studies.

Figure 25. NF- κ B and p38MAPK are activated during aneuloidy-induced senescence and p38MAPK is required for the expression of some SASP factors. (A-B) (A) RPE-1 and (B) HCT116 cells were treated with Noc for 16 h or 1 μ M Rev for 24 h as described previously. Cells were collected at indicated days and whole cell lysates were subjected to Western blotting analyses. Shown are indicated proteins with GAPDH as loading control. (C) Nuclear fractions were isolated from RPE-1 and HCT116 cells treated as per (A) and (B) and Day 3 samples were harvested and analysed by Western blotting.

Nup153 (Nucleoporin 153) was used as loading control. **(D)** RPE-1 cells were treated with Noc for 16 h and mitotic cells collected by shake-off were cultured for indicated number of days with or without 10 μ M SB203580 (SB). Western blotting analyses of indicated proteins were shown. GAPDH was used as loading control. **(E)** RPE-1 cells treated with Noc for 16 h were cultured for 5 days in the absence or presence of SB. Expression of SASP genes was measured by qRT-PCR. β -actin was used for normalising gene expression. Data are mean $\pm$ SD (n=3). * P <0.05, ** P <0.01, *** P <0.001, and ns, not significant by Student's *t*-test.

In addition to studying the molecular pathways that regulate the SASP itself, we were also interested in identifying the molecular mechanisms underlying the establishment of senescence, although these two types of pathways may overlap such as NF- κ B (224). To this end, we looked into our RNA-seq data for potential targets whose down- or up-regulation may possibly mediate senescence induction. Among the significantly down-regulated genes in aneuploid cells immediately following chromosome mis-segregation, one gene of interest is FoxM1 (Fig. 9G), which has been reported to be down-regulated in senescent cells and its constitutive activation bypassed senescence arrest (224). We further found that FoxM1 was expressed at much lower levels in senescent cells (Day 5 samples) compared to euploid control in RPE-1 cells by qRT-PCR analyses (Fig. 26A and 26B). Consistently, Western blotting analyses also displayed dramatically decreased protein expression of FoxM1 upon Noc 16 h and 1 μ M Rev treatments in RPE-1 and HCT116 cells (Fig. 25A and 25B), leading to our hypothesis that down-regulation of FoxM1 might play a role in senescence induction. Interestingly, a recent report studying aneuploidy in the aging context showed FoxM1 repression in aged primary human fibroblasts which exhibited increased chromosome mis-segregation and aneuploidy (225). Importantly, they found that depletion of FoxM1 in young fibroblasts increased mitotic defects and induced senescence, and restoration of FoxM1 in elderly fibroblasts prevented aneuploidy and delayed the onset of senescence, leading to their conclusion that FoxM1 levels regulate aneuploidy and senescence. This study further lends support to the idea that FoxM1 repression might contribute to senescence in our context.

Apart from FoxM1, another candidate EZH2, a PcG (Polycomb group) histone methyltransferase that mediates the trimethylation of lysine 27 on histone H3 (226), is also among the significantly down-

regulated genes in aneuploid cells from our RNA-seq data, which was confirmed by qRT-PCR analysis (Fig. 9G, 26A and 26B). EZH2 expression decreases during senescence and its down-regulation causes DNA damage and increases p21 level, leading to premature senescence exhibiting features of SASP (227). It is reasonable to propose that severely-aneuploid cells enter senescence in part through down-regulation of EZH2. Future study would be to delineate the role of EZH2 in senescence induction.

Besides the down-regulated genes in aneuploidy-induced senescent cells, we also found up-regulation of genes, including ATF3 (activating transcription factor 3) (Fig. 26A and 26B). ATF3 is a common stress responder and has been shown to bind to p53 and therefore prevent p53 from degradation (228), suggesting its potential role in contributing to senescence induction by stabilising p53 in our context. It remains to be determined whether these genes play a role in senescence induction.

Figure 26. Suppression of FoxM1 and EZH2 and increase of ATF3 in aneuploidy-induced senescence in RPE-1 cells. (A-B) qPCR analyses of indicated genes in RPE-1 cells treated with (A) Noc for 16 h or (B) 1 μ M Rev and cultured in drug-free media for 5 days. Relative mRNA levels were normalised to actin. Data represent three independent experiments (mean $\pm$ SD). * P <0.05, ** P <0.01, *** P <0.001 and **** P <0.0001 by Student's t -test.

4. Discussion

Chromosomal instability (CIN) and aneuploidy are both regarded as the universal features of cancer. Elevated CIN and aneuploidy are reported to be associated with poor prognosis and drug resistance (167, 169, 229, 230), strongly suggesting the role of abnormal ploidy in contributing to tumourigenesis (2, 231-233). Nevertheless, how CIN and aneuploidy affect cell physiology and tumourigenesis remains largely unknown. Here, the purpose of this study was to investigate the consequences of aneuploidy on cell physiology and its potential role in tumourigenesis. We generated different levels of random aneuploidies using chemical and genetic intervention mechanisms. Our results indicate that mildly aneuploid state (with < 5 chromosomes lost or gained) was well-tolerated and cells tend to continue proliferation. On the contrary, severely aneuploid populations (with ≥ 5 chromosomes lost or gained and polyploidy) ceased to divide and ultimately entered senescence, and produced a SASP (senescence-associated secretory phenotype) that engendered pro-tumourigenic effects via paracrine action both *in vitro* and *in vivo*. Our results showed distinct levels of aneuploidy differentially affect cell physiology, and we suggest a new mechanism of how chromosome mis-segregation could exert pro-tumourigenic functions.

4.1. Is p53 an obligatory outcome of chromosome mis-segregation?

In this study, we observed heightened expression of p53 in response to both Noc- and Rev-induced chromosome mis-segregation. Notably, severely aneuploid cells with massive amount of chromosome imbalances and alterations displayed greater accumulation of p53 than their mildly aneuploid counterparts (Fig. 12C and 12D). This observation led us to speculate that p53 might be a general response to the aneuploid state, which correlated with the level of aneuploidy. Elevated expression of p53 has also been detected in the central nervous system from patients with Down syndrome (234, 235). Also, Thompson *et al.* previously reported chromosome mis-segregation induced by monastrol washout in diploid human cells led to activation of p53 in cell culture (132). Monastrol is an

inhibitor of the mitotic kinesin Eg5 (236), a motor protein important for bipolar spindle assembly, and causes formation of monopolar spindle and mitotic arrest (237). However, subsequent study revealed that prolonged prometaphase arrest (> 1.5 h) induced by microtubule-targeting drugs (i.e. nocodazole, monastrol and taxol) triggered p53 activation in the daughter cells irrespective of whether chromosomes mis-segregated or not in their mother cells (238). This led to their proposal of the existence of a cellular mechanism that cells could somehow sense the duration of prometaphase. Moreover, studies from other groups where they generated aneuploidy from otherwise diploid cells using methods that did not cause prolonged mitosis also reported that chromosome mis-segregation *per se* did not always lead to a p53 response in aneuploid daughter cells by tracking the cell fate of single cells post chromosome mis-segregation through live-cell imaging (138, 239). Given these observations, we reasoned that the increased p53 protein levels after Noc treatment for 8 h or 16 h may be potentially affected by the prolonged prometaphase arrest (> 1.5 h) to some extent. This also made us realize that agents that cause increased mitotic duration may not be the ideal method to induce chromosome mis-segregation and aneuploidy. It is also important to keep the mitotic duration within 1.5 h to eliminate any possible adverse effects of prolonged mitotic arrest on the fitness of daughter cells.

Apart from Noc, we also used Rev to induce chromosome mis-segregation. Different from Noc, Rev inactivates SAC and therefore accelerates rather than delays mitotic progression. RPE-1 cells treated with 0.2 μ M and 1 μ M Rev both triggered p53 activation with the latter showing stronger p53 response (Fig. 12D), suggesting the correlation between the level of aneuploidy and p53 activation. The greater increase of p53 upon 1 μ M Rev treatment could be a result of severe chromosome mis-segregation on one hand. However, on the other hand, this may also be in part owing to the higher percentage of cells with abnormal mitosis induced by 1 μ M Rev treatment as compared to 0.2 μ M Rev treatment, as shown by the live-cell imaging (Fig. 7B). While it seems that chromosome mis-segregation, especially severe chromosome segregation errors, tends to induce a p53 response, it does not support the idea of p53 activation in every single aneuploid daughter cell. The fact that the majority of mildly aneuploid cells

induced by both Noc and Rev continued to proliferate after an initial transient G1 cell cycle arrest suggests the idea that aneuploidy may not always provoke p53 activation. This notion is also supported by the observation that trisomic MEFs did not bring about a p53 response by others (126). Also, deficiency of the SAC protein Mad2 was tolerated in murine interfollicular epidermal cells, indicative of weak activation of p53 (240). Alternatively, depending on the severity of chromosome mis-segregation and level of aneuploidy, or even combinations of certain chromosomes, the amount of p53 accumulated in aneuploid cells may determine whether or not a p53-dependent cell cycle arrest occurs. Taken together, it is not evident from our results that p53 activation is an obligatory outcome of chromosome mis-segregation.

While most of the mildly aneuploid cells were still able to continue proliferating, a significant amount of cells in the severely aneuploid populations (Noc 16 h and 1 μ M Rev) entered a stable proliferative arrest, namely senescence, and were associated with strong and persistent p53 activation even 5 days after chromosome mis-segregation (Fig. 12C and 12D). What are the mechanisms responsible for the persistent p53 activation and cell cycle arrest in these severely aneuploid cells? It has been proposed that the answer may not be the aneuploid state *per se*, but the cellular stresses associated with chromosome mis-segregation and aneuploidy such as DNA damage, oxidative stress and proteotoxic stress and so forth (125, 130, 138, 241). In agreement with this notion, indeed, we observed strong DNA damage in highly aneuploid cells induced by Noc 16 h and 1 μ M Rev treatments (Fig. 12). However, it has not escaped our attention that prolonged mitotic arrest has been reported to induce telomere deprotection and DNA damage foci at telomeres, as well as a p53-dependent cell cycle arrest in the subsequent G1 phase (242), which suggest that DNA damage observed in highly aneuploid cells after Noc treatment in our study might not only be attributed to chromosome mis-segregation and the aneuploid state. This argues against the idea that DNA damage induced by Noc 16 h treatment is purely a result of the aneuploid state *per se*, and therefore it is less convincing that aneuploidy-associated DNA damage contributes to p53 activation based on these data. Nevertheless, we did observe increased DNA damage

after 1 μ M Rev treatment (Fig. 12D and 12E), arguing that DNA damage associated with chromosome mis-segregation may contribute to p53 activation. The sources of DNA damage may be the lagging chromosomes which can be trapped at the cleavage furrow (171). Micronuclei is another major source of DNA damage due to defects in DNA replication, DNA repair and rupture of nuclear envelope induced by lamin disorganization (172, 243), which is supported by our IF data (Fig. 12A). Moreover, premature entry into anaphase induced by SAC inhibition could result in anaphase onset before the complete DNA replication or decatenation, leading to DNA damage in daughter cells. To conclude, chromosome mis-segregation has the potential but is not sufficient to activate p53.

4.2. Mild chromosome mis-segregation can be tolerated and propagation of severe aneuploidy is restricted to p53-deficient cells

Our results demonstrated that while low level of aneuploidy following mild chromosome mis-segregation continued to proliferate although p53 stabilisation and a transient G1 arrest were observed, severe forms of aneuploidy (including polyploidy) induced a much stronger p53 response and eventually entered senescence (Fig. 10). These findings suggest that the amount of mis-segregated chromosomes and level of aneuploidy may represent an important determinant of their cell fate. Similar to our observation, the Amon lab also reported senescence induction in RPE-1 cells with complex karyotypes (138). Moreover, studying in the aging context, a previous study by the Montagna group also demonstrated that whole chromosome instability was sufficient to induce senescence in human fibroblast (133). Although aneuploidy-associated cellular stresses such as DNA damage and oxidative stress have been proposed to potentially contribute to senescence induction (133), how CIN and aneuploidy induce senescence remains to be addressed. Here, we showed that this anti-proliferative property of aneuploidy was p53-dependent as p53 depletion abrogated the senescence phenotype in both normal and cancer cells (Fig. 13 and 14). It is likely that only when p53 was activated to a certain threshold can aneuploid cells be prevented from further proliferation. As has been shown previously, the degree of aneuploidy correlated with the level of cellular stresses (107, 125), it is plausible that when the cellular stresses exceed a certain level, p53

becomes activated to the extent that prohibits cell proliferation. Alternatively, we could not rule out the possibility that particular combinations of certain chromosomes may cause cellular imbalances that interfere with essential cellular processes, leading to either cell cycle arrest or apoptosis. Given that p53 deficiency is sufficient to override the senescence growth arrest as a result of severe chromosome segregation defects, this may give rise to descendants with complex karyotypes that are rapidly proliferating and have enhanced oncogenic potential. In fact, genetically engineered mice with CIN accelerated tumour development drastically when p53 was depleted (130). Thus, it will be intriguing to test whether these severely-aneuploid cells in the absence of p53 would form tumours when injected into nude mice.

Although we have shown that propagation of severe aneuploidy was limited in a p53-proficient setting, it has come to our attention that ablation of p53 was not able to completely rescue the proliferation defects, especially in 1 μ M Rev-induced severely aneuploid cell populations (Fig. 13 and 14). Possibly, when the cellular stresses exceed a certain level in those extremely aneuploid cells, p53 may no longer be able to counteract those anti-proliferative factors. While it remains unclear why p53 depletion has a stronger anti-senescent effect in Noc 16 h-induced severe aneuploidy than 1 μ M Rev-treated populations, these data suggest the existence of other factors and pathways that prevent the proliferation of severely aneuploid cells. Besides p53/p21 pathway, Rb/p16 tumour-suppressor pathway has been involved in oncogene-induced senescence (244), replicative senescence and DNA damage-induced senescence (245). However, we observed no significant difference in p16 expression at the protein levels in senescent RPE-1 cells in comparison to euploid control cells (data not shown), suggesting p16 pathway may not play a role in senescence induction in this context. Unfortunately, we were unable to detect p16 expression in HCT116 cells by immunoblotting either due to low expression of p16 or the antibody used. However, our data imply that persistent DNA damage exhibited by severely aneuploid cells may act as a driver for the establishment of senescence. Unfortunately, this could not be tested in our study since inhibition of the DNA damage response by either knockdown of ATM or ATR,

or treatment with ATM inhibitor KU-55933 or caffeine which inhibits both ATR and ATM, could not override aneuploidy-induced senescence, but induced cellular apoptosis or senescence instead. Although direct evidence of DNA damage in contributing to aneuploidy-induced senescence is lacking, our study raises the possibility of DNA as a potential mechanism responsible for senescence induction. Additionally, our preliminary data suggest increased ROS, another well-known senescence inducer, in severely aneuploid cells. Although our current data do not prove the causative role of ROS in senescence induction, it is possible that ROS may represent another source by which aneuploidy could induce senescence.

Additionally, we found reduced expression of the transcription factor FoxM1 in severely aneuploid cells, whose repression has been recently shown to lead to senescence during aging (225). The polycomb group (PcG) histone H3 lysin-27 methyltransferase EZH2, another down-regulated candidate gene in aneuploidy-induced senescent cells, has been reported to be suppressed in senescent cells and its down-regulation promotes senescence (227). These studies indicate that FoxM1 and EZH2 might cooperate with aneuploidy-associated stresses to trigger senescence in our context. Moreover, the up-regulation of the p53 activator ATF3 following severe chromosome mis-segregation probably be partly responsible for inducing senescence. It would be intriguing to investigate the potential role of FoxM1, EZH2 and ATF3 in aneuploidy-induced senescence in the future.

Taken together, our data suggest that the severity of chromosome mis-segregation might determine whether cells are arrested or continue propagation, and that p53 depletion could overcome these growth-inhibiting barriers at least in a subset of aneuploid cells that would otherwise be permanently arrested in a p53-proficient background.

4.3. The dark side of aneuploidy-induced senescence and SASP in tumourigenesis

In this study, a significant proportion of severely-aneuploid cells were observed to eventually cease proliferation and enter a state of permanent cell cycle arrest and exhibit features of senescence such

as increased SA- β -gal activity. Although senescent cells remain metabolically active, senescence is recognized as a potent cancer-protective mechanism since it permanently arrests cells at risk for malignant transformation (246). As such, senescence has been considered as a desirable outcome for anti-cancer therapy, and efforts have been made to find compounds that induce senescence in tumour cells (247). These types of drugs have shown promise for the treatment of various cancers in clinical studies (248-250). Studies in mice also supported the tumour-suppressive role of senescence induction in tumour cells (251-253). In our study, however, further characterisation of the senescent cells induced by chromosome mis-segregation revealed functional consequences for tumourigenesis. Paradoxically as it may seem, senescence can promote tumour development through SASP (207, 254, 255) that consists of a variety of inflammatory cytokines, chemokines, growth factors, extracellular matrix and matrix metalloproteinases. Secreted SASP factors can alter the tumour microenvironment and affect adjacent cells. Depending on the context and target cells, the SASP can have tumour-suppressing capabilities by activating the immune response (251-253, 256), inducing paracrine senescence (257, 258) or enforcing senescence in an autocrine manner (205, 206, 259). Conversely, the SASP can also promote tumour aggressiveness, metastasis, cancer initiation and relapse (260-262), and cancer stemness (263). Here, aneuploidy-induced senescent cells were found to secrete SASP factors that promoted cancer cell migration, invasion and angiogenesis in neighbouring cells in a paracrine fashion (Fig. 17-21). Given the aneuploid nature of most solid tumours, it is tempting to speculate that aneuploidy-induced senescence may represent a cell non-autonomous mechanism in promoting tumour development and cancer recurrence.

By investigating the relevance of aneuploidy-induced senescence and level of aneuploidy *in vivo*, we found aneuploidy and senescence were present at a higher incidence in the tumour region than the matched normal region in invasive ductal breast carcinomas (Fig. 22 and 23). Very interestingly, higher degree of aneuploidy, increased incidence of senescence and SASP were localized at the invasive front of the tumoural region relative to the intratumoural center area (Fig. 22A and 22B). These findings are

supported by a recent study in papillary thyroid carcinoma (PTC) illustrating the frequent presence of senescent cells at the invasive border, and higher invasive ability of the senescent tumour cells than non-senescent tumour cells via SASP expression (214). Interestingly, in addition to the paracrine capabilities, senescent cells themselves could lead the collective invasion, a process that tumour cells invade as a cohesive multicellular unit as opposed to single-cell migration, and increase cancer cell survival in PTC (214). Similar to PTC, breast cancers also display features of collective invasion (264). Thus, it will be of particular interest to determine whether senescent cells induced by aneuploidy also lead the collective invasion in breast carcinomas. It will also be of great importance to investigate the extent to which aneuploidy can impact senescence at the tumour invasive front. Overall, our results add a new dimension on how aneuploidy and CIN could potentially contribute to tumour development.

The strong association between CIN, aneuploidy and tumourigenesis also prompted Santaguida and colleagues to investigate the pathways that prevent aneuploid cells from becoming cancerous. They found that cells with complex karyotypes senesced and produced pro-inflammatory signals that promoted the clearance of these senescent aneuploid cells by the natural killer (NK) cells *in vitro* (138). Although aneuploidy-induced senescent cells may be cleared by the immune system during tumour development, our study shows that the SASP factors from cells harbouring a higher degree of aneuploidy confer pro-tumourigenic effects via paracrine action. How do we reconcile these seemingly contradicting results? One can speculate that once the SASP factors were produced, they may promote the clearance of aneuploidy-induced senescent cells by attracting immune cells, together with the enhanced expression of immune recognition molecules present on senescent cells. In the meanwhile, these SASP factors could stimulate tumourigenic effects on bystander cancer cells. This could mirror the good and dark sides of the SASP in tumourigenesis. In a recent large-scale analysis study that analysed data from over 5000 tumour versus normal samples representing twelve human cancer types from the TCGA (The Cancer Genome Atlas) project reported that aneuploidy correlated with immune evasion and inversely correlated with response to immunotherapy (265). They found that most of the tumours that exhibit extensive aneuploidy,

which displays highly abnormal number of whole chromosomes and chromosomal segments, decreased cytotoxic immune infiltration. They also showed that melanoma patients displaying highly aneuploid tumours showed poor survival after immunotherapy compared to patients with more normal karyotypes in their tumours. These apparently contradictory findings (immune recognition versus immune evasion) raise the possibility that the immunogenic trait of aneuploidy could be transformed to an immune evasive feature at some point during cancer development, eventually leading to the correlation between aneuploidy and immune evasion. Alternatively, the paracrine pro-tumourigenic effects of aneuploidy-associated SASP become more prominent only within an immunosuppressive tumour microenvironment.

Intriguingly, although p53 is required for the growth arrest following chromosome mis-segregation, ablation of p53 was observed to amplify the expression of SASP factors (Fig. 16). How to reconcile these seemingly contradictory results? Firstly, one needs to understand that not all senescent cells develop a SASP. The SASP is not an inevitable consequence of the senescence growth arrest and not tied to the growth arrest and the SA- β -gal expression (203, 266, 267). For example, ectopic expression of p16 induces senescence growth arrest but is not able to promote SASP like DNA damage (266), and the authors suggested that SASP is not a consequence of senescence *per se*, but rather a response to damage that is separate from the growth arrest. Consistent with this idea, p53 inactivation that reversed (203) or bypassed replicative senescence (268) driven cell proliferation with a concomitant increase in DNA damage and enhanced SASP expression than senescent cells with intact p53, further lending support to the notion that SASP could be a result of stress response. Based on this notion, we speculated that although p53 depletion permitted the proliferation of severely aneuploid cells, aneuploidy-associated stresses may not be abated if not exacerbated. Hence, this raises the interesting possibility that loss of p53 enhanced SASP expression by causing more damage in these non-senescent cells. Future research on the mechanisms of how p53 modulates SASP expression would provide more insights in the role of p53 in cancer. On the other hand, a recent study showed that cells with extra centrosomes that were negative for SA- β -gal staining and displayed high level of proliferating cells induced paracrine invasion via secretion

of pro-invasive factors such as IL-8, ANGPTL4, GDF15 and PAI, which were induced by oxidative stress (269), suggesting that the secretory phenotype that is a characteristic of senescence could be induced in damaged non-senescent cells. Consistent with this idea, our RNA-seq and qRT-PCR data (Fig. 9C and 9E) also showed that severely aneuploid cells 24 h after chromosome mis-segregation already displayed increased expression of pro-inflammatory genes before entering senescence, although the expression levels of these genes were lower than senescent cells on day 5 (Fig. 15A). Our result is in line with previous reports describing p53 restrains the expression of SASP components during DNA damage-induced senescence, replicative senescence as well as oncogene-induced senescence (203, 221), even though the mechanisms are currently unknown.

It will be interesting to test whether CM derived from severely aneuploid cells in the absence of p53 confer stronger paracrine pro-tumourigenic properties than those produced from p53 intact cells. The function of p53 in restraining SASP suggests that p53 may suppress tumourigenesis partly by inhibiting the development of a pro-inflammatory tissue microenvironment. In fact, over half of all human tumours harbour p53 mutations that compromise its function (270). It is conceivable that inhibition or loss of p53 function, on one hand, permits cell survival and promotes proliferation by preventing cell apoptosis and senescence; on the other hand, it aids the development of a pro-inflammatory microenvironment that potentially fuels tumourigenesis.

Although we have found aneuploidy-associated SASP factors tend to promote the invasiveness on transformed cancer cells, another area of research that we have not tested yet but is equally exciting would be to see how these SASP factors influence normal bystander cells. It has been shown previously that senescent human fibroblasts, regardless of how senescence was induced, stimulated the proliferation of malignant and premalignant but not normal epithelial cells and greatly accelerated tumourigenesis, at least in part owing to the various factors secreted by senescent fibroblasts (207). This study indicates that the secretome of senescent cells differentially affect normal cells and malignant cells. Hence, the authors suggested a link between aging and cancer: as senescence and somatic mutations both increase with age

(184, 271, 272), senescent cells may create a microenvironment that stimulates the proliferation and progression of cells with oncogenic mutations. In line with our data, the Montagna group demonstrated that CIN-driven senescence in human primary fibroblasts promoted the development of SASP (133). They hypothesize that this may contribute to aging and age-related diseases as the CIN-associated secretome could potentially elicit growth arrest in neighbouring diploid normal cells via the SASP-mediated paracrine senescence effect. Based on these reports as well as our results, it will be of interest to study the functional consequences of aneuploidy-associated SASP on normal cells. We postulate that the SASP of aneuploidy-induced senescent cells might possibly cause senescence in bystander normal cells, as opposed to the pro-tumourigenic effects on preneoplastic and malignant cells. However, if this is the case, it raises the possibility that the secreted SASP factors from severely aneuploid cells may induce paracrine senescence in the aneuploid cells themselves during the course of the experiment in our study. To test this possibility, one solution would be to determine whether culture of their parent diploid cells with senescent CM leads to paracrine senescence.

As mentioned above, CIN and aneuploidy have been implicated in aging and aging-related pathologies, including cancer. Aneuploidization has been shown to increase with age in various mammalian tissues such as the brain (100) and the liver (96, 273). Some mouse models of CIN also demonstrated that aneuploidy caused premature aging phenotypes (163, 274, 275). In addition to CIN and aneuploidy, cellular senescence has also been recognized as a hallmark of aging (276), and it has been proposed to contribute to aging through its associated pro-inflammatory secretome. Also, suppressed immune surveillance has been observed in the elderly, which can increase cancer occurrence (277). Thus, aneuploidy-induced senescence may represent a mechanism by which cancer risk increases with age. Therefore, an interesting area of research would be to illuminate the links between aging and cancer through aneuploidy-induced SASP from the tissue microenvironment perspective.

4.4. Potential role of polyploidy in senescence and tumourigenesis

It is important to point out that the severely aneuploid cells we generated in this study consisted of a subset of polyploid cells including tetraploid and even octoploid cells (Fig. 6B, 7G and 8C). Indeed, published data from our lab demonstrated that antimitotic drugs (e.g. Noc)-enforced mitotic slippage (cells "slipped" into next G1 phase without cytokinesis) and subsequent multinucleated polyploidy in U2OS cells induced senescence as well as a SASP which affords pro-tumourigenic effects (278). During the development of many human carcinomas, tetraploid cells are often found in pre-cancerous lesions and early stages of tumour development in many human carcinomas (279-282). Analyses of almost 5000 cancers from the TCGA dataset showed that up to 37% of all human cancers underwent whole-genome doubling at some point during tumour progression (283). In light of the tumourigenic potential of tetraploid cells, tumour suppressor mechanisms have evolved to limit their proliferation, leading to either cell cycle arrest or apoptosis (284, 285). However, proliferating polyploid cells are genetically unstable and are thought to act as a transient intermediate to sub-tetraploid or highly aneuploid cells with oncogenic potential (77, 286). Additionally, p53-null mouse mammary epithelial cells caused marked increase in chromosomal abnormalities and promoted tumour formation in comparison to their genetically matched diploid cells (287). Similarly, telomere dysfunction-induced tetraploidization also promoted tumourigenesis (286). Not surprisingly, polyploid tumour cells have emerged as critical drug target in the treatment of cancer.

Importantly, we also observed the enhanced occurrence polyploid tumour cells at the breast tissues, especially at the invasive front (Fig. 23), supporting the potential role of polyploidy in tumour development. A recent study reported that near-tetraploid cancer cells exhibited elevated invasiveness and displayed CIN (215), lending support to our findings. Our results therefore describe another mechanism by which polyploidization *per se* has the potential to contribute to tumourigenesis through senescence and SASP. Unfortunately, our current data did not indicate the extent to which polyploid cells contribute to senescence induction in severely aneuploid populations.

4.5. Potential mechanisms that regulate SASP in aneuploidy-induced senescence

In view of the tumourigenic potential of SASP proteins, it is of crucial importance to identify the molecular pathways that influence the development of SASP in aneuploidy-induced senescent cells. The senescence cell-cycle arrest and the SASP are often regulated coordinately, but their pathways do not overlap completely. For example, p53 is a well-known inducer of senescence, yet it restrains SASP. Likewise, ectopic expression of p16 is sufficient to induce a senescence stable growth arrest without a SASP (266). Persistent DNA damage response (DDR), not transient DDR, is one important regulator of the SASP. Proteins of the DDR signalling cascade that sense and repair DNA damage such as ATM (Ataxia-telangiectasia mutated), NBS1 and CHK2 (Checkpoint kinase 2) are found to be essential for the secretion of a subset of pro-inflammatory cytokines, including IL-6 (268). The observation of persistent DNA damage in severely aneuploid cells generated in our study implies that DNA damage may trigger the expression and secretion of some SASP factors. The fact that elevated expression of pro-inflammatory factors was observed before the onset of senescence supports this hypothesis. However, to dissect the possible contribution of DDR to the expression of SASP factors, it is necessary to examine the SASP expression upon inhibition or blocking of the DDR signalling cascade. If this is indeed the case, aneuploidy-associated oxidative stress that leads to DNA damage may indirectly enhance the expression and secretion of the SASP.

In addition to causing genomic DNA damage, elevated ROS has been demonstrated to contribute to the secretion of pro-inflammatory factors. For example, recent work showed that elevated ROS in multinucleated breast cancer cells stabilise HIF-1 α , contributing to markedly increased secretion of a variety of cytokines, chemokines and growth factors, including VEGF and MIF (macrophage migration inhibition factor), which led to chemoresistance (288). Godinho and colleagues recently demonstrated that high levels of ROS in cells with supernumerary centrosomes induce secretion of pro-invasive factors that mediate paracrine invasion (269). These findings prompted us to postulate that increased ROS levels may be responsible for the secretion of SASP in severely aneuploid cells. It would be interesting to further delineate the role of ROS in SASP expression in this context.

Previous studies have demonstrated that the NF- κ B signalling is a key regulator of the SASP, in addition to its central role in promoting senescence (217, 224). The Campisi lab has also identified the p38MAPK pathway as a novel DDR-independent regulator of the SASP in response to genotoxic stress (221). Although p38MAPK is a stress-inducible kinase that is important for induction of the senescence growth arrest owing to its ability to activate p53 and Rb/p16 pathways directly or indirectly (289, 290), they suggest a divergent role of p38MAPK in controlling SASP via NF- κ B. In this study, our preliminary results demonstrated increased protein levels of both p-p65 and p-p38, as well as enhanced accumulation of p65 in the nuclear fractions in aneuploidy-induced senescent cells (Fig. 25A-C), indicative of a potential link between NF- κ B and p38MAPK pathways and SASP development. Although inhibition of p38MAPK by SB was observed to suppress the expression of IL-6 and CCL7 in Noc 16 h-treated RPE-1 cells, this also increased the transcript levels of IL-1 β , IL-8 and CCL3 (Fig. 25E). Therefore, whether p38MAPK and NF- κ B pathways have a direct causative role to play in the development of SASP and to what extent require further investigation.

Another mechanism that may drive the SASP is the cGAS-cGAMP-STING pathway, which belongs to the innate immune system and functions to sense the presence of double-stranded DNA (dsDNA) in the cytosol. The cytoplasmic protein cGAS (cyclic guanosine monophosphate (GMP)-adenosine monophosphate (AMP) (cGAMP) synthase) is activated by cytosolic dsDNA, leading to the formation of cGAMP, which in turn binds and activates the endoplasmic reticulum membrane protein STING (stimulator of interferon genes), eventually resulting in the activation of inflammatory pathways such as the NF- κ B pathway to induce the transcription of type I interferons as well as the SASP (291). It has been demonstrated elegantly by several groups that micronuclei represent a substantial source to trigger the inflammatory signalling (292, 293). The nuclear envelope of micronuclei are prone to collapse due to defective nuclear lamina organization, which enables the access of cGAS to DNA from the micronuclei (243). The formation of micronucleated and multinucleated cells that we observed following

chromosome mis-segregation, particularly severe chromosome segregation errors, strongly suggests that the cGAS-STING pathway may be activated and mediate the transcription of some SASP genes.

The molecular mechanisms as discussed above may coexist, and are not necessarily mutually exclusive. Possibly, the initiation and maintenance of SASP in aneuploidy-induced senescent cells may depend on, not limited to, the interplay of these pathways at different stages during SASP development. Modulation or perturbation of such pathways may provide opportunities in targeting the cell non-autonomous functions of SASP by which aneuploid cells contribute to tumour development. Another area of interest related to cancer therapy is to investigate whether aneuploidy-induced senescent cells give rise to "senescence revertants", which refer to a subset of senescent cells that have resumed proliferation. Such revertants of senescent tumour cells have been reported to lead to increased cell migration and invasion as well as enhanced tumourigenic potentials (294). Delineating this could further shed light on the molecular processes by which aneuploidy-induced senescent cells contribute to tumour development and potentially facilitate the development of therapies for tumours with high-grade aneuploidy.

4.6. Limitations of the present study

Based on our results, there are potential areas of research that we wish to explore but could not due to limited time for this study. One such area is how aneuploidy-induced senescence affects the innate immune system, particularly macrophages. Although the Amon lab has demonstrated nicely that senescent cells with complex karyotypes promote their own clearance by the NK cells, but the effects of these aneuploid senescent cells on macrophage polarization and differentiation remain unclear. While the populations of macrophages in the tumour microenvironment play a critical role in tumour development (295), macrophages differentiation from monocyte precursors also depend on the local tissue microenvironment. It would be very interesting to investigate whether and how aneuploidy-induced senescent cells impact macrophage polarization via its secretory phenotype. A better understanding of this

question will reveal novel mechanisms on how aneuploidy contributes to cancer by modulating the immune system, which could potentially lead to discovery of new drug targets.

Another limitation of this study is the small size of our patient breast samples. Due to the short period of time, we could only obtain 5 pairs of patient samples. Analyses of more patient samples would definitely make our conclusion more sound and solid.

Thirdly, while we have demonstrated the paracrine pro-tumorigenic effects of aneuploidy-induced senescent cells and the correlation of high level of aneuploidy and senescence at the invasive front in breast tumour samples, the significance of these senescent tumour cells in tumour development is unknown. One way is to take advantage of the cancer database and perform data mining on the relationship between a senescent signature, stages of cancer and patient prognosis. This will provide more insights in deciphering the role of aneuploidy and senescence in tumour progression, relapse and prognosis.

5. Conclusion and Perspectives

Chromosomal instability (CIN) and aneuploidy are common characteristics of cancer. Despite the strong association between aneuploidy and tumourigenesis, the role of aneuploidy in tumourigenesis remains a chicken-and-egg situation until now. Although the mechanisms by which aneuploidy impacts tumourigenesis remain elusive, it is becoming crystal clear that aneuploidy is not just a by-product during malignant transformation, but rather directly involved in tumour progression. Previous studies have extensively investigated the cellular consequences of defined aneuploidy in culture, however, the effects of random complex aneuploidy, which mimics the karyotypic heterogeneity in most tumours, have not been examined very well. Hence, it is of crucial importance to determine how random chromosome mis-segregation affects cell physiology and the underlying mechanisms by which they promote tumour development. This study has demonstrated that differential levels of chromosome mis-segregation distinctively affect cell physiology and confer functional non-equivalence on tumourigenesis. We suggest a cell non-autonomous mechanism by which aneuploidy-induced senescence and SASP can alter the tumour microenvironment to promote tumourigenesis.

We first looked at the transcriptional response to relatively low and high degrees of random aneuploidy, and found random aneuploidy led to a cellular stress response, as has been reported in defined aneuploid mouse and human cell lines. Notably, we found that the level of aneuploidy or degree of chromosome mis-segregation correlated with cellular stress levels, which may underlie our further observation that mildly aneuploid cells continued to proliferate, while their highly aneuploid counterparts ceased to divide and senesced in a p53-dependent manner. Hence, the first aim of this study which is to identify possible mechanistic differences between different levels of random aneuploidies is roughly met.

We further identified a SASP of aneuploidy-induced senescent cells that elicited pro-tumourigenic paracrine effects both *in vitro* and *in vivo*. While the Amon lab has described the immunosurveillance function of senescent cells with complex karyotypes in promoting their own

clearance by the NK cells via the pro-inflammatory signals, our findings add a new dimension on how aneuploidy-induced SASP, once produced, could affect neighbouring cancer cells. Furthermore, we showed a correlative presence of a higher degree of aneuploidy, senescence and SASP in human breast carcinomas, particularly at the invasive fronts. To our knowledge, this is first time that the association of aneuploidy, senescence and SASP is investigated in tumours. Hence, our study has suggested a new mechanism on how aneuploidy could contribute to cancer cell migration, invasion and metastasis by modulating tumour microenvironment via senescence and SASP. These data show that the second aim of this study that is to characterize the role of aneuploidy in tumourigenesis and the underlying mechanisms is also completed.

Apart from cancer, our findings also shed light on the potential role of age-associated accumulation of ploidy changes and senescence in aging phenotypes and age-related pathologies, including cancer. Genomic instability and cellular senescence are among the hallmarks of aging (276), and our study indicates that aneuploid cells that accumulate with age may undergo senescence and produce SASP that has the potential to cause inflammation and tissue degeneration. Hence, an interesting area of research would be to elucidate the links between aneuploidy and aging from the tissue microenvironment perspective.

Future Research

While this study has aimed to attain a better understanding of the cell fate consequences of chromosome mis-segregation and its potential role in tumourigenesis, it has opened up several new avenues for further investigation:

(1) Firstly, it would be interesting to determine the dominant SASP components that elicit the pro-tumourigenic effects and the mechanisms through which they contribute to enhanced cell invasion and migration. Identification of such factors would provide useful targets in controlling the paracrine pro-tumourigenic effects of the SASP. To this end, one strategy would be to block or inhibit the biological

activities of specific SASP components using either neutralizing antibodies or small inhibitors. However, neutralizing antibodies may cause cytotoxicity and influence the immune system, and small inhibitors may have off-target effects. Therefore, another attractive approach is antisense oligonucleotides (AONs) which are 15-25 nt DNA sequences designed to bind the complementary RNA target and lead to cleavage of the RNA/DNA hybrid by RNase H, ultimately resulting in specific knockdown of gene expression (296). AONs are highly specific and their production can be standardized and at large scale, and do not involve the challenges of other genome editing therapeutics which require viral vector for delivery (297). Based on our qRT-PCR and Luminex data, the potential candidate cytokines to test could include but not limited to, IL-6, IL-8, IL-1 β , MMP1, CCL20, CCL5 and so forth.

(2) In this study, we have examined the tumourigenic potentials of the SASP proteins secreted by aneuploidy-induced senescent cells, but the molecular mechanisms and pathways that are crucial for the establishment and maintenance of senescence and/or SASP remain poorly explored. A better understanding of such mechanisms may be beneficial in preventing senescence and SASP to achieve a more desirable outcome such as cell apoptosis, or selectively killing senescent cells in tumours. Our preliminary data implicate the potential role of FoxM1, EZH2 and ATF3 in contributing to senescence in response to severe chromosome mis-segregation, and p38MAPK and NF- κ B pathways in regulating SASP in aneuploidy-induced senescent cells. However, further investigation is required to test these hypotheses.

(3) Our study also raises the interesting question of how aneuploidy-induced SASP affects normal bystander cells. The answer to this question may provide clues to the effects of senescent tumour cells (naturally occurring or therapy-induced) on adjacent normal cells such as epithelial cells in tumours. An extrapolation from this is what is the functional consequence of senescent cells accumulated in the elderly on tissue function, aging and age-related diseases. The answer to these questions may help in intervening in aging phenotype and age-related diseases.

(4) Senescent cells secrete a variety of inflammatory cytokines and chemokines that can trigger an immune response by modulating cellular phenotypes such as differentiation of monocytes, promoting recruitment of leukocytes and affecting lymphocytic populations in the tumour (202). Although the Amon lab has demonstrated the immunogenic potential of aneuploidy-induced senescent cells where the pro-inflammatory signals produced by these senescent cells promoted their own clearance by NK cells *in vitro*, it would be interesting to see whether aneuploidy-induced SASP has the ability to create an immunosuppressive environment that promotes tumour progression. One simple method is to examine if CM from aneuploidy-induced senescent cells skew the polarization of macrophages towards the M2 immune-suppressive macrophages, which are the main population of tumor-associated macrophages (TAMs), instead of an otherwise pro-inflammatory M1 state.

(5) Although senescence is generally regarded as a form of permanent cell-cycle arrest, studies have revealed that senescence is not necessarily irreversible (298). Senescent tumour cells have been shown to be able to resume proliferation and yield aggressive variants (294). Therefore, it would be interesting to see if aneuploidy-induced senescent cells give rise to "revertants". Thus far, this has only been studied in the context of therapy-induced senescence (263). Studying this could shed further insights into how aneuploidy-induced senescence facilitates tumour progression, invasion as well as cancer recurrence.

In closing, our study has attempted to investigate the consequences of aneuploidy on cell physiology as well as the mechanisms that aneuploidy contributes to tumourigenesis. Many outstanding questions remain to be answered: what are the other pathways that suppress proliferation of aneuploid cells? Through what mechanisms and under what circumstances does aneuploidy promote or inhibit tumour formation? Identifying traits common to aneuploidy but not euploidy perhaps could be exploited to specifically kill aneuploid cancer cells. Further insights into these crucial questions may open up a new window of opportunity for the treatment of a variety of aneuploid cancers.

Table 10. List of down-regulated genes common to Noc 8 h vs Ctrl and Noc 16 h vs Ctrl.

Ensemble ID	Gene Symbol	Ensemble ID	Gene Symbol	Ensemble ID	Gene Symbol
ENSG00000006634	DBF4	ENSG00000121621	KIF18A	ENSG00000162063	CCNF
ENSG00000007968	E2F2	ENSG00000121957	GPSM2	ENSG00000162607	USP1
ENSG00000011426	ANLN	ENSG00000122952	ZWINT	ENSG00000162692	VCAM1
ENSG00000012048	BRCA1	ENSG00000122966	CIT	ENSG00000163507	KIAA1524
ENSG00000013810	TACC3	ENSG00000123080	CDKN2C	ENSG00000163535	SGOL2
ENSG00000014138	POLA2	ENSG00000123219	CENPK	ENSG00000163596	ICA1L
ENSG00000022267	FHL1	ENSG00000123374	CDK2	ENSG00000163808	KIF15
ENSG00000024526	DEPDC1	ENSG00000123473	STIL	ENSG00000163923	RPL39L
ENSG00000031691	CENPQ	ENSG00000123485	HJURP	ENSG00000164032	H2AFZ
ENSG00000035499	DEPDC1B	ENSG00000123975	CKS2	ENSG00000164087	POC1A
ENSG00000040275	SPDL1	ENSG00000124212	PTGIS	ENSG00000164104	HMGB2
ENSG00000049541	RFC2	ENSG00000124795	DEK	ENSG00000164105	SAP30
ENSG00000051180	RAD51	ENSG00000125319	C17orf53	ENSG00000164109	MAD2L1
ENSG00000051341	POLQ	ENSG00000125885	MCM8	ENSG00000164611	PTTG1
ENSG00000058804	NDC1	ENSG00000125898	FAM110A	ENSG00000164754	RAD21
ENSG00000066279	ASPM	ENSG00000126787	DLGAP5	ENSG00000165071	TMEM71
ENSG00000068489	PRR11	ENSG00000127564	PKMYT1	ENSG00000165244	ZNF367
ENSG00000072571	HMMR	ENSG00000128383	APOBEC3A	ENSG00000165304	MELK
ENSG00000072864	NDE1	ENSG00000128944	KNSTRN	ENSG00000165480	SKA3
ENSG00000073111	MCM2	ENSG00000129173	E2F8	ENSG00000166451	CENPN
ENSG00000075218	GTSE1	ENSG00000129195	FAM64A	ENSG00000166483	WEE1
ENSG00000075702	WDR62	ENSG00000129534	MIS18BP1	ENSG00000166508	MCM7
ENSG00000076003	MCM6	ENSG00000129810	SGOL1	ENSG00000166803	KIAA0101
ENSG00000076382	SPAG5	ENSG00000130816	DNMT1	ENSG00000166851	PLK1
ENSG00000077152	UBE2T	ENSG00000131153	GIN52	ENSG00000166881	NEMP1
ENSG00000079616	KIF22	ENSG00000131351	HAUS8	ENSG00000167325	RRM1
ENSG00000080839	RBL1	ENSG00000131747	TOP2A	ENSG00000167513	CDT1
ENSG00000080986	NDC80	ENSG00000132182	NUP210	ENSG00000167900	TK1
ENSG00000081052	COL4A4	ENSG00000132780	NASP	ENSG00000168077	SCARA3
ENSG00000085840	ORC1	ENSG00000133119	RFC3	ENSG00000168078	PBK
ENSG00000087586	AURKA	ENSG00000133739	LRRCC1	ENSG00000168874	ATOX8
ENSG00000088325	TPX2	ENSG00000134057	CCNB1	ENSG00000169607	CKAP2L
ENSG00000089685	BIRC5	ENSG00000134222	PSRC1	ENSG00000169679	BUB1
ENSG00000090889	KIF4A	ENSG00000134690	CDCA8	ENSG00000170312	CDK1
ENSG00000091651	ORC6	ENSG00000135451	TROAP	ENSG00000170540	ARL6IP1
ENSG00000092470	WDR76	ENSG00000135476	ESPL1	ENSG00000171241	SHCBP1
ENSG00000092853	CLSPN	ENSG00000136108	CKAP2	ENSG00000171408	PDE7B
ENSG00000093009	CDC45	ENSG00000136492	BRIP1	ENSG00000171848	RRM2
ENSG00000094804	CDC6	ENSG00000136824	SMC2	ENSG00000172197	MBOAT1

ENSG00000095739	BAMBI	ENSG00000136943	CTSV	ENSG00000172244	C5orf34
ENSG00000097046	CDC7	ENSG00000136982	DSCC1	ENSG00000172403	SYNPO2
ENSG00000100162	CENPM	ENSG00000137193	PIM1	ENSG00000173207	CKS1B
ENSG00000100297	MCM5	ENSG00000137310	TCF19	ENSG00000174371	EXO1
ENSG00000100479	POLE2	ENSG00000137404	NRM	ENSG00000175063	UBE2C
ENSG00000100526	CDKN3	ENSG00000137804	NUSAP1	ENSG00000175643	RMI2
ENSG00000100629	CEP128	ENSG00000137807	KIF23	ENSG00000176208	ATAD5
ENSG00000100749	VRK1	ENSG00000138160	KIF11	ENSG00000176244	ACBD7
ENSG00000101003	GIN51	ENSG00000138180	CEP55	ENSG00000176887	SOX11
ENSG00000101057	MYBL2	ENSG00000138182	KIF20B	ENSG00000176890	TYMS
ENSG00000101224	CDC25B	ENSG00000138346	DNA2	ENSG00000177084	POLE
ENSG00000101412	E2F1	ENSG00000138587	MNS1	ENSG00000178999	AURKB
ENSG00000101447	FAM83D	ENSG00000138778	CENPE	ENSG00000179750	APOBEC3B
ENSG00000101639	CEP192	ENSG00000139354	GAS2L3	ENSG00000181938	GIN53
ENSG00000101868	POLA1	ENSG00000139734	DIAPH3	ENSG00000182010	RTKN2
ENSG00000102384	CENPI	ENSG00000140451	PIF1	ENSG00000182481	KPNA2
ENSG00000103196	CRISPLD2	ENSG00000140525	FANCI	ENSG00000183763	TRAIP
ENSG00000104147	OIP5	ENSG00000140534	TICRR	ENSG00000183814	LIN9
ENSG00000104738	MCM4	ENSG00000141401	IMPA2	ENSG00000183856	IQGAP3
ENSG00000105011	ASF1B	ENSG00000141753	IGFBP4	ENSG00000184661	CDCA2
ENSG00000105486	LIG1	ENSG00000142731	PLK4	ENSG00000185347	C14orf80
ENSG00000106462	EZH2	ENSG00000142945	KIF2C	ENSG00000185432	METTL7A
ENSG00000106804	C5	ENSG00000143228	NUF2	ENSG00000185480	PARBPB
ENSG00000108423	TUBD1	ENSG00000144354	CDCA7	ENSG00000186185	KIF18B
ENSG00000109255	NMU	ENSG00000144476	ACKR3	ENSG00000186193	SAPCD2
ENSG00000109674	NEIL3	ENSG00000144554	FANCD2	ENSG00000186767	SPIN4
ENSG00000109805	NCAPG	ENSG00000145386	CCNA2	ENSG00000187266	EPOR
ENSG00000111206	FOXMI	ENSG00000145861	C1QTNF2	ENSG00000187456	NA
ENSG00000111247	RAD51AP1	ENSG00000146143	PRIM2	ENSG00000187741	FANCA
ENSG00000111602	TIMELESS	ENSG00000146410	MTFR2	ENSG00000187951	ARHGAP11B
ENSG00000111665	CDCA3	ENSG00000146670	CDCA5	ENSG00000188486	H2AFX
ENSG00000112029	FBXO5	ENSG00000146918	NCAPG2	ENSG00000188610	FAM72B
ENSG00000112118	MCM3	ENSG00000148773	MKI67	ENSG00000188613	NANOS1
ENSG00000112312	GMNN	ENSG00000149503	INCENP	ENSG00000189057	FAM111B
ENSG00000112742	TTK	ENSG00000149636	DSN1	ENSG00000196584	XRCC2
ENSG00000112984	KIF20A	ENSG00000151725	CENPU	ENSG00000197299	BLM
ENSG00000113368	LMNB1	ENSG00000151849	CENPJ	ENSG00000197977	ELOVL2
ENSG00000113810	SMC4	ENSG00000152253	SPC25	ENSG00000198331	HYLS1
ENSG00000114346	ECT2	ENSG00000153044	CENPH	ENSG00000198826	ARHGAP11A
ENSG00000116962	NID1	ENSG00000154146	NRGN	ENSG00000198830	HMGN2
ENSG00000117399	CDC20	ENSG00000154839	SKA1	ENSG00000198901	PRC1

ENSG00000117632	STMN1	ENSG00000156509	FBXO43	ENSG00000203760	CENPW
ENSG00000117650	NEK2	ENSG00000156802	ATAD2	ENSG00000203817	NA
ENSG00000117724	CENPF	ENSG00000156876	SASS6	ENSG00000205208	C4orf46
ENSG00000118193	KIF14	ENSG00000156970	BUB1B	ENSG00000213186	TRIM59
ENSG00000118513	MYB	ENSG00000157456	CCNB2	ENSG00000213853	EMP2
ENSG00000118898	PPL	ENSG00000158164	TMSB15A	ENSG00000214357	NEURL1B
ENSG00000119397	CNTRL	ENSG00000159231	CBR3	ENSG00000215784	FAM72D
ENSG00000119403	PHF19	ENSG00000160298	C21orf58	ENSG00000221829	FANCG
ENSG00000119969	HELLS	ENSG00000160949	TONSL	ENSG00000221869	CEBPD
ENSG00000120802	TMPO	ENSG00000161692	DBF4B	ENSG00000228716	DHFR
ENSG00000121152	NCAPH	ENSG00000161800	RACGAP1	ENSG00000237649	KIFC1
ENSG00000121211	MND1	ENSG00000162062	C16orf59	ENSG00000268310	NA

Table 11. List of down-regulated genes in Noc 16 h vs Ctrl.

Ensemble ID	Gene Symbol	Ensemble ID	Gene Symbol	Ensemble ID	Gene Symbol
ENSG00000000460	C1orf112	ENSG00000123080	CDKN2C	ENSG00000163507	KIAA1524
ENSG000000005189	AC004381.6	ENSG00000123219	CENPK	ENSG00000163535	SGOL2
ENSG000000006634	DBF4	ENSG00000123374	CDK2	ENSG00000163584	RPL22L1
ENSG000000007968	E2F2	ENSG00000123473	STIL	ENSG00000163596	ICA1L
ENSG000000010292	NCAPD2	ENSG00000123485	HJURP	ENSG00000163808	KIF15
ENSG000000011426	ANLN	ENSG00000123975	CKS2	ENSG00000163923	RPL39L
ENSG000000012048	BRCA1	ENSG00000124212	PTGIS	ENSG00000164032	H2AFZ
ENSG000000013297	CLDN11	ENSG00000124795	DEK	ENSG00000164045	CDC25A
ENSG000000013573	DDX11	ENSG00000125319	C17orf53	ENSG00000164087	POC1A
ENSG000000013810	TACC3	ENSG00000125378	BMP4	ENSG00000164104	HMGB2
ENSG000000014138	POLA2	ENSG00000125885	MCM8	ENSG00000164105	SAP30
ENSG000000021645	NRXN3	ENSG00000125898	FAM110A	ENSG00000164109	MAD2L1
ENSG000000022267	FHL1	ENSG00000126453	BCL2L12	ENSG00000164118	CEP44
ENSG000000024526	DEPDC1	ENSG00000126787	DLGAP5	ENSG00000164611	PTTG1
ENSG000000025770	NCAPH2	ENSG00000126803	HSPA2	ENSG00000164649	CDCA7L
ENSG000000029993	HMGB3	ENSG00000127564	PKMYT1	ENSG00000164754	RAD21
ENSG000000031691	CENPQ	ENSG00000128383	APOBEC3A	ENSG00000164761	TNFRSF11B
ENSG000000035499	DEPDC1B	ENSG00000128617	OPN1SW	ENSG00000165071	TMEM71
ENSG000000035664	DAPK2	ENSG00000128944	KNSTRN	ENSG00000165113	GKAP1
ENSG000000040275	SPDL1	ENSG00000128951	DUT	ENSG00000165244	ZNF367
ENSG000000049541	RFC2	ENSG00000129173	E2F8	ENSG00000165304	MELK
ENSG000000051180	RAD51	ENSG00000129195	FAM64A	ENSG00000165480	SKA3
ENSG000000051341	POLQ	ENSG00000129484	PARP2	ENSG00000165490	DDIAS
ENSG000000058056	USP13	ENSG00000129534	MIS18BP1	ENSG00000166106	ADAMTS15
ENSG000000058804	NDC1	ENSG00000129810	SGOL1	ENSG00000166451	CENPN

ENSG00000065328	MCM10	ENSG00000130816	DNMT1	ENSG00000166483	WEE1
ENSG00000066279	ASPM	ENSG00000131153	GIN52	ENSG00000166508	MCM7
ENSG00000068489	PRR11	ENSG00000131351	HAUS8	ENSG00000166803	KIAA0101
ENSG00000069493	CLEC2D	ENSG00000131470	PSMC3IP	ENSG00000166851	PLK1
ENSG00000071539	TRIP13	ENSG00000131747	TOP2A	ENSG00000166881	NEMP1
ENSG00000072571	HMMR	ENSG00000132182	NUP210	ENSG00000166896	XRCC6BP1
ENSG00000072864	NDE1	ENSG00000132436	FIGNL1	ENSG00000167325	RRM1
ENSG00000073111	MCM2	ENSG00000132780	NASP	ENSG00000167513	CDT1
ENSG00000075218	GTSE1	ENSG00000133110	POSTN	ENSG00000167900	TK1
ENSG00000075702	WDR62	ENSG00000133119	RFC3	ENSG00000168077	SCARA3
ENSG00000076003	MCM6	ENSG00000133739	LRRCC1	ENSG00000168078	PBK
ENSG00000076382	SPAG5	ENSG00000134013	LOXL2	ENSG00000168386	FILIP1L
ENSG00000077152	UBE2T	ENSG00000134057	CCNB1	ENSG00000168393	DTYMK
ENSG00000077684	JADE1	ENSG00000134222	PSRC1	ENSG00000168411	RFWD3
ENSG00000079616	KIF22	ENSG00000134291	TMEM106C	ENSG00000168874	ATOH8
ENSG00000079931	MOXD1	ENSG00000134690	CDCA8	ENSG00000169607	CKAP2L
ENSG00000080839	RBL1	ENSG00000134871	COL4A2	ENSG00000169679	BUB1
ENSG00000080986	NDC80	ENSG00000135045	C9orf40	ENSG00000170312	CDK1
ENSG00000081052	COL4A4	ENSG00000135451	TROAP	ENSG00000170540	ARL6IP1
ENSG00000081320	STK17B	ENSG00000135476	ESPL1	ENSG00000171241	SHCBP1
ENSG00000082482	KCNK2	ENSG00000135486	HNRNPA1	ENSG00000171320	ESCO2
ENSG00000085840	ORC1	ENSG00000136108	CKAP2	ENSG00000171408	PDE7B
ENSG00000085999	RAD54L	ENSG00000136492	BRIP1	ENSG00000171848	RRM2
ENSG00000087301	TXNDC16	ENSG00000136824	SMC2	ENSG00000172197	MBOAT1
ENSG00000087586	AURKA	ENSG00000136943	CTSV	ENSG00000172244	C5orf34
ENSG00000088305	DNMT3B	ENSG00000136982	DSCC1	ENSG00000172403	SYNPO2
ENSG00000088325	TPX2	ENSG00000137193	PIM1	ENSG00000173085	COQ2
ENSG00000089685	BIRC5	ENSG00000137310	TCF19	ENSG00000173207	CKS1B
ENSG00000090889	KIF4A	ENSG00000137404	NRM	ENSG00000173227	SYT12
ENSG00000091651	ORC6	ENSG00000137563	GGH	ENSG00000174059	CD34
ENSG00000091986	CCDC80	ENSG00000137573	SULF1	ENSG00000174371	EXO1
ENSG00000092140	G2E3	ENSG00000137804	NUSAP1	ENSG00000175063	UBE2C
ENSG00000092470	WDR76	ENSG00000137807	KIF23	ENSG00000175643	RMI2
ENSG00000092853	CLSPN	ENSG00000137812	CASC5	ENSG00000176208	ATAD5
ENSG00000093009	CDC45	ENSG00000138092	CENPO	ENSG00000176244	ACBD7
ENSG00000094804	CDC6	ENSG00000138160	KIF11	ENSG00000176619	LMNB2
ENSG00000095002	MSH2	ENSG00000138180	CEP55	ENSG00000176887	SOX11
ENSG00000095739	BAMBI	ENSG00000138182	KIF20B	ENSG00000176890	TYMS
ENSG00000096060	FKBP5	ENSG00000138193	PLCE1	ENSG00000176974	SHMT1
ENSG00000097046	CDC7	ENSG00000138346	DNA2	ENSG00000177084	POLE
ENSG00000100162	CENPM	ENSG00000138587	MNS1	ENSG00000177706	FAM20C

ENSG00000100242	SUN2	ENSG00000138778	CENPE	ENSG00000178177	LCORL
ENSG00000100297	MCM5	ENSG00000138796	HADH	ENSG00000178202	KDELC2
ENSG00000100479	POLE2	ENSG00000139354	GAS2L3	ENSG00000178295	GEN1
ENSG00000100526	CDKN3	ENSG00000139618	BRCA2	ENSG00000178878	APOLD1
ENSG00000100629	CEP128	ENSG00000139675	HNRNPA1L2	ENSG00000178966	RMI1
ENSG00000100749	VRK1	ENSG00000139734	DIAPH3	ENSG00000178999	AURKB
ENSG00000101003	GIN51	ENSG00000140451	PIF1	ENSG00000179750	APOBEC3B
ENSG00000101057	MYBL2	ENSG00000140525	FANCI	ENSG00000180998	GPR137C
ENSG00000101224	CDC25B	ENSG00000140534	TICRR	ENSG00000181544	FANCB
ENSG00000101412	E2F1	ENSG00000141401	IMPA2	ENSG00000181938	GIN53
ENSG00000101447	FAM83D	ENSG00000141753	IGFBP4	ENSG00000182010	RTKN2
ENSG00000101639	CEP192	ENSG00000142731	PLK4	ENSG00000182054	IDH2
ENSG00000101868	POLA1	ENSG00000142945	KIF2C	ENSG00000182481	KPNA2
ENSG00000101945	SUV39H1	ENSG00000143127	ITGA10	ENSG00000182963	GJC1
ENSG00000102098	SCML2	ENSG00000143228	NUF2	ENSG00000183087	GAS6
ENSG00000102384	CENPI	ENSG00000143401	ANP32E	ENSG00000183137	CEP57L1
ENSG00000103196	CRISPLD2	ENSG00000143476	DTL	ENSG00000183150	GPR19
ENSG00000103888	CEMIP	ENSG00000143815	LBR	ENSG00000183763	TRAIP
ENSG00000104147	OIP5	ENSG00000144354	CDCA7	ENSG00000183814	LIN9
ENSG00000104738	MCM4	ENSG00000144395	CCDC150	ENSG00000183856	IQGAP3
ENSG00000105011	ASF1B	ENSG00000144476	ACKR3	ENSG00000184661	CDCA2
ENSG00000105486	LIG1	ENSG00000144554	FANCD2	ENSG00000185347	C14orf80
ENSG00000105894	PTN	ENSG00000145375	SPATA5	ENSG00000185432	METTL7A
ENSG00000105968	H2AFV	ENSG00000145386	CCNA2	ENSG00000185480	PARPBP
ENSG00000106268	NUDT1	ENSG00000145604	SKP2	ENSG00000186185	KIF18B
ENSG00000106399	RPA3	ENSG00000145861	C1QTNF2	ENSG00000186193	SAPCD2
ENSG00000106462	EZH2	ENSG00000146143	PRIM2	ENSG00000186767	SPIN4
ENSG00000106484	MEST	ENSG00000146410	MTFR2	ENSG00000187266	EPOR
ENSG00000106804	C5	ENSG00000146670	CDCA5	ENSG00000187456	NA
ENSG00000107518	ATRNL1	ENSG00000146918	NCAPG2	ENSG00000187498	COL4A1
ENSG00000108106	UBE2S	ENSG00000147536	GIN54	ENSG00000187741	FANCA
ENSG00000108423	TUBD1	ENSG00000148019	CEP78	ENSG00000187951	ARHGAP11B
ENSG00000108691	CCL2	ENSG00000148773	MKI67	ENSG00000187955	COL14A1
ENSG00000109084	TMEM97	ENSG00000149503	INCENP	ENSG00000188486	H2AFX
ENSG00000109255	NMU	ENSG00000149596	JPH2	ENSG00000188610	FAM72B
ENSG00000109674	NEIL3	ENSG00000149636	DSN1	ENSG00000188613	NANOS1
ENSG00000109685	WHSC1	ENSG00000149929	HIRIP3	ENSG00000188783	PRELP
ENSG00000109805	NCAPG	ENSG00000151466	SCLT1	ENSG00000189057	FAM111B
ENSG00000111206	FOXM1	ENSG00000151725	CENPU	ENSG00000189403	HMGB1
ENSG00000111247	RAD51AP1	ENSG00000151849	CENPJ	ENSG00000196081	ZNF724P
ENSG00000111602	TIMELESS	ENSG00000152253	SPC25	ENSG00000196584	XRCC2

ENSG00000111665	CDCA3	ENSG00000152455	SUV39H2	ENSG00000197299	BLM
ENSG00000112029	FBXO5	ENSG00000153044	CENPH	ENSG00000197977	ELOVL2
ENSG00000112118	MCM3	ENSG00000154146	NRGN	ENSG00000198331	HYLS1
ENSG00000112312	GMNN	ENSG00000154678	PDE1C	ENSG00000198374	NA
ENSG00000112742	TTK	ENSG00000154839	SKA1	ENSG00000198554	WDHD1
ENSG00000112984	KIF20A	ENSG00000156509	FBXO43	ENSG00000198826	ARHGAP11A
ENSG00000113368	LMNB1	ENSG00000156689	GLYATL2	ENSG00000198830	HMG2
ENSG00000113810	SMC4	ENSG00000156802	ATAD2	ENSG00000198901	PRC1
ENSG00000114107	CEP70	ENSG00000156876	SASS6	ENSG00000203760	CENPW
ENSG00000114346	ECT2	ENSG00000156970	BUB1B	ENSG00000203817	NA
ENSG00000115252	PDE1A	ENSG00000157456	CCNB2	ENSG00000204536	CCHCR1
ENSG00000115380	EFEMP1	ENSG00000158164	TMSB15A	ENSG00000204899	MZT1
ENSG00000115461	IGFBP5	ENSG00000158402	CDC25C	ENSG00000205208	C4orf46
ENSG00000116962	NID1	ENSG00000158427	TMSB15B	ENSG00000205221	VIT
ENSG00000117399	CDC20	ENSG00000159231	CBR3	ENSG00000213186	TRIM59
ENSG00000117407	ARTN	ENSG00000160298	C21orf58	ENSG00000213347	MXD3
ENSG00000117632	STMN1	ENSG00000160352	ZNF714	ENSG00000213390	ARHGAP19
ENSG00000117650	NEK2	ENSG00000160949	TONSL	ENSG00000213853	EMP2
ENSG00000117724	CENPF	ENSG00000160957	RECQL4	ENSG00000214357	NEURL1B
ENSG00000118193	KIF14	ENSG00000161547	SRSF2	ENSG00000215784	FAM72D
ENSG00000118513	MYB	ENSG00000161692	DBF4B	ENSG00000221829	FANCG
ENSG00000118898	PPL	ENSG00000161800	RACGAP1	ENSG00000221869	CEBPD
ENSG00000119326	CTNNA1	ENSG00000161888	SPC24	ENSG00000228716	DHFR
ENSG00000119397	CNTRL	ENSG00000162062	C16orf59	ENSG00000231924	PSG1
ENSG00000119403	PHF19	ENSG00000162063	CCNF	ENSG00000237649	KIFC1
ENSG00000119946	CNNM1	ENSG00000162174	ASRGL1	ENSG00000244414	CFHR1
ENSG00000119969	HELLS	ENSG00000162267	ITIH3	ENSG00000250081	CTD-2116N20.1
ENSG00000120802	TMPO	ENSG00000162591	MEGF6	ENSG00000251259	AC004069.2
ENSG00000121152	NCAPH	ENSG00000162607	USP1	ENSG00000259070	LINC00639
ENSG00000121211	MND1	ENSG00000162645	GBP2	ENSG00000260260	SNHG19
ENSG00000121621	KIF18A	ENSG00000162692	VCAM1	ENSG00000262477	AC021224.1
ENSG00000121957	GPSM2	ENSG00000162745	OLFML2B	ENSG00000268310	NA
ENSG00000122367	LDB3	ENSG00000163002	NUP35	ENSG00000268313	NA
ENSG00000122952	ZWINT	ENSG00000163006	CCDC138	ENSG00000272761	NA
ENSG00000122966	CIT	ENSG00000163431	LMOD1		

Table 12. List of up-regulated genes in Noc 16 h vs Ctrl.

Ensemble ID	Gene Symbol	Ensemble ID	Gene Symbol	Ensemble ID	Gene Symbol
ENSG00000003147	ICA1	ENSG00000131015	ULBP2	ENSG00000171860	C3AR1

ENSG00000006075	NA	ENSG00000131080	EDA2R	ENSG00000172137	CALB2
ENSG00000010379	SLC6A13	ENSG00000131459	GFPT2	ENSG00000172572	PDE3A
ENSG00000019549	SNAI2	ENSG00000131737	KRT34	ENSG00000172748	ZNF596
ENSG00000026103	FAS	ENSG00000132185	FCRLA	ENSG00000172831	CES2
ENSG00000026559	KCNG1	ENSG00000132429	POPDC3	ENSG00000172995	ARPP21
ENSG00000029534	ANK1	ENSG00000133083	DCLK1	ENSG00000173846	PLK3
ENSG00000040731	CDH10	ENSG00000133134	BEX2	ENSG00000174236	REP15
ENSG00000042832	TG	ENSG00000134107	BHLHE40	ENSG00000174792	C4orf26
ENSG00000048052	HDAC9	ENSG00000134548	SPX	ENSG00000175426	PCSK1
ENSG00000059758	CDK17	ENSG00000134955	SLC37A2	ENSG00000175600	SUGCT
ENSG00000064300	NGFR	ENSG00000135114	OASL	ENSG00000176046	NUPR1
ENSG00000067798	NAV3	ENSG00000135679	MDM2	ENSG00000176058	TPRN
ENSG00000069431	ABCC9	ENSG00000136114	THSD1	ENSG00000176531	PHLDB3
ENSG00000069812	HES2	ENSG00000136531	SCN2A	ENSG00000177076	ACER2
ENSG00000070669	ASNS	ENSG00000136826	KLF4	ENSG00000177283	FZD8
ENSG00000072041	SLC6A15	ENSG00000137033	IL33	ENSG00000178695	KCTD12
ENSG00000073737	DHRS9	ENSG00000137975	CLCA2	ENSG00000178726	THBD
ENSG00000073756	PTGS2	ENSG00000138166	DUSP5	ENSG00000179388	EGR3
ENSG00000078237	TIGAR	ENSG00000138678	AGPAT9	ENSG00000180596	HIST1H2BC
ENSG00000087494	PTHLH	ENSG00000139318	DUSP6	ENSG00000180921	FAM83H
ENSG00000089127	OAS1	ENSG00000139410	SDSL	ENSG00000181634	TNFSF15
ENSG00000090020	SLC9A1	ENSG00000140323	DISP2	ENSG00000181773	GPR3
ENSG00000091490	SEL1L3	ENSG00000140961	OSGIN1	ENSG00000183558	NA
ENSG00000091844	RGS17	ENSG00000141682	PMAIP1	ENSG00000183578	TNFAIP8L3
ENSG00000099338	CATSPERG	ENSG00000143217	PVRL4	ENSG00000183691	NOG
ENSG00000100292	HMOX1	ENSG00000143556	S100A7	ENSG00000184792	OSBP2
ENSG00000100867	DHRS2	ENSG00000144136	SLC20A1	ENSG00000184995	IFNE
ENSG00000101384	JAG1	ENSG00000146674	IGFBP3	ENSG00000185519	FAM131C
ENSG00000102003	SYP	ENSG00000146678	IGFBP1	ENSG00000185567	AHNAK2
ENSG00000102312	PORCN	ENSG00000147168	IL2RG	ENSG00000186642	PDE2A
ENSG00000102554	KLF5	ENSG00000147614	ATP6V0D2	ENSG00000187837	HIST1H1C
ENSG00000103316	CRYM	ENSG00000148803	FUOM	ENSG00000188100	FAM25A
ENSG00000104368	PLAT	ENSG00000149968	MMP3	ENSG00000189001	SBSN
ENSG00000104381	GDAP1	ENSG00000151640	DPYSL4	ENSG00000189060	H1FO
ENSG00000104419	NDRG1	ENSG00000153233	PTPRR	ENSG00000189129	PLAC9
ENSG00000104689	TNFRSF10A	ENSG00000154134	ROBO3	ENSG00000196152	ZNF79
ENSG00000104833	TUBB4A	ENSG00000154734	ADAMTS1	ENSG00000196208	GREB1
ENSG00000105048	TNNT1	ENSG00000154822	PLCL2	ENSG00000196562	SULF2
ENSG00000105419	MEIS3	ENSG00000155961	RAB39B	ENSG00000196872	KIAA1211L
ENSG00000106123	EPHB6	ENSG00000155974	GRIP1	ENSG00000197093	GAL3ST4
ENSG00000108342	CSF3	ENSG00000156453	PCDH1	ENSG00000197191	CYSRT1

ENSG00000109321	AREG	ENSG00000157150	TIMP4	ENSG00000197249	SERPINA1
ENSG00000110092	CCND1	ENSG00000158373	HIST1H2BD	ENSG00000197261	C6orf141
ENSG00000110169	HPX	ENSG00000158406	HIST1H4H	ENSG00000197457	STMN3
ENSG00000111679	PTPN6	ENSG00000159388	BTG2	ENSG00000197461	PDGFA
ENSG00000112297	AIM1	ENSG00000161249	DMKN	ENSG00000197632	SERPINB2
ENSG00000112715	VEGFA	ENSG00000161513	FDXR	ENSG00000198074	AKR1B10
ENSG00000113070	HBEGF	ENSG00000161570	NA	ENSG00000198573	SPANXC
ENSG00000115008	IL1A	ENSG00000162490	DRAXIN	ENSG00000198910	L1CAM
ENSG00000115009	CCL20	ENSG00000162643	WDR63	ENSG00000203812	HIST2H2AA3
ENSG00000115129	TP53I3	ENSG00000162772	ATF3	ENSG00000203849	NA
ENSG00000115290	GRB14	ENSG00000162981	FAM84A	ENSG00000203926	SPANXA2
ENSG00000116396	KCNC4	ENSG00000163071	SPATA18	ENSG00000204219	TCEA3
ENSG00000116717	GADD45A	ENSG00000163235	TGFA	ENSG00000212724	KRTAP2-3
ENSG00000118785	SPP1	ENSG00000163536	SERPINI1	ENSG00000221852	KRTAP1-5
ENSG00000119508	NR4A3	ENSG00000163814	CDCP1	ENSG00000224592	RP5-884C9.2
ENSG00000119514	GALNT12	ENSG00000163932	PRKCD	ENSG00000225383	SFTA1P
ENSG00000119630	PGF	ENSG00000164070	HSPA4L	ENSG00000225667	LINC00505
ENSG00000119771	KLHL29	ENSG00000164171	ITGA2	ENSG00000227234	SPANXB1
ENSG00000120833	SOCS2	ENSG00000164237	CMBL	ENSG00000228251	AC012442.6
ENSG00000122641	INHBA	ENSG00000164283	ESM1	ENSG00000231672	DIRC3
ENSG00000122877	EGR2	ENSG00000166396	SERPINB7	ENSG00000232164	AC092669.3
ENSG00000123689	G0S2	ENSG00000166592	RRAD	ENSG00000232274	NA
ENSG00000124102	PI3	ENSG00000166840	GLYATL1	ENSG00000235385	GS1-600G8.5
ENSG00000124225	PMEPA1	ENSG00000167178	ISLR2	ENSG00000235604	NA
ENSG00000124635	HIST1H2BJ	ENSG00000167772	ANGPTL4	ENSG00000236453	AC003092.1
ENSG00000124762	CDKN1A	ENSG00000168062	BATF2	ENSG00000237753	AC079922.3
ENSG00000125538	IL1B	ENSG00000168274	NA	ENSG00000238261	NA
ENSG00000125845	BMP2	ENSG00000168306	ACOX2	ENSG00000241163	LINC00877
ENSG00000127325	BEST3	ENSG00000168918	INPP5D	ENSG00000242732	RGAG4
ENSG00000128564	VGF	ENSG00000169064	ZBBX	ENSG00000244509	APOBEC3C
ENSG00000128604	IRF5	ENSG00000169067	ACTBL2	ENSG00000249992	TMEM158
ENSG00000128805	ARHGAP22	ENSG00000169306	IL1RAPL1	ENSG00000251562	MALAT1
ENSG00000128965	CHAC1	ENSG00000170044	ZPLD1	ENSG00000253161	LINC01605
ENSG00000129910	CDH15	ENSG00000170469	SPATA24	ENSG00000253746	RP11-527N22.2
ENSG00000130066	SAT1	ENSG00000170537	TMC7	ENSG00000260910	LINC00565
ENSG00000130477	UNC13A	ENSG00000170909	OSCAR	ENSG00000273142	RP11-458F8.4
ENSG00000130766	SESN2	ENSG00000171303	KCNK3	ENSG00000273143	RP11-525A16.4
ENSG00000130822	PNCK				

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