Université de Montréal

Causes and Consequences of Chromosome Segregation Errors in the Mouse Preimplantation Embryo

New Insights into Mosaic Aneuploidy in Embryos

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Résumé

La division cellulaire est un processus biologique universel nécessaire à la reproduction, au développement, à la survie cellulaire ainsi qu'à la réparation des tissus. Une ségrégation chromosomique exacte pendant la mitose est essentielle pour une répartition égale des chromosomes répliqués entre les cellules filles. Des erreurs dans la ségrégation des chromosomes mènent à une condition appelée aneuploïdie, définie par un nombre inadéquat de chromosomes dans une cellule. L'aneuploïdie est associée à une altération de la santé cellulaire, la tumorigénèse, des malformations congénitales et l'infertilité. Contre toute attente, les embryons préimplantatoires de mammifères, dont les humains, consistent souvent en un mélange de cellules euploïdes et de cellules aneuploïdes. Ce mosaïcisme est inexorablement causé par des erreurs dans la ségrégation des chromosomes au cours des divisions mitotiques suivant la fécondation et est associé à un potentiel de développement réduit lors des traitements de fertilité. Malgré sa découverte il y a 25 ans, les mécanismes qui sous-tendent l'apparition de l'aneuploïdie mosaïque dans les embryons préimplantatoires sont toujours méconnus.

Pour explorer les causes et les conséquences des erreurs de ségrégation chromosomique, des approches d'imagerie de fine pointe ont été utilisées sur des embryons préimplantatoires murins. L'analyse de la dynamique de la ségrégation des chromosomes via l'imagerie de cellules vivantes a permis d'identifier les chromosomes retardataires, lors de l'anaphase, comme la forme la plus répandue des erreurs de ségrégation. Ces chromosomes retardataires entraînent fréquemment une encapsulation de chromosome unique dans une structure appelée micronoyau. D'autres expériences d'imagerie par immunofluorescence sur des cellules vivantes ou fixées ont révélé que les chromosomes des micronoyaux subissent des dommages importants à l'ADN et sont mal répartis de manière récurrente lors des divisions cellulaires subséquentes dans la phase préimplantatoire. D'autres approches ont aussi permis d'examiner l'efficacité du mécanisme de contrôle de l'assemblage du fuseau mitotique, (SAC pour Spindle Assembly Checkpoint). Les résultats obtenus attestent que le SAC fonctionne, cependant la signalisation liée au SAC n'est pas efficace et ne permet pas de différer l'anaphase, malgré la présence de chromosomes retardataires et ce indépendamment de la taille des cellules. Les résultats présentés révèlent aussi qu'une inhibition partielle d'une cible du SAC, le complexe de promotion de l'anaphase (APC/C), cause une mitose prolongée et une réduction des erreurs de ségrégation. En outre, les études présentées démontrent que la

fonction déficiente du SAC pendant le développement préimplantatoire est la cause principale d'une forte incidence de chromosomes retardataires qui entraînent une mauvaise ségrégation chromosomique répétée et qui causent une aneuploïdie mosaïque dans l'embryon. De plus, ce travail fournit la preuve que la modulation pharmacologique de la signalisation SAC-APC/C permet d'éviter les erreurs de ségrégation des chromosomes dans les embryons précoces.

En conclusion, ces résultats apportent de nouvelles perspectives sur les causes et la nature des erreurs de ségrégation chromosomique dans les embryons. De plus, ce travail apporte de nouvelles explications mécanistiques sur l'apparition du mosaïcisme dans les embryons ce qui aura des implications importantes dans la détection et la prévention thérapeutique potentielle de l'aneuploïdie mosaïque dans les embryons préimplantatoires.

Mots-clés :

Embryons préimplantatoires • Mitose • Ségrégation chromosomique • Aneuploïdie mosaïque • Chromosomes retardataires • Micronoyaux • Mécanisme de contrôle de l'assemblage du fuseau mitotique

Abstract

Cell division is a universal biological process necessary for reproduction, development, cell survival and the maintenance and repair of tissues. Accurate chromosome segregation during mitosis is essential to ensure replicated chromosomes are partitioned equally into daughter cells. Errors in chromosome segregation often result in cells with abnormal numbers of chromosomes, a condition termed aneuploidy, which is associated with impaired cellular health, tumorigenesis, congenital defects and infertility. Counterintuitively, preimplantation embryos from many mammalian species, including humans, often consist of a mixture euploid and aneuploid cells. Such mosaic aneuploidy in embryos is inexorably caused by errors in chromosome segregation during mitotic divisions following fertilization and has been associated with reduced developmental potential in fertility treatments. However, ever since its discovery 25 years ago, how and why mosaic aneuploidy arises in the preimplantation embryo has remained elusive.

To explore the causes and consequences of embryonic chromosome segregation errors, advanced imaging approaches were employed in the mouse preimplantation embryo. Live cell imaging analysis of chromosome segregation dynamics identified lagging anaphase chromosomes as the most prevalent form of chromosome mis-segregation in embryos. Lagging chromosomes frequently result in the encapsulation of single chromosomes into micronuclei, which occur in embryos *in vitro* and *in vivo.* Further live imaging and immunofluorescence experiments revealed chromosomes within micronuclei are subject to extensive DNA damage and centromeric identity loss, failing to assemble functional kinetochores and being recurrently mis-segregated during ensuing cell divisions in preimplantation development. To uncover the underlying causes for the increased propensity for chromosome mis-segregation in embryos, live imaging and loss-of-function approaches were used to examine the effectiveness of the mitotic safeguard mechanism, the Spindle Assembly Checkpoint (SAC). These studies demonstrated that the SAC normally functions to prevent segregation errors during preimplantation development but SAC signaling at misaligned chromosomes fails to delay anaphase. Moreover, SAC failure in embryos is most evident during mid-preimplantation development, independent of cell size. Partial inhibition of SAC target, the Anaphase Promoting Complex (APC/C), extended mitosis and reduced chromosome segregation errors in embryos.

These studies have uncovered deficient SAC function during preimplantation development as a major cause for the high incidence of lagging chromosomes in embryos, which result in repeated mis-segregation of single chromosomes in a manner that necessarily causes mosaic aneuploidy. Additionally, this work provides proof-of-principle demonstration that pharmacological modulation of SAC-APC/C signalling can avert chromosome segregation errors in the early embryo. Altogether, these findings present new insights into the causes and nature of chromosome mis-segregation in embryos, providing novel mechanistic explanations for the occurrence of mosaicism that will have substantial implications for the detection and potential therapeutic prevention of aneuploidy in preimplantation embryos.

Keywords :

Preimplantation embryo • Mitosis • Chromosome segregation • Mosaic aneuploidy • Lagging chromosomes • Micronuclei • Spindle Assembly Checkpoint

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List of Abbreviations

3D	Three-Dimensional
4D	Four-Dimensional
53BP1	p53-Binding Protein 1
ACA	Anti-Centromere Antibodies
ANOVA	Analysis-Of-VAriance
APC/C	Anaphase Promoting Complex/Cyclosome
ART	Assisted Reproduction Technology
ATRX	Alpha Thalassemia/mental Retardation syndrome X-linked
AU	Arbitrary Units
BAF	Barrier-to-Autointegration Factor
BSA	Bovine Serum Albumin
BUB1	Budding Uninhibited by Benzimidazoles 1
BUB3	Budding Uninhibited by Benzimidazoles 3
BUBR1	Budding Uninhibited by Benzimidazoles-Related 1
C. elegans	Caenorhabditis elegans
Cdc20	Cell Division Cycle 20
Cdc27	Cell Division Cycle 27
CDK1	Cyclin-Dependent Kinase 1
CDK2	Cyclin-Dependent Kinase 2
Cdt1	Cdc10-Dependent Transcript 1 protein
CENP-A	CENtromere Protein-A
CENP-B	CENtromeric Protein-B
CENP-C	CENtromeric Protein-C
CENP-E	CENtromere Protein-E
CENP-N	CENtromeric Protein-N
CENP-T	CENtromeric Protein-T
CENP-U	CENtromeric Protein-U
CENP-W	CENtromeric Protein-W
CIN	Chromosomal INstability
CO_2	Carbon dioxide
CPC	Chromosomal Passenger Complex

CREST	Calcinosis, Raynaud phenomenon, Esophageal dysmobility, Sclerodactyly and Telangiectasia
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
cRNA	Complimentary RiboNucleic Acid
CUEDC2	CUE-Domain-Containing protein 2
DMSO	DiMethylSulfOxide
DNA	DeoxyriboNucleic Acid
DSB	(DNA) Double Strand Break
DSN1	Dosage Suppresor of NNF1
EDTA	EthyleneDiamineTetra-Acetic acid
EM	Electron Microscopy
ESC	Embryonic Stem Cells
FISH	Fluorescence In Situ Hybridization
FUCCI	Fluorescent Ubiquitination-based Cell Cycle Indicator
GAP43	Growth Associated Protein 43
GFP	Green Fluorescent Protein
GFP	Green Fluorescent Protein
H2B	Histone H2B
H3.3	Histone H3.3, variant
hCG	human Chorionic Gonadotrophin
HDR	Homology-Directed Repair
HJURP	Holliday JUnction-Recognition Protein
HyD	Hybrid Detector
ICM	Inner Cell Mass
ICSI	Intra-Cytoplasmic Sperm Injection
IgG	Immunoglobulin G
INCENP	INer CENtromere Protein
IU	International Units
IVF	In Vitro Fertilization
IVT	In Vitro Transcription
KI-67	antigen identified by monoclonal antibody Ki-67
KIF2B	KInesin Family member 2B
KIF2C	KInesin Family member 2C

KNL1	Kinetochore NulL1
KSOM	Potassium(K)-supplemented Simplex Optimised Medium
LSD1	Lysine-Specific histone Demethylase 1A
MAD1	Mitotic Arrest Deficient 1
MAD2	Mitotic Arrest Deficient 2
MajSat	Major Satellite
MCAK	Mitotic Centromere Associated Kinesin
MCC	Mitotic Checkpoint Complex
MCM2	Minichromosome Maintenance Complex Component 2
MN	Micronucleus
MPS1	MonoPolar Spindle 1 kinase
MTOCS	MicroTubule-Organizing Centers
NA	Numerical Aperture
NDC80^{Hec1}	Nuclear Division Cycle 80$^{Highly\ Expressed\ in\ Cancer\ protein\ 1}$
NE	Nuclear Envelope
NEBD	Nuclear Envelope BreakDown
NGS	Next Generation Sequencing
NHEJ	Non-Homologous End Joining
NLS	Nuclear Localization Signal
PBE	Polar Body Extrusion
PBS	Phosphate Buffered Solution
PCAF	P300/CBP-Associated Factor /lysine(K) AcetylTransferase 2B (KAT2B)
PFA	ParaFormAldehyde
PGS	Preimplantation Genetic Screening
PLK1	Polo-Like Kinase 1
PLK4	Polo-Like Kinase 4
PMSG	Pregnant Mare’s Serum Gonadotrophin
PMT	PhotoMultiplier (Tubes)
PN	Principal Nucleus
Poly(A)	PolyAdenylation
PP1	Protein Phosphatase 1
PP2A	Protein Phosphatase 2 A
PTM	Post-Translational Modification

RFP	Red Fluorescent Protein
RNAi	RNA interference
RNApol II	RNA polymerase 2
ROD	ROugh Deal
RZZ complex	ROD-ZW10-ZWILCH complex
SAC	Spindle Assembly Checkpoint
SCNT	Somatic Cell Nuclear Transfer
SEM	Standard Error of the Mean
SNP	Single Nucleotide Polymorphism
STED	Stimulated Emission Depletion
TALE	Transcription Activator-Like Effector
TE	TrophEctoderm
TRIP13	Thyroid Hormone Receptor Interacting Protein 13
UV	UltraViolet
VRK1	Vaccinia-Related Kinase 1
ZW10	Zeste White-10
γH2AX	phosphorylated(γ) Histone 2A variant X

A mi abuelo.

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General organization of chapters

Chapter 1 consists of a review I wrote with my supervisor and was published in the journal Reproduction in January 2018. It details the mechanisms of chromosome segregation and error-avoidance pathways in embryos and also discusses how these may contribute to mosaic aneuploidy as well as the biological and clinical impacts of mosaicism in the early mammalian embryo. This publication will serve as general introduction to the topic of my thesis.

Chapter 2 presents my first author paper in the journal PNAS, published in January 2016. This study demonstrated a novel and unusual mode of chromosome segregation error in the mouse preimplantation embryo. Through live-cell imaging and immunofluorescence approaches we showed that lagging anaphase chromosomes typically result in the encapsulation of single chromatids into micronuclei. Importantly, micronucleus-enclosed chromosomes display defective nuclear envelope structure and nuclear import function and are subject to extensive DNA damage and loss of centromeric identity. These findings explain observations that micronucleus-enclosed chromosomes fail to assemble functional kinetochores causing their repeated inheritance during the following cell cycles. While not formally validated, this pattern of single chromosome mis-segregation and inheritance in preimplantation embryo development prevents reincorporation of heavily damaged micronucleus chromosomes into the embryonic genome, avoiding deleterious chromosomal translocations while inextricably causing a subset of daughter cells during subsequent divisions to bear aneuploid chromosome complements.

Chapter 3 presents an invited book chapter I wrote for Methods in Molecular Biology: Chromothripsis, which was published in March 2018. This methods article outlines in exhaustive detail the embryo collection, micromanipulation and imaging techniques that were employed for the vast majority of experiments contained within this thesis. Specifically, this article highlights the use of sequential live imaging and immunofluorescence to correlate chromosome segregation dynamics with endogenous protein subcellular localization. We were the first group to apply this correlative technique in a mammalian embryo. This approach was critical to demonstrate that micronuclei typically contain single chromatids and lack functional kinetochores during subsequent cell cycles in our study published in PNAS, 2016 (Chapter 2).

Chapter 4 presents my first author paper that is currently in preparation for submission. Having previously demonstrated that mouse embryos typically mis-segregate chromosomes, we next decided to investigate the underlying molecular causes of lagging chromosomes and mosaic aneuploidy in embryos. This study examines the function, sensitivity and robustness of the main error-avoidance mechanism in mitosis, the Spindle Assembly Checkpoint (SAC), in the mouse preimplantation embryo. We show that while the SAC is present, and normally acts to prevent errors, its strength is compromised during mid-preimplantation development, failing to arrest mitosis in the presence of chromosome alignment errors. We further demonstrate that unlike other systems of large cellular size explored to date, in the mouse embryo SAC strength is unaltered by natural and experimental reduction of cell size. Moreover, we show partial inhibition of SAC-target, the APC/C, can subtly extend mitosis and reduce segregation errors. This study represents the first mechanistic examination of the SAC in the early mammalian embryo and provides proof-of-principle evidence that pharmacological modulation of SAC signalling dynamics may reduce segregation errors and therefore potentially avert mosaic aneuploidy.

In **Chapter 5** I discuss the major contributions of the studies presented in this thesis to the chromosome segregation and reproductive biology research fields and will describe potential implications of our findings for clinical embryo culture, testing and selection methods in assisted reproduction technologies and infertility treatment in humans. Furthermore, current technical challenges of studying preimplantation cell division and outstanding questions will be discussed.

CHAPTER 1

INTRODUCTION

Publication

CAUSES AND CONSEQUENCES OF CHROMOSOME SEGREGATION ERROR IN MOUSE PREIMPLANTATION EMBRYOS

Review article published in Reproduction (2018) Jan;155(1): R63-R76,
PMID: 29109119

1.1 Preface

This review article was published in the journal Reproduction in January 2018. It gives an overview of the mechanisms of chromosome segregation and details the current knowledge of chromosome segregation error dynamics in the mammalian preimplantation embryo. It integrates recent findings from studies in animal models (mostly murine) with clinical and research observations in human embryos. Dr. Greg FitHarris and I wrote and edited the main text. I performed the live cell imaging experiments and produced all diagrams and figures presented in this article.

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CAUSES AND CONSEQUENCES OF CHROMOSOME SEGREGATION ERROR IN PREIMPLANTATION EMBRYOS

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Short title: Causes and Consequences of Embryo Mosaicism

1.2 Abstract

Errors in chromosome segregation are common during the mitotic divisions of preimplantation development in mammalian embryos, giving rise to so-called ‘mosaic’ embryos possessing a mixture of euploid and aneuploid cells. Mosaicism is widely considered to be detrimental to embryo quality, and is frequently used as criteria to select embryos for transfer in human fertility clinics. However, despite the clear clinical importance, the underlying defects in cell division that result in mosaic aneuploidy remain elusive. In this review, we summarize recent findings from clinical and animal model studies that provide new insights into the fundamental mechanisms of chromosome segregation in the highly unusual cellular environment of early preimplantation development and consider recent clues as to why errors should commonly occur in this setting. We furthermore discuss recent evidence suggesting that mosaicism is not an irrevocable barrier to a healthy pregnancy. Understanding the causes and biological impacts of mosaic aneuploidy will be pivotal in the development and fine-tuning of clinical embryo selection methods.

1.3 Introduction

1.3.1 The advent of the mosaic embryo

Preimplantation development is initiated by the fusion of highly specialised gametes, the sperm and the oocyte, resulting in the formation of a totipotent zygote. Faithful execution of the first several cell divisions after fertilisation is fundamental to the establishment of a healthy pregnancy. Following fertilisation, the male and female genomes form pronuclei in the zygote, whose subsequent breakdown marks the onset of the first mitotic division. Preimplantation mitotic cell divisions are 'reductive', meaning unaccompanied by cellular growth, thereby producing progressively smaller cells (Fleming and Johnson, 1988; Tsichlaki and FitzHarris, 2016). Simultaneously, numerous developmentally important events take place. For example, zygotic genome activation at the 4- to 8-cell stage in humans and 2- to 4-cell stage in mouse means that the embryo no longer relies on maternally stockpiled mRNAs and proteins and can synthesise these factors from the embryonic genome (Lee et al., 2014; Niakan et al., 2012). Compaction at the 8-cell stage brings the dividing cells into close adherence with each other, and cavitation at the 16- to 32-cell stage creates a fluid-filled cavity marking arrival at the blastocyst stage of development (Fleming and Johnson, 1988). Blastocyst formation is also accompanied by the clear delineation of the first two cell fate lineages in the embryo; the trophectoderm which will give rise to extraembryonic structures, and the inner cell mass which will constitute all embryonic tissues (Morris and Zernicka-Goetz, 2012; Niakan et al., 2012; Rossant, 2016), (**Fig. 1.1,** p.10)**.** This complex and coordinated series of events all take place as the embryo travels down the fallopian tubes from the ovary en-route to the implantation site, the endometrium. Thus, preimplantation development presents a highly unusual cellular context in which to execute such a critical succession of cell divisions.

Given that the early mitoses form the small number of cells from which the entire organism develops, one might imagine that these mitoses should be heavily safeguarded to ensure genetic fidelity is maintained. Rather, early mammalian development is synonymous with cell division errors. It has long been recognised that aneuploidy, when cells have an abnormal number of chromosomes, may be linked to reduced fertility since genetic aberrations are common in tissues from spontaneous miscarriages and most unisomy and trisomy aneuploid karyotypes are non-viable (Hassold and Hunt, 2001; Jones and Lane, 2013; Webster and

Schuh, 2017). Whole-embryo single chromosome copy number aberrations are predominantly due to chromosomes segregation defects in oocyte meiosis, which markedly increase with advanced maternal age (Hassold and Hunt, 2001). However, the introduction and development of improved culture methods and assisted reproduction technologies such as *in vitro* fertilization (IVF) in the last four decades provided an opportunity to study the chromosomal status of cells during early human development, and led to the realisation that while meiotically-derived whole-embryo aneuploidies occur in some embryos, 'mosaic' aneuploidy, where only a subset of blastomeres within an embryo are aneuploid, is more prevalent (Taylor et al., 2014a). This phenomenon was first reported in 1993 using simple Fluorescent In Situ Hybridisation (FISH) to label and count the copies of a limited number of chromosomes, and subsequent studies using whole-genome hybridization and modern sequencing approaches have revealed single chromosome gains or losses as the predominant genetic anomaly in mosaic embryos, occurring in up to 90% of embryos, depending upon the study (Delhanty et al., 1993; Echten-Arends et al., 2011; Munné et al., 1993, 2017; Vera-Rodriguez and Rubio, 2017). Additionally, polyploid and segmental mosaic aneuploidies are observed, albeit at much lower incidences (Echten-Arends et al., 2011) (**Fig. 1.2**, p.11). Chromosomal mosaicism has also been reported in non-human primate, porcine, bovine and murine embryos, suggesting it may be a wide-reaching phenomenon (Bolton et al., 2016; Dupont et al., 2010; Elaimi et al., 2012; Hornak et al., 2012, 2016). Chromosomal mosaicism must originate from mitotic errors during preimplantation development (Mantikou et al., 2012; Taylor et al., 2014a), but why the early mammalian embryo is inherently susceptible to mitotic errors, and precisely how these errors come about is very poorly understood.

Many excellent studies have used observed chromosome complements in spare embryos from the clinical setting to attempt to extrapolate the series of events that lead to embryo mosaicism (Coonen et al., 2004; Fragouli et al., 2013; Mantikou et al., 2012; Taylor et al., 2014a). However, understanding the aetiology of mitotic errors requires visualisation of the events in real time, and interventional experiments to probe the role of molecular players - experiments that are hard to tackle in the clinical setting. Therefore, the underlying cellular mechanisms through which chromosome segregation errors arise in early human development remain mostly elusive. Since many reviews of clinical literature cover the incidence and characteristics of preimplantation mosaicism (Echten-Arends et al., 2011; Munné et al., 2017; Taylor et al., 2014a), we here discuss what is known about the mechanisms of cell division in mammalian

embryos, highlighting recent key advances that point towards perspectives on the aetiology and impact of mitotic chromosome segregation errors in early embryos, and then go on to consider their consequences. For reviews of other types of genomic errors occurring in embryos such as segmental aneuploidies, chromosomal rearrangements, microdeletions and duplications, see (Capalbo et al., 2017; Morin et al., 2017; Treff and Franasiak, 2017).

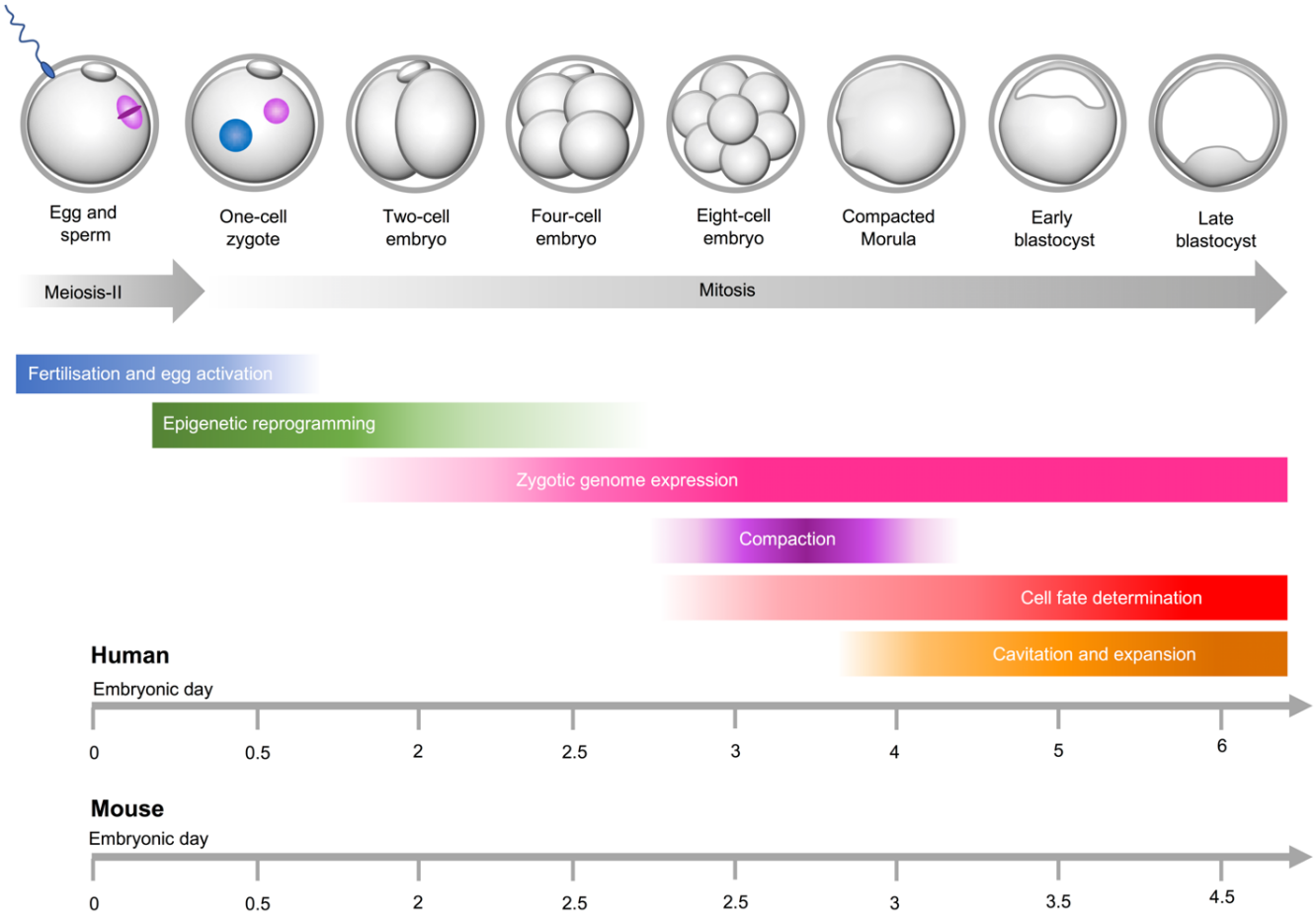

Figure 1.1. Human and mouse preimplantation embryo development

Diagrammatic representation of the major stages in mammalian preimplantation development. Key biological processes occurring during early development and timings for human and mouse embryos are indicated below.

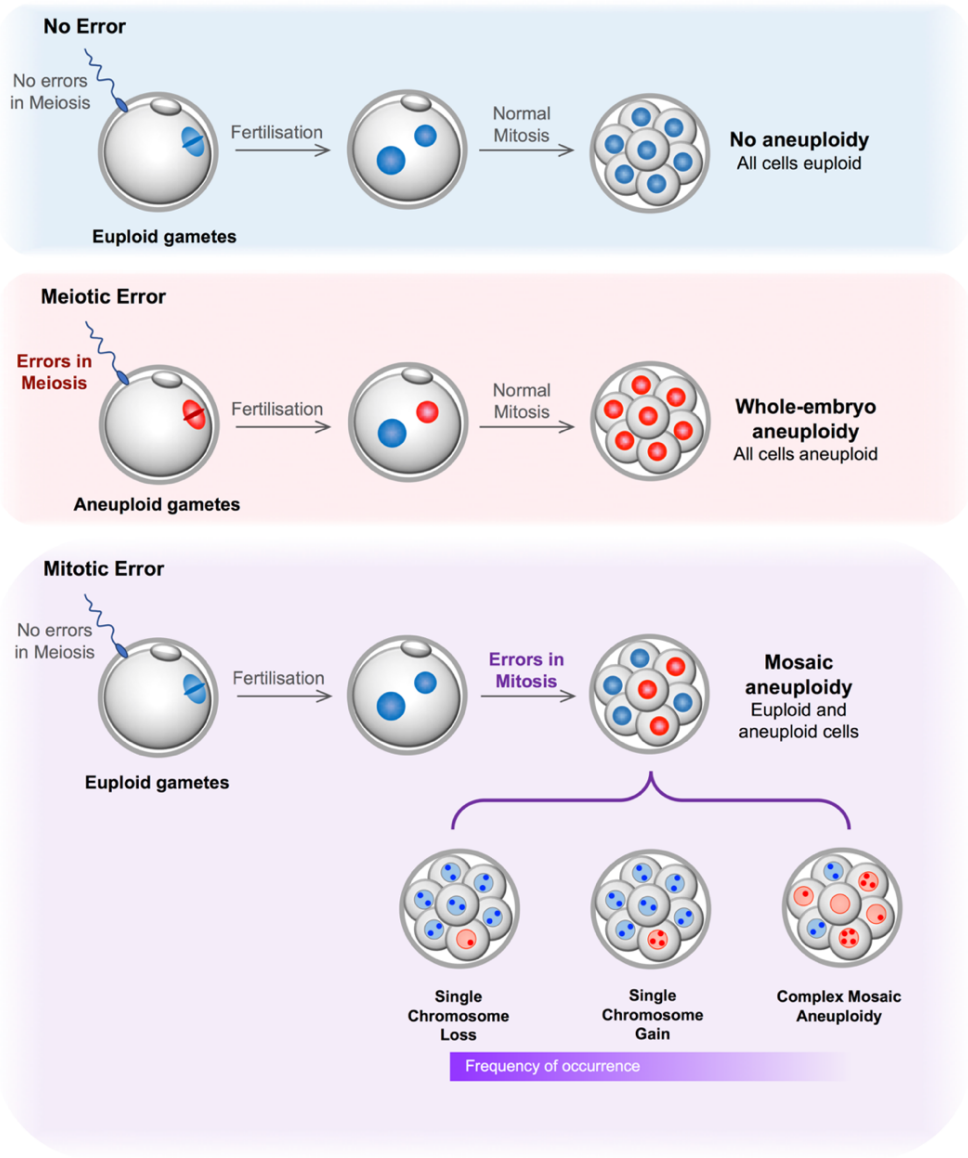

Figure 1.2. Aneuploidy in the preimplantation embryo originated from meiotic and mitotic chromosome segregation errors

Top panel: Normal fertilization of euploid gametes and error-free progression of meiosis-II and embryonic mitosis results in embryos in which all cells are euploid. Middle panel: Meiotic errors rendering gametes aneuploid (note that female meiosis-I error is represented, but may also occur during meiosis-II and male meiosis), result in embryos comprised of homogeneously aneuploidy cells. Bottom panel: Normal fertilization of euploid gametes and faithful completion of meiosis-II produce a diploid, chromosomally-balanced zygote. Errors in mitosis during embryonic cell divisions lead to a mixture of euploid and aneuploid cells. Diploid-aneuploid mosaicism is most common, with single chromosome copy number losses as the most prevalent defect, followed by single chromosome gains and complex or 'chaotic' mosaic aneuploidies.

1.3.2 The mechanics of chromosome segregation in mammalian embryos

1.3.2.1 Form and function of the mammalian spindle

Accurate chromosome segregation at the time of cell division is essential to preserve genetic integrity, and is achieved through a highly coordinated series of events. As cells enter mitosis the nuclear envelope breaks down and chromatin becomes further condensed into mitotic chromosomes. Simultaneously, microtubule-organizing centers (MTOCs) form, usually around two separate pairs of centrioles that nucleate and promote the polymerization of spindle microtubules. Kinetochores, complex multi-protein structures, become fully assembled at centromeric regions of chromosomes and act as a binding platform for spindle microtubules. Thus, by the stochastic attachment of microtubules emanating from two MTOCs results in the formation of a fusiform spindle, along whose equator chromosomes become progressively attached and aligned (Compton, 2000; Heald and Khodjakov, 2015; Petry, 2016).

Following the correct alignment and attachment of chromosomes, cohesin complexes affixing sister chromatids are cleaved, allowing replicated sister chromatids to be separated and pulled apart towards spindle poles in a process termed anaphase. In most cells anaphase comprises two components that co-ordinately separate the chromosomes, termed anaphase A and anaphase B. Anaphase A consists of a shortening of kinetochore bound microtubules, thereby lessening the distance from the chromosome to the pole. Anaphase B describes a spindle elongation that separates the spindle poles and thus further separates the chromosomes (Asbury, 2017; Maiato and Lince-Faria, 2010). Key to accurate segregation of chromosomes is that they be correctly attached at the bi-polar spindle, with sister kinetochores binding microtubules emanating from opposite spindle poles. Errors in attachment in somatic cells lead to segregation error and aneuploidy (Cimini et al., 2001a; Thompson and Compton, 2011). It is thus perhaps unsurprising that most cells possess mechanisms for ensuring correct kinetochore-microtubule attachment. Extensive work in somatic cells has elucidated two major and interconnected mechanisms that operate to prevent chromosome segregation errors during mitosis; kinetochore-microtubule error correction (‘error correction’), and the Spindle Assembly Checkpoint (SAC), both of which are outlined below. Anaphase is followed by the partitioning of the cytoplasm (a process termed cytokinesis) and decondensation of mitotic

chromosomes and formation of daughter nuclei (termed telophase) giving rise to two daughter cells with newly formed interphase nuclei (**Fig. 1.3**, p.13).

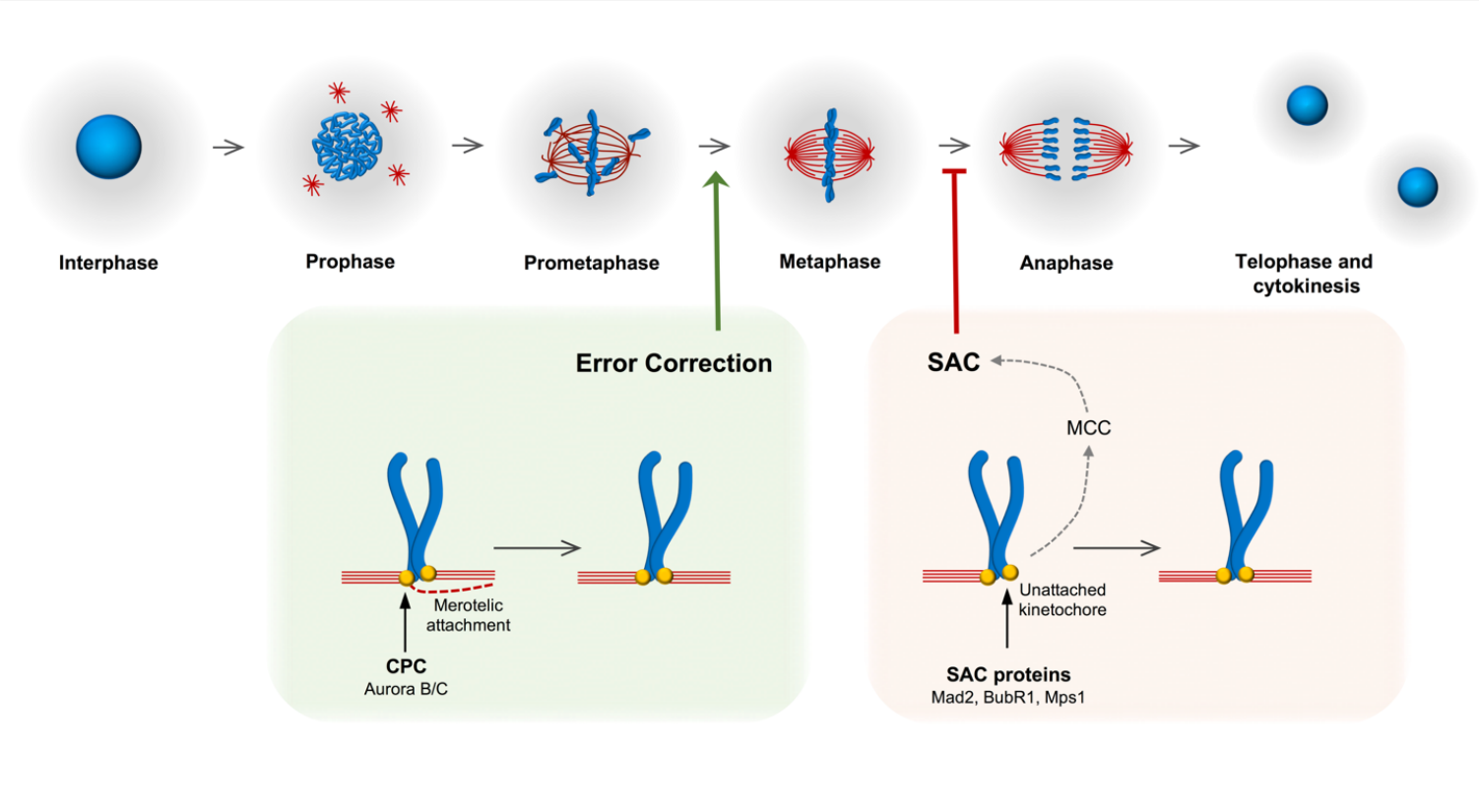

Figure 1.3. Chromosome segregation during mitosis and mechanisms preventing errors

Top: Schematic of the major physical events during mitosis. Condensation of the interphase nucleus into mitotic chromosomes (blue) as the nuclear envelope breaks down and microtubule organising centers (MTOCs) emerge (red). This is followed by MTOC clustering, bipolar spindle assembly and chromosome attachment to spindle microtubules (red). This is followed by chromosome congression and alignment at the metaphase plate as correct kinetochore-microtubule attachments are establish. Sister chromatids are pulled apart during anaphase and separation of the cytoplasm during cytokinesis ensues the formation of daughter cells. Bottom left: The error correction mechanism relies on the enrichment of Chromosomal Passenger Complex (CPC), the activity of Aurora kinases B and C and downstream effectors to destabilize incorrect kinetochore (yellow)- microtubule attachments, such as merotelic attachments. Bottom right: Spindle Assembly Checkpoint (SAC) proteins are recruited to unattached kinetochores, catalysing the formation of the mitotic checkpoint complex (MCC) which acts to prevent the metaphase-to-anaphase transition.

1.3.2.2. Spindle idiosyncrasies in the early mammalian embryo

The mechanisms of mitosis have been intensely studied in a variety of cellular contexts, demonstrating that even slight variations in spindle dynamics may result in dramatic chromosome segregation defects (Thompson et al., 2010). The reductive divisions of early development are highly idiosynchratic, and understanding spindle behaviour in this cellular system cannot rely upon extrapolation from somatic cells. Importantly, a small number of recent studies employing live imaging approaches to understand how spindles are assembled in embryos, largely utilising mouse as a model for mammalian embryogenesis, have begun to reveal some of the challenges faced by embryo cell divisions.

In the context of dramatically changing cell size, with each division approximately halving cell size, how spindle structure is regulated during embryo mitosis presents an interesting cell biological conundrum. In general, spindle length is considered to be controlled by two classes of forces; the dynamics of the microtubules and associated motor proteins as well as the physical properties of chromosomes are termed 'intrinsic' influences, whereas 'extrinsic' influences describe external influences on the spindle, such as whether the cell size limits the length of the spindle (Dumont and Mitchison, 2009; Goshima and Scholey, 2010; Levy and Heald, 2012). The spindle in the zygote is substantially shorter than the diameter of the cell, suggesting length regulation is entirely intrinsic. But from the second mitosis onwards, as cell size decreases, spindle size approaches the diameter of the cell, indicating extrinsic regulation (Yamagata and FitzHarris, 2013), as has been seen in lower vertebrate embryos (Good et al., 2013; Wühr et al., 2008). Cytoplasmic removal experiments in mouse illustrated this point elegantly; moderate reductions in cell size during the first few cell divisions, when the spindle is far shorter than the cell, has little affect upon spindle length. Contrastingly, cytoplasmic removal in later preimplantation divisions (4-8 cell and onwards), when spindle length is similar to cell length, shortens the spindle (Courtois et al., 2012). Similarly, whereas 1- and 2-cell embryos exhibit a pronounced anaphase-B spindle elongation, the extent and speed of spindle elongation during anaphase is decreased thereafter as the size of the cell becomes a limiting factor to spindle elongation (Yamagata and FitzHarris, 2013). Hence, reductive divisions impose ever-changing limits on spindle structure and dynamics during preimplantation development. Curiously, the spindle in the first mitotic division is proportionally smaller than in the second division, despite a far greater cell size (Courtois et al., 2012; Yamagata and

FitzHarris, 2013), suggesting spindle length during the first mitosis is subject to different, perhaps more meiotic-like, regulatory mechanisms, than later divisions. Whether this is mediated by remnants of cytostatic factor components that may persist in the first mitosis, remains to be seen (Kubiak and Ciemerych, 2001; Maller et al., 2002). The shift in the mode of spindle length regulation in metaphase and anaphase described above exemplifies an emerging theme of cell division during early development; that progression from zygote to blastocysts is accompanied by gradual developmental shifts, rather than an abrupt switch in the way in which blastomeres approach a canonical mode of mitosis. Another such example is that maintenance of spindle bipolarity in metaphase is critically dependent on the mitotic motor Kinesin-5 during the first three mitosis but not later divisions, reflecting a shift in the role of the motor from its oocyte role, where it is essential in metaphase, to its somatic cell role, where it is not (FitzHarris, 2009). How changing cell size may impact the occurrence of segregation errors is further discussed in the context of error avoidance pathways below.

1.3.2.3 Unusual centriole behaviour in early development

Centrioles form the focal point of the spindle-organising centrosome in most mammalian cells. Since one centriole pair is inherited by each daughter cell, centrioles must replicate each S-phase to provide two pairs in the subsequent mitosis (Loncarek and Khodjakov, 2009; Nigg and Raff, 2009). Making sure that centriole replication happens once-and-only-once is important, since over-replication causes multipolar spindles, which cause chromosome instability as seen in cancer cells (Ganem et al., 2009; Gönczy, 2015; Kwon et al., 2008). To avoid too many centrioles after fertilisation, oocytes of most species degrade their centrioles. In many species, including humans, a single centriole and centrosome is thus inherited from the sperm at fertilisation. Pronuclear removal experiments in tri-pronuclear and diploid human zygotes and somatic cell nuclear transfer in mouse zygotes demonstrated that the sperm or donor centrosome is functional and directs spindle assembly in the first mitotic divisions (Kalatova et al., 2015; Palermo et al., 1994; Van Thuan et al., 2006). However, little is known about how these function during the remainder of early human preimplantation development, and some intriguing observations allude that centriole dysregulation may occur in embryos. Firstly, studies of fresh and vitrified human embryos have reported multipolar spindles and tri-directional anaphases (Chatzimeletiou et al., 2012; Vera-Rodriguez et al., 2015), which in somatic cells is symptomatic of too many centrosomes (Ganem et al., 2009). Secondly, a recent

genome wide association study identified single nucleotide polymorphisms in the sequence of PLK4, a key regulator of centriole duplication, to be associated with embryonic mitotic errors (McCoy et al., 2015). Thus, the idea that centrosome/centriole dysregulation contributes to human embryo mosaicism requires further attention.

In the mouse embryo the role and regulation of centrioles is even more intriguing. In addition to the oocyte lacking centrioles, the mouse sperm also destroys its centrioles during the elongating spermatid phase (Sathananthan, 1997), such that most of mouse preimplantation development then occurs in the absence of classical centrosomes until the ~64-cell stage, when centrioles mysteriously emerge (Courtois et al., 2012; Gueth-Hallonet et al., 1993; Houliston et al., 1987; Howe and FitzHarris, 2013). This unexplained series of events uncovers another gradual shift in spindle microtubule behaviour in embryos. Spindle assembly in the first few mitoses was seen to rely on recruiting several cytoplasmic MTOCs, which form a multipolar spindle, that is later focused and achieves bipolarity, reminiscent of meiotic spindles (Courtois et al., 2012; Gueth-Hallonet et al., 1993; Schatten et al., 1986; Schuh and Ellenberg, 2007), but from the eight-cell stage, while centrioles and canonical centrosomes are still absent, spindle assembly is mediated by MTOCs that arise exclusively at the nuclear periphery and along the spindle in multipolar intermediates (Courtois et al., 2012). This MTOC clustering within the spindle during mid-preimplantation development was shown to be dependent on the Augmin complex, distinct from its role in other systems (Watanabe et al., 2016). Additionally, PLK4 has been shown to be essential for bipolar spindle assembly in mouse preimplantation embryos, despite the fact that centrioles are not present, further indicating non-canonical roles of well-characterised spindle proteins in early development (Arquint and Nigg, 2016; Coelho et al., 2013). Although centrioles later emerge at blastocyst stage, it remains unclear whether they are fully functional, since they appear then to lack canonical microtubule-organising ability in interphase (Howe and FitzHarris, 2013). To summarise, multiple lines of evidence both from mouse and human embryos highlight the importance of understanding mechanisms of centriole and centrosome function in preimplantation development, and multiple clues indicate that the way in which they function is likely distinct from better-studied somatic cells. Further work is needed to elucidate how centrosome function is regulated during development.

1.4 How it all goes wrong: the dynamics of errors in embryos

1.4.1 Micronuclei as a possible catalyst for mosaicism

Manifold studies of *in vitro* cultured human embryos have attempted to use the observed chromosome complements of blastomeres at various developmental stages to extrapolate how chromosome segregation errors originated in those embryos. These analyses resulted in various potential explanations as to how mosaicism might arise including; non-disjunction of sister chromatids at anaphase, disappearance of a sister chromatid (often termed 'anaphase lag' in the human embryo literature), and inappropriate repeated occurrence of a single chromatid (sometimes referred to as endoreplication). While these studies highlight important principles of how errors could arise, unravelling the true nature of mis-segregation will require coupling current and emerging genetic screening techniques, such as next-generation genome sequencing, with direct observation of errors to determine the temporal and genetic dynamics of mosaic aneuploidy in the early embryo. Perhaps unsurprisingly, live imaging experiments of chromosome segregation errors are yet to be presented in human embryos. However, recent live imaging of chromosome segregation errors in mouse embryos suggest that micronuclei, small additional nuclei containing one or few chromosomes separate from the main nucleus which, importantly, are well-known to be prevalent in human embryos, may play a critical and previously unrecognized role in the generation of mosaicism (Fragouli et al., 2013; Vázquez-Diez et al., 2016).

By extensive imaging of embryonic mitoses in mouse it was shown that micronuclei arise because of single sister chromatids that remain separate from the main group in anaphase (termed 'lagging chromosomes'), which then form their own nuclear membrane in the resulting daughter cells (**Fig. 1.4**, p.18). Following encapsulation into micronuclei, the chromosomes become extensively damaged and lose centromeric identity indicated by the centromere marker CENP-A, and therefore fail to assemble a kinetochore and be segregated by the spindle during subsequent cells divisions (Vázquez-Diez et al., 2016). A similar series of events has been observed in somatic cells (Crasta et al., 2012; Guerrero et al., 2010; Huang et al., 2012; Zhang et al., 2015a), and it is thought that the defective nuclear envelope on the micronucleus, leading to failed nuclear compartmentalisation, underpins the DNA damage to the chromosome enclosed (Hatch et al., 2013). Strikingly, in embryos, the same heavily damaged chromosome re-appears as a micronucleus following each subsequent cell division (Vázquez-Diez et al.,

2016). Although live imaging of this type in human embryos is yet to be reported, human embryo immunofluorescence data shows the occurrence of single anaphase lagging chromosomes, micronuclei bearing high levels of DNA damage and lacking centromeric identity, and micronucleus-like chromosomes in mitosis devoid of kinetochore staining (Kort et al., 2016). These findings strongly allude that the same sequence of events likely occurs in human embryos. Importantly, as elaborated in **Box1**, this series of events provides a mechanism for the high occurrence of single chromosome copy number variation in mosaic embryos, and, provide for the first time a coherent explanation as to why single chromosome losses outnumber gains in early embryos (*see* **Box 1,** p.19, **Fig. 1.5**, p.21). This mechanism may be specific to early embryos, as micronuclei are typically re-incorporated back into the main nucleus in other cell types (Crasta et al., 2012; Hatch et al., 2013; Leibowitz et al., 2015; Ly et al., 2017; Zhang et al., 2015).

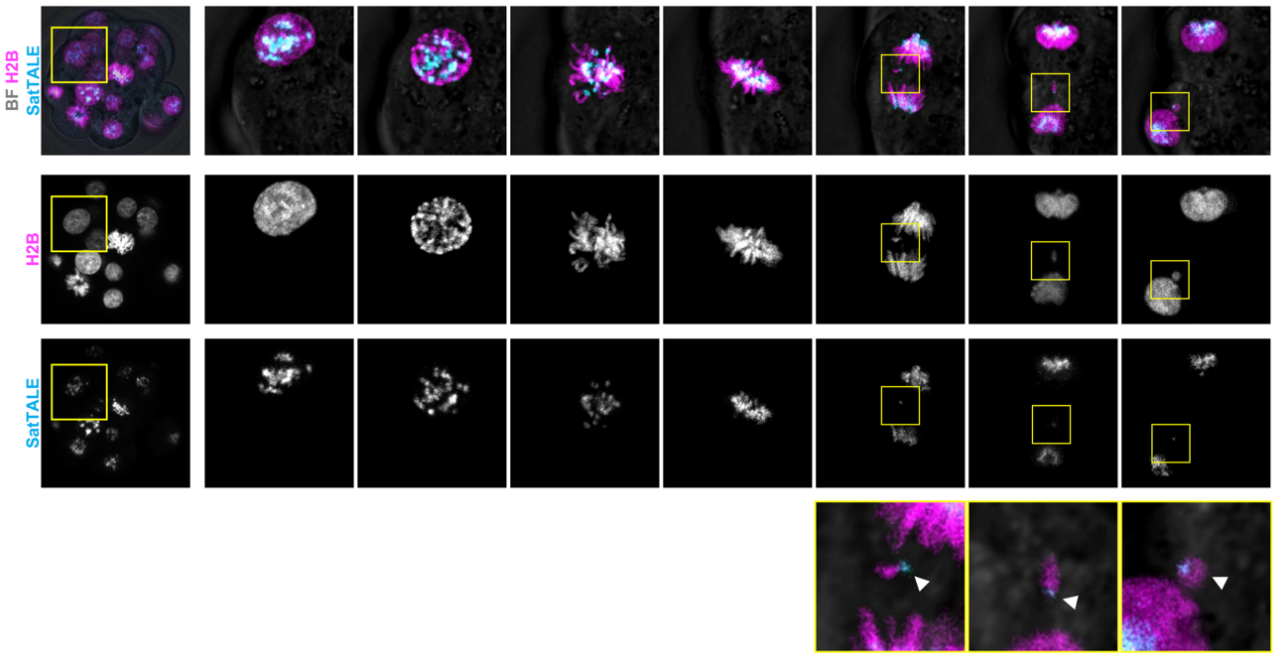

Figure 1.4. Lagging chromosomes cause micronuclei in mouse preimplantation embryos

Confocal time-lapse frames of a H2BRFP and MajSatTALEmClover-expressing morula, labelling chromatin and centromeric regions, respectively. Lagging anaphase chromosomes result in the encapsulation sister chromatids into micronuclei (white arrowheads).

BOX 1. Micronucleus and inheritance can explain the most prevalent forms of mosaic aneuploidy in embryos

Live imaging in mouse embryos revealed that after the formation of micronuclei by a lagging chromosome (**Fig. 1.4**, p.18), the micronucleus is unilaterally inherited at each subsequent cell division (Vázquez-Diez et al., 2016). This can explain two of the most commonly observed features of mosaicism in the clinic.

Firstly, these events provide a coherent explanation for single chromosome losses, which are by far the most common genetic abnormality present in embryos - mosaic unisomies being up to seven times more frequent than trisomies (Coonen et al., 2004; Taylor et al., 2014a) (**Fig. 1.5**, p.21). During cell division, a lagging chromosome causes a single chromatid to be incorporated into a micronucleus in only one of the two daughter cells. In a balanced scenario, the lagging chromosome initially forms a micronucleus in the correct daughter cell, such that the complete chromosome complement is present taking into account both the main nucleus and micronucleus. However, although both sister blastomeres are initially formally euploid, the main nucleus of the micronucleus-containing cell harbours a single chromosome copy loss. In the subsequent division of that cell, the daughter cell that does not receive the MN will necessarily be hypoploid (**Fig. 1.5,** left panel). Alternatively, if the lagging chromosome generates a micronucleus in the daughter cell whose main nucleus already contains a euploid chromosome content (ie the initial segregation error is unbalanced), then the micronucleus-free sister blastomere will lack a single chromosome. Subsequently all of the progeny of this cell will display single chromosome loss (**Fig. 1.5,** right panel). Thus, regardless of the initial 'direction' of lagging chromosomes, micronucleus inheritance provides a potential explanation for a high incidence of single chromosome losses.

Secondly, micronucleus inheritance can explain single chromosome gains, the second most common genetic abnormality in mosaic embryos (Echten-Arends et al., 2011; Taylor et al., 2014a). Specifically, if the lagging chromosome forms a micronuclei in the daughter cell whose main nucleus already harbours a complete chromosome complement, this will result in a single (micronucleus-enclosed) chromosome gain (**Fig. 1.5,** right panel). While such single chromosome gains are usually attributed to a classical non-disjunction event, wherein an extra sister chromatid arrives within a single newly-forming nucleus (Echten-Arends et al.,

2011; Mantikou et al., 2012; Taylor et al., 2014a), lagging chromosomes leading to formation of micronuclei in non-complementary cells should have a similar impact.

Importantly, note that in the literature single chromosome losses have often been attributed to ‘anaphase lag’, without the mechanism for this being clear. Within our model the lagging anaphase chromosome acts as a trigger point by generating a micronucleus, but it is the clonal propagation of the blastomeres following a micronucleus formation event (rather than the lagging chromosome per se) that accounts for the high incidence of cells exhibiting a chromosome loss.

Finally, note that this model unexpectedly provides a possible mechanism for aneuploid cells to ‘self-correct’. During cell division of a trisomic cell, if the additional chromatid is enclosed in a micronucleus, the micronucleus-free daughter of that (formally aneuploid) cell will be euploid, and should therefore have the potential to generate a clone of euploid blastomeres (Fig. 5. right panel, arrows). Whilst some preimplantation genetic screening approaches can detect DNA within micronuclei on at least some occasions, how reliable MN detection is by such methods after several cell divisions is unclear, and will be important to clarify.

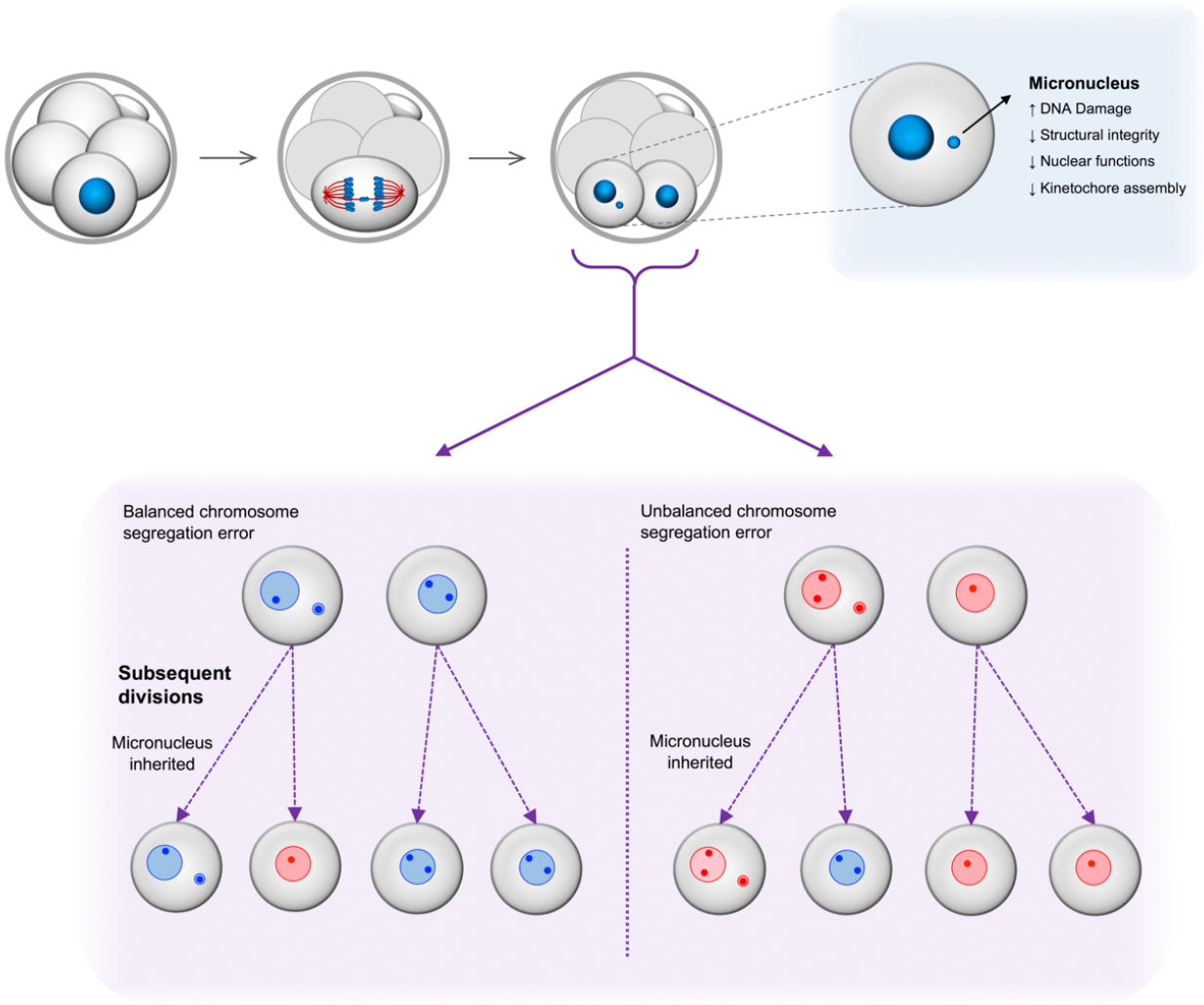

Figure 1.5. Micronucleus inheritance explains the most prevalent defects in mosaic embryos

Lagging anaphase chromosomes lead to the isolation of single chromosomes into micronuclei, which display defective nuclear structure and function, wherein chromosomes are subject to high levels of DNA damage. Likely as a consequence of DNA damage, micronuclei-enclosed chromosomes fail to assemble a kinetochore and are inherited during subsequent divisions. Regardless of whether the micronucleus initially forms in the chromosomally-balanced sister (left) or not (right), this pattern of inheritance inevitably generates aneuploid daughters (red nuclei) during following cell cycles. Notably, this model explains the higher occurrence of single chromosome losses and the less frequent incidence of single chromosome gains observed in human embryos.

1.4.2 Chromosomal abnormalities unexplained by micronucleus formation

Other chromosomal composition abnormalities have been described in preimplantation embryos including ploidy defects and complex or 'chaotic' abnormalities, albeit at lower frequencies (Echten-Arends et al., 2011). Furthermore, nucleation status abnormalities such as binucleation and multinucleation are also common in preimplantation embryos (Royen et al., 2003). Tetraploidy, when a cell has two copies the normal chromosome complement, is also observed commonly in humans. How tetraploidy emerges is not clear but could conceivably arise from cell fusion, although cytokinesis failure or mitotic slippage - where the cells exits mitosis in the absence of sister chromatid separation – are more plausible explanations. In the human embryo, these tetraploid conditions are likely accompanied by extra centrosome copies, that could lead to multipolar spindle formation and multidirectional anaphases, both scenarios resulting in aneuploidy and multi-nucleation. Whether binucleated and tetraploid embryos exhibit multipolar metaphases and tri-directional cell divisions has not been confirmed. However, in human somatic cells the SAC delays mitosis in the presence of multipolar spindles (Kwon et al., 2008), and aneuploid human embryos show delayed first mitotic divisions, together alluding that super-numerary centrioles may exist (Vera-Rodriguez et al., 2015).

1.5 Why it all goes wrong: the embryo as a permissive environment for errors?

Given the importance of maintaining genetic fidelity, it is perhaps unsurprising that most mammalian cells possess multiple mechanisms for averting errors. Indeed, in somatic tissues the rate of aneuploidy is thought to be only 2% (Knouse et al., 2014). Given the high incidence of mosaic aneuploidy and prevalence of mitotic errors in mammalian preimplantation development, it has long been suggested such mechanisms may be compromised or absent during mitotic cleavage divisions (Albertini, 2016; Echten-Arends et al., 2011; Mantikou et al., 2012; Taylor et al., 2014a). Here we discuss the limited data on whether and how these pathways function in early embryos.

1.5.1 Kinetochore-microtubule attachment error correction

The error correction mechanism acts locally on mis-attached chromosomes and involves the targeted recruitment of factors that will destabilize improper kinetochore microtubule

attachments. This mechanism is mediated by the Chromosomal Passenger Complex (CPC), a multi-protein assembly typically composed of Aurora B, INCENP, Survivin and Borealin, which localizes to centromeres during prometaphase (Krenn and Musacchio, 2015; Musacchio and Salmon, 2007). At mis-attached chromosomes, Aurora B kinase phosphorylates several kinetochore components ($Ndc80^{Hec1}$, Knl1 and Dsn1) to reduce their microtubule-binding affinity. Furthermore, Aurora B also recruits kinesin-13 family members KIF2B and MCAK/KIF2C, which have microtubule depolymerising activities (Krenn and Musacchio, 2015). The error correction mechanism disrupts improper kinetochore-microtubule attachments, promoting the formation of new, correct, end-on attachments. Error correction is particularly important for the detection and correction of syntelic (where both sister kinetochores are bound to microtubules emanating from the same spindle pole) and merotelic attachments (in which a single kinetochore is bound to microtubules originating from both spindle poles) (**Fig. 1.3**, p.13).

Several recent lines of evidence reveal embryo-specific particularities of the error-correction pathway that could conceivably contribute to the increased rates of mis-segregation in early embryos. While in somatic cells CPC error correction function is mediated by Aurora B kinase (Krenn and Musacchio, 2015), recent studies suggest a major role for a less-well characterised Aurora kinase isoform, Aurora C Kinase. Human and murine preimplantation embryos exhibit higher relative abundance of Aurora C in early development, with Aurora B abundance increasing by blastocyst stage, both in mouse and human embryos (Avo Santos et al., 2011; Li et al., 2017b; Schindler et al., 2012). Moreover, knockout of Aurora B kinase in mouse embryos results in normal developmental rates but fail to develop beyond blastocyst stage (Fernández-Miranda et al., 2011). On the other hand, Aurora C knockout significantly impairs development, reducing blastocyst formation rates, and knockdown of both Aurora B and C together result in increased rates of mitotic arrest and chromosome segregation defects (Fernández-Miranda et al., 2011), perhaps suggesting a more significant role of Aurora C kinase in error correction in embryonic mitoses (Fernández-Miranda et al., 2011; Schindler et al., 2012). Additional evidence implicating Aurora kinases in regulation of embryonic mitosis comes from the identification of two Aurora B and C single nucleotide variants in humans, one of which is associated with reduced rates of aneuploidy in women advanced maternal age and which promotes correct chromosome alignment when expressed in mouse oocytes (Nguyen et al., 2017). Further studies using immunofluorescence and chemical inhibition approaches in human tri-pronuclear and diploid embryos have revealed an unusual CPC component

localization in zygotes due to increased activity of Aurora-activator Haspin kinase (van de Werken et al., 2015). Determining how this unexpected expression pattern of Aurora kinases regulate downstream CPC effectors such as KIF2B and MCAK to modulate chromosome segregation dynamics and microtubule interactions at the kinetochore, will be important to establish the role of these kinesins in the early embryo.

1.5.2 The Spindle Assembly Checkpoint (SAC)

The SAC is a near-ubiquitous signalling pathway that operates to arrest cells in metaphase of mitosis until all chromosomes have been successfully attached. SAC signalling is initiated at mis-attached kinetochores through the recruitment proteins such as Mps1, Mad1, Mad2, Bub1, Bub3, BubR1. These in turn catalyze the production of the Mitotic Checkpoint Complex (MCC), composed of Mad2/BubR1/Bub3/Cdc20 (Lara-Gonzalez et al., 2012; Musacchio and Salmon, 2007). Kinetochore-produced MCC acts as a diffusible signal in the cytoplasm that targets the Anaphase Promoting Complex (APC/C) to inhibit its ubiquitin ligase activity and prevent degradation of Securin and Cyclin B, hence preventing cohesin cleavage and delaying anaphase onset (Collin et al., 2013a; Lara-Gonzalez et al., 2012; Musacchio and Salmon, 2007). The SAC coordinates achievement of correct kinetochore-microtubule attachment of all chromosomes with sister chromatid separation and mitotic exit (**Fig. 1.3**, p.13). It is perhaps unsurprising therefore that absent SAC activity has recurrently been forwarded as a candidate explanation for the high incidence of mitotic errors in embryos (Echten-Arends et al., 2011; Fragouli et al., 2013; Mantikou et al., 2012; Taylor et al., 2014a). In support of such an idea, SAC component transcript levels are low during human cleavage stages (Wells et al., 2005).

A recent study in human embryos revealed that the spindle poison nocodazole can arrest cell divisions in mitosis, which is a first line of evidence that the SAC might in fact operate (Jacobs et al., 2017). Whether these effects are a bona fide indicator of SAC function remains to be determined and will require direct investigation of SAC components. In mouse, deletion of SAC components Mad2 or BubR1 subtly affects preimplantation development, but direct information of SAC activity is currently lacking (Dobles et al., 2000; Wang et al., 2004). In the meantime, there are strong clues that, analogous to error correction, the function of the SAC may be non-canonical. Significantly, landmark recent studies revealed that in the mouse oocyte the SAC

not only surveys attachment of kinetochores to spindle microtubules, but unexpectedly also specifically arrests meiosis-I in response to DNA damage (Collins et al., 2015; Lane et al., 2017; Marangos et al., 2015). Interestingly, DNA damage during mitosis has been shown to directly alter microtubule dynamics in somatic cells (Bakhoum et al., 2014). Whether DNA damage may influence spindle dynamics and SAC function in mammalian embryos remains elusive. Classic studies of SAC function in other vertebrate embryos, including *Xenopus* and Zebrafish, demonstrated that early cleavage divisions occur in the absence of a functional SAC, which appears later at the mid-blastula transition (Hara et al., 1980; Zhang et al., 2015b). Indeed, a role for changing cell size in SAC function has been underlined by two recent studies. In *C. elegans* embryos it was shown that early embryos possess a weak checkpoint, and the SAC becomes increasingly robust as development progresses and cells become smaller (Galli and Morgan, 2016). Analogously, in mouse oocytes, experimental manipulation of oocyte size found that larger cells had less robust SAC function (Kyogoku and Kitajima, 2017; Lane and Jones, 2017). Whether a similar cell size-dependent shift in SAC function operates in the early mammalian embryo and might contribute to the error-prone nature of the mammalian embryo has not yet been tested.

1.6 Consequences of errors: Is mosaicism such a bad thing?

1.6.1 The impact of mosaicism on embryo quality

In the light of the prevalence of mosaic aneuploidy in mammalian preimplantation development, it is essential to understand its impact to developmental potential and embryo viability, particularly in the clinical setting. Whilst early studies of early embryo mosaicism relied on FISH to analyse the presence or absence of a restricted number of chromosomes, the advent of advanced methods such as comparative genomic hybridisation and so-called next generation sequencing to assess all chromosomes has improved our understanding of the impact of mosaicism (Munné et al., 2017). Blastocysts classified mosaic after biopsying and analysing a moderate number of cells (often ~5 or so) are less likely to implant and more likely to miscarry than ‘euploid’, controls. Importantly, however, the same study also provides strong evidence indicating mosaic embryos can be viable (Munné et al., 2017). Specifically, diploid-aneuploid mosaic embryos had implantation rates comparable to euploid embryos, and blastocyst with up to 60% aneuploidy in biopsied cells resulted in successful pregnancy outcomes (Fragouli

et al., 2017; Munné et al., 2017). These findings suggest that mosaic aneuploidy does not necessarily end developmental potential. One caveat of this conclusion is that biopsy studies are naturally hampered by random selection of cells from embryos, of which a large proportion likely comprise at least some aneuploid cells (Vanneste et al., 2009). Importantly, it is becoming increasingly clear that preimplantation genetic screening results of a few cells is not necessarily a reliable judge of the extent of mosaicism within an embryo (Gleicher et al., 2016, 2017). Nonetheless, the emerging view is that, clinically, embryos judged to be likely mosaic are embryos of intermediate favourability for patient transfer – less favourable than euploid, more than uniformly aneuploid embryos – but consistency of practice between clinics remains lacking (Munné et al., 2017).

Detailed and elegant experiments in mouse corroborate the notion that mosaic embryos are not necessarily doomed, and provide the beginnings of an explanation. Embryo transfer experiments using two different mouse models of mosaicism demonstrated that degree of mosaicism does not affect implantation rates (Bolton et al., 2016; Lightfoot et al., 2006). Furthermore, embryos with moderate but not extensive levels of mosaicism can fully develop to term (Bolton et al., 2016), suggesting a 'threshold' degree of mosaicism could be tolerated provided that a critical number of healthy inner cell mass cells are preserved. Indeed, it has been proposed that the mammalian fetus is derived from as little as three inner cell mass cells (Markert and Petters, 1978), leading to the hypothesis that only a small portion of euploid cells are necessary to sustain human fetal development - a notion that is supported by the observation that frozen-thawed human embryos which have lost almost half of blastomeres during the cryopreservation procedure are still viable and result in live births (Zheng et al., 2008). This may be explained by two major non-mutually exclusive mechanisms; the preferential proliferation of euploid cells and the negative selection of aneuploid cells in the inner cell mass (Echten-Arends et al., 2011; Fragouli et al., 2013). Live imaging experiments in mouse embryos revealed that some aneuploid cells within the inner cell mass undergo apoptosis, presumably to increase the proportion of euploid cells, though no evidence was seen of aneuploid cells being 'directed' to the trophectoderm as previously posited (Bolton et al., 2016). Increased rates of apoptosis were also seen in mosaic embryos, such that the embryo cylinder from embryo chimaeras is largely composed of normal cells by embryonic day 7.5 (Bolton et al., 2016; Lightfoot et al., 2006). Coherently, in human embryos, rates of mosaicism are lower in blastocyst than morula stages and are further reduced post-implantation (Echten-

Arends et al., 2011; Mantikou et al., 2012; Taylor et al., 2014). Thus, whilst much is left to be learned about the consequences and fate of aneuploid cells in the mosaic embryo, evidence to date suggests that apoptotic pathways and other mechanisms may act in a targeted cell-type specific manner before and after implantation to remove abnormal cells from mosaic embryos (Albertini, 2016), and that provided sufficient euploid cells are present in the inner cell mass to form the embryo-proper, mosaic aneuploidy can be compatible with healthy, full term development and live birth.

1.6.2 An unexpected advantage: mosaicism as an evolutionary benefit?

As described above, it appears increasingly plausible that mosaic aneuploidy is, perhaps counterintuitively, a normal feature of preimplantation development across multiple mammalian species (Bolton et al., 2016; Dupont et al., 2010; Hornak et al., 2012, 2016; Mizutani et al., 2012; Vázquez-Diez et al., 2016). In which case, it becomes reasonable to ask; could chromosome missegregation in the early embryo present some positive biological benefit?

Unlike other animals which produce tens to hundreds of embryos *ex vivo*, mammals produce fewer offspring. Due to the physical and time investment in gestation, lactation and nursing required by their offspring, mammalian reproduction should be selective of the parental genomes, environment and maternal health status. One possible way to achieve such selection could be to exploit the highly atypical environment of preimplantation embryo development that likely poses strains on the cellular resources of the embryo during development. Having mitotic divisions that are inherently error-prone and sensitive to environmental perturbations may be physiologically advantageous since upon undesired situations such as poor maternal health, low gamete quality, or deleterious environment (poor nutrition for example), a 'mosaicism threshold' can be surpassed and chances of full term development in an unfavourable biological context are reduced. In other words, the mosaicism threshold could serve as a sensor for whether an ongoing pregnancy is desirable. Alternatively, it was recently suggested that increasing oocyte aneuploidy with maternal age may serve a positive benefit in humans by causing an extended period of subfertility in the 40's, prior to the complete cessation of ovulatory cycles (menopause) which occurs at ~50. Since the loss of all oocytes is associated with a decline in oestrogen that precipitates osteoporosis and other changes to detrimental to

general health, age-related oocyte aneuploidy potentially provides a mechanism to allow older females to maintain a baseline level of oestrogen that is beneficial to health, while averting pregnancy (Sirard, 2011). This notion is consistent with the well-known grandmother hypothesis, wherein older females are considered to increase their chances of passing genes to future generations by helping raise their children's offspring, rather than undergoing further potentially hazardous pregnancies (Alvarez, 2000; Hawkes and Coxworth, 2013; Hawkes et al., 1998). Within such a model one might imagine that an inherently fragile ooplasm susceptible to increased mis-segregation in the face of age-related ovarian changes may be crucial, and that segregation errors in the embryo shortly after fertilisation are simply a manifestation of this fragility. Whilst much of the above is speculative, we consider it at least conceivable that mosaicism of the preimplantation embryo may not be purely a pathology, but may play a positive role that is yet to be fully realised.

1.7 Concluding remarks

Mosaic aneuploidy is a common phenomenon in mammalian preimplantation embryos that arises due to errors in mitosis during embryonic divisions. The underlying mechanisms responsible for chromosome segregation errors are yet to be fully elucidated but the emerging theme is that the unique cellular environment and idiosyncrasies of cell division in the early embryo are at the heart of the story. For this reason, unravelling the aetiology of these errors cannot rely on extrapolation from somatic cells and other traditional systems, but will require direct examination in mammalian embryos, and the advent of advanced imaging systems to observe these events will no doubt advance our understanding. Furthermore, it becomes increasingly clear that whilst a high level of mosaic aneuploidy is detrimental to embryo viability, some levels can be compatible with successful pregnancy and full term development, but the biological reasons for this remain far from clear. We envisage a continuing shift in how mosaicism is viewed and interpreted in the clinical context in the coming years.

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C.V.-D. and G.F. wrote the manuscript. C.V.-D. performed the live imaging experiment and produced the figures. We thank Karen Schindler and David Albertini for valuable comments and discussion of the manuscript. We apologise to any authors whose work was not cited for space reasons. Work in G.F.'s laboratory is funded by CIHR, NSERC, and Fondation Jeanne et Jean-Louis Lévesque. G.F. is an Editor-In-Chief of Reproduction but was not involved in the appraisal of this paper in any way.

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CHAPTER 2

Publication

MICRONUCLEUS FORMATION CAUSES PERPETUAL UNILATERAL CHROMOSOME INHERITANCE IN MOUSE EMBRYOS

Research article published in PNAS (2016) Jan;113(3): 626-31, PMCID: PMC4725495

2.1 Preface

Both mosaic aneuploidy and micronuclei are commonly observed in human embryos, however how these arise and relate to each other was not clear. Additionally, a series of recent studies identified DNA damage in cancer cell micronuclei as a critical mechanism for chromothripsis, a phenomenon where cancer cells commonly harbour extensive DNA rearrangements typically confined to a single chromosome. Despite a certain degree of speculation, whether chromothripsis also takes place in the mammalian embryo was elusive. I performed and analysed live cell imaging and immunofluorescence experiments revealing that in embryos lagging anaphase chromosomes are the major cause of micronucleus formation, where they are subject to defective nuclear structure and function, DNA damage and centromeric identity loss (Figs. 2.1, S2.1, 2.2, S2.2, S2.6 and S2.7). I also performed and analysed experiments demonstrating that micronuclei are predominantly inherited rather than reincorporated during mitosis in a random manner during development (Figs. 2.4, S2.5). Dr. Greg FitzHarris devised the study and performed experiments showing lagging anaphase chromosomes in embryos usually consist of single chromatids and micronucleus chromosomes lack functional kinetochores (Figs. 2.1C, 2.2B, S2.3). Through a collaboration with Dr. Kazuo Yamagata, who performed the experiments of 4D live imaging throughout preimplantation development, we acquired the image data sets which were annotated by Shardul Trivedi and analysed by Dr. Jenna Haverfield (Figs. 2.3, S2.4). Dr. Greg FitzHarris wrote the manuscript and I assembled the figures and produced all illustrations.

MICRONUCLEUS FORMATION CAUSES PERPETUAL UNILATERAL CHROMOSOME INHERITANCE IN MOUSE EMBRYOS

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2.2 Abstract

Chromosome segregation defects in cancer cells lead to encapsulation of chromosomes in micronuclei (MN), small nucleus-like structures within which dangerous DNA rearrangements termed chromothripsis can occur. Here we uncover a strikingly different consequence of MN formation in preimplantation development. We find that chromosomes from within MN become damaged and fail to support a functional kinetochore. MN are therefore not segregated, but are instead inherited by one of the two daughter cells. We find that the same MN can be inherited several times without rejoining the principal nucleus, and without altering the kinetics of cell divisions. MN motion is passive, resulting in an even distribution of MN across the first two cell lineages. We propose that perpetual unilateral MN inheritance is a novel mode of chromosome missegregation, which could contribute to the high frequency of aneuploid cells in mammalian embryos, but simultaneously may serve to insulate the early embryonic genome from chromothripsis.

2.3 Significance Statement

Early mammalian embryos frequently constitute a mixture of euploid and aneuploid cells, termed embryo mosaicism. Though this is considered a major cause of fertility problems, the mechanistic explanation for the mitotic errors that give rise to the aneuploid cells within mosaic embryos remains mysterious. Here using long-term live imaging of chromosome segregation in mouse embryos we show that individual chromosomes are frequently encapsulated within small nucleus-like structures called micronuclei. We show that micronucleus-enclosed chromosomes lack proper kinetochores and are therefore unable to be correctly segregated, causing them to be randomly inherited by just one of the daughter cells during subsequent embryonic cell divisions. This unexpected pattern of chromosome inheritance provides a novel explanation for mosaicism in early embryos.

Accurate chromosome segregation is achieved by correct attachment of spindle microtubules to kinetochores, complex proteinaceous structures that assemble on centromeric DNA. Misattachment can cause so-called lagging chromosomes during anaphase [1], which are a hallmark of chromosomally unstable cells [2], and can result in micronuclei (MN) – small nucleus-like bodies that form if a chromosome remains separate from the main group of chromosomes at the time of nuclear envelope reformation. MN have long been used as a marker of genetic fidelity. For example, MN may be predictive of tumorigenicity, and can be used to screen chemicals for genotoxicity [3-5]. However, the cellular impact of MN formation is poorly understood. Recent studies found that chromosomes within MN become heavily damaged, causing DNA rearrangements [6-9]. Paired with reincorporation of the MN chromosome into the main nucleus (principal nucleus; PN) during the next cell cycle [7,9,10], this provided an elegant explanation for chromosome-specific extensive rearrangements seen in many cancer cells, termed chromothripsis [11,12]. In the present study we show that the outcome of MN formation is markedly different in the preimplantation mammalian embryo.

Chromosomal mosaisicm is common in mammalian preimplantation embryos, up to 50% of human embryos produced in fertility clinics containing some aneuploid cells resulting from early mitotic errors [13-16], for which there is currently no clear cellular explanation. Simultaneously, early embryos frequently exhibit MN, the causes and consequences of which are unknown [17,18]. Here we pair long-term live 4D microscopy with high resolution fixed embryo analysis

to analyse the causes and consequences of naturally-occurring MN in mouse embryos. Our experiments reveal an unexpected series of events in which chromosomes from within MN do not rejoin the principal nucleus but are repeatedly inherited by only one daughter cell. We propose that this mechanism should generate a cascade of aneuploid cells, whilst protecting the genome from chromothripsis-like rearrangements.

2.4 Results

2.4.1 Micronucleus formation in mouse embryos

The preimplantation mouse embryo develops from a fertilised zygote to a 16-32 cell morula and then a 64-128 cell blastocyst without intervening cell growth over the course of ~4 days, providing a tractable setting in which to examine cell divisions. We first analysed MN occurrence using fixed cell analysis of embryos cultured *in vitro* under standard conditions. MN were rare in early embryos, but from the 16-cell stage onwards embryos typically possessed 1-5 MN-containing cells (**Fig. 2.1A, Fig. S2.1**, p.44,45). Embryos that developed *in vivo* and were fixed for examination immediately after isolation at morula stage exhibited a similar number of MN as *in vitro* cultured embryos, revealing that *in vitro* culture does not affect MN abundance (**Fig. 2.1A**). MN could potentially arise via a variety of mechanisms [5,19-21]. To determine how MN form in embryos we performed medium-term (20 hour duration) live confocal 4D time-lapse imaging of H2B:RFP-expressing morulae, analysing the dynamics of chromosome segregation in a total of 453 cell divisions. The vast majority (70%) of divisions occurred without obvious defect, the two sets of sister chromatids moving apart synchronously in anaphase. 15% of divisions exhibited mildly lagging chromosomes, which did not normally result in MN formation. However, in 10% of cell divisions one or more severely lagging chromosomes were detected that resulted in MN formation in one of the daughter cells (**Fig. 2.1B**). Immunolabelling of embryos fixed in mid-anaphase revealed that lagging chromosomes almost always possessed CREST-labelled kinetochores at their leading edge (27/29 cases; **Fig. 2.1C**), indicating that anaphase lagging chromosomes in embryos are usually intact chromatids as opposed to DNA fragments, and thus that newly formed MN generally contain single whole chromatids. Consistent with this, MN diameter varied little between cells or developmental stage (1.74±0.06μm in diameter in morula, 1.63±0.11μm in blastocysts). Recent studies in cancer cells revealed that DNA in MN is subject to high levels of damage during the subsequent S-phase as a result of defective MN nuclear envelope function [6,7]. Analogous to this we found that MN in morulae exhibit faint or absent staining for the nuclear envelope structural component nuclear lamin B1 (**Fig. 2.1D**), and also for LSD1, a marker of nuclear import [6], suggesting that MN nuclear envelope function is defective. Labelling with γH2AX antibodies revealed very high levels of damage in approximately half of all MN (**Fig. 2.1D**), consistent with DNA damage occurring in S-phase, as in cancer cells [6,7,22]. In summary, lagging whole chromatids are a normal feature of preimplantation development in mouse, giving rise to MN within which DNA is subject to damage.

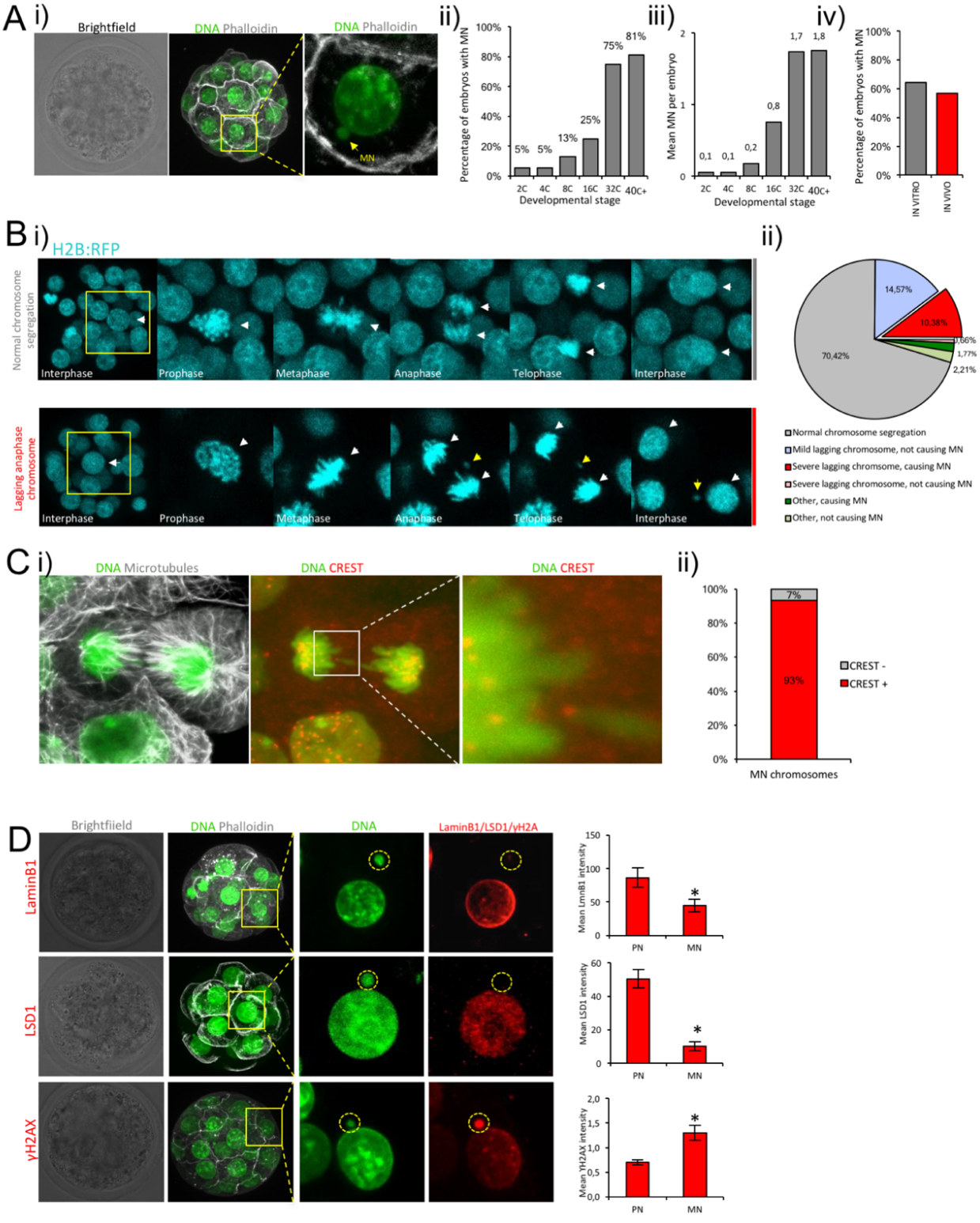

Figure 2.1. Cause and impact of MN formation in mouse embryos

(Ai) Typical example of a fixed morula illustrating the appearance of a MN (arrow). (ii, iii) Fixed cell analysis of MN number in in vitro cultured embryos during development, illustrating the emergence of MN at the 16-32 cell stage. (iv) Contemporaneous comparison of MN abundance in morulae that were either cultured from 2-cell stage or flushed from the uterus at morula stage. Note no difference in MN abundance. 16-60 embryos per datapoint. (B) Live imaging of cell division using H2B:RFP. (i) Examples of chromosome segregation (white arrows), illustrating anaphases with and without lagging chromosomes that cause a MN (yellow arrows). (ii) Analysis of 453 cell divisions from 58 embryos. (Ci) Example of embryo fixed for kinetochore examination in mid-anaphase. (ii) Note that lagging chromosomes possessed clear CREST-labelled kinetochores in 27/29 cases. (D) Immunofluorescence analysis of MN structure and function using Lamin B1, LSD1 and γH2AX antibodies. Yellow circles highlight the MN. Quantitative fluorescence intensity analysis of PN vs MN is presented for each. Minimum 20 MN-containing cells per group. Error bars represent SEM. t-tests were used where appropriate, asterisks indicating P<0.01.

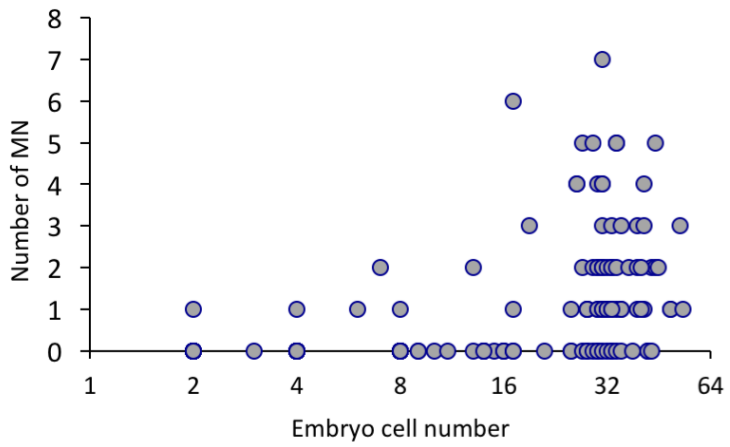

Figure S2.1. MN abundance during early embryo development

Analysis of MN abundance in fixed embryos. Dataset the same as presented in Figure 1A, presented so as to illustrate the number of MN-containing cells per embryo at different developmental stages. Total of 153 embryos examined.

2.4.2 Micronuclei are unilaterally inherited at the time of cell division

In cancer cells MN chromosomes are segregated along with chromosomes from the PN in the next mitosis, such that a MN often persists for only one cell cycle [7,10]. To explore the fate of MN during early development we examined the behavior of MN in live H2B:RFP-expressing morulae. In contrast to cancer cells, in mouse embryos we found that the MN was segregated normally along with the main chromosome mass in only 2 of 34 cases (6%) (**Fig. S2.2**, p.48). In the other 94% of MN-containing cell divisions, the MN was inherited by only one of the two daughter cells and persisted as a MN in that daughter cell (**Fig. 2.2A**, p.47). In most cases the MN remained visibly separate from the main chromosome mass throughout the whole of M-phase, and was continuously observed to travel into one daughter (**Fig. 2.2A**). We saw no evidence of MN fragmentation that might lead to the reincorporation of a portion of MN DNA back into the PN, but cannot formally exclude very small DNA fragments below the imaging resolution limit. To determine why the chromosomal content of MN is not segregated normally, H2B:RFP-expressing embryos were observed with live 4D confocal microscopy, and individual embryos fixed for examination by immunofluorescence when a MN-containing cell entered mitosis. Chromosomes from within MN exhibited a rounded hyper-condensed appearance in ~50% of cases (**Fig. 2.2B**, p.47), whereas the normal condensed chromatin structure typical of mitosis was evident in the other ~50% (**Fig. S2.3**, p.48). Chromosomes from MN were usually spatially divorced from the spindle with no evidence of spindle microtubule interaction (**Fig. 2.2B**). Notably, regardless of the morphology of the DNA, chromosomes from MN lacked prominent CREST staining in all cases, indicating a failure to maintain a proper kinetochore, whereas prominent CREST-labelled kinetochores were evident on other chromosomes (**Fig. 2.2B, Fig. S2.3**). Since lagging chromosomes that form MN possess clear kinetochores (**Fig. 2.1C**, p.47), this indicates that the ability to support a functional kinetochore is lost while the chromatid is enclosed within the MN, concomitant with DNA damage. As a result, chromosomes from MN fail to be segregated in the subsequent mitosis, and are inherited by only one of the daughter cells.

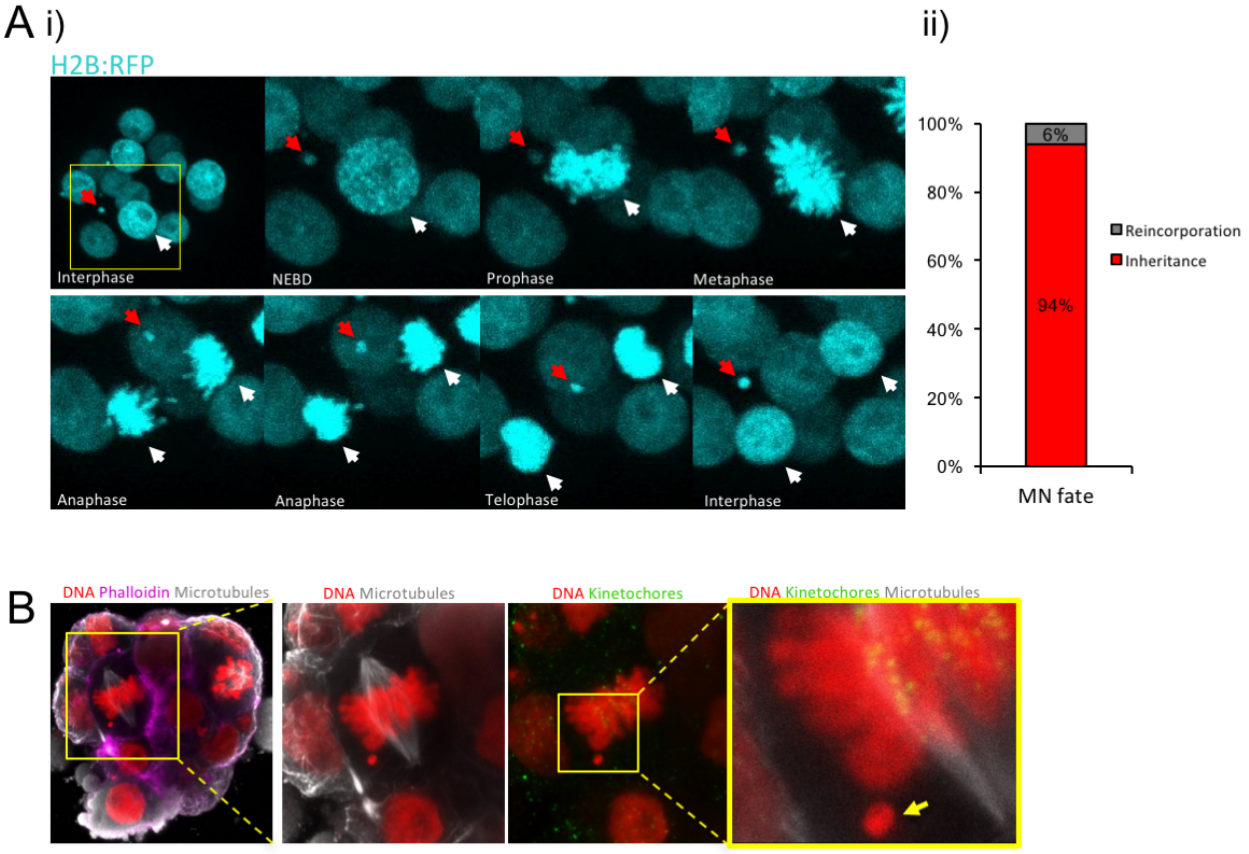

Figure 2.2. MN are unilaterally inherited during embryogenesis

(Ai) Example of MN inheritance observed with H2B:RFP imaging. Note that the MN (red arrow) remains separate from the rest of the chromosomes (white arrow) during M-phase, and is inherited by one daughter cell. (ii) Analysis is of 34 MN-containing cell divisions. (B) H2B:RFP embryos were observed using live imaging, and then fixed when a MN-containing blastomere was observed to enter mitosis. The embryo was then immunolabelled for microtubules and CREST. Note that the MN chromosome (yellow arrow) lacks clear CREST-labelled kinetochores, and is separate from the spindle.

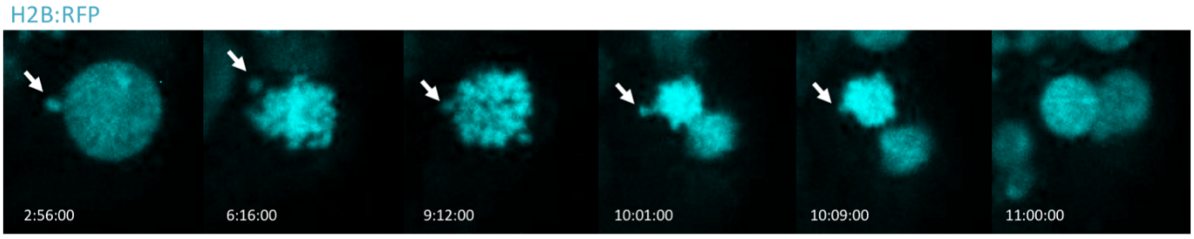

Figure S2.2. MN reincorporation

As described in the text the vast majority of MN-containing cell divisions result in the persistence of the MN and its inheritance into one daughter cell. Displayed here is a rare example of the alternate outcome, where a MN reincorporates into the PN following the subsequent mitosis, highlighted with a white arrow.

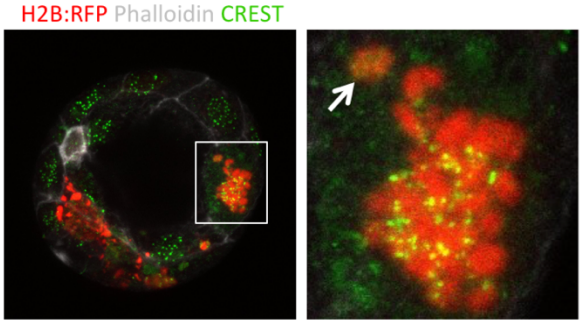

Figure S2.3. Absence of kinetochore staining on MN chromosomes in metaphase

A second example (supplemental to that in Fig2B), in which the MN chromosome assumes a normal mitotic condensed chromosome appearance, but nonetheless lacks clear evidence of a CREST-labelled kinetochore (arrow). Note clear CREST-labelled kinetochores on all other chromosomes.

2.4.3 Micronucleus inheritance does not prevent preimplantation development

An advantage of the early mouse embryo as a model for analysing cell divisions is that all cells remain constrained within a ~100 μm sphere throughout preimplantation development, enabling observation of sequential cell divisions following MN formation. We therefore analysed MN inheritance using datasets comprising complete Z-projections acquired every 225 seconds using spinning disk microscopy for 90 hours, encompassing development from 1-cell stage through to blastocyst (**Fig. 2.3**, p.50). This provides the spatial and temporal resolution to observe the genesis and fate of all MN, without affecting the health of the embryo, as determined by the ability to generate live pups after embryo transfer [23]. We performed a comprehensive analysis of every cell division within each embryo, recording the incidence of lagging chromosomes and MN, as well as cell cycle durations, cell fate, and lineage relationships during preimplantation development. Consistent with the previous experiments, chromosomes from MN were not segregated in the subsequent cell cycle, but were unilaterally inherited resulting in a single MN in one daughter cell (**Fig. 2.3A**). Importantly, these long-term datasets allowed us to continuously track the same MN over the course of several cell divisions. Taking advantage of this, we observed that second generation MN were similarly inherited by one daughter cell, and MN were continually inherited in this manner up to four times within the observed period of preimplantation development (**Fig. 2.3A**, **Fig. S2.4**, p.50, 51). Analysis of cell cycle timings across our datasets revealed that MN had no impact upon the duration of M-phase (**Fig. 2.3B**), consistent with the lack of coherent kinetochores on MN chromosomes. Interphase durations were also unaltered, indicative of a failure to activate DNA-damage responses (**Fig. 2.3B**). Moreover, cell cycle durations were similar in cells containing first or subsequent generation MN (**Fig. 2.3B**). For example, the 16-32 cell mitosis was 51.6±7.7mins in cells containing first generation MN, and 55±7.9mins in cells containing second generation MN ($P>0.1$). Thus, once formed, MN persist throughout preimplantation development, with their DNA contents being inherited at each subsequent mitosis without apparent impact upon the durations of subsequent cell divisions.

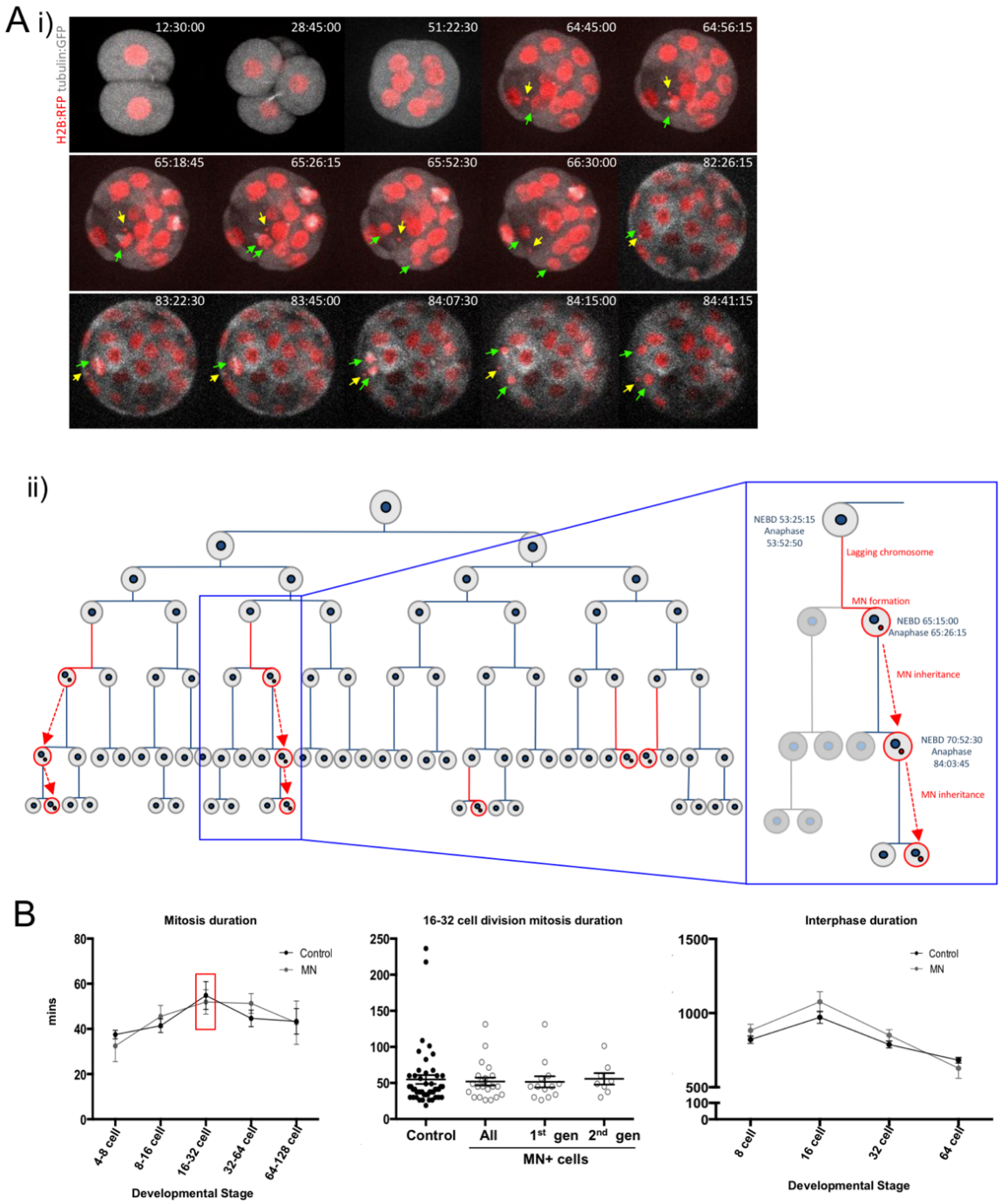

Figure 2.3. Long term 4D imaging reveals repeated MN inheritance

(Ai) Live imaging of H2B:RFP-expressing embryos throughout preimplantation development, and (Aii) lineage analysis of the same embryo. Blue box shows timing details of the divisions illustrated in the images in Ai. Note that in this example the MN which is generated by a lagging chromosome, is then unilaterally inherited in the next two divisions. (B) Analysis of cell cycle durations in MN containing cells compared to MN-free cells with no history of lagging chromosomes. Analysis of 12 examples of imaging complete continuous development from 1-cell stage to blastocyst. Error bars represent SEM.

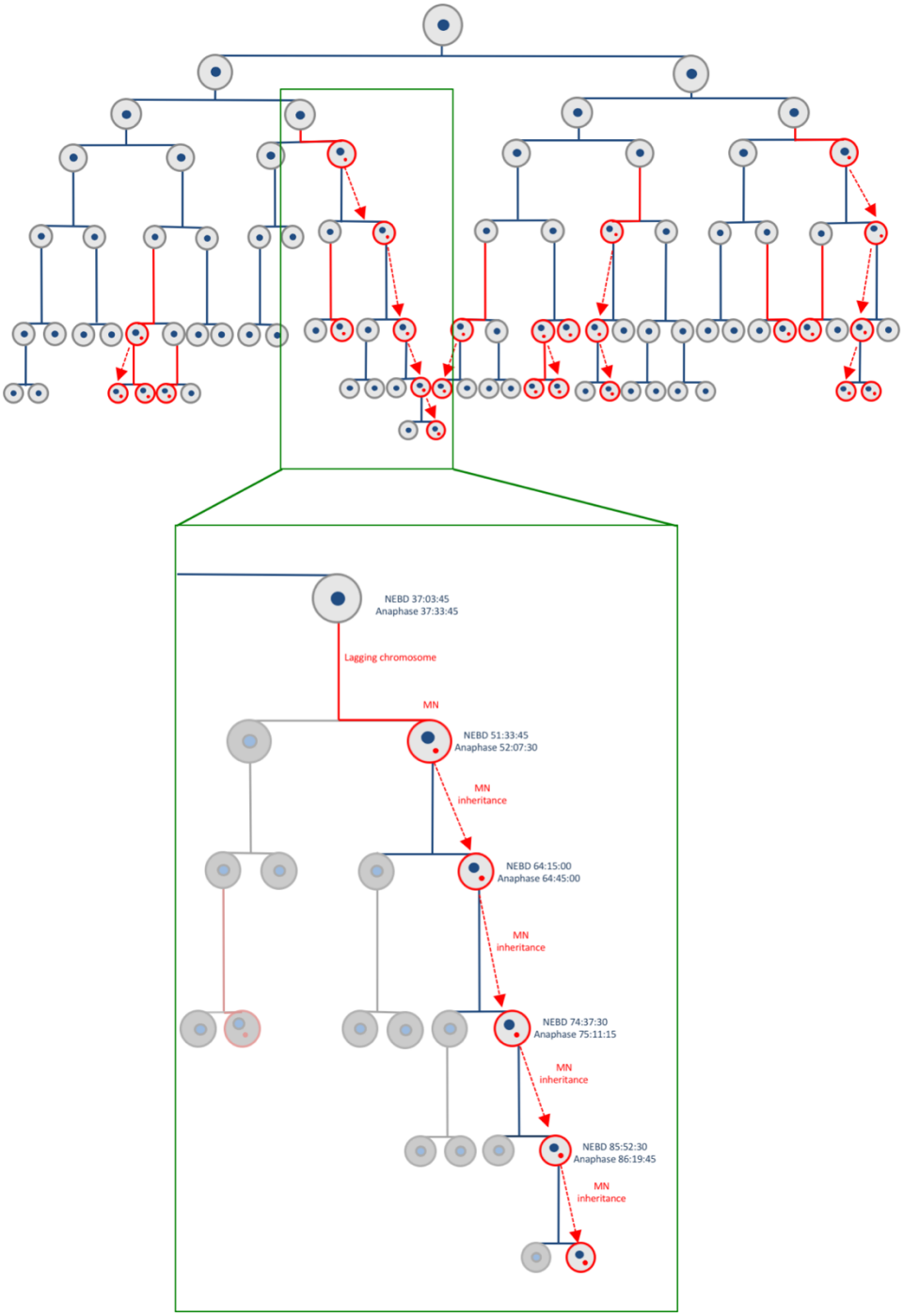

Figure S2.4. Repetitive MN inheritance

An additional example of lineage tracking following live imaging from 1-cell through to blastocyst stage, similar to that shown in ***Fig. 2.3****. Note that in this example, a MN is formed as a result of a lagging chromosome, and is subsequently inherited four times.*

2.4.4 Passive inheritance results in randomly distributed micronuclei in embryos

Finally, we performed three series of experiments to understand the spatiotemporal dynamics of MN inheritance during cell division. First we analysed the 3D positioning of MN within live embryos by co-labelling chromosomes and the plasmalemma using H2B:RFP and CAAX:GFP respectively. In interphase MN oscillated over small distances in three dimensions, but exhibited little or no net displacement over time. No obvious inheritance bias was detected based on cell morphology, the MN being equally likely to be inherited by the larger or smaller daughter cell, or similarly the daughter cell with the larger or smaller nucleus (**Fig. S2.5**, p.53). Second, as a further way of tracking the movement of MN, H2B:RFP-expressing embryos were co-injected with inert fluorescent beads to provide a passive maker of cytoplasmic dynamics during cell division. MN movement in anaphase closely mirrored that of nearby beads, suggesting that MN move passively as a result of normal cytoplasmic dynamics, rather than by specific cytoskeleton-directed mechanisms (**Fig. 2.4A**, p.54). Thirdly, we analysed the distribution of MN in ~128 cell stage blastocyst-stage embryos, and found no difference in the proportion of MN-containing cells in the inner cell mass (ICM; 3.6±0.8% of cells with MN) and the trophectoderm (TE; 3.2±0.5%; $P>0.1$) (**Fig. 2.4B**, p.54). Together these experiments indicate that MN movement in embryos is undirected, resulting in an even distribution of MN between the two major cell lineages at the end of preimplantation development.

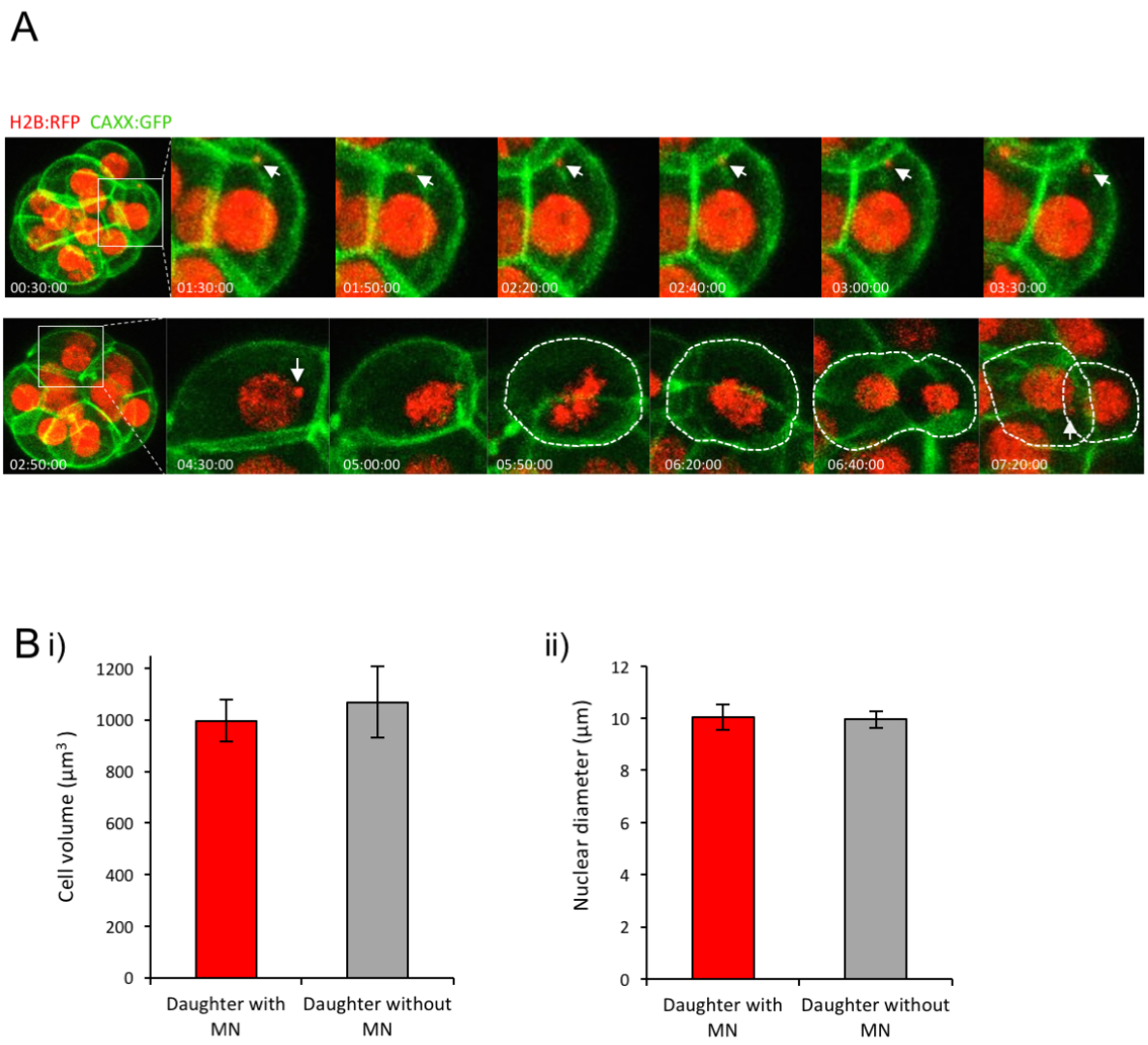

Figure S2.5. Monitoring MN behaviour in relation to cell shape

2-cell stage embryos were microinjected with cRNA encoding H2B:RFP to label chromosomes, and CAAX:GFP to label plasmalemma, and live imaging performed using confocal microscopy, as elsewhere in the study. (A) Timecourse illustrating interphase motion of MN. Note that the MN exhibits little to no net movement during the period of imaging. (B) Example of mitosis in an embryo expressing CAAX:GFP and H2B:RFP (n=11). The dotted lines highlight the two daughter cells. (C) Quantitative analysis of cell and nucleus size in relation to MN inheritance. Note that the MN is equally likely to be inherited by the larger or smaller cell.

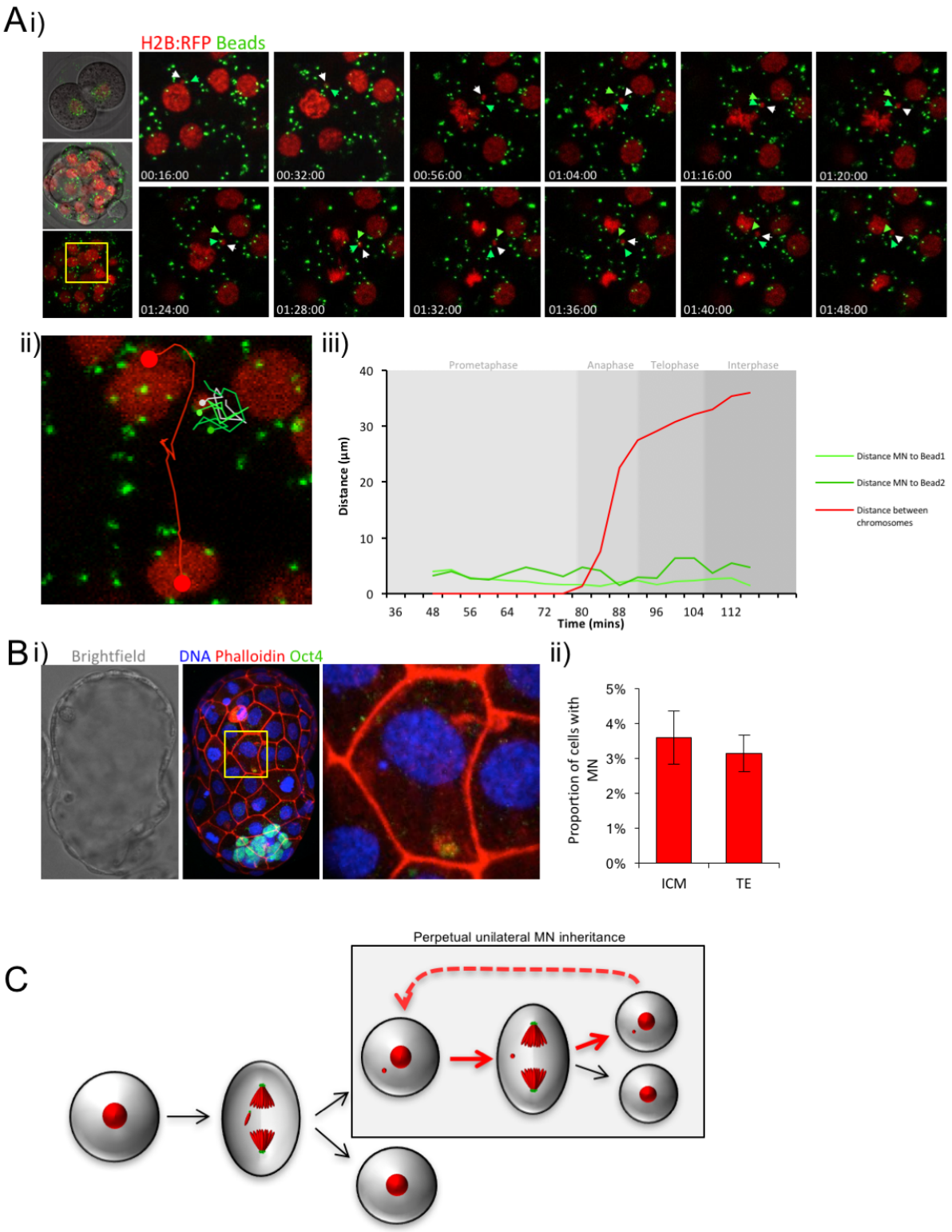

Figure 2.4. Passive MN inheritance and even distribution of MN in blastocysts

(A) Analysis of anaphase MN motion relative to inert Dragon-green beads (n=9). (Ai) Individual time-points are shown charting the division of a MN-containing cell within a morula. Note that

MN motion closely matches that of nearby beads (white and green arrowheads, respectively), indicating that MN motion is not directed by specific mechanisms. (Aii) Tracking analysis from the same cell showing the movement of anaphase chromosomes (red) the MN (grey) and the same two beads highlighted in Ai (green). Bold circles mark positions at the end of the experiment. Note that the movement of the MN reflects that of nearby beads. (Aiii) Quantitative analysis of distances between the beads and MN. Distance between anaphase chromosomes shown on the same chart to illustrate timings. (Bi) Example MN location analysis using Oct4 antibodies to label the inner cell mass (ICM; green). Trophoectoderm (TE) nuclei appear blue as they are unlabelled by Oct4 antibodies. Note the blastocyst shown is hatching, and therefore takes a ´figure-of-8´ appearance. Arrow marks MN. (ii) Analysis is of 34 embryos. Error bars represent SEM. (C) Cartoon depiction of perpetual unilateral inheritance. See further description in text.

2.5 Discussion

Our experiments show that, in mouse embryos, encapsulation within a MN leads to DNA damage and an apparently irreversible loss of the ability to assemble a normal kinetochore. As a result, MN chromosomes repeatedly fail to be segregated, and are instead perpetually inherited (**Fig. 2.4C**, p.54). Although we have not formally counted chromosomes in embryo cells here, we suggest that repeated MN inheritance must result in whole chromosome aneuploidy (depicted in **Fig. S2.6**, p.57). If the lagging chromosome is initially correctly inherited such that first-generation MN-containing cells possess the correct number of chromosomes (albeit one within a MN) as is frequently the case in cancer cell lines [10,24], then unilateral inheritance would render MN-free progeny of that cell hypoploid. Alternatively, if the MN first forms in the incorrect cell, then the MN-containing cell and its MN-containing progeny would be hyperploid, while the original MN-free cell and its progeny would be hypoploid (**Fig. S2.6**, p.57). Perpetual MN inheritance thus presents means of generating a cascade of aneuploid cells from a single initial lagging chromosome. Direct observation of the ploidy impacts of MN inheritance will require new methods for counting chromosomes in individual blastomeres in live embryos *in situ*. However, we suggest that this pattern of chromosome dynamics may provide at least a partial explanation for the high level of mitotic chromosome segregation errors and mosaicism detected in human embryos.

Whether the apparent absence of kinetochores on DNA from MN in embryos involves specific mechanisms, or is a serendipitous by-product of extreme chromosome damage, remains to be

explored. In addition to CREST-labelling being absent on chromosomes from within MN, we have also found that MN typically lack foci of the centromeric histone CENP-A, which are readily detectable in the principal nucleus in embryos [25] (**Fig. S2.7**, p.57). A simple explanation therefore is that damage to the MN chromosome may lead to loss of centromeric identity, precluding proper kinetochore assembly. Our data thus raise the broader question of whether lower levels of DNA damage, perhaps causing more subtle kinetochore assembly defects, may cause de-novo segregation defects and thus be a more general driver of segregation errors in some contexts [26].

We conclude that perpetual unilateral MN inheritance is a novel mode of chromosome segregation error, and joins chromothripsis as a direct consequence of MN formation. In addition, we speculate that MN inheritance may simultaneously serve an important genome protective role in early development. Although we did see MN rejoining the main group of chromosomes on a very small number of occasions (6%; **FigS2.2**, p.48) and so do not exclude the possibility that some low level of chromothripsis-like rearrangement could occur in early embryos [27], our data show clearly that in the vast majority of cases MN chromosomes do not rejoin the PN during mitosis in preimplantation development. We also observed no evidence in any of our datasets of MN re-absorption into the PN in interphase. Indeed, our data raise the possibility that MN observed in embryonic stem cells [28] reflect segregation errors in preimplantation development. Thus, whereas MN in somatic cells rejoin the genome and risk cell transformation, MN in embryos generally do not. Instead, MN inheritance generates a small cascade of aneuploid cells, that likely undergo apoptosis later in development [29]. MN inheritance may therefore provide a means of sacrificing a modest number of cells in order to prevent the incorporation of damaged DNA into the genome, thereby insulating the early embryo from chromothripsis. Whether some somatic tissues retain this strategy remains to be determined.

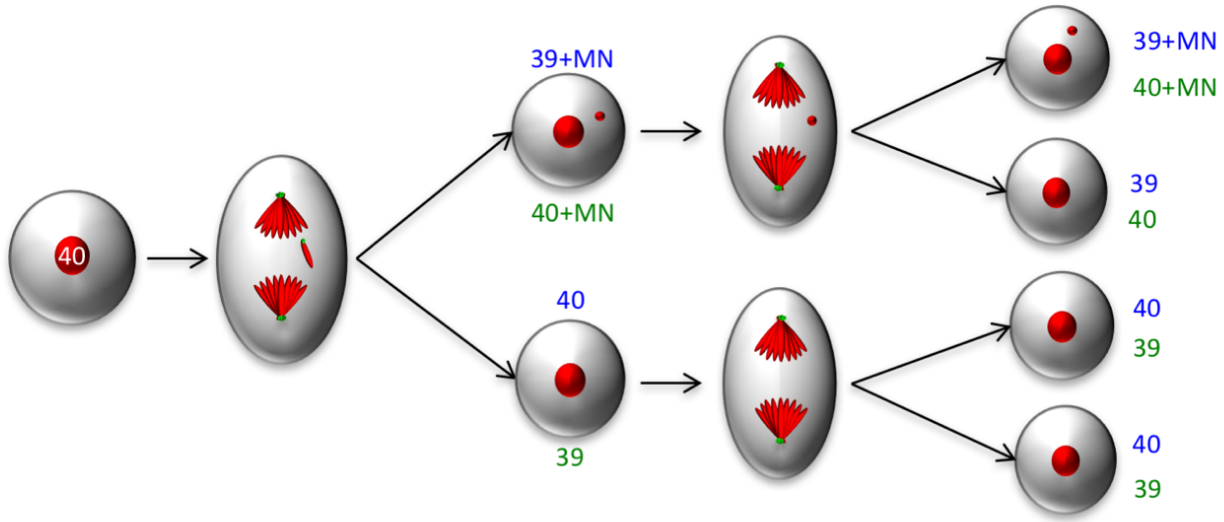

Figure S2.6. Cartoon explanation of hypothetical ploidy outcomes following unilateral MN inheritance

The cartoon depicts two possible scenarios, depending upon whether the MN is initially formed in the 'correct' cell (blue numerals indicate chromosome numbers), or whether the MN is initially formed in the wrong cell (green numerals). For further explanation see text.

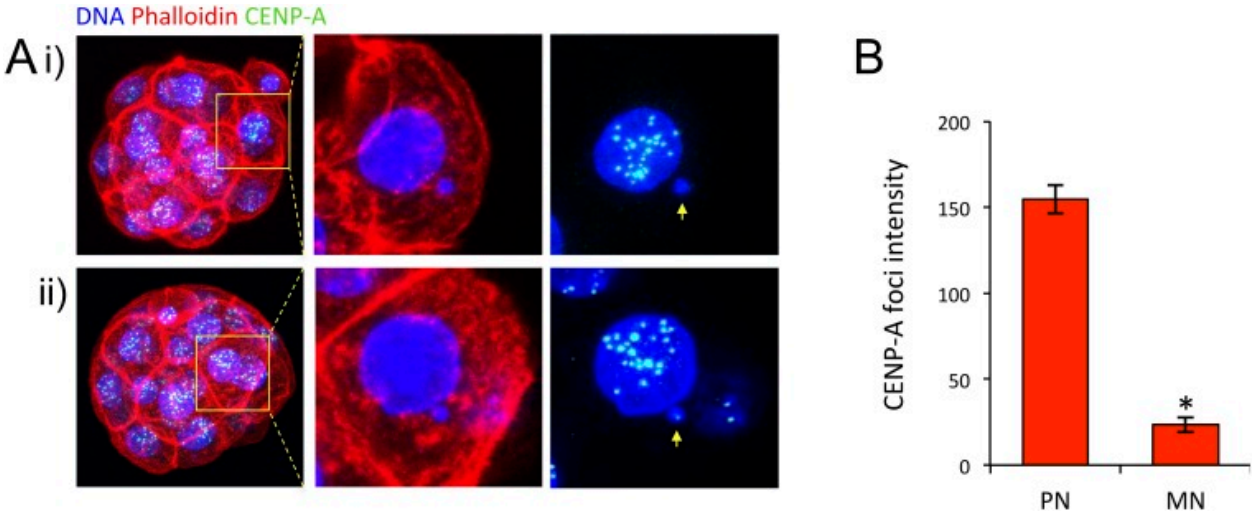

Figure S2.7. MN lack CENP-A foci

Immunofluorescence analysis using CENP-A antibodies. Ai shows a typical confocal image of a MN lacking a pronounced CENP-A signal, while clear discrete foci are observed in the PN. Aii shows a rare example (~10% of cases) where a clear, albeit reduced, CENP-A signal was observed in the MN. Arrows highlight MN. (B) Quantification of CENP-A foci intensity in PN and MN across all MN-containing cells. Data from a total of 55 MN from 63 embryos.

2.6 Materials and methods

2.6.1 Embryo culture and microinjection

Embryos were harvested from super-ovulated BDF1 female mice mated with BDF1 males, and cultured in KSOM media in 5% CO_2 at 37°C. mRNA was manufactured using Ambion mMessage Machine according to the manufacturer's instructions, microinjected into embryos using a picopump (WPI) and micromanipulators (Narishige) mounted on a Leica DMI4000 inverted microscope, as previously described [30]. Plasmids used were CAAX:GFP in pcDNA3.1 (from Guillaume Charras), H2B:RFP in pRN4 (from Alex McDougall). Dragon-green beads were purchased from Bangs Laboratories and injected at a 1:5 dilution.

2.6.2 Immunofluorescence and live imaging

Embryos were fixed using 4% paraformaldehyde (PFA; 40 mins) and permeablised by Triton-X (0.25%, 10 mins) [31]. Primary antibodies used as follows: CREST (Gift from William Earnshaw, 1:200), α-tubulin (Sigma, 1:1000), γH2AX (Trevigen, 1:800), Oct3/4 (SantaCruz, 1:300), LSD1 (Cell Signaling Technology 1:400). Where CENP-A antibodies were used (Cell Signalling Technology 1:200) embryos were fixed with 2% PFA. Alexa-labelled secondary antibodies were from Life Technologies. Imaging was performed on a Leica SP8 confocal microscope fitted with a 20x 0.75NA objective and a *HyD* detector. Imaging was performed for 20 hours at the morula stage, and embryos were included for analysis only following morphologically normal cavitation, which typically occurred in ≥50% of embryos in any given experiment. Complete 4D datasets of embryo development (as in Fig3) were obtained as previously described [23,32]. Briefly, 61 optical sections were obtained at 225 second intervals for 90 hours using a CSU10 Yokagawa Nipkow disk system, mounted on an Olympus IX-71 inverted microscope.

2.6.3 Analysis and statistics

All data analysis was performed using ImageJ/Fiji. For tracking, positional coordinates were extracted from Z-stack datasets to calculate distances in 3D using the TrackMate Fiji plugin. Where shown, error bars represent SEM. *t*-tests were used where appropriate, asterisks indicating $P<0.01$.

2.7 Acknowledgements

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2.8 Author Contributions

Experiments were performed by CV, GF and KY. Data was analysed by CV, JH, GF and ST. GF devised the study and wrote the manuscript. We have no competing financial interests.

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CHAPTER 3

Publication

CORRELATIVE LIVE IMAGING AND IMMUNOFLUORESCENCE FOR ANALYSIS OF CHROMOSOME SEGREGATION IN MOUSE PREIMPLANTATION EMBRYOS

Book chapter published in Methods in Molecular Biology Chromothripsis (2018) Mar;1769:319-335, PMID: 29564833

3.1 Preface

Following the study we published in PNAS in 2016, demonstrating that unlike in somatic and cancer cells, in embryos, micronucleus-enclosed chromosomes do not reincorporate into daughter cell nuclei during following cell divisions, hence safeguarding the embryonic genome from chromothripsis, we were invited to write a methods book chapter for a series of Methods in Molecular Biology in Chromothripsis. We describe an approach of sequentially combined live imaging and immunofluorescence techniques, that was pivotal to demonstrating deficient kinetochore assembly in micronucleus chromosomes during mitosis. I wrote the main text with guidance and comments from Dr. Greg FitzHarris, I performed all experiments and produced all figures presented herein.

CORRELATIVE LIVE IMAGING AND IMMUNOFLUORESCENCE FOR ANALYSIS OF CHROMOSOME SEGREGATION IN MOUSE PREIMPLANTATION EMBRYOS

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Running head: Correlative live imaging and immunofluorescence in mouse embryos

3.2 Summary

Chromothripsis is a phenomenon observed in cancer cells, wherein a single or few chromosome(s) exhibit vast genomic rearrangements. Recent studies elucidated a striking series of events in which defective segregation of chromosomes causes their incorporation into micronuclei, where they are subject to extensive DNA damage prior to re-joining the main mass of chromosomes in a subsequent cell cycle, which provide an appealing mechanism for the aetiology of chromothripsis. Micronuclei are well known to be common in human preimplantation embryos. We recently showed that, unlike in cancer cells, in mouse preimplantation embryos the micronuclei are maintained during multiple cell generations and apparently fail to re-join the main set of chromosomes. This unexpected finding could safeguard the early embryonic genome from chromothripsis. Here, we describe an approach that combines live and immunofluorescence imaging methods that was pivotal in that study to reveal the lack a functional kinetochore in chromosomes from mouse embryo micronuclei.

Key Words

Mitosis, Live cell Imaging, Chromosome Segregation, Preimplantation Development, Immunofluorescence, Micronuclei

3.3 Introduction

A recently elucidated feature of cancer in both primary tumours and immortalized cells lines are clustered genomic rearrangements restricted to one or few chromosomes, a phenomenon termed chromothripsis (Adey et al., 2013; Kloosterman et al., 2011; Stephens et al., 2011) While conventionally it is considered that mutations and translocations are progressively acquired over time, the nature of rearrangements observed in chromothripsis, restricted to a defined genomic region and showing limited changes in gene copy number, suggests that these emerge concurrently over a short period of time, possibly in a single catastrophic event (Forment et al., 2012; Stephens et al., 2011). Such rapid and complex genomic rearrangements have the potential to simultaneously activate oncogenes while disabling checkpoints, hence posing an important novel pathway for cellular transformation.

Micronuclei (MN) are a hallmark of chromosomal instability and cancer, and may arise through a variety of different mechanisms (Storchova and Kloosterman, 2016). A common cause of MN in human cancer cells are lagging anaphase chromosomes (Cimini et al., 2001; Thompson and Compton, 2011), leading to the encapsulation of single chromosomes into a MN at the time of nuclear envelope (NE) formation. Recently it has been shown that during the subsequent cell cycle MN-enclosed chromosomes acquire DNA double strand breaks during S/G2 phases, resulting from atypical nuclear membrane function (Crasta et al., 2012; Hatch et al., 2013; Ly et al., 2017; Zhang et al., 2015). MN chromosomes are then reincorporated into daughter cells during the subsequent mitosis (Crasta et al., 2012; Zhang et al., 2015). Elegant experiments showed that MN-free daughter cells of MN-containing cells harbour extensive genomic rearrangements usually confined to a single chromosome as revealed by a combined live imaging and single cell whole genome sequencing approach (Zhang et al., 2015). Hence, MN have emerged as the likely structural intermediate between chromosome mis-segregation and the emergence of chromothripsis.

Chromosomal abnormalities, such as mosaic aneuploidy and MN have been observed at high incidences in human preimplantation embryos (up to 70% of embryos have mosaic aneuploidy) (Fragouli et al., 2013; Kort et al., 2016). We recently found that, in cleavage stage mouse embryos, MN-enclosed chromosomes are rarely reincorporated into the genome as they have been shown to in cancer cells. Instead, MN are repeatedly inherited by daughter cells during

subsequent divisions of preimplantation development as a result of faulty kinetochores (Vázquez-Diez et al., 2016). Central to this discovery was a series of experiments in which we combined live imaging and fixed cell immunofluorescence in the same embryo to show that lagging chromosomes destined for MN possess an apparently normal kinetochore, but that the ability to subsequently assemble a functional kinetochore is lost while the chromosome is in the MN, and kinetochores are not observed on the MN-chromosome in subsequent mitoses.

Live imaging is an essential tool for the analysis of chromosome segregation dynamics during cell division, providing single cell and even single chromosome tracking of mitotic events over time (Rieder and Khodjakov, 2003). However, live cell imaging usually requires over-expression of fluorescent fusion proteins to look at subcellular localization of proteins of interest during cell division. In some cases, fusion protein overexpression may have biological effects causing artefacts, or the addition of the reporter eg. GFP may cause the protein to mis-localize or loose function. To circumvent these limitations, some somatic cell studies have observed chromosome segregation dynamics using live imaging and followed by fixation of single cells at defined moments of mitosis to perform correlative immunofluorescence and analyse localization of endogenous proteins (Gregan et al., 2011; Hinchcliffe et al., 2016; Hornick et al., 2011). Here, we describe such an approach, applied to preimplantation mouse embryos, to investigate the origin and fate of MN-enclosed chromosomes during mitosis.

3.4 Materials

3.4.1 Materials and reagents for production of synthetic cRNA

1. H2B:RFP plasmid DNA in pRN3 vector backbone (*see* **Note 1**)
2. Restriction enzyme for plasmid linearization (*see* **Note 2**)
3. Agarose
4. 1x TAE buffer (Tris-Acetate 0.04M, EDTA 0.01M)
5. DNA loading dye (NEB) or equivalent
6. SYBR Safe DNA Gel Stain (Thermo Fisher Scientific)
7. DNA 1kb ladder (NEB)
8. Gel electrophoresis tank and power supply
9. UV gel illuminator to visualise DNA

10. Sterile scalpels
11. DNA Gel Extraction Kit (QIAGEN) or equivalent
12. mMessenger Machine ® Kit (Thermo Fisher Scientific, SP6/T3/T7) (*see* **Note 3**)
13. PolyA Tailing kit (Thermo Fisher Scientific)
14. RNAeasy Clean up kit (QIAGEN) or equivalent
15. Molecular biology-grade ethanol
    RNase Away reagent (Invitrogen) or equivalent

3.4.2 Materials and reagents for embryo collection, culture and microinjection

1. Male and female CD-1 mice of approximately 6-12 weeks of age (Charles River Laboratories) (*see* **Note 4**)
2. Pregnant Mare's Serum Gonadotrophin (PMSG) (*see* **Note 5**)
3. Human Chorionic Gonadotrophin (hCG) (*see* **Note 5**)
4. Phosphate buffered saline (PBS) tablets
5. 27-Gauge needles
6. M2 medium (Sigma-Aldrich) (*see* **Note 6**)
7. KSOM medium (EMD/Millipore) (*see* **Note 6**)
8. Mineral oil
9. 0.22 µm syringe filter
10. 10 ml syringe to filter media
11. Digital dry bath at 37ºC
12. Petri dishes (BD Falcon, 35 x 10 mm)
13. Pasteur pipette (Fisher scientific, Soda lime glass 9 inch) (*see* **Note 7**)
14. Mouth controlled aspiration tube (*see* **Note 7**)
15. Cell culture incubator at 37 ºC, 5% CO_2
16. Ice
17. Centrifuge (Eppendorf centrifuge 5430) or equivalent
18. Oocyte and embryo microinjection station, with components and set-up as previously described (Nakagawa and FitzHarris, 2016). Inverted light microscope (Leica DMIL-LED), fitted micromanipulators and injectors (Narishige), a pneumatic picopump (World precision instruments), and an intracellular amplifier electrometer (Harvard apparatus). The apparatus is assembled and placed on an anti-vibration table (*see* **Note 8**).
19. Holding pipettes (*see* **Note 9**).
20. Vertical micropipette puller for the injection pipette (Sutter instrument company)

21. Glass capillaries with filament for cRNA injection (Harvard apparatus, 1.5 mm OD x 0.86 mm ID x 150 mm L)

3.4.3 Materials and reagents for confocal live imaging

1. Leica SP8 confocal fitted with HyD detectors or equivalent live cell imaging scopes with highly sensitive emitted light detection and/or reduced illumination such as spinning disk or structured illumination microscopes may also be used for this purpose.
2. Temperature-controlled microscope-stage incubator with 5% CO_2 supply
3. 50 mm uncoated glass bottom culture dishes (MatTek corporation)
4. KSOM medium (EMD/Millipore) (*see* **Note 6**)
5. Mineral oil

3.4.4 Materials and reagents for embryo fixation, immunofluorescence and confocal imaging

1. The following working solutions should be prepared. The solutions should be filtered and are stable for several weeks at 4 °C.
    a. 4% Paraformaldehyde (PFA) in PBS for fixing
    b. 0.25% Triton X-100 in PBS for permeablisation
    c. 1% Bovine serum albumin (BSA) in PBS for washing
    d. 3% BSA in PBS for blocking
2. 96-well plates with round bottom
3. 35 mm Glass bottom culture dishes (MatTek corporation) (*see* **Note 10**)
4. Mouse anti-α-tubulin primary antibody (Sigma-Aldrich)
5. Human CREST or ACA primary antibody (*see* **Note 11**)
6. Alexa 405 anti-Mouse IgG secondary antibody (Thermo Fisher scientific)
7. Alexa 488 anti-Human IgG secondary antibody (Thermo Fisher scientific)
8. Alexa 647-Phalloidin Thermo Fisher scientific)
9. A confocal microscope equipped with three different coloured lasers, a 63x oil immersion lens, and a computer-controlled Z-motor allowing to take Z-stacks.

3.5 Methods

The workflow for this experimental approach follows four defined stages, which are described herein; production of cRNA for H2B:RFP, embryo collection and microinjection, live imaging of embryo mitosis, fixation and reimaging by immunofluorescence**.**

3.5.1 Production of cRNA

Here, we briefly describe our standard approach for producing cRNAs. We use QIAGEN kits for DNA and RNA purification and Ambion kits for IVT and Poly(A) tailing. These steps are performed according to manufacturer's instructions.

3.5.1.1 Linearization and purification of plasmid DNA

Incubate (3-5 µg) of H2B:RFP plasmid DNA with the appropriate restriction enzyme according to manufacturer's instructions (*see* **Notes 1, 2** and **12**). Mix reactions with appropriate volumes of loading dye and load on a 1% agarose gel supplemented with SYBR-DNA dye in an electrophoresis tank. Run gel at 100 V for 40 minutes. Visualize DNA using a UV gel illuminator. Using a clean scalpel, excise the gel fragment containing linearized DNA. Purify linearized DNA following the QIAGEN Gel Extraction kit protocol (*see* **Note 13**). We recommend using a NanoDrop to measure concentration and purity of DNA.

3.5.1.2 IVT of linearized plasmid DNA

Use the mMessage mMachine ® in vitro transcription kits (Ambion) to produce capped RNA from the linearized template plasmid. Reaction is performed according to manufacturer's instructions (*see* **Note 14**).

3.5.1.3 Poly(A) Tailing

To increase cRNA stability and enhance translation efficiency, following IVT, perform a 3' polyadenylation reaction, using the Poly(A) Tailing kit (Ambion) (*see* **Note 15***).*

3.5.1.4 RNA Purification

Purify the resulting capped and polyadenylated H2B:RFP cRNA using the RNeasy Mini kit Clean up Protocol (QIAGEN), and elute in 30 µl of RNase-free water. We recommend

using a NanoDrop to measure the concentration and purity. Aliquot H2B:RFP cRNA in 1 µl batches in RNAase-free tubes, and store at -80 °C.

3.5.2 Two-cell embryo collection, culture and microinjection

3.5.2.1 Two-cell embryo collection

1. ~96 hours before collection of two-cell embryos, stimulate 2-3 month-old CD-1 female mice with an intra-peritoneal injection of 5 IU of PMSG. 40 hours before collection of two-cell embryos, inject the same mice with 5 IU of hCG (*see* **Note 16***)*, and transfer immediately into cages with a male mouse for mating (1 female:1 male).
2. To collect two-cell embryos, sacrifice the female mice using isoflurane anesthesia followed by cervical dislocation and dissect out the reproductive tract (*see* **Note 17**). For more detailed description see (Nagy, 2003).
3. Using a dissection microscope: In a culture dish with ~2 ml of M2 media use the forceps and needles to isolate the oviducts, leaving the ovaries, uterine horns and other tissues in this dish (*see* **Figure 3.1A**, p.73) (*see* **Note 18**).
4. Transfer the oviducts into a fresh dish with ~2 ml M2 media. Carefully tear open the oviducts using forceps and a needle or two needles, so that the embryos are released into the media (*see* **Note 19**).
5. At higher magnification, find and collect two-cell embryos using a pulled glass Pasteur of approximately 80-100 µm in diameter, mounted on a mouth-controlled aspiration tube (*see* **Note 7**) and transfer into a dish containing ~40 µl drops of M2 covered by mineral oil.
6. Wash the embryos from residual tissues from dissection by consecutively transferring them into fresh M2 drops. This process is usually repeated three times or until no more tissue debris is found in the media drop containing the embryos.
7. For embryo culture, transfer the embryos into a dish containing ~40 µl drops of pre-equilibrated KSOM. Wash away any residual M2 medium by transferring embryos through multiple KSOM drops. Place the dish in an incubator with 5% CO_2 at 37 ºC (*see* **Note 20**), (*see* **Figure 3.1A**, p.73).

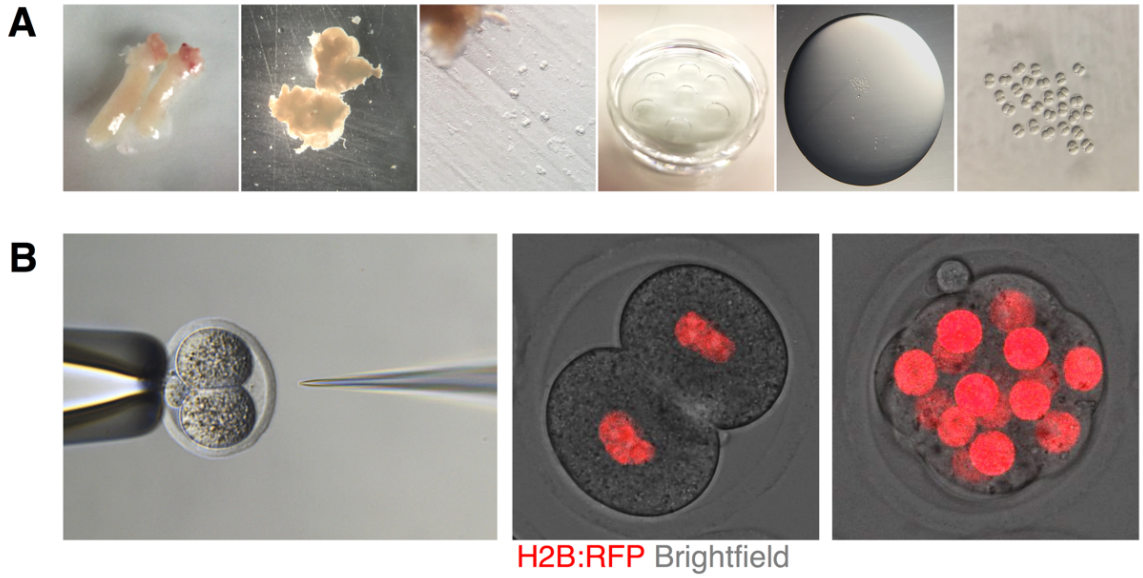

Figure 3.1. Embryo collection, culture and microinjection
(A) *From left to right: Mouse female reproductive tracts, isolated oviducts, torn oviducts with embryos released, 35 mm culture dish with 40 µl drops of KSOM and mineral oil, KSOM drop containing embryos for culture, magnified image of embryos within a media drop.* ***(B)*** *From left to right: Microinjection of two-cell embryos, with holding and microinjection piepettes. Two-cell embryo expressing H2B:RFP. Morula-stage embryo expressing H2B:RFP.*

3.5.2.2 Embryo microinjection

Our standard microinjection components, set up, and procedure have been described in extensive details elsewhere (Nakagawa and FitzHarris, 2016), therefore here we will briefly describe the protocol and variations required for two-cell embryo microinjections (**Figure 3.1B**, p.73).

1. Set up the microinjection station: Prepare the microinjection chamber using 600 to 800 µl M2 media covered with mineral oil on an upturned lid of a 35 mm tissue culture dish and place on the microscope stage. Prepare and place holding pipette in the field of view just above the bottom surface of the microinjection chamber. Pull fresh microinjection pipettes using a vertical pipette puller.
2. Pipette embryos into the M2 media in the microinjection chamber (*see* **Note 21**).
3. Keep cRNA on ice, dilute to appropriate concentration as necessary. Centrifuge briefly before opening. Using a microloader tip backload ~1 µl of cRNA into a microinjection

pipette and mount it on the injection probe (Nakagawa and FitzHarris, 2016) (*see* **Note 22**).

4. Focus on the embryos, and lower holding pipette until it becomes levelled with the embryos, vary the holding pressure such that the embryo is stably held, then lift the then raise the holding pipette and embryo 100-200 µm above the chamber surface (*see* **Note 23**).
5. Using micromanipulators lower the microinjection pipette into the M2 media such that the tip of the pipette is in the same plane as the embryos (*see* **Note 24**).
6. Insert the tip of the injection pipette into the cytoplasm of one of the blastomeres through the *zona pellucida* and apply a short pulse of negative capacitance with the intracellular electrometer and microinject the H2B:RFP cRNA by applying a timed pressure using the picopump. Successful microinjection should be evidenced by cytoplasmic displacement. Then remove the pipette smoothly. Repeat procedure for the second blastomere (*see* **Note 25**).
7. Once the settings have been adjusted for the desired injection size, use the same settings to microinject the main group of embryos to be used for the experiment, to ensure consistent microinjection volume and comparable cRNA translation levels between blastomeres and embryos (*see* **Note 26**).
8. Following microinjection, wash the embryos through three drops of pre-equilibrated KSOM media using a mouth controlled pipette. Culture embryos in KSOM in an incubator with 5% CO_2 at 37ºC for 2-3 hours and check fluorescent protein expression using an inverted epifluorescence microscope equipped with fluorescence optics. Return embryos to the incubator and culture until the desired developmental stage is reached (*see* **Note 20**).

3.6 Live cell imaging set-up for mouse embryos

We aim to observe the dynamics of chromosome segregation in embryos using confocal live cell imaging conditions as non-damaging as practically possible. To this end, we use a temperature-controlled incubator with precise 5% CO_2 concentration supply mounted on the microscope stage, and use imaging protocols designed to minimize light exposure to embryos. The set-up of the live cell imaging chamber is described below and depicted in **Figure 3.2**, p.76.

1. Fit the top-stage incubator on the microscope stage, turn on the temperature controller device, and set temperature at 37 ºC (*see* **Note 27**). Connect the lid of the incubator to a 5% CO_2 supply (*see* **Note 28**).
2. Place a 50 mm glass bottom culture dish on the stage incubator, pipette a 5 µl drop of KSOM in the center of and cover with warm mineral oil pre-equilibrated with 5% CO_2 (*see* **Notes 29** and **30**).
3. At a low magnification (5x, 10x), focus on the KSOM drop and pipette H2B:RFP cRNA microinjected embryos into the middle of the drop. Positioning the embryos closely together will allow to image multiple embryos within the same frame (*see* **Note 31**).
4. Embryos are imaged using a 0.75 NA 20x lens and 552 nm laser at 0.1-0.5% power, collecting 560-700 nm emission with a HyD detector and spectral detection system. Select an optical section of 2 µm, a Z-stack size of ~60 µm and step size of 2 µm. Acquire images bi-directionally at 400 Hz speed in 1024x1024 pixel format at an 8-bit depth, two-scan averaging may be used to further increase image clarity. A bright-field image is simultaneously acquired by means of a PMT_{trans} detector. To capture the dynamic events of chromosome segregation in H2B:RFP-expressing embryos, we perform time lapse imaging using a time interval of 3-5 minutes (*see* **Notes 32** and **33**).
5. Adjust imaging settings such that minimal 552 nm laser power is used to achieve a clear unsaturated image and start three-dimensional (Z-stack) time lapse imaging.
6. To investigate the fate of MN-chromosomes in preimplantation embryos, identify embryos with blastomeres containing MN and monitor cells entering mitosis, this is first evident by condensation of chromatin into chromosomes during nuclear envelope breakdown (NEBD). Mitotic divisions at this stage have a duration of 40-60 minutes, so we recommend systematically and periodically (every 10 minutes) examine the Z-slices for each embryo for NEDB.
7. Upon observation of a MN-containing cell entering mitosis, closely monitor progression into mitosis until most chromosomes are aligned at the metaphase plate, in most cases the MN is distinctly separate from the metaphase plate.
8. At this time, stop the image acquisition, switch back to a lower magnification lens, and using a mouth-controlled pipette individually collect the embryo of interest for immediate fixation.

9. For later identification and relation in correlative immunofluorescence, for each selected embryo, it is useful to draw a map of the layout of the embryos, and mark the relative position selected embryo. It is also recommended to annotate the live imaging file name, last time-point and Z-slice in which the MN-containing metaphase cell is located (see **Note 34**).

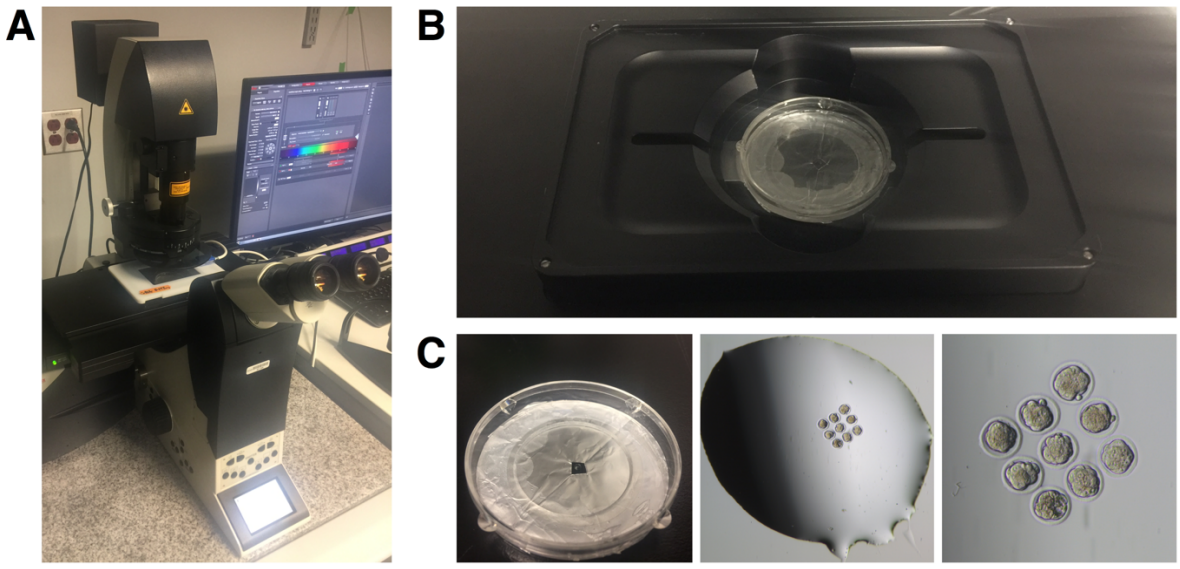

Figure 3.2. Live imaging confocal and top-stage incubator set-up
(A) *Confocal microscope fitted with top-stage incubator chamber.* ***(B)*** *50 mm glass-bottom culture dish and aluminium foil bottom for live cell imaging in top-stage incubator.* ***(C)*** *From left to right: 50 mm glass-bottom culture dish with a 5 µl drop of KSOM covered with mineral oil, embryos positionaed in 5 µl drop of KSOM, magnified image of embryos in culture dish.*

3.7 Embryo fixation and immunofluorescence

Prior to initiation of live imaging, prepare a round-bottom 96-well plate for immunofluorescence staining, arranging solutions in vertical columns, and individual selected embryos in horizontal rows. Place 50 µl of solution in each well, covering each 50 µl filled-well with a drop of mineral oil, except wells containing 0.25% Triton-X. Stain embryos with CREST and tubulin antibodies to label kinetochores and microtubules, respectively. Alexa-Phalloidin staining will enable visualization of filamentous actin at the cell cortex, hence delineating individual blastomeres within the embryo.

1. Using a bent Pasteur pipette mounted on the mouth-controlled aspiration tube, transfer the embryo into the first column containing 4% PFA in PBS. Incubate for 30 minutes at room temperature. Subsequent steps described below entail moving embryos to successive wells in the 96-well plate using the mouth-controlled pipette.
2. Permeabilize embryos with 0.25% Triton-X in PBS for 10 minutes at room temperature.
3. Wash with 3% BSA in PBS three times.
4. Block in 3% BSA in PBS for 60 minutes at 37ºC or overnight at 4ºC.
5. Incubate with CREST primary antibody for 60 minutes at 37ºC (*see* **Note 35**).
6. Wash with 1% BSA in PBS three times to remove residual primary antibody.
7. Incubate with secondary antibody (anti-human IgG Alexa) for 60 minutes at 37 ºC. From this step onwards, wrap the plate with aluminum foil to protect it from light exposure (*see* **Note 36**).
8. Wash with 1% BSA in PBS three times to remove residual secondary antibody.
9. Incubate with mouse anti-α-tubulin primary antibody for 60 minutes at 37 ºC (see **Note 35**).
10. Wash with 1% BSA in PBS three times to remove residual primary antibody.
11. Incubate with secondary antibody (anti-mouse IgG Alexa) for 60 minutes at 37 ºC (*see* **Note 36**).
12. Wash with 1% BSA in PBS three times to remove residual secondary antibody.
13. Incubate with Alexa-conjugated Phalloidin for 60 minutes at 37ºC (*see* **Note 36**).
14. Wash with 1% BSA in PBS three times to remove residual Alexa-Phalloidin staining.
15. DNA-staining dyes such as DAPI/Hoechst are not required since chromosomes are fluorescently labeled with H2B:RFP.

3.8 Correlative immunofluorescence confocal imaging

This section describes our standard method for confocal immunofluorescence imaging to obtain high resolution three-dimensional images of individual embryos and individual mitotic cells (*see* **Note 37**). Hence allowing to analyse the localization of kinetochore proteins and interaction with microtubules of MN-chromosomes during mitosis (**Figure 3.3**, p.79).

1. For confocal immunofluorescence imaging, place a 5 µl drop of 1% BSA in PBS in a 35 mm glass bottom culture dish, and cover it with mineral oil.
2. Transfer the embryo into the imaging dish using the mouth controlled pipette.
3. Using a confocal microscope, locate the embryo using a lower magnification (5x, 10x lens). After having applied a small amount of immersion oil on the objective lens, switch to a 63x (1.40 NA) oil immersion lens and perform immunofluorescence confocal imaging (*see* **Note 37**).
4. Manually rotate and reposition of the embryo by means of a mouth-controlled pulled glass pipette to place the embryo in a similar orientation to that in the initial live imaging experiment (*see* **Note 34**).
5. Increase the zoom further to make the cell of interest fit the image frame. Adjust the optical section to 1 µm, and excitation laser power for the different channels as necessary. Use the Alexa-Phalloidin signal marking the cell cortex to set a Z-stack front-end to the back-end of the cell of interest, with a step size of 1 µm.

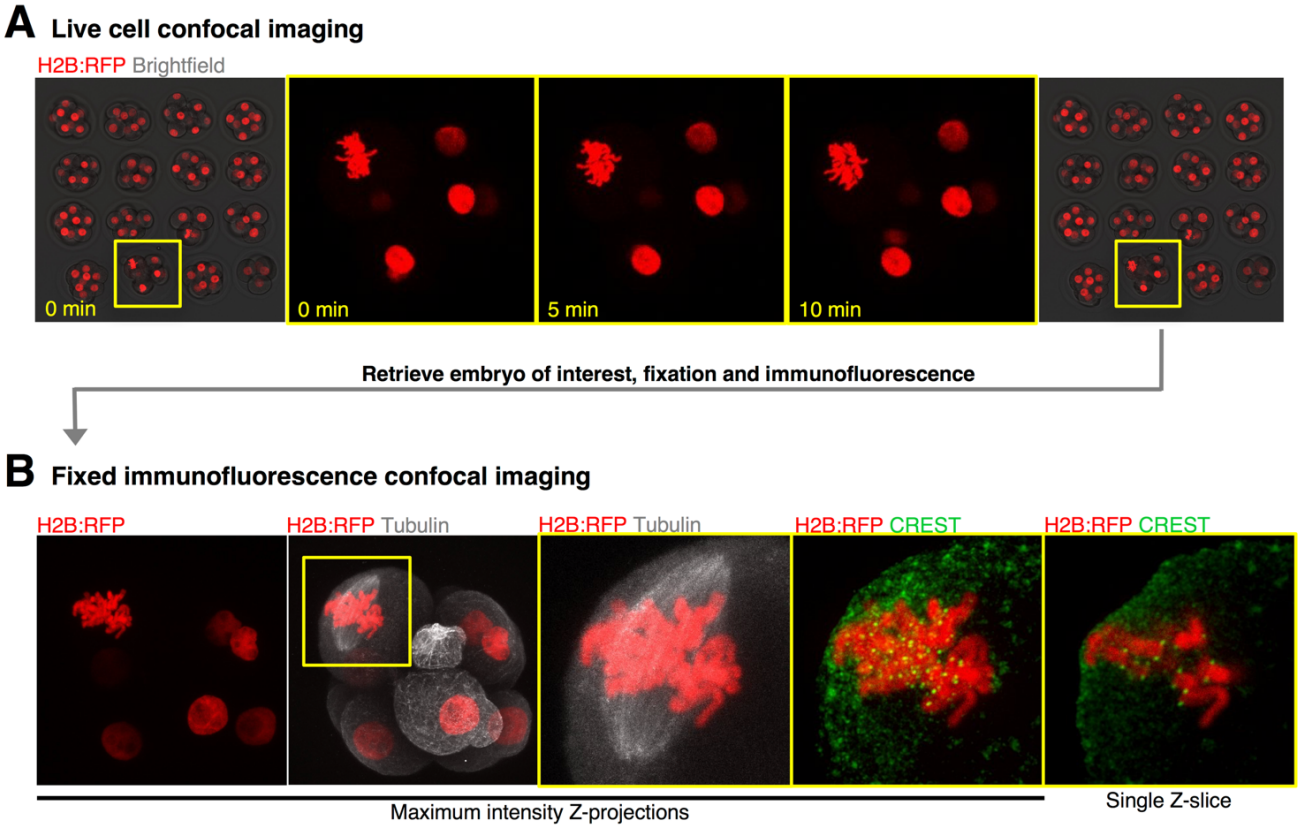

Figure 3.3. Correlative live and immunofluorescence imaging

(A) *Sill frames from time-lapse live cell confocal imaging of H2B:RFP expressing embryos. Yellow boxes indicate the embryo of interest.* ***(B)*** *Immunofluorescence imaging of the embryo of interest in* ***(A)*** *selected by live imaging. Chromosomes/DNA appear in red (H2B:RFP), microtubules appear in gray scale (anti-α-tubulin), and kinetochore appear in green (CREST). Yellow boxes indicate cell of interest.*

3.9 Notes

1. The H2B:RFP plasmid we routinely use is a gift from Alex McDougall, (Observatoire Océanologique de Villefranche-sur-Mer, Villefranche Sur Mer, France) (Vázquez-Diez et al., 2016). However, equivalent alternatives are available from Addgene: pCS-H2B-mRFP1 Plasmid #53745, H2B-mCherry Plasmid #20972. (https://www.addgene.org/53745/, https://www.addgene.org/20972/)
2. The restriction enzyme required depends on the DNA plasmid to be used as a template. Choice of an appropriate restriction enzyme for DNA plasmid linearization requires a unique restriction site that must lie downstream of the promoter and fusion protein.
3. mMessage mMachine ® kit used must match the promoter present on template plasmid DNA: T3, T7 or SP6.
4. We typically use CD-1 mice. However, different mouse strains may be used. Animals should be acquired and stored in accordance with local animal welfare regulations.
5. Hormones are prepared in sterilized filtered PBS on ice (we use a dilution of 25 international units (IU) per ml). 1ml aliquots are stored at -20 ºC. Hormones are injected into mice immediately after thawing.
6. We normally use commercially provided M2 and KSOM media, but these solutions can also be made in-house and stored frozen at -20°C, supplementing with the appropriate amount of BSA after thawing (Erbach et al., 1994; Gardner and Leese, 1986).
7. Pasteur pipettes need to be pulled to appropriate width using an alcohol lamp or Bunsen burner. Pipettes with an internal diameter of ~80-100 µm are recommended for embryo collection. Pasteur pipettes are attached to a mouth-controlled aspiration tube to allow embryos to be transferred between drops. Moving embryos with a mouth-controlled Pasteur pipette takes several weeks' practice, but once mastered allows very precise and controlled movement. As an alternative to mouth pipetting, manual pipettes may also be used (Cook Medical).
8. Further details on components and set-up of our microinjection system can be found in (Nakagawa and FitzHarris, 2016). Precise micromanipulation set-up may vary. The amplifier and negative capacitance pulse is essential when performing injections into metaphase-stage eggs and embryos, which are extremely sensitive to injection. An anti-vibration assembly is essential, such as commercial vibration isolation tables, though a marble slab mounted on tennis or squash balls can also suffice.

9. Holding pipettes can be made in-house using a pipette puller and microforge. Alternatively, we recommend investigators with limited micromanipulation experience to use commercially available pre-made holding pipettes that may be purchased from Hunter scientific http://www.hunterscientific.com.
10. We normally use petri dishes fitted with No. 0 cover glass (0.085-0.13mm) for immunofluorescence microscopy.
11. Our ACA antibody is derived from CREST patients' human sera and obtained from Marvin J Fritzler from University of Calgary. However, CREST antibodies are also commercially available.
12. We typically assemble control and restriction enzyme digestion reactions with final volumes of 10 µl and 50 µl respectively. To verify complete digestion run 5 µl of each reaction on a 1% agarose gel, a single band is observed if plasmid is completely linearized. Use a DNA ladder to check the linearized fragment is the correct size.
13. Linearized DNA template may be purified by alternative methods such as DNA clean up and DNA precipitation.
14. Before starting IVT and following steps ensure that all surfaces and equipment to be used have been cleaned with 70% ethanol and then wiped with RNase decontamination solution reagent. Use certified RNase-free or autoclaved filter tips. It is also recommended to change gloves before each step.
15. Prior to the Poly(A) tailing step, incubate the IVT reaction mix with Turbo DNase to remove the DNA template.
16. We recommend optimizing hormone doses for the chosen mouse strain and age.
17. The method of euthanasia must be performed in accordance with local animal welfare regulations.
18. M2 media must be pre-warmed to 37 ºC on a dry bath. During the dissection process, dishes should be kept on the dry bath when not being used on the dissection microscope.
19. Alternatively, embryos can be retrieved by oviduct flushing (Nagy, 2003).
20. In our hands, CD-1 two-cell embryos will be four cells ~60 hours post hCG and mating (post-hCG+m), eight to sixteen cells ~72 hours post-hCG+m, morula ~96 hours post-hCG+m and blastocysts ~120 hours post-hcG+m.
21. To wash residual KSOM medium, transfer embryos through three drops of M2 before pipetting into the M2 media in the microinjection chamber.

22. We typically inject H2B:RFP cRNA at a concentration of 400-800ng/µl (microinjection pipette concentration), however these may be adjusted for different cRNA batches to achieve efficient expression. Optimal cRNA concentrations and injection size can be tested by microinjection as described in (Nakagawa and FitzHarris, 2016) (*see* **Note 25**).

23. Orienting two-cell embryos such that both cells are in the same focus plane is advised to facilitate successful microinjection of both blastomeres.

24. Upon contact of the microinjection tip with the M2 media, the negative capacitance circuit should be closed and displayed on the intracellular electrometer as described in (Nakagawa and FitzHarris, 2016) (*see* **Note 8**).

25. Since microinjection volume may vary with pipette tip diameter, use a small group of embryos to adjust the pressure and time to achieve a modest, but visible cytoplasmic displacement. Using a small cohort of test embryos, inject separate groups with different injection sizes, by adjusting the ejection pressure and injection time as described in (Nakagawa and FitzHarris, 2016). Confirm injection size is not detrimental to embryo health by culturing two-cell embryos in KSOM for ~72 hours and compare blastocyst rates in non-injected vs water-injected embryos. Cell death directly attributable to the micro-injection technique should not exceed 15% for standard microinjections.

26. We recommend applying gated pressure pulses before and after every microinjection to prevent backflow of media into the pipette and ensure consistent content of microinjection.

27. We advise to test and calibrate the temperature of the top-stage incubator required to maintain the drop of media at 37ºC by using a thermocouple.

28. We use centrally-supplied CO_2 which is equilibrated at 5% using a proportional gas mixer. CO_2 gas cylinders may also be purchased, and concentration is adjusted using a pressure regulator. In any case, we recommend the use of a CO_2 meter to confirm CO_2 concentration is 5% before starting an experiment.

29. For optimal heat transfer throughout the culture dish we place a piece of aluminum foil on the bottom of the dish, a small hole is cut out, and the 5 µl drop of KSOM is placed right above it to allow light transmission and imaging of embryos. A sparing amount of Vaseline on the dish circumference may be used to better affix the aluminum foil to the dish.

30. We recommend the live cell imaging chamber to be set up at least 30 minutes before starting the experiment, to allow sufficient time for temperature and pH equilibration. This will also prevent focus shifts during live cell imaging should there be any expansion of the live cell imaging chamber as the temperature equilibrates.

31. Before beginning experiments, it is important to verify that the live cell imaging conditions are suitable for embryo development. This can be done by setting up the chamber and placing a small group of 10-15 two-cell embryos on the microscope stage (without any imaging) for two or three days and compare developmental rates with embryos placed in a similar dish in the cell culture incubator.

32. Our confocal settings have been optimized to reduce embryo light exposure while still yielding high resolution images. For this reason, the RFP fluorophore is most suitable, since shorter wavelengths may cause photo-toxicity and longer wavelengths may cause sample heating. HyD detectors as opposed to conventional PMT detectors are preferable due to their superior sensitivity, hence requiring reduced amounts of laser power and light exposure to achieve a high contrast image with minimal noise.

33. To validate that the imaging is not causing damage to the embryos we recommend performing a live imaging run for 8-12 hours at the earlier stages two-cell up to eight-cell, and then placing embryos back in the culture incubator. Compare developmental rates to blastocyst with control embryos that were left in the incubator and not imaged. The settings, and in particular the 552 nm laser power, might have to be slightly modified on an experiment-to-experiment basis to obtain bright but unsaturated images

34. In the case that more than one cell is in mitosis in a given embryo, it is advised to take notes of other features of the embryo that may later help identifying the dividing MN-containing blastomere. For example, the other mitotic cell being in a different stage of mitosis, prometaphase/anaphase. Positional information such as proximity to polar body, metaphase plate orientation, inner/outer location of blastomere within the embryo may also be valuable. The bright-field image will aid in discerning which blastomere the MN belongs. When performing confocal immunofluorescence imaging, the H2B:RFP signal may be used to positively identify the cell of interest within the embryo. Manipulating embryo orientation, comparison to the last time-point of the live cell imaging Z-stack of that same embryo and the use of previous annotations of embryo features may be required to facilitate identification of the MN-containing cell at metaphase.

35. Optimal fixation method, antibody dilution, incubation times and temperatures should have been previously and empirically determined on similar developmental stage embryos. Alternative fixing methods that may be used include: (1) using 2% PFA in PBS fixation, (2) a brief (5 seconds) pre-permeabilization in 0.5% Triton-X in PHEM buffer (60mM Pipes, 25mM HEPES, 10mM EGTA, 2mM MgCl2, pH 6.9) prior to fixation in 4% PFA in PHEM

followed by normal permeabilization, or (3) simultaneously fixing and permeabilizing in 4% PFA + 0.25% Triton-X in PHEM buffer.

36. Use different Alexa fluorophores to distinguish the microtubules from CREST and Phalloidin. After performing the immunofluorescence staining, embryos may be stored in 1% BSA in PBS solution at 4 ºC, protected from light. In our experience, Alexa-Phalloidin staining provides best results when applied immediately prior to confocal imaging.

37. For whole embryo immunofluorescence imaging, we typically acquire 1024x1024 format images at a speed of 400 Hz with bi-directional scanning, and an optical section of 1.5 µm. Different channels are sequentially acquired per frame with two-scan averaging. Excitation laser power is adjusted as required and emission collection selected based on emission spectra of each fluorophore, preferentially using a HyD over PMT detectors, since they provide a more defined image with a better signal-to-noise ratio. Even if using sequential scanning, we set the non-overlapping collection spectra to prevent crosstalk between acquired channels. We recommend having the live cell imaging Z-stack for the last time-point of the embryo to be analyzed, open and easily available on the software simultaneously. The digital zoom is adjusted such that the whole embryo fits the image frame. The Z-stack is set to span the whole embryo, starting from the side that's closest to the glass coverslip, using a step size of 1.5 µm. It is recommended to manually reposition the embryo if necessary, such that spindles are parallel to the cover slip to obtain a high-quality image and facilitate analysis of CREST and microtubule localization to mitotic MN-chromosomes.

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CHAPTER 4

Publication

SPINDLE ASSEMBLY CHECKPOINT FAILURE UNDERLIES CHROMOSOME SEGREGATION ERROR IN THE MOUSE PREIMPLANTATION EMBRYO

Manuscript in preparation for submission

4.1 Preface

Having identified that the relatively high prevalence of lagging chromosomes in embryos is the major cause of micronuclei, which in turn act as a catalyst for mosaic aneuploidy, Dr Greg FitzHarris and I wanted to investigate the mechanisms underlying the increased propensity for embryos to mis-segregate chromosomes. We chose to focus on the Spindle Assembly Checkpoint since it is the major fail-safe mechanism in mitosis that functions to prevent segregation errors and mechanistic studies of this pathway in embryos were lacking. Dr Greg FitzHarris and I devised the study and designed experiments, I performed all experiments, data analysis and produced all figures and illustrations. Dr. Greg FitzHarris and I wrote the manuscript presented in this thesis.

SPINDLE ASSEMBLY CHECKPOINT FAILURE UNDERLIES CHROMOSOME SEGREGATION ERROR IN THE MOUSE PREIMPLANTATION EMBRYO

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4.2 Summary

Chromosome segregation errors in early embryos may jeopardise development and cause reproductive failure in humans. However, why segregation errors are common in early mammalian embryos is unknown. Here we apply live imaging, micromanipulation, gene knockdown, and pharmacological approaches in mouse embryos to examine the function of the Spindle Assembly Checkpoint (SAC), a failsafe mechanism that prevents errors in other cell types by inhibiting the Anaphase Promoting Complex (APC/C). We show that SAC signalling is functional in embryos and limits the number of segregation errors. However, misaligned chromosomes fail to prevent anaphase onset, despite active SAC signalling at kinetochores. The failure of SAC to delay anaphase is not attributable to the large size of early embryo cells, and SAC-mediated anaphase delay is weakest in mid-preimplantation development. We show that mild chemical inhibition of APC/C can extend mitosis, thereby allowing more time for chromosome alignment, and reduces segregation errors. Our data provide the first mechanistic explanation for frequent segregation error in mammalian embryos, and provide proof of principle that modulation of the SAC-APC/C axis can increase the likelihood of faithful chromosome segregation.

4.3 Introduction

Accurate chromosome segregation during cell division is a highly coordinated series of events resulting in equal distribution of replicated chromosomes into formed daughter cells. One would expect stringent mitotic fidelity during early cleavage divisions of the newly fertilized mammalian preimplantation embryo given that these are responsible for the establishment of all fetal and extraembryonic tissues. Yet strikingly, mosaic aneuploidy in embryos, where some blastomeres harbour abnormal chromosome complements, has been reported across multiple mammalian species, and is remarkably frequent in human embryos (Dupont et al., 2010; Hornak et al., 2012, 2016; Mizutani et al., 2012; Vanneste et al., 2009). However, the underlying cellular cause for the inherent tendency of embryonic blastomeres to mis-segregate chromosomes remains unknown.

The major cellular safeguard preventing chromosome segregation errors in somatic cells is the Spindle Assembly Checkpoint (SAC), a near-ubiquitous signalling pathway that delays the anaphase onset until all chromosomes are correctly attached to spindle microtubules. SAC signaling at mis-attached kinetochores catalyses the generation of the Mitotic Checkpoint Complex (MCC), a diffusible signal that inhibits Anaphase Promoting Complex (APC/C) activity, preventing Cyclin B and Securin degradation and delaying anaphase until correct kinetochore-microtubule attachment is achieved (Lara-Gonzalez et al., 2012). Whilst the high incidence of mosaic aneuploidy in the early mammalian embryo has led to speculation that the SAC may be absent in embryos, a mechanistic examination is yet to be presented.

SAC function has been well studied in mammalian oocytes, where it is only weakly efficient in preventing segregation errors. This is likely due in part to the need for diffusible MCC to inhibit APC/C throughout the cytoplasm in an extraordinarily large cell (Gui and Homer, 2012; Jones and Lane, 2013; Kyogoku and Kitajima, 2017; Nagaoka et al., 2011). Consistently, in *C. elegans* embryos the SAC is ineffective in early embryos, and becomes more effective as cells reduce in size during early development (Galli and Morgan, 2016). Here, we use a range of approaches to analyse SAC function in the early mammalian embryo, and test the hypothesis that large cell size hampers the checkpoint in the earliest stages of development. Unexpectedly, we report that frequent segregation error in the mouse embryo is permitted by a cell size-independent failure of SAC signalling to prevent anaphase. We show that mildly inhibiting the APC/C can improve the probability of correct alignment being achieved before the completion of cell division, and lowers the number of segregation errors.

4.4 Results

4.4.1 SAC signalling fails to prevent chromosome segregation errors in embryos

Mouse embryos progress from a fertilised 1-cell embryo to a 16-32 cell stage morula and 64-128 cell blastocyst in 4-5 days, blastomeres approximately halving in size at each division. To begin to investigate SAC effectiveness during these divisions, we examined chromosome segregation in live H2B:RFP-expressing embryos. Classically in mitotic cells, a single misaligned chromosome is sufficient to sustain robust SAC signalling and prevent anaphase (Rieder et al., 1995). In morula-stage embryos, chromosomes were fully aligned at the time of anaphase onset in 75% of divisions, anaphase occurring without any obvious defect. However, a significant subset of divisions progressed into anaphase despite the presence of mildly (20%) or severely (6%) misaligned chromosomes (**Fig. 4.1A and B**, p.93). Notably, there was no significant difference in the duration of mitosis regardless of whether divisions exhibited misalignments at anaphase onset (**Fig. S4.1**, p.93), alluding to an absence of a fully-functional SAC.

To more directly determine whether SAC functions during normal development, we inhibited SAC using two approaches; AZ3146, a highly specific inhibitor of Mps1 kinase (Hewitt et al., 2010), and by microinjecting morpholino oligonucleotides (MO) against Mad2 (**Fig. S4.2**, p.94). While development to blastocyst was unaffected by either treatment (**Fig. 4.2A**, p.95), the number of micronuclei per cell in blastocysts, which in mouse embryos is a direct indication of accumulated segregation errors (Vázquez-Diez et al., 2016), increased from ~0.025 in controls to 0.051 in AZ3146 and 0.050 in MO-injected embryos (**Fig. 4.2B and C**). Analysis of chromosome segregation dynamics in live embryos revealed that AZ3146 shortened mitosis from 53±3 mins to 33±1 mins, and caused a marked increase in chromosomes remaining misaligned at anaphase onset (63% compared to 24%), and lagging anaphase chromosomes (47% compared to 35%) (**Fig. 4.2D and E**). Together these data show that SAC signalling is in operation in embryos and generally serves to prolong mitosis, thereby allowing more time for complete alignment, but that cells frequently enter anaphase before chromosome alignment is complete.

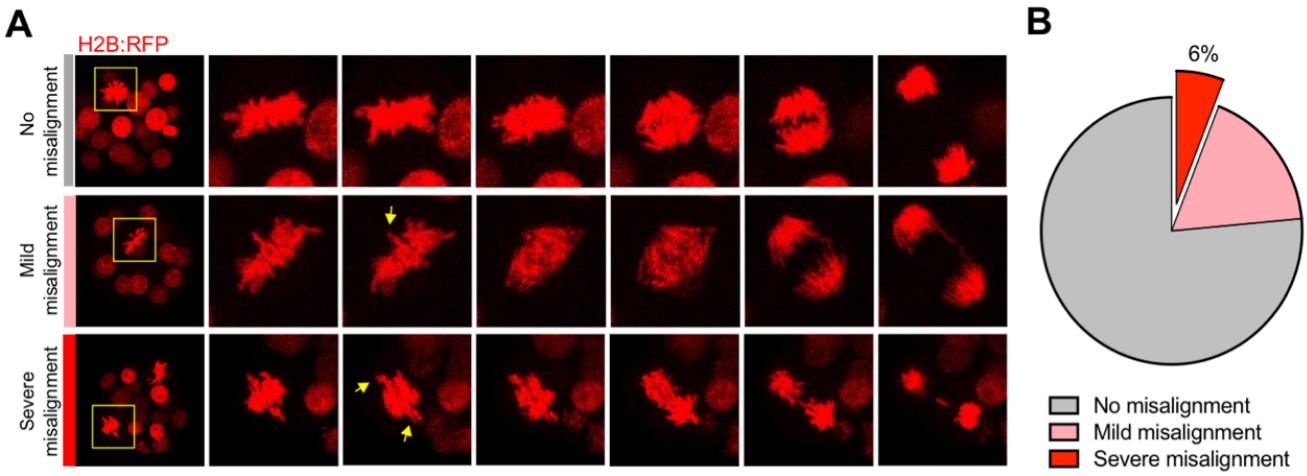

Figure 4.1. Mouse preimplantation embryo cleavage divisions occur in the presence of misaligned chromosomes

A and B: *Representative images and analysis of chromosome alignment prior to anaphase from live imaging of H2BRFP-expressing (red) embryos. Complete Z-stack every 30 seconds. Data from 100 divisions at the 16- to 32-cell transition. Yellow arrows indicate misaligned chromosomes in the frame previous to anaphase onset.*

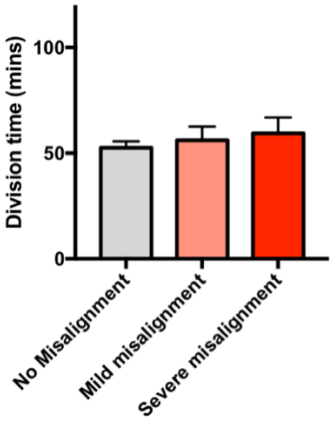

Figure S4.1. Misalignments at anaphase onset do not affect the duration of mitosis

Quantification of NEBD to Anaphase time in divisions with or without misalignments at anaphase onset in H2B:RFP-expressing embryos (mean and SEM, n= 42 divisions in total, one-way ANOVA P= 0.78).

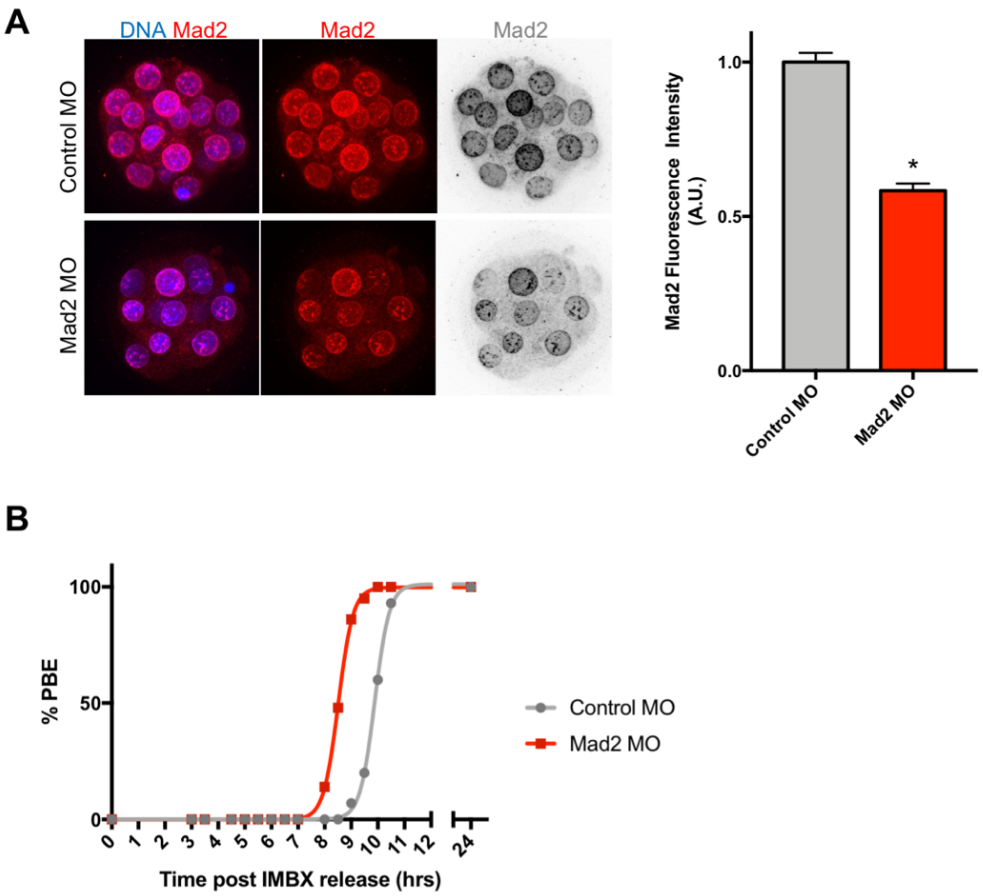

Figure S4.2. Validation of Mad2 knockdown with Morpholino Oligonucleotides

A: *Representative confocal images and fluorescence intensity quantification of Mad2 immunofluorescence in embryos microinjected with Control and Mad2 morpholino oligonucleotides (MO) (mean and SEM, n= >108 cells per group, t-test, P= <0.0001).* ***B:*** *Polar body extrusion (PBE) of meiosis-I in oocytes microinjected with Control and Mad2 morpholino oligonucleotides (MO).*

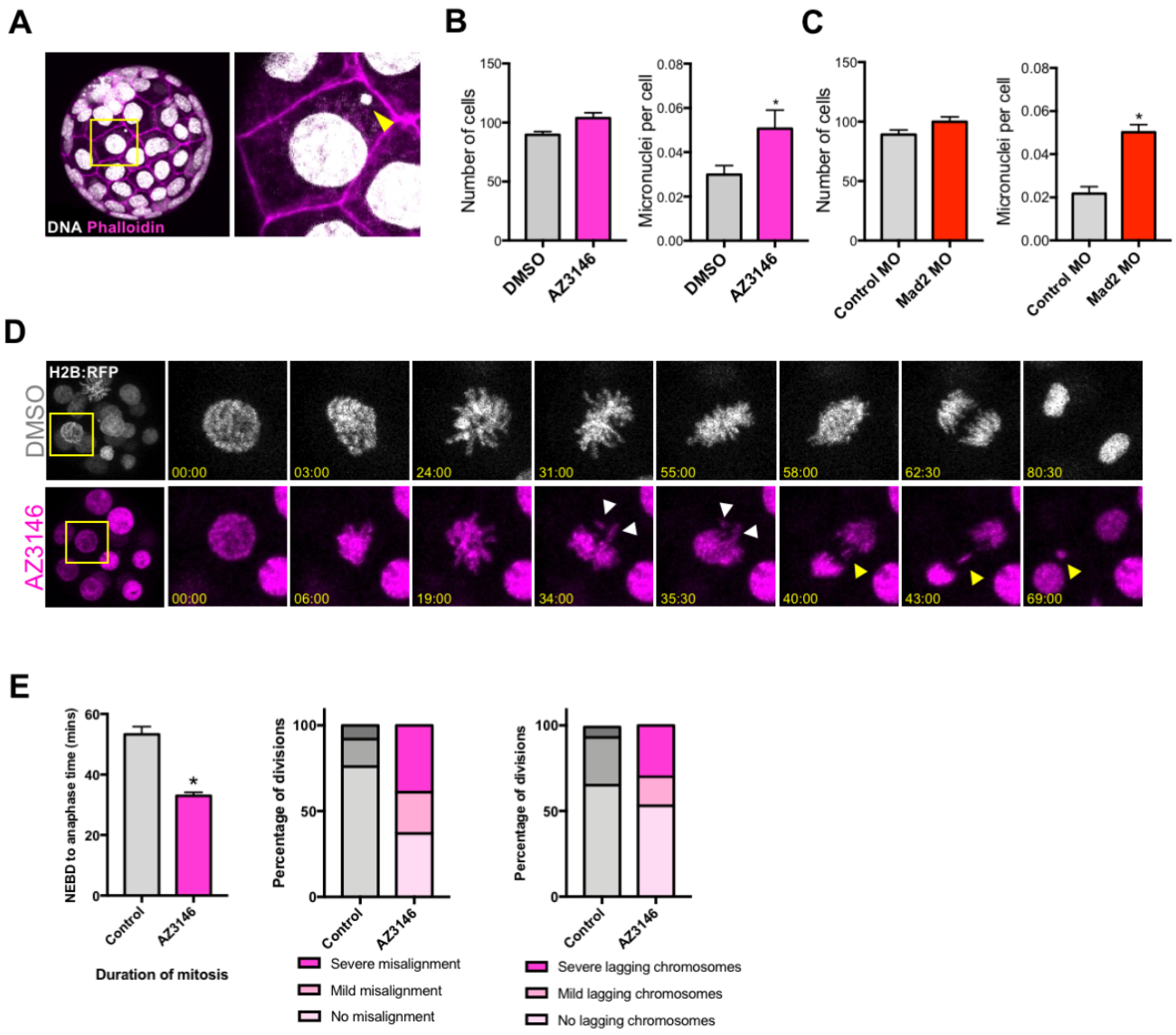

Figure 4.2. Inhibition of SAC constituents causes chromosome alignment and segregation defects

A: *Representative images of a blastocyst and a micronucleus (indicated by the yellow arrowhead).* ***B and C:*** *Quantification of cell numbers and micronuclei per cell in DMSO and AZ3146-treated blastocyst embryos and Control MO and Mad2 MO blastocysts (mean ± SEM, n=>20 embryos/group, t-test, P<0.05).* ***D:*** *Representative images of chromosome segregation dynamics in 16-32C transition of H2B:RFP embryos in control DMSO and 20μM AZ3146. White arrows indicate pre-anaphase misaligned chromosomes; yellow arrows indicate anaphase lagging chromosome resulting in the formation of a micronucleus.* ***E:*** *Duration of mitosis (mean ± SEM,), incidence of pre-anaphase misaligned chromosomes and anaphase lagging chromosomes in DMSO and 20μM AZ3146 (n=>40 divisions/group, t-test, P <0.05).*

4.4.2 Small numbers of misaligned chromosomes fail to prevent anaphase

We next set out to determine the ability of the SAC to respond to spindle defects and misaligned chromosomes. Treating embryos with the spindle poison nocodazole caused a pronounced mitotic arrest (mitotic index 40% and 95% in 100nM and 1μM nocodazole, respectively) (**Fig. 4.3A, B and C**, p.97). Mad2 was evident at kinetochores in nocodazole-treated cells, and the arrest was reversible by AZ3146 (**Fig. 4.3D and E**). Thus, severe spindle damage can elicit a SAC arrest in early embryos.

To more discerningly appraise the SAC's capacity to respond to chromosome alignment we employed GSK923295, a specific inhibitor of chromokinesin CENP-E (Bennett et al., 2015). Fixed cell analysis revealed that 500nM and 5μM of GSK923295 resulted in multiple misaligned chromosomes in almost all metaphases in morulae without disrupting spindle architecture (93% and 100% respectively) (**Fig. 4.4A and B**, p.98). However, embryos displayed only a moderate increase in mitotic index (23% and 12% of cells in prometaphase) (**Fig. 4.4C**). Live imaging of the 8-16 cell transition in GSK923295 revealed anaphase onset occurred with at least one severely misaligned chromosomes in all cases (mean of 3.6±0.3 per cell) (**Fig. 4.4D, E and F** and **S4.3**, pg.98).

The failure of misaligned chromosomes to prevent anaphase suggests either that the misaligned chromosomes fail to initiate a SAC signal, or that the SAC fails to prevent APC/C activation. To distinguish between these two scenarios, we employed Mad1:EGFP, an extensively used reporter of SAC activity at kinetochores (Kruse et al., 2014; Lane et al., 2017). Mad1:GFP did not affect the timing of cell divisions or development of embryos to blastocyst stage (data not shown) and displayed expected spatiotemporal dynamics, accumulating in the nucleus in interphase, then on kinetochores shortly after nuclear breakdown, leaving kinetochores as chromosomes align (**Fig. S4.4**, p.99). We performed live imaging of H2B:RFP and Mad1:EGFP-expresing embryos in 500nM GSK923295, again observing multiple misaligned chromosomes at anaphase onset (mean 3.5±0.4). Notably, 80% of misaligned chromosomes bore detectable Mad1:EGFP signal on kinetochores at anaphase onset (**Fig. 4.5A and B**, p.100), suggesting that misaligned chromosomes mount a SAC signal, but that SAC signalling from a limited number of chromosomes is insufficient to prevent anaphase onset.

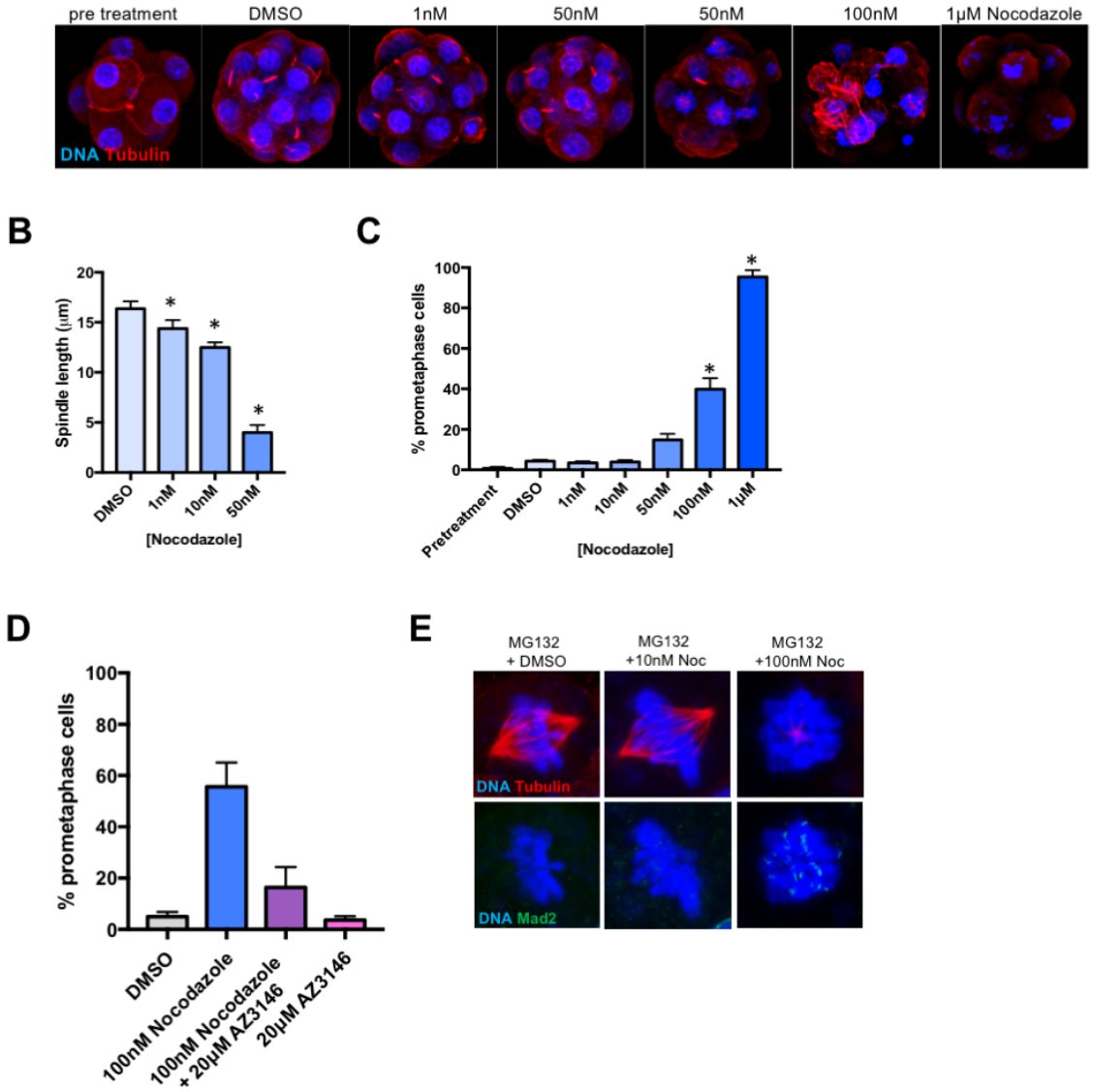

Figure 4.3. Severe spindle damage induces a robust SAC-mediated mitotic arrest

A: *Representative images of embryos before and after a 16hour exposure to the vehicle (DMSO) or different concentrations of the spindle poison Nocodazole.* ***B:*** *Measurements of spindle length in DMSO- and Nocodazole-treated embryos (mean and SEM, n= >7 metaphases per group).* ***C:*** *Quantification of the percentage of prometaphase cells in embryos exposed to different Nocodazole concentrations (mean and SEM, n= >20 embryos per group, one-way ANOVA, P<0.05).* ***D:*** *Quantification of the percentage of mitotic cells in embryos treated with DMSO or 100nM Nocodazole and/or 20µM AZ3146. (mean and SEM, n= >13 embryos per group, one-way ANOVA, P<0.0001).* ***E:*** *Representative images of SAC protein Mad2 recruitment to metaphase spindles in the presence of different concentrations of Nocodazole.*

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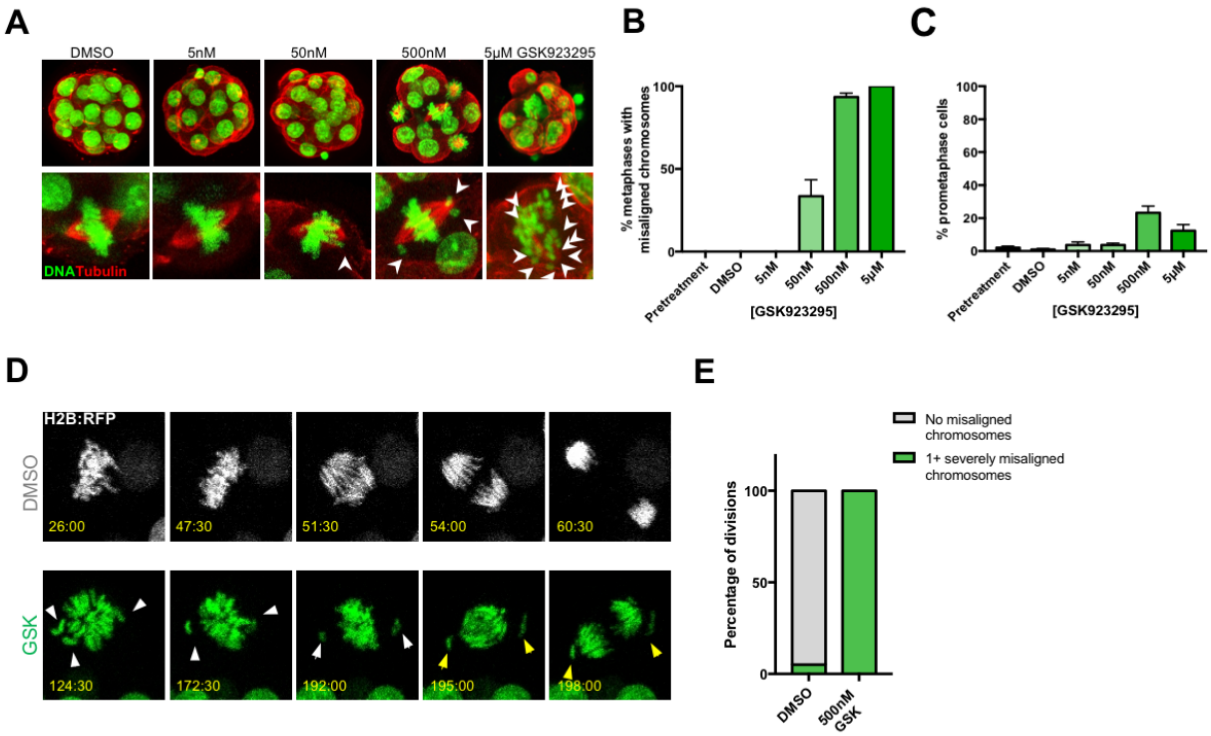

Figure 4.4. The SAC fails to sustain a metaphase arrest in the presence of misalignments

A: *Representative images of embryos and spindles after a 16hour exposure to GSK933295. White arrowheads indicate misaligned chromosomes* ***B:*** *Percentage of metaphases containing severely misaligned chromosomes (mean and SEM, n=>7 metaphases/group).* ***C:*** *Percentage of prometaphase cells in embryos exposed to GSK923295 (mean and SEM, n= >7 embryos/group, total 129).* ***D:*** *Confocal images of chromosome segregation dynamics in H2BRFP-expressing embryos in DMSO and 500nM GSK923295. White and yellow arrowheads indicate misaligned chromosomes prior to and during anaphase, respectively.* ***E:*** *Proportion of divisions initiating anaphase in the presence of at least one severely misaligned chromosome (n=>50 divisions/group).*

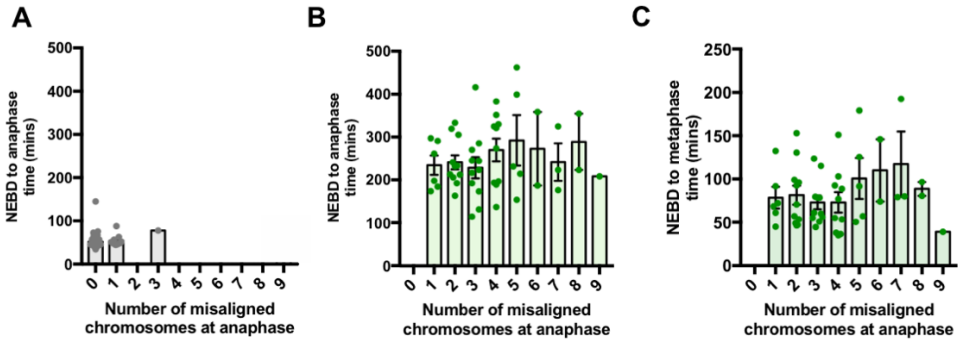

Figure S4.3. Anaphase onset is unaffected by the number of misaligned chromosomes.

A and B: *Quantification of NEBD to anaphase time number of misaligned chromosomes at anaphase onset in DMSO and 500nM.* ***C:*** *Quantification of NEBD to metaphase time and number of misaligned chromosomes at anaphase onset in 500nM GSK (mean and SEM, n>51 divisions per group).*

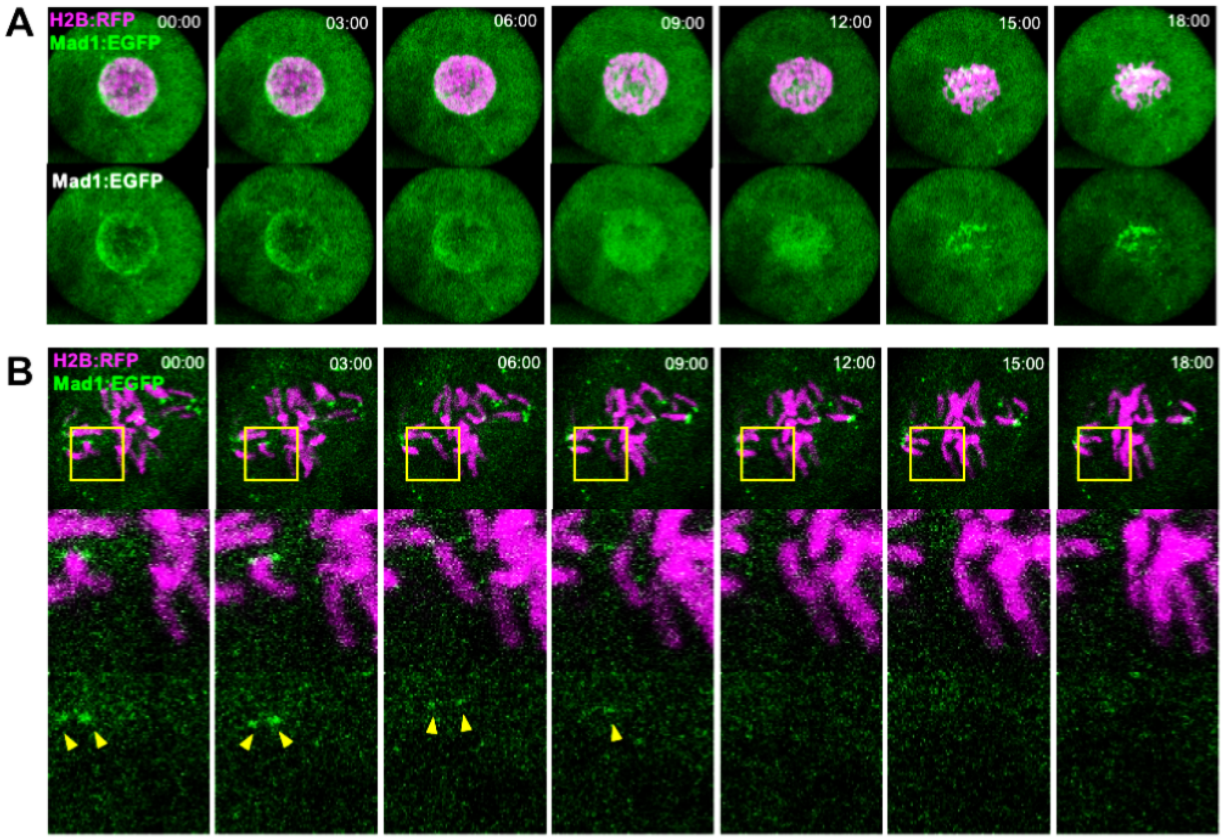

Figure S4.4. Mad1:EGFP is dynamically recruited to chromosomes

Consecutive time-points of confocal time-lapse imaging of the four- to eight-cell division in H2B:RFP- Mad1:EGFP expressing embryos during ***A:*** *nuclear envelope breakdown and* ***B:*** *chromosome alignment. White arrows indicate aligning chromosome, yellow arrows indicate kinetochore Mad1:EGFP signal.*

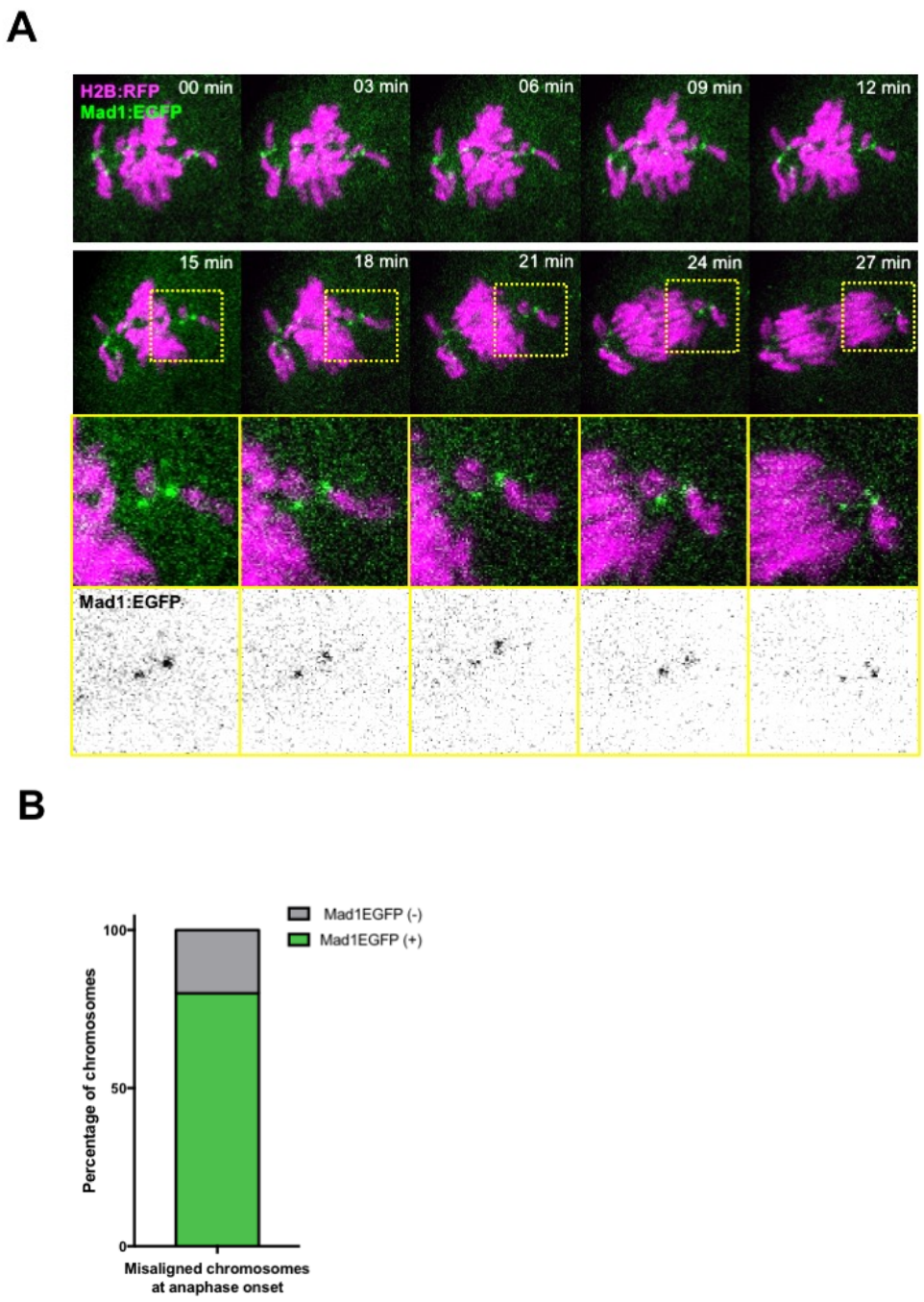

Figure 4.5. Misaligned chromosomes harbour active SAC signalling at anaphase entry
A: *Representative confocal images of a metaphase with misaligned chromosomes undergoing anaphase in the four- to eight-cell mitosis in H2B:RFP-Mad1:EGFP- expressing embryos. Yellow squares delineated amplified panels.* ***B:*** *Quantification of the proportion of misaligned chromosomes displaying detectable Mad1:EGFP (+) signal at anaphase onset (n=59 misaligned chromosomes from 17 divisions).*

4.4.3 Developmental stage-specific SAC strength is unaffected by cell size

Recent work in mouse oocytes and C. elegans embryos has revealed that SAC is inefficient at preventing APC/C activation in large cells (Galli and Morgan, 2016; Kyogoku and Kitajima, 2017). Preimplantation mitotic divisions are unaccompanied by cell growth, causing cells to decrease in size ~40-fold from ~200pL 1-cell stage, similar to the oocyte, to ~5pL at blastocyst stage (Courtois et al., 2012; Tsichlaki and FitzHarris, 2016). We thus hypothesised that failure of SAC to arrest mitosis may relate to cell size, especially in the first few mitoses, and SAC efficiency might strengthen as cells decrease in size during development. We tested this hypothesis with two series of experiments. First we examined SAC strength throughout preimplantation development. We performed live imaging of H2B:RFP-expressing embryos (**Fig. 4.6A**, p.102) in the presence of 10nM nocodazole to induce a mild spindle challenge and thus stimulate SAC activity as a measure of SAC effectiveness (Galli and Morgan, 2016; Lane and Jones, 2017). Strikingly, nocodazole prolonged mitosis at the two-cell and blastocyst stages but not at morula (**Fig. 4.6B**). Accordingly, and consistent with a failure of the SAC, anaphase lagging was most common in nocodazole-treated morulae (19% of divisions; **Fig. 4.6C and D**). Thus, SAC efficiency varies during development, but is at its weakest in morula, where cells are smaller than two-cell but larger than blastocyst stage. Secondly, we used micromanipulation techniques to experimentally alter cell size (**Fig. 4.6E**). Up to 40% of cytoplasm was removed from interphase two-cell embryos using a cytoplasm-aspiration pipette. Mitosis was then examined in the presence and absence of a nocodazole challenge at the 2-4 cell transition and in morulae. Importantly, embryos co-expressed H2B:RFP and GAP43:GFP, allowing the dynamics of chromosome segregation and cell volume to be simultaneously analysed on a cell-by-cell basis, and data was analysed by correlation and linear regression analysis. As previously, nocodazole extended mitosis at the two-cell stage but not in morula (**Fig. 4.6I and J**). However, there was no association between cell size and SAC strength at either developmental stage, regardless of the presence or absence of nocodazole. Similar results were obtained in a separate series of experiments in which only a single blastomere was manipulated (**Fig. S4.5**, p.103). Together these results demonstrate that the inability of SAC signalling to arrest the cell cycle in preimplantation embryos is not attributable to cell size.

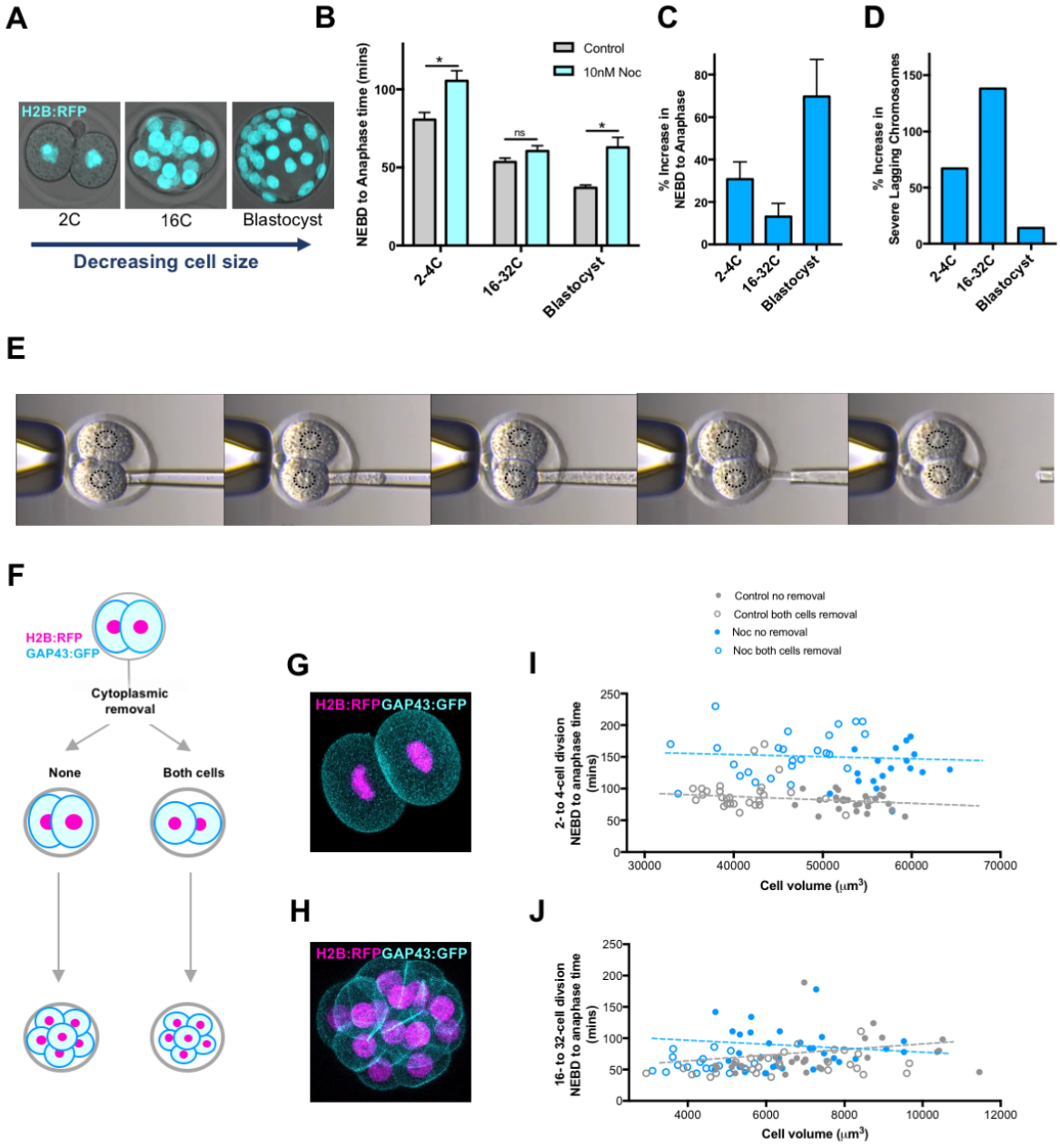

Figure 4.6. SAC strength is unaffected by cell size during early mammalian development

A: *Representative images of H2BRFP-expressing embryos at different developmental stages, two-cell (2C), morula (16C) and blastocyst.* ***B:*** *Analysis and quantification of mitosis duration at the different developmental stages examined in Control and 10nM of Nocodazole (mean and SEM, n= >24 divisions per group, t-test p<0.05).* ***C:*** *Representation of the percentage change in mitosis duration in 10nM Nocodazole vs. control at different developmental stages.* ***D:*** *Percentage increase in the rate of severe lagging chromosomes in 10nM Nocodazole vs. control at different developmental stages.* ***E:*** *Representative images of the cytoplasmic*

aspiration and removal technique in two-cell stage embryos, dashed circles indicate nuclei. ***F:*** *Diagrammatic representation of experimental design.* ***G and H:*** *Representative images of H2B:RFP- and GAP43:GFP-expressing embryos at the two-cell (2C) and morula (16C) stages, respectively.* ***I and J:*** *Scatterplot of mitosis duration and cell volume measured at metaphase for individual cells of embryos subjected to no manipulation or cytoplasmic removal in one or both cells in Control or 10nM Nocodazole conditions at the 2-4C and 16-32C divisions (correlation analysis, r= -0.11 and -0.17 at the 2C-stage, r= 0.33 and -0.24 at the 16C-stage, in Control and 10nM Nocodazole, respectively).*

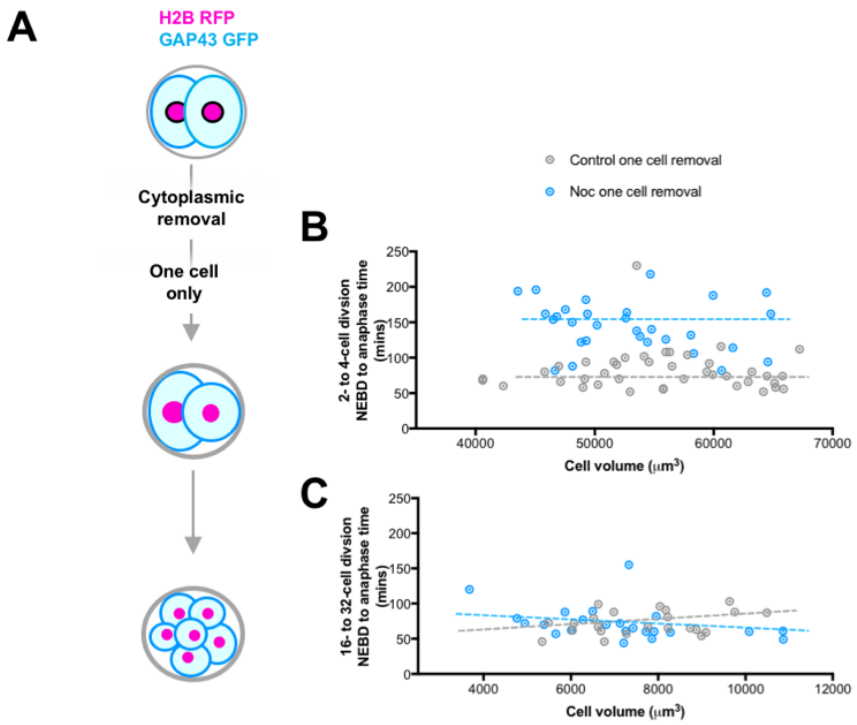

Figure S4.5. SAC strength is unaltered by experimental cell size reduction

A: *Diagrammatic representation of the experimental design. Cytoplamic removal was performed in one cell of two-cell embryos expressing H2B:RFP and GAP43:GFP.* ***B and C:*** *Analysis of cell volume measured at metaphase, and mitosis duration in control or 10nM Nocodazole conditions, at the two-cell and morula- stages, respectively (n= >24 divisions per group).*

4.4.4 Partial APC/C inhibition can reduce chromosome segregation errors

The SAC's failure to prevent anaphase in the presence of misaligned chromosomes suggests SAC signalling from small numbers of kinetochores is insufficient to effectively inhibit the APC/C. We thus reasoned that experimentally curtailing APC/C activity could improve chromosome segregation fidelity in the embryo. To test this, we employed proTAME, a cell-permeable specific inhibitor of the APC/C that directly binds APC/C and prevents its interaction with cofactors. In HeLa cells, micromolar concentrations of proTAME cause a mitotic arrest as a result of robust APC/C inhibition, whereas nanomolar concentrations can extend mitosis but allow mitosis to be completed (Zeng et al., 2010). We wondered whether low concentrations of proTAME would extend mitosis in embryos, and in doing so may allow more time for correct chromosome alignment to be achieved. Therefore, embryos were cultured in various concentrations of proTAME from the 2-cell stage onwards. 1-10μM concentrations of proTAME caused a prolonged mitotic arrest analogous to somatic cells (Zeng et al., 2010), and therefore prevented preimplantation development. In contrast, 0.01nM-100nM proTAME were permissive of development to blastocyst (**Fig. S4.6**, p.106) and did not affect blastocyst cell number (**Fig. 4.7A and B**, p.105). Strikingly, embryos cultured from the 2-cell stage to blastocyst in 1nM and 10nM proTAME exhibited a substantial reduction in micronuclei from 0.022 micronuclei per cell to 0.012 and 0.013, respectively ($P<0.05$) (**Fig. 4.7C**). Live imaging of control and proTAME-treated embryos at morula stage revealed that 10nM proTAME increased the duration of mitosis from 52.1±1.4 min to 57.9±1.4 min ($P<0.01$), and accordingly reduced the incidence of misaligned chromosomes at anaphase onset from 28% to 16% (**Fig. 4.7E and F**). A similar reduction in micronuclei was observed in experiments employing low concentrations of APCin (10μM), a second APC/C inhibitor which acts by binding the mitotic APC/C cofactor cdc20 (**Fig. S4.7**, p.106)(Sackton et al., 2014). Hence, mild pharmacological APC/C inhibition extends mitosis, allowing time for more chromosomes to align before anaphase, and reduces segregation errors in embryos.

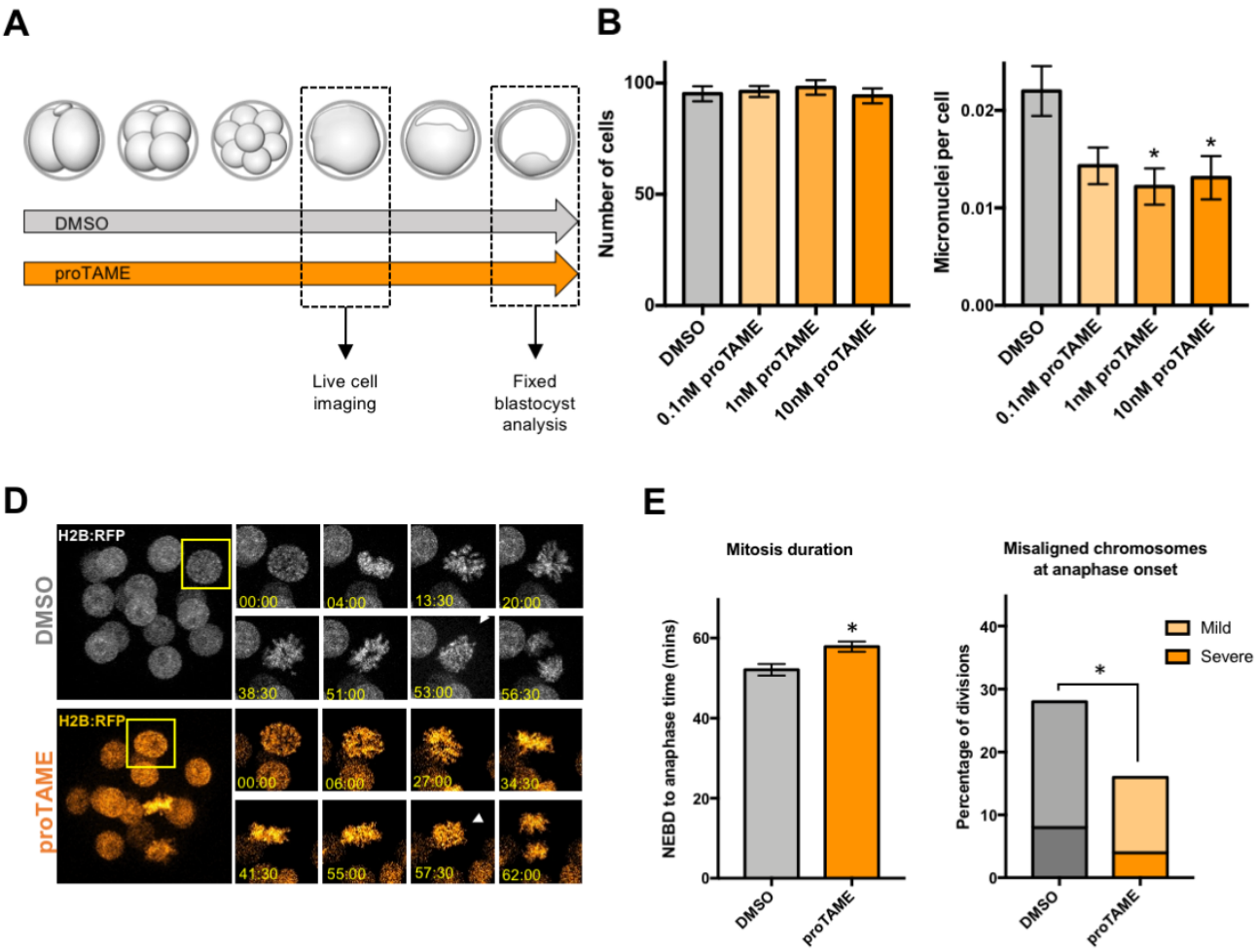

Figure 4.7. Mild APC/C inhibition enhances SAC function and reduces chromosome segregation errors

Diagrammatic representation of the experimental design. Two-cell embryos were cultured to blastocyst in DMSO or different concentrations of proTAME, for immunofluorescence analysis, or previously microinjected with H2B:RFP for live cell imaging at morula stage. ***B:*** *Number of cells in blastocysts treated with proTAME (mean and SEM, n=>26 embryos/group).* ***C:*** *Number of micronuclei per cell in blastocyst exposed to different proTAME concentrations (mean ± SEM, n=>26 embryos/group, one-way ANOVA, P=0.006, Tukey's multiple comparisons test, asterisks denote P<0.01).* ***D:*** *Representative confocal images of H2B:RFP-expressing embryos in DMSO and 10nM proTAME. Yellow squares delineate cells zoomed in pannels, white arrowheads indicate anaphase onset.* ***E:*** *Quantification of mitosis duration in DMSO and 10nM proTAME (mean ± SEM, n=>150 divisions/group, t-test, P<0.05).* ***F:*** *Analysis of incidence of mild and severely misaligned chromosomes at anaphase onset in DMSO and 10nM proTAME (n= >150 divisions/group, Fischers exact test, p<0.05).*

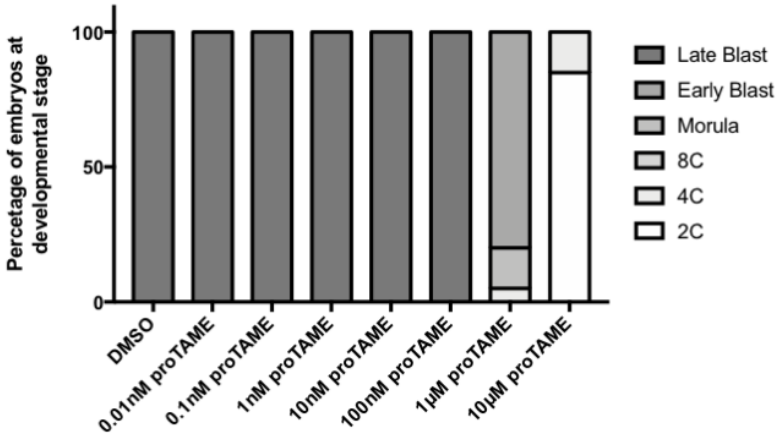

Figure S4.6. Effect of proTAME on blastocyst development

Scoring of developmental stages of embryos at E4.5 following culture in various concentrations of proTAME from the two-cell stage (n=>17 embryos per group).

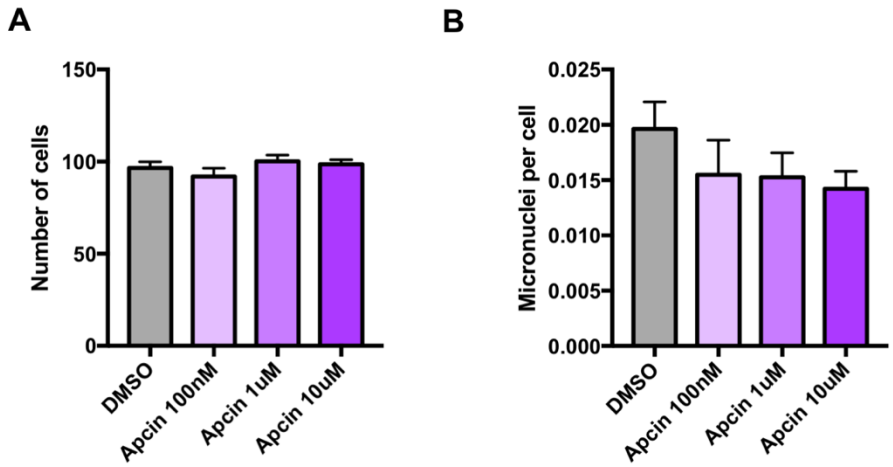

Figure S4.7. APCin exposure during preimplantation development reduces the occurrence of micronuclei

A *and* ***B:*** *Quantification of the number of cells and micronuclei per cells in blastocyst cultured in the presence of different concentrations of APC/C inhibitor APCin (mean and SEM, n=>19 embryos per group).*

4.5 Discussion

Deficient SAC function has been repeatedly speculated as an explanation for the near-ubiquitous reports of mosaic aneuploidy in the early mammalian embryo (Echten-Arends et al., 2011; Taylor et al., 2014), but mechanistic experiments to probe the effectiveness of the SAC have been lacking. We demonstrate that knockdown of components of the canonical SAC pathway (Mad2 and Mps1) does not impair preimplantation development to blastocyst (**Fig. 4.2**, p.94), consistent with findings in Mad2 and BubR1 knockout mice (Dobles et al., 2000; Wang et al., 2004), and that these function to extend mitosis, such that their depletion shortens mitosis and exacerbates error. We find SAC function is compromised at mid-development, failing to extend the duration of mitosis in response to a mild spindle challenge (**Fig. 4.6C**, p.102). Interestingly, similar findings have been reported in human embryos, where a significant Nocodazole-induced mitotic arrest is not observed only at Day 4 of development (Jacobs et al., 2017), alluding to SAC failure as an intrinsic defect to mammalian mid-preimplantation development.

Current perspectives suggest that SAC function is not a binary switch but rather a graded ability of SAC signalling at kinetochores to inhibit APC/C activity (Collin et al., 2013; Galli and Morgan, 2016). In line with this, we report that in embryos, complete spindle disruption effectively mounts a SAC-mediated metaphase arrest, but that the early embryo is unable to prolong metaphase in the face of moderate numbers of misaligned chromosomes. This scenario is similar to the mammalian oocyte, where recent work suggests that extreme cell size hampers a diffusible kinetochore SAC signal from inhibiting the APC/C (Kyogoku and Kitajima, 2017). Our data show that Mad1:GFP is frequently evident on kinetochores at the point of anaphase onset (**Fig. 4.5**, p.100), which would be consistent with such a notion in embryos. Importantly however, the SAC-APC/C axis could not be strengthened by a substantial cell size reduction, suggesting a different mechanism of SAC-APC/C disconnect in the embryo.

Non-matching SAC and APC/C activities could explain SAC failure in embryos as a decompensated stoichiometry of SAC-APC/C signalling components could arise as maternal and embryonic transcripts and proteins are differentially synthesised and destroyed during the first few mitoses after fertilisation. Indeed, transcript levels of SAC component Mad2 are low in human embryos (Wells et al., 2005). Further support for a SAC-APC/C mismatch comes from our observation that low doses of the APC/C inhibitors proTAME and APCin can extend embryo mitosis and reduce segregation errors (**Fig. 4.7, S4.7**, p.105,106). Our data, together with

findings that partial APC/C inhibition may also extend mitosis to rescue segregation errors and cell viability in somatic and cancer cells with compromised SAC function (Sansregret et al., 2017, Thu et al., 2018, Wild et al., 2016), lead to an emerging model of mismatched SAC and APC/C activities as a cause of SAC failure (**Fig. S4.8**, p.108). To our knowledge these studies provide the first example of a specific chemical intervention improving ploidy outcomes in cultured embryos. Whilst the data allude that modulation of the cell cycle may in the future present a viable approach for improving embryo quality in some specific scenarios, manipulation of early embryos can have undesired effects, and such an approach should not be attempted clinically prior to exhaustive testing of safety and efficacy.

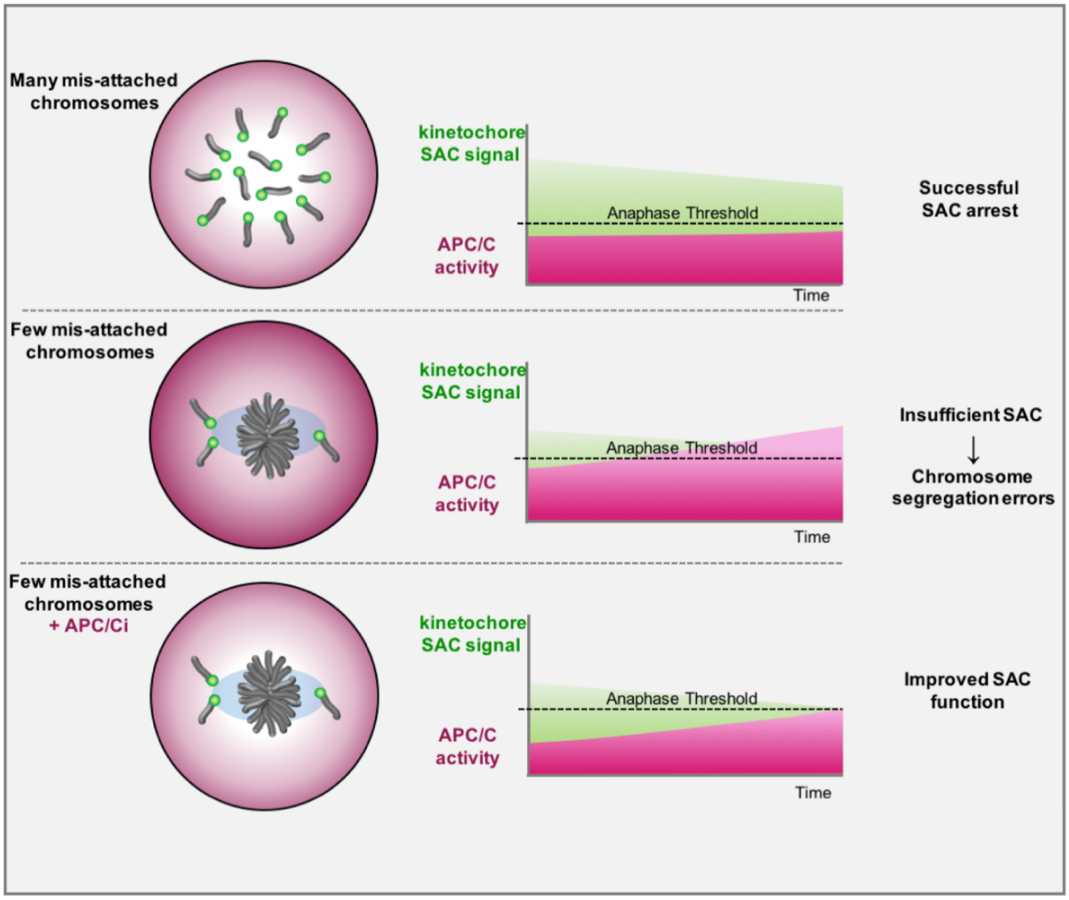

Figure S4.8. Model for mismatched SAC and APC/C activities in the mammalian embryo
Top: SAC signalling from a large proportion of chromosomes is sufficient to effectively inhibit APC/C activity and prevent anaphase. Middle: SAC signalling from a reduced fraction of chromosomes is insufficient to inhibit APC/C activity. Bottom: When the APC/C is subject to mild chemical inhibition, SAC signalling from a limited number of chromosomes can inhibit APC/C to prevent anaphase onset and reduce chromosome segregation errors.

4.6 Author contributions

G.F. and C.V.-D. designed the study, C.V.-D. performed all experiments, image and data analysis, produced all figures. C. V.-D. and G.F. wrote the manuscript.

4.7 Acknowledgements

We thank Michael Lampson for the pIVT-Mad1:2xEGFP plasmid, Yojiro Yamanaka for the pCS2-GAP43:GFP plasmid. This work was supported by research grants to G.F. from La Fondation Jean-Louis Lévesque, Canadian Foundation for Innovation (CFI) and Canadian Institutes of Health Research (CIHR).

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4.9 Methods Details

All reagents and materials are listed in **Table 4.9.**

4.9.1 Embryo collection, culture and chemical treatments

All experiments were approved by the Centre de Recherche du Centre Hospitalier de l'Université de Montréal (CRCHUM) Comité Institutionnel de Protection des Animaux (CIPA). Two cell embryos were obtained from superovulated 2-3 month old CD-1 female mice mated with CD-1 males. Embryos were collected in M2 media and cultured in KSOM media drops covered in mineral oil at 37ºC in 5%CO_2. For chemical treatments, media was supplemented with the following compounds: AZ3146 (20μM), GSK923295 (various concentrations), Nocodazole (various concentrations), Cytochalasin B (5μg/ml), MG132 (25μM), proTAME (various concentrations). All drug stocks are dissolved in DMSO. For experiments involving proTAME and APCin, embryos were cultured without oil in 500μL of media in 4-well plates.

4.9.2 Cytoplasmic removal and microinjection

All micromanipulations were performed on a Leica DMI4000 inverted microscope fitted with Narashige micromanipulators. In-house glass pipettes with paraffin liquid hydraulic control mounted on a piezo-electric drill were employed to perforate the *zona pellucida* and subsequently aspirate varying amounts of cytoplasm from interphase two-cell embryos in M2 media containing 10μM Nocodazole and 5μg/mL Cytochalasin B. Embryos where thoroughly washed in over a dozen drops of M2 media prior to transfer and incubation in equilibrated KSOM medium for at least 2 hours prior to imaging, allowing them to recover from the cytoplasmic removal procedure. Microinjections were performed as described previously (Nakagawa and FitzHarris, 2016; Vázquez-Diez and FitzHarris, 2018; Vázquez-Diez et al., 2016). cRNA was synthesized using mMessenger Machine kit (Ambion) as previously (Nakagawa and FitzHarris, 2017; Vázquez-Diez and FitzHarris, 2018), from the following plasmids; pRN4-H2B:RFP, psC2-GAP43:GFP, MajSatTALE:mClover (pTALYM3B15) (Miyanari et al., 2013).

4.9.3 Live cell imaging and immunofluorescence

All imaging was performed on a Leica SP8 confocal microscope with HyD detectors. For live imaging embryos were placed in a heated top-stage incubator with CO_2 supply and imaged with a 20x 0,75NA objective as previously (Vázquez-Diez et al., 2016). All imaging was

performed in 2µL drops under mineral oil, for experiments involving proTAME, embryos were cultured in Ibidi micro-insert wells mounted on a glass-bottom dish with distilled water and our setup was modified to provide humidified CO_2 gas supply. Z-stack images of embryos (~50µm) with step size 2µm were obtained at a time interval of 30 seconds for experiments concerning H2BRFP only, 2 min for H2B:RFP-GAP43:GFP, and 3 min H2B:RFP-Mad1:EGFP experiments. For immunofluorescence embryos were fixed in 2% PFA in PBS (20min) and permeabilised in 0.25% Triton-X100 in PBS (10min), for Mad2 immunofluorescence embryos were fixed for 15 min in PHEM buffer containing 2% PFA and 0.05% Triton X-100, and permeabilised in 0.05% Triton-X in PHEM buffer for 15min. Blocking was performed in 3% bovine serum albumin in PBS, for 1 hr at 37ºC or overnight at 4ºC. Primary antibodies used are rabbit anti-Mad2 (1:300), mouse anti-alpha-Tubulin (1:1000), anti-rabbit and anti-mouse AlexaFluor secondaries were used at 1:1000 dilution. Alexa-555-Phalloidin (1:300) and Hoechst (1:1000) were used to visualize F-actin and DNA, respectively.

4.9.4 Image analysis and statistics

All image analysis was performed using Fiji software. Time-lapse Z-stacks were examined to determine mitosis duration and identify chromosome alignment and segregation errors. Spindle length measurements were performed measuring the distance between spindle poles, using Pythagoras' theorem where spindle poles were located on different Z-slices. Mad2 Immunofluorescence was quantified by subtracting nuclear envelope and background maximum grey values. To calculate cell volume at metaphase, areas delineated by the cell membrane (GAP43:GFP signal) were manually traced and measured at each Z-slice, the sum of the areas was multiplied by the step size (2µm) to approximate cell volume for each cell. All data analysis was performed using GraphPad Prism, statistical test used are accordingly noted in figures, α=0.05.

Table 4.9 Key reagents and resources

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti-Mad2	Biolegend	924601
Mouse anti-beta-Tubulin	Sigma	T4026
Alexa Fluor 488 anti-Rabbit	ThermoFisher	A-11008
Alexa Fluor 488 anti-Mouse	ThermoFisher	A-11029
Alexa Fluor 568 anti-Mouse	ThermoFisher	A-11031
Alexa Fluor 555 Phalloidin	ThermoFisher	A-34005
Hoechst	ThermoFisher	H-2399
Bacterial and Virus Strains		
Not applicable		
Biological Samples		
Not applicable		
Chemicals, Peptides, and Recombinant Proteins		
Pregnant Mare's Serum Gonadotrophin	Genway biotech	GWB-2AE30A
Human Chorionic Gonadotrophin	Sigma	CG10
M2 Media	Sigma	M7167
KSOM Embryo Culture Media	Millipore Sigma	MR-020P-5F
AZ3146	Calbiochem	531976
Nocodazole	Calbiochem	487928
GSK923295	Cayman Chemical	18389
Cytochalasin B	Sigma	C6762
MG132	Calbiochem	474790
proTAME	R&D Systems Inc.	I-440-01M
Apcin	Tocris	5747
Critical Commercial Assays		
mMessage Machine Kit	Ambion	SP6 AM1336, T3 AM1348, T7 AM1344
Experimental Models: Organisms/Strains		
Mouse: CD-1 IGS	Charles River Laboratories	-
Oligonucleotides		
Control Morpholino Oligonucleotide	Gene tools LLC	5'-CCTCTTACCTCAGTTACAATTTATA -3'
Mad2 Morpholino Oligonucleotide	Gene tools LLC	5'-GCTCTCGGGCGAGCTGCTGTGCCAT-3'
Recombinant DNA		
pRNA-H2B:RFP	Gift from Alex McDougall	Laboratoire de Biologie du Développement de Villefranche-sur-mer (LBDV), FRANCE

GAP43:GFP	Gift from Yojiro Yamanaka	McGill University, CANADA
MajSatTALEmClover	Addgene	47878
pIVT-Mad1:2xEGFP	Gift from Michael Lampson	University of Pennsylvania, USA
Software and Algorithms		
Fiji	Schindelin, J.; Arganda-Carreras, I. & Frise, E. et al. (2012), "Fiji: an open-source platform for biological-image analysis", Nature methods 9(7): 676-682, PMID 22743772, doi:10.1038/nmeth.2019	
Excel	Microsoft Office professional Plus Excel 2010	
GraphPad Prism	GraphPad Prism version 7.00 for MAC/Windows, GraphPad Software, La Jolla California USA, www.graphpad.com	
Other		
4-well micro-insert	Ibidi	80489

4.9.5 Supplementary References

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Nakagawa, S., and FitzHarris, G. (2017). Intrinsically Defective Microtubule Dynamics Contribute to Age-Related Chromosome Segregation Errors in Mouse Oocyte Meiosis-I. Curr. Biol. CB *27*, 1040–1047.

Vázquez-Diez, C., and FitzHarris, G. (2018). Correlative Live Imaging and Immunofluorescence for Analysis of Chromosome Segregation in Mouse Preimplantation Embryos. Methods Mol. Biol. Clifton NJ *1769*, 319–335.

Vázquez-Diez, C., Yamagata, K., Trivedi, S., Haverfield, J., and FitzHarris, G. (2016). Micronucleus formation causes perpetual unilateral chromosome inheritance in mouse embryos. Proc. Natl. Acad. Sci. 201517628.

CHAPTER 5

DISCUSSION AND CONCLUSIONS

5.1 Discussion

Accurate chromosome segregation during cell division is essential to maintain genetic fidelity in single-celled and multicellular organisms. Errors in chromosome segregation usually result in aneuploidy, where daughter cells possess abnormal chromosome complements, which has been associated with cancer cell transformation, metastasis and congenital abnormalities (Crawford and Steiner, 2015; Funk et al., 2016). Stringent control of mitotic fidelity should be especially critical in a cellular system such as the newly fertilized mammalian preimplantation embryo, where a single cell, the zygote, undergoes multiple cleavage divisions to produce a blastocyst, composed of ~100 cells, from which all fetal and extraembryonic tissues are derived (Niakan et al., 2012). Nonetheless, counterintuitively, the early mammalian embryo frequently mis-segregates chromosomes and often contains a subset of aneuploid cells as well as chromosomal abnormalities such as micronuclei. Mosaic aneuploidy was first reported almost 25 years ago in human assisted reproduction technology (ART) cycles, and has ever since become a critical factor determining the selection of embryos in fertility treatment (Delhanty et al., 1993; Munné et al., 1993; Vanneste et al., 2009). However, only recently have clinical and animal research studies started to unravel the causes and impact of mitotic errors and mosaic aneuploidy in the early embryo.

5.1.1 Chromosomal instability in the early mammalian embryo

Chromosomal instability (CIN) is the tendency for cells to mis-segregate chromosomes during cell division, regardless of whether they were initially euploid or aneuploid. CIN is a hallmark of tumorigenesis, is typically evidenced by single chromosome gains and losses and has been the focus of extensive research in cancer cells (Funk et al., 2016; Thompson and Compton, 2008; Thompson et al., 2010). In human cells, the major cause of CIN is a specific type of kinetochore-microtubule attachment error, termed merotelic attachment, where a single sister kinetochore is bound to microtubules emanating from opposite spindle poles. If uncorrected prior to anaphase onset, merotelic attachments exert opposing forces on kinetochores causing lagging chromosomes during anaphase (**Fig. 5.1A**, p.124) (Cimini et al., 2001; Thompson and Compton, 2011; Thompson et al., 2010).

Interestingly, whole genome analyses in preimplantation genetic screening (PGS), where a few cells are removed from the embryo and analysed for chromosome copy numbers and abnormalities, have revealed that mosaic aneuploidy in human embryos predominantly occurs as single chromosome losses and gains (Vanneste et al., 2009). However, given the short duration of anaphase in the cell cycle, whether lagging chromosomes occurred in the early mammalian embryo had remained elusive until recently. Live cell imaging studies in mouse embryos have demonstrated abnormal chromosome segregation, including lagging chromosomes, occurs in embryos generated by intracytoplasmic sperm injection (ICSI) and embryos cloned by somatic cell nuclear transfer (SCNT) (Mizutani et al., 2012). Importantly, our work has shown that the most common chromosome segregation defect in *in vivo*-fertilized embryos cultured *in vitro* are lagging anaphase chromosomes, often resulting in the formation of micronuclei, which we have also found to occur at comparable rates in embryos developed entirely *in vivo* (Vázquez-Diez et al., 2016) (*see* Chapter 2, **Fig. 2.1**, p.44). In addition, although similar live cell imaging and analysis of chromosome segregation dynamics in human embryos is yet to be reported, immunofluorescence studies have revealed the occurrence of whole chromosomes lagging in anaphase and micronuclei in cryopreserved good quality human embryos (Kort et al., 2016). Findings that, as in mouse embryos, human embryo micronuclei bear high levels of DNA damage and frequently lack centromeric identity marker CENP-A, strongly allude to similar events taking place in human embryos, pointing towards a common mode of chromosome mis-segregation causing mosaic aneuploidy resulting in a far more prevalent incidence of single chromosome copy number losses than gains (**Fig. 5.1B**, p.124) (*see* Chapter 2, **Fig. 2.1**) (Kort et al., 2016; Taylor et al., 2014; Vázquez-Diez and FitzHarris, 2018; Vázquez-Diez et al., 2016). Further studies combining live cell imaging, cell tracking and single-cell whole genome analysis approaches will be required to empirically prove whether lagging chromosomes and micronucleus formation catalyse chromosome copy number losses in the early mammalian embryo.

5.1.2 Mechanisms and impact of micronucleus formation in embryos

Micronuclei are small nuclear like structures containing chromosomal DNA that are a hallmark of cancer and a *bona fide* indicator of CIN (Balmus et al., 2015; Fenech, 2007). Micronuclei have been reported to occur frequently in human preimplantation embryos in ART cycles (Kort et al., 2016; Meriano et al., 2004). Since micronuclei can arise through a variety of different

mechanisms in mammalian cells (Fenech et al., 2011; Storchová and Kloosterman, 2016), it was not well understood what events lead to micronucleus formation in the early mammalian embryo. Our findings have established lagging anaphase chromosomes, where chromatids are delayed during anaphase and become excluded from the main nucleus when the nuclear envelope reforms, as the major cause of micronuclei in mouse preimplantation embryos. Importantly, we have shown lagging chromosomes occur at a much higher frequency (~10%) in embryos than in stable diploid cultured cells (~1%), and approaches rates observed in CIN-positive cancer cells (Rao et al., 2008; Salmon et al., 2005). Why mammalian embryos mis-segregate chromosomes is not fully understood. Although merotelic attachments, which give rise to lagging chromosomes, are believed to evade detection by the SAC (Cimini et al., 2001), impaired SAC function results in increased rates of lagging chromosomes in embryos and somatic cells (*see* Chapter 4, **Fig. 4.2**, p.95) (Michel et al., 2001) and thus poses a considerable cause of CIN embryos that will be discussed in more detail below (*see* Section **5.1.4**).

Additional factors that may contribute to increased CIN include reduced error correction by the chromosomal passenger complex (CPC) and the non-canonical mode of spindle assembly in embryos, which are discussed in detail in Chapter 1, but whether other factors than spindle-based mechanisms of chromosome segregation may contribute to an increased propensity for micronucleus formation in embryos is not known. A recent genetic screen for spindle-independent factors that promote formation of a single nucleus in somatic cells identified barrier-to-autointegration factor (BAF) as a key protein preventing micro- and multi-nucleation (Samwer et al., 2017). During late anaphase, BAF crosslinks DNA of adjacent chromosomes stiffening their outer surface to limit nuclear envelope formation at inter-chromosomal regions. During early mitosis phosphorylation by VRK1 kinase prevents BAF's localisation to chromosomes while PP2A-mediated dephosphorylation during anaphase promotes it. Similarly, H3.3 is phosphorylated in mitotic chromosomes arms by Aurora B kinase in the CPC at metaphase and removed during anaphase by PP1 and PP2A phosphatases (Krenn and Musacchio, 2015; Li et al., 2017a). However, BAF function remains to be explored in the context of mis-segregated lagging anaphase chromosomes, which are more exposed to the kinase activity of mid-zone localized Aurora B and show increased H3.3 phosphorylation (Hinchcliffe et al., 2016). Whether this could potentially antagonise PP2A activity and prevent BAF localization to lagging chromosomes is yet to be determined. Thus, in addition to the spatial separation caused by anaphase lagging itself, the difference in chromosomes' mechanical

properties caused by a hypothetically reduced BAF localisation to lagging chromosomes could potentially constitute a further impediment for these to coalesce with the main chromosome mass during late anaphase, promoting their exclusion from the main nucleus.

Another hypothesis is that given the altered cell cycle dynamics in embryos, with proportionally reduced interphase durations, several processes that are essential for chromosome segregation such as DNA replication, epigenetic centromere specification and inner kinetochore assembly may be regulated differently in embryos. However, whether the rapid divisions in the mammalian preimplantation embryo, with shortened G1 and G2 phases unaccompanied by cell growth (Abdelalim, 2013), compromise the fidelity or efficiency of these processes is elusive. Evidence in somatic cells shows that replication stress can increase the incidence of lagging anaphase chromosomes (Utani et al., 2010), although it is not clear if these are whole chromatids or fragments caused by replicative stress-induced DNA double strand breaks (DSB). Notably, in G1 and G2, HJURP, a chaperone for the centromeric histone CENP-A, protects it from degradation and promotes its incorporation at centromeres where kinetochore proteins CENP-B, CENP-C, CENP-N, CENP-T and CENP-W are pre-assembled during G2 (Müller and Almouzni, 2017). Depletion of HJURP in cancer cells has been shown to increase rates of micronuclei (Filipescu et al., 2017). Thus, shortened growth phases in the preimplantation cell cycle and reduced loading of kinetochore proteins could perhaps result in impaired kinetochore function and increased incidence of lagging chromosomes and micronuclei in the early embryo but remains to be explored.

We have found that mouse embryo micronuclei display high levels of DNA damage as revealed by DSB-marker γH2AX (*see* Chapter 2, **Fig. 2.1**, p.44) and others have similarly highly increased levels of γH2AX in human embryo micronuclei (Kort et al., 2016) (**Fig. 5.1**, p.124). Likewise, cancer cell micronuclei and induced micronuclei in chromosomally stable cell lines have also been reported to have extensive DNA damage (Crasta et al., 2012; Hatch et al., 2013; Ly et al., 2017). Mouse embryo micronuclei have reduced localization of nuclear envelope and nuclear import markers LaminB1 and LDS1, respectively. It was demonstrated in somatic cells that impaired nuclear envelope repair following rupture and the acquisition of DNA damage coincide with entry into S-phase (Crasta et al., 2012; Hatch et al., 2013). However, it is unclear how S-phase causes nuclear envelope disruption and whether DNA damage arises from aberrant DNA replication and/or impaired DNA damage repair in

micronucleus-enclosed chromosomes following nuclear rupture (Crasta et al., 2012; Hatch et al., 2013). Additionally, since compacted G1 nuclear chromatin expands and relaxes during S-phase (Dolby et al., 1979), it is tempting to speculate that this expansion coupled with impaired nuclear envelope repair capabilities in micronuclei could cause persistent nuclear envelope disruption in micronuclei.

In contrast to mouse embryos, where micronuclei remain separate from the main mass of chromosomes, lack kinetochore structures and are passed down randomly into the cytoplasm of one of the daughter cells during the next mitoses (*see* Chapter 2, **Fig.2.2** and **2.3**, p.47,50), in somatic cells, micronucleus chromosomes either reincorporate as a single unified mass into one of the daughter cell nuclei or become shattered into many fragments upon mitotic entry (Crasta et al., 2012; Ly et al., 2017). Because DNA damage repair is defective in micronuclei but not the main cell nucleus, it was hypothesised that reassembly of pulverized chromosomes occurred following reincorporation in daughter cell nuclei where DNA repair machinery is active. Indeed, this has been demonstrated by elegant studies by two different groups. First, experiments using an inducible system to selectively mis-segregate the Y-chromosome have shown that micronucleus-enclosed chromosomes shatter upon mitotic entry and are repaired in the next cell cycle by non-homologous end joining (NEHJ) but not homology-directed repair (HDR) (Ly et al., 2017). Second, correlative single-cell live imaging, DNA sequencing and copy number analysis of daughter cells following a micronucleus reincorporation event, revealed one of the daughter cells often displays copy number losses and extensive chromosomal rearrangements restricted to the lost chromosome (Zhang et al., 2015a). These studies have provided compelling evidence for a micronucleus-mediated mechanism causing chromothripsis. Since mouse embryo micronuclei neither fragment upon mitotic entry nor reincorporate into daughter cell nuclei, this unexpected series of events likely shelters the early embryonic genome from chromothripsis, albeit at the cost of persistent chromosome copy number losses.

Why mouse embryo micronuclei don't undergo shattering upon mitotic entry is puzzling. We find mouse embryo micronuclei have similar functional and structural properties to somatic cell micronuclei and also exhibit extensive DNA damage suggesting a similar scenario. The lack of mitotic chromosome pulverization may be due to intrinsic differences in chromatin structure and cell cycle progression during embryonic mitosis, such as a hypothetically lower rate and extent

of chromatin compaction into mitotic chromosomes. Additionally, increased adhesion between chromosomes, or chromosome fragments in micronuclei, could potentially prevent their dispersion in mitosis. It has been recently demonstrated that the widely-used proliferation marker Ki-67, encoded by the *MKI67* gene, coats chromosomes in mitosis to prevent excessive chromosome clustering, facilitating chromosome movement and interactions with the spindle apparatus (Cuylen et al., 2016). However, whether reduced Ki-67 in embryos or somatic cells increases chromatin association to reduce or avert micronucleus fragmentation in mitosis has not been explored but could provide an attractive explanation for the observed difference in embryos.

Another key difference between embryos and somatic cells is that micronuclei are recurrently inherited during the ensuing cell cycles. This cannot be solely explained by the apparent relaxation of p53-mediated checkpoints in embryos since the somatic cell experiments were necessarily performed in p53-null cells to prevent a G1 arrest (Crasta et al., 2012; Hatch et al., 2013; Hinchcliffe et al., 2016; Zhang et al., 2015a). We have demonstrated that in embryos, micronucleus-enclosed chromosomes fail to assemble functional kinetochores in subsequent mitoses presumably due to the loss of centromeric identity marker CENP-A (*see* Chapter 2). Interestingly, findings that somatic cell micronuclei have detectable, albeit seemingly weaker, ACA staining in G2 and un-pulverized micronuclei frequently reincorporate into daughter nuclei (Crasta et al., 2012) suggest centromeric identity is preserved in these cells despite comparable nuclear structure and function impairment and high levels of DNA damage. Such discrepancy could be explained by the reduced duration of G1 and G2 phases and a potentially reduced centromere maintenance function in mouse embryos, as mentioned above. Altered maintenance, epigenetic state or structure of centromeric loci in embryos could not only increase vulnerability to DSBs but also reduce repair. Hence, a speculative role for impaired centromere homeostasis in embryos could underlie a propensity for chromosome mis-segregation and repeated micronucleus inheritance.

Our findings that micronuclei do not rejoin the genome in embryos suggest chromothripsis does not originate during preimplantation development. However, chromothripsis has been reported to occur in the germline, as reciprocal translocations affecting multiple chromosomes (Kloosterman and Cuppen, 2013). Reports of increased levels of DNA damage in sperm with advanced paternal age which are believed to be repaired after fertilization in the ooplasm

(Belloc et al., 2014), and the strong paternal bias in SNP positions of heterozygous chromothripsis, including deletions, complex translocations and rearrangements (De Gregori et al., 2007), point towards a paternal origin of chromothripsis in the germline that is transmitted but not exacerbated during preimplantation mitotic divisions.

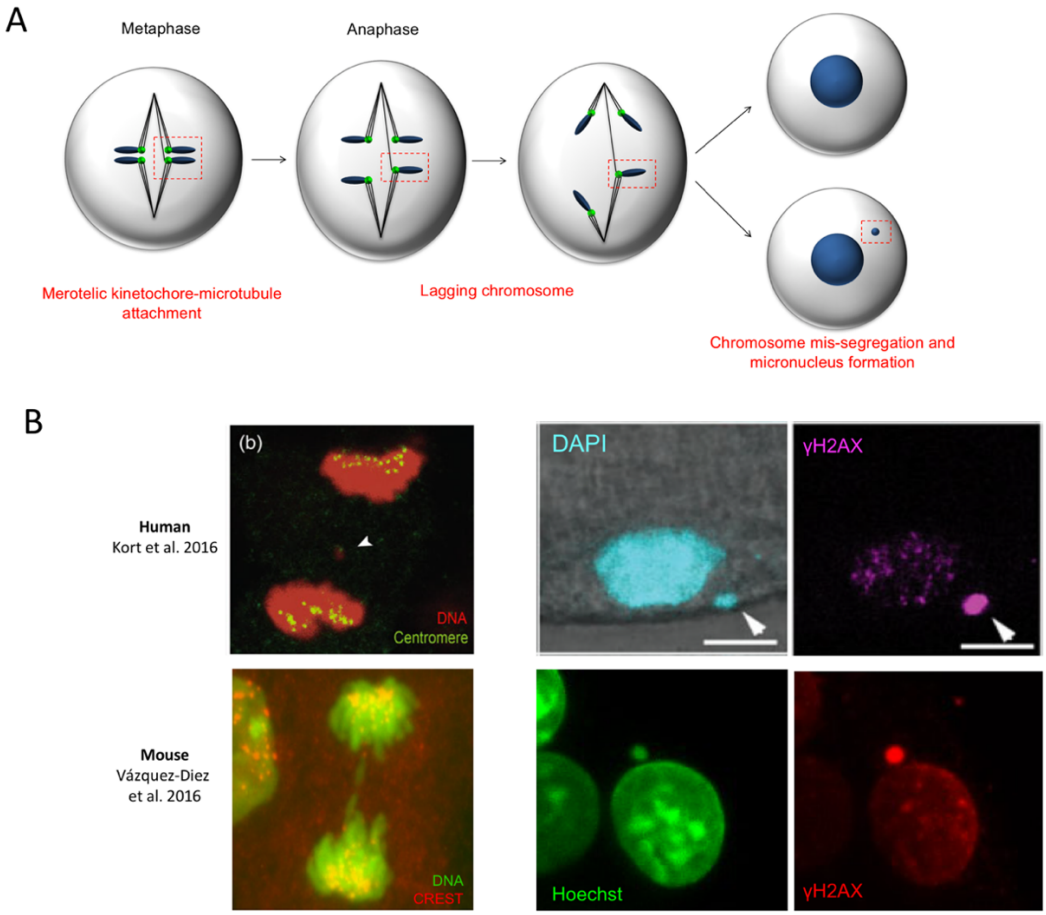

Figure 5.1. Lagging chromosomes and micronuclei in human and mouse embryos.

(A) Diagrammatic representation of merotelic attachments causing lagging chromosomes resulting in micronucleus formation. (B) Immunofluorescence images of lagging chromosomes (left) and micronuclei with high levels of DNA damage (right) in human and mouse embryos (above and below, respectively). Images adapted from Kort et al., 2016 and Vázquez-Diez et al., 2016.

5.1.3 Micronucleus inheritance as a catalyst of mosaic aneuploidy in embryos

Mosaic aneuploidy has been reported to occur in up to 70% of human embryos (Echten-Arends et al., 2011; McCoy, 2017; Taylor et al., 2014; Vanneste et al., 2009). However, what constitutes a mosaic embryo has been categorized inconsistently between studies, as sometimes this includes entirely yet heterogeneously aneuploid embryos. Diploid-aneuploid mosaics consist of a mixture of cells containing correct and abnormal chromosome complements, among which, the most common abnormality reported are single chromosome losses, followed by single chromosome gains and less frequently, more complex abnormalities (*see* Chapter 1) (Coonen et al., 2004; Munné and Wells, 2017; Taylor et al., 2014). Until recently, there was no clear mechanistic explanation for how these patterns of chromosomal abnormalities come about in embryos and why chromosome losses occur at a much higher incidence.

The mouse preimplantation embryo provides a confined system allowing to track micronuclei over multiple cell cycles revealing their persistent inheritance during early development (*see* Chapter 2, **Fig. 2.3**, p.50). While it cannot be formally excluded that this phenomenon may also take place in other cell types for which micronucleus tracking over several generations is less amenable, it is unlikely this would be the case in somatic cells since a G1 arrest is elicited in p53-functional cells upon micronucleus formation and they usually become reincorporated in the subsequent divisions when the G1 checkpoint is overridden (Crasta et al., 2012; Hinchcliffe et al., 2016; Ly et al., 2017). Although it is yet to me demonstrated in human embryos, it is likely that similar to mouse embryos, micronuclei are likely also inherited during human preimplantation development as micronuclei have been reported to occur frequently, usually bearing high levels DNA damage, and in some cases lacking centromeric identity (Kort et al., 2016; Meriano et al., 2004). The lack of a chromosome mis-segregation-induced G1 arrest in embryos enables these chromosome copy losses to be perpetuated during subsequent cell divisions resulting in the generation of additional aneuploid cells downstream of the initial segregation error and micronucleus formation. Furthermore, while chromosome encapsulation into micronuclei in embryos may not immediately result in numerical chromosome loss, it may also effectively cause a ‘functional’ chromosome loss since impaired import of nuclear substrates likely compromises transcriptional and replicative activities. In line with this, somatic cell micronuclei with similarly defective nuclear structure and function, display reduced

recruitment of replication factors such as MCM2 and RNApol-II and display impaired incorporation of thymidine analog EdU (Crasta et al., 2012; Hatch et al., 2013). Thus, the failure to translate and replicate chromosomes in micronuclei could explain the more prevalent incidence of single chromosome copy number losses in human embryos.

CIN and lagging chromosomes in embryos have been recurrently speculated to be the cause of the high incidence of mosaicism and single chromosome losses in embryos (Coonen et al., 2004; Vanneste et al., 2009). We have found that lagging anaphase chromosomes occur in ~10% of divisions in mouse embryos and typically result in the formation of micronuclei (*see* Chapter 2). However, it is hard to conceive how this mechanism alone could contribute to the high mosaicism rates reported in human embryos (20-80%). Crucially, the failure to replicate and segregate micronucleus-enclosed chromosomes, together with the lack of an aneuploidy-induced G1 arrest in embryos, causes repeated micronucleus inheritance resulting in a subset of daughter cells exhibiting chromosome copy number losses (discussed in detail in Chapter 1*,* **Fig. 1.5**, p.21). Thus, while mosaicism is initiated by lagging chromosomes and their subsequent entrapment into micronuclei, the dynamics of micronucleus inheritance, rather than lagging chromosomes *per se*, contribute to further increased levels of mosaic aneuploidy in the early mammalian embryo.

5.1.4 Impaired SAC function as a major cause of chromosome segregation errors in preimplantation development

Merotelic attachments are generally considered to evade detection by the SAC because they fail to significantly delay anaphase onset and the majority of CIN cell lines and solid tumours appear to have robust SAC function (Cimini, 2008; Thompson et al., 2010). However, premature anaphase onset in cells with impaired SAC function may limit error-correction and increase their propensity for segregation errors and CIN. Indeed, some tumours display reduced levels of SAC component Mad2 and mutations in Mad2 and Bub1 have been reported in breast and gastric cancer cell lines (Cahill et al., 1998; Li and Benezra, 1996; Percy et al., 2000). Moreover, deletion of one copy of Mad2 in a stable diploid cell line increases chromosome mis-segregation and heterozygous mutation of Mad2 in mice, causes increased rates of aneuploidy and spontaneous development of lung tumours (Michel et al., 2001). Additionally, mutation of BubR1 in humans is associated with recurrent pregnancy loss and when viable causes mosaic variegated aneuploidy syndrome, which is characterized by growth retardation, childhood cancers and death at a young age, with highly elevated aneuploidy rates in patient tissues and lymphocytes commonly exhibiting chromosome segregation defects (Schmid et al., 2014; Hanks et al., 2004; Matsuura et al., 2006). Moreover, BubR1 deletion in mice causes single chromosome copy gains and losses in the early embryo at E 8.5 (Schmid et al., 2014). Thus, loss of robust SAC function may exacerbate CIN by compromising mitotic fidelity.

By performing functional analyses of SAC strength at different stages of preimplantation development we have identified a particularly deficient SAC response to mild spindle disruption during the 16- to 32-cell division, at morula stage (*see* Chapter 4, **Fig. 4.6**, p.102). Interestingly, this impaired SAC strength coincides with a more pronounced emergence of micronuclei at the 32-cell stage (*see* Chapter 2, **Fig. 2.1, S2.1**, p.44,45). Together with findings that SAC loss-of-function by chemical inhibition or RNAi component knockdown in preimplantation mouse embryos increases the incidence of lagging chromosomes and micronuclei (*see* Chapter 4, **Fig. 4.2**, p.95) (Bolton et al., 2016; Wei et al., 2011), this data argues that at least in the mouse embryo, an inherently reduced SAC strength at morula could provide a permissive window for increased CIN, chromosome mis-segregation, micronuclei and generation of aneuploid cells during mid-development. A similar impairment to elicit a mitotic arrest in response to spindle poisons has been reported in Day 4 cleavage-stage human embryos, when the highest rates

of mosaic aneuploidy are reported (Echten-Arends et al., 2011; Jacobs et al., 2017; Taylor et al., 2014). However, whether this increase in mosaic aneuploidy in human embryos results directly from deficient SAC function and extremely error-prone divisions or is generated downstream of chromosome segregation errors via a micronucleus inheritance-mediated mechanism remains to be tested. Nevertheless, SAC insufficiency during mid-development provides plausible mechanistic explanation for the observed frequency of mosaic aneuploidy during cleavage stages in human embryos. However, restored SAC function at blastocyst stage on its own cannot fully account for the lower incidence of mosaic aneuploidy reported in human blastocysts (Echten-Arends et al., 2011; Taylor et al., 2014), as this would at best stably maintain the levels of mosaicism. Live imaging studies in mouse embryos have demonstrated that aneuploid cells in the inner cell mass (ICM) undergo apoptosis and an increased proportion of TUNEL-positive apoptotic cells is observed in human embryos following treatment with spindle poisons at late blastocyst (Day 6) (Bolton et al., 2016; Jacobs et al., 2017). Thus, in addition to an increased stringency of SAC function ensuring more accurate chromosome segregation, aneuploid cells may be removed post-mitotically by apoptosis to lower the overall occurrence of mosaic aneuploidy in the late mammalian preimplantation embryo (**Fig. 5.2**, p.128).

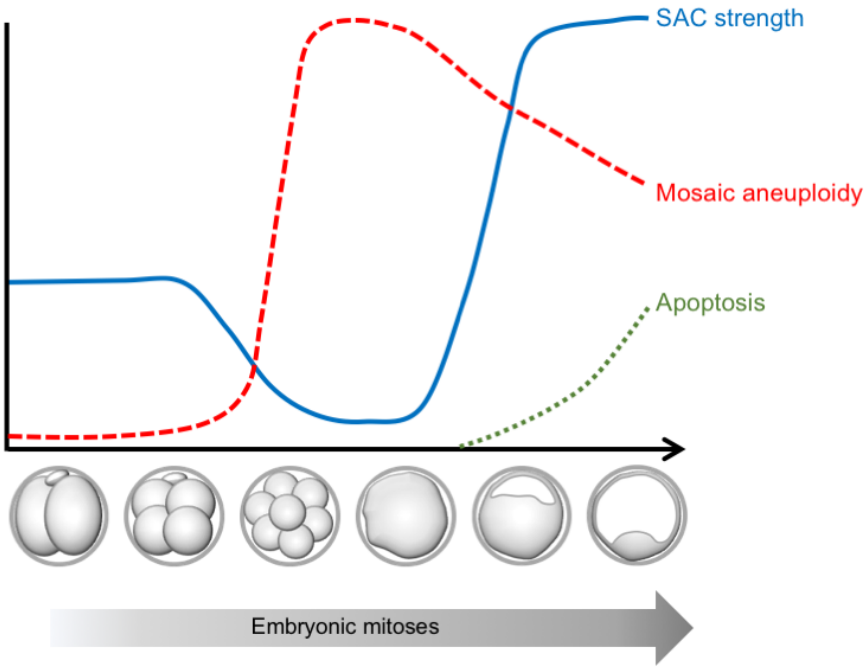

Figure 5.2. Diagrammatic representation of SAC strength, apoptosis and level of mosaic aneuploidy during preimplantation development

5.1.5 Graded SAC robustness in mouse embryo blastomeres

Although initially regarded as a binary switch, recent studies are supporting the perspective that SAC function during mitosis is not an all-or-nothing effect but rather a graded response to MCC produced by actively signalling kinetochores and its ability to inhibit the APC/C (Collin et al., 2013; Galli and Morgan, 2016; Wild et al., 2016). In line with this hypothesis, we find that in cleavage-stage mouse embryos complete spindle depolymerisation resulting in many actively signalling kinetochores induces a robust SAC-mediated arrest while SAC signalling from a subset of misaligned chromosomes fails to prevent anaphase (*see* Chapter 4, **Fig. 4.3, 4.4 S4.3 and 4.5**, p.97,98,100). This SAC defect in embryos could be explained by at least three non-mutually exclusive phenomena: (1) inadequate catalytic activity and MCC production at kinetochores, (2) compromised MCC stability in the cytoplasm and (3) insufficient MCC-mediated inihibition of APC/C activity, that will be discussed in more detail below (**Fig. 5.3**, p.134).

5.1.5.1 Potentially altered kinetochore-SAC signalling

In somatic cells, the sequence in which SAC components are recruited to kinetochores has not been fully elucidated but involves recruitment of Mps1 to mis-attached kinetochores, mediating phosphorylation of MELT repeats of the outer kinetochore protein Knl1, which in turn are recognised by Bub3 to promote the recruitment of Bub1:Bub3, BubR1: Bub3 and Mad1:Mad2 complexes (Musacchio, 2015a). Additionally, Mad1 is recruited to the outer kinetochore through its interaction with Rod, ZW10 and Zwilch subunits (RZZ complex) (Karess, 2005). While in the mouse embryo Mad1 and Mad2 are recruited to kinetochores and Mps1 functions in SAC signalling (*see* Chapter 4), it is unclear whether additional SAC proteins are efficiently recruited to kinetochores and the extent to which these may compromise MCC production remains elusive. Aurora B kinase has been shown to promote the recruitment of Mps1, Bub1 and BubR1 SAC proteins to kinetochores (Krenn and Musacchio, 2015). Recent findings have revealed that overexpression of Aurora B, but not Aurora C, extends mitosis duration and reduces Securin degradation in mouse two-cell embryos (Li et al., 2017b). It is therefore likely that in the early embryo, where Aurora B is scarce (*see* Chapter 1, p.23), Aurora C cannot fully compensate in SAC signalling regulation, posing a plausible yet unconfirmed cause of impaired SAC signalling at kinetochores in embryos.

An additional factor that may alter SAC recruitment to kinetochores in embryos is kinetochore composition and function itself. There is some evidence to suggest that embryonic cells may have different requirements for kinetochore function as the inner kinetochore protein CENP-U is necessary for kinetochore assembly in mouse embryonic stem cells (ESC) but dispensable in mouse fibroblasts (Kagawa et al., 2014). Furthermore, in line with a potentially compromised centromere homeostasis in embryos mentioned above (*see* Section **5.1.2**), epigenetic modification of centromeric histones CENP-A and H3.3 could also influence kinetochore assembly and function. This may be particularly important in the context of preimplantation development and ESCs, where the genome undergoes extensive epigenetic modification and chromatin reorganization (Canovas and Ross, 2016; Gieni et al., 2008; Musacchio and Desai, 2017; Turinetto and Giachino, 2015). In support of this notion, genetic deletion or loss-of-function of H3.3 or reduction of its chaperone, ATRX in mouse embryos result in chromosome segregation errors (Baumann et al., 2010; Lin et al., 2013; Santenard et al., 2010). Moreover, it has been recently demonstrated that H3.3 shows different genome-wide turnover rates in mouse ESCs cells than in differentiated cells (Deaton et al., 2016), raising the possibility that centromeric chromatin in embryonic cells may also display altered H3.3 dynamics. Thus, dynamic epigenetic regulation of centromeric regions and divergent kinetochore requirements in embryonic cells provide an attractive but highly speculative explanation for reduced SAC signalling at kinetochores in mouse embryos (**Fig. 5.3**, p.134).

5.1.5.2 Reduced MCC abundance and stability compromises SAC strength

The MCC tetramer is assembled from the interaction of Mad2, BubR1 and Bub3 SAC components with the APC/C co-activator Cdc20 (Musacchio, 2015b). Although kinetochores act as a catalytic platform for MCC production, it is currently unclear whether these are responsible for the entire MCC production or whether MCC generation in the cytosol also contributes to SAC signalling. At kinetochores, Mps1 phosphorylates Mad1:Mad2 complexes to stimulate their function as a template promoting the conversion of open-Mad2 to closed-Mad2 and its incorporation into the MCC, which has been proposed to be the sole rate limiting step for MCC assembly (Faesen et al., 2017). Our findings of SAC failure in the presence of few actively signalling kinetochores are consistent with a kinetochore-based mode of MCC production in mitosis. This view is supported by laser microsurgery experiments in somatic cells demonstrating that SAC signalling from a single unattached chromosome inhibits the APC/C

less efficiently than a large majority of unattached chromosomes (*see* Chapter 4) (Dick and Gerlich, 2013). Because continuous MCC degradation is required for dynamic silencing of the SAC upon achievement of correct kinetochore-microtubule attachment (Musacchio and Ciliberto, 2012), our findings point towards a scenario where in embryos, the steady-state levels of MCC produced by a single or few kinetochores are insufficient to fully inhibit the APC/C.

MCC disassembly in the cytoplasm is achieved through specific degradation of MCC-bound Cdc20 via APC/C-mediated ubiquitination in manner that is SAC-independent and requires the Apc15 subunit of the APC/C. Depletion of Apc15 in somatic cells causes a mitotic arrest despite fully aligned metaphase chromosomes and increased levels of APC/C-bound MCC (Foster and Morgan, 2012; Mansfeld et al., 2011; Uzunova et al., 2012). Whether increased levels of Apc15 in somatic cells or embryos may exacerbate MCC disassembly and compromise SAC signalling is not known but could be an additional factor contributing to insufficient SAC strength in the early embryo. Additionally, TRIP13 and $p31_{comet}$ can specifically bind MCC-bound Mad2 in the cytoplasm and extract it from the MCC downstream of kinetochores (Brulotte et al., 2017; Eytan et al., 2014; Westhorpe et al., 2011). Similarly, CDK1-phosphorylated CUEDC2 has also been reported to stimulate MCC disassembly by directly binding Cdc20 and promoting Mad2 release from APC/C-bound MCC (Gao et al., 2011). While all MCC subunits are subject to phosphorylation by mitotic kinases during cell division and multiple other post-translational modifications (PTM) have been annotated, detailed analysis of how these affect MCC assembly or stability is scarce (Liu and Zhang, 2016). Phosphorylation of Mad2 at serine 195 has been reported to preclude its conformational change into closed-Mad2 thereby preventing its interaction with Cdc20 and effective APC/C inhibition (Kim et al., 2010; Wassmann et al., 2003). Other PTMs could further regulate MCC rates of assembly and disassembly, for example, Cdc20 becomes polyubiquitinated and targeted for degradation by the APC/C itself, as mentioned above, but this activity may be counteracted by USP44-mediated deubiquitination of Cdc20 (Stegmeier et al., 2007). In addition, BubR1 acetylation by PCAF is thought to promote BubR1 degradation by the APC/C and SAC silencing (Choi et al., 2009). Thus, hypothetically altered levels and activity of Apc15, TRIP13, $p31_{comet,}$ CUEDC2, PCAF and PTMs on MCC subunits could potentially compromise cytoplasmic MCC stability and SAC robustness in the preimplantation embryo (**Fig. 5.3**, p.134).

5.1.5.3 Insufficient inhibition of APC/C activity

SAC strength ultimately depends on the ability of kinetochore-generated MCC to effectively overcome the threshold to fully inhibit APC/C activity and delay anaphase. Thus, increased APC/C activity in mouse embryos could cause a SAC deficit. Recent evidence in support of this model comes from experiments in *C. elegans* embryos where by altering cell size and injecting centromeric DNA it was shown that the ratio between the number of actively signalling kinetochores and cytoplasmic size dictates SAC strength in this system (Galli and Morgan, 2016). Similarly, experimental reduction of cell size prior to meiosis-I resumption in mouse oocytes reduces basal APC/C activity and strengthens SAC function (Kyogoku and Kitajima, 2017). However, cell size reduction in mouse oocytes has no impact on SAC strength if cytoplasm is removed after nuclear envelope breakdown (Kyogoku and Kitajima, 2017; Lane and Jones, 2017), and this effect could be attributed to MCC preassembly at the nuclear envelope in interphase (Rodriguez-Bravo et al., 2014) presumably causing increased SAC signalling at kinetochores as well as a reduction in the abundance of cytoplasmic APC/C. Interestingly, in the mouse embryo, SAC strength is unaffected by neither experimental reduction of cell size in interphase or the naturally decreasing cell size with cleavage divisions (*see* Chapter 4, **Fig. 4.6**, p.102), demonstrating that kinetochore-to-cytoplasmic size ratio is not a determinant factor of SAC strength and suggesting that compromised SAC function in embryos could instead arise from altered levels of cytoplasmic regulators of APC/C activity.

The APC/C is a E3 ubiquitin ligase multiprotein complex constituted by at least 15 subunits. Importantly, APC/C subunit composition itself may dramatically affect SAC robustness, since the presence or absence of certain subunits can alter the activity and substrate specificity of the APC/C (Izawa and Pines, 2011; Uzunova et al., 2012). Perhaps the most well-established regulator of APC/C activity is CDK1, which has long been known to phosphorylate the APC/C enhancing its affinity for Cdc20 and stimulating its ubiquitin ligase function (Fang et al., 1998; Kramer et al., 1998; Sudakin et al., 1995). In fact, the molecular mechanism has recently been elucidated: the Apc1 subunit has an autoinhibitory loop that prevents binding of Cdc20 to the Apc8 subunit. CDK1- and PLK1-mediated phosphorylation of the Apc1 loop causes a conformational change that allows Cdc20-Apc8 binding (Fujimitsu et al., 2016; Zhang et al., 2016a). How CDK1 and PLK1 levels in preimplantation development impact APC/C activity and SAC function is not fully understood but some insight has been provided from experiments in zygotes. Overexpression of PLK1 in mouse one-cell embryos stimulates APC/C activity and

degradation of Cyclins A2 and B1 while inhibition prevents APC/C activation causing a metaphase arrest (Ajduk et al., 2017; Baran et al., 2016). Similarly, partial depletion of PLK1 in human zygotes impairs progression through the first mitosis and PLK1-null mice display severely delayed cleavage divisions culminating in complete arrest after three cleavages, at the eight-cell stage, ensued by fragmentation (Lu et al., 2008; Zhao et al., 2010).

CDK1 activator Cyclin B1 but not Cyclin B2 is essential for mouse development (Brandeis et al., 1998), and thus seems to play a more pivotal role regulating mammalian meiosis and preimplantation development. Similar to PLK1, CDK1 is present in the early preimplantation embryo and its genetic ablation in mice abolishes development to the two-cell stage (Baatout et al., 2007; Santamaría et al., 2007). Findings that CDK1 activity in ESCs is essential for self-renewal and pluripotency maintenance through repression of TE fates (Li et al., 2012; Zhang et al., 2011) raise the possibility that elevated CDK1 activity in the early mammalian embryo, necessary to maintain an undifferentiated state, could result in a collateral hyperactivity of the APC/C, thus compromising SAC function. Furthermore, the CDK2 activator Cyclin B3 has been implicated in the regulation of anaphase timing with contradicting results: Overexpression of Cyclin B3 in HeLa cells and depletion in *Drosophila* and *C. elegans* embryos both delay anaphase onset (Nguyen et al., 2002; Yuan and O'Farrell, 2015), perhaps reflecting its different functions in somatic and embryonic cell types. Findings that in mouse oocytes, like in fly and worm embryos, partial Cyclin B3 depletion results in a metaphase arrest independent of the SAC (Zhang et al., 2015b), and that Cyclin B3 is present in cleaving embryos from multiple invertebrate species (Deyter et al., 2010; Lozano et al., 2010; Yuan and O'Farrell, 2015) allude to a potential role for elevated Cyclin B3 in early preimplantation development as a key factor stimulating APC/C activity. Additionally, 53BP1 has been recently reported to interact with the APC/C and its co-activators to regulate its activity, as revealed by increased APC/C activity and compromised SAC function upon 53BP1 depletion in somatic cells (Kucharski et al., 2017). Interestingly, the observed abundance and cellular localization of 53BP1 in mouse embryos, which is low during early development becoming accumulated in the cytoplasm at blastocyst stage (Ziegler-Birling et al., 2009), coincide with our findings of increased SAC function at blastocyst stage (*see* Chapter 4), and suggest a yet to be confirmed role for 53BP1-mediated regulation of APC/C activity in the early embryo (**Fig. 5.3**, p.134).

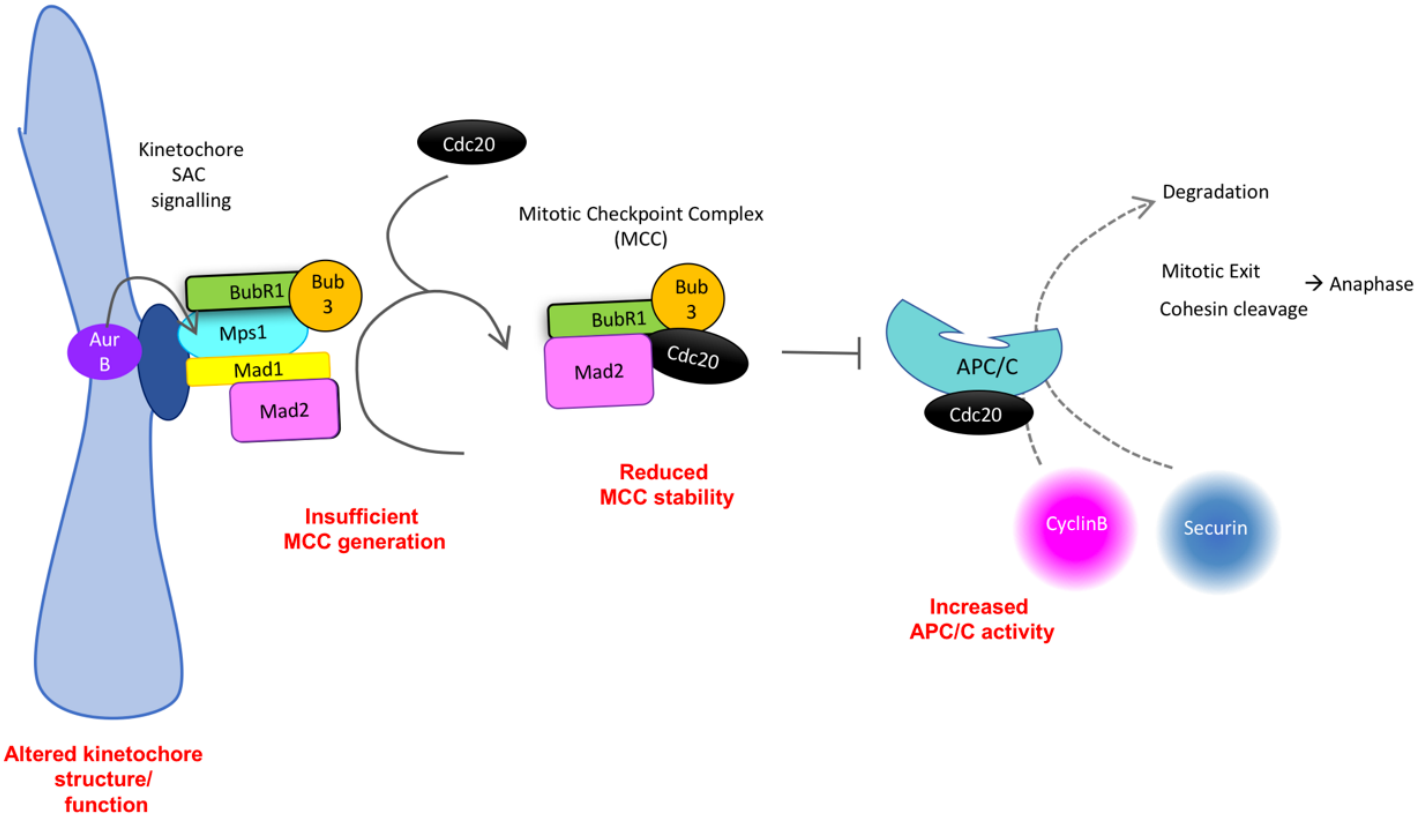

Figure 5.3. Potential factors contributing to weakened SAC function in embryos

5.1.6 Mismatched SAC and APC/C activities as an emerging mechanism for CIN

Our findings that mild APC/C inhibition with proTAME reduces chromosome segregation errors in embryos (*see* Chapter 4, **Fig. 4.7**) supports a model of decompensated SAC signalling and APC/C activities that ultimately compromise SAC strength in preimplantation development. Further evidence for non-matching SAC and APC/C activities as a cause of CIN come from recent somatic and cancer cell studies. First, APC/C subunits, appear to be frequently mutated in several human cancer cell types and loss of APC/C subunits has been correlated with poor survival, increased risk of colorectal cancer recurrence and metastasis in prostate cancer (Kim et al., 2017; Liu et al., 2018; McGranahan et al., 2015). Secondly, reports that APC/C inhibition in somatic cells with compromised SAC signalling rescues cell viability strongly suggest a reduction in CIN (Wild et al., 2016). Lastly, genetic screens in somatic and cancer cells subjected to SAC inhibition have identified APC/C subunits whose loss confers a proliferative advantage. Further experiments demonstrated that partial APC/C subunit knockdown or pharmacological inhibition extended mitosis duration to prevent induced and naturally-occurring segregation errors (Thu et al., 2018; Sansregret et al., 2017). Thus, it has been suggested that partial loss of APC/C function in cancer may serve as an adaptation to limit excessive CIN, maintain cellular fitness and develop resistance to anti-mitotic agents. These novel perspectives lead to an emerging model where SAC function is not exclusively defined by SAC signalling itself but by its concerted activity with APC/C-mediated degradation of Cyclin B and Securin.

5.1.7 Clinical Implications for embryo culture, selection and testing in fertility treatment

There is an ever increasing need for Assisted Reproduction Technology (ART) treatments for age-related infertility as the age at which both men and women in developed countries have their first child is increasing (Belloc et al., 2014; Crawford and Steiner, 2015; Navot et al., 1994). Moreover, advances in ART are providing fertility preservation options for young cancer patients undergoing chemo- and radiotherapy, many of which rely on storing frozen oocytes and embryos (Kim et al., 2016). Importantly, despite major advances in ART, birth rates from IVF and ICSI cycles have not significantly improved over the last decade (Chambers et al., 2016). Embryo selection in fertility clinics is based on embryo morphology, developmental kinetics and preimplantation genetic screening (PGS) results (Wong et al., 2014). However, neither of these alone or in combination are sufficient to predict an ongoing pregnancy

(Friedenthal et al., 2018; Munné and Wells, 2017). Therefore, current embryo selection methods and perhaps the general quality of embryos, are major limitations to improving the success of ART and IVF cycles. Moreover, the usefulness of PGS is still being debated, with contradicting reports on whether it improves live birth rates and some studies even showing that the PGS procedure itself may negatively impact embryo health (Dahdouh et al., 2015; Friedenthal et al., 2018).

A potential explanation for the limited predictive power of PGS is technology failure, where current detection methods perhaps fail to accurately detect all abnormalities. This may be the case for micronucleus-enclosed chromosomes, where extensively damaged DNA and impaired DNA repair (*see* Chapter 2) (Crasta et al., 2012; Ly et al., 2017) would result in a profound degree of DNA fragmentation and whether such fragments can be detected by current PGS technologies is not clear. While there is some indication that micronucleus-enclosed DNA may be detected by whole-genome SNP analysis in some cases, as micronuclei have been associated with chromosome gains in blastomeres from an early cleavage stage human embryo (Kort et al., 2016), it remains to be tested whether PGS methods can reliably detect chromosomes in micronuclei over the next cell cycle(s) after micronucleus formation as it is conceivable that the highly fragmented DNA in micronuclei could lead to failed amplification during sample preparation and/or exclusion in the PGS results processing algorithms. Undetected chromosomes in micronuclei may potentially compromise the overall accuracy and significance of PGS results quite dramatically as PGS is typically performed on only 5-7 blastomeres biopsied from the TE, and thus may not necessarily reflect the overall degree of mosaicism in the embryo (Gleicher et al., 2017). The application of other possible markers of embryo health as PGS parameters has also been explored. Thus far, studies investigating DNA in the blastocoel cavity of blastocyst-stage embryos and media metabolite consumption have found no improvement to ART success (Poli et al., 2013; Uyar and Seli, 2014; Zhang et al., 2016b). Interestingly, mitochondrial DNA (mtDNA) quantification has revealed higher levels of mtDNA in aneuploid embryos, and that euploid embryos with increased mtDNA levels above a certain threshold fail to implant, but nevertheless this technology cannot predict implantation for embryos with below-threshold levels of mtDNA (Fragouli et al., 2015). Thus, a whole-embryo comprehensive and non-invasive *bona fide* reporter of embryo viability is yet to be identified and current techniques only serve to identify and select against embryos unlikely to implant but fail identify embryos that will implant and lead to a successful pregnancy.

Oocyte and embryo culture conditions in ART may not only influence the epigenetic and transcriptional profiles of embryos but also the rates of implantation, pregnancy and live births (Feuer et al., 2016; Kleijkers et al., 2016; Lazaraviciute et al., 2014). These findings suggest the ART culture environment may profoundly impact embryo quality. Our work has demonstrated that supplementation of culture media with proTAME can achieve a moderate prolongation of mitosis and reduce chromosome segregation errors and micronuclei in mouse preimplantation embryos (*see* Chapter 4), implying that pharmacological modulation of the SAC-APC/C axis could potentially reduce mosaic aneuploidy in human embryos. Although extensive testing, optimisation and validation in embryos donated to research will be required prior to its clinical application, APC/C inhibitors may present a therapeutic means to improve the overall quality of human embryos in fertility treatments. Thus, while improved PGS methods and the optimal culture conditions are under way, pharmacological enhancement of SAC function may effectively increase the overall quality of embryos and may therefore potentially serve to increase the effectiveness of ART treatments in the future.

5.1.8 Technical challenges of studying cell cycle dynamics in the preimplantation embryo

The research findings discussed above have greatly advanced our understanding of how and why preimplantation embryos are prone to chromosome segregation errors and mosaic aneuploidy. However, there are still numerous outstanding questions. Importantly, many of these will require the development and application of innovative approaches to overcome current technical limitations in this cellular system and uncover previously inaccessible aspects of mitosis and cell cycle regulation in the early mammalian embryo.

The correlative live imaging and immunofluorescence technique described in Chapter 3, which we applied for the first time in a preimplantation embryo, was instrumental to demonstrate that micronuclei in the subsequent cell cycles lack functional kinetochores, hence explaining their repeated mis-segregation and inheritance during development. This approach combined live cell imaging and immunofluorescence to enable the detection of endogenous protein localization at a defined time point in mitosis in the unperturbed embryo (*see* Chapters 2 and 3). Future variations of this approach could include, but are not limited to, correlative live imaging in combination with photo-labeling and single cell DNA or RNA sequencing to relate cell cycle dynamics to chromosome copy number and/or gene transcript levels, respectively. In fact, this type of correlative live cell imaging and single-cell DNA sequencing has already been employed to prove micronuclei cause chromothripsis in somatic cells (Zhang et al., 2015a). Additionally, some barcode-based technologies, such as NanoString™ 3D Biology allow for simultaneous detection and quantification of the levels of a predefined set of DNA, RNA and proteins that could potentially allow to examine the relationship between cell cycle dynamics, aneuploidy, gene expression and protein levels in embryos. Furthermore, while live cell imaging and immunofluorescence are indispensable tools for the study of cell division, these are usually limited by the optical resolution of conventional microscopes. The combination of current live cell imaging techniques with super-resolution approaches would enable to link particular dynamics during mitosis with kinetochore or spindle pole structures and compositions. This could be achieved by stimulated emission depletion (STED) microscopy, expansion microscopy or the recently reported correlative light and electron microscopy (EM) for improved structural resolution (Chen et al., 2015; Sydor et al., 2015; Wolff et al., 2016) (**Fig. 5.4**, p.141).

A less exhaustive alternative to correlative live imaging and DNA sequencing for whole genome copy number variation analysis would involve developing a system where at least one chromosome is specifically and differentially labelled to provide a continuous readout, allowing tracking of aneuploidy. This has been previously done in somatic cells by random integration of the bacterial operon sequence lacO and the coding sequence for its cognate receptor, LacI, fused to GFP (Thompson and Compton, 2011). Such approach could be accomplised more efficiently in mouse embryos by site-directed transgenesis into a safe harbour locus, such as the Rosa26 locus, using CRISPR/Cas9 gene editing technology. Integration of the LacO-LacI:GFP cassette into a given chromosome in the mouse genome would cause LacI-GFP expression and binding to that locus, therefore enabling to label and track a single chromosome in the preimplantation embryo but also in the developing fetus and the adult mouse (**Fig. 5.4**, p.141). Moreover, this system would allow examination of the natural generation, elimination and persistence of mosaic aneuploidy throughout mammalian development, for the first time, without mitotic disruption or gene depletion experimental interventions. Another method that could be employed to label specific chromosomes in embryos involves the use of a nuclease-dead Cas9 (dCas9) and specific gRNAs that will direct its localization to discrete genomic loci. Two variations of this approach have been demonstrated in somatic cells: either by using dCas9 variants that will associate with specific gRNAs, fused to different fluorophores, or by employing unlabeled dCas9 and fluorophore-conjugated gRNAs (Anton et al., 2016; Ma et al., 2016). While both are feasible in mouse embryos, requiring microinjection of dCas9 and gRNA reagents, the latter offers more flexibility since fluorophores on gRNAs can be multiplexed, allowing to simultaneously label up to 7 different chromosomes (**Fig. 5.4**, p.141) It must be noted that successful chromosome-labeling by this approach will ultimately depend on the identification of sufficient chromosome-specific sequence repeats that would yield a bright enough and distinct signal for detection by conventional fluorescence microscopy.

Microinjection of cRNA and RNAi can be used in embryos to achieve overexpression and knockdown of specific proteins. However, in cases where there is rapid protein turnover and/or cell cycle-dependent degradation of proteins, overexpression approaches are limited to the first couple of cell cycles in preimplantation development because microinjection of blastomeres beyond the four-cell stage is technically very challenging. For this reason, measurement of Securin-GFP and Geminin-GFP degradation as a direct measure of APC/C activity has only been successfully performed in mouse zygotes and two-cell embryos (Ajduk et al., 2017; Li et

al., 2017a). Alternatively, monitoring APC/C activity at later stages of preimplantation development would require constitutive overexpression of these fusion proteins. This has been achieved with transgenic systems such as Fluorescent Ubiquitination-based Cell Cycle Indicator (FUCCI), in which fluorescently tagged Cdt1 and Geminin provide direct measurement of APC/C activity and cell cycle progression (Abe et al., 2013; Sakaue-Sawano et al., 2008). While a FUCCI mouse has been generated and is available (Abe et al., 2013), it does not allow to monitor the degradation of other APC/C substrates during cell division. A potential practical solution to this problem would be the use of a single transgenic mouse line, containing a LoxP site at the Rosa26 locus, where upon microinjection of embryos with Cre recombinase and donor DNA encoding the protein of interest and a fluorophore flanked by LoxP sites, this would be integrated into the genome in a targeted manner to provide constitutive expression. Importantly, because embryos in which successful recombination has taken place will be identifiable by conventional fluorescence microscopy, the generation and maintenance of a new transgenic line of mice may not be necessary depending on integration efficiency (**Fig. 5.4**, p.141).

A current caveat of RNAi and cRNA microinjection in embryos is the lack of temporal control, due to the technical difficulty in performing microinjections beyond the four-cell stage, proteins cannot be acutely overexpressed or downregulated at defined times during development. This could be achieved by slight modification of the system described above, using the same Rosa26-LoxP mice, but the donor construct would additionally contain a reverse tetracycline-controlled transactivator (rtTA), a reporter of integration (eg. RFP), a tetracycline response element (TRE), before the desired RNAi or cDNA sequence to be expressed and a second expression reporter (GFP). In this system, in the absence of doxycycline, only rtTA and the reporter of integration are expressed, thus allowing selection of embryos with successful integration. Upon addition of doxycycline, rtTA would bind the TRE to stimulate expression of the desired siRNA or cDNA sequences (Haenebalcke et al., 2013). This inducible system for overexpression and downregulation would provide increased temporal control over the level of specific proteins in the preimplantation embryo. Alternatively, preimplantation embryos can be successfully transfected by electroporation (Chen et al., 2016), and could present a technically simpler approach to transiently express fusion protein cDNAs and siRNAs albeit with potentially more variability in the extent of transfection (**Fig. 5.4**, p.141).

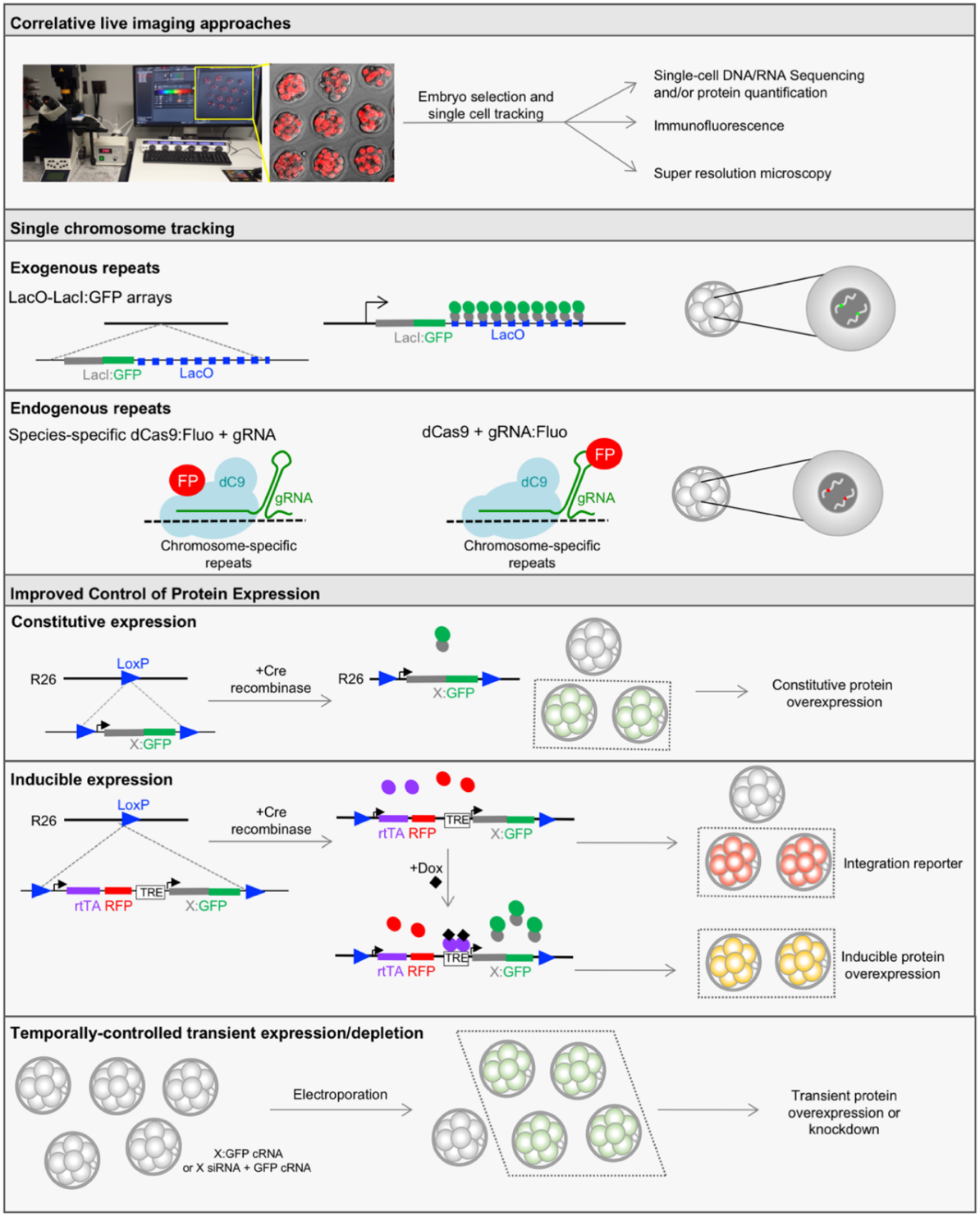

Figure 5.4. Proposed technical approaches to overcome current experimental limitations in mouse preimplantation embryos

R26: Rosa26 locus, rtTA: reverse tetracyclin transactivator, TRE: Tetracyclin responsive element, Dox: Doxycycline, X: cDNA or siRNA for gene of interest.

A major hurdle in the study of chromosome segregation in human preimplantation embryos is that it requires the introduction of fluorescent labels for chromatin or DNA and intensive imaging, which is too invasive for embryos in fertility clinics and ethically controversial in some research settings. Thus, it is not surprising that live imaging studies of chromosome segregation in human embryos have not yet been reported. Future imaging advances might enable non-invasive and label-free visualization of chromatin in intact human embryos. If these allow the detection of micronuclei and chromosome segregation dynamics, such technologies have the potential to provide more comprehensive and non-invasive readouts of mosaic aneuploidy in the whole embryo than current PGS methods, and may therefore not only replace the highly-praised but not very informative Embryoscope-like approaches in fertility clinics but may also obviate the need for PGS (Chawla et al., 2015). Innovative application of current and future techniques will provide more stringent spatiotemporal control of protein expression, increased imaging resolutions, reporters of cell cycle progression and aneuploidy, as well as non-invasive imaging methods will lead to ground-breaking insights into chromosome segregation during preimplantation embryo and perhaps also improved embryo selection methods.

5.2 Conclusions

The mammalian preimplantation embryo often exhibits mosaic aneuploidy, where embryos are comprised of cells bearing normal and abnormal chromosome complements. Surprisingly, mosaic aneuploidy is remarkably common in human embryos, has been associated with impaired developmental potential, implantation failure and miscarriage, and is therefore frequently selected against in fertility treatments. However, although mosaic aneuploidy in embryos is inextricably caused by chromosome segregation errors during embryonic divisions, the causes, nature and impact of chromosome mis-segregation in the early mammalian embryo were poorly understood. In this thesis, we have applied advanced imaging techniques to the mouse preimplantation embryo to characterize chromosome segregation dynamics and probe the effectiveness of the main mitotic safeguard mechanism, the SAC. We have identified lagging chromosomes as the most prevalent form of chromosome mis-segregation in embryos, which lead to the formation of micronuclei, wherein chromosomes are subject to severe DNA damage and centromeric identity loss, resulting in their recurrent mis-segregation. This pattern of repeated micronucleus inheritance necessarily causes the propagation of aneuploid cells over subsequent cell cycles. Moreover, through mechanistic examination of SAC function in embryos we have demonstrated that while the SAC is functional in embryos, SAC signalling fails to prevent anaphase in mitoses with misaligned chromosomes. Furthermore, we have found this deficit in SAC strength is weakest at mid-preimplantation development independent of cell size, and have provided proof-of principle evidence that pharmacological modulation of SAC-APC/C signaling can reduce segregation errors in the mouse embryo. These studies have demonstrated impaired SAC function is a major underlying cause for the high incidence of chromosome segregation error, micronuclei and mosaic aneuploidy in mouse embryos. Thus, this work has uncovered previously lacking mechanistic explanations for the cause, occurrence and impact of chromosomal instability in the mammalian preimplantation embryo. These novel findings are therefore likely to have profound implications for the detection, selection and potential prevention of human embryo mosaic aneuploidy in fertility treatments.

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