

Diversity in cytokinesis regulation across cancerous and healthy human cell types

Cécilia Brancheriau

A Thesis

In the Department

of

Biology

Presented in Partial Fulfillment of the Requirements

For the Degree of

Doctor of Philosophy (Biology) at

Concordia University

Montreal, Quebec, Canada

January 2026

© Cécilia Brancheriau, 2026

CONCORDIA UNIVERSITY
School of Graduate Studies

This is to certify that the thesis prepared

By: Cécilia Brancheriau

Entitled: Diversity in cytokinesis regulation across cancerous and healthy human cell types
and submitted in partial fulfilment of the requirements for the degree of

Doctor of Philosophy (Biology)

complies with the regulations of the University and meets the accepted standards with respect to its originality and quality.

Signed by the final Examining Committee:

_____ Chair

Dr. Christopher Brett

_____ External Examiner

Dr. Jean-Claude Labbé

_____ External Examiner

Dr. Elena Kuzmin

_____ Examiner

Dr. Brandon Helfield

_____ Examiner

Dr. Anan Chen

_____ Supervisor

Dr. Alisa Piekny

Approved by _____

Dr. Robert Weladji, Graduate Program Director

_____ 2026

Pascale Sicotte, Dean of Faculty

ABSTRACT

Diversity of cytokinesis regulation across cancerous and healthy human cell types

Cécilia Brancheriau

Cytokinesis is the physical separation of a cell into two daughter cells through the ingression of a contractile ring. Failure of cytokinesis can alter cell fate or ploidy, leading to developmental abnormalities and disease. To ensure fidelity, cytokinesis is tightly regulated by multiple spatiotemporal pathways associated with the mitotic spindle and chromatin. However, much of our current understanding of human cytokinesis derives from HeLa cells, and the requirement for these regulatory pathways likely varies across cell types. Our group recently demonstrated such diversity by comparing the localization of cytokinetic proteins in five distinct human cell lines, highlighting the need to study cytokinesis in both cancerous and healthy contexts. This work aims to investigate how chromatin-sensing regulates cytokinesis in cells with different ploidy and to uncover the mechanisms that control cytokinesis in normal human cells. In Chapter 2, we examined whether importins respond to the chromatin-associated Ran-GTP gradient to regulate contractile ring assembly. Using endogenous tagging, live-cell imaging, optogenetic perturbations, FRET-FLIM, and computational modeling, we found that importin- β 1 becomes equatorially enriched and is required for cytokinesis in hypotriploid HeLa cells but not in euploid HCT 116 cells. A predictive model of Ran-GTP and Ran-free importin- β 1, validated experimentally, identified factors such as the chromatin-to-cell size ratio that modulate this chromatin-sensing pathway. These findings suggest that highly aneuploid cancer cells depend on importin-mediated anillin recruitment, revealing a potential vulnerability that distinguishes them from diploid cells. In Chapter 3, we investigated cytokinesis in human induced pluripotent stem cells (iPSCs) with endogenously tagged cytokinetic proteins to elucidate mechanisms of ring assembly. We found that ring constriction is asymmetric in iPSCs, with anillin enriched at the leading edge of the furrow. Astral microtubules were shown to play dual roles: an outer pool bundled by central spindle proteins promotes ring assembly, while asters restrict the contractile ring to a defined cortical zone. Comparison with differentiated endoderm cells revealed broader contractile rings, suggesting that cytokinesis regulation changes during differentiation. RNA sequencing further showed that spindle-associated pathways are downregulated as iPSCs differentiate into hepatoblast progenitors and hepatocyte-like cells, potentially leading to defective ring assembly and cytokinesis failure. Overall, this work provides new insight into the diversity of cytokinetic regulation in human cells and represents a first step toward building a comprehensive atlas of cytokinesis mechanisms across healthy and cancerous cell types.

ACKNOWLEDGEMENTS

First of all, I would like to thank Alisa Piekny, my supervisor. I joined her lab because she told me that if I wasn't happy, I could leave. Well, thank you for creating a healthy environment conducive to learning and science, which makes me wake up with motivation every day. Thank you for supporting me, letting me express myself, try and make mistakes.

I would also like to thank my committee members for their support, their useful feedback and for taking the time to evaluate my thesis. Especially Dr. Chen who pushed me to do an experiment which I postponed for a long time because the potential outcome frightened me.

I would also like to acknowledge the support of the teams from the CMCI, the Genome Foundry at Concordia and ABIF at McGill without whom this work would never have been possible. Thank you to Chris Law, who has been a life saver with the numerous microscopes I have used and with the tissue culture room maintenance. I also would like to thank him for our thoughtful discussion. Thank you to Angela Quach and Kevin Jacquet for their help and their generosity with the stem cells culture. I would also like to acknowledge Joel Ryan for his help with FLIM-FRET images collecting and processing.

I would like to take a moment to acknowledge Robert Carson, who is the lab technician of BIOL 368. I liked teaching, but Robert made me love it!

Last but not least, I would like to thank the whole Piekny lab crew. Kevin Larocque, Imge Ozugergin, Mathieu Husser and Nhat Pham were my mentor by either providing me technical guidance, knowledgeable advice about lab and life and especially great time! The future of the lab lies in the hands of Natasha Letourneau, Alexandra Perlman, Ioanna Tountas, Gabrielle Schick, Juliette Gennaro and Christina Dembegiotis. I'm not worried at all because they are talented, kind, and lively women who have given me wonderful years and whom I will miss more than anything. A special thank you for Ioanna Tountas who endured my supervision. Thank you for being such a good trainee and friend, you taught me more than you can imagine.

I ran out of words and tears. Thank you everyone for making science possible and enjoyable.

DEDICATION

I would like to dedicate this thesis to my beloved family, Sophie, Loïc et Anaïs Brancheriau, whom I can't wait to see again.

I also would like to dedicate it to Harold Sassi, who was an amazing stay-at-home dad for our cat, Luna. Even though he refused to marry me before the end of my PhD so I could be Dr. Sassi.

CONTRIBUTIONS OF AUTHORS

Chapter 2: Importin-regulation of cytokinesis depends on ploidy

Alisa Piekny supervised and conceptualized the project and edited the manuscript. Mathieu Husser generated HeLa mNG:ANLN and HCT 116 p53^{-/-} mNG:ANLN cells. Andrew Cormier designed and built the DNA plasmids with anillin shRNA and for the rescue experiments. Kevin Larocque conceptualized, designed and conducted the experiments on the hyperploid HCT 116 cells for Figure 10D, E and G. Alexandra Perlman conducted experiments on hypotonic HeLa cells for Figure 10D, E and G. Ioanna Tountas performed experiments on HCT 116 cells for Figure 7C and on hypotonic HeLa cells 10A. Cécilia R. Brancheriau wrote the manuscript including all figures, conceptualized, designed and conducted the remaining experiments including analysis and entirely did the computational modelling.

Chapter 3: Characterization of mechanisms regulating iPSC cytokinesis

Alisa Piekny supervised the project and edited the manuscript. Mathieu Husser generated smNG2:ANLN iPSCs and smNG2-P₁₋₁₀ iPSCs. Ioanna Tountas designed and conducted the experiments for Figure 14B and F and Figure 17B and performed the analysis for Figure 14D and E and Figure 17C, D and E. Gabrielle Schick designed and built the DNA plasmids for this project. Cécilia R. Brancheriau wrote the manuscript including all figures, designed and conducted the remaining experiments including analysis.

List of Figures

Figure 1. Mitosis and cytokinesis.....	2
Figure 2. Pathways regulating cytokinesis.....	5
Figure 3. Ran regulates spindle assembly and ring position.	11
Figure 4. Polyploidization in cells during normal tissue development and pathologies.....	17
Figure 5. Importin- β 1 is transiently enriched equatorially in anaphase HeLa but not HCT 116 cells ..	30
Figure 6. The equatorial enrichment of importin- β 1 is anillin-dependent.	33
Figure 7. Importin-binding is required for anillin's equatorial localization and function in HeLa but not HCT 116 cells.	36
Figure 8. Importin- β 1 is temporally required during anaphase for cytokinesis	38
Figure 9. Ran-free importin- β 1 is equatorially enriched in anaphase HeLa but not HCT 116 cells....	41
Figure 10. Chromatin to size ratio affects the localization of importin- β 1 and anillin.....	44
Figure 11. The contractile ring ingresses asymmetrically with asymmetric anillin distribution in iPSCs	56
Figure 12. Anillin is required for cytokinesis in iPSCs.....	57
Figure 13. Importin- β 1 is not equatorially enriched in anaphase iPSCs.....	58
Figure 14. iPSCs have two pools of astral microtubules and a weak central spindle during cytokinesis	60
Figure 15. Astral microtubules regulate anillin's localization in the ring	63
Figure 16. Model of regulation of cytokinesis in iPSCs compared to HeLa cells.....	64
Figure 17. Ring assembly and ingression occurs differently in endoderm cells compared to iPSCs	66
Figure 18. Differences in the regulation of cytokinesis in stem cells and cancer cells	73

List of Supplemental Figures

Supplementary Figure S1. Genotyping of importin- β 1:mNG tagged cells	91
Supplementary Figure S2. Anillin localizes differently in mitotic HeLa and HCT 116 cells	92
Supplementary Figure S3. Method used to image and evaluate the fluorescence lifetime	93
Supplementary Figure S4. Fluorescence lifetime is dependent on the levels of Rango-3	94
Supplementary Figure S5. Reactions used to calculate the Ran gradient in the predictive model ...	95
Supplementary Figure S6. Cell measurements used in the predictive model	96
Supplementary Figure S7. RNA-seq data analysis for Ran and Ran regulators in HeLa and HCT 116 cells.....	97
Supplementary Figure S8. Method for model refinement	98
Supplementary Figure S9. Impact of changing the levels of Ran or Ran regulators on the predicted Ran-free importin- β 1 concentration	99
Supplementary Figure S10. Generation of hypotonic HeLa cells and hyperploid HCT 116 cells	100
Supplementary Figure S11. The spindle scales with cell size	101
Supplementary Figure S12. Endogenously tagging ezrin and importin- β 1 using the split mNeonGreen system in iPSCs	102
Supplementary Figure S13. Anillin forms aggregates in anaphase iPSCs	104
Supplementary Figure S14. Cyk4 localization in iPSCs during cytokinesis	105
Supplementary Figure S15. Plk1 localization in iPSCs during cytokinesis	106
Supplementary Figure S16. Endoderm differentiation of FOXA2:smNG2 iPSCs	107

List of Supplemental Tables

Supplementary Table S1. Rango-3 fluorescence intensity in individual HeLa cells	108
Supplementary Table S2. Diffusion coefficients for Ran and Ran regulators	108
Supplementary Table S3. Reaction rates and kinetic constants for Ran and Ran regulators	108
Supplementary Table S4. Measurements of components in metaphase and anaphase HeLa and HCT 116 cells	109
Supplementary Table S5. Concentrations of Ran and Ran regulators in HeLa and HCT 116 cells ...	109
Supplementary Table S6. Initial conditions for the predictive Ran-free importin- β 1 gradient model	110
Supplementary Table S7. Measurements for anaphase hypotonic HeLa and hyperploid HCT116 cells	110
Supplementary Table S8. List of primers used for cloning	111

List of Abbreviations

C1	Protein kinase C conserved lipid binding region 1
C2	Protein kinase C conserved lipid binding region 2
Cdk1	Cyclin-dependent kinase 1
cDNA	Complementary DNA
CLIP170	Cytoplasmic linker protein 170
CPC	Chromosomal passenger complex
C-term	C-terminus or carboxyl-terminus
Cyk4	Cytokinesis defect 4 [or MgcRacGAP (Male germ cell RacGAP (RacGTPase activating protein)) rho-family GAP in humans; RacGAP50C in <i>Drosophila</i>]
DIAPH1	Diaphanous-related formin 1
DIAPH3	Diaphanous-related formin 3
DMEM	Dulbecco's Modified Eagle Medium (DMEM)
DNA	Deoxyribunucleic acids
DOCK7	Dedicator of cytokinesis
Ect2	Epithelial cell transforming 2
EGFP	Enhanced Green fluorescent protein
ERM	Ezrin/Radixin/Moesin
ESC	Embryonic stem cell
FLIM	Fluorescence-lifetime Imaging Microscopy
FRET	Förster resonance energy transfer
GDP	Guanosine diphosphate
GEF	Guanosine nucleotide exchange factor
GTP	Guanosine triphosphate
GTPase	Guanosine triphosphatase
HCC	Hepatocellular carcinoma
HEK293	Human embryonic kidney 293

HeLa	Henrietta Lacks (uterine cells derived from)
HFF-1	Human foreskin fibroblast 1
HLC	Hepatocyte-like cell
iPSC	Induced pluripotent stem cell
Kif4	Kinesin Family Member 4
KPNB1	Karyopherin- β 1 or importin- β 1
MP-GAP	Mitotic-phase GTPase activating protein
mCherry	Monomeric cherry fluorescent protein
MKLP1	Mitotic kinesin like protein 1
mNeonGreen	monomeric Neon Green
mScarlet	monomeric Scarlet
MT	Microtubules
n.s.	Not significant
NLS	Nuclear Localization Signal
N-term	N-terminus or NH ₂ -terminus
NuMA	Nuclear Mitotic Apparatus protein
PDE	Partial differential equation
PH	Pleckstrin homology
Plk1	Polo-like kinase 1
PP1-Sds22	Protein phosphatase 1 and its regulatory subunit Sds22
PRC1	Protein Regulator of Cytokinesis 1
RacGAP	Rac GTPase activating protein
Ran	Ras-related nuclear protein GTPase
RanGAP	Ran GTPase activating protein
RBD	Rho-binding domain
RCC1	Regulator of chromosome condensation 1
RhoA	Ras homolog family member A
RhoGAP	Ras homolog family member A GTPase-Activating Protein

RNA	Ribonucleic Acid
RNAi	Ribonucleic Acid interference
ROCK	Rho-associated protein kinase
ROI	Region of interest
SAF	Spindle assembly factor
Scc1	Sister chromatid cohesion 1
SD	Standard deviation
shRNA	short-hairpin RNA
siRNA	Small interfering ribonucleic acid
STC	S-trityl-L-cysteine
TPX2	Targeting Protein for Xklp2
XCTK2	Xenopus Cytoplasmic Kinesin-related Protein 2
Xklp2	Xenopus kinesin-like protein 2

Table of contents

ABSTRACT	iii
ACKNOWLEDGEMENTS	iv
DEDICATION.....	v
CONTRIBUTIONS OF AUTHORS.....	vi
List of Figures.....	vii
List of Supplemental Figures	viii
List of Supplemental Tables.....	ix
List of Abbreviations.....	x
Chapter 1	1
1.1 Overview	1
1.2 Cell cycle and cytokinesis	1
1.3 Pathways regulating cytokinesis	3
1.3.1 Spindle-dependent pathways	3
1.3.2 Spindle-independent pathways.....	7
1.4 Ran-dependent pathways	8
1.4.1 Ran and importins	8
1.4.2 Spindle assembly and scaling	8
1.4.3 Ring assembly	12
1.5 Cytokinesis diversity	13
1.5.1 Diversity among animal cells and human cells.....	13
1.5.2 Stem cell division	14
1.6 Cytokinesis failure and polyploidy: wanted or unwanted?	15
1.7 Thesis overview.....	18
Chapter 2	19
Importin- β 1 functions as a chromatin sensor to position the contractile ring for cytokinesis	19
2.1 Highlights	19
2.2 Summary.....	19
2.3 Introduction.....	20
2.4 Materials & Methods	22
2.5 Results	29
2.5.1 Importin- β 1 is transiently enriched equatorially in anaphase HeLa but not HCT 116 cells	29
2.5.2 Importin- β 1 equatorial enrichment in HeLa cells is dependent on anillin	32

2.5.3 Importin- β 1 is required for ring assembly in HeLa cells but not in HCT 116 cells	35
2.5.4 Ran-GTP free importin- β is equatorially enriched in HeLa but not HCT 116 cells.....	39
2.5.5 Chromatin to size ratio affects the equatorial enrichment of Ran-free importin- β 1 and anillin..	43
2.6 Discussion.....	46
Chapter 3	48
3.1 Summary.....	48
3.2 Introduction.....	48
3.3 Materials and Methods.....	50
3.4 Results	54
3.4.1 The contractile ring ingresses asymmetrically in iPSCs	54
3.4.2 Importin- β 1 is not equatorially enriched in anaphase iPSCs.....	58
3.4.3 iPSCs have two pools of astral microtubules and a weak central spindle during cytokinesis....	59
3.4.4 Astral microtubules regulate the breadth of the ring in iPSCs	62
3.4.5 Ring assembly and ingression occurs differently in endoderm cells compared to iPSCs	65
3.5 Discussion.....	68
Chapter 4	71
4.1 Overview	71
4.2 The chromatin-sensing pathway requirement differs among cell types	71
4.3 Cytokinesis regulation in stem cells: differences and similarities with other models	74
4.4 Cytokinesis changes during differentiation.....	75
4.5 Future directions.....	75
References	78
Supplemental figures	91
Supplemental tables.....	108

Chapter 1

Introduction

1.1 Overview

Cytokinesis occurs during mitosis to physically split a cell into two daughter cells. This process requires the assembly and ingression of a contractile ring at the equator of the cell (Piekny & Glotzer, 2008; Green et al., 2012). Failure of cytokinesis can lead to changes in cell fate or ploidy, which can cause pathologies, but is also a normal step in the differentiation of healthy polyploid cells, such as hepatocytes (Lacroix & Maddox, 2012). Cytokinesis is spatiotemporally controlled by multiple pathways associated with mitotic spindle and chromatin among others (Glotzer, 2013; Green et al., 2012). Our group recently helped discover a chromatin-sensing pathway which positions the ring between the segregating chromosomes (Kiyomitsu and Cheeseman, 2013; Beaudet et al., 2017; 2020; Ozugergin et al., 2022). However, some of the molecular components of this pathway remain to be characterized, and we predict the requirement of this pathway to vary with cell types. Additionally, most of our knowledge of human cytokinesis was generated using a few diseased and/or transformed cell lines, and we lack knowledge of how healthier human cells divide. This emphasizes the importance of studying cytokinesis in more meaningful cell types.

1.2 Cell cycle and cytokinesis

The cell cycle can be separated into interphase and mitosis. Interphase consists of multiple phases: G1, S and G2. G1 is a growth phase prior to cell cycle entry, and it is during this phase that the decision is made to divide or remain in G0, quiescence. S phase is when DNA is replicated, while G2 is another gap/growth phase when the cell prepares to enter mitosis (Wenzel et al., 2018). Mitosis is composed of prophase, metaphase, anaphase and telophase during which sister chromatids and cytosolic components (e.g., organelles) undergo changes to be faithfully segregated by a bipolar spindle. Cytokinesis occurs during mitotic exit to physically separate the cell into two daughters.

Mitotic entry and exit are controlled by cyclin-dependent kinase 1 (Cdk1)/Cyclin B activity (Schafer et al., 1998). Cdk1 phosphorylates substrates that mediate chromosome condensation, cell rounding, nuclear envelope breakdown and spindle assembly (Fig. 1) (Hégarat et al., 2016; El Dika et al., 2023). As the kinetochores associated with the sister chromatids attach to the spindle microtubules from their respective poles to satisfy the spindle checkpoint, mitotic exit is triggered by separase-mediated degradation of cohesin Scc1 and cyclin B degradation. Importantly, mitotic exit requires Cdk1 activity to decrease and substrates to be dephosphorylated (Forester et al., 2007; Hégarat et al., 2016). This includes permitting microtubule depolymerization for sister chromatid segregation, chromosome decondensation, nuclear envelope reassembly, central spindle assembly (antiparallel bundled microtubules that arise between the segregating chromosomes), and formation and ingression of a contraction ring for cytokinesis. The contractile ring is composed of actin and myosin as well as crosslinkers and anchoring proteins including anillin, formins, septins and the ERM proteins (ezrin, radixin and moesin) (Green et al. 2012). Notably, the master regulator of

the ring is RhoA, which is a GTPase that is activated by the RhoA GEF Ect2 – exchanges GDP for GTP. Ect2 is activated by binding to Cyk4, which is part of the centralspindlin complex that regulates central spindle assembly (Somers & Saint, 2003; Yüce et al., 2005; Bement et al., 2005; Kamijo et al., 2006). Centralspindlin and Ect2 are each negatively regulated by Cdk1 phosphorylation. Thus, RhoA activation is spatiotemporally coordinated with a decrease in Cdk1 activity (Glotzer, 2009; Green et al. 2012). Active RhoA leads to the formation of actin filaments by regulating formins, and activates myosin via regulation of RhoA kinase, which phosphorylates myosin light chain. Active RhoA also recruits anillin to the equatorial cortex, where it can bind to actin, myosin, phospholipids, microtubules, among other proteins. ERM proteins can also respond to RhoA to help stabilize F-actin, while septins are recruited by anillin. Once the ring assembles, ingression occurs to pinch in the membrane and subsequently forms a midbody as it condenses around the central spindle microtubules. The midbody then acts as the site of abscission, the final stage of cell division. Abscission requires well-orchestrated changes in involved regulators and endocytic trafficking to physically seal the daughter cell membranes (Schiel & Prekeris, 2013). During this final act, ring components are shed, depolymerized or sequestered by the nucleus making way for new components including regulators of vesicle delivery (Steigemann & Gerlich, 2009; Pham et al., 2025). Some components, like anillin, MKLP1 and Cyk4, remain and interact with new partners for later steps of cytokinesis (Simon et al., 2008; Steigemann & Gerlich, 2009).

Multiple spindle-dependent and -independent pathways control ring assembly and position, which are described in the next section. Dysregulation of cytokinesis can lead to aneuploidy causing cancer or other diseases. Cytokinesis failure can also cause binucleation, which can lead to genomic instability in diseased cells, or in some healthy cells can lead to polyploidization, which is required for their differentiation and function (Chapter 1.6).

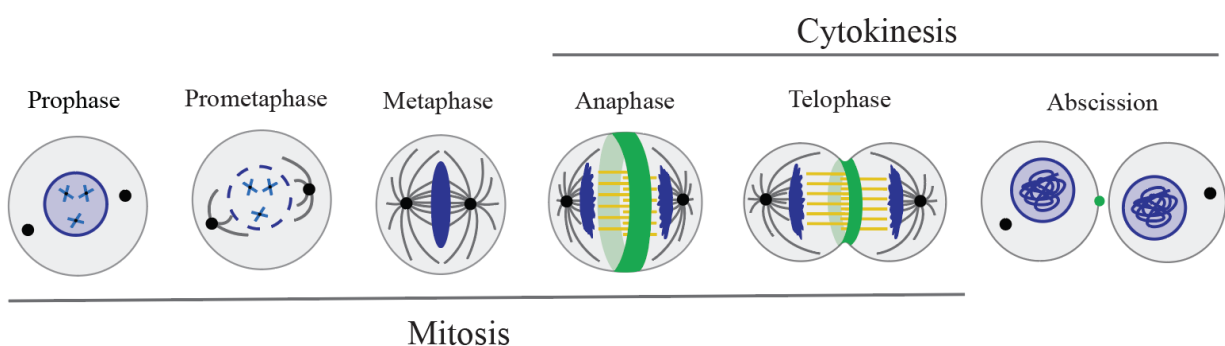


Figure 1. Mitosis and cytokinesis. During **prophase**, duplicated chromosomes (blue) condense into distinct structures, and centrosomes (black) move to opposite poles of the cell. In **prometaphase**, the nuclear envelope breaks down, and spindle forms (grey) from the centrosomes and attach to the kinetochore. In **metaphase**, chromosomes align at the metaphase plate. During **anaphase**, sister chromatids are pulled toward opposing poles, the central spindle forms (yellow), and the contractile ring (green) assembles at the equatorial cortex. In **telophase**, the nuclear envelope re-forms, and the ring ingresses then transitions into a midbody with the microtubules. Finally, **abscission** severs on one or both sides of the intercellular bridge, physically separating the daughter cells.

1.3 Pathways regulating cytokinesis

1.3.1 Spindle-dependent pathways

As described above, the anaphase spindle consists of the central spindle that forms between segregating chromosomes and astral microtubules that emanate from the centrosomes toward the polar cortex (Fig. 2A) (Mishima et al., 2002; Glotzer, 2009). Live imaging of microtubule plus ends and regrowth assays revealed that central spindle microtubules initially arise independently during anaphase and depends on the activity of HURP and the augmin complex for nucleation (Uehara & Goshima, 2010). These microtubules are initially bundled by PRC1 and Kif4, which then recruits centralspindlin and the Chromosomal passenger complex (CPC) for further organization (Mishima et al., 2002; Glotzer, 2009; Lee et al., 2012; Basant et al., 2015). These distinct microtubule networks—astral and central spindle—create separate but coordinated signals that determines the site of furrow formation in *C. elegans* and cultured human cells (Dechant and Glotzer, 2003; Bringmann & Hyman, 2005; Lewellyn et al., 2010; van Oostende-Triplet et al., 2014). Selective perturbation of either asters or the central spindle supports a two-step model in which astral microtubules first restrict cortical contractility at the poles, while midzone-derived signals subsequently specify the cleavage plane (Dechant and Glotzer, 2003; Bringmann & Hyman, 2005; Lewellyn et al., 2010; van Oostende-Triplet et al., 2014).

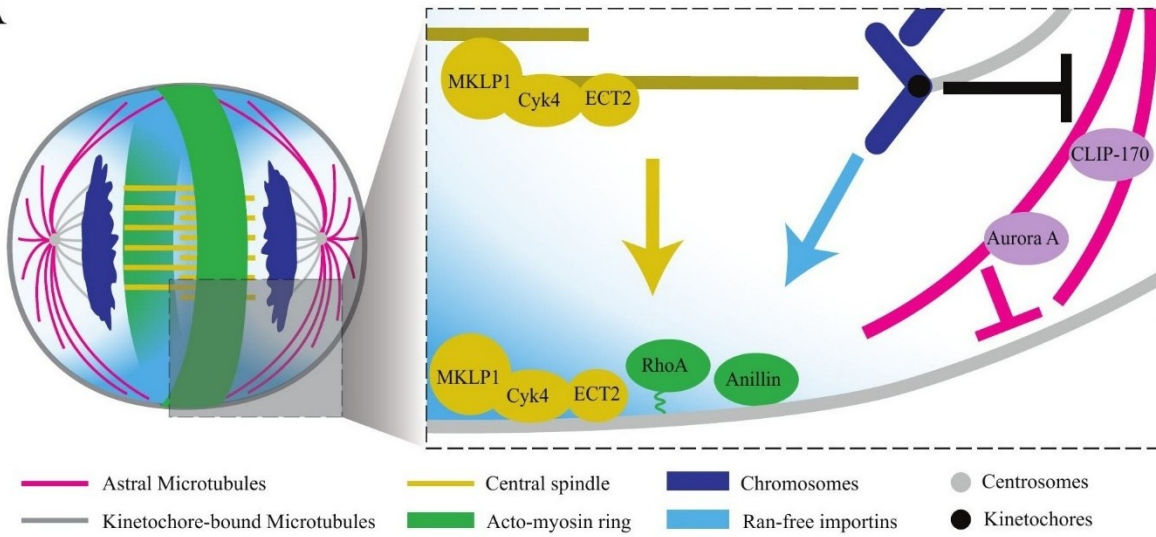
The central spindle functions as a signaling hub that directs activation of RhoA (Fig. 2A and B). As described previously, active RhoA (RhoA-GTP) generates actomyosin filaments and promotes the recruitment of ring regulators including anillin. Centralspindlin is composed of a kinesin-6 motor (MKLP1) and the RhoGAP Cyk4 (MgcRacGAP), and Cyk4-binding to Ect2 is required for ring assembly (Mishima et al., 2002; Somers & Saint, 2003; Yüce et al., 2005; Bement et al., 2005; Kamijo et al., 2006). As described previously, a cascade of kinase-mediated events ensures that this pathway is activated precisely at anaphase. Cdk1 phosphorylation negatively regulates Ect2 via promoting intramolecular interactions that prevent accessibility to binding partners and RhoA (Saito et al., 2004; Niiya et al., 2006). Polo-like kinase 1 (Plk1) phosphorylation of Cyk4 is required to bind to the N-terminal BRCT domains of Ect2 through electrostatic interactions, which may help to relieve this autoinhibition (Petronczki et al., 2007; Wolfe et al., 2009; Gómez-Cavazos et al., 2020). In support of this, inhibition of Plk1 prevents Ect2's localization to the central spindle and causes cytokinesis failure, although MKLP1 localization remains intact (Petronczki et al., 2007). More recently, Plk1 was shown to exert dual functions: in addition to activating centralspindlin, it limits PRC1 activity to release centralspindlin from the central spindle to the overlying cortex where it can regulate Ect2 (Adriaans et al., 2019). In addition, Aurora B kinase, a component of the CPC regulates centralspindlin oligomerization and membrane association by phosphorylating and removing 14-3-3, a negative regulator (Douglas et al., 2010; Basant et al., 2015). Importantly, Cyk4 and Ect2 generation of active RhoA requires membrane localization, while the central spindle is dispensable. Ect2 localizes to the membrane through its pleckstrin homology (PH) domain and polybasic cluster (PBC), and loss of these domains or point mutations in the PH domain abolishes Ect2's membrane localization, reduces its ability to generate active RhoA and prevents ring assembly (Su et al., 2011; Frenette et al., 2012; Kotýnková et al., 2016). The C1 domain of Cyk4 also mediates lipid binding and

helps to anchor centralspindlin to the plasma membrane—but this interaction may play a stronger role in later stages of cytokinesis (Lekomtsev et al., 2012).

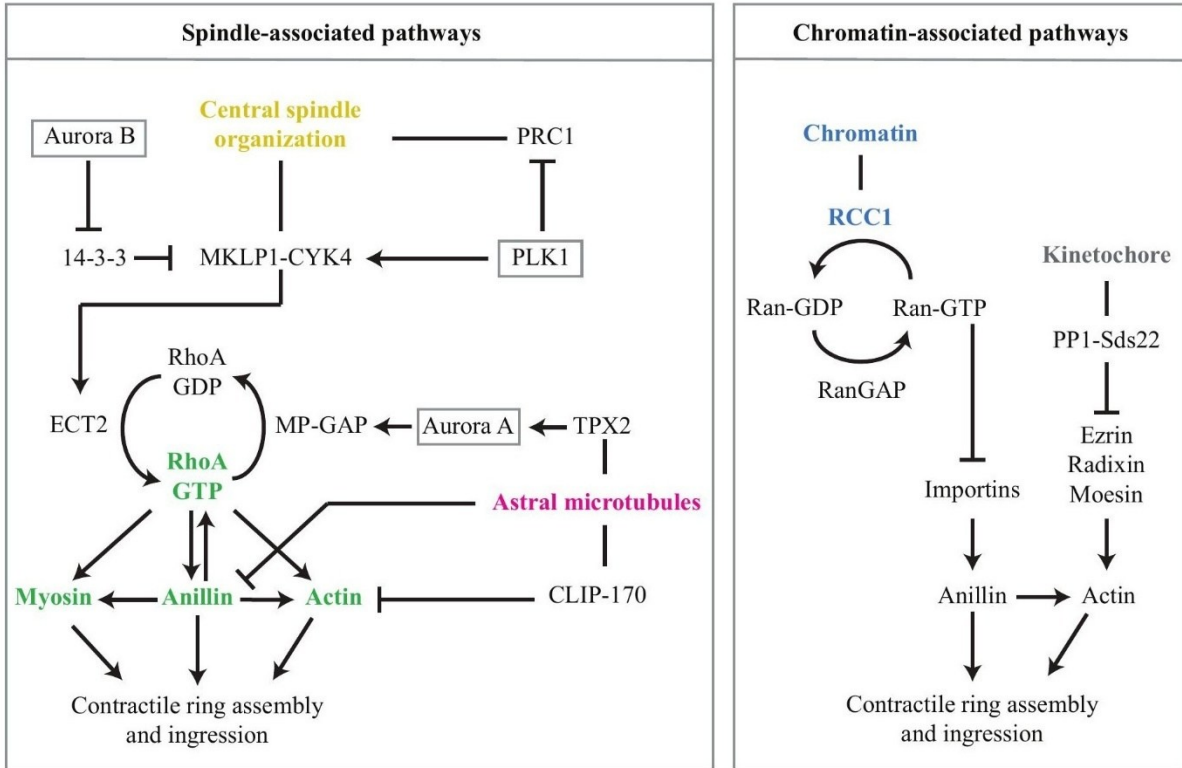
Astral microtubules also define the location of the ring by inhibiting contractility at the cell poles (polar relaxation; Fig. 2A and B). In *C. elegans* embryos, laser ablation of the asters induces ectopic furrow ingression, whereas delaying aster separation causes a delay in cytokinesis and aberrant cortical accumulation of anillin and myosin II (Bringmann & Hyman, 2005; Lewellyn et al., 2010). Similarly, partial depletion of γ -tubulin, which reduces aster formation, results in monopolar spindles whose microtubule tips locally clear myosin from the cortex (Werner et al., 2007). In mammalian cells, depletion of astral microtubules broadens the cortical zones enriched in RhoA, myosin II, and anillin, while enhancing aster length has the opposite effect, narrowing these zones and restricting contractility to the equator (Murthy & Wadsworth, 2008; Zanin et al., 2013; van Oostende-Triplet et al., 2014). Together, these findings demonstrate that asters exert long-range inhibitory influences on the cortex, thereby complementing midzone-derived activation signals to define the cleavage plane with high spatial precision. Despite their importance, the molecular mechanisms by which astral microtubules mediate cortical inhibition are only beginning to emerge. One key component is MP-GAP, a conserved Rho GTPase-activating protein that limits RhoA activity during mitosis and cooperates with microtubule asters to spatially confine active RhoA at the equator (Zanin et al., 2013). MP-GAP activity is itself regulated by Aurora A kinase, which relieves its autoinhibition; Aurora A, in turn, is activated by the spindle assembly factor TPX2, a microtubule-associated protein essential for aster organization (Mangal et al., 2018; Wolff et al., 2023). These findings link the classical polar relaxation pathway to the centrosome- and aster-based signaling network centered on Aurora A. Additional mechanisms may further reinforce this inhibition at the poles. Previous studies revealed also that anillin interacts with microtubules, suggesting that this interaction could compete with its ring anchoring function (van Oostende-Triplet et al., 2014; Bareja et al., 2025). Moreover, astral microtubules can modulate actin organization by delivering CLIP-170, which locally inactivates the formin DIAPH1, leading to depletion of γ -actin networks at the poles (Chen et al., 2021). These regulatory layers converge to dampen cortical contractility where astral microtubules contact the plasma membrane.

Together, these studies establish that the central spindle serves as a dynamic signaling platform that links microtubule organization to cortical contractility. Through the coordinated action of PRC1, centralspindlin, Plk1, Aurora B, and Ect2, central spindle-derived cues precisely position and activate the RhoA-dependent contractile machinery. This intricate regulatory network ensures that ring assembly is linked to spindle position, which in turn also regulates chromosome segregation and the distribution of organelle components associated with the centrosomes. In addition, astral microtubules establish inhibitory gradients that shape the cortical landscape during cytokinesis. By locally suppressing RhoA activity and contractile components at the poles while central spindle-derived pathways activate RhoA at the equator, cells achieve a robust spatial balance of forces. This complementary interplay between astral and midzone signaling ensures that cleavage furrow ingression occurs precisely between the segregating chromosome masses, thereby safeguarding the fidelity of cell division in metazoans.

A



B



C

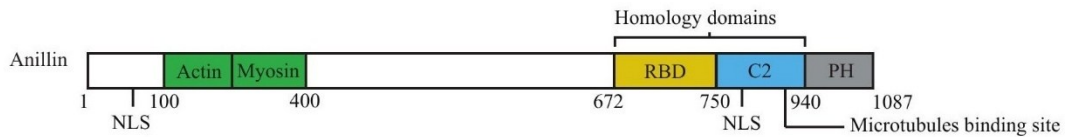


Figure 2. Pathways regulating cytokinesis. (A) The schematic shows a cell at anaphase, where astral microtubules are represented in pink, the central spindle is in yellow, chromatin is in dark blue, the contractile ring is in green and Ran-free importins are in cyan. The left box is a zoomed-in representation of how contractile proteins are recruited to the cortex by the different pathways. (B) The components of the different pathways are shown. **Spindle-associated pathways:** MKLP1-Cyk4 and PRC1 bundle microtubules. Aurora B phosphorylates and inactivates 14-3-3 to relieve inhibition of centralspindlin. Plk1 phosphorylates Cyk4 for Ect2-binding to activate RhoA for ring assembly. Astral microtubules regulate Aurora A via TPX2, which in turn regulates MP-GAP to downregulate RhoA. CLIP-170 also regulates polar actin via formin DIAPH-1. Anillin binding to the microtubules also causes its removal/prevents recruitment to the polar cortex. **Chromatin-associated pathways:** Kinetochore-associated PP1-Sds22 downregulates ERM (ezrin, radixin and meosin) at the poles, clearing F-actin. Chromatin-associated RCC1 activates Ran and binds to importins, while importins are free to bind to anillin equatorially and promote its recruitment to the equatorial cortex. (C) A schematic shows the structure of human anillin with binding domains as indicated. RBD: RhoA-GTP Binding Domain. PH: Pleckstrin Homology domain. NLS: Nuclear Localization Signal.

1.3.2 Spindle-independent pathways

In addition to microtubule-derived cues, signals originating from cortically localized proteins and chromatin also contribute to cytokinesis. Remarkably, cleavage furrow formation can occur even in the absence of microtubules. Indeed, cells treated with microtubule-depolymerizing agents still exhibit cortical contractility and, in some cases, successful ingression (Canman et al., 2000; Cabernard et al., 2010). In *Drosophila* neuroblasts, for example, the cortical Pins complex orchestrates myosin recruitment to the basal cortex, driving asymmetric contractility independently of the spindle (Cabernard et al., 2010).

Beyond localized contractility, cortical flows—actomyosin-driven surface movements—play a central role in positioning the contractile ring. These flows transport and concentrate actin and myosin toward the equator, promoting the local assembly of a constricting ring (Reymann et al., 2016; Bement et al., 2008). In *C. elegans* and *Drosophila* embryos, polarized cortical flows have been shown to align actin filaments and bias furrow placement, often acting in concert with spindle-derived signals (Munro, 2007; Roubinet et al., 2017; Oon et al., 2019). Moreover, asymmetries in cortical flow or contractility can shift the furrow position during asymmetric divisions, emphasizing the mechanical contribution of cortical dynamics to cytokinetic geometry (Connell and Cabernard, 2011; Sehring et al., 2015).

A crucial element reinforcing these contractile and positional cues is anillin, a conserved scaffolding protein that stabilizes active RhoA and integrates feedback within the cortical network. Anillin interacts with actin, myosin, RhoA, septins, and membrane phospholipids (Fig. 2C), and is required for proper ring positioning across multiple metazoan systems (Field and Alberts, 1995; Piekny and Glotzer, 2008; Piekny and Maddox, 2010; Budnar et al., 2019). Its recruitment to the cortex depends on active RhoA, to which it binds cooperatively along with phospholipids (Piekny and Glotzer, 2008; Sun et al., 2015). Once localized, Anillin promotes the formation of lipid nanodomains and enhances the membrane retention of RhoA, thereby amplifying local RhoA activity and facilitating downstream activation of effectors such as Rho kinase that drive actomyosin assembly (Budnar et al., 2019). This positive feedback loop between Anillin and RhoA not only stabilizes the contractile machinery but also couples biochemical signaling with mechanical flows to ensure robust furrow positioning.

Chromatin can also influence cortical polarity (Fig. 2A and B). The proximity of DNA to the cortex modulates the distribution of cytoskeletal regulators: the kinetochore-associated PP1–Sds22 phosphatase complex promotes local actin clearance by dephosphorylating the actin crosslinker Moesin when kinetochores approach the cell cortex (Rodrigues et al., 2015). Similarly, chromatin proximity has been shown to direct the cortical localization of Myosin II and Anillin (Kotadia et al., 2012; Kiyomitsu and Cheeseman, 2013; Beaudet et al., 2017). The underlying molecular mechanism involves the Ran-GTP/importins system, which will be discussed in the following section.

1.4 Ran-dependent pathways

1.4.1 Ran and importins

The Ran machinery is best known for its role in nuclear transport (Chang et al., 2021). In interphase, active Ran (Ran-GTP) is enriched in the nucleus because the guanine nucleotide exchange factor (GEF) for Ran, RCC1, is tethered to histones, while inactive Ran (Ran-GDP) is generated by RanGAP in the cytosol. In the cytoplasm, importins bind to proteins with nuclear localization signals (NLSs) and transport them through the nuclear pore complex into the nucleus, where Ran-GTP binds to importins and releases the cargo. Importins recognize a wide range of NLS motifs with variable affinities. A heterodimer of importin- α and - β typically bind to classical mono- or bipartite sequences, although each protein can also bind to NLS as a monomer (Dingwall and Laskey, 1992; Palmeri and Malim, 1999; Lee et al., 2006). While importin- β 1 is known as the major isoform of the importin- β family, there are at least seven importin- α isoforms that are partly redundant but display distinct subfamily-specific preferences (Oka et al., 2018).

Importins also modulate other cellular functions. Importins can function as chaperones, suppressing nonspecific interactions of aggregation-prone proteins and thereby preventing the formation of aberrant liquid–liquid phase separations. For instance, importins protect ribosomal proteins, ribonucleoproteins, and histones from aggregation, ensuring protein solubility and functional availability (Jäkel et al., 2002). Importins are also key players during mitosis. In metazoans, which undergo open mitosis, the nuclear envelope disassembles at the onset of cell division, but the Ran gradient persists because RCC1 remains bound to chromatin (Moore et al., 2002; Kaláb et al., 2008; Furuta et al., 2016). This creates an inverse gradient of importins where they are bound to active Ran near chromatin and are free to bind to NLS-containing proteins away from chromatin. This distribution transforms importins into molecular sensors of chromatin position, coordinating the recruitment of factors required for spindle assembly, nuclear envelope reformation, and contractile ring formation (Kaláb et al., 2008; Chang et al., 2021; Beaudet et al., 2017; Beaudet et al., 2020). Thus, importins function as central hubs that integrate spatial information from the Ran gradient for various cellular processes.

1.4.2 Spindle assembly and scaling

The mitotic spindle is a bipolar structure composed of microtubules nucleated primarily from centrosomes, which align and segregate chromosomes during mitosis. Microtubules are also nucleated and polymerized around chromatin via Ran-GTP, which establishes a spatially restricted region of spindle assembly factors (SAFs; Fig. 3A). The gradient of Ran-GTP in mitotic cells was first revealed by Förster resonance energy transfer (FRET) -based RANGO probes (Kaláb et al., 2006; Kaláb et al., 2010; Soderholm et al., 2011; Hasegawa et al., 2013). The RANGO probes contain an importin- β -binding (IBB) domain sandwiched between two fluorescent proteins that transfer photons when in close proximity (Kaláb et al., 2010; Soderholm et al., 2011). FRET occurs when Ran-GTP levels are high and importin- β cannot bind to the IBB domain (Kaláb et al., 2010; Soderholm et al., 2011). However, FRET cannot occur when Ran-GTP levels are low and importin- β is free to bind to the IBB domain (Kaláb et al., 2010; Soderholm et al., 2011). Studies using RANGO probe variants

revealed that the steepness of the Ran-GTP gradient in metaphase varies across cell types (Hasegawa et al., 2013). In hypotriploid HeLa cells, the Ran gradient is steeper than in diploid HFF-1 fibroblasts (Hasegawa et al., 2013). This steeper gradient correlates with changes in spindle morphology and mitotic progression (Hasegawa et al., 2013). Experimentally reducing the gradient's steepness in HeLa cells caused severe chromosome congression and segregation defects, whereas chromosomal gain in HFF-1 cells increased gradient steepness (Hasegawa et al., 2013). These findings suggest that cells with higher ploidy rely on a steeper Ran gradient to ensure accurate mitotic progression. Importins can bind to and inhibit the function of SAFs, which are released by Ran-GTP near chromatin (Fig. 3A). These factors are responsible for microtubule nucleation, stabilization, and organization. Among the best-characterized SAFs regulated by importins are TPX2, NuMA, and XCTK2 (Kaláb and Heald, 2008; Ozugergin and Piekny, 2021).

Active ran mediates microtubule nucleation at the kinetochore through TPX2 (Targeting Protein for Xklp2), which facilitates recruitment of the remaining factors to microtubules (Valdez et al., 2023; Fig. 3A). Briefly, importin- β sequesters TPX2 in the cytoplasm, preventing its premature association with microtubules. Upon Ran-GTP binding to importin- β near chromatin, TPX2 is released and stimulates microtubule nucleation by recruiting nucleation complexes to already formed microtubules. TPX2 also regulates Aurora A kinase activity, ensuring robust spindle microtubule stabilization (Gruss and Vernos, 2004). Interestingly, importin- β localizes to the spindle, which is abolished by TPX2 knock-down suggesting that importin- β localization is dependent on its target (Ciciarello et al., 2004). Although it is not clear which importin- β isoform was used for this study, immunostaining of importin- β 1 revealed that it localizes to the spindle (Di Francesco et al., 2018). Another key factor is the kinesin XCTK2 (kinesin-14), which is required for spindle pole organization. A study revealed that importin α/β -binding prevents binding to microtubules (Ems-McClung et al., 2004). Ran also controls spindle orientation by regulating astral microtubule attachment to the cell cortex (Fig. 3A). Importin- α can be post-translationally modified by palmitoylation, which inserts the protein into the plasma membrane (Brownlee and Heald, 2019). At the polar cortex this pool of importin- α can interact with NuMA to capture and stabilize astral microtubules, although NuMA is also part of the dynein-dynactin complex and it is not clear if this is an independent role for NuMA (Merdes et al., 1996; Sutton et al., 2025).

As above, by regulating proteins such as TPX2 and XCTK2, which control microtubule nucleation and spindle pole cohesion, the Ran-gradient may regulate many aspects of the spindle to coordinate its size with other aspects of the cell (Hazel et al., 2013; Rieckhoff et al., 2019). Across species, spindle length scales with cell diameter in large embryonic systems, such as *C. elegans* and *Xenopus laevis*, but this scaling relationship diminishes in smaller mammalian somatic cells (15–30 μ m), where spindle size appears constrained by intrinsic biochemical control rather than physical cell volume (Rieckhoff et al., 2019). The Ran-GTP gradient is proposed to be one of the key biochemical mechanisms underlying spindle size regulation. For example, membrane-sequestered importin- α also controls the levels of importins in the cytosol to scale spindle size in *Xenopus laevis* (Brownlee and Heald, 2019).

In parallel, Ran signaling has also been implicated in controlling nuclear size and chromosome compaction. Jevtić et al. (2019) demonstrated that the Ran system couples nuclear import capacity to nuclear size: higher Ran-GTP activity promotes nuclear expansion by facilitating import of nuclear structural and functional components, whereas disruptions in Ran or importin function reduce nuclear growth. This suggests that, just as Ran gradients spatially regulate spindle assembly during mitosis, they may also define nuclear size and composition during interphase through their control of nucleocytoplasmic transport (Jevtić et al., 2019; Ladouceur et al., 2015).

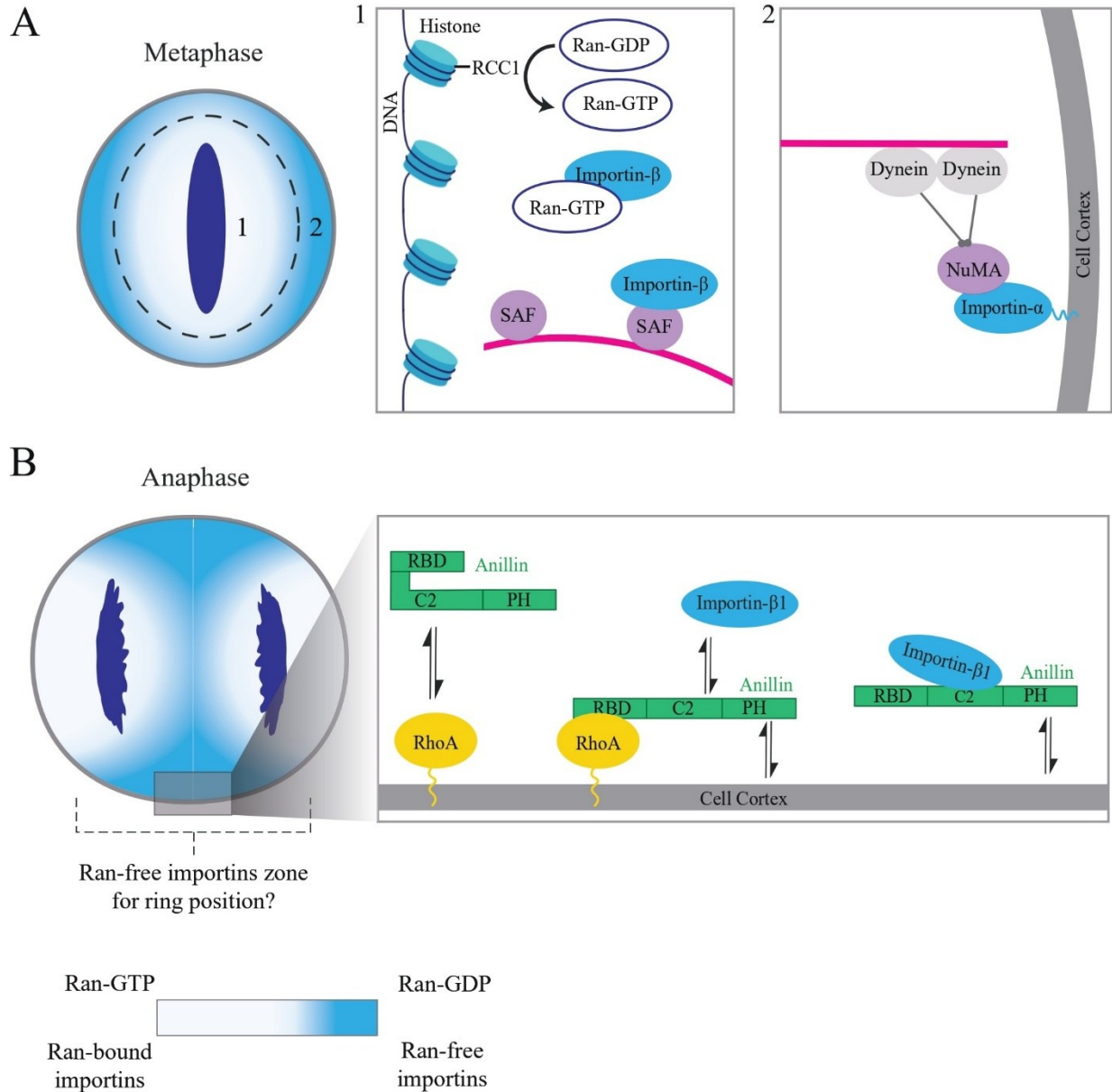


Figure 3. Ran regulates spindle assembly and ring position. (A) The cartoon shows a cell at metaphase. Chromatin is in dark blue, and Ran is shown as a gradient from white (high Ran-GTP) to cyan (low Ran-GTP). The black dotted circle represents the limit defined by the Ran-bound importins gradient for spindle assembly. The right boxes show assembly (1) and orientation (2) for zoomed-in portions of the spindle (1). Near chromatin, RCC1 activates Ran-GTP and spindle assembly occurs due to the release of spindle assembly factors (SAFs) from importin-β. Palmitoylated importin-α anchors NuMA to the cortex to stabilize microtubules via dynein. (B) A cartoon shows the Ran gradient in anaphase, where a depleted zone of Ran-GTP between the segregating chromosomes could position the ring. The right box shows how importin-β1 is suspected to interact with anillin for its cortical recruitment.

1.4.3 Ring assembly

Our group and others revealed that Ran functions as a chromatin-sensing pathway for ring positioning (Fig. 2B and 3B) (Kiyomitsu and Cheeseman, 2013; Beaudet et al., 2017; Beaudet et al., 2020). Kiyomitsu and Cheeseman (2013) found that when the anaphase spindle is mispositioned in HeLa cells, the cortex elongates to restore symmetry, thereby preventing unequal division. This response correlated with the proximity of chromatin and the local clearing of anillin from cortical regions adjacent to the nearby chromosomes. In *Drosophila melanogaster* neuronal stem cells, elongation of the membrane also occurred in response to chromatin (Kotadia et al., 2012). We subsequently showed that ring position was Ran-dependent in HeLa and baby hamster kidney (BHK) epithelial cells, and that anillin was regulated by importin-binding (Beaudet et al., 2017). In *C. elegans* embryos, changes in ploidy and depletion of RCC1 or importins induced changes in the rate of ring assembly in AB and P1 daughter cells (Ozugergin et al., 2022). These findings highlight a conserved feedback system that couples chromosome segregation with ring assembly or position, suggesting that the Ran-GTP gradient may play a central role in defining the division plane.

Evidence for the involvement of the Ran pathway in anillin regulation comes from studies by our group perturbing Ran activity. Reduction of active Ran levels at the cortex using a temperature-sensitive allele of RCC1 caused anillin to ectopically localize to the polar cortex in BHK cells (Beaudet et al., 2017). In addition, targeting constitutive active Ran to the equatorial membrane in HeLa cells disrupted contractile ring assembly and caused the ring to oscillate similar to anillin depletion (Beaudet et al., 2017). These observations imply that local changes in Ran-GTP concentration modulate anillin dynamics at the cortex, possibly through the release or sequestration of importins.

The Ran gradient regulates the ring by spatially defining where importins could regulate contractile proteins for ring assembly (Fig. 3B). Anillin contains two NLSs that mediate distinct interactions with importins (Fig. 2C). The N-terminal NLS is required for nuclear localization during interphase and binds to importin- β 2 (Chen et al., 2015). We found that a second NLS, non-classical, in the C-terminal region located in the C2 domain, which is adjacent to a binding site for $PI_{4,5}P_2$, is not involved in nuclear transport but rather controls anillin's function in anaphase (Sun et al., 2015; Beaudet et al., 2020). Importin-binding is required for anillin's recruitment to the equatorial cortex during anaphase and point mutations that disrupt importin-binding delay anillin's recruitment and causes cytokinesis failure in HeLa cells (Beaudet et al., 2017; Beaudet et al., 2020). Notably, the C2 NLS is autoinhibited and prevented from binding to importins until after active RhoA binds to the neighboring RBD, temporally coupling importin-binding to anaphase (Beaudet et al., 2020). Mutations at the RBD-C2 interface phenocopy importin-binding mutants and combining these mutants completely blocks ring assembly (Beaudet et al., 2020). These findings support a model where importin-binding to the C2 is required to retain anillin in an accessible conformation for phospholipid-binding. By modulating importin-cargo interactions in a position-dependent manner, the Ran gradient may define cortical zones permissive to contractile protein accumulation during anaphase, coupling ring assembly with chromosome segregation. Despite these advances, the function of importins during anaphase remains poorly understood, and elucidating how their

regulatory roles extend beyond nuclear transport could reveal additional mechanisms coupling nuclear and cytoskeletal organization during cell division.

1.5 Cytokinesis diversity

1.5.1 Diversity among animal cells and human cells

Extensive knowledge of cytokinesis was generated using a variety of models revealing how mechanisms regulating cytokinesis are conserved but also highlight diversity among organisms and within different human cell lines.

Differences in the composition of ring components also influence ring kinetics. Myosin isoforms vary in their motor and crosslinking activities, while different actin isoforms can assemble into branched or unbranched filaments through distinct formins (Chen et al., 2017; Wang et al., 2020; Chen et al., 2021). In *C. elegans*, larger cells contain higher levels of contractile proteins than smaller cells, suggesting that variability in cytokinesis efficiency within an organism can arise from differences in protein concentration (Carvalho et al., 2009). Moreover, germline-fated cells in *C. elegans* embryos display slower ring assembly and contain less linear F-actin and myosin than somatic cells, operating closer to threshold requirements (Davies et al., 2018; Ozugergin et al., 2022). Requirements for key cytokinetic proteins also differ across species—for instance, myosin's necessity in cytokinesis varies between mice and *C. elegans* (Ma et al., 2012; Osório et al., 2019). Additionally, our group recently revealed that anillin ring breadth and ingression varies in different human cell lines as well as differences in anillin requirement (Husser et al., 2022; Kevin Larocque, unpublished observations).

Asymmetric ring ingression is observed in various cell types and species, but the underlying mechanism is not clear. A model is proposed where curvature-mediated filament alignment breaks ingression symmetry, speeding up ring closure while conserving energy (Dorn et al., 2016). Anillin and septins are also known to play a role in asymmetrical ring ingression (Maddox et al., 2007; Chan et al., 2019). In embryonic cells in mice and *C. elegans*, the ring ingresses asymmetrically (Maddox et al., 2007; Chan et al., 2019; Paim and FitzHarris, 2022). Among HeLa, HepG2, and HCT 116 cells, which are human cancer cell lines, the ring ingresses more symmetrically, while it is asymmetric in canine kidney epithelial (MDCK) cells (Husser et al., 2022).

Differences in upstream regulators contribute to cell type-specific variations in contractile ring dynamics during cytokinesis. The RhoGEF Ect2 and its ortholog Pbl localize to both microtubules and the equatorial cortex in HeLa and *Drosophila* cells, while in *C. elegans* zygotes, ECT-2 is mainly cortical (Prokopenko et al., 1999; Yüce et al., 2005; Verma and Maresca, 2019; Gomez-Cavazos et al., 2020). Both Ect2 and its partner Cyk4 require membrane association to activate RhoA (Su et al., 2011; Frenette et al., 2012; Lekontsev et al., 2012; Basant et al., 2015), and their cortical recruitment may be particularly critical when the central spindle is distant from the cortex.

Anillin also exhibits cell type-specific localization and function. It is cytosolic in early *C. elegans* and *Drosophila* embryos, but is nuclear in differentiated *Drosophila* (e.g., S2) and human (e.g., HeLa) cell lines (Piekny and Maddox, 2010). While its depletion disrupts cytokinesis in many systems, *C. elegans* neuroblasts, *Xenopus* embryos, and *Drosophila* S2 cells, it is dispensable in the *C. elegans*

zygote, indicating variable functional thresholds or compensatory mechanisms (Maddox et al., 2005; Straight et al., 2005; Hickson and O'Farrell, 2008; Piekny and Glotzer, 2008; Fotopoulos et al., 2013; Reyes et al., 2014). This variability in sensitivity to anillin depletion has been also reported in human cell lines (Kevin Larocque, unpublished observations). Despite viable anillin-deficient animals, such as Dalmatians with early nonsense mutations (Holopainen et al., 2017), residual or alternatively spliced isoforms may sustain partial function. Anillin contributes to multiple stages of cytokinesis including ring positioning, constriction, and midbody formation. Anillin also regulates RhoA signaling. Previous studies showed that RhoA's equatorial localization decreased after anillin depletion in HeLa cells (Piekny and Glotzer, 2008), which could be due to a role for anillin in scaffolding RhoA effectors (Budnar et al., 2019) or by promoting inactivation through feedback loops in *C. elegans* (Rehain-Bell et al., 2017; Bell et al., 2020). Beyond scaffolding, anillin can crosslink and slide actin filaments independently of myosin (Kucera et al., 2021), underscoring its versatile role in generating and stabilizing contractile forces.

Altogether, these findings emphasize that cytokinesis, although built upon a conserved molecular framework, is a highly adaptable process fine-tuned to the geometry, size, and lineage of each cell type. Since our knowledge of cytokinesis in human cells is largely from studies using diseased and/or transformed cell lines, it is critical to use more meaningful cell types to reveal how the mechanisms regulating cytokinesis change with cell type. In this thesis, I shed light on mechanisms that regulate cytokinesis in induced pluripotent stem cells (iPSCs), providing a foundation for others to build on. Understanding this adaptive landscape will be key to elucidating how cells maintain division precision under varying developmental, mechanical, and pathological conditions.

1.5.2 Stem cell division

Pluripotent stem cells are self-renewing, undifferentiated cells capable of giving rise to any cell type of the body. Two major types of pluripotent stem cells are used to study tissue development: embryonic stem cells (ESCs), derived from the inner cell mass of blastocyst-stage embryos (Lee and Lee, 2011), and iPSCs, which are reprogrammed from adult somatic cells into a pluripotent state (Takahashi et al., 2007). iPSCs closely mimic the biological and functional properties of ESCs, although subtle transcriptional and epigenetic differences have been reported (Bilic and Izpisua Belmonte, 2012; Bock et al., 2011; Puri and Nagy, 2012; Shi et al., 2017). Because iPSCs can be generated from any individual without the ethical concerns associated with using embryonic material, they have become an indispensable tool for biomedical research. These cells possess a stable genome, robust proliferative capacity, and the ability to differentiate into derivatives of all three germ layers under controlled in vitro conditions (Williams et al., 2012; Shi et al., 2017).

Despite intensive study of stem cell pluripotency and differentiation, very little is known about cytokinesis in healthy human stem cells or tissues. Stem and progenitor cells sustain tissue homeostasis by balancing self-renewal and differentiation. This balance is achieved through two division modes: symmetric divisions, which produce two identical daughter cells and expand the stem cell pool, and asymmetric divisions, which generate one stem cell and one differentiated progeny, thereby promoting cell-type diversity (Morin and Bellaiche, 2011). The decision between

symmetric and asymmetric division is critical for development and regeneration and is often modulated by intrinsic polarity cues and extrinsic niche signals.

There is little knowledge of cytokinesis during cell differentiation. Recent work has begun to bridge this gap. In *C. elegans*, our group and others characterized key differences between AB and P1 cells, while another group compared cytokinesis between ABa, ABp, P2, and EMS cells, revealing differences in ring composition, ring kinetics, and gene requirements (Ozugergerin et al., 2022; Connors et al., 2024; Mauro et al., 2024; Pimpale et al., 2020). Our group also established iPSC lines expressing fluorescently tagged cytokinetic proteins, enabling direct comparison of cytokinesis in stem cells versus HeLa cells (Husser et al., 2023). This analysis revealed striking structural differences: iPSCs had narrower contractile rings, robust astral microtubules, and a less prominent central spindle compared to HeLa cells, suggesting they rely on distinct mechanisms for cytokinesis. In another example, mesenchymal stem cells were found to complete cytokinesis even in suspension, which contrasts with the strong adhesion dependence observed in most somatic cell lines (Rani et al., 2025). These findings point to potential adaptations of the cytokinetic machinery that support the unique division modes and physical constraints of stem cells. Additionally, a previous study revealed that the outer cells of a mouse embryo have apical polarity and slower ring ingression compared to inner cells, which is independent of cell fate, suggesting that other parameters such as positional information based on cues within developing tissues could also influence cytokinesis (Paim et al., 2022). Indeed, studies of cytokinesis in epithelial cells within tissues of *Xenopus* or *Drosophila* revealed extrinsic mechanisms like tension and mechanical forces from neighbor cells that influence ring position and kinetics (Pinheiro & Bellaïche, 2017; di Pietro et al., 2023; Landino et al., 2025).

Understanding how cytokinetic mechanisms vary in stem cells and during differentiation will not only clarify how tissues maintain homeostasis but may also reveal vulnerabilities that distinguish normal renewal from pathological proliferation.

1.6 Cytokinesis failure and polyploidy: wanted or unwanted?

Polyploidy is a process that contributes to tissue differentiation and functional specialization in several organs, notably the liver, heart, placenta and bone marrow (Lacroix and Maddox, 2012; Fig. 4). In these contexts, polyploidization is thought to provide adaptive advantages, such as enhanced metabolic capacity or increased resistance to stress. Polyploid cells can arise through diverse mechanisms, including cell fusion, endoreduplication, or cytokinesis failure. For instance, megakaryocytes, specialized bone marrow cells, are suspected to fail cytokinesis through Rho regulation (Lacroix and Maddox, 2012). Another example is hepatocytes, which can have up to 16n ploidy and represent 70% of the liver cells (Gentric and Desdouets, 2014). The generation of polyploidization through cytokinesis failure happens during post-natal development, then the number of polyploid cells increases slowly until the age of 50 years, where the process intensifies (Kudryavtsev et al., 1993). This process seems to respond to physiological conditions. In rat liver, low insulin levels cause less polyploid hepatocytes correlating with suckling-to-weaning transition (Celton-Morizur et al., 2010). The same group also showed that cytokinesis fails due to defective ring assembly: weak microtubules are observed as well as poor recruitment of RhoA, actin, myosin and

Cyk4 at the cortex (Margall-Ducos et al., 2007; Gentric and Desdouets, 2014). However, a more recent study used spheroids to better mimic the *in vivo* microenvironment and found that cytokinesis failure can occur after ingression through the loss of anchoring proteins (Darmasaputra et al., 2024). Additionally, Anillin is up-regulated in hepatocellular carcinoma (HCC) cells, and its depletion blocked tumour growth in mice, suggesting that HCC cells escape their fate by completing cytokinesis (Zhang et al., 2017). Thus, the mechanism causing cytokinesis failure in hepatocytes in human livers remains to be determined but could be critical for designing novel therapies to treat HCC.

Conversely, when cytokinesis failure occurs in diploid somatic cells, causing binucleation and whole-genome duplication, this could lead to disease (Fig. 4). Tetraploid intermediates were once considered terminal or senescent, but growing evidence shows that cells could re-enter the cell cycle (Uetake & Sluder, 2004). Upon further divisions, their supernumerary chromosomes and centrosomes promote mitotic errors, resulting in poly-aneuploidy and chromosomal instability (CIN) which can lead to cancer (Ganem et al., 2009; Lacroix and Maddox, 2012). Beyond cancer, cytokinesis failure has been linked to blood disorders and developmental abnormalities, underscoring its broad physiological impact (Lacroix and Maddox, 2012).

Current anti-cancer therapies largely target rapidly dividing cells by interfering with mitotic processes. However, their lack of specificity leads to collateral toxicity in normal tissues, while paradoxically sparing some proliferative cells for reasons that remain unclear (Lacroix and Maddox, 2012). These limitations emphasize the need for more selective therapeutic strategies. Cytokinesis components have emerged as promising candidates: for example, anillin depletion reduces liver cancer progression and can even promote tissue recovery toward a more differentiated phenotype (Zhang et al., 2018; Chen et al., 2022). Anillin appears particularly attractive as a target because it is largely dispensable for other cellular processes, though it may also play a role in translational regulation (Naydenov et al., 2021; Cao et al., 2025). Notably, not all cell types respond equally to anillin depletion (Kevin Larocque, unpublished observations), raising the question of whether healthy tissues might tolerate cytokinetic inhibition differently.

A deeper understanding of cytokinesis mechanisms across healthy tissues is therefore critical for identifying tissue-specific vulnerabilities. Stem cells provide a valuable model system for studying cytokinesis in the context of differentiation, offering insight into how division mechanics adapt to cell fate and developmental state.

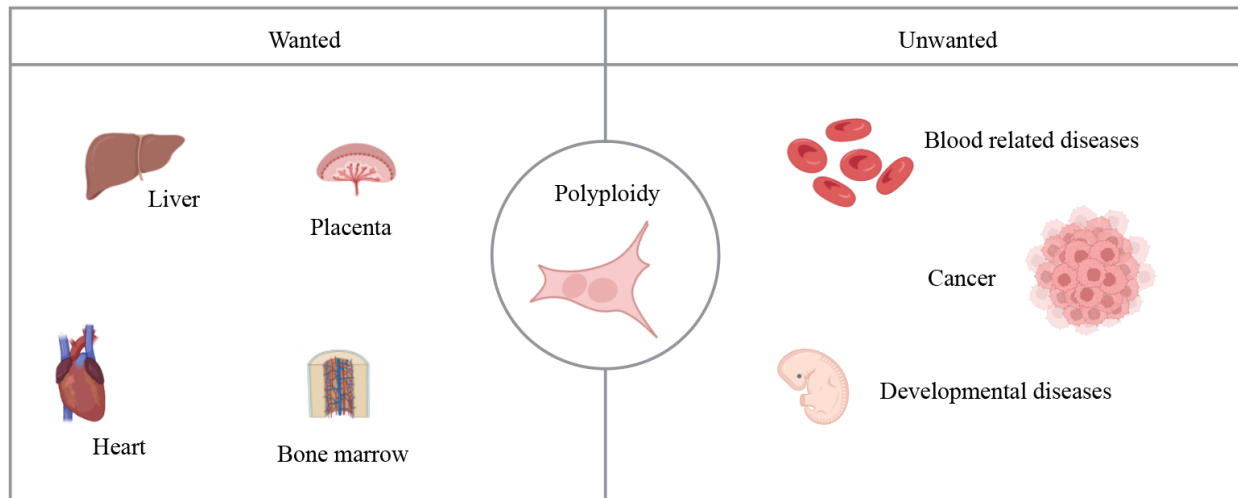


Figure 4. Polyploidization in cells during normal tissue development and pathologies. A schematic shows that polyploidization occurs in cells in the liver, bone marrow, heart and placenta, but also arises in cells causing blood related diseases, developmental diseases and cancers.

1.7 Thesis overview

Cytokinesis is essential for successful cell division, to ensure proper fate determination and to prevent genomic instability. Certain pathways have been extensively studied, establishing RhoA and its cascade activation signals as the master regulator of cytokinesis. A chromatin-associated pathway helps define the division plane in some cell types, but its requirement in others could vary with ploidy and cell fate. Additionally, most of our knowledge of human cytokinesis was generated using a handful of transformed or cancer cell lines, yet our bodies contain billions of cells with diverse fates and ploidy, highlighting the need to study cytokinesis in normal, diverse cell types.

In Chapter 2, we wanted to show how the chromatin-sensing pathway works by investigating importin- β 1 function and revealing pathway requirements in two human cell lines. Ran activity changes the contractile ring machinery and point mutations in anillin that disrupt importin-binding cause changes in its localization and function, but we lacked evidence that importins respond to Ran in anaphase to mediate the changes in anillin. We hypothesized that as chromosomes segregate, a zone depleted of active Ran permits importins to be free to bind to anillin and promote its recruitment to the equatorial cortex. We also hypothesized that reliance on the chromatin sensing pathway could increase with ploidy, as the steepness of the Ran-gradient should correlate with ploidy. All together, my findings revealed that importin- β 1 indeed serves as a key mediator between Ran activity and anillin, and that the chromatin-to-cell size ratio impacts the requirement of the chromatin-sensing pathway during cytokinesis.

In Chapter 3, we uncovered mechanisms controlling iPSCs' cytokinesis. Our previous studies showed that iPSCs have prominent astral microtubules and narrow anillin localization, which differed from HeLa cells with more prominent central spindles and broad anillin (Husser et al., 2022). I hypothesized that the narrow ring in iPSCs could be caused by the astral microtubules restricting anillin, which may play a stronger role compared to the weak central spindle. Exciting preliminary findings support my hypothesis by revealing that astral microtubules restrict anillin localization, but also that the outer part of the central spindle is derived from astral microtubules which can be bundled by Cyk4 and could locally control RhoA activation. Thus, astral microtubules may play dual roles in providing both stimulatory and inhibitory cues for ring assembly. Notably, preliminary studies reveal differences in cytokinesis between iPSCs and endoderm cells, laying the groundwork for future studies of how mechanisms change during differentiation.

These studies provide new knowledge of the mechanisms required for successful cytokinesis in several different human cell types, and parameters that could be used to predict requirements for different pathways. This knowledge is critical for developing novel therapies that could be more selective for specific cell types.

Chapter 2

Importin- β 1 functions as a chromatin sensor to position the contractile ring for cytokinesis

2.1 Highlights

- Ran-free Importin- β 1 becomes enriched between segregating chromosomes in anaphase HeLa cells but not in HCT116 cells.
- Importin-binding is required for anillin localization and function in cytokinesis in HeLa cells but not in HCT 116 cells.
- Importin- β 1 disruption at anaphase causes furrow oscillation and cytokinesis failure in HeLa cells.
- Importin- β 1 is not equatorially enriched and anillin is broader in HeLa cells with a lower chromatin-to-cell-size ratio.
- Importin- β 1 is equatorially enriched and anillin is narrower in HCT 116 cells with a higher chromatin-to-cell-size ratio.

Keywords: cytokinesis, importin- β 1, anillin, Ran-gradient, optogenetic, FRET, FLIM.

2.2 Summary

Cytokinesis, the final step of cell division, relies on the ingression of a carefully positioned actomyosin ring. Chromatin-associated Ran-GTP helps position this ring although the mechanism is not clear. We hypothesize that a depleted zone of Ran-GTP between the segregating chromosomes causes importins to become equatorially enriched and promote the recruitment of the scaffold protein anillin. However, the role of importins during anaphase remains unclear. Here, we tested if importins respond to the chromatin-associated Ran-GTP gradient to regulate ring assembly. Using endogenous tagging, live-cell imaging, and optogenetic perturbations, we found that importin- β 1 becomes equatorially enriched and is required for cytokinesis in hypotriploid HeLa cells, but not in euploid HCT 116 cells. A predictive model of the Ran-GTP gradient and Ran-free importin- β 1, validated experimentally by FLIM-FRET, identified factors that modulate this chromatin sensing pathway including chromatin-to-cell size ratio. Our findings suggest that highly aneuploid cancer cells may depend on importin-mediated anillin recruitment, representing a targetable weakness that distinguishes them from diploid cells.

Summary blurb

Importin enrichment at the equator promotes anillin recruitment during cytokinesis in a ploidy-dependent manner, linking Ran-GTP gradients to cell division.

2.3 Introduction

Cytokinesis is the physical separation of a cell into two daughter cells. This process occurs through the ingression of a RhoA-dependent contractile ring, which is spatiotemporally regulated by multiple pathways associated with the mitotic spindle and chromatin. Failed cytokinesis can lead to aneuploidy and altered cell fate, contributing to a range of diseases. Our group and others previously identified a chromatin-sensing pathway that coordinates contractile ring position with chromosome segregation for successful cytokinesis (Kiyomitsu and Cheeseman, 2013; Rodrigues et al., 2015; Beaudet et al., 2017; 2020).

Chromatin sensing relies on the importin-mediated binding and regulation of proteins with nuclear localization signals (NLSs) (Cavazza and Vernos, 2016). The release of NLS-proteins from importins is spatially controlled by active Ran, which is enriched near chromatin since its activator RCC1 (RanGEF) is bound to histones, while RanGAP inactivates Ran in the cytoplasm (Moore et al., 2016; Kaláb and Heald, 2008; Furuta and Hori, 2016). Importin- β 1 is essential and known as the major isoform of importin- β , while the seven importin- α isoforms appear partly redundant, though they exhibit subfamily-specific preferences (Oka et al., 2018). Although importin- β 1 is often thought to function indirectly through importin- α , several cargos can bind directly to importin- β 1. Since the goal of this study was to assess the role of importins in regulating cytokinesis, we chose to focus on importin- β 1. During anaphase, we predict that active Ran remains associated with the segregating chromosomes creating an equatorially depleted zone where importins are free to bind to NLS-proteins. Since several contractile proteins have NLS's, we hypothesize that importins promote the recruitment of contractile proteins to form a well-positioned ring. In support of this hypothesis, targeting Ran-GTP to the equatorial membrane displaced ring proteins in HeLa cells, while reducing Ran-GTP levels during anaphase caused ring positioning defects in baby hamster kidney epithelial (BHK) cells (Beaudet et al., 2017).

We found that the contractile ring protein anillin is a target of the Ran-importin pathway (Beaudet et al., 2017; 2020). Anillin crosslinks actomyosin with the plasma membrane to stabilize the ring and contains a conserved C-terminal NLS that does not control nuclear localization but is essential for anillin localization and function in HeLa cells (Oegema et al., 2000; Piekny and Glotzer, 2008; Piekny and Maddox, 2010; Beaudet et al., 2017). Importin- β 1 binds directly to anillin, and mutations that reduce importin- β 1 binding impair anillin recruitment to the equatorial cortex in anaphase HeLa cells (Beaudet et al., 2020). These findings suggest that importins respond to changes in active Ran to coordinate ring positioning with chromatin by regulating anillin. However, the timing and breadth of anillin's localization at the equatorial cortex differs in HCT 116 and HEK293 cells compared to HeLa cells (Husser et al., 2022) supporting that the mechanisms regulating ring assembly change with cell type. Different factors could affect Ran-GTP and its ability to regulate chromatin-cortical signaling.

It is not clear which factors could affect the ability of the Ran-importin pathway to regulate the ring. Presumably ploidy could cause a change in the steepness and/or extent of the gradient of active Ran. We previously found that mild perturbation of Ran regulators or importins had different effects on ring kinetics in AB compared to P1 cells in early *C. elegans* embryos (Ozugergin et al., 2022). These cells have different sizes and fates. Further, increasing their ploidy from 2n to 4n caused them to

increase in size by 1.5-fold, and altered both ring kinetics and reliance on Ran regulation (Ozugergin et al., 2022). This sublinear correlation with ploidy and cell size means that chromatin could potentially be closer to the cortex in cells with higher ploidy. Further, the levels of Ran, importins, RCC1 or RanGAP could also vary with cell type, and/or post-translational modifications could alter their localization. FRET imaging previously showed that the metaphase Ran-GTP gradient is steeper in cancer cells with higher ploidy compared to diploid HFF-1 cells (Hasegawa et al., 2013). A model was developed to predict the location of the active Ran gradient in metaphase HeLa cells (Caudron et al., 2005) This model revealed that the GTP/GDP ratio as well as the levels and localization of RCC1 were key parameters that determine the gradient. However, their model was not used to make predictions about active Ran in anaphase.

Importantly, importins have not been extensively studied in anaphase, yet we speculate that they respond to the Ran gradient to regulate contractile ring assembly. Here, we investigated if importins play a role in cytokinesis by responding to changes in the Ran gradient, and how these changes could affect ring assembly using both experimental approaches and a predictive model. First, we compared the regulation of cytokinesis via the Ran-importin pathway in hypotriploid HeLa cells and euploid HCT 116 cells. Then, using endogenous tagging and optogenetic perturbations, we found that importin- β 1 is equatorially enriched and is functionally required for cytokinesis in HeLa cells, but not in HCT 116 cells. These findings were supported by FLIM analysis and a predictive model of the Ran-GTP gradient during anaphase in both cell types. We then used the model to identify factors that could modulate the Ran-importin pathway's role in cytokinesis and validated these predictions experimentally. We found that the pathway is required in cells with higher chromatin-to-cell size ratio. Since many cancer cells have high aneuploidy with a net gain in chromosomes, their potential dependence on chromatin sensing for ring assembly may represent a therapeutic vulnerability that distinguishes them from euploid cells.

2.4 Materials & Methods

Cell culture, transfection and drug treatment. HeLa cells were cultured in Dulbecco's modified Eagle medium (DMEM; Wisent) media supplemented with 10% Cosmic calf serum (CCS; Cytoviva). HCT116 (p53^{-/-}) cells were cultured in McCoy's media (Wisent) supplemented with 10% CCS. The cells were maintained in 10 cm dishes in incubators at 37°C with 5% CO₂. For plating, cells were counted using Countess 3 Automated Cell Counter (Thermofisher).

For transfection, HeLa cells were plated in DMEM media without antibiotics (penicillin and streptomycin: PS; Thermofisher) and transfected with DNA (cDNA and shRNA) or DNA and siRNA using Lipofectamine 3000 (Invitrogen) according to the manufacturer's protocol. We modified the amount of DNA or siRNA to maintain molar ratios as recommended. Specifically, we used 2 µl of 20 µM of KPNB1 siRNA for 2 ml of media and incubated the cells for 48h. In addition, HCT116 cells were transfected in suspension before plating.

To induce monopolar cytokinesis, 2 µM of S-trityl-L-cysteine (STC; Millipore-Sigma) was added to cells for 8-16h, arresting them in prometaphase, after which Purvalanol A (Millipore-Sigma) was added to a final concentration of 22.5 µM to inhibit Cdk for mitotic exit (Hu et al., 2008; Beaudet et al., 2017). Cells were imaged immediately after Cdk inhibition. To arrest mitotic cells lacking microtubules, 100 nM of nocodazole (Sigma-Aldrich) was added to cells for 3–4 h, then Purvalanol A was added to induce mitotic exit as described above.

To increase ploidy, HCT116 (p53^{-/-}) cells were treated with 100-150 µM CoCl₂ (Millipore-Sigma) for 48h as described previously (Lopez-Sánchez et al., 2014). The media was changed 3-4h before imaging. The FACSMelody cell sorter (BD Biosciences) was used to measure changes in ploidy in the population. After CoCl₂ treatment, cells were dissociated and resuspended in Hank balanced salt solution (HBSS). They were then treated with 5 µM Vybrant™ DyeCycle™ Ruby (Invitrogen) for 30 minutes at 37°C with 5% CO₂ to stain chromatin according to manufacturer's instructions. The proportion of cells with different intensities were measured using the FACSMelody and the analysis was done using FlowJo (BD).

The cytosolic volume of HeLa cells was increased using hypotonic media. Cells were plated as described above, then the media was changed to DMEM diluted with 25% of water and 10% of CCS. Cells were imaged after 12h of incubation.

Cloning. mScarlet:anillin and rescue plasmids, pGG-mScarlet:Anillin-shANLN1 and pGG-mScarlet:Anillin-NLS-mutant-shANLN1, were generated using Golden Gate assembly (Engler et al., 2009). Briefly, coding sequences of anillin and anillin with mutations in the C terminus NLS (887KK888-DE) were amplified from previously generated constructs (Piekny and Glotzer, 2008; Beaudet et al., 2017) and cloned into the pYTK001 backbone (Addgene #65108; Lee et al., 2015) by Golden Gate using BsmBI (New England Biolab), then they were assembled with mScarlet into a modified pX459V2.0-HypaCas9 vector (Addgene #108294; Kato-Inui et al., 2018) using BsaI (New England Biolab) for mammalian expression. A shRNA sequence targeting endogenous anillin, designed after the siRNA described previously (Beaudet et al., 2017) was cloned into the modified pX459V2.0-HypaCas9 vector beforehand.

mCherry-CRY2clust cDNA was obtained by PCR from mCherry-CRY2clust (Addgene plasmid #105624; Park et al., 2017). Importin- β 1 cDNA was amplified by PCR from a previously generated construct (Beaudet et al., 2017). We generated three silent mutations (E760 and L761) to make the cDNA sequence resistant to KPNB1 siRNA using PCR-based site-directed mutagenesis. Importin- β 1 and mCherry-CRY2clust sequences were assembled using Golden Gate assembly as described above. All constructs were transformed into *Escherichia coli* DH5 α and sequenced using Plasmidsaurus. Primers can be found in Supplementary Table S8.

Genome editing. The sgRNA spacer sequence used to endogenously tag KPNB1 was used previously by OpenCell CZ BIOHUB (Cho et al., 2022). The sgRNA spacer was cloned into the pX459V2.0-HypaCas9-mRuby2 plasmid (Addgene plasmid #183872) as described previously (Husser et al., 2022). To build the repair template, the homology arms were amplified from genomic DNA extracted from HeLa cells. mNeonGreen was amplified independently using primers that match part of the homology arms (15–51 bp). The repair template was assembled into the pYTK089 (Addgene plasmid #65196) vector using NEBuilder[®] HiFi DNA Assembly (New England Biolab). pX459-HypaCas9-mRuby2 (CRISPR-Cas9 construct) and the repair template plasmid were transfected into HeLa cells using Lipofectamine 3000 as described above. Transfection in HCT 116 (p53^{-/-}) was done by electroporation using the SE Cell Line 4D-Nucleofector[™] X Kit (Lonza) as per the manufacturer's protocol. Cells were treated with NHEJ inhibitors NU7441 (2 μ M; Tocris Bioscience) and SCR7 (1 μ M; Xcess Biosciences) 4h before transfection and for 48h after transfection to improve editing efficiency. Cells were monitored for the appearance of mNeonGreen fluorescence after ~5 days.

Single cells were collected by sorting 7 days after transfection using the FACSMelody cell sorter (BD Biosciences). To do this, cells were dissociated and resuspended in FACS buffer composed of PBS with 1 mM EDTA, 25 mM HEPES pH 7.0 and 1% FBS. The gates were set to capture single fluorescent cells which were recovered in a 96-well plate containing DMEM or McCoy's with 20% of CCS and penicillin-streptomycin (50 units ml⁻¹ penicillin and 50 μ g ml⁻¹ streptomycin; Wisent). Cells were left to recover and were supplemented with media as needed.

Editing was confirmed by PCR and heterozygous clones were selected. PCR products were sequenced by Plasmidsaurus to confirm no alteration of KPNB1 and mNG sequence.

Microscopy. For live-cell imaging, cells were seeded onto acid-etched round coverslips (25 mm, no. 1.5) in 6-well plates and incubated at 37°C with 5% CO₂ for 24-72h. To visualize DNA, SYTO Deep Red Nucleic Acid Stain (ThermoFisher) was added to a final concentration of 0.25-2.5 μ M. To visualize microtubules, SiR-tubulin (Cytoskeleton Inc) was added to a final concentration of 1.25 μ M. The coverslips were transferred to a magnetic chamber (Quorum) with 1 ml of media before imaging. Imaging was performed using an inverted Nikon Eclipse Ti microscope (Nikon) equipped with a Livescan Sweptfield scanner (Nikon), Piezo Z stage (Prior), IXON 879 EMCCD camera (Andor), and 488, 561 and 640 nm lasers (100mW, Agilent) using the 100 $\times$ /1.45 NA objective. The cells were kept at 37°C and 5% CO₂ during imaging in an INU-TiZ-F1 chamber (MadCityLabs). Z-stacks of 1 μ m thickness were acquired every 1-2 min using NIS Elements software (Nikon, v.4.0). We also used Zeiss Axio Observer microscope (Zeiss) equipped with a Cicero spinning disk (CrestOptics), a Hamamatsu Flash 4.0 LT camera (Hamamatsu) and 488, 561 and 640 nm LDI-5 laser lasers (89North)

using the 63×/1.4 NA oil immersion objective (Zeiss; pixel size 0.26µm). The cells were kept at 37°C and 5% CO₂ during imaging in an environmental chamber for long-term live imaging. Z-stacks of 1 µm thickness were acquired every 1-2 min using Volocity software (Quorum). Pictures of the spindle in cells with SiR-tubulin dye were acquired using the Nikon Super-Resolution Spinning Disk (SR-SD) which is an inverted Nikon Eclipse Ti2 microscope with a CSU-X1 spinning disk (Yokogawa), GATACA Live-SR unit, using the 60×/1.4 CFI PLAN APO VC oil immersion objective (Nikon), with a piezo Z stage (MadCityLabs), and with the Zyla camera (Andor). All image files were exported as TIFFs and were opened with Fiji (NIH).

Optogenetics. HeLa cells with endogenous mNeonGreen-tagged importin-β1 were seeded at 30% confluency onto acid-etched round coverslips (25 mm, no. 1.5) in 6-well plates. The next day, they were co-transfected with KPNB1 RNAs (5' CUGAAUGAGCUAAGGGAAA 3'; Horizon), KPNB1:mCherry:CRY2 using Lipofectamine 3000 as described above. Cells were filmed 45-50h after siRNA transfection. CRY2 was activated upon anaphase onset by acquisition of green fluorescence signal (laser wavelength: 488nm) which also confirmed endogenous importin-β1 depletion. The laser power was set at 15% with an exposure time of 800ms and 16 slices of 1 µm were acquired. Cells with at least 80% depletion of endogenous importin-β1 were selected for analysis.

Fluorescence Lifetime Microscopy (FLIM). Cells seeded in 35 mm glass bottom dishes with 20 mm µ-wells (Cellvis) were transfected with pK135, which encodes Rango-3 (Soderholm et al., 2011) 12 hours after transfection, FLIM images were acquired using a Leica Stellaris-8 inverted laser scanning confocal microscope equipped with a HC PL APO CS2 100x/1.40 oil objective, and 80 MHz pulsed white light laser, a time-correlated HyD S detector, with LAS X software and a Falcon module for phasor analysis of photon arrival time. Cells were maintained at 37°C and 5% CO₂ using a Tokai Hit stagetop incubator. Images of 512 x 512 pixels with a lateral size of 0.076 x 0.076 µm were acquired by unidirectional scanning with the white light laser set to 470 nm and 2% total intensity, an emission window of 490-600 nm and the detector set to photon counting mode, with a scan rate of 400 Hz, a pixel dwell time of 2.825 µs and 8 - 16x line accumulation, with transmitted light simultaneously acquired using a TransPMT. Photon arrival times for each pixel were calculated by the phasor plot, and false colouring for lifetime information was generated by drawing a line through the main pixel population in the phasor plot, using a blue-to-red colour scheme reflecting shorter to longer lifetimes (Supp. Fig. S3). Cells with similar expression levels were used for analysis as fluorescence intensity is proportional to Rango-3 concentration (Supp. Fig. S4 and Supp. Table S1).

Image analysis and quantification. All images were opened in Fiji (v.2.3, NIH). Images of importin-β1 acquired by the Nikon Eclipse Ti microscope (Nikon) equipped with a Livescan Sweptfield scanner (Nikon) were deconvolved using AutoQuant (Media cybernetics). To reveal anillin's cortical localization, linescans of anillin fluorescence intensity were performed at the desired time point on a max z-projection of three z-slices (1 µm per slice) by drawing a 3-pixel-wide line around the cortex of the cell. To calculate the breadth to size ratio, the breadth of the peak was measured as the number of pixels above 50% of the maximum value, which was subsequently divided by the length of the cell. To determine the ratio of cortical to cytosolic anillin fluorescence, the background was first subtracted from the image, then three regions of interest (ROI) were drawn in the cytosol or at the

cortex and averaged, then the cortical average value was divided by the cytosolic average value. To reveal subcortical importin- β 1 localization, linescans of importin- β 1 fluorescence intensity were performed at the desired time point on a sum z-projection of three z-slices (1 μ m per slice) by drawing a 10-pixel-wide line around the cortex of the cell. Importin- β 1 linescans were plotted together by normalizing them by their median. The breadth of importin- β 1 was determined as per anillin but measuring the number of pixels above 60% of the peak maximum value.

To measure the correlation between enriched anillin and importin- β 1, maximum z-projections of three z-slices (1 μ m per slice) were used for anillin and sum z-projections of three z-slices (1 μ m per slice) were used for importin- β 1. A mask was created and applied to each projection to define the zones of enrichment. The angle between the equatorial axis and the boundary of the breadth of enrichment was measured and plotted for each protein.

Measurements including the length and width of chromatin and the length and width of cells, spindle length and the distance between the segregating chromosomes, were done on images acquired using the Nikon Super-Resolution Spinning Disk (SR-SD). Spindle length was measured from centrosome to centrosome, and the distance between chromosomes was measured from the middle of one set of chromosomes to the middle of the other set. The means of each parameter from 10 cells were used for the computational model.

All data was exported as Excel (Microsoft) files for analysis. All graphs and statistics were generated using Prism (v.9.3, GraphPad). Statistical significance was tested using a Welch t-test.

Ran-gradient modeling. The Ran-gradient model is based on a 1-D reaction-diffusion model from Caudron et al. (2005) converted into a model with 2-D geometry. The partial differential equations were solved using the PDE toolbox of MATLAB (R2025a).

Description of the reaction and diffusion coefficients

The cell was divided into two zones: cytosol and chromatin (Supp. Fig. S5 and S6). We assume that the guanine nucleotide exchange from Ran-GDP to Ran-GTP happens only in the chromatin zone since the guanine nucleotide exchange factor (GEF), RCC1, is bound to histone and does not diffuse (Moore et al., 2002). We assume that Ran-GTP, Ran-GDP, GTP and GDP can freely diffuse through all zones. The hydrolysis of Ran-GTP into Ran-GDP and the interaction between Ran-GTP and importin- β take place in the cytosol. The RanBP1 interaction with Ran-GTP can be neglected due to the rapid hydrolysis of GTP, according to Caudron et al. (2005). We used the same diffusion coefficients as well as reaction rates and kinetic constants as Caudron et al. (2005) (Supp. Table S2 and S3).

Description of the equations

We describe the reaction in a 2-D space representing a cell at metaphase or anaphase. In this geometry, the concentration of the proteins depends on their position (x and y) within the cell and time. The reaction-diffusion equation for a concentration u (x,y,t) is defined as the following:

$$\frac{\partial u(x,y,t)}{\partial t} = D \cdot \Delta u(x,y,t) - a \cdot u(x,y,t) + f$$

$$\text{Time evolution} = \text{Diffusion} + \text{Reaction} = \text{Diffusion} - \text{Consumption} + \text{Production}$$

In the chromatin zone

$$\frac{\partial[\text{RanGTP} - \text{RCC1}]}{\partial t} = r3. [\text{GTP}]. [\text{Ran} - \text{RCC1}] + r5. [\text{RCC1}]. [\text{RanGTP}] - (r4 + r6). [\text{RanGTP} - \text{RCC1}]$$

$$\frac{\partial[\text{Ran} - \text{RCC1}]}{\partial t} = r2. [\text{RanGDP} - \text{RCC1}] + r6. [\text{RanGTP} - \text{RCC1}] - (r7. [\text{GDP}] + r3. [\text{GTP}]). [\text{Ran} - \text{RCC1}]$$

$$\frac{\partial[\text{RanGDP} - \text{RCC1}]}{\partial t} = r7. [\text{GDP}]. [\text{Ran} - \text{RCC1}] + r1. [\text{RCC1}]. [\text{RanGDP}] - (r2 + r8). [\text{RanGDP} - \text{RCC1}]$$

$$\frac{\partial[\text{RanGTP}]}{\partial t} = Dr. \Delta[\text{RanGTP}] + r4[\text{RanGTP} - \text{RCC1}] - r5. [\text{RCC1}]. [\text{RanGTP}]$$

$$\frac{\partial[\text{RanGDP}]}{\partial t} = Dr. \Delta[\text{RanGDP}] + r8. [\text{RanGDP} - \text{RCC1}] - r1. [\text{RCC1}]. [\text{RanGDP}]$$

$$\frac{\partial[\text{RCC1}]}{\partial t} = r8. [\text{RanGDP} - \text{RCC1}] + r4. [\text{RanGTP} - \text{RCC1}] - (r1. [\text{RanGDP}] + r5. [\text{RanGTP}]). [\text{RCC1}]$$

In the cytosol zone

$$\begin{aligned} \frac{\partial[\text{RanGTP}]}{\partial t} = & Dr. \Delta[\text{RanGTP}] + koff. [\text{RanGTP} - \text{importin}\beta] - kon. [\text{importin}\beta]. [\text{RanGTP}] \\ & - \frac{kcat. \text{RanGAP}}{kM + [\text{RanGTP}]} \cdot [\text{RanGTP}] \end{aligned}$$

$$\frac{\partial[\text{RanGDP}]}{\partial t} = Dr. \Delta[\text{RanGDP}] + \frac{kcat. \text{RanGAP} \cdot [\text{RanGTP}]}{kM + [\text{RanGTP}]}$$

$$\frac{\partial[\text{importin}\beta]}{\partial t} = Di. \Delta[\text{importin}\beta] + koff. [\text{RanGTP} - \text{importin}\beta] - kon. [\text{RanGTP}]. [\text{importin}\beta]$$

$$\begin{aligned} \frac{\partial[\text{RanGTP} - \text{importin}\beta]}{\partial t} \\ = D2. \Delta[\text{RanGTP} - \text{importin}\beta] + kon. [\text{importin}\beta]. [\text{RanGTP}] - koff. [\text{RanGTP} - \text{importin}\beta] \end{aligned}$$

The calculations were done using the Partial Differential Equation (PDE) solver of Matlab®.

Description of geometry and boundary conditions

There is a reflective condition (no flux) at the edge of the cell (cortex, cytosol) or chromatin for proteins except Ran-GTP and Ran-GDP, as they cannot escape the cell or their respective zone (Supp. Table S2).

The parameters used for the equations above come from measurements of metaphase and anaphase HeLa and HCT116 cells (Supp. Figure S6). These include cell and chromatin length and width, as well as spindle length. Our measurements revealed that the cell and chromatin volume did not vary significantly between metaphase and anaphase cells, therefore we assumed no change in concentration between metaphase and anaphase. Volume errors were calculated using standard analytical propagation: $\frac{\sigma V^2}{V} = \frac{\sigma L^2}{L} + \frac{\sigma D^2}{D} + \frac{\sigma W^2}{W}$.

The mesh is shown in Supplementary Figure S6. The spatial steps were chosen to be small enough to capture the important features of the solution, but not too small that the computation becomes excessive and difficult to run on a standard computer. The maximum size of the mesh in the cytosol is 1 μm , while in the chromatin zone, it is 0.1 μm . The mesh was defined to include 15 grid points across the chromatin width, to prevent numerical instability due to boundary conditions or the creation of a sharp gradient. $\Delta x = \frac{L}{N}$, where L is the smallest distance used, and N is the number of grid points.

RNA sequencing analysis and estimation of the initial conditions of the computational model.

The total cellular concentrations of Ran, RanGAP1 and importin- β in HeLa cells are 6 μM , 0.4 μM and 3 μM , respectively, which were calculated in previous studies (Bischoff and Ponstingl, 1991; Ribbeck, Lipowsky et al., 1998). Assuming that RanGAP and importin- β are restricted to the cytosol, their local concentrations are 0.7 μM and 5.25 μM , respectively. Caudron et al. (2005) assumed that RCC1 concentration is the same in HeLa cells and *Xenopus* eggs at 0.24 μM . However, since RCC1 is localized to chromatin, it is ~10 to 100-fold more concentrated at this location compared to the cytosol (Bischoff and Ponstingl, 1991; Ribbeck, Lipowsky et al., 1998).

Since such estimates of protein concentrations are not available for HCT 116 cells, we used RNA sequencing data for HeLa and HCT 116 cells from Wu Y. et al. (2024) with GEO2R (GEO accession number: GSE220879) to measure the expression of RAN, RCC1, RANGAP1 and KPNB1. The total concentrations are similar between HeLa and HCT 116 cells with some small but significant changes in RanGAP1 and KPNB1 as indicated in (Supp. Fig. S7). Using these values and the measurements of cell volume (Supp. Table S4), we estimated the concentrations of Ran, RanGAP1, RCC1 and importin- β in HCT 116 cells (Supp. Table S5). Note that errors of concentrations were calculated using standard analytical propagation: $\frac{\sigma_C^2}{C} = \frac{\sigma_V^2}{V} + \frac{\sigma_A^2}{A}$.

We acknowledge that RNA sequencing represents only the levels of mRNA and there could be differences in protein levels. However, mRNA can be used as a proxy for protein. We compared the fold-change to Ran in HeLa cells, revealing that the RNA sequencing analysis matches with the approximations of RanGAP1, RCC1 and importin- β in HeLa cells in previous studies (Bischoff and Ponstingl, 1991; Ribbeck, Lipowsky et al., 1998).

Deterministic computational models are imperfect as their results are based on input. We are forced to make assumptions due to technical limitations, but these assumptions must be physically plausible. The calculated errors for the initial conditions (Ran, RCC1, RanGAP1 and importin- β concentrations) provide a range for each parameter to be tested experimentally (Supp. Table S5). We tested different conditions and compared the output to the FLIM data to refine the model (Supp. Fig. S8 and Supp. Table S6). In addition, we evaluated the impact of each parameter on the system, which helped us to make the adjustments shown in Supp. Fig. S9.

Estimation of equilibrium

The time steps needed to be small enough to capture the dynamics of the system, but not so small that they require unnecessary computational needs. For $\Delta t \leq \frac{\Delta x}{2.D}$, D is the greatest diffusion rate. The dynamics of the system can be resolved using a time step of 0.000227 seconds.

The model starts from a state of non-equilibrium: all of Ran is inactive and free, RCC1 and RanGAP1 are free within their respective zones, and all importin- β is free. The model is stopped when the system has reached an equilibrium. We picked t_{end} as the difference between t_{end} and $t_{\text{end}-1}$ is nul.

Establishing changes in parameters that impact the cytosol to chromatin ratio

To model HeLa cells with a decrease in chromatin-to-cell-size ratio based on an increase in cytosol volume, we adjusted the length and width of the cell (Supp. Table S6). We did not change the distance between chromatin or chromatin shape. The concentrations of proteins were adjusted accordingly (Supp. Table S7).

To model HCT116 cells with an increase in chromatin-to-cell-size ratio based on an increase in ploidy, we doubled the DNA content and increased both the cell and chromatin volumes by 1.5 (Supp. Table S6). The concentrations of proteins were adjusted accordingly (Supp. Table S7).

Resource availability

The MATLAB code for the predictive computational model of Ran-free importin- β is available at: <https://github.com/CytokinesisCB/RanGradientAnaphase>

2.5 Results

2.5.1 Importin- β 1 is transiently enriched equatorially in anaphase HeLa but not HCT 116 cells

Importins have not been extensively studied during anaphase, but previous studies by our group suggests that they control anillin localization for cytokinesis in HeLa cells (Fig. 5A) (Beaudet et al., 2017; 2020). To visualize where importins localize during mitotic exit, we endogenously tagged importin- β 1 with mNeonGreen using CRISPR/Cas9 in hypotriploid HeLa cells from cervical adenocarcinoma from a Black female, and euploid HCT 116 cells from colorectal cancer from a Caucasian male (Fig. 5B and Supp. Fig. S1). As shown in Figure 5C, importin- β 1 is cytosolic, but we observed a transient enrichment of importin- β 1 near the equatorial cortex in anaphase HeLa cells, but not in HCT 116 cells, where the localization was more uniform. These observations were quantified using linescans drawn just under the cortex from one pole to the other during anaphase to reveal differences in fluorescence intensity in a population of cells (Fig. 5D). We also used linescans to measure fluorescence over time in HeLa cells, which revealed that the equatorial enrichment of fluorescence relative to the poles occurs between 2-6 minutes after anaphase onset and is uniform before and after this time (Fig. 5E). Notably, the timing of importin- β 1's equatorial enrichment in HeLa cells appears as chromosomes segregate (Fig. 5F). Measurements revealed that indeed there was a positive correlation in the breadth of enriched importin- β 1 and the distance between the chromosomes (Fig. 5G). Thus, these data show that importins become transiently equatorially enriched in anaphase HeLa cells as chromosomes segregate, but not HCT 116 cells. Notably, the timing of enrichment correlates with when anillin is first visibly enriched at the equatorial cortex in HeLa cells (Supp. Fig. S2) (Beaudet et al., 2017; 2020).

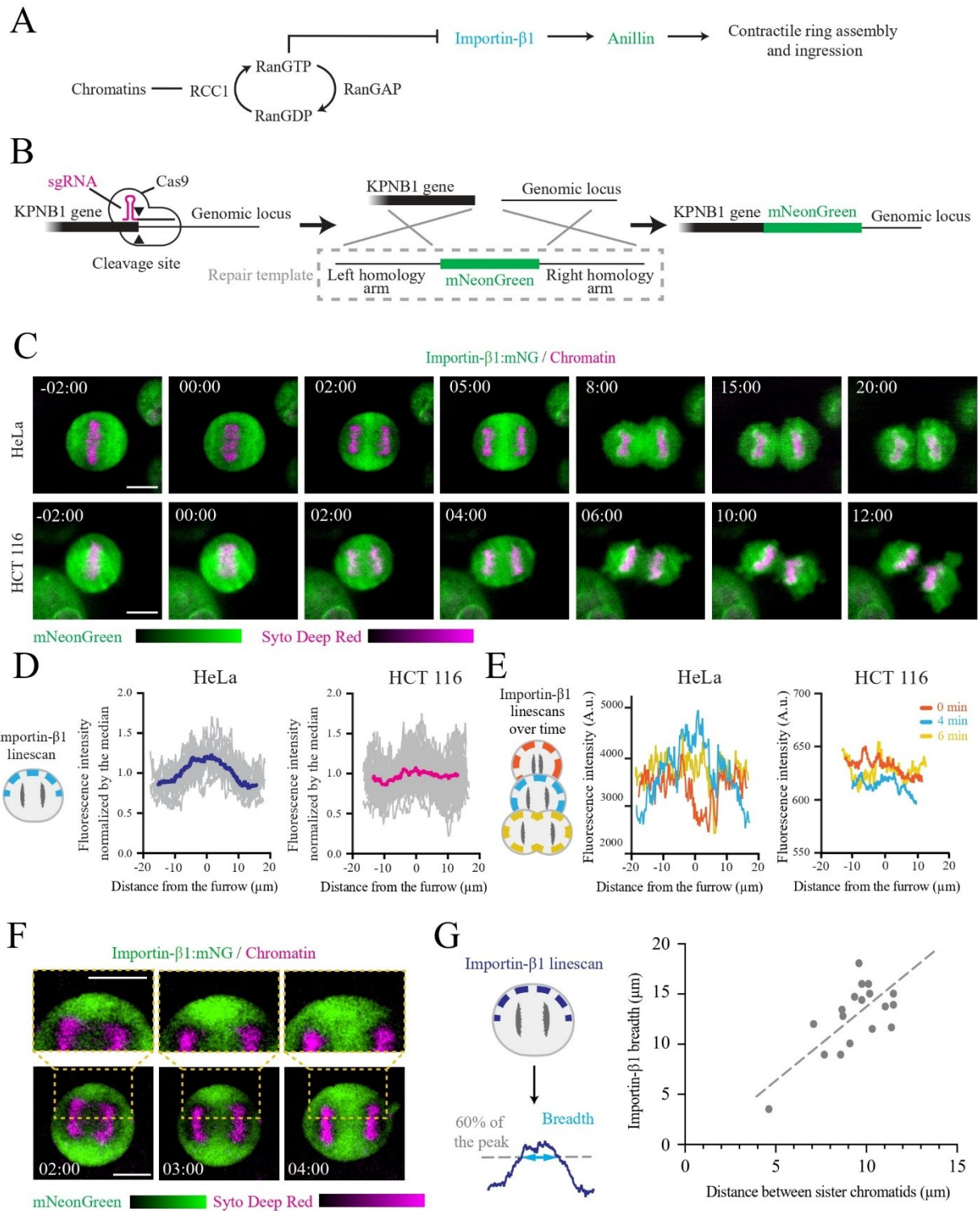


Figure 5. Importin- β 1 is transiently enriched equatorially in anaphase HeLa but not HCT 116 cells.

(A) A pathway shows how chromatin sensing via Ran-GTP could control ring assembly by the importin-mediated regulation of anillin. (B) A schematic shows how genome editing was performed in HeLa and HCT 116 cells to insert mNeonGreen at the C terminus of importin- β 1. (C) Timelapse images show the localization of endogenous importin- β 1 (green) co-stained for chromatin (Syto Deep Red; magenta) in HeLa cells (top) or HCT 116 cells (bottom). The colour scales show levels (darker colours indicate lower fluorescence intensity compared to brighter colours). Time is shown in minutes, and the scale bar is 10 μ m. (D) The cartoon cell shows how importin- β 1 was measured in anaphase cells. A thick line (2 μ m) was drawn subcortically from one pole to the other and the intensity along this line was plotted. Graphs show the linescans of the fluorescence intensity of importin- β 1 in HeLa (blue) or HCT 116 (pink) cells (n=20 for each) normalized by their median over the length of the cell (shown as distance from the furrow). (E) Cartoon cells show how linescans were done at anaphase onset (0 min, cyan), late anaphase (4 min, orange) and early telophase (6 min, yellow). The fluorescence intensity over distance from the furrow is shown in the graphs for a HeLa (left) and HCT 116 cell (right). (F) Timelapse images show endogenous importin- β 1 (green) in a HeLa cell (bottom) from anaphase onset co-stained for chromatin (Syto Deep Red; magenta). The images above show a zoomed-in view of the yellow boxes. Time is indicated in minutes and the scale bar is 10 μ m. (G) A graph shows the correlation between segregated sister chromatids (μ m) and the breadth of equatorially enriched importin- β 1 (μ m). The dashed line shows linear regression ($r^2 = 0.43$; n=20 cells).

2.5.2 Importin- β 1 equatorial enrichment in HeLa cells is dependent on anillin

The timing and breadth of importin- β 1's equatorial enrichment appears to correlate with anillin (Beaudet et al., 2017; 2020). To test this, we imaged HeLa cells with endogenous importin- β 1:mNG co-expressing mScarlet:anillin (Fig. 6A). Indeed, the enrichment of importin- β 1 correlated with when anillin became visibly enriched at the equatorial cortex. To compare their breadth, we measured the angle between the equatorial axis and the boundary of importin or anillin enrichment in anaphase cells (Fig. 6B). Although importin- β 1 enrichment was broader than anillin, there was a positive correlation between both proteins (Fig. 6B). This finding suggests that the breadth of anillin increases with the breadth of importin- β 1 enrichment or vice versa.

We also tested if importin- β 1 enrichment correlates with anillin in cells that are induced to undergo monopolar cytokinesis. We previously showed that anillin is asymmetrically enriched at the polarized cortex in cells undergoing monopolar cytokinesis, as well as in the absence of polymerized microtubules (Beaudet et al., 2017). HeLa cells were treated with 2 μ M of S-trityl-L-cysteine (STC) to block centrosome separation and mitotic exit was induced by inhibiting Cdk1 with 22.5 μ M of Purvalanol A (Hu et al., 2008). We observed the transient enrichment of importin- β 1 near the polarized cortex away from chromatin, and where anillin became cortically enriched (Fig. 6C). We noticed that importin- β 1 enrichment occurred at 4 minutes after Cdk inhibition, while anillin's peak enrichment occurred at 6 minutes, with noticeable cortical ingression after 8 minutes. Importin- β 1 and anillin were also enriched near the polarized cortex in HeLa cells treated with 33 nM nocodazole to depolymerize microtubules and induced to exit mitosis by Cdk inhibition (Fig. 6D). Next, we measured the symmetry ratios four minutes into monopolar cytokinesis or after polarization of cells lacking microtubules, which was 1.5 ± 0.2 and 2.5 ± 0.5 or 1.4 ± 0.1 and 2.1 ± 0.7 for importin- β 1 and anillin, respectively (Fig. 6E). These findings suggest that importin- β 1 also becomes enriched in polarized cells and precedes anillin recruitment to the nearby cortex.

Next, we tested if importin- β 1's equatorial enrichment is dependent on anillin. Importin- β 1 was previously shown to localize to the metaphase spindle via its cargo, TPX2 and depletion of its cargo led to the loss of importin- β 1 at the spindle (Ciciarello et al., 2004). Since anillin was previously shown to bind to importin- β 1, we speculated that importin- β 1's equatorial enrichment in anaphase cells could be dependent on anillin. Indeed, depletion of anillin by expressing shRNAs in HeLa cells caused a reduction in the transient enrichment of importin- β 1 (Fig. 6F and G). There is a small amount of importin- β 1 enrichment that remains, which could reflect other targets that are also regulated by importin- β 1 for ring assembly. This finding shows that the transient anaphase enrichment of importin- β 1 is at least partly due to anillin, which is consistent with anillin being a target of importin- β 1 binding.

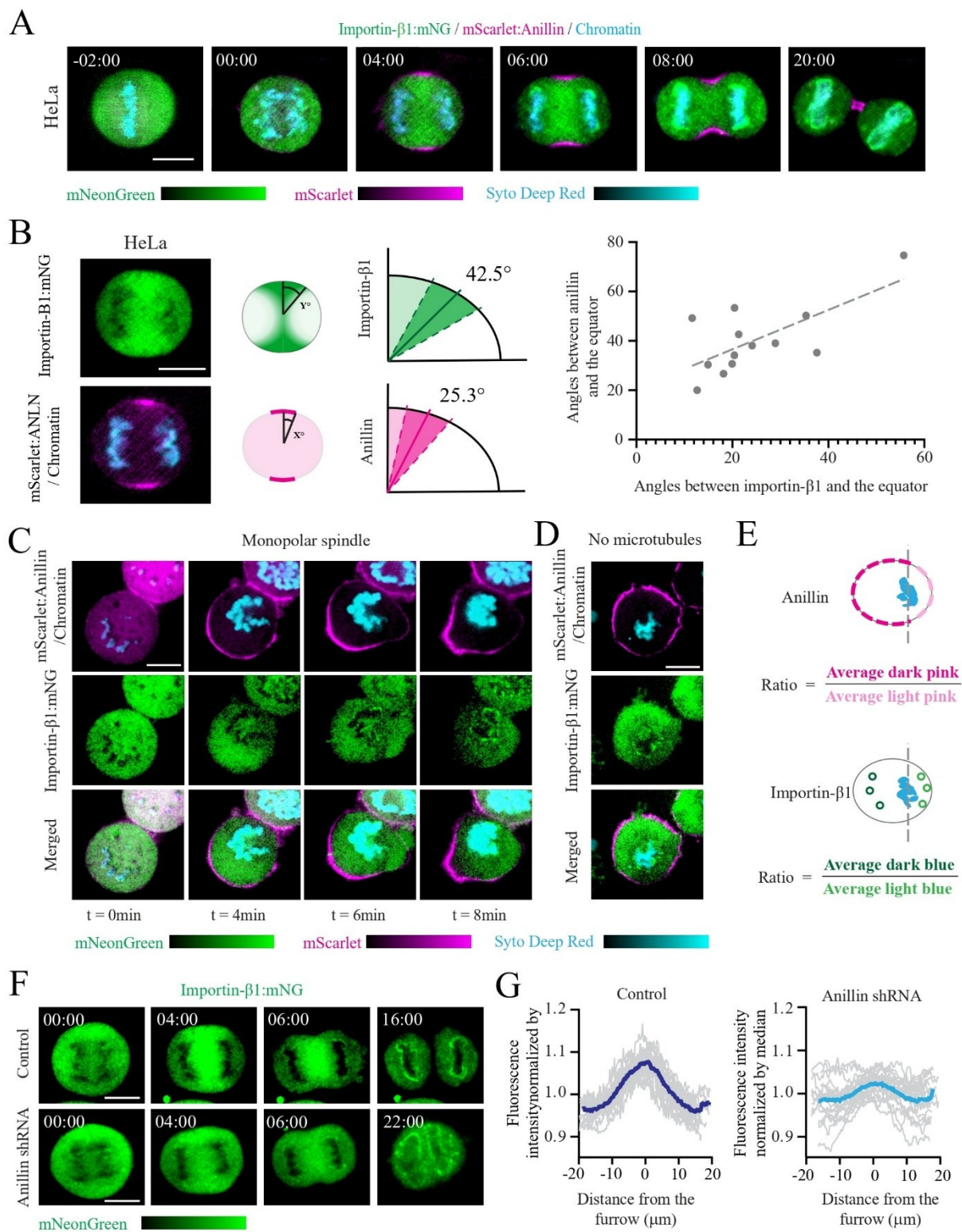


Figure 6. The equatorial enrichment of importin- β 1 is anillin-dependent. (A) Timelapse images endogenous mNG:importin- β 1 (green) in a HeLa cell co-expressing mScarlet:anillin (magenta) and stained for chromatin (Syto Deep Red, cyan). Time is indicated in minutes, and the scale bar is 10 μ m. (B) Images show endogenous mNG:importin- β 1 (green, top) co-expressing mScarlet:anillin (magenta, bottom) and stained for chromatin (Syto Deep Red, cyan) at 4 minutes after anaphase onset. The scale bar is 10 μ m. Cartoon cells show how angles between the equatorial axis and the boundary of anillin (green) and importin- β 1 (pink) enrichment were used to measure correlation. The origin is at the center of the cell, while the vertical axis is at the equator, and the horizontal axis is at poles. Values are indicated in the graphs to the right, where the darker colored area represents the standard deviation. A graph shows the correlation between angles corresponding to anillin (y-axis) and importin- β 1 (x-axis) enrichment. The dashed line shows linear regression ($r^2 = 0.52$; $n = 13$ cells). (C) Timelapse images show endogenous mNG:importin- β 1 (green) in a HeLa cell co-expressing mScarlet:anillin (magenta) and stained for chromatin (Syto Deep Red; cyan) induced to undergo monopolar cytokinesis. Time is shown in minutes, and the scale bar is 10 μ m. (D) Images show a single time point of endogenous mNG:importin- β 1 (green) in a HeLa cell co-expressing mScarlet:anillin (magenta) and stained for chromatin (Syto Deep Red; cyan) after microtubule depolymerization and polarization. The scale bar is 10 μ m. (E) Schematics show how the symmetry of importin- β 1 and anillin was measured. The average intensity of importin- β 1 in regions of interest (ROI) in the cytosol away from chromatin was divided by the region near chromatin. The average intensity of a cortical linescan of anillin in a region of the cortex away from chromatin was divided by the region near chromatin. (F) Timelapse images show endogenous mNG:importin- β 1 (green) in a control HeLa cell (top) or after anillin depletion by shRNAs (bottom). The colour scale shows intensity (darker colours indicate lower fluorescence intensity compared to brighter colours). Time is indicated in minutes, and the scale bar is 10 μ m. (G) Graphs show the linescans of the fluorescence intensity of importin- β 1 in control HeLa cells (dark blue) or after anillin RNAi (light blue; $n = 10$ for each) normalized by their median over the length of the cell (shown as distance from the furrow).

2.5.3 Importin- β 1 is required for ring assembly in HeLa cells but not in HCT 116 cells

We previously found that mutating the NLS in anillin reduces importin- β 1-binding and causes cytokinesis failure in HeLa cells (Beaudet et al., 2017). However, anillin localization in mitotic HCT 116 cells is different compared to HeLa cells, and importin- β 1 is not equatorially enriched. To determine if anillin's localization relies on importin- β 1 in HCT 116 cells, we depleted endogenous anillin using shRNAs and co-expressed RNAi-resistant mScarlet:anillin or mScarlet:anillin-NLS-mutant (Fig. 7A). First, we determined if importin-binding to anillin is required for cytokinesis in HCT 116 cells. Cytokinesis failure was evaluated by counting the proportion of binucleate cells in the rescued control or NLS mutant populations (Fig. 7B). In HeLa cells, $37 \pm 7\%$ of the cells rescued with the NLS mutant failed cytokinesis compared to $6 \pm 2\%$ for the non-mutant control or non-treated cells ($4 \pm 0.5\%$), while in HCT 116 cells, there was no significant change in cytokinesis failure for the NLS mutant compared to controls ($5 \pm 0.75\%$ vs. 4 ± 2.5 or $2.5 \pm 1\%$ for non-treated cells). As shown in Figure 7C, anillin was not cortically localized in HeLa cells until mid-late anaphase, when it became enriched at the equatorial cortex. The NLS mutant was recruited later in anaphase and localized to a narrower region at the equatorial cortex, similar to what was previously reported (Beaudet et al., 2017). In HCT 116 cells, anillin localized to the cortex prior to mitotic exit and was more broadly localized at the equatorial cortex (Fig. 7C). NLS mutant anillin was no longer cortical during metaphase and early anaphase, but by mid-anaphase was equatorially enriched with a breadth similar to control cells (Fig. 7C). We quantified these observations by measuring the cortical to cytosol ratio of anillin in metaphase HCT 116 cells and found that while this ratio was above one for control cells, the ratio was close to 1 in cells rescued with NLS mutant anillin (Fig. 7D). We also used linescans to measure the breadth to cortex length ratio of anillin in anaphase HeLa and HCT 116 cells and found that while the ratio was indeed reduced in HeLa cells as expected, the average breadth was similar, although more variable, in HCT 116 cells (Fig. 7E). Importantly, the localization and breadth-to-cell-size ratio of endogenous anillin and exogenous RNAi-resistant mScarlet:anillin co-expressed with shRNAs in HeLa and HCT 116 cells are identical suggesting that changes in localization are not caused by over-expression (Supp. Fig. S2 and Fig. 7). Overall, although importin- β 1-binding is required for anillin's cortical localization in metaphase HCT 116 cells, it is not required for regulating anillin's localization at the equatorial cortex or for its function in cytokinesis. Thus, other pathways likely regulate anillin's localization and function for cytokinesis in HCT 116 cells.

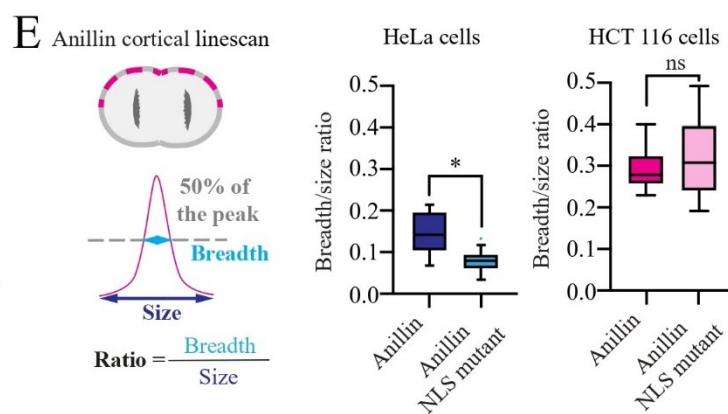
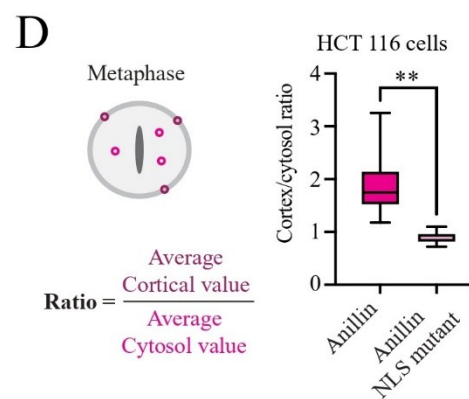
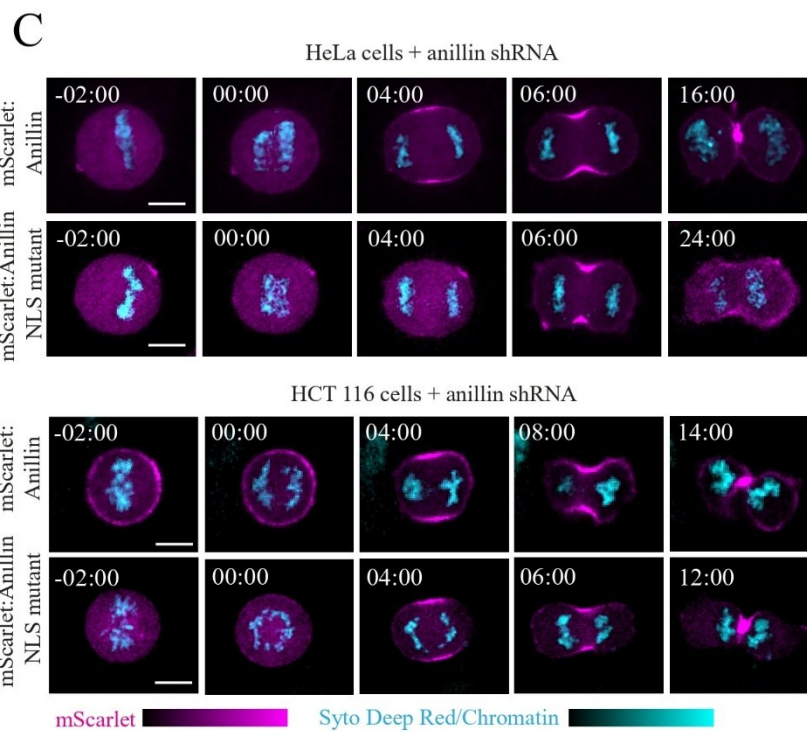
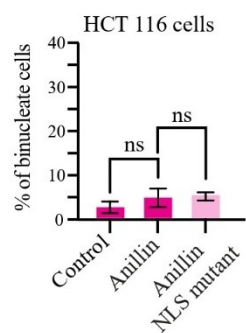
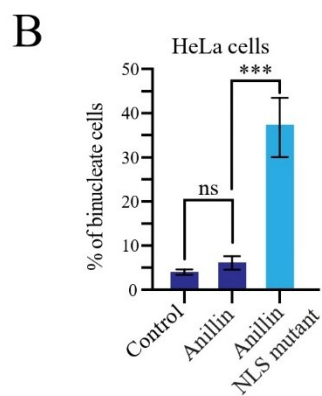
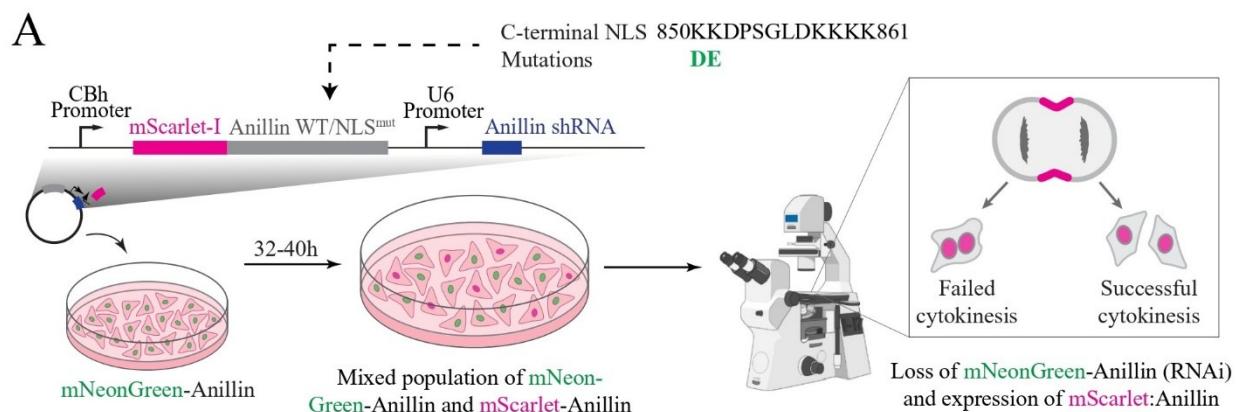


Figure 7. Importin-binding is required for anillin's equatorial localization and function in HeLa but not HCT 116 cells. (A) A schematic shows the workflow to deplete endogenous anillin with shRNAs while expressing RNAi-resistant anillin or the NLS mutant fused to mScarlet. Cells were imaged 32-40 hours after transfection. The point mutations in the C terminus NLS are indicated (residue numbers are for the isoform that is 1087aa). (B) Bar graphs show the percentage of binucleate cells in non-treated HeLa (dark blue) or HCT 116 cells (dark pink), after rescue with mScarlet:anillin HeLa (dark blue) or HCT 116 (dark pink) and after rescue with mScarlet:anillin-NLS-mutant in HeLa (light blue) or HCT 116 cells (light pink; n=100 cells per replicate; N = 3). Asterisks (***) indicate significance ($p \leq 0.0001$) while ns refers to non-significance. (C) Timelapse images show HeLa (top) or HCT 116 cells (bottom) rescued with mScarlet:anillin or mScarlet:anillin-NLS-mutant (magenta) stained for chromatin (Syto Deep Red; cyan). Time is in minutes, and the scale bar is 10 μ m. (D) A cartoon cell shows how the ratio of cortical to cytosolic anillin fluorescence was measured using three regions of interest (ROI) at the cortex and in the cytosol and dividing the averages. A bar graph shows the cortical to cytosolic ratios in metaphase HCT 116 cells (n = 15) rescued with mScarlet:anillin (dark pink) or mScarlet:anillin-NLS-mutant (light pink). Asterisks (**) indicate significance $p \leq 0.001$. (E) A cartoon cell shows how the breadth to size ratio was measured 6 minutes after anaphase onset. A line was drawn along the cortex from one pole to the other and fluorescence intensity along the line was plotted. The breadth of the anillin peak was measured as the number of pixels >50% of the maximum value, which was then divided by the length. The bar graphs show the breadth to size ratio of mScarlet:anillin (dark blue) and mScarlet:anillin-NLS-mutant (light blue) in anaphase HeLa cells (n=10) and in HCT 116 cells (n=15; dark pink and light pink, respectively). Asterisks (*) indicate significance ($* p \leq 0.005$) while ns refers to non-significance.

Next, we wanted to determine if importin- β 1 is required for ring assembly in HeLa cells. Since importin- β 1 has pleiotropic functions (Lombardi et al., 2025), we designed an optogenetic tool to disrupt its function with high temporal precision. The optogenetic tool expresses importin- β 1 fused to the fluorescent protein mCherry and CRY2clust (Fig. 8A). CRY2clust is a modified version of CRY2 with enhanced homo-oligomerization (Park et al., 2017). Clustering of CRY2clust was previously shown to occur rapidly in response to 400-500 nm illumination (Bugaj et al., 2013; Huang et al., 2023). HeLa cells with endogenous importin- β 1:mNG were transfected with importin- β 1 siRNAs to deplete the endogenous protein while co-expressing RNAi-resistant importin- β 1:mCherry:CRY2clust (Fig. 8). Since exposure to 488 nm can activate CRY2clust and excite mNeonGreen, the tool was activated while imaging for the depletion of endogenous importin- β 1 at anaphase onset (Fig. 8A). Cells with $\geq 70\%$ depletion of endogenous importin were used for analysis. In addition, since our group previously showed that high over-expression of importin- β 1 causes changes in anillin localization (Beaudet et al., 2017), cells were selected with mid to low expression of importin- β 1:mCherry:CRY2clust. When not activated, importin- β 1:mCherry:CRY2clust localizes similar to endogenous importin- β 1 (Fig. 8B). When the tool was activated at anaphase onset by exposure to 488 nm of light, we observed clustering of importin- β 1 followed by oscillation of the contractile ring and 2 out of 5 cells failed cytokinesis (Fig. 8B). These results suggest that importin- β 1 is required to stabilize the ring for cytokinesis.

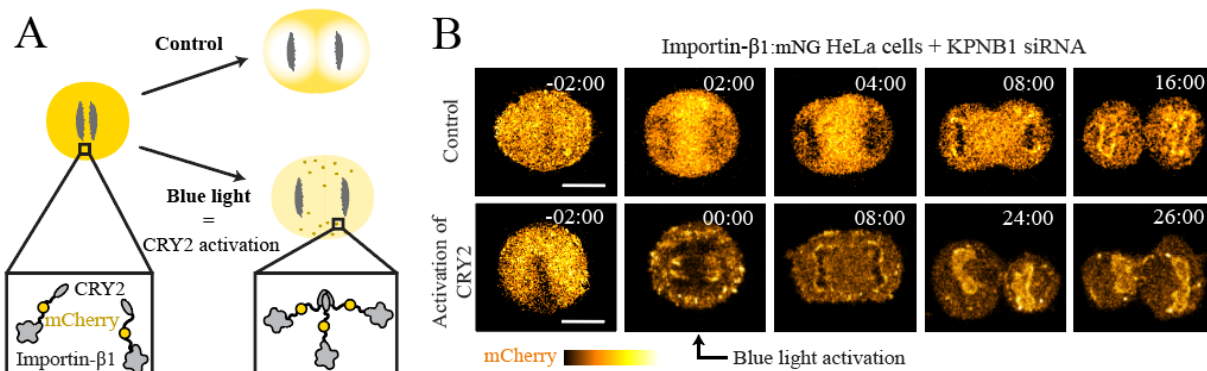


Figure 8. Importin- β 1 is temporally required during anaphase for cytokinesis. (A) A cartoon schematic shows the design and regulation of importin- β 1:mCherry:CRY2clust. Before activation, importin- β 1:mCherry:CRY2clust localizes similar to endogenous importin- β 1. After exposure to 488 nm of light for 800 ms at anaphase onset, importin- β 1:mCherry:CRY2clust clusters due to the oligomerization of CRY2clust. (B) Timelapse images show importin- β 1:mCherry:CRY2clust (orange hot LUTs: black indicates low fluorescence and yellow indicates high fluorescence) localization in importin- β 1:mNG HeLa cells after treatment with importin- β 1 RNAi. The top panel shows a control cell and the bottom panel shows a cell in which importin- β 1:mCherry:CRY2clust was activated by exposure to 488 nm of light at anaphase onset. Time is shown in minutes, and the scale bar is 10 μ m.

2.5.4 Ran-GTP free importin- β is equatorially enriched in HeLa but not HCT 116 cells

Our data shows that importin- β 1 is enriched equatorially and controls anillin's cortical localization for ring assembly in HeLa cells, but not in HCT 116 cells. Importins should be enriched where active Ran is low, and we observed correlation between importin-enrichment and chromatin position in HeLa cells. To reveal where Ran-free importin- β 1 is localized in HeLa and HCT 116 cells, FLIM imaging was performed using Rango-3, a FRET probe designed by the Káláb laboratory (Soderholm and Káláb, 2010; Hasegawa et al., 2013; Drutovic et al., 2020; Hayes et al., 2020). Rango-3 has EGFP as the donor and dsREACH (non-fluorescent) as the acceptor, and the two proteins are separated by the IBB domain from snurportin, which binds to importin- β 1 (Soderholm and Káláb, 2010). The lifetime of EGFP decreases when FRET occurs, as photons are transferred to dsREACH. When importin- β 1 is bound to Ran-GTP, it is not free to bind to the IBB and FRET occurs between EGFP and dsREACH. Consequently, regions with low lifetime indicate active FRET and *low levels* of Ran-free importin- β 1, whereas regions with higher lifetime reflect the absence of FRET and *greater levels* of Ran-free importin- β 1 (Fig. 9A). The lifetime of Rango-3 was assessed using the phasor plot generated by fast-FLIM (fluorescence lifetime imaging) and highlighting a specific region of the plot to capture the lifetime variation on a picture (Supp. Fig. S3). As shown in Supplemental Figure S4 and Supplemental Table S1, we observed high variation in the EGFP lifetime measurements among cells due to differences in the expression levels of Rango-3. Specifically, the lifetime became more uniform and decreased with higher expression of the Rango-3 probe, likely due to saturation of importin- β 1-binding. Given the challenges in imaging cells with low Rango-3 expression, cells where the Rango-3 signal is sufficient for acquisition and image analysis could be close to saturation meaning that the levels of Ran-free importin- β 1 could be underestimated. First, we performed FLIM imaging of Rango-3 in metaphase HeLa and HCT 116 cells and measured the distribution of lifetime changes along a radius from chromatin to the cortex (Fig. 9B and C). As expected, there was a comparable change in lifetime from the chromatin toward the cortex in both cell types indicating a switch from low to high Ran-free importin- β near the cortex (Fig. 9B and C). However, in HCT 116 cells, the overall lifetime was shorter (1.23 – 1.33 ns) suggesting lower concentrations of Ran-free importin- β 1 compared to HeLa cells (Fig. 9B and C). Next, we imaged Rango-3 in anaphase cells and measured the changes in lifetime along a thick line drawn subcortically from one pole to the other (Fig. 9B and C). In HeLa cells we saw longer lifetimes in the equatorial region (1.43ns) compared to the poles (1.35ns), while the lifetimes were shorter in HCT 116 cells and more uniformly distributed (between 1.23 to 1.3ns). Thus, there are overall lower levels of Ran-free importin- β 1 that are more uniformly distributed in HCT 116 cells compared to HeLa cells, which have a high pool of Ran-free importin- β 1 in the equatorial region.

Next, we generated a computational model to predict the Ran-free importin- β 1 gradient in metaphase and anaphase HeLa and HCT 116 cells derived from a previously published 1-D model (Caudron et al., 2005). As described in the methods, we used the partial differentiation equation toolbox in MATLAB to adapt the model to a 2-D geometry which represents a cell in metaphase or anaphase. The model works by solving a series of diffusion-reaction equations using the

concentrations of Ran, RanGAP, RCC1, importin- β 1 and GTP/GDP, reaction rates, kinetic constants and diffusion rates in defined zones: 1) chromatin, where RCC1-mediated exchange of GDP for GTP activates Ran, and 2) cytosol, where RanGAP hydrolyzes Ran-GTP, and Ran-GTP and importin- β 1 can form complexes (Fig. 9D, Supp. Fig. S5 and Supp. Table S2 and S3). To generate a gradient, we artificially sequestered RCC1 complexes in the chromatin zone and RanGAP and importin- β 1 complexes in the cytosolic zone, where only Ran-GTP and Ran-GDP can cross the artificial boundary (Fig. 9D and Supp. Fig. 5). To determine values for the geometrical parameters, we measured cell and chromatin length and width (Supp. Fig. S6 and Supp. Table S4). The biochemical parameters and protein concentrations for HeLa cells were taken from previous studies (Supp. Tables S2 and S3) (Caudron et al., 2005). We used publicly available RNA sequencing data comparing HeLa and HCT 116 cells (Wu et al., 2024) to make an approximation of the protein concentrations in HCT 116 cells (Supp. Fig. S7 and Supp. Table S5). To refine the predictive model, we solved the system of reaction-diffusion equations for HeLa and HCT 116 cells in metaphase and anaphase cells and compared the results to the experimental data, then made minor adjustments to the protein concentrations, while respecting measured values (Supp. Fig. S8 and Supp. Table S6). Among the conditions tested, we found that the concentrations of Ran and importin- β 1 have greater impacts on the Ran-free importin- β 1 gradient compared to RCC1 and RanGAP (Supp. Fig. S9). Using these parameters, the model predicted trends for the localization of Ran-free importin- β 1 that were similar to what was obtained by the FLIM data (Fig. 9C, E and F). In particular, there was an overall lower level of Ran-free importin- β 1 in HCT 116 cells compared to HeLa cells, and there were higher levels of Ran-free importin- β 1 in the equatorial region of anaphase HeLa cells compared to the poles, while the distribution was more uniform in anaphase HCT 116 cells (Fig. 9C and F). Thus, our model provides a good estimate of the levels and location of Ran-free importin- β 1 in cells.

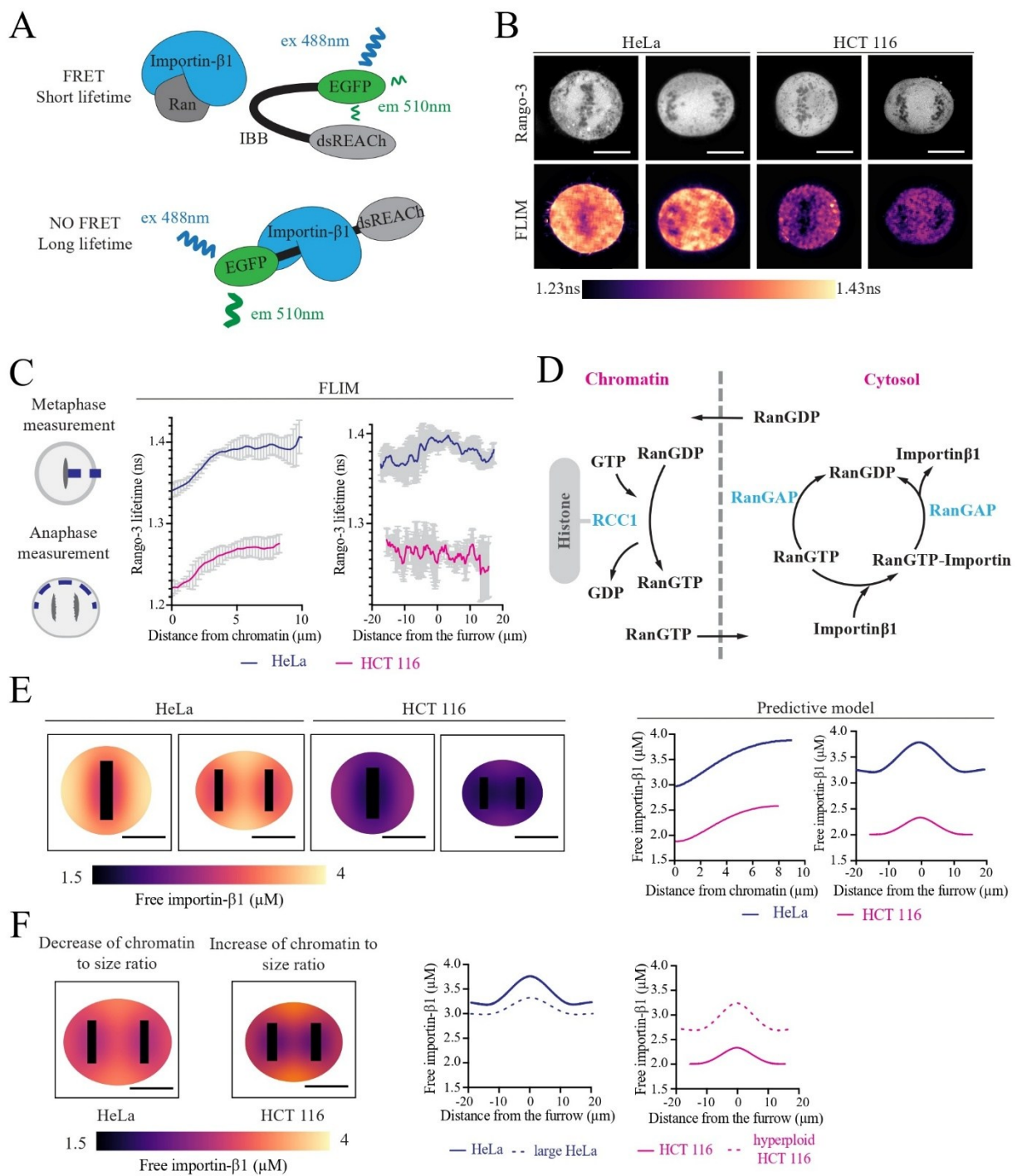


Figure 9. Ran-free importin- β 1 is equatorially enriched in anaphase HeLa but not HCT 116 cells. (A) A schematic shows the Rango-3 probe used to measure Ran-free importin- β 1 in cells. EGFP is fused to the importin- β binding domain (IBB) and dsREACH, a non-fluorescent acceptor. Binding of importin- β to the IBB prevents EGFP from interacting with dsREACH decreasing FRET, and resulting in higher EGFP lifetime compared to when importin- β is not bound. (B) Images show Rango-3 fluorescence (grey, top panel) and FLIM (ns; magma LUTs: black indicates short lifetime; pale yellow indicates long lifetime) in HeLa and HCT 116 cells at metaphase and anaphase. The scale bar is 10 μ m. (C) Cartoons on the left show where lines were drawn in metaphase and anaphase cells to measure lifetime from FLIM data or concentration from the models. The graphs show lifetime (ns) over distance (μ m) from chromatin (metaphase) or the furrow (anaphase) for HeLa (blue) and HCT 116 cells (pink). (D) The reactions used for the computational model are shown. The model solves 8 partial differential equations in a metaphase or anaphase cell. Chromatin and cytosol are separated by a fictive limit which Ran-GTP and Ran-GDP can cross freely. RCC1 and relevant complexes are fixed in the chromatin subdomain. Other species have their own diffusion coefficients and are in the cytosol. (E) Images show the predicted levels of Ran-free importin- β 1 (μ M; magma LUTs: black indicate low concentrations; pale yellow indicates high concentrations) in metaphase and anaphase HeLa and HCT 116 cells. The graphs on the right show Ran-free importin- β concentration (μ M) over distance (μ m) from chromatin (metaphase) or the furrow (anaphase) for HeLa (blue) and HCT 116 cells (pink). (F) Images show the predicted levels of Ran-free importin- β 1 (μ M; magma LUTs: black indicate low concentrations; pale yellow indicates high concentrations) in anaphase HeLa and HCT 116 cells after increasing cell volume and increasing ploidy, respectively. The graphs show the changes in concentrations in Ran-free importin- β 1 (μ M) over distance from the furrow in anaphase HeLa (blue lines) and HCT 116 cells (pink cells) after increasing cell volume and increasing ploidy, respectively, compared to control cells.

2.5.5 Chromatin to size ratio affects the equatorial enrichment of Ran-free importin- β 1 and anillin

We identified protein concentrations that accurately predicts the Ran-free importin- β 1 gradients in metaphase and anaphase HeLa and HCT 116 cells. Another parameter that could affect the Ran-GTP gradient is the chromatin to cytosolic ratio. As previously reported, the steepness of the gradient is greater in metaphase cells with higher ploidy, which could impact the mechanisms regulating spindle assembly (Hasegawa et al., 2013). Since HeLa cells have higher ploidy compared to HCT 116 cells, this could cause differences in the concentration and distribution of Ran-free importin- β 1 in anaphase cells. The relationship with ploidy and cell size is sublinear, where an increase in ploidy by a factor of 2 causes a 1.5 increase in cell size (Park et al., 2011). Thus, as ploidy increases, there could be a greater chance for active Ran to reach the cortex and create differences in the equatorial enrichment of importin- β 1. To test this, we used the computational model to predict how increasing the ploidy of HCT 116 cells impacts the distribution of Ran-free importin- β 1 during anaphase (Supp. Table S6 and S7). Indeed, the model predicted an equatorial enrichment of Ran-free importin- β 1, similar to what we observe for HeLa cells (Fig. 9F). We also speculated that increasing cytoplasmic volume in HeLa cells to reduce the chromatin to cytosol ratio, could have the opposite effect and reduce the equatorial enrichment of Ran-free importin- β 1. Indeed, using the model to predict the impact of increasing cell volume relative to ploidy in anaphase HeLa cells resulted in a more uniform distribution of Ran-free importin- β 1 (Fig. 9F).

Next, we determined if experimentally altering the chromatin to cytosolic ratio in HeLa and HCT 116 cells could affect importin- β 1 and anillin localization. To increase the ploidy of HCT 116 cells, they were treated with CoCl_2 as described in the methods (Supp. Fig. S10) (Lopez-Sánchez et al., 2014; Tripathi et al., 2019). The volume of HeLa cells was increased using hypotonic media supplemented with FBS (Supp. Fig. S10). Spindle size scales with cell size and is constrained by intrinsic biochemical regulation, and should not be significantly altered by our treatments (Rieckhoff et al., 2019). However, since volume was increased by hypotonic conditions, this could affect the concentrations of free tubulin, which could impact microtubule length. To test this, we imaged microtubules in hypotonic treated HeLa cells and did not observe any changes in the spindle in comparison to control cells (Supp. Fig. S11). In hypotonic HeLa cells, importin- β 1 was more uniformly distributed during anaphase, while we observed an equatorial enrichment of importin- β 1 in hyperploid HCT 116 cells (Fig. 10A). We measured the levels of importin- β 1 relative to the furrow and indeed, there was no change in the distribution of importin- β 1 in hypotonic HeLa cells, which remained similar over time (Fig. 10B and C). In hyperploid HCT 116 cells, there was a measurable increase in importin- β 1 in the furrow region, which appeared at 4 minutes after anaphase onset (Fig. 10B and C). Changing the chromatin to cytosol ratio also had an impact on anillin localization. In hypotonic HeLa cells, anillin was cortically localized during metaphase, and in anaphase became more broadly localized along the equatorial cortex compared to control cells (Fig. 10D-H). In hyperploid HCT 116 cells, anillin was not cortically localized in metaphase, and in anaphase localized to a narrower region along the equatorial cortex compared to control cells (Fig. 10D-H).

These results show that the chromatin to cytosol ratio affects the localization of importin- β 1 and cortical anillin.

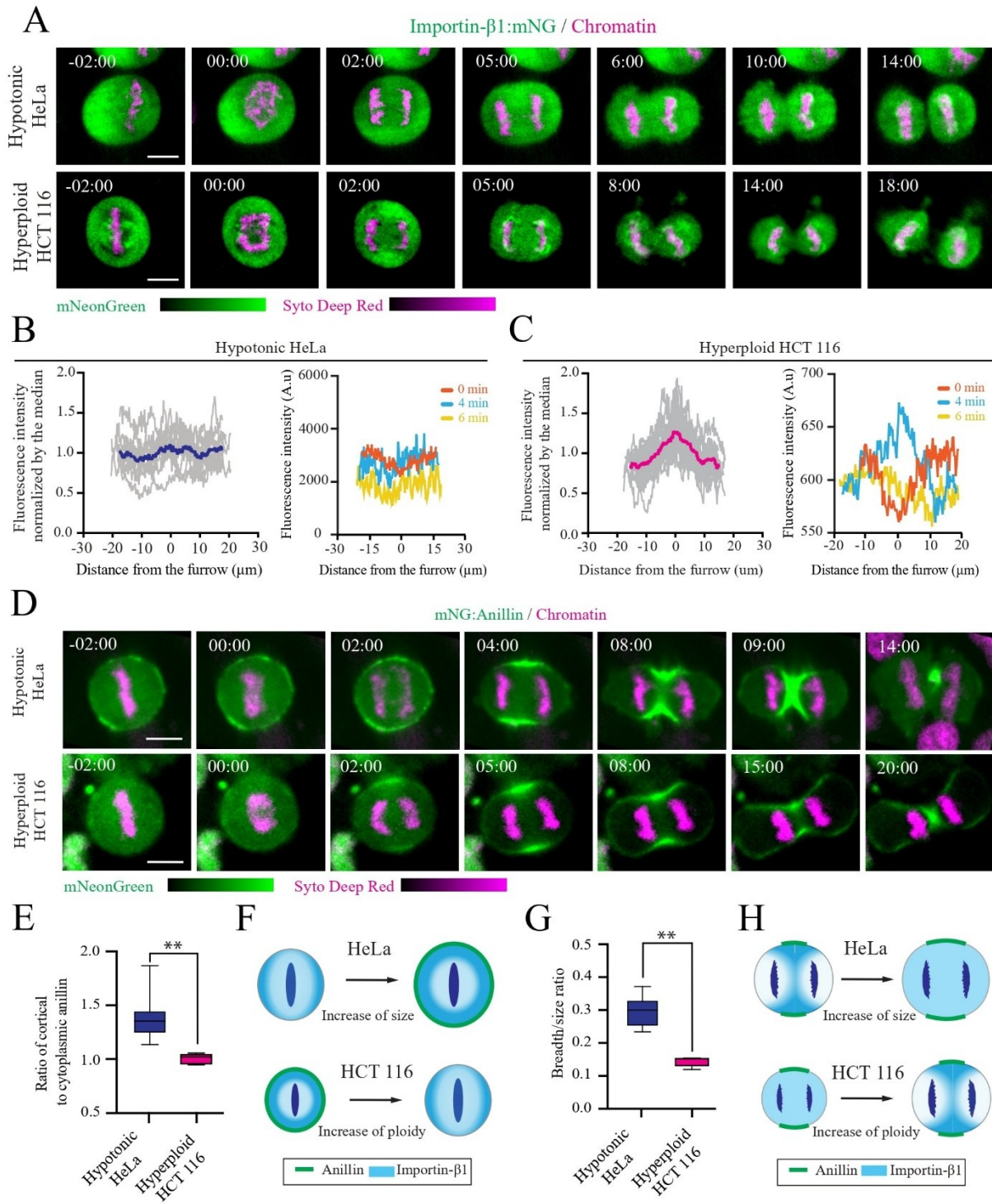


Figure 10. Chromatin to size ratio affects the localization of importin- β 1 and anillin. (A) Timelapse images show endogenous mNG:importin- β 1 (green), co-stained for DNA (Syto Deep Red; magenta) in hypotonic HeLa cells (top) or hyperploid HCT 116 cells (bottom). Time is in minutes, and the scale bar is 10 μ m. (B) Graphs show importin- β 1 fluorescence intensity at 4 minutes after anaphase onset (left) and over time (right; 0 min in red, 4 min in blue, 6 min in yellow) in hypotonic HeLa cells (blue; n=10), and (C) in hyperploid HCT 116 cells (pink; n=10). (D) Timelapse images show endogenous mNG:Anillin (green), co-stained for DNA (Syto Deep Red; magenta) in hypotonic HeLa cells (top) or hyperploid HCT 116 cells (bottom). Time is in minutes, and the scale bar is 10 μ m. (E) Graphs show the average cortical to cytosolic ratio for anillin in metaphase hypotonic HeLa cells (blue; n=10) and hyperploid HCT 116 cells (pink; n=15; **: $p \leq 0.001$). Bars indicate standard deviation. (F) Cartoon cells show the changes in cortical anillin in HeLa and HCT 116 cells after treatment to increase size or ploidy, respectively (Anillin in green, importin- β 1 in blue). (G) Graphs indicate the anillin breadth to size ratio in anaphase hypotonic HeLa (blue; n=10) and hyperploid HCT 116 cells (pink; n=15; **: $p \leq 0.001$). Bars indicate standard deviation. (H) Cartoon cells show the changes in anillin breadth in anaphase HeLa and HCT 116 cells after treatment to increase size or ploidy, respectively (Anillin in green, importin- β 1 in blue).

2.6 Discussion

Here we show that importin- β 1 regulates anillin's cortical recruitment during mitosis, and depending on the cell type, controls the enrichment of equatorial anillin to coordinate ring position with the segregating chromosomes in anaphase. While previous data suggested that importins regulate anillin function for ring assembly in response to Ran, importins themselves had not been extensively studied during earlier stages of mitotic exit. In HeLa cells, we show that both the total and Ran-free pools of importin- β 1 become transiently equatorially enriched between the segregating chromosomes and correlates with where anillin accumulates at the overlying equatorial cortex. Importin- β 1 was previously reported to have localization patterns that reflect its cargo during mitosis (Ciciarello et al., 2004), and we found that importin- β 1's equatorial enrichment is anillin-dependent. Disruption of importin- β 1 localization during anaphase caused ring oscillations similar to anillin depletion and point mutations in the NLS of anillin that reduce importin-binding caused cytokinesis failure in HeLa cells (Beaudet et al., 2017). However, despite this requirement for importin-binding in HeLa cells, importin- β 1-binding is not required for anillin localization or function in cytokinesis in HCT 116 cells. Additionally, both the total and Ran-free pools of importin- β 1 are not enriched in the equatorial region of anaphase HCT 116 cells. HeLa and HCT 116 cells differ in cell fate as well as ploidy. Changing the concentrations of Ran and importin- β 1 or altering the chromatin to cytosol ratio using our computational model predicted differences in the equatorial enrichment of Ran-free importin- β 1. Importantly, experimentally changing the chromatin to cytosol ratio by changing ploidy or cytoplasmic volume caused both importin- β 1 and anillin localization in HeLa and HCT 116 cells to change and resemble the other cell type. Thus, these parameters could be used to predict when the Ran-importin pathway plays a critical role in chromatin sensing for ring assembly during cytokinesis.

The model that was used in this research was inspired by a previously published 1D model predicting the Ran gradient in metaphase cells (Caudron et al., 2005) and extending it to a 2D representation of both metaphase and anaphase cells. The Ran-free importin- β 1 gradient was selected as the model output, which was confirmed using FLIM imaging of Rango-3. While our model made predictions on the levels and distribution of Ran-free importin- β 1 that reflected the FLIM data, the prediction showed more enrichment in the equatorial plane of HeLa cells than what was observed. As previously mentioned, Rango-3 is likely close to saturation, which could dampen the equatorial values. In addition, our model does not include importin- α , which could cause differences in the distribution of importin- β 1 (Sutton et al., 2025). Further, importin- β 1 localization is influenced by its targets TPX2 in metaphase (Ciciarello et al., 2004), and anillin in anaphase, which were not considered in the model. The levels of anillin could define a threshold of importin- β 1 that is required for regulation, and future improvements to the model could incorporate anillin binding affinities to predict this. Given that predicted Ran-free importin- β gradients in metaphase and anaphase HeLa and HCT 116 cells are similar to what was found experimentally, our findings support that chromatin to size ratio is one of the key factors that could be used to predict whether importin- β 1 is required for cytokinesis. Further, since the total pool of importin- β 1 localizes similar to the Ran-free pool,

importin- β 1 localization could be used as a proxy for predicting the role of the Ran- importin- β 1 pathway in cytokinesis.

As highlighted above, our study did not test a role for importin- α in cytokinesis. The classical bipartite NLS in anillin is predicted to bind to a heterodimer of importin- α and importin- β 1. Our studies focused on importin- β 1 because there are multiple importin- α isoforms that could bind to classical NLS's, while importin- β 1 is predicted to be the prominent importin- β (Yang et al., 2023). Depending on the importin- α , post-translational modifications that influence localization or stability could affect the levels of the heterodimer and their location, which could also impact their role in ring assembly. It will be important to investigate which importin- α binds to importin- β 1 to regulate cytokinesis and/or if importin- β 1 functions separately.

Our model and experimental data revealed that several factors can affect the Ran-importin pathway and its ability to regulate the ring during cytokinesis. Notably, a previous study showed that chromosomal gain increases chromatin-associated RCC1 and the steepness of the Ran gradient, which affects spindle assembly in cultured human cells (Hasegawa et al., 2013). This steeper gradient is predicted to remain with the segregated chromosomes in anaphase, creating a greater differential enrichment of equatorial importin- β 1 compared to cells with less steep gradients. Interestingly, changing RCC1 alone in the model did not have as great an impact as changing Ran or importin- β 1 levels, indicating that changes in the concentration of Ran components likely also contribute to the steepness of the gradient.

Our findings support that the requirement for the Ran-importins pathway in regulating the ring for cytokinesis varies with cell type. This is consistent with our previous findings studying this pathway in *C. elegans* embryos. We found that increasing ploidy or changing the levels of active Ran by depletion of RCC1, RanGAP, importin- α and/or - β caused changes in ring kinetics, but there were differences depending on the cell type (Ozugergin et al., 2022) AB cells are larger and give rise to multiple tissues of the adult worm, while P1 cells are smaller and give rise to the germline. Interestingly, we found that anillin was not a target of the Ran pathway in P1 cells, suggesting that it has other targets that affect ring assembly (Ozugergin et al., 2022). Thus, differences in size and fate could support different requirements for the cytokinetic machinery including regulation by Ran-importins in distinct cell types. Determining which cell types rely on this pathway, and how threshold levels of anillin or other effectors influence pathway engagement, will be an important direction for future studies. Given that many progressive cancers have aneuploidy with a net gain in chromosome content, targeting the chromatin-sensing pathway or its mediators such as importin- β 1 may provide new strategies to restrict their proliferation.

In summary, we uncover a central role for importin- β 1 as a readout for chromosome position and Ran activity to coordinate ring position via anillin, and we define the chromatin-to-size ratio as a key determinant of when cells require the chromatin-sensing pathway for successful cytokinesis.

Chapter 3

Characterization of mechanisms regulating iPSC cytokinesis

3.1 Summary

Cytokinesis is the physical separation of a cell into two daughters. Cytokinesis occurs due to the ingression of a contractile ring that is spatiotemporally regulated by multiple pathways. Cytokinesis has been studied in a handful of transformed human cell lines, and we lack knowledge of how most human cells divide. Studying cytokinesis in more diverse and healthy cell types is critical to identify tissue-specific vulnerabilities. In this study, we used human induced pluripotent stem cells (iPSCs) with endogenously tagged cytokinetic proteins to characterize cytokinesis and reveal the mechanisms regulating ring assembly. We found that ring constriction is asymmetric in iPSCs, with anillin enriched at the leading side of the ring. We also found that astral microtubules play dual roles in ring assembly, with an outer pool that is bundled by central spindle proteins to promote ring assembly, and asters that restrict the ring to a narrow zone. Finally, comparison with differentiated endoderm cells revealed that they have a broader ring, suggesting that the mechanisms regulating ring assembly differ. RNA sequencing analysis suggests that spindle-associated pathways are downregulated as iPSCs differentiate into hepatoblast progenitors and hepatocyte-like cells which could cause defective ring assembly and cytokinesis failure. Overall, this work represents a first step towards generating an atlas of cytokinesis regulation in normal human cells.

3.2 Introduction

Cytokinesis occurs due to the assembly and ingression of a contractile ring that pinches the cell into two daughter cells. This process is tightly controlled by multiple pathways associated with the spindle or chromatin. Failure of cytokinesis leads to genomic instability causing diseases like cancer, blood disorders and developmental abnormalities (Ganem et al., 2009; Lacroix and Maddox, 2012). Conversely, cytokinesis failure is critical to increase ploidy for the differentiation of cells in the liver and bone marrow, for tissue specialization and enhanced metabolic capacity (Davoli and de Lange, 2011; Lacroix and Maddox, 2012). Most of our knowledge of cytokinesis is from transformed human cells, and it is critical to study normal cell types. Notably, the mechanisms regulating the ring are predicted to vary with ploidy and cell fate.

Multiple pathways regulate cytokinesis. These pathways lead to the assembly of a well-positioned RhoA-dependent actomyosin ring (Green et al., 2012). RhoA activation is stimulated by signals from the central spindle. The centralspindlin complex is composed of a heterotetramer of MKLP1 and Cyk4, which bundles central spindle microtubules (Mishima et al., 2002). Cyk4-binding recruits Ect2 to the equatorial region of the cell and is required for Ect2 to become active (Yüce et al., 2005; Green et al., 2012). Prior to Ect2-binding, Cyk4 needs to be phosphorylated by Plk1, a kinase which also inhibits PRC1, a core regulator of central spindle bundling (Petronczki et al., 2007; Wolfe et al., 2009; Gómez-Cavazos et al., 2020). Active RhoA binds to effectors to generate actin and myosin filaments, as well as anillin, which crosslinks actomyosin and binds to phospholipids to anchor the ring to the

membrane (Piekny and Maddox, 2010). In some cell types, anillin can also be recruited to the equatorial cortex by importin- β 1, which is enriched between the segregating chromosomes (Beaudet et al., 2017 and 2020)). Ring assembly also relies on the clearance of contractile proteins from the poles, which is achieved by signals coming from kinetochores and astral microtubules. The ERM (ezrin, radixin, moesin) proteins crosslink and stabilize F-actin and are negatively regulated by kinetochore-associated PP1-Sds22 phosphatase complex to promote local actin clearance when the spindle comes close to the polar cortex (Rodrigues et al., 2015). RhoA can be negatively regulated at the poles by the spindle assembly factor TPX2, which increases MP-GAP activity via Aurora A kinase (Zanin et al., 2013; Mangal et al., 2018; Wolff et al., 2023). Astral microtubules also associate with another protein, CLIP-170, which negatively regulates actin at the poles through the formin DIAPH1 (Chen et al., 2021). Finally, astral microtubules can also directly bind to anillin and remove it from the polar cortex where active RhoA is low (van Oostende Triplet et al., 2014).

Cytokinesis has been extensively studied in embryonic cells in *C. elegans*, *Xenopus*, and *Drosophila* in addition to diseased and/or transformed human cell lines. The core cytokinesis machinery is highly conserved, but the mechanisms regulating this machinery vary. Depending on the cell type and organism, there are differences in ring kinetics, ring breadth, central spindle and astral microtubules, chromatin (ploidy), and differences in the expression of ring components and ring regulators, among others (Chen et al., 2017; Davies et al., 2018; Wang et al., 2020; Chen et al., 2021; Ozugergin et al., 2022). The contractile ring can also be positioned asymmetrically to give rise to two daughter cells of different sizes and fates, or ingress asymmetrically (Fig. 13A). Asymmetric ingression is observed in early embryos in *C. elegans* and mice, but also in canine kidney epithelial (MDCK) cells and *Drosophila* epithelial cells, while in human cell lines including HEK293T, HeLa, HepG2 and HCT 116 cells, ingression is symmetrical (Maddox et al., 2007; Pinheiro et al., 2017; Chan et al., 2019; Paim and FitzHarris, 2022; Husser et al., 2022). Diversity in cytokinesis among cell types highlights the need for studies of cytokinesis in normal human cells (Husser et al., 2020; Ozugergin and Piekny, 2023).

Despite intensive study of stem cell pluripotency and differentiation, very little is known about cytokinesis in healthy human stem cells or tissues. Stem and progenitor cells sustain tissue homeostasis by balancing self-renewal and differentiation, which is often modulated by intrinsic polarity cues and extrinsic niche signals (Horvitz and Herskowitz, 1992; Morin and Bellaïche, 2011). Studies of epithelial cells within tissues of *Xenopus* or *Drosophila* revealed how extrinsic mechanisms like tension and mechanical forces from neighbor cells influence ring position and kinetics (Pinheiro & Bellaïche, 2017; di Pietro et al., 2023; Landino et al., 2025). However, there is little knowledge of how cytokinesis changes during cell differentiation. Recent work has begun to bridge this gap. In *C. elegans*, our group and others characterized key differences between cells in *C. elegans* embryos, revealing differences in ring composition, ring kinetics, and gene requirements (Ozugergin et al., 2022; Connors et al., 2024; Mauro et al., 2024; Pimpale et al., 2020).

Recently, we generated tools to study cytokinesis in human induced pluripotent stem cells (iPSCs). iPSCs are self-renewing, undifferentiated cells capable of giving rise to any cell within the body (Shi et al., 2017; Williams et al., 2012). iPSCs are reprogrammed from adult somatic cells into a

pluripotent state (Takahashi et al., 2007). They closely mimic the biological and functional properties of embryonic stem cells (ESCs). We created a split tag system in iPSCs to endogenously tag cytokinetic proteins with mNeonGreen, enabling studies of cytokinesis (Husser et al., 2024). Characterization of RhoA and anillin localization, as well as microtubules revealed that iPSCs had narrower contractile rings, robust astral microtubules, and what appeared to be a less prominent central spindle compared to HeLa cells. This led us to hypothesize that iPSCs rely more heavily on astral microtubules compared to the other pathways to regulate ring assembly.

Here, we characterized cytokinesis in iPSCs in more depth by imaging ezrin, actin and central spindle proteins, and revealed that astral microtubules regulate ring assembly. Importantly, we found that while the asters strongly restrict anillin localization, a distinct pool of astral microtubules is bundled by centralspindlin near the cortex and likely promote ring assembly in that location. Further, we found that the chromatin-sensing pathway is likely not required by iPSCs as importin- β 1 does not differentially accumulate during anaphase. Additionally, we revealed that the ring ingresses asymmetrically, with anillin concentrated on the leading side of the ring. Finally, we differentiated iPSCs into endoderm cells, and observed differences in anillin localization, suggesting that the mechanisms regulating the ring assembly change during differentiation. Using RNA sequencing data, we speculate that as cells further differentiate into hepatoblasts, polyploidization could occur due to the downregulation of spindle-dependent pathways required for ring assembly. These findings provide new knowledge of cytokinesis in iPSCs and lay the foundation for further studies of cytokinesis in diverse normal human cell types.

3.3 Materials and Methods

Cell culture, drug treatment and transfection

Culture. smNG2-P₁₋₁₀ iPSCs, which constitutively expresses the mNG₁₁ fragment and derived from 201B7 iPSCs (ATCC, catalog number ACS-1023), were used in this study, as well as previously generated lines: FOXA2:smNG2 iPSCs, smNG2:Anillin iPSCs and smNG2:Actin iPSCs (Husser et al., 2024). Cells were cultured in mTeSR Plus media (STEMCELL Technologies) in 6-well or 12 well plates coated with iMatrix-511 Silk (Iwai North America Inc) as per the manufacturer's protocol. The cells were passaged every 3-4 days by dissociating them using Accutase (STEMCELL Technologies) according to the manufacturer's protocol. After dissociation, the cells were resuspended and transferred into freshly coated plates (25,000-50,000 cells per well of a 12-well plate; 50,000-100,000 cells per well of a 6-well plate) containing mTeSR Plus media with 10 μ M Y-27632 (ROCK inhibitor, STEMCELL Technologies). After plating, cells were incubated in media without ROCK inhibitor, which was changed daily. The cells were incubated at 37°C and 5% CO₂.

Drug treatment. 1,000 to 3000 cells were plated in 96-well optical-compatible polymer-based plate (Thermofisher). After 4 days, cells just after anaphase onset were treated with 30nM nocodazole (Sigma-Aldrich) to selectively disrupt astral microtubules.

Transfection. Cells were transfected in suspension. For RNAi, 10,000 cells were transfected and plated in a coated 96-well optical-compatible polymer-based plate (Thermofisher). First, 5nM of

anillin siRNAs (Dharmacon Horizon Discovery) were mixed with 0.6 μ L of Lipofectamine 3000 (ThermoFisher) or Oligofectamine (ThermoFisher) and 18.4 μ L of Opti-MEM (ThermoFisher) and incubated at room temperature for 15-20 minutes. The mix was added to cells in 200 μ L of mTeSR Plus media with 10 μ M ROCK inhibitor (Y-27632; STEMCELL Technologies). The next day, the media was replaced with fresh media. The cells were incubated at 37°C and 5% CO₂ and imaged within 48 hours after transfection, to assess the number of binucleate cells. For DNA plasmids, 500,000 cells were transfected and plated in a coated 35 mm dish with a 20 mm micro-well and #1.5 glass-like polymer coverslip in a chamber (Cellvis). For transfection, 2 μ g of DNA was mixed with 3 μ L of p3000 and 100 μ L of Opti-MEM and incubated for 5 minutes. Then, 100 μ L of Opti-MEM was mixed with 3 μ L of Lipofectamine 3000 for 5 minutes, then added to the first mix and incubated for another 15-20 minutes. The transfection mix was added to cells in 2 mL of mTeSR Plus media and 10 μ M ROCK inhibitor. The next day, media was replaced with fresh media. The cells were incubated at 37°C and 5% CO₂ for and imaged about 48 hours after transfection.

Cloning

The cDNA for human Plk1 and Cyk4 was amplified from the hORFeomeV7.1 library (Horizon). These cDNAs, as well as mScarlet cDNA, were each cloned into the pYTK001 backbone (Addgene #65108; Lee et al., 2015) by Golden Gate assembly (Engler et al., 2009) using BsmBI (New England Biolab), then Plk1 and Cyk4 were each assembled with mScarlet into a modified pX459V2.0-HypaCas9 vector (Addgene #108294; Kato-Inui et al., 2018) using BsaI (New England Biolab). Constructs were transformed into *Escherichia coli* DH5 α . All plasmids were sequenced by Plasmidsaurus.

Gene editing

Editing was done by CRISPR/Cas9 using freshly prepared ribonucleoprotein and nucleofection as previously published (Husser et al., 2024). 4h before nucleofection, NHEJ inhibitors: AZD7688 (Tocris Bioscience) and PolQ_i2 (generously provided by the Genome Foundry, Concordia University; Dacquay et al., 2025), were added to 500,000 cells to a final concentration of 0.5 μ M and 1.5 μ M, respectively. 2h before nucleofection, Clone R2 (STEMCELL Technologies) was added according to the manufacturer's protocol. The cells were dissociated using Accutase (STEMCELL Technologies), then centrifuged at 400g for 5 minutes, and supernatant was replaced with PBS (Wisent). The cells were centrifuged again and resuspended in 20 μ L of P3 nucleofection buffer (Lonza), before transferring to a nucleofection cuvette (Lonza). 30.5 pmoles of spCas9 2NLS Nuclease (Synthego) and 91.5 pmoles of gRNA (Synthego) were mixed and incubated at room temperature for 15-60 minutes, before adding the mix to the cells in a nucleofection cuvette. The guide RNAs used to target ezrin and importin- β 1 were previously successfully used by Open Cell biohub (Cho et al., 2022). The repair templates containing the small fragment of mNG (48bp) and 100bp of gene-specific sequence before and after the targeted site were built as 248bp ssODNs (Synthego). For nucleofection, 82 pmoles of the ssODN was added to cells in a nucleocuvette and nucleofected (4D-Nucleofector; Lonza) using the DN100 program. After nucleofection, the cells were left at room temperature for 5 minutes before transferring them to a well in a coated 24-well plate containing 0.5ml of mTeSR Plus media (STEMCELL Technologies) with 1% of Clone R2 (STEMCELL Technologies). Pen-Strep (50 units/mL Penicillin and 50 μ g/mL Streptomycin; Wisent) was added to the cells 1 hour after

nucleofection. For two days, the media was changed daily with mTeSR Plus media and Clone R2, then daily with mTeSR Plus media only.

Fluorescence-activated cell sorting and screening

Fluorescence-activated cell sorting. Cells were sorted using a FACSMelody cell sorter (BD Biosciences) 8 days after nucleofection. Briefly, cells were dissociated using Accutase (STEMCELL Technologies), resuspended in PBS (Wisent), and then passed through a 40 μ m strainer to remove large cell clumps. Cells were sorted using gates set to capture individual cells into wells of a 96-well plate containing mTeSR Plus media (STEMCELL Technologies) with CloneR2 (STEMCELL Technologies) reagent and Pen-Strep (50 units/mL Penicillin and 50 μ g/mL Streptomycin; Wisent). To recover clones, cells were kept in media for 6 days with media changes on days 2 and 4. The media was then changed daily with mTeSR Plus until day 11. On day 11, the cells were passaged into fresh 96-well plates and grown to confluency before screening and freezing.

Screening. The colonies from single cell sorting were imaged using a Cytation 5 microscope (Agilent) to assess fluorescence. Cells from positive clones were subsequently passaged into two 96-well plates; one for freezing and one for lysis to sequence for successful integration of the small mNG fragment. The cells for lysis were washed with PBS and resuspended in 50 μ L of QuickExtract reagent (ThermoFisher) per well. The cells were then incubated at 65°C for 15 minutes, then mixed and placed at 98°C for 15 minutes. Integration of the mNG₁₁ fragment was verified by amplifying 1kb fragments. PCR reactions were prepared by mixing 10 μ L GC buffer (Thermo Fisher Scientific), 1 μ L 10 mM dNTP mix (Thermo Fisher Scientific), 1 μ L DMSO (Thermo Fisher Scientific), 0.25 μ L of each primer at 100 μ M, 5 μ L cell lysate, 0.5 μ L Phusion polymerase (Thermo Fisher Scientific) and 32 μ L water. The following touchdown cycles were then performed: 98°C for 3 min, followed by 40 cycles of: 98°C for 10s, initial annealing at 72°C and decreasing by 1°C every cycle until down to 55°C, and 72°C for 30s per kb; followed by a final extension at 72°C for 10min and then held at 12°C. The PCR products were run on an agarose gel and extracted using the GeneJET gel extraction kit (Thermo Fisher Scientific) for sequencing by Plasmidsaurus. The sequencing data was analysed using Snapgene. Positive clones used for subsequent analysis were homozygous for insertion at the ezrin locus, and heterozygous at the importin- β 1 (KPNB1) locus (Supp. Fig. S12).

Endoderm differentiation

The STEMdiff™ Definitive Endoderm Kit (STEMCELL Technologies) was used to differentiate iPSCs into endoderm cells as per the manufacturer's protocol. On day 0, 5,000 cells were seeded as single cells per well in a coated 96-well plate containing mTeSR Plus media (STEMCELL Technologies) with 10 μ M Y-27632 (ROCK inhibitor; STEMCELL Technologies). On day 1, media was replaced with STEMdiff™ Endoderm Basal Medium containing STEMdiff™ Definitive Endoderm Supplement MR and STEMdiff™ Definitive Endoderm Supplement CJ. On day 2 to 5, media was replaced with with STEMdiff™ Endoderm Basal Medium containing STEMdiff™ Definitive Endoderm Supplement CJ. Differentiation was assessed by cell morphology and FOXA2 expression using FOXA2:smNG2 cells.

Microscopy

For live-cell imaging, cells were seeded on a coated 35 mm dish with 20 mm micro-wells and #1.5 glass-like polymer coverslip (Cellvis) or 96-well optical-compatible polymer-based plate (Thermofisher) and incubated at 37°C with 5% CO₂. To visualize DNA, Syto Deep Red (Thermofisher) was added to a final concentration of 0.25 – 2.5 µM. To visualize microtubules, SiR-tubulin (Thermofisher) was added to the cells to a final concentration of 1.25 pM. To visualize the cell membrane, Orange CellMask (Thermofisher) was added as per the manufacturer's protocol. The cells were imaged using an inverted Nikon Eclipse Ti2 microscope with a CSU-X1 spinning disk (Yokogawa), using the 60×/1.4 CFI PLAN APO VC oil immersion objective (Nikon), with a piezo Z stage (MadCityLabs), and Zyla camera (Andor). The 488 nm laser was used at 15% of its power with a 400 ms exposure time. The 638 nm laser was used at 10% of its power with a 300 ms exposure time. Images were acquired every 2min for 20-40min and 15 z stacks with a 1.5µm step were acquired for each time point.

For super-resolution imaging, a commercial structured illumination microscope (MI-SIM, CSR Biotech) was used to acquire and reconstruct the cell images. The procedure for imaging was performed using previously published protocols (Lal et al., 2018). To further improve the resolution and contrast in reconstructed images, sparse deconvolution was performed as previously published (Zhao et al., 2021).

RNA sequencing analysis

RNA sequencing data generated from human iPSCs, hepatoblasts and hepatocyte-like cells (HLCs) was obtained from the accession number GSE152390 (Gene Expression Omnibus), generated by Dr. Paganelli's group (Cuomo et al., 2022). RNA sequencing data of human iPSCs and endoderm cells was obtained from the accession number GSE248820 generated by a different group (Okubo et al., 2025). The analysis of genes in cytokinesis was used to plot the log₂ fold change of the normalized gene expression changes for iPSCs vs. hepatoblasts, iPSCs vs. HLCs or iPSCs vs. endoderm cells.

Image analysis

All image files were exported as TIFFs and opened in Fiji (NIH). To obtain ZY views, we used the orthogonal view tool of Fiji. To measure fluorescence intensity of anillin, actin or ezrin, a 3-pixel-wide line was drawn and measured on the leading or lagging region of the ring. The region of the ring that was in contact with the coverslip was not included. The average fluorescence intensity ratio was calculated by dividing the average fluorescence intensity of the leading by the lagging regions of the ring. The asymmetry ratio was calculated by measuring the furthest vs. the smallest distance between the closed ring and the cortex before constriction. Linescans of anillin and Cyk4 were performed at ~4 minutes after anaphase onset on a maximum z-projection of 3 z-slices (1 µm each) by drawing a 5-pixel-wide line around the cortex of the cell and plotting anillin or Cyk4 intensity along this line, respectively. The breadth of the peak was measured as the number of pixels >50% of the maximum value and divided by the length of the cell to get the breadth to size ratio. The enrichment of anillin and Cyk4 over time was measured by drawing a small region of interest (ROI) at the equatorial cortex at 0, 2, 4 and 6 minutes after anaphase onset and the average fluorescence intensity in the ROI was plotted over time. For Cyk4, we also performed a linescan through the

equatorial plane and measured the distance between its localization near the cortex and astral microtubules compared to the central region of the spindle. Linescans of importin- β 1 were performed by drawing a 10-pixel-wide line just under the cortex of the cell at ~2 minutes after anaphase onset on a sum z-projection of 3 z-slices (1 μ m each) and plotting importin- β 1 intensity. To plot the linescans of multiple replicates together, each linescan was normalized by its median. To determine the ratio of cortical to cytosolic anillin fluorescence, the background was first subtracted from the image, then three regions of interest (ROI) were drawn in the cytosol or at the cortex and averaged, then the cortical average value was divided by the cytosolic average value. The average cytosolic anillin fluorescence was measured as a proxy for levels of abundance.

All data was exported as compatible files that were opened in Excel (Microsoft) for analysis. All graphs and statistics were generated using Prism (v.9.3, GraphPad). Statistical significance was assessed using a Welch t-test.

3.4 Results

3.4.1 The contractile ring ingresses asymmetrically in iPSCs

We had previously shown that anillin localizes to a narrow breadth at the cortex of dividing iPSCs, which also have prominent astral microtubules (Husser et al., 2024). To further characterize the ring in iPSCs, we imaged anillin during cytokinesis. To do this we used smNG2-P₁₋₁₀ iPSCs where endogenous anillin is tagged with the small fragment of mNG₁₁ and collected z-planes to provide an end-on view of the ring (ZY) at the equatorial axis (Fig. 11A). As shown in the images in Figure 11B, the ring appeared to close asymmetrically. Measurements revealed that the distance between the leading side of the ring just before ingression and the midbody was 4.1 ± 1.3 times larger than the distance between the lagging side of the ring and the midbody (Fig. 11C). Interestingly, asymmetric ingression was not dependent on adhesion to the coverslip, as we observed ring ingression away from the coverslip in 14 cells and toward the coverslip in 16 cells, suggesting that intrinsic signals regulate asymmetric ingression. Further analysis of anillin localization revealed that it was enriched at the leading portion of the ingressing ring (Fig. 11B). We then imaged iPSCs with endogenous mNeonGreen tagged ezrin, which is part of the Ezrin/Radixin/Moesin (ERM) family of F-actin-binding and crosslinking proteins, and is regulated by kinetochore signaling (Rodrigues et al., 2015). As shown in Fig. 11B, ezrin appeared to be cytosolic at anaphase onset, and then became weakly enriched at the equatorial cortex or remained cytosolic (4 out of 14 cells, not shown). In cells where ezrin was enriched, it was initially asymmetrically localized, but then became evenly distributed during ingression (Fig. 11B). We also imaged F-actin during cytokinesis. As a core ring component, we did not expect it to be asymmetrically distributed, but both anillin and ezrin can crosslink F-actin and could influence its distribution. As shown in Figure 11B, actin was uniformly distributed within the ingressing ring. Indeed, quantitative measurements of the fluorescence intensity of anillin, ezrin and actin revealed that there was asymmetric distribution of anillin between the leading and lagging side of the ring, while ezrin and F-actin were evenly distributed (Fig. 11D). Therefore, ring components are not asymmetrically distributed per se, and since anillin is asymmetrically distributed it could influence mechanical properties that impact constriction.

Ring ingression is also asymmetric in the first zygotic division in *C. elegans*, which is dependent on anillin, but anillin depletion does not cause cytokinesis failure (Maddox et al., 2007). To determine if anillin is required for cytokinesis in iPSCs, smNG2:Anillin iPSCs were transfected with anillin siRNAs and cytokinesis failure was assessed by counting the proportion of bi (or multi) nucleated cells (Fig. 12). We observed that $64.5 \pm 5.5\%$ cells failed cytokinesis after anillin depletion (3 biological replicates of $n = 100$ cells each; Fig. 12). Therefore, unlike *C. elegans*, the requirement is more like what was reported for HeLa cells (Piekny and Glotzer, 2008; Beaudet et al., 2017). This is not a feature of growing human cells *in vitro*, since we observed significantly lower requirements for anillin in other cultured human cell lines (e.g., HFF-1, HCT 116; Kevin Larocque, unpublished observations).

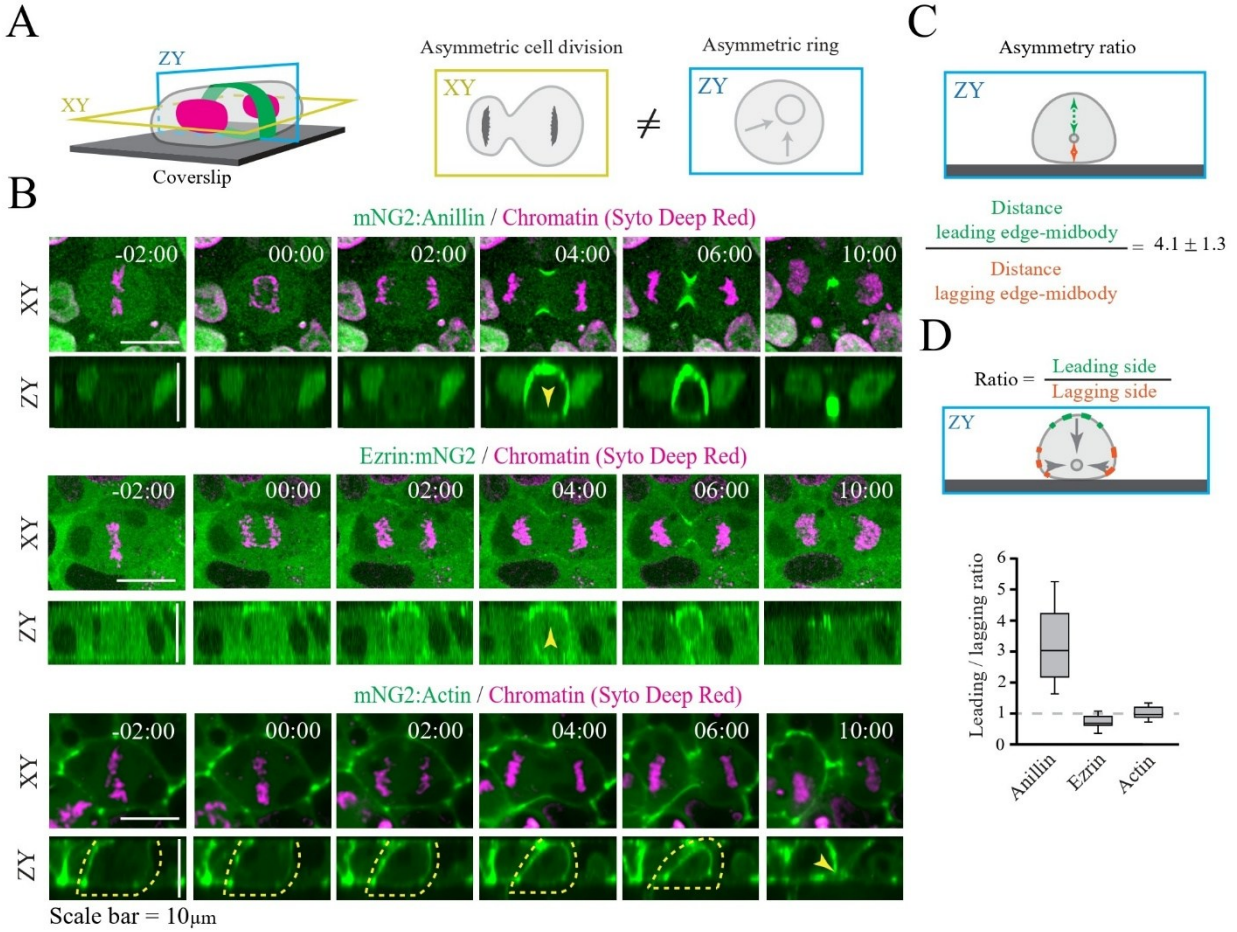


Figure 11. The contractile ring ingresses asymmetrically with asymmetric anillin distribution in iPSCs. (A) A cartoon shows an adherent cell in 3D and the XY (yellow) and ZY (cyan) planes. Chromatin is shown in pink and the ring is in green. To the right, cartoon cells show the difference between an asymmetrically positioned ring and asymmetric ingression using XY and ZY views. (B) Timelapse images show XY and ZY views of endogenous smNG2:Anillin (green), Ezrin:smNG2 (green) and smNG2:Actin (green) in iPSCs co-stained for chromatin (Syto Deep Red; magenta). In the ZY images of smNG2:Actin, dashed yellow lines outline the ingressing ring. Yellow arrowheads point to the lagging side of the ring. Time is in minutes, and scale bars are 10 μ m. (C) A cartoon shows how the ratio of asymmetry was measured for the ring, by dividing the distance from the leading side of the ring to the midbody by the lagging side (n= 30 cells). (D) A cartoon shows how the average fluorescence of the leading to the lagging side ratio was measured for each tag. The graph below shows the ratio of anillin (n=10 cells), ezrin (n= 10 cells) and actin (n= 10 cells) in the leading vs. lagging side of the ring. Bars indicate standard deviation.

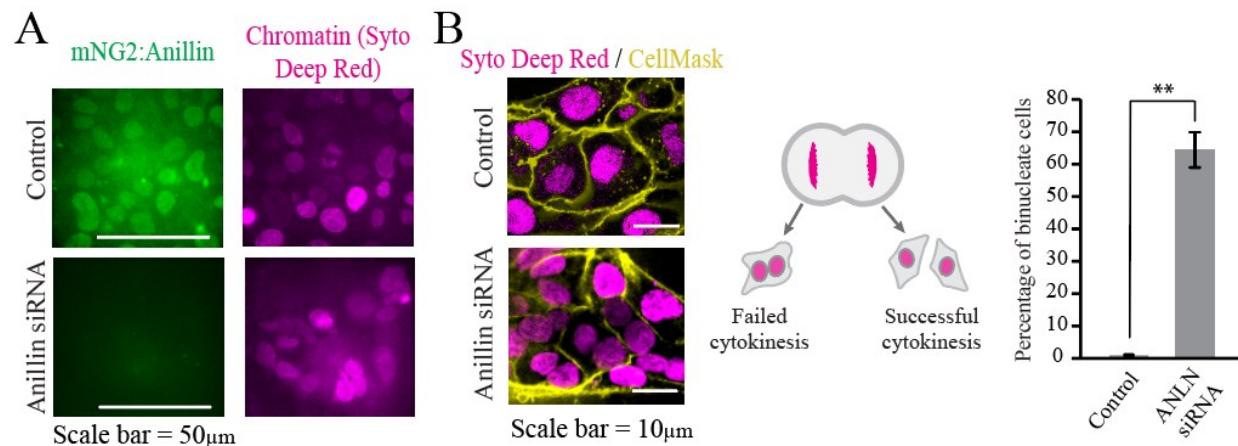


Figure 12. Anillin is required for cytokinesis in iPSCs. (A) Images show a field of view of multiple iPSCs with smNG2:Anillin (green) or after anillin RNAi co-stained for chromatin (Syto Deep Red; magenta). The scale bar is 50 μ m. (B) Images show iPSCs with smNG2:Anillin or after anillin RNAi co-stained for chromatin (magenta) and the cell membrane (Orange Cell Mask; yellow). The scale bar is 10 μ m. The cartoon cells show how cytokinesis failure leads to the formation of binucleate cells. On the right, the bar graph shows the percentage of binucleate cells in control cells or with anillin RNAi (N = 3 biological replicates of n= 100 cells each). The bars show standard deviation. Asterisks (**) indicate significance (** $p \leq 0.0001$).

3.4.2 Importin- β 1 is not equatorially enriched in anaphase iPSCs

As indicated previously, chromatin-sensing is a mechanism that regulates ring positioning in HeLa cells. A role for this pathway can be predicted if there is an enrichment of importin- β 1 localization in the equatorial plane during anaphase. In cells with higher chromatin to cytosol ratios, the steeper Ran gradient leads to the accumulation of importin- β 1 in the region between the segregating chromosomes, where it promotes the recruitment of anillin for cytokinesis (Beaudet et al., 2020; manuscript in progress, Chapter 2). Although we did not expect to see a requirement for the chromatin sensing pathway in diploid iPSCs, we still wanted to test this by imaging importin- β 1 during cytokinesis. To do this, we endogenously tagged importin- β 1 with the mNG₁₁ fragment in smNG2-P₁₋₁₀ iPSCs (Supp. Fig. S12). Imaging revealed that there was no equatorial enrichment of importin- β 1 in iPSCs during cytokinesis (Fig. 13A). To verify this, we measured the levels of importin- β 1 fluorescence by drawing a thick subcortical line from one pole to the other ~2-4 minutes after anaphase onset. As shown in Figure 13B, importin- β 1 was not equatorially enriched in iPSCs. This finding suggests that the chromatin-sensing pathway may not be required for cytokinesis in iPSCs, although this needs to be tested.

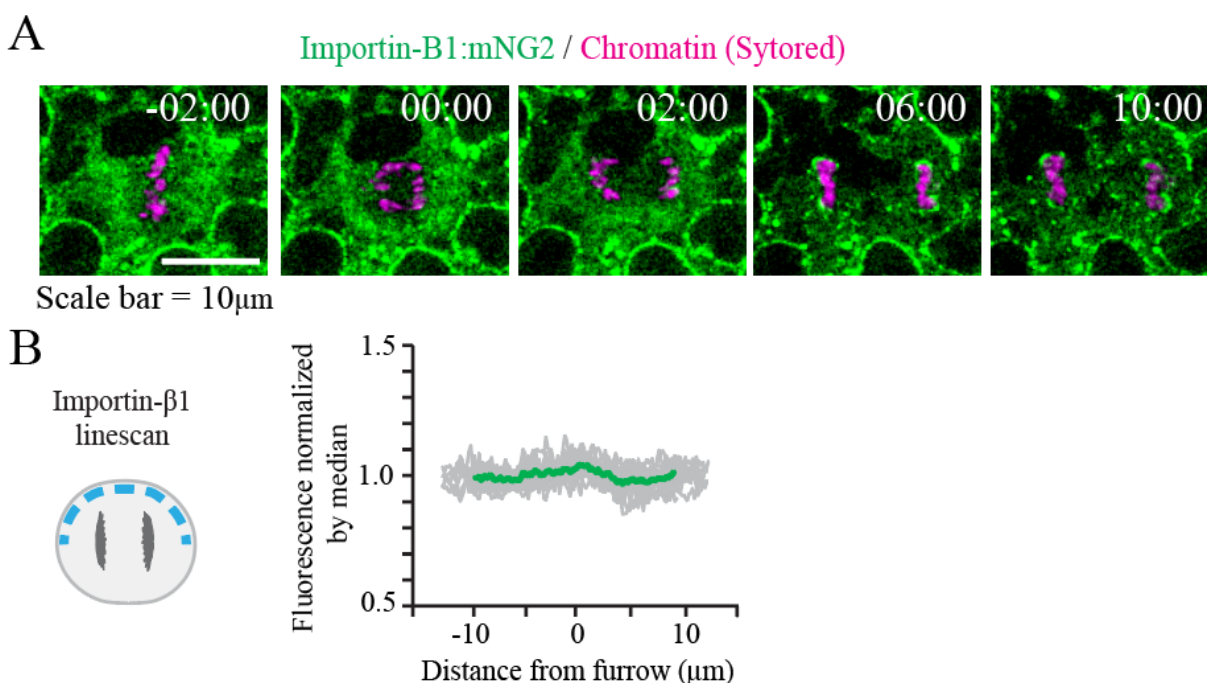


Figure 13. Importin- β 1 is not equatorially enriched in anaphase iPSCs. (A) Timelapse images show iPSCs with endogenous Importin- β 1:smNG2 (green) co-stained for chromatin (Syto Deep Red; magenta). Time is shown in minutes and the scale bar is 10 μ m. (B) A cartoon cell shows how importin- β 1 fluorescence was measured in anaphase cells. A thick line (2 μ m) was drawn subcortically from one pole to the other and the intensity along this line was plotted. Graphs show linescans of the fluorescence intensity of importin- β 1 (n=15) normalized by their median over the length of the cell (shown as distance from the furrow).

3.4.3 iPSCs have two pools of astral microtubules and a weak central spindle during cytokinesis

Previous studies suggested that iPSCs have prominent asters and a weak central spindle, however this was speculated based on lower resolution images of endogenously tagged microtubules (Husser et al., 2024). To further characterize the spindle in iPSCs and HeLa cells, we obtained higher resolution images using Structured Illumination Microscopy (SIM) with smNG2:Anillin iPSCs and HeLa cells with endogenous mNG:Anillin co-stained for Sir-tubulin to visualize microtubules. The region where the central spindle is predicted to arise looks strikingly different between iPSCs (left) and HeLa cells (right; $n=5$ cells for each; Fig. 14A). In the XY and the ZY views, the central spindle is sparse and difficult to discern from the asters in iPSCs, while it is very dense and clearly distinct from the asters in HeLa cells (Fig. 14A). Notably, astral microtubules can be observed in the equatorial plane where they come into close proximity to anillin in iPSCs, while there is a larger space separating the spindle and anillin in HeLa cells. In the zoomed-in images of the ZY planes (end-on view of the ring), we can see that anillin aggregates can be visualized in iPSCs and in close proximity to microtubules, while it is more uniform in HeLa cells (Fig. 14A; Supp. Fig. S13).

To determine if the astral microtubules that come near the cortex in iPSCs are bundled and could potentially stimulate ring assembly, we imaged the central spindle proteins Cyk4 and Plk1. Cyk4 is part of centralspindlin that bundles central spindle microtubules and activates Ect2 for ring assembly (Yüce, et al., 2005; Glotzer, 2009). Co-expression of mScarlet:Cyk4 with Sir-tubulin or smNG2:Anillin in anaphase iPSCs revealed distinct pools of Cyk4 near the cortex and in more central locations (Fig. 14B; Supp. Fig. S14). Drawing a linescan through the equatorial plane of the cell revealed an overlap between Cyk4 and microtubules as expected with three different sets of peaks that correspond to more cortical and central locations (Fig. 14C). Notably, more Cyk4 localized to the central location compared to those near the cortex. We measured the distance between the cortical microtubules and the centrally located microtubules and found that they were separated by a distance $3.9 \pm 1.5 \mu\text{m}$ ($n=7$ cells; Fig. 14C). This finding suggests that a pool of astral microtubules extends to the equatorial cortex and become bundled by centralspindlin. Indeed, breadth measurements of Anillin and Cyk4 at the equatorial cortex ~ 4 minutes after anaphase onset reveals that their peaks align within $0.4 \pm 0.2 \mu\text{m}$ ($n=3$ cells; Fig. 14C). Both signals also correlate over time as Cyk4 and anillin fluorescence appear at the equatorial plane simultaneously ($n=7$ cells for Cyk4 and 10 cells for anillin; Fig. 14E).

Cyk4 requires Plk1 phosphorylation to bind to Ect2, and Plk1 regulates PRC1 at the central spindle (Yüce et al., 2005; Glotzer, 2009). Thus, we also imaged mScarlet:Plk1 in iPSCs co-stained for microtubules (Fig. 14F; Supp. Fig. S15). We observed Plk1 at the kinetochores during metaphase as expected, and then at the central spindle as well as near the equatorial cortex where Cyk4 and anillin were enriched. Surprisingly, Plk1 appeared to be more diffusely localized at the central spindle and was retained at kinetochores. This likely reflects that the *de novo* central spindle is weak, since Plk1 localization on the central spindle is driven by PRC1 which bundle microtubules (Hu et al., 2012). The small pool of Plk1 may not need to release as much Cyk4 from the central spindle if there is already sufficient Cyk4 at the equatorial plane bundling astral microtubules (Adriaans et al., 2019).

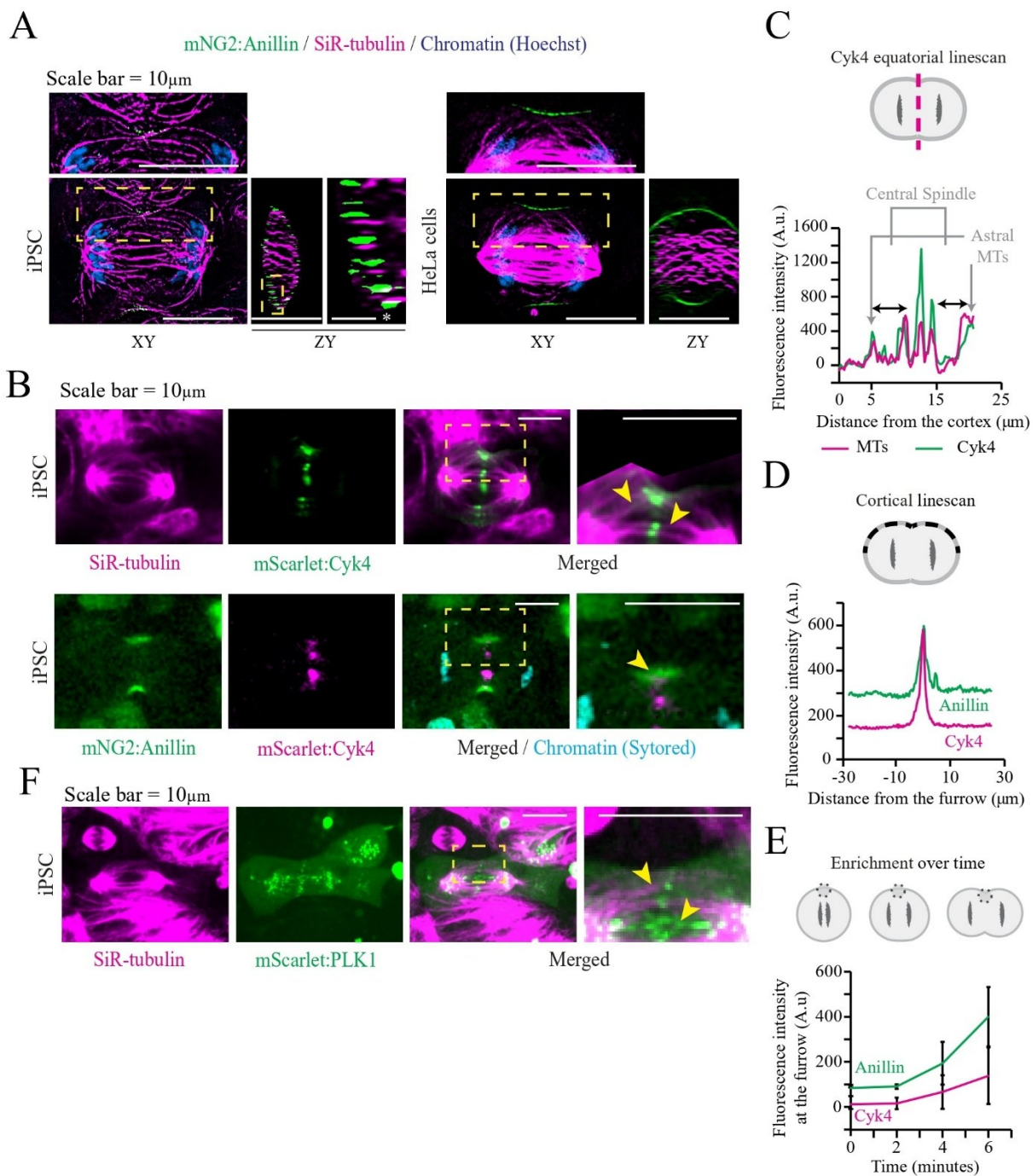


Figure 14. iPSCs have two pools of astral microtubules and a weak central spindle during cytokinesis.

(A) Super-resolution images show endogenous anillin (green) in iPSC and HeLa cells co-stained for tubulin (Syto Deep Red; magenta) and chromatin (blue). The yellow boxes indicate the zoomed in regions of the XY (above) or ZY (right) images. The scale bars are 10 μm , and the bar labeled with an asterisk (*) is 2 μm . (B) Images show iPSCs transfected with mScarlet:Cyk4 (green; top or pink; bottom) co-stained for tubulin (magenta; top) or mNeonGreen:Anillin (green; bottom). The yellow boxes indicate the zoomed in regions (right). The yellow arrowheads point to bundled microtubules from the astral and central spindle microtubules (top), and where anillin overlaps with Cyk4 (bottom). The scale bars are 10 μm . (C) A cartoon cell shows how Cyk4 and microtubules (MTs) were measured by a linescan drawn through the equatorial plane at ~4 min after anaphase onset. The graph shows the average fluorescence intensity of Cyk4 (green) or microtubules (magenta) along this line (distance to one side of the cortex) in a single cell. Similar trends were seen for 7 cells. The grey arrows show the location of the astral microtubules, while the black arrows highlight the distance between the asters and the central spindle. (D) A cartoon cell shows how the average fluorescence intensity of anillin and Cyk4 was measured by linescans drawn along the cortex at ~4 minutes after anaphase onset. The graph shows anillin (green) and Cyk4 (magenta) fluorescence relative to the furrow in a single cell. Similar trends were seen for 3 cells. (E) A cartoon shows how the average fluorescence intensity of anillin and Cyk4 was measured over time using a region of interest (circle). The graph shows changes in the average intensity of anillin (green) and Cyk4 (magenta) in the circle at 0, 2, 4 and 6 minutes after anaphase onset (n= 3 cells for Cyk4; n= 10 cells for anillin). The bars show standard deviation. (F) Images show mScarlet:Plk1 (green) in iPSCs co-stained for tubulin (magenta). The yellow arrowhead points to bundled microtubules. The scale bars are 10 μm .

3.4.4 Astral microtubules regulate the breadth of the ring in iPSCs

Our data suggests that there are two pools of astral microtubules that could regulate ring assembly in anaphase iPSCs. One pool becomes bundled at the equatorial plane where they could promote the recruitment of ring proteins, while the non-bundled astral microtubules could restrict ring proteins from localizing outside of this region. To test the impact of microtubule disruption, we treated iPSCs with endogenous smNG2:Anillin with 30 nM nocodazole just after anaphase onset (Fig. 15). Nocodazole depolymerizes microtubules, and adding lower amounts (e.g., <100 nM) can more selectively disrupt dynamic astral microtubules (van Oostende Triplet et al., 2014). In nocodazole treated cells, anillin cortical enrichment appears sooner and is broader than in control cells (Fig. 15A). In addition, we noticed that asymmetrical enrichment of anillin along the furrow is exacerbated in nocodazole treated cells (Fig. 15A & B). We quantified these observations by measuring anillin fluorescence intensity along the cortex and measuring the ratio of ring breadth to length (Fig. 15C & D). Indeed, the breadth to length ratio significantly increased after nocodazole treatment compared to control cells and was barely detectable along the other region of the cortex (Fig. 15C & D). Therefore, the astral microtubules play a role in restricting the ring, similar to other human cells, but also may be critical for recruitment (Fig. 16). Given that anillin remained asymmetrically distributed suggests that other pathways may influence the asymmetric distribution of anillin in the ring.

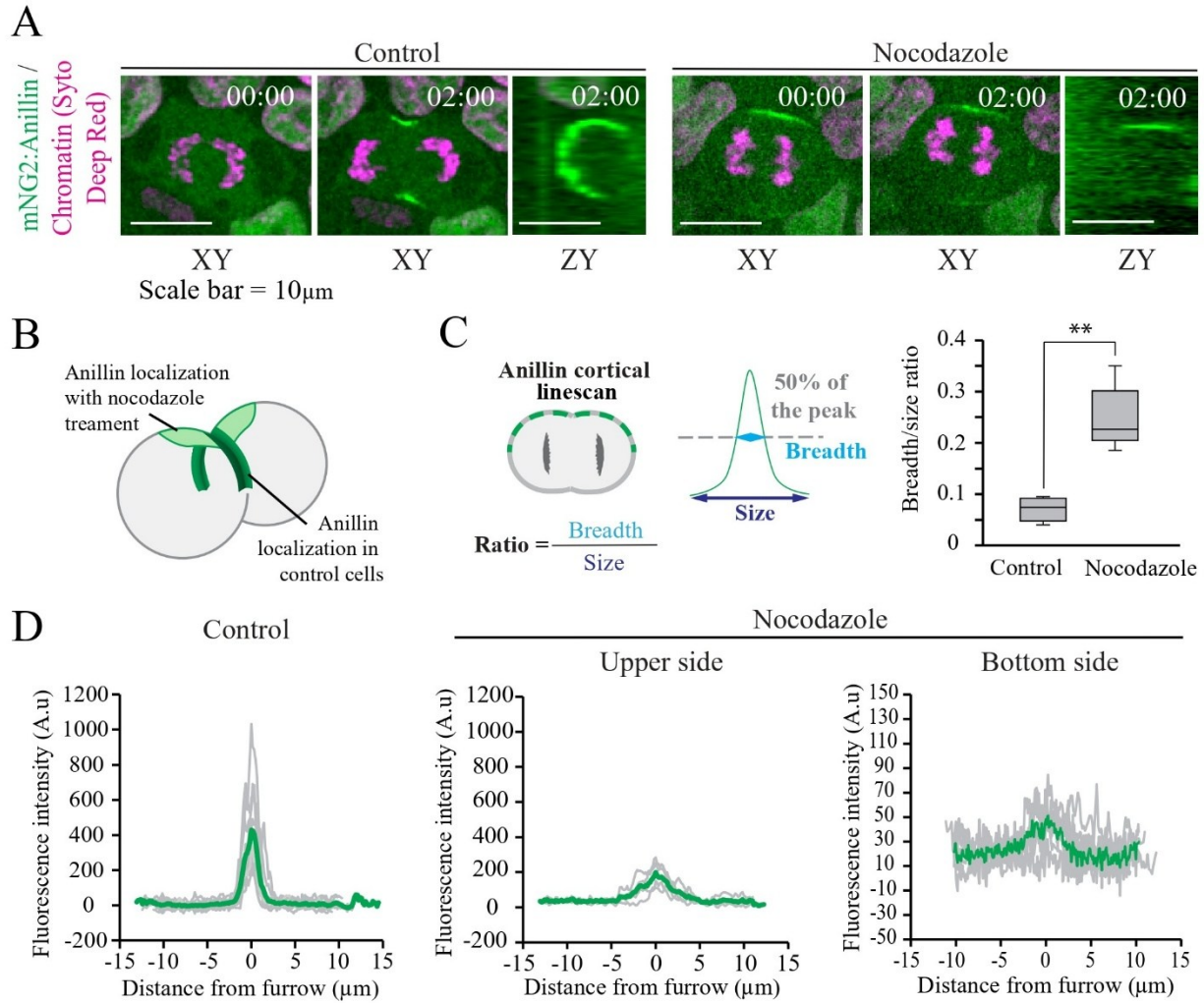


Figure 15. Astral microtubules regulate anillin's localization in the ring. (A) Images show XY and ZY views of endogenous smNG2:Anillin (green) in control iPSCs (left) co-stained for Syto Deep Red to visualize chromatin (magenta) or after the addition of 30 nM of nocodazole (right) at anaphase onset (t=0 min). The scale bars are 10 μ m. (B) A cartoon shows anillin localization in a 3D cell in control (dark green) or after nocodazole treatment (light green). (C) A schematic shows how the breadth (blue) of anillin to cell size (purple) was measured. Fluorescence was measured by a linescan drawn along the cortex from one pole to the other, then the breadth was determined as the number of pixels >50% of the maximum value, which was then divided by the length. A bar graph shows the breadth to size ratio for anillin in control (n=10 cells) and nocodazole-treated cells (n=5 cells). Asterisks (**) indicate significance (** p $\leq$ 0.0001). (D) The graphs show the changes in anillin fluorescence along the cortex in control cells and in nocodazole treated cells. The graph in the middle shows the region of the cortex where anillin enrichment was more detectable, while the graph on the right shows the other side.

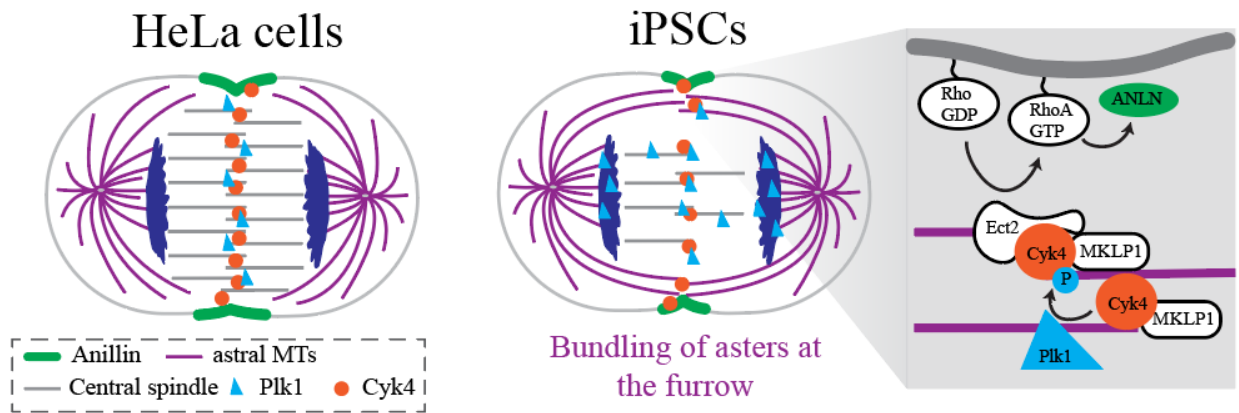


Figure 16. Model of regulation of cytokinesis in iPSCs compared to HeLa cells. The schematics show a HeLa cell and an iPSC at late anaphase. Anillin is green, the central spindle is grey, astral microtubules are purple, chromatin is dark blue, Plk1 is shown as blue triangles and Cyk4 is indicated by orange circles. To the right, the box shows the regulation of RhoA activation where Plk1 phosphorylates Cyk4 for binding to Ect2, which becomes active at the equatorial cortex to activate RhoA.

3.4.5 Ring assembly and ingression occurs differently in endoderm cells compared to iPSCs

The advantage of using iPSCs as a model for cytokinesis is that they can be used to reveal how the mechanisms regulating cytokinesis change with differentiation. In particular, we are interested in how these mechanisms change as endoderm differentiates into hepatocytes. Hepatocytes gain ploidy due to cytokinesis failure, although the underlying mechanism remains unclear (Lacroix and Maddox, 2012; Gentric and Desdouets, 2014). iPSCs can be differentiated into hepatocyte-like cells (HLCs) through sequential induction from iPSCs into definitive endoderm cells and then hepatoblast progenitors (Raggi et al., 2022; Fig. 17A). Since cytokinesis has not been studied in endoderm or hepatoblasts, to our knowledge, as a first step, we looked to see how cytokinesis occurs in endoderm cells. We differentiated iPSCs with endogenous smNG2:Anillin into definitive endoderm and imaged them during cytokinesis (Fig. 17B). Prior to doing this, we confirmed the successful generation of endoderm cells by assessing the expression of endogenous FOXA2:smNG2 (Supp. Fig. S16). Imaging anillin in endoderm cells revealed several differences compared to iPSCs. First, ring ingression was faster in endoderm cells (9 ± 1 min) than in iPSCs (16 ± 2 min) (Fig. 17C). We also observed some anillin at the cortex in metaphase endoderm cells, which was not detectable in iPSCs (Fig. 17B). We noticed that the overall levels of anillin appeared stronger in endoderm cells, which was confirmed by measuring the levels in the cytosol (Fig. 17D). RNA sequencing of endoderm and iPSCs revealed that anillin expression was not significantly different in the two cell types (Okubo et al., 2025; Fig. 17F), suggesting that this change could come from changes in cytosolic volume, translation efficiency, or protein stability. In anaphase and early telophase, anillin was also more broadly localized in endoderm cells compared to iPSCs (Fig. 17B and E). Overall, these findings indicate that cytokinesis is remodeled during differentiation from iPSCs to endoderm cells. Furthermore, transcriptomic analyses of iPSCs, hepatoblast progenitors and HLCs demonstrated a significant decrease in the expression of key regulators of cytokinesis (Raggi et al., 2022; Fig. 17E). While RhoA was not significantly different, anillin, microtubule regulators, and several tubulin genes were all downregulated. The similarity in expression between hepatoblast progenitors and HLCs suggests that cytokinesis failure could occur as early as the hepatoblast stage. Overall, this work is the start of a larger study to reveal mechanisms driving the remodeling of cytokinesis during hepatocyte differentiation.

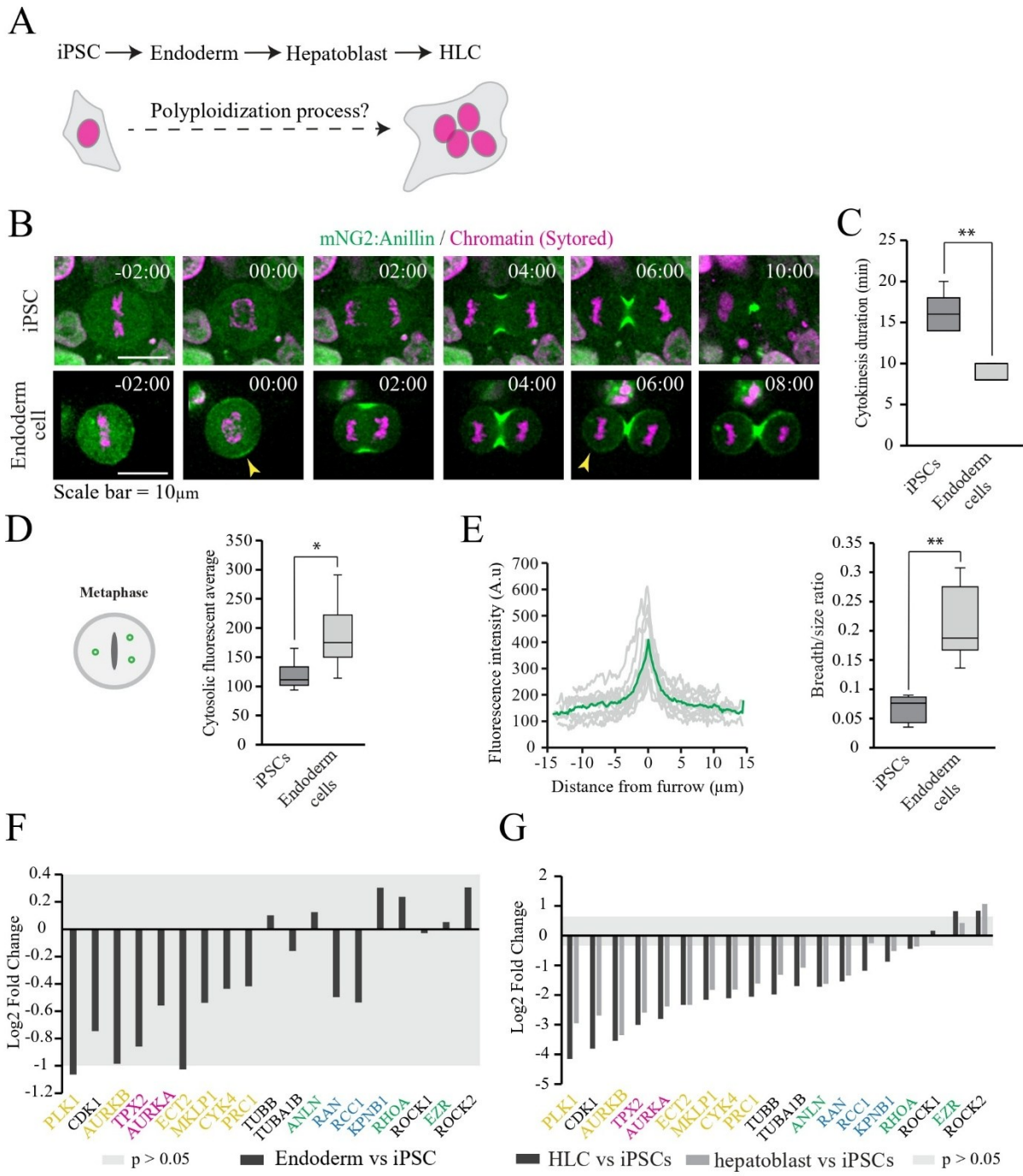


Figure 17. Ring assembly and ingression occurs differently in endoderm cells compared to iPSCs. (A) A schematic shows the steps of iPSC differentiation into definitive endoderm cells, then hepatoblast progenitors and finally into polyploid hepatocyte-like cells (HLCs). (B) Images show endogenous sm2NG:Anillin (green) in an iPSC (top) and endoderm cell (bottom) co-stained with Syto Deep Red (chromatin; magenta). The yellow arrowheads point to cortical enrichments of anillin in endoderm cells. Time is in minutes, and the scale bars are 10 μm . (C) A box and whisker graph shows the average duration of ring closure in minutes from anaphase onset in iPSCs (n= 10 cells) and endoderm cells (n= 5 cells). Bars indicate standard deviation, and asterisks (**) indicate $p \leq 0.0001$. (D) A cartoon shows how cytosolic anillin fluorescence was measured using three regions of interest (ROI). The box and whisker graphs show cytosolic anillin fluorescence average in iPSCs (n= 10 cells) and endoderm cells (n= 5 cells). Bars indicate standard deviation, and the asterisk (*) indicates $p \leq 0.001$. (E) A graph shows anillin fluorescence in cortical linescans over distance relative to the furrow in endoderm cells (average is shown in green). The box and whisker graph shows the anillin breadth to size ratio in iPSCs (n= 10 cells) and endoderm cells (n= 5 cells). Bars indicate standard deviation, and asterisks (**) indicate $p \leq 0.0001$. (F) A bar graph shows the log2 fold change in the expression of cytokinesis genes between endoderm cells and iPSCs. Normalized RNA sequencing data is from Okubo et al. (2025), with defined thresholds at $|\log_2 \text{fold change (FC)}| > 1$ and adjusted $p < 0.05$. Bars outside of these thresholds are statistically significant. (G) A bar graph shows the log2 fold change in the expression of cytokinesis genes between hepatoblasts and iPSCs and between HLCs and iPSCs. Normalized RNA sequencing data comes from Raggi et al. (2022), with defined thresholds at $|\log_2 \text{fold change (FC)}| > 0.5$ and adjusted $p < 0.05$. Bars outside of these thresholds are statistically significant

3.5 Discussion

Cytokinesis studies using various model systems have uncovered the core cytokinesis machinery, but also the diversity in how this machinery is regulated. This, combined with the fact that most of our knowledge of human cell cytokinesis comes from diseased and/or transformed cell lines emphasizes the importance of studying cytokinesis in more normal, diverse human cell types. This work aims to bridge this gap by characterizing how cytokinesis is regulated in iPSCs and revealing that cytokinesis changes in endoderm cells derived from iPSCs. Our group initiated studies to characterize cytokinesis in iPSCs by endogenously tagging a variety of cytokinetic proteins using the split mNG system (Husser et al., 2024). Stem cells are particularly sensitive to phototoxicity, but with recent advances in imaging technology we were able to perform live cell imaging studies of cytokinesis with improved spatiotemporal resolution in iPSCs and endoderm cells.

We found that anillin is recruited to a narrow zone in late anaphase iPSCs, which matches with our previously published data (Husser et al., 2024; Fig.15). Here, we also show that ring ingression is asymmetric and that anillin is enriched on the leading side of the ingressing ring, while actin and ezrin are symmetrically distributed (Fig. 11). Since anillin can crosslink actin and myosin, we hypothesize that anillin could facilitate constriction in regions of the ring where it accumulates. Two mechanisms have been proposed for asymmetric ingression in *C. elegans* embryos (Maddox et al., 2007; Dorn et al., 2016). They rely on positive feedback from anillin and septins, which stochastically crosslinks actomyosin filaments, or from cortical flow of contractile proteins that fuel the leading side of the furrow. Depletion of anillin and/or septins caused a decrease in asymmetric ingression in *C. elegans* embryos (Maddox et al., 2007). Thus, in cells with a narrow ring like iPSCs, which may be predicted to struggle to overcome resistive forces to pinch in the cortex, asymmetric ingression could make the process more energy efficient (Maddox et al., 2007; Dorn et al., 2016). Notably, in *C. elegans* embryos, ingression is not dependent on anillin or septins, while iPSCs are very sensitive to anillin depletion (Fig. 14). This suggests that anillin plays a stronger role in ring constriction in iPSCs.

We also found that astral microtubules may have dual roles in ring assembly. Previously, our group revealed that iPSCs have strong astral microtubules, and possibly a weaker central spindle compared to HeLa cells, although this was not definitively shown (Husser et al., 2024). Here, we used super-resolution imaging to reveal that the central spindle was indeed very sparse in iPSCs compared to HeLa cells (Fig. 14A). A pool of astral microtubules also seemed to bundle near the furrow, and a zoomed-in view of the ring showed distinct aggregates of anillin adjacent to microtubules. In HeLa cells, anillin was more uniformly distributed and separated from the microtubules. Further, the astral and central spindle microtubules were more clearly distinct. In iPSCs, Cyk4 is localized to the astral microtubules near the equatorial cortex where it overlapped with anillin in the ring (Fig. 14B and Supp. Fig. S14). Since Cyk4 typically bundles central spindle microtubules and binds to Ect2 to generate active RhoA, we hypothesize that Cyk4 bundles these astral microtubules to promote RhoA signalling at the nearby cortex (Glotzer, 2013). In HeLa cells, astral microtubules restrict the localization of contractile proteins from the poles to define a zone for ring assembly (Murthy & Wadsworth, 2008; Zanin et al., 2013; van Oostende-Triplett et al., 2014). The selective disruption of dynamic astral microtubules by a low concentration of nocodazole also

caused anillin to be more broadly localized in iPSCs (Fig. 15). Therefore, our findings suggest that there are two sets of astral microtubules that promote ring assembly and polar relaxation in iPSCs. The bundling of astral microtubules during anaphase has been reported for different *Drosophila* cells, where central spindle regulators bundled the microtubules and promoted ring assembly (Thomas et al., 2021). It is interesting to speculate that this is a conserved feature of metazoans that changes in human cells as they differentiate.

We also investigated Plk1 localization in iPSCs (Fig. 14C and Supp. Fig. S15). Plk1 localizes similar to Cyk4 but takes longer to appear on the spindle and is weaker near the cortex. Surprisingly, a high portion of Plk1 stays associated with the kinetochores during cytokinesis, unlike what was previously reported in HeLa cells (Petronczki et al., 2007; Wolfe et al., 2009; Adriaans et al., 2019). Plk1 translocation from the kinetochores to the central spindle depends on PRC1, the master regulator of central spindle assembly (Neef et al., 2007). The slow transition of Plk1 from kinetochores to the midzone supports that the central spindle is weak. It would be interesting to determine if the remaining pool of Plk1 at the kinetochores has a function in iPSCs.

To explore how cytokinesis regulation changes during differentiation, we compared iPSCs with their endoderm derivatives. This differentiation path was chosen because hepatocyte-like cells (HLCs) are derived from endoderm and undergo cytokinesis failure leading to polyploidy (Lacroix and Maddox, 2012; Gentric and Desdouets, 2014). A group previously showed that mouse hepatocytes have weak microtubules and poor recruitment of RhoA, actin, myosin and Cyk4 at the cortex preventing robust ring assembly (Margall-Ducos et al., 2007; Gentric and Desdouets, 2014). However, a more recent study used spheroids to better mimic the *in vivo* microenvironment and found that cytokinesis failure occurs after ingression (Darmasaputra et al., 2024). Thus, the mechanisms underlying hepatocyte polyploidization remains unclear. By differentiating iPSCs with endogenously tagged anillin into endoderm cells, we visualized cytokinesis for the first time in this lineage. Endoderm cells displayed weak cortical localization during metaphase, increased anillin levels, a broader anillin ring, and faster ingression compared to iPSCs (Fig. 17). These observations suggest that cytokinesis changes during differentiation. Interestingly, RNA sequencing revealed no significant changes in the expression of cytokinesis genes between iPSCs and endoderm cells, indicating that differences in anillin levels might result from changes in cytoplasmic volume, translation efficiency, or protein stability. Previous reports showed that there is extensive cytoskeletal remodeling during endoderm differentiation (He et al., 2021; Lim et al., 2021), which could contribute to these alterations. This highlights the importance of studying proteins to complement transcriptomic data. Furthermore, RNA-seq predictions across later differentiation stages revealed that while anillin expression decreases in hepatoblasts and HLCs, RhoA levels remain stable. However, genes associated with spindle pathways are strongly downregulated. The similarity in expression patterns between hepatoblasts and HLCs suggests that cytokinesis failure and the onset of polyploidization may originate as early as the hepatoblast stage.

In conclusion, this work provides new insights into the mechanisms of cytokinesis in human iPSCs and their endoderm derivatives. We reveal that iPSCs employ a distinct mode of cytokinesis characterized by asymmetric furrow ingression and bundled astral microtubules that actively

contribute to furrow positioning and assembly. During differentiation, cytokinesis becomes remodeled, likely reflecting adaptations in cytoskeletal organization and altered signaling. By establishing iPSCs as a non-transformed, genetically tractable model, this study opens avenues to investigate how cytokinesis changes with differentiation and mechanisms causing polyploidization or disease states.

Chapter 4

Discussion

4.1 Overview

Separation of the daughter cells during mitosis is a crucial event which is controlled by a variety of pathways studied over the last century. Thanks to studies in various organisms such as *C. elegans*, *Drosophila*, *Xenopus* eggs and cancerous human cell lines, a model was established in which signalling pathways associated with various components of the cell including the anaphase spindle. As discussed in Chapter 1, cues from the central spindle promotes ring assembly and ingression while cues from the astral microtubules inhibits contractility at the poles. However, the strength of these pathways changes with cell type – astral microtubules are predominant in eggs and embryos, while central spindles are predominant in highly differentiated cell types. Further, spindle-independent pathways may play stronger roles in cells depending on their fate and ploidy, or via extrinsic cues. When we compare cytokinesis in different organisms and cell types, we observe differences in the expression of ring components, ring kinetics, and symmetry, yet knowledge of cytokinesis in diverse healthy human cell types is lacking.

The field needs to turn toward a comparative approach to reveal pathway requirements across more diverse cell types so that this knowledge can be applied, such as in the development of tissue-specific therapies. The goal of this work was to take a comparative approach to study cytokinesis pathways in stem cells, and aneuploid vs. euploid cancer cells. In Chapter 2, we investigated the chromatin-sensing pathway and how its requirement changes with cell fate and chromatin to cytosol ratio. In Chapter 3, we characterized cytokinesis in iPSCs revealing that the mechanisms regulating cytokinesis change with differentiation.

4.2 The chromatin-sensing pathway requirement differs among cell types

Chapter 2 describes the role of importin- β 1 function during cytokinesis. Previous studies showed that ring position is coordinated with chromosome segregation, which is Ran-dependent (Cheeseman and Kiyomitsu, 2013; Beaudet et al., 2017 and 2020). Point mutations in a conserved NLS in the C-terminus of anillin revealed that importin-binding is required for anillin's localization and function in ring position in HeLa cells, but importins have not been directly studied in cytokinesis to our knowledge (Beaudet et al., 2017 and 2020). In Chapter 2, we found that importin-binding is not required for anillin's function in cytokinesis in HCT 116 cells. Notably, the cortical localization of anillin in metaphase was lost with the importin-binding mutation, suggesting that importins play a general role in anillin's cortical recruitment, and not necessarily in restricting anillin to the equatorial cortex to control the ring. Indeed, the requirement for importin's regulation of anillin for cytokinesis correlates with importin- β 1 enrichment at the equatorial region during anaphase. In support of this, the selective disruption of importin- β 1 at anaphase onset is sufficient to cause furrow oscillation, confirming that it is a key component of the chromatin-sensing pathway.

We speculated that the requirement for the chromatin-sensing pathway changes with the chromatin to cytosolic ratio. We found that Ran-free importin- β 1 is more concentrated in the equatorial region compared to the poles in HeLa cells, while there is no difference in HCT 116 cells. A computational model predicted that increasing the volume of HeLa cells to decrease the chromatin to cytosol ratio caused the distribution of Ran-free importin- β 1 to be more like HCT 116 cells, while increasing ploidy in HCT 116 cells to increase the chromatin to cytosol ratio caused Ran-free importin- β 1 to be more like HeLa cells. These predictions were supported by experimental outcomes, including changes in anillin and importin- β 1 localization. Our findings demonstrate that the chromatin-to-cell-size ratio contributes to cytokinesis regulation through the chromatin-sensing pathway. Importantly, this mechanism may be conserved. We previously found that in *C. elegans* embryos, the AB cell, which is larger and gives rise to multiple somatic tissues, has faster ring assembly compared to the smaller P1 cell, which is fated to become the germline (Ozugergin et al., 2022). Increasing ploidy causes 4n P1 cells to have the same size and ring assembly kinetics as 2n AB cells (Ozugergin et al., 2022). However, gene expression differences could also affect the Ran-gradient. The predictive model revealed that the levels of the regulators RCC1 and RanGAP had less of an impact on the Ran-gradient compared to Ran and importin- β 1 (Supp. Fig. S9). In addition, the GTP/GDP ratio was previously reported to have an impact on the steepness of the Ran gradient (Caudron et al., 2005). Since GTP synthesis is enhanced in cancerous cell lines, they could have a steeper gradient compared to other cell types (Kofuji et al., 2020). Hasegawa et al. (2013) showed that chromosomal gain also increases the steepness of the Ran-gradient due to higher chromatin-associated RCC1. Further, post-translational modifications that affect the activity and/or localization of RCC1 or RanGAP could also affect the Ran-gradient.

Overall, our findings suggest that cells with higher ploidy could rely more on the chromatin-sensing pathway than euploid cells (Fig. 18). Progressive cancers typically have genomic instability and aneuploidy with net chromosome gains (Zhou et al., 2023). It is exciting to speculate that cancer cells could rely more heavily on the chromatin-sensing pathway for cytokinesis, which could be targeted as their Achilles' heel. However, this pathway could also be required in healthy cells. There are diverse cell types with differences in polarity, ploidy and fate that could rely on different mechanisms for regulating cytokinesis. In Chapter 3, the characterization of cytokinesis in iPSCs revealed that importin- β 1 is not equatorially enriched during anaphase suggesting that the chromatin-sensing pathway is not required in stem cells (Fig. 13). However, endoderm cells have different ring kinetics compared to iPSCs suggesting that they could rely on different mechanisms to control cytokinesis. Further, decreasing Ran regulators or importins in AB and P1 cells within the early *C. elegans* embryo caused changes in ring assembly kinetics, suggesting that the chromatin sensing pathway plays a role in regulating the ring in this model system (Ozugergin et al., 2022). These studies show that more studies of cytokinesis are needed in more diverse human cell types to determine when the chromatin-sensing pathway is required, and if it could be exploited in aneuploid cancer cells for targeted therapies.

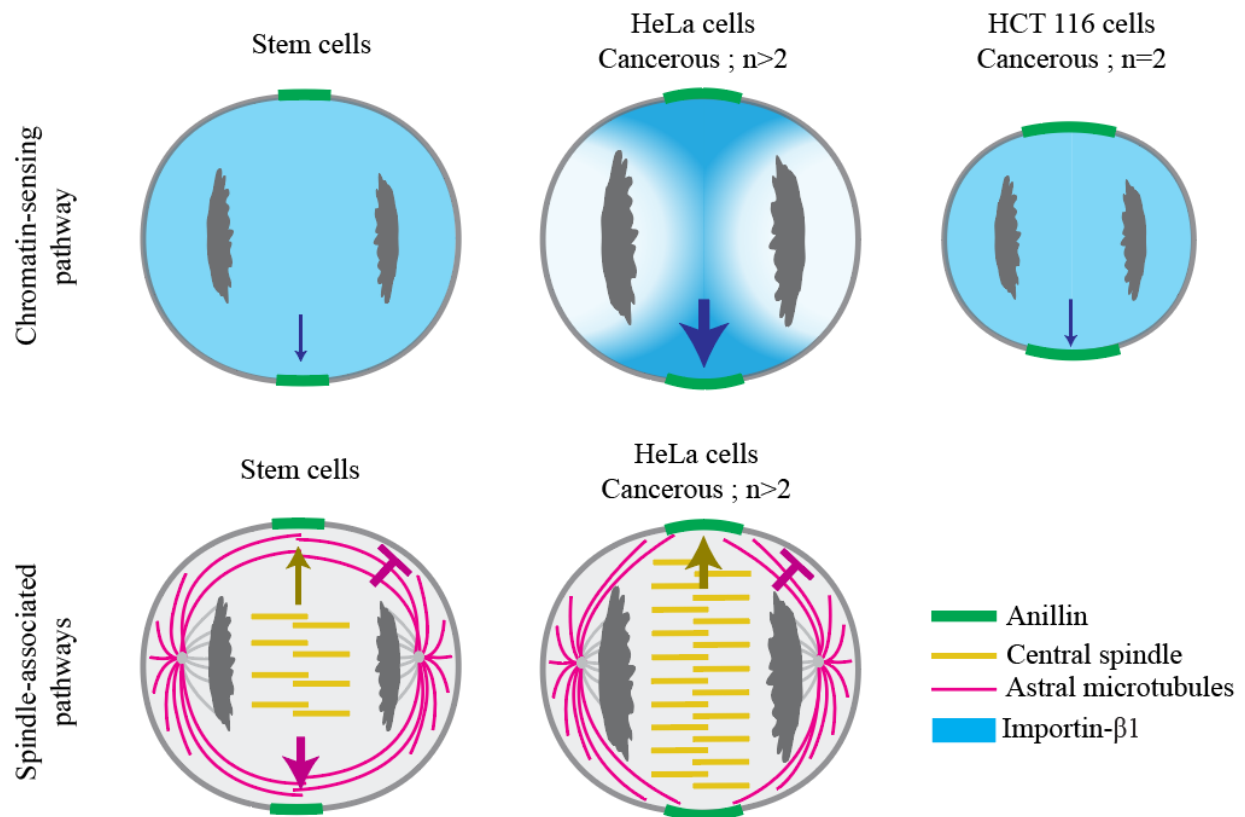


Figure 18. Differences in the regulation of cytokinesis in stem cells and cancer cells. At the top, cartoons show importin-β1 (cyan) localization in a HeLa cell, an HCT 116 cell and an iPSC. The dark blue arrow shows the contribution of the chromatin-sensing pathway in ring assembly, which is more strongly required in HeLa cells compared to HCT 116 cells or iPSCs. The size of the arrow reflects the contribution (larger indicates greater). Below, cartoons show the spindle in a HeLa cell and an iPSC. Astral microtubules are shown in pink, and the central spindle is in yellow. The pink and yellow arrows show promotion or inhibition of ring assembly from the astral microtubules and central spindle, respectively. Anillin localization is in green.

4.3 Cytokinesis regulation in stem cells: differences and similarities with other models

Cytokinesis has been studied in numerous models, from *C. elegans* embryos to mammalian cultured cells, revealing both conserved mechanisms and striking diversity. In this context, pluripotent stem cells represent a physiologically relevant yet underexplored system. Our group previously generated iPSC lines with cytokinesis genes fused to endogenous fluorescent protein tags, enabling live imaging studies of cytokinesis (Husser et al., 2024).

In Chapter 3, we found that iPSCs have very prominent astral microtubules and a sparse central spindle compared to HeLa cells (Fig. 14). They also display asymmetric furrow ingression, unlike HeLa cells, with anillin enriched at the leading side of the furrow (Fig. 11). Super resolution imaging revealed that astral microtubules were bundled in proximity to the furrow where anillin was localized (Fig. 14). Spindles with large asters and small central spindles are found in *Drosophila* cells and in larger embryonic cells, including *C. elegans* embryos (Galli et al., 2008; Lee & Mishima, 2012; Thomas et al., 2021). While in *Drosophila*, a pool of outer astral microtubules moves into the equatorial plane where they are bundled, in *C. elegans*, the astral microtubules remain distinct from the central spindle (Inoue et al., 2004; Galli et al., 2008; Thomas et al., 2021). Pools of astral microtubules can be detected in the equatorial region although it is not clear how they could promote ring assembly (Motegi et al., 2006). In HeLa and in other cultured cancer cells, the central spindle is prominent and extends toward the cortex (Frenette et al., 2012; Lee & Mishima, 2012; van Oostende et al., 2014; Orr et al., 2021). Despite these differences, these different cell types rely on both the central spindle and astral pathways to position the ring within the equatorial region of the cell. We investigated the localization of a component of the centralspindlin complex, Cyk4, and found that it colocalizes with both astral microtubules and the central spindle, confirming that astral microtubules bundle near the cortex where they could promote ring assembly. Thus, cytokinesis in human iPSCs more closely resembles cells from model systems compared to human cancer cells. Further, we also found that the ring ingresses asymmetrically in iPSCs, similar to in *C. elegans* embryos (Maddox et al., 2007; Galli et al., 2008). Despite this similarity, anillin is required for asymmetric ingression, but not for cytokinesis (with ~5% of levels compared to controls; Maddox et al., 2007), while we found that anillin is strongly required for cytokinesis in iPSCs. Therefore, our studies revealed properties that are shared with other cell types, while others are inherent to iPSCs.

Additionally, we investigated Plk1 localization. Plk1 activates Cyk4, while also negatively regulating PRC1, the key regulator of central spindle assembly (Fig. 14; Petronczki et al., 2007; Wolfe et al., 2009; Gómez-Cavazos et al., 2020). Plk1 localized weakly to peripheral microtubules and to the central spindle, unlike in HeLa cells where Plk1 is strongly localized to the central spindle and overlaps with centralspindlin (Neef et al., 2007; Adriaans et al., 2019). These findings support that the central spindle is weak. Plk1 was also retained at the kinetochores throughout cytokinesis, which has not been reported for other cells. It is not clear if this phenomenon was caused by overexpression, and future studies will use alternative approaches to determine if Plk1 is indeed retained at kinetochores, and why.

4.4 Cytokinesis changes during differentiation

The value of using iPSCs to study cytokinesis is to reveal how cytokinesis changes through differentiation. Development and tissue homeostasis relies on the differentiation of progenitor cells (Tian et al., 2023). Despite intensive studies of factors regulating pluripotency and differentiation, cytokinesis has not been studied in most human cell types (Horvitz and Herskowitz, 1992; Morin and Bellaiche, 2011). Our findings begin to address this knowledge gap by studying cytokinesis in endoderm cells. Endoderm cells displayed broader anillin localization, higher anillin levels, and faster furrow ingression compared to iPSCs (Fig. 17). Interestingly, these changes occurred without significant transcriptional variation in cytokinesis genes, as revealed by RNA sequencing. This suggests that post-transcriptional changes, protein stability or structural factors, such as changes in cytoplasmic volume, or cytoskeletal organization, underlie the observed remodeling.

4.5 Future directions

By revealing how cytokinesis is regulated in cancerous, healthy and differentiating cells, this thesis provides a framework to interpret cytokinesis dysregulation in disease and reveal potential targets for therapies.

A complete understanding of the chromatin-sensing pathway will require additional studies to reveal if importins regulate anillin as a heterodimer of importin- α and importin- β 1. While importins typically bind to classical bipartite sequences as a heterodimer, importin- β 1 can also bind directly to some NLS-containing proteins, bypassing importin- α (Dingwall and Laskey, 1992; Lee et al., 2006; Palmeri and Malim, 1999). The heterodimer could make different contacts in the target protein compared to importin- β 1, which could have different impacts on protein function or localization. In addition, palmitoylation of importins is a post-translational modification of importins providing an affinity for the cortex (REF). The palmitoylated importin- α pool is not visible using fluorescent tag, but disruption or enhancement of palmitoylation affects spindle orientation (Brownlee et al., 2019; Sutton et al., 2025).

Another remaining question is how importin-binding regulates anillin function. The NLS is located in a disordered region within the C2 domain which also contains binding sites for phospholipids and microtubules (Oegema et al., 2000; Sun et al., 2015). Disordered regions could adopt different conformations that affect binding affinity to these different components. We hypothesize that importin-binding stabilizes the conformation of this region to favour binding to phospholipids for anillin's cortical recruitment (Beaudet et al., 2020). Mutations that prevent importin-binding delayed anillin's cortical localization in both HeLa and HCT 116 cells. In addition, over-expression of importin- β 1 also reduced anillin's cortical localization (Beaudet et al., 2017). Thus, too much importin can also be a problem – likely due to competition, suggesting that there is an optimal concentration of importins that bind and regulate anillin. It would also be important to determine which pathways control anillin localization to the equatorial cortex in HCT 116 cells, since this is not dependent on the chromatin sensing pathway.

Another question that remains is how RhoA changes anillin's conformation to relieve autoinhibition of the C2 domain. It is not clear which residues are involved in the inhibition, and how RhoA binding relieves this. Functionally, this likely coordinates anillin's cortical recruitment to mitosis when it is needed for ring assembly. We hypothesized that Active RhoA binding to the RBD could cause a change of conformation making the C2 domain more accessible for interaction with phospholipids or with importin- β 1 (Beaudet et al., 2020). Importin binding could subsequently stabilize this conformation that favors interactions for cortical recruitment.

Further, other contractile ring regulators could also be regulated by importin-binding. Preliminary studies from our group revealed that Ect2 has a conserved NLS in the C-terminus that is not required for its nuclear localization and that mutations in this site prevented Ect2's localization to the equatorial cortex in HeLa cells (Nhat Pham, unpublished observations). We also found several cortical regulators that can bind to importin- β 1 during mitotic exit, including the formin DIAPH3 and the dedicator of cytokinesis DOCK7 (Nhat Pham, unpublished observations).

This thesis also characterized cytokinesis in iPSCs, shedding light on mechanisms of regulation. We found that the ring ingresses asymmetrically. Asymmetric furrow ingression may make ring ingression more efficient by concentrating anillin on the one side of the ring where it could crosslink actomyosin. As mentioned above, this also occurs in early *C. elegans* embryos and could be a property of pluripotent cells. Prior studies speculated that this could help ensure that sister chromatids have less of a chance of going back into the other daughter cell (Maddox et al., 2007; Dorn et al., 2016). We also hypothesize that astral microtubules have a stronger role in regulating ring assembly compared to the central spindle. Notably, these asters are still bundled by central spindle proteins and could promote ring assembly in the overlying cortex. As mentioned above, this could be an evolutionary conserved mechanism that occurs in cells across metazoans. Further, disruption of astral microtubules caused anillin localization to spread along the cortex but did not disrupt its asymmetric distribution. Studies are needed to determine the mechanism causing this asymmetric distribution. It could be caused by stochastic changes that feedback to reinforce anillin in locations where it first accumulates (Dorn et al., 2016). However, asymmetric flows of actomyosin filaments could also affect anillin's distribution, and these could be affected by both intrinsic and extrinsic cues or mechanical forces (Dorn et al., 2016; Pimpale et al., 2020; di Pietro et al., 2023).

We started to characterize cytokinesis in endoderm cells derived from iPSCs. To determine why anillin localization changes in endoderm cells, we need to further analyze the various pathways controlling cytokinesis. Staining microtubules revealed that they are more robust in the central spindle region and could extend to the cortex unlike iPSCs, however, they may not be as prominent as in HeLa cells (Ioanna Tountas, unpublished observations). Due to limitations with transfection and differentiation, I recommend endogenously tagging central spindle proteins like Cyk4 or Ect2 with fluorescent protein tags in iPSCs to be able to visualize them in endoderm cells. In this thesis, I tagged ezrin and importin- β 1 in iPSCs to be able to study the kinetochore and chromatin-sensing pathways, respectively, and differentiating these cells into endoderm could be useful in determining their contribution to cytokinesis.

Importantly, iPSCs can be differentiated into a plethora of cell types to study how cytokinesis changes with differentiation. The endoderm cells can be further differentiated into the hepatocyte lineage to reveal when and how cytokinesis fails to give rise to hepatocytes with increased ploidy (Gentric and Desdouets, 2014). While previous studies revealed that cytokinesis fails in hepatocytes to cause polyploidization, the mechanism causing failure is not clear. An earlier study using mouse hepatocytes obtained from livers during prenatal stages of development revealed that ring assembly was defective causing early failure (Margall-Ducos et al., 2007; Gentric and Desdouets, 2014), while a more recent study using spheroids with human hepatocytes showed that the ring assembles and ingresses, but then regresses suggesting failure is caused by a defective midbody (Darmasaputra et al., 2024). Critically, hepatocytes tend to de-differentiate when isolated *in vitro* and a protocol to properly differentiate them *in vitro* was recently described (Raggi et al., 2022). RNA sequencing analysis of iPSCs, hepatoblasts and hepatocyte-like cells from this study revealed that many cytokinetic genes are down regulated at the hepatoblast stage, especially genes involved in the central spindle pathway (Fig. 17). By differentiating iPSCs into endoderm and then into hepatoblasts, we could uncover the mechanisms causing cytokinesis failure, which could be critical information to successfully generate *in vitro* liver tissue for transplants. In addition, this would help to understand better how liver cancer cells escape their fate and proliferate, driving the discovery of novel targets to treat liver cancer.

References

- Adriaans, I. E., Basant, A., Ponsioen, B., Glotzer, M., & Lens, S. M. A. (2019). PLK1 plays dual roles in centralspindlin regulation during cytokinesis. *The Journal of cell biology*, 218(4), 1250–1264.
- Bareja, I., Kučera, O., Petitjean, I. I., Orozco Monroy, B. E., Sabo, J., Braun, M., Lansky, Z., Koenderink, G. H., & Dogterom, M. (2025). Anillin directly crosslinks microtubules with actin filaments. *The EMBO journal*, 44(17), 4803–4824.
- Basant, A., Lekomtsev, S., Tse, Y. C., Zhang, D., Longhini, K. M., Petronczki, M., & Glotzer, M. (2015). Aurora B kinase promotes cytokinesis by inducing centralspindlin oligomers that associate with the plasma membrane. *Developmental cell*, 33(2), 204–215.
- Beaudet, D., Akhshi, T., Phillipp, J., Law, C., & Piekny, A. (2017). Active Ran regulates anillin function during cytokinesis. *Molecular biology of the cell*, 28(24), 3517–3531.
- Beaudet, D., Pham, N., Skaik, N., & Piekny, A. (2020). Importin binding mediates the intramolecular regulation of anillin during cytokinesis. *Molecular biology of the cell*, 31(11), 1124–1139.
- Bell, K. R., Werner, M. E., Doshi, A., Cortes, D. B., Sattler, A., Vuong-Brender, T., Labouesse, M., & Maddox, A. S. (2020). Novel cytokinetic ring components drive negative feedback in cortical contractility. *Molecular biology of the cell*, 31(15), 1623–1636.
- Bement, W. M., Benink, H. A., & von Dassow, G. (2005). A microtubule-dependent zone of active RhoA during cleavage plane specification. *The Journal of cell biology*, 170(1), 91–101.
- Bilic, J., & Izpisua Belmonte, J. C. (2012). Concise review: Induced pluripotent stem cells versus embryonic stem cells: close enough or yet too far apart? *Stem cells (Dayton, Ohio)*, 30(1), 33–41.
- Bischoff, F. R., & Ponstingl, H. (1991). Catalysis of guanine nucleotide exchange on Ran by the mitotic regulator RCC1. *Nature*, 354(6348), 80–82.
- Bock, C., Kiskinis, E., Verstappen, G., Gu, H., Boulting, G., Smith, Z. D., Ziller, M., Croft, G. F., Amoroso, M. W., Oakley, D. H., Gnirke, A., Eggan, K., & Meissner, A. (2011). Reference Maps of human ES and iPS cell variation enable high-throughput characterization of pluripotent cell lines. *Cell*, 144(3), 439–452.
- Bringmann, H., & Hyman, A. A. (2005). A cytokinesis furrow is positioned by two consecutive signals. *Nature*, 436(7051), 731–734.
- Brownlee, C., & Heald, R. (2019). Importin α Partitioning to the Plasma Membrane Regulates Intracellular Scaling. *Cell*, 176(4), 805–815.e8.
- Budnar, S., Husain, K. B., Gomez, G. A., Naghibosadat, M., Varma, A., Verma, S., Hamilton, N. A., Morris, R. G., & Yap, A. S. (2019). Anillin Promotes Cell Contractility by Cyclic Resetting of RhoA Residence Kinetics. *Developmental cell*, 49(6), 894–906.e12.
- Bugaj, L. J., Choksi, A. T., Mesuda, C. K., Kane, R. S., & Schaffer, D. V. (2013). Optogenetic protein clustering and signaling activation in mammalian cells. *Nature methods*, 10(3), 249–252.

- Cabernard, C., Prehoda, K. E., & Doe, C. Q. (2010). A spindle-independent cleavage furrow positioning pathway. *Nature*, 467(7311), 91–94.
- Canman, J. C., Hoffman, D. B., & Salmon, E. D. (2000). The role of pre- and post-anaphase microtubules in the cytokinesis phase of the cell cycle. *Current biology : CB*, 10(10), 611–614.
- Cao, Y. F., Wang, H., Sun, Y., Tong, B. B., Shi, W. Q., Peng, L., Zhang, Y. M., Wu, Y. Q., Fu, T., Zou, H. Y., Zhang, K., Xu, L. Y., & Li, E. M. (2025). Nuclear ANLN regulates transcription initiation related Pol II clustering and target gene expression. *Nature communications*, 16(1), 1271.
- Carvalho, A., Desai, A., & Oegema, K. (2009). Structural memory in the contractile ring makes the duration of cytokinesis independent of cell size. *Cell*, 137(5), 926–937.
- Caudron, M., Bunt, G., Bastiaens, P., & Karsenti, E. (2005). Spatial coordination of spindle assembly by chromosome-mediated signaling gradients. *Science (New York, N.Y.)*, 309(5739), 1373–1376.
- Cavazza, T., & Vernos, I. (2016). The RanGTP Pathway: From Nucleo-Cytoplasmic Transport to Spindle Assembly and Beyond. *Frontiers in cell and developmental biology*, 3, 82.
- Celton-Morizur, S., Merlen, G., Couton, D., & Desdouets, C. (2010). Polyploidy and liver proliferation: Central role of insulin signaling. *Cell Cycle*, 9(3), 460–466.
- Chang, C. C., & Hsia, K. C. (2021). More than a zip code: global modulation of cellular function by nuclear localization signals. *The FEBS journal*, 288(19), 5569–5585.
- Chen, A., Akhshi, T. K., Lavoie, B. D., & Wilde, A. (2015). Importin β 2 Mediates the Spatio-temporal Regulation of Anillin through a Noncanonical Nuclear Localization Signal. *The Journal of biological chemistry*, 290(21), 13500–13509.
- Chen, A., Arora, P. D., McCulloch, C. A., & Wilde, A. (2017). Cytokinesis requires localized β -actin filament production by an actin isoform specific nucleator. *Nature communications*, 8(1), 1530.
- Chen, A., Ulloa Severino, L., Panagiotou, T. C., Moraes, T. F., Yuen, D. A., Lavoie, B. D., & Wilde, A. (2021). Inhibition of polar actin assembly by astral microtubules is required for cytokinesis. *Nature communications*, 12(1), 2409.
- Cho, N. H., Cheveralls, K. C., Brunner, A. D., Kim, K., Michaelis, A. C., Raghavan, P., Kobayashi, H., Savy, L., Li, J. Y., Canaj, H., Kim, J. Y. S., Stewart, E. M., Gnann, C., McCarthy, F., Cabrera, J. P., Brunetti, R. M., Chhun, B. B., Dingle, G., Hein, M. Y., Huang, B., ... Leonetti, M. D. (2022). OpenCell: Endogenous tagging for the cartography of human cellular organization. *Science (New York, N.Y.)*, 375(6585), eabi6983.
- Ciciarello, M., Mangiacasale, R., Thibier, C., Guarguaglini, G., Marchetti, E., Di Fiore, B., & Lavia, P. (2004). Importin beta is transported to spindle poles during mitosis and regulates Ran-dependent spindle assembly factors in mammalian cells. *Journal of cell science*, 117(Pt 26), 6511–6522.

- Connell, M., Cabernard, C., Ricketson, D., Doe, C. Q., & Prehoda, K. E. (2011). Asymmetric cortical extension shifts cleavage furrow position in *Drosophila* neuroblasts. *Molecular biology of the cell*, 22(22), 4220–4226.
- Connors, C. Q., Martin, S. L., Dumont, J., Shirasu-Hiza, M., & Canman, J. C. (2024). Cell type-specific regulation by different cytokinetic pathways in the early embryo. *microPublication biology*, 2024, 10.17912/micropub.biology.001316.
- Coward, J., & Harding, A. (2014). Size Does Matter: Why Polyploid Tumor Cells are Critical Drug Targets in the War on Cancer. *Frontiers in oncology*, 4, 123.
- Cuomo, A. S. E., Seaton, D. D., McCarthy, D. J., Martinez, I., Bonder, M. J., Garcia-Bernardo, J., Amatya, S., Madrigal, P., Isaacson, A., Buettner, F., Knights, A., Natarajan, K. N., HipSci Consortium, Vallier, L., Marioni, J. C., Chhatiwala, M., & Stegle, O. (2020). Single-cell RNA-sequencing of differentiating iPS cells reveals dynamic genetic effects on gene expression. *Nature communications*, 11(1), 810.
- Dacquay, L. C., Antoniou, P., Mentani, A., Selfjord, N., Mårtensson, H., Hsieh, P. P., Mustfa, S., Thom, G., Wimberger, S., Firth, M., Akrap, N., Maresca, M., & Peterka, M. (2025). Dual inhibition of DNA-PK and Pol Θ boosts precision of diverse prime editing systems. *Nature communications*, 16(1), 4290.
- Darmasaputra, G. S., Geerlings, C. C., Chuva de Sousa Lopes, S. M., Clevers, H., & Galli, M. (2024). Binucleated human hepatocytes arise through late cytokinetic regression during endomitosis M phase. *The Journal of cell biology*, 223(8), e202403020.
- Davies, T., Kim, H. X., Romano Spica, N., Lesea-Pringle, B. J., Dumont, J., Shirasu-Hiza, M., & Canman, J. C. (2018). Cell-intrinsic and -extrinsic mechanisms promote cell-type-specific cytokinetic diversity. *eLife*, 7, e36204.
- Davoli, T., & de Lange, T. (2011). The causes and consequences of polyploidy in normal development and cancer. *Annual review of cell and developmental biology*, 27, 585–610.
- Dechant, R., & Glotzer, M. (2003). Centrosome separation and central spindle assembly act in redundant pathways that regulate microtubule density and trigger cleavage furrow formation. *Developmental cell*, 4(3), 333–344.
- Di Francesco, L., Verrico, A., Asteriti, I. A., Rovella, P., Cirigliano, P., Guarguaglini, G., Schininà, M. E., & Lavia, P. (2018). Visualization of human karyopherin beta-1/importin beta-1 interactions with protein partners in mitotic cells by co-immunoprecipitation and proximity ligation assays. *Scientific reports*, 8(1), 1850.
- Dingwall, C., & Laskey, R. (1992). The nuclear membrane. *Science (New York, N.Y.)*, 258(5084), 942–947.
- di Pietro, F., Osswald, M., De Las Heras, J. M., Cristo, I., López-Gay, J., Wang, Z., Pelletier, S., Gaugué, I., Leroy, A., Martin, C., Morais-de-Sá, E., & Bellaïche, Y. (2023). Systematic

- analysis of RhoGEF/GAP localizations uncovers regulators of mechanosensing and junction formation during epithelial cell division. *Current biology : CB*, 33(5), 858–874.e7.
- Dorn, J. F., Zhang, L., Phi, T. T., Lacroix, B., Maddox, P. S., Liu, J., & Maddox, A. S. (2016). A theoretical model of cytokinesis implicates feedback between membrane curvature and cytoskeletal organization in asymmetric cytokinetic furrowing. *Molecular biology of the cell*, 27(8), 1286–1299.
- Douglas, M. E., Davies, T., Joseph, N., & Mishima, M. (2010). Aurora B and 14-3-3 coordinately regulate clustering of centralspindlin during cytokinesis. *Current biology : CB*, 20(10), 927–933.
- Drutovic, D., Duan, X., Li, R., Kalab, P., & Solc, P. (2020). RanGTP and importin β regulate meiosis I spindle assembly and function in mouse oocytes. *The EMBO journal*, 39(1), e101689.
- El Dika, M., Dudka, D., Kloc, M., & Kubiak, J. Z. (2023). CDC6 as a Key Inhibitory Regulator of CDK1 Activation Dynamics and the Timing of Mitotic Entry and Progression. *Biology*, 12(6), 855.
- Engler, C., Gruetzner, R., Kandzia, R., & Marillonnet, S. (2009). Golden gate shuffling: a one-pot DNA shuffling method based on type IIs restriction enzymes. *PloS one*, 4(5), e5553.
- Field, C. M., & Alberts, B. M. (1995). Anillin, a contractile ring protein that cycles from the nucleus to the cell cortex. *The Journal of cell biology*, 131(1), 165–178.
- Forester CM, Maddox J, Louis JV, Goris J, Virshup DM. Control of mitotic exit by PP2A regulation of Cdc25C and Cdk1. *Proc Natl Acad Sci U S A*. 2007 Dec 11;104(50):19867-72. Epub 2007 Dec 4.
- Fotopoulos, N., Wernike, D., Chen, Y., Makil, N., Marte, A., & Piekny, A. (2013). *Caenorhabditis elegans* anillin (ani-1) regulates neuroblast cytokinesis and epidermal morphogenesis during embryonic development. *Developmental biology*, 383(1), 61–74.
- Frenette P, Haines E, Loloyan M, Kinal M, Pakarian P, Piekny A. An anillin-Ect2 complex stabilizes central spindle microtubules at the cortex during cytokinesis. *PLoS One*. 2012;7(4):e34888. Epub 2012 Apr 13.
- Furuta M, Hori T, Fukagawa T. Chromatin binding of RCC1 during mitosis is important for its nuclear localization in interphase. *Mol Biol Cell*. 2016 Jan 15;27(2):371-81.
- Galli M, van den Heuvel S. Determination of the cleavage plane in early *C. elegans* embryos. *Annu Rev Genet*. 2008;42:389-411.
- Ganem, N. J., Godinho, S. A., & Pellman, D. (2009). A mechanism linking extra centrosomes to chromosomal instability. *Nature*, 460(7252), 278–282.
- Gentric, G., & Desdouets, C. (2014). Polyploidization in liver tissue. *The American journal of pathology*, 184(2), 322–331.
- Glotzer M. Cytokinesis: centralspindlin moonlights as a membrane anchor. *Curr Biol*. 2013 Feb 18;23(4):R145-7.

- Glotzer M. The 3Ms of central spindle assembly: microtubules, motors and MAPs. *Nat Rev Mol Cell Biol.* 2009 Jan;10(1):9-20.
- Gómez-Cavazos JS, Lee KY, Lara-González P, Li Y, Desai A, Shiau AK, Oegema K. A Non-canonical BRCT-Phosphopeptide Recognition Mechanism Underlies RhoA Activation in Cytokinesis. *Curr Biol.* 2020 Aug 17;30(16):3101-3115.e11. Epub 2020 Jul 2.
- Green RA, Paluch E, Oegema K. Cytokinesis in animal cells. *Annu Rev Cell Dev Biol.* 2012;28:29-58. Epub 2012 Jul 9.
- Gruss, O. J., & Vernos, I. (2004). The mechanism of spindle assembly: functions of Ran and its target TPX2. *The Journal of cell biology*, 166(7), 949–955.
- Hasegawa K, Ryu SJ, Kaláb P. Chromosomal gain promotes formation of a steep RanGTP gradient that drives mitosis in aneuploid cells. *J Cell Biol.* 2013 Jan 21;200(2):151-61. Epub 2013 Jan 14.
- Hayes, L. R., Duan, L., Bowen, K., Kalab, P., & Rothstein, J. D. (2020). C9orf72 arginine-rich dipeptide repeat proteins disrupt karyopherin-mediated nuclear import. *eLife*, 9, e51685.
- Hazel J, Krutkramelis K, Mooney P, Tomschik M, Gerow K, Oakey J, Gatlin JC. Changes in cytoplasmic volume are sufficient to drive spindle scaling. *Science.* 2013 Nov 15;342(6160):853-6.
- He Y, Zhang L, He Y, Yu H, Li S, Li Q. Involvement of LIMK2 in actin cytoskeleton remodeling during the definitive endoderm differentiation. *In Vitro Cell Dev Biol Anim.* 2021 May;57(5):493-500. Epub 2021 May 11.
- Hégarat N, Rata S, Hochegger H. Bistability of mitotic entry and exit switches during open mitosis in mammalian cells. *Bioessays.* 2016 Jul;38(7):627-43. Epub 2016 May 27.
- Hickson, G. R., & O'Farrell, P. H. (2008). Rho-dependent control of anillin behavior during cytokinesis. *The Journal of cell biology*, 180(2), 285–294.
- Holopainen, S., Hytönen, M. K., Syrjä, P., Arumilli, M., Järvinen, A. K., Rajamäki, M., & Lohi, H. (2017). ANLN truncation causes a familial fatal acute respiratory distress syndrome in Dalmatian dogs. *PLoS genetics*, 13(2), e1006625.
- Horvitz, H. R., & Herskowitz, I. (1992). Mechanisms of asymmetric cell division: two Bs or not two Bs, that is the question. *Cell*, 68(2), 237–255.
- Huang Z, Benman W, Dong L, Bugaj LJ. Rapid optogenetic clustering of a cytoplasmic BcLOV4 variant. *bioRxiv [Preprint].* 2023 Sep 17:2023.09.14.557726.
- Hu CK, Coughlin M, Field CM, Mitchison TJ. Cell polarization during monopolar cytokinesis. *J Cell Biol.* 2008 Apr 21;181(2):195-202.
- Husser, M. C., Pham, N. P., Law, C., Araujo, F. R. B., Martin, V. J. J., & Piekny, A. (2024). Endogenous tagging using split mNeonGreen in human iPSCs for live imaging studies. *eLife*, 12, RP92819.

- Husser MC, Ozugergin I, Resta T, Martin VJJ, Piekny AJ. Cytokinetic diversity in mammalian cells is revealed by the characterization of endogenous anillin, Ect2 and RhoA. *Open Biol.* 2022 Nov;12(11):220247. Epub 2022 Nov 23.
- Inoue, Y. H., Savoian, M. S., Suzuki, T., Mathe, E., Yamamoto, M. T. and Glover, D. M. (2004). Mutations in orbit/mast reveal that the central spindle is comprised of two microtubule populations, those that initiate cleavage and those that propagate furrow ingression. *J. Cell Biol.* 166, 49-60.
- Jevtić, P., Schibler, A. C., Wesley, C. C., Pegoraro, G., Misteli, T., & Levy, D. L. (2019). The nucleoporin ELYS regulates nuclear size by controlling NPC number and nuclear import capacity. *EMBO reports*, 20(6), e47283.
- Jäkel, S., Mingot, J. M., Schwarzmaier, P., Hartmann, E., & Görlich, D. (2002). Importins fulfil a dual function as nuclear import receptors and cytoplasmic chaperones for exposed basic domains. *The EMBO journal*, 21(3), 377–386.
- Kaláb P, Pralle A, Isacoff EY, Heald R, Weis K. Analysis of a RanGTP-regulated gradient in mitotic somatic cells. *Nature*. 2006 Mar 30;440(7084):697-701.
- Kaláb P, Heald R. The RanGTP gradient - a GPS for the mitotic spindle. *J Cell Sci.* 2008 May 15;121(Pt 10):1577-86.
- Kaláb P, Soderholm J. The design of Förster (fluorescence) resonance energy transfer (FRET)-based molecular sensors for Ran GTPase. *Methods*. 2010 Jun;51(2):220-32. Epub 2010 Jan 22.
- Kamijo K, Ohara N, Abe M, Uchimura T, Hosoya H, Lee JS, Miki T. Dissecting the role of Rho-mediated signaling in contractile ring formation. *Mol Biol Cell*. 2006 Jan;17(1):43-55. Epub 2005 Oct 19.
- Kato-Inui, T., Takahashi, G., Hsu, S., & Miyaoka, Y. (2018). Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 with improved proof-reading enhances homology-directed repair. *Nucleic acids research*, 46(9), 4677–4688.
- Kiyomitsu T, Cheeseman IM. Cortical dynein and asymmetric membrane elongation coordinately position the spindle in anaphase. *Cell*. 2013 Jul 18;154(2):391-402.
- Kofuji S, Sasaki AT. GTP metabolic reprogramming by IMPDH2: unlocking cancer cells' fuelling mechanism. *J Biochem*. 2020 Oct 1;168(4):319-328.
- Kotadia, S., Montembault, E., Sullivan, W., & Royou, A. (2012). Cell elongation is an adaptive response for clearing long chromatid arms from the cleavage plane. *The Journal of cell biology*, 199(5), 745–753.
- Kotýnková K, Su KC, West SC, Petronczki M. Plasma Membrane Association but Not Midzone Recruitment of RhoGEF ECT2 Is Essential for Cytokinesis. *Cell Rep*. 2016 Dec 6;17(10):2672-2686.

- Kudryavtsev BN, Kudryavtseva MV, Sakuta GA, Stein GI. Human hepatocyte polyploidization kinetics in the course of life cycle. *Virchows Arch B Cell Pathol Incl Mol Pathol*. 1993;64(6):387-93.
- Kučera, O., Siahaan, V., Janda, D., Dijkstra, S. H., Pilátová, E., Zatecka, E., Diez, S., Braun, M., & Lansky, Z. (2021). Anillin propels myosin-independent constriction of actin rings. *Nature communications*, 12(1), 4595.
- Lacroix B, Maddox AS. Cytokinesis, ploidy and aneuploidy. *J Pathol*. 2012 Jan;226(2):338-51.
- Ladouceur, A. M., Dorn, J. F., & Maddox, P. S. (2015). Mitotic chromosome length scales in response to both cell and nuclear size. *The Journal of cell biology*, 209(5), 645–651.
- Lal, A., Shan, C., Zhao, K., Liu, W., Huang, X., Zong, W., Chen, L., & Xi, P. (2018). A frequency domain SIM reconstruction algorithm using reduced number of images. *IEEE transactions on image processing : a publication of the IEEE Signal Processing Society*, 27(9), 4555–4570.
- Landino, J., Misterovich, E., van den Goor, L., Adhikary, B., Chumki, S., Davidson, L. A., & Miller, A. L. (2025). Neighbor cells restrain furrowing during *Xenopus* epithelial cytokinesis. *Developmental cell*, 60(16), 2139–2148.e6. DOI: 10.1016/j.devcel.2025.03.010.
- Lee, H. G., Ueda, M., Miyamoto, Y., Yoneda, Y., Perry, G., Smith, M. A., & Zhu, X. (2006). Aberrant localization of importin alpha1 in hippocampal neurons in Alzheimer disease. *Brain research*, 1124(1), 1–4.
- Lee, J. E., & Lee, D. R. (2011). Human embryonic stem cells: derivation, maintenance and cryopreservation. *International journal of stem cells*, 4(1), 9–17.
- Lee, M. E., DeLoache, W. C., Cervantes, B., & Dueber, J. E. (2015). A Highly Characterized Yeast Toolkit for Modular, Multipart Assembly. *ACS synthetic biology*, 4(9), 975–986.
- Lee KY, Davies T, Mishima M. Cytokinesis microtubule organisers at a glance. *J Cell Sci*. 2012 Aug 1;125(Pt 15):3495-500.
- Lekomtsev S, Su KC, Pye VE, Blight K, Sundaramoorthy S, Takaki T, Collinson LM, Cherepanov P, Divecha N, Petronczki M. Centralspindlin links the mitotic spindle to the plasma membrane during cytokinesis. *Nature*. 2012 Dec 13;492(7428):276-9.
- Lewellyn L, Dumont J, Desai A, Oegema K. Analyzing the effects of delaying aster separation on furrow formation during cytokinesis in the *Caenorhabditis elegans* embryo. *Mol Biol Cell*. 2010 Jan 1;21(1):50-62. Epub 2009 Nov 4.
- Lim HYG, Plachta N. Cytoskeletal control of early mammalian development. *Nat Rev Mol Cell Biol*. 2021 Aug;22(8):548-562. Epub 2021 Apr 29.
- Lombardi, Z., Gardini, L., Kashchuk, A. V., Menconi, A., Lulli, M., Tusa, I., Tubita, A., Maresca, L., Stecca, B., Capitanio, M., & Rovida, E. (2025). Importin subunit beta-1 mediates ERK5 nuclear translocation, and its inhibition synergizes with ERK5 kinase inhibitors in reducing cancer cell proliferation. *Molecular oncology*, 19(1), 99–113.

- Lopez-Sánchez, L. M. et al. CoCl₂, a mimic of hypoxia, induces formation of polyploid giant cells with stem characteristics in colon cancer. *PLoS One* 9, e99143 (2014).
- Maddox AS, Lewellyn L, Desai A, Oegema K. Anillin and the septins promote asymmetric ingression of the cytokinetic furrow. *Dev Cell*. 2007 May;12(5):827-35.
- Mangal, S., Sacher, J., Kim, T., Osório, D. S., Motegi, F., Carvalho, A. X., Oegema, K., & Zanin, E. (2018). TPXL-1 activates Aurora A to clear contractile ring components from the polar cortex during cytokinesis. *The Journal of cell biology*, 217(3), 837–848.
- Margall-Ducos, G., Celton-Morizur, S., Couton, D., Brégerie, O., & Desdouets, C. (2007). Liver tetraploidization is controlled by a new process of incomplete cytokinesis. *Journal of cell science*, 120(Pt 20), 3633–3639. DOI: 10.1242/jcs.016907.
- Mauro, M. S., Martin, S. L., Dumont, J., Shirasu-Hiza, M., & Canman, J. C. (2024). Patterning, regulation, and role of FoxO/DAF-16 in the early embryo. *bioRxiv : the preprint server for biology*, 2024.05.13.594029.
- Ma X, Kovács M, Conti MA, Wang A, Zhang Y, Sellers JR, Adelstein RS. Nonmuscle myosin II exerts tension but does not translocate actin in vertebrate cytokinesis. *Proc Natl Acad Sci U S A*. 2012 Mar 20;109(12):4509-14. Epub 2012 Mar 5.
- Merdes, A., Ramyar, K., Vechio, J. D., & Cleveland, D. W. (1996). A complex of NuMA and cytoplasmic dynein is essential for mitotic spindle assembly. *Cell*, 87(3), 447–458.
- Mishima M, Kaitna S, Glotzer M. Central spindle assembly and cytokinesis require a kinesin-like protein/RhoGAP complex with microtubule bundling activity. *Dev Cell*. 2002 Jan;2(1):41-54.
- Moore W, Zhang C, Clarke PR. Targeting of RCC1 to chromosomes is required for proper mitotic spindle assembly in human cells. *Curr Biol*. 2002 Aug 20;12(16):1442-7.
- Morin X, Bellaïche Y. Mitotic spindle orientation in asymmetric and symmetric cell divisions during animal development. *Dev Cell*. 2011 Jul 19;21(1):102-19.
- Motegi, F., Velarde, N. V., Piano, F., & Sugimoto, A. (2006). Two phases of astral microtubule activity during cytokinesis in *C. elegans* embryos. *Developmental cell*, 10(4), 509–520.
- Munro E. (2007). The microtubules dance and the spindle poles swing. *Cell*, 129(3), 457–458.
- Murthy K, Wadsworth P. Dual role for microtubules in regulating cortical contractility during cytokinesis. *J Cell Sci*. 2008 Jul 15;121(Pt 14):2350-9. Epub 2008 Jun 17.
- Naydenov, N. G., Koblinski, J. E., & Ivanov, A. I. (2021). Anillin is an emerging regulator of tumorigenesis, acting as a cortical cytoskeletal scaffold and a nuclear modulator of cancer cell differentiation. *Cellular and molecular life sciences : CMLS*, 78(2), 621–633.
- Neef R, Gruneberg U, Kopajtich R, Li X, Nigg EA, Sillje H, Barr FA. Choice of Plk1 docking partners during mitosis and cytokinesis is controlled by the activation state of Cdk1. *Nat Cell Biol*. 2007 Apr;9(4):436-44. Epub 2007 Mar 11.

- Niiya, F., Tatsumoto, T., Lee, K. S., & Miki, T. (2006). Phosphorylation of the cytokinesis regulator ECT2 at G2/M phase stimulates association of the mitotic kinase Plk1 and accumulation of GTP-bound RhoA. *Oncogene*, 25(6), 827–837.
- Oegema, K., Savoian, M. S., Mitchison, T. J., & Field, C. M. (2000). Functional analysis of a human homologue of the *Drosophila* actin binding protein anillin suggests a role in cytokinesis. *The Journal of cell biology*, 150(3), 539–552.
- Oka, M., & Yoneda, Y. (2018). Importin α : functions as a nuclear transport factor and beyond. *Proceedings of the Japan Academy. Series B, Physical and biological sciences*, 94(7), 259–274.
- Okubo C, Nakamura M, Sato M, Shichino Y, Mito M, Takashima Y, Iwasaki S, Takahashi K. EIF3D safeguards the homeostasis of key signaling pathways in human primed pluripotency. *Sci Adv*. 2025 Apr 11;11(15):eadq5484. Epub 2025 Apr 9.
- Oon, C. H., & Prehoda, K. E. (2019). Asymmetric recruitment and actin-dependent cortical flows drive the neuroblast polarity cycle. *eLife*, 8, e45815.
- Orr, B., De Sousa, F., Gomes, A. M., Afonso, O., Ferreira, L. T., Figueiredo, A. C., & Maiato, H. (2021). An anaphase surveillance mechanism prevents micronuclei formation from frequent chromosome segregation errors. *Cell reports*, 37(6), 109783.
- Osório DS, Chan FY, Saramago J, Leite J, Silva AM, Sobral AF, Gassmann R, Carvalho AX. Crosslinking activity of non-muscle myosin II is not sufficient for embryonic cytokinesis in *C. elegans*. *Development*. 2019 Nov 12;146(21):dev179150.
- Ozugerin, I., & Piekny, A. (2021). Complementary functions for the Ran gradient during division. *Small GTPases*, 12(3), 177–187.
- Ozugerin, I., & Piekny, A. (2022). Diversity is the spice of life: An overview of how cytokinesis regulation varies with cell type. *Frontiers in cell and developmental biology*, 10, 1007614.
- Ozugerin I, Mastronardi K, Law C, Piekny A. Diverse mechanisms regulate contractile ring assembly for cytokinesis in the two-cell *Caenorhabditis elegans* embryo. *J Cell Sci*. 2022 Feb 1;135(3):jcs258921. Epub 2022 Feb 8.
- Paim LMG, FitzHarris G. Cell size and polarization determine cytokinesis furrow ingression dynamics in mouse embryos. *Proc Natl Acad Sci U S A*. 2022 Mar 22;119(12):e2119381119. Epub 2022 Mar 16.
- Palmeri, D., & Malim, M. H. (1999). Importin beta can mediate the nuclear import of an arginine-rich nuclear localization signal in the absence of importin alpha. *Molecular and cellular biology*, 19(2), 1218–1225.
- Park H, Kim NY, Lee S, Kim N, Kim J, Heo WD. Optogenetic protein clustering through fluorescent protein tagging and extension of CRY2. *Nat Commun*. 2017 Jun 23;8(1):30.
- Park SU, Choi ES, Jang YS, Hong SH, Kim IH, Chang DK. [Effects of chromosomal polyploidy on survival of colon cancer cells]. *Korean J Gastroenterol*. 2011 Mar;57(3):150-7. Korean.

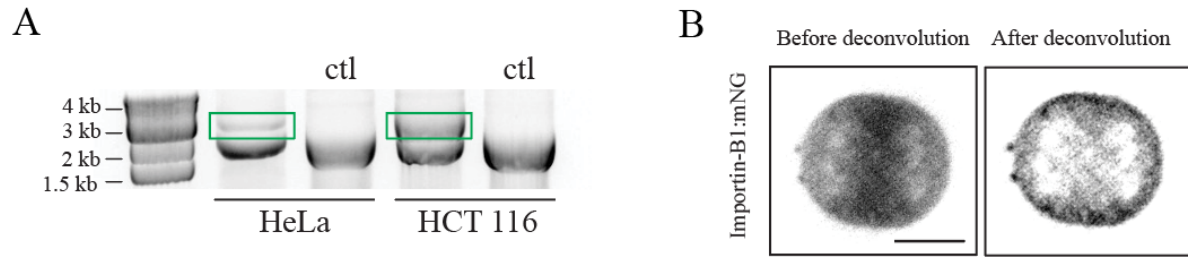
- Petronczki M, Glotzer M, Kraut N, Peters JM. Polo-like kinase 1 triggers the initiation of cytokinesis in human cells by promoting recruitment of the RhoGEF Ect2 to the central spindle. *Dev Cell*. 2007 May;12(5):713-25.
- Pham NP, Schick G, Del Corpo J, Piekny A. The nuclear localization of Ect2 is required for cytokinesis. *bioRxiv* 2025.09.04.674117.
- Piekny AJ, Glotzer M. Anillin is a scaffold protein that links RhoA, actin, and myosin during cytokinesis. *Curr Biol*. 2008 Jan 8;18(1):30-6.
- Piekny AJ, Maddox AS. The myriad roles of Anillin during cytokinesis. *Semin Cell Dev Biol*. 2010 Dec;21(9):881-91.
- Pimpale, L. G., Middelkoop, T. C., Mietke, A., & Grill, S. W. (2020). Cell lineage-dependent chiral actomyosin flows drive cellular rearrangements in early *Caenorhabditis elegans* development. *eLife*, 9, e54930. DOI: 10.7554/eLife.54930.
- Pinheiro, D., & Bellaïche, Y. (2017). Studying cytokinesis in *Drosophila* epithelial tissues. *Methods in cell biology*, 137, 73–84. DOI: 10.1016/bs.mcb.2016.04.013.
- Prokopenko, S. N., Brumby, A., O'Keefe, L., Prior, L., He, Y., Saint, R., & Bellen, H. J. (1999). A putative exchange factor for Rho1 GTPase is required for initiation of cytokinesis in *Drosophila*. *Genes & development*, 13(17), 2301–2314.
- Puri, M. C., & Nagy, A. (2012). Concise review: Embryonic stem cells versus induced pluripotent stem cells: the game is on. *Stem cells* (Dayton, Ohio), 30(1), 10–14.
- Raggi C, M'Callum MA, Pham QT, Gaub P, Selleri S, Baratang NV, Mangahas CL, Cagnone G, Reversade B, Joyal JS, Paganelli M. Leveraging interacting signaling pathways to robustly improve the quality and yield of human pluripotent stem cell-derived hepatoblasts and hepatocytes. *Stem Cell Reports*. 2022 Mar 8;17(3):584-598. Epub 2022 Feb 3.
- Rani, B., Qian, H., & Johansson, S. (2025). Cytokinesis in Suspension: A Distinctive Trait of Mesenchymal Stem Cells. *Cells*, 14(12), 932.
- Rehain-Bell, K., Love, A., Werner, M. E., MacLeod, I., Yates, J. R., 3rd, & Maddox, A. S. (2017). A Sterile 20 Family Kinase and Its Co-factor CCM-3 Regulate Contractile Ring Proteins on Germline Intercellular Bridges. *Current biology : CB*, 27(6), 860–867.
- Reyes, C. C., Jin, M., Breznau, E. B., Espino, R., Delgado-Gonzalo, R., Goryachev, A. B., & Miller, A. L. (2014). Anillin regulates cell-cell junction integrity by organizing junctional accumulation of Rho-GTP and actomyosin. *Current biology : CB*, 24(11), 1263–1270.
- Reymann, A. C., Staniscia, F., Erzberger, A., Salbreux, G., & Grill, S. W. (2016). Cortical flow aligns actin filaments to form a furrow. *eLife*, 5, e17807.
- Ribbeck, K., Lipowsky, G., Kent, H. M., Stewart, M., & Görlich, D. (1998). NTF2 mediates nuclear import of Ran. *The EMBO journal*, 17(22), 6587–6598.

- Rieckhoff EM, Ishihara K, Brugués J. How to tune spindle size relative to cell size? *Curr Opin Cell Biol.* 2019 Oct;60:139-144.
- Rodrigues NT, Lekomtsev S, Jananji S, Kriston-Vizi J, Hickson GR, Baum B. Kinetochore-localized PP1-Sds22 couples chromosome segregation to polar relaxation. *Nature.* 2015 Aug 27;524(7566):489-92.
- Roegiers F, Jan YN. Asymmetric cell division. *Curr Opin Cell Biol.* 2004 Apr;16(2):195-205.
- Roubinet, C., Tsankova, A., Pham, T. T., Monnard, A., Caussinus, E., Affolter, M., & Cabernard, C. (2017). Spatio-temporally separated cortical flows and spindle geometry establish physical asymmetry in fly neural stem cells. *Nature communications*, 8(1), 1383.
- Saito, S., Liu, X. F., Kamijo, K., Raziuddin, R., Tatsumoto, T., Okamoto, I., Chen, X., Lee, C. C., Lorenzi, M. V., Ohara, N., & Miki, T. (2004). Dereglulation and mislocalization of the cytokinesis regulator ECT2 activate the Rho signaling pathways leading to malignant transformation. *The Journal of biological chemistry*, 279(8), 7169–7179.
- Schafer KA. The cell cycle: a review. *Vet Pathol.* 1998 Nov;35(6):461-78.
- Schiel, J. A., Childs, C., & Prekeris, R. (2013). Endocytic transport and cytokinesis: from regulation of the cytoskeleton to midbody inheritance. *Trends in cell biology*, 23(7), 319–327.
- Schmitz MH, Held M, Janssens V, Hutchins JR, Hudecz O, Ivanova E, Goris J, Trinkle-Mulcahy L, Lamond AI, Poser I, Hyman AA, Mechtler K, Peters JM, Gerlich DW. Live-cell imaging RNAi screen identifies PP2A-B55alpha and importin-beta1 as key mitotic exit regulators in human cells. *Nat Cell Biol.* 2010 Sep;12(9):886-93. Epub 2010 Aug 15.
- Sehring, I. M., Recho, P., Denker, E., Kourakis, M., Mathiesen, B., Hannezo, E., Dong, B., & Jiang, D. (2015). Assembly and positioning of actomyosin rings by contractility and planar cell polarity. *eLife*, 4, e09206.
- Shi, Y., Inoue, H., Wu, J. C., & Yamanaka, S. (2017). Induced pluripotent stem cell technology: a decade of progress. *Nature reviews. Drug discovery*, 16(2), 115–130.
- Simon, G. C., Schonteich, E., Wu, C. C., Piekny, A., Ekiert, D., Yu, X., Gould, G. W., Glotzer, M., & Prekeris, R. (2008). Sequential Cyk-4 binding to ECT2 and FIP3 regulates cleavage furrow ingression and abscission during cytokinesis. *The EMBO journal*, 27(13), 1791–1803.
- Soderholm JF, Bird SL, Kalab P, Sampathkumar Y, Hasegawa K, Uehara-Bingen M, Weis K, Heald R. Importazole, a small molecule inhibitor of the transport receptor importin- β . *ACS Chem Biol.* 2011 Jul 15;6(7):700-8. Epub 2011 Apr 21.
- Somers WG, Saint R. A RhoGEF and Rho family GTPase-activating protein complex links the contractile ring to cortical microtubules at the onset of cytokinesis. *Dev Cell.* 2003 Jan;4(1):29-39.
- Spradling A, Drummond-Barbosa D, Kai T. Stem cells find their niche. *Nature.* 2001 Nov 1;414(6859):98-104.

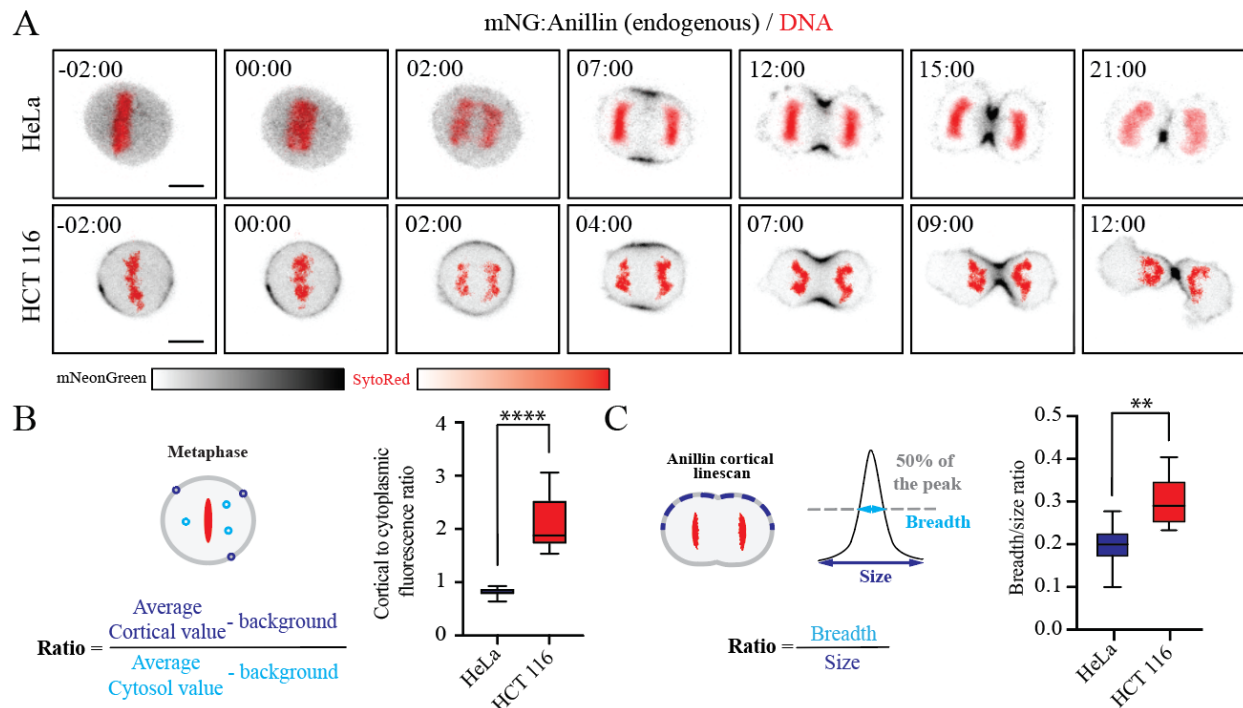
- Steigemann, P., & Gerlich, D. W. (2009). Cytokinetic abscission: cellular dynamics at the midbody. *Trends in cell biology*, 19(11), 606–616.
- Straight 2005 anillin.
- Su KC, Takaki T, Petronczki M. Targeting of the RhoGEF Ect2 to the equatorial membrane controls cleavage furrow formation during cytokinesis. *Dev Cell*. 2011 Dec 13;21(6):1104-15.
- Sun, L., Guan, R., Lee, I. J., Liu, Y., Chen, M., Wang, J., Wu, J. Q., & Chen, Z. (2015). Mechanistic insights into the anchorage of the contractile ring by anillin and Mid1. *Developmental cell*, 33(4), 413–426.
- Sutton PJ, Mosqueda N, Brownlee CW. Palmitoylated importin α regulates mitotic spindle orientation through interaction with NuMA. *EMBO Rep*. 2025 Jul;26(13):3280-3304. Epub 2025 May 27.
- Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., & Yamanaka, S. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell*, 131(5), 861–872.
- Thomas A, Gallaud E, Pascal A, Serre L, Arnal I, Richard-Parpaillon L, Savoian MS, Giet R. Peripheral astral microtubules ensure asymmetric furrow positioning in neural stem cells. *Cell Rep*. 2021 Oct 26;37(4):109895.
- Tian Z, Yu T, Liu J, Wang T, Higuchi A. Introduction to stem cells. *Prog Mol Biol Transl Sci*. 2023;199:3-32. Epub 2023 Mar 15.
- Tripathi, V. K., Subramaniyan, S. A. & Hwang, I. Molecular and Cellular Response of Co-cultured Cells toward Cobalt Chloride (CoCl₂)-Induced Hypoxia. *ACS Omega* 4, 20882–20893 (2019).
- Uehara R, Goshima G. Functional central spindle assembly requires de novo microtubule generation in the interchromosomal region during anaphase. *J Cell Biol*. 2010 Oct 18;191(2):259-67. Epub 2010 Oct 11.
- Uetake, Y., & Sluder, G. (2004). Cell cycle progression after cleavage failure: mammalian somatic cells do not possess a "tetraploidy checkpoint". *The Journal of cell biology*, 165(5), 609–615.
- Valdez VA, Neahring L, Petry S, Dumont S. Mechanisms underlying spindle assembly and robustness. *Nat Rev Mol Cell Biol*. 2023 Aug;24(8):523-542. Epub 2023 Mar 28.
- van Oostende-Triplett, C., Jaramillo Garcia, M., Haji Bik, H., Beaudet, D., & Piekny, A. (2014). Anillin interacts with microtubules and is part of the astral pathway that defines cortical domains. *Journal of cell science*, 127(Pt 17), 3699–3710. DOI: 10.1242/jcs.147504.
- Verma and Maresca 2019 ect2.
- Wang D, Naydenov NG, Dozmorov MG, Kobinski JE, Ivanov AI. Anillin regulates breast cancer cell migration, growth, and metastasis by non-canonical mechanisms involving control of cell stemness and differentiation. *Breast Cancer Res*. 2020 Jan 7;22(1):3.

- Wenzel ES, Singh ATK. Cell-cycle Checkpoints and Aneuploidy on the Path to Cancer. *In Vivo*. 2018 Jan-Feb;32(1):1-5.
- Werner, M., Munro, E., & Glotzer, M. (2007). Astral signals spatially bias cortical myosin recruitment to break symmetry and promote cytokinesis. *Current biology : CB*, 17(15), 1286–1297.
- Williams, E. C., Zhong, X., Mohamed, A., Li, R., Liu, Y., Dong, Q., Ananiev, G. E., Mok, J. C., Lin, B. R., Lu, J., Chiao, C., Cherney, R., Li, H., Zhang, S. C., & Chang, Q. (2014). Mutant astrocytes differentiated from Rett syndrome patients-specific iPSCs have adverse effects on wild-type neurons. *Human molecular genetics*, 23(11), 2968–2980.
- Wolfe BA, Takaki T, Petronczki M, Glotzer M. Polo-like kinase 1 directs assembly of the HsCdk-4 RhoGAP/Ect2 RhoGEF complex to initiate cleavage furrow formation. *PLoS Biol*. 2009 May 5;7(5):e1000110. Epub 2009 May 26.
- Wolff F, Srinivasan S, Nöcker MC, Mangal S, Mikeladze-Dvali T, Zanin E. Spatially distinct inputs modulate the amount of active Mitotic-phase GAP to locally restrict RhoA signaling for successful cell division. *bioRxiv* 2023.08.08.552464; doi:.
- Wu Y, Lin Y, Shen F, Huang R, Zhang Z, Zhou M, Fang Y, Shen J, Fan X. FBXO38 deficiency promotes lysosome-dependent STING degradation and inhibits cGAS-STING pathway activation. *Neoplasia*. 2024 Mar;49:100973.
- Yang, Y., Guo, L., Chen, L., Gong, B., Jia, D., & Sun, Q. (2023). Nuclear transport proteins: structure, function, and disease relevance. *Signal transduction and targeted therapy*, 8(1), 425.
- Yüce O, Piekny A, Glotzer M. An ECT2-centralspindlin complex regulates the localization and function of RhoA. *J Cell Biol*. 2005 Aug 15;170(4):571-82.
- Zanin E, Desai A, Poser I, Toyoda Y, Andree C, Moebius C, Bickle M, Conradt B, Piekny A, Oegema K. A conserved RhoGAP limits M phase contractility and coordinates with microtubule asters to confine RhoA during cytokinesis. *Dev Cell*. 2013 Sep 16;26(5):496-510. Epub 2013 Sep 5.
- Zhang S, Nguyen LH, Zhou K, Tu HC, Sehgal A, Nassour I, Li L, Gopal P, Goodman J, Singal AG, Yopp A, Zhang Y, Siegwart DJ, Zhu H. Knockdown of Anillin Actin Binding Protein Blocks Cytokinesis in Hepatocytes and Reduces Liver Tumor Development in Mice Without Affecting Regeneration. *Gastroenterology*. 2018 Apr;154(5):1421-1434. Epub 2017 Dec 21. Erratum in: *Gastroenterology*. 2018 Dec;155(6):2027.
- Zhao, W., Zhao, S., Li, L., Huang, X., Xing, S., Zhang, Y., Qiu, G., Han, Z., Shang, Y., Sun, D. E., Shan, C., Wu, R., Gu, L., Zhang, S., Chen, R., Xiao, J., Mo, Y., Wang, J., Ji, W., Chen, X., ... Chen, L. (2022). Sparse deconvolution improves the resolution of live-cell super-resolution fluorescence microscopy. *Nature biotechnology*, 40(4), 606–617.
- Zhou, J., Sun, H., Wang, Z., Cong, W., Zeng, M., Zhou, W., Bie, P., Liu, L., Wen, T., Kuang, M., Han, G., Yan, Z., Wang, M., Liu, R., Lu, L., Ren, Z., Zeng, Z., Liang, P., Liang, C., Chen, M., ... Fan, J. (2023). Guidelines for the Diagnosis and Treatment of Primary Liver Cancer (2022 Edition). *Liver cancer*, 12(5), 405–444.

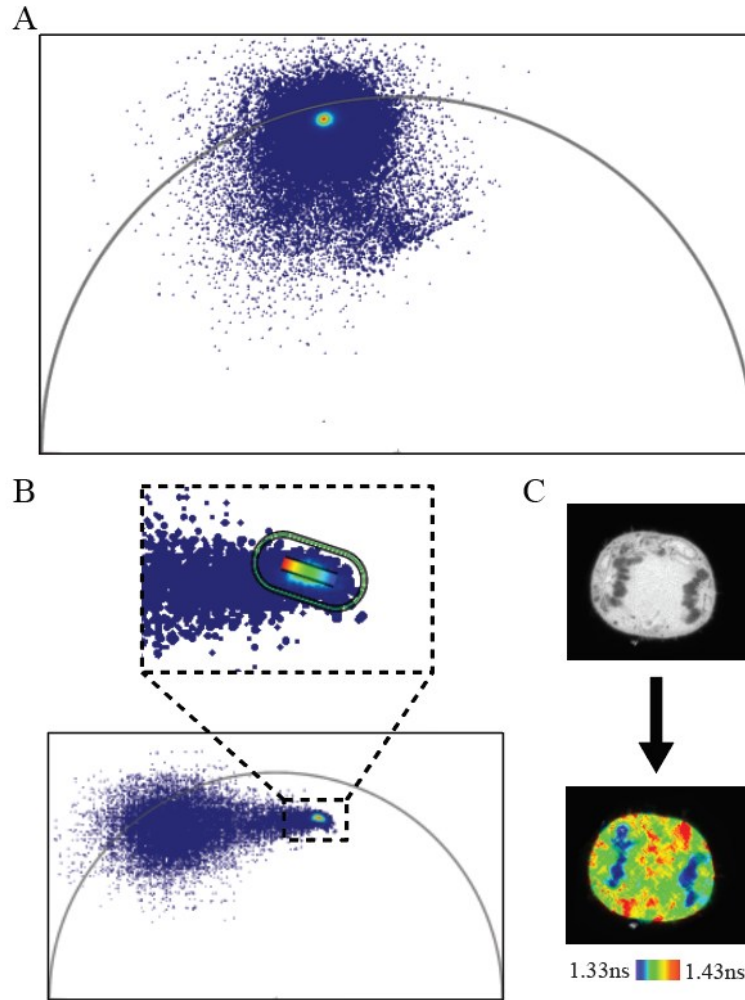
Supplemental figures



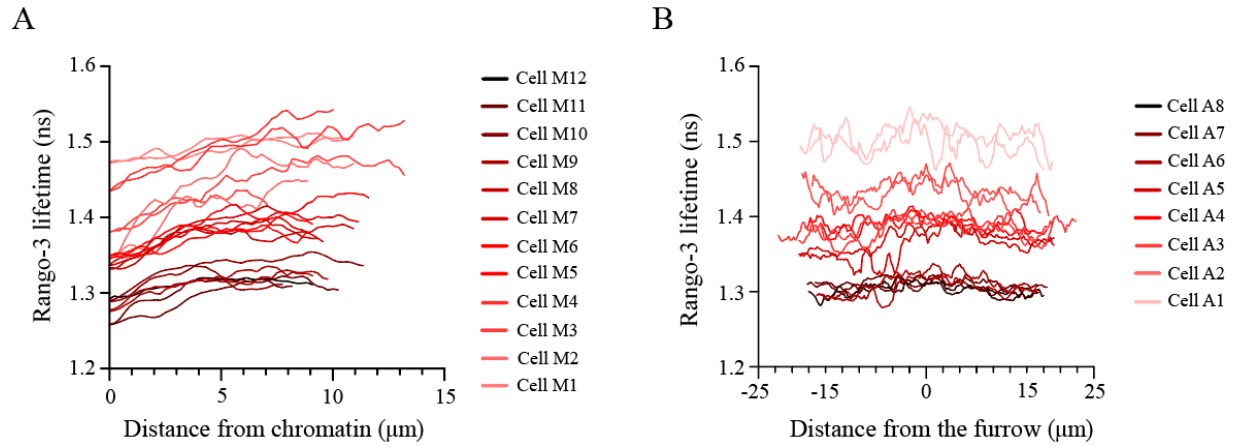
Supplementary Figure S1. Genotyping of importin-β1:mNG tagged cells. (A) An image shows an agarose gel with PCR-amplified DNA for the KPNB1 locus and mNG insertion in HeLa and HCT 116 cells. Amplification of clones with the mNG insertion produced a 3kb fragment (green box), while non-tagged control cells produced a 2kb fragment. Note that the intensity of the mNG-inserted fragment in HeLa cells and HCT 116 cells indicates that the clones are heterozygous. (B) Images show the importin-β1:mNG in an anaphase HeLa cell before (left) and after deconvolution (right).



Supplemental Figure S2. Anillin localizes differently in mitotic HeLa and HCT 116 cells. (A) Timelapse images show endogenous anillin tagged with mNG (greyscale) co-stained for chromatin (Hoechst; red) in HeLa (top) and HCT 116 cells (bottom). The colour bar indicates the intensity of signal from light (low levels) to bright (high levels). Time is in minutes, and the scale bars are 10 μm . (B) A cartoon cell shows how the ratio of cortical to cytosolic anillin fluorescence was measured. Three regions of interest (ROI) were drawn in the cytosol or at the cortex and averaged, then the cortical average value was divided by the cytosolic average value. A bar graph shows the average cortical vs. cytoplasmic fluorescence ratio of anillin in metaphase HeLa (n=17) and HCT 116 cells (n=20; ****: $p \leq 0.0001$). (C) A schematic shows how the breadth of anillin in anaphase cells was measured using linescans, and the number of pixels >50% of the maximum peak value. The breadth was then divided by the length of the line to calculate the ratio of breadth to length (size). The graph shows the average ratio of breadth to size of anillin (>50% peak signal) vs. size at HeLa (n=17) and HCT 116 cells (n=20; **: $p \leq 0.005$).

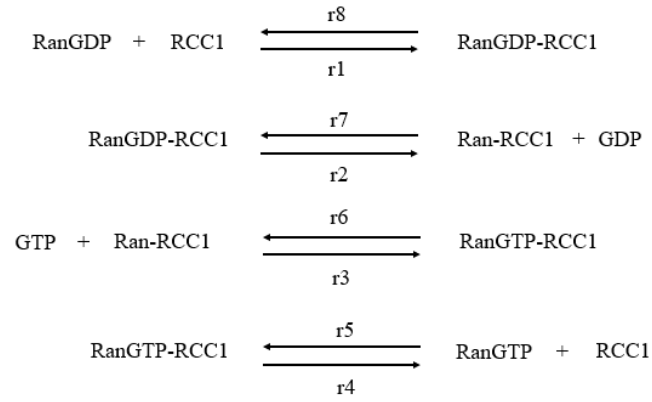


Supplementary Figure S3. Method used to image and evaluate the fluorescence lifetime. (A) A graph shows phasor plots of EGFP, which represents the fluorescence decay for each pixel. Mono-exponential decay occurs along the semi-circle. Signals on the left indicated higher lifetime compared to those on the right. Multi-exponential decay occurs inside the semi-circle. Signals closer to the centre are higher in complexity and reflect a mix of fluorophores. The density cloud indicates the where signal is located in the semi-circle. (B) A graph shows the phasor plot of a HeLa cell with Rango-3 at anaphase. The picture on top is a zoomed-in image of the signal. A mask was created (coloured line) on the image. (C) Images show Rango-3 fluorescence in the cell (top) and the measured lifetimes (bottom; shorter; blue to longer; red).

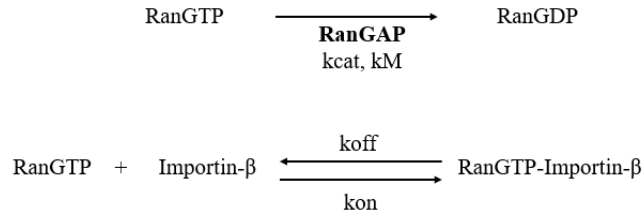


Supplementary Figure S4. Fluorescence lifetime is dependent on the levels of Rango-3. (A) A graph shows Rango-3 lifetime measurements in nanoseconds along a line drawn from chromatin to the cortex in metaphase HeLa cells (n = 12). (B) A graph shows Rango-3 lifetime measurements in nanoseconds along the cortex of anaphase HeLa cells from pole to pole (n = 8). The color scale is based on the intensity of Rango-3 fluorescence from dark (high levels) to light (low levels).

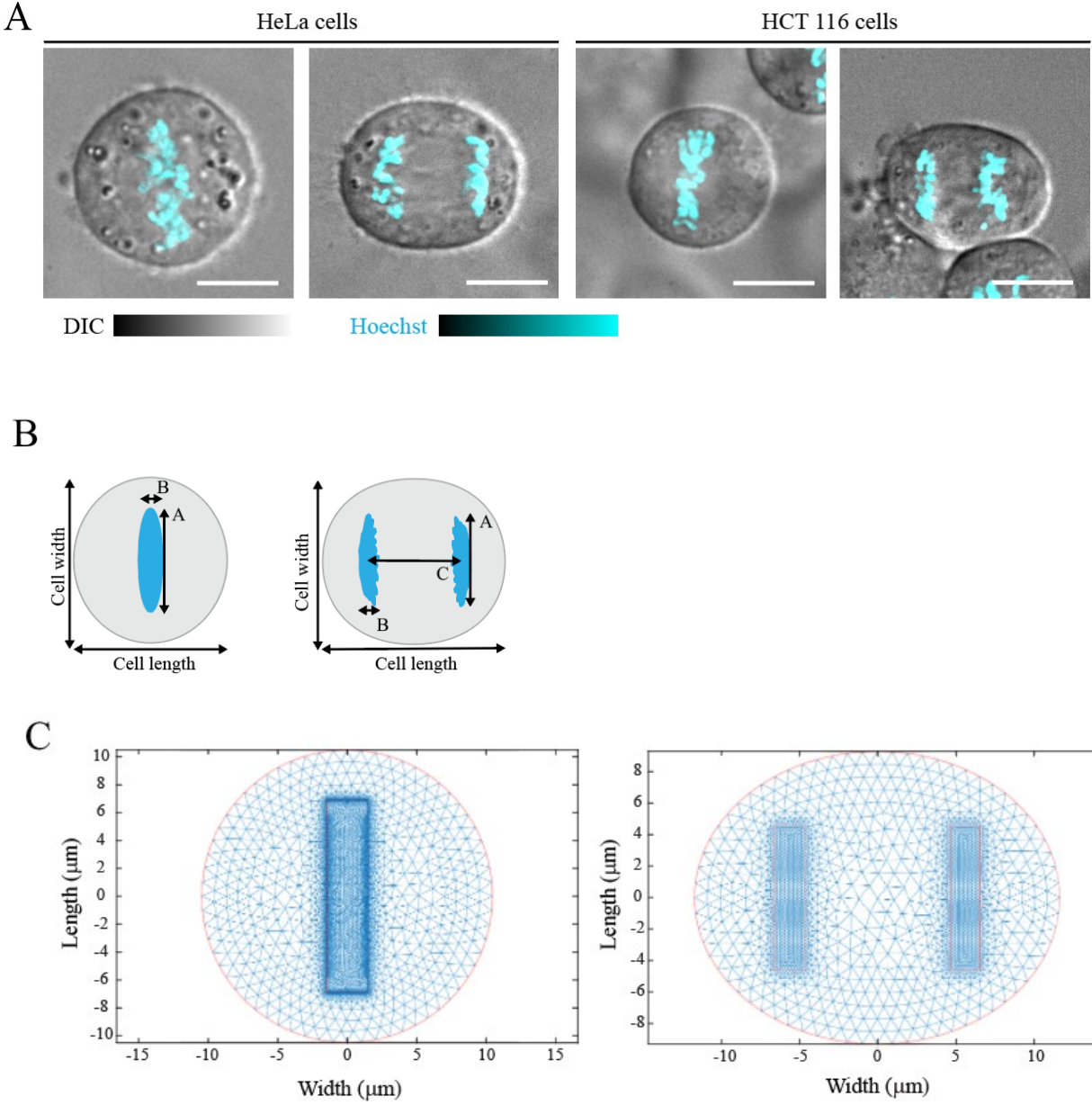
A



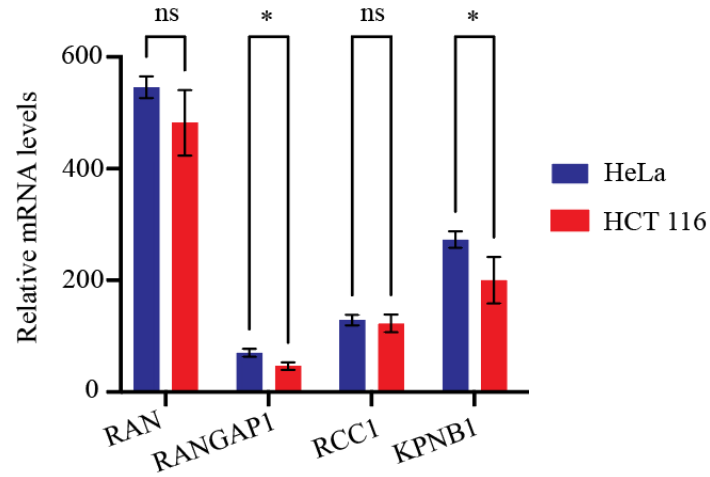
B



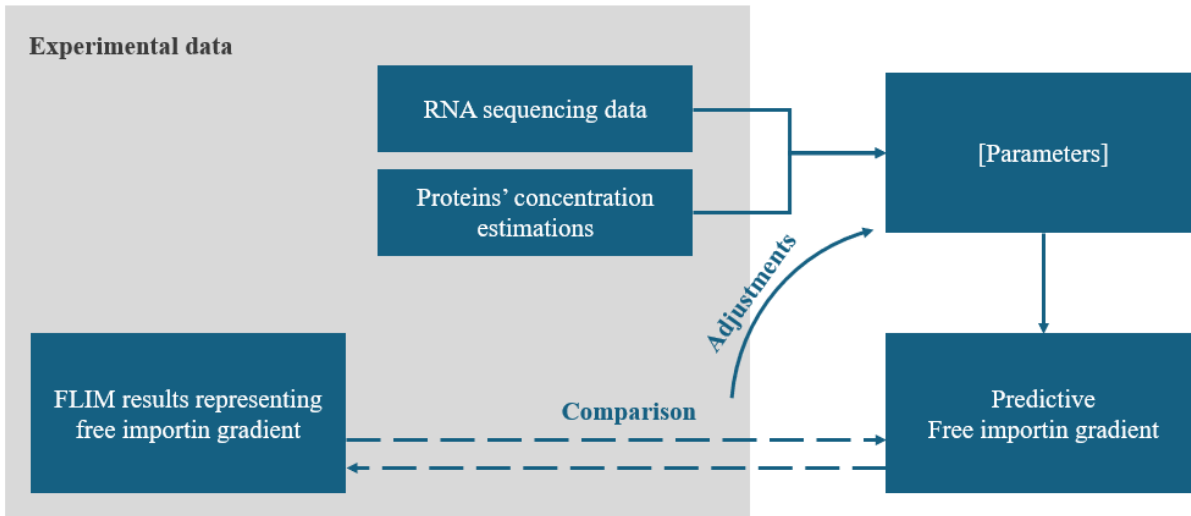
Supplementary Figure S5. Reactions used to calculate the Ran gradient in the predictive model. (A) Reactions for the RCC1-mediated guanine nucleotide exchange between Ran-GTP and Ran-GDP. These equations take place in the region near chromatin. The reaction rates and kinetic constants are listed in Table S2. (B) Reactions for the hydrolysis of Ran-GTP by RanGAP, and Ran-GTP interaction with importin- β . These equations take place in the cytosol.



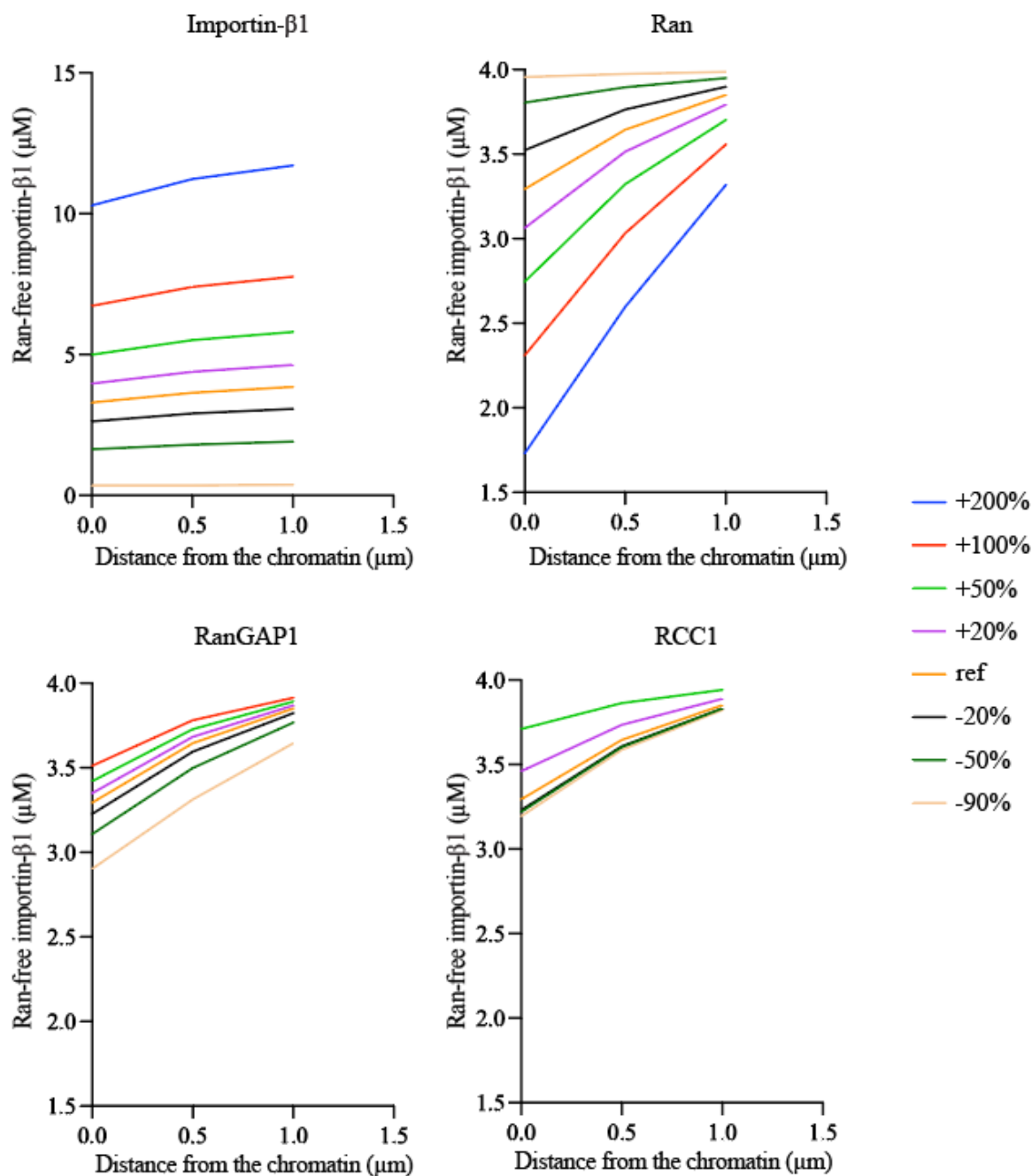
Supplementary Figure S6. Cell measurements used in the predictive model. (A) Images show DIC metaphase and anaphase HeLa and HCT 116 cells stained with Sir Tubulin (red) and Hoechst (chromatin, cyan). The colour scales indicate fluorescence intensity from dark (low levels) to bright (high levels). (B) A schematic shows how components of the cells were measured (A indicates chromatin length; B indicates chromatin width; C indicates the distance between sister chromatids). (C) The mesh used for the 2D model is shown for a HeLa cell at metaphase (left; $n = 10$) and anaphase (right; $n = 10$). The red line shows the outer boundary of the cell, while the blue hatch marks indicate the equations solved in the cytosol, and the lines show those near chromatin.



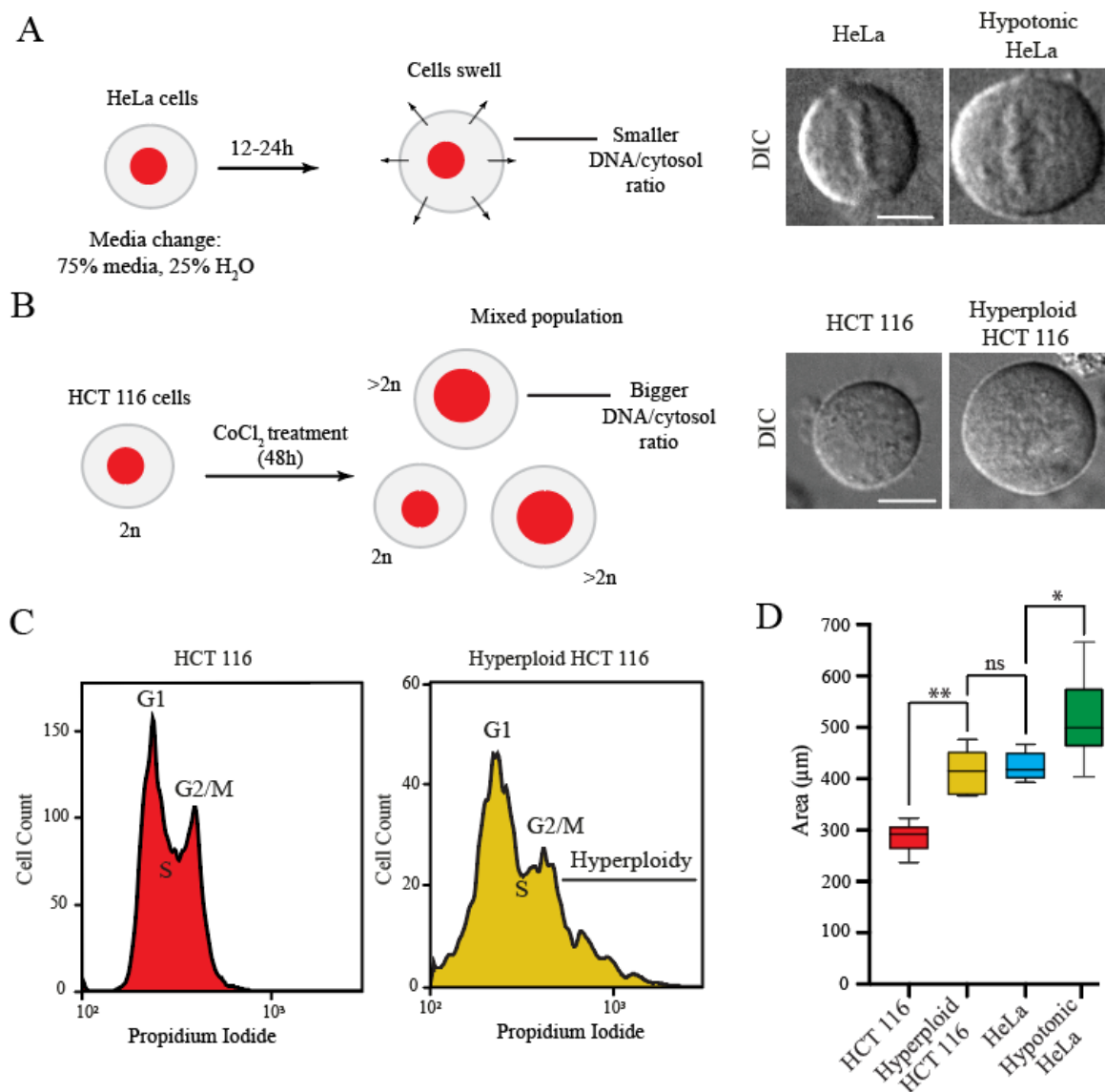
Supplementary Figure S7. RNA-seq data analysis for Ran and Ran regulators in HeLa and HCT 116 cells. The graph shows normalized mRNA expression levels for Ran, RanGAP1, RCC1 and KPNB1 in HeLa (blue) and HCT 116 cells (red; * $p < 0.05$; ns = non-significance) using the RNA sequencing data from Wu et al., 2024 (GSE220879).



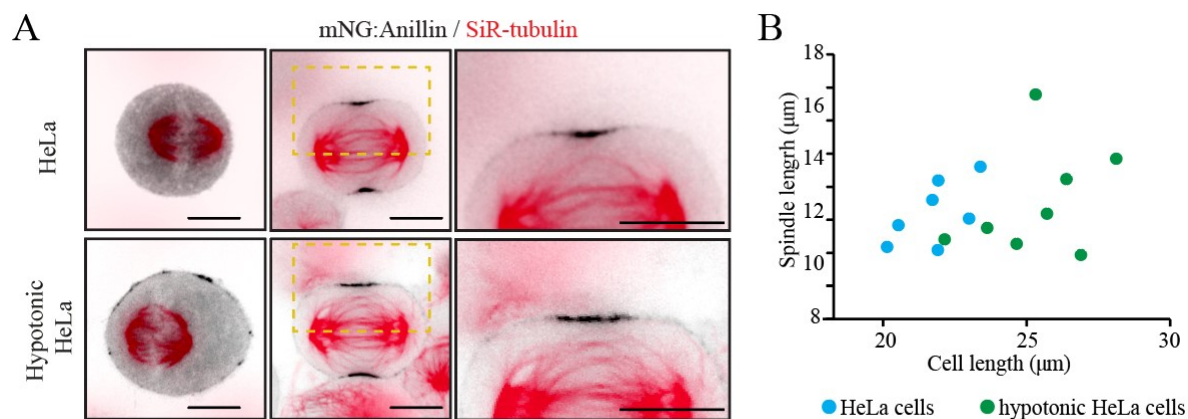
Supplementary Figure S8. Method for model refinement. A cartoon shows the method used to find an estimation of conditions that match experimental findings. Parameters implemented in the predictive model include cell measurements, kinetic constants, reaction rates and protein concentrations. These values were taken from published data or measured and adjusted in the model using this workflow.



Supplementary Figure S9. Impact of changing the levels of Ran or Ran regulators on the predicted Ran-free importin- β 1 concentration. Graphs show the Ran-free importin- β 1 concentrations (mM) in metaphase cells from the chromatin to the cortex in response to changes in the amounts of Ran, RCC1, RanGAP and importin- β 1. The conditions vary between -90% and 200% in relation to the concentrations calculated based on the RNA sequencing data (ref; reference).

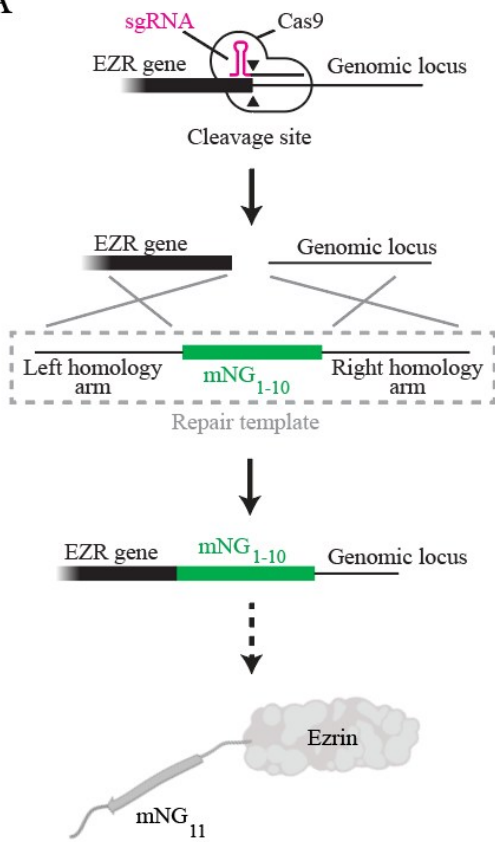


Supplemental Figure S10. Generation of hypotonic HeLa cells and hyperploid HCT 116 cells. (A) The schematic on the left shows the workflow to generate hypotonic HeLa cells. Cells were cultured in 75% hypotonic media supplemented with FBS for 12h resulting in large HeLa cells. The DIC images show a metaphase HeLa cell in isotonic media (left) and in hypotonic media (right). The scale bar is 10 μm. (B) The schematic on the left shows the workflow to generate hyperploid HCT 116 cells. Cells were treated with CoCl₂ for 48h resulting in a mixed population of euploid and hyperploid cells. The DIC images on the right show a metaphase euploid HCT 116 cell (left) and hyperploid cell (right). The scale bar is 10 μm. (C) The histograms show the DNA content of HCT 116 cells (left) and after treatment to increase ploidy (right). DNA was stained with propidium iodide. The cell cycle stages are indicated in the graphs. (D) The graph shows the area of metaphase HCT 116, hyperploid HCT 116, HeLa, and hypotonic HeLa cells (n=15 for each category; *p<0.005; **p<0.0001; n.s. = not significance).

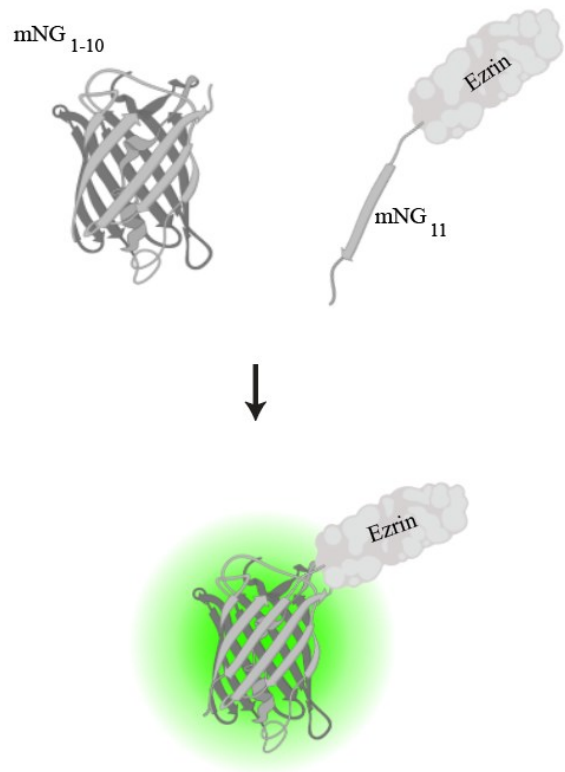


Supplementary Figure S11. The spindle scales with cell size. (A) Images show HeLa cells and hypotonic HeLa cells at metaphase (left) and anaphase (right) with endogenous anillin tagged with mNG (greyscale) and co-stained for tubulin (SiR-tubulin; red). The images on the right are zoomed-in views of the yellow boxes from anaphase cells. The scale bars are 10 μm . (B) A graph shows the correlation between the spindle length and cell length in metaphase cells. The colours of the dots show the different cell types (HeLa cells; $n=7$; green, hypotonic HeLa cells; $n=8$).

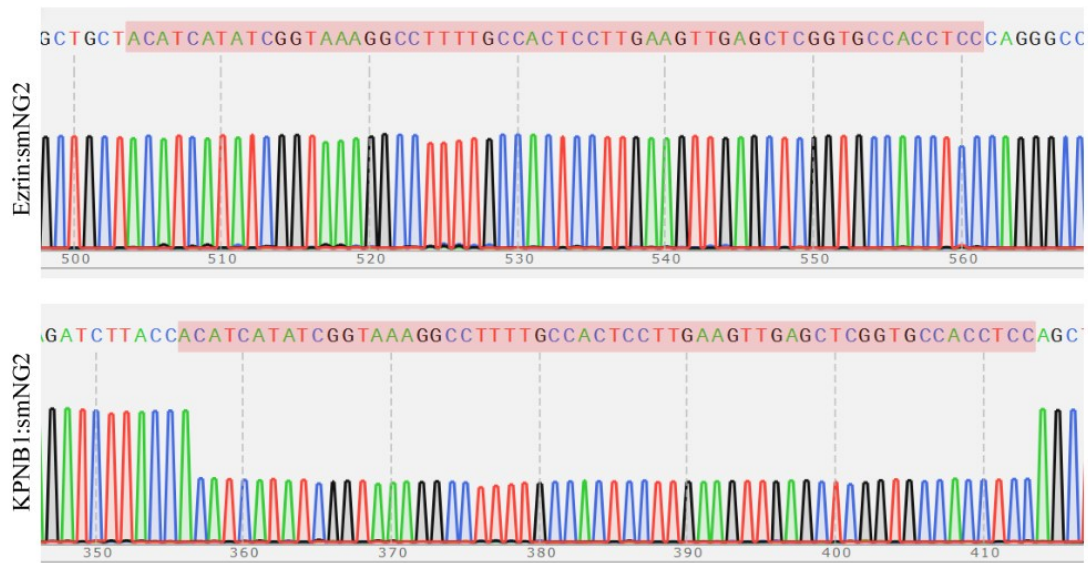
A



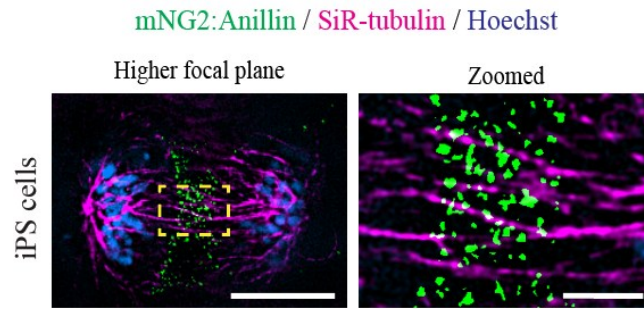
B



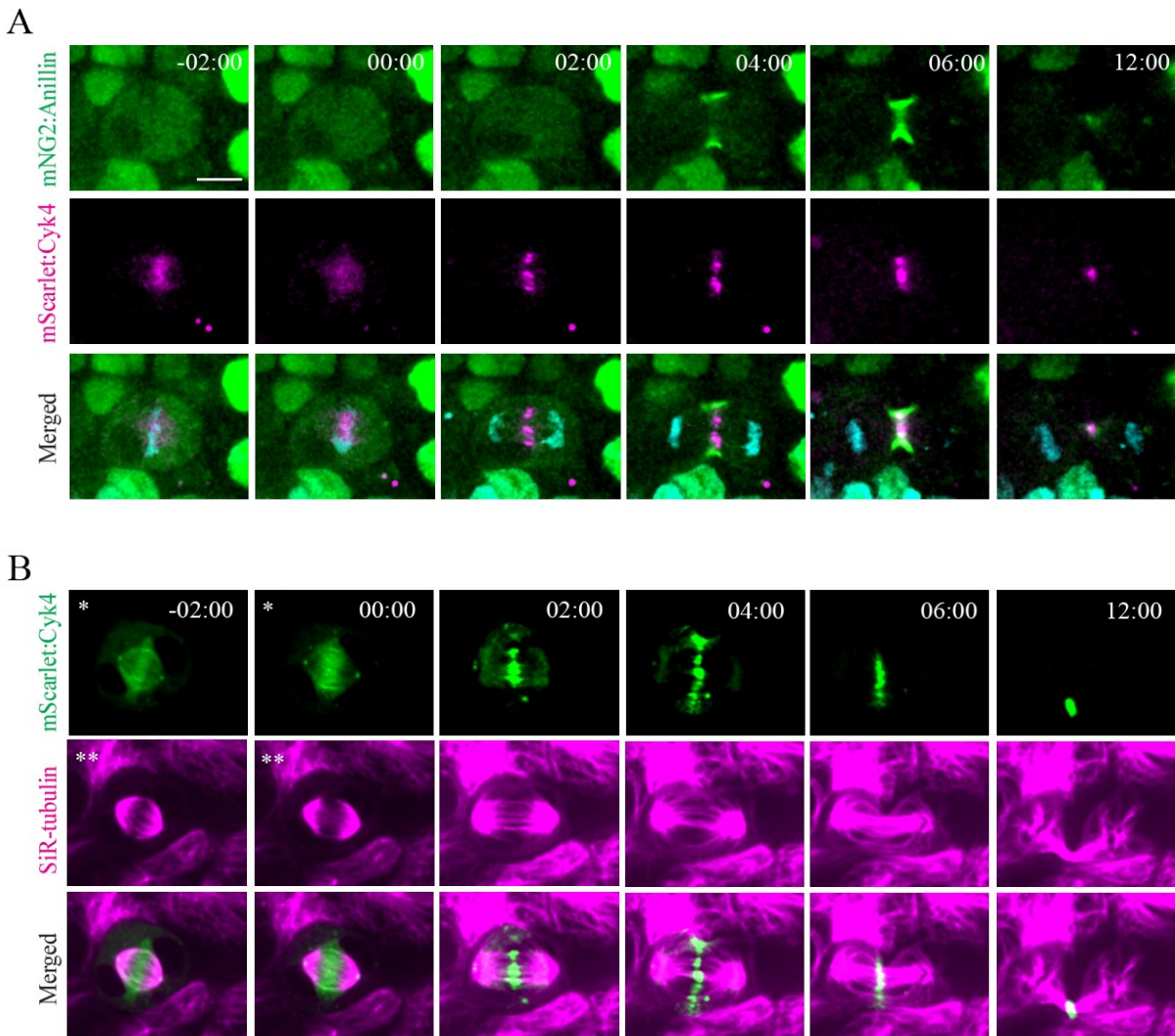
C



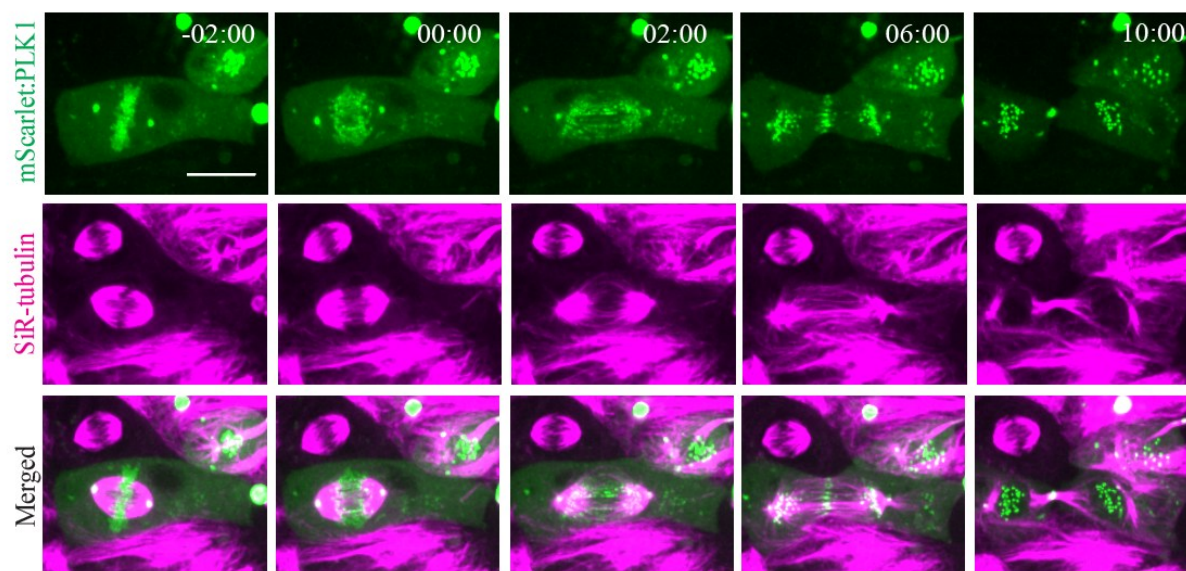
Supplementary Figure S12. Endogenously tagging ezrin and importin- β 1 using the split mNeonGreen system in iPSCs. (A) A schematic shows how genome editing was performed in smNG2-P iPSCs to insert the small mNeonGreen (mNG) fragment, smNG₁₁, at the C terminus of ezrin or importin- β 1. The sgRNA guide is shown in pink, and the small fragment is shown in green. (B) A cartoon shows how the small fragment complements the large fragment, which is in the AAVSII locus, to generate mNeonGreen fluorescence. (C) Sequence chromatograms show the insertion of the small mNG fragment (highlighted in red) in the ezrin (top) and importin- β 1 (bottom) locus.



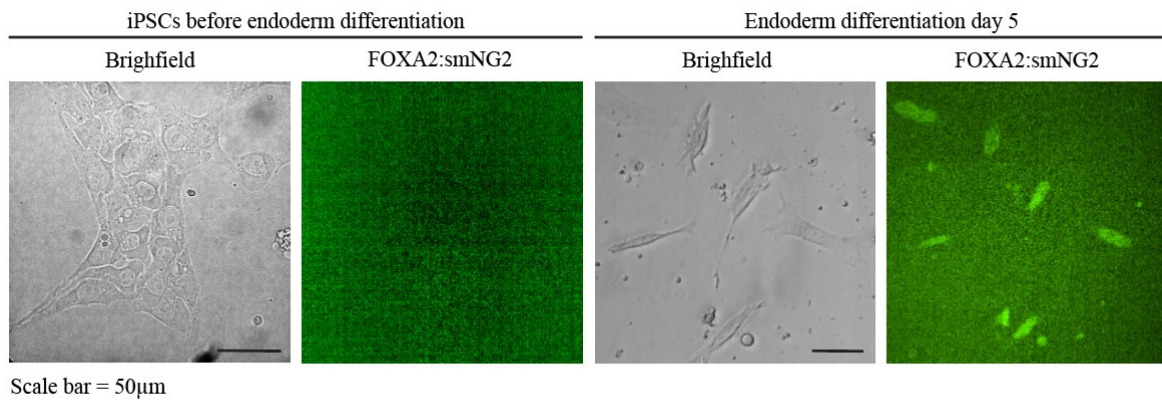
Supplementary Figure S13. Anillin forms aggregates in anaphase iPSCs. The image on the left shows a higher focal plane of mNG2:Anillin (green) in an anaphase iPSC co-stained with SiR-tubulin (microtubules; magenta) and Hoechst (chromatin; blue). The scale bar is 10 μm . The image on the right is a zoomed-in view of the yellow box from the image on the left to highlight the anillin aggregates. The scale bar is 2 μm .



Supplementary Figure S14. Cyk4 localization in iPSCs during cytokinesis. (A) Timelapses show mNG2:anillin (green; top panel) in an iPSC co-expressing mScarlet:Cyk4 (magenta; middle panel). Merged images show the overlay of anillin (green) and Cyk4 (magenta), also co-stained for chromatin (Syto Deep Red; Cyan). Time is in minutes. The scale bar is 10 μ m. (B) Timelapse images show mScarlet:Cyk4 (green; top panel) in an iPSC during cytokinesis and co-stained with SiR-tubulin (microtubules; magenta; middle panel). Merged images show the overlay of Plk1 (green) and microtubules (magenta; bottom panel). Time is in minutes. The scale bar is 10 μ m.



Supplementary Figure S15. Plk1 localization in iPSCs during cytokinesis. Timelapse images show mScarlet:Plk1 (green; top panel) in an iPSC during cytokinesis and co-stained with SiR-tubulin (microtubules; magenta; middle panel). Merged images show the overlay of Plk1 and microtubules (bottom panel). Time is in minutes. The scale bar is 10 μ m.



Supplementary Figure S16. Endoderm differentiation of FOXA2:smNG2 iPSCs. Images show fluorescence (green) and brightfield images of endogenous FOXA2:smNG2 in iPSCs (left) and after differentiation into endoderm (right). The scale bar is 50 μm.

Supplemental tables

Supplementary Table S1. Rango-3 fluorescence intensity in individual HeLa cells. Values indicate the average fluorescence intensity of Rango-3 in metaphase and anaphase HeLa cells. Grey boxes indicate the cells used for analysis in the main text.

Metaphase cells	Fluorescence intensity	Anaphase cells	Fluorescence intensity
Cell M1	195.12	Cell A1	247.006
Cell M2	205.1	Cell A2	501.532
Cell M3	531.982	Cell A3	626.57
Cell M4	586.031	Cell A4	1038.823
Cell M5	556.447	Cell A5	1283.053
Cell M6	603.82	Cell A6	1685.009
Cell M7	1142.071	Cell A7	2219.852
Cell M8	1777.825	Cell A8	2052
Cell M9	2231.665		
Cell M10	2122.818		
Cell M11	1632.743		
Cell M12	4068.93		

Supplementary Table S2. Diffusion coefficients for Ran and Ran regulators.

Symbol in the code	Proteins	Localization	Diffusion coefficient name	D ($\mu\text{m}^2/\text{sec}$) in the cytoplasm
u1	RanGTP-RCC1	Chromatins, immobile	NA	NA
u2	Ran-RCC1	Chromatins, immobile	NA	NA
u3	RanGDP-RCC1	Chromatins, immobile	NA	NA
u4	RanGTP	Chromatins/cytosol/spindle	Dr	22
u5	RanGDP	Chromatins/cytosol/spindle	Dr	22
u6	RCC1	Chromatins, immobile	NA	NA
u7	Importin- β	Cytosol/spindle	Di	14
u8	RanGTP-ImportinB	Cytosol/spindle	D2	13

Supplementary Table S3. Reaction rates and kinetic constants for Ran and Ran regulators.

In chromatins	In cytosol
$r1 = 74.4 \mu\text{M}^{-1} \cdot \text{s}^{-1}$ $r2 = 21.1 \text{ s}^{-1}$ $r3 = 0.65 \mu\text{M}^{-1} \cdot \text{s}^{-1}$ $r4 = 55 \text{ s}^{-1}$ $r5 = 102 \mu\text{M}^{-1} \cdot \text{s}^{-1}$ $r6 = 19 \text{ s}^{-1}$ $r7 = 11.4 \mu\text{M}^{-1} \cdot \text{s}^{-1}$ $r8 = 55 \text{ s}^{-1}$	$k_{\text{cat}} = 10.6 \text{ s}^{-1}$ $k_{\text{M}} = 0.7 \mu\text{M}$ $k_{\text{cat}2} = 10.8 \text{ s}^{-1}$ $k_{\text{M}2} = 0.1 \mu\text{M}$ $k_{\text{on}} = 0.45 \mu\text{M}^{-1} \cdot \text{s}^{-1}$ $k_{\text{off}} = 0.00045 \text{ s}^{-1}$

Supplementary Table S4. Measurements of components in metaphase and anaphase HeLa and HCT 116 cells. Measurements were made using images ($n = 10$ cells each).

	HeLa cells		HCT 116 cells	
	Metaphase	Anaphase	Metaphase	Anaphase
Distance between the chromatin (μm)	NA	11.5 ± 0.7	NA	8.4 ± 0.5
Length of chromatin (μm)	13.8 ± 1	9.5 ± 0.7	12 ± 1	7.5 ± 0.6
Width of chromatin (μm)	3 ± 0.5	2 ± 0.3	3 ± 0.5	2 ± 0.1
Depth of chromatins (μm)	20 ± 1.6	19 ± 2.4	24 ± 0.6	19 ± 1.6
Cell length (μm)	21 ± 3.9	22.6 ± 1.1	17.4 ± 2.4	18.8 ± 1.19
Cell width (μm)	21 ± 3.9	18.6 ± 1.3	17.4 ± 2.4	15.6 ± 0.76
Cell depth (μm)	21 ± 1.4	21 ± 2.7	24 ± 2.5	22 ± 1.9
Cell volume (μm^3)	4920 ± 900	4850 ± 750	3920 ± 780	3440 ± 320
Chromatin volume (μm^3)	830 ± 110	720 ± 140	670 ± 79	570 ± 82

Supplementary Table S5. Concentrations of Ran and Ran regulators in HeLa and HCT 116 cells. Concentrations are in μM and were evaluated using published RNA sequencing data.

	HeLa cells	HCT 116 cells
Ran (total concentration)	6 ± 1.6	6.6 ± 2.4
RanGAP1 (cytosol)	0.84 ± 0.3	0.56 ± 0.4
RCC1 (chromatins' zone)	7.7 ± 1.2	7 ± 1.6
Importin- β 1 (cytosol)	3.8 ± 1.3	3.8 ± 1.8

Supplementary Table S6. Initial conditions for the predictive Ran-free importin- β 1 gradient model.
Concentrations are in μM .

	HeLa cell	HeLa cell with increased cytosol	HCT 116 cell	Tetraploid HCT 116 cell
Ran (total)	6.7	6.2	9.1	12.1
RanGAP1 (cytosol)	1	0.87	0.9	1.24
RCC1 (chromatin zone)	8.7	8.7	8.7	11.6
Importin- β 1 (cytosol)	4	3.7	2.75	3.7

Supplementary Table S7. Measurements for anaphase hypotonic HeLa and hyperploid HCT116 cells.

	Large HeLa cells	Hyperploid HCT116 cells
Distance between the chromatins (μm)	11.5	9.6
Length of chromatins (μm)	9.5	8.6
Width of chromatin (μm)	2	2.3
Cell length (μm)	24	21.5
Cell width (μm)	19.1	17.9
Cell depth (μm)	22	25
Total cell volume (μm^3)	5380	5160
Chromatins Volume (μm^3)	830	860

Supplementary Table S8. List of primers used for cloning.

Final Plasmids	Gene target / name	Fwd/ Rev	Sequence
KPNB1:mCherry:CRY2	KPNB1	FR W	CTGAATGAATTGAGGGAAAGCTGCTTGGAAGCCTA TACT
	KPNB1	REV	TTTCCCTCAATTCATTCAGATAATCCACCATGTCAT AGTCTGACTT
	mCherry:CRY2clust	FR W	GCATCGTCTCATCGGTCTCAGGGGGTTCAGGAGGG TCTatggtgagcaagggcgaggagg
	mCherry:CRY2clust	REV	ATGCCGTCTCAGGTCTCActcatcagttatctagatccggtggatcc
	KPNB1	FR W	GCATCGTCTCATCGGTCTCAccATGGAGCTGATCAC CATTCTCGAGA
	KPNB1	REV	ATGCCGTCTCAGGTCTCACCCCCTTCAGTTTCCTCA GTTCTTTTGT
p63R	N/A	FR W	CACCGAGGCAAGATCTTACCAAGCT
	N/A	REV	AAACAGCTTGGTAAGATCTTGCCTC
KPNB1 repair template	Genomic DNA	FR W	GACAGCTGCATCAGACCttctc
	Genomic DNA	REV	AGCTTGGTTCTTCAGTTTCCTCAG
	Genomic DNA	FR W	TGGTAAGATCTTGCCTCCACTGT
	Genomic DNA	REV	aggaggctgaggtgggtg
	mNeonGreen	FR W	AAAGAACTGAGGAACTGAAGAACCAAGCTGGAG GTTCAGGAGGCTCCatggtgagcaagggcgag
	mNeonGreen	REV	AAAGAGGACAGTGGAGGCAAGATCTTACCActgtaca gctcgtccatgcc
	pYTK089	FR W	ttttaggtgatccaccacctcagcctcctgattatcaaaaaggatcttcacctagatcc ttt
	pYTK090	REV	aaaagagagagaaGGTCTGATGCAGCTGTCacggttatccacagaa tcagggg
pGG_mScarlet:Anillin, pGG-mScarlet:Anillin-shANLN1 and pGG-mScarlet:Anillin(	Anillin	FR W	GCATCGTCTCATCGGTCTCAGTCTatggatccgtttacggagaa actg
	Anillin	REV	ATGCCGTCTCAGGTCTCActcaaggctttccaataggtttgtagcaa
	N/A	FR W	caccgGGCGATGCCTCTTTGAATAAATTCAGAGATT TATTCAAAGAGGCATCGCC
	N/A	REV	aaaaGGCGATGCCTCTTTGAATAAATCTCTTGAATTT ATTCAAAGAGGCATCGCCc

NLSmutant)- shANLN1	mScarlet	FR W	GCATCGTCTCATCGGTCTCAccatGGTGAGCAAGGGC GAG
	mScarlet	REV	ATGCCGTCTCAGGTCTCAAGACCCTCCTGAACCCCC CTTGTACAGCTCGTCCATGCC