Separase cleaves the kinetochore protein Meikin to direct the meiosis I/II transition

by

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Abstract

To generate haploid gametes, meiotic cells must undergo two consecutive rounds of chromosome segregation without an intervening gap phase. Importantly, because homologous chromosomes are segregated in meiosis I, but sister chromatids are segregated in meiosis II, this requires a dramatic rewiring of the cell division machinery between the two divisions. How meiotic cells coordinate this rapid and substantial change to the cell division machinery is a central mystery at the heart of proper fertility and reproduction.

Our work reveals a new paradigm that rewires key cell division processes at the meiosis I/II transition through the action of the protease Separase, which we demonstrate acts by cleaving the meiosis-specific kinetochore protein Meikin. Cleavage of Separase substrates such as cohesin results in their potent and complete inactivation. In contrast, we find that Separase cleavage of Meikin acts as a molecular “scalpel,” providing an elegant mechanism to precisely and irreversibly modulate Meikin activity between the two meiotic divisions without inactivating Meikin function. Our results demonstrate that the C-terminal Meikin cleavage product generated by Separase proteolysis retains substantial activity such that it localizes to kinetochores, binds to Plk1 kinase, and promotes downstream activities such as the cleavage of the meiosis-specific cohesin subunit Rec8, similar to full length Meikin.

Importantly, we demonstrate that both the failure to cleave Meikin or the complete inactivation of Meikin at the meiosis I/II transition each result in dramatic defects in the proper execution of meiosis II. Our functional analysis in mouse oocytes demonstrates that precise Meikin cleavage is critical to differentially control meiosis I and II. Thus, in contrast to previous models, Meikin is not just a regulator of meiosis I-specific activities, but differentially coordinates chromosome segregation across both meiotic divisions. Our discovery of Meikin as a new substrate for Separase cleavage represents a novel mechanism for the regulatory control of the meiosis I/II transition.

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Table of Contents

Abstract........................................................................................................................................2
Acknowledgements .........................................................................................................................3
Table of Contents ............................................................................................................................4

Chapter 1: Introduction .......................................................................................................................6
Sexual Reproduction ............................................................................................................................7
Chromosome Segregation and Kinetochore Function .........................................................................9
Overview of Meiosis ..........................................................................................................................9
  Figure 1......................................................................................................................................11
Diversity of Sexual Reproduction at the Organismal and Molecular Level ........................................11
MOKIRS and Meiosis I Activities Across Organisms ........................................................................14
  Figure 2......................................................................................................................................15
  Figure 3......................................................................................................................................18
Driving Cell Cycle Transitions with the Kinases Cdk and Plk1 ............................................................18
Driving Cell Cycle Transitions with Degradation by the E3 Ligase APC/C ........................................19
Driving Cell Cycle Transitions with the Protease Separase ................................................................21
  Figure 4......................................................................................................................................25
Open Questions Motivating Thesis Work ..........................................................................................25
References ........................................................................................................................................27

Chapter 2: Separase cleaves the kinetochore protein Meikin to direct the meiosis I/II transition .........................................................................................................................34
Summary ........................................................................................................................................35
Introduction .....................................................................................................................................36
Results ............................................................................................................................................37
  Meikin is proteolytically cleaved by Separase during anaphase I ....................................................38
    Figure 1...................................................................................................................................39
    Figure 2...................................................................................................................................42
  The Meikin C-terminus is necessary and sufficient for kinetochore localization ...............................43
    Figure 3...................................................................................................................................44
    Figure 4...................................................................................................................................47
  Plk1 displays phospho-dependent binding to the Meikin C-terminal region .................................48
    Figure 5...................................................................................................................................49
    Figure 6...................................................................................................................................52
    Figure 7...................................................................................................................................53
  Meikin-Plk1 complexes promote Rec8 cleavage when present in close proximity .........................53
  A sensitized assay for Plk1 interactions reveals distinct behaviors for
    full-length and C-Meikin ...........................................................................................................55
    Figure 8...................................................................................................................................57
  Separase cleavage of Meikin is required for chromosome alignment during meiosis II ...............58
    Figure 9...................................................................................................................................59
  C-Meikin is required during meiosis II for proper chromosome alignment ..................................60
    Figure 10..................................................................................................................................62
| Section                                                                 | Page |
|------------------------------------------------------------------------|------|
| Discussion                                                             | 63   |
| Acknowledgements                                                       | 65   |
| Experimental Procedures                                                | 66   |
| References                                                             | 81   |
| Chapter 3: Discussion and Future Directions                            | 84   |
| Summary of Thesis Work                                                 | 85   |
| How Does Meikin Affect Plk1 Activity Throughout Meiosis?              | 85   |
| How Do Meikin-Plk1 Complexes Regulate Meiosis?                         | 86   |
| How is Meikin Localization Regulated Throughout Meiosis?             | 87   |
| Implications for Meikin-like Proteins in Other Organisms              | 89   |
| Implications for Other Separase Substrates                             | 89   |
| Conclusion                                                             | 90   |
| References                                                             | 91   |
Chapter 1:

Introduction
Sexual Reproduction

Sexual reproduction is remarkably prevalent in the living world and its effects on life of all kinds are ubiquitous. However, sexual reproduction is not a given. In fact, entire kingdoms of life eschew sexual reproduction as commonly defined. Why then is sexual reproduction so prevalent?

As defined here, sexual reproduction involves alternating haploid and diploid cell types through cell-cell fusion and meiosis coupled with the shuffling of genetic material. This definition excludes the “parasexual” processes of genetic exchange common among prokaryotes. Evidence of sexual reproduction has been found in all major eukaryotic lineages suggesting that the last eukaryotic common ancestor was sexual (Goodenough and Heitman, 2014). Sexual reproduction is the norm in multicellular metazoan species and even a majority of single-celled eukaryotes utilize sexual reproduction at certain points in their life cycles (Colegrave, 2012).

The ubiquity of sex has long puzzled biologists because the costs of this reproductive strategy are high. Except for self-fertile hermaphrodite species, sexual reproduction requires expending energy finding a mate, energy that could otherwise be used to grow or reproduce directly. In species that compete for mates, significant energy is spent in this competition and sexual traits are selected that may make individuals less fit. For example, male peacock tails and cricket chirping make them easy targets for predators (Colegrave, 2012). In species, such as humans, that produce two morphologically disparate gametes (a condition known as anisogamy), a further cost of sex arises. In these species, up to half of the reproductive resources of any individual female are wasted producing male progeny who cannot themselves reproduce (Barton and Charlesworth, 1998).

At the molecular level, there are also significant costs to sexual reproduction. The production of gametes through meiotic cell division is a slow process, taking considerably longer
in most organisms than mitotic cell division. As described below, meiosis requires complex modifications to the mitotic cell division process. This additional complexity creates more points of failure and the possibility of cell division defects, which can lead to infertility. In species in which not all of the chromosomes from the diploid progenitor are transmitted to the haploid gamete (as is true for female meiosis in animals), there is significant opportunity for “cheating” genetic elements to tip the scales in their favor, which results in the evolution of costly anti-cheating elements to rebalance the scales (Henikoff et al., 2001). Meiosis has also been proposed as a key factor in the development and maintenance of linear chromosomes in eukaryotes as recombination and crossing over between circular chromosomes generates unstable circular dicentrics (Goodenough and Heitman, 2014). Linear chromosomes themselves entail evolutionary costs requiring mechanisms to protect and replicate the DNA ends.

Despite all of these costs, sexual reproduction appears to be evolutionarily favored as it has been maintained across eukaryotes for over 2 billion years (Goodenough and Heitman, 2014). The most prevalent argument is that sex generates offspring that are genetically distinct from either parent. This creates variability in the population upon which natural selection can act and allow a species to adapt to their environment (Barton and Charlesworth, 1998). This appears to be especially true when modelling the types of environments often seen in the real world, where small, fragmented populations face multiple changing selective pressures at the same time (Otto, 2009). Indeed, the preference for sexual reproduction under fluctuating environmental pressures is easily observed in species who switch between sexual and asexual reproduction. These species often reproduce asexually when conditions are ripe for growth, but switch to a sexually reproductive state under stressful overcrowded conditions when the disadvantages of sex are relatively low (Burt, 2000).
Ultimately, the exact circumstances leading to the evolution of sexual reproduction will never be known. However, the prevalence of sex in the natural world and its widespread effects on biology demand a thorough understanding of its mechanisms and complexities.

**Chromosome Segregation and Kinetochore Function**

During both mitotic and meiotic cell division, microtubule polymers organize into a bipolar spindle structure. To be properly segregated, chromosomes must attach to this microtubule spindle. The kinetochore is a macromolecular structure that connects chromosomes to the microtubules. To accurately segregate chromosomes, kinetochores must not only transduce microtubule forces to the chromosomes, they must also serve as signaling and regulatory platforms integrating multiple signals to ensure that chromosome segregation occurs with the correct timing and fidelity. Work from numerous labs has found that kinetochores are complex structures composed of over 100 different proteins, most in multiple copies per kinetochore structure (reviewed in Cheeseman, 2014). Our understanding of how these many components are arranged and organized is growing with recent structural and reconstitution efforts (Pesenti et al., 2016). However, most of our understanding of kinetochore structure and regulation comes from mitotic cells. How the kinetochore is modulated to fit the requirements of alternative cell cycles, such as meiosis, remains obscure.

**Overview of Meiosis**

Meiosis requires major modifications to the cell division cycle. Meiotic prophase is significantly extended compared to mitotic prophase. This prolonged stage provides time for homologous recombination to occur (Zickler and Kleckner, 2015). The activity of the cell cycle kinases that mediate transitions between cell cycle stages must be modulated to allow the cell to undergo two
divisions concurrently without an intervening gap or DNA synthesis phase (Marston and Amon, 2004). In many organisms, germ cells must maintain an arrested meiotic state for extended periods of time until triggered to complete meiosis (Von Stetina and Orr-Weaver, 2011).

Meiosis requires three main modifications to the chromosome segregation machinery (Marston and Amon, 2004). First, physical linkages must be created between homologous chromosomes. This requires a complex process of homolog pairing, synapsis, and recombination (Zickler and Kleckner, 2015). The end-product of this activity is homologous chromosomes that are physically linked into bivalents. Multiple meiosis-specific genes are required to accomplish this process such as those of the synaptonemal complex (Cahoon and Hawley, 2016) and the Spo11 endonuclease (Keeney et al., 1997). This multi-stage mechanism spans meiotic prophase and accounts for much of the increased length of meiosis compared to mitosis.

Second, meiosis requires that sister chromatid cohesion be relieved in a stepwise pattern. Cohesin is a ring-shaped protein complex whose primary role in mitotic cells is maintaining physical linkage of replicated sister chromatids (Gruber et al., 2003). Cohesin also has important functions in regulating transcription, double-strand break repair (Dorsett and Ström, 2012), and assembly of the synaptonemal complex (Cahoon and Hawley, 2016). During meiosis, different populations of cohesin have distinct behaviors and make different contributions. Cohesin on the chromosome arms is critical for linking recombined homologous chromosomes. This population of molecules must be removed during anaphase I to allow for homolog separation. However, cohesin complexes proximal to the centromeres are critical for holding sister chromatids together and must be retained until anaphase II (Marston and Amon, 2004). This two-step pattern of cohesin removal requires precise regulation of protease activity across space and time (see below).
Third, sister kinetochores must attach to spindle microtubules emanating from the same spindle pole during meiosis I, a process called co-orientation (or mono-orientation) (Marston and Amon, 2004). This is in contrast to mitosis where sister kinetochores adopt a bi-oriented state. Importantly, kinetochore co-orientation must be reversed during meiosis II to allow for the proper segregation of sister chromatids during the second meiotic division. Thus, to reduce cell ploidy with high fidelity, complex molecular innovations to the standard mitotic cell division cycle have evolved.

Figure 1: Chromosome segregation patterns and mechanisms in mitosis versus meiosis. Diagram showing loss of cohesion and separation of sister chromatids in mitosis. During meiosis, homologous chromosomes pair and recombine at chiasma in prophase. Sister kinetochores are co-oriented at metaphase I but bi-oriented in metaphase II. Centromeric cohesion is protected at anaphase I but lost in anaphase II.

Diversity of Sexual Reproduction at the Organismal and Molecular Level

Meiosis highlights the incredible diversity in the natural world. Even within the same species, meiosis in males and females occurs in cells of dramatically different size. In most animals, the
regulation of oocyte size is intricately tied to the arrest of the meiotic cell cycle at prescribed points. A prolonged arrest during prophase I of meiosis provides an opportunity for growth and differentiation of the oocyte. The length of this arrest also varies widely across the animal kingdom, lasting from a few days in short-lived species, such as Drosophila, to decades in long-lived species like humans. Additionally, many organisms have a secondary arrest point in oocyte meiosis. In Drosophila, this secondary arrest occurs at metaphase I, but in vertebrates the arrest occurs in metaphase II. Starfish oocytes arrest in G1 immediately after completion of meiosis, and *C. elegans* lacks a secondary arrest. The intrinsic and extrinsic cues that signal the onset, maintenance, and release from these arrests differ widely across species, further highlighting the incredible plasticity of the meiotic cell cycle (reviewed in (Von Stetina and Orr-Weaver, 2011)).

Meiosis represents a particularly complex and unique challenge for species that lack regional centromeres. Such holocentric species build their kinetochore structures across the entire length of their chromosomes. Holocentricity has evolved independently multiple times and has been identified in over 700 species spanning the eukaryotic tree including select clades of insects, arachnids, plants, and worms. This broad and sporadic occurrence of holocentricity suggests that holocentric chromosomes evolved in monocentric species at least 13 independent times (Melters et al., 2012).

Although holocentricity may help organisms tolerate double-stranded breaks to prevent the loss of DNA, a main disadvantage to holocentric kinetochores is their behavior during meiosis. Holocentric chromosomes can attach to multiple microtubules across their length impairing the coordination between sister chromatids to co-orient their kinetochores during meiosis I. Furthermore, cohesin complexes in the pericentromeric region must be preferentially protected during anaphase I to retain sister chromatid cohesion into meiosis II, while cohesin distal to the
kinetochore is removed to allow recombined homologous chromosomes to segregate. However, holocentric chromosomes have no defined centromere site and thus have no basis for discriminating between these cohesin complexes (Maddox et al., 2004).

Holocentric species have evolved a number of mechanisms for solving this problem. A straightforward solution found in some holocentric species is to restrict kinetochore activity during meiosis to one end of the chromosome, therefore creating a functionally monocentric chromosome. In *C. elegans*, meiotic bivalents assume a capsule morphology and kinetochore proteins are restricted to a cup shape around the long arms of each cruciform bivalent. However, the relative contribution of kinetochore proteins compared to other microtubule binding proteins, such as molecular motors and CLASPs, towards the movement of *C. elegans* meiotic chromosomes is a matter of continued debate (Monen et al., 2005). Cohesion along the short arms, distal to the chiasmata, is released to allow anaphase I segregation. A number of holocentric insect species utilize a similar mechanism where kinetochore activity is restricted to chromosome ends during meiosis. Interestingly, there is evidence that the chromosome end that is designated as a kinetochore is random and can switch between meiosis I and meiosis II (Pérez et al., 1997).

A more extreme solution to the problem of meiosis in holocentric organisms is to perform meiosis II sister chromatid separation before meiosis I homologue separation. This “inverted meiosis” has been discovered in both animal and plant holocentric species (Viera et al., 2009). During meiosis I, sister chromatids face opposite poles and kinetochore activity is retained along the full length of the chromosomes. All cohesion between sisters is lost during anaphase I similar to mitosis. This necessitates that homologous chromosomes re-pair prior to the second division. These alternative chromosome segregation strategies further highlight the incredible diversity of
meiotic processes in the natural world. Importantly, many of the molecular mechanisms involved in these alternative strategies remain poorly studied.

**MOKIRS and Meiosis I Activities Across Organisms**

Although a large number of meiosis-specific proteins have been identified that contribute to homolog pairing, synapsis, and recombination, the number of meiosis-specific proteins implicated in sister-kinetochore co-orientation and sister-chromatid cohesion protection is relatively small. Work from model organisms has identified a group of proteins that is critical to these two meiotic processes, referred to collectively as Meiosis One Kinase Regulators (MOKIRs) (Galander and Marston, 2020). MOKIRs include Spo13 (budding yeast), Moa1 (fission yeast), Matrimony (Mtrm, fruit fly), and Meikin (mammals). This group of proteins is remarkably diverse, sharing little primary protein sequence conservation or identifiable domains. However, all of these members are crucial for meiosis I and control the activity of meiotic kinases.

All MOKIRs regulate the step-wise loss of cohesin during meiosis. The loss of Meikin, Spo13, Moa1, or Mtrm leads to premature sister chromatid separation defects (Bonner et al., 2020; Katis et al., 2004b; Kim et al., 2015; Lee et al., 2004; Shonn et al., 2002; Yokobayashi and Watanabe, 2005). In meiotic cells, meiosis-specific cohesion subunits are incorporated into cohesin complexes. Importantly, the meiosis-specific protein Rec8 replaces the Rad21/Scc1 kleisin subunit of cohesin in most organisms (Buonomo et al., 2000; Tachibana-Konwalski et al., 2010). Rec8 cleavage by the protease Separase requires prior phosphorylation, but the kinase responsible for this phosphorylation differs among organisms (Brar et al., 2006; Ishiguro et al., 2010; Katis et al., 2010; Kudo et al., 2009). To create the spatial control of cohesin cleavage, the pericentromeric cohesin population is protected from phosphorylation and thus cleavage by the
activity of Shugoshin/MEI-S332 (Katis et al., 2004a; Kerrebrock et al., 1992; Kitajima et al., 2004; Marston et al., 2004). Shugoshin is targeted to pericentromeres through the phosphorylation of histone 2A by Bub1 (Kawashima et al., 2010; Kiburz et al., 2005; Kitajima et al., 2005; Tang et al., 2004). Shugoshin in turn recruits protein phosphatase 2A (PP2A) to pericentromeres where PP2A dephosphorylates cohesin, inhibiting its cleavage by Separase (Kitajima et al., 2006; Riedel et al., 2006).

**Figure 2: Role of Shugoshin and PP2A in protecting centromeric cohesion.** Cohesin molecules linking sister chromatids are phosphorylated by cohesin kinases. Shugoshin in complex with PP2A localizes to centromere regions and inhibits this phosphorylation.

MOKIRs have been proposed to regulate the activity of meiotic kinases, tipping the balance of kinase and phosphatase activities at the pericentromere to favor cohesin protection. However, how exactly individual MOKIRs achieve this is still unclear. Meikin null mutants have reduced levels of Sgo2 at centromeres suggesting that Meikin’s effects on cohesin protection are mediated by modulating Shugoshin localization (Kim et al., 2015), thus increasing phosphatase activity at
metaphase I pericentromere regions. In budding yeast, fission yeast, and fruit flies, MOKIRs may act instead to inhibit meiotic kinases. Shugoshin localizes correctly in spo13, moa1, and mtrm mutants (Bonner et al., 2020; Galander et al., 2019; Lee et al., 2004; Miyazaki et al., 2017). Spo13 interacts either directly or indirectly with the meiotic cohesin kinases CK1, DDK, and Cdc5 (Galander et al., 2019; Matos et al., 2008) and inhibition of any of these kinases in spo13 null cells restores cohesion protection (Galander et al., 2019); although the contributions of each interaction pair to cohesion protection is unclear. Drosophila MTRM promotes the sequestration of active Polo kinase away from centromeres to the spindle (Bonner et al., 2020), and similar to budding yeast, a reduction of Polo activity rescues cohesion protection in mtrm mutants (Xiang et al., 2007). Importantly, inhibition of these kinases may have a two-fold effect in protecting cohesion. MOKIRs may directly reduce the ability of these kinases to phosphorylate cohesin, and additionally, kinase activity mediates removal of Shugoshin during anaphase I in both budding yeast and Drosophila (Clarke et al., 2005; Galander et al., 2019). Spo13 and Mtrm may thus prevent premature removal of pericentromeric phosphatase activity.

MOKIRs also promote sister kinetochore co-orientation such that the kinetochores of sister chromatids attach to microtubules from the same pole promoting the separation of homologous chromosomes in meiosis I (Galande and Marston, 2020). In many organisms, this process appears to involve the fusion of sister kinetochores into a single microtubule-binding entity (Chiang et al., 2010; Goldstein, 1981; Li and Dawe, 2009; Sarangapani et al., 2014), but how this fusion occurs seems to differ based on the nature of the kinetochore. Budding yeast have point centromeres that assemble upon a single CENP-A nucleosome (Furuyama and Biggins, 2007). Sister kinetochore fusion is established through the incorporation of a meiosis-specific protein complex, Monopolin (Sarangapani et al., 2014). Structural studies have found that the Monopolin complex forms a V-
shaped homodimer and interacts directly with the kinetochore, presumably crosslinking the two sister kinetochores into a single functional unit (Corbett and Harrison, 2012; Corbett et al., 2010; Plowman et al., 2019; Sarkar et al., 2013). Spo13 promotes monopolin recruitment to kinetochores, although the precise mechanism of this recruitment is still unclear (Glander et al., 2019; Katis et al., 2004b; Lee et al., 2004).

In organisms without point centromeres, the mechanism of kinetochore fusion appears to be distinct as no monopolin-like complex has been identified. In many organisms, cohesin seems to facilitate sister kinetochore co-orientation (Chelysheva et al., 2005; Chiang et al., 2010; Severson et al., 2009; Waizenegger et al., 2002). Cohesin complexes at the core centromere may geometrically restrict sister kinetochores to act as a single unit. Experiments in fission yeast suggested the importance of such core centromeric cohesin complexes in promoting co-orientation (Sakuno et al., 2009; Yokobayashi and Watanabe, 2005; Yokobayashi et al., 2003). Recent work in mouse germ cells has also identified a population of Rec8-containing cohesin molecules at the core centromere that appear to be important for co-orientation (Ogushi et al., 2020). If this cohesin-based model is correct, an open question of how MOKIRs promote the establishment or maintenance of these centromeric cohesin complexes remains, but genetic evidence that both fission yeast Moa1 (Yokobayashi and Watanabe, 2005) and mouse Meikin (Kim et al., 2015) contribute to co-orientation is clear.
Figure 3: Mechanisms of meiosis I sister kinetochore co-orientation. During mitosis sister chromatids are linked by cohesin complexes. Kinetochores on sister chromatids are bi-oriented. During meiosis I, centromeric cohesin in fission yeast and vertebrates geometrically constrains sister kinetochores to bi-orient. In budding yeast, the monopolin complex fuses sister kinetochores.

Driving Cell Cycle Transitions with the Kinases Cdk and Plk1

The cell cycle fundamentally represents an ordered series of events with regulated and irreversible transition points. Cells ensure progress through these transitions with a variety of molecular mechanisms. Cell cycle kinases play a central role in coordinating the division cycle. Throughout the cell cycle, the activity of a group of serine/threonine protein kinases named cyclin dependent kinases (Cdk) is particularly important. Crucially, the activity of Cdks are regulated by binding of specific cyclin regulatory subunits. Thus, modulation of cyclin protein levels through transcription, translation, and protein stability is an important mechanism of specifying cell cycle stage (Morgan,
In mammals, distinct Cdks and cyclins are active at different cell-cycle stages. However, there is a significant overlap between the different Cdks and cyclins (Hochegger et al., 2008). The sequential activation of different cyclin-Cdks and the orderly phosphorylation of their numerous targets drives important cell cycle transitions. In particular, the activity of Cdk1 associated with cyclin A and cyclin B is a major regulator of the G2/M transition and the maintenance of the mitotic state requires continued activity of Cdk1-cyclin B (Norbury and Nurse, 1992).

Another key kinase relevant to the work presented here is Polo-like kinase 1 (Plk1), which plays important roles during the mitotic stage of the cell cycle. Plk1 ensures proper spindle assembly, centrosome maturation, kinetochore-microtubule attachment, and cytokinesis (Petronczki et al., 2008). The activity of kinases can be modulated by the cell through the action of specific inhibitor proteins, such as CKIs, subcellular localization, such as the relocalization of Plk1 to the anaphase spindle, or through the phosphorylation status of the kinase itself, including phosphorylation of Cdk by the inhibitory kinase Wee1 or the activator kinase CAK (Morgan, 1995). Thus, given the importance of cell cycle kinases to the initiation, maintenance, and transition of cell cycle stages, organisms utilize a variety of mechanisms to regulate kinase activity.

**Driving Cell Cycle Transitions with Degradation by the E3 Ligase APC/C**

An alternative mechanism for driving irreversible cell cycle transitions is the selective degradation of critical proteins. The main pathway of protein degradation is the ubiquitin-proteasome system in which target proteins are modified by covalent attachment of the ubiquitin polypeptide. These marks serve as a recognition motif for degradation by the proteasome complex. To start this process, ubiquitin molecules are linked to an E1 ubiquitin-activating enzymes. The activated ubiquitin is then transferred to a E2 ubiquitin conjugating enzyme. The E2 enzyme then transfers the ubiquitin to a lysine residue on a target protein. Target specificity is established by
collaboration of the E2 with an E3 ubiquitin ligase, which brings E2 enzymes and target proteins into close proximity and stimulates catalytic activity of the E2. Thus, recognition of target-proteins by E3 ligases serves as the critical regulatory step mediating ubiquitin conjugation. Ubiquitin molecules can be added to a single lysine residue, multiple different lysine residues, or added as chains of ubiquitin molecules in a variety of linkage arrangements. In some cases, ubiquitination can modulate protein function independently of degradation (reviewed in (Teixeira and Reed, 2013)).

The most critical E3 ligase during both mitosis and meiosis is the anaphase-promoting complex/cyclosome (APC/C). The APC/C is a multi-subunit protein complex that recognizes short destructions motifs (degrons) on target substrates. The best characterized APC/C degrons are the D-box and KEN box motifs (Glotzer et al., 1991; Pfleger and Kirschner, 2000), but other target sequences and determinants have been described (Littlepage and Ruderman, 2002). During mitosis, the APC/C initiates chromatid separation by targeting the Separase inhibitor, Securin, for destruction (Ciosk et al., 1998; Cohen-Fix et al., 1996). Mitotic exit is promoted by the APC/C through degradation of cyclins A and B (King et al., 1995; Sudakin et al., 1995). At the beginning of G1, the APC/C is also responsible for degrading mitotic kinases, such as Aurora A (Honda et al., 2000) and Plk1 (Lindon and Pines, 2004), and the continued maintenance of a low Cdk activity state. Other important targets of the APC/C include mitotic spindle proteins, such as TPX2 (Stewart and Fang, 2005) and PRC1 (Jiang et al., 1998).

Given the irreversible nature of ubiquitin-mediated proteolysis, it is not surprising that the activity of E3 ligases is highly regulated by the cell. The APC/C in particular is subject to numerous distinct modes of regulation. To achieve full activity, the core APC/C complex associates with a coactivator, either Cdc20 or Cdh1 (Fang et al., 1998; Schwab et al., 1997; Visintin et al., 1997).
Choice of coactivator is cell cycle controlled and determines substrate specificity. Unattached kinetochores generate the mitotic checkpoint complex (MCC), a diffusible APC/C inhibitor containing the checkpoint proteins Mad2, BubR1, and Bub3, as well as Cdc20 (Sudakin et al., 2001). MCC-bound APC/C cannot bind substrates. Proper attachment of every kinetochore to the mitotic spindle satisfies the spindle assembly checkpoint leading to disassembly of the MCC and productive binding of freed Cdc20 to the APC/C allowing destruction of mitotic targets to initiate anaphase (Musacchio, 2015). In late mitosis, Cdc20 itself is targeted by the APC/C for degradation mediating a switch to the Cdh1 coactivator (Foe et al., 2011; Prinz et al., 1998).

Other mechanisms of APC/C regulation include phosphorylation and binding of inhibitor proteins. Cdk1 phosphorylates a number of APC/C subunits promoting activation of APC/C-Cdc20 (Rudner and Murray, 2000), but APC/C-Cdh1 activity is inhibited by Cdk phosphorylation (Jaspersen et al., 1999; Zachariae et al., 1998), further contributing to the switch in APC/C coactivator during mitotic exit. Phosphorylation of target substrates near degron motifs can also modulate APC/C affinity (Davey and Morgan, 2016). The inhibitor protein Emi1 directly binds the APC/C and prevents substrate binding (Reimann et al., 2001). Specific protein degradation mediated by E3-ubiquitin ligases provides an essential and irreversible mechanism for driving cell cycle transitions.

**Driving Cell Cycle Transitions with the Protease Separase**

Proteases are essential to numerous biological processes. Protein maturation and homeostasis often involves protease activity. For example, many proteins require trimming of N-terminal methionine (Giglione et al., 2004) or signal peptides (Paetzel et al., 2002) for their proper folding and function. Numerous proteins are produced as inactive proproteins that require proteolytic processing to a
biologically active form. This type of processing is especially common for growth factors, peptide hormones, and proteases (Seidah and Prat, 2012).

The use of proteolytic cleavage in signaling pathways is widespread. Proteolysis is an irreversible process that can be used to drive a pathway away from equilibrium. For example, protease cascades regulate coagulation (Chapin and Hajjar, 2015), complement activation (Merle et al., 2015), and apoptotic cell death (Pop and Salvesen, 2009). Membrane-associated and extracellular proteases play important roles in cell-cell signaling by cleaving and releasing ectodomains of signaling factors. For example, ligand-mediated activation of the Notch receptor induces proteolytic cleavage of the receptor by extracellular ADAM proteases. This cleavage releases the Notch intracellular domain to enter the nucleus and promote transcription of downstream targets (Kopan and Ilagan, 2009).

Proteases also play key roles during the cell cycle to drive the cell forward through the distinct stages. Specifically, the cysteine protease Separase initiates anaphase chromosome separation by cleaving the kleisin subunit of the cohesin complex. Cleavage of Scc1/Rad21 opens the cohesin ring allowing the separation of sister chromatids (Hauf et al., 2001; Uhlmann et al., 2000; Waizenegger et al., 2000). Cleaved cohesin molecules are then subsequently degraded by the proteasome (Rao et al., 2001). Separase also cleaves Pericentrin/Kendrin during anaphase (Lee and Rhee, 2012; Matsuo et al., 2012), which, along with Separase-mediated cleavage of centrosome-localized cohesin (Schöckel et al., 2011), is important for centriole disengagement. Although the C-terminal Kendrin fragment is rapidly degraded by the N-end rule, the N-terminal fragment appears to remain associated with centrosomes until mitotic exit. However, the functional role of this retained fragment is unclear (Matsuo et al., 2012). Separase cleavage of the anti-apoptotic proteins Mcl-1 and Bel-XL causes release and activation of the pro-apoptotic protein
Bak and leads to cell death if Separase is activated too early in mitosis. Interestingly, the Mcl-1 C-terminal cleavage product also directly promotes cell death through formation of pores in the mitochondrial outer membrane (Hellmuth and Stemmann, 2020). In budding yeast, Separase cleaves the kinetochore protein Slk19 during anaphase. The C-terminal Slk19 cleavage product relocates at this time to the anaphase spindle where it stabilizes the anaphase spindle. However, cleavage is not required either for Slk19 spindle association or stability (Sullivan et al., 2001).

A number of meiosis specific substrates of Separase have been found. As described above, Rec8 replaces Rad21 in meiosis-specific cohesin complexes and is cleaved by Separase to facilitate meiotic chromosome segregation (Buonomo et al., 2000; Tachibana-Konwalski et al., 2010). In C. elegans, the N-terminal tail of the meiosis-specific CENP-A variant CPAR-1 is cleaved by Separase during meiosis I; however, the functional significance of this cleavage event is not known (Monen et al., 2015). During male meiosis in Drosophila, the protein Uno participates in an alternative homologous chromosome conjunction system which is independent of recombination. Cleavage of Uno by Separase is required for separation of bivalents at meiosis I (Weber et al., 2020).

Because Separase initiates chromosome separation, its activity must be carefully controlled to prevent premature cohesion loss and chromosome mis-segregation. A number of mechanisms for regulating Separase activity have been identified. The first identified mechanism of Separase regulation is binding of its inhibitor Securin (Ciosk et al., 1998; Funabiki et al., 1996; Zou et al., 1999). Securin is produced in excess of Separase and binds to Separase while the protease is still being translated (Hellmuth et al., 2015a). This ensures that essentially every Separase molecule is inhibited. Surprisingly, Securin knockout mice are viable with only minor subfertility phenotypes (Mei et al., 2001; Wang et al., 2001). Similarly, tissue-culture cells depleted of securin undergo
largely normal cell divisions (Pfleghaar et al., 2005). Thus, additional mechanisms of Separase regulation must be present. Shugoshin 2 in complex with the spindle assembly checkpoint protein Mad2 binds and inhibits Separase similarly to Securin in human mitotic cells providing a redundant pathway of Separase inhibition (Hellmuth et al., 2020).

Separase appears to be aggregation prone, limiting its activity unless stabilized (Hellmuth et al., 2015a). Phosphorylation of Securin-free Separase by mitotic kinases promotes proline isomerization by the Pin1 peptidyl-prolyl cis/trans isomerase, which is hypothesized to contribute to this aggregation (Hellmuth et al., 2015b). In vertebrates, isomerization of Separase also promotes its binding to cyclin B/Cdk1 (Hellmuth et al., 2015b), which appears to serve a similar role to Securin in preventing Separase aggregation and inhibiting Separase proteolytic activity (Hellmuth et al., 2015a). Thus, Cdk may serve as a Separase inhibitor in cell types where Securin plays less of a role (Huang et al., 2009; Huang et al., 2008). Interestingly, the association of Cdk with Separase also inhibits Cdk activity, and this inhibitory effect on a small pool of Separase-bound Cdk appears to be important for proper anaphase events (Gorr et al., 2005).

Regulated localization may also serve to regulate Separase activity. Separase is excluded from the nucleus prior to mitotic or meiotic entry, limiting access of the protease to interphase chromosomes (Holland and Taylor, 2006; Sun et al., 2006). After nuclear envelope breakdown, Aurora B kinase activity promotes the localization of Separase to chromatin (Yuan et al., 2009). DNA binding may also promote Separase catalytic activity (Sun et al., 2009).

Separase activation in vertebrates leads to autocleavage of the protease yielding two major fragments that remain associated (Stemmann et al., 2001; Waizenegger et al., 2002; Zou et al., 2002). Autocleavage is not required for proteolytic activity in vitro (Waizenegger et al., 2002; Zou et al., 2002), but is required for proper mitotic entry and spindle assembly (Papi et al., 2005). How
Separase autocleavage regulates the cell cycle is unclear, but a role for autocleavage in preventing binding of the PP2A phosphatase to Separase may be a potential mechanism (Holland et al., 2007). Autocleavage is also known to play similar regulatory roles in the activation of other cysteine proteases, such as caspases (Pop and Salvesen, 2009). Thus, given the importance of Separase to driving the metaphase-anaphase cell cycle transition, Separase activation is a tightly regulated molecular event.

**Figure 4: Different mechanisms of driving cell cycle transitions.** Molecules responsible for distinct cell cycle transitions are depicted. Inhibitors of each molecule are marked in red, promoters of each are marked in blue. Cyclin dependent kinase phosphorylates mitotic substrates. The APC/C promotes ubiquitination and degradation of substrates. Separase cleaves target proteins.

**Open Questions Motivating Thesis Work**

As discussed above, to generate haploid gametes, meiotic cells must undergo two consecutive rounds of chromosome segregation without an intervening gap phase. Importantly, because homologous chromosomes are segregated in meiosis I, but sister chromatids are segregated in meiosis II, this requires a dramatic rewiring of the cell division machinery between the two
divisions. How meiotic cells coordinate this rapid and substantial change to the cell division machinery is a central mystery at the heart of proper fertility and reproduction. In fact, very little is known about the fundamental transition between meiosis I and II despite decades of research. Given the identification of Meikin as a meiosis-specific kinetochore factor that controls key aspects of meiosis I chromosome segregation in vertebrates (Kim et al., 2015), a thorough understanding of Meikin’s interactions, activities, and regulation is needed. My work reveals a new paradigm that rewires key cell division processes at the meiosis I/II transition through the action of the protease Separase, which I demonstrate acts by cleaving the meiosis-specific kinetochore protein Meikin. I find that Separase cleavage of Meikin acts as a molecular “scalpel,” providing an elegant mechanism to precisely and irreversibly modulate Meikin activity between the two meiotic divisions without inactivating Meikin function.
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Chapter 2:

Separase cleaves the kinetochore protein Meikin to direct the meiosis I/II transition
Separase cleaves the kinetochore protein Meikin to direct the meiosis I/II transition

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Summary

To generate haploid gametes, germ cells undergo two consecutive meiotic divisions requiring key changes to the cell division machinery. Here, we explore the regulatory mechanisms that differentially control meiotic events. We demonstrate that the protease Separase rewires key cell division processes at the meiosis I/II transition by cleaving the meiosis-specific protein Meikin. In contrast to cohesin, which is inactivated by Separase proteolysis, cleaved Meikin remains functional, but results in a distinct activity state. Full-length Meikin and the C-terminal Meikin Separase-cleavage product both localize to kinetochores, bind to Plk1 kinase, and promote Rec8 cleavage, but our results reveal distinct roles for these proteins in controlling meiosis. Mutations that prevent Meikin cleavage or that conditionally inactivate Meikin at anaphase I both result in defective meiosis II chromosome alignment. Thus, Separase cleavage of Meikin creates an irreversible molecular switch to rewire the cell division machinery at the meiosis I/II transition.
Introduction

The generation of haploid germ cells from diploid precursors requires a specialized cell division, termed meiosis, in which two successive rounds of chromosome segregation occur without undergoing intervening DNA replication (Miller et al., 2013). In most organisms, the first round of chromosome segregation during meiosis I reduces the chromosome number by half, requiring three major modifications relative to the mitotic cell division program. First, homologous chromosomes pair and become physically linked through synapsis and recombination. Second, instead of binding to opposing spindle poles, the kinetochores of sister chromatids co-orient to connect to microtubules from the same spindle pole. Third, cohesin complexes adjacent to centromeres are retained through to the second meiotic division even as cohesin along the chromosome arms is removed, allowing sister chromatids to remain associated and co-segregate to the same pole. Each of these modifications to meiosis I requires the activity of meiosis-specific proteins whose expression is restricted to germ cells. For example, the vertebrate factor Meikin (meiosis-specific kinetochore protein) plays important roles in meiosis I sister kinetochore co-orientation and the protection of centromeric cohesion (Kim et al., 2015a), with Meikin-null mice exhibiting meiotic defects and sterility. Current functional evidence suggests that Meikin plays an analogous role to that of Spo13 and Moa1 in yeast meiosis (Galandier et al., 2019b; Kim et al., 2015a; Miyazaki et al., 2017).

As germ cells transition from meiosis I to meiosis II, meiosis I-specific activities must be reversed to allow for sister chromatid segregation during this second equational meiotic division. However, the limited time between the two meiotic stages in many organisms (Kishimoto, 2003) restricts the ability of transcriptional or translational control to broadly rewire the cell division apparatus to distinguish these events. How meiotic cells coordinate this rapid and substantial
change to the cell cycle machinery is a critical unanswered question for our understanding of meiosis. Here, we sought to define the mechanisms that enable the switch between meiosis I and II by analyzing the regulatory control of Meikin activity during the different stages of meiosis. Our results demonstrate that Separase cleavage of Meikin acts to rewire cell division activities and create distinct behaviors for each meiotic stage.
Results

Meikin is proteolytically cleaved by Separase during anaphase I

To define how germ cells accomplish two distinct cell divisions in rapid succession, it is critical to determine how the activities of meiosis-specific factors, such as Meikin, are precisely controlled. Although Meikin expression is normally restricted to the germline, we found that ectopically-expressed mNeonGreen-tagged Meikin localized to kinetochores during both interphase and mitosis in mitotically-dividing human HeLa cells (Fig. 1A). The ability of Meikin to localize to mitotic kinetochores suggests that its kinetochore binding partners are at least partially retained between meiosis and mitosis, providing an experimentally-tractable system to analyze Meikin behavior. Consistent with the delocalization of Meikin that occurs during anaphase I of meiosis (Kim et al., 2015a), mNeonGreen-Meikin localization was lost from mitotic kinetochores during anaphase (Fig. 1B). The loss of Meikin localization at anaphase onset could reflect changes to its phosphorylation, anaphase-specific degradation, proteolytic-processing, or structural changes to Meikin or its kinetochore binding partners. Interestingly, as cells progressed into anaphase, Western blotting revealed the formation of a faster migrating form of Meikin, suggestive of proteolytic cleavage (Fig. 1C).
Figure 1: Meikin is cleaved by Separase during anaphase. A. Deconvolved immunofluorescence images of HeLa cells stably expressing 2xmNeonGreen-hMeikin. Kinetochores are stained with ACA. Images are not scaled equivalently. Insets, 5 µm. B. Montage of deconvolved time-lapse images of HeLa cells expressing 2xmNeonGreen-hMeikin entering anaphase. Numbers indicate minutes relative to anaphase onset. C. Western blot showing Meikin mobility during mitotic exit. EGFP-hMeikin expression was induced in HeLa cells by doxycycline treatment. Cells were arrested in mitosis with nocodazole then forced to exit from mitosis by
treatment with Mps1i and analyzed at the indicated timepoints. Lower mobility EGFP-hMeikin bands are due to mitosis-specific phosphorylation (see Fig. 5). A high-mobility fragment of EGFP-hMeikin, indicated with the arrow, appears upon mitotic exit. Cyclin B1 degradation indicates mitotic exit. Tubulin was blotted as a loading control. D. Schematic of dual-color H2B-cleavage sensors and montage of time-lapse images of HeLa cells expressing the indicated sensor. A Meikin fragment (amino acids 1-332) excluding the kinetochore localization sequence (see Fig. 3) was used. Proteolytic cleavage of Meikin leads to release and diffusion of C-terminal mNeonGreen, but retention of the N-terminal H2B-mScarlet on the DNA. Numbers indicate minutes relative to anaphase onset. E. Quantification of Meikin H2B-cleavage sensor. The DNA mass was segmented and the ratio of mNeonGreen:mScarlet within the DNA mass was quantified and normalized to the -15 min timepoint. Error bars indicate 95% confidence interval. 15 cells were analyzed per condition. F. Sequence alignment of the Separase cleavage site in Meikin in various vertebrates. Fully conserved amino acids are indicated in blue. Separase is predicted to cleave after the arginine residue in the ExxR motif. G. Mouse oocytes injected with the indicated 3xEGFP-mMeikin mRNA were observed in meiosis I and meiosis II for Meikin localization to kinetochores. Images taken at the same timepoint are scaled equivalently. Scale bars, 10 μm. See also Figure 2.

To determine if Meikin is indeed proteolytically cleaved during anaphase, we adapted a previously described protease cleavage sensor (Shindo et al., 2012) in which we targeted Meikin to chromatin through an N-terminal fusion to histone H2B, with fluorescent proteins placed at the Meikin N- and C-termini (Fig. 1D). In this system, Meikin cleavage would result in the delocalization of the mNeonGreen tag, but the chromosomal retention of the H2B-mScarlet tag. Time-lapse imaging revealed that the H2B-mScarlet tagged portion of Meikin remained localized to chromatin as cells exited mitosis. In contrast, the mNeonGreen tagged Meikin fragment progressively delocalized from chromatin during anaphase (Fig. 1D-E). Together, these data indicate that Meikin is proteolytically processed during anaphase when expressed in mitotically-dividing cells.

We next sought to define the protease responsible for Meikin cleavage. Separase is a cysteine protease that cleaves cohesin to initiate sister chromatid separation (Hauf et al., 2001; Uhlmann et al., 1999; Uhlmann et al., 2000). Separase is specifically activated at the metaphase-anaphase transition, and the behavior of the Meikin cleavage sensor mirrors that of a sensor for
the established Separase substrate, Rad21/Scc1 (Shindo et al., 2012) (Fig. 2A-B). Indeed, we found that depletion of Separase by RNAi inhibited Meikin proteolysis (Fig. 2C). The consensus cleavage motif for Separase cleavage is ExxR (Hauf et al., 2001; Uhlmann et al., 1999), and charge-swap mutations (RxxE) of this motif in the cohesin subunit Rad21 eliminate its proteolytic processing (Hauf et al., 2001; Shindo et al., 2012) (Fig. 2A-B). Human Meikin contains three ExxR consensus sequences, but only one of these sequences (amino acids 151-154) is conserved across vertebrates (Fig. 1F). We found that charge-swap mutations in this conserved Meikin Separase motif (ELFR to RLFE) eliminated Meikin proteolysis based on analysis of its electrophoretic mobility (Fig. 1C) and our H2B-targeted cleavage sensor (Fig. 1D-E). For some previously established substrates, Separase recognizes an acidic or phosphorylated residue at the P6 position relative to the cleavage site to enhance proteolysis (Alexandru et al., 2001; Hauf et al., 2001). Meikin contains a conserved serine in the P6 position (amino acid 149, Fig. 1F) matching a proline-directed cyclin-dependent kinase (Cdk) phosphorylation motif. Mutation of this residue to alanine reduced Meikin cleavage (Fig. 2D). Together, these data indicate that Meikin is cleaved at anaphase onset by Separase when expressed in mitotic cells.

To determine whether Separase also cleaves Meikin during the meiosis I/II transition in germ cells, we tested the localization of N-terminally tagged murine Meikin in mouse oocytes using mRNA injection. 3xEGFP-Meikin localized to kinetochores in meiosis I, but was lost from kinetochores in meiosis II (Fig. 1G). In contrast, Separase-resistant 3xEGFP-Meikin (E156R, R159E) localized to kinetochores during both meiosis I and meiosis II (Fig. 1G). This suggests that cleavage of Meikin by Separase occurs during anaphase I of meiosis. Together, these data demonstrate that Meikin is processed by the Separase protease at anaphase onset in meiotically dividing cells.
Figure 2: Dual color H2B-cleavage sensors monitor Separase activity at anaphase. A. Schematic of dual-color hRad21 H2B-cleavage sensor. A fragment of hRad21 (amino acids 142-476) including the predicted Separase cleavage sites was used. Proteolytic cleavage of Rad21 leads to the release and diffusion of C-terminal mNeonGreen, but retention of the N-terminal H2B-mScarlet on the DNA. Montage of time lapse images of cells expressing the indicated sensor. Numbers indicate minutes relative to anaphase onset. B. Quantification of the mNeonGreen:mScarlet ratio of Rad21 H2B cleavage sensors. The increase in mNeonGreen signal after +10 min in the wild-type sensor coincides with DNA decondensation and likely reformation of the nuclear envelope. Thus, we hypothesize that this increase is due to nuclear import of the cleaved C-terminal fragment. C. HeLa cells were treated with the indicated siRNA for 24 hr then
induced to express EGFP-hMeikin by treatment with doxycycline and arrested in mitosis by treatment with nocodazole. Cells were forced to exit from mitosis by treatment with Mps1i. At the indicated timepoint, cells were collected, lysed, and analyzed by Western blotting. Percent cleavage was calculated by densitometry analysis of the GFP blot. D. Quantification of the mNeonGreen:mScarlet ratio of Meikin mutant H2B cleavage sensors as in Fig. 1E. The number of cells analyzed per condition were: Rad21 (19 cells), Rad21(separase-resistant) (23 cells), Meikin(S149A) (15 cells), Meikin(8A mutant) (23 cells). Data for wild type (15 cells) and Separase-resistant (15 cells) Meikin sensors are duplicated from Fig. 1. Error bars indicate 95% confidence intervals. Scale bars, 10 µm.

The Meikin C-terminus is necessary and sufficient for kinetochore localization

Meikin plays a key role in the meiosis I-specific processes of kinetochore co-orientation and sister chromatid cohesion protection (Kim et al., 2015a), critical activities that must be reversed to enable the events associated with meiosis II. Our discovery that Meikin is specifically targeted by Separase during anaphase I provides an attractive model for how Meikin activity is restricted to meiosis I. Thus, we hypothesized that Separase cleavage acts to fully inactivate Meikin, similar to the effect of Separase proteolysis to inactivate cohesin complexes and promote sister chromatid separation (Uhlmann et al., 1999; Uhlmann et al., 2000). To test this hypothesis, we analyzed the consequences of Separase cleavage to Meikin’s known molecular functions. Separase-mediated Meikin cleavage is predicted to produce two protein fragments - N-Meikin (amino acids 1-154) and C-Meikin (amino acids 155-373). Based on the delocalization of the mNeonGreen fluorescence in our cleavage sensor (Fig. 1D), these fragments do not remain associated after proteolysis, but whether these fragments retain any activity is unclear.
Figure 3: The Meikin C-terminus is necessary and sufficient for kinetochore localization and CENP-C binding. A. Deconvolved immunofluorescence images of HeLa cells stably expressing...
the indicated 2xNeonGreen-hMeikin constructs. Kinetochores are stained with ACA. Images are not scaled equivalently. B. Immunofluorescence images of germinal vesicle intact mouse oocytes injected with the indicated mNeonGreen-mMeikin mRNA. Kinetochores are stained with mouse CENP-A antibody. Images are not scaled equivalently. C. Recombinant sfGFP-Meikin and GST-CENP-C protein fragments were bound and complexes analyzed by size exclusion chromatography. Fractions corresponding to elution volumes 1.0 to 2.0 mL were analyzed by SDS-PAGE and Coomassie staining. The Meikin C-terminus containing the isoleucine motifs is sufficient for binding to CENP-C as indicated by co-elution of the two proteins. A contaminating protein in the Meikin prep is indicated with an asterisk. D. Recombinant sfGFP-Meikin containing alanine mutations in the isoleucine motifs and GST-CENP-C fragments were analyzed by gel filtration as in Fig. 3C. E. Immunofluorescence images of mouse oocytes injected with mMeikin-3xEGFP mRNA and fixed at the indicated stage. Kinetochores are stained with mouse CENP-C antibody. Images are scaled equivalently. F. Immunofluorescence images of mouse oocytes injected with mMeikin-3xEGFP mRNA and fixed at the indicated stage. Kinetochores are stained with mouse CENP-C antibody. Images are scaled equivalently. Scale bars, 10 µm. See also Figure 4.

To understand how this processing event alters Meikin function at the meiosis I/II transition, we first sought to define the consequences of Meikin cleavage to its kinetochore localization. By analyzing a series of truncation mutants, we identified a Meikin C-terminal domain (amino acids 328-373) that is both necessary and sufficient for kinetochore localization when expressed ectopically in HeLa cells (Fig. 3A). Similarly, we found that the equivalent C-terminal domain of murine Meikin (amino acids 387-434) was sufficient to localize to meiosis I kinetochores in mouse oocytes (Fig. 3B). Previous yeast two-hybrid assays (Kim et al., 2015a) and our affinity purifications of Meikin from mitotic cells (Fig. 4A) both identified interactions with centromere protein C (CENP-C), which is present constitutively at both meiotic and mitotic centromeres (Earnshaw et al., 1989; Kitajima et al., 2011; Tanaka et al., 2009). Meikin also co-localizes with CENP-C to the inner kinetochore (Fig. 4B). Using recombinant proteins, we found that the Meikin C-terminal region was sufficient to bind to CENP-C directly in vitro (Fig. 3C). Mutation of two C-terminal isoleucine-rich motifs (I333CCII and I367DIII) previously implicated in Meikin localization in spermatocytes (Kim et al., 2015a) abolished Meikin’s kinetochore
localization in both HeLa cells and mouse oocytes (Fig. 3A-B) and its interaction with CENP-C in vitro (Fig. 3D). In reciprocal experiments, we identified a minimal C-terminal fragment of CENP-C (amino acids 808-943) that is sufficient for Meikin binding (Fig. 4C). As we found that increased salt concentrations promoted Meikin-CENP-C binding (Fig. 4D), we tested whether their interface requires hydrophobic interactions. We identified two hydrophobic patches within CENP-C (835IILM or 865PFF) that are required for Meikin binding (Fig. 4E-F), possibly by partnering with the Meikin isoleucine-rich motifs. This binding site for Meikin on CENP-C does not overlap with established CENP-C-interaction partners (Klare et al., 2015), suggesting that CENP-C interacts with Meikin without disrupting its other kinetochore interfaces.

As the Meikin C-terminus is sufficient for kinetochore localization and its interaction partner CENP-C is present throughout meiosis, this suggests that the C-terminal Meikin fragment generated by Separase cleavage would retain the ability to target to kinetochores. Indeed, although N-terminally tagged Meikin in oocytes is lost from kinetochores in meiosis II (Fig. 1G), we found that C-terminally tagged Meikin localizes to kinetochores during both meiosis I and meiosis II (Fig. 3E), and is only removed from kinetochores upon completion of meiosis during anaphase II (Fig. 3F). Thus, while the N-terminal fragment of Meikin is lost from kinetochores following Separase cleavage (Fig. 1G), the Meikin C-terminal cleavage fragment (C-Meikin) remains associated with kinetochores in meiosis II through its interaction with CENP-C, creating the possibility that this fragment could retain some activities at meiosis II kinetochores.
Figure 4: Meikin binds to CENP-C through hydrophobic patches on both proteins. A. GFP-immunoprecipitates from HeLa cells expressing EGFP-hMeikin were analyzed by mass-spectrometry. The percent sequence coverage and number of total peptides of the indicated proteins is shown. Data is the sum of multiple mass-spectrometry experiments. B. Metaphase HeLa cells stably expressing 2xmNeonGreen-hMeikin were fixed in ice cold methanol for 10 min then stained for CENP-C or the Ndc80 complex. Linescan analysis of the boxed sister-kinetochore pairs.
is shown. Meikin localizes to the inner kinetochore overlapping with CENP-C. Scale bar is 1 µm. Deconvolved immunofluorescence images are shown. Linescan analysis was performed before deconvolution. C. Diagram of known kinetochore protein interaction sites within CENP-C. Recombinant sfGFP-tagged Meikin and GST-tagged CENP-C protein fragments were bound and complexes analyzed by gel filtration. Fractions corresponding to elution volumes of 1.0 to 2.0 mL were analyzed by SDS-PAGE and Coomassie staining. D. Recombinant sfGFP-tagged Meikin and GST-tagged CENP-C protein fragments were bound and complexes analyzed by gel filtration. Binding reactions and gel filtration were performed in buffer with the indicated KCl concentration. E. Sequence alignment of CENP-C from selected vertebrates with conserved hydrophobic patches indicated. Amino acid hydrophobicity is indicated in blue. F. Recombinant Meikin and CENP-C fragments containing mutations in conserved hydrophobic patches analyzed by gel filtration as above.

**Plk1 displays phospho-dependent binding to the Meikin C-terminal region**

Although Meikin kinetochore localization is retained following Separase cleavage, it is possible that other Meikin interaction partners are affected. Prior work suggested that Meikin promotes meiosis I sister kinetochore co-orientation and the protection of centromeric cohesin through its direct interaction with Polo-like kinase 1 (Plk1) (Kim et al., 2015a), an essential cell cycle kinase that controls multiple aspects of mitosis and meiosis (Petronczki et al., 2008). We found that immunoprecipitation of ectopically-expressed Meikin from HeLa cells arrested in mitosis isolated both CENP-C and Plk1 (Fig. 4A). Plk1-Meikin complexes co-purified from HeLa cells display similar kinase activity on an artificial substrate to Plk1 isolated on its own (Fig. 5A). In addition, ectopic Meikin expression in HeLa cells results in increased Plk1 localization to mitotic kinetochores and increased phosphorylation of Plk1-dependent kinetochore substrates (Fig. 5B-C). The combination of these data suggests that Meikin acts as a targeting subunit for Plk1 to recruit Plk1 to meiotic kinetochores, where it can then phosphorylate downstream targets.
Figure 5: Meikin binds active Plk1. A. Michaelis-Menten parameters derived from kinase assays of Plk1 or Plk1-Meikin complexes immunoprecipitated from HeLa cells. Standard error is
represented in brackets. B. Deconvolved immunofluorescence images of HeLa cells (control) or HeLa cells stably expressing hMeikin-mNeonGreen and stained for Plk1 (pre-extracted in PBS + 0.5% Triton-X100 for 5 min; pre-extraction disrupts the bipolar spindle and centrosome staining of Plk1; kinetochores are co-stained with CENP-C antibody) or phosphorylated S311 on CENP-C (fixed in ice-cold methanol at -20°C for 20 min; centrosome pCENP-C staining is non-specific; kinetochores are co-stained with CENP-A antibody). Images of similarly stained cells are scaled identically. Scale bars, 10 μm. C. Quantification of kinetochore intensity of mitotic HeLa cells stably expressing hMeikin-mNeonGreen. Values were normalized to the mean of the control. Means and 95% confidence intervals are presented. Specificity of the pS311 CENP-C antibody was demonstrated by treatment of cells with Plk1i for 2 hr prior to staining. ****P < 0.0001, two-tailed t-test. n = Plk1: control (27 cells, 2,911 kinetochores), hMeikin (22 cells, 2,626 kinetochores); control vs hMeikin (t = 29.24, df = 5535). n = pCENP-C: control (21 cells, 1,093 kinetochores), hMeikin (27 cells, 1,185 kinetochores), control + Plk1i (25 cells, 2,302 kinetochores); control vs hMeikin (t = 23.55, df = 2276); control vs control + Plk1i (t = 12.50, df = 3393). Quantification was performed before deconvolution. D. HeLa cells stably expressing 2xmNeonGreen-hMeikin were treated with the indicated inhibitors. Whole cell lysates were incubated with or without lambda-phosphatase and analyzed by Western blot. E. GFP-immunoprecipitates from mitotic HeLa cells expressing EGFP-hMeikin were analyzed by mass-spectrometry. Phosphorylation sites identified across multiple experiments are indicated on the Meikin protein sequence.

To determine whether Meikin cleavage affects its binding to Plk1, we first sought to define the basis for the Meikin-Plk1 interaction. Plk1 typically binds to its substrates and targeting factors via its phosho-peptide binding domain (Polo-box domain; PBD). We found that Meikin is extensively phosphorylated when expressed in HeLa cells (Fig. 1C; Fig. 5D) on both Cdk ([pT/pS]-P) and Plk1 ([D/E/N]-x-[pT/pS]) consensus phosphorylation sites (Fig. 5E). Chemical inhibition of Plk1 kinase activity eliminates the Meikin-Plk1 interaction (Fig. 7A). Human Meikin contains three S-[pT/pS]-P (STP) motifs (T251, T264, T276), which provide potential docking sites for the Plk1 PBD (Elia et al., 2003), and which have been implicated previously in Plk1 binding (Kim et al., 2015a). Mutation of all three STP motifs (STP to SAP) reduced, but did not eliminate the Meikin-Plk1 interaction (Fig. 6A). However, a Meikin mutant that eliminates the STP motifs together with multiple consensus sites for Plk1 (S175, T176, T180, S181, S196), which enhance kinase docking interactions in other substrates (Lee et al., 2008), abrogated Plk1 binding
(8A mutant; Fig. 6A). Notably, this Meikin-8A mutant is not cleaved efficiently by Sepa
rase (Fig. 2D), consistent with other Sepa
rase substrates that require phosphor-
ylation for their proteolysis (Alexandru et al., 2001; Hauf et al., 2005; Hornig and Uhlmann, 2004; Kim et al., 2015b; Kudo et
al., 2009). We conclude that Meikin binding to Plk1 requires its phosphorylation at multiple sites.

To determine whether Meikin cleavage affects its binding to Plk1, we next defined the
minimal region required for the Meikin-Plk1 interaction. The N-terminal Meikin fragment
generated by Sepa
rase cleavage (N-Meikin; amino acids 1-154) did not bind to Plk1 (Fig. 6B). In contrast, the C-terminal cleavage fragment (C-Meikin; amino acids 155-373) was still capable of
interacting with Plk1 (Fig. 6C). As an alternative strategy to detect Meikin-Plk1 interactions, we
targeted Meikin to chromatin using an H2B fusion. Expression of H2B fusions with either full
length Meikin or the C-Meikin Sepa
rase fragment recruited endogenous Plk1 to chromatin (Fig. 7B). In contrast, an N-Meikin-H2B fusion failed to recruit Plk1 (Fig. 7B). Together, these data suggest that Sepa
rase cleavage does not prevent the binding of C-Meikin to Plk1.
Figure 6: Full length Meikin and the C-Meikin cleavage fragment bind Plk1 and promote Rec8 cleavage similarly. A. HeLa cells expressing the indicated EGFP-hMeikin C-terminal truncation constructs under the control of a doxycycline inducible promoter were induced and arrested in mitosis by STLC treatment. Cells were lysed and GFP-immunoprecipitation (IP) was performed and analyzed by Western blotting. 175-181 4A mutant includes S175A, T176A, T180A, S181A mutations. STP mutant includes T251A, T264A, T276A mutations. 8A mutant includes S175A, T176A, T180A, S181A, S196A, T251A, T264A, T276A mutations. B. Western blot
analysis of GFP immunoprecipitates from cells expressing EGFP-hMeikin C-terminal truncation mutants as in Fig. 6A. C. Western blot analysis of GFP immunoprecipitates from cells expressing EGFP-hMeikin N-terminal truncation mutants as in Fig. 6A. D. Schematic of dual-color Rec8 H2B-cleavage sensors and montage of time-lapse images of HeLa cells expressing the indicated sensor. The sensor contains a fragment of hRec8 (amino acids 297-506) including the predicted Separse cleavage sites and a fragment of hMeikin with Separse-resistant mutations (amino acids 1-332, E151R, R154E). Numbers indicate minutes relative to anaphase onset. Scale bars, 10 µm. E. Quantification of Rec8 H2B-cleavage sensor analyzed and represented as in Figure 1. The number of cells analyzed per condition were: Rec8(separase-null)-Meikin (26 cells), Rec8 (30 cells), Rec8-Meikin(8A mutant) (29 cells), Rec8-Meikin (22 cells), Rec8-C-Meikin (22 cells). See also Figure 5 and Figure 7.

Figure 7: Full length Meikin and the C-Meikin cleavage fragment bind Plk1. A. HeLa cells expressing EGFP-hMeikin were induced with doxycycline and arrested in mitosis by STLC or Plk1i treatment. Cells were lysed and GFP-immunoprecipitation (IP) was performed and analyzed by Western blot. B. Deconvolved immunofluorescence images of HeLa cells induced to express EGFP-hMeikin-H2B by treatment with doxycycline. A Meikin fragment excluding the C-terminal kinetochore binding domain was used. Cells were stained for Plk1. Images are not scaled equivalently. Scale bars, 10 µm.

**Meikin-Plk1 complexes promote Rec8 cleavage when present in close proximity**

We next sought to assess whether Meikin cleavage affects its downstream activities. Current models suggest that Meikin-Plk1 complexes regulate key features of both kinetochore co-
orientation and the protection of pericentric cohesin (Kim et al., 2015a). To define the substrates responsible for these activities, we analyzed the meiosis-specific kleisin subunit of cohesion Rec8 (Buonomo et al., 2000; Tachibana-Konwalski et al., 2010). Centromere-localized Rec8 has been proposed to play a critical role in promoting kinetochore co-orientation in many organisms (Chelysheva et al., 2005; Sakuno et al., 2009; Severson et al., 2009; Watanabe and Nurse, 1999). In this model, the centromeric Rec8 population would need to be eliminated at the meiosis I/II transition to allow for proper chromosome alignment at metaphase II. In contrast, the pericentric population of Rec8-containing cohesin must be protected from Separase cleavage to ensure that sister chromatids remain associated until anaphase II. Indeed, recent work suggests that the centromeric, pericentric, and chromosome arm populations of Rec8 are differentially regulated at the meiosis I/II transition in mammalian oocytes (Ogushi et al., 2020).

To monitor Rec8 cleavage, we adapted the H2B-based cleavage sensor in HeLa cells. In contrast to the Rad21 cleavage sensor (Fig. 2B), a Rec8 fragment containing its established Separase cleavage sites did not show any proteolysis upon anaphase onset in mitotic cells (Fig. 6D-E). To test whether Meikin-Plk1 complexes could promote Rec8 cleavage when present in close proximity, we created an in-frame Rec8-Meikin fusion. Strikingly, fusion of Meikin to the Rec8 fragment resulted in efficient Rec8 cleavage (Fig. 6D-E). Charge-swap mutation of the conserved Separase cleavage site in Rec8 (E401R, R404E) (Kudo et al., 2009) eliminated this proteolysis (Fig. 6E). Rec8 cleavage was also dependent on the interaction between Meikin and Plk1, as a Meikin mutant that does not bind Plk1 (8A mutant; Fig. 6A) does not potentiate Rec8 cleavage (Fig. 6E). This suggests that kinetochore-targeted Meikin-Plk1 complexes could phosphorylate Rec8 to promote the cleavage of adjacent centromere-proximal Rec8-cohesin at anaphase I. Consistent with this, prior work found that Plk1 phosphorylation of mammalian Rec8
enhances Separase-mediated cleavage in vitro (Kudo et al., 2009). These data suggest that Meikin-Plk1 complexes potentially activate proximal Rec8 for cleavage by Separase, providing a mechanism to reverse kinetochore co-orientation following anaphase I.

To determine whether Separase-cleavage inhibits Meikin’s ability to promote Rec8 cleavage, we next created an in-frame fusion between the C-Meikin cleavage product and Rec8 in our H2B sensor. Similarly to full length Meikin, C-Meikin induced efficient cleavage of the Rec8 sensor (Fig. 6E). This suggests that retention of C-Meikin at kinetochores would further promote the elimination of centromeric Rec8 during late anaphase, ensuring complete reversal of kinetochore co-orientation before meiosis II chromosome alignment. Importantly, these data indicate that full length Meikin and the C-Meikin Separase cleavage product display similar activities with respect to their kinetochore localization, Plk1 binding, and ability to promote Rec8 cleavage. Thus, in contrast to other substrates, Separase cleavage does not fully inactivate Meikin function.

A sensitized assay for Plk1 interactions reveals distinct behaviors for full-length and C-Meikin

Given the similar activities observed for full-length Meikin and C-Meikin in the assays described above, we sought to generate a sensitized assay for Meikin activity. Stable expression of Meikin at low levels in mitotically dividing cells results in a modest increase in misaligned chromosomes (Fig. 8A). In contrast, increased Meikin expression (under the control of a doxycycline-inducible promoter), caused a potent mitotic arrest with misaligned chromosomes and monopolar spindle structures (Fig. 8A-B), phenotypes consistent with an effect on Plk1 activity. The ability of Meikin overexpression to disrupt cell division provides a sensitized assay to monitor the behaviors of
Meikin mutants. For example, although individual Meikin phosphorylation site mutants were not sufficient to disrupt Meikin-Plk1 binding (Fig. 6A), these mutants did not promote a mitotic arrest (Fig. 8C). This suggests that a direct interaction between Meikin and Plk1 is required, but not sufficient for the mitotic arrest phenotype. Utilizing this assay, we defined a minimal domain (amino acids 124-332) spanning the N- and C-Meikin Separase cleavage fragments that is sufficient to induce a mitotic arrest (Fig. 8C). Importantly, neither the N- nor C-terminal Separase cleavage fragment of Meikin was sufficient to generate a mitotic arrest (Fig. 8C), despite an interaction between C-Meikin and Plk1 (Fig. 6C). Based on these data, we conclude that full-length Meikin and C-Meikin both localize to kinetochores and bind to Plk1, but display different efficiency or properties for their Plk1 interactions. Thus, Separase cleavage has the potential to differentially control Meikin-Plk1 interactions at the meiosis I/II transition.
Figure 8: Meikin expression causes chromosome misalignment and monopolar spindles. A. Quantification of mitotic defects observed in HeLa cells stably expressing Meikin or acutely induced to express Meikin from a doxycycline-inducible promoter. 100 mitotic cells were analyzed for each condition. B. Representative immunofluorescence images of mitotic HeLa cells stained
for DNA and microtubules. High Meikin levels were induced in HeLa cells using a doxycycline inducible promoter. Scale bar, 10 µm. C. Schematic of the human Meikin protein with conserved residues (as measured by Consurf (Ashkenazy et al., 2016)) indicated in black. The indicated EGFP-Meikin constructs were expressed in HeLa cells by doxycycline induction. Cells were fixed and stained for the mitotic marker phosphorylated histone 3. The mitotic index of GFP-positive cells was measured by flow cytometry. The minimal region sufficient for Plk1 binding and for the mitotic arrest phenotype are indicated. D. Mouse oocytes injected with the indicated 3xEGFP-mMeikin mRNA were matured to meiosis II and stained for Plk1 or Bub1. Kinetochoreps were stained with mouse CENP-C antibody. Images of similarly stained cells are scaled identically. Insets, 5 µm. Scale bars, 10 µm. E. Quantification of kinetochore intensity of Plk1 and Bub1 in meiosis II oocytes injected with the indicated mMeikin mRNA. Values were normalized to the mean of wild-type. Means and 95% confidence intervals are presented. ****P < 0.0001, two-tailed t-test. n = Plk1 (pool of 2 independent experiments, t = 16.53, df = 890): wild-type (362 kinetochores), Separase-resistant (530 kinetochores), Bub1 (pool of 3 independent experiments, t = 29.55, df = 1344): wild-type (449 kinetochores), Separase-resistant (897 kinetochores).

Separase cleavage of Meikin is required for chromosome alignment during meiosis II

Based on the results described above, we sought to directly test whether Separase-mediated Meikin proteolysis is required for the proper execution of meiosis. To determine the functional consequences of Meikin cleavage, we ectopically expressed the Separase-resistant Meikin mutant in mouse oocytes, causing full length Meikin to persist at kinetochores into meiosis II (Fig. 1G; Fig. 9A). Despite the presence of endogenous Meikin, the induced retention of Meikin at kinetochores during meiosis II in this Separase cleavage mutant caused severe chromosome alignment defects during the second meiotic division (Fig. 9B-C). Separase-resistant Meikin expression also resulted in increased kinetochore levels of Plk1 and Bub1 at meiosis II kinetochores (Fig. 8D-E). As Plk1 and Bub1 regulate kinetochore-microtubule interactions (Petronczki et al., 2008; Watanabe, 2012), improper kinetochore localization of Plk1 and Bub1 in meiosis II oocytes expressing Separase-resistant Meikin likely contributes to the observed chromosome alignment defects (Fig. 9B-C). Thus, Meikin cleavage during anaphase I is critical to enable a proper meiosis II division.
Figure 9: Separase cleavage of Meikin is required for proper meiosis II chromosome alignment. A. Schematic of murine Meikin constructs injected and their expected behaviors at meiosis II. Separase-resistant Meikin contains a charge swap mutation in the Separase cleavage site (E156R, R159E). B. Representative immunofluorescence images of chromosome misalignment defects observed in meiosis II oocytes. C. Quantification of meiosis II chromosome misalignment defects observed in oocytes injected with the indicated 3xEGFP-mMeikin construct. D. Schematic of murine Meikin constructs injected. E. Representative immunofluorescence images of mouse oocytes injected with the indicated mNeonGreen-mMeikin mRNA and matured to meiosis II. Insets show separated chromatids. F. Quantification of premature sister chromatid separation defects observed in meiosis II oocytes expressing the indicated Meikin mRNA. n
represents the number of meiosis II oocytes analyzed. Kinetochores are stained with Hecl antibody. Scale bars, 10 µm. Insets, 5 µm. See also Figure 8.

**C-Meikin is required during meiosis II for proper chromosome alignment**

Our results indicate that the failure to cleave Meikin results in a defective meiosis II, but that cleaved C-Meikin retains at least partial activity based on its kinetochore localization, Plk1 binding, and ability to promote Rec8 cleavage. Thus, instead of abolishing Meikin function, our work suggests that Separase processing modulates Meikin activity at anaphase I to promote additional functions during meiosis II. To test this, we sought to disrupt Meikin function by creating dominant-negative Meikin alleles. For these experiments, we first expressed Meikin mutants that retain its CENP-C interaction (allowing them to displace endogenous Meikin at kinetochores), but are defective for Plk1 binding (Fig. 9D). Oocytes ectopically expressing wild-type Meikin using mRNA microinjection progressed to metaphase II similarly to mock-injected oocytes. In contrast, expression of the minimal C-terminal murine Meikin kinetochore targeting domain (amino acids 387-434; corresponding to amino acids 328-373 in human Meikin), which lacks Plk1 binding (Fig. 6C), caused premature-sister chromatid separation during meiosis II (Fig. 9E-F). Similarly, expression of full length Meikin with a mutation in the STP motif also caused premature-sister chromatid separation during meiosis II (Fig. 9E-F). These phenotypes suggest that the Meikin-Plk1 interaction acts during meiosis I to protect the pericentric cohesion that holds sister chromatids together until anaphase II.

To test Meikin function during meiosis II, we next developed a conditional strategy to fully eliminate Meikin activity at anaphase I onset, allowing us to circumvent the consequences of the dominant negative Meikin mutants that disrupt meiotic behaviors in meiosis I (Fig. 9D-F). To do this, we altered the position of Separase proteolysis by inserting the Rad21 Separase cleavage site
between the Meikin Plk1 binding and kinetochore targeting domains defined by our analysis (Fig. 3A-B, Fig. 6B-C), forming a Meikin allele with two potential cleavage sites (Fig. 10A). Introducing inactivating mutations at either the endogenous Meikin cleavage site or the inserted cleavage site enabled us to control the position of Separase proteolysis to generate either a product equivalent to C-Meikin or a product completely lacking Plk1 binding activity. Importantly, both alleles retain full Meikin activity during meiosis I, allowing us to determine the functional consequences of specifically inactivating Meikin during meiosis II. Expression of the control Meikin construct which is cleaved at the endogenous Meikin proteolysis site (amino acid 160) resulted in normal meiosis II oocytes (Fig. 10B-C). In contrast, targeting Separase to the introduced downstream site (amino acid 387) to eliminate Meikin-Plk1 interactions during meiosis II resulted in chromosome alignment defects in meiosis II oocytes (Fig. 10B-C). These data indicate that C-Meikin is not simply an inactive form of Meikin, but is required for proper chromosome alignment after anaphase I. Thus, instead of Meikin acting as a meiosis I-specific factor as proposed by prior work (Kim et al., 2015a), Meikin remains active throughout meiosis with Separase cleavage at anaphase I generating a distinct form of Meikin with different activity. Full length Meikin acts to enable meiosis I events, such as kinetochore co-orientation and the protection of pericentric cohesin (Kim et al., 2015a), with the C-Meikin cleavage product retaining modified activities that are critical to enable meiosis II events (Fig. 10D).
Figure 10: The C-Meikin cleavage fragment is required for meiosis II chromosome alignment. A. Schematic of murine Meikin constructs injected and their expected behaviors at meiosis II. A fragment of hRad21 (amino acids 142-275) was inserted between the Plk1 binding and kinetochore localization domains of Meikin. Charge-swap mutations in the Separse-cleavage sites of Meikin (E156R, R159E) or Rad21 (E169R, R172E) were used to direct the location of Separate cleavage during anaphase I. B. Representative immunofluorescence images of chromosome misalignment defects observed in meiosis II oocytes. Kinetochores are stained with mouse CENP-C antibody. Scale bars, 10 µm. C. Quantification of meiosis II chromosome misalignment defects observed in oocytes injected with the indicated 3xEGFP-mMeikin construct. n represents the number of meiosis II oocytes analyzed. D. Model for differential Meikin activity at distinct stages of meiosis.
Discussion

Our data reveal the intricate regulatory control by which the two meiotic divisions are coordinated, providing a mechanism to rewire the cell division machinery between meiosis I and II. We find that the protease Separase acts as a molecular “scalpel” to precisely and irreversibly modulate Meikin activity between the two meiotic divisions. Instead of proteolytic cleavage completely inactivating the protein, full-length Meikin and the C-Meikin Separase cleavage product both localize to kinetochores and bind Plk1, but differ in their functional activities. Importantly, the activity of C-Meikin during meiosis II is required for proper chromosome alignment and cannot be replaced with full-length Meikin. Both the failure to cleave Meikin (Fig. 9A-C) or the complete inactivation of Meikin at anaphase I (Fig. 10A-C) result in defective chromosome alignment during meiosis II. Thus, Separase cleavage of Meikin is critical for mammalian meiosis. Notably, Meikin counterparts in other organisms have distinct mechanisms of regulation that restrict their activities to meiosis I. For example, the budding yeast meiosis I factor Spo13 is degraded at anaphase I by the APC/C (Sullivan and Morgan, 2007), which results in its elimination.

Our data support a model in which kinetochore-localized Meikin establishes a gradient of Plk1 activity during meiosis I centered on the kinetochore, which leads to different fates for cohesin complexes based on their location within the gradient. Meikin is targeted to kinetochores through its binding to CENP-C (Fig. 3A-D), allowing Meikin to recruit Plk1 (Fig. 5C-D). Centromere-localized Meikin-Plk1 complexes differentially regulate the centromeric and pericentromeric populations of Rec8 cohesin during meiosis, with opposing effects on these two pools. By increasing the concentration of centromere-localized Plk1, Meikin-Plk1 complexes direct phosphorylation of centromere-bound Rec8 to promote its efficient cleavage during anaphase I (Fig. 6D-E), thereby reversing meiosis I kinetochore co-orientation. In parallel, Meikin-
Plk1 activity recruits Bub1 (Fig. 8D-E), which in turn recruits Sgo2-PP2A complexes to the pericentromere to reverse Rec8 phosphorylation and protect pericentromeric Rec8 from Separase cleavage (Fig. 9E-F) (Galander et al., 2019a; Galander et al., 2019b; Marston, 2015; Miyazaki et al., 2017). Thus, during anaphase I, Separase cleaves centromere-proximal and phosphorylation-primed Rec8, but not pericentromeric Rec8, eliminating kinetochore co-orientation while maintaining sister chromatid cohesion. At anaphase I, Separase also cleaves Meikin (Fig. 1G), resulting in a C-terminal Meikin fragment that remains associated with kinetochores (Fig. 3E). However, while retaining key activities, this cleaved version is less potent in its ability to bind to or regulate Plk1 (Fig. 8B-C), leading to reduced Plk1 at kinetochores in meiosis II compared to meiosis I (Fig. 8D-E) (Kim et al., 2015a). This reduced Plk1 activity leads to a corresponding reduction in pericentromeric Bub1 and Sgo2, allowing for Rec8 phosphorylation and cleavage at anaphase II. This model is consistent with recent findings that Separase activity at meiosis I is required for deprotection of peri-centromeric cohesin (Ogushi et al., 2020). Finally, as germ cells exit meiosis, Meikin localization is completely eliminated (Fig. 3F), returning kinetochore regulation to a mitotic state.

In conclusion, Meikin is a novel Separase substrate that is cleaved specifically during anaphase of meiosis I. Despite the identification of Separase more than two decades ago and extensive efforts to identify additional targets, the number of confirmed Separase substrates remains low. Our data show that Meikin cleavage by Separase in anaphase I does not inactivate the protein, but rather modulates its activity to allow Meikin to transition from meiosis I to meiosis II specific functions. This provides an elegant mechanism to rapidly reverse the meiosis I-specific modifications to the cell cycle machinery and coordinate the sequential meiotic divisions.
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All requests for data and materials should be addressed to Iain Cheeseman (icheese@wi.mit.edu). All plasmids and cell lines generated in this study are available upon request from the lead contact. The datasets and code used in this study and not included in the text are available upon request from the lead contact.

Author contributions were as follows: Conceptualization – NKM, IMC, MAL; Methodology – NKM, JM; Validation – NKM, JM; Investigation – NKM, JM; Writing - Original Draft Preparation – NKM, IMC; Writing – Review & Editing – NKM, IMC, MAL; Visualization – NKM, JM; Supervision: IMC, MAL; Funding Acquisition: IMC, NKM, MAL

The authors declare that they have no competing interests.
**Experimental Procedures**

**Cell Culture**

HeLa cells (transformed human female cervical epithelium) were cultured in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and streptomycin, and 2 mM L-glutamine at 37°C with 5% CO₂. Doxycycline inducible cell lines were cultured in medium containing FBS certified as tetracycline free and were induced by addition of doxycycline to 1 µg/mL for 16 hr. Other drugs used on human cells were kinesin Eg5 inhibitor (S-trityl-L-cysteine, STLC, 10 µM), nocodazole (0.3 µM), AZ-3146 (MPS1i, 3 µM), BI-2536 (Plk1i, 10 µM), RO-3306 (Cdk1i, 10 µM). Hela cells were regularly monitored for mycoplasma contamination using commercial detection kits.

**Mouse oocyte collection and culture**

8-14 week-old female mice used in this study were purchased from Envigo (Strain: NSA(CF-1)). All animal experiments were approved by the University of Pennsylvania Institutional Animal Care and Use Committee and were consistent with the National Institutes of Health guidelines. Female mice were hormonally primed with 5U of Pregnant Mare Serum Gonadotropin (PMSG, Calbiochem, cat# 367222) 44-48 hr prior to oocyte collection. Germinal vesicle intact oocytes were collected in M2 medium (Sigma, M7167), denuded from cumulus cells, and cultured in CZB medium (Sigma, MR-019-D) covered with mineral oil (Sigma, M5310) in a humidified atmosphere of 5% CO₂ in air at 37°C. During collection, meiotic resumption was inhibited by addition of 2.5 µM milrinone (Sigma, M4659).
Cell line generation

The cell lines used in this study are described in Table 2. pBABE derivatives were transfected with Effectene (Qiagen) according to the manufacturer’s protocol along with VSVG packaging plasmid into 293-GP cells for generation of retrovirus as described (Morgenstern and Land, 1990). Supernatant-containing retrovirus was sterile filtered, supplemented with 20 µg/mL polybrene (Millipore) and used to transduce HeLa cells. Doxycycline-inducible cell lines were generated by homology-directed insertion into the AAVS1 “safe-harbor” locus. Donor plasmid containing selection marker, the tetracycline-responsive promoter, the transgene, and reverse tetracycline-controlled transactivator flanked by AAVS1 homology arms (Qian et al., 2014) was transfected using Effectene with a pX330-based plasmid (Cong et al., 2013) expressing both spCas9 and a guide RNA specific for the AAVS1 locus (pNM220, gRNA sequence – 5’-GGGGCCACTAGGGACAGGAT). Two days post-transfection or transduction, cells were selected with the appropriate antibiotic (puromycin at 0.5 µg/mL or blasticidin at 2 µg/mL, Life Technologies). Where indicated, clonal lines were obtained by fluorescence activated cell-sorting single cells into 96 well plates. Fluorescence enriched lines were generated by bulk sorting a polyclonal population for fluorescence positive cells.

Immunofluorescence and microscopy of mitotic cells

Cells for immunofluorescence were seeded on poly-L-lysine (Sigma-Aldrich) coated coverslips and fixed in PBS plus 4% formaldehyde for 10 min unless otherwise noted in figure legends. Coverslips were washed with PBS plus 0.1% Triton X-100 and blocked in Abdil (20 mM Tris-HCl, 150 mM NaCl, 0.1% Triton X-100, 3% bovine serum albumin, 0.1% NaN₃, pH 7.5). Primary antibodies used in this study are described in Table 1 and were diluted in Abdil. Cy3- and Cy5-
conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) were diluted 1:300 in PBS plus 0.1% Triton X-100. DNA was stained with 1 µg/mL Hoechst-33342 (Sigma-Aldrich) in PBS plus 0.1% Triton X-100 for 10 min. Coverslips were mounted using PPDM (0.5% p-phenylenediamine, 20 mM Tris-HCl, pH 8.8, 90% glycerol). Images were acquired on a DeltaVision Core deconvolution microscope (Applied Precision) equipped with a CoolSnap HQ2 charge-coupled device camera and deconvolved where appropriate. All images are maximal projections in z unless otherwise indicated. Image analysis was performed in Fiji (ImageJ, NIH) (Schindelin et al., 2012). Integrated fluorescence intensity of mitotic kinetochores was measured with a custom CellProfiler pipeline (McQuin et al., 2018). The median intensity of a 5-pixel wide region surrounding each kinetochore was used to background subtract each measurement.

Live-cell imaging of mitotic cells

For live-cell imaging, cells were seeded into 8-well glass-bottomed chambers (Ibidi) and moved into CO₂-independent media (Life Technologies) before imaging at 37°C. For certain movies, DNA was stained with SiR-DNA (Cytoskeleton Inc) at 0.2 µM. Images were acquired on a DeltaVision Core deconvolution microscope (Applied Precision) equipped with a CoolSnap HQ2 charge-coupled device camera and deconvolved where appropriate. Image analysis was performed in Fiji (ImageJ, NIH). For quantification of H2B cleavage sensors, cells undergoing anaphase were selected in Fiji then analyzed using a custom CellProfiler pipeline (McQuin et al., 2018). The pipeline segmented cells based on SiR-DNA signal, performed background subtraction, and measured mNeonGreen and mScarlet signal in each cell. The mNeonGreen/mScarlet ratio was normalized to the first timepoint (t = -15 min).
RNAi treatment

siRNAs against ESPL1 (5’-GCUUGUGAUGCCAUCCUGAUU) (Waizenegger et al., 2002) and non-targeting control pool (D-001810-10) were obtained from Dharmacon. 5 µL of 20 µM stock siRNA was mixed with 5 µL of Lipofectamine RNAiMax (Life Technologies) and diluted in 500 µL of OptiMem (Life Technologies). The reaction was applied to cells in a 6-well dish. Transfection media was changed after 24 hr.

Mitotic index determination

Meikin expressing cells were induced with 1 µg/mL doxycycline for 24 hr. Cells were collected by incubation for 10 min in PBS + 5 mM EDTA, washed once in PBS, then fixed in PBS + 2% formaldehyde for 10 min at room temperature. Cells were blocked in Abdil for 30 min followed by immunostaining for phosphorylated S10 on histone 3 (antibody details in Table 1) followed by Cy-5 conjugated secondary antibody. The proportion of GFP-positive single cells also staining positive for H3pS10 was determined on an LSRFortessa (BD Biosciences) flow cytometer and analyzed with FACSDiva software (BD Biosciences). Over 5,000 GFP-positive cells were analyzed per condition.

GFP immunoprecipitation and Mass-spectrometry

IP-MS experiments were performed as described previously (Cheeseman and Desai, 2005). Harvested cells were washed in PBS and resuspended 1:1 in 1X Lysis Buffer (50 mM HEPES, 1 mM EGTA, 1 mM MgCl₂, 100 mM KCl, 10% glycerol, pH 7.4) then drop frozen in liquid nitrogen. Cells were thawed after addition of an equal volume of 1.5X lysis buffer supplemented with 0.075% Nonidet P-40, 1X Complete EDTA-free protease inhibitor cocktail (Roche), 1 mM
phenylmethylsulfonyl fluoride, 20 mM beta-glycerophosphate, 1 mM sodium fluoride, and 0.4 mM sodium orthovanadate. Cells were lysed by sonication and cleared by centrifugation. The supernatant was mixed with Protein A beads coupled to rabbit anti-GFP antibodies (Cheeseman lab) and rotated at 4°C for 1 hr. Beads were washed five times in Wash Buffer (50 mM HEPES, 1 mM EGTA, 1 mM MgCl₂, 300 mM KCl, 10% glycerol, 0.05% NP-40, 1 mM dithiothreitol, 10 µg/mL leupeptin/pepstatin/chymostatin, pH 7.4). After a final wash in Wash Buffer without detergent, bound protein was eluted with 100 mM glycine pH 2.6. Eluted proteins were precipitated by addition of 1/5th volume trichloroacetic acid at 4°C overnight. Precipitated proteins were reduced with TCEP, alkylated with iodoacetamide, and digested with mass-spectrometry grade Lys-C and trypsin (Promega). Digested peptides were cleaned up using C18 spin columns (Pierce) according to the manufacturer’s instructions. Samples were analyzed on an LTQ XL Ion Trap mass spectrometer (Thermo Fisher) coupled with a reverse phase gradient over C18 resin. Data were analyzed using SEQUEST.

GFP immunoprecipitation and Western blot

For IP-Western experiments, cells were harvested, washed once in PBS, then lysed on ice for 15 min in Lysis Buffer (50 mM HEPES, 1 mM EGTA, 1 mM MgCl₂, 100 mM KCl, 10% glycerol, 1% Triton X-100, 0.05% NP-40, 1 mM dithiothreitol, 1X Complete EDTA-free protease inhibitor cocktail (Roche), 1 mM phenylmethylsulfonyl fluoride, 20 mM β-glycerophosphate, 1 mM sodium fluoride, and 0.4 mM sodium orthovanadate, pH 7.4). Cellular debris was removed by centrifugation. Protein concentrations in each sample were measured using Bradford reagent (Bio-Rad), and sample concentrations were normalized before addition of Protein A beads (Bio-Rad) coupled to affinity-purified rabbit anti-GFP polyclonal antibodies (Cheeseman lab). After 1 hr
incubation at 4°C, beads were washed 3X with Wash Buffer (50 mM HEPES, 1 mM EGTA, 1 mM MgCl₂, 100 mM KCl, 10% glycerol, 0.05% NP-40, 1 mM dithiothreitol, 10 µg/mL leupeptin/pepstatin/chymostatin, pH 7.4). Beads were then incubated in an equal volume of Laemmli buffer for 5 min at 95°C to remove bound protein. Samples were analyzed by SDS-PAGE and Western blotting. Samples were separated by SDS-PAGE and semidry transferred to nitrocellulose. Membranes were blocked for 1h in Blocking Buffer (5% milk in TBS + 0.1% Tween-20). Primary antibodies were diluted in Blocking Buffer + 0.2% NaN₃ and applied to the membrane for 1 hr. HRP-conjugated secondary antibodies (GE Healthcare) were diluted 1:10,000 in TBS + 0.1% Tween-20 and applied to the membrane for 1 hr. After washing in TBS + 0.1% Tween-20, Clarity enhanced chemiluminescence substrate (Bio-Rad) was added to the membrane according to the manufacturer’s instructions. Membranes were imaged with a KwikQuant Imager (Kindle Biosciences). Membranes were stripped (55°C, 1 hr) in stripping buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 100 mM β-mercaptoethanol) and re-blocked before re-probing.

Anaphase synchronization and cell lysis

For synchronization in anaphase, cells were arrested by treatment with STLC for 16 hr followed by addition of Mps1i for the times indicated in the figure legends. Cells were harvested and washed once in PBS. Cell pellets were resuspended in radioimmunoprecipitation buffer (RIPA, ThermoFisher) supplemented with 1X Complete EDTA-free protease inhibitor cocktail (Roche), 1 mM phenylmethylsulfonyl fluoride, 20 mM β-glycerophosphate, 1 mM sodium fluoride, and 0.4 mM sodium orthovanadate. Cells were lysed on ice for 15 min, and cellular debris was removed by centrifugation at >10,000 g for 10 min at 4°C. Protein concentrations were measured and
normalized using a bicinchoninic protein assay (Pierce). Samples were analyzed by SDS-PAGE and Western blot.

**Phosphatase treatment of lysates**

Cells treated with the drugs indicated in the Fig. legends were collected, washed once with PBS then lysed in Lysis Buffer without phosphatase inhibitors (50 mM HEPES, 1 mM EGTA, 1 mM MgCl₂, 100 mM KCl, 10% glycerol, 0.05% NP-40, 1 mM dithiothreitol, 1X Complete EDTA-free protease inhibitor cocktail (Roche), 1 mM phenylmethylsulfonyl fluoride, pH 7.4) for 10 min on ice. Cellular debris was removed by centrifugation and protein concentrations were measured by Bradford assay (Bio-Rad) and normalized. Cell lysates were supplemented with 1X Protein MetalloPhosphatase buffer (New England Biolabs) and 1 mM MnCl₂. 1 µL of Lambda Protein Phosphatase (New England Biolabs) or 1 µL of phosphatase inhibitor mix (20 mM β-glycerophosphate, 1 mM sodium fluoride, and 0.4 mM sodium orthovanadate) was added to each 50 µL reaction. After incubation at 30°C for 30 min, reactions were stopped by addition of 2X Laemmli buffer. Samples were analyzed by SDS-PAGE and Western blot.

**In vitro Plk1 activity assays**

Plk1 and Plk1/Meikin complexes were immunoprecipitated from HeLa cells. Cells were induced to express EGFP-Plk1 or EGFP-Meikin by treatment with doxycycline and arrested in mitosis by STLC for 16 hr. Cells were harvested, lysed and GFP-immunoprecipitated as above for mass-spectrometry. Bound proteins were eluted by addition of recombinant Tobacco Etch Virus (TEV) protease (Cheeseman lab) and incubation at 4°C for 16 hr which leads to cleavage between EGFP and the tagged-protein. The amount of Plk1 in each elution was normalized by Western blot.
Enzyme kinetic parameters were measured in a continuous read fluorescence assay (PhosphoSens CSKS-AQT0691K, Assay Quant) according to manufacturer directions in a SpectraMax iD3 plate reader (Molecular Devices) in 96-well format at 30°C with reads every 60 sec. Substrate concentrations were varied from 1-35 µM. Initial reaction rates were fit to the Michaelis-Menten equation in GraphPad Prism.

Recombinant protein expression and purification

Plasmids used for recombinant protein expression were based on the pGEX-6P1 backbone and are described in Table 3. BL21(DE3) LOBSTR cells (Andersen et al., 2013) carrying the pRARE tRNA plasmid were transformed with the appropriate plasmid and plated on Luria-Bertani (LB)-agar plates containing the appropriate antibiotic. Overnight liquid cultures of LB supplemented with antibiotics and 4% glucose were grown overnight at 37°C from single colonies. The saturated overnight culture was diluted 1:100 and grown to an OD600nm of 0.6-0.7 at 30°C. Cells were shifted to 16°C and induced with 0.3 mM isopropyl β-D-1-thiogalactopyranoside and incubated for 16 hr. Cells were collected by centrifugation, resuspended in lysis buffer, and flash frozen in liquid nitrogen. Cell pellets were resuspended in Lysis buffer (1X PBS supplemented with 250 mM NaCl, 0.1% Tween-20, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride). Cells were disrupted by sonication, and the lysate was cleared by centrifugation. The lysate was applied to 0.5 mL of glutathione agarose (Sigma-Aldrich) per liter of culture for 1 hr at 4°C. Agarose was washed three times in Lysis Buffer, and proteins were eluted using Elution Buffer (50 mM Tris-HCl, 75 mM KCl, 10 mM reduced glutathione, pH 8.0). For certain proteins, the glutathione S-transferase (GST) purification tag was removed by incubation overnight at 4°C with 1 mg HRV-3C protease (Cheeseman lab) per 50 mL elution fraction. Final polishing was performed by gel-
filtration on a Superdex 200 16/60 coupled to an AktaPurifier system (GE Healthcare) into Binding Buffer (50 mM HEPES, 400 mM KCl, 10% glycerol, 0.1% Tween-20, 1 mM EDTA, 1 mM dithiothreitol, pH 7.4). Peak fractions were concentrated in Vivaspin concentrators (GE Healthcare), aliquoted, and snap-frozen in liquid nitrogen.

In vitro binding assays and gel filtration

50 µL binding reactions were prepared with recombinant proteins diluted to 3.5 µM each in Binding Buffer (50 mM HEPES, 400 mM KCl, 10% glycerol, 0.1% Tween-20, 1 mM EDTA, 1 mM dithiothreitol, pH 7.4). Reactions were incubated on ice for 1 hr before clearing by centrifugation at >10,000 g for 10 min. Cleared samples were run over a Superdex 200 3.2/300 column on an Akta Micro FPLC system (GE Healthcare) pre-equilibrated in Binding Buffer. 40 µL fractions were analyzed by SDS-PAGE followed by staining with Acquastain (Bulldog Bio). For examination of the binding interaction under variable salt concentrations, the KCl concentration in the Binding Buffer was altered according to Fig. legends.

Phospho-antibody generation

The CENP-C pS311 phosphospecific antibody was generated against a synthesized phosphopeptide with the following amino acid sequence: CNLRNEE(pS)VLLFTQ (New England Peptide; Covance). Peptide was coupled to SulfoLink Coupling Resin (Thermo Fisher Scientific). Serum from immunized rabbit was depleted against the unphosphorylated peptide and affinity purified against the phosphorylated peptide.
Mouse oocyte injection

Germinal vesicle intact oocytes were microinjected with ~5 pL of cRNAs in M2 medium containing milrinone at room temperature with a micromanipulator TransferMan NK 2 (Eppendorf) and picoinjector (Medical Systems Corp.). Injected oocytes were kept in CZB with milrinone for 6-12 hr to allow protein expression, before switching to milrinone-free CZB medium to develop to meiosis I (6.5 hr in vitro maturation) or meiosis II (16 hr in vitro maturation). Oocytes were checked for germinal vesicle breakdown 1.5 hr after milrinone washout, and those that did not breakdown the germinal vesicle were discarded. Meiosis II eggs were activated to progress to anaphase II in CZB medium containing 5mM SrCl$_2$ and 2 mM EGTA for 1 hr as previously described (Kishigami and Wakayama, 2007). Plasmids for cRNA generation were generated in the pCS2+ backbone (von Dassow et al., 2009) and are described in Table 4. cRNAs were synthesized using the T7 mScript Standard mRNA Production System (Cell Script) and injected at ~125 ng/µL final concentration.

Oocyte immunocytochemistry and imaging

For immunocytochemistry, samples were fixed in 2% paraformaldehyde in PBS for 30 min at room temperature. The cells were then permeabilized for 15 min in PBS containing 0.2% Triton X-100, blocked in PBS containing 0.2% immunoglobulin G-free bovine serum albumin and 0.01% Tween-20 for 30 min (blocking solution) and then incubated with the primary antibody for 1 hr at room temperature. Antibody details are described in Table 1. After four 15 min washings in blocking solution, samples were incubated for 1 hr with either Alexa Flour 594-conjugated (1:500, Invitrogen) or Cy5-conjugated (1:100, Jackson ImmunoResearch) secondary antibody diluted in blocking solution. After an additional three 15-min washings in blocking solution, the samples
were mounted in Vectashield mounting solution containing DAPI (Vector Laboratories). Confocal images were collected with a microscope (DMI4000 B; Leica) equipped with a 63× 1.3 NA glycerol-immersion objective lens, an x-y piezo Z stage (Applied Scientific Instrumentation), a spinning disk confocal scanner (Yokogawa Corporation of America), an electron multiplier charge-coupled device camera (ImageEM C9100-13; Hamamatsu Photonics), and an LMM5 laser merge module with 488- and 593-nm diode lasers (Spectral Applied Research) controlled by MetaMorph software (Molecular Devices). Confocal images were collected as z-stacks at 1 µm intervals to visualize the entire meiotic spindle. To quantify Plk1 and Bub1 signal intensity at kinetochores, CENP-C staining was used to select kinetochores. For each kinetochore, a maximum intensity projection of the optical Z-sections containing CENP-C signal was performed using Fiji/Image J. A circle was drawn around the CENP-C signal, and the same circle was used to quantify Plk1 or Bub1 signal intensity. The mean signal intensity was measured for each circle after subtracting background signal from the surrounding area.

Quantification and statistical analysis

Fiji/ImageJ (NIH) was used for image manipulation and kinetochore quantification. Where indicated in the method details, a custom CellProfiler pipeline was used. Statistical tests and analysis of enzyme kinetics were performed in Graphpad Prism. All statistical details including type of test and exact value of n is included in the figure legends.

| Antibody target | Source | Raised in | Dilution |
|-----------------|--------|-----------|----------|
| α-Tubulin       | Sigma Aldrich #T6199 RRID: AB 477583, lot 078M5796V Mouse clone DM1A | 1:3000 (IF) |
| α-Tubulin       | Abcam #ab52866, RRID: AB 869989, lot GR3241238-2 Rabbit clone EP1322Y | 1:500 (IF) |
| **α-Tubulin (HRP-conjugated)** | Abcam #ab40742, RRID:AB_880625, lot GR3229214-1 | Mouse clone DM1A | 1:5000 (WB) |
|---------------------------------|-----------------------------------------------|----------------|-------------|
| GFP                             | Roche #11814460001, RRID: AB_390913, lot 14442000 | Mouse clones 13.1 and 7.1 | 1:1000 (WB) |
| mNeonGreen                      | Chromotek #32F6, RRID: AB_2827566, lot 71108021 | Mouse clone 32F6 | 1:1000 (WB) |
| Anti-centromere (ACA)           | Antibodies Inc #15234, RRID: AB_2687472, lot 1CK37 | Human polyclonal | 1:100 (IF) |
| CENP-A                          | Abcam #ab13939, RRID: AB_300766, lot GR3265183-3 | Mouse clone 3-19 | 1:1000 (IF) |
| Mouse CENP-A                    | Cell Signaling Tech #2048S, RRID: AB_1147629 | Rabbit clone C51A7 | 1:200 (Oocyte IF) |
| CENP-C                          | Cheeseman lab(Gascoigne et al., 2011), #pBB280 | Rabbit polyclonal | 1 µg/mL (IF) |
| Mouse CENP-C                    | Cheeseman lab(Swartz et al., 2019), #pKG137 | Mouse clone 35-206 | 1:1000 (Oocyte IF) |
| pS311 CENP-C                    | Cheeseman lab, #85B, this study | Rabbit polyclonal | 1 µg/mL (IF) |
| Plk1                            | Santa Cruz Biotech #sc17783, RRID: AB_628157, lot D1219 | Mouse clone F-8 | 1:200 (IF, WB) |
| Plk1                            | Sigma Aldrich #05-844, RRID: AB_310836 | Mouse clone 35-206 | 1:200 (Oocyte IF) |
| Ndc80 “Bonsai” complex          | Cheeseman lab(Schmidt et al., 2012) | Rabbit polyclonal | 1 µg/mL (IF) |
| Hec1                            | Santa Cruz Biotech #sc515550 | Mouse clone C-11 | 1:100 (Oocyte IF) |
| Mouse Bub1                      | Gift of Yoshinori Watanabe (UTokyo)(Kawashima et al., 2010) | Mouse polyclonal | 1:100 (Oocyte IF) |
| Separase                        | Abcam #ab16170, RRID: AB_2101815, lot GR44153-1 | Mouse clone XJ11-1B12 | 1:500 (WB) |
| Cyclin B1                       | Cell Signaling Tech #4138, RRID: AB_2072132, lot 3 | Rabbit polyclonal | 1:1000 (WB) |
| Histone 3 pS10                  | Abcam #ab5176, RRID: AB_304763, lot GR3217296-1 | Rabbit polyclonal | 1:3000 (FC) |

IF = immunofluorescence, WB = Western blot, FC = flow cytometry
| Name       | Description                        | Expression  | Source                                          |
|------------|------------------------------------|-------------|------------------------------------------------|
| cNM216-7  | 2xmNeonGreen-hMeikin               | Constitutive| Retroviral transduction with pNM481, clonal    |
| cNM239-14 | 2xmNeonGreen-hMeikin (328-373)     | Constitutive| Retroviral transduction with pNM512, clonal    |
| cNM240-4  | 2xmNeonGreen-hMeikin (1-332)       | Constitutive| Retroviral transduction with pNM513, clonal    |
| cNM238-3  | 2xmNeonGreen-hMeikin (ICCII/DIII mutant) | Constitutive| Retroviral transduction with pNM511, clonal    |
| cNM396-15 | hMeikin-mNeonGreen                 | Constitutive| Retroviral transduction with pNM795, clonal    |
| cNM087    | EGFP                               | Dox-Inducible| AAVS1 Safe-harbor insertion of pNM280          |
| cNM102    | EGFP-hMeikin                       | Dox-Inducible| AAVS1 Safe-harbor insertion of pNM299          |
| cNM220    | EGFP-hMeikin (104-373)             | Dox-Inducible| AAVS1 Safe-harbor insertion of pNM480          |
| cNM244    | EGFP-hMeikin (124-373)             | Dox-Inducible| AAVS1 Safe-harbor insertion of pNM549          |
| cNM271    | EGFP-hMeikin (155-373)             | Dox-Inducible| AAVS1 Safe-harbor insertion of pNM603          |
| cNM142    | EGFP-hMeikin (184-373)             | Dox-Inducible| AAVS1 Safe-harbor insertion of pNM335          |
| cNM143    | EGFP-hMeikin (199-373)             | Dox-Inducible| AAVS1 Safe-harbor insertion of pNM336          |
| cNM134    | EGFP-hMeikin (227-373)             | Dox-Inducible| AAVS1 Safe-harbor insertion of pNM325          |
| cNM123    | EGFP-hMeikin (1-332)               | Dox-Inducible| AAVS1 Safe-harbor insertion of pNM310          |
| cNM145    | EGFP-hMeikin (1-310)               | Dox-Inducible| AAVS1 Safe-harbor insertion of pNM338          |
| cNM124    | EGFP-hMeikin (1-301)               | Dox-Inducible| AAVS1 Safe-harbor insertion of pNM311          |
| cNM226    | EGFP-hMeikin (1-226)               | Dox-Inducible| AAVS1 Safe-harbor insertion of pNM502          |
| cNM311    | EGFP-hMeikin (1-154)               | Dox-Inducible| AAVS1 Safe-harbor insertion of pNM680          |
| cNM273    | EGFP-hMeikin (124-332)             | Dox-Inducible| AAVS1 Safe-harbor insertion of pNM611          |
| cNM172    | EGFP-hMeikin (175-181 4A mut)      | Dox-Inducible| AAVS1 Safe-harbor insertion of pNM400          |
| cNM170    | EGFP-hMeikin (S196A)               | Dox-Inducible| AAVS1 Safe-harbor insertion of pNM398          |
| cNM119    | EGFP-hMeikin (STP mutant)          | Dox-Inducible| AAVS1 Safe-harbor insertion of pNM300          |
| cNM272  | EGFP-hMeikin (8A mutant) | Dox-Inducible | AAVS1 Safe-harbor insertion of pNM604 |
|---------|--------------------------|---------------|-------------------------------------|
| cNM321  | EGFP-hMeikin (E151R, R154E) | Dox-Inducible | AAVS1 Safe-harbor insertion of pNM726 |
| cNM371  | EGFP-hMeikin (1-332)-H2B | Dox-Inducible | AAVS1 Safe-harbor insertion of pNM801 |
| cNM469  | EGFP-hMeikin (1-154)-H2B | Dox-Inducible | AAVS1 Safe-harbor insertion of pNM888 |
| cNM366  | EGFP-hMeikin (155-332)-H2B | Dox-Inducible | AAVS1 Safe-harbor insertion of pNM802 |
| cNM370  | EGFP-hMeikin (1-332, STP mutant)-H2B | Dox-Inducible | AAVS1 Safe-harbor insertion of pNM804 |
| cNM372  | EGFP-hMeikin (1-332, 8A mut)-H2B | Dox-Inducible | AAVS1 Safe-harbor insertion of pNM803 |
| cNM426e | H2B-mScarlet-hMeikin(1-332)-mNeonGreen | Constitutive | Retroviral transduction with pNM848, fluorescence enriched |
| cNM427e | H2B-mScarlet-hMeikin(1-332, E151R, R154E)-mNeonGreen | Constitutive | Retroviral transduction with pNM849, fluorescence enriched |
| cNM428e | H2B-mScarlet-hMeikin(1-332, S149A)-mNeonGreen | Constitutive | Retroviral transduction with pNM850, fluorescence enriched |
| cNM430e | H2B-mScarlet-hMeikin(1-332, 8A mut)-mNeonGreen | Constitutive | Retroviral transduction with pNM852, fluorescence enriched |
| cNM431e | H2B-mScarlet-hRad21(142-476)-mNeonGreen | Constitutive | Retroviral transduction with pNM853, fluorescence enriched |
| cNM432e | H2B-mScarlet-hRad21(142-476, E169R, R172E, E447R, R450E, E457R, R460E)-mNeonGreen | Constitutive | Retroviral transduction with pNM854, fluorescence enriched |
| cNM478e | H2B-mScarlet-hRec8(297-506)-mNeonGreen | Constitutive | Retroviral transduction with pNM937, fluorescence enriched |
| cNM486e | H2B-mScarlet-hRec8(297-506)-hMeikin(1-332, E151R, R154E)-mNeonGreen | Constitutive | Retroviral transduction with pNM869, fluorescence enriched |
All cell lines listed are derived from HeLa cells

Table 3 – Recombinant Proteins used in this study

| Protein                                      | Plasmid                  |
|----------------------------------------------|--------------------------|
| GST-CENP-C (700-943)                         | pKM138                   |
| GST-CENP-C (808-943)                         | pNM221                   |
| GST-CENP-C (775-943, 835-838A)              | pNM232                   |
| GST-CENP-C (775-943, 865-867A)              | pNM271                   |
| *GST-sfGFP-Meikin (328-373)                 | pNM190                   |
| *GST-sfGFP-Meikin (328-373, ICCII/DIII mutant) | pNM201           |
| *GST-sfGFP-Meikin (302-373)                 | pNM175                   |
| * = GST-tag removed during purification      |                          |

Table 4 – Plasmids used for oocyte experiments

| Description                                                                 | Plasmid |
|----------------------------------------------------------------------------|---------|
| 3xEGFP-mMeikin                                                             | pNM530  |
| 3xEGFP-mMeikin (E156R, R159E)                                             | pNM606  |
| 3xEGFP-mMeikin(1-391, E156E, R159E) - hRad21(142-275) - mMeikin(387-434)   | pNM962  |
| 3xEGFP-mMeikin(1-391) - hRad21(142-275, E169R, R172E) - mMeikin(387-434)   | pNM963  |
| mMeikin-3xEGFP                                                             | pNM739  |
| mNeonGreen-mMeikin                                                         | pNM474  |
| mNeonGreen-mMeikin (387-434)                                               | pNM477  |
| mNeonGreen-mMeikin (392ICCI, 425DII → 392AAAAA, 425AAA)                   | pNM476  |
| mNeonGreen-mMeikin (T335A)                                                 | pNM475  |
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Chapter 3:
Discussion and Future Directions
Summary of Thesis Work

Meiosis requires two consecutive cell divisions. The significant differences between these two divisions necessitates a major rewiring of the chromosome segregation machinery. Meikin was previously found to control critical aspects of meiosis I chromosome segregation (Kim et al., 2015). However, how Meikin activity was modulated to enable meiosis II events was unclear. The thesis work presented here demonstrates that Meikin is required during both meiosis I and meiosis II. Loss of Meikin activity at meiosis I leads to premature sister chromatid separation, and loss of Meikin activity at meiosis II leads to chromosome misalignment on the metaphase II spindle. Critically, Separase-mediated proteolysis of Meikin at a conserved cleavage site generates a C-Meikin cleavage fragment during anaphase I. C-Meikin retains many of the activities of full-length Meikin including kinetochore localization and Plk1 binding. However, C-Meikin and full-length Meikin are not interchangeable as retention of full-length Meikin at meiosis II kinetochores leads to meiotic defects highlighting the important role for Separase-mediated cleavage of Meikin during meiosis. C-Meikin is ultimately lost from kinetochores at anaphase II. These data suggest three distinct Meikin activity states, full-length, C-Meikin, and no Meikin corresponding to the three distinct cell divisions faced by germ cells, meiosis I, meiosis II, and mitosis.

How Does Meikin Affect Plk1 Activity Throughout Meiosis?

My work found that full-length Meikin could not substitute for C-Meikin at meiosis II kinetochores, as oocytes expressing non-cleavable Meikin had significant chromosome misalignment defects. This demonstrates that a critical difference between full-length Meikin and C-Meikin exists. However, the nature of this difference is unclear. Both forms of Meikin are capable of kinetochore localization and Plk1 binding. We found a small, but significant increase
in Plk1 kinetochore localization when ectopically expressing full-length Meikin in mitotic cells or during meiosis II. However, the functional effect of this increased localization is not known. The Meikin-like Drosophila protein Mtrm has been suggested to directly inhibit Polo kinase (Bonner et al., 2020; Xiang et al., 2007). The phenotype of HeLa cells inducibly expressing Meikin does share some similarity with the phenotype observed upon chemical inhibition of Plk1 (Lénárt et al., 2007). Thus, it is tempting to speculate that Meikin also directly inhibits Plk1. However, our enzymatic assays using immunoprecipitated Meikin-Plk1 complexes found no such inhibitory effect, suggesting that Meikin acts instead as a targeting subunit. Full-length Meikin was able to induce a potent mitotic arrest when overexpressed in HeLa cells, but C-Meikin did not induce an arrest. This suggests that the two Meikin forms interact with Plk1 in distinct manners. Whether the two proteoforms of Meikin induce subtle, but important differences in Plk1 enzymatic activity or substrate preference remains to be seen.

How Do Meikin-Plk1 Complexes Regulate Meiosis?

We note that our data does not define how sister kinetochore co-orientation is established before the first meiotic division despite the genetic evidence that Meikin plays a role in establishment of co-orientation (Kim et al., 2015). It is possible that loading of core centromeric Rec8-cohesin complexes requires the activity of Meikin-Plk1. However, this establishment phase of co-orientation likely occurs during meiotic prophase when Meikin is present at kinetochores (Kim et al., 2015), but before our manipulations in this study.

Confusingly, the outcome of expressing full-length Meikin during meiosis II and eliminating C-Meikin during meiosis II is the same - chromosome misalignment. It is possible that these phenotypes are due to distinct activities. Mis-regulation of many kinetochore processes can
lead to chromosome misalignment. Future studies that investigate these defects in further depth may shed light on these distinctions.

**How is Meikin Localization Regulated Throughout Meiosis?**

Our study found that C-Meikin is localized on kinetochores during meiosis II. However, previous work observed no Meikin localization during this time (Kim et al., 2015). This discrepancy may be due to differences in detection strategies. We directly detected fluorescently-tagged Meikin expressed from exogenous mRNA. Prior work (Kim et al., 2015) raised antibodies against Meikin and utilized indirect immunofluorescence against the endogenous protein. It is possible that the antibodies used in that study do not efficiently recognize epitopes in the C-terminal portion of the protein leading to a failure to detect C-Meikin during meiosis II. Alternatively, our fluorescently tagged Meikin may be expressed at higher levels than the endogenous protein allowing us to see the protein at kinetochores during meiosis II. Importantly, our functional studies demonstrate that C-Meikin is required at meiosis II for proper chromosome alignment, supporting our observation that a Meikin fragment is retained at kinetochores during the second division.

Our work identified a critical portion of CENP-C that is required for Meikin binding, which encompasses a C-terminal CENP-C fragment. CENP-C is a critical scaffold for kinetochore assembly. Importantly, this Meikin-binding fragment does not contain binding sites for established CENP-C interaction partners, such as CENP-A or Mis12 (Klare et al., 2015). However, this C-terminal cupin domain is required for CENP-C dimerization (Cohen et al., 2008). Whether Meikin binding alters CENP-C oligomerization and the effect of the interaction on higher order kinetochore structure remains to be seen. Interestingly, the fission yeast MOKIR, Moa1, interacts
with the cupin domain of the *S. pombe* CENP-C homolog Cnp3 (Chik et al., 2019; Tanaka et al., 2009). It is possible that the two MOKIRs share similar binding modes with CENP-C.

Finally, we found that C-Meikin is ultimately removed from kinetochores at meiotic exit. How this is achieved remains mysterious. Whatever processes control this removal must be specific for anaphase II as Meikin remains at kinetochores through anaphase I. Meikin cleavage during anaphase I could facilitate Meikin degradation during anaphase II through exposure of a degron. N-terminal degrons are known to mediate degradation of other proteins after proteolytic cleavage (Gibbs et al., 2014). However, the neo-N-terminal residue acid exposed after Separase cleavage of Meikin is not conserved and in many organisms is predicted to be a stabilizing amino acid.

A major difference between anaphase I and anaphase II is the magnitude of the drop in Cdk activity. During anaphase I, Cdk levels decrease to allow for chromosome segregation and cytokinesis. However, they remain elevated compared to G1 to prevent the germ cell from leaving meiosis. During anaphase II, Cdk levels fall substantially to allow the cell to enter interphase (Iwabuchi et al., 2000). It is unclear at this time whether C-Meikin is degraded as cells enter anaphase II or simply dissociates from the kinetochore. Our in vitro analysis of Meikin-CENP-C binding found that the two proteins can bind in the absence of phosphorylation. Furthermore, we observed Meikin kinetochore localization in HeLa cells during interphase when mitotic kinase activity is low. These results suggest that Cdk phosphorylation does not affect binding between Meikin and CENP-C. Alternatively, it is possible that residual Cdk activity during anaphase I inhibits degradation of C-Meikin until the second division. A compelling candidate E3-ligase for this degradation is the APC/C, which is activated during anaphase. There are known APC/C substrates whose degradation is controlled by phosphorylation (Davey and Morgan, 2016). Meikin
contains a degenerate D-box APC/C recognition motif in its C-terminal region (318-LxExxxN). Intriguingly, a similar motif is found in other MOKIR proteins (Galander and Marston, 2020). Future work should analyze the effects on Meikin localization during anaphase II of mutations to this motif.

**Implications for Meikin-like Proteins in Other Organisms**

Meikin-like proteins in other eukaryotic clades face a similar challenge of restricting or modulating their activity between meiotic divisions. There is no evidence that Spo13, Moa1, or Mtrm are Separase substrates. Spo13 is subject to APC/C-mediated degradation at anaphase I. However, stabilization of Spo13 during meiosis II does not lead to significant meiotic defects suggesting that additional mechanisms for altering Spo13 activity must exist (Sullivan and Morgan, 2007). Mtrm is also an APC/C substrate, but is not degraded until the oocyte/embryo transition. Mtrm degradation is required for proper mitotic cell division in the early embryo (Whitfield et al., 2013). Moa1 centromere localization and cellular protein levels decline after the first meiotic division. However, the mechanism and consequences of this decline are not known (Yokobayashi and Watanabe, 2005). It is possible that Separase-cleavage and activity at meiosis II are molecular innovations unique to Meikin.

**Implications for Other Separase Substrates**

Although Separase was identified decades ago, only a handful of substrates have been identified. Our work identifies Meikin as a new meiosis-specific substrate for Separase and opens the door for additional Separase substrates either in all dividing cells or just a subset. The minimal ExxR consensus motif for Separase is found in many proteins, but very few of these motifs serve as
Separse cleavage sites. Despite work describing local (Sullivan et al., 2004) and distal (Rosen et al., 2019) sequences which promote Separase cleavage, predicting whether a particular ExxR motif is cleaved by Separase remains difficult. The identification of new Separase substrates, like Meikin, and new methods of producing active Separase for biochemical studies (Rosen et al., 2019) will hopefully shed light on Separase substrate choice. Future work using high-throughput proteomics techniques for investigating protease substrates, such as TAILS (Kleifeld et al., 2011), may identify additional Separase substrates.

**Conclusion**

In conclusion, meiosis is a highly plastic process where the key function, a reduction in ploidy, is coupled to the developmental and evolutionary requirements of a particular species. The two meiotic divisions require dramatic rewiring of the chromosome segregation machinery. Studies in multiple organisms have identified Meikin-like proteins as critical for promoting meiosis I specific modifications to the cell division machinery. However, these MOKIRs are quite distinct at both the primary sequence and functional level. A coordinating theme across these molecules is the ability to regulate cell cycle kinases (Galande and Marston, 2020).

Importantly, this thesis work indicates that mammalian Meikin is not a meiosis I-specific protein as previously believed. Our work demonstrates that Meikin has critical roles during meiosis II. Thus, Meikin serves as a general regulator of meiotic chromosome segregation. Importantly, this work finds that Meikin is a novel Separase substrate and highlights a critical role for specific proteolysis to modulate Meikin function between the two meiotic divisions.
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