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Synaptonemal complex disassembly activates Rad51-mediated double strand break repair during budding yeast meiosis

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Evelyn Prugar

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Evelyn Prugar

We, the dissertation committee for the above candidate for the

Doctor of Philosophy degree, hereby recommend

acceptance of this dissertation.

Nancy Marie Hollingsworth, Ph.D.– Dissertation Advisor
SUNY Distinguished Teaching Professor
Department of Biochemistry and Cell Biology

Aaron Neiman, Ph.D- Chairperson of Defense
Professor
Department of Biochemistry and Cell Biology

Bruce Futcher, Ph.D- Committee member
Professor
Department of Molecular Genetics and Microbiology

Ed Luk, Ph.D- Committee member
Assistant Professor
Department of Biochemistry and Cell Biology

Andreas Hochwagen, Ph.D- Committee member
Assistant Professor of Biology
NYU Department of Cell Biology

This dissertation is accepted by the Graduate School

Nancy Goroff

Interim Dean of the Graduate School

Abstract of the Dissertation

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Meiosis is a highly conserved specialized cell division that occurs in many organisms, including budding yeast and mammals. Meiosis divides the chromosome number of the cell in half to create gametes for sexual reproduction. A single round of chromosome duplication is followed by two rounds of chromosome segregation, Meiosis I (homologs segregate) and Meiosis II (sister chromatids segregate). Proper segregation at Meiosis I requires that homologs are connected by both crossovers and sister chromatid cohesion. Crossovers are formed by the repair of double strand breaks (DSBs) preferentially by the homolog. The choice of repair template is determined at the time of strand invasion, which is mediated by two recombinases, Rad51 and the meiosis-specific Dmc1. Rad51 is necessary for Dmc1 to function properly but its strand exchange activity is inhibited both by Dmc1 and Mek1, a meiosis-specific kinase, which is activated by DSBs. Mek1 suppresses interaction between Rad51 and its accessory factor Rad54 in two ways. First, phosphorylation of Rad54 lowers its affinity for Rad51. Second, phosphorylation stabilizes Hed1, a meiosis-specific protein that binds to Rad51 and excludes Rad54. Although *RAD54* is not required for wild-type levels of interhomolog recombination, *rad54*Δ diploids exhibit decreased sporulation and spore viability, indicating the presence of unrepaired DSBs. My thesis tested the idea that Mek1 kinase activity is down-regulated after interhomolog recombination to allow Rad51-mediated repair of any remaining DSBs.

Meiotic recombination occurs in the context of a proteinaecous structure called the synaptonemal complex (SC). The SC is formed when sister chromatids condense along protein cores called axial elements (AEs) comprised of the meiosis-specific

proteins, Hop1, Red1 and Rec8. AEs are brought together by interhomolog recombination, which creates stable connections and the gluing together of the AEs by the insertion of the transverse filament protein, Zip1, in a process called synapsis. Pachynema is the stage of meiotic prophase in which chromosomes are fully synapsed and where interhomolog recombination has proceeded to the double Holliday junction (dHJ) stage.

Meiotic progression requires transcription factor *NDT80*, a middle meiosis transcription factor required to express >200 genes, including the polo-like kinase, *CDC5* (required for Holliday junction resolution and SC disassembly) and *CLB1* (required for meiotic progression). Diploids deleted for *NDT80* arrest in pachynema with unresolved dHJs. I used an inducible version of *NDT80* (*NDT80-IN*) to separate prophase into two phases: pre-*NDT80*, when interhomolog recombination occurs and post-*NDT80*, when it is proposed that inactivation of Mek1 allows intersister recombination to repair residual DSBs. *RAD54* is sufficient to function after interhomolog recombination, as inducing both *RAD54* and *NDT80* simultaneously rescues the spore inviability defects observed in *NDT80-IN rad54*Δ diploids. Using an antibody specific for phosphorylated Hed1 as an indicator of Mek1 kinase activity, I showed that Mek1 is constitutively active in *ndt80*-arrested cells and that induction of *NDT80* is sufficient to abolish Mek1 activity. Furthermore, inactivation of Mek1 by Ndt80 can occur in the absence of interhomolog strand invasion and synapsis. Mek1 inactivation correlates with the appearance of *CDC5* and the degradation of Red1. My work demonstrates that the sole target of *NDT80* responsible for inactivating Mek1 is *CDC5*.

Unrepaired DSBs trigger the meiotic recombination checkpoint resulting in prophase arrest, which requires Mek1 and works by sequestering Ndt80 in the cytoplasm. Mek1 also delays meiotic progression in wild-type cells, likely through inactivation of Ndt80. My work shows that Ndt80 in turn negatively regulates Mek1. Based on my observations, as well as published work showing that synapsis results in the removal of Mek1 from chromosomes, I propose that recombination and meiotic progression are coordinated by regulation of Mek1.

Dedication Page

Dedicated in the memory of my grandfather Edward James Eaton, Sr.

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List of Abbreviations

AE:	Axial element
APC:	Anaphase-promoting complex
as:	analog sensitive
CO:	Crossover
CDK:	cyclin-dependent kinase
dHJ:	Double Holliday Junction
DAPI:	4’6-diamidino-2-phenylindole
DNA:	Deoxyribonucleic acid
D-loop:	Displacement loop
DSB:	Double strand break
DDR:	DNA damage response
ED:	β-estradiol
EDTA:	Ethylenediamine tetraacetic acid
hr:	Hour
IH:	Interhomolog
IS:	Intersister
JM:	Joint molecule
LE:	Lateral element
MI:	Meiosis I
MII:	Meiosis II
MMS:	methyl methanesulfonate
ml:	milliliter
μl:	microliter
μM:	micromolar
μs:	microsecond
NCO:	Noncrossover
OD:	Optical density
P:	Parental fragment

RPA:	Replication Protein A
SC:	Synaptonemal complex
SDSA:	Synthesis dependent strand annealing
ss:	Single strand
ssDNA:	Single strand DNA
ZMM:	Zip1-4, Msh4/5, Mer3
Spo:	Sporulation
1-NA-PP1:	4-amino-1-*tert*-butyl-3-(1 naphthyl) pyrazolo [3,4-*d*] pyrimidine

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CHAPTER ONE: INTRODUCTION

Significance of chromosome segregation in meiosis

Sexually reproducing eukaryotes, including humans, plants, worms and fungi, undergo a specialized cell division called meiosis to produce reproductive cells (Petronczki et al., 2003). In diploid organisms, the purpose of meiosis is to ensure that every haploid gamete receives one copy of each chromosome. To achieve this goal, a diploid progenitor mother cell creates haploid daughter cells by first duplicating the chromosomes and then having two consecutive rounds of chromosome segregation to reduce the chromosome number by half. The two meiotic divisions are called Meiosis I (MI) and Meiosis II (MII). MI is referred to as a reductional division because homologous chromosomes segregate to opposite spindle poles. MII is an equational division, similar to mitosis where sister chromatids separate to opposite spindle poles (Figure 1). The resulting haploid nuclei are packaged into gametes referred to as eggs and sperm in humans and spores in yeast. The fusion of two gametes results in a zygote, thereby restoring the chromosome number of the progenitor cell. When chromosome segregation errors occur in meiosis, chromosome imbalances or aneuploidy may result in infertility, miscarriages or genetic disorders. For example, Trisomy 21, also known as Down syndrome, is the leading cause of mental retardation in the United States (Champion and Hawley, 2002; Hassold and Hunt, 2001; Roeder, 1997).

Sister chromatid cohesion and crossover formation ensure proper segregation at Meiosis I

Proper segregation of chromosomes during both mitosis and meiosis requires that chromosomes align accurately during metaphase. The cell uses sister chromatid cohesion to assist in the correct alignment of chromosomes to the spindle. During DNA replication, sister chromatids are connected by protein complexes known as cohesins (Guacci et al., 1997; Michaelis et al., 1997; Uhlmann and Nasmyth, 1998) (Figure 1B). In vegetatively growing cells, the multi-subunit cohesin complex contains four proteins: Scc1/Mcd1, Scc3, Smc1, and Smc3 (Berchowitz and Copenhaver, 2010; Nasmyth and Haering, 2005; Strunnikov et al., 1995). Smc1 and Smc3 are structural components of the complex that are ATPases and govern loading and unloading (Losada et al., 1998; Toth et al., 1999). Mcd1 is a member of the kleisin family. The cohesin complex forms a ring structure around the DNA to prevent chromosome segregation until the onset of anaphase (Nasmyth, 2001). The spindle checkpoint monitors attachment of microtubules to the kinetochores of sister chromatids (Lew and Burke, 2003; Musacchio and Salmon, 2007). When two sister kinetochores are attached to microtubules from opposite poles (known as biorientation), tension results from the pulling forces of the microtubules being counteracted by sister chromatid cohesion. When all the chromosomes are bi-oriented, a ubiquitin ligase called the anaphase-promoting complex (APC) is activated which targets the degradation of securin, the inhibitory subunit of separase, by the proteasome (Hornig et al., 2002). Separase is a cysteine protease that cleaves the Mcd1 subunit of the cohesin complex and triggers anaphase (Ciosk et al., 1998; Nasmyth et al., 2000; Toth et al., 1999).

To enable alignment of homologous pairs of sister chromatids at Metaphase I, meiosis-specific adaptations have evolved. First, sister kinetochores function as a single unit, binding microtubules from just one spindle pole (called mono-orientation) (Hauf and Watanabe, 2004; Petronczki et al., 2003). In yeast, a fork shaped protein complex called monopolin serves this purpose (Corbett and Harrison, 2012; Rabitsch et al., 2003; Toth et al., 2000) (Figure 1C). Second, the cohesion complex has a meiosis-specific α-kleisin subunit, Rec8, in place of Mcd1 (Klein et al., 1999). Two proteins, Spo13 and Shugoshin (Sgo1), prevent cleavage of Rec8 cohesin complexes at the centromeres during MI by inhibiting the phosphorylation that promotes Rec8 cleavage (Katis et al., 2004; Kitajima et al., 2004; Klein et al., 1999; Lee et al., 2004). Rec8 is therefore cleaved only on chromosome arms during Anaphase I (Figure 1D) (Buonomo et al., 2000). At MII, monopolin is removed so that sister kinetochores now biorient and cleavage of centromeric cohesin triggers the onset of anaphase II (Figure 1E). Sister chromatid cohesion and monopolin alone are not sufficient to connect homologs during meiosis. A third meiosis-specific adaptation is the formation of crossovers due to recombination between the non-sister chromatids of homologous chromosomes. It is the combination of crossovers and sister chromatid cohesion during MI that allows for the homologs to segregate reductionally (Figure 1C).

Mitotic versus meiotic recombination

Double strand breaks (DSBs) occur both in mitotic and meiotic cells. During vegetative growth DSBs can arise from errors during DNA replication or from exogenous DNA damage caused by ultraviolet light, X-rays, and chemicals (Friedberg et al., 2004; Hoeijmakers, 2001). Cells have adapted to repair DSBs preferentially using

sister chromatids which maintains genomic integrity because sister chromatids have identical DNA sequences (Bzymek et al., 2010; Hunter and Kleckner, 2001; Kadyk and Hartwell, 1992; Moynahan and Jasin, 2010; Paques and Haber, 1999). In mitotic cells a RecA-like recombinase, Rad51, mediates repair of DSBs using intersister recombination (Paques and Haber, 1999). DSBs are processed by resection of the 5' ends of the DNA to produce a 3' single stranded (ss) tails. These ssDNA ends are then bound by the single strand binding heterotrimeric protein complex, replication protein A (RPA) (Zou et al., 2006). RPA removes secondary structures that the ssDNA may have formed. The mediator proteins, Rad52 and Rad55/57, assist in replacing RPA with Rad51 (Gasior et al., 1998). Rad54 is a member of the Swi2/Snf2 family of DNA motor proteins and an important accessory factor that physically interacts with Rad51 (Dresser et al., 1997; Petukhova et al., 2000; Petukhova et al., 1999; Raschle et al., 2004). Rad54 functions in stabilization of the Rad51 filament, stimulation of Rad51-mediated strand invasion and the removal of Rad51 from the DNA once the joint molecules have been formed (Heyer et al., 2006; Petukhova et al., 1998; Petukhova et al., 2000; Shinohara et al., 1992; Sugawara et al., 2003).

In mitotically dividing cells, DSBs are not desired and can be lethal. However in meiosis, DSBs are deliberately created with the purpose of making interhomolog (IH) crossovers (COs). Both the creation and repair of meiotic DSBs are therefore carefully regulated. DSBs are introduced into the DNA at the beginning of meiotic prophase by Spo11, a topoisomerase like protein that cuts at preferred sites in the genome called "hot spots" (Keeney et al., 1997; Lam and Keeney, 2015)(Figure 2B). Spo11 hot spots have been shown to occur primarily within nucleosome free regions, independently of

DNA sequence (Blitzblau et al., 2007; Buhler et al., 2007; de Massy and Nicolas, 1993; Keeney, 2001; Wu and Lichten, 1995). Spo11 initiated DSBs cannot be processed until there is an endonucleolytic event that removes Spo11 linked to a short oligonucleotide (Neale et al., 2005). Similar to mitosis, the 5' ends of the DSBs are resected by Sae2/Com1 and the Rad50/Mre11/Xrs2 endonuclease complex, leaving 3' single strand DNA ends bound by RPA (Alani et al., 1990; McKee and Kleckner, 1997; Prinz et al., 1997) (Figure 2B). The recombinases that bind to the 3' ends in meiosis are the RecA orthologs Rad51 and the meiosis-specific Dmc1 (Neale and Keeney, 2006; Sheridan and Bishop, 2006). Dmc1 is loaded onto ssDNA by the accessory proteins, Mei5, Sae3, Hop2, Mnd1, and Rdh54 (Rad54 paralog) (Hayase et al., 2004; Petukhova et al., 2005; Shinohara et al., 2000). Rad51 and Dmc1 colocalize to DSBs during meiosis, and are both required for normal levels of IH recombination, (Bishop, 1994; Schwacha and Kleckner, 1997; Shinohara et al., 1997a). In Arabidopsis, Rad51 binds to one side of the DSB while Dmc1 is located on the other side (Kurzbauer et al., 2012). Unlike Arabidopsis, in yeast the filaments formed on both sides of a DSB are seeded with Rad51 and Dmc1 (Brown et al., 2015). Rad51 promotes loading of Dmc1 onto DSBs and functions as an accessory factor for Dmc1 strand exchange activity *in vitro* (Bishop, 1994; Brown et al., 2015; Cloud et al., 2012). Invasion of one of the 3' ends into a homologous duplex of DNA displaces the strand of like polarity to create a displacement or D-loop (Figure 2C). While the presence of the Rad51 protein is necessary for strand invasion to occur preferentially with the homolog, it is the strand exchange activity of Dmc1 that mediates the bulk of meiotic recombination (Cloud et al., 2012; Lao et al., 2008; Schwacha and Kleckner, 1997).

Rad54 and Rdh54/Tid1, members of the Swi2/Snf2 family of DNA motor proteins, are important accessory factors that interact with Rad51 and Dmc1, respectively (Dresser et al., 1997; Petukhova et al., 2000; Petukhova et al., 1999; Raschle et al., 2004). Rdh54/Tid1 stimulates IH recombination, and facilitates the removal of Dmc1 from uncut double stranded DNA (Holzen et al., 2006). Both Rad54 and Rdh54/Tid1 can have cross talk in meiosis as *rad54*Δ *rdh54*Δ double mutants have a more severe phenotype than either single mutant (Shinohara et al., 1997b). However, genetic experiments have shown that in meiosis Rad51 interacts primarily with Rad54 mediating IS repair, while Dmc1 interacts primarily with Rdh54/Tid1 for IH recombination (Arbel et al., 1999; Bishop et al., 1999; Shinohara et al., 1997a). These results are consistent with biochemical experiments using Dmc1, Rad51, Rad54, and Rdh54/Tid1 proteins that demonstrated that Rad51-Rad54 and Dmc1-Rdh54/Tid1 work as functionally distinct pairs in strand invasion (Nimonkar et al., 2012).

After strand invasion, processing of the intermediates can result in the formation of either COs or noncrossovers (NCOs). NCOs result when DSBs are repaired without an exchange of chromosome arms, in contrast to COs. CO formation involves double Holliday junction (dHJ) intermediates, which are joint molecules (JMs) that can be physically detected by two-dimensional electrophoresis (Hunter and Kleckner, 2001). Upon extension of the invading end by DNA synthesis, the D-loop undergoes enlargement with the displaced single strand ultimately annealing to the other side of the break. Ligation of the nicks then results in the formation of a dHJ intermediate (Figure 2D). Resolution of the HJs occurs by the nicking and ligation of strands of like polarity for each HJ. If the same strands are ligated for both HJs, a NCO is produced.

In contrast, if different pairs of strands are nicked and ligated, a CO is produced (Figure 2E). In wild-type yeast, resolution of meiotic dHJs results primarily in COs (Allers and Lichten, 2001). This occurs when strand invasion intermediates are processed using a group of functionally diverse proteins collectively called the “ZMMs” (Borner et al., 2004) (Figure 2D). The ZMM complex is a diverse group of proteins with different functions necessary for proper synapsis and CO formation. Zip1 is the transverse filament protein of the synaptonemal complex that works in combination with Zip2, Zip3, Spo22/Zip4, a SUMO E3 ligase. In addition the ZMM complex includes Mer3, a helicase, Msh4 and Msh5, a heterodimeric meiosis-specific complex orthologous to the mismatch repair protein MutS, and Spo16 all required for CO formation (Cheng et al., 2006; Chua and Roeder, 1998; Hollingsworth et al., 1995; Mazina et al., 2004; Ross-Macdonald and Roeder, 1994; Shinohara et al., 2008). Resolution of dHJs formed using the ZMM pathway requires both *MLH1* and *MLH3* (Wang et al., 1999; Zakharyevich et al., 2012). A minor fraction of dHJs formed are created in the absence of the ZMM pathway. These intermediates are resolved equally into COs or NCOs by alternative resolvases such as the structure-specific endonuclease Mus81/Mms4 or Yen1 (Borner et al., 2004; de los Santos et al., 2003; Jessop and Lichten, 2008; Oh et al., 2007).

In wild-type meiosis, COs and NCOs are temporally and mechanistically distinct (Allers and Lichten, 2001). Most NCOs result from synthesis-dependent strand annealing (SDSA), and do not involve dHJs. SDSA occurs when there is a disruption of the D-loop after the invading strand has been extended by DNA synthesis, which leads to the ssDNA being displaced (Figure 2F). A protein complex containing Sgs1, a

member of the RecQ family of 3’ to 5’ helicases, Top3 and Rmi1, is able to unwind and displace the single-stranded DNA from the homolog (De Muyt et al., 2012; Kaur et al., 2015; Mimitou and Symington, 2008; Oh et al., 2007; Oh et al., 2008; Tang et al., 2015). After displacement the ssDNA anneals to the other side of the DSB where there is repair synthesis and ligation to generate a NCO (McMahill et al., 2007) (Figure 2F).

The synaptonemal complex

During meiosis, the bias for DSB repair is changed from sister chromatids to homologs. This IH bias depends on a meiosis-specific chromosome structure called the synaptonemal complex (SC) (Page and Hawley, 2004). During meiotic prophase sister chromatids condense to form loops that are attached to protein cores called axial elements (AEs) (Figure 3A). In many organisms such as yeast and mammals, stable pairing and synapsis of homologs is dependent upon recombination between homologs (Villeneuve and Hillers, 2001) (Figure 3B). The SC is formed when homologous AEs are connected by proteins that comprise the central region (Zip1 in yeast) in a process called synapsis (Dong and Roeder, 2000; Page and Hawley, 2004; Sym et al., 1993; Sym and Roeder, 1995; Tung and Roeder, 1998) (Figure 3C). In yeast, synapsis requires the genes encoding the ZMM proteins (Borner et al., 2004). The fully formed SC is a zipper-like structure consisting of the two AEs which are now referred to as lateral elements (LEs) that align in parallel to the central element (Zickler and Kleckner, 1999). The pachytene stage of prophase I in meiosis is when all of the chromosomes are fully synapsed and dHJs have been formed (Allers and Lichten, 2001; Page and Hawley, 2004).

The Ndt80 transcription factor controls HJ resolution, SC disassembly and exit from pachytene

Exit from pachytene into Metaphase I requires induction of *NDT80*, a gene that encodes a transcription factor that activates expression of over 200 middle meiosis genes (Chu and Herskowitz, 1998; Xu et al., 1995). *ndt80*Δ diploids arrest in pachytene with unresolved dHJs (Allers and Lichten, 2001; Xu et al., 1995). *NDT80* is also required for turning off Spo11 activity, as DSBs continue to be made and IH JMs to accumulate in *ndt80*Δ-arrested cells (Allers and Lichten, 2001; Carballo et al., 2013; Subramanian et al., 2016). One target of Ndt80 is *CDC5*, which encodes a polo-like kinase required for dHJ resolution, Red1 removal from chromosomes, and SC disassembly (Clyne et al., 2003). Furthermore ectopic induction of *CDC5* in the *ndt80*Δ background is sufficient for HJ resolution and SC disassembly, indicating it is the sole target of *NDT80* responsible for these events (Sourirajan and Lichten, 2008).

DSB formation occurs in association with AEs after premeiotic DNA replication

In yeast, IH bias arises in part from the suppression of meiotic intersister (IS) DSB repair, which is dependent on the genes encoding the meiosis-specific AE proteins Red1, and Hop1 (Bishop et al., 1999; de los Santos and Hollingsworth, 1999; Kim et al., 2010; Thompson and Stahl, 1999; Xu et al., 1997). Orthologs of Hop1 in other species, such as nematodes and mice, appear to share this phenotype (Couteau et al., 2004; Handel and Schimenti, 2010; Li et al., 2011; Zetka et al., 1999). *RED1* is

essential for AE formation, however, *hop1Δ* mutants can form some pieces of the AEs but the chromosomes fail to synapse (Hollingsworth et al., 1990; Loidl et al., 1994).

Prior to DSB formation, hotspot sequences within DNA loops are recruited to the axes to form tethered loop axis complexes, where DSBs are generated (Figure 3A) (Acquaviva et al., 2013; Panizza et al., 2011; Sommermeyer et al., 2013). There exists a temporal regulation that coordinates the formation of DSBs with replication (Borde et al., 2000; Lam and Keeney, 2015; Pan et al., 2011). This temporal regulation depends on cell-cycle kinases (Lam and Keeney, 2015). Cdc28 is the catalytic subunit of the cell cycle cyclin-dependent kinase (CDK). Cdc28 associates with different cyclins throughout the cell cycle to target diverse substrates for phosphorylation (Loog and Morgan, 2005; Ubersax et al., 2003). Cdc7 is a serine/threonine protein kinase that is regulated by the Dbf4: the complex is referred to as Dbf4-dependent kinase or DDK (Dowell et al., 1994; Hartwell, 1976; Jackson et al., 1993). Both CDK and DDK are involved in the phosphorylation of the axis protein, Mer2 (Henderson et al., 2006; Li et al., 2006; Sasanuma et al., 2008; Wan et al., 2008). DDK travels with the replication fork and phosphorylates Mer2 (Murakami and Keeney, 2014). This phosphorylation results in the recruitment of Rec114 and Mei4 to form the RMM (Rec114, Mer2, Mei4) complex on the axis (Li et al., 2006; Maleki et al., 2007; Sasanuma et al., 2008). Spo11 dimerization and association with DSB sites relies on the interaction with the Rec102 and Rec104 subcomplex (Kee et al., 2004; Prieler et al., 2005). Spo11 cleaves DNA sequences located in the chromatin loops, while the proteins necessary to cleave the hot spot are on the chromosome axes (Panizza et al., 2011). Hot spot sequences are brought to the chromosome axes through the action of Spp1. Spp1, a component of the

histone H3K4 methyltransferase Set1 complex, is associated with chromosome axes during early prophase and interacts with phosphorylated Mer2 (Sommermeyer et al., 2013). The PHD finger domain of Spp1 interacts with methylated H3K4 adjacent to hotspot DNA sequences, which promotes the tethering of hot spots to the chromosome axes where Spo11 cleavage can occur (Acquaviva et al., 2013; Brachet et al., 2012; Sommermeyer et al., 2013).

The meiosis-specific kinase Mek1 mediates suppression of meiotic intersister DSB repair

The fact that DSB cleavage occurs on the axis enables the repair of the breaks to be regulated via meiosis-specific adaptations to the DNA damage response (DDR) (Subramanian and Hochwagen, 2014). In mitotic cells, DSB formation and resection trigger the DDR by allowing the assembly of the "9-1-1" PCNA-like clamp onto the resected ends (Clerici et al., 2014; Gray et al., 2013; Shinohara et al., 2003). In yeast this clamp is comprised of Ddc1, Rad17 and Mec3, and is necessary for the recruitment of the ATR-related checkpoint kinase, Mec1. Mec1 phosphorylation of the adaptor protein Rad9, results in recruitment and activation of the checkpoint kinase, Rad53 (Gilbert et al., 2001; Majka and Burgers, 2003; Shinohara et al., 2003; Sweeney et al., 2005). In meiotic cells, Spo11-generated DSBs activate a checkpoint response mediated by Mec1 which monitors the repair of the DSBs (Subramanian and Hochwagen, 2014). An important difference is that in meiosis Mec1 phosphorylates Hop1 instead of Rad9 (Carballo et al., 2008). The meiosis-specific serine-threonine kinase, Mek1, then binds to phosphorylated Hop1 via its FHA domain where Mek1 activates itself by phosphorylation *in trans* (Carballo et al., 2008; Niu et al., 2007; Wan

et al., 2004). (Figure 3B). Mek1 kinase activity is required to suppress IS repair (Kim et al., 2010; Niu et al., 2005). In the absence of Mek1 kinase activity, DSBs are repaired primarily using sister chromatids and as a result produce predominantly inviable spores (Bailis and Roeder, 1998; Hollingsworth et al., 1995; Leem and Ogawa, 1992; Niu et al., 2005; Thompson and Stahl, 1999).

Mek1 is also required for the meiotic recombination checkpoint (Xu et al., 1997). This checkpoint delays meiotic progression until DSB repair is complete (Subramanian and Hochwagen, 2014). This checkpoint is triggered by the presence of unrepaired DSBs, such as occurs in *dmc1*Δ mutants, and arrests cells in meiotic prophase (Lydall et al., 1996; Roeder and Bailis, 2000; Xu et al., 1997). An analog sensitive version of *MEK1*, called *mek1-as*, allows inactivation of the kinase by the addition of a purine analog, 1-NA-PP1, to the sporulation (Spo) medium (Wan et al., 2004). Using this conditional *mek1* mutant, it was shown that Mek1 kinase activity is required constitutively to maintain the prophase arrest in *dmc1*Δ diploids (Niu et al., 2005; Wan et al., 2004). Ndt80 is a central target for the meiotic recombination checkpoint (Acosta et al., 2011; Bailis and Roeder, 1998; Pak and Segall, 2002). When the meiotic recombination checkpoint is triggered, Ndt80 is prevented from mediating transcription by being sequestered in the cytoplasm (Wang et al., 2011).

Four *in vivo* substrates of Mek1 have been identified: Mek1 T327, Histone H3 T11, Rad54 T132 and Hed1 T40 (Callender, 2016; Govin et al., 2010; Niu et al., 2007). Phosphorylation of Mek1 T327 is important for activation of the kinase (Niu et al., 2007), while the function of H3 T11 phosphorylation is unknown. Hed1 is a meiosis-specific protein that binds to Rad51, thereby inhibiting Rad51-Rad54 complex formation

(Busygina et al., 2008; Tsubouchi and Roeder, 2006). Phosphorylation of Rad54 and Hed1 by Mek1 are independent mechanisms that suppress Rad51-mediated DSB repair using sister chromatids as templates in *dmc1*Δ diploids (Callender, 2016) (Figure 3B). Mek1 phosphorylation of Rad54 T132 reduces the binding affinity of Rad54 to Rad51, while Hed1 T40 phosphorylation prevents Hed1 degradation (Callender, 2016; Niu et al., 2009).

The *rad51-II3A* mutant is defective for strand exchange activity *in vitro* yet has no effect on IH recombination *in vivo*, leading to the idea that Dmc1 is solely responsible for IH recombination during meiosis (Cloud et al., 2012). However, the fact that Rad51 strand exchange activity is down-regulated during meiosis by the dynamic modification of phosphorylation raises the question as to whether Rad51 activity may be important later in prophase, after the bulk of DSBs have been repaired using Dmc1. This idea is further supported by the observation that *rad54*Δ diploids exhibit wild-type levels of IH recombination, but decreased sporulation and spore viability (Schmuckli-Maurer and Heyer, 2000; Shinohara et al., 1997b). These results suggest that there may be DSBs that remain unrepaired in the absence of *RAD54*; perhaps DSBs that would normally have been repaired by sister chromatids.

The goal of my research was to test the hypothesis that Rad51 strand exchange activity is regulated by Mek1 to allow the repair of residual DSBs after IH recombination has occurred to connect homologous chromosomes. For my experiments, meiotic prophase was divided into two temporally distinct phases using an inducible allele of the *NDT80* transcription factor, *NDT80-IN* (Benjamin et al., 2003). This system places a gene of interest under the control of the *GAL1* promoter (Figure 1-4). Additionally

engineered into this strain is the *GAL4* transcription factor fused to human estrogen hormone binding domain (abbreviated Gal4-ER), which is under control of the constitutively active *GPD1* promoter (Benjamin et al., 2003; Carlile and Amon, 2008). For simplicity, I refer to the P_{GAL1}-*GENEX* P_{GPD1}-*GAL4(848).ER* genotype as *GENEX-IN*. *GENEX-IN* can be induced by the addition of β-estradiol (ED) to the Spo medium (Benjamin et al., 2003; Carlile and Amon, 2008). Before activation by ED, Gal4.ER is inactive in the cytoplasm by associating with the Hsp90 chaperone complex (Figure 1-4). ED diffuses through the cell membrane and binds to the estrogen hormone binding domain, resulting in a release of Gal4.ER from the Hsp90 chaperone complex and localization to the nucleus. Once, in the nucleus Gal4.ER binds to the *GAL1* promoter and activates transcription of *GENEX* (McIsaac et al., 2011) (Figure 1-4). In my thesis I used this approach to create conditional alleles of *NDT80*, *CDC5* and *RAD54*.

Using an antibody specific for a Mek1-dependent phosphosite on Hed1 as a marker for Mek1 activity, I have shown that induction of *NDT80*, and specifically of *CDC5*, inactivates Mek1, thereby allowing Rad51-mediated DSB repair. Furthermore, Mek1 inactivation by *NDT80* does not require strand invasion or synapsis but instead is correlated with degradation of Red1, leading to the proposal that it is the disassembly of the SC mediated by Cdc5 that triggers Rad51-mediated recombination late in prophase to repair any remaining DSBs prior to entry into the meiotic divisions.

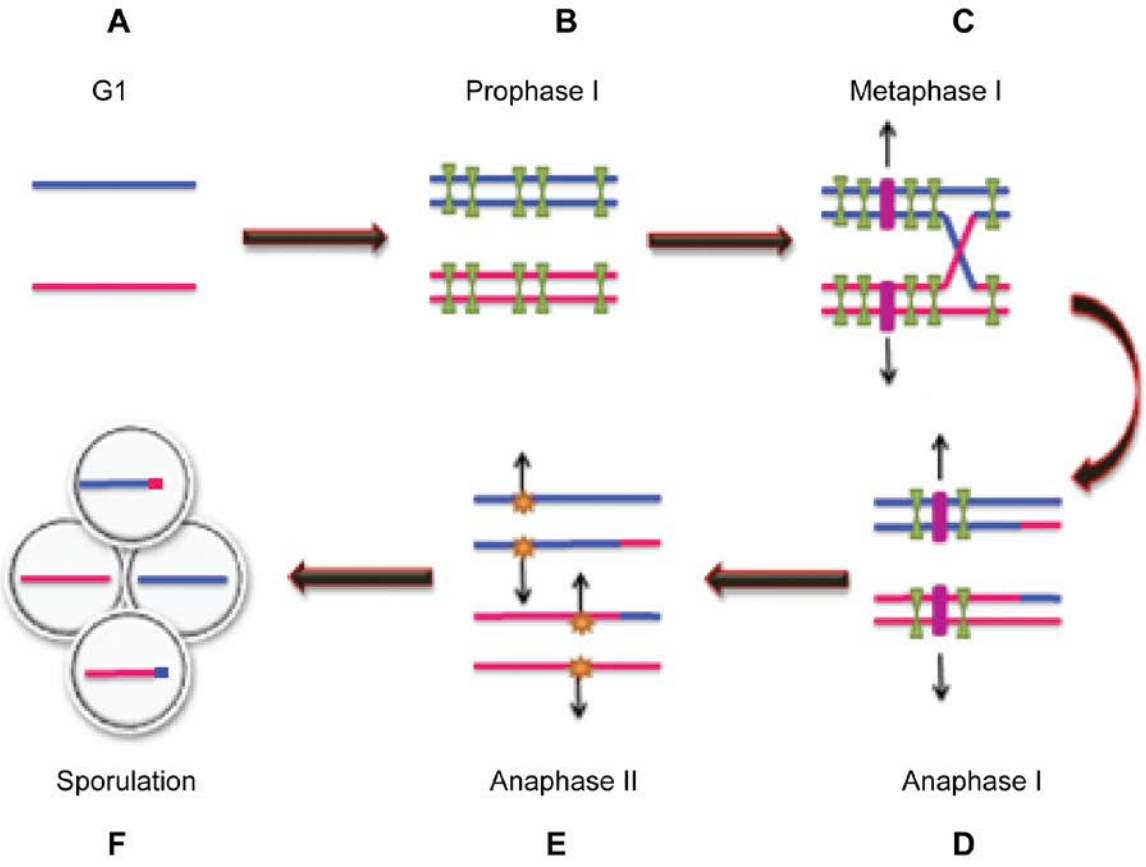


Figure 1-1. Chromosome segregation in meiosis

Blue and pink lines represent two homologous chromosomes, respectively. (A) A diploid cell before entering meiosis containing a single pair of homologous chromosomes. (B) During pre-meiotic DNA replication, pairs of sister chromatids are connected by cohesion complexes containing the meiosis-specific kleisin subunit, Rec8 (Green bowties). (C) A crossover between non-sister chromatids, in combination with sister chromatid cohesion, physically connects the homologs. Mono-oriented sister kinetochores are indicated by purple rectangles (D) At Anaphase I, arm cohesion is removed allowing homologs to segregate to opposite poles. (E) At MII, centromeric cohesion is lost allowing bi-oriented sister chromatids to segregate to opposite poles. Orange stars indicate individual kinetochores. (F) In yeast the four meiotic products are packaged into spores that are contained within a sac called a ascus.

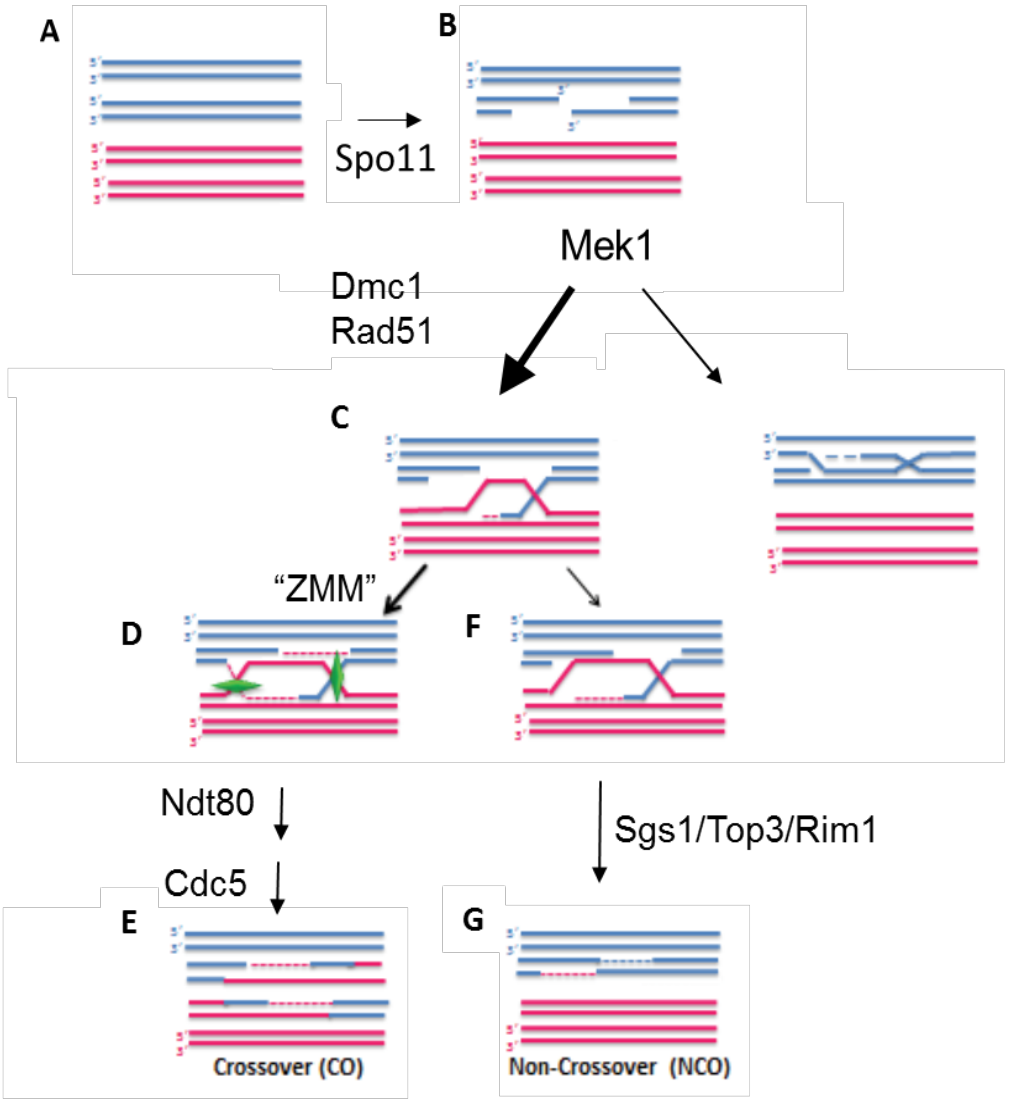


Figure 1-2. A model for meiotic recombination

(A) Pre-meiotic DNA replication of a pair of homologous chromosomes results in four chromatids. Each chromatid is represented as a duplex of DNA. (B) DSB formation on one of the four chromatids is catalyzed by Spo11. The 5' ends of the DSB are then resected to produce 3' single-strand (ss) tails. (C) The Rad51 and Dmc1 recombinases bind to the 3' ss ends where they preferentially mediate strand invasion of the homolog due to Mek1 to form a displacement or D-loop. (D) Extension of the invading strand by DNA synthesis (indicated by a dotted line) displaces the strand of like polarity, which then anneals to the ss DNA on the other side of the break. Ligation then results in the formation of a double Holliday junction (dHJ) intermediate. (Green diamonds indicates strands that are cut and ligated). (E) Induction of *NDT80* results in transcription of the polo-like kinase *CDC5* gene, which in turn triggers HJ resolution. dHJ intermediates formed via the ZMM pathway are resolved to give COs by cutting the two HJs on opposite strands. (F) Extension of the invading strand by DNA synthesis. (G) NCOs may occur when the Sgs1-Top3-Rim1 complex displaces the extended strand thereby allowing it to anneal to the other side of the break.

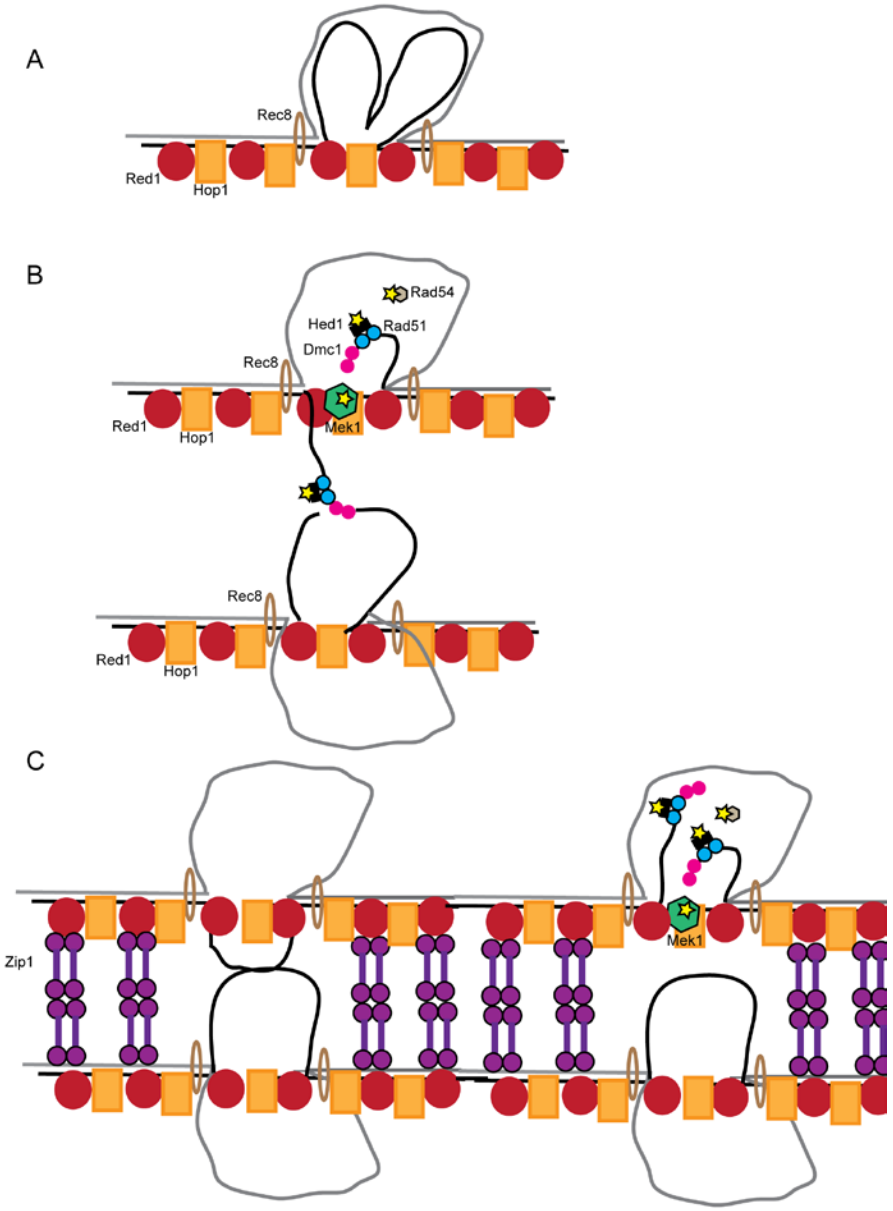


Figure 1-3. Recombination and synaptonemal complex formation. (A) Sister chromatids (black and gray lines) condense to form loop structures that are attached to an axial element containing Hop1 (orange rectangle), Red1 (red circle) and Rec8 (gold ring). Rec8 is part of the cohesin complex that holds sister chromatids together. Hot spot sequences are recruited to the axis where DSB cleavage by Spo11 occurs. (B) DSB formation results in recruitment and activation of Mek1 (green hexagon) by autophosphorylation (gold star) on the axis where it phosphorylates Hed1 (black rectangle) and Rad54 (gray hexagon) to repress Rad51 activity. Dmc1 (pink circle) and Rad51 (blue circle) bound to the ssDNA ends of the break mediate strand invasion of the homolog. When strand invasion occurs by the ZMM pathway, stable interhomolog connections are formed. (C) Axial elements are held together by the insertion of the transverse filament protein, Zip1 (purple dumbbells) to form the SC. Recombination progresses to the dHJ stage (left side). (Figure courtesy of Nancy Hollingsworth.)

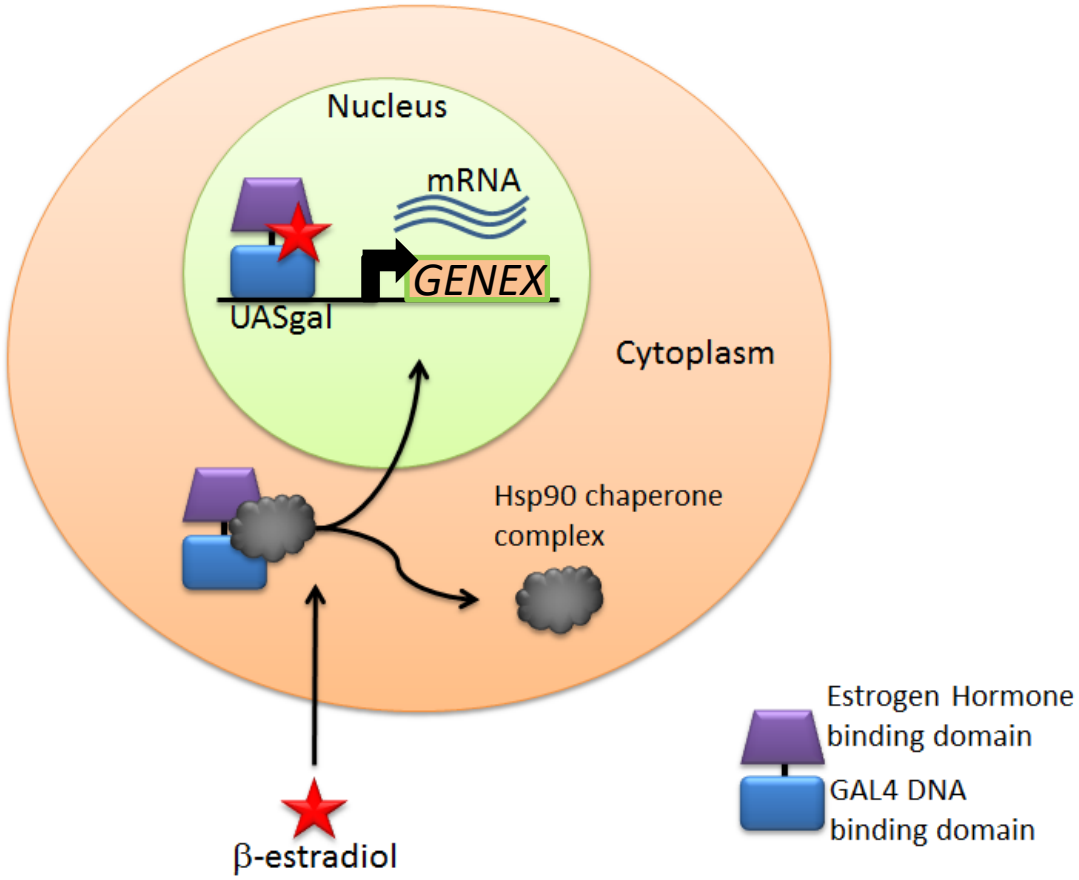


Figure 1-4. Schematic overview of *GENEX-IN* induction using Gal4.ER

Schematic of the Gal4.ER overexpression system in budding yeast. [Adapted from (McIsaac et al., 2011)]. *GAL4.ER* is constitutively expressed from the *GPD1* promoter (Gal4 blue box, ER purple quadrant). Before activation by ED (Red star), Gal4.ER is inactive in the cytoplasm, associating with the Hsp90 chaperone complex (Grey cloud). ED diffuses through the cell membrane and binds to the Gal4.ER estrogen receptor domain, resulting in release of Gal4.ER from the Hsp90 chaperone complex. Gal4.ER then localizes to the nucleus and binds to the upstream activating sequence (UAS) present in the *GAL1* promoter, thereby activating transcription of *GENEX.*

CHAPTER TWO: RESULTS

RAD54-IN is a conditional allele in mitotic and meiotic cells

To determine the timing of *RAD54* function relative to *NDT80* induction, a conditional allele of *RAD54* (*RAD54-IN*) was created that can be transcriptionally induced by the introduction of ED to the sporulation (Spo) medium. *RAD54* is a member of the *RAD52* epistasis group in which mutants are sensitive to the alkylating agent, methyl methanesulfonate (MMS) (Game and Mortimer, 1974; Shinohara et al., 1997b). In the absence of inducer (no ED or galactose), *RAD54-IN* diploids containing either *NDT80* or *NDT80-IN* were sensitive to MMS (Figure 2-1A). To induce *RAD54-IN*, overnight cultures were diluted in YPD and incubated on a roller drum for two hours at 30ºC. ED was added to a final concentration of 1 μM, followed by an additional two hour incubation prior to plating. The cell density in each culture was normalized to an OD_{600} = 2.0, and ten-fold serial dilutions were spotted onto YPD, YPD + 0.04% MMS or YPgalactose + 0.04% MMS. Induction with ED and galactose rescued the MMS sensitivity of *RAD54-IN* but not *rad54*Δ (Figure 2-1A).

In meiosis, *rad54* mutants exhibit a delay in meiotic progression, as well as decreased sporulation and spore viability (Shinohara et al., 1997b). To determine if *RAD54-IN* shares these phenotypes, *RAD54, rad54*Δ*, and RAD54-IN* diploids were grown to log phase and transferred to Spo medium. After three hours, the *RAD54-IN* culture was divided between two flasks, to which either ethanol or ED was added. In the absence of inducer, *RAD54-IN* exhibited a delay in meiotic progression and

decreased levels of sporulation and spore viability equivalent to the *rad54*Δ (Figure 2-1B, C and E). The pattern of spore lethality for both *rad54*Δ and *RAD54-IN* suggests that inviability is not due to Meiosis I non-disjunction, which results in decreased numbers of tetrads with four viable spores and increased numbers of tetrads with 2 and 0 viable spores (Hollingsworth et al., 1995) (Figure 2-1F). Instead increased numbers of tetrads with 1, 2 or 3 viable spores suggest that the spore lethality was due to unrepaired DSBs, similar to what is observed in *mms4*Δ diploids (de los Santos et al., 2001). *RAD54-IN* is therefore a non-functional allele in both mitotic and meiotic cells in the absence of inducer.

Induction of *RAD54* resulted in an increase in spore viability to nearly wild-type levels, with over 80% of the tetrads exhibiting four viable spores (Figure 2-1E and F). Unexpectedly, addition of ED delayed meiotic progression in the *RAD54-IN* diploid (Figure 2-1C). Immunoblot analysis confirmed induction of Rad54 protein (Figure 2-1C). For reasons that are not clear, Ndt80 was not detected by 10 hr in the *RAD54-IN* diploid, even though cells were observed to enter the meiotic divisions (Figure 2-1G). *RAD54-IN* had a strong delay in meiotic progression which may be caused by overexpression of *RAD54* or the addition of ED to meiotic cells at 3 hours. *RAD54-IN* is therefore a conditional mutant in both mitotic and meiotic cells.

Rad54 activity is required after *NDT80* induction

Diploids deleted for *NDT80* arrest in pachytene with fully synapsed chromosomes and unresolved dHJs (Allers and Lichten, 2001; Xu et al., 1995). The *ndt80* arrest therefore provides a potential transition point from IH to IS recombination.

The fact that *RAD54* is not required for IH recombination, yet exhibits reduced sporulation and spore viability, suggests that it may function after *NDT80* induction (Schmuckli-Maurer and Heyer, 2000; Shinohara et al., 1997b). The timing of *RAD54* function relative to *NDT80* induction was therefore tested.

NDT80-IN cells were arrested in prophase by incubating cells in Spo medium for seven hours prior to induction using 1 μM ED. Ndt80 protein was observed 1 hr after induction, at which time cells proceeded relatively synchronously through the meiotic divisions, resulting in ~60% sporulation with >90% viable spores (Figure 2-1B,D and H) (Benjamin et al., 2003). Deletion of *RAD54* in the *NDT80-IN* background exhibited a similar decrease in spore viability as *rad54*Δ (Figure 2-1E). Meiotic progression was delayed in *NDT80-IN rad54*Δ, despite similar timing and levels of Ndt80 protein compared to *NDT80-IN* (Figure 2-1D and H). Co-induction of *NDT80-IN RAD54-IN* rescued the delay in meiotic progression and restored spore viability to nearly wild-type levels in two SK1 diploids constructed using different parents (Figure 2-1D and E). Complementation of the *rad54* sporulation defect was observed in one of these diploids, but not the other (Figure 2-1B, green vs. speckled bars). The difference of sporulation rescue is reflective of sporulation as an insufficient assay for the *NDT80-IN* system due to complications with spore wall formation. The difference in sporulation levels can be attributed to the finicky nature of the *NDT80-IN* inducible system and not a reflection of meiosis (personal communications with Luke Berchowitz). Meiotic progression and spore viability are superior assays to monitor meiosis when using the *NDT80* inducible allele. In conclusion these results collectively demonstrate that *RAD54* functions either

during the *ndt80* arrest or after *NDT80* induction to promote meiotic progression and spore viability.

Mek1 is constitutively active at the *ndt80* arrest

Since Rad54 is an accessory factor for Rad51, the post-*NDT80* requirement for *RAD54* in generating viable spores suggests that Rad51 has a role in recombination after Dmc1 has repaired the bulk of the DSBs by IH recombination (Schmuckli-Maurer and Heyer, 2000; Shinohara et al., 1997b). Mek1 inhibits Rad51-Rad54 complex formation during meiosis in two independent ways: (1) phosphorylation of Rad54 T132 and (2) phosphorylation of Hed1 T40 (Callender et al., 2016; Niu et al., 2009). Therefore activating Rad51 can be achieved by inactivating Mek1. Previous work has suggested that Mek1 is active in *ndt80*Δ-arrested cells. First, Mek1 is activated by phosphorylation of T327 in the T loop of the kinase and this modification is detected on Mek1 isolated from *ndt80*Δ mutants (Niu et al., 2007; Wu et al., 2010). Second, IH JMs have been observed to accumulate at several different hotspots during the *ndt80*Δ arrest, consistent with Mek1 promoting interhomolog bias to the repair of the DSBs that continue to be formed in the absence of *NDT80* (Allers and Lichten, 2001). However, it has recently been proposed that synapsis is the signal that inactivates Mek1 (Subramanian et al., 2016). This idea was based in part, on the observation that phosphorylation of H3 T11, an *in vivo* target of Mek1, decreases when cells are held at the *ndt80*Δ arrest (Govin et al., 2010; Subramanian et al., 2016). Given that the function of this modification is not known, we examined phosphorylation of Hed1 T40 as an alternative Mek1 target known to downregulate Rad51 activity in *dmc1*Δ mutants (Callender et al., 2016).

The *mek1-as* allele encodes an analog-sensitive version of Mek1 that can be inactivated by the addition of the 1-NA-PP1 inhibitor to Spo medium (Wan et al., 2004). A *NDT80-IN mek1-as* diploid was transferred to Spo medium and separated into three flasks. No Mek1-as inhibitor was added to one flask, while 1-NA-PP1 was added either at 0 or 7 hours to the other flasks, respectively. Cells were taken at various time points and protein extracts probed with antibodies to detect various proteins. In the absence of ED, low levels of Ndt80 protein were observed, but these amounts were not sufficient to induce transcription of *CDC5*, as no Cdc5 protein was detected (Figure 2-2A, -ED). Hop1 phosphorylation is an indirect indicator of meiotic DSB formation, as well as being required for Mek1 activation (Carballo et al., 2008; Niu et al., 2005). Phosphorylated Hop1 results in slower migrating species that can be detected on SDS polyacrylamide gels. In the absence of inducer, phosphorylated Hop1 persisted, regardless of whether Mek1 was inhibited (Figure 2-2A). This observation is consistent with the fact that Hop1 phosphorylation occurs prior to Mek1 activation and that DSBs continue to occur at the Ndt80 arrest (Goldfarb and Lichten, 2010).

Total Hed1 levels were constant for up to 12 hours in absence of ED (Figure 2-2A). Probing with an antibody specific for the phosphorylated form of Hed1 T40 revealed Hed1 phosphorylation similarly persists until at least 12 hours. No Hed1 T40 phosphorylation was observed when Mek1-as was inactivated immediately after transfer to Spo medium, consistent with previous experiments demonstrating that Mek1 directly phosphorylates Hed1 T40 (Callender et al., 2016). Inactivation of Mek1-as in the *ndt80*-arrested cells resulted in the disappearance of Hed1 T40 phosphorylation within 1 hour,

proving that constitutive Mek1 activity is needed to maintain Hed1 in the phosphorylated state in pachytene-arrested cells (Figure 2-2A).

NDT80 induction inactivates Mek1 and promotes Red1 and Rec8 degradation

Induction of *NDT80-IN* by the addition of ED resulted in the production of Cdc5 and a loss of Hop1 phosphorylation, suggesting that DSBs have been repaired (Figure 2-2A, +ED). The disappearance of Hed1 T40 phosphorylation similarly correlated with the presence of Cdc5. Hed1 T40 phosphorylation was lost more quickly than total Hed1 protein, suggesting dephosphorylation precedes Hed1 degradation, consistent with previous work showing that a negative charge at Hed1 T40 promotes protein stability (Figure 2-2A)(Callender et al., 2016). Inactivation of Mek1 by *NDT80* induction could be due to degradation of the Mek1 protein, whose disappearance also correlated with the production of Cdc5.

After induction of Ndt80, Hop1 levels slowly decreased, while both Red1 and Rec8 proteins were degraded within two hours (Figure 2-2A, +ED). Hyperphosphorylation of Rec8 was observed, consistent with previous work showing that phosphorylation of Rec8 promotes cleavage by separase at Anaphase I (Brar et al., 2006; Katis et al., 2010). Red1 and Rec8 degradation was not due to the loss of Mek1 kinase activity, because steady state levels of both proteins were unchanged in the *ndt80* arrested cells in which Mek1-as was inactivated by addition of inhibitor (Figure 2-2A, -ED).

Mek1 kinase activity is required before, but not after, *NDT80* induction for the formation of viable spores

Because *MEK1* is required to suppress IS recombination during meiosis, diploids lacking Mek1 activity make dead spores due to chromosome missegregation at MI (Kim et al., 2010; Rockmill and Roeder, 1991; Wan et al., 2004) (Figure 2-2B). Execution point experiments using *mek1-as* have shown that Mek1 kinase activity is required only in prophase to produce viable spores (Wan et al., 2004). To further resolve the timing of Mek1 function in meiotic prophase, an *NDT80-IN mek1-as* diploid was incubated in Spo medium in the presence or absence of 1-NA-PP1 for either 5 or 7 hours. *NDT80* was then induced by the addition of ED and the resulting tetrads dissected to determine spore viability. A *mek1-as* diploid sporulated in the absence of 1-NA-PP1 exhibited 97.8% viable spores, compared to 5.1% in the presence of inhibitor (Figure 2-2B). The inhibitor exhibited no effect on the spore viability of an *NDT80-IN MEK1*, confirming the specificity of the inhibitor for Mek1-as (Figure 2-2B). In contrast, inhibiting Mek1-as immediately after transfer to Spo medium significantly reduced the spore viability when *NDT80* was induced at either 5 or 7 hours compared to the no inhibitor control (χ^2 test, $p<0.0001$) (Figure 2-2B). The spore viability was not as low as the *mek1-as* + I control, however, and increased the longer the cells were arrested prior to *NDT80* induction (Figure 2-2B) suggesting that prolonged time at the *ndt80* arrest allows increasing amounts of IH recombination to occur in the absence of *MEK1*. This result is consistent with physical analyses of JMs in *mek1*Δ *ndt80*Δ diploids that showed that IH JMs increase with time at the *ndt80*Δ arrest (Goldfarb and Lichten, 2010).

Inactivation of Mek1-as at the same time as *NDT80* induction resulted in wild-type levels of viable spores after the 7 hour arrest (χ^2 test, $p<0.142$) (Figure 2-2B). A similar result was observed for the 5 hour arrest, although spore viability was slightly

reduced compared to the no inhibitor control (χ^2 test, $p<0.0001$) (Figure 2-2B). These data support the idea that Mek1-as kinase activity is required prior to *NDT80* induction while the bulk of interhomolog recombination is occurring, but is not required after *NDT80* induction, as expected given that the kinase is naturally inactivated at this time.

The addition of inhibitor at time of induction +I at 7 hr +ED at 7 hr did not effect meiotic progression as compared to no inhibitor –I +ED at 7 hr (Figure 2-2C). In contrast addition of inhibitor at time of transfer to Spo medium + I at 0 hr +ED at 7 hr showed a reproducible slight delay in progression. The delay in meiotic progression may be attributed to an increase in DSBs as indirectly measured by the increase in phospho-Hop1.

Mek1 kinase inactivation by *NDT80* induction does not require IH strand invasion

A previous study proposed that synapsis is the signal for alleviating Mek1 activity to allow any repair (Subramanian et al., 2016). However the fact that Hed1 phosphorylation requires constitutive Mek1 kinase activity in *ndt80*-arrested cells when cells are fully synapsed, suggests that Mek1 activity may be reduced in two stages: first by synapsis and then by Ndt80 induction (Figure 2-2A). To test whether synapsis is a prerequisite for the Mek1 inactivation mediated by Ndt80, Mek1 activity was monitored in *dmc1*Δ diploids after *NDT80-IN* induction. In the absence of *DMC1*, DSBs are made and resected but are not repaired, thereby preventing SC formation and triggering the meiotic recombination checkpoint (Bishop et al., 1992; Lydall et al., 1996; Xu et al., 1997).

Previous work using the BR strain background showed that overexpression of *NDT80* partially suppresses the *dmc1*Δ checkpoint arrest (Tung et al., 2000). This predicted that induction of *NDT80-IN* by ED would also bypass this arrest. However, this was not the case in the SK1 strains used in my experiments. Although high levels of Ndt80 protein were observed in the *NDT80-IN dmc1*Δ *mek1-as* diploid after addition of ED, only a slight increase in meiotic progression was observed. Transcription of *CDC5* and *CLB1* during meiosis is dependent on *NDT80*, but neither protein was detected, indicating that the Ndt80 generated during the checkpoint arrest was not competent for activating transcription (Chu et al., 1998; Chu and Herskowitz, 1998; Clyne et al., 2003) (Figure 2-3A and C). This result is consistent with Ndt80 activity being post-transcriptionally regulated by the meiotic recombination checkpoint by sequestration of Ndt80 protein in the cytoplasm (Wang et al., 2011). Hed1 T40 phosphorylation and Mek1 protein persisted, confirming that active Ndt80 is necessary to inactivate Mek1.

Inhibition of Mek1-as in *dmc1*Δ diploids results in IS repair of DSBs, thereby allowing meiotic progression by eliminating the unrepaired DSBs that trigger the checkpoint (Niu et al., 2005; Wan et al., 2004). Similarly, when Mek1-as was inhibited either immediately after transfer to Spo medium or at the time of *NDT80* induction, both Cdc5 and Clb1 were observed, Hed1 T40 phosphorylation disappeared, and cells progressed efficiently through the meiotic divisions (Figure 2-3A and C). The robust checkpoint inhibition of Ndt80 activity precluded testing whether *NDT80* induction inactivates Mek1 in the absence of *DMC1* in this diploid.

The meiotic recombination checkpoint requires the PCNA-like "9-1-1" clamp encoded by the *DDC1*, *RAD17* and *MEC3* genes (Lydall et al., 1996; Subramanian and Hochwagen, 2014). To eliminate the checkpoint inhibition of Ndt80 in the *dmc1*Δ background, *RAD17* was deleted from the *NDT80-IN dmc1*Δ *mek1-as* strain. In the absence of *RAD17*, addition of ED resulted in the production of transcriptionally active Ndt80, with both Cdc5 and Clb1 proteins appearing within an hour after Ndt80 protein was observed (Figure 2-3D). As was true for the *DMC1* diploid, the timing of Cdc5 production correlated with dephosphorylation of Hed1 T40, indicating that Mek1 was inactivated (Figure 2-3D). Therefore *NDT80*-dependent inactivation of Mek1 does not require IH recombination or synapsis.

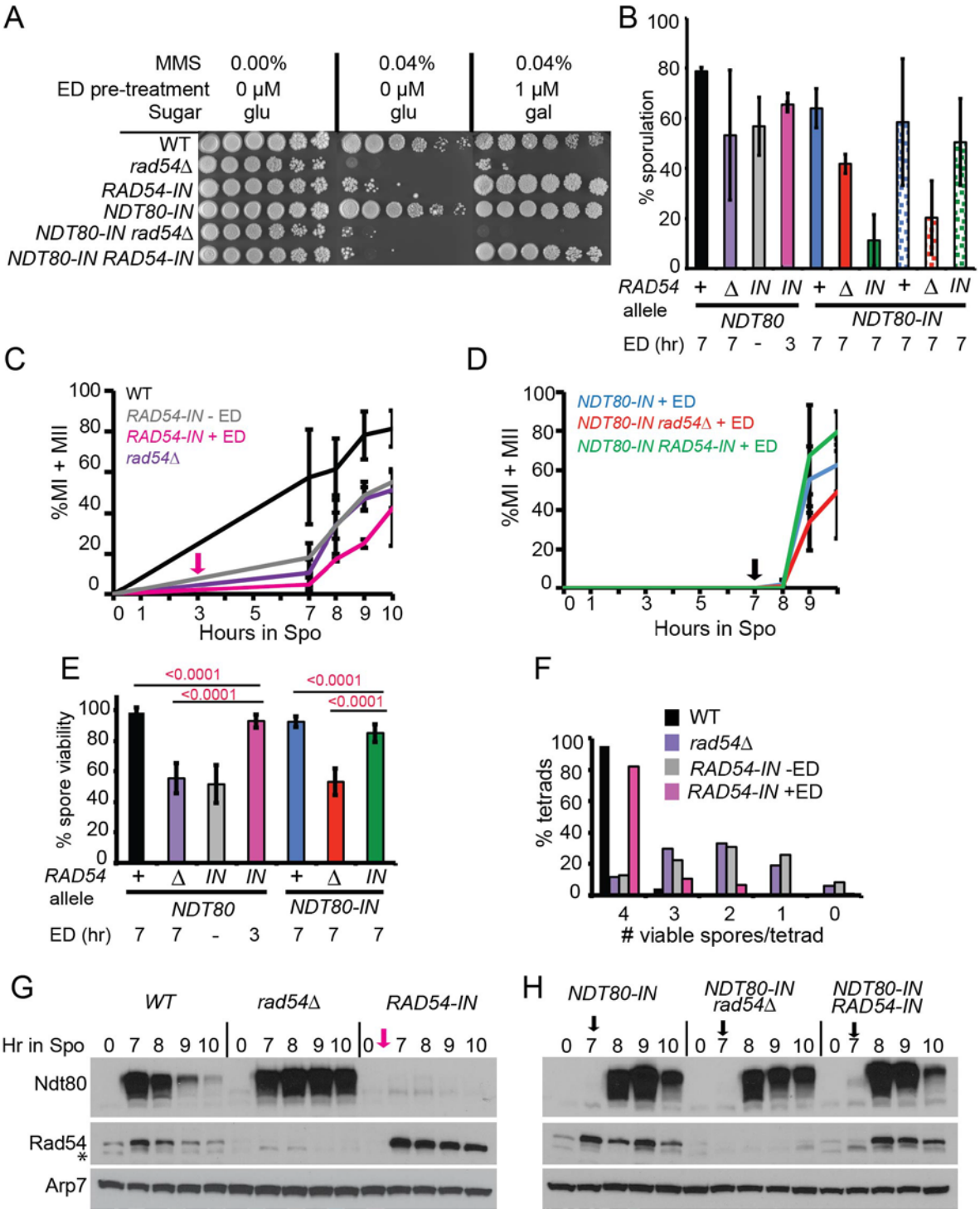
NDT80-dependent inactivation of Mek1 promotes DSB repair in *dmc1*Δ mutants

Induction of *NDT80* in the *dmc1*Δ *rad17*Δ background abolished Hop1 phosphorylation, suggesting that DSBs were repaired (Figure 2-3D). Alternatively, the loss of Hop1 phosphorylation could be the consequence of the loss of Red1. To test this more directly, Southern blot analysis was performed using cells from the same time course to look at repair of DSBs at the *HIS4/LEU2* hotspot. This hotspot is flanked by XhoI restriction sites and DSBs can therefore be detected by probing XhoI digested DNA (Hunter and Kleckner, 2001; Oh et al., 2009). In the *NDT80-IN dmc1*Δ *mek1-as* diploid, DSBs were unrepaired both before and after *NDT80* induction, consistent with Mek1 remaining active (Figure 2-4A). Artificially inactivating Mek1-as by the addition of inhibitor either at 0 hr or 5 hr resulted in repair of the breaks (Figure 2-4A). In contrast, in the *NDT80-IN dmc1*Δ *mek1-as rad17*Δ strain, DSBs persisted in the absence of ED, but disappeared upon expression of *NDT80* (Figure 2-4B, 4C). Therefore induction of

NDT80 in the d*mc1*Δ *rad17*Δ background results in inactivation of Mek1 and Rad51-mediated repair of DSBs.

CDC5 induction is sufficient to inactivate Mek1

Ndt80 activates the transcription of >200 genes, one of which encodes the polo-like kinase, Cdc5, which is required for JM resolution and SC disassembly (Clyne et al., 2003). *CDC5* is the sole Ndt80 target responsible for these phenotypes as ectopic expression of *CDC5* in the *ndt80*Δ background is sufficient for JM resolution and SC disassembly (Sourirajan and Lichten, 2008). To see whether *CDC5* is the sole Ndt80 target responsible for inactivation of Mek1, a diploid homozygous for *ndt80*Δ and containing an inducible allele of *CDC5* (*CDC5-IN*) was constructed. In the absence of ED, a slow decline in Hed1 T40 phosphorylation was observed while Hed1 and Red1 steady state levels remained fairly constant (Figure 2-5A). In contrast, induction of *CDC5* results in loss of both Red1 and Hed1 T40 phosphorylation, as well as a decrease in total Hed1 protein levels. To test directly if *CDC5* induction resulted in DSB repair, DNA from the same time course was digested with BglII and probed to detect breaks at the *YCR048w* hotspot (Callender and Hollingsworth, 2010). In the samples without *CDC5* induction, DSBs persist, however, upon addition of +ED the DSBs disappeared (Figure 2-5B). Quantification for the DSBs Southern blots (Figure 2-5C). In addition to these data, Michael Lichten via personal communication has data showing induction of *CDC5* at a different hotspot is able to repair DSBs. Therefore *CDC5* is the gene responsible for inactivating Mek1 and enabling DSB repair.


A
MMS
ED pre-treatment
Sugar
0.00%
0 µM
glu
0.04%
0 µM
glu
0.04%
1 µM
gal
WT
rad54Δ
RAD54-IN
NDT80-IN
NDT80-IN rad54Δ
NDT80-IN RAD54-IN
B
% sporulation
RAD54 allele
NDT80
NDT80-IN
ED (hr)
C
%MI + MII
WT
RAD54-IN - ED
RAD54-IN + ED
rad54Δ
Hours in Spo
D
%MI + MII
NDT80-IN + ED
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RAD54 allele
NDT80
NDT80-IN
ED (hr)
F
% tetrads
WT
rad54Δ
RAD54-IN -ED
RAD54-IN +ED
viable spores/tetrad
G
WT
rad54Δ
RAD54-IN
Hr in Spo
Ndt80
Rad54*
Arp7
H
NDT80-IN
NDT80-IN rad54Δ
NDT80-IN RAD54-IN

Figure 2-1. *RAD54* functions after *NDT80* induction to promote meiotic progression and the formation of viable spores. (A) MMS sensitivity. Wild-type (NH144), *rad54*Δ (NH2136), *RAD54-IN* (NH2319), *NDT80-IN* (NH2127), *NDT80-IN rad54*Δ (NH2126), and *NDT80-IN RAD54-IN* (NH2185) diploids were grown to log phase in YPD. For one set of strains, β-estradiol (ED) was added to a final concentration of 1 μM for two hours prior to plating. Ten-fold serial dilutions were spotted onto YP plates containing either 2% glucose (glu) or galactose (gal) with or without 0.04% MMS as indicated. (B) Sporulation. Wild-type (NH144), *rad54*Δ (NH2136), *RAD54-IN* (NH2319) –ED or +ED, *NDT80-IN* (NH2127 and NH2232), *NDT80-IN rad54*Δ (NH2126 and NN2240), and *NDT80-IN RAD54-IN* (NH2185 and NH2245) were transferred to sporulation medium. For the *RAD54-IN* and the *NDT80-IN* containing strains, a final concentration of 1 μM ED was added at the indicated timepoints. The *NDT80-IN* containing strains were constructed using two different sets of SK1 parents, one set is shown with solid colors while the other is shown using stippled colors. The averages of at least 7 biological replicates are plotted with error bars indicating the standard deviations. (C) Meiotic progression in *NDT80* diploids. WT, *rad54*Δ, and *RAD54-IN* diploids were transferred to Spo medium and incubated at 30ºC. After 3 hours the *RAD54-IN* culture was split with a final concentration of 1 μM ED added to one of the *RAD54-IN* culture (indicated by pink arrow). Meiotic progression was assayed using fluorescent microscopy of fixed DAPI-stained nuclei to determine the percentage of binucleate (MI) and tetranucleate (MII) cells. Two hundred cells were counted for each strain at each time point. The average values from three biological replicates are shown. Error bars indicate the standard deviations. (D) Meiotic progression in *NDT80-IN* diploids. ED was added at 7 hours (black arrows) and progression was monitored as in B. (E) Spore viability. ED was added to a final concentration of 1 μM at the indicated timepoints. Lines indicate the *p* values using a χ^2 test. *p* values in red are statistically significant. Average values are shown with error bars indicating the standard deviations. For the *NDT80-IN* strains two independently constructed diploids using different SK1 parents exhibited similar results and therefore the data were combined. For each strain the number of biological replicates: number of tetrads is: NH144 (3:120), NH2136 (4:162), NH2319 -ED (7:185), NH2319 +ED at 3 hr (7:172), NH2127 + NH2232 (14:524), NH2126 + NH2240 (10:423), NH2185 + NH2245 (10:318). (F) Distribution of viable spores in tetrads for WT, *rad54*Δ, *RAD54-IN* without and with ED from the dissections presented in Panel B. (G) Induction of Rad54 and Ndt80 in *NDT80* strains was monitored by immunoblots. Protein extracts from one of the timecourses shown in panel C were probed with antibodies against Ndt80 and Rad54. The asterisk indicates a non-specific band. Arp7 was used as a protein loading control (Sourirajan and Lichten, 2008). The pink arrow indicates addition of ED at 3 hours after transfer to Spo medium. (H) Induction of Rad54 and Ndt80 in *NDT80-IN* diploids analyzed as in Panel G. The black arrows indicate addition of ED at 7 hours after transfer to Spo medium. The timecourses in Figures G and H were performed three times with similar results.

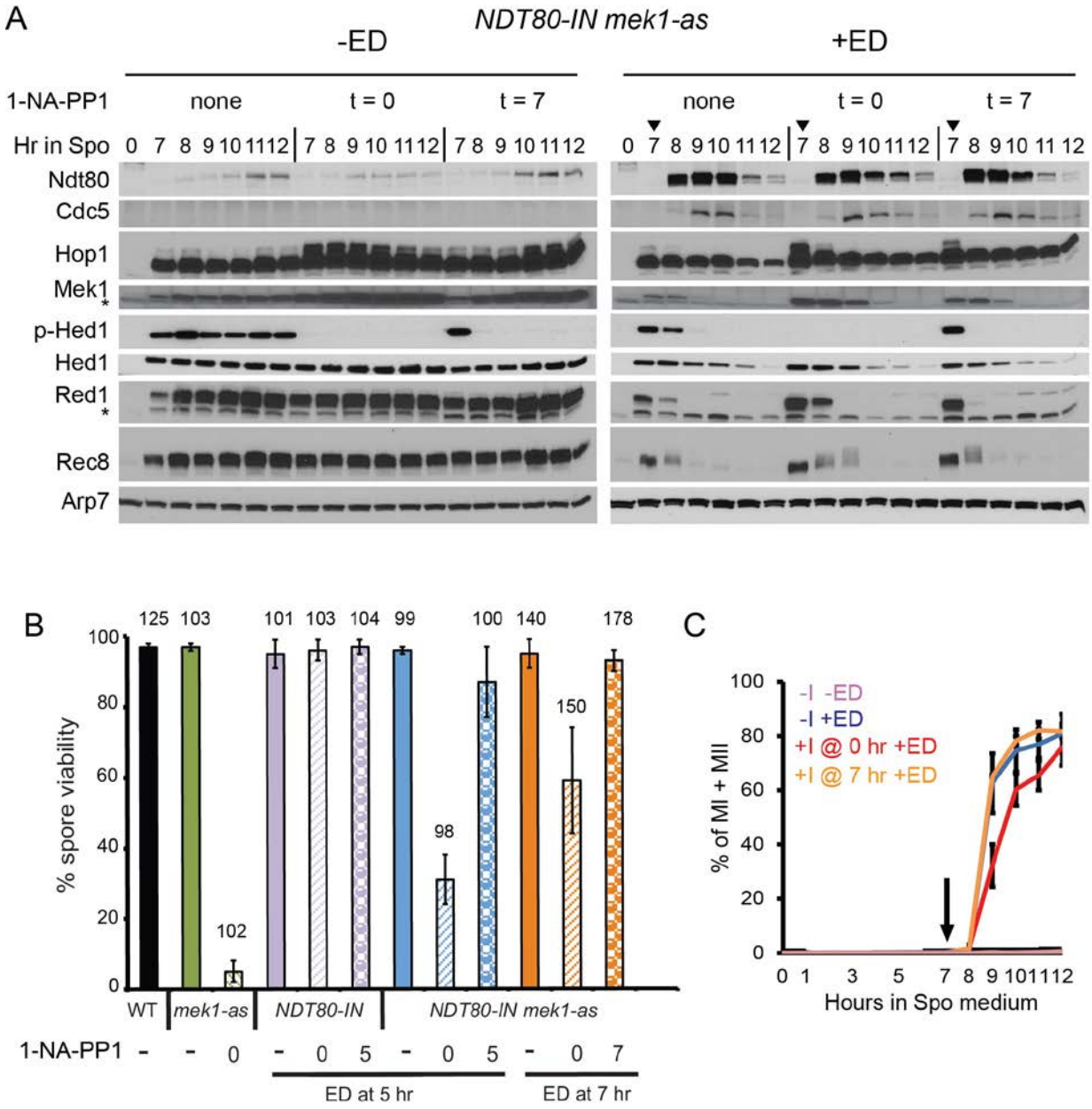

Figure 2-2. Mek1 inactivation resulting from *NDT80* induction correlates with the presence of Cdc5. (A) The *NDT80-IN mek1-as* diploid, NH2122::pJR2, was transferred to Spo medium and incubated for seven hours at which time the culture was split in half, and either DMSO (-ED) or a final concentration of 1 μM ED (+ED) was added at 7 hours (indicated by arrowheads). The Mek1-as inhibitor, 1-NA-PP1 was added at a final concentration of 1 μM at the indicated times. Protein extracts were then probed with antibodies against Ndt80, Cdc5, Hop1, Mek1, phospho-Hed1 threonine 40 (p-Hed1), Hed1, Red1, Rec8 and Arp7 as a loading control. Asterisks indicate non-specific bands. Three biological replicates of this experiment gave similar results. (B)

Spore viability. Wild-type (NH144), *mek1-as* (NH729::pJR2), *NDT80-IN* (NH2033), and *NDT80-IN mek1-as* (NH2122::pJR2) were transferred to Spo medium. *NDT80* was induced using 1 μM ED at the indicated times and Mek1-as was inhibited using 1-NA-PP1 at the indicated times. The resulting tetrads were dissected to determine the percent viable spores. The averages of at least three biological replicates are shown with error bars indicating the standard deviation. (C) Meiotic Progression. *NDT80-IN mek1-as* diploid, NH2122::pJR2, was transferred to Spo medium and incubated for seven hours at which time the culture was split in half, and either DMSO (-ED) or a final concentration of 1 μM ED (+ED) was added at 7 hours (indicated by arrowhead). The Mek1-as inhibitor, 1-NA-PP1 was added at a final concentration of 1 μM at the indicated times. Meiotic progression was assayed using fluorescent microscopy of fixed DAPI-stained nuclei to determine the percentage of binucleate (MI) and tetranucleate (MII) cells. Two hundred cells were counted for each strain at each time point. The average values from three experiments were plotted with error bars to indicate the range.

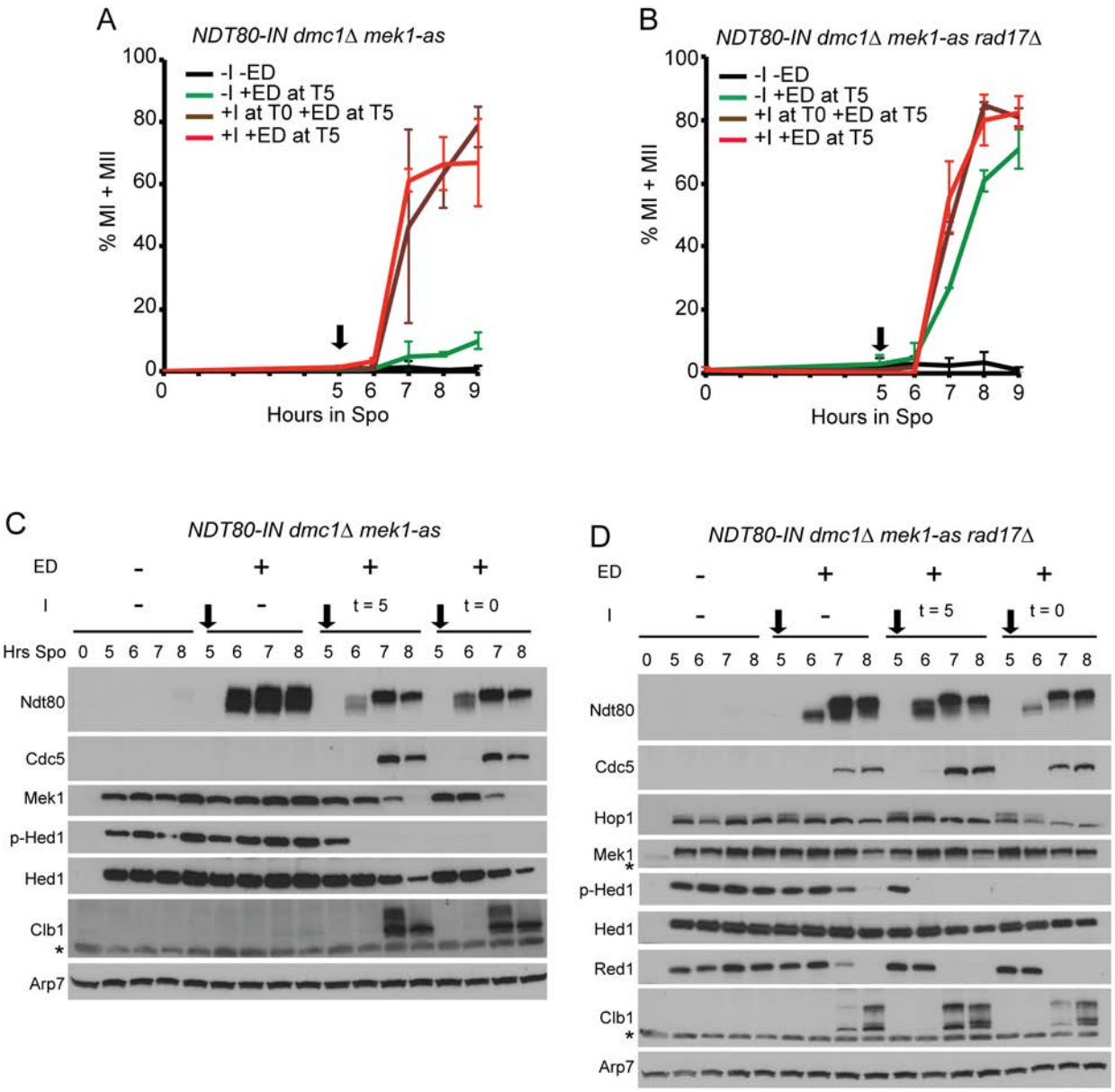

Figure 2-3. *NDT80*-dependent inactivation of Mek1 is independent of strand invasion and synapsis. Meiotic progression. (A) The *NDT80-IN dmc1Δ mek1-as* diploid (NH2278) was transferred to Spo medium for 5 hours and a final concentration of 1 μM 1-NA-PP1 and/or ED were added at the indicated times. Throughout black arrows indicate the time of ED addition. Meiotic progression was assayed using fluorescent microscopy of fixed DAPI-stained nuclei to determine the percentage of binucleate (MI) and tetranucleate (MII) cells. Two hundred cells were counted for each strain at each time point. The average values from two experiments were plotted with error bars indicated the range. (B) Meiotic progression in the *NDT80-IN dmc1Δ mek1-as rad17Δ* diploid (NH2365) was analyzed as in Panel A. (C) Immunoblot analysis of various proteins from extracts of cells taken from one of the *NDT80-IN dmc1Δ mek1-as* timecourses shown in Panel A. Asterisks indicate non-specific bands. Two biological replicates of this experiment gave similar results. (D) Similar to Panel C using extracts from one of the *NDT80-IN dmc1Δ mek1-as rad17Δ* timecourses shown in Panel B.

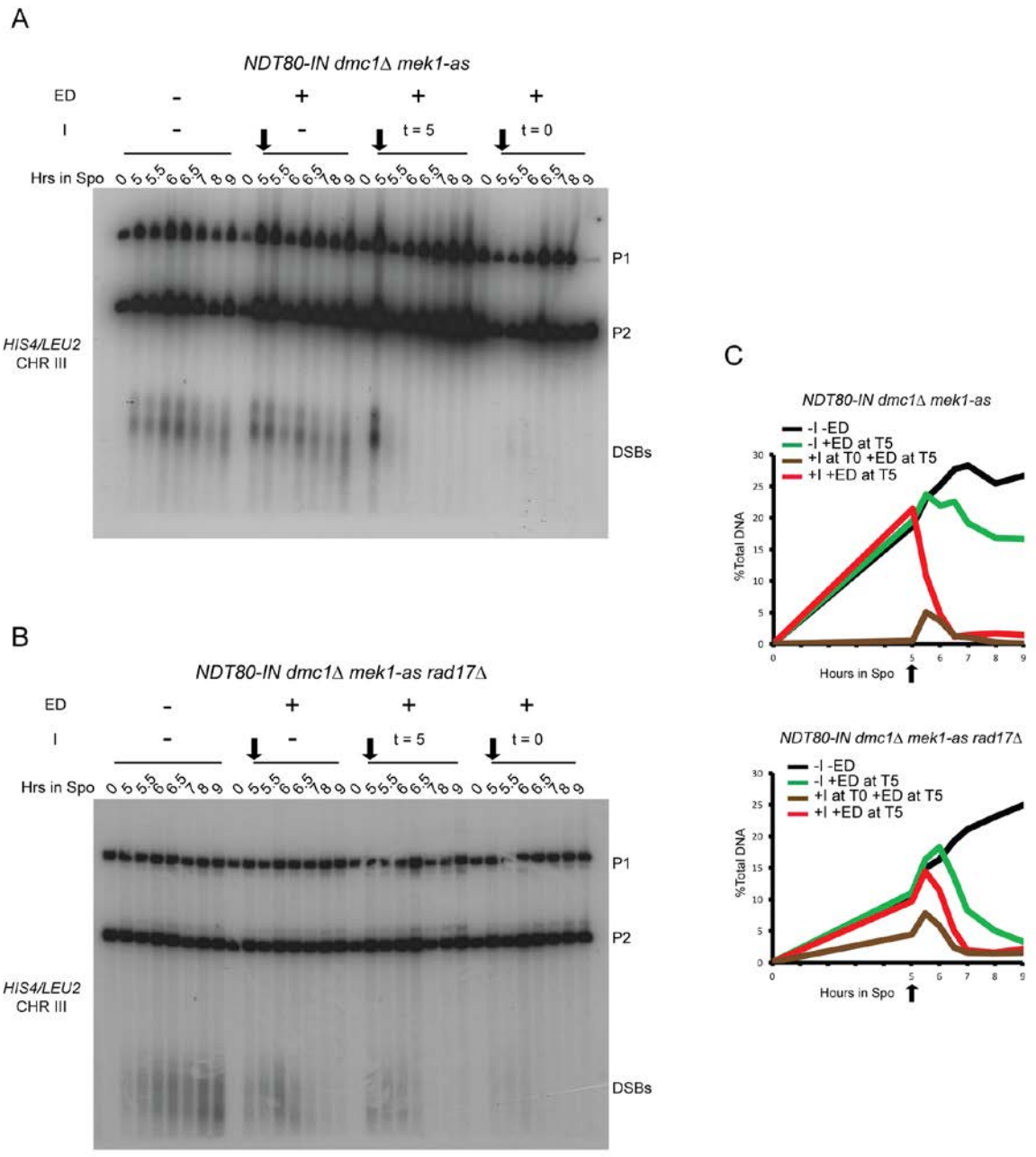

Figure 2-4. Physical analysis of DSBs in *NDT80-IN* inducible diploids at the *HIS4/LEU2* hotspot on chromosome III. (A) The *NDT80-IN dmc1Δ mek1-as* diploid (NH2278) was transferred to Spo medium for 5 hours and a final concentration of 1 μM 1-NA-PP1 and/or ED were added at the indicated times. Black arrows indicate ED addition. DSBs detected by XhoI digestion of genomic DNA was probed with a fragment to detect the *HIS4::LEU2* hotspot located on chromosomes III and was created by insertion of a 2.8kb LEU2 segment adjacent to the *HIS4* locus in the SK1 strain background. Parental bands are indicated P1 and P2. To detect the *HIS4::LEU2*

hotspot , a 0.6 kb gel purified AgeI/BglII fragment from pNH90 was used as a probe (Hunter and Kleckner, 2001) (B) *NDT80-IN dmc1*Δ *mek1-as rad17*Δ diploid (NH2365) was analyzed as in Panel A. (C) Graphs indicate the percent of total DNA constituted by the DSB fragment.

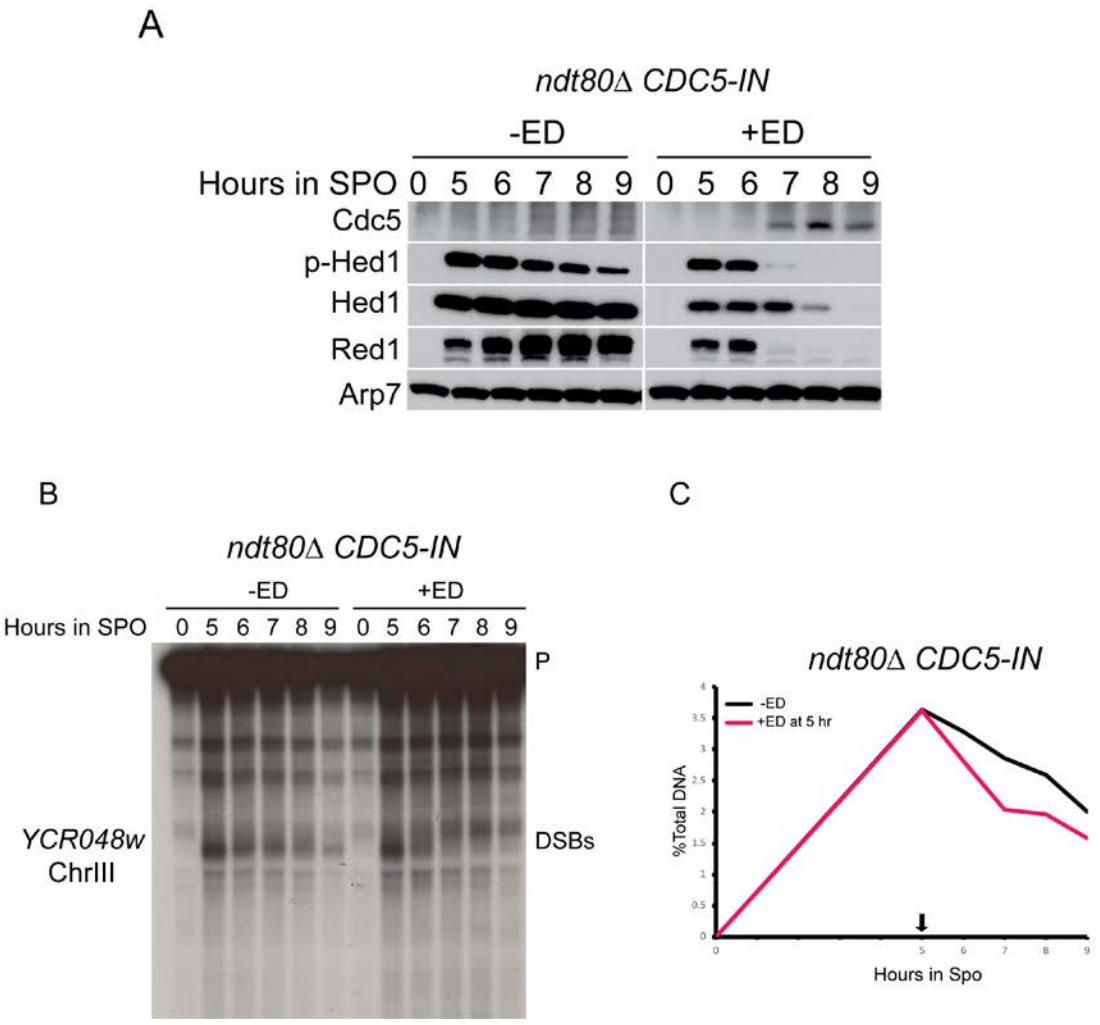

Figure 2-5. *CDC5* is sufficient to inactivate Mek1 in *ndt80Δ* arrested cells. The *CDC5-IN ndt80Δ* diploid, NH2296 was transferred to Spo medium and incubated for five hours at which time the culture was split in half, and either DMSO (-ED) or a final concentration of 1 μM ED (+ED) was added at 5 hours. (A) Protein extracts were then probed with antibodies against, Cdc5, phospho-Hed1 threonine 40 (p-Hed1), Hed1, Red1, and Arp7 as a loading control. I performed this experiment once but it has been repeated >3 times by other people in the Hollingsworth lab. (B) DSBs monitored by Bgl II digestion of genomic DNA was probed with a fragment to detect the *YRC048w* hotspot located on chromosomes III, and detected by a 0.9kb HindIII fragment from pME1210 (Wu and Lichten, 1994). Parental band indicated by P. Southern blot analysis was performed by Dr. Xiangyu Chen. (C) Graphs indicate the percent of total DNA constituted by the DSB fragment.

CHAPTER THREE: METHODS

Media

YPD plates contain 1% yeast extract, 2% peptone, 2% dextrose, and 2% agar, while YPGAL plates have 2% galactose in place of glucose. YPglycerol plates contain 1% yeast extract, 2% peptone, 3% glycerol (w/v), and 2% agar. Dropout plates contain 0.7% yeast nitrogen base, 2% glucose, 2% agar and 2 g of the appropriate dropout powder per liter (for formulation of the dropout powder see (Lo and Hollingsworth, 2011)). YPA is 1% yeast extract, 2% peptone, and 2% potassium acetate and liquid Spo medium is 2% potassium acetate (K0Ac). Spo plates contain 0.1% yeast extract, 0.5% dextrose, 1% potassium acetate, and 2% agar. 10% Methyl methanesulfonate (MMS) (Sigma 129925) was added to YPD medium at a final concentration of 0.04% immediately prior to pouring the plates. Plates were left in the hood with the lid slightly ajar under aluminum foil overnight and used the next day. Sodium thiosulfate (5%) (Sigma 217247) was used to inactivate the MMS by soaking overnight.

Plasmids

The plasmid, pKB80 contains *pGPD-GAL4.ER* in a *URA3* integrating plasmid (Benjamin et al., 2003). The fusion gene was moved into a *TRP1* integrating plasmid in two steps. First, site-directed mutagenesis of pRS304 was used to change T to C at basepair 529 of the *TRP1* open reading frame (ORF) in pRS304 to generate pEP102. This mutation creates an NheI site, but does not alter the amino acid specified by the codon (Leu 178). Second, a fragment containing *pGPD-GAL4.ER* and engineered to

have NsiI and KpnI ends was amplified by PCR using pKB80 as template and cloned into NsiI/KpnI-digested pEP102 to create pEP105. This plasmid can be targeted to integrate at the *trp1-5'*Δ locus using NheI. To enable making *GAL1* promoter fusions using *hphMX4* as the selectable marker, the*GAL1* promoter region from pFA6a-kanMX6-P.was amplified and digested with BglII and PacI and subcloned into pAG32 to make pEP104 (Goldstein and McCusker, 1999; Longtine et al., 1998). pJR2 is a mek1-as URA3 integrating plasmid (Callender et al., 2016).

Chromosome III hotspot probes were derived from pME1210 (*YCR048w)* (Wu and Lichten, 1994), and pNH90 (*HIS4::LEU2)* (Hunter and Kleckner, 2001).

Yeast strains

The complete genotypes of all strains used in this work can be found in Table 3-1. Strains are derivatives of the fast sporulating SK1 background and genetic experiments were performed at 30ºC unless otherwise noted. Gene deletions and *GAL1* fusions were created by polymerase chain reaction (PCR)-based methods using *kanMX6*, *natMX4*, and *hphMX4* which confer resistance to G418, nourseothricin, and Hygromycin B, respectively (Goldstein and McCusker, 1999; Longtine et al., 1998; Tong and Boone, 2006). In addition, some genes were deleted using the *Kluyveromyces lactis URA3* gene as a selectable marker (pKlU, Aaron Neiman, Stony Brook University). Unless stated otherwise, deletions and gene fusions were confirmed by PCR. Deletions of *RAD54* were also confirmed phenotypically by sensitivity to 0.04% MMS.

To make the *RAD54-IN* diploid, NH2319, the *GAL1* promoter was fused to the *RAD54* ORF in 14154, thereby creating 14154 RAD54-IN. This haploid was then mated to SKY371 and a *MAT*α, Ura$^+$ (*ura3::P$_{GPD1}$-GAL4(848).ER::URA3*), Trp$^-$ (*NDT80*), G418^R (*kanMX6-P$_{GAL1}$-RAD54*) segregant was crossed to RKY1145::pKB80 rad54Δ. RKY1145::pKB80 rad54Δ was generated by first integrating pKB80 digested with NheI at the ura3 locus to introduce *P$_{GDP}$-GAL4(848).ER*, followed by deletion of *RAD54* with *natMX4*.

The *NDT80-IN* diploid, NH2127, was derived from mating segregants from a cross between 14154 and 28417, while the *NDT80-IN rad54*Δ diploid, NH2126, was made by mating segregants from a cross between 14154 rad54 and 28417. The *MAT*α parent of NH2126, NH2111-12-3, was crossed to 14154 RAD54-IN to make the *NDT80-IN RAD54-IN* diploid, NH2185. The *NDT80-IN* diploid, NH2033, was created by mating 14154 with S2683 ndt80. To create an isogenic diploid containing mek1-as, *MEK1* was deleted using *natMX4* in 14154 and S2683 ndt80. The S2683 ndt80 mek1 haploid was then transformed with pJR2 digested with RsrII to target integration downstream of the *MEK1* ORF and mated to 14154 mek1 to make NH2122::pJR2.

The *CDC5-IN ndt80*Δ diploid, NH2296, was created by mating S3363 with RKY1145 ndt80::hph. Diploids were selected for Arg$^+$ is$^+$ and being non-maters. S3363 strain is *ndt80Δ::hphMX4* haploid carrying the *GAL4.ER* gene with a plasmid pMJ830 carrying *P$_{GAL1}$-CDC5* integrated at the *CDC5* locus (a gift provided by Michael Lichten, National Cancer Institute).

The *NDT80-IN*, *NDT80-IN rad54*Δ and *NDT80-IN RAD54-IN* genotypes were also introduced into isogenic diploids containing the *HIS4-LEU2* hotspot as follows. Because the NHY1210 and NHY1215 haploids contain a deletion of *URA3*, thereby preventing integration of the *URA3 GAL4-ER* plasmid, the *TRP1 GAL4-ER* integrating plasmid, pEP105, was used instead. To allow use of *TRP1* as a selectable marker, the first 222 bp of the *TRP1* ORF were substituted with *natMX4* to make NHY1210 trp1 and NHY1215 trp1 (Chen et al., 2015). The *GAL1* promoter was fused to *NDT80* using pFA6a-kanMX6-pGAL1 in both and the strain was then transformed with pEP105 digested with NheI (Goldstein and McCusker, 1999; Longtine et al., 1998). The resulting haploids, NHY1210 trp1 P_{GAL1}-NDT80::pEP105 and NHY1215 trp1 P_{GAL1}-NDT80::pEP105, were mated to make NH2232. The *rad54*Δ derivative was created by deleting *RAD54* with *hphMX4* in the parents of NH2232, and mating to create NH2240. For the *NDT80-IN RAD54-IN* diploid, a *GAL1-RAD54* fusion was generated in the genome using pEP104 NHY1210 trp1 NDT80-IN::pEP105 which was then mated to NHY1215 trp1 NDT80-IN::pEP105 to make NH2245.

To make the *NDT80-IN mek1-as* diploid, NH2290, *MEK1* was first deleted using *hphMX4* from NHY1210 trp1 P_{GAL1}-NDT80::pEP105 and NHY1215 trp1 P_{GAL1}-NDT80::pEP105. The *mek1-as URA3* plasmid, pJR2 was digested with RsrII to target integrate downstream of the *mek1*Δ deletion and transformed into each haploid (Callender and Hollingsworth, 2010). The pJR2 was then popped out using 5'-FOA to select for Ura^- candidates (Boeke et al., 1987). $5\text{-}FOA^R$ colonies were screened for sensitivity to HygB to identify those popouts in which the *mek1-as* allele remained in the chromosome. The resulting haploids were crossed to make NH2290. For the *dmc1*Δ

NDT80-IN mek1-as diploid, NH2278, the second exon of *DMC1* was deleted using *hphMX4* from NHY1210 trp1 mek1-as P_{GAL1}-NDT80::pEP105 and NHY1215 trp1 mek1-as P_{GAL1}-NDT80::pEP105 and the resulting haploid were mated. The *dmc1*Δ *NDT80-IN mek1-as rad17*Δ diploid, NH2365 was created by deleting *RAD17* from NHY1210 trp1 dmc1 mek1-as P_{GAL1}-NDT80::pEP105 and NHY1215 trp1 dmc1 mek1-as P_{GAL1}-NDT80::pEP105 using *K. lactis URA3* and the resulting haploids were mated.

MMS sensitivity assay

Single colonies for each strain were inoculated into three ml YPD and grown overnight on a roller at 30ºC. The OD_{600} of each culture was measured and cells were diluted to an OD_{600} of 0.6 in two ml YPD in two test tubes. The cells were incubated on a roller drum at 30ºC for 2 hours, at which time 5 mM ED was added to a final concentration of 1 μM to one tube for each pair, and an equal volume of 100% ethanol was added to the other tube. After two more hours at 30ºC, the OD_{600} was determined, and the volume of cells equivalent to 2 OD_{600} units from each culture was transferred to 1.5 ml microcentrifuge tubes. Cells were pelleted by centrifugation at 13,000 rpm for one minute at room temperature (RT) and then washed with one ml of sterile deionized distilled water (ddH_2O). The pellets were resuspended in 100 μL ddH_2O and tenfold serial dilutions were spotted onto YPD, YPD+0.04% MMS, and YPGAL+0.04% MMS plates. The YPD plates were incubated for one day at 30ºC, while the YPD+0.04% MMS, and YPGAL 0.04%MMS were incubated for two days at 30ºC before scoring.

Meiotic Time Courses

The protocol for sporulation was adapted from (Lo and Hollingsworth, 2011) as follows. All experiments were carried out at 30ºC. Diploids were inoculated into five ml of YPD in 15 ml test tubes and put on a roller drum for 24 hours. A portion of each colony was patched to YPGlycerol plates to ensure that the colonies were competent for respiration (Lo and Hollingsworth, 2011). In addition, these patches were replica plated to Spo medium for one day to check for sporulation and spore viability in *NDT80* diploid strains. For liquid sporulation, YPD overnight cultures were diluted into YPA (1/300 and 1/500) in Erlenmeyer flasks. For cultures in both YPA and Spo medium, the ratio of the volume of cells to air was always at least 1:5, and preferably 1:10. Immediately after dilution, the cell density of each culture was determined. Typically the 1/300 and 1/500 dilutions exhibited OD_{660} values of 0.04 and 0.03, respectively. The YPA cultures were then grown for 16 ~ 18 hours on a shaker at 250 rpm until the OD_{660} was between1.5 to 1.8. Cells were harvested using a GSA rotor in a Sorvall RC-5B centrifuge at an angular velocity of 5000 revolutions/minute (rpm) for ten minutes. Each pellet was resuspended in 100 ml sterile ddH2O and centrifuged again under the same conditions as before. The resulting pellets were resuspended in liquid Spo medium (2% K0AC) to a cell density of 3 x 10^7/ml using this formula [((#cells/2)xYPA culture volume in ml)/3x10^7 cells/ml] (Table 3-2) in an Erlenmeyer flask (typically 200 ml culture/2L flask or 100 ml culture/1L flask and incubated with shaking at 250 rpm. To induce transcription of the *RAD54-IN*, *NDT80-IN* and *CDC5* alleles ED from a 5 mM stock was added to a final concentration of 1 µM at the indicated times. Cells at various timepoints were taken and processed as follows. For meiotic progression, 0.5 ml of

culture was fixed in 3.7% formaldehyde and stored at 4ºC. The cells were vortexed for approximately 15 seconds. Cells were subsequently spotted onto wells of 5 μl of 1 X PBS on lysine coasted slides (Carlson Scientific, #101204). The slides were left at room temperature for 5 minutes to allow the cells to settle to the bottom of the wells. After 5 minutes the PBS solution was removed by aspiration. The cells were washed three times using 1X PBS. After the final wash the slide was left at RT for 10 minutes to allow for the cells to air dry. 1 μl of mounting medium containing 1.5 μg/ml 4,6-diamidino-2-phenylindole (DAPI) (Vector, #H-1200) was added to each well. A 24 x 60 mm cover slip was placed on the slide. The weight of the cover slip allowed for the DAPI solution to spread within the wells. Clear nail polish (Sally Hansen Hard as Nails bought from Walgreens) was used to seal the cover slip to the slide. The number of bi-nucleate (Meiosis I) and tetranucleate (Meiosis II) cells was determined using fluorescence microscopy using and Axioskop 2 Plus that has attached the AxioCAm MRM camera which works with Zeiss imagining software ZEN. The 40 X objective lenses was used with a 75 ms exposure time for the camera.

For protein analysis, five ml of culture were harvested using a swinging bucket rotor Beckman Coulter Allegra X-15R centrifuge at 3,000 rpm for 1 minute at 4ºC. The cells were washed once with one ml ddH_2O and the pellets resuspended in one ml 20% Trichloroacetic acid (TCA) (Sigma 91228). After pelleting the cells by centrifugation as before, each pellet was weighed and resuspended in 200 μl 20% TCA and stored at -80ºC. For Southern blot analysis of DSBs, ten ml of sporulating culture was transferred to a 50 ml conical tube containing 10 ml 100% ethanol and 2 ml 0.5 M EDTA and stored at -20ºC. Sporulation of each culture was scored after 24 hours by light microscopy

using a Zeiss AxioScope microscope. Two hundred cells were counted for each time point.

For tetrad dissection 100 μl of Spo culture was added to 1 ml sterile $_{dd}H_2O$ in a 1.5 ml microcentrifuge tube. The tube was gently mixed by inverting approximately 5 times. 3 μl of 10 mg/ml zymolyase was added and the cells incubated at 37ºC for 15 minutes and then placed on ice. 10 μl from the tube was dropped onto a thin YPD plate with the plate at an angle to allow for the formation of a streak down the center of the plate. Tetrads were dissected using Zeiss tetrad dissecting microscope and the spores incubated at 30ºC for approximately 3 days.

Physical analysis of DSBs

Physical analysis was performed using method described in (Oh et al., 2009). DNA was isolated using the MasterPure Yeast DNA Purification kit (Epicentre, Cat. #MPY80200). DNA was digested with XhoI or BglII, and probed after fractionation on a one-dimensional gel. DSBs were quantified using the Multi-Gauge Software with a FujiFilm FLA 7000 Phosphoimager.

Western blots

TCA (Sigma 91228) protein extracts were prepared from five ml of sporulating culture as described in (Acosta et al., 2011). Protein gels were hand cast using TGX FastCast acrylamide gel solutions as described by the manufacturer (7.5% or 12%, BioRad #1610171 and #1610175, respectively). Protein samples were fractionated using sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a

Criterion Cell midi-format vertical electrophoresis cell (BioRad #1656001) with a PowerPac Basic power supply (BioRad #1645050) at a constant voltage of 200V for 40 minutes. Proteins were transferred to polyvinylidine fluoride (PDVF) membrane (Millipore #IPVH00010) using a Criteron Blotter with Plate Electrodes (BioRad #1704070).

Antibodies

Primary antibodies were incubated overnight between 16 hr to 20 hrs at 4ºC, except α-Ndt80 and α-Hop1 which were incubated at RT for two hours. Secondary antibodies were incubated at RT for 1 hour. Antibody dilutions are in Table 3-3.

Table 3-1. *Saccharomyces cerevisiae* strains.

Strain	Genotype	Source
S2683	*MATα leu2-k arg4-nsp ura3 lys2 hoΔ::LYS2*	(de los Santos and Hollingsworth, 1999)
S2683 ndt80	same as S2683 only *ndt80Δ::kanMX6*	this work
S2683 ndt80 mek1	same as S2683 only *ndt80Δ::kanMX6 mek1Δ::natMX4*	this work
RKY1145	*MAT***a** *leu2::hisG his4-x ura3 lys2 ho::LYS2*	(de los Santos and Hollingsworth, 1999)
NH144	*MAT***a** *leu2::hisG his4-x ARG4 ura3 lys2 ho::LYS2* *MATα leu2-k HIS4 arg4-Nsp ura3 lys2 ho::LYS2*	(Hollingsworth et al., 1995)
NH2136	Same as NH144 except *rad54Δ::kanMX6*	this work
14154	*MAT***a** *leu2::hisG lys2 hoD::LYS2 ura3::pGPD-GAL4(848).ER::URA3 his3::hisG trp1::hisG ndt80::TRP1-P_{GAL}-NDT80*	(Benjamin et al., 2003)
14154 RAD54-IN	same as 14154 only *kanMX6-P_{GAL1}-RAD54*	this work
14154 rad54	same as 14154 only *rad54Δ::natMX4*	this work

14154 mek1	same as 14154 only *mek1Δ::natMX4*	Callender et al., 2016
SKY371	*MATα leu2::hisG his4-X::LEU2 lys2 hoΔ::LYS2 ura3 trp1::hisG arg4-nsp*	S. Keeney
RKY1145::pKB80 rad54	*MAT***a** *leu2ΔhisG his4-X lys2 ho::LYS2 ura3::pGPD-GAL4(848).ER::URA3 rad54Δ::natMX4*	this work
NH2319	*MATα leu2::hisG HIS4 ho::LYS2 lys2* *MAT***a** *leu2::hisG his4-x ho::LYS2 lys2* *ura3::pGPD-GAL4(848).ER::URA3 his3::hisG* *ura3::pGPD-GAL4(848).ER::URA3 HIS3* *trp1::hisG kanMX6-P$_{GAL1}$-RAD54 arg4* *TRP1 rad54Δ::natMX4 ARG4*	this work
28417	*MATα leu2::hisG ho::LYS2 lys2 ura3 his::hisG trp1::hisG ndc80-1*	A. Amon
NH2127	*MATα leu2::hisG ho::LYS2 lys2* *MAT***a** *leu2::hisG ho::LYS2 lys2* *ura3::pGPD-GAL4(848).ER::URA3 his3::hisG trp1::hisG* *ura3::pGPD-GAL4(848).ER::URA3 his3::hisG* *trp1::hisG ndt80::TRP1-P$_{GAL}$-NDT80*	this work

	ndt80::TRP1-P_{GAL}-NDT80	
NH2111-12-3	*MATα leu2::hisG ho∆::LYS2 lys2 ura3::pGPD-GAL4(848).ER::URA3 his3::hisG trp1::hisG ndt80::TRP1-P_{GAL}-NDT80 rad54∆::natMX4*	this work
NH2126	*MATα leu2::hisG ho∆::LYS2 lys2 MAT**a** leu2::hisG ho∆::LYS2 lys2 ura3::pGPD-GAL4(848).ER::URA3 his3::hisG trp1::hisG ura3::pGPD-GAL4(848).ER::URA3 his3::hisG trp1::hisG ndt80::TRP1-P_{GAL}-NDT80 rad54∆::natMX4 ndt80::TRP1-P_{GAL}-NDT80 rad54∆::natMX4*	this work
NH2185	*MATα leu2::hisG ho::LYS2 MAT**a** leu2::hisG ho::LYS2 ura3::pGPD-GAL4(848).ER::URA3 his3::hisG trp1::hisG ura3::pGPD-GAL4(848).ER::URA3 his3::hisG trp1::hisG ndt80::TRP1-P_{GAL}-NDT80 rad54∆::natMX4 ndt80::TRP1-P_{GAL}-NDT80 kanMX4∆::P_{GAL1}-RAD54*	this work
NH2033	*MAT**a** leu2::hisG lys2 ho∆::LYS2*	this work

	MATα leu2-K lys2 hoΔ::LYS2 *ura3::P_{GPD}-GAL4-ER::URA3 his3::hisG trp1::hisG* *ura3 HIS3 TRP1* *ndt80::TRP1::P_{GAL1}-NDT80 ARG4* *ndt80Δ::kanMX6 arg4-Nsp*	
NH2122::pJR2	same as NH2033 only *mek1Δ::natMX4::URA3::mek1-as* *mek1Δ::natMX4*	Callender et al., 2016
NHY1210	*MAT**a** leu2::hisG HIS4::LEU2-(Bam+ ori) ho::hisG ura3(ΔSma-Pst)*	(Callender and Hollingsworth, 2010)
NHY1210 trp1	Same as NHY1210 except *trp1-5'Δ::natMX4*	this work
NHY1215	*MATα leu2::hisG his4-X::LEU2-(NgoMIV + ori) ho::hisG ura3(ΔSma-Pst)*	(Callender and Hollingsworth, 2010)
NHY1215 trp1	Same as NHY1215 except *trp1-5'Δ::natMX4*	this work
NH716	*MAT**a** leu2::hisG HIS4::LEU2-(Bam+ ori)*	(Callender and

	MATα leu2::hisG his4-X::LEU2-(NgoMIV + ori) *ho::hisG ura3(∆Sma-Pst)* *ho::hisG ura3(∆Sma-Pst)*	Hollingsworth, 2010)
NH729::pJR2	Same as NH716 except *mek1∆::natMX4::URA3-mek1-as* *mek1∆::natMx4*	(Callender and Hollingsworth, 2010)
NHY1210 trp1 P_{GAL1}-NDT80	Same as NHY1210 except *trp1-5'∆::natMX4 kanMX6-P_{GAL1}-NDT80*	this work
NHY1210 trp1 P_{GAL1}-NDT80::pEP105	Same as NHY1210 except *trp1-5'∆::natMX4::pGPD-GAL4.ER::TRP1 kanMX6-P_{GAL1}-NDT80*	this work
NHY1210 trp1 P_{GAL1}-NDT80 rad54 ::pEP105	Same as NHY1210 except *trp1-5'∆::natMX4::pGPD-GAL4.ER::TRP1 kanMX6-P_{GAL1}-NDT80 rad54∆::hphMX4*	this work
NHY1210 trp1 P_{GAL1}-NDT80 P_{GAL1}-RAD54 ::pEP105	Same as NHY1210 except *trp1-5'∆::natMX4::pGPD-GAL4.ER::TRP1 kanMX6-P_{GAL1}-NDT80 hphMX4-P_{GAL1}-NDT80*	this work

NHY1210 trp1 P_{GAL1}-NDT80 dmc1 mek1-as ::pEP105	Same as NHY1210 except *trp1-5'Δ::natMX4::pGPD-GAL4.ER::TRP1 kanMX6-P_{GAL1}-NDT80 mek1-as dmc1Δ::hphMX4*	this work
NHY1210 trp1 P_{GAL1}-NDT80 dmc1 mek1-as rad17::pEP105	Same as NHY1210 except *trp1-5'Δ::natMX4::pGPD-GAL4.ER::TRP1 kanMX6-P_{GAL1}-NDT80 mek1-as dmc1Δ::hphMX4 rad17Δ::URA3*	this work
NHY1215 trp1 P_{GAL1}-NDT80	Same as NHY1215 except *trp1-5'Δ::natMX4 kanMX6-P_{GAL1}-NDT80*	this work
NHY1215 trp1 P_{GAL1}-NDT80 ::pEP105	Same as NHY1215 except *trp1-5'Δ::natMX4::pGPD-GAL4.ER::TRP1 kanMX6-P_{GAL1}-NDT80*	this work
NHY1215 trp1 P_{GAL1}-NDT80 rad54 ::pEP105	Same as NHY1215 except *trp1-5'Δ::natMX4::pGPD-GAL4.ER::TRP1 kanMX6-P_{GAL1}-NDT80 rad54Δ::hphMX4*	this work
NHY1215 trp1 P_{GAL1}-NDT80 dmc1 mek1-as ::pEP105	Same as NHY1215 except *trp1-5'Δ::natMX4::pGPD-GAL4.ER::TRP1 kanMX6-P_{GAL1}-NDT80 mek1-as dmc1Δ::hphMX4*	this work
NHY1215 trp1 P_{GAL1}-NDT80	Same as NHY1215 except *trp1-5'Δ::natMX4::pGPD-GAL4.ER::TRP1 kanMX6-P_{GAL1}-NDT80 mek1-as*	this work

dmc mek1-as rad17 ::pEP105	*dmc1Δ::hphMX4 rad17Δ::URA3*	
NH2232	*MAT**a** leu2::hisG HIS4::LEU2-(Bam+ ori)* *MATα leu2::hisG his4-X::LEU2-(NgoMIV + ori)* *ho::hisG ura3(ΔSma-Pst)* *ho::hisG ura3(ΔSma-Pst)* *kanMX6-P$_{GAL1}$-NDT80* *kanMX6-P$_{GAL1}$-NDT80* *trp1-5'Δ::natMX4::pGPD-GAL4.ER::TRP1* *trp1-5'Δ::natMX4::pGPD-GAL4.ER::TRP1*	this work
NH2240	Same as NH2232 except *rad54Δ::hphMX4* *rad54Δ::hphMX4*	this work
NH2245	Same as NH716 except *trp1-5'Δ::natMX4::pGPD-GAL4.ER::TRP1* *trp1-5'Δ::natMX4::pGPD-GAL4.ER::TRP1* *kanMX6-P$_{GAL1}$-NDT80* *kanMX6-P$_{GAL1}$-NDT80* *hphMX4-P$_{GAL1}$-RAD54*	this work

	rad54Δ::hphMX4	
NH2290	Same as NH716 except *trp1-5'Δ::natMX4::pGPD-GAL4.ER::TRP1* *trp1-5'Δ::natMX4::pGPD-GAL4.ER::TRP1* *kanMX6-P_{GAL1}-NDT80* *kanMX6-P_{GAL1}-NDT80* *mek1Δ::natMX4::URA3-mek1-as* *mek1Δ::natMX4::URA3-mek1-as*	this work
NH2278	Same as NH716 except *trp1-5'Δ::natMX4::pGPD-GAL4.ER::TRP1* *trp1-5'Δ::natMX4::pGPD-GAL4.ER::TRP1* *kanMX6-P_{GAL1}-NDT80* *kanMX6-P_{GAL1}-NDT80* *mek1-as dmc1Δ::hphMX4* *mek1-as dmc1Δ::hphMX4*	this work
NH2365	Same as NH716 except *trp1-5'Δ::natMX4::pGPD-GAL4.ER::TRP1* *trp1-5'Δ::natMX4::pGPD-GAL4.ER::TRP1*	this work

	kanMX6-P_{GAL1}-NDT80 *kanMX6-P_{GAL1}-NDT80* *mek1-as dmc1Δ::hphMX4 rad17Δ::URA3* *mek1-as dmc1Δ::hphMX4 rad17Δ::URA3*	
S3363	*MATα leu2-R::URA3-tel1-ARG4 His4 ura3::P_{GPD1}-GAL4.(848).ER::URA3 lys2 ho::LYS2 ndt80Δ::kanMX6 P_{CDC5}-CDC5-pFA6a-hphMX4-P_{GAL1}-CDC5 arg4Δ(eco47III-hpaI)*	Gift from Michael Licthen
RKY1145 ndt80::hph	*MAT**a** leu2::hisG his4-x ura3 lys2 ho::LYS2 ndt80Δ::hphMX4*	(Chen et al., 2015)
NH2296	*MAT**a** leu2::hisG his4-x* *MATα leu2-R::URA3-tel1-ARG4 His4* *ura3 lys2 ho::LYS2* *ura3::P_{GPD1}-GAL4.(848).ER::URA3 lys2 ho::LYS2* *ndt80Δ::hphMX4 CDC5* *ndt80Δ::kanMX6 P_{CDC5}-CDC5-pFA6a-hphMX4-P_{GAL1}-CDC5*	this work

	ARG4 *arg4Δ(eco47III-hpaI)*	

Table 3-2. Conversion of optical density$_{660}$ (OD_{660}) values to cell density.

OD_{660}	Cells/ml ($X10^7$)
0.95	1.67
1.00	1.85
1.15	2.47
1.20	2.71
1.25	2.95
1.30	3.22
1.35	3.50
1.40	3.82
1.45	4.13
1.55	4.85

Table 3-3. Primary and Secondary antibodies.

Protein	Primary	dilution	source	Secondary	Dilution	Source
Arp7	α-Arp7	1:10,000	Santa Cruz #sc-8961	α-Goat	1:10,000	Santa Cruz #sc-2020
Cdc5	α-Cdc5	1:500	Santa Cruz #sc-6733	α-Goat	1:15,000	Santa Cruz #sc-2020
Clb1	α-Clb1	1:300	Santa Cruz #sc-7647	α-Goat	1:10,000	Santa Cruz #sc-2020
Hop1	α-Hop1	1:10,000	N.M. Hollingsworth	α-Rabbit	1:10,000	Santa Cruz #sc-2004
Hed1	α-Hed1	1:50,000	P. Sung	α-Rabbit	1:10,000	Santa Cruz #sc-2004
Hed1 phospho-T40	α-p-Hed1	1:20,000	N. M. Hollingsworth	α-Rabbit	1:10,000	Santa Cruz #sc-2004
Mek1	α-Mek1	1:5,000	N. M. Hollingsworth	α-Guinea Pig	1:10,000	Santa Cruz sc-2903
Ndt80	α-Ndt80	1:15,000	Courtesy of M. Lichten	α-Rabbit	1:15,000	Santa Cruz #sc-2004

Rad54	α-Rad54	1:5,000	N.M. Hollingsworth	α-Rabbit	1:5,000	Santa Cruz #sc-2004
Rec8	α-Rec8	1:500,000	N.M. Hollingsworth	α-Guinea Pig	1:10,000	Santa Cruz sc-2903

CHAPTER FOUR: DISCUSSION

NDT80 induction activates Rad51-mediated recombination during yeast meiosis

During budding yeast meiosis Rad51 is downregulated by Dmc1, as well as Mek1 phosphorylation of Hed1 and Rad54 (Callender et al., 2016; Callender and Hollingsworth, 2010; Lao et al., 2013; Liu et al., 2014; Niu et al., 2009). *rad54Δ* mutants have wild type levels of IH recombination, yet there is reduced spore viability and sporulation (Schmuckli-Maurer and Heyer, 2000; Shinohara et al., 1997b). When *rad54Δ* mutants complete the meiosis cell cycle the resulting distribution of viable spores suggests that the inviability is not due to meiosis I non-disjunction which has a particular viable spore pattern of an elevated number of 2- and 0- spore viable tetrads, but instead to unrepaired DSBs. The distribution pattern of 4-, 3-, 2- and 0- spore viable tetrads in a *rad54Δ* indicates that the unrepaired DSBs within the population of sporulating cells vary between individual cells. Taken together these facts suggest that there is a role for Rad51 during budding yeast meiosis, perhaps in the repair of DSBs after IH recombination has occurred. The idea of two rounds of recombination has been proposed for budding yeast (Niu et al., 2009; Sheridan and Bishop, 2006), but has not yet been demonstrated. Furthermore, in nematode meiosis there has been an observation that two rounds of recombination exist, first IH and then IS within the context of synapsed chromosomes (Hayashi et al., 2007; Zetka et al., 1999).

The meiosis-specific transcription factor, Ndt80, is required for dHJ resolution, disassembly of SCs and meiotic progression (Allers and Lichten, 2001; Xu et al., 1995).

Diploids deleted for *NDT80* arrest in pachytene, at which time homologs are fully synapsed and most DSBs have been processed either into dHJ intermediates (on the way to forming COs) or NCOs. Meiotic prophase can therefore be divided into two phases. Phase one occurs prior to *NDT80* induction where IH recombination occurs in the presence of an IH bias imposed by Mek1. Phase two is initiated by the activation of Ndt80. I propose that Ndt80 abolishes the restraints on Rad51-Rad54 interaction by inhibiting Mek1 activity.

I have established a timing of *RAD54* function relative to *NDT80* by creating diploids containing ED-inducible alleles of both genes. Co-induction of *NDT80* and *RAD54*, rescued the spore inviability of a *rad54*Δ, which indicates that Rad54 is necessary upon *NDT80* induction. However, recent work suggests there are two types of DSBs; those that occur those in early pachytene that are under Mek1 imposed IH bias, and a second class of DSBs that are made at the *ndt80*Δ arrest (Subramanian et al., 2016). My results indicate that *RAD54*, and by extension, Rad51, are required either during the *ndt80*Δ arrest or after *NDT80* induction to repair DSBs. This explains why *RAD54* has no effect on IH recombination, but still has defects in sporulation and spore viability.

Given that *RAD54* is required post-*NDT80* a reasonable hypothesis is that *NDT80* induction inactivates Mek1. Inactivation of Mek1-as prior to *NDT80* induction reduces spore viability by allowing IS repair at the time that IH recombination is occurring. In contrast, inhibition of Mek1-as at the same time as *NDT80* induction had little effect on spore viability, as predicted if the kinase is no longer active. In *NDT80-IN mek1-as* experiments, phosphorylation of Hed1 T40 was used as a downstream

molecular marker for Mek1 kinase activity. In the absence of inducer (-ED) the phospho-Hed1 signal was present, and yet when inhibitor (+I) was added after 7 hours in Spo medium, within one hour the phospho-Hed1 signal was lost. This indicates that Mek1 is constitutively active during an *ndt80* pachytene arrest. When *NDT80* was induced after 7 hour in Spo medium, phosphorylated Hed1 disappeared within two hours, demonstrating that *NDT80* induction is sufficient to inactivate Mek1 kinase activity. There were other interesting correlations occurring upon *NDT80-IN* induction. The appearance of Cdc5 correlated with loss of Hed1 T40 phosphorylation, as well as Mek1 and Red1 degradation.

A recent paper has suggested that synapsis results in the removal of Mek1 from chromosomes, thereby reducing its activity (Subramanian et al., 2016). To test whether IH strand invasion and synapsis are required for the Ndt80-mediated inhibition of Mek1, I initially used an *NDT80-IN dmc1Δ mek1-as* diploid to prevent IH recombination. Although *NDT80-IN* induction resulted in the production of abundant phosphorylated Ndt80, it was not transcriptionally functional as indicated by the lack of downstream molecular markers, *CDC5* and *CLB1.* Hed1 phosphorylation and Red1 protein persisted and DSBs remained unrepaired. Although a previous study showed that over-expression of *NDT80* was able to partially rescue the meiotic recombination checkpoint induced arrest in a different strain background (Tung et al., 2000), that was not the case in my experiments as cells did not enter the meiotic divisions. This arrest was dependent upon *MEK1*, as inhibiting Mek1-as either at t = 0 hour or t = 5 hour in SPO the cells were able to progress. My work clearly shows that the *MEK1*-dependent mechanism for inactivating Ndt80 when the checkpoint is active is post-translational and

is consistent with the observation that Ndt80 is sequestered into the cytoplasm when the recombination checkpoint is triggered (Wang et al., 2011).

To test whether Ndt80 is sufficient to inactivate Mek1 without strand invasion and synapsis occurring, it was necessary to abrogate the checkpoint. This was accomplished by deleting *RAD17* (Grushcow et al., 1999; Lydall et al., 1996). When the checkpoint was abolished by *rad17*Δ, active Ndt80 protein was produced as indicated by the appearance of Cdc5 and Clb1 and the cells progressed into MI and MII. Red1 was degraded and Mek1 was inactivated as Hed1 phosphorylation disappeared and DSBs were repaired. Therefore IH strand invasion and synapsis are not a prerequisite for Ndt80-mediated inactivation of Mek1.

CDC5 is the target of *NDT80* responsible for Mek1 inactivation

The Ndt80 transcription factor is required to express >200 genes, one of which is *CDC5*. Given the correlation between the appearance of Cdc5 and the loss of Hed1 T40 phosphorylation and Red1 degradation, and the observation that induction of *CDC5* in an *ndt80*Δ background is sufficient to remove Red1 from chromosomes to disassemble the SC (Sourirajan and Lichten, 2008), I tested whether *CDC5* is the sole Ndt80 target required to inactivate Mek1 using *CDC5-IN ndt80*Δ diploid. In fact, induction of *CDC5* was sufficient to inactivate Mek1, resulting in Hed1 dephosphorylation, Red1 degradation and DSB repair (Figure 2-5A, 2-5B).

Coordination of recombination and meiotic progression is mediated by Mek1

Meiotic DSBs result in activation of Mek1, which post-translationally regulates the low levels of Ndt80 produced by Ime1 and has been suggested that this occurs through

sequestering Ndt80 to the cytoplasm (Wang et al., 2011). My work shows that Ndt80 in turn, negatively regulates Mek1. For this to occur, Mek1 activity must be reduced so that sufficient Ndt80 is activated to allow it to bind to the *NDT80* promoter and start the positive feedback loop. How do cells know when to make this transition? I propose that there are two waves of Mek1 inactivation during meiosis. First synapsis removes the bulk of Mek1 from chromosomes thereby lowering the overall amount of Mek1 kinase activity in the cell below the threshold needed to keep Ndt80 inactive (Figure 4-1A) (Subramanian et al., 2016). As a result, Ndt80 is able to transcribe itself, as well as target genes such as *CDC5* and *CLB1* (Figure 4-1B). The resulting disassembly of the SCs through degradation of Red1 then eliminates any remaining Mek1 activity, allowing the repair of residual DSBs (Figure 4-1C).

Support for this model comes from several previously published papers. In haploid cells where there are no homologs to allow synapsis, DSBs are made on the haploid chromosomes but are not repaired, and the chromosomes do not progress. Inactivation of Mek1-as in haploid cells results in DSB repair and meiotic progression (Callender and Hollingsworth, 2010; De Massy et al., 1994). I propose that in haploid cells the global levels of Mek1 remain above the threshold due to the absence of synapsis. Additionally, in diploid meiosis it has been shown that when synapsis occurs the level of Mek1 on chromosome is diminished. For some DSBs, IS recombination now occurs, while at others IH bias persists, suggesting that varying degrees of sensitivity to amount of Mek1 activity are exhibited by different hotspots (Subramanian et al., 2016). For the less sensitive hotspots, Mek1 must be eliminated by its total inactivation resulting from *CDC5*-dependent disassembly of the SC to allow IS DSB repair.

Further support of this model is that overexpression of *CDC5* in meiosis results in premature induction of *NDT80* (Acosta et al., 2011). Since the model proposes two waves of inactivating Mek1 with the final inhibition dependent on *CDC5* transcription by Ndt80, it would predict that overexpression of *CDC5* independent of *NDT80* expression would inactivate Mek1 and thus allow for functional Ndt80. This model also predicts that deleting genes that either do not trigger or are required for the meiotic recombination checkpoint such as *SPO11, RED1,* and *HOP1* should exhibit faster meiotic progression because there is a lack of post-translational control of Ndt80. Indeed deletions of these genes progress faster into the meiotic divisions than WT diploids in the SK1 strain background (Malone et al., 1991; Mao-Draayer et al., 1996).

This work has identified how Mek1 is able to coordinate DSB repair and synapsis with the timing of meiotic progression. However, it has not addressed how Ndt80 is inactivated by the meiotic recombination checkpoint. One possibility is that Ndt80 has a post-translational modification that affects nuclear localization, causing sequestration to the cytoplasm. The addition of nuclear localization signals to different parts of Ndt80 can test if it is the exclusion of Ndt80 from the nucleus that is causing the non-functional phenotype. Additionally, Wang et al., 2011, found that an internal in-frame deletion in *NDT80* encoding amino acids 346 to 402 allowed Ndt80 to enter the nucleus under checkpoint inducing conditions and allow meiotic progression. Interestingly, this region contains a Mek1 consensus phosphorylation site (RXXT) (Suhandynata et al., 2016). It will be interesting to mutate this site and see if the inability to phosphorylate Ndt80 at this position bypasses the *dmc1*Δ arrest and allows inactivation of Mek1. Alternatively, Ndt80 itself may not be the reason of the lack of middle meiosis gene transcription

under checkpoint conditions. Instead, Sum1 may be targeted by the recombination checkpoint thus preventing Ndt80 from binding to the middle sporulation element within the promoter region of the middle meiosis genes such as *CDC5*.

In conclusion, my thesis has shown that there is a late role for Rad51-Rad54 during budding yeast meiosis. Mek1 kinase functions in creating an IH bias during early prophase by the inhibition of Rad51-Rad54 complex formation. It is upon SC formation that the kinase activity is dampened allowing for a functional Ndt80 to induce cell progression, which my studies revealed is necessary for complete inactivation of Mek1 and thus relieving the IH bias imposed at DSBs.

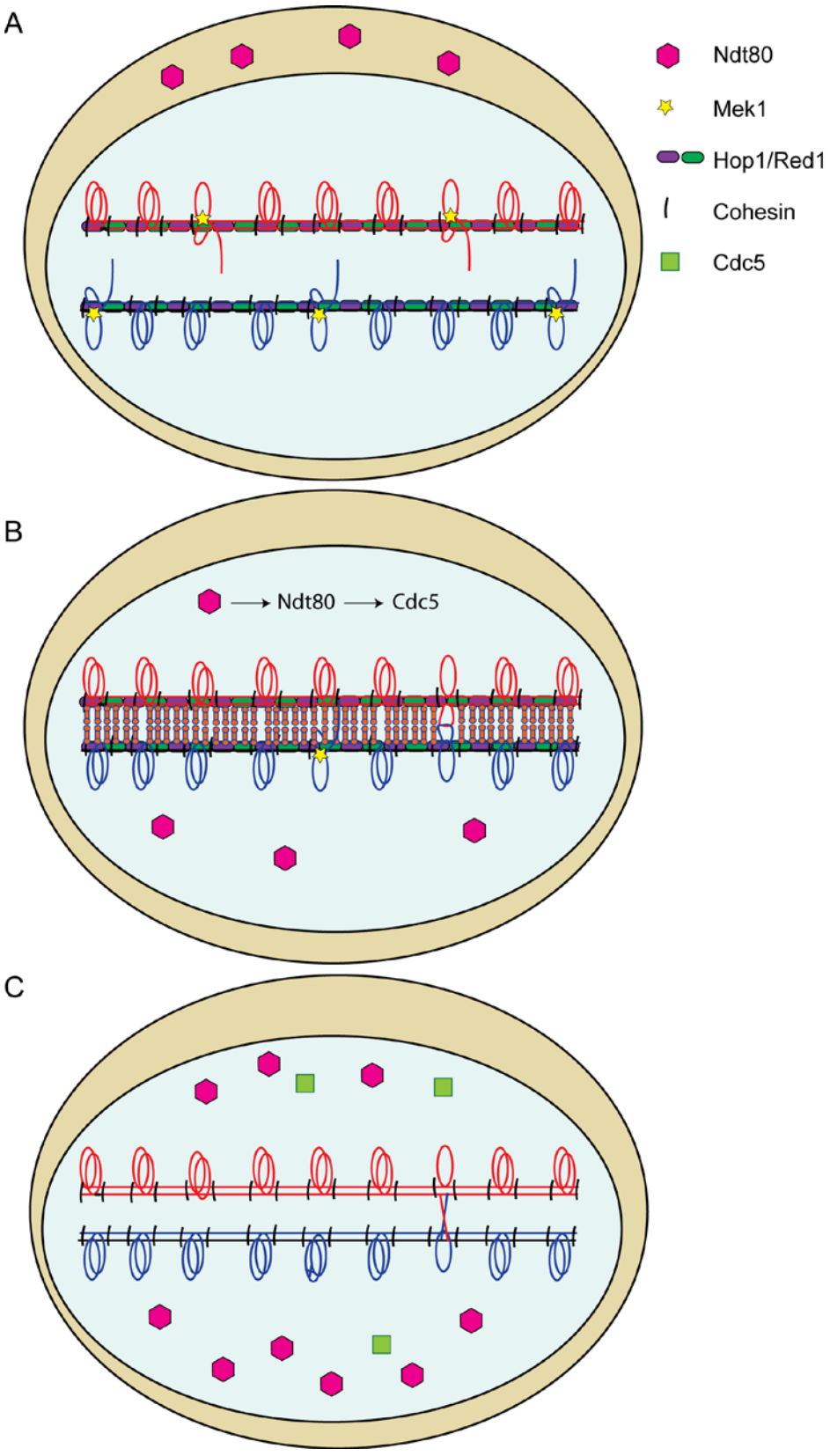
A
B
C
Ndt80
Mek1
Hop1/Red1
Cohesin
Cdc5
Ndt80
Cdc5

Figure 4-1. Mek1 kinase activity is regulated in two steps. A. A single pair of replicated homologous chromosomes is shown, one is blue and the other is red. The nucleus is the light blue oval while the cytoplasm is indicated by the tan oval. Early in prophase, DNA is cleaved by Spo11 on the chromosome axes, resulting in the recruitment and activation of Mek1. Ndt80 is transcribed at a low level by Ime1. The high global levels of Mek1 activity result in the inhibition of Ndt80, perhaps by promoting sequestration or export of Ndt80 to the cytoplasm. B. Stable interhomolog connections mediated by the ZMM proteins to form dHJs also result in the synapsis of AEs by the insertion of the transverse filament protein, Zip1. Synapsis results in the loss of Mek1, thereby lowering the levels of Mek1 kinase activity below the threshold required to inhibit Ndt80. Ndt80 therefore enters the nucleus where it promotes transcription of itself to start the positive feedback loop to produce more Ndt80, as well as the transcription of Ndt80-regulated genes such as *CDC5*. Any DSBs occurring during this transition result in recruitment and activation of Mek1 which locally promotes IH bias. C. Cdc5 promotes the degradation of Red1 and Zip1 resulting in SC disassembly and the inactivation of Mek1. Any unrepaired DSBs are then repaired by Rad51 using the sister chromatid as the template. (Figure courtesy of Nancy Hollingsworth).

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