Mutation in Mouse *Hei10*, an E3 Ubiquitin Ligase, Disrupts Meiotic Crossing Over

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Crossing over during meiotic prophase I is required for sexual reproduction in mice and contributes to genome-wide genetic diversity. Here we report on the characterization of an *N*-ethyl-*N*-nitrosourea-induced, recessive allele called *mei4*, which causes sterility in both sexes owing to meiotic defects. In mutant spermatocytes, chromosomes fail to congress properly at the metaphase plate, leading to arrest and apoptosis before the first meiotic division. Mutant oocytes have a similar chromosomal phenotype but in vitro can undergo meiotic divisions and fertilization before arresting. During late meiotic prophase in *mei4* mutant males, absence of cyclin dependent kinase 2 and mismatch repair protein association from chromosome cores is correlated with the premature separation of bivalents at diplonema owing to lack of chiasmata. We have identified the causative mutation, a transversion in the 5' splice donor site of exon 1 in the *mouse ortholog of Human Enhancer of Invasion 10 (Hei10)*; also known as *Gm288* in mouse and *CCNB1IP1* in human), a putative B-type cyclin E3 ubiquitin ligase. Importantly, orthologs of *Hei10* are found exclusively in deuterostomes and not in more ancestral protostomes such as yeast, worms, or flies. The cloning and characterization of the *mei4* allele of *Hei10* demonstrates a novel link between cell cycle regulation and mismatch repair during prophase I.

Citation: Ward JO, Reinholdt LG, Motley WW, Niswander LM, Deacon DC, et al. (2007) Mutation in mouse *Hei10*, an E3 ubiquitin ligase, disrupts meiotic crossing over. PLoS Genet 3(8): e139. doi:10.1371/journal.pgen.0030139

**Introduction**

The successful completion of meiosis I (MI) in the vast majority of multicellular eukaryotes requires replication of the genome, proper signals from somatic support cells, synapsis, and crossing over between homologous chromosomes. The progression of the distinct phases of MI is thought to be orchestrated by periodic synthesis and degradation of cyclins, the activity of cyclin dependent kinases (CDKs), the correct establishment of the synaptonemal complex (SC), and the proper functioning of the DNA repair machinery during replication and recombination. The accuracy of the meiotic cell cycle in mammals is monitored both during recombination via the pachytene checkpoint in prophase I or during the spindle checkpoint at metaphase I (reviewed in [1,2]). While meiocytes of both sexes are sensitive to early recombinational repair defects, the pachytene checkpoint in males is more sensitive to synopsis defects per se than in females [2]. The components of the mammalian pachytene checkpoint and the nature of the fundamental molecular communications triggering cell cycle arrest after recognition of DNA anomalies in prophase I remain elusive.

The DNA repair machinery is essential for meiotic progression. Genetically programmed double strand breaks (DSBs) are generated and processed by SPO11 [3,4]. DSB formation is followed by recombinational repair facilitated by the RecA homologs RAD51 and DMC1, whose protein products colocalize with ~300 early recombination nodules on chromosome cores during leptotene [5,6]. Both are subsequently replaced by the single-stranded binding protein replication protein A (RPA) [5,6]. Foci containing the MutS homolog MSH4 colocalize with RAD51 foci during zygonema and persist into the onset of early pachynema [7,8]. Roughly half of MSH4 foci appear to be involved in reciprocal recombinations between homologous chromosomes, while the others result in non-crossover gene conversion events [9]. MSH4 and MSH5 form a heterodimeric complex thought to mediate recognition of the Holliday junction [10]. Both the *Msh4−/−* and *Msh5−/−* knockout mice fail to synapse appropriately and arrest in pachynema [7]. Temporally, Msh4 and Msh5 mark the first appearance of the mismatch repair (MMR) system in prophase I.

During pachynema, the protein products of the MutL homologs *Mlh3* and *Mlh1* [6,11,12] colocalize with the sites of reciprocal recombination and mark the second appearance of the MMR system in prophase I. *MLH3/MLH1* foci appear in...

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early pachytene and are present until early diplonema. Targeted deletion of Mlh3 causes arrest at metaphase I and prevents MLH1 recruitment to the chromosome [11]. Meiocytes deficient in MLH1 fail to complete meiosis owing to loss of bivalent maintenance and disorganized univalents at metaphase I [13–16]. As evidenced by their respective knockout phenotypes, the spatiotemporal progression from foci containing MSH4/MSH5 to those containing MLH3/MLH1 appears to be a critical event in the transition from susceptibility to a pachytene checkpoint to that of a spindle checkpoint. Interestingly, little is known about the cell cycle-related molecular mechanisms that are responsible for this transition.

In addition to the role of the DNA repair machinery, meiosis is regulated in part by the synthesis and ubiquitin-mediated degradation of cyclins and their functional relationship with CDKs. There are three cyclins (A1, B1, and B3) that are necessary for or are specifically expressed during mammalian meiosis [reviewed in [17,18]]. Several CDKs have been implicated in the progression of meiosis, including CDK1, CDK2, CDK4, and CDK6 [19]. CDK2 and CDK4 are essential for proper meiosis as both Cdk2−/− and Cdk4−/− mice are sterile [20–22]. Cdk2−/− spermatocytes arrest in mid-pachynema, whereas Cdk2+/− oocytes are lost perinatally [20]. Cdk4−/− mice have reduced viability and display degeneration of primary spermatocytes [21]. Interestingly, CDK2 colocalizes with MLH1 at sites of reciprocal recombination [23]. Beyond this, the role of CDK2 during the progression of MMR in prophase I remains unclear.

Ubiquitin-mediated proteolysis is thought to ensure the properly timed involvement of the cyclins in the meiotic cell cycle. For example, limited proteolytic degradation of meiotic cyclin B1 (CCNB1) is necessary for homolog disjunction and release from MI and the exit from MI in yeast and mammals [17,24]. Ubiquitin-mediated proteolysis involves transfer of ubiquitin by a ubiquitin-activating enzyme (E1) to a ubiquitin conjugating enzyme (E2), which in turn assembles a ubiquitin chain onto a substrate recruited by a substrate-specific E3 ligase (reviewed in [25]). To date however, very few interactions have been identified between substrate-specific E3 ligases and meiotic cell cycle regulators. One such potential interaction involves the anaphase-promoting complex and CCNB1. The anaphase promoting complex is a large multiprotein complex containing E3-ubiquitin ligase activity thought to be responsible for degradation of meiotic cyclins, including CCNB1 [26].

Evidence is emerging that ubiquitin conjugation by non-anaphase promoting complex-related pathways occurs during gametogenesis. Male sterility results from targeted disruptions of the ubiquitin-like DNA repair gene Rad23b or the Ubr2 ubiquitin ligase via failure to initiate spermatogenesis and failure to complete MI, respectively [27,28]. Further, mutations in Hr6b (mouse RAD6 homolog) [29], Siah1a [30], and F-box protein family members result in MI defects [31], particularly in males. Despite these phenotypes, none of the above mentioned genes have been directly connected phenotypically or mechanistically to the progression of the cell cycle machinery during meiosis.

Previously, we reported the results of forward genetic screens to identify mutant alleles of genes necessary for the early stages of MI that lacked recognizable orthologs in other model organisms [32]. Here we report on the characterization of one of these alleles called mei4. Mutant spermatocytes are defective in maintaining interhomolog associations at MI due to a lack of crossing over. Positional cloning led to the identification of a mutation in the mouse ortholog of Human Enhancer of Invasion 10 (Hei10). This gene encodes a putative E3 ligase that has not previously been implicated in meiotic recombination. Defects in Hei10 result in aberrant MMR protein and CDK localization. We proposed that the function of Hei10 during MI reveals a link between cell cycle regulation and the accurate resolution of reciprocal recombination intermediates.

Author Summary

Human infertility and reproductive complications have devastating social and monetary costs. Errors in meiosis during reproduction may lead to birth defects, spontaneous abortion, or infertility. Many of the genes essential for meiosis function in DNA repair and mutations in several of these genes have been shown to contribute to cancer. The identification of the genes necessary for normal meiosis is an important goal and will potentially influence the fields of reproductive and cancer biology. In this study, genetic screens in mice have generated the mutation mei4. mei4 causes male and female sterility by disrupting meiosis and altering the function of the DNA repair system known as mismatch repair. We have identified the causative mutation behind the mei4 phenotype in a gene called Human Enhancer of Invasion 10 or Hei10. This work demonstrates that Hei10 is essential for the completion of meiosis and that it functions to coordinate the DNA repair system and the progression of the cell cycle during meiosis.
Figure 1. The mei4 Mutation Inhibits Normal Meiotic Chromosome Alignment at Metaphase I in Males and Females and Is Associated with Apoptosis in Spermatocytes

(A) Heterozygous (mei4/+) seminiferous tubule cross sections stained with hematoxylin and eosin show normal telophase and metaphase I structures (black arrow and arrowheads, respectively) as well as postmeiotic round spermatids and elongating differentiating spermatids (white arrows).

(B) Homozygous mutant (mei4/mei4) sections show abnormal metaphase and anaphase I-like spermatocytes with disrupted chromosome congression to the metaphase plate and aberrant chromosome distribution on the spindle (black arrows).

(C and D) mei4/mei4 mutant seminiferous tubules have significantly greater numbers of apoptotic cells (brown cell, black arrow) than +/+ tubules (D versus C, respectively) as determined by TUNEL assay. The average number of TUNEL positive cells per 0.146-mm² field in tubules that contained apoptotic cells was 140 for mei4/mei4 versus 9.6 for +/+ (p = 3.28 × 10⁻¹⁵).

(E and F) The ovaries of mei4/mei4 females appear histologically normal. Hematoxylin and eosin-stained cross sections reveal follicles and oocytes in all stages of development in normal (E) and mutant animals (F).

(G and H) Hoechst-stained metaphase I chromosome structures occur normally in mei4/+ oocytes (white arrow, G) and abnormally (asterisks, H) in mei4/mei4 oocytes. Infrequently, normal metaphase chromosomes structures are seen in mutant oocytes (white arrow, [H]). Spindles in (G and H) are labeled with an anti-β tubulin antibody.

doi:10.1371/journal.pgen.0030139.g001
developmental competency of embryos derived from mei4 mei4 oocytes was lost between the two-cell and blastocyst stage.

Defective Interhomolog Associations in Mutant Spermatocytes

To determine the cause of the aberrant MI in mei4/mei4 spermatocytes, we prepared metaphase spreads. Whereas nineteen metaphase bivalents were present in mei4/+ spreads (Figure 2A), mei4/mei4 spreads contained primarily univalents (Figure 2B). Bivalents were rarely present (Figure 2B, white arrow). The total number of condensed chromosomes (univalents + bivalents) approached 40. These observations suggest that either homologous chromosomes never paired and/or synapsed or did so but failed to remain attached upon entry to metaphase.

To examine synapsis, we used antibodies to detect the distribution of SC proteins along mutant and wild-type prophase I chromosomes. SYCP3 marks the axial elements of the SC [33]. Wild-type (+/+ and mei4/mei4 spermatocyte chromosome spreads labeled with anti-SYCP3 antibodies revealed the presence of pachytene stage nuclei containing 19 paired chromosomes plus an apparent X-Y pair (Figure 2C and 2D, respectively). However, there was premature homolog separation in diplo micronucleus mei4/mei4 spermatocytes (Figure 2E) versus +/+ (Figure 2F). The distribution of SYCP1, a SC transverse element protein that is indicative of synapsis [6,34], was normal in mei4/mei4 versus mei4/+ spermatocytes (Figure 2H versus 2G).

Recombination Initiates Normally, but mei4 Spermatocytes Fail to Form Chiasmata

Since mutant spermatocytes are capable of homolog synapsis but defective in maintaining interhomolog attachment, we examined the progress of recombination in these nuclei. RAD51 foci were distributed similarly along zygotene chromosome cores in control and mei4/mei4 spermatocyte nuclei (Figure 3A and 3B), indicating that DSBs formed and that recombination repair was initiated. We then examined the distribution of two markers of recombination nodules, the MMR molecules MLH3 and MLH1 [6,11]. Mid-pachytene mei4/+ spermatocytes had 1–2 MLH3 foci on each synapsed chromosome (Figure 3C) and ~23 per nucleus, typical for wild-type meioses [6]. However, no MLH3 foci were evident in mei4/mei4 (Figure 3B). Similar results were obtained with MLH1 (Figure 3E and 3F).

Candidate Gene Identification and Positional Cloning of mei4

mei4 was previously mapped to a large region on Chromosome 14 between the markers D14Mit101 and D14Mit266 (Figure 4) [32]. In this study, we refined the genetic region containing mei4 to a 0.7-cM (four recombinants out of 572 meioses) region corresponding to 2.33 Mb between the markers D14Mit101 and D14Mit183 on Chromosome 14 (Figure 4A). The mouse Ensembl database (http://www.ensembl.org, version 43, NCBI36) annotates 62 Refseq genes (Table S1) and 11 other Ensembl annotated genes in this region including a cluster of 28 olfactory receptors. Potential candidate genes for mei4 were prioritized on the basis of known or predicted meiotic function and/or germ tissue expression pattern. For novel genes or genes of unknown function, protein-protein BLAST (blastp) comparisons were made to identify functional domains or regions of homology to known genes or motifs. Three genes warranted further attention under these criteria: poly ADP ribosylase 2 (Parp2), apurinic/apyrimidinic endonuclease 1 (Apex1), and the Ensembl novel gene Gm288 (Table S1, numbers 33, 35, and 41 in bold-face type). We performed reverse-transcripase (RT)-PCR on testis RNA from mei4/+ and mei4/mei4 animals to detect possible expression variations in Parp2 and Apex1 (Figure 4B, rows 2 and 3, respectively). Primer pairs spanning multiple exons were used for both genes. Both genes appeared to be expressed normally in control and mutant testes. We sequenced all exons of both genes and no mutations were found.

The mei4 Allele of Hei10 Contains a Splice Site Mutation

Gm288 encodes the mouse ortholog of human CCNB1 (CCNB1 interacting protein 1), also called HEI10 [35]. Using RT-PCR, we found an altered length transcript present in both mei4/+ and mei4/mei4 mutants versus +/+ animals (Figure 4B, row 4). Using primers specific to exons 1 and 2, the expected 430-bp product was seen in +/+ and mei4/+ animals. In addition, a 358-bp fragment was also present in mei4/+ heterozygotes (Figure 4C). In mei4/mei4 testes, we observed only the 358-bp product (Figure 4C). RT-PCR analysis of multiple tissue types revealed that Hei10 is transcribed prominently in the testes and 17-d embryo (corresponding to prophase I in females) and to a much lesser degree in other tissues (Figure 4D).

Sequencing of Hei10 genomic DNA from control and mutant mice revealed a G>T transversion at bp 298 (from the start of the first coding exon, G298T) in mei4/mei4 animals (Figure 5A), which corresponds to the 5’ splice donor site at the 3’ end of exon 1 (Figure 5B, Transcript). Sequencing of cDNA from control and mutant animals showed that G298T causes the use of a cryptic splice donor site 72 bp upstream (GT in position 226) in exon 1. This results in an in-frame, 24 amino acid deletion in the predicted protein product (Figure 5B, Protein, amino acids 76–100) that removes a putative cyclin/CDK interaction domain, the RXL motif (Figure 5B, Protein, amino acids 76–100). We will refer to the above-described allele as Hei10mei4.

Abnormal Localization of CDK2 in mei4/mei4 Spermatocytes

To investigate the possibility of an error in the progression of the cell cycle, we examined the localization of CDK2, a CDK essential for meiosis [20], on spermatocyte chromosomes (Figure 6). CDK foci are normally observed at the telomeres, at one to two interstitial sites on each synapsed bivalent, and on the asynapsed portions of the X and Y chromosomes in males [23]. Further, CDK2 colocalizes on
Pachytene chromosomes with MLH1 [23]. Mid-pachytene +/+ spermatocytes displayed CDK2 foci at interstitial sites (Figure 6A, white arrows), telomeres (Figure 6A, open arrowheads), and the X-Y body (Figure 6A, closed arrowheads). In contrast, CDK2 foci were seen at telomeric sites and on the X-Y body on mei4/mei4 pachytene chromosomes (Figure 6B, open arrowheads and white arrow, respectively) but were rarely seen at interstitial sites.

Figure 2. mei4/mei4 Spermatocytes Synapse in Prophase I but Have Few Intact Bivalents at Metaphase I

(A and B) Metaphase chromosome spreads were prepared from normal (mei4/+ [A]) and mutant (mei4/mei4 [B]) males and stained with DAPI. In (A) mei4/+ spreads exhibit the expected number of bivalent recombination structures. (B) depicts a mei4/mei4 spread with few chiasmata (white arrow) and with most chromosomes present as univalents.

(C–F) Chromosome spreads from normal (+/+) and mutant (mei4/mei4) spermatocytes were labeled with antibodies to SYCP3 and counter-stained with DAPI at various stages of MI. Pachytene (C and D) +/+ and mei4/mei4 spermatocytes are similar. At diplonema, +/+ (E) spreads show pairs of homologous chromosomes associated at sites of recombination (white arrows) and or centromeres. In contrast, in mei4/mei4 spreads (F) many chromosomes are univalents associated neither at the centromere nor sites of recombination (white arrowheads). The loss of bivalent cohesion is not complete however, and some chiasmata are seen in mei4/mei4 preparations.

(G and H) Chromosome spreads from heterozygote (mei4/+) ([G]) and homozygous recessive (mei4/mei4 [H]) animals were labeled (red) with antibodies to SYCP1, a marker of the central element and synapsis. In both cases SYCP1 is present indicating that the transverse elements of the SC are forming and that chromosomes in mei4/mei4 spermatocytes synapse normally in pachynema.

doi:10.1371/journal.pgen.0030139.g002
Discussion

Failure to Form Chiasmata Is Correlated with Defects in the MMR System

In this study, we have demonstrated that mei4/mei4 spermatocytes undergo a uniform early metaphase I arrest marked by aberrant chromosome congression (Figure 1). Mutant oocytes show a similar configuration but fail to arrest at early metaphase I and are competent, postfertilization, to reach the two-cell embryonic stage. Immunocytochemical labeling of microspread spermatocytes and 4′, 6-diamidino-
2-phenylindole, dihydrochloride (DAPI) labeling of metaphase chromosomes demonstrates that maintenance of bivalents is lost in all but a few chromosome pairs. Our data show that the high prevalence of univalents in late diplonema and metaphase I are due to a lack of chiasmata. Failure to form chiasmata can arise from defects in the DSB system, the MMR system, or in the complexes required for proper SC formation [4,11,15]. Normal localization of RAD51 and SYCP3/SYCP1 in mei4/mei4 spermatocyte nuclei indicates that DSB repair and SC formation are not disrupted. However, MLH3 and MLH1 fail to form foci in mei4/mei4 spermatocyte nuclei, suggesting that defects in the MMR system cause chiasma failure and subsequent metaphase arrest in males (Figure 3).

Loss of the HEI10 RXL Domain May Lead to Inability to Associate with Cyclins

Sequencing of Hei10mei4 cDNA identified a shortened transcript produced in the testes. When translated, this mRNA would produce a protein missing 24 amino acids (residues 76–100) in a highly conserved portion of the N-terminal region of HEI10 (Figure 7). This missing region includes a RXL motif (asterisks, amino acids 91–93, RAL) with predicted cyclin/CDK-binding activity [35]. RXL domains are found in CDK substrates such as p107, p21 CIP1, and the retinoblastoma protein [36,40–44]. Deletion or mutation of the RXL domain or peptides containing the RXL motif inhibit phosphorylation of the CDK substrate and/or eliminate binding. On the basis of data from a cyclin A/CDK2 crystal structure, the RXL domain contacts a highly conserved remote (relative to the active site) binding domain on the cyclin partner [45]. While we cannot rule out the contributions of the other residues in the region, deletion of the RXL domain in Hei10mei4 would abrogate binding of the E3 to its presumed target, a B-type cyclin.

Hei10mei4 and the Cyclins

Alternatively, HEI10 may function as a cell cycle regulator that acts between early and late pachynema. The loss of MLH3/
MLH1 foci may be secondary to a possible cell cycle error such as premature exit from G2 before chiasmata are established. As mentioned, cyclins A1, B1, and B3 are essential for meiosis. Deletion of the germ-cell specific cyclin A1 results in a MI spermatogenic arrest in males [46,47]. Cyclin A1 mRNA and protein are expressed primarily from late pachynema through diplonema of prophase I [46,47]. CCNB1 is expressed from mid-pachynema through postmeiotic spermiogenesis [48], and regulation of partial proteolytic degradation of this cyclin is necessary for homolog disjunction in mouse oocytes [24]. In oocytes, CCNB1 is essential for the successful completion of MI as loss of Ccnb1 expression causes accelerated polar body extrusion and inability to enter G2–metaphase meiosis II [49]. Recently, a third mammalian B-type cyclin, cyclin B3 (Ccnb3) has been identified that is highly expressed in prepachytene spermatocytes and the fetal ovary [18].

HEI10 has been shown to associate with exogenously expressed human CCNB1 in mitotic cells and in yeast two-hybrid assays utilizing a somatic cell-derived library [35]. Golemis and colleagues suggested that the human protein product of HEI10 functions as a mitotic E3 ubiquitin ligase for CCNB1, ensuring its degradation and thus transition from metaphase I to interphase of meiosis II [35]. In the male germline, however, CCNB1’s activity is highest in postmeiotic spermatids [48], which is temporally inconsistent with a potential role of HEI10 as a CCNB1 E3 ligase. Further, since Ccnb1 and Hei10 are expressed in many somatic cell types (Hei10, Figure 4D) and mutation of Ccnb1 causes embryonic lethality [50], one might expect a more pleiotropic somatic phenotype in the Hei10mei4 mutant mice if CCNB1 were the primary target.

Koff and colleagues identified mammalian CCNB3, a meiotic B-type cyclin that is expressed maximally during the leptotene to zygotene transition [18], a point just prior to the first observed phenotypic defect in Hei10mei4 homozygotes. This raises the possibility that CCNB3 is the target of HEI10’s B-type cyclin E3 ligase activity in meiosis. Hei10mei4 has a deletion in the putative cyclin-B interaction domain that contains an RXL motif. We speculate that this mutation abrogates an association between HEI10 and CCNB3. This scenario may explain the observed defects in crossing over, homolog attachment, and congression to the metaphase

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Figure 5. Genomic Sequencing of Hei10 Reveals a Mutation in the Consensus GT 5′ Splice Site in Intron 1

(A) Genomic DNA from +/+; mei4/+; and mei4/mei4 animals was sequenced, and a single base transversion (g > t, gray shading) was found in the 5′ splice site consensus GT sequence. Exon sequence is denoted with capital letters and intron sequence with lower case.

(B) The 5′ splice site of intron 1 contains the consensus GT dinucleotide (Hei10). The splice site mutation described in (A) (g > t, red t indicates mutant form) causes the splicing machinery to select a GT site 72 bp upstream of the original site (dashed line). The 72-bp in-frame section of exon 1 is removed with intron 1 and is not present in the mature transcript (sequence not shown). The predicted protein sequence of HEI10 in mei4/mei4 animals contains a deletion of amino acids (a.a.) 76–100 including a conserved putative cyclin binding domain (red RAL).

doi:10.1371/journal.pgen.0030139.g005

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Roles of HEI10 in Meiosis versus Mitosis

The genetic and cytological data presented here are supportive of a key role for Hei10<sup>mei4</sup> in mouse meiosis. Toby and colleagues previously demonstrated a role for (human) HEI10 in the mitotic cell cycle [35]. Our data show that Hei10 is transcribed in many somatic tissues but at considerably lower levels than in testes (Figure 4D). While the effect of the mutation in meiosis is dramatic, we have not identified any obvious defects in Hei10<sup>mei4</sup> mice other than infertility. One possible explanation is that while Hei10 is expressed in many tissues at lower levels than in testis, its hypothesized in vivo target (CCNB3) is primarily expressed in germ cells. Thus, the effects in somatic cells may be subtle.

Alternatively, as a putative B-type E3 ligase, HEI10 may play an essential role maintaining accurate euploid segregation at the meiotic prophase I-metaphase I boundary and the mitotic G2-metaphase boundary. Errors in either, even if relatively rare, could lead to aneuploid segregation and/or neoplasia. Notably, Mine and colleagues have shown that HEI10 resides at a fusion breakpoint in some uterine leiomyomas, a benign tumor of the reproductive system [32]. We have not noticed an elevated tumor incidence in Hei10<sup>mei4</sup> mice.

Hei10 Is Conserved among Deuterostomes

Several years ago, we initiated a forward genetic screen with the goal of identifying novel genes required for gametogenesis, with an emphasis on those required for meiosis [32]. Although meiosis is a highly conserved process, it is noteworthy that two (mei4 and Mei1 [53]) mutations cloned from these screens do not have direct orthologs in Saccharomyces cerevisiae, Drosophila melanogaster, or Caenorhabditis elegans. Hei10 is conserved among deuterostomes but has not been found in protostomes or unicellular organisms and may serve as a marker of the modification of sexual reproduction during a major transition in the animal lineage. Among deuterostomes, sequence identity is highest in the N-terminal portion of the protein containing the RING finger and a coiled coil domain (Figure 7, amino acids 1–197). The C-terminal portion (residues 198 to the end) of the protein exhibits reduced sequence identity when compared to the N terminus. Interestingly, Hei10 orthologs have not been found in Gallus gallus or Taeniopygia guttata (zebra finch). These results may reflect incomplete sequence coverage in the Hei10 syntenic region in chicken or zebra finch or may be the result of divergent meiotic evolution in avian taxa. The identification of Hei10<sup>mei4</sup> and Mei1 validate the efficacy of N-ethyl-N-nitrosourea screens in mammals as a tool to identify novel genes required for meiosis.

CDK2 and Prophase I

The Hei10<sup>mei4</sup> phenotype sheds light on the role of CDK2 in meiotic progression. Cdk2<sup>−/−</sup> mice fail to complete MI because of an arrest in early prophase I [20] by presumably triggering the pachytenite checkpoint. CDK2 foci are normally found at telomeric sites from zygonema through diplonema but at
interstitial crossover sites and the unsynapsed portion of the X and Y chromosomes only from mid-pachynema through early-diplonema [23]. In mei4/mei4 males, which arrest at metaphase I, CDK2 is normally localized to the telomeres and sex chromosomes but, interestingly, is absent at interstitial sites (Figure 6B). Therefore, in the mei4/mei4 background, the presence of CDK2 at the telomeres and/or the XY body, but not at the interstitial sites, is necessary to bypass the pachytene checkpoint. The correlation of the loss of CDK2 and MLH3/MLH1 foci at interstitial sites on mei4/mei4 spermatocyte chromosome cores suggests a role for CDK2 mediation of MMR during prophase I.

Figure 7. ClustalW Protein Sequence Alignment of HEI10 Orthologs from Deuterostome Lineages

Sequence from Homo sapiens (human) and Mus musculus (mouse) have been experimentally determined, while the sequences from Canis familiaris (dog), Bos taurus (cow), Monodelphis domestica (opossum), Ornithorhynchus anatinus (platypus), Xenopus tropicalis (clawed frog), Gasterosteus aculeatus (stickleback fish), Danio rerio (zebrafish), Ciona intestinalis (transparent sea squirt), and Strongylocentrotus purpuratus (California purple sea urchin) were available in databases as predicted proteins (accession numbers are listed below). A candidate RING domain, a potential RXL-type B-type cyclin interaction motif (RAL), the deleted portion of the Hei10mei4 allele (asterisks), and a central coiled-coil domain are indicated with text above the sequences. Using database and BLAST searches, HEI10 orthologs could not be identified in the chicken, yeast, or in any protostomes such as D. melanogaster or C. elegans.

doi:10.1371/journal.pgen.0030139.g007

Hei10 and Crossing Over in Mice

CDK2 and Recombination Nodules

Hei10med provides evidence of a functional connection between the cell cycle machinery via CDK2 (and possibly via
B-type cyclins) and the DNA repair machinery during prophase I. The Hei10<sup>mei4</sup> variant has a deletion in the proposed B-type cyclin interaction domain (RXL). CCNB3 has been shown to interact with CDK2 [18]. CDK2 also interacts with MLH1 at sites of recombination in mid-late pachynema [23] and is essential for meiosis [20]. We envision a mechanism by which HEI10 mediates the destruction of CCNB3, permitting CDK2 association with MLH1 or other MMR proteins in recombination nodules on the chromosome cores (Figure 8A). As suggested by Ashley and colleagues, this interpretation is consistent with CDK2 having a substrate in the recombination nodules [23]. In our model, failure to correct mismatches during Holiday junction resolution leads to incomplete recombination intermediate resolution and arrest at the metaphase I spindle checkpoint.

doi:10.1371/journal.pgen.0030139.g008

Figure 8. Proposed Mechanism: HEI10<sup>mei4</sup> Mediated Failure to Degrade CCNB3 Leads to Inability to Recruit MLH3 and MLH1 Resulting in Failed Recombination

(A) During normal Prophase I, subsequent to DSB repair via RAD51, HEI10 mediates the degradation of CCNB3 (B3) freeing CDK2 to associate with interstitial sites on chromosome cores in pachynema. Subsequently (or contemporaneously), MLH3 and MLH1 are recruited to recombination nodules containing CDK2. In diplonema, MMR has occurred, MLH3, MLH1, and CDK dissociate from the cores and chiasmata maintain homolog association until the onset of anaphase I.

(B) In mei4/mei4 animals, DSB formation and RAD51 foci occur normally. Inability of HEI10<sup>mei4</sup> to associate with B3 leads to the accumulation or mislocalization of B3 and the titration of the available CDK2. CDK2 is unable to associate with sites of recombination, as are MLH3 and MLH1. In our model, failure to correct mismatches during Holiday junction resolution leads to incomplete recombination intermediate resolution and arrest at the metaphase I spindle checkpoint.
competence. These results provide new insights into the mechanistic control and the cell cycle regulation of mammalian meiotic DNA repair systems.

Materials and Methods

Mice. Mice were obtained from and maintained at The Jackson Laboratory and the Middlebury College Research Animal Facility according to the procedures outlined by the IACUC committees at both institutions.

Oocyte culture and assessment of meiotic progression. Oocytes were removed from females 44 h after treatment of 5-wk-old female mice with equine chorionic gonadotropin (Organon, http://www.organon.com) and manually disrupted to release oocytes. The released oocytes were cultured as described to test for spontaneous resumption of meiosis [54,55]. The oocytes were examined using a stereomicroscope after 15 h of culture and assessed for germinal vesicle breakdown, indicative of the resumption of meiosis and the presence of a polar body, which is usually characteristic of progression to metaphase II.

Fertilization and blastocyst culture. Oocytes at metaphase II were washed three times in minimum essential medium (MEM) supplemented with 5% BSA. In vitro fertilization and culture were performed as described previously [56,57]. Eggs were removed from fertilization drops after 4–6 h, rinsed twice in 2.5 ml MEM, and cultured overnight in a bovine serum tube with 1 ml of fresh medium. At 24 h postfertilization, cleavage-stage embryos were rinsed twice with KSOM medium supplemented with essential and nonessential amino acids and 1 mg/ml BSA (KSOM/AA) and cultured to the blastocyst stage in 1 ml KSOM/AA medium in bovine serum tubes.

Histology. Testes were fixed in Bouin’s solution for >24 h before being embedded in paraffin. We cut 5-mm sections, and they were stained with hematoxylin and eosin (H & E). Digital images were obtained with either an Olympus BX51 upright microscope fitted with an Olympus MagniFire CCD (The Jackson Laboratory) or a Zeiss Axiioskop 2 plus microscope fitted with a Zeiss Axiocam MRm digital camera using AxioVision 4.4 software (Middlebury College Imaging Facility).

Immunocytochemistry. Testes from prepubertal males 17–24 d postpartum (dpp) were microprepared and immunolabeled as described previously [58,59] and counterstained with DAPI (Molecular Probes, http://probes.invitrogen.com) to visualize DNA. Antibodies used were anti-β tubulin (Sigma, http://www.sigmaaldrich.com), anti-SYCP3 (0.8 ng/ml) (Abcam, http://www.abcam.com), anti-MLH1 (1:500) (gift from P. E. Cohen), anti-MLH1 (1:500) (BD Biosciences, http://www.bdbiosciences.com), anti-CDK2 (1:300) (Santa Cruz Biotechnology, http://www.scbt.com), anti-SYCP1 (1:500) (Abcam), and anti-RAD51 (1:600) antibodies (http://www.ensembl.org). Immunofluorescent detection of the oocyte meiotic spindle assembly. Oocytes were collected, cultured (described above), fixed, and stained for DNA and β-tubulin as previously described [60].

Detection of apoptosis. Testes were obtained from +/+ and mei4/mei4 mutant mice that were between 2 and 4 mo old. Apoptosis in paraffin-embedded testes was assessed using TUNEL Plus Peroxidase In Situ Apoptosis Detection Kit (Chemicon, http://www.chemicon.com). Tissue section images were taken using an Axiolvert 200 microscope and Axiocam MRc camera using AxioVision 4.4 software. Apoptotic cells were imaged and counted in 0.146-mm² fields of view (200X total magnification) that were representative of the highest level of apoptosis for each section. A total of six such images from each animal for each genotype were compared from two independent and replicable experiments.

Genetic mapping. Linkage was implicated by the association of phenotypic polymorphism between C57BL/6J and CAST/Ei. wild-type CAST/Ei animals to take advantage of the higher degree of phenotype with homozygosity for C57B6/J, the parental strain that contributed to the writing of the paper.

Acknowledgments

We would like to thank the members of the Ward laboratory and Catherine Cobboldes for critical reading of the manuscript and preparation of the manuscript. JCS contributed to the writing of the paper.
supported by the Bicentennial Fund at Middlebury College and the Vermont chapter of Sigma Xi. LBG was supported by the Janet C. Curry ’49 Research Fellowship at Middlebury College. This work was supported by NIH grant GM45415 to JCS. A Cancer Center Grant supported by NIH grant GM45415 to JCS. A Cancer Center Grant

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