

MARKERS OF THE PHYSIOLOGICALLY INDUCED DNA DOUBLE STRAND BREAKS IN MOUSE SPERMATOCYTES AND YEAST MEIOSIS

Emad A. Ahmed

Laboratory of Immunology and Molecular Physiology, Zoology Department, Faculty of Science, Assiut University, Egypt

email: emad20us@yahoo.com; Tel: 00966568331887

Received: 17/3/2019 **Accepted:** 19/5/2019 **Available Online:** 7/7/2019

The integrity of the human genome is constantly under attack by several exogenous and endogenous sources of DNA damage. Among all types of DNA damage, DNA double-strand break (DSB) is considered to be of the most cytotoxic lesions. During meiosis, DNA DSBs is induced physiologically by the topoisomerase II like protein (Spo11). These induced breaks are repaired by a physiological and complicated repair process known as meiotic recombination (MR). Here, I analyzed and compared the expression of two of these highly conserved proteins, the recombinase Rad51 and the DSB marker phosphorylated histone variant γ -H2AX, which involve in the MR process during mouse and yeast meiosis. Consistent with previous studies, the expression analysis of γ -h2ax fluorescent (FL) foci formation during mouse MR show that the induced DSBs during leptotene stage of meiosis are fully repair at the end of pachytene stage except for spontaneous foci that represent persistent un-repaired ones. The recombinase Rad51 clearly marks the MR progression, where foci appear at early zygotene stage and disappear at the end recombination process when scynaptonaemal complex (SC) proteins signal degraded. However, both western blot and foci kinetics analysis of γ -H2A (human and mouse homologue) and Rad51 in WT and Spo11 knockout strains, revealed that γ -H2A is not a good marker for DSBs in during MR of yeast meiosis.

Keywords: mouse and yeast meiosis, Spermatocytes, γ -h2ax, Rad51, Meiotic recombination

INTRODUCTION

Unlike cancer where cell division is uncontrolled, meiosis is a special type of programmed cell division is needed to halve the genetic material of the diploid parental genome to produce haploid

gametes i.e. spores in fungi or egg and sperm cells in other eukaryotes; (reviewed in Hunter, 2015). This is achieved through one round of DNA replication followed by two rounds of chromosome segregation. The 1st segregation occurs between homologous chromosomes and the 2nd between sister chromatids. For correct segregation of chromosomes during meiosis, a programmed DNA double strand breaks (DSBs) are induced during leptotene stage by the topoisomerase-like Spo11 protein (reviewed in Lam and Keenly, 2015). The induced DSBs are repaired by a complicated process known as meiotic recombination (MR), during which maternal and paternal chromosomes are paired establishes physical connections (chiasmata) and the formation of synaptonemal complex (SC) proteins between chromosomes takes place. This is followed by reciprocal exchange of maternal and paternal genetic material (crossovers). Once programmed DSB is formed by Spo11, resection occurs by the MRX complex and other nucleases. The resection process results in single strand ends at 3' and thus both recombinases Rad51 and Dmc1 are loaded to the single strands forming a nucleoprotein filament. This is followed by strand invasion of one of the 3' ends and D-loops formation. The repair of the DSBs induced by SPO11 takes 7 days in mice (Moens et al., 2002). In synchronization with the biochemical changes during MR, cytological changes occur, as shown in Figure 1 for budding yeast and mice meiosis. The bounded Rad51 at the single strand is seen as foci that marks the hotspots at which chromosomal breaks are repaired and crossover and non-crossover may occur. At the same time, at the site of Spo11 induced DSBs, the histone variant H2AX is phosphorylated at serine 139 (in S129, in yeast), after which it is called γ -H2AX, leading to the formation of nuclear foci at the breaks sites. Hence, γ -H2AX is used as a marker for DNA DSBs. Studies on mouse germ cells have shown that the kinetics of γ -H2AX loss (reduction of foci numbers) is related to DSB repair activity during mice meiosis (Ahmed et al., 2010& 2013; Chicheportiche et al., 2007). The genes involve in MR process are highly conserved among human and various eukaryotic

models such as mouse and yeast. Therefore, Recognizing the function of genes and proteins associated with the repair of DNA DSBs after physiological or exogenous damage will help to understand mechanisms involved in DNA repair and genome protection. In the current study, the expression of two highly conserved proteins (Rad51 and γ -H2AX), in mice and yeast was analyzed. The recombinase Rad51, which involve in the MR processin yeast meiosis marks the recombination process, as shown by western blot analysis and IF but not the phospho-H2A.

MATERIALS AND METHODS

Animals and samples collection

Testes of albino mice (8–10 week of age) were used to check DSB repair markers in male germ cells. Mice were maintained under optimal conditions of humidity and temperature for one week at Biological Science department, King Faisal University Saudi Arabia. Nucleus spreads of spermatogenic cells were prepared (see below). The animals were used and maintained according to regulations provided by the animal ethical rules and no specific treatment has been done for mice except for isolating testes from scarified mice.

Induction of meiosis in yeast

A single colony of WT and Spo11 knockout budding yeast strains were inoculated and grown overnight in 5 ml of yeast extract peptone dextrose (YPD) media. Then, the suspension was diluted to an OD600 of ~0.3 in (buffered yeast extract tryptone acetate) BYTA and incubated with aeration for 16 h. These synchronized cultured cells (at G1-phase) were harvested by centrifugation, washed with 200 ml 1% potassium acetate, re-suspended in 250 ml SPM (0.3% potassium acetate, 0.02% raffinose) with appropriate supplements to a final OD600 of ~1.9, and incubated with vigorous aeration. In the SPM, sporulation in budding yeast (one cell gives 4 spores) occurs within 8 h (Hunt et al., 2019). Between 1 h and 8 h time points, samples were taken and were used to prepare meiotic

spreads for subsequent immunostaining or frozen to be processed later for western blot analysis.

Surface spread preparations

TesticularNucleus spreads were prepared as previously described by Ahmed et al., 2010. Briefly, a suspension of spermatogenic cells in MEM- α was obtained, followed by incubation with a hypotonic buffer (17 mM sodium citrate, 50 mM sucrose, 30 mM TrisHCl, pH 8.2). After centrifugation (1000 rpm for 5 min), the pellet was carefully resuspended in a 100 mM sucrose solution and applied over a paraformaldehyde (PFA) covered glass slide (1% PFA, 0.15% Triton-X-100, pH 9.5). The slides were kept in a humidified atmosphere in a box to slow down drying out. After 1.5 hr the box was opened and the slides were washed in 0.08% photo-flo (Sigma P7417, St. Louis, USA). Meiotic spreads preparation from testicular yeast cells (from time course points between 1 and 8 h of Zip1 tagged WT) was done as described (Oh et al., 2009, Hunt et al., 2019).

Fluorescent immunostaining

For immunofluorescence, nuclei were washed in PBS and incubated for 10 min in PBS including 0.04% Triton-X-100 and then incubated with blocking solution (10% low fat milk powder 10% BSA in PBS for 30 min). After blocking, primary antibodies were applied, diluted in blocking solution, for 2 h at room temperature. The slides were washed in PBS and incubated with the diluted secondary antibodies in blocking solution for 1 h at room temperature. Then, the nuclei were stained with DAPI (for 10 min), mounted in VECTAshield (Vector lab. H-1000) and viewed with an Olympus microscope. Images were recorded digitally.

Protein extraction and western blots

The frozen pellet was resuspended in alkaline solution (n 150 μ l of 1.85 M NaOH) and , 7.5% (v/v) β -mercaptoethanol to break the yeast cell wall. Protein suspension in sample buffer (250 μ l of 200 mM Tris-HCl at pH 6.5, 8 M urea, 5% (w/v) SDS, 1 mM EDTA, 0.02% bromophenol blue, 5% (v/v) β -mercaptoethanol with 10 μ l of

25× protease inhibitor stock (Roche), heat shock (d at 65 °C for 10 min), loading to SDS-PAGE gel, western immunoblotting were done according to our recent submitted paper (Hunt et al., 2019). Membranes were incubated in 5% (w/v) skimmed milk in TBS and subsequently incubated with primary antibody in 1% (w/v) milk in TBS. Membranes were washed 3x in PBS, 5 min each at RT, and incubated in horseradish peroxidase-conjugated secondary antibodies. Membranes were washed 3x in PBS, 5 min each at RT, incubated with 2 ml high sensitivity ECL detection solution (Millipore), and blots were visualized on a GeneGnomechemiluminescence imaging system. The Rad51 and γ -H2A signal were quantified relative to the house keeping protein PSTAIR using image J software (<https://imagej.net/Fiji/>).

Antibodies

The primary antibodies were rabbit polyclonal anti-SCP3 (1:400, ab15092, Abcam) and mouse monoclonal anti-phospho-H2AX[Ser139] (1:400 Upstate Biotechnology USA). Rabbit anti Rad51 (Santa Cruz, 1:2000); anti mouse PSTAIR, (Sigma, 1:2500) and anti-goat H2A-129 Santa Cruz (1:200). The secondary antibodies for IF, goat anti-rabbit (Alexa fluor 488, A-11008, Breda, Netherlands), goat anti-mouse (Alexa fluor 488, A-21121 and 594, A-21125, Breda, Netherlands) and Texas Red-labeled goat anti-mouse (T6134), were obtained from Jackson ImmunoResearch (West Grove, USA). The secondary antibodies for western were the appropriate horse reddish peroxidase (HRP) anti-goat, anti-mouse and anti Rabbit Santa Cruz antibodies and used and analyzed as described (Oh et al., 2009, Hunt et al., 2019).

RESULTS

γ -H2AX foci marks the physiologically induced DSBs during mouse meiotic recombination

The IF staining of early meiotic prophase testicular cells during mouse meiosis has shown that γ -H2AX displays staining at leptotene stage. In Zygotene stage, the signals start to be restricted at the autosomes and sex chromosomes and then as cells grow from

early to late pachytene the foci at autosomes pachytene stage are repaired and hence disappear as the meiotic recombination progress and crossover and non-crossover occur (Fig. 1). These data confirm that γ -H2AX is an ideal marker for DSBs during meiosis.

Rad51 and γ -H2A showed different kinetics during yeast meiosis

In yeast, the IF staining data revealed that γ -H2A is not clearly expressed as foci but showed non specific staining (Fig 2 B-E). These foci are not overlapped with Rad51 foci (Fig 2 B,C). On the other hand, Rad51 clearly marks the hotspot sites at autosomes and forms foci in early zygotene, zygotene and pachytene stages. Those foci disappear by the end of pachytene stage when the SC (ZIP1) protein degraded (Fig 2). These data indicated different kinetics between Rad51 and γ -H2A in yeast meiosis.

Western blot analysis of Rad51 and γ -H2AX during yeast meiosis in WT and Spo11 knockout strains.

Western blot analysis of Rad51 and γ -H2AX relative to the PSTAIR cyclin-dependent kinase during yeast meiosis in WT and Spo11 knockout strains reveals that Rad51 signals are reduced at longer time points during meiosis. However, γ -H2A signal is not reduced by time. Although in Spo11 knockout stain the physiological DNA DSBs are not induced, γ -H2A bands were seen which clearly indicate that this phospho-Histon variant is not good marker of DSBs in yeast.

DISCUSSION

In the present study, the expression of the DSB maker γ -H2AX (γ -H2A) at the chromosomal level by IF staining during meiotic recombination in mice and yeast has been analysed. In addition the expression of both γ -H2A and Rad51 have been analyzed by western analysis during yeast meiosis. γ -H2AX form foci at the spermatocytes' autosomes hotspots, all these foci are dephosphorylated as MR progresses, and disappear at the end of early meiotic prophase leaving only few persistent foci (Fig 1). These DSBs are induced during leptotene stage of mouse meiosis

when the SC protein are formed (SCP1, SCP2 and SCP3, the later is shown the figure 1 to ensure accurate chromosomal recombination and segregation (Keeney et al., 1997). However, during sporulation, that occurs in synchronized budding yeast cell within 8 hours, IF staining data reveals that γ -H2A is not expressed as foci and shows non specific staining (Fig 2 B-E). Although in mice, Rad51 and γ -H2AX are partially overlapped (Ahmed et al., 2007) in yeast γ -H2A foci are not overlapped with Rad51 foci (Fig 2 B,C). The recombinase Rad51 clearly marks the hotspot site at autosomes and forms foci in early zygotene, zygotene and pachytene stages. Those foci disappear by the end of pachytene stage when the SC (Zip1) protein degraded (Fig 2). In mice, similar kinetics of Rad51 formation and disappearance have been shown and partially overlapped with γ -2AX (Ahmed et al., 2007). Foci kinetics analysis of γ -H2AX and Rad51 in yeast, revealed that γ -H2A is not a good marker for DSBs in during MR of yeast meiosis. However, the recombinase Rad51 is a good marker for MR progression in yeast and mice.

To further check the reliability of γ -H2A as a DSB marker during yeast meiosis, western blot analysis of the phosphorylated H2A-S129 has been conducted and compared with Rad51 expression. Data indicated that γ -H2A is expressed in synchronized pre -meiotic cells (at G1-phase), its level increased as cells grow from early Zygotene to pachytene stage, but only slightly decreased at the end of meiosis (at 8h where meiosis I and II are completed). The higher expression of γ -H2A at the end of meiosis is consistent with the finding of Lo et al., (2014). To clarify if the expression of γ -H2A is dependent on Spo11 induced DSB, Spo11 knockout stain has been used to check the phosphorylated protein expression. Interestingly, signals of γ -H2A, but not for Rad51, were seen in the absence of Spo11 during yeast meiosis.

CONCLUSION

Both western blot and foci kinetics analysis of γ -H2A and Rad51 in WT and Spo11 knockout strains, revealed that γ -H2A is not a good

marker for DSBs in during MR of yeast although it is a reliable mouse and Human meiosis DSB marker.

REFERENCES

- Ahmed EA, Philippens ME, Kal HB, de Rooij DG, de Boer P. Genetic probing of homologous recombination and non-homologous end joining during meiotic prophase in irradiated mouse spermatocytes. *Mutat Res.* 2010 Jun 1;688(1-2):12-8.
- Ahmed EA, Rosemann M, Scherthan H. NHEJ Contributes to the Fast Repair of Radiation-induced DNA Double-strand Breaks at Late Prophase I Telomeres. *Health Phys.* 2018 Jul;115(1):102-107.
- Ahmed EA, Sfeir A, Takai H, Scherthan H. Ku70 and non-homologous end joining protect testicular cells from DNA damage. *J Cell Sci.* 2013 Jul 15;126(Pt 14):3095-104.
- Chicheportiche A, Bernardino-Sgherri J, de Massy B, Dutrillaux B. Characterization of Spo11-dependent and independent phospho-H2AX foci during meiotic prophase I in the male mouse. *J Cell Sci.* 2007 May 15;120(Pt 10):1733-42.
- Oh, S.D., Jessop, L., Lao, J.P., Allers, T., Lichten, M., and Hunter, N. (2009). Stabilization and electrophoretic analysis of meiotic recombination intermediates in *Saccharomyces cerevisiae*. *Methods MolBiol* 557, 209-234.
- Hunt L, Ahmed E A, Kaur H, Abuja J, Hulme L, Chou T, Lichten M and Goldman A. *S. cerevisiae* Srs2 helicase ensures normal recombination intermediate metabolism during meiosis and prevents accumulation of Rad51 aggregates, *Chromosoma*, 2019.
- Hunter N. Meiotic Recombination: The Essence of Heredity. *Cold Spring Harb Perspect Biol.* 2015 Oct 28;7(12). Review.
- Lo YH et al. Pch2 prevents Mec1/Tel1-mediated Hop1 phosphorylation occurring independently of Red1 in budding yeast meiosis. *PLoS One.* 2014 Jan 21;9(1):e85687.
- Moens PB, Kolas NK, Tarsounas M, Marcon E, Cohen PE, Spyropoulos B. The time course and chromosomal localization of recombination-related proteins at meiosis in the mouse are compatible with models that can resolve the early DNA-DNA

interactions without reciprocal recombination. *J Cell Sci.* 2002 Apr 15;115(Pt 8):1611-22.

Keeney S, Giroux CN, Kleckner N. Meiosis-specific DNA double-strand breaks are catalyzed by Spo11, a member of a widely conserved protein family. *Cell.* 1997 Feb 7;88(3):375-84.

Figures legend

Fig. 1. γ -H2AX foci marks the physiologically induced DSBs during mouse meiotic recombination. (A and B) Leptotene cell, B) SCP3 protein is red and γ -H2AX signals in green, (C) early pachytene cells, foci at autosomes (green) and sex body (arrow-head), (D) mid-Pachytene stage, (E) Late pachytene (persistent foci, arrow) and (F) Diplotene stage. Magnified images for autosomes (C', C'' and E')

Fig. 2. Rad51 and γ -H2A kinetics during yeast meiosis. (A) Progression of yeast cells tagged with ZIP1 protein during meiosis. (C) Rad51 foci mark hotspots at early zygotene stage and pachytene stages (E). γ -H2AX appear to be not specific (B,D).

Fig. 3. Western blot analysis of Rad51 and γ -H2AX during yeast meiosis in WT and Spo11 knockout strains. (A) Western data included the house keeping PSTAIR protein (cyclin-dependent kinase 1 (CDK1) and (B) Image J analysis of relative expression to PSTAIR.

دلالات التكسر الفسيولوجي للحامض النووي المزدوج الشريط في الخلايا المنوية
الابتدائية في الفئران والخلايا المنقسمة ميوزيا في الخميرة

عماد عبدالعزيز أحمد

معمل المناعة والفسيولوجيا الجزيئية - قسم علم الحيوان - كلية العلوم - جامعة أسيوط

يتعرض الجينوم البشري باستمرار للعديد من العوامل المسببة لتكسر أو تلف أجزاء من الحامض النووي. ويعتبر تكسر الحامض النووي المزدوج من أخطر الأنواع المسببة للطفرات والسرطان، وقد يتكسر الحامض النووي بصورة طبيعية فسيولوجية وذلك أثناء الانقسام الاختزالي (الميوزي) للخلايا التناسلية

حيث يقوم بروتين يسمى Spo11 (بروتين متواجد في الانسان والفئران والخميرة) بكسر الحامض النووي علي مستوي الشريط المزدوج مما يحفز الخلايا اثناء نموها (الانقسام الميوزي الأول) (علي اصلاح هذا التكسير بواسطة عملية معقدة تشترك فيها العديد من البروتينات حيث يحدثتزاوج الكروموسومات الغير شقيقة يعقبه التداخل ثم العبور ليؤدي ذلك إلي تبادل للمادة الوراثية . هذا ويرتبط البروتين المحفز للتداخل والعبور هو Rad51 مع النهاية المفردة للحامض النووي الناتجة عن الكسر ويوجه هذه العملية المعقدة. كما تتم فسفرة نظير الهستون H2A (H2A ويكون بؤر علي الكروموسومات عند مواقع هذا التكسر الفسيولوجي. تهدف هذه الدراسة إلي تحليل ومقارنة التعبير البروتيني لكلا من Y-H2A , Rad51 اثناء الانقسام الاختزالي في خلايا الفئران وخلايا الخميرة التي تنقسم ميوزيا في وجود ظروف نقص الغذاء والنيتروجين. تماشيا مع الدراسات السابقة، أظهر تحليل تعبير تكوين بؤر γ -H2AX في الخلايا المنوية الابتدائية لخصية الفار وكان واضحا ان هذا البروتين قد يستخدم كدلالة علي الاصلاح الفسيولوجي للحامض النووي وبقاء البور يعكس التكسر الذي لم يتم اصلاحه ويدلل علي خلل ما بالخلية قد يؤثر علي انفصال وتباعد الكروموسومات اثناء الطور الانفصالي من الانقسام الميوزي. علي الجانب الاخر فيما يتعلق بنظير الهستون المماثل لهذا البروتين في الخميرة لا تؤيد الدراسة الحالية استخدامة لدلالة لتكسر الحامض النووي واصلاحه في الابحاث التي تستخدمها كنموذج مهم لحقيقيات النواة في مجال البيوتكنولوجي. بينما كان البروتين المحفز للتبادل الكروموسومي والعبور Rad51 ذو دلالة واضحة.

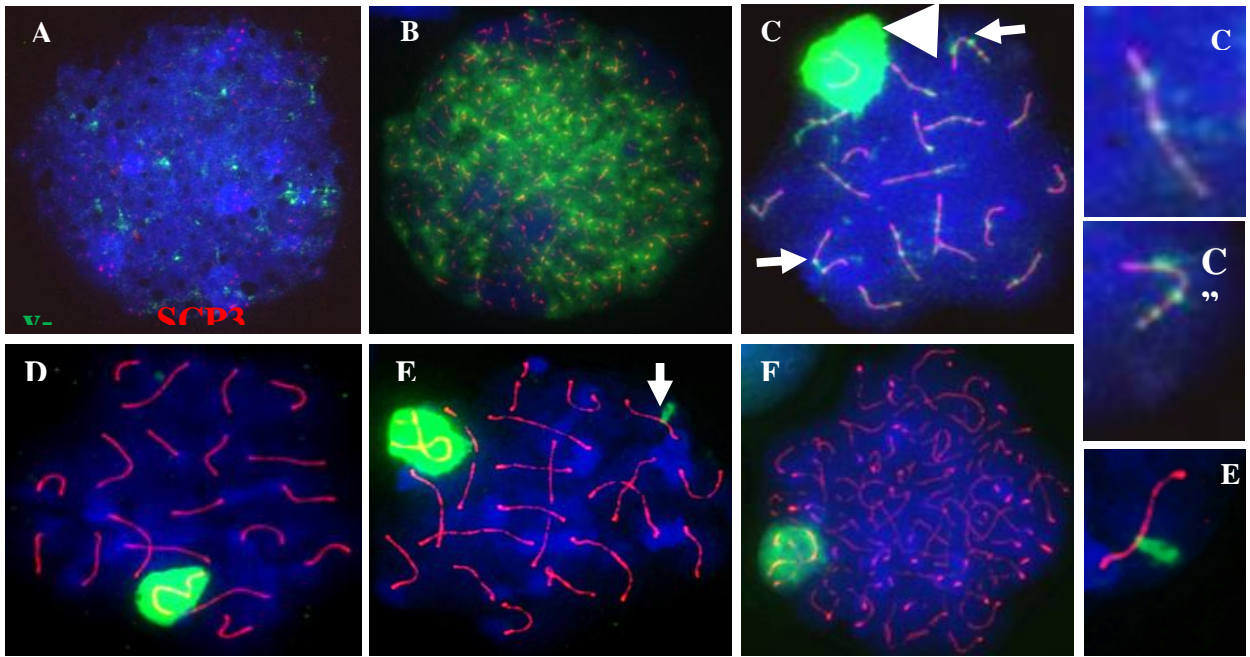


Fig. 1. γ -H2AX foci marks the physiologically induced DSBs during mouse meiotic recombination. (A and B) Leptotene cell, B) SCP3 protein is red and γ -H2AX signals in green, (C) early pachytene cells, foci at autosomes (green) and sex body (arrow-head), (D) mid-Pachytene stage, (E) Late pachytene (persistent foci, arrow) and (F) Diplotene stage. Magnified images for autosomes (C', C'' and E')

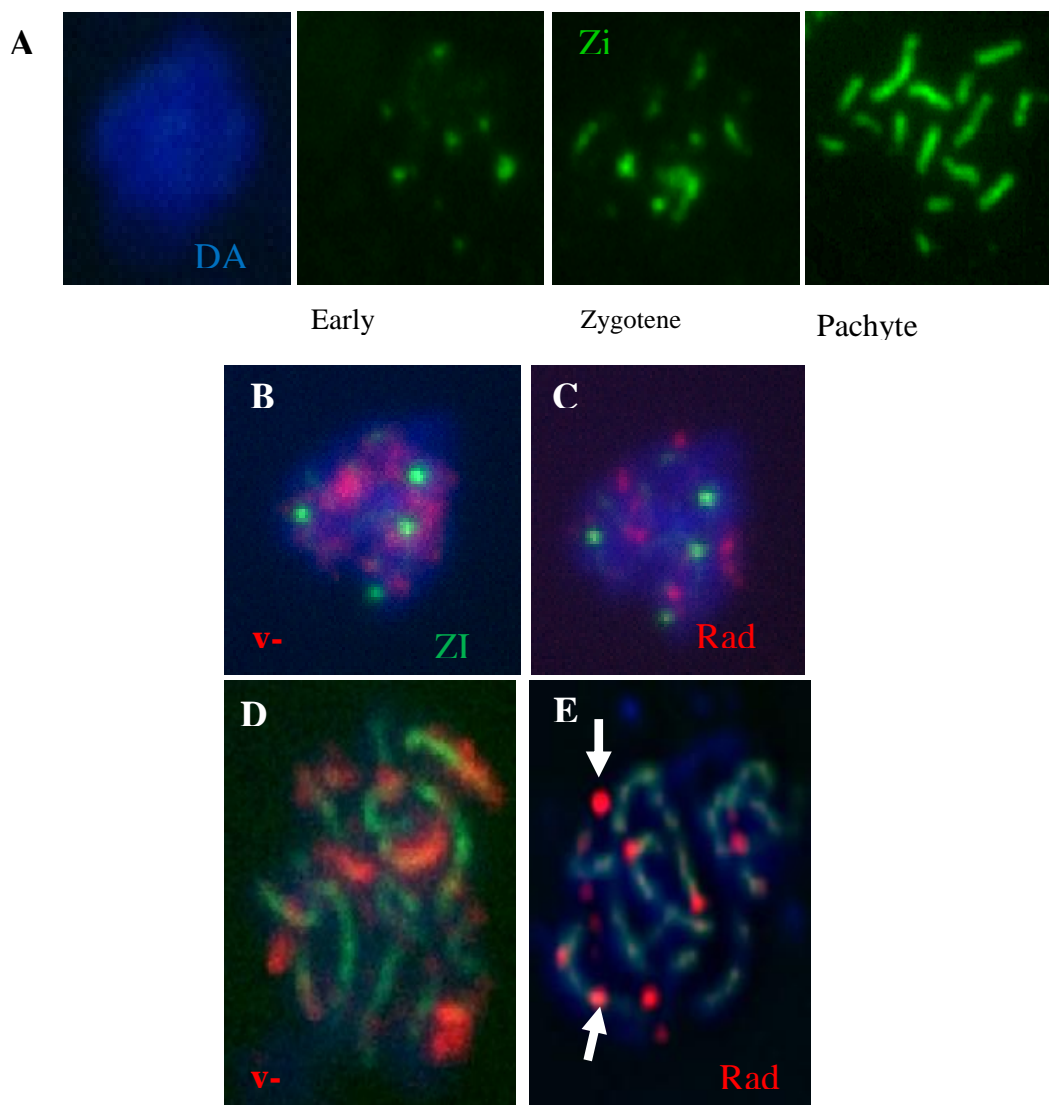


Fig. 2. Rad51 and γ -H2A kinetics during yeast meiosis. (A) Progression of yeast cells tagged with ZIP1 protein during meiosis. (C) Rad51 foci mark hotspots at early zygotene stage and pachytene stages (E). γ -H2AX appear to be not specific (B,D).

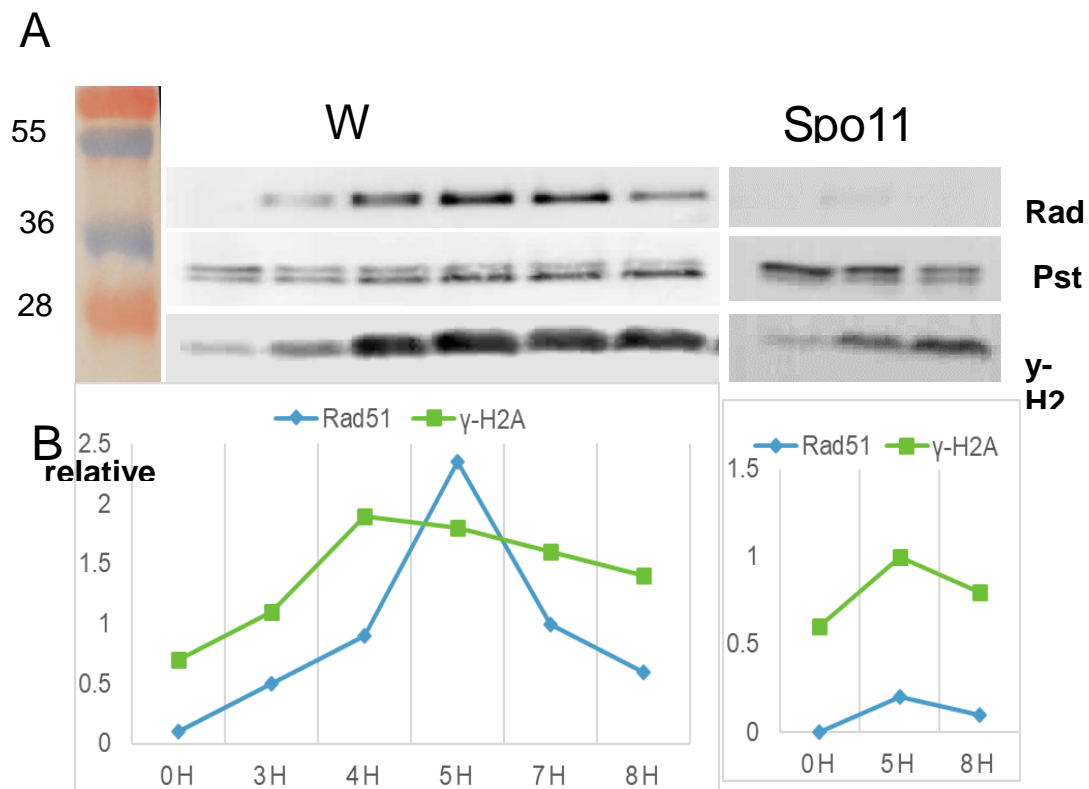


Fig. 3. Western blot analysis of Rad51 and γ -H2AX during yeast meiosis in WT and Spo11 knockout strains. (A) Western data included the house keeping PSTAIR protein (cyclin-dependent kinase 1 (CDK1) and (B) Image J analysis of relative expression to PSTAIR.