Role of HP1β during spermatogenesis and DNA replication

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Abstract
Heterochromatin protein 1β (HP1β), encoded by the Cbx1 gene, has been functionally linked to chromatin condensation, transcriptional regulation, and DNA damage repair. Here we report that testis-specific Cbx1 conditional knockout (Cbx1 cKO) impairs male germ cell development in mice. Depletion of HP1β negatively affected sperm maturation and increased seminiferous tubule degeneration in Cbx1 cKO mice. In addition, the spermatogonia have elevated γ-H2AX foci levels as do Cbx1 deficient mouse embryonic fibroblasts (MEFs) as compared to wild-type (WT) control MEFs. The increase in γ-H2AX foci in proliferating Cbx1 cKO cells indicates defective replication-dependent DNA damage repair. Depletion or loss of HP1β from human cells and MEFs increased DNA replication fork stalling and firing of new origins of replication, indicating defective DNA synthesis. Taken together, these results suggest that loss of HP1β in proliferating cells leads to DNA replication defects with associated DNA damage that impact spermatogenesis.

Keywords HP1β · Spermatogenesis · DNA replication · Fork stability · Homologous recombination

Introduction
Normal germ cell progression through meiosis depends upon homologous chromosomes undergoing accurate pairing, synapsis, recombination, and proper segregation. Gametogenesis generates a large number of base mismatches, single-strand and double-stranded DNA breaks (DSBs) which are repaired through the base excision repair (BER), mismatch repair (MMR), homologous recombination (HR), and non-homologous end joining (NHEJ) pathways (Garcia-Rodriguez et al. 2018). Unlike somatic cells, spermatogonia stem cells undergo multiple cycles of DNA synthesis/cell divisions to produce spermatogonia cells which undergo a final meiotic cell division to produce mature, haploid spermatoocytes. During spermatogenesis, a high frequency of DSBs is generated in meiotic prophase I. Double-strand breaks that persist until pachynema are more likely to be repaired through crossovers (Allers and Lichten 2001) and non-crossovers (Cohen and Pollard 2001; Roeder 1997).

Heterochromatin proteins (HP1) are a highly conserved family of proteins, which contain a characteristic chromodomain and play a critical role in establishing and maintaining heterochromatic domains (Wang et al. 2000). In mammals, three HP1 isoforms are known (HP1α, HP1β, and HP1γ) which are located at different chromosomal sites (Chevillard et al. 1993). Each mammalian HP1 contains a chromodomain and a chromo shadow domain separated by a hinge region (Eissenberg and Elgin 2000; Lorentz et al. 1994; Wang et al. 2000). The HP1 proteins are small, less than 200 amino acids, with molecular masses of approximately 25 kDa.

In somatic cells, heterochromatin proteins (HP1s) are fundamental units of heterochromatin regions (Bosch-Presegue et al. 2017). Heterochromatin proteins are non-histone chromatin components that interact with a variety of proteins which
contribute to chromatin remodeling and transcriptional silencing (Ma et al. 2001). Furthermore, HP1 proteins function in epigenetic gene regulation and DNA damage repair by HR (Bosch-Presegue et al. 2017; Horikoshi et al. 2019; Kalousi et al. 2015; Kumar et al. 2012; Legartova et al. 2019; Lomberk et al. 2012; Sharma et al. 2003). During male germ cell development, heterochromatin regions undergo a series of remodeling events before the final maturation of spermatozoa (Bruner 2014). Previous studies have shown that chromatin modifiers/DNA repair proteins have an essential role in the gametogenesis process (Guo et al. 2018; Simhadri et al. 2014; Sun et al. 2016; Ward et al. 2003) as loss of HR repair-related proteins, such as 53BP1, RIF1, BRCA1, ATM, and MOF, adversely affects spermatogenesis (Bartkova et al. 2001; Jiang et al. 2018; Marcet-Ortega et al. 2017; Pandita et al. 1999; Schwab et al. 2013; Sun et al. 2016). Our prior studies reported that mice lacking HP1β due to gene deletion (Cbx1−/−) suffer perinatal death due to severe alveoli lung damage as well as abnormal neuromuscular and cerebral cortex tissue development (Aucott et al. 2008). In addition, Cbx1−/− MEFs displayed increased genomic instability (Aucott et al. 2008). In this study, we generated testis-specific conditional Cbx1 knock out mice as a means to examine the in vivo effect of Cbx loss in a rapidly dividing cell population that requires accurate DNA repair for functional development. Deletion of Cbx1 in rapidly dividing germ cells was found to cause DNA replication defects and DNA damage that affected cell survival, spermatogenesis, and male fertility.

Materials and methods

Ethics statement

Cbx1m1a KOMP–CSD allele mice were obtained from the European Mutant Mouse Archive (EMMA) consortium. Gt(Rosa)26 Sor tm1(FLP) DYM/RainJ and Tg(Str8-icre)1Reb/J (FLPeR) mice (Farley et al. 2000) were obtained from Jackson Laboratory. All animal experiments were performed according to guidelines of the Institutional Animal Care Committee of the Houston Methodist Research Institute, USA.

Cell isolation

Cbx1 WT and Cbx1 cKO mice were sacrificed by cervical dislocation/CO2 asphyxiation. Testes were excised and washed three times with PBS. Isolated testis were cut into small pieces and re-suspended in 2 ml of TIM buffer containing 40 mg of collagenase. After 1 h, samples were incubated with 1 mg/ml trypsin (T8003; Sigma) for 20 min. DMEM + 10% FBS media was added to neutralize. These single cell suspensions were washed twice with PBS and re-suspended in 0.1 M sucrose solution. Then, 20 µl of cellular suspension mixed with 65 µl of 1% PFA + 0.1% Triton X-100 spread on a slide uniformly. Air-dried slides were stored at −80 °C until utilized (Zelazowski et al. 2017).

Immuno-fluorescence staining was performed by incubating tissue sections with primary antibodies (γ-H2AX, 1:200, 05–636, Millipore; SYCP3, 1:200, AB15093, Abcam; RPA, 1: 250, AB2175, Abcam) overnight, then washed three times with PBS, before incubating with secondary antibodies (anti-rat Alexa Fluor 488–conjugated [1:100 dilution] and anti-mouse Alexa Fluor 568–conjugated [1:100 dilution] antibodies) for 2 h. Each slide was washed 3 times with PBS. Images were captured on an Axio Imager 2.0 microscope and analyzed by Image J software.

Cell survival assay

Cbx1WT and Cbx1−/− MEF cells were seeded onto separate 60-mm dishes in triplicate, incubated for 16 h, and then treated with either cisplatin for 1 h or hydroxyurea (HU) for 24 h. Treated cells were grown for 13 additional days and the surviving colonies were stained and counted.

DNA replication restart assay and fork protection assay

The DNA fiber assay was performed as previously described (Singh et al. 2013; Mattoe et al. 2017). Exponentially growing Cbx1WT and Cbx1−/− MEF cells were pulse labeled with 150 mM 5-chlorodeoxyuridine (CldU) for 30 min before stalling with 2 mM HU for 2 h, then washed twice with PBS and incubated in medium containing 450 mM iodoxyuridine (IdU) for 1 h (Chakraborty et al. 2018; Horikoshi et al. 2016).

Replicative fork progression, stability assays

Exponentially growing cells were incubated in fresh medium containing 50 mM 5-chlorodeoxyuridine (CldU) for 20 min, washed in PBS three times, and then incubated in media containing 450 mM IdU for 30 min. These labeled cells were treated with 2 mM HU and DNA fibers were prepared at 0 h, 4 h, and 6 h later (Schlacher et al. 2012). DNA fiber spreads were fixed and washed 3 times with PBS, blocked with 5% BSA for 1 h, before incubation for 1 h with anti CldU (1:150) and anti IdU (1:150) antibody. After washing in PBS, the fibers were incubated for 1 h in secondary anti-rat Alexa Fluor 488–conjugated (1:100) and anti-mouse Alexa Fluor 568–conjugated (1:100) antibody solution. Each slide was then washed three times with PBS. Mounted cell images were acquired by using an Axio Imager 2.0. DNA fiber length was measured by using Image J software. For each data set, the length of about 300 nascent CldU-labeled fibers was measured. Results were summarized in bar graphs.
Hematoxylin and eosin staining and TUNEL staining

Harvested testes were immediately fixed in 4% paraformaldehyde for immunohistochemistry. For hematoxylin and eosin (H&E) staining, testis tissue sections were deparaffinized, rehydrated, and stained with H&E (Kumar et al. 2011; Jiang et al. 2018). TUNEL staining was performed on testis sections according to the instructions provided with the cell death detection kit (11684795910, Roche). To reduce experimental variations, Cbx1 WT and Cbx1 cKO mice were processed simultaneously. Images were captured using an Axio Imager microscope equipped with a CCD camera and analyzed with Image J software.

Sperm counting

The epididymis and vasa differentia were removed from Cbx1 WT and Cbx1 cKO mice, incised several times, and then incubated in 1 ml PBS at 37 °C to release the sperms. The
number of sperms per testis was counted by using a hemocytometer.

Statistical analysis

All data are presented as mean ± standard deviation (SD). The Mann-Whitney test was applied to compare the number of γ-H2AX foci, RPA foci, and SCP3 between Cbx1 WT and Cbx1 cKO mice spermatocytes. Student’s t test was used for TUNEL and sperm count assays.

Results

Generation of Cbx1 conditional knockout mice

To generate cKO HP1β (Cbx1) mice for the study, we first deleted the LacZ/neomycin gene region in the EMMA Cbx1tm1a mice by crossing with FLPeR (flipper) mice (Farley et al. 2000). Homozygous Cbx1EFT mice were then generated by backcrossing the Cbx1EFT/+ mice (Fig. 1a) and tissue-specific Cbx1 knockout in testis then generated by crossing Cbx1EFT mice with Stra8 CreERT2 mice using the strategy described previously (Gupta et al. 2013; Kumar et al. 2011). Cre expression deleted the second exon and shifted the Cbx1 gene reading frame, which ultimately resulted in loss of functional HP1β protein in testis (Fig. 1a).

Generation of Cbx1EFT in mice was confirmed by PCR (Fig. 1b). Deletion of testis-specific Cbx1 EKO in mice was confirmed by western blot analysis (Fig. 1c). All Cbx1 WT, Cbx1EFT+/CreERT2 (Cbx1 heterozygous), and conditional testis-specific Cbx1 cKO mice were viable with no visibly apparent defects. Cbx1 WT and Cbx1 heterozygous male mice displayed similar fertility levels, whereas in Cbx1 cKO mice produced half the litter size as compared to Cbx1 WT mice. Cbx1 cKO mice had 3.07 pups per litter and a 1/1.7 male/female ratio (Table 1) while wild-type mice had 7.87 pups per litter and a 1/1.28 male/female ratio. These results suggest testis-specific loss of Cbx1 caused a male specific sub-fertility effect as well as a skewed sex ratio.

Loss of HP1β impairs spermatogenesis and sperm maturation

The testis size of Cbx1 WT and Cbx1 cKO mice was compared and Cbx1 cKO testes found to be about 20% smaller in size than Cbx1 WT testis (Fig. 1d) and also weighed less (Fig. 1e). Depletion of HP1β impairs chromosome repair in human cells (Sharma et al. 2003) and loss of DNA repair proteins in vivo has been linked with germ cell loss and seminiferous tubule degeneration that result in physiological manifestations (Gunes et al. 2015; Xu et al. 1996). We, therefore, examined whether Cbx1 loss in testis cause similar defects. Tubular vacuolation and partial degeneration of spermatogonia were increased in Cbx1 cKO testes (Fig. 1f) in seminiferous tubule degeneration which is known to affect spermatogenesis and mature sperm production (Vyas et al. 2013). We harvested epididymis sperms from Cbx1 WT and Cbx1 cKO mice and found that Cbx1 loss reduced sperm counts by 50% as compared to WT mice (Fig. 1g). Moreover, the Cbx1 cKO mice had a 5-fold increase in abnormal sperm including sperm lacking hook or having a folded banana-shaped head morphology (Fig. 1h, i). Since we did not observe HP1β in post pachytene stage cells in either wild-type or Cbx1 cKO testes by immunofluorescence analysis (Fig. 2a), these results suggest that HP1β has a critical role in the early stages of meiosis. To further investigate HP1β function in spermatogenesis,

Table 1 Average litter size of Cbx1 WT, Cbx1 heterozygous and Cbx1 cKO mice

| Genotype (male × female) | Cbx1EFT CreERT2 × Cbx1EFT | Cbx1EFT+/CreERT2 × Cbx1EFTCreERT2 | Cbx1EFT+/CreERT2 × Cbx1EFT |
|-------------------------|---------------------------|----------------------------------|---------------------------|
| No. of breedings        | 8                         | 7                                | 13                        |
| Average pups (total pups) | 7.87 (63)                 | 6.57 (46)                        | 3.07 (40)                 |
| Average male pups/total pups | 0.437                    | 0.42                            | 0.37                      |
| Average female pups/total pups | 0.56                    | 0.58                            | 0.63                      |
| Male/female ratio       | 1.28                      | 1/1.38                          | 1/1.70                    |

Fig. 2 Loss of Cbx1 increased γ-H2AX and apoptosis in testis. a Immunofluorescence staining for HP1β (red) and γ-H2AX (green) in Cbx1 WT and Cbx1 cKO mouse testis sections. b Immuno-staining SYCP3 (green) and γ-H2AX (red) in Cbx1 WT and Cbx1 cKO spermatocytes. c Frequencies of spermatocytes in the Cbx1 WT and Cbx1 cKO meiotic stages. d Percentage of spermatocytes containing >5 γ-H2AX in Cbx1 WT and Cbx1 cKO. e Representative immunofluorescence staining images of RPA (red) and SYCP3 (green) in spermatocytes stages in Cbx1 WT and Cbx1 cKO, spermatogonia (top panel), and pachytene (low panel). f Average mean number of spermatocytes with >10 RPA foci. g Images of TUNEL-stained sections of Cbx1 WT and Cbx1 cKO mice (arrow indicating cells undergoing apoptosis). h Quantification of the TUNEL assay results is then represented as the percentage of apoptotic cells. Data analyzed and represented as mean ± SEM. **P < 0.01, ***P < 0.001, Student’s t test.
spermatocytes were prepared from Cbx1 WT and Cbx1 cKO testes and stained for synaptonemal complex protein 3 (SYCP3) which revealed that loss of HP1β increased spermatocyte arrest at the spermatogonia stage (Fig. 2b, c).

**Elevated γ-H2AX and apoptosis levels in spermatogonia cells lacking HP1β**

HP1β plays an integral role in gene repression and DNA repair (Bosch-Presegue et al. 2017; Horikoshi et al. 2019; Kalousi et al. 2015; Kumar et al. 2012; Legartova et al. 2019; Lomberk et al. 2012; Sharma et al. 2003). The basal level of γ-H2AX foci in seminiferous tubules of Cbx1 cKO and Cbx1 WT testes was measured. Loss of Cbx1 increased γ-H2AX foci formation in the initial stages of spermatogenesis. While we observed increased γ-H2AX foci in spermatogonia and pachytene cells, there was no comparable difference observed in the leptotene and zygotene phases (Fig. 2b, d). One major function of γ-H2AX is to recruit proteins required for DSB repair, after which it is removed. Phosphorylated H2AX...
(γ-H2AX) recruits MDC1 and further mediates binding of DNA end resection proteins (Chakraborty et al. 2018; Guo et al. 2018). To determine whether the increased γ-H2AX foci in Cbx1 cKO spermatogonia cells was due to defective DNA damage repair, we examined RPA foci in Cbx1-deficient spermatoocytes. Interestingly, we observed that Cbx1 cKO had decreased RPA foci formation in spermatogonia cells and at the pachytene stage of meiosis (Fig. 2e, f). The elevated level of γ-H2AX (Fig. 2d) correlated with decreased RPA foci (Fig. 2e, f) in spermatogonia cells in Cbx1 cKO mice. Apoptosis was more pronounced in seminiferous tubules of Cbx1 cKO mouse testicles compared with Cbx1 WT mouse testicles (Fig. 2g, h). Cbx1 cKO mice also had spermatocyte degeneration in mouse testicles (Fig. 2e, f). In spermatogonia cells in Cbx1 cKO mice, apoptosis was due to defective DNA damage repair, we examined RPA foci in Cbx1-deficient spermatocytes. Interestingly, we observed that Cbx1 cKO had decreased RPA foci formation in spermatogonia cells and at the pachytene stage of meiosis (Fig. 2e, f). The elevated level of γ-H2AX (Fig. 2d) correlated with decreased RPA foci (Fig. 2e, f) in spermatogonia cells in Cbx1 cKO mice. Apoptosis was more pronounced in seminiferous tubules of Cbx1 cKO mouse testicles compared with Cbx1 WT mouse testicles (Fig. 2g, h). Cbx1 cKO mice also had spermatocyte degeneration in the seminiferous tubules (Figs. 1f and 2g).

Cbx1 depletion affects replication fork progression

The in vivo studies suggested that HP1β function could be critical in replicating cells and therefore the DNA replication process was studied in mouse embryonic fibroblasts (MEFs). Cbx1+/− MEFs were prepared and Cbx1−/− MEFs were subsequently generated by infection with adeno-cre lentivirus (Fig. 3a). Deletion of Cbx1 decreased cell proliferation, as determined by cell count, as compared to Cbx1+/+ MEFs (Fig. 3b). To determine whether the decreased cell growth is due to DNA damage, we studied spontaneous γ-H2AX and 53BP1 foci in Cbx1−/− MEFs (Fig. 3c) and observed MEFs with Cbx1 deletion had higher frequency of γ-H2AX (Fig. 3d) and 53BP1 foci (Fig. 3e) as compared to MEFs with Cbx1. To determine whether the increased γ-H2AX and 53BP1 foci observed in Cbx1 depleted MEFs could be due to impaired DNA fork progression, we labeled the cells with CldU for 30 min and compared the fiber length between cells with and without Cbx1 (Fig. 3f). Cbx1−/− MEFs had a reduced nascent fork length, from 12 to 7 μm (Fig. 3g), and the fork speed decreased from 1.02 to 0.7 kb/min (Fig. 3h).

Replication fork dynamics in cells were measured with and without hydroxyurea treatment (Fig. 4a) and fork dynamics was compared in cells with and without Cbx1 (Fig. 4b). We observed that MEFs without Cbx1 have higher spontaneous stalled fork (Fig. 4c). To determine whether human or MEF cells depleted for HP1β have higher frequency of stalled frequency, HP1β was depleted with small interfering RNA (siRNA) in HeLa cells. We observed a decreased percentage of restart in HP1β-depleted cells (Fig. 4d, e). Both MEFs as well as HeLa cells with HP1β depletion had a higher frequency of stalled replication forks (Fig. 4f, g) and new origin firings (Fig. 4f, h, i).

We determined the role of HP1β in fork stability in Cbx1 MEFs under replicative stress (HU treatment) by using the DNA fiber assay. Cells were labeled with CldU followed by 2 mM HU treatment. DNA fibers were prepared at 0 h, 4 h, and 6 h after recovery from HU treatment. Control cells at 0 h had an average mean nascent DNA strand fork length of 11.5 μm and the length gradually decreased to 6.2 μm by 6 h post HU treatment (Fig. 4j). Cbx1 deletion reduced mean average nascent DNA fork length, from 8.3 to 6.15 μm after HU treatment (Fig. 4k). These results suggest HP1β is important for nascent DNA fork progression and stability.

To determine the role of HP1β in fork degradation, we screened for interacting DNA repair proteins. HP1β contains two conserved domains, namely a chromodomain (CD) and a chromo shadow domain (CSD). The CSD recognizes and interacts with proteins containing a PxVxL motif and we searched for potential HP1β interacting (PxVxL motif containing) proteins. The Motif search analysis (www genome.jp/tools/motif) identified 2916 human PxVxL motif-containing proteins, among which we found several exonuclease proteins (Table 2), one of which (DNA2) was known to be involved in fork degradation (Pawlovska et al. 2017; Rossi et al. 2018; Thangavel et al. 2015; Zheng et al. 2019). Depletion of either HP1β or DNA2 reduces fork degradation, but measurement of nascent DNA fork length in HP1β, DNA2 co-depleted cells did not detect an additive impact on fork degradation in HU-treated cells (Fig. 4l). These results suggest that HP1β and DNA2 function epistatically in fork degradation (Fig. 4).

Cbx1−/− MEFs are sensitive to drug-induced replicative stress

To evaluate the role of Cbx1 in the response to drug-induced replication stress, we measured cell survival of Cbx1−/− and Cbx1+/− MEF cells treated with increasing concentrations of HU or cisplatin. Either deletion of Cbx1 in MEFs or depletion of HP1β in HeLa cells increased cell death after HU or cisplatin treatment (Fig. 5a–c). Measurement of chromosomal aberrations in the cells at metaphase after the drug treatments revealed increased S-phase-specific chromosomal aberrations like radials and breaks (Fig. 5d–g). These results indicate HP1β is important in repairing chromosome damage in S-phase cells (Fig. 5).

Discussion

During germ cell production, epigenetic chromatin modifications such as methylation, acetylation, phosphorylation, and ubiquitination play major roles (Gannon et al. 2014). Any alteration in epigenetic modifications could modulate critical gene expression patterns and lead to pathological outcomes such as male infertility and failure of embryonic development (Aston et al. 2012; Cho et al. 2003; Gupta et al. 2008). Depletion of RNase F or MOF (both epigenetic modifiers) results in a sub- or infertile phenotype and affected spermatogenesis (Jiang et al. 2018; Guo et al. 2018). Altered DNA repair mechanisms are also linked to infertility as ATM-deficient mouse germ cells arrest during the leptotene and zygotene stages (Pandita et al. 1999;
Scherthan et al. (2000), whereas BRCA1 deletion causes arrest in diplotene stage of meiosis I (Xu et al. 2003). Mutations of another important DNA repair protein, Mre11 affect meiosis I during the meiotic recombination phase (Cherry et al. 2007), whereas Cbx1 deletion affects the maturation of spermatocytes during the spermatogonia phase. Taken together, our results show that the reduced number of offspring from Cbx1 cKO mice is due to underlying replicative defect in male germ cell spermatogenesis.

The reduced testicular weight and size in Cbx1 cKO mice are likely a manifestation of the increased tubular vacuolation and spermatocyte degeneration that further decreases sperm production. The sperm reduction is likely due to unrepaired DNA damage as evidenced by the higher frequency of γ-H2AX foci in spermatogonia from Cbx1−/− mice. During meiotic prophase phase I, the DSBs produced by SPO11 lead to H2AX phosphorylation and subsequent accumulation of repair proteins (Pandita and Richardson 2009). Recombinases such as RAD51/DMC1/RPA-MEIOB-SPATA22 facilitate direct strand invasion to generate recombinant intermediates and these intermediates are resolved by crossovers or non-crossovers (Luo et al. 2013; Zhang et al. 2019). Similar to KU70 knockout mice, Cbx1 deletion also increased γ-H2AX accumulation in spermatogonia cells (Ahmed et al. 2009). Recombinases such as RAD51/DMC1/RPA-MEIOB-SPATA22 facilitate direct strand invasion to generate recombinant intermediates and these intermediates are resolved by crossovers or non-crossovers (Luo et al. 2013; Zhang et al. 2019). Similar to KU70 knockout mice, Cbx1 deletion also increased γ-H2AX accumulation in spermatogonia cells (Ahmed et al. 2009).

**Table 2** DNA repair proteins with P-x-V-x-L-containing proteins

| hsa:107986217 | RNA polymerase II |
| hsa:107303344 | SETDB2-PHF11 |
| hsa:5896 | RAG1, RN174 |
| hsa:114827 | FHAD1 |
| hsa:9577 | BRCA1 |
| hsa:3908 | LAMA2, LAMM, MDC1A |
| hsa:5591 | PRKDC, DNA-PKC |
| hsa:2189 | FANCG, FAG, XRCC9 |
| hsa:56897 | WRNIP1, WHIP, BA420G6.2 |
| hsa:9101 | USP8, HumORF8, PITA4, SPG59, UBPY |
| hsa:23210 | JMD16, PSR, PTDSR |
| hsa:5579 | PRKCB, PKC-beta |
| hsa:3910 | LAMA4, LAM3, CMD1JJ |
| hsa:5897 | RAG2 |
| hsa:9656 | MDC1, NFBD1 |
| hsa:10388 | SYCP2, SCP-2 |
| **hsa:1763** | **DNA2** |
| hsa:81426 | RAB28, CORD18 |
| hsa:9985 | ATRIP |
| hsa:675 | REC8, HR21spB |
| hsa:50485 | BRCA2, BRC2, BRC2A2, FACD, FANCD1, GLM3 |
| hsa:5885 | SMARCAL1, HARP |
| hsa:5980 | RAD21, CDLS4, HR21 |
| hsa:29072 | POLZ, REV3 |
| hsa:7486 | SETD2, HBP231 |
| hsa:23135 | WRN, RECQ3 |
| hsa:10036 | KDM6B, JMJ3 |
| hsa:3981 | CHAF1A, CAF1 |
| hsa:4361 | LIG4 |
| hsa:2475 | MRE11 |
| hsa:10014 | MTO1 |
| hsa:10721 | HDAC5 |
| hsa:9400 | HDLCQ, PRO0327 |
| hsa:57697 | RECL5 |
| hsa:5093 | FANC8 |
| hsa:9759 | HNRPE1, HNRPX |
| hsa:10155 | HDAC4, AH05 |
| hsa:55215 | TRIM28, KAP1 |
| hsa:596 | FANC1, KIAA1794 |

**BCL2**
The increased frequency of γ-H2AX foci and decrease in RPA foci seen in Cbx1 cKO mice is consistent with a role for Cbx1 during DNA repair in replicating cells. Unlike ATM, BRCA1, and MEIOB, our data detected no comparable difference in γ-H2AX in leptotene and zygotene stages; however, there was difference in γ-H2AX and RPA foci in the pachytene stage. It is based on the fact that defect in DNA repair in cells from Cbx1 cKO mice accumulated DNA damaged, and subsequently undergo apoptosis, thus resulting in reduction of sperm production and sub-fertility.

Our results are consistent with the current literature that HP1β is involved in DSB repair (Ayoub et al. 2008; Kalousi et al. 2015; Lee et al. 2013; Sharma et al. 2003), and in addition plays a role in the resolution of the stalled replication forks like that of the chromatin-modifying factor MOF (Singh et al. 2018). Cbx1 deletion in MEFs decreased fork speed progression and reduced cellular proliferation compared to wild-type MEF cells, thus supporting the argument that like depletion of MOF or FANCD2, HP1β loss also showed fork progression even in the absence of replicative stress (Singh et al. 2018; Zhu et al. 2015). Similar to loss of MOF, FANCD2, and other HR proteins, HP1β depletion increased stalled forks and new origins in HU or cisplatin-treated cells, supporting a role for HP1β in HR repair, which is also critical for germ cell development in order to maintain DNA fidelity. Based on the in vitro fiber assay and in vivo DNA damage analysis, the potential reason for aberrant spermatogenesis is due to replication stress caused by the absence of HP1β, a novel function for the protein.

**Author contributions** V.C., C.R.H, and T.K.P. directed the study. V.C., C.R.H., and T.K.P. contributed to the design. V.C., A.T., R.K.P., and T.K.P. performed the experiments. V.C., C.H., and T.K.P wrote the paper.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no competing interests.
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