Maternal BCAS2 protects genomic integrity in mouse early embryonic development

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SUMMARY

Mammalian early embryos maintain accurate genome integrity for proper development within a programmed timeline despite constant assaults on their DNA by replication, DNA demethylation and genetic defects transmitted from germ cells. However, how genome integrity is safeguarded during mammalian early embryonic development remains unclear. BCAS2 (Breast Carcinoma Amplified Sequence 2), a core component of the PRP19 complex involved in pre-mRNA splicing, plays an important role in the DNA damage response through the RPA complex, a key regulator in the maintenance of genome integrity. Currently, the physiological role of BCAS2 in mammals is unknown. We now report that BCAS2 responds to endogenous and exogenous DNA damage in mouse zygotes. Maternal depletion of BCAS2 compromises the DNA damage response in early embryos, leading to developmental arrest at the two- to four-cell stage accompanied by the accumulation of damaged DNA and micronuclei. Furthermore, BCAS2 mutants that are unable to bind RPA1 fail in DNA repair during the zygotic stage. In addition, phosphorylated RPA2 cannot localize to the DNA damage sites in mouse zygotes with disrupted maternal BCAS2. These data suggest that BCAS2 may function through the RPA complex during DNA repair in zygotes. Altogether, our results reveal that maternal BCAS2 maintains the genome integrity of early embryos and is essential for female mouse fertility.
INTRODUCTION

Organisms and cells have evolved complex systems for responding to DNA damage to ensure the survival and faithful transmission of genetic materials to subsequent generations. Depending on the type of DNA lesion, cells generally arrest cell cycle progression by activating the DNA damage response (DDR), which includes sensing DNA lesions and triggering cascades mediated by the ATM (Ataxia telangiectasia mutated) and ATR (Ataxia telangiectasia and Rad3 related) kinases to repair damaged DNA (Ciccia and Elledge, 2010; Jazayeri et al., 2006). RPA (Replication protein A), an eukaryotic single stranded DNA (ssDNA)-binding protein, plays critical roles in DNA repair and replication stress to maintain genome integrity (Fanning et al., 2006). ssDNA can be recognized by RPA, which recruits the ATR/ATRIP complex and generates a platform to activate the ATR signalling cascade (Zou and Elledge, 2003). Defects in DDR affect genome instability and cell survival (Jazayeri et al., 2006).

Genome instability arising from DDR defects in early embryos or transmitted from germ cells may result in human diseases, including cancers and abnormal reproductive outcomes (O'Driscoll and Jeggo, 2006). Due to the lack of efficient DNA damage checkpoints, the mouse oocyte is comparatively tolerant of the accumulation of DNA lesions (Marangos and Carroll, 2012). Genetic lesions induced by DNA damaging agents in mouse sperm are repaired in fertilized eggs (Generoso et al., 1979). DNA demethylation is
accompanied by the appearance of the DNA damage signal in mouse zygotes (Hajkova et al., 2010; Wossidlo et al., 2010). Thus, DDR may contribute to mouse early embryonic development by cooperating with important events within the programmed implantation window (Kojima et al., 2014; Zeng and Schultz, 2005).

DDR depends on maternal mRNAs and proteins in newly fertilized zygotes, which almost lack transcription activity (Brandriff and Pedersen, 1981; Li et al., 2010). DDR can be detected almost immediately after fertilization and as early as the G1 phase (Toyoshima et al., 2005). Disabling the RPA complex from binding to DNA by mutating RPA1 results in embryonic lethality at the blastocyst stage (Wang et al., 2005). Genetic mutations in Atr and other important DNA damage repair genes such as Chk1 (Checkpoint kinase 1) and Rad50 (RAD50 homolog) lead to early embryonic lethality during the perimplantation stage (Brown and Baltimore, 2000; Liu et al., 2000; Luo et al., 1999; Takai et al., 2000). The embryonic lethality of these mutants impedes the investigation of the maternal function of DDR genes. Until now, only maternal PMS2 (Postmeiotic segregation increased 2), a mammalian homolog of a bacterial DNA mismatch repair protein, is reported to play a role in DDR in mouse development (Larson et al., 2004). Embryos lacking maternal UBE2A (Ubiquitin-conjugating enzyme E2A), a homologue of the ubiquitin-conjugating enzyme RAD6 involved in DNA repair, arrest development at the two-cell stage. However, whether UBE2A is involved in DNA repair in mouse early embryos
has not been analysed (Roest et al., 2004). Currently, how maternal DDR functions in early mouse development remains elusive.

\textit{Bcas2 (breast carcinoma amplified sequence 2)} was originally identified by gene amplification in human breast cancer cells (Nagasaki et al., 1999). Subsequently, BCAS2 has been identified as a core component of PRP19, a pre-mRNA splicing complex (Ajuh et al., 2000). BCAS2 down-regulates p53 (Transformation related protein 53) in the presence of DNA damage in MCF-7 cells (Kuo et al., 2009). BCAS2 interacts directly with RPA in DNA damage repair in HEK293T and HeLa cells (Marechal et al., 2014; Wan and Huang, 2014). However, the physiological roles of BCAS2 in mammals are unknown. In the current study, we find that BCAS2 responds to endogenous and exogenous DNA damage in mouse zygotes and demonstrate its vital role in the maintenance of genomic integrity during mouse preimplantation development, potentially through RPA.
Materials and Methods

Mouse maintenance and gene targeting at the Bcas2 locus

In compliance with the guidelines of the Animal Care and Use Committee of the Institute of Zoology at the Chinese Academy of Sciences, the Bcas2loxp/loxp;Zp3-Cre mouse line was maintained on a mixed background (129/C57BL/6). To construct a conditional mouse line for targeting the Bcas2 allele, exons 3 and 4 of Bcas2 were flanked by LoxP sites (Fig. S2 and Tab. S1). The recombined ES cells were screened by PCR with two primers (Neo-F and screen-R, Tab. S1), further confirmed by sequencing (data not shown), and injected into C57BL/6 blastocysts. One chimera transplanted the targeted allele through the germ line, and the Neo cassette was deleted by mating Bcas2+/-Neo and Rosa26-Flp mice to produce Bcas+/- mice (Fig. S2). Mice were genotyped using tail DNA as PCR templates with three oligo-primers (F-WT, F-KO, R, Tab. S1; Fig. S3A). The conditional mouse line was established by crossing the Bcas2fl/fl mouse with the Zp3-Cre mouse to specifically ablate BCAS2 in growing oocytes (Fig. S2). Bcas2mNull embryos were obtained by mating Bcas2fl/fl;Zp3-Cre females with normal males.

Fertility assessment and ovarian histology

Fertility assessment was conducted by 2:1 caging of each Bcas2fl/+;Zp3-Cre and Bcas2fl/fl;Zp3-Cre female (n > 5) with wild-type males for over three months. Successful mating was verified by the presence of a post-coital
vaginal plug, after which mice were caged separately. For histology analysis, ovaries from adult females were fixed in Bouin’s solution, sectioned, rehydrated, and stained with hematoxylin and 1% eosin (ZSGB-BIO).

In vitro fertilization, culture and collection of mouse oocytes and early embryos

For in vitro fertilization (IVF), MII oocytes were harvested with M2 medium from 7- to 8-week-old females after injections of PMSG and hCG (Ningbo Second Hormone Factory) and transferred into human tubular fluid (HTF) buffer. Next, 5 x 10⁵/ml capacitation sperms were added to HTF containing MII oocytes, which were then cultured in a humidified atmosphere of 5% CO₂ for the next 6 hrs and then transferred to KSOM at the designated stages (Fraser and Drury, 1975).

For harvesting oocytes or embryos, females were intraperitoneally injected with PMSG and hCG. Oocytes or embryos were manipulated in M2 at the indicated time points after hCG injection: MII oocytes, 13 hrs; zygotes, 23-30 hrs; two-cell embryos, 33-48 hrs; morula, 71 hrs; blastocyst, 98 hrs. GV oocytes were obtained 48 hrs after PMSG.

Fertilization of UV-irradiated mouse MII oocytes and chemical treatment of early embryos

MII oocytes were manipulated with M2 and transferred to HTF media covered with mineral oil. UV irradiation was performed using UV-C lamps (254 nm).
After UV irradiation, MII oocytes were transferred to another HTF drop and fertilized in vitro with normal sperms. For UV irradiation two-cell embryo experiment, embryos were obtained at 37 hrs post-hCG, irradiated for 1 min, and cultured 3 hrs prior to the fixation and staining.

For Pifithrin-α (Sigma) treatment, Bcas2\textsuperscript{mNull} zygotes from IVF were cultured for 15 hrs, treated with either 0.1% DMSO or 100 μM Pifithrin-α for 43 hrs, and imaged. At 48 hrs post-hCG, 25 Bcas2\textsuperscript{mNull} two-cell stage embryos that were treated with either 0.1% DMSO or 100 μM Pifithrin-α were collected for qRT-PCR. For HU and TDRL-505 treatment, control and Bcas2\textsuperscript{mNull} zygotes were recovered at 25 hrs post-hCG and exposed to 4 mM HU (Sigma) for 3 hrs or 100 μM TDRL-505 (Calbiochem) for 7-8 hrs. Zygotes were either fixed for immunostaining with phosphor-RPA2 or γH2AX antibody or cultured for 48 hrs post-hCG for assessing the percentage of embryos progressing to the two-cell stage.

**Immunofluorescence staining, microscopy and image analysis**

For PT (PFA–Triton) conditions, embryos were fixed in 4% PFA (paraformaldehyde) for 30 mins at 37 °C, washed three times with PBS (phosphate buffered saline), and permeabilized with 0.5% Triton X-100 in PBS (5 mins, RT). Embryos were washed three times with PBST (PBS with 0.1% Triton X-100), blocked with 5% donkey serum (1 hr, RT), incubated in primary antibodies (Tab. S2, overnight at 4 °C), washed with PBST, and incubated with
secondary antibody (Tab. S2, 1 hr at RT). The images were obtained with the LSM780 (Zeiss).

For TP (Triton–PFA) conditions, the zona pellucida of embryos were removed with acid Tyrode’s solution (Sigma). Embryos were permeabilized with 0.1% Triton X-100 in PBS (PBST) for 1 min on ice and then fixed with 4% PFA (37 °C, 30 mins). The embryos were stained as described above.

Immunofluorescence images were analysed with ZEN lite 2011 (Zeiss). A Z-series was performed to cover the maximal radius of individual pronuclei. For data presentation, the separated images of both pronuclei were merged.

**Western blotting of oocytes and preimplantation embryos**

Mouse oocytes or early embryos were washed with PBS before freezing in 10 μl RIPA containing protease inhibitor cocktail (Roche) and stored at -80 °C. The samples were separated on NuPage 4-12% Bis-Tris gels with MOPS SDS running buffer and transferred to PVDF membranes (Invitrogen). The blots were pre-treated with Superblock Blocking Buffer (Thermo Fisher Scientific), incubated with primary antibodies (Tab. S2, overnight at 4 °C), washed six times with PBST (5 mins), incubated with horseradish peroxidase-conjugated secondary antibodies (Tab. S2, 1 hr at RT), detected with SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific) and imaged with a LAS-3000 (FujiFilm).
**EdU incorporation and TUNEL assay of mouse embryos**

Embryos were recovered at E0.5 or E1.5 and cultured in KSOM containing 50 mM EdU at 37 °C for 30 mins prior to fixation. Detection of incorporated EdU was performed using the Cell-Light™ EdU Apollo®567 In Vitro Imaging Kit (RiboBio) according to the manufacturer’s protocol. Immunofluorescent staining was performed as described above. Control and BCAS2<sup>mNull</sup> zygotes were examined by TUNEL assay according to the manufacturer’s protocol (Roche).

**mRNA microinjection of mouse zygotes**

*Bcas2* and *Rpa2* were amplified from mouse ovary cDNA with specific primers (Tab S3). The *Bcas2* and *Rpa2* cDNAs were inserted into the modified *pEGFP-N1* and *Pcs2<sup>+</sup>-myc* vectors, respectively. The *Rpa2* point mutations were generated by mutating *Rpa2* (Tab. S3) in the *Rpa2-pcs2<sup>+</sup>-myc* vector (Toyobo). Linearized plasmid was used for *in vitro* transcription with the mMESSAGE mMACHINE Kit (Ambion) and purified with an RNeasy MinElute Cleanup kit (Qiagen). The mRNAs were dissolved in nuclease-free water at 100 ng/μl. Mouse zygotes before the PN3 stage were microinjected with 5-10 pl of mRNA and cultured until the next processes.
**Quantitative real-time RT-PCR**

The mRNA was isolated from oocytes or embryos using the Dynabeads mRNA Direct Micro Kit (Abion). Reverse transcription of RNA was performed using PrimeScript RT reagent Kit (Takara). Quantitative PCR was performed using EvaGreen qPCR MasterMix (Applied Biological Materials) with specific primers (Tab. S4). Measurements were performed with three independent biological replicates.

**Analysis of γH2AX foci in zygotes and two-cell embryos**

Mouse zygotes and two-cell embryos from control and Bcas2<sup>−/−</sup> females were stained as described above. Z-series images were obtained to cover the maximal radius of individual pronuclei (nuclei) and were merged for each embryo by ZEN lite 2011 (Zeiss). Each picture was processed with Enhanced Contrast by saturated pixels 0.4% using ImageJ software. The foci in each picture were counted with Find Maxima by altering Noise Tolerance. The data were presented as the means ± SEM.

**Statistical analyses**

Quantitative analyses were carried out with GraphPad Prism software with at least 3-5 independent biological samples and expressed as the means ± SEM. P-values of comparisons between two groups were calculated using Student’s t-test. Embryo developing beyond two-cell stage was the dichotomous
dependent variable and pifithrin-α treatment was covariates in the model using binary logistic repression analysis of SPSS software. P-values < 0.05 was considered as significant.
RESULTS

BCAS2 responds to DNA damage in mouse zygotes

The Bcas2 mRNA and protein profiles were examined by quantitative RT-PCR (qRT-PCR) and western blot in mouse oocytes and preimplantation embryos. The Bcas2 transcript and protein levels were comparatively high in germinal vesicle (GV) oocytes, decreased in metaphase II (MII) oocytes, further reduced after fertilization until the two-cell stage, elevated in morulas, and increased in blastocyst stage embryos (Fig. 1A, B). BCAS2 protein localization in mouse oocytes and preimplantation embryos was further investigated by immunofluorescent staining. BCAS2 localized to nuclei in oocytes and at all stages of mouse preimplantation embryos except for MII oocytes (Fig. 1C).

BCAS2 is involved in pre-mRNA splicing and DDR as described above. However, splicing is not inefficient in the mouse zygote (Abe et al., 2015). To determine whether BCAS2 was involved in DNA repair in mouse zygotes, the correlation between BCAS2 and \( \gamma \)H2AX, a marker of DNA damage, was assessed in zygotes of relevant pronuclear stages (PN) by staining. Mouse zygotes at various PN stages were obtained 23 hours (hrs, PN2), 25 hrs (PN3), 28 hrs (PN4), and 30 hrs (PN5) post-hCG at embryonic day 0.5 (E0.5) and stained with traditional staining conditions (PFA-Triton, PT condition). BCAS2 appeared in PN2-5 stage embryos, and BCAS2 staining intensity was stronger in paternal pronuclei than in maternal pronuclei in PN3 to PN5 zygotes, which
correlated with γH2AX staining (Fig. 2A). Furthermore, under TP (Triton–PFA) conditions, BCAS2 foci colocalized with γH2AX in the pronuclei of PN3 to PN5 zygotes (Fig. 2B), suggesting its potential role in DDR in mouse zygotes.

Because γH2AX and BCAS2 foci colocalized more frequently in the paternal pronuclei of mouse zygotes, we induced DSBs in MII oocytes to examine whether BCAS2 responds to exogenous DNA damage in zygotes (Fig. 2C). MII oocytes were treated with ultraviolet light (UV), a common treatment to induce DSBs, and fertilized with normal sperm (Bradley and Taylor, 1981; Masui and Pedersen, 1975). Few mouse zygotes developed into two-cell embryos when MII oocytes were irradiated with a high dosage of UV irradiation (0.42J/cm²) (data not shown). After lowering the UV dosage (0.21J/cm²), the majority of zygotes underwent the pronuclear stage and developed into two-cell embryos with a severe delay (data not shown). UV-induced DSBs were evidenced by increased staining of γH2AX, Ser15-phosphorylated p53 and CHK1 in maternal pronuclei (Fig. S1). Having determined the proper UV dosage (0.21J/cm²), irradiated MII oocytes were fertilized with normal sperms. Subsequently, zygotes were cultured for 10 hrs and stained with BCAS2 and γH2AX antibodies. Compared with the controls, BCAS2 and γH2AX foci accumulated in the maternal pronuclei of zygotes (Fig. 2D). These data show that BCAS2 responds to UV-induced DSBs by localizing to DNA damage sites, supporting the potential role of BCAS2 in DNA repair in mouse zygotes.
**BCAS2 is critical for female mouse fertility**

To investigate whether BCAS2 functions in DNA repair in early embryos, a mouse line with a conditionally targeted *Bcas2* allele was established by flanking its exons 3 and 4 with LoxP sites (Fig. S2). *Bcas2*^fl-Neo^ (fl, flox) mice were viable and fertile. However, no *Bcas2*^fl-Neo/fl-Neo^ mice were available after mating *Bcas2*^fl-Neo/fl-Neo^ males and females (data not shown), suggesting that the inserted Neo cassette might disrupt BCAS2 function and result in embryonic lethality. After deleting the Neo cassette from *Bcas2*^fl-Neo^ mice by mating with *Rosa26-Flip* mice (Rodriguez et al., 2000), *Bcas2*^fl/fl^ mice were obtained.

To investigate BCAS2 function in mouse oocytes, *Bcas2*^fl/fl^ females were mated with *Zp3-Cre* males (Lewandoski et al., 1997). *Bcas2*^fl/fl^;*Zp3-Cre* mice were obtained by mating *Bcas2*^fl/+;Zp3-Cre^ mice and genotyped with PCR (Fig. S2, Fig. S3A). *Bcas2*^fl/fl^;*Zp3-Cre^ females and males grew to adults and appeared grossly normal. However, *Bcas2*^fl/fl^;*Zp3-Cre^ females did not deliver offspring after mating with normal males over three months (Fig. 3A). Compared with controls, the *Bcas2* mRNA level was reduced by 95% (Fig. 3B), and BCAS2 protein was almost absent in *Bcas2^mNull^* oocytes (Fig. 3C, D). These data show that BCAS2 plays important roles in female mouse fertility. Unless otherwise stated, the *Bcas2*^fl/+;Zp3-Cre^ and *Bcas2*^fl/fl^;*Zp3-Cre^ oocytes and embryos are designated control and *Bcas2*^mNull^, respectively.
Maternal BCAS2 is required for mouse preimplantation development

To examine the potential role of BCAS2 in oogenesis, ovaries from control (Bcas2^{fl/+};Zp3-Cre) and Bcas2^{fl/fl};Zp3-Cre females were fixed and sectioned for histological analysis. Ovaries from Bcas2^{fl/fl};Zp3-Cre females included all stage follicles and corpora lutea and were indistinguishable from controls (Fig. S3B). In addition, superovulation resulted in similar MII oocyte morphology and number between the control and Bcas2^{mNull} groups (Fig. S3C, D). These data suggest that BCAS2 may be not essential for oocyte maturation and ovulation in Bcas2^{fl/fl};Zp3-Cre females.

Next, mouse embryos were recovered at E0.5, E1.5, E2.5, and E3.5 after mating Bcas2^{fl/+};Zp3-Cre and Bcas2^{fl/fl};Zp3-Cre females with wild-type males. Compared with controls, Bcas2^{mNull} zygotes and two-cell embryos were almost normal in morphology and number (Fig. 3E, F at E0.5 and E1.5). However, the majority of Bcas2^{mNull} embryos arrested at the two- to four-cell stages at E2.5 and E3.5, when most control embryos were at the morula and blastocyst stages, respectively (Fig. 3E, F at E2.5 and E3.5).

To further examine the defects, embryos were recovered at E0.5 and E1.5 and cultured in vitro to develop into the next cleavage stage. The rate of Bcas2^{mNull} zygotes progressing into the two-cell stage was similar to controls, but the rate of two-cell embryos progressing into the four-cell stage was much lower than in controls (Fig. 3G, H), suggesting a delayed transition from the two- to the
four-cell stage in $Bcas2^{mNull}$ embryos. These results indicate that maternal BCAS2 is required for mouse preimplantation development.

**DNA damage repair is defective in $Bcas2^{mNull}$ early embryos**

BCAS2 responding to DNA damage in mouse zygotes prompted us to investigate the role of BCAS2 in DNA repair. To address this, two-cell embryos were recovered at 33 (G0), 37 (mid/late S-phase), and 48 hrs post-hCG (late G2 phase) at E1.5 (Artus and Cohen-Tannoudji, 2008) and stained with $\gamma$H2AX antibody. The $\gamma$H2AX staining was mostly diffuse in the nuclei of control (n=10) and $Bcas2^{mNull}$ two-cell (n=12) embryos recovered at 33 hrs post-hCG (Fig. 4A). At 37 hrs post-hCG, $\gamma$H2AX foci increased in both control and $Bcas2^{mNull}$ two-cell embryos (Fig. 4A and B). The number of $\gamma$H2AX foci was similar in control (n=13) and $Bcas2^{mNull}$ embryos (n=34). However, large $\gamma$H2AX foci were frequently observed in $Bcas2^{mNull}$ embryos (Fig. 4A and B). At 48 hrs post-hCG, $\gamma$H2AX foci were dramatically reduced in control embryos (n=65) but remained at relatively high levels in $Bcas2^{mNull}$ embryos (n=46) (Fig. 4A and B).

To investigate whether the increased $\gamma$H2AX in $Bcas2^{mNull}$ two-cell embryos was caused by developmental delay, we stained $Bcas2^{mNull}$ embryos (48 post-hCG) with phosphor-H3S10, a marker of late G2/M-phase (Goto et al., 1999). Compared with controls (n=30), 75% of $Bcas2^{mNull}$ embryos (n=30) shared a similar intensity of phosphor-H3S10 signal (Fig. 4C and D),
suggesting that the majority of *Bcas2*<sup>mNull</sup> two-cell embryos entered G2-phase at 48 post-hCG with damaged DNA. Together, these data suggest the BCAS2 plays an important role in DNA repair in mouse early embryos.

**DNA damage activates checkpoints in *Bcas2*<sup>mNull</sup> two-cell embryos**

The damaged DNA in *Bcas2*<sup>mNull</sup> two-cell embryos might activate p53 to delay cell cycle progression (Shiloh, 2001). To test this, *Bcas2*<sup>mNull</sup> embryos recovered at 48 hrs post-hCG were stained with phosphorylated p53 antibody. Compared with control, phosphorylated p53 increased in *Bcas2*<sup>mNull</sup> two-cell embryos (Fig. 5A). Western blotting further confirmed the increased level of \( \gamma \)-H2AX and phosphorylated p53 (Fig. 5B). In response to DNA damage, the activation of p53 usually triggers the expression of *p21* (Cyclin-dependent kinase inhibitor 1A) and *Gadd45a* (Growth arrest and DNA-damage-inducible 45 alpha) to arrest the cell cycle (Brugarolas et al., 1995; Siafakas and Richardson, 2009). Thus, p53 targets in *Bcas2*<sup>mNull</sup> embryos were examined by qRT-PCR. Compared with controls, the expression of *Gadd45a* was not significantly changed. However, *p21* mRNA significantly increased in *Bcas2*<sup>mNull</sup> two-cell embryos (Fig. 5C).

Subsequently, we tested whether p53 inhibition could alleviate the two-cell arrest of *Bcas2*<sup>mNull</sup> embryos. To this end, *in vitro* fertilized *Bcas2*<sup>mNull</sup> zygotes were treated with Pifithrin-\( \alpha \), a specific inhibitor of p53-dependent transcriptional activation, and cultured for 43 hrs. Compared with 0.1% DMSO
treatment (control), Pifithrin-α significantly decreased the percentage of arrested \textit{Bcas}^{mNull} two-cell embryos (Fig. 5D and E). Furthermore, Pifithrin-α significantly decreased the expression of \textit{p21} and \textit{Gadd45a} in \textit{Bcas}^{mNull} two-cell embryos (Fig. 5F). These results suggest that the delayed or arrested development of \textit{Bcas}^{mNull} two-cell embryos is partially due to damaged DNA and the subsequent activation of p53.

Due to the relatively limited ability of Pifithrin-α to alleviate the developmental arrest of \textit{Bcas}^{mNull} two-cell embryos, other pathways might also be involved in the arrest of \textit{Bcas}^{mNull} embryos. DNA damage activates the Wee1/Cdc25 pathway that is regulated by CHK1, which arrests the cell cycle at interphase by stimulating the CDC2-cyclinB complex (OConnell et al., 1997; Sanchez et al., 1997). In normal mouse two-cell embryos, CHK1 was phosphorylated at S-phase and then decreased at the late G2 stage during cell cycle progression (Fig. S4). To examine whether CHK1/CDC25 was activated in \textit{Bcas}^{mNull} two-cell embryos, control and \textit{Bcas}^{mNull} two-cell embryos recovered at 48 hrs post-hCG were immunostained with pCHK1. pCHK1 staining was almost absent in controls (n=30) but was observed in 48% of \textit{Bcas}^{mNull} two-cell embryos (n=25) (Fig. 5G).

Unrepaired DNA damage endangers cells with risks of genome instability, including abnormal micronuclei (Takai et al., 2000). To examine if micronuclei were present in \textit{Bcas}^{mNull} embryos, DNA was stained with Hoechst. Micronuclei were not observed in control embryos. However, 40% (n=40) of
BCAS2 functions in DNA repair in mouse zygotes

To examine DNA repair defects in mouse zygotes, embryos were recovered at 23, 25, 28, and 30 hrs post-hCG (PN2, PN3, PN4, PN5, respectively) and stained with γH2AX antibody. In control zygotes, γH2AX foci initially appeared at PN2, dramatically increased at PN3 and PN4, and almost disappeared at late PN5 (Fig. 6A). The number of γH2AX foci did not significantly increase during PN2 to PN4 (data not shown). However, enlarged γH2AX foci were observed in Bcas2mNull PN3 zygotes (Fig. 6A). Strikingly, compared with the control at late PN5 in which γH2AX foci almost disappeared, γH2AX foci persisted in Bcas2mNull zygotes (Fig. 6A, B). DNA fragmentation was further confirmed in Bcas2mNull PN5 zygotes by TUNEL assay (Fig. S5A).

Next, we tested whether re-expression of BCAS2 could repair the damaged DNA in Bcas2mNull zygotes. Bcas2mNull zygotes were recovered at 23 hrs post-hCG and microinjected with EGFP-tagged Bcas2 mRNA or control (EGFP). The injected zygotes were cultured for 7 hrs until the PN5 stage and stained with γH2AX antibody. γH2AX foci were partially reduced in the Bcas2mNull zygotes injected with EGFP-Bcas2 mRNA but not in the Bcas2mNull
zygotes injected with *EGFP* (Fig. 6C, D). These results further support a BCAS2 function in DNA repair in mouse zygotes.

**BCAS2-mediated DNA repair facilitates replication in mouse zygotes**

DNA replication mainly initiates at PN3 in mouse zygotes (Ferreira and Carmo-Fonseca, 1997). The observation of enlarged γH2AX foci at PN3 in *Bcas2<sup>mNull</sup>* zygotes (Fig. 7A left panel) led us to suspect that the defects in DNA repair might disrupt DNA replication in *Bcas2<sup>mNull</sup>* zygotes. To test this, control and *Bcas2<sup>mNull</sup>* zygotes were recovered at 25, 28, and 30 hrs post-hCG, and pronuclear formation was examined. Most control and *Bcas2<sup>mNull</sup>* zygotes developed into PN3 (25 hrs), PN4 (28 hrs) and PN5 (30 hrs). Then, these embryos were cultured with EdU for 30 mins prior to fixation and stained with EdU. In controls, the EdU signal was almost equally diffused in both parental pronuclei at PN3 and in the peripheral regions of both pronuclei at PN4 (Fig. 7A left panel), consistent with a previous report (Wossidlo et al., 2010). However, the EdU signal was either weak or absent in PN3 and PN4 *Bcas2<sup>mNull</sup>* zygotes (Fig. 7A right panel, and 5B). PN3 and PN4 zygotes labelled with EdU were further quantified. In controls, 87% (n=32) of PN3- and 83% (n=45) of PN4 zygotes were labelled with EdU. However, only 51% (n=26) of PN3 and 38% (n=31) of PN4 *Bcas2<sup>mNull</sup>* zygotes were labelled with EdU (Fig. 7B). At the late PN5 stage, control zygotes (n=15) were not labelled with EdU. However, 64% (9/14) of *Bcas2<sup>mNull</sup>* zygotes were still labelled with EdU (Fig. 7B). These data demonstrate that *Bcas2<sup>mNull</sup>* embryos have impaired DNA
replication, suggesting that unrepaired DNA breaks cause replication stress during pronuclear stages in mouse zygotes.

To further investigate the role of BCAS2-mediated DNA repair in recovering from replication stress, zygotes were treated with HU, a general DNA damaging agent that stalls or breaks replication forks. Control and $Bcas2^{mNull}$ zygotes were recovered at 25 hrs post-hCG and exposed to HU for 3 hrs. After HU release, these embryos were cultured for 23 hrs to develop into two-cell embryos. Approximately 55% of control zygotes ($n=121$) developed into two-cell embryos, whereas only 22% of $Bcas2^{mNull}$ zygotes ($n=132$) progressed to the two-cell stage (Fig. 7C). These data suggest that the capability of recovering from replication stress is impaired in $Bcas2^{mNull}$ zygotes.

**BCAS2 functions in mouse zygotes through the RPA complex**

The RPA complex, a well-known sensor in response to replication stress, contains RPA1, RPA2 and RPA3 (Fanning et al., 2006; Zou and Elledge, 2003). BCAS2 lacking the 139 N-terminal bases fails to interact with RPA1, prompting us to examine whether the interaction between BCAS2 and RPA1 was required for DNA repair in mouse zygotes (Wan and Huang, 2014). Normal zygotes at PN2 were injected with $Bcas2$ mRNA lacking the N-terminal 139 bases (referred as $\Delta N-EGFP$), cultured until PN5 and stained with $\gamma H2AX$. Compared with controls injected with $EGFP$ mRNA or full-length $Bcas2-EGFP$, zygotes injected with $\Delta N-EGFP$ exhibited increased $\gamma H2AX$ foci, suggesting
that BCAS2 lacking the interaction with RPA1 might function in DNA repair in mouse zygote as a dominant-negative mutant (Fig. 6C, D). Furthermore, γH2AX foci were not reduced by the injection of ΔN-EGFP in Bcas2<sup>mNull</sup> zygotes compared with Bcas2<sup>mNull</sup> zygotes injected with normal Bcas2-EGFP mRNA (Fig. 6C and E). These data suggest that the BCAS2 interaction with RPA1 is important for DNA repair in mouse zygotes.

In response to DNA damage, RPA2 is phosphorylated at Ser33, Ser4/Ser8, Ser12, and Thr21, promoting the binding of the RPA complex to DNA to activate DNA damage checkpoints (Anantha et al., 2007; Block et al., 2004; Oakley et al., 2003; Shi et al., 2010; Wang et al., 2001). Mutated Ser33A, Ser4/Ser8 and Thr21A phosphorylation sites in RPA2 lead to defective recovery from replication stress (Ashley et al., 2014; Olson et al., 2006). Decreased BCAS2 affects the phosphorylation of RPA2 Ser4/Ser8 (Wan and Huang, 2014), which is phosphorylated after Thr21 and Ser33 phosphorylation (Anantha et al., 2007; Liu et al., 2012). To further investigate whether BCAS2 is involved in DNA repair through the RPA complex, the activity of RPA2 in response to DNA damage was investigated in control and Bcas2<sup>mNull</sup> zygotes treated with HU. The phosphorylated RPA2 formed numerous foci that colocalized with γH2AX in control zygotes (n=20) (Fig. 8A upper panel), indicating that RPA2 is effectively activated in normal zygotes. However, few phosphorylated RPA2 foci were observed in Bcas2<sup>mNull</sup> zygotes (n=19) (Fig. 8A lower panel). Compared with control, phosphorylated RPA2 foci were
significantly decreased in \(B\text{cas}2^{m\text{Null}}\) zygotes (Fig. 8B). These data suggest a defect of RPA activity in responding to replication stress in the \(B\text{cas}2^{m\text{Null}}\) zygotes.

To examine the involvement of the RPA complex in DNA repair at the first mouse cell cycle, normal zygotes were recovered at 23 hrs post-hCG (PN2) and either injected with \(R\text{pa}2\) mutant (S4A/S8A, Thr21A and S33A) mRNA or treated with TDRL-505, an inhibitor blocking the formation of RPA-ssDNA (Anciano Granadillo et al., 2010; Shuck and Turchi, 2010). After culturing for 7-8 hrs, the embryos were stained with \(\gamma\text{H2AX}\). Zygotes injected with normal \(R\text{pa}2\) mRNA (control) had few \(\gamma\text{H2AX}\) foci (n=51). However, embryos injected with mutant Ser4A/Ser8A (n=22), Thr21A (n=28) or Ser33A (n=23) mRNA had increased \(\gamma\text{H2AX}\) foci in both parental pronuclei (Fig. 8C, D). In addition, TDRL-505 treatment dramatically increased \(\gamma\text{H2AX}\) foci in PN5 zygotes (Fig. 8E, F). TDRL-505 treatment also delayed zygote development into two-cell embryos, accompanied by accumulated \(\gamma\text{H2AX}\) foci (Fig. S6A, B and C). These data suggest that the RPA complex plays important roles in DNA repair in mouse zygotes.

Together, our data suggest that BCAS2 protects mouse zygotes from replication stress and DNA damage by influencing RPA function during the first cell cycle in mouse development.
DISCUSSION

BCAS2 plays important roles in alternative splicing and DNA repair in cells. Knockdown of BCAS2 by siRNA in HEK293T and HeLa cells exhibits no obvious defects in DNA repair, but cells are defective in responses to camptothecin (CPT) induced DNA lesions (Wan and Huang, 2014). In the present study, we demonstrated that BCAS2 responds to both exogenous and endogenous DNA lesions in mouse zygotes. Oocyte-specific ablation of BCAS2 results in the developmental arrest of cleavage-stage embryos accompanied by accumulated DNA damage and defects in DNA replication. Thus, our results suggest that BCAS2-mediated DNA repair is important to maintain the genome integrity of mouse early embryos. Persistent DNA damage in Bcas2mNull zygotes and two-cell embryos but not in BCAS2 knockdown cells suggests that early mouse embryos may be more sensitive to damaged DNA than normal cells.

We previously showed that BCAS2 responds to CPT induced DNA damage by interacting with RPA (Wan and Huang, 2014). Consistently, BCAS2 mutants unable to bind RPA1 failed to repair damaged DNA in mouse zygotes. Furthermore, phosphorylated RPA2 did not form foci in response to replication stress in Bcas2mNull zygotes. In addition, disruption of the activity of the RPA complex resulted in accumulated DNA damage in mouse zygotes. Our data suggest that the maternal BCAS2 pathway potentially protects genomic integrity through the RPA complex in mouse early embryonic development.
In somatic cells, deficient DNA repair usually activates cell cycle checkpoints such as the CHK1 and p53-p21-cyclin-CDKs pathways (Arias-Lopez et al., 2006; Eldeiry et al., 1992; Espinosa et al., 2003; Jazayeri et al., 2006; Levine, 1997; Macleod et al., 1995; Miyashita and Reed, 1995). Although many mutant strains display embryonic lethality at the perimplantation stage, few mutant mouse lines exhibit embryonic lethality at early cleavage stages (Friedberg and Meira, 2006). Due to the maternal contribution of these genes, the embryonic lethal phenotypes observed in mutants deficient in DDR may underscore the importance of DDR during preimplantation development. Our finding that both the p53 and CHK1 pathways are activated in response to damaged DNA in \textit{Bcas2}\textsuperscript{mNull} two-cell embryos suggests that mouse early embryos could arrest development in response to DNA damage via these pathways as early as the two-cell stage. \textit{Bcas2}\textsuperscript{mNull} zygotes cleaved into two-cell embryos without obvious developmental delays but arrested at the zygotic stage with increased DNA damage upon HU treatment, supporting the notion that the checkpoint is activated in a dosage dependent manner in mouse zygotes (Adiga et al., 2007; Grinfeld and Jacquet, 1987). Thus, it is possible that damaged DNA does not activate the checkpoint in \textit{Bcas2}\textsuperscript{mNull} zygotes, which cleave into two-cell stage embryos. As DNA lesions accumulate and checkpoints gradually activate at the two-cell stage, \textit{Bcas2}\textsuperscript{mNull} embryonic development eventually arrests at late cleavage stages.

In addition to checkpoints, malfunction of zygotic genome activation (ZGA)
also causes two-cell stage arrest (Bultman et al., 2006; Posfai et al., 2012; Wu et al., 2003). When the expression of ZGA genes (Zeng et al., 2004; Zeng and Schultz, 2005) was examined by quantitative RT-PCR, the mRNA levels of ZGA genes such as Tdpoz1, Tdpoz3, Tdpoz4 increased. However, the expression of other ZGA genes (Gm13043 and Ccnj1) decreased (Fig. S7) or remained unaffected (Zscan4a, H2-Q6 and Prss8, data not shown), suggesting that ZGA is partially affected in Bcas2mNull two-cell embryos and may also contribute to delayed or arrested development.

Because BCAS2 is involved in multiple functions in cells and due to the limitations of current models and analyses, we could not rule out the possibility that BCAS2 may also indirectly affect the RPA function in DNA repair in mouse zygotes or the roles of BCAS2 during oogenesis and early development. However, established mouse embryos lacking maternal BCAS2 not only allow us to study the physiological function of BCAS2 in DNA repair during early embryonic development but may also provide useful models for elucidating the roles of BCAS2 in ZGA, DNA demethylation and other important events involved in mouse early embryogenesis.
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CONFLICT of INTEREST DISCLOSURE

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

Q.X. designed and performed the major experiments, analysed the data and wrote the manuscript. F.W. established the Bcas2 transgenic mouse line and analysed its major phenotypes. Y.X. performed the microinjections. X.Z., Z.Z., Z.G., W.L., X.L., Y.L, and X.Y. contributed to mouse maintenance, embryo isolation and vector construction. H.W., J.H. and Z.Y. analysed the data. S.G. and L.L. initiated, organized and designed the study, analysed the data and wrote the manuscript. All authors commented on the manuscript.
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Fig. 1. The dynamics of BCAS2 expression in mouse oocytes and early embryos. (A) Quantitative RT-PCR analysis of germinal vesicle (GV) oocytes, metaphase II (MII) oocytes, zygotes (1C), two-cell (2C), morula (MO), and blastocyst (BL) stage embryos. RNA expression in GV oocytes was set as one. The error bars represent the standard error of mean (SEM) from three independent experiments. (B) Oocytes and preimplantation embryos (50) were analysed by western blotting. (C) Oocytes and early embryos at various stages were immunostained with the BCAS2 antibody. Scale bar, 20 μm.
Fig. 2. BCAS2 responses to endogenous and exogenous DNA damage in mouse zygotes. (A and B) Zygotes were obtained from the oviduct at 23, 25, 28, and 30 hrs post-hCG, stained with antibodies to BCAS2 and γH2AX using PT or TP conditions, and imaged. (C) Illustration of UV-induced DNA damage in MII oocytes. (D) MII oocytes were irradiated by UV for 5 mins and subjected to in vitro fertilization. Zygotes were cultured for 10 hrs and fixed after pre-extraction (at least three independent IVFs). Male and female pronuclei were indicated with ♂ and ♀, respectively. Scale bar, 20 μm.
Fig. 3. Maternal depletion of BCAS2 leads to developmental arrest. (A) Wild type (Wt), Bcas2fl/- (Het) and Bcas2fl/fl;Zp3-Cre (Mut) females were mated with normal fertile males over three months. The average number of pups per litter from the females (n=9) was analysed. The error bars represent the standard deviation (SD). (B) Total RNA samples were prepared with 50 control and Bcas2mNull oocytes and subjected to real-time RT-PCR. Error bars represent SEM. (C) Western blotting was performed with control and Bcas2mNull oocytes with specific antibodies. (D) Oocytes were fixed without pre-extraction and stained with the BCAS2 antibody. (E and F) Bcas2fl/+;Zp3-Cre and Bcas2fl/fl;Zp3-Cre females were superovulated and mated with normal fertile males. Control and Bcas2mNull embryos were flushed
from the oviducts of fertilized females at E0.5, E1.5, E2.5 and E3.5. The developmental stages of embryos were affirmed according to blastomere number. Average numbers of embryos are shown at different stages derived from five $Bcas2^{+/+};Zp3$-Cre and $Bcas2^{+/};Zp3$-Cre females. (G) Zygotes were flushed from the oviducts at 26 hrs post-hCG and cultured in KSOM to develop into two-cell embryos. Developmental rates were determined from five females. (H) Two-cell embryos were flushed from oviducts at 52 hrs post-hCG and cultured in KSOM into the four-cell stage. Developmental rates were calculated without the amount of arrested two-cell embryos. Error bars represent SEM. Scale bar, 20 $\mu$m.
Fig. 4. DNA damage accumulates in Bcas2<sup>mNull</sup> two-cell embryos. (A) Representative images of control and Bcas2<sup>mNull</sup> two-cell embryos obtained at 33, 37, and 48 hrs post-hCG and stained with γH2AX and BCAS2 antibodies. Large γH2AX foci were indicated by white arrowhead. (B) Two-cell embryos obtained at 37 and 48 hrs post-hCG were stained as in (A). γH2AX foci in embryos were quantified with ImageJ software. (C) Representative images of two-cell embryos (48 hrs post-hCG) stained with phosphor-H3S10 using TP conditions. (D) Quantification of phosphor-H3S10 intensity normalized against DNA staining by Hoechst and analysed with ImageJ. The error bars represent the SEM from three independent experiments. *** P < 0.001. Scale bar, 20 μm.
Fig. 5. Checkpoints are activated in Bcas2mNull two-cell embryos. (A) Control and Bcas2mNull two-cell embryos were harvested at 48 hrs post-hCG and stained with p53 (p15S) and γH2AX antibodies. (B) Control and Bcas2mNull two-cell embryos were recovered at 48 hrs post-hCG and subjected to western blotting. (C) Levels of p21 and Gadd45a mRNA were analysed in two-embryos obtained at 48 hrs post-hCG by qRT-PCR. (D) Bcas2mNull zygotes (N=743) from IVF were cultured in KSOM containing either 0.1% DMSO or 100 μM Pifithrin-α for 43 hrs and imaged. Pifithrin-α treatment did not alter the
development of normal embryos compared with DMSO treatment. (E) 

*Bcas2*<sup>mNull</sup> two-cell embryos from (D) were counted in individual culture drops. The percentage of two-cell embryos was calculated by dividing the number of all embryos in an individual drop. Binary logistic repression was used to analyze statistical significance ($P < 0.001$). (F) Levels of *p21* and *Gadd45a* mRNA were measured in *Bcas2*<sup>mNull</sup> two-cell embryos obtained at 48 hrs post-hCG from (D) using qRT-PCR with specific primers (Table S 4). (G) Control and *Bcas2*<sup>mNull</sup> two-cell embryos were harvested at 48 hrs post-hCG and stained with phosphor-CHK1 (S345) and γH2AX antibodies. (H) Control and *Bcas2*<sup>mNull</sup> embryos were obtained at E2.5 and stained with Hoechst. Arrowheads indicate micronuclei. Scale bar, 20 μm. The data are represented as the mean ± SEM calculated from three independent experiments.
Fig. 6. BCAS2 is required for DNA repair in mouse zygote. (A) Control and Bcas2<sup>mNull</sup> zygotes were obtained at 23, 25, 28, and 30 hrs post-hCG and stained with the γH2AX antibody. White arrowhead indicates large γH2AX foci. The number of analysed zygotes at indicated stages was given in parenthesis. (B) γH2AX foci were quantified in both pronuclei of individual zygotes obtained at 30 hrs post-hCG. (C) Wild type (WT) and Bcas2<sup>mNull</sup> zygotes were obtained
at 23 hrs post-hCG and microinjected with EGFP, ΔN-EGFP or EGFP-tagged Bcas2 mRNA. Microinjected zygotes were cultured for 7-8 hrs and stained with γH2AX antibody. (D and E) γH2AX foci were quantified in individual zygotes from (C). The error bars represent the SEM from three independent experiments. *, P < 0.05. ***, P < 0.001. Scale bar, 20 μm.
Fig. 7. BCAS2-mediated DNA repair is involved in DNA replication in mouse zygotes. (A) Control and \( Bcas2^{mNull} \) zygotes were obtained at 25, 28, and 30 hrs post-hCG, cultured with EdU for 30 mins prior to fixation, stained and imaged. (B) The zygotes were treated as in (A). EdU positive zygotes were counted. (C) Control and \( Bcas2^{mNull} \) zygotes were exposed to HU for 3 hrs and cultured for 23 hrs until the late two-cell stage. Numbers of two-cell embryos were counted in control and HU treated groups. The error bars
represent the SEM from three to five independent experiments. *, $P < 0.05$. **, $P < 0.01$. Scale bar, 20 μm.
Fig. 8. BCAS2-mediated DNA repair functions through RPA. (A) Control and Bcas2<sup>mNull</sup> zygotes were obtained at 25 hrs post-hCG and exposed to HU for 5 hrs. Zygotes were fixed and stained with γH2AX antibody. (B) The zygotes were obtained and stained as in (A). Phosphor-RPA2 foci were quantified in both pronuclei of control and Bcas2<sup>mNull</sup> zygotes. (C) Normal zygotes were injected with either RPA2 or RPA2 mutation mRNA (Thr21A),
cultured for 7-8 hrs in KSOM prior to fixation and stained with γH2AX antibody. (D) Normal zygotes were injected with either RPA2 or RPA2 mutation mRNA (Ser4A/8A, Thr21A and Ser33A) and stained as in (C). γH2AX foci in both pronuclei of zygotes were quantified. (E) Zygotes were harvested 25 hrs post-hCG, cultured for 5 hrs in KSOM containing 50 μM TDRL-505, fixed, stained with γH2AX antibody and imaged. (F) Zygotes were obtained and treated as in (E). γH2AX foci of both pronuclei in zygotes were quantified. The error bars represent the SEM from three independent experiments. *** P < 0.001. Scale bar, 10 μm.