A novel IncRNA Discn fine-tunes replication protein A (RPA) availability to promote genomic stability

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RPA is a master regulator of DNA metabolism and RPA availability acts as a rate-limiting factor. While numerous studies focused on the post-translational regulations of RPA for its functions, little is known regarding how RPA availability is controlled. Here we identify a novel IncRNA Discn as the guardian of RPA availability in stem cells. Discn is induced upon genotoxic stress and binds to nucleolin (NCL) in the nucleolus. This prevents NCL from translocation into nucleoplasm and avoids undesirable NCL-mediated RPA sequestration. Thus, Discn-NCL-RPA pathway preserves a sufficient RPA pool for DNA replication stress response and repair. Discn loss causes massive genome instability in mouse embryonic stem cells and neural stem/progenitor cells. Mice depleted of Discn display newborn death and brain dysfunctions due to DNA damage accumulation and associated inflammatory reactions. Our findings uncover a key regulator of DNA metabolism and provide new clue to understand the chemoresistance in cancer treatment.

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Stem cells (SCs, including pluripotent embryonic stem cells and tissue stem cells) are cellular basis for organism development and tissue homeostasis. Genetic lesions in SCs usually induce the loss of stem cell identity, causing numerical or functional perturbations in SCs. These perturbations can have catastrophic consequences for tissue and organisal homeostasis, resulting in embryonic lethality, developmental defects, degenerative disorders, and oncogenesis. In adaptation to their functional significance, SCs are able to maintain superior stable genome. The genome maintenance ability in SCs lies in their unique DNA metabolic properties in aspects of DNA replication and repair. Limited pioneer works showed that mouse embryonic stem cells (mESCs) utilize ZSCAN4-mediated DNA recombination-based pathway to lengthen the telomere. ESCs do not use TRF2 to protect the telomere, whereas TRF2 is essential for telomere protection in somatic cells. Compared to somatic cells, ESCs have superior abilities to resolve replication stress and repair DNA damages. Several stem cell-specific proteins, including SALL4, ILIA, and ILIA-FLOPED protein complex were identified as the underlying regulators. Despite these progresses, how SCs efficiently replicate and repair DNA during frequent cell proliferation still remains largely unclear. Comprehensive elucidation of the underlying mechanisms would largely expand our knowledge on the regulations of DNA metabolism and genomic stability maintenance in stem cells, and help understand the relevant pathophysiological conditions.

During DNA metabolism including DNA replication, repair and recombination, single-stranded DNA (ssDNA) is frequently generated and rapidly coated by replication protein A (RPA) complex. RPA is composed of three subunits RPA70, RPA32, and RPA14, and is the major ssDNA-binding protein in eukaryotic cells. RPA binding to ssDNA not only protects the naked ssDNA from nucleolytic degradation and prevents the secondary structure formation in ssDNA, but also serves as a platform to launch downstream events in all DNA metabolic processes. Thus, RPA acts as a master regulator in DNA metabolism. RPA perturbations, for instance RPA haploinsufficiency, depletion, or exhaustion, can result in DNA replication catastrophe, DNA repair defects and genomic instability. Given its central roles, RPA has emerged as a focus for cellular responses to genotoxic stress.

To date, the majority of studies investigated the regulations of RPA functions in different aspects of DNA metabolism, and revealed the post-translational modifications including phosphorylation, SUMOylation, and ubiquitination as important regulators. Compared to post-translational modifications, however, little is known on how cells regulate RPA availability, which is a rate-limiting factor in RPA functions.

DNA replication stress and DNA DSBs can generate massive ssDNA, which rapidly exhausts RPA reservoir. Increasing RPA availability is essential to improve cellular resistance to these stresses. Compared to somatic cells, ESCs are more tolerant to DNA DSBs and replication stress. This raised a hypothesis that ESCs may develop unique strategies to better sustain free RPA pool. In this study, we aimed to understand the mechanisms regulating the resistance of ESCs to DNA replication stress and DNA DSBs. We focused on long noncoding RNAs (IncRNAs), which can modulate and fine-tune gene expression and protein functions through interacting with DNA, RNAs, and proteins in many cellular events. By comparing the RNA expression profiles of mouse ESCs and their differentiated progenies under unperturbed and genotoxic treatment conditions, we identified a short list of IncRNAs that were specifically induced in ESCs upon both replication stress and DNA DSBs. We then characterized in detail an un-annotated IncRNA Discn (DNA damage-induced stem cell specific noncoding RNA), whose expression displayed the most robust increase in response to DNA replication stress and DSBs. Our results showed that Discn ensured sufficient RPA availability via Discn-NCL-RPA pathway to promote genome stability. Notably, knockout of Discn in mice caused newborn death as well as brain dysfunctions, demonstrating its important physiological functions in normal embryonic development.

**Results**

A novel IncRNA Discn responds to genotoxic stress in stem cells. To explore if there exist unique IncRNAs responsive to DNA replication stress and DNA DSBs in stem cells, we performed RNA-seq and compared the genome-wide RNA expression profiles of mouse ESCs to their differentiated progenies cultured under unperturbed or genotoxic conditions (hydroxyurea or etoposide treatment). The cellular identity of differentiated progenies was validated by the expression levels of pluripotency genes and three germ layer markers (Supplementary Fig. 1a). We identified a short list of IncRNAs (Supplementary Fig. 1b), which were predominantly expressed in ESCs and significantly stimulated by genotoxic treatments. Among them, an un-annotated IncRNA displayed the most robust response to genotoxic stress, with expression level increasing more than twenty fold after hydroxyurea, etoposide, mitomycin C, or camptothecin treatment (Fig. 1a). We, therefore, named this IncRNA as Discn (DNA damage-induced stem cell specific noncoding RNA). We also treated ESCs with hydroxyurea plus ATR inhibitor VE-821, which can robustly enhance the unscheduled origin firing and ssDNA formation under replication stress. Intriguingly, inhibition of ATR signaling during replication stress further stimulated the expression of Discn in a dose-dependent manner (Fig. 1b). This expression pattern suggested that Discn was responsive to the generation of ssDNA, and might be involved in regulation of genomic stability via ssDNA-related events.

To better understand Discn, we obtained its full length sequence (GenBank accession number: MZ269527) using 3’- and 5’-end RACE (rapid amplification of cDNA ends) (Supplementary Fig. 1c). Discn is located on chromosome 11 and contains two exons (Supplementary Fig. 1d). Although several potential coding frames are present in the sequence (Supplementary Fig. 1c), they did not translate into proteins or peptides (Supplementary Fig. 1e). Thus, Discn is a potential noncoding RNA. We also observed the expression of Discn in several available mouse tissue stem/progenitor cell samples (e.g., cultured mouse spermatogonia stem cells, mouse neural stem/progenitor cells (NSPCs), mammary stem cells) (Supplementary Fig. 1f). However, Discn was not detected in many organs except of brain and testis (Supplementary Fig. 1g). In addition, Discn is conserved among mammalian species (Supplementary Fig. 1h). These data collectively proposed a potential and probably a general role of Discn in regulating stem cell genomic stability.

**Discn safeguards genomic stability of cultured stem cells.** To find out if Discn is required to maintain genomic stability of stem cells, we first evaluated its functions in in-vitro cultured stem cells. Discn was efficiently knocked down (KD) via two independent short hairpin RNAs (shRNAs) in mESCs (Supplementary Fig. 1i). Under unperturbed culture condition, Discn KD mESCs displayed elevated level of DNA DSBs, as monitored by increased amount of γH2AX (Fig. 1c). The severe DNA DSBs were further validated by neutral comet assay (Fig. 1d), which measures the DSBs at single cell resolution. In addition, many chromosome instability (CIN) phenotypes including aneuploidy (Fig. 1e), chromosome translocation measured by sister chromatid exchange (SCE) (Fig. 1f), and micronuclei formation (Fig. 1g), were prevalent in Discn KD mESCs. However, the
Differentiated cell origin elicits coordinated reactions, which locally protect and repair the DNA.

Discn regulates replication stress response and HR-mediated DNA repair. We next went on to elucidate the functions and mechanisms of Discn using mouse ESC as a model. Endogenous DNA DSBs frequently arise when the DNA replication and/or discn was lost, although its initial activation was grossly normal (Fig. 2a).

We then utilized DNA fiber assay to closely examine the behaviors of replication forks. Compared to Discn proficient cells, the stalled forks in Discn KD cells were prone to degradation under hydroxyurea treatment (Fig. 2b) and difficult to restart after release from stress (Fig. 2c). This indicated that the local responses to protect/repair stalled forks were compromised when Discn was absent. Notably, more dormant replication origins were detected in Discn KD ESCs following hydroxyurea treatment and examined the local and global responses of cells. ATR-CHK1 signaling plays a central coordination role in replication stress response.

Fig. 1 Discn safeguards genomic stability of mouse ESCs. a Mouse ESCs and their differentiated isogenic cells were cultured in normal condition (Nor) or treated with 2 mM hydroxyurea (HU), 10 μM etoposide (Et), 1 mM camptothecin (CPT), or 8 μg/mL mitomycin C (MMC) for 4 h. The relative expression level of Discn was determined by quantitative RT-PCR. The amount in ESCs under normal condition was set as 1.0. n = 3 biologically independent samples.

Discussion: Discn depletion compromises DNA replication stress response, we induced fork stalling by hydroxyurea treatment and examined the local and global responses of cells. ATR-CHK1 signaling plays a central coordination role in replication stress response. In WT ESCs, ATR kinase was robustly activated and efficiently sustained, as monitored by the persistent phosphorylation of its downstream effector CHK1 at Ser345. However, ATR signaling failed to be well maintained when Discn was lost, although its initial activation was grossly normal (Fig. 2a). We then utilized DNA fiber assay to closely examine the behaviors of replication forks. Compared to Discn proficient cells, the stalled forks in Discn KD cells were prone to degradation under hydroxyurea treatment (Fig. 2b) and difficult to restart after release from stress (Fig. 2c). This indicated that the local responses to protect/repair stalled forks were compromised when Discn was absent. Notably, more dormant replication origins were fired (Fig. 2d) and significantly higher level of ssDNA was detected in Discn KD ESCs following hydroxyurea treatment (Fig. 2e), demonstrating that the global response to suppress unscheduled origin firing was also impaired by Discn KD. As a result, Discn KD ESCs were less tolerant to replication stress and generation. To find out if Discn depletion compromises DNA replication stress response
produced more DSBs at different time-points of hydroxyurea treatment when compared to Discn proficient ESCs (Fig. 2f).

Because Discn expression was also induced by etoposide treatment which generates DNA DSBs, we examined its possible involvement in DSB repair. DNA DSBs are repaired by two major pathways: homologous recombination (HR) and classical nonhomologous end-joining (NHEJ) pathways, both of which can be monitored through engineered GFP-based reporters. In the reporter cells, site-specific DSBs are generated in a cassette after expression of endonuclease I-SceI. Repair of the DSBs by respective HR or NHEJ pathway restores the functional RFP or GFP gene and the numbers of RFP- or GFP-expressing cells counted by flow cytometry provide quantitative measure of HR or NHEJ efficiency. To this end, we efficiently knocked down Discn in these reporter mESC lines by using the aforementioned shRNAs, and measured the respective HR and NHEJ repair efficiency. In Discn KD cells, HR repair was suppressed, whereas NHEJ pathway was inversely stimulated probably due to repair compensation (Fig. 2g). To further validate the impairment of HR pathway, we examined the recruitment of recombinase RAD51 to

**Fig. 2 Discn regulates DNA replication stress response and HR-mediated DNA DSB repair.** a Mouse ESCs were treated with hydroxyurea (HU) to induce replication stress. ATR-CHK1 kinase activation failed to be well sustained in two Discn knockdown cells (KD-1 and KD-2) when compared to the knockdown control (KD-C). Three independent experiments were repeated with similar results. b DNA fiber assay revealed that the stalled forks in Discn KD cells were prone to undergo degradation after HU treatment. 200 fibers from three independent replications were analyzed. c Discn KD compromised the stalled fork restart. At least 200 fibers from three independent replications were analyzed. d Discn KD enhanced dormant origin firing as indicated by the reduced mean fork spacing. At least 50 continuous fibers from three independent replications were analyzed. e Discn KD increased the content of ssDNA as measured by the native BrdU incorporation assay at S phase. Scale bar, 10 μm. Quantification is shown on the right. At least 20 images were analyzed in each condition. f Compared to control ESCs (KD-C), Discn KD cells were more sensitive to hydroxyurea treatment and accumulated more DNA DSBs as measured by neutral comet assay. Data were from three replications and shown as mean ± SEM. g FACS analysis of reporter ESCs showed that Discn KD suppressed HR-mediated DNA DSB repair, but inversely stimulated NHEJ repair pathway. Data were from three independent experiments. h DNA DSBs were generated by laser microirradiation. Discn KD impaired the recruitment of Rad51 to DSB sites labeled with γH2AX (++)), indicating the suppression of HR pathway. Right panel showed the proportions of S phase cells capable of HR repair (three replicates, 50 cells in each replicate). Scale bar, 5 μm. i Immunoblotting showed a decrease in chromatin-bound Rad51 in Discn KD ESCs synchronized at S phase. Three independent experiments were repeated with similar results. b, c, f–h Data were shown as mean ± SEM, two-tailed Student’s t-test. d, e Data were representative of individual values with box and whiskers plots showing the median, upper and lower quartiles, and minimum and maximum. Two-tailed Student’s t-test.
DSB sites, which is essential for HR repair\(^\text{19}\). Following laser micro-irradiation, much less Discn KD cells recruited RAD51 to DSB sites (labeled with γH2AX) at S-phase of cell cycle when compared to WT ESCs (Fig. 2h). In concordance, the amounts of chromatin-bound RAD51 proteins were significantly reduced by Discn KD in ESCs synchronized at S phase and treated with etoposide (Fig. 2i). Taken together, these data support that Discn regulates DNA replication stress response and HR-mediated DNA DSB repair.

**Discn accumulates in nucleolus and binds to nucleolin (NCL).**

To understand how Discn regulates replication stress response and HR-mediated DNA repair, we first investigated its subcellular localization under normal and stressed conditions by RNA fluorescence in situ hybridization (RNA-FISH). No specific distribution pattern of Discn was detected when using conventional staining procedures. However, following extraction of soluble Discn with Triton X-100, we observed faint staining of Discn in nucleolus labeled with fibrillarin (FBL) under normal culture condition. Hydroxyurea treatment significantly stimulated the accumulation of Discn in nucleolus (Fig. 3a). Fractionation of nucleolus (Supplementary Fig. 3a) followed by quantitative RT-PCR examination verified the increased amount of Discn in nucleolus after hydroxyurea treatment (Fig. 3b). Of note, Discn exhibited the same subcellular localization in response to etoposide treatment (Fig. 3a, b), suggesting that a common mechanism underlies the dual regulations of Discn on replication stress response and HR-mediated DNA repair.

Next, we performed in vitro RNA pulldown followed by mass spectrometry analysis to identify potential interaction proteins of Discn (Supplementary Fig. 3b). Among the list of candidates, a nucleolus protein nucleolin (NCL) showed relatively high score (Supplementary Fig. 3c). Immunoblotting examination of NCL on in vitro RNA pulldown samples validated the association of Discn with NCL (Supplementary Fig. 3d). Because in vitro binding can yield false-positive interaction, we performed in vivo cross-linking followed by RNA pulldown to validate their interaction. Notably, while weak interaction was detected in nuclear fraction under unperturbed condition, strong association was observed after hydroxyurea or etoposide treatment (Fig. 3c).

To further confirm the association of Discn with NCL, we conducted RNA immunoprecipitation to examine whether Discn can be co-immunoprecipitated with NCL under native condition. As shown, very few Discn RNAs were detected in immunoprecipitated samples in unperturbed condition. However, genotoxic treatments robustly increased the amount of Discn associated with NCL (Fig. 3d, e). In line with their association, immunostaining showed the co-localization of Discn with NCL in nucleolus upon genotoxic stress (Fig. 3f). Collectively, these data support that Discn binds to NCL in nucleolus and their association is enhanced by genotoxic stress.

We went on to map the NCL binding sites on Discn. NCL has binding motif in target RNAs. The motif is G-rich and has stem-loop secondary structures. NCL is able to associate with RNAs bearing this motif in the absence of other proteins\(^\text{20}\). Using RegRNA 2.0 online tools (http://regrna2.mbc.nctu.edu.tw/index.html)\(^\text{21}\), we identified several putative NCL binding sites predominantly at the 5’ end of Discn. We then constructed the Discn mutants containing either the 5’ end (2–156 bp), 3’ end (625–823 bp), or central part (157–630 bp). In vitro RNA pull down assay revealed that the NCL binding sites were predominantly localized at the 5’ end (Fig. 3g).

**Discn-NCL interaction does not require genotoxic stimuli.**

Genotoxic stress stimulated the expression of Discn as well as its association with NCL. To elucidate whether the enhanced Discn-NCL interaction was simply due to the increased Discn expression or required genotoxic stimuli, we stably expressed Discn in WT mESCs (Supplementary Fig. 3e) and re-examined the Discn-NCL association. Intriguingly, under unperturbed culture conditions Discn localized in nucleolus (Supplementary Fig. 3f) and associated with NCL as detected by in vivo RNA pulldown (Supplementary Fig. 3g). Notably, genotoxic treatment did not further increase their association (Supplementary Fig. 3g).

To gain more evidence, we ectopically expressed Discn in mouse embryonic fibroblasts (MEFs) (Supplementary Fig. 3h). Concordantly, Discn appeared in nucleolus of MEFs (Fig. 3h) and associated with NCL as detected by in vivo RNA pulldown assay (Fig. 3i). Of note, the association was not affected by genotoxic treatments (Fig. 3i). Thus, localization of Discn in nucleolus as well as Discn-NCL interaction does not require the genotoxic signaling and rather it seems cell autonomous.

**Discn sequesters NCL in nucleolus to preserve the free RPA pool.**

NCL predominantly resides in nucleolus under unperturbed conditions. However, two studies on somatic cells reported that upon heat shock or genotoxic stress, part of the nucleolar NCL pool translocates into the nucleoplasm, where it binds to RPA\(^\text{22,23}\). The formation of NCL-RPA protein complex in nucleoplasm reduces the free RPA level, thereby counteracting the RPA functions.

Based on the above knowledge, we wondered whether Discn regulates genomic stability by targeting the NCL-RPA complex. To this end, we first evaluated the functional significance of Discn-NCL interaction in nucleolus. In line with previous reports, hydroxyurea or heat stress did not change NCL protein expression in MEFs. However, these treatments drastically increased the relocation NCL into nucleoplasm, and conversely decreased the retention of NCL in nucleolus, as determined by the fractionation of nucleolus and nucleoplasm combined with immunoblotting analysis (Fig. 4a).

Intriguingly, we observed a distinctive pattern of NCL distribution in mESCs, in which the NCL protein level in nucleoplasm remained comparable before and after genotoxic treatments (Fig. 4b). Concordantly, NCL retention in nucleolus was not obviously affected by genotoxic stress (Fig. 4b). Thus, unlike in differentiated cells, genotoxic stress does not evoke obvious NCL translocation from nucleolus to nucleoplasm in mESCs.

We then examined if Discn loss influenced NCL distribution in mESCs. Unlike in Discn proficient ESCs, genotoxic treatment caused part of the nucleolar NCL pool relocation to nucleoplasm in Discn KD ESCs, as manifested by a substantial increase of NCL level in nucleolasm. Due to the high abundance of NCL in nucleolus, release of part of the nucleolar NCL pool into nucleoplasm caused a mild but visible reduction of NCL level in nucleolus (Fig. 4b). The translocation of NCL from nucleolus to nucleoplasm in Discn KD ESCs was further verified by immunofluorescence analysis (Supplementary Fig. 3i). Concordantly, ecotype expression of Discn in MEFs significantly attenuated the translocation of NCL from nucleolus to nucleoplasm (Fig. 4c). We also expressed the three Discn fragments (5’ end, 3’ end, and central part), respectively, in MEFs and examined their influence on NCL translocation. In line with their ability to bind to NCL (Fig. 3g), 5’ end fragment displayed the higher efficiency to prevent NCL translocation to nucleoplasm than 3’ end, whereas central part had no effect (Fig. 4d). These data altogether supported that Discn-NCL interaction sequestered NCL in nucleolus.

In somatic cells, NCL forms protein complex with RPA in nucleoplasm\(^\text{23}\). Given that the NCL protein level increased drastically in nucleoplasm of Discn KD ESCs (Fig. 4b), we speculated that NCL-RPA association was subsequently enhanced. Indeed, co-
immunoprecipitation using antibody against RPA32, one of the RPA subunits[^24], revealed a significant induction of NCL-RPA32 interaction in Discn KD mESCs compared to Discn proficient ESCs upon hydroxyurea treatment (Fig. 4e). Conversely, ectopic expression of Discn in MEFs significantly decreased the NCL-RPA32 complex formation (Fig. 4f). Because the total RPA protein expression was not influenced by Discn depletion (Supplementary Fig. 3j), we concluded that Discn KD led to the reduction of free RPA level in ESCs.

**Overexpression of RPA rescues the genomic instability of Discn KD cells.** Because RPA is a master regulator of DNA
metabolism and critical guardian of genomic integrity, we reasoned that insufficient RPA surplus in Discn KD SCs might underpin the genomic instability defects. To test this hypothesis, we over-expressed the three subunits of RPA complex (RPA70, RPA32, and RPA14) in Discn KD ESCs (Supplementary Fig. 4a), and examined if this could rescue the defects. Notably, the cellular responses to hydroxyurea or etoposide treatment were fully restored. These included the ALT-CHK1 signaling (Fig. 5a), the stalled fork stabilization (Fig. 5b) and restart (Fig. 5c), the suppression of unscheduled origin firing (Fig. 5d) and ssDNA formation (Fig. 5e), the tolerance to hydroxyurea-induced replication fork breaking (Fig. 5f), and the HR-mediated DSB repair (Fig. 5g). Concordantly, the overall DNA DSB contents (Supplementary Fig. 4b) and the cell proliferation rates (Supplementary Fig. 4c) of Discn KD ESCs were recovered to normal by overexpression of RPA. We also examined if the same mechanism operated in NSPCs. Similar to the observations in mESCs, overexpression of the RPA complex in cultured Discn KD NSPCs rescued the genomic instability phenotypes (Supplementary Fig. 5). Collectively, these data support that Discn-NCL-RPA pathway functions generally in SCs. Protein expression level also serves as a regulatory layer. We found that ESCs express more RPA proteins than NSPCs and MEFs, whereas NSPCs and MEFs had comparable RPA expression (Supplementary Fig. 4d). Thus, Discn adds a fine-tune regulatory layer to RPA surplus.

In conclusion, our data proposed a unique mechanism in SCs, in which lncRNA Discn is robustly induced by genotoxic stress and localizes in nucleolus where it interacts with NCL and sequesters NCL in nucleolus. Interaction of Discn-NCL in nucleolus reduces the undesirable NCL-RPA association in nucleoplasm, thereby achieving a sufficient RPA surplus crucial for DNA replication stress response and repair (Fig. 5h). In line with this model, ectopic expression of Discn in MEFs reduced the NCL-RPA association (Fig. 4i), and conferred protection against genotoxic stress (Supplementary Fig. 4e–i). However, expression of mutant Discn fragments which do not efficiently bind to NCL (3’ end and central part) failed to protect MEFs against genotoxic damage (Supplementary Fig. 4j). A recent study reported that lncRNAs, which are generally out-numbered by their associated RNA binding proteins (RBPs), can efficiently regulate the activity of interacting RBPs by driving their phase separation. Discn was expressed at about 65–160 copies per mouse ESC under genotoxic conditions (Supplementary Fig. 4j). Future works are required to clarify whether lncRNA-driven phase separation underlies Discn-mediated regulation.

Discn knockout mice show newborn death and brain dysfunctions. Stem cells are the cellular basis for embryo development and tissue homeostasis. Genomic instability in pluripotent stem cells or tissue stem cells could impair embryo development, cause premature ageing or cancer susceptibility. The phenotypes that we observed in cultured stem cells prompted us to further examine the physiological functions of Discn. To this end, we generated Discn knockout (KO) mice via CRISPR/Cas-mediated gene targeting strategy (Supplementary Fig. 6a). Two mouse lines from the same targeting design were established and the success of Discn KO was validated by Sanger sequencing of PCR-amplified targeting regions (Supplementary Fig. 6b), as well as the loss of Discn expression in brain tissues of newborn KO mice (Supplementary Fig. 6c).

Heterozygous mutant mice (Discn+/−) were viable and no obvious abnormality was observed. Crossings of Discn+/− mice in line 1 produced pups with expected Mendelian ratios (Fig. 6a). Similar results were obtained from mouse line 2 (Fig. 6a). To exclude a possible maternal effect of Discn on early embryonic development, we mated Discn−/− females with Discn+/− males. Pups were born in normal Mendelian ratios (Fig. 6b), confirming...
that loss of Discn did not cause embryonic lethality. Consistently, crossing of Discn−/− mice produced litters with grossly normal sizes (Fig. 6b). Thus, maternal-zygotic loss of Discn does not cause embryonic lethality. However, high rates of newborn death occurred in pups obtained from Discn−/− crossings. More than half of them died within one week after birth (Fig. 6c).

We further investigated if Discn loss displayed long-term influences on the survived adults. Given that Discn was expressed in brain tissue and regulated the genomic integrity of cultured NSPCs, we thus focused on the brain functions. No structural defect in brain tissue and regulated the genomic integrity of cultured in wild-type (WT) and Discn−/− mice. Discn−/− mice displayed mood and anxiety-related disorders as determined by the following tests. In open field experiment, Discn−/− mice spent significantly less time in the center (Fig. 6d) and crossed into the center for fewer times (Fig. 6e) than the WT littermates. Consistently, mutant mice stayed in the center (Fig. 6d) and crossed into the center for fewer times (Fig. 6e) than the WT littermates. Consistently, mutant mice stayed in the center (Fig. 6d) and crossed into the center for fewer times (Fig. 6e) than the WT littermates. Consistently, mutant mice stayed in the center (Fig. 6d) and crossed into the center for fewer times (Fig. 6e) than the WT littermates.

We next conducted Morris water maze to evaluate the brain functions on learning and spatial memory. Adult littermates swam in similar speeds (Supplementary Fig. 6e), and had more self-grooming behaviors (Fig. 6g, h) than WT littermates. Consistently, mutant mice stayed in the center (Fig. 6d) and crossed into the center for fewer times (Fig. 6e) than the WT littermates. Consistently, mutant mice stayed in the center (Fig. 6d) and crossed into the center for fewer times (Fig. 6e) than the WT littermates. Consistently, mutant mice stayed in the center (Fig. 6d) and crossed into the center for fewer times (Fig. 6e) than the WT littermates. Consistently, mutant mice stayed in the center (Fig. 6d) and crossed into the center for fewer times (Fig. 6e) than the WT littermates.

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...and balance abilities. Discn−/− mice stayed on the rotating rod without falling for shorter time than WT littermates when the speed reached 20 rpm (Fig. 6), indicating the reduced coordination and balance. Because Discn−/− neonates generated from Discn−/− crossings displayed more severe phenotype than those generated from Discn+/−/− crosses, we suspected that Discn−/− adults from Discn−/− crossings could have worse brain functions. To test this hypothesis, we compared their coordination and balance abilities. Indeed, Discn−/− adults from Discn−/− crossings had worse performance than those from Discn+/−/+ crossings in rotarod test (Supplementary Fig. 6i). Collectively, our data show that Discn plays important physiological functions and its loss in mice causes newborn death as well as brain dysfunctions in survived adults.

**Discn−/− mouse brains have massive DNA DSBs and inflammation.** NSPCs are the cellular basis for neurogenesis at fetal and postnatal stages. Accumulations of DNA DSBs and cytosolic double-strand DNA (dsDNA) in NSPCs and their progenies not only impaired neurogenesis and brain functions, but also evoked innate immune and inflammation responses leading to animal death. Based on the function of Discn in vitro cultured NSPCs, we hypothesized that Discn KO might cause accumulations of DSBs and cytosolic dsDNA in brain cells of neonates and adults, and the damages and associated immune responses underlie the newborn death and brain dysfunctions. Indeed, the brain cells of Discn−/− pups (one day old) contained higher level of DNA DSBs measured by γH2AX staining and neutral comet assay (Fig. 7a, b), and cytosolic dsDNA (Fig. 7c) compared to WT pups. Notably, KO pups from Discn−/− crossings had more abundant DSBs and cytosolic dsDNA than those from Discn+/− crossings (Fig. 7b, c). Similar results were obtained from adult mouse brains (Supplementary Fig. 7a-c).

DNA DSBs and cytosolic dsDNA can activate the STING pathway inducing the type I interferon expression and downstream immune responses. Cytosolic dsDNA also stimulates the AIM2 inflammasome activation leading to caspase 1 (CASP1) cleavage, pro-interleukin-1β (IL-1β) maturation and cell death via gasdermin D-mediated pyroptosis. STING pathway activation is monitored by phosphorylation of the key components TBK1 and IRF3, and the expression of type 1 interferons (IFNα and IFNβ). Compared to the WT counterparts, adult Discn KO mice, in particular those from Discn−/− crossings, showed drastic STING activation in brain cells (Fig. 7d). Moreover, cleavage of CASP1 and maturation of IL-1β were elevated in KO mouse brains (Fig. 7e). Intriguingly, the DNA DSBs and immune reactions were not detected in lung tissue of KO mice (Supplementary Fig. 7d). This suggested that the influences of Discn loss might be confined to certain tissues/organs where tissue stem cells rely on Discn for genome stability. Severe immune and inflammation reactions usually cause animal death. We then asked if the genomic instability-driven inflammation accounted for the newborn death of Discn KO mice. To this end,
we administrated ibuprofen, which is able to alleviate inflammation, in drinking water to pregnant Discn−/− females mated with Discn−/− males. Notably, ibuprofen administration partially rescued the defect of newborn death (Fig. 6c), supporting that inflammation is a causative factor for animal death.

We finally examined the whole-genome gene expression changes in Discn KO mouse brains by RNA sequencing. Brain tissues of WT and Discn−/− mice, which were derived from crossings of Discn−/− and Discn−/−, respectively, were analyzed. We identified 3535 differentially expressed genes (DEGs) whose expressions were consistently decreased or increased (fold change ≥ 2) in two types of Discn KO mouse brains compared to the WT control (Fig. 7f, Supplementary data). Of note, Discn−/− mice born from Discn−/− crossings displayed the most drastic changes when compared to the other groups. Gene Ontology (GO) term analyses revealed that DEGs down-regulated in Discn−/− were mostly involved in the regulations of neurogenesis, neuronal activities and functions (Fig. 7g, Supplementary Fig. 7e, f). DEGs upregulated in Discn−/− mice were enriched in terms of negative regulations of neurogenesis and neuronal functions, DNA damage responses, positive regulation of autophagy and cell differentiation (Fig. 7g, Supplementary Fig. 7g). Consistently, term of innate immune response was enriched (Fig. 7g, Supplementary Fig. 7h, i).

**Discussion**

Stem cells are the cellular basis for embryo development and tissue homeostasis, and are known to have much more stable...
genome than differentiated somatic cells. Only recently was it recognized that stem cells employed unique DNA metabolic pathways to efficiently maintain stable genome. Although several pioneer works reported stem-cell specific regulations on telomere lengthening, DNA damage response, and repair, it remains largely unknown regarding the specificity of DNA metabolism and their relevance to genomic stability in stem cells. LncRNAs play increasingly important functions in many cellular events. Whether lncRNAs are involved in stem-cell specific regulations of DNA metabolism is unexplored. In this study, we identified a list of ESC-specific lncRNAs whose expressions are responsive to genotoxic stress. By analyzing one un-annotated lncRNA Discn, we identified a novel regulatory pathway Discn-NCL-RPA which preserves a sufficient RPA pool for DNA replication stress response and repair.

During continuous cell proliferation, DNA replication frequently encounters endogenous and exogenous barriers which stall the replication forks and generate replication stress. During stress, ssDNA is persistently generated due to the continuous firing of new origins even the checkpoint is active. The ssDNA is not converted into DNA breaks as long as it is coated and protected by RPA. Thus, the RPA surplus plays a central part and is considered as a common denominator for avoiding replication catastrophe. Cells usually express more RPA than needed during the normal unperturbed replication. However, excessive ssDNA generated by replication stress can rapidly exhaust RPA reservoir. Therefore, efficient sustaining the RPA pool is vital to survive the replication stress. Increasing the expression of RPA is one simple strategy that is employed by pluripotent stem cells. Here we reported a novel mechanism in which lncRNA Discn sustains the free RPA pool by sequestering NCL in nucleolus and counteracting its association with RPA. The Discn-NCL binding in nucleolus seems cell autonomous and does not require stress stimuli. Discn is induced by genotoxic stress and its expression level is adjusted by the strength of stress (for instance hydroxyurea treatment plus ATR deficiency). Therefore, Discn fine-tunes the RPA reservoir in response to replication stress.

Discn depletion causes severe DNA DSBs in cultured mouse ESCs which are in vitro counterparts of the undifferentiated epiblast cells in peri-implantation embryos. Previous study reported that induction of DSBs at gastrulation stage (E6.5-E7.5) could result in apoptosis and embryonic lethality. However, Discn KO did not cause prenatal death immediately after gastrulation. This could be due to the fact that the pluripotent epiblast cells exist transiently in vivo (from embryonic E4.5-E5.5) and undergo limited rounds of DNA replication during this time window. The extent of DNA replication-associated DSBs could therefore be mild and fail to evoke lethal phenotype. In contrast, NSPC-based neurogenesis starts from ~E8.5 and persists throughout the fetal stage and even until after birth. The long time periods of NSPC proliferation renders the neural system susceptible to the replication-associated DNA damage. Indeed, we detected increased levels of DNA DSB and cytostatic dsDNA in brain cells of Discn KO mice. Moreover, the DSB level was significantly higher in KO mice derived from Discn−−/− crosses than in those derived from Discn−/+ crosses. Concordantly, Discn KO mice displayed variable extents of developmental defects ranging from brain dysfunctions to newborn death, depending on the genotypes of their parents.

The genomic instability acted as a causative factor in the developmental defects of Discn KO mice. Previous studies including ours have shown that in NSPCs, replication stress-induced DNA DSBs frequently arise in replication fragile sites and form recurrent DSB clusters (RDCs). Many genes critical for neurogenesis in NSPCs harbor RDCs and are susceptible to replication stress-associated perturbations. Thus, replication-associated DNA DSBs in NSPCs directly impacted neurogenesis. In addition, DNA DSBs and in particular cyttoplasmic dsDNA can be activated to evoke innate immune response and inflammatory reactions. Immune reaction alone in developing brain imparts lifelong neuropathology and altered behaviors. Adult neural stem cells are also altered by systemic inflammation. Severe inflammation is able to cause pyroptotic cell death. Indeed, Discn KO induced the STING pathway activation leading to the production of type-1 interferons. Inflammamosomes were also activated in KO brains as evidenced by the cleavage of caspase 1 and maturation of pro-inflammatory cytokine IL-1β. Similar to the tendency of DNA DSB and cytoplasmic dsDNA accumulation, the immune and inflammatory reactions in KO mice from Discn−−/− crosses were more severe, leading to the newborn death. It is intriguing to know why KO mice from Discn−−/− crosses accumulated more DNA DSBs than those from Discn−/+ crosses. One possible explanation is that oocytes and sperms from Discn−−/− mice might contain more DSBs, which heightened genomic instability of embryos.

In summary, our study uncovered a novel stem cell-specific pathway Discn-NCL-RPA which regulates RPA availability and genomic stability, and plays physiological role in mouse neurogenesis. Our attempts to identify the tissue expression pattern of human DISCN using publicly available RNA-seq data were unsuccessful. Future works are needed to investigate the human DISCN expression patterns and functions, and its relevance to human diseases.

Methods
Mouse embryonic fibroblasts (MEF) preparation and transfection. MEF was isolated from embryos of C57BL/6J at embryonic day 13.5 (E13.5) as previously described. Briefly, pregnant mice were euthanized and the embryos were dissected. The head and internal organs were removed. Then the fetus tissue was minced thoroughly and digested with 3 ml of 0.25% trypsin-EDTA at 37 °C for 10 min. Trypsin was neutralized with DMEM (Gibco, 11965) supplemented with 10% FBS. The suspension was transferred to a 50 ml conical tube to allow the undissociated tissue pellet with gravity for 10 min. MEF in supernatant was transferred to 100 mm dishes for further culture. MEF was cultured in DMEM supplemented with 10% fetal bovine serum. To prepare feeder cells for ESC culture, MEF were expanded for three passages and treated with 5 μg/ml mitomycin C (Sigma, M2487) for 4 h. Inactive MEF was frozen in liquid nitrogen. For ectopic expression of Discn in MEF, cells were transfected with lipofectamine 2000 according to the manufacturer's instructions.

Mouse ESC culture and induced differentiation. Mouse ESCs (derived in our lab) were routinely maintained on activated MEF in the following culture condition: Dulbecco’s Modified Eagle’s Medium/Nutrient Mixture F-12 (DMEM/F12) medium supplemented with 20% Knockout serum replacement (Gibco, 10828028), 1 mM sodium pyruvate (Gibco, 11360070), 2 mM L-glutamine (Sigma, G8540), 1 non-essential amino acids (Gibco, 11140-035), 0.1 mM β-mercaptoethanol (Sigma; M7572), penicillin (100 U/mL), streptomycin (100 μg/mL) (Gibco, 15140-122) and 1000 units/mL mouse leukemia inhibitory factor (LIF) (Millipore, ES1107). For ATR inhibition, cells were treated for 4 h with 10 μM VE-821 (Selleck, S8007).

To obtain differentiated cells, mouse ESCs were passaged with 0.05% Trypsin-EDTA (Gibco, 25200072) and seeded onto 0.1% gelatin-coated dishes twice to remove feeders. Culture medium was replaced with differentiation medium: culture media of MCDB153 (JRH Biosciences) supplemented with 0.5 μM retinoic acid (Sigma; R2625). After one week induction, the differentiated cells were harvested for RNA extraction.

Neural stem/progenitor cell (NSPC) culture. Mouse NSPC was previously derived from WT mice at postnatal day 5 (P5). Cells were seeded in 35-mm dishes pre-coated with laminin (Sigma, L2020) and maintained in complete medium: StemCELL Technologies, 05702 supplemented with 10 ng/mL basic fibroblast growth factor (bFGF) (STEMCELL Technologies, 02634), 20 ng/mL recombinant human epidermal growth factor (rhEGF) (STEMCELL Technologies, 02633), and 2 μg/mL heparin (STEMCELL Technologies, 07980).

Generation of Discn Knockout mice. The Discn−−/− mouse strain was generated on the C57Bl/6J genetic background by Cyagen Company (https://www.cyagen.com). Briefly, a pair of gRNA located on both sides of mouse Discn gene and Cas9 mRNA were co-injected into mouse zygotes to generate targeted knockout offsprings. The gRNA target sequences were CAATTGACGTTTCTAGGAGG and ATAGGG...
CAGCTAGATTGGGG. Discn knockout was validated by Sanger sequencing of PCR-amplified fragment. Genotyping primers, P1, P2-Mut, and F3-WT, were listed in Supplementary Table 1. PCR-based genotyping was performed using genomic DNA from mouse tails at the following program: 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 15 s, annealing at 64°C for 30 s, and extension at 68°C for 36 s. Mice were housed in accordance to the Animal Care and Use Committee rules and guidelines of the Kunming Institute of Zoology, Chinese Academy of Sciences. Mice were kept under 12 h dark/light cycle, with daylight from 8:00 to 20:00, ambient temperature 20 to 25°C, and 40 to 70% humidity. All works on mice were carried out in accordance with the guidelines of the Animal Care and Use Committee of the Kunming Institute of Zoology, Chinese Academy of Sciences. All the studies on mice received ethical approval of the Animal Care and Use Committee of the Kunming Institute of Zoology, Chinese Academy of Sciences.

**RNA sequencing and data analyses.** Total RNAs were extracted from cultured cells or from mouse brains using Trizol (Tiangen, DP424). The cDNA libraries were constructed using the TrueSeq™ RNA Sample Preparation Kit (Illumina) and sequenced using an Illumina HiSeq™ 3000 or HiSeq X Ten platform. To identify novel IncRNAs, the clean reads were mapped to mouse reference genome (mm9) using TopHat2 software (Version 2.0.8). The values of gene expression were calculated using Cufflinks (Version 2.1.1)55. Non-coding transcript annotation file was downloaded from the NONCODE database (http://www.noncode.org/) (Version 4.0). Novel IncRNAs were identified by Coding-Non-Coding Index (CNCI) software (Version 2.1). Gene ontology enrichment was performed using an online tool (http://geneontology.org/)56. The heatmaps were created by the “ggplot2” R packages with default parameter. The RNA-seq data have been deposited in the Gene Expression Omnibus (GEO) database (accession number: GSE161998).

**Rapid amplification of cDNA ends (RACE).** The RACE protocol is based on published Smart-seq2 protocol57. The oligo-dt primer, template-switching oligos (TSO) and ISPCR primers were synthesized as described in the Smart-seq2 method. For 5' end amplification of Discn, reverse transcription was performed using gene specific primer 1 (GSP1). 5' end was amplified by PCR using gene specific primer 1 and ISPCR primer. For 3' end of Discn, reverse transcription was performed using oligo-dt primer. 3' end was amplified by PCR using gene specific primer 2 (GSP2) and ISPCR primer. The products were cloned into TOPO-TA Vector (Invitrogen) and validated by the Sanger sequencing. The primers used for PCR were listed in Supplementary Table 1.

**Expression vector construction.** To construct the shRNA expression plasmids, shRNA oligos targeting to Discn were designed through an online tool of Ambion siRNA Target Finder. The nucleotide sequences, shRNA-control, shRNA#, shRNA2#, were listed in Supplementary Table 1. The shRNA oligos were synthesized by Tsingke Company (Beijing, China), annealed and inserted into pLKO.1 plasmid using EcoR I and Age I sites.

**Expression vector construction** for Discn or Discn mutants inducible expression plasmids, Discn or Discn mutants were inserted into mcherry expression plasmids, Discn or Discn mutants expressed using a mcherry promoter vector. The Discn constructs were packaged into lentivirus for transfection. For transient transfection, RPA70-P2A-PA2A-RPA14 was constructed as made previously. Coding sequences for three RPA subunits (RPA70, RPA32, and RPA14) were obtained from cDNA transcribed from mRNA of mESCs by standard PCR methods. For 5' end amplification of Discn, reverse transcription was performed using gene specific primer 1 (GSP1). 5' end was amplified by PCR using gene specific primer 1 and ISPCR primer. For 3' end of Discn, reverse transcription was performed using oligo-dt primer. 3' end was amplified by PCR using gene specific primer 2 (GSP2) and ISPCR primer. The products were cloned into TOPO-TA Vector (Invitrogen) and validated by the Sanger sequencing. The primers used for PCR were listed in Supplementary Table 1.

**Neutral comet assay.** The neutral comet assay was performed as previously described58. Briefly, cells were dissociated into single cells at the concentration of 1 × 10⁶ cells/mL. Cells were then mixed with low-melting agarose at a ratio of 1:8 and spread onto the slides. Cells were lysed in lyser buffer (2.5 M NaCl, 100 mM Tris-HCl, pH 10, 10 mM EDTA, 1% Triton X-100) at room temperature for 1 h. Electrophoresis was performed in electrophoresis buffer (300 mM sodium acetate, 100 mM Tris, pH 8.3) once. Then the slides covered with lens paper were exposed coverslip-side-up in 55°C 2 × SSC buffer to 365 nm UV light at a distance of 1 cm for 30 min. The slides were immersed in 1 × SSC and incubated for 1 h at room temperature. The slides were stained with 10% Giemsa solution (Gibco, 1892798) for 10 min at 37°C. After washing with water and dried overnight at room temperature, SCEs images were captured using an Olympus confocal microscopy system.

**Immunofluorescence staining in cells.** For immunofluorescence staining, cells were fixed in 4% paraformaldehyde (PFA), permeabilized in 0.5% Triton X-100, blocked with 2% BSA for 2 h with 0.2% Tween 20/PBS and then incubated with rabbit anti-Discn (optimal cutting temperature compound) for sectioning. H&E staining and immunostaining were performed following the standard protocol.

**Brain section, hematoxylin and eosin (H&E) staining, and immunostaining.** After induction of deep anesthesia, mice were transcardially perfused with 10 mM Ribonucleoside Vanadyl Complex (10 mM) to remove cytoplasm detach from nuclei. Cells were lysed by a 0.45-mm clearance 1-mL pipette. Cells were then harvested and resuspended in ice-cold phosphate-buffered saline (PBS) with 4% paraformaldehyde (PFA) in PBS. Then the brains were harvested and fixed in 4% PFA overnight at 4 °C. After sunk in 15 and 30% sucrose in PBS, the brains were embedded in OCT (optimal cutting temperature compound) for sectioning. H&E staining and immunostaining were performed following the standard protocol.

**Nucleolar and nucleoplasm fractionation.** Nucleoli were isolated as previously described59. Briefly, 2 × 10⁶ cells g were harvested and resuspended in ice-cold hypotonic buffer (10 mM NaCl, 1 mM MgCl₂, 10 mM HEPES, pH = 7.4) supplemented with Roche complete protease inhibitor (Roche Applied System) and Ribonucleoside Vanadyl Complex (10 mM) (New England BioLabs, S1402S) to have cytoplasm detach from nuclei. Cells were lysed by a 0.45-mm clearance 1-mL Dounce homogenizer. Nuclei were purified and broken by sonication in ice-cold tissue homogenizer and then were washed through a sucrose cushion.

Nucleoplasm fractionation was performed as previously described59. Briefly, cells were washed once with ice-cold PBS, and then fixed in 1% formaldehyde for 15 min. Cells were lysed in lysis buffer (25 mM Tris, pH 7.4, 0.1% Triton X-100, 85 mM KCl). The nuclei were pelleted by centrifuging for 5 min at 2300 x g. Then the nuclei were washed with 0.2% SDS and 0.1% Triton X-100 for 5 min. The supernatant was collected as nucleoplasm.
RNA fluorescence in situ hybridization (FISH) and immunostaining. For RNA-FISH of Discn, DNA probes and RNA-FISH hybridization kit were obtained from Guangzhou Ribobio Co., LTD, and labeled with biotin using PierceTM RNA 3′ Transcription Kit (Thermo, T7491) according to the manufacturer’s instructions. In vitro RNA pulldown was performed as previously described46. Briefly, 50 pmol of labeled RNA in RNA structure buffer (10 mM Tris pH 7.5, 0.1 mM KCl, 10 mM MgCl2) was heated to 95 °C for 2 min, stand on ice for 3 min, then left at room temperature (RT) for 30 min to allow proper secondary structure formation. Samples were subjected to RNA pulldown using PierceTM Magnetic RNA-Protein Pull-Down Kit (Thermo, 20164). Beads were eluted and boiled in 2× SDS protein loading buffer. The recovered proteins were subjected to gradient gel electrophoresis and mass spectrometry (MS) for protein identification or detection by western blot.

In vivo RNA pulldown. DNA probes for in vivo pulldown were ordered from Guangzhou Ribobio Co., LTD and labeled with biotin using PierceTM RNA 3′ End Destinybiotinylatination Kit (Thermo, 102163) according to the manufacturer's instructions. In vitro RNA pulldown was performed as previously described44. Briefly, 2× 10^6 cells were washed with ice-cold PBS once, cross-linked with 265 mM UV light at 400 mJ energy in ice-cold PBS, treated with CSKT buffer supplemented with 1 nM PMSF and SuperRasen for 14 min. After centrifugation for 10 min at 12000 g, the pellet was re-suspended in 3 mL of DNaase I buffer (30 mM Tris pH 7.5, 0.5% Nonident-P 40, 0.1% sodium lauroyl sarcosine, 1× protease inhibitors, SUPERaseIn, 600 U RNase free DNaase I, 10 mM vanadyl ribonucleoside complex) and incubated at 37 °C to dissolve the pellet followed by a spin. The supernatant was pre-cleaned by 50 μL of M-28 streptavidin Dynabeads (Thermo, 00781251) at room temperature for 20 min, and beads were discarded. Pre-cleaned lysate, 100 pmol of probes and 160 μL beads were combined, incubated at room temperature for 20 min, preheated to 65 °C for 15 min and followed by slowly cooling to 37 °C for 1 h. The beads were washed 5 min for three times with wash buffer 1 (50 mM Tris, pH 7.5, 0.3 M LiCl, 1% SDS, 0.5% Nonident-P 40, 1 mM DTT, 1 mM PMSF, 1× protease cocktail inhibitors (Roche)) at 37 °C, treated with 20 U of DNaase I in 300 μL of wash buffer 2 (1% SDS, 1 mM DTT, 5 μM EDTA, 150 mM NaCl, 1 mM PMSF) once. Proteins were eluted and boiled in 2× protein loading buffer at 100 °C for 5 min.

RNA immunoprecipitation (RIP). RIP was performed as previously described26,27 with minor modifications. Briefly, 2× 10^6 cells were lysed in lysis buffer (10 mM HEPPS pH 7.0, 100 mM KCl, 5 mM MgCl2, 0.5% NP40, 1 mM DTT, plus 0.5 U SUPERasen (Thermo, AM2694) and proteinase inhibitors) at −80 °C overnight. The lysates were centrifuged at 15,000 × g for 15 min at 4 °C. The supernatants were incubated with protein A/G agarose (Abmart, A10001) precoated either with 2.5 μg of normal rabbit IgG antibody (Sigma, I5006) or with 2.5 μg of NCL anti-β-I-SceI were kindly provided by professor An-yong Xie, School of Medicine, Zhejiang University. The cells were incubated with 1 μg of isotype IgG (Sigma, I5006) per sample was performed using Protein G Dynabeads (Thermo, 88847) according to the manufacturers' protocol. For western blot, total protein was extracted using RIPA buffer (Beyotime, P00133) supplemented with 1× protease inhibitor cocktail containing 40 μg/mL (Beyotime, P0013J). Proteins were subjected to 4%-12% SDS-PAGE gels for separation and transferred to polyvinylidene fluoride (PVDF) membrane (Roche, 03010004001). The membrane was blocked with 5% non-fat milk at room temperature for 2 h and then incubated with primary antibodies at 4 °C overnight. After incubation with anti-mouse or rabbit HRP-conjugated secondary antibodies for 1 h at room temperature, the bands were detected using ECL reagent (Beyotime, P0018FS) and the membranes were video-tracked and their trace was analyzed using the SMART 3.0 software (Panlab Harvard, MA, USA).

DNA fiber assay. Cells were labeled with 50 μM 5-iodo-2′-deoxyuridine (IdU; Sigma, I7125) for 30 min and then incubated with 50 μM 5-chloro-2′-deoxyuridine (CldU; Sigma, C6891) for 30 min, with or without treatment of hydroxyurea (HU, Selleck, S1896). DNA fibers were made as described26. Briefly, cells were harvested and suspended in PBS at a concentration of 10^6/mL. 2500 cells were lysed in 12 μL of spreading buffer (0.5% SDS, 50 mM EDTA, 200 mM Tris, pH 7.4) on one end of the glass slide. DNA fibers were spread along the slide by tilting the slides, fixed with freshly prepared methanol-acetic acid (3:1), and treated with 2.5 M hydrochloric acid. For detection of labeled fibers, rat anti-BrdU/CldU (B1/75) monoclonal antibody (Novus, NB300-169) and mouse anti-idU monoclonal antibody (BD, 347580) were used as the primary antibody. The secondary antibodies were AlexaFlor 488-conjugated goat anti-mouse IgG or Cy3-conjugated goat anti-rat IgG. Images were captured from Olympus confocal microscope. The length of labeled fibers were measured using the Image J software, and values were averaged from at least four consecutive tracks were selected for measurement. At least 50 DNA fibers were analyzed.

New replication origin firing analysis. Origin firing analysis was performed as described28. Briefly, cells were pulse-labeled with 50 μM CIdU for 10 min without HU or with 0.1 mM HU for 20 min. DNA fibers including at least four consecutive tracks were selected for measurement. At least 50 DNA fibers were analyzed.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR). RNA was reverse transcribed using PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara, RR037A), according to the manufacturer’s protocol. qRT-PCR was performed on CFX96™Real Time System (BioRad) using the TB Green™ Premix Ex Taq™ II kit (Takara, RR820A). The sequences of the primers used were listed in Supplementary Table 1.

Discn copy number measurement. The cells were counted and lysed in TRNZol Universal (Tiangen, DPA24). The External RNA Controls Consortium (ERCC) RNA Spike-In Control Mix I (Thermo, 4456740) was added into the cell lysate as control. RNAs were isolated for downstream qRT-PCR. ERCC-00042 was used to generate a standard curve for absolute quantification of ERCC-00042 abundance in recovered RNAs. The efficiency of RNA recovery was calculated as the ratio of recovered RNA to ERCC-00042 abundance. Copy numbers of Discn were determined by comparing Ct values of Discn to the Ct values of ERCC-00042, followed by calibration with the RNA recovery efficiency.

Laser micro-irradiation. Cells were plated on coverslips pre-coated with matrigel. A 405-nm laser of Olympus FV1000 confocal microscope was used for micro-irradiation. Cells were allowed to recover for 2 h. To label S phase of cells, 50 μM BrdU were added in the final 30 min. Then cells were fixed in 4% PFA and permeabilized for immunostaining. 50 cells per experiment were analyzed. Three independent experiments were performed.

DNA repair assay using HR-RFP and NHEJ-GFP reporter cells. Mouse ESC lines containing NHEJ reporter and HR reporter, and the I-cis expression plasmid, pcDNA3-I-cis were kindly provided by professor An-yong Xie, School of Medicine, Zhejiang University. mESC transfectants were transfected with pcDNA3-I-cis plasmid using Lipofectamine (Invitrogen). At least three days post-transfection, cells were analyzed for RFP+ or GFP+ frequencies using a FACs LSFortessa flow cytometer (BD), BD FACSDiva software (Version 8.0.2), and Flowjo software (Version 7.6). The gating strategies for HR-RFP+ cells and NHEJ-GFP+ cells were presented in Supplementary Fig. 8.

Immunoprecipitation and western blotting. Cells were lysed in RIPA buffer (Beyotime, P00133) supplemented with 1× protease inhibitor cocktail containing 40 μg/mL (Beyotime, P0013J). Immunoprecipitation with 2.5 μg of RPA32 antibody or 2.5 μg of isotype IgG (Sigma, I5006) per sample was performed using Protein G Dynabeads (Thermo, 88847) according to the manufacturers’ protocol.

For western blot, total protein was extracted using RIPA buffer (Beyotime, P00133), Proteins were subjected to 4%-12% SDS-PAGE gels for separation and transferred to polyvinylidene fluoride (PVDF) membrane (Roche, 03010004001). The membrane was blocked with 5% non-fat milk at room temperature for 2 h and then incubated with primary antibodies at 4 °C overnight. After incubation with anti-mouse or rabbit HRP-conjugated secondary antibodies for 1 h at room temperature, the bands were detected using ECL reagent (Beyotime, P0018FS). Antibodies used were listed in Supplementary Table 2.

Mouse behavioral tests. All behavioral tests were performed using two months old male mice from littermates. All experiments and data analyses were performed in a blinded manner. The behavioral studies including open-field test, light–dark shuttle box test, Morris water maze test and self-grooming test were performed as previously reported29. Briefly, Morris water maze test was carried out in a tank with 120-cm diameter and 50-cm depth. The tank was equipped with a 10-cm diameter platform submerged 1 cm under the water surface masked using white beads. Mice were trained to find the platform within 60 s and stay on the platform for 15 s. Training was terminated once one of the groups succeeded in landing on the platform within 10 s. At 4 and 72 h following the last training, the platform was removed and mice were tested to locate the platform within 60 s in the pool. Mice were video-recorded and their trace was analyzed using the SMART 3.0 software (Panlab Harvard, MA, USA).
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Author contributions
L.W., J.Z.L., and W.D. Z. performed the experiments, analyzed the data, and participated in the experimental design and manuscript preparation. H.Z. and J.G. performed mass spectrometry analysis. P.Z. supervised the study and wrote the manuscript.

Competing interests
The authors declare no competing interests.

Additional information

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