Sirt1 regulates acrosome biogenesis by modulating autophagic flux during spermiogenesis in mice

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ABSTRACT

Sirt1 is a member of the sirtuin family of proteins and has important roles in numerous biological processes. Sirt1−/− mice display an increased frequency of abnormal spermatozoa, but the mechanism of Sirt1 in spermiogenesis remains largely unknown. Here, we report that Sirt1 might be directly involved in spermiogenesis in germ cells but not in steroidogenic cells. Germ cell-specific Sirt1 knockout mice were almost completely infertile: the early mitotic and meiotic progression of germ cells in spermatogenesis were not obviously affected after Sirt1 deletion, but subsequent spermiogenesis was disrupted by a defect in acrosome biogenesis, which resulted in a phenotype similar to that observed in human globozoospermia. In addition, LC3 and Atg7 deacetylation was disrupted in spermatids after knocking out Sirt1, which affected the redistribution of LC3 from the nucleus to the cytoplasm and the activation of autophagy. Furthermore, Sirt1 deletion resulted in the failure of LC3 to be recruited to Golgi apparatus-derived vesicles and in the failure of GOPC and PICK1 to be recruited to nucleus-associated acrosomal vesicles. Taken together, these findings reveal that Sirt1 has a novel physiological function in acrosome biogenesis.

KEY WORDS: Sirt1, Map1lc3, Acrosome biogenesis, Globozoospermia, Deacetylation, Autophagy, Mouse

INTRODUCTION

Infertility is estimated to affect up to 15% of couples of reproductive age (Boivin et al., 2007). Globozoospermia is a rare but severe disorder causing male infertility that is characterized by round-headed spermatozoa and an absent or highly abnormal acrosomal compartment (Dam et al., 2007; Kullander and Rausing, 1975; Lalonde et al., 1988; Singh, 1992). The acrosome is a cap-shaped, Golgi-derived organelle that is located over the anterior part of the sperm nucleus. The morphogenic changes of acrosome biogenesis during spermiogenesis have been well documented; numerous proacrosomal granules derived from trans-Golgi stacks accumulate in the concave region near the trans-Golgi stacks and fuse with each other while they bind to the acroplaxome (a cytoskeletal structure attached to the nuclear envelope) (Kierszenbaum et al., 2003) and then generate the acrosomal vesicle. Additional vesicle fusion increases acrosome size and, together with nucleus reformation, the acrosome adopts its characteristic shape (Abou-Haila and Tulsiani, 2000). Genetically modified mouse models have been helpful in understanding the molecular mechanisms underlying acrosome biogenesis, and several genes have been identified as associated with globozoospermia, including Csnk2a2 (Xu et al., 1999), Hrb (Aggl1) (Kang-Decker et al., 2001), Gapc (Yao et al., 2002), Gba2 (Yildiz et al., 2006), Zphpl (Lin et al., 2007), Pick1 (Xiao et al., 2009), Hsp90b1 (Audouard and Christians, 2011), Vps54 (Paiardi et al., 2011), Spacal (Fujihara et al., 2012) and Dpy19L2 (Pierre et al., 2012). Recently, we found that Atg7 is involved in the transportation/fusion of Golgi-derived proacrosomal vesicles to form the nucleus-associated acrosome (Wang et al., 2014). However, much of the aetiology of globozoospermia remains unknown, and the mechanism underlying acrosome biogenesis remains elusive.

Sirt1 is a member of the mammalian sirtuin gene family and is the closest relative of yeast Sir2 in mammalian cells (Brachmann et al., 1995; Smith et al., 2000). By deacetylating a number of substrates, Sirt1 regulates numerous important physiological processes, including glucose metabolism, cell survival, mitochondrial respiration and histone modification. Sirt1 is not essential to survival in mice; however, Sirt1−/− male mice are infertile (McBurney et al., 2003). It has been proposed that Sirt1 might regulate spermatogenesis at postnatal stages by controlling hypothalamus-pituitary gonadotropin (HPG) signalling (Kolthur-Seetharam et al., 2009). A detailed analysis of the functions of Sirt1 in the germ line of Sirt1−/− mice (Coussens et al., 2008) and meiotic-specific Sirt1 knockout mice revealed that Sirt1 might be involved in the differentiation of spermatogenic stem cells and the transition from histone to protamine (Bell et al., 2014). In addition, the researchers found an increased proportion of abnormal spermatozoa in the Sirt1-deficient mice; however, the mechanism by which Sirt1 deficiency in germ cells leads to malformed spermatozoa and the identity of its specific substrate during spermiogenesis remain largely unknown.

To study the role of Sirt1 during spermiogenesis, we generated steroidogenic cell-specific and germ cell-specific Sirt1 knockout mouse lines by crossing Sirt1F/F mice with SF1-Cre and Tnap-Cre mice, respectively (Bingham et al., 2006;洛美利 et al., 2000). We found that the malformed spermatozoa in Sirt1−/− deficient mice were primarily caused by Sirt1 deficiency in germ cells rather than in the steroidogenic cells. Further investigation revealed that Sirt1 deletion in germ cells led to the accumulation of acetylated LC3 in the spermatid nucleus, and this affected the recruitment of several acrosome biogenesis-related proteins to nucleus-associated acrosomal vesicles. Therefore, our study reveals a novel function for Sirt1 during acrosome biogenesis.
RESULTS
Spermatogenesis is not disrupted in steroidalogenic cell-specific Sirt1 knockout mice
To test whether Sirt1 deficiency in steroidalogenic cells disrupts spermatogenesis in testsis, we generated a steroidalogenic cell-specific Sirt1 knockout mouse line by crossing Sirt1F/F with SF1-Cre mouse strains. SF1-Cre [in which Cre is driven by steroidalogenic factor 1 (SF1; also known as Nr5a1)] is predominately expressed in pituitary, adrenal gland and Leydig cells in testsis (Bingham et al., 2006), and its expression resulted in steroidalogenic cell-specific Sirt1 knockout in Sirt1F/F; SF1-Cre mice. Western blotting showed that the protein level of Sirt1 was dramatically reduced in the Leydig cells of Sirt1F/F; SF1-Cre compared with Sirt1F/F mice (Fig. S1A).

As Sirt1 has been reported to regulate testosterone synthesis by controlling HPG signalling (Kolthur-Seetharam et al., 2009), we assessed mating efficiency and testosterone concentrations in Sirt1F/F and Sirt1F/F; SF1-Cre male mice. Both were decreased in Sirt1F/F; SF1-Cre male mice compared with control groups (Fig. S1B,C). However, no significant difference in testis size (Fig. S1D) was observed between Sirt1F/F and Sirt1F/F; SF1-Cre mice. Histological analysis of testsis and epididymis also showed no obvious defect compared with control mice (Fig. S1E,F). When spermatooza released from the cauda epididymis were assessed, no abnormal spermatooza were found in either the Sirt1F/F or Sirt1F/F; SF1-Cre mice (Fig. S1G). Furthermore, the average epididymal sperm count of Sirt1F/F; SF1-Cre mice was similar to that of Sirt1F/F mice (Fig. S1H), and this was accompanied by high sperm motility (Fig. S1I).

These results suggest that the disruption of Sirt1 in steroidalogenic cells might affect sexual behaviour, but not spermatogenesis, indicating that the malformed spermatooza defect of Sirt1F/F−/− mice might not be caused by Sirt1 deficiency in steroidalogenic cells.

Germ cell-specific Sirt1 knockout causes infertility in male mice
To address the function of Sirt1 during spermatogenesis, Sirt1 was specifically knocked out in germ cells by crossing Sirt1F/F mice with Tnap-Cre mouse strains. Tnap-Cre [in which Cre is driven by tissue non-specific alkaline phosphatase (Tnap; also known as Alpl)] is specifically expressed in primordial germ cells (Lomeli et al., 2000), which resulted in germ cell-specific Sirt1 knockout in Sirt1F/F; Tnap-Cre mice. The Sirt1 protein level was dramatically reduced in the testis of Sirt1F/F; Tnap-Cre compared with Sirt1F/F mice (Fig. 1A), confirming Sirt1 deficiency in these germ cells.

To test male fertility after germ cell-specific Sirt1 knockout, a breeding assay was conducted by mating Sirt1F/F or Sirt1F/F; Tnap-Cre male mice with Sirt1F/F females over a 2 month period. As shown in Fig. 1B, Sirt1F/F; Tnap-Cre male mice were severely subfertile; only 19.83% of the females mating with Sirt1F/F; Tnap-Cre male mice became pregnant compared with 82.67% of those mating with Sirt1F/F males. Both the size and weight of the testes of Sirt1F/F; Tnap-Cre mice were reduced compared with those of Sirt1F/F mice (Fig. 1C,D). In addition, although histological analysis of the testsis did not show any obvious defects, the diameters of round seminiferous tubules in Sirt1F/F; Tnap-Cre mice were smaller than in Sirt1F/F mice (Fig. 1E,F).

Sirt1 is not essential to mitosis and meiosis during spermatogenesis
Cre recombinase is expressed in primordial germ cells in Sirt1F/F; Tnap-Cre mice. Therefore, to investigate the mechanism underlying the subfertility of Sirt1F/F; Tnap-Cre male mice, we first examined the germ cells by immunostaining promyelocytic leukaemia zinc-finger protein (PLZF; also known as Zbtb16), which is a marker for early spermatogonial stem cells (SSCs). The number and distribution of PLZF-positive cells were comparable in Sirt1F/F;
**Abnormal acrosome biogenesis in Sirt1F/F; Tnap-Cre mice**

To characterize and confirm the defects in acrosome formation in Sirt1F/F; Tnap-Cre mice, TEM analysis was performed. In Sirt1F/F mice, all four acrosome biogenesis phases, including Golgi phase, cap phase, acrosome phase and maturation phase (Abou-Halia and Tulasi, 2000), could be identified by their standard characteristics (Fig. 3A,C,E,G). However, in Sirt1F/F; Tnap-Cre mouse testis, multiple acrosomal structures could be detected in both Golgi phase and cap phase (Fig. 3B,D). These results suggest that the malformed acrosomes in Sirt1F/F; Tnap-Cre mice might result from the failure of proacrosomal vesicles to fuse or to be transported to the preacrosomal structure. In the subsequent stages of acrosome biogenesis, vacuolated or irregularly shaped acrosomes were observed in the acrosome phase and maturation phase of Sirt1-deficient spermatids (Fig. 3F,H). Together, these results indicate that acrosome biogenesis was affected in Sirt1F/F; Tnap-Cre mice.

To further examine how Sirt1 knockout affects acrosome biogenesis, the acrosome was labelled with an antibody against Sp56 at the early stages of spermiogenesis. As shown in Fig. 4A, a single acrosomal vesicle was attached to one end of the nucleus in Sirt1F/F spermatids, indicating that proacrosomal vesicles derived from the Golgi apparatus were fused. By contrast, in Sirt1F/F; Tnap-Cre mice, fragmented or unfused Sp56-positive structures were observed in the perinuclear region, and ~20% of the spermatids were observed with multiple small vesicles without fusing (Fig. 4B), which was similar to observations from TEM analysis. These results indicate that Sirt1 is involved in the transportation/fusion of Golgi-derived proacrosomal vesicles to form the nucleus-associated acrosome.

Previously, we found that autophagy is involved in acrosome biogenesis and that the autophagic molecular marker microtubule-associated protein light chain (LC3; also known as Map1lc3a) localizes on Golgi apparatus-derived vesicles (Wang et al., 2014). We examined the localization of LC3 and its relationship with Golgi apparatus-derived vesicles by immunofluorescence labelling of LC3 and TGN38 (a trans-Golgi network marker; also known as Tgoln1/2). At the early stages of spermiogenesis in Sirt1F/F mice, LC3 colocalized with TGN38 in spermatids (Fig. 4C). This colocalization was significantly reduced in Sirt1F/F; Tnap-Cre mice (Fig. 4C,D). These data suggested that LC3 transfer to the Golgi-derived vesicles was affected in Sirt1-deficient testes. Interestingly, in Sirt1F/F; Tnap-Cre mice, Sirt1 deficiency in germ cells led to an accumulation of LC3 in the nucleus (Fig. 4C,E), and some spermatids with LC3 in the nucleus showed no colocalization between LC3 and TGN38 (Fig. 4C). These results indicate that the nuclear accumulation of LC3 might affect proacrosomal granule fusion or the delivery of the granules to the nucleus-associated acrosomal vesicles.

**Autophagic flux is partially disrupted in the testis of Sirt1F/F; Tnap-Cre mice**

Recently, it was reported that LC3 can be deacetylated by Sirt1 in the nucleus, which mediates the redistribution of LC3 to the cytoplasm, where it can conjugate to Atg7 and drive autophagy initiation (Huang et al., 2015). To test whether the nucleocytoplasmic distribution of LC3 in spermatids is regulated by Sirt1, the acetylation status of LC3 in testis was determined by immunoprecipitation with an antibody to LC3 followed by western blotting with an anti-acetyl-lysine antibody. As shown in Fig. 5A, compared with Sirt1F/F mice, the acetylation of LC3 in testis was obviously increased after Sirt1 deletion in the Sirt1F/F; Tnap-Cre mice. To further examine whether the absence of Sirt1 impairs spermiogenesis, we first examined the cauda epididymis by histological analysis. In Sirt1F/F; Tnap-Cre mice, abnormal spermatozoa were found and spermatid density was decreased in the cauda epididymis compared with Sirt1F/F mice (Fig. 2A). Quantitative analysis showed that the total number of spermatozoa in the cauda epididymis was dramatically reduced in Sirt1F/F; Tnap-Cre compared with Sirt1F/F mice (3.18±1.12×10⁶ versus 22.15±0.73×10⁶; Fig. 2B). In addition, sperm motility, as measured by computer-assisted semen analysis (CASA), was severely decreased compared with Sirt1F/F mice (10.80±0.53% versus 91.33±0.49%; Fig. 2C). All other parameters that we assessed also showed severe defects in Sirt1F/F; Tnap-Cre compared with Sirt1F/F mice (Fig. 2D-G), including the percentage of progressive spermatozoa (1.27±0.12% versus 21.17±0.65%), average path velocity (VAP; 16.10±0.20 μm/s versus 93.22±1.41 μm/s), straight line velocity (VSL; 14.15±0.13 μm/s versus 60.90±1.34 μm/s) and curvilinear velocity (VCL; 37.36±0.55 μm/s versus 181.0±1.95 μm/s). Further morphological evaluation found that in Sirt1F/F; Tnap-Cre mice, many spermatozoa had irregularly shaped or round heads (Fig. 2H,J). This phenotype is very similar to that found in human globozoospermia, a serious fertility disorder that is characterized by round-headed spermatozoa with malformed or deformed heads (Dam et al., 2007). To examine whether the absence of Sirt1 impairs spermiogenesis, we examined the single-sperm immunofluorescence of Sp56 (Zp3r), an acrosome-specific marker. In Sirt1F/F mice, most spermatozoon acrosomes exhibited a typical crescent moon shape (Fig. 2). By contrast, the acrosomes of Sirt1F/F; Tnap-Cre mice exhibited various defects, including deformation, mislocalization and fragmentation. Transmission electron microscopy (TEM) analysis of the cauda epididymis in Sirt1-deficient mice also showed malformed acrosomes (Fig. 2K). Together, these findings suggest that Sirt1 might be involved in acrosome formation and the pathogenesis of globozoospermia.
mice; this explains the accumulation of acetylated LC3 in the nucleus of the spermatids. As Sirt1 could interact with and deacetylate several essential components of the autophagy machinery, such as Atg5 and Atg7, to modulate the autophagy processes (Lee et al., 2008), we assessed the acetylation status of Atg7 in germ cells using acetyl-lysine immunoprecipitation followed by western blotting with an anti-Atg7 antibody. We found that the acetylation of Atg7 in Sirt1F/F; Tnap-Cre mice dramatically increased compared with Sirt1F/F mice (Fig. 5B). Thus, Sirt1 might regulate the activation of autophagy by modulating the acetylation status of LC3 and Atg7 in testis.

Sirt1 might be involved in acrosome biogenesis through the modulation of autophagy. To test this, we first evaluated the effect of Sirt1 deficiency on autophagic flux in the testis. Immunoblotting analysis showed that the total levels of the autophagic substrate SQSTM1 (p62) were clearly elevated in the testis of Sirt1F/F; Tnap-Cre compared with Sirt1F/F mice (Fig. 5C); the unconjugated LC3 form was also slightly increased (Fig. 5C), and a similar SQSTM1

Fig. 2. Defects of Sirt1F/F; Tnap-Cre spermatozoa.
(A) Histology of the cauda epididymis of Sirt1F/F and Sirt1F/F; Tnap-Cre mice. Boxed regions are magnified to the right. Arrows indicate abnormal spermatozoa. (B) The total number of sperm in Sirt1F/F; Tnap-Cre cauda epididymis (3.18±1.32×10⁸) was significantly reduced compared with that of Sirt1F/F mice (22.15±0.73×10⁶). (C) Motile sperm in Sirt1F/F (91.33±4.99%) and Sirt1F/F; Tnap-Cre (10.80±0.53%) mice. (D) Progressive sperm in Sirt1F/F (21.17±0.65%) and Sirt1F/F; Tnap-Cre (1.27±0.12%) mice. (E) The average path velocity (VAP) of sperm from Sirt1F/F (93.22±1.41 μm/s) and Sirt1F/F; Tnap-Cre (16.10±0.20 μm/s) mice. (F) The straight line velocity (VSL) of sperm from Sirt1F/F mice (60.90±1.34 μm/s) and Sirt1F/F; Tnap-Cre (14.15±0.13 μm/s) mice. (G) The curvilinear velocity (VCL) of sperm from Sirt1F/F (181.0±1.95 μm/s) and Sirt1F/F; Tnap-Cre (37.36±0.55 μm/s) mice. (H) Confocal images showing round-headed spermatozoa in Sirt1F/F; Tnap-Cre mice. (I) Defects of the acrosome in Sirt1F/F; Tnap-Cre mice. Acrosomes were visualized by immunofluorescence of the acrosome-specific marker Sp56 (green); nucleus is in blue. (J) The percentage of abnormal spermatozoa with globozoospermia-like morphology was higher in Sirt1F/F; Tnap-Cre (41.60±3.71%) than in Sirt1F/F (1.41±0.43%) mice. Data are presented as the mean±s.e.m. ***P<0.001. (K) TEM analysis of spermatozoa from Sirt1F/F and Sirt1F/F; Tnap-Cre cauda epididymis. The Sirt1F/F; Tnap-Cre images illustrate spermatozoa with an irregular acrosome (left), with a separate acrosome (middle) or with cytoplasm (right). Nu, nucleus; Ac, acrosome.
accumulation was also observed by immunofluorescence (Fig. 5D,E). To confirm this defect, we performed TEM analysis in testis. The large double-membrane vacuoles filled with cargos could be detected in Sirt1F/F mouse testes (Fig. 5F, upper panel), which is the typical appearance of autolysosomes (Eskelinen et al., 2011; Gerland et al., 2004). However, in the Sirt1F/F; Tnap-Cre mice, only a few electron-dense lysosomes surrounded by some unfused small vesicles were observed (Fig. 5F, lower panel), and few autophagosomes were found in these spermatids (Klionsky and Eskelinen, 2014). These results indicate that autophagic flux is partially disrupted in the germ cells of Sirt1-deficient mice.

GOPC and PICK1 fail to be recruited to the acrosome in Sirt1-deficient germ cells

To examine whether the absence of Sirt1 affects the transportation/fusion of several acrosome biogenesis-related proteins during formation of the nucleus-associated acrosome, the localization of GOPC (golgi associated PDZ and coiled-coil motif containing protein), beclin 1 (an autophagy-related protein; also known as Atg6) and Sp56 was examined in the spermatids by immunofluorescence. GOPC is proposed to be involved in vesicle trafficking from the Golgi apparatus to the acrosome and interacts with beclin 1 (Yao et al., 2002; Wang et al., 2014). As shown in Fig. 6A, in both Sirt1F/F and Sirt1F/F; Tnap-Cre mice, beclin 1 predominately colocalized with Sp56. GOPC colocalized with beclin 1 in the acrosomal region in round spermatids of Sirt1F/F mouse but failed to be recruited to the acrosomal region of Sirt1F/F; Tnap-Cre mice. Statistical analysis showed that GOPC and Sp56 colocalization was significantly reduced in Sirt1F/F; Tnap-Cre mice (48.77±5.28%) compared with Sirt1F/F mice (81.63±2.49%) (Fig. 6B); this suggests that GOPC fails to be recruited to the acrosome in Sirt1-deficient germ cells.

We then examined the localization of another GOPC-associated protein, PICK1, which is a highly expressed peripheral membrane protein in round spermatids that is localized to Golgi-derived proacrosomal granules (Xiao et al., 2009) according to immunofluorescence studies. PICK1 predominantly colocalized with Sp56 in Sirt1F/F mouse Fig. 6C. By contrast, many PICK1

Fig. 3. Ultrastructural analysis of acrosome biogenesis in Sirt1F/F and Sirt1F/F; Tnap-Cre mice. (A,B) The ultrastructures of Golgi phase from Sirt1F/F and Sirt1F/F; Tnap-Cre mouse testis, showing three proacrosomal centres (arrows) around the nucleus in Sirt1F/F; Tnap-Cre testis. (C,D) Cap phase, showing that there were two additional acrosomal structures (arrows) around the nucleus in Sirt1F/F; Tnap-Cre mouse testis. (E,F) Acrosome phase, showing that there were vacuolated (asterisks) or irregularly shaped acrosomes in Sirt1F/F; Tnap-Cre spermatids. (G,H) Maturation phase, showing irregularly shaped acrosomes in Sirt1F/F; Tnap-Cre spermatids. Boxed regions are magnified to the right. Nu, nucleus; Ac, acrosome.
molecules failed to be recruited to the acrosomal region in the spermatids of Sirt1<sup>F/F</sup>; Tnap-Cre mice (Fig. 6C,D).

Taken together, these findings indicate that the acrosome biogenesis defect in Sirt1<sup>F/F</sup>; Tnap-Cre mice might be due to a failure to recruit GOPC and PICK1 to nucleus-associated acrosomal vesicles.

**DISCUSSION**

Sirt1 has multiple physiological functions, most of which are dependent on the acetylation status of specific substrates (Haigis and Guarente, 2006; Martinez-Redondo and Vaquero, 2013; Saunders and Verdin, 2007). Given that Sirt1 is induced by calorie restriction and that Sirt1 activity is necessary for the induction of starvation-induced autophagy (Lee et al., 2008), the relationship between Sirt1 and autophagy has been known for some years (Cui et al., 2006; Kume et al., 2010; Ng and Tang, 2013; Zeng et al., 2012). Sirt1 co-immunoprecipitates with Atg5, Atg7 and LC3/LC3b (Map1lc3b or Atg8), thereby establishing the connection between Sirt1 and autophagy (Lee et al., 2008). Under starvation conditions, Atg5,
Atg7 and LC3b can be deacetylated by Sirt1 (Lee et al., 2008); the deacetylation of LC3 by Sirt1 in the nucleus is a prerequisite for LC3 redistribution to the cytoplasm, and the nucleocytoplasmic transport of LC3 is essential for autophagosome formation (Huang et al., 2015). However, until now, the biological relevance of LC3 nucleocytoplasmic transportation has largely remained unexplored. Here, we show that the depletion of Sirt1 in germ cells leads to the accumulation of acetylated LC3 in the nucleus and to a partial disruption of autophagic flux in spermatids. Thus, it is possible that during spermiogenesis Sirt1 deacetylates LC3 in the nucleus, thereby mediating LC3 nucleocytoplasmic transport. Once in the cytoplasm, LC3 is activated by Atg7 and is then transferred to Golgi-derived vesicles to promote acrosome biogenesis (Fig. 7). Therefore, our investigations discovered a novel physiological function for Sirt1-mediated LC3 nucleocytoplasmic transportation in the biogenesis of a spermatozoa-specific organelle. Thus, it is possible that during spermiogenesis Sirt1 deacetylates LC3 in the nucleus, thereby mediating LC3 nucleocytoplasmic transport. Once in the cytoplasm, LC3 is activated by Atg7 and is then transferred to Golgi-derived vesicles to promote acrosome biogenesis (Fig. 7). Therefore, our investigations discovered a novel physiological function for Sirt1-mediated LC3 nucleocytoplasmic transportation in the biogenesis of a spermatozoa-specific organelle. In addition, Sirt1 could regulate the acetylation status of Atg7 in testis, and our previous work also indicated that Atg7 is required for acrosome biogenesis during spermatogenesis in mice (Wang et al., 2014). Thus, Sirt1 might participate in acrosome biogenesis through the autophagy pathway. The germ cell–specific Sirt1 knockout mice had abnormal spermatozoa, with irregularly shaped or rounded heads and less acrosome (Fig. 2H,J), which was very similar to the round-headed spermatozoa with malformed acrosome characteristic of human globozoospermia (Dam et al., 2007). The proportion of round-headed spermatozoa in Sirt1-deficient mice was 41.60±3.71%, which is similar to partial globozoospermia (Dam et al., 2011), suggesting Sirt1 as a potential globozoospermia pathogenic gene. The partial globozoospermia phenotype might result from either the activation of alternative pathways or gene redundancy. In addition to acrosome biogenesis defects, we observed a decrease in sperm count; this appears to be a common phenotype for a variety of genetic defects that perturb the normal programme of mouse spermiogenesis (Kang-Decker et al., 2001; Venables and Cooke, 2000; Wang et al., 2014). Bell et al. (2014) found that the histone to protamine transition was disrupted in Sirt1-deficient spermatids, and this might partially account for the decrease in sperm count. An alternative explanation relates to the functional diversity of Sirt1 during spermatogenesis. In the testes of Sirt1F/F; Tnap-Cre mice, we observed many TUNEL signals in the germ cells (Fig. S3), which was consistent with the findings of some previous studies (Bell et al., 2014; Kolthur-Seetharam et al., 2009). In yeast, Sir2 can inhibit ribosomal DNA recombination and relocalize to the sites of DNA breaks; the absence of Sir2 activity leads to silencing defects, sensitivity to DNA damage and increased genomic instability (Denu, 2003; Guarente and Picard, 2005). In mammalian cells, Sirt1 promotes DNA repair by...
deacetylating various DNA repair factors, including Nijmegen breakage syndrome protein 1 (NBS1; also known as nibrin) (Yuan et al., 2007), Werner syndrome protein (WRN) (Lee et al., 2015), Ku70 (Xrcc6) (Jeong et al., 2007), xeroderma pigmentosum complementation group A (XPA) (Fan and Luo, 2010) and Krüppel-associated box (KRAB)-associated protein 1 (KAP1; also known as Trim28) (Lin et al., 2015). In addition, Sirt1 can be recruited to DSBs in an ATM-dependent manner, and deacetylates and stimulates HDAC1, thus facilitating the dynamic regulation of HDAC1 activity, which is essential for DSB repair through non-homologous end joining (Dobbin et al., 2013). The role of Sirt1 in DNA repair has been substantiated in Sirt1−/− embryos, which exhibit
and the SF1-Cre mice were bred from 08:00 and 20:00. All animal experiments were approved by the Animal with free access to food and water, and illumination was provided between

Sirt1F/F; SF1-Cre Mice

MATERIALS AND METHODS

increased chromosomal aberrations and impaired DNA repair (Wang et al., 2008). Because most of the above-mentioned molecules are involved in meiotic recombination, it is likely that cell death in the tubule lumens of the testes is dependent on impaired meiotic recombination after Sirt1 depletion. This indicates that Sirt1 might be involved in more than one process during spermatogenesis.

In the Sirt1+/−; Tnap-Cre mice, several characteristic phenotypes of Sirt1−/− mice (including a small, feeble body with significantly increased post-natal mortality rates) disappeared, and only defects in spermiogenesis (mainly malformed spermatooza) were observed. This indicates that Sirt1 performs various functions that are dependent on cell type or developmental stage.

Taken together, our findings demonstrate that the malformed spermatooza defects in Sirt1−/− mice were mainly caused by a deficiency of Sirt1 in germ cells rather than in steroidogenic cells, and impaired acrosome biogenesis might be the main cause of the observed male infertility. Further investigation revealed that Sirt1 can deacetylate nuclear LC3, and the deacetylated LC3 is then transported to the cytoplasm, where it interacts with autophagic effectors and initiates autophagy. After this sequence of events, the entire autophagic molecular machinery participates in the fusion and transportation of Golgi-derived proacrosomal vesicles to the acrosome to promote acrosome biogenesis.

isolation and primary culture of Leydig cells

Leydig cells were isolated as previously described (Svechnikova et al., 2011). Briefly, the 2-month-old mice were euthanized by cervical dislocation, and the testes were removed and washed three times with PBS. The testes were then decapsulated and incubated with 1 mg/ml collagenase IV in DMEM/F12 in a shaking water bath (120 cycles/min) at 37°C for 15 min. After incubation, cold DMEM/F12 containing 5% fetal bovine serum (FBS; 10270, Gibco) was added to stop the action of collagenase IV. Seminiferous tubules were separated from the interstitial cells by gravity sedimentation. The cells were collected by centrifugation (300 g for 6 min) and resuspended in 2 ml DMEM/F12 containing 5% FBS. To obtain purified Leydig cells, this suspension was loaded onto a discontinuous Percoll gradient consisting of layers of 30%, 40%, 50% and 60% Percoll dissolved in Hank’s Balanced Salt Solution and centrifuged at 600 g at 4°C for 28 min. Leydig cells were primarily distributed from fraction 50% to 60% Percoll in Hank’s Balanced Salt Solution and centrifuged at 600 g at 4°C for 28 min. Leydig cells were primarily distributed from fraction 50% to 60% Percoll in Hank’s Balanced Salt Solution and centrifuged at 600 g at 4°C for 28 min. Leydig cells were primarily distributed from fraction 50% to 60%

Hormone measurement

Serum levels of mouse testosterone were measured using a radioimmunoassay kit (Beijing Sinouk Institute of Biological Technology) as previously described (Mason-Garcia et al., 1985).

Assessment of mating efficiency

Males of different genotypes (8-9 weeks) were used for mating efficiency measurement. Each male mouse was caged with two wild-type CD1 female mice (7-8 weeks) and their vaginal plugs were checked every morning. Each male underwent 10-15 cycles of the above measurement with different female mice.

Assessment of fertility

Males of different genotypes (8-9 weeks) were used for the breeding assay. Each male mouse was caged with two wild-type CD1 females (7-8 weeks) and their vaginal plugs were checked every morning. The number of pups in
each cage was counted within a week of birth. Each male underwent four cycles of the above breeding assay with different females.

**Epididymal sperm count assays**

Epididymal sperm count assays were performed as previously described (Jimenez et al., 2010; Kullander and Rausing, 1975). Briefly, the cauda epididymis was dissected from adult mice. Spermatozoa were squeezed from the cauda epididymis and incubated for 30 min at 37°C under 5% CO₂. The medium was then diluted 1:500 and transferred to a hemocytometer for counting. Non-fixed spermatozoa were spread on precoated slides for morphological observation or immunostaining.

**Sperm motility assays**

Sperm motility assays were performed as previously described (Jimenez et al., 2010; Kullander and Rausing, 1975). Briefly, the cauda epididymis was dissected from adult mice and spermatozoa were allowed to exude from incisions of the cauda epididymis for 30 min at 37°C under 5% CO₂. Then, 10 μl of the exudate was placed into a glass cell chamber (Leja Products BV, Nieuw-Vennep, The Netherlands). The chambers were maintained at 37°C on a heated platform, and the spermatozoa were viewed using an Olympus BX51 microscope through a 20× phase objective. Viewing areas on each chamber were imaged using a CCD camera (Olympus). The samples were analyzed using CASA (CEROS v.12, Hamilton Thorne Research) implemented using the Minutipe Sperm Vision Digital Semen Evaluation System (12500/1300, Minitube Group, Tiefenbach, Germany). Various sperm motility parameters were analyzed, including total motility, progressive sperm, average path velocity (VAP), straight line velocity (VSL) and curvilinear velocity (VCL).

**Tissue collection and histological analysis**

Testes and cauda epididymides were dissected immediately following euthanization by cervical dislocation. The tissues were then fixed in 4% paraformaldehyde (PFA; pH 7.5) overnight at 4°C, dehydrated and embedded in paraffin; sections (5 μm) were cut and mounted on glass slides. Following deparaffinization, the slides were stained with Haematoxylin and Eosin (H&E) for histological analysis.

**Transmission electron microscopy**

Adult mouse testes were dissected and fixed with 2.5% (v/v) glutaraldehyde in 0.2 M cacodylate buffer (pH 7.4) overnight. After washing in 0.2 M cacodylate buffer, tissues were cut into pieces of ∼1 mm³ and immersed in 1% OSO₄ for 2 h at 4°C. Then, the samples were dehydrated through a graded ethanol series and embedded in resin (Low Viscosity Embedding Media Spur’s Kit, EMS, 14300). Ultrathin sections were cut on an ultramicrotome, stained with uranyl acetate and lead citrate, and observed using a JEM-1400 transmission electron microscope (JEOL).

**Immunofluorescence**

Testes were immediately embedded in OCT compound (Tissue-Tek) and cut into 8 μm sections using a microtome-cryostat (CM1950, Leica). Sections were fixed in 4% PFA for 10 min and then rinsed in PBS three times (pH 7.4), treated with 0.1% Triton X-100 for 10 min and then rinsed in PBS three times, blocked in 3% bovine serum albumin (BSA) in PBS for 30 min, and then incubated with primary antibody at 4°C overnight. After three rinses in PBS, the sections were incubated with FITC-conjugated secondary antibody at 1:200 for 1 h at 37°C. DAPI (D3571, Life Technologies) was used to label nuclei. Images were captured immediately using an LSM 780/710 microscope (Zeiss). To examine sperm immunofluorescence, the spermatozoa were washed with PBS three times, plated on coated cover slips, fixed, and then stained as described above.

**Immunohistochemistry**

Testes were fixed in 4% PFA at 4°C overnight, stored in 70% ethanol, and embedded in paraffin. Sections (5 μm) were deparaffinized and rehydrated, followed by antigen retrieval in 10 mM sodium citrate buffer (pH 6.0) for 15 min. After blocking in 3% BSA and 10% normal goat serum in PBS for 30 min, the sections were incubated with primary antibody at 4°C overnight and then washed in PBS three times. The sections were then treated with 3% H₂O₂ to eliminate internal peroxidase activity. After washing in PBS three times, the sections were stained with HRP-conjugated secondary antibody for 1 h at 37°C. Finally, the sections were stained with 3,3′-diaminobenzidine (DAB) and Haematoxylin. Images were recorded using a Nikon 80i inverted microscope equipped with a CCD camera (Nikon).

**Immunoprecipitation and western blotting**

Testes extracts were homogenized in lysis buffer [50 mM Tris-HCl pH 7.4, 420 mM NaCl, 0.1% Triton X-100, 10% glycerol, 0.5% Nonidet P-40, protease inhibitors (Roche), 1 mM PMSF, 1 mM DTT, 10 mM nicotinamide, 10 μM trichostatin A (TSA), 100 μM E6-527 (E7034, Sigma)] using a Dounce homogenizer (1234F35, Thomas Scientific). For immunoprecipitation analysis, the lysates were mixed with antibodies at 4°C overnight, followed by the addition of protein A-Sepharose (GE Healthcare, 17-1279-03). The resulting immunocomplexes were then washed four times with lysis buffer and subjected to western blot analysis.

For the western blot analysis, proteins obtained from lysates or immunoprecipitates were separated by SDS-PAGE and electrotransferred to nitrocellulose membranes. The membranes were then incubated in 5% (w/v) BSA or nonfat milk and stained with the appropriate primary and secondary antibodies. The membrane was scanned using an Odyssey infrared imaging system (Li-Cor Biosciences).

**Statistical analysis**

All experiments were repeated at least three times and the results are presented as the mean±s.e.m. The distribution of treatment and control groups was analyzed assuming a normal distribution; a homogeneity test of variance showed that their variance was equal. Differences between the treatment and control groups were analyzed using analysis of variance (ANOVA), and differences were calculated using Tukey’s test. P<0.05 was considered significant.

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**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

W.L., F.G., J.J. and Y.G. conceptualized and designed the experiments; C.L., Z.S., F.G., J.J. and Y.G. performed the experiments; W.L., C.L. and Z.S. wrote and revised the manuscript. All authors discussed the results and commented on the manuscript.

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**Supplementary information**

Supplementary information available online at http://dev.biologists.org/lookup/doi/10.1242/dev.147074.supplemental

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Figure S1

A

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<table>
<thead>
<tr>
<th>Genotype</th>
<th>Mating Efficiency</th>
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<tbody>
<tr>
<td>Sirt1F/F</td>
<td>93 (140)</td>
</tr>
<tr>
<td>Sirt1F/F;SF1-Cre</td>
<td>25 (124)</td>
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Fig. S1. Spermatogenesis was not affected in Sirt1F/F; SF1-Cre male mice. (A) Sirt1 protein levels were dramatically reduced in the Leydig cells of Sirt1F/F; SF1-Cre mice. (B) The mating efficiency was reduced in Sirt1 deficient male mice. (C) Testosterone production was significantly reduced in Sirt1 deficient mice. In Sirt1F/F; SF1-Cre mice (white column), the concentration of serum testosterone was 2.88 ± 0.28 ng/ml, whereas the serum testosterone concentration in Sirt1F/F mice was 0.60 ± 0.30 ng/ml (black column). (D) Testes of 8-week-old Sirt1F/F and Sirt1F/F; SF1-Cre mice. (E) Histology of the seminiferous tubules of Sirt1F/F and Sirt1F/F; SF1-Cre mice. (F) Histology of the cauda epididymis of Sirt1F/F and Sirt1F/F; SF1-Cre mice. (G) Spermatozoa of 8-week-old Sirt1F/F and Sirt1F/F; SF1-Cre mice cauda epididymis. (H) The total number of sperm from the cauda epididymis in Sirt1F/F and Sirt1F/F; SF1-Cre mice. (I) Sperm motility of Sirt1F/F and Sirt1F/F; SF1-Cre mice.
**Fig. S2. Mitotic and meiotic progression of germ cells during spermatogenesis in Sirt1^{F/F} and Sirt1^{F/F}; Tnap-Cre mice.** (A) PLZF immunohistochemistry (IHC) of Sirt1^{F/F} and Sirt1^{F/F}; Tnap-Cre mice testes. (B) WT1 immunofluorescence (IF) of Sirt1^{F/F} and Sirt1^{F/F}; Tnap-Cre mice testes. (C) Sirt1^{F/F} and Sirt1^{F/F}; Tnap-Cre spermatocytes stained for SYCP3 (red) and γH2Axd (green). Leptotene, zygotene and pachytene spermatocytes were stained. (D) Sirt1^{F/F} and Sirt1^{F/F}; Tnap-Cre spermatocytes stained for SYCP3 (red) and RAD51 (green). Leptotene, zygotene and pachytene spermatocytes were stained. (E) Sirt1^{F/F} and Sirt1^{F/F}; Tnap-Cre pachytene spermatocytes stained for SYCP3 (red) and SYCP1 (green). The area bounded by an ellipse indicates the XY body. (F) Sirt1^{F/F} and Sirt1^{F/F}; Tnap-Cre pachytene spermatocytes stained for SYCP3 (red) and MLH1 (green). Merged (top), MLH1 (bottom). (G) Quantitation of the number of MLH1 foci per cell in Sirt1^{F/F} (n=29) and Sirt1^{F/F}; Tnap-Cre (n=29) pachytene spermatocytes.
Fig. S3. Increased numbers of apoptotic germ cells are present in the seminiferous tubules of Sirt1^{F/F}; Tnap-Cre mice. (A) TUNEL assays of testis cross sections from Sirt1^{F/F} and Sirt1^{F/F}; Tnap-Cre mice. (B) The number of apoptotic cells per TUNEL-positive tubule in Sirt1^{F/F} and Sirt1^{F/F}; Tnap-Cre mice.