Germline Deletion of Huntingtin Causes Male Infertility and Arrested Spermiogenesis in Mice

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Abstract

Human Huntingtin (HTT), a Huntington’s disease gene, is highly expressed in the mammalian brain and testis. Simultaneous knockout of mouse Huntingtin (Htt) in brain and testis impairs male fertility, providing evidence for a link between Htt and spermatogenesis; however, the underlying mechanism remains unclear. To understand better the function of Htt in spermatogenesis, we restricted recombination specifically to the germ cells using the Cre/loxP site-specific recombination strategy and found that the mice manifest smaller testes, azoospermia, and complete male infertility. The meiotic chromosome spread experiments showed that the process of meiosis is normal in the absence of Htt. Notably, we found that Htt-deficient round spermatids did not progress beyond step 3 during post-meiotic phase when round spermatids differentiate into mature spermatozoa. Using iTRAQ-based quantitative proteomic assay, we found that knockout of Htt significantly altered the testis protein profile. The differentially expressed proteins exhibited a remarkable enrichment for proteins involved in translation regulation and DNA packaging, suggesting that Htt may play a role in spermatogenesis by regulating translation and DNA packaging in the testis.

Keywords

Huntingtin, spermiogenesis, translation, spermatogenesis, DNA packaging
Introduction

Human Huntingtin gene (HTT) is a disease gene linked to Huntington’s disease (HD), a neurodegenerative disorder characterized by loss of striatal and cortical neurons (Gil and Rego, 2008). Since expansion of a CAG triplet repeat encoding polyglutamine within the HTT gene causes HD, most studies on mouse Huntingtin (Htt) were focused on elucidating the underlying mechanism(s) of how the mutant Htt (mHtt) leads to neuronodegeneration. Little is known about the cellular function of wild-type (WT) Htt. The complete inactivation of Htt in mice causes embryonic death before day 8.5 (Nasir et al., 1995; Zeitlin et al., 1995; Dragatsis et al., 2000), indicating that Htt is essential for embryonic development (White et al., 1997; Auerbach et al., 2001). Notably, the human mutant HTT (mHTT) does not seem to abrogate the developmental functions of HTT, as HD patients only start to manifest symptoms years after birth. Moreover, human mHTT can compensate for the absence of endogenous Htt gene, by rescuing the embryonic lethality of Htt-nullizygous mice for a targeted disruption of the endogenous Htt gene (Leavitt et al., 2001). Htt is a large protein with unknown function that is expressed ubiquitously at low levels during early development and at high levels in the testis and in neurons of the brain (Van Raamsdonk et al., 2007). Within the cell, mammalian Htt is associated with a variety of organelles, including the nucleus, endoplasmic reticulum, Golgi complex, and mitochondrion. This widespread subcellular localization indicates its various functions, including anti-apoptosis, facilitation of vesicular transport, control of BDNF production, neuronal gene transcription and synaptic transmission (Cattaneo et al., 2005). The role of Htt in post-transcriptional regulation has been postulated to link to HD pathogenesis. Htt localizes in P granules, which are centers of mRNA storage, degradation, and small RNA-mediated gene silencing. Consistently, Htt associates with argonaute 2 (Ago2, the catalytic component of the RNA-induced silencing complex) (Savas et al., 2008) and is also involved in RNA transport in cultured cortical neurons (Savas et al., 2010). Recently, both WT and mHtt were found to co-purify with several translation-related proteins, and co-fractionate with ribosomes. Furthermore, Htt overexpression inhibits cap-dependent translation of a
reporter mRNA in an *in vitro* system (Culver et al., 2012).

Interestingly, employing the Cre/*loxP* site-specific recombination strategy to inactivate Htt expression in the mouse forebrain, Ioannis Dragatsis, et al. found that the generated conditional knockout mice display reduced Htt expression in testis and severe spermatogenesis arrest. The mutant seminiferous tubules were disorganized and contained fewer spermatocytes and round spermatids compared with controls. They also observed a corresponding reduction in the number of mature motile sperm in the lumen of the mutant epididymis (Dragatsis et al., 2000). These results hint that Htt may play an essential role during spermatogenesis in testis. However, considering that the Camk2a-cre lines they employed can induce a simultaneous deletion of floxed *Htt* gene in the brain and male germ cells (Choi et al., 2014), it is necessary to generate a germ cells conditional knockout mouse (CKO) model to directly confirm the possibility.

Spermatogenesis is a major function of mammalian testis, covering the process from spermatagonia to mature sperm, which is characterized by three phases, mitosis, meiosis and spermiogenesis. During meiosis, spermatocytes pass through six different stages, distinguished by the morphology of the meiotic chromosomes. While in spermiogenesis, round spermatids pass through 16 stages to differentiate into spermatozoa with specialized organelles for motility and fertilization (Oakberg, 1956). During spermatogenesis, translational regulation plays an important role in the development of spermatocytes and spermatids (Paronetto and Sette, 2010; Nguyen-Chi and Morello, 2011; Idler and Yan, 2012; Kleene, 2013). Many mRNA transcribed in spermatocytes and round spermatids are stored in a translationally inactive state for several days to more than two weeks, before their protein products could be detected in elongating and elongated spermatids. Additionally, translational control directs the onset and the end of the first meiotic phase, with most of the mRNAs in pachytene spermatocytes and round spermatids partially repressed by an unknown mechanism.
In this report we generated the conditional knockout mouse (CKO) with the promoter of stra8 to specifically promote the expression of Cre in the male germ cells. We show that the germ line-specific ablation of \( Htt \) in mouse testes results in a male infertility, with a specific defect in spermiogenesis. In order to study the molecular mechanism of Htt in spermatogenesis, we employed a quantitative proteomic approach based on isobaric tags for relative and absolute quantitation (iTRAQ) to investigate the differentially expressed proteins in testis in the absence of Htt. We found that knockout of \( Htt \) significantly altered the testis protein profile. The differentially expressed proteins were mainly enriched in the pathway involved in translation regulation and DNA packaging, suggesting that male infertility in \( Htt \) CKO mice was partially attributable to the role of Htt in protein synthesis and chromatin remodeling.
Methods and Materials

Mice

Mouse harboring two conditional *Htt* alleles (*Htt*\(^{\text{flox/flox}}\)) was generated as described previously, in which LoxP sequences were inserted 1.3 kb upstream of the *Htt* transcription initiation site and within intron 1 (Dragatsis et al., 2000). The *Stra8-cre* transgenic mice were purchased from the Jackson Laboratory. The breeding of the *Htt*\(^{\text{flox/flox}}\) with *Stra8-cre* mice to generate the *Htt* conditional knockout mice was described in Fig. 1A. Specifically, to achieve specific deletion of *Htt* gene in germ cells, the female mice which were homozygous for *Htt* flox alleles, were crossed with male *Stra8-cre* mice. The heterozygous progenies, with the genotype of *Htt*\(^{\text{flox/+}}\)/*Stra8-cre* were inbred to obtain the female *Htt*\(^{\text{flox/Δ}}\)/*Stra8-cre* mice, which were further crossed with male *Htt*\(^{\text{flox/flox}}\) mice to produce male *Htt*\(^{\text{flox/Δ}}\)/*Stra8-cre* mice, *Htt*\(^{\text{flox/flox}}\)/*Stra8-cre* mice, *Htt*\(^{\text{flox/Δ}}\) mice and *Htt*\(^{\text{flox/flox}}\) mice. The male *Htt*\(^{\text{flox/Δ}}\)/*Stra8-cre* mice and *Htt*\(^{\text{flox/flox}}\)/*Stra8-cre* mice were used as CKO groups, while the male *Htt*\(^{\text{flox/Δ}}\) mice and *Htt*\(^{\text{flox/flox}}\) mice were used as control groups. The PCR genotyping primers for WT, floxed and Cre alleles of *Htt* are listed below: For WT allele, forward 5‘-CGGGCTTTATACCCCTACAGT-3’ and reverse 5‘-AAGCCAAGCAGTGATAGAACACA-3’; for the floxed allele, forward sequence 5‘-CTAAAGCGCATGCTCCAGACTG-3’ and reverse 5‘-AGATCTCTGAGTTATAGGTCAGC-3’; for delta allele (Δ), forward 5‘-CTAAAGCGCATGCTCCAGACTG-3’ and reverse 5‘-CTGGCTGGCCTGACCCGGCT-3’; for Cre allele, forward 5‘-GTGCAAGCTGAACAACAGGA-3’ and reverse 5‘-AGGGACACAGCATTGGAGTC. All the mice were maintained on a C57BL/6; 129/SvEv mixed genetic background. The mice were housed in cages under a 12/12 h light/dark cycle. All the animal procedures were reviewed and approved by the Institute of Zoology, Institutional Animal Care and Use Committee and were conducted according to the committee’s guidelines.
**Fertility and epididymal sperm counts**

For fertility testing, 8-week old *Htt* CKO and control male mice were singly housed with wild-type 129 females. Copulatory plugs were monitored daily, and plugged females were moved to separate cages for monitoring pregnancy. The female would be replaced by a new one if it isn’t pregnant within 2 weeks. The mating process was lasted for 3 months. Viable pups were counted on the first day of life.

Caudal epididymides from each 8-week old mouse were minced with fine forceps in a petri dish with 37°C phosphate-buffered saline [PBS], and assessed under microscope. Sperm was counted with a haemocytometer under a light microscope after fixed in 10% neutral-buffered formalin.

**Histology, immunostaining and TUNEL**

Testis cell preparations were generated from 3, 6 or 8-week-old mice. Usually, one testis was used for immunohistochemistry while the other was processed for surface spreading according to established protocol (Moens et al., 2000). For histology, testes were fixed with Bouin’s fixatives (Polysciences) overnight at 4°C, dehydrated in an ethanol series, and embedded in paraffin wax. Paraffin sections (5 μm) were cut and then stained with hematoxylin-eosin or periodic acid schiff (PAS)-hematoxylin to visualize the acrosome. Stages of seminiferous epithelium cycle and steps of spermatid development were determined as described (Ahmed and de Rooij, 2009).

For immunostaining, paraffin-embedded sections of testis were used for staining Plzf (Millipore, 100105, 1:400, USA), PCNA (Santa Cruz, sc-7907, 1:800, USA), Dazl (AbD Serotec, MCA2336, 1:1000, UK), γH2AX (Millipore, 16-193, 1:1000, USA), Protamine 2 (Prm2) (Santa, sc23104, 1:200, USA), RPL29 (Proteintech, 15799-1-AP, 1:400, USA) and SPG20 (Proteintech, 13791-1-AP, 1:500, USA). After deparaffinization and rehydration, slides were incubated with boiling 0.01 M sodium citrate (pH 6.0) for 10 min to retrieve the antigens before immunostaining. Standard immunostaining procedures were used. For detection of apoptotic cells (TUNEL assays), slides were fixed in 4% (w/v) paraformaldehyde (PFA) in PBS for 15 min at room temperature after deparaffinization and rehydration. TUNEL-positive cells were detected using the In Situ Cell Death Detection Kit, Fluorescein (Roche, Switzerland),
and the sections were counterstained with 0.1% (w/v) 4,6-diamidino-2-phenylindole-dihydrochloride (DAPI) from Sigma-Aldrich. The images were taken using a Nikon TiE microscope and a confocal laser microscope (LSM510, Carl Zeiss, Oberkochen, Germany) with an argon ion laser.

**Germ cell nuclear spreads preparations and immunofluorescent staining**

Nuclear spreads destined for immunostaining were obtained from testes at 6 weeks of age to enrich for meiosis stages and were carried out as described previously (Peters et al., 1997; Cai et al., 2011). Slides were incubated with indicated primary antibodies diluted in PBST (PBS containing 10% goat serum, 3% bovine serum albumin, 0.05% Triton X-100) for about 24 h at 37°C in a humid chamber. Sequentially, slides were incubated with according secondary antibodies for 90 min at 37°C in the dark. The images were taken with a Leica fluorescence microscope. The primary antibodies include mouse γH2AX (Millipore, 16-193, 1:1000, USA), rabbit SYCP1 (abcam, ab15090, 1:200), rabbit SYCP3 (abcam, ab15093, 1:200, UK), goat SYCP3 (abcam, sc-20845, 1:200, UK), mouse MLH1 (BD Pharmingen, 551091, 1:50, USA), human CREST (Immunovision, HSM0101, 1:800, USA). The secondary antibodies include Alexa 488-labeled donkey anti mouse, Alexa 488-labeled donkey anti rabbit, Alexa 568-labeled donkey anti-goat (Molecular Probes, Eugene, OR), Dylight 405-labeled donkey anti human (Abbkine, CA, USA). The stages of prophase I meiosis were categorized based on the distribution of the synaptonemal complex proteins SYCP3 and SYCP1 together with the kinetics of CREST. The experiment was repeated at least three times. The immunofluorescence images were taken with a Nikon/Perkin-Elmer spinning disc confocal microscopy system.

**Tissue lysis, protein extraction, protein digestion and iTRAQ labeling**

Testes from Htt CKO mice and the controls (n=6) at 5-week-old were harvested and fast frozen in liquid nitrogen. Samples were ground and precipitated with prechilled TGA and acetone for several times until it turned to be white. The prepared testis tissues were dissolved in the lysis buffer (8 M urea, 30 mM HEPES, 1 mM PMSF, 2 mM EDTA, 10 mM DTT) and sonicated. The homogenate was centrifuged at 20,000 g for 30 min. For the iTRAQ experiments, the testis proteins were reduced
with 10 mM DTT at 56°C for 1 h, and alkylated with 55 mM iodoacetamide (IAM) at room temperature for 1 h in the dark. The treated proteins were precipitated in acetone at -20°C for 3 h. After centrifugation at 20,000 g for 30 min, the protein pellet was resuspended and ultrasonicated in pre-chilled 50% TEAB buffer with 0.1% SDS for 3 min. The proteins were finally harvested after centrifugation at 20,000 g for 30 min.

Equal amounts of proteins from each testis in each group were mixed and grouped into two pools. Then the digestion was performed by adding sequencing-grade trypsin (Promega, Madison, WI) at 37°C overnight. The tryptic peptides were desalted and labeled with iTRAQ 4 plex reagents (Applied Biosystem) according to the manufacturer’s protocol. The testis peptides from control groups and knockout groups were labeled with iTRAQ reagent 113, 114, 118 and 119 respectively.

**Two-dimensional chromatography to separate the iTRAQ labeled peptides followed by identification through TripleTOF MS**

The labeled peptides were pooled and dried by vacuum centrifugation. SCX chromatography was performed with a Shimadzu HPLC system connected to a Phenomenex Luna SCX column (25 cm x 4.6 mm, 5 mm, 100A). Equal amounts of the iTRAQ labeled peptides from all the four samples were mixed, and the pH of the peptide mixture was adjusted to 3 and was loaded onto the SCX column which was equilibrated with buffer A (10 mM KH2PO4 and 25% acetonitrile, pH 3.0). Then the peptide mixture was eluted with buffer A for 10 mins, a gradient program with the elution buffer B (10 mM KH2PO4, 25% acetonitrile and 2 M KCl, pH 3), 0–5% within 36 min, 5–30% within 56 min, 30-50% within 61 min, 50-60% within 66 min and 50–100% within 81 min. The flow rate of HPLC was 1 mL/min. The peptide elution was monitored at 214 nm. A total of 30 eluted fractions were collected and desalted with a Strata X C18 column (Phenomenex), and vacuum-dried. TripleTOF MS/MS (Q Enactive, Thermo Fisher Scientific, USA) with a NanoLC system was used for peptide identification.

**RNA extraction and real-time PCR**

Testes were harvested in TRIzol Reagent (1 ml Reagent/50-100 mg testis; Invitrogen, USA). RNA was extracted according to the manufacturer’s instructions and quantified
by measurement of the optical density at 260 nm. Reverse transcription was carried out following standard procedures using random primers and Quant Reverse Transcriptase (TIANGEN, China). Real-time PCR was done with an ABI Prism 7000 device (Applied) using UltraSYBR Mixture (CWBIO, China) and the primers given in the supplementary Table S4. Three samples from different mice for each group were used. Quantifications were made in triplicate for each sample from individual testes. For analysis of the mRNA expression, the comparative Ct method ($\Delta\Delta$CT) with Gapdh RNA as the internal control (Livak and Schmittgen, 2001).

**Data processing**

The data files of each fraction were combined together to perform searching against Mus musculus protein database (uniprot2014_mus) using PD software (Protein Discover 1.3, thermo). The searching parameters were set as, 1) missed cleavage, carboxymethylation of cysteine as fixed modification, oxidation of methionine, N-terminal iTRAQ 8 plex mass addition lysine and iTRAQ 8 plex mass addition on tyrosine, and N-terminal pyroglutamyl as variable modifications. The software of Mascot (version 2.3.0, http://www.Matrixscience.com/search_form_select.html) was employed to calculate iTRAQ quantitation with the Mascot files. In order to achieve high quality MS signal for quantification, the peptides served as the quantitative evaluation should meet two criteria, 1) a target false discovery rate (FDR) threshold is set≤1% at the peptide level, 2) The minimum required peptide length was set to 6 amino acids, and 3) a protein contains at least one tag-labeled unique peptides.

Gene Ontology (GO) annotation of the identified proteins was done by searching DAVID Web site (http://david.abcc.ncifcrf.gov). GO terms enrichment analysis of the differentially expressed proteins was done with Cytoscape and its plugin EnrichmentMap (version 2.0.1), which is a java-based tool used to visualize the results of gene-set enrichment as a network. To better understand these differentially expressed proteins in relation to published literature, interactions among these proteins regarding function and disease, and biological pathway were determined using IPA (www.ingenuity.com). Uniprot accession was used as the identifier and Ingenuity knowledge gene database was used as a reference for the pathway analysis.
**Statistical analysis**

Chi-squared analysis was carried out to test differences in meiotic stage distribution in mutant and WT spermatocytes. A nonparametric or independent sample T-test was used to determine significant differences of testis weight, the number of MLH1 foci per spermatocyte and the number of apoptotic cells per tubule between the control and CKO groups. For all tests, statistical significance was taken as $P \leq 0.05$. At least three mice per genotype were used for each experiment.
Results

Specific deletion of Htt in the male germ cells results in severe infertility and impairs spermatogenesis

To determine the role of Htt in spermatogenesis and male fertility, the Cre/loxP system was utilized to inactivate Htt in male germ cells. We generated a germ cell-specific Htt conditional knockout mice model by mating Htt\textsuperscript{flox/flox} mice harboring two loxP sites inserted 1.3 kb upstream of the Htt transcription initiation site and within intron 1 with Stra8-cre mice (Figure 1A) (Dragatsis et al., 2000). The Stra8 promoter drives Cre expression in germ cells starting from primitive spermatogonia at P5, then, from P10 onwards, becomes prominent in preleptotene spermatocytes, and is not detected in other tissues examined (Vernet et al., 2006). When Stra8-cre transgenic males are bred with female mice containing a Htt floxed sequence, Cre-mediated recombination will result in deletion of the Htt, specifically during these stages of spermatogenesis. Since loss of one allele of Htt in testes has no apparent effects on spermatogenesis and fertility, Htt\textsuperscript{flox/flox} and Htt\textsuperscript{Δ/Δ} were together used as controls. Htt\textsuperscript{flox/flox}/Stra8-cre mice and Htt\textsuperscript{Δ/Δ}/Stra8-cre mice were used as conditional knockout group (CKO) (Figure 1A).

To test the fertility of Htt deficient males, we mated CKO male mice with WT females and monitored the breeding capacity within 3 months of mating. The percentage of females plugged was similar between the controls and Htt CKO male mice, suggesting that inactivation of Htt expression in germ cells has no effect on mating capacity. However, no Htt CKO mice were able to father litters, while the average litter number for the control male mice was 8±1.4. Meanwhile, epididymal sperm counts of CKO animals yielded no detectable sperm, indicating that the CKO mice were azoospermic (Table 1).

To understand the underlying mechanism for the male infertility in Htt-deficient mice, we harvested the testes and performed histological staining. We found that depletion of Htt results in smaller testes in adult mice. The testis weights were significantly reduced in Htt CKO mice as early as postnatal week 6, suggesting that Htt deficiency might impair the first wave of spermatogenesis (Fig. 1B-C). The testes from Htt CKO
mice display an obvious abnormality in histological analysis, with many tubules filling with large vacuolated spaces and lacking late-stage germ cells. Additionally, a more disordered arrangement of germ cells in seminiferous tubules was noticed in Htt CKO mice, with scattered multinucleated germ cells and Eosinophilic materials in the epithelial layer and lumen of CKO testes. In contrast, the testes from the control littermates contain germ cells of all developmental stages: spermatogonia, meiotic spermatocytes, postmeiotic round spermatids, and elongating spermatids (Fig.1 D-G). Consistently, Prm2 immunofluorescence assay revealed that the signal of Prm2 was significantly reduced in Htt CKO mice compared to the control mice (Fig. S1). Only degenerating spermatozoa was detected in Htt KO epididymides when we examined the histology of the adult epididymides (Fig.1 H-I). Taken together, these data indicate that Htt is required for male fertility and spermatogenesis.

**Loss of Htt does not impair the proliferation and differentiation of spermatogonia**

Since the number of germ cells within CKO seminiferous tubules was substantially reduced, we were wondering whether it was attributable to the reduction of spermatogonia amount. Analyzing the staining of PLZF, whose expression is restricted to undifferentiated spermatogonia and required for stem cell self-renewal (Costoya et al., 2004), we could not detect any significant difference in the proportion of PLZF-positive spermatogonia between WT and CKO testes (Fig. 2A, B). Meanwhile, both control and CKO tubules exhibited a basal layer of PCNA (a mitotic proliferation marker)-positive cells, suggesting that mitotic proliferation is unaffected in CKO tubules (Fig. 2C, D). After mitosis, spermatogonia will differentiate to enter into meiotic prophase beginning at preleptotene spermatocytes. We want to know whether the onset of meiosis is affected in Htt CKO mice. The expression of Dazl1, which is expressed in the cytoplasm of spermatocytes (Lifschitz-Mercer et al., 2002) was examined in CKO mice. We could not detect any abnormality in spermatocytes of 3-week-old CKO testes, indicating that spermatogonia can differentiate into meiotic spermatocytes in the absence of Htt (Fig. 2E, F).
**Loss of Htt does not affect DNA double strand break formation, homologous chromosome synapsis and crossover in meiosis**

During meiosis, a large number of DNA double strand breaks (DSBs) are produced. We thus monitored the signal of the phosphorylated form of histone variant H2AX (γH2AX), which is a well-defined DSB marker. As reported previously, γH2AX was expressed in all preleptotene to zygotene spermatocytes, whereas in pachytene spermatocyte γH2AX only presented in the sex vesicle (Hamer et al., 2003) (Fig. 3E). No significant differences in γH2AX localization were detected in zygotene and pachytene spermatocytes from WT and Htt CKO mice at 3-week-old (Fig. 3A, B). However, several seminiferous tubules contained more than one layer of zygotene or pachytene spermatocytes in 6-week-old Htt CKO mice (Fig. 3C-F).

To check whether Htt inactivation affects key events of meiotic prophase, we performed meiotic chromosome spreads from WT and Htt CKO mice. No significant differences in γH2AX localization in pachytene and diplotene spermatocytes could be observed (Fig. 4A). To determine whether deleting Htt in the CKO testis results in meiotic defects or spermatocytes depletion, we examined the dynamics of chromosome synapsis and recombination using antibodies directed against synaptonemal complex (SC) proteins-SYCP1 and SYCP3, components of the meiotic nodules and chromatin markers. During spermatogenesis, SYCP3 proteins begin to assemble along each sister-chromatid pair at leptonema to form the axial element, which represents the precursor of the SC and subsequently, becomes a major component of the SC lateral elements during zygonema. SYCP1, which appears at zygonema, is a major component of the SC central element, and is used as a marker of fully synapsed chromosome segments. In control and CKO pachytene spermatocytes, SYCP1 and SYCP3 decorated the axes of all 19 completely synapsed autosomes. Based on the dynamic signaling of SYCP1 and SYCP3, no apparent difference was seen in the formation and dissolution of the synaptonemal complex from Htt CKO mice compared with those of control mice (Fig. 4B). Meanwhile, we did not detect any significant change in the proportion of leptotene, zygotene, pachytene and diplotene between control and Htt CKO mice at 6-week-old (Fig. 4C). These data
suggest that Htt deficiency does not impair the formation and disassembly of SC.
We further probed meiotic chromosomes with an antibody directed against the mismatch repair protein MLH1, a marker of chiasmata. We found that the mid-pachytene chromosomes from WT and CKO mice displayed one or two MLH1 foci per synapsed homologue. The average number of MLH1 foci in every pachytene exhibited no significant difference between control and CKO pachytenes (Fig. 4D). Therefore, no defect in prophase I of meiosis could be detected in Htt CKO mice (indicated by the hollow arrow in Fig. 5I, J).

**Spermiogenesis is interrupted at the Golgi phase upon Htt deficiency**

Since Htt CKO mice lack elongated spermatid in testis (Fig. 1D-I), we next examined whether spermiogenesis during which round spermatids differentiate into mature spermatozoa be affected upon Htt deficiency. The process of spermiogenesis lasts about 13 days in mice and is divided into 16 developmental steps based on the morphology of the nucleus and acrosome, as well as associations between germ cells at different stages of spermatogenesis (Oakberg, 1956; L. D. Russell, 1990). It usually covers four phases: Golgi (steps 1-3), cap (steps 4-7), acrosomal (steps 8-12), and maturation (steps 13-16) (Leblond and Clermont, 1952; Oakberg, 1956). To pinpoint the exact arrest point in Htt CKO mice, we stained the puberal testis sections with the acrosome marker equatorin, sperm acrosome formation associated factor (AFAF) (Li et al., 2006). As shown in Fig. 5, the acrosome was detected as a purple-magenta signal on the nuclear envelope of round spermatid as well as along one edge of the elongated spermatid head in the control mice (Fig. 5 C, E, G and I). Intriguingly, in Htt CKO mice, round spermatids were present in stage I-Ⅲ tubules, but by stage Ⅱ-Ⅲ not all spermatids had the normal acrosome granule indicative of step 3 at the Golgi phase, and many had two pre-acrosomal vesicles that failed to fuse (indicated with arrowhead in Fig. 5D). Additionally, the acrosome of spermatids showed a range of malformations inside seminiferous tubules after stage III. Most spermatids had earlier step 2 acrosomes or two pre-acrosomal vesicles, and only a few had developed to step 7, but no more steps further (indicated with arrowhead in Fig. 5F, H, J). We also observed that spermiogenesis was blocked at the step 3 by periodic acid Schiff’s
staining (PAS) (Fig. 5K, L). These results indicated that most of the round spermatids do not develop beyond step 3 of spermiogenesis in Htt CKO mice.

As indicated above, multinucleated giant cells were present in Htt CKO testis, which is indicative of apoptosis (Nantel et al., 1996). We also examined whether depletion of Htt provokes germ cells apoptosis through TUNEL staining. We found the amount of apoptotic germ cells was similar between WT and Htt CKO mice at 3-weeks of age. Interestingly, Htt CKO testes manifest a significant increase in the amount of apoptotic germ cells compared to the controls at 6-weeks of age (Fig. 6), most abundantly at multinucleated round spermatids (Fig. 6B). These results suggested that germ cell development in the Htt CKO mice was impaired at the early round spermatid stage.

**Differentially expressed protein identification through iTRAQ analysis**

To elucidate the underlying mechanism of how Htt affects spermiogenesis, we compared the global protein expression profiles between the control and Htt KO testes from 5-week-old mice using iTRAQ-based quantitative proteomic approach. To ensure reliable identification, the following conditions were set to ensure reliable analysis: 1) FDR<1%; 2) only proteins identified with at least one unique peptide were accepted. As a result, the MS/MS analysis identified a total of 328,455 mass spectra. After data filtering to exclude low-scoring spectra, 74,233 unique spectra matched to special peptides were obtained. Searching using Mascot 2.3.0 identified a total of 20,598 peptides from 3,481 proteins (Table S1). Considering that iTRAQ quantification usually underestimates the “real” fold change between samples (Karp et al., 2010; Zhang et al., 2014), expression differences greater than 1.2-fold with a p value<0.05 were applied to classify protein of interests and potential significance for further investigations. Results showed that 119 proteins (42 up-regulated and 77 down-regulated) exhibited significant differential expression in Htt deficient testes, which included the significantly downregulated Htt (Table S1).

**Functional annotation of differentially expressed proteins in the absence of Htt**

We used the web tool provided by the DAVID (http://david.abcc.ncifcrf.gov/) to search for functional annotation terms (FATs) that are enriched in the above
differentially expressed proteins (DEPs) (Table S2 and Fig. 7A-C). GO categories were ranked by their corrected (Corr) p-value, which indicated the degree of overrepresentation in the significantly DEPs compared with the complete Mus musculus proteome. We found that the DEPs were enriched in biological processes such as “translation” and “DNA packaging”, as well as male fertility (Table S2 and Fig. 7A). A large number of proteins involved in translation were differentially expressed upon Htt inactivation, including 18 ribosomal proteins (RL28, RS16, RS8, RL36A, RS9, RL24, RL4, RL27, RL13, RL34, RL26, RL7, RL15, RL18A, RL29, RL18, RL6, RL14) and a tRNA ligase protein (SYAM). Consistently, “Ribosome” and “structural constituent of ribosome” were the most significantly enriched cellular components and molecular functions respectively (Table S2 and Fig. 7B, C). Additionally, Htt deficiency activated the expression of 6 histone proteins (H11, H4, H1T, H15, H14, H12) while repressed the other 4 proteins (EP400, SMC2, PRM3, KDM3B) related to DNA packaging (Table S2). In line with the enrichment of “Chromosome remodeling” in the biological process, “Chromosome” was significantly enriched in cellular components (Table S2 and Fig. 7B).

To understand how Htt deletion affects spermatogenesis, we performed comprehensive bioinformatic analysis using Ingenuity pathway (IPA) to assess integrated function and pathways of these DEPs. The most significant functions and diseases related to these proteins analyzed in IPA belonged to a network consisting 27 proteins involved in sperm disorder, apoptosis of hematopoietic progenitor cells, cell cycle progression, the infection of kidney cell lines, the infection of epithelial cell lines, the infection of embryonic cell lines, fertility and azoospermia (Fig. 7D). In particular, the markedly down-expressed proteins upon Htt depletion, such as ODF1, Smcp, ACRBP, CLIP1, EPS8, TYRO3 and BAK1, have been reported to be directly linked to spermatogenesis and male fertility (Baba et al., 1994; Oldereid et al., 2001; Nayernia et al., 2002; Akhmanova et al., 2005; Xiong et al., 2008; Cheng et al., 2011; Yang et al., 2012). Thus, knockout of Htt might impair spermatogenesis through altering the expression of these proteins. We also annotated the mouse phenotype related to male reproduction of DEPs from the MGI (Mouse Genome Informatics)
We next investigated post-transcriptional regulation of gene expression in Htt CKO testes by comparing changes at protein levels with changes at transcript levels of these 27 DEPs (Fig. S2A, B). We found that the mRNA levels of 8 down-regulated proteins in Htt CKO were significantly lower than those of their counterparts in WT controls and the mRNA levels of 6 down-regulated proteins in Htt CKO were not significantly changed (Fig. S2A). Among the 11 up-regulated proteins, the mRNA levels of two proteins were significantly lower in Htt CKO than in control, while the mRNA levels of 8 proteins did not change significantly (Fig. S2B). To further verify our iTRAQ data, we selected two up-regulated proteins for western blot analysis. As shown in Fig. S2C, the expression of SPG20 and RPL29 proteins was significantly up-regulated in Htt CKO testes compared to the controls (Fig. S2C).

IPA results showed that the 119 DEPs were mainly enriched in Eif2 signaling and Granzyme A signaling pathways (Fig. 7E), which are related to translation and DNA packaging respectively. Based on the analysis of DEPs with Molecule activity predictor (MAP) in IPA, the elongation of translation was predicted to be hyperactivated in Htt knockout testis (Fig. 7F). Eif2 signaling was predicted to be activated based on the upregulation of 17 ribosomal proteins. This translation activation is likely induced by knockout of Htt, as WT Htt functions with argonaute 2 (Ago2, the catalytic component of the RNA induced silencing complex) in a post-transcriptional gene silencing pathway and the overexpression of it strongly inhibited translation in HeLa cell extracts (Savas et al., 2008; Culver et al., 2012).

Taken together, the protein expression profiles between control and Htt CKO testes were compared and those differentially expressed genes were applied to DAVID and IPA. The enrichment of gene clusters related to translation and chromosome remodeling was identified. IPA results showed that translation was predicted to be hyperactivated in Htt CKO testis, which is consistent with previous studies (Savas et al., 2008; Culver et al., 2012). Hence, there might be a link between the abnormal translation and male infertility in absence of Htt.
Discussion

Although a number of genes are known to be involved in spermatogenesis, only a few possess clean-cut arrest phenotypes indicative of their role in the global regulation of key spermatogenic steps. In this study, we have presented evidence that *Htt* is such a spermatogenic gene for the first time. Here, the phenotype of male sterility, azoospermia, and spermiogenesis arrest in *Htt* CKO mouse reveals the essential role of Htt in spermiogenesis.

A previous work from Dr. Scott Zeitlin’s group had detected a reduction of Htt expression in testis and male fertility following Cre-mediated recombination using a Camk2a-cre transgene (Dragatsis et al., 2000). Given that the Camk2a-cre lines they used can induce a simultaneous deletion of floxed *Htt* gene in the brain and male germ cells (Choi et al., 2014), it is hard to exclude the possibility that the sterility they observed could be due to an impaired hypothalamic-pituitary-testis axis caused by the Cre-mediated recombination in the brain. In this study, we generated a CKO mouse which had conditional knockout of *Htt* specifically in the male germ cells. The *Htt* CKO mouse showed complete sterility, loss of germ cell and spermatogenesis arrest at early spermatid in seminiferous tubules. These results indicated that Htt has a critical function in spermatogenesis. It is known that HD patients and animal model have specific testicular pathology with reduced numbers of germ cells and abnormal seminiferous tubule morphology (Van Raamsdonk et al., 2007). We speculate that the testicular degeneration in HD patients and mouse models might attribute to the partial loss of Htt normal function.

To further elucidate the molecular mechanisms underlying spermatogenesis defects in *Htt*-deleted testes, we undertook a comparative proteomics analysis and discovered 119 differentially expressed proteins (Table S4). Eleven up-regulated proteins and sixteen down-regulated proteins were previously shown to be essential for spermatogenesis (Fig. 7D). The knockout of *Htt* might impair spermatogenesis through disturbing the expression of these spermatogenesis regulators. In addition, some other differentially expressed proteins might also contribute to the *Htt* deletion-induced male fertility.
Htt localizes in P granules and is involved in RNA storage in germ cell (Culver et al., 2012). It also associates with Ago2, the catalytic component of the RNA-induced silencing complex, with involvement in RNA transport in cultured cortical neurons (Savas et al., 2008; Savas et al., 2010). Recently, both WT and mHtt were found to co-purify with several translation-related proteins and co-fractionate with ribosomes. Furthermore, the overexpression of Htt inhibits cap-dependent translation of a reporter mRNA in an *in vitro* system (Culver et al., 2012). During spermiogenesis, translational control is essential since de novo protein production is needed for the terminal steps of germ cell differentiation which occur after transcription has ended. In our study, Gene Ontology analysis of those *Htt* deletion-induced DEPs revealed a statistically significant enrichment for proteins involved in translation among multiple categories (Fig. 7). The elongation of translation was predicted to be hyperactivated in *Htt* knockout testis through further analysis of DEPs in IPA (Fig. 7 F). Thus we speculate that Htt may function as a regulator of post-transcription required for spermiogenesis. This possibility is suggested not only from the translation association but also from the existence of three RNA binding proteins, RBM28, RBMX2 and RBM19, and three E3 ligases, RNF167, LTN1, MYCB2 involved in proteosome degradation identified among our differentially expressed proteins (Table S3). This proteomic analysis will be a valuable resource to help characterize important proteins involved in spermatogenesis and in revealing their mechanisms of action.

In addition to arrest at the round spermatid stage, some tubules of 6-week-old *Htt* CKO mice show more than one layer of zygotene or pachytene spermatocytes (Fig. 3C-F). Given that no other appreciable meiotic defects could be detected in *Htt* CKO mice, we speculate that this abnormality might be an indirect effect of prolonged spermiogenetic arrest. In support of this interpretation, TUNEL analysis showed a striking increase of apoptotic germ cells in *Htt* CKO mice, limited to round spermatids. Similar to nonspecific defects, apoptosis, for example, is frequently seen in spermatid-arrested mutants (Martianov et al., 2001; Zhang et al., 2001; Deng and Lin, 2002).

In our study, knockout of *Htt* in male germ cells results in infertility and
spermatogenesis arrest at round spermatid stage. A range of proteins regulated by Htt was identified through comparative proteomics. These Htt regulated proteins are involved in translation and DNA packaging. Thus, Htt could be important for regulating the translation of crucial genes and DNA packaging at specific times during spermatogenesis. Mice deficient in Htt expression protein could serve as a model system for study male reproduction and fertility control. A better understanding of mouse Htt will shed light on the role of HTT in human infertility and HD disease.
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Conflict of interest

The authors declare no conflict of interest.
References


Table 1. Male fertility data from *Htt* CKO mice and control mice at 8 weeks age

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>Plugs</th>
<th>Fertility</th>
<th>##Litter size</th>
<th>#Sperm Count</th>
<th>#Sperm Motility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctrl</td>
<td>3</td>
<td>100%</td>
<td>100%</td>
<td>8.0±1.4</td>
<td>2.0<em>10^7±1.2</em>10^6</td>
<td>Yes/moving well</td>
</tr>
<tr>
<td>CKO</td>
<td>3</td>
<td>100%</td>
<td>0</td>
<td>0</td>
<td>No sperms</td>
<td>No sperms</td>
</tr>
</tbody>
</table>

## The mean of litter size from all the female mice (± SD). # Count and Motility of isolated sperms from epididymides was assessed under microscope. Sperm count is the mean of 3 mice per group (±SD).
Fig. 1 The generation and phenotype of male Htt CKO mice

(A) The breeding scheme for the Htt conditionally knockout mice. Htt^flox/flox mice
were mated with Stra8-cre mice to generate mice as conditional knockout group (CKO) and Control group (Ctrl). The genotypes of last row were used as Ctrl and CKO group in this study. The genetic background of the parental mouse strain is indicated within the block diagram. (B) Photographs of testes from 5-week and 5-month old Htt CKO and control mice from Stra8-cre and Htt-Flox crosses, respectively. The scale bar=1 cm. (C) Weights of testes from control mice and CKO mice (3 to 32-week-old). (D-E) Histological analysis of seminiferous tubules in 6-week-old mice at low magnifications. (F-G) Histological analysis of seminiferous tubules in 6-week-old mice at high magnifications. (H-I) Hematoxylin and Eosin-stained sections of epididymides from 8-week-old mice. Scale bar: 100 μm (D, E); 50 μm (F, G); 100 μm (H, I).
Fig. 2 Loss of Htt shows normal number and proliferation of spermatogonia

Sections from 8-week-old control and CKO mouse testes were stained with the anti-Plzf (A-B), anti-PCNA (C-D). Sections from 3-week-old control and CKO mouse testes were stained with the anti-DAZL (E-F). Scale bar: 50 μm.
Fig. 3 Loss of Htt does not affect the production of DNA double strand breaks during meiosis

Sections from 3 and 6-week-old control and CKO mouse testes were stained with the anti-γH2AX. Pachytene spermatocytes were identified by the γH2AX positive sex body (cells inside rectangle of E). Zygote spermatocytes were marked by the strong γH2AX signaling over the whole nucleus (cells inside ellipse of E). Scale bar: 100 μm (A-D); 50 μm (E, F).
Fig. 4 Loss of \( Htt \) does not affect homologous chromosome synapsis and crossover in meiosis.
Immunofluorescence staining was performed on spread chromosomes from 6-week-old male primary spermatocytes, and cells were imaged by laser confocal microscopy. (A) The γH2AX signal doesn’t manifest appreciable differences between the control and CKO testes. Testicular cells were spread and then stained with anti-γH2AX (green), anti-SYCP3 (red) and anti-Crest (blue) antibody. SYCP3 accumulates on chromosomes beginning in leptotene and is present along their full length during pachytene. γH2AX accumulates from leptotene to zygotene and be restricted to the sex chromosomes at pachytene and diplotene stages. (B) The assembly and disassembly of synaptonemal complex are not affected by knockout of Htt. Testicular cells were spread and then stained with anti-SYCP1 (green) and anti-SYCP3 antibody (red). The developmental stages of meiotic prophase based on SYCP1 and SYCP3 kinetics were indicated in each panel. (C) The ratio of each meiotic phase cells in the control and CKO testes. (D) Testicular cells were spread and then stained with anti-MLH1 (green) and anti-SYCP3 antibody (red). The mean numbers of MLH1 foci per pachytene are from 3 mice (± SD).
Fig. 5 Spermiogenesis was arrested at step 3 spermatid in CKO testes

(A-J) Immunohistochemistry for the acrosome marker AFAF in 6-week-testis sections.
Representative control and conditional knockout sections showing stage I (A and B),
stage II-III (C and D), stage IV (E and F), stage VII (G and H) and stage XI-XII (I and J) seminiferous tubules. Black arrows indicate spermatids with normal acrosome. Black arrowheads indicate round spermatid with abnormal acrosome (C-J). Hollow arrowheads indicate metaphase spermatocytes (I and J). (K-L) PAS/H-stained cross sections of seminiferous from 6-week-old control and Htt CKO mice. The black arrowhead indicates spermatid at about step 3 (L). Scale bar: 100 μm (D, E); 25 μm (K, L).
Fig. 6 TUNEL staining of testes sections from the control and Htt CKO mice

TUNEL staining images of testes sections from 6-week-old control and CKO mice (A, B). Cells in the square frame in (B) indicate the characteristic multinucleated giant cells and cells with abnormal nuclear structure. Most multinucleate round spermatids and some isolated round spermatid were apoptotic, while no increased apoptosis was detected in other type of cells. The number of TUNEL-positive cells per tubule cross section in control and Htt CKO mice aged 3 weeks or 6 weeks (C). There is a significant difference in the number of apoptotic cells per tubule between the control and Htt CKO mice at 6-weeks of age (n=3, unpaired t test; P<0.05). Scale bar: 50 μm.
Fig. 7 Functional annotations of proteins involving spermiogenesis impairment induced by knockout of *Htt* revealed the enrichment for translation and DNA
packaging proteins

(A-C) Overview of GO Slim generic distribution of differentially expressed proteins in mouse testis induced by knockout of Htt using Enrichment (version 2.44), a plugin of Cytoscape. (A) Biological process analysis; (B) Cellular component analysis; (C) Molecular function analysis. Each annotation is color-coded according to its corrected (Corr) p-value (see color bar), which indicates degree of overrepresentation in data compared to complete Mus musculus proteome. (D) Diseases and disorders analyses of differentially expressed proteins in IPA. (E) The most significant canonical pathways in which the differentially expressed genes were enriched. 119 differentially expressed genes were applied to Ingenuity Pathway analysis (IPA) software, and the most significant canonical pathways were shown. (F) Eif2 signaling was predicted to be activated in Htt-deficient testis by IPA. The prediction was based on the expression of associated proteins in comparison of proteomics data. The red 40S and 60S were detected to be activated in Htt-deficient testes. Then the Molecule Activity Predictor (MAP) propagated the effects to neighboring molecules. The blue color indicates predicted inhibition (or decreased activity) and the orange color represents predicted activation (or increased activity). The intensity of color indicates the confidence of the prediction. If confidence is high, nodes will be darker orange or blue; as confidence decreases, the shade of orange/blue becomes paler. The arrowheads indicate the expected effect from the literature, and the edge color signifies the effect the upstream molecule has on the downstream molecule, given the activity of those molecules. The prediction legend of F is the same to D. p < 0.005, FDR q < 0.1, overlap cutoff > 0.5.