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***Haprin*-deficient spermatozoa are incapable of *in vitro* fertilization**

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Abstract

Haprin (TRIM36) is a ubiquitin-protein ligase that mediates ubiquitination and subsequent proteasomal degradation of target proteins. It is expressed in the testes in both mice and humans and is thought to be involved in spermiogenesis, the acrosome reaction and fertilization. However, the functional role of Haprin is poorly

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understood. The aim of this study was to investigate the physiological role of Haprin in fertility. Homozygous *haprin*-deficient mice were generated and these mice, and their spermatozoa, were analyzed to detect morphological and fertility-related abnormalities. In these models, normal spermatogenesis was observed but sperm quality was reduced with *haprin*-deficient mice having poorer sperm morphology and motility than wild-type mice. Interestingly, *haprin*-deficient mice showed normal *in vivo* fertility but could not fertilize oocytes under standard *in vitro* fertilization (IVF) conditions. In conclusion, this study demonstrated that Haprin deficiency causes morphological abnormalities in spermatozoa, indicating that Haprin is involved in spermiogenesis.

Graphical Abstract

Haprin (TRIM36) is a ubiquitin-protein ligase that mediates ubiquitination and subsequent proteasomal degradation of target proteins. The aim of this study was to investigate the physiological role of haprin in fertility. Homozygous *haprin*-deficient mice were generated and these mice, and their spermatozoa, were analyzed to detect morphological and fertility-related abnormalities. In these models, normal spermatogenesis was observed but sperm quality was reduced with *haprin*-deficient mice having poorer sperm morphology and motility than wild-type mice.

Interestingly, haprin-deficient mice showed normal *in vivo* fertility but could not fertilize oocytes under standard *in vitro* fertilization (IVF) conditions.

Keywords: TRIM36, RBCC protein, spermatogenesis, acrosome

Introduction

TRIM36 is a haploid germ cell-specific RBCC containing protein (Haprin)

(Kitamura et al., 2003). The tripartite (TRIM) motif has three zinc binding domains

(RING, B-box-type 1 and B-box-type 2) and a coiled-coil region (also referred to

as a RBCC motif). The TRIM motif mediates protein-protein interactions and is

implicated in several multi-protein complexes (Saurin et al., 1996). The TRIM motif

is located at the N-terminus of the protein and associated with either one or two

C-terminal domains which differentiate the TRIM superfamily (~70 members in the

human genome) (James et al., 2007; Nisole et al., 2005; Reymond et al., 2001;

Sardiello et al., 2008; Short & Cox, 2006). Haprin (TRIM36) belongs to a sub-family

of proteins that are associated with the microtubule cytoskeleton (Meroni, 2012).

Notable member of this sub-family include TRIM9, TRIM46, MIDI1, MIDI2 and

TNL (Meroni, 2012). TRIM9 is mainly expressed in the cerebral cortex, where it is

localized in neurons. It regulates synaptic vesicle exocytosis by modulating its

binding partner SNAP25 (Tanji et al., 2010) which is an essential component of the

SNARE complex that mediates vesicle fusion. Expression of just the coiled-coil domain of TRIM9, without the RING domain, has been shown to inhibit Ca^{2+} -dependent exocytosis in PC12 cells (Cahill et al., 2006). The acrosome reaction in spermatozoa shares many structural and functional features with exocytic events at the synaptic vesicle.

The acrosome is a sperm-specific structure formed from the Golgi apparatus during spermiogenesis. The acrosome is a discrete membrane-bound structure sitting between the plasma and nuclear membranes on the sperm head. The SNARE (soluble N-ethylmaleimide sensitive factor attachment protein receptor) complex also regulates the acrosome reaction in spermatozoa following the interaction of the sperm protein

GalT G with the zona pellucida glycoprotein ZP3. The acrosome reaction involves multiple fusions between the outer acrosomal membrane and the plasma membrane. This exocytotic event releases numerous proteins contained in the acrosome, such as hyaluronidase and acrosin, and exposes the inner acrosomal membrane for subsequent binding with the plasma membrane of the egg (Yanagimachi, 1994).

In humans, *Haprin* transcripts are expressed in the testis, brain, prostate, kidney, heart and lung (Balint et al., 2004). The Haprin protein is expressed after the completion of meiosis in late-stage spermatids and mature spermatozoa (Kitamura et al., 2003) and the RBCC motif has been used as a marker of haploid germ cells (Geijsen et al., 2004). Haprin is localized to the acrosomal region of mature

spermatozoa in acrosome-intact cells and is not observed in acrosome reacted cells

(Kitamura et al., 2003). Inhibition experiments using antibodies that target the RING

finger domain of Haprin inhibited the acrosome reaction in permeabilized

spermatozoa, suggesting that protein interactions mediated by the TRIM motif of

Haprin may be involved in the acrosomal exocytosis process (Kitamura et al., 2003).

Finally, a immunofluorescence study also showed that Haprin interacts with the

kinetochore protein centromere protein-H (CENP-H), colocalizes with α -tubulin

(Miyajima et al., 2009) and its overexpression decelerates the cell cycle and attenuates

cell growth (Miyajima et al., 2009). Transient expression of TRIM36 in HeLa and

LN229 cells has also been shown to cause microtubule disruption, disorganized

spindles, loosely arranged chromosomes, abnormal cytokinesis, reduced cell

proliferation and increased apoptosis (Singh et al., 2017). These findings suggest, in a

reproductive context, TRIM 36 may be involved in microtubule organization during

spermatogenesis.

Many genes specifically expressed in haploid germ cells have been taken from a

subtracted cDNA library for cloning and further characterization (Fujii et al., 2002;

Iguchi et al., 1999; Tanaka et al., 1994). To test the hypothesis that Haprin is involved

in spermiogenesis or the acrosome reaction we generated homozygous

haprin-deficient mice from this cDNA library (Kitamura et al., 2003).

Materials and methods

Breeding of mice

All animal experiments conformed to the Guide for the Care and Use of Laboratory

Animals and were approved by the Ethics Review Committee and the Institutional

Committee of Laboratory Animal Experimentation of Nagasaki International

University and the Ethics Review Committee for Animal Experimental of Osaka

University School of Medicine. C57BL/6 mice were purchased from CLEA Japan,

Inc. (Tokyo, Japan). The *haprin* gene-targeted mice were mated with C57BL/6 or

enhanced green fluorescent protein (EGFP) transgenic mice (Nakanishi et al., 1999).

Positive male offspring from this cross were used for experiments. Mice were

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maintained at 22°C under a 12-h light-dark cycle (light on from 7:00 to 19:00) and provided with food and water ad libitum in the animal experimental facility. Mice were sacrificed by an intraperitoneal injection of pentobarbital (200 mg/kg) or cervical dislocation.

Construction of the targeting vector and the production of haprin targeted mice

The *haprin* targeting construct was created by the amplification of a homologous 3.5-kb 5'-arm and 12.0-kb 3'-arm using C57BL/6 genomic DNA as the template (Fig. 1a). The primers used to amplify the arms were designed to incorporate synthetic enzyme sites at both ends. The amplified fragments were digested to create sticky ends and the clone was sequentially ligated into the poly-linker cloning sites on either

side of the neomycin resistance gene in the targeting vector. The targeting vector contained the neomycin resistance gene and a thymidine kinase gene that were both under the control of the phosphoglycerate kinase promoter. The vector plasmid was linearized by NotI digestion prior to electroporation into C57BL/6 derived embryonic stem (ES) cells. The G418 ganciclovir-resistant clones were screened by Southern blot analysis and two were found to have undergone homologous recombination correctly. The targeted cell lines were injected into C57BL/6 blastocysts, resulting in the birth of male chimeric mice. Highly chimeric males were mated with C57BL/6 wild-type females to generate F1 offspring, half of which were heterozygous for the targeted allele. Heterozygous F1 males were then crossed with C57BL/6 females to

obtain heterozygous F2 animals. The heterozygous F2 animals were bred to produce homozygous mutants and to check for Mendelian inheritance.

Genotyping

Genomic DNA was extracted from the tails of the mice using standard procedures.

Southern blotting was performed on SacI digested DNA using a 5' external probe by standard procedures. The expected wild-type band is 8.7 kb and the targeted band is 7.7 kb.

The mice were genotyped by the polymerase chain reaction (PCR) using two sets of primers as follows: one set of primers (5'-CCTCAGAAGAACTCGTCAAG-3' and 5'-ATCTGGACGAAGAGCATCAG-3') to amplify the Neo gene and one set of

primers (5'- GTGCAAGGAGCTGTTTACCCACCC-3' and

5'-TGTCGATTTTATCCATGCTAGGGG-3') for exon 2 of *haprin*. The expression of

Haprin was examined by northern blotting analysis according to the manufacturer's

instructions by PerfectHyb (Toyobo, Osaka, Japan) using a reported *haprin* probe

(Kitamura et al., 2003). Western blotting was performed according to a previously

described protocol (Kitamura et al., 2003).

Observation of haprin-deficient spermatozoa in the testes and epididymis

For morphological observation, testes were fixed in Bouin's solution, embedded in

paraffin and sectioned at a thickness of 8 µm. Deparaffinized sections were stained

with hematoxylin and eosin. Spermatozoa from the cauda epididymis were cultured in

TYH medium (119 mM NaCl, 4.8 mM KCl, 1.7 mM CaCl₂, 1.2 mM KH₂PO₄, 1.0

mM MgSO₄, 25 mM NaHCO₃, 5.6 mM glucose, 0.5 mM sodium pyruvate, 4 mg/ml

BSA, pH 7.4), spotted and dried onto glass slides, and fixed with 70% methanol at –

20°C for 5 min.

Observation of the acrosome reaction in haprin-deficient spermatozoa

For examination of the acrosome reaction, EGFP transgenic mice expressing EGFP in

the acrosome were mated to *haprin* mutant mice (Nakanishi et al., 1999).

Spermatozoa expressing the acrosin/EGFP transgene were analyzed by

fluorescence-activated cell sorting (FACS) analysis using a FACSCalibur flow

cytometer (Becton Dickinson Co. Ltd.). Viability was determined by counter-staining

the spermatozoa with 15 μ M propidium iodide (PI; Invitrogen). A 515-545 nm band path filter and a 650 nm band path filter were used for GFP and PI, respectively.

In vitro fertilization assays

Female C57BL/6 mice were superovulated by intraperitoneal injections of 5 international units (IU) of pregnant mare serum gonadotropin, followed 48 h later by the injection of 5 IU of human chorionic gonadotropin (hCG). The follicles were recovered 24 h after hCG injection. Spermatozoa were isolated from the cauda epididymis of each male, capacitated in TYH medium at 37°C for 1 h, added to the follicles, and incubated for 6 h at 37°C in 5% CO₂ covered with mineral oil. After 24 h, two-cell stage embryos were counted (Nakanishi et al., 1999).

Computer-assisted sperm analysis

Spermatozoa in HTF media (LifeGlobal Group, Sofia, Bulgaria) with 4 mg/ml BSA

were transferred to Standard Count 2 chamber slides (100 μ M; Leja, GN

Nieuw-Vennep, The Netherlands) for analysis of sperm movement using an

HTM-IVOS Ver.10 (Hamilton Thorne Research, Beverly, MA, USA). Spermatozoa

were analyzed after incubation periods of 2, 3 and 5 h at 37°C. At least 100

spermatozoa were counted per sample. Parameters examined were total motility,

progressive motility, average path velocity (VAP), straight-line velocity (VSL),

curvilinear velocity (VCL), amplitude of lateral head displacement (ALH), beat cross

frequency (BCF), straightness ($STR = VSL/VAP \times 100$), and linearity ($LIN =$

VSL/VCL $\times$ 100) (Hirano et al., 2003). VAP, VSL, STR and LIN are indicators of

sperm progression, whereas VCL, ALH, and BCF are indicators of sperm vigor. STR

and LIN are also used to describe the sperm swimming pattern.

Statistical analyses

Data are expressed as means $\pm$ standard deviation. Means were compared between treatment groups using Student's *t*-test; significant differences were determined at the level of $P < 0.05$.

Results

Generation of haprin-deficient mice

Mice that were homozygous for an inactivating mutation of the *haprin* gene were

generated to investigate the physiological role of Haprin. Gene trapping was used to

generate ES cell clones that were heterozygous for the *haprin* mutation (Fig. 1a).

Targeted ES cells were injected into blastocysts and implanted in pseudopregnant mice to raise chimeric mice. Construction of the trapping vector and the production and detection of mutant mice by Southern blot analysis and PCR are shown in Figure 1b, c. Northern blot analysis showed that *haprin* mRNA was not detectable in homozygous mutant testes (Fig. 1d). The antiserum recognized 46-kDa Haprin in the testes of normal adult mice, but no positive signals were detected for Haprin in homozygous *haprin* mutant testes (Fig. 1e). These results indicate that homozygous *haprin* mutant mice express neither the transcripts nor the translation products of *haprin*.

Fertility rates and body weight among the adult mutant mice

The proportion of wild-type, heterozygous and homozygous offspring was not significantly different from the expected Mendelian pattern of inheritance. Body weight (Table 1), growth rates of the newborn pups and various organ weights including testes and seminal vesicles of adult mice were not significantly different between *haprin*-mutant homozygous, heterozygous and wild-type mice (data not shown). No abnormal development and breeding of *haprin* mutant mice were observed (data not shown).

Microscopic observation of the testicular sections did not show any significant differences among homozygous, heterozygous and wild-type mice with

normal spermatogenetic function in mice with the homozygous *haprin* mutation observed (Fig. 2a). There was also a similar number of spermatozoa recovered from the cauda epididymides of homozygous and heterozygous mutant males (Table 2). However, light microscopic observation of sperm sections showed that almost all spermatozoa from *haprin*-deficient males had morphological abnormalities such as kinks in their tails and/or abnormal head shapes (Fig. 2b).

Observation of the acrosome reaction in haprin-deficient spermatozoa

Given the localization of Haprin to the acrosome, we examined the effect of *haprin* deficiency on the acrosome reaction. We used standard culture conditions for mouse *in vitro* fertilization (IVF) using spermatozoa from C57/BL6 mice. The percentage of

spermatozoa who had undergone the acrosome reaction at each tested time point (1, 1.5 and 4 h) did not differ in *haprin*-mutant homozygous, heterozygous and wild-type mice (Table 3).

The analysis of sperm motility in haprin-deficient mice

The motility of the *haprin*-deficient spermatozoa was analyzed in detail using the computer-assisted semen analysis (CASA) system (Hamilton Thorne, Beverly, MA).

The *haprin*-deficient spermatozoa had significantly lower total and progressive motility compared with spermatozoa from wild-type littermates (Table 2, Fig. 3).

Haprin-deficient spermatozoa also showed reduced velocity (VAP, VSL, VCL) and

reduced complexity in their motility patterns (ALH decreased) compared with the control after 3 hours (Table 2, Fig. 3).

Spermatozoa from the haprin-deficient mice was fertile in vivo but could not fertilize oocytes in vitro.

We performed IVF experiments to examine whether spermatozoa from *haprin*-deficient mice could fertilize oocytes. Twenty-four hours after the introduction of spermatozoa almost all eggs had developed to the 2-cell stage in wild-type mice whereas no 2-cell embryos were observed in *haprin*-deficient mice (Fig. 4).

Discussion

During spermiogenesis, haploid germ cells undergo a remarkable transformation that includes nuclear condensation, flagellum development and acrosome biogenesis (Tanaka & Baba, 2005). Many genes specifically expressed in

haploid germ cells have been cloned and characterized to better understand their contribution to sperm function and fertility (Fujii et al., 2002; Iguchi et al., 1999; Tanaka et al., 1994). In this study, sperm production, quality and fertility of *haprin*-deficient mouse models were assessed to examine the physiological role of Haprin in these reproductive processes. In these mouse models, sperm quality was reduced compared with their wild-type counterparts. *Haprin*-deficient spermatozoa showed lower total and progressive motility and less complex motility patterns (reduced velocity, reduced head amplitude) than wild-type controls (Table 2) and a greater number of morphological abnormalities. The reason for reduced head amplitude may be due to abnormal morphology of motile spermatozoa. However,

time-series analysis of the occurrence of the acrosome reaction in *haprin*-deficient spermatozoa showed no significant difference to that of spermatozoa from wild-type mice (Table 3). This result is somewhat surprising because of i) its putative function in acrosomal exocytosis due to its structural similarities to the vesicular fusion protein TRIM9 and ii) previous studies showing that Haprin is localized to the acrosomal region of mature spermatozoa, and that blocking of the RING finger domain of Haprin inhibited the acrosome reaction in permeabilized spermatozoa (Kitamura et al., 2003). The normal acrosome reaction observed for *haprin*-deficient spermatozoa may be explained by hyperactivation of these spermatozoa.

Aside from poor sperm quality *haprin*-deficient mice displayed minimal physical differences compared with wild-type mice. No differences in body weights, growth and development of the newborn pups or organ weight (e.g. testes, seminal vesicles) were noted and microscopic observation of the teste sections did not show any significant differences in spermatogenetic function (Table 1 and Fig. 2a). There was also a similar number of spermatozoa recovered from the cauda epididymides of homozygous and heterozygous mutant males (Table 3). Most importantly, when males were placed with females for a period of at least 3 months, all of the *haprin*-deficient mice were fertile, and showed similar litter sizes to wild-type mice (Table 1). Similar results were not found when the fertility of *haprin*-deficient spermatozoa was

assessed *in vitro* (Fig. 4). Almost all of the oocytes developed to the 2-cell stage in

wild-type mice ($95 \pm 1.7\%$) whereas no 2-cell embryos were observed in

haprin-deficient mice. The reason why *haprin*-deficient can fertilize *in vivo*, when

given multiple months to mate, but not *in vitro*, under a shorter fertilization window

of opportunity, is unknown. These results suggests that the factors inhibiting

fertilization *in vitro* are compensable *in vivo*; potentially by components in the female

tract that may compensate for the absence of active Haprin *in vivo* (Yamashita et al.,

2008). As the occurrence of the acrosome reaction in *haprin*-deficient mice is similar

to that of wild-type mice under IVF conditions it is likely that IVF failure is not

because the acrosome reaction does not occur normally. Collectively, the results show

that Haprin is not required for normal acrosome function but its absence does reduce sperm quality, potentially because of impaired spermiogenesis. These changes inhibit *in vitro* fertilization but not *in vivo* fertility. Further investigation of potential compensable substances that allow *haprin*-deficient mice to fertilize under IVF conditions is warranted to better understand the role of Haprin in fertility and how IVF procedures can better mimic *in vivo* fertility.

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Authors' contributions

YA designed the study and wrote the initial draft of the manuscript. HT contributed to analysis and interpretation of data and assisted in the preparation of the manuscript.

All other authors have contributed to data collection and interpretation, and critically reviewed the manuscript. All authors approved the final version of the manuscript,

and agree to be accountable for all aspects of the work in ensuring that questions

related to the accuracy or integrity of any part of the work are appropriately

investigated and resolved.

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Disclosures

Conflict of interest: None of the authors declare competing financial interests.

Animal Rights: The protocol for the research was approved by the Institutional

Review Board of the Nagasaki International University, Japan. All institutional and

national guidelines for the care and use of laboratory animals were followed.

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Figures

Figure 1 Screening strategy to identify clones that are targeted correctly at the 5' end of *haprin* in the targeting vector. Primary screening was performed on *Sac*I-digested DNA using a 5' external probe. The expected wild-type band was 13.6 kb and the targeted band was 22 kb. The *haprin*-deleted mouse was created by homologous recombination in embryonic stem (ES) cells (a). The mice were genotyped by the polymerase chain reaction using two primers sets for *haprin* exon 2 and the neomycin resistance gene (b). Transcription and translation of haprin were absent in mice homozygous for disrupted *haprin* (c, d).

Figure 2 Observation of *haprin*-deficient spermatozoa. No histological differences

were observed compared with wild-type mice (a). Some morphological abnormalities

were observed in almost all *haprin*-deficient sperm (b)–(h). Sperm counts from

epididymides were normal compared with wild type. Each magnified sperm in panel

(b) are shown in (c)–(h). Arrows indicate subtle morphological abnormalities in

sperm. Wild indicates the sperm head from a wild type mouse.

Figure 3 Computer-assisted semen analysis (CASA) of *haprin*-deficient spermatozoa at the end of the incubation period. The percentage of motility and amplitude of lateral head displacement (ALH) decreased significantly after 3 h. No change in the beat cross frequency (BCF) and linearity (LIN) was observed.

Figure 4 Sperm samples were collected from the cauda epididymides of each male, capacitated in TYH medium at 37°C for 1 h, and then introduced into oocytes with cumulus cells. The inseminated oocytes (~20) were placed in 0.3 mL of TYH droplets, covered with mineral oil and incubated at 37°C in 5% CO₂. After 24 h, 2-cell embryos were counted.

Table 1 Fertility rates and body weight among the adult mutant mice.

Genotype		+/+	+/-	-/-
	Fertility (no. of fertile males/no. of males)	10/10	10/10	10/10
Male	Litter size (avg. no. of newborn pups)	6.9 ± 2.0	6.8 ± 1.9	6.6 ± 1.9
	Body weight of adults (g)	29.2 ± 2.2	28.7 ± 2.2	29.2 ± 2.1

Fertility (no. of fertile males/no. of females)		10/10	10/10	10/10
Female	Litter size (avg. no. of newborn pups)	6.6 ± 1.8	6.3 ± 0.5	6.5 ± 1.1
Body weight of adults (g)		25.1 ±	24.9 ±	25.2 ± 2.2
		2.2	2.3	

All entries in the table are means ± SD; Mice are 12 weeks old.

Table 2 Computer aided semen analysis of *haprin*-deficient spermatozoa at the end of a 3-h incubation period.

Characteristics	Wild-type	<i>Haprin</i> -deficient
CASA estimates		
Concentration (10 ⁶ /mL)	89.1 ± 32.6	75.3 ± 40.1
Motility (%)	84.0 ± 6.1	60.6 ± 9.1*
Rapid (%)	68.3 ± 1.5	41.0 ± 5.3*

Sperm motion variables

VCL ($\mu\text{m/s}$)	151.8 ± 6.4	$87.9 \pm 10.7^*$
VSL ($\mu\text{m/s}$)	52.2 ± 5.1	$30.2 \pm 6.0^*$
VAP ($\mu\text{m/s}$)	82.3 ± 2.7	$47.2 \pm 7.7^*$
ALH (μm)	8.5 ± 0.3	$6.7 \pm 0.6^{**}$
BCF (Hz)	24.7 ± 1.6	27.0 ± 0.6
STR (VSL/VAP)	58.7 ± 4.1	58.8 ± 2.5

LIN (VSL/VCL)	33.7 ± 3.2	32.3 ± 1.9
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Entries represent the mean ± SD.

P* < 0.01, *P* < 0.05.

Table 3 The percent rates of the acrosome reaction.

Genotype	1 h	1.5 h	4 h
+/+	25 ± 3.5	33 ± 2.2	67 ± 8.7
+/-	25 ± 2.9	30 ± 2.4	67 ± 7.4

—/—

23 ± 2.6

33 ± 2.6

60 ± 6.6

Values are mean $\pm$ SD; $n = 3$ assays.

Propidium iodide-negative spermatozoa were measured by flow cytometry and fluorescence microscopy.

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