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Role of FYN Kinase in Spermatogenesis: Defects Characteristic of *Fyn*-Null Sperm in Mice[1]

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ABSTRACT

FYN kinase is highly expressed in the testis and has been implicated in testis and sperm function, yet specific roles for this kinase in testis somatic and germ cells have not been defined. The purpose of the present investigation was to identify aspects of spermatogenesis, spermiation, or sperm fertilizing capacity that required FYN for normal reproductive function. Matings between *Fyn*-null males and wild-type females resulted in normal litter sizes, despite the fact that *Fyn*-null males exhibited reduced epididymal size and sperm count. Morphological analysis revealed a high frequency of abnormal sperm morphology among *Fyn*-null sperm, and artificial insemination competition studies demonstrated that *Fyn*-null sperm possessed reduced fertilizing capacity. *Fyn*-null sperm exhibited nearly normal motility during capacitation in vitro but reduced ability to undergo the acrosome reaction and fertilize oocytes. The typical pattern of capacitation-induced protein tyrosine phosphorylation was slightly modified in *Fyn*-null sperm, with reduced abundance of several minor phosphoproteins. These findings are consistent with a model in which FYN kinase plays an important role in proper shaping of the head and acrosome within the testis and possibly an additional role in the sperm acrosome reaction, events required for development of full fertilizing capacity in sperm.

capacitation, gamete biology, gametogenesis, kinase, spermatogenesis

INTRODUCTION

The *Src* protein tyrosine kinase (PTK)-encoding gene family includes nine members that share extensive structural homology, yet with a highly variable N-terminal domain conferring unique character to each family member. The *Src* gene family kinases have been associated with multiple aspects of sperm and testis function [1–3], and an understanding of their specific actions in the testis and in mature sperm may present unique opportunities for contraceptive development and provide insight into the cause of human male infertility. The *Src* gene family kinases exhibit distinct expression profiles in the testis, and the four (*Src*, *Yes*, *Fyn*, and *Hck*) gene family members have been implicated in various aspects of testis or sperm function. In the testis, both the SRC and the structurally similar YES kinases are associated with basal and apical ectoplasmic specializations (ES junctions) of Sertoli cells and are important for junctional dynamics [4–6]. FYN kinase is known to be expressed in two isoforms in the testis: a catalytically active 59-kDa form present in Sertoli cells and spermatozoa and a 22-kDa truncated form (tr-FYN) found in Sertoli cells and spermatids [7]. FYN also is associated with ES junctions in association with actin filaments bundled within these junctions, and structural analysis of the *Fyn*-null testis has revealed significant ultrastructural defects in apical ES junctions [8], suggesting that FYN may play a role in maintaining junction stability. In addition, expression and localization of tr-FYN in round and elongating spermatids led to the hypothesis that tr-FYN may function in sperm head morphogenesis and acrosomal development [7]. While the above-described functional roles of FYN are supported by circumstantial evidence, no functional evidence has confirmed either role. To date, functional studies of the role of *Src* gene family kinases during spermatogenesis, sperm capacitation, and acrosome reaction have relied on the use of kinase inhibitors [4, 6, 9–11]. While pharmacological evidence supports a role for the *Src* gene family PTKs in ES junctions, the limited specificity of these inhibitors does not allow one to distinguish between the specific roles of SRC, YES, or FYN kinases. Single-gene knockout mouse models provide a more specific approach to defining the functions of individual *Src* gene family members, and, while an earlier study demonstrated that *Fyn*-null mice exhibit slower testis development and ES junctional defects [8], the fact that simple mating trials failed to detect infertility in *Fyn* and other *Src* family knockout males has brought into question whether this kinase family plays an important role in spermatogenesis or in sperm function. The objective of the present study was to apply more definitive tests of sperm function to determine whether sperm from *Fyn*-null males exhibited normal fertilizing capacity in competitive artificial insemination and in vitro fertilization (IVF) trials. Results showed that while *Fyn*-null males are technically fertile, a significant fraction of the sperm they produce exhibits morphological and functional defects, resulting in decreased fertilizing capacity.

MATERIALS AND METHODS

Animal Source and Care

CF1 female mice, 6–7 wk old, were obtained from Harlan Sprague Dawley, Inc. (Frederick, MD). *Fyn*-null mice (B6;129S7-*Fyn*^{tm1Sor}/*J* strain) [12] and the wild-type (WT) mice used as controls (B6;129SF2/J strain) were obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained as described previously [13]. Housing standards and policies, as well as standard operating procedures regarding handling of mice, were approved by the Institutional Animal Care and Use Committee at the University of Kansas Medical Center

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and were in accordance with the National Research Council's *Guide for the Care and Use of Laboratory Animals*.

Male Breeding Test

Four *Fyn*-null or WT males, 8–15 wk old, were each caged with two 8-wk-old CF1 females for a period of 14 days. Females were then euthanized on day 14 after breeding to determine whether they were pregnant and to quantify the number of fetuses produced.

Sperm Competition In Vivo

Sperm competition assays were performed via artificial insemination to evaluate the ability of sperm from *Fyn*-null males to negotiate the female reproductive tract and fertilize eggs in a way that ruled out other physical and behavioral factors typical of this strain of mice. Cauda epididymal sperm from WT and *Fyn*-null males (approximately 10 wk old) were released into 0.4 ml of 0.85% sodium chloride by squeezing tubules with a 26-gauge needle. Sperm were incubated in air at 25°C for 10 min and then counted in a hemocytometer. For artificial insemination, a 1:1 mixture of *Fyn*-null and WT sperm was prepared at a final concentration of 100×10^6 sperm cells/ml. CF1 females were induced to ovulate by intraperitoneal injection of 5 IU of pregnant mare serum gonadotropin (Sigma-Aldrich, St. Louis, MO), followed 48 h later by 5 IU of human chorionic gonadotropin (Sigma-Aldrich, St. Louis, MO). Artificial insemination was carried out using 50 μl of sperm mixture per female as described previously [14, 15]. Sperm mixtures from three pairs of *Fyn*-null and WT males were used to artificially inseminate a total of 19 WT females, 7 of which became pregnant. At day 15 after artificial insemination, all females were euthanized to determine whether they were pregnant. Any fetuses present were collected for genotyping, which was performed as suggested by the Jackson Laboratory by using the sense primer 5′-CTT GGG TGG AGA GGC TAT TC-3′ (oIMR0013) and antisense primer 5′-AGG TGA GAT GAC AGG AGA TC-3′ (oIMR0014) to amplify a 280-bp fragment of the neomycin gene from *Fyn*-null sperm-derived fetus samples. As an internal control, the sense primer 5′-TGT GTG TCC TAC TGT GAA ACC C-3′ (oIMR0172) and antisense primer 5′-GCA TCC TTG ACC TAG TTT CAC-3′ (oIMR0173) were used to generate a 103-bp fragment in exon 7 of the *Yes1* gene (National Center for Biotechnical Information accession no. NM_009535.2) from all samples. Fetuses containing the neomycin insert were considered derived from *Fyn*-null sperm and were classified as *Fyn*-null heterozygotes ($Fyn^{+/-}$).

Scanning Electronic Microscopy

Cauda epididymal sperm were collected in Tyrode solution [16] without bovine serum albumin (BSA), incubated at 37°C for 10 min, and then diluted and loaded onto a coverslip coated with 0.01% poly-L-lysine (Sigma-Aldrich, St. Louis, MO) and incubated at room temperature for 10 min to allow sperm to attach to the coverslips. After coverslips were washed to remove unbound sperm, 1 ml of potassium simplex optimized medium with amino acids (Millipore Corp., Phillipsburg, NJ) was applied to each coverslip, followed by 1 ml of ice-cold 2% glutaraldehyde in 0.15 M cacodylate buffer (pH7.4). Samples were coded and scored blindly as described previously [17] and then examined using scanning electron microscopy (SEM) as previously described [18].

In Vitro Fertilization

Cauda epididymal sperm from adult *Fyn*-null or WT mice were collected, capacitated, and used to perform IVF of WT oocytes as described previously [18]. Standard (1×10^5 sperm/ml–3×10^5 sperm/ml) or higher (8-fold) concentrations of *Fyn*-null sperm were used during IVF to assess the functionality of sperm. Degrees of sperm-zona binding and -zona penetration and rates of fertilization were determined by confocal fluorescence microscopy examination of oocytes recovered from IVF. Meiotic status was monitored by staining chromatin with ethidium homodimer to label DNA. The position of bound sperm heads relative to the egg surface was monitored by staining the eggs with rhodamine-phalloidin (Molecular Probes-Invitrogen, Eugene, OR) to stain the cortical actin layer.

Computer-Assisted Sperm Analysis

Cauda epididymal sperm released from *Fyn*-null or WT males were suspended in 0.5 ml of noncapacitating medium (Tyrode solution without $NaHCO_3$ and BSA) or capacitating medium (Tyrode solution containing 25 mM Na_2HCO_3 and 4 mg/ml BSA [Sigma-Aldrich]). Samples were incubated for 90 min at 37°C in a humidified atmosphere of 5% CO_2 in air and labeled with 156 nM SYTO 21 live-cell nucleic acid stain (Molecular Probes, Inc., Eugene, OR). To prevent sperm from binding to chambered slides (Leja Products B.V., The Netherlands), 0.2 mg/ml polyvinyl alcohol (average molecular weight, 30 000–70 000 [Sigma-Aldrich]) was added to the samples. Sperm (2 μl) were loaded into the slide chambers and analyzed using the Sperm Vision computer-assisted sperm analysis (CASA) system operated by Sperm Vision PRISM version 3.5 software (Minitube of America, Inc.,Verona, WI). The percentages of sperm (average, 1700 spermatozoa per sample analyzed) displaying total, progressive, local, and hyperactive motility were recorded.

Acrosome Reaction

The capacity of cauda epididymal sperm to undergo spontaneous acrosome reaction was compared with their response to calcium ionophore-induced (A23187; Sigma-Aldrich) acrosome reaction by analysis with a FACSCalibur cytometer (Becton Dickinson) as described previously [19]. Briefly, sperm isolated in Tyrode solution containing 4 mg/ml BSA were incubated for 90 min at 37°C in a humidified atmosphere of 5% CO_2 in the presence or absence of 10 μM A23187 to induce acrosome reaction. After incubation, sperm were collected by centrifugation and labeled with 1 μg/ml peanut agglutinin-fluorescein isothiocyanate (PNA-FITC; Sigma-Aldrich) and then with PBS containing 8 μg/ml propidium iodide (PI; Molecular Probes). Percentages of PI-negative but PNA-FITC-positive sperm were recorded as having undergone the acrosome reaction.

Flow Cytometric Analysis of Spermatogenesis

Cytometric analysis was performed as previously described [20]. Briefly, testes from 8- to 9-wk-old *Fyn*-null and WT males ($n = 5$) were sequentially digested, first with 1 mg/ml collagenase (type IA; Sigma-Aldrich) and then with 1× trypsin-EDTA solution (Sigma-Aldrich, St. Louis, MO), to dissociate individual cells. After cells were fixed in 70% ethanol, they were stained with 50 μg/ml PI in the presence of 100 μg/ml RNase A (DNase and protease-free; Fermentas, Inc., Glen Burnie, MD) for 30 min at 37°C. Samples were filtered through a 70-μm cell strainer (BD Biosciences Discovery Labware, Bedford, MA), and cell subpopulations in 50 000 events per sample according to DNA content were analyzed with a FACSCalibur cytometer (Becton Dickinson). Quantitation of testicular cells in condensed haploid (the population of elongated spermatids with reduced uptake of stain due to condensation of nuclear DNA), haploid, diploid, S phase, and tetraploid categories was recorded.

Western Blotting

Testis lysates were prepared from *Fyn*-null and WT males, 8–10 wk old, by homogenization in 2.0 ml of RIPA buffer (containing 10 mM Tris-HCl, pH7.5, 158 mM NaCl, 1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 5 mM EDTA, 1mM Na_3VO_4, 0.1 mM diisopropylfluorophosphate, and a protease inhibitor cocktail [Roche Diagnostics, Indianapolis, IN]). Homogenates were incubated on ice for 30 min and then centrifuged at $25\,000 \times g$ for 30 min at 4°C, and supernatants were collected for Western blot analysis. Antibodies used to probe the blots included rabbit anti-FYN (1:1000 dilution; FYN3; Santa Cruz Biotechnology, Inc.), mouse anti-PLZF (1:200 dilution; Calbiochem), rat anti-GCNA1 (1:50 dilution; a kind gift from Prof. George C. Enders, University of Kansas Medical Center), rabbit anti-DDX4 (1 μg/ml; Abcam, Cambridge, MA), mouse antiphosphotyrosine (anti-P-Tyr; 1:1000 dilution; clone 4G10; Millipore, Billerica, MA), mouse anti-beta tubulin (1:1000 dilution; Sigma-Aldrich), and mouse anti-GAPDH (1:2000 dilution; Abcam).

Statistical Analysis

Statistical variance between *Fyn*-null and WT samples was analyzed by *t*-test performed with SigmaStat software (Jandel Scientific, San Rafael, CA) to determine whether sample values were significantly different (a P value of ≤ 0.05 was considered significant).

RESULTS

Effect of Fyn Knockout on the Testis

The B6;129S7-*Fyn*^{tm1Sor}/*J* strain is fertile, allowing continuation of the homozygous *Fyn*-null mouse line. However, because a detailed analysis of *Fyn*-null oocytes revealed significant defects in oocyte quality that provided insight into the function of this kinase in the female gamete [13], it was important to identify potential defects in gametogenesis and

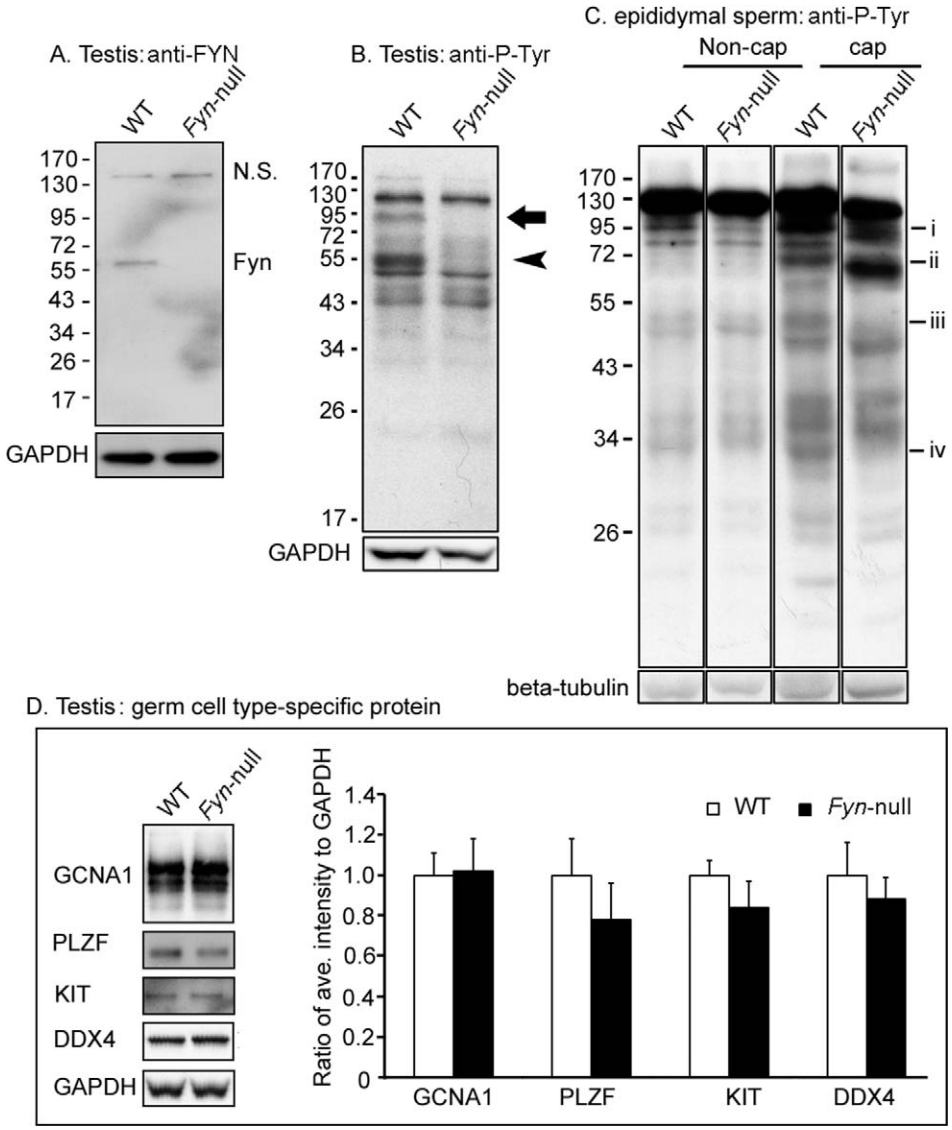

FIG. 1. Western blot analysis of *Fyn*-null testis and spermatozoa is shown. Testes were homogenized in RIPA buffer, and sperm were lysed in Laemmli sample buffer. After centrifugation, soluble protein was analyzed by Western blotting as described in *Materials and Methods*. **A**) The expression levels of FYN kinase in the testis of WT and *Fyn*-null mice were detected with an anti-FYN antibody (NS, nonspecific band). **B**) Patterns of P-Tyr-containing proteins in WT and *Fyn*-null testes were detected with anti-P-Tyr antibody. Anti-GAPDH was used as a loading control. An arrow (close to 95 kDa) and arrowhead (approximately 55 kDa) indicate P-Tyr-containing proteins in the WT but not in the *Fyn*-null sample. **C**) Patterns of P-Tyr-containing proteins in WT and *Fyn*-null sperm were detected in noncapacitating (Non-cap) and capacitating (cap) sperm by using an anti-P-Tyr antibody. P-Tyr-containing proteins at ~95 kDa (i), ~72 kDa (ii), ~50 kDa (iii), or ~30 kDa (iv) exhibited reduced antibody binding in *Fyn*-null capacitating sperm relative to those of WT. **D**) Analysis of the expression levels of different germ cell type-specific proteins in the testis is shown. Testis proteins from WT and *Fyn*-null males were analyzed by Western blotting with antibodies to GCNA1 (representing germ cells, except for elongating spermatids), PLZF (spermatogonial stem cells or undifferentiated spermatogonia), KIT and DDX4 (differentiating/differentiated germ cells), and GAPDH (a loading control). The bar graph represents the ratios between average intensity of identified protein band and that of GAPDH and WT control. Values represent means ± SD for six testes in a *Fyn*-null or WT group. Comparison by *t*-test indicated that individual expression levels of the four analyzed proteins of *Fyn*-null mice were not significantly different from those of WT mice.

gamete quality in the *Fyn*-null male as well. Western blot analysis confirmed that testicular cells from the *Fyn*-null mice lacked detectable levels of the 59-kDa FYN protein (Fig. 1A), as has been previously demonstrated in other tissues [12]. To characterize the reproductive organs involved in gamete production in *Fyn*-null males, we analyzed testis and epididymal weight as well as epididymal sperm counts from 7- to 8-wk-old males. Results presented in Table 1 demonstrate the fact that testis weight in *Fyn*-null males was not significantly different from that in controls; however, epididymal weight and sperm count were significantly lower than those of controls.

TABLE 1. Comparison of testis and epididymis weight and epididymal sperm counts.[a]

Genotype	Testis (mg)	Epididymis (mg)	Epididymal sperm ($\times 10^6$)
WT	89.0 ± 4.1	8.3 ± 0.8	16.8 ± 2.3
Fyn-null	82.8 ± 7.4	6.1 ± 0.8[b]	7.2 ± 1.9[b]

[a] Testes and epididymides from three age-matched *Fyn*-null or WT males 7–8 wk of age were used for comparison. Values represent the mean ± SD.

[b] Value is significantly different from WT as determined by *t*-test ($P = 0.001$).

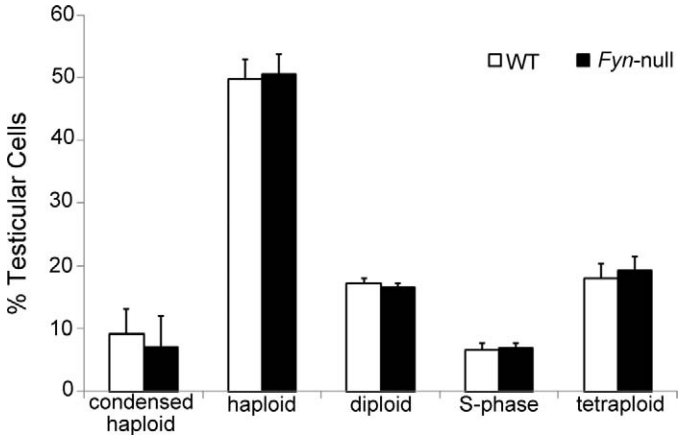

FIG. 2. DNA flow cytometric analysis of *Fyn*-null testicular cell composition is shown. *Fyn*-null and WT testes (five testes from five mice in each group) were digested to single cells. Testicular cells were then fixed with ethanol and stained with PI. Flow cytometric analysis revealed five populations categorized according to cellular DNA content: condensed haploid (elongated spermatids), haploid (round spermatids), diploid (spermatogonia/somatic cells), S phase (S-phase spermatogonia), and tetraploid (primary spermatocytes/G_2 spermatogonia). Percentages of each population (50 000 events per sample analyzed) in the *Fyn*-null testis were compared with those in the WT testis by *t*-test and found not to be significantly different ($n = 5$; $P > 0.05$). Bars indicate means ± SD.

Functional evidence that knockout of FYN kinase had biochemical effects on the testis was provided by Western blot analysis of potential targets for tyrosine kinases, as assessed with an anti-P-Tyr antibody. The pattern of P-Tyr-containing testis proteins from *Fyn*-null males was characterized by the absence of 55- and 95-kDa bands that were easily detectable in the WT testis (Fig. 1B). Similar analysis of epididymal sperm revealed reduced P-Tyr content of a ~95-kDa band in sperm from *Fyn*-null males (Fig. 1C). Capacitation of *Fyn*-null sperm in vitro triggered a general increase in the abundance of P-Tyr in many proteins, as normally seen in WT sperm. However, capacitated *Fyn*-null sperm did exhibit minor differences such as decreases in P-Tyr levels in bands of approximately 95, 50, and 30 kDa. Surprisingly, the P-Tyr content of a 72-kDa band was higher in capacitated *Fyn*-null sperm than in WT sperm (Fig. 1C). This result suggests that FYN kinase phosphorylates a select group of sperm proteins during capacitation but that these proteins appear to be targets of other PTKs as well because they contained significant amounts of P-Tyr even in the absence of FYN kinase activity.

Histological analysis of postpubertal testis by using light microscopy revealed no major structural differences between *Fyn*-null and WT genotypes. However, to more carefully characterize the possible impact of ablation of FYN kinase on meiosis, the relative abundance of cells representing different stages of spermatogenesis was studied using Western blot analysis with cell type-specific antibodies and DNA flow cytometry. Semiquantitative analysis of Western blots probed

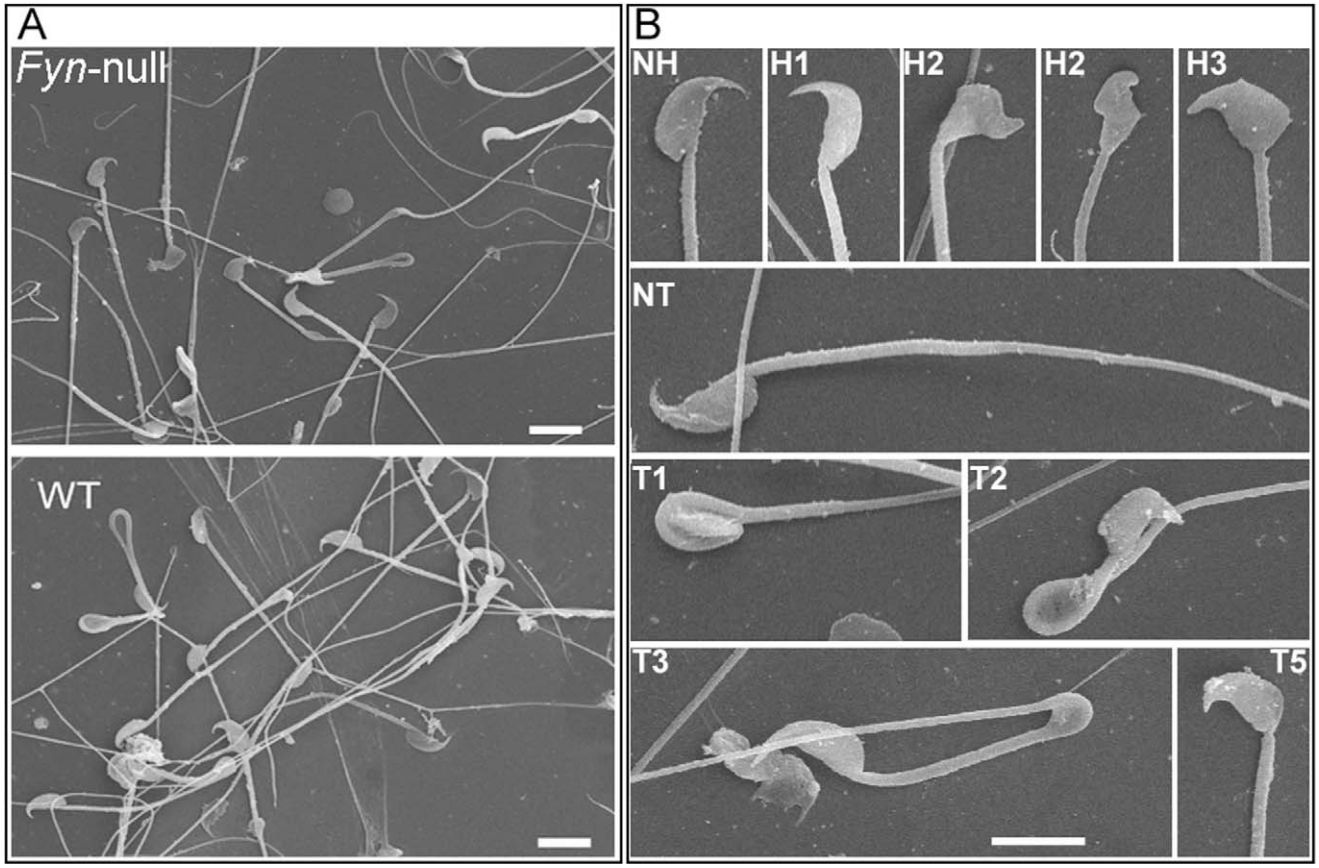

FIG. 3. Characterization of *Fyn*-null sperm morphology is shown. Cauda epididymal sperm from *Fyn*-null and WT males were fixed and prepared for SEM as described in *Materials and Methods*. Normal heads (NH) and tails (NT) and abnormal heads (coded, H1–H3) and tails (coded, T1–T5) were coded as described in *Materials and Methods*. **A**) A representative SEM image from samples of *Fyn*-null or WT sperm is shown. **B**) Examples of coded groups used to categorize head (H) and tail (T) morphologies are shown. Bar = 6 μm. Percentages of *Fyn*-null and WT sperm classified within each coding group are summarized in Table 2.

TABLE 2. Morphological abnormalities of *Fyn*-null sperm.[a]

	Head morphology (%)				Tail morphology (%)				
Genotype	NH[b]	H1	H2[b]	H3[b]	NT[b]	T1	T2	T3	T5[b]
Fyn-null	36.2 (7.6)	9.2 (4.8)	15.2 (8.0)	39.4 (1.3)	43.8 (6.4)	2.4 (2.6)	5.2 (3.8)	15.1 (7.0)	33.5 (1.3)
WT	92.7 (8.7)	4.5 (4.3)	1.1 (2.0)	1.7 (2.9)	71.7 (6.4)	2.2 (3.9)	9.9 (1.3)	15.0 (4.0)	1.1 (1.9)

[a] The morphology of epididymal sperm from adult *Fyn*-null or WT control males was imaged by Hoffman modulation contrast or by SEM and categorized according to the published criteria [17]. NH, normal head; H1, thin, elongated head; H2, club-shaped head; H3, triangle-shaped head; NT, normal tail; T1, bent head; T2, looping midpiece; T3, folded mid- and principle piece; T5, incorrect head-neck connection. Values represent means ± (SD), calculated from analysis of samples from three males of each group.
[b] Values determined by *t*-test to be significantly different from WT ($P < 0.05$).

with antibodies to GCNA1, representing spermatogonia, spermatocytes, and round spermatids [21]; to PLZF, representing spermatogonial stem cells [22, 23]; to KIT, representing types A1 to A4 spermatogonia and spermatocytes [24, 25]; and to DDX4, representing spermatocytes and round spermatids [26] revealed that the abundance of these proteins in the *Fyn*-null testis was not significantly different from that in controls (Fig. 1D). Testicular cell composition analysis by DNA flow cytometry also failed to detect significant differences in the relative levels of the five major cell categories that can be differentiated by this technique, including spermatogonia/somatic cells (diploid), S-phase spermatogonia (S phase), primary spermatocytes/G_2 spermatogonia (postreplication), round spermatids (haploid), or elongated spermatids (condensed haploid) (Fig. 2).

Epididymal sperm morphology was investigated by Hoffman modulation contrast microscopy [27] and by SEM. Sperm from *Fyn*-null males contained a high frequency of morphological defects that were classified by the morphological assessment system reported previously [17] (Fig. 3). Common defects included club-shaped and triangular head morphologies as well as incorrect head-to-neck connections. Quantitative analysis of these defects based on Hoffman modulation contrast microscopy and SEM revealed that most *Fyn*-null sperm had morphological defects (Table 2), indicating that the later stages of spermatogenesis in which the physical shaping of the sperm is completed were negatively impacted by the absence of FYN kinase.

Fertility Analysis of Fyn-*Null Male Mice*

In order to assess the impact of *Fyn* ablation on male fertility, a breeding study was performed in which the ability of *Fyn*-null males to produce fetuses when mated with CF1 females was compared with that of WT males. Females caged with either *Fyn*-null males or WT controls were all pregnant at day 14 after breeding. As shown in Figure 4A, females mated with *Fyn*-null males produced normal numbers of fetuses that appeared grossly normal, in agreement with previously published results, which indicated that *Fyn*-null mice were fertile [12]. In order to more precisely assess the ability of *Fyn*-null sperm to transit the female reproductive tract and fertilize eggs, an in vivo competition assay was performed in which equal numbers of WT and *Fyn*-null epididymal sperm were mixed and used to artificially inseminate females. Genotype analysis of the resulting fetuses revealed that the percentage of fetuses produced by *Fyn*-null sperm was significantly lower than that produced by WT sperm because only 14.4% of the total 54 fetuses were derived from *Fyn*-null sperm (Fig. 4B). This result demonstrates that *Fyn*-null sperm exhibit impaired fertilizing capacity, which may reflect problems with transiting the female reproductive tract or with fertilizing oocytes. Apparently, the reduced fertilizing capacity of *Fyn*-null sperm was compensated for during matings by the fact that an excess of sperm is delivered into the female reproductive tract via copulation.

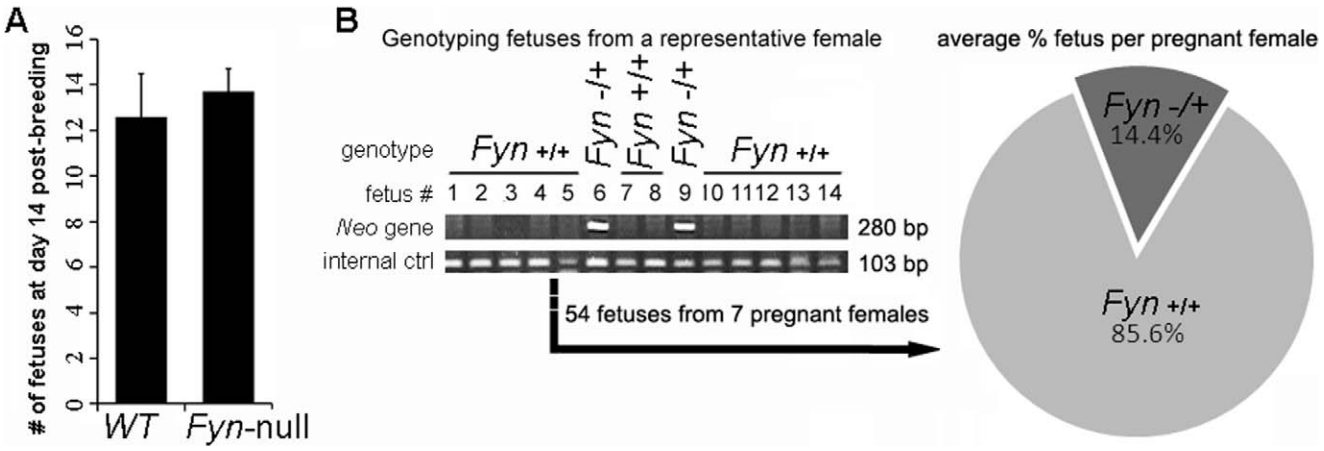

FIG. 4. In vivo analysis of male fertility and sperm fertilizing capacity is shown. **A**) A limited mating trial was performed to determine whether *Fyn*-null males were fertile. Adult *Fyn*-null or WT males (n = 4) were each caged with two CF1 females, as described in *Materials and Methods*. The mean ± SD number of fetuses per female is indicated by vertical bars. Analysis by *t*-test indicated that differences between means were not significant ($P > 0.05$). **B**) The relative fertilizing capacities of sperm from three WT and *Fyn*-null males were tested by artificial insemination of CF1 females by using an equal mixture of *Fyn*-null and WT sperm. Genotype analysis of fetuses resulting from artificial insemination detected the presence of the neomycin insert in those fetuses produced from *Fyn*-null sperm (center). The percentage of fetuses per female derived from *Fyn*-null sperm is presented as dark grey in the pie chart (right). The percentage of fetuses derived from *Fyn*-null sperm was significantly lower than that derived from WT sperm, as determined by *t*-test ($P < 0.001$).

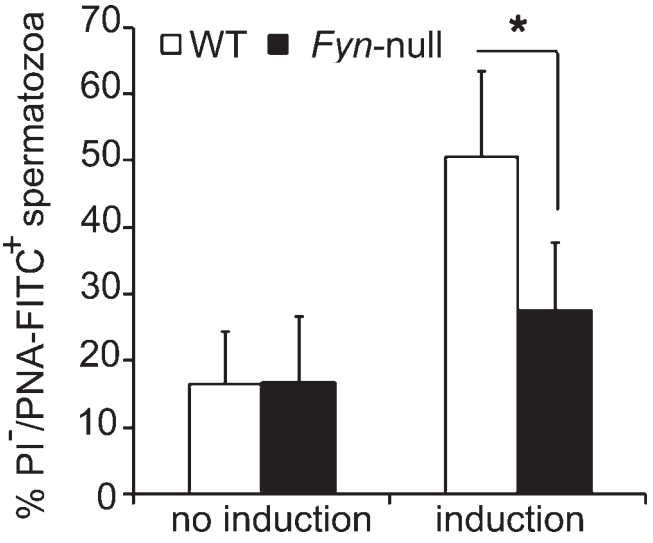

FIG. 5. Percentage of acrosome reactions of *Fyn*-null sperm following capacitation in vitro is shown. *Fyn*-null and WT mice (five males in each group) were used to isolate epididymal sperm. Isolated sperm were incubated for 90 min in capacitating medium containing 25 mM $NaHCO_3$ and 4 mg/ml BSA in the absence (no induction) or presence (induction) of 10 μM calcium ionophore A23187 to test sperm acrosome reaction. Flow cytometric analysis (20 000 events per sample) was performed after live sperm were stained with PNA-FITC and PI. The mean ± SD incidence of PNA-FITC-positive but PI-negative (representing those sperm that had successfully undergone acrosome reaction) *Fyn*-null sperm was compared with that of WT sperm. Asterisk indicates significant difference ($P < 0.05$).

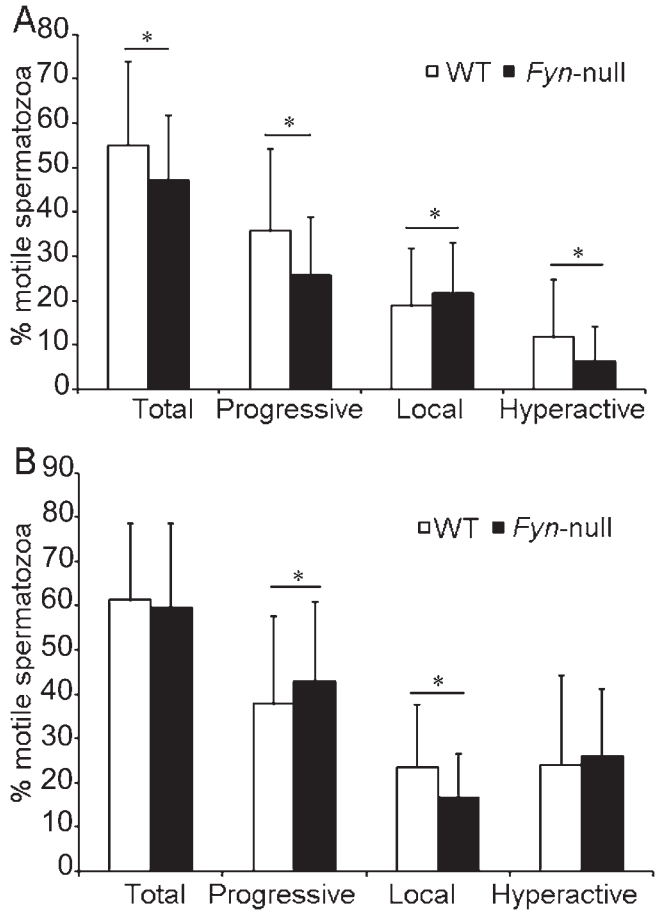

FIG. 6. Motility analysis of *Fyn*-null and WT sperm is shown. CASA motility analysis was performed with cauda epididymal sperm from five different *Fyn*-null and WT males following incubation under non-capacitating conditions lacking $NaHCO_3$ and BSA (**A**) or capacitating conditions (**B**), as described in *Materials and Methods*. Motility values represent the mean percentage ± SD of sperm within each classification category (1700 spermatozoa ± SD per sample were analyzed). In order to determine whether values for WT and *Fyn*-null sperm were significantly different, means were compared using a *t*-test (n = 5). Asterisk indicates significantly different values ($P < 0.05$).

In Vitro Analysis of Sperm Function

In order to directly test the fertilizing capacity of *Fyn*-null sperm without complications related to mating and passage through the female reproductive tract, in vitro capacitation and fertilization studies were performed. *Fyn*-null cauda epididymal sperm exhibited the same low frequency of spontaneous acrosome reactions as WT sperm following capacitation in vitro (Fig. 5). However, the frequency of A23187-induced acrosome reactions in *Fyn*-null sperm was significantly lower than in WT sperm (Fig. 5). CASA analysis demonstrated that *Fyn*-null sperm exhibited small decreases in total, progressive, and hyperactive motility and higher local (twitching, nonprogressive) motility before capacitation. After capacitation, *Fyn*-null sperm demonstrated normal total and hyperactive motility, a slight decrease in progressive motility, and little reduction in local motility (Fig. 6, A and B). Together, these data suggest that the major defects responsible for reduced fertilization capacity of *Fyn*-null sperm correlate more with lower frequency of acrosome reaction than with flagellar motility of sperm.

To test the capacity of *Fyn*-null sperm to penetrate the zona pellucida and fertilize eggs, IVF assays were performed in which capacitated WT or *Fyn*-null sperm were used to inseminate WT CF1 oocytes in vitro. As shown in Table 3, *Fyn*-null sperm at concentrations of 1×10^5 to 3×10^5 sperm/ml were unable to fertilize zona-intact oocytes during the 5-h incubation period, while WT sperm at this concentration range fertilized 67.3% of the oocytes. At higher concentrations (8-fold), the *Fyn*-null sperm were able to fertilize 19.0% of the oocytes, demonstrating that they still retained some fertilizing capability. Microscopic analysis was performed for all oocytes to determine the frequency of sperm bound to the zona pellucida as well as the frequency of sperm in the perivitelline space or in the ooplasm. These observations revealed that *Fyn*-null sperm were able to bind to the zona pellucida at the same frequency as that of controls but exhibited a significantly reduced ability to penetrate the zona pellucida, as indicated by the lower frequency of sperm retained in the perivitelline space or fused with the ooplasm (Table 3). These results indicate that the reduced fertilizing capacity of *Fyn*-null sperm in vitro is largely due to reduced ability to undergo the acrosome reaction and penetrate the zona pellucida.

DISCUSSION

Previous studies have provided evidence that the *Src* gene family kinases play a role in several aspects of testis and sperm function [1, 3, 8–11, 28]. However, the use of chemical inhibitors that lack absolute specificity and the absence of detailed fertility analysis of mouse models in which *Src* gene family PTKs have been knocked out have limited the interpretations of the functional studies to date. The present work, based on the use of *Fyn*-null mice, indicated that *Fyn* expression is important for development of sperm with normal morphology and normal fertilizing capacity. The lack of *Fyn* expression did not result in morphological changes in the testis and epididymis, as detected macroscopically or by light

TABLE 3. In vitro fertilization of oocytes with *Fyn*-null or WT sperm.[a]

Sperm genotype	Sperm concentration	Cumulus-enclosed oocytes			Zona-free oocytes	
		Fertilized (%)	Sperm bound	Sperm penetrated	Fertilized (%)	Sperm penetrated
WT	Standard[b]	67.3 ± 5.7	7.8 ± 2.1	1.3 ± 0.1	83.3 ± 16.7	0.9 ± 0.1
Fyn-null	Standard[b]	0[c]	5.3 ± 3.7	0[c]	50.0 ± 25.0	0.5 ± 0.3
	8 × Standard	19.0 ± 0.6[c]	8.6 ± 0.8	0.2 ± 0.1[c]	–	–

[a] Sperm from WT and *Fyn*-null males were capacitated in vitro and used to fertilize WT cumulus-enclosed eggs or cumulus- and zona-free eggs as described in *Materials and Methods*. Oocytes were scored as fertilized if two or more expanded pronuclei were present. The number of sperm bound to the zona pellucida (bound) or within the perivitelline space/ooplasm (penetrated) were determined by confocal fluorescence microscopy. Values represent the mean from three separate experiments ± SD.

[b] Standard sperm concentration in IVF: 1–3 × 10^5/ml.

[c] Mean value is significantly different from WT as determined by *t*-test ($P < 0.05$).

microscopy. This finding agrees with that of an earlier study in *Fyn*-null testes that described only ultrastructural defects consisting of alterations in the basal and apical Sertoli cell junctions [8]. Loss of *Fyn* expression apparently had little effect on differentiation of the male germ cells, as neither stage-specific protein expression profiles nor DNA flow cytometry analysis detected significant differences between the relative abundance of stages comprising the germ cell compartment of the testis. In contrast, our analysis of sperm showed lower sperm counts in the epididymis of the *Fyn* knockouts; and associated with this reduction of sperm count was an increase in the frequency of various aberrant sperm morphologies. Most of these defects were localized to the sperm head, suggesting that FYN kinase or the tr-FYN protein is critical for the development of the sperm head structure during spermiogenesis. This finding agrees with a model based on subcellular localization studies in the testis proposed by Kierszenbaum [29] and Kierszenbaum et al. [30], in which FYN and FER would function in acrosome-axoplaxome biogenesis. FYN and FER have been found to function as part of a common pathway, modulating cytoskeletal and cell-cell contacts in other cell types [31], two events that would seem to be integral to the sperm head shaping process.

While functional studies of *Src* gene family kinases using chemical inhibitors have drawbacks due to limited specificity within the kinase family, single-gene knockout studies suffer from compensation by other *Src* gene family members that might be expressed in Sertoli cells and/or germ cells (e.g., the *Src* gene) [32]. The wide range of morphological defects which appeared in *Fyn*-null sperm may well reflect the modest level of success with which the other *Src* gene family members can compensate for the loss of FYN. Given the fact that *Fyn*-null males exhibited normal fertility, it was of particular importance to determine whether the defects resulting from the loss of FYN kinase activity were significant enough to result in reduced sperm fertilizing capacity. Functional defects in sperm from *Fyn*-null mice were demonstrated most conclusively in competitive artificial insemination studies, where *Fyn*-null sperm were found to produce embryos at a rate approximately one-sixth that of controls (14.4% vs. 85.6%, respectively). The very significant deficiency in fertilizing capacity detected by using a competitive artificial insemination trial with WT and *Fyn*-null sperm mixed in an artificial buffer, in contrast to the lack of effect seen with natural matings, highlights the limitations of mating trials in assessing certain parameters that negatively impact sperm function. Most probably, the biology of mating has evolved to provide an oversupply of sperm that can mask significant heterogeneities in sperm function.

Our use of in vitro techniques to characterize the fertility defects in *Fyn*-null sperm was not ideal in that it exposed the sperm to the stresses of in vitro culture. However, it did allow us to quantify the percentage of acrosome reaction and zona pellucida penetration relative to those of WT sperm, and under identical conditions with adequate statistical power. *Fyn*-null sperm exhibited only minor differences in motility parameters but exhibited a greatly reduced rate of A23187-induced acrosome reaction following in vitro capacitation. This agrees with previous findings made with chemical inhibitors [10, 11] and suggests that in addition to a potential role in sperm head morphogenesis, FYN kinase may also play a significant role in signaling mechanisms leading to the successful completion of the acrosome reaction.

As mentioned above, the propensity for compensation among *Src* gene family members can reduce the effectiveness of the single-gene knockout approach. For example, Western blot analysis of P-Ty phosphorylation patterns in *Src*-null sperm failed to detect differences from WT sperm even after capacitation [1]. Similar Western blot analyses of P-Tyr-containing proteins in *Fyn*-null sperm reported here demonstrated that WT and *Fyn*-null epididymal sperm contained nearly identical patterns of P-Tyr labeling. However, *Fyn*-null sperm consistently exhibited reduced P-Tyr labeling in a 95-kDa band. Following capacitation, *Fyn*-null sperm exhibited reduced P-Tyr content in several (~95-, ~50-, and ~30-kDa) bands and increased P-Tyr labeling in a ~72-kDa band. In the absence of data indicating the identity of these P-Tyr-containing bands, it is not possible to be certain whether changes reflect alterations in phosphorylation state or in amounts of each protein expressed intact in the capacitated sperm. However, taken together, these findings suggest that FYN actively phosphorylates multiple proteins during capacitation. Close structural similarities among *Src* gene family members raise the possibility that FYN could potentially compensate for the loss of SRC kinase, which may explain the demonstrated lack of an effect of *Src* deletion on sperm phosphorylation patterns [1].

In summary, the loss of FYN kinase in the testis resulted in the development of morphologically defective sperm, leading to structural defects and reduced capacity to undergo the acrosome reaction and penetrate the zona pellucida in vitro. Whether the observed functional defects resulted primarily from the loss of FYN in Sertoli cells, which resulted in defective acrosomal organization and structure, or from the absence of FYN in the sperm, which interrupted a key signaling pathways involved in the acrosome reaction, has not been resolved by this study. Future work with Sertoli or spermatid-targeted *Fyn* ablation could potentially answer that question. Results presented here do clearly demonstrate that FYN kinase plays an important role(s) in the organization and shaping of sperm and potentially in the acrosome reaction that

cannot be fully compensated by other *Src* gene family members. Further biochemical studies may ultimately identify the specific targets of FYN in the testis and in sperm, which would help to identify the specific pathways in which FYN participate.

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