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A physiological function of serum proteoglycan bikunin: The chondroitin sulfate moiety plays a central role

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Bikunin is a small chondroitin sulfate proteoglycan that occurs in blood as the light chain of inter-α-trypsin inhibitor (ITI) family members. The relatively short chondroitin sulfate chain of bikunin shows a characteristic pattern of sulfation in both the linkage region and the chondroitin sulfate backbone. To the internal *N*-acetylgalactosamines in the lower sulfated portion near the non-reducing end, up to two "side" proteins could bind covalently via a unique ester bond to form "core protein-glycosaminoglycan-side protein" complexes, the ITI family. ITI molecules are synthesized in hepatocytes, and then secreted into circulation at high concentrations. In the presence of yet unidentified factors, the side proteins are transferred from chondroitin sulfate to hyaluronan by a transesterification reaction to form what has been described as the Serum-derived Hyaluronan-Associated Protein (SHAP)-hyaluronan complex. The formation of this complex is required for the stabilization of the extracellular matrix of fibroblasts, mesothelial cells, and cumuli oophori. When the gene for bikunin is inactivated, female mice exhibit severe infertility as a consequence of a defect of the side protein precursor in forming a complex with the hyaluronan in cumulus oophorus before ovulation. Therefore, the chondroitin sulfate moiety of bikunin is essential for presenting SHAP to hyaluronan, which is indispensable for ovulation and fertilization in mammals.
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Bikunin, sometimes called urinary trypsin inhibitor or the light chain of inter-α-trypsin inhibitor (ITI), was shown to be a small proteoglycan with a single chondroitin sulfate chain in 1986 [1]. However, it was often overlooked in publications on proteoglycans, probably because: (1) it is rarely found in an extracellular matrix, (2) its core protein shows a higher sequence homology to serine protease inhibitors than to any known proteoglycan, and (3) its association with ITI heavy chains rather masks the small glycosaminoglycan moiety. In the 1990s, after the cloning of cDNAs, the molecular structure of bikunin was extensively characterized. Recently, the functional scenario of bikunin also emerged. All these new findings demonstrate that as a proteoglycan, bikunin is structurally and functionally so unusual that it deserves a special chapter in books on the subject.

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Bikunin is a major circulating proteoglycan

Bikunin is principally synthesized and secreted by liver, and occurs in blood and urine as the major chondroitin sulfate proteoglycan [2]. In circulation, bikunin is often present at relatively high levels, 30–100 μg/ml, whereas in urine the level is usually lower than 5 μg/ml in healthy individuals, but is liable to elevate, even over one hundred-fold, in patients with disseminated cancers, renal failure, rheumatoid arthritis, and infection [3–5].

Bikunin consists of about 140 amino acid residues that are arranged into two tandem Kunitz-type protease inhibitory domains, (after which the name "bi-kun-in" was cast [6]), and extensions at both *N*- and *C*-termini. The *N*-terminal extension covers about 25 residues, of which the serine residue at position 10 initiates the synthesis of a typically *O*-linked chondroitin sulfate chain [7,8] (Figure 1). It is reported that the bikunin deglycosylated natural protein or the unglycosylated recombinant protein shows much higher acceptor activity for xylosylation by a chondrocyte-secreted β-D-xylosyltransferase

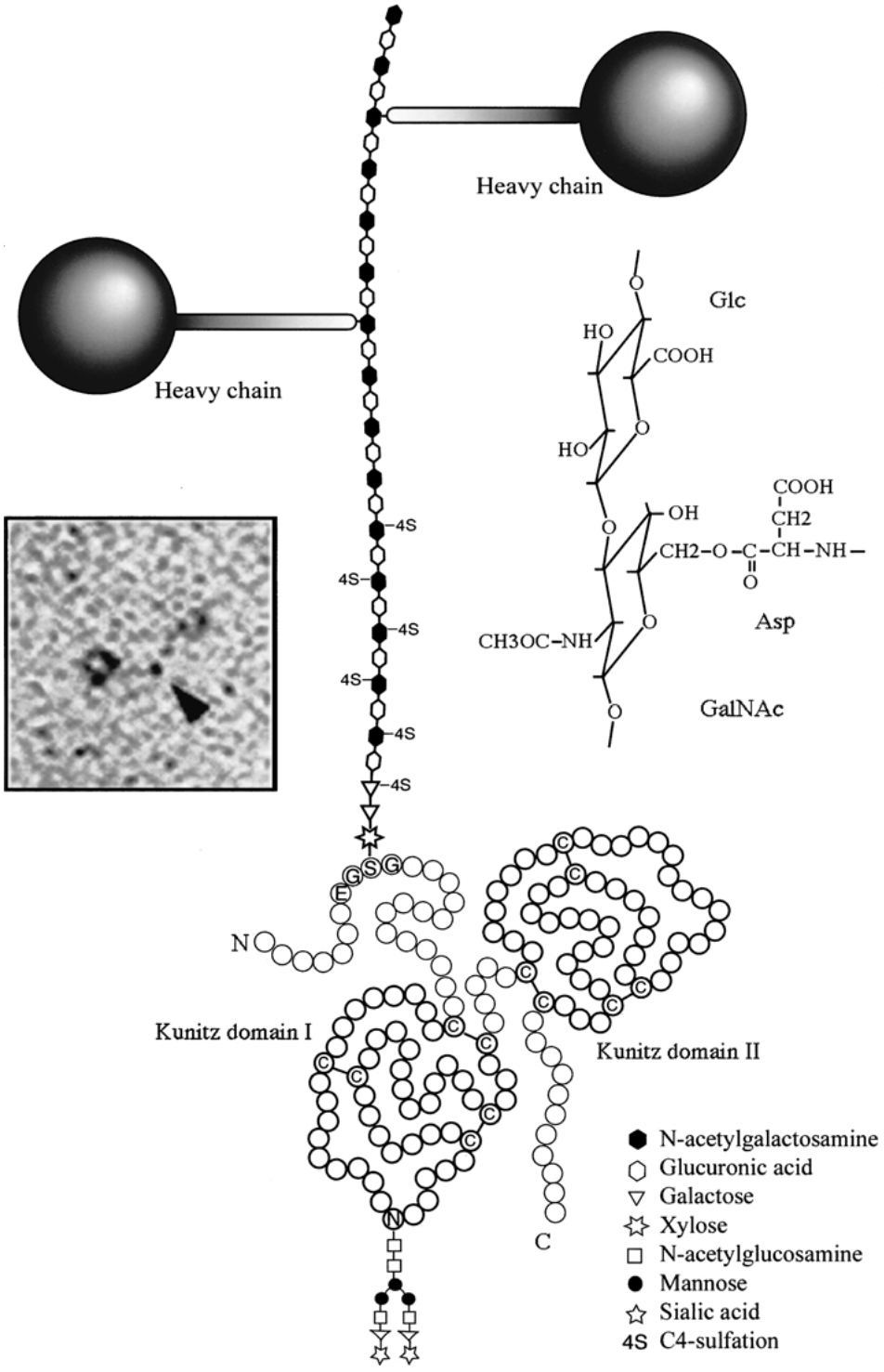

Figure 1. Schematic representation of the structure of bikunin. The core protein of bikunin consists of about 140 amino acid residues that are arranged into the *N*-terminal extension, the *C*-terminal extension, and two tandem Kunitz-type protease inhibitory domains. A chondroitin-4-sulfate chain composed of 15 disaccharide repeats on average is synthesized at the serine residue at position 10, of which the proximal portion is more commonly sulfated. Up to two side proteins is associated with the circulating bikunin to form the complexes collectively known as inter-α-trypsin inhibitor family. The side proteins are linked to the distal portion of chondroitin sulfate via an ester bond between the *C*6-hydroxyl group of an internal *N*-acetylgalactosamine and the α-carboxyl group of their *C*-terminal aspartic acid residues (the right inset). Under an electron microscope, bikunin looks like a small sphere (the arrowhead in the left inset), and the side proteins have an appearnce of a globular domain with a thin tail. [Modified from references 7,10,11,29,33].

than chemically deglycosylated aggrecan and silk fibroin (Km = 0.9 μM vs. 155 μM and 545 μM, respectively) [9]. The strong acceptor activity of bikunin may not just be a coincidence because the glycosaminoglycan moiety is indispensable to the function of bikunin, which will be discussed later in this review. Consistently, the peptide sequence of the chondroitin sulfate attachment site, Glu-Gly-Ser-Gly, is well conserved in bikunin from all animal species examined to date.

The chondroitin sulfate chain of bikunin is relatively short, having a molecular weight of about 8,000 and consisting of 12–18 repeats of the GlcA-GalNAc disaccharide in addition to a conventional linkage region GlcAβ1-3Galβ1-3Galβ1-4Xylβ1-O-Ser [10,11]. Under inflammatory conditions, the chain length of bikunin in both blood and urine may be increased by roughly eight disaccharide repeats [12]. The sulfation of the chondroitin backbone occurs at the *C*-4 hydroxyl group of GalNAc and its extent is about 30%. Despite a certain amount of heterogeneity [11,13], clustered sulfation was found in the proximal portion, probably with the innermost five disaccharide units fully sulfated [10]. In the linkage region, the Gal residue next to GlcA is uniformly sulfated, in sharp contrast to the heterogeneity found in cartilaginous chondroitin sulfate proteoglycans [14,15] and in decorin [16]. In addition to the chondroitin sulfate, there is a "complex type" biantennary N-glycan on Asn45, which seems to be homogenous in all bikunin molecules [11,17] (Figure 1).

The gene for bikunin is also unique. It codes simultaneously another serum protein, α-1-microglobulin, which seems to have neither a structural nor a functional relation with bikunin [18]. The two proteins are synthesized as a precursor polypeptide, and then released by proteolytic cleavage at the *trans*-Golgi compartment after the chondroitin sulfate of bikunin has been synthesized [19,20]. Transfection of CHO cells with the precursor cDNA and bikunin cDNA alone results in a different glycosylation and sulfation of bikunin, suggesting an interesting regulatory role for the α-1-microglobulin portion in the posttranslational modification of bikunin [21].

The "side" protein of bikunin

The most surprising feature of bikunin is that there are covalently bound proteins on its chondroitin sulfate. These proteins are usually referred to as the heavy chains of ITI family molecules. Here we propose to call them "side" proteins in contrast to the "core" protein of bikunin proteoglycan.

ITI was originally isolated as a plasma globulin with 10% hexose in 1961 [22]. For a long period, it had been thought to be a single-peptide glycoprotein. In the late 1980s, the cloning of cDNAs unambiguously indicated that ITI was a complex of multipeptides, and that the sequence of the light chain was in complete agreement with that of urinary trypsin inhibitor, which was already shown to be a chondroitin sulfate proteoglycan [23–25]. Soon after, it was found that the multipeptides are cross-linked by the glycosaminoglycan of the light chain bikunin [26–28]. Further study revealed a novel ester bond between the *C*-6 hydroxyl group of an internal *N*-acetylgalactosamine of the chondroitin sulfate of bikunin and the α-carboxyl group of the asparate residue at the C-terminus of a side protein (Figure 1). In the present, three side proteins have been identified to form the protein-glycosaminoglycan-protein complex with bikunin via such an ester bond [29–31]. These proteins derive from three different genes, but their sequence homology suggests a common origin [32]. In particular, the sequence immediately after the esterifying aspartic acid residue, Pro-His-Phe-Ile-Ile, is well conserved among all side proteins in all species examined to date. The side proteins have molecular weights of 65,000–90,000. A bikunin molecule is able to accept up to two side proteins in the undersulfated distal portion of its chondroitin sulfate chain [11]. Under an electron microscope, the side proteins have the appearance of a globular domain with a thin "tail" (diameter and length of about 11 nm and 15 nm, respectively), and bikunin looks like a small sphere [33] (Figure 1).

The coupling of a side protein to bikunin occurs in the *trans*-Golgi compartment of hepatocytes, just before the cleavage of α-1-microglobulin/bikunin precursor polypeptide [34]. However, the coupling mechanism remains largely unknown. A recent study suggested a low pH-triggered autocatalytic cleavage mechanism for the exposure of the *C*-terminal active group of the side protein [35]. Whether this is involved in the physiological coupling remains to be examined.

At present, bikunin is the only example of a physiologically occurring proteoglycan that carries covalently bound side proteins at the glycosaminoglycan moiety. Using cell culture systems, some authors have shown that cells other than hepatocytes, for example COS-1 and CHO cells, also have machinery for synthesizing the protein-glycosaminoglycan-protein complex, and that proteoglycan other than bikunin, decorin, is able to accept the side proteins of bikunin [36,37]. Therefore, it might be possible that other proteoglycans with a side protein exist.

Transfer of the "side" protein to hyaluronan

A further surprise is that the side proteins of bikunin were also found being associated to hyaluronan, the free glycosaminoglycan neither sulfated nor bound to a core protein. The side protein-hyaluronan complex was first isolated from the hyaluronan-rich extracellular matrix of cultured mouse dermal fibroblasts. Because the complex formed between the cell-secreted hyaluronan and ITI molecules present in the serum-supplemented culture medium, we have designated it the SHAP-hyaluronan complex [38,39]. This complex is also present in large amounts in pathological synovial fluid from patients with rheumatoid arthritis. Structural analysis of the synovial SHAP-hyaluronan complex revealed that the SHAP was bound to an internal *N*-acetylglucosamine residue of hyaluronan via an ester bond equivalent to those found in bikunin [40]. No bikunin could be found in the purified complexes, indicating that it was released during the formation of the complex. Therefore, the complex seems to form via a trans-esterification

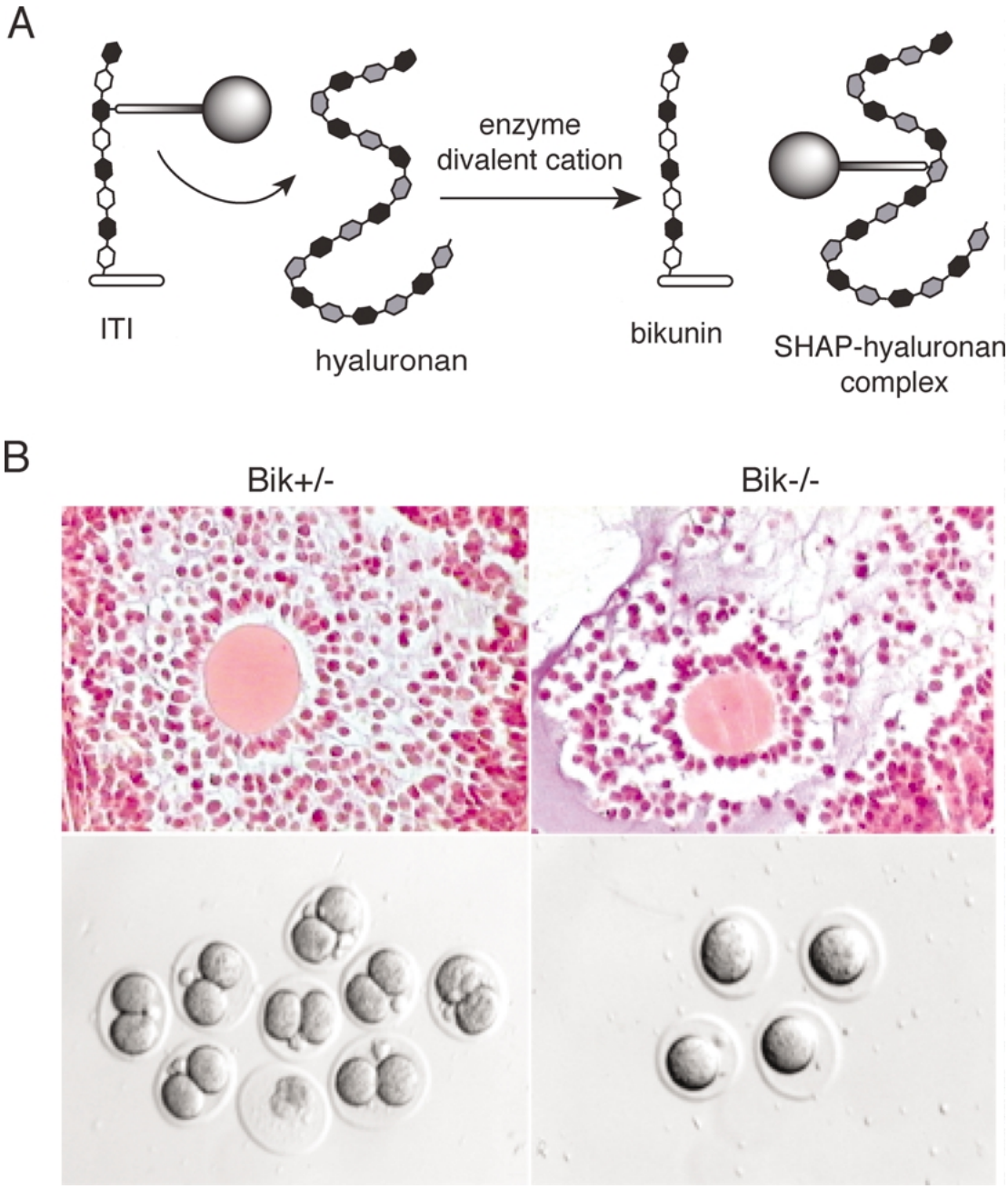

Figure 2. The chondroitin sulfate moiety of bikunin is important for its physiological function. (A) Schematic diagram of the transfer of the side proteins of bikunin (the heavy chains of ITI family molecules) to hyaluronan to form the SHAP-hyaluronan complex. (B) When the gene for bikunin is inactivated in mice, the side proteins of bikunin are secreted at their precursor forms, which are unable to form the SHAP-hyaluronan complex with hyaluronan. Consequently, the construction of the hyaluronan-rich cumulus matrix in preovulatory follicles is severely impaired (upper panel), leading to a significant defect in ovulation and fertilization (lower panel) [47].

reaction where hyaluronan substitutes for the chondroitin sulfate chain of bikunin to bind to the side proteins (Figure 2A). All three side proteins have been found to be involved in the formation of the SHAP-hyaluronan complex.

The complex can be formed simply by incubating hyaluronan with serum [39]. However, the incubation of hyaluronan with purified ITI did not result in complex. In addition, the presence of chelating reagents like EDTA inhibited the reaction [39]. Therefore, a divalent cation-dependent enzymatic factor(s) is required for the trans-esterification reaction, which, however, remains to be identified. The reaction specifically requires hyaluronan as substrate. The hyaluronan octasaccharide or longer oligomers are competitive inhibitors, but other glycosaminoglycans including chondroitin, chondroitin sulfate,

dermatan sulfate, heparan sulfate, and dextran sulfate are neither a substrate nor a competitor [38]. The presence of versican enhanced the formation apparently via the simultaneous interaction of the *N*-terminal globular domain with hyaluronan and the side protein of bikunin [41]. The synovial fluid-derived complex has on average 3-5 SHAP proteins per hyaluronan chain with a molecular weight of about 2×10^6. Transfer of the side proteins from bikunin to hyaluronan did not bring about a significant change in molecular appearance under the electron microscope [50].

The important role of the chondroitin sulfate moiety of bikunin

Many cells are able to form hyaluronan-rich extracellular matrix when cultured in the presence of serum. In some cells, such as fibroblasts and mesothelial cells, ITI was found to be able to replace serum to induce the matrix formation, indicating a role for ITI in the stabilization of the extracellular hyaluronan-rich matrix [42]. Since the SHAP-hyaluronan complex has been isolated from the matrix of fibroblasts [38], it is plausible to assume that the formation of this complex between ITI and cell-secreted hyaluronan underlies the matrix-stabilizing activity of ITI.

A similar observation was made in the *in vitro* expansion of the cumulus-oocyte complex. During ovarian folliculogenesis, a sub-population of granulosa cells differentiates into cumulus cells that are arranged into several cell layers around the oocyte. Following the ovulatory gonadotropin surge, cumulus cells secrete large amounts of hyaluronan and other components that are assembled into an extensive extracellular matrix, leading to a significant expansion of the cumulus-oocyte complex. The process can be induced *in vitro*, where serum was found to be dispensable for hyaluronan synthesis but absolutely necessary for hyaluronan retention in the matrix [43,44]. Again, ITI family molecules were able to substitute for serum in this system [45,46].

To clarify the physiological significance of bikunin and the formation of the SHAP-hyaluronan complex, we selectively inactivated the bikunin gene in the α-1-microglobulin-bikunin precursor gene. The bikunin-null mice exhibited severe female infertility due to impaired ovulation and fertilization [47]. Histological examination of the ovaries revealed that folliculogenesis progressed normally in these mice but defective cumulus oophorus expansion occurred during the preovulatory time. After the ovulatory gonadotropin surge, cumulus cells were irregularly arranged around the oocyte and even scattered in the antral cavity of follicles. Importantly, the formation of corona radiata, which is characterized by the elongation of cells in the innermost layer, was absent (Figure 2B). As a consequence, the ovulated cumuli oophori were hardly associated with the oocytes, resulting in the abnormal occurrence of denuded oocytes in the oviducts. Hyaluronan-bound SHAP was present in the matrix of cumulus-oocyte complexes from wild type mice, but is undetectable in those from bikunin-null mice. These results established that the formation of the SHAP-hyaluronan complex is of great importance in the processes of ovulation and fertilization.

In the absence of *in vivo* expression of bikunin, the side proteins were detected in blood in the precursor forms. However, incubation of hyaluronan with the serum from bikunin-null mice did not result in the formation of the SHAP-hyaluronan complex, indicating that the coupling of the side proteins to the chondroitin sulfate of bikunin is essential for the subsequent transfer to hyaluronan [47]. Bikunin is neither a component of wild type cumulus matrix, nor it able to rescue the cumulus defect in bikunin-null mice, indicating that bikunin is not directly involved in the construction of the cumulus matrix [47]. In contrast, intraperitoneal administration of ITI corrected the cumulus defect. Therefore, bikunin plays a role in these processes by presenting the side proteins to hyaluronan. The glycosaminoglycan moiety of bikunin is indispensable for such a function.

Future prospects

Two steps are needed for the SHAPs to exert their function: to be loaded on the chondroitin sulfate chain of bikunin in the Golgi compartments of hepatocytes, and then to be launched to hyaluronan in the extracellular matrix of remote target tissues. In contrast to the accumulation of knowledge about structure, the reaction mechanisms underlying these steps are largely unknown. Because they are the keys to the control of related processes, they are undoubtedly the most urgent question to be answered.

To the best of our knowledge, bikunin is at present the only known proteoglycan with a side protein, and SHAP is the only characterized protein that binds to a glycosaminoglycan via an ester bond. It is unclear whether these features are unique to bikunin or of general importance. It has been shown that decorin is also able to accept the side proteins of bikunin, suggesting that the selectivity is partially due to the compartmentation of bikunin synthesis in hepatocytes, where other known proteoglycans are rarely synthesized, or to the predominance of bikunin in the circulating chondroitin sulfate proteoglycans. In addition, some authors have suggested that an arthritis-associated hyaluronan binding protein (TSG-6) might covalently associate to the chondroitin sulfate chain of bikunin under inflammatory conditions [48]. It would be interesting to look for other members of the side protein-carring proteoglycans.

Except for the infertility in females, bikunin-null mice are as healthy as wild type mice under normal conditions. Given the high level of bikunin-side protein complex in circulation, and the transfer of the side proteins to hyaluronan in order to become functional in cumulus oophorus expansion, it is very likely that such a reaction plays a role in the body defense system or some pathological processes. Actually, many activities of bikunin and ITI family molecules have been suggested [for review, see 49]. It will be interesting to examine their physiological importance using the bikunin-null mice.

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