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Review

Why Would Spermatozoa Require Autophagy? A Narrative Review

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Abstract

Autophagy, a highly conserved cellular mechanism that maintains cell survival and homeostasis by degrading and recycling damaged biomolecules, organelles, and other cytoplasmic components, is poorly understood with regard to male fertility and in spermatozoa. In the interplay between canonical autophagy and the different stages of spermatozoa, there is an important challenge: to understand the physiological role of autophagy-related proteins amid diverse sperm scenarios that involve fertility, different species, developmental stages, and experimental contexts. The objective of our work is to highlight the dynamics of the autophagy process along with changes in the related proteins in sperm while trying to connect them to fertility. Our narrative review gathers recent advances in the understanding of the role of major proteins involved in macroautophagy and its associated structures, including mTOR, LC3 (we review changes in its expression, localization and lipidation), p62, TFEB, Sirt1, PINK1, TOM20, ULK1, and members of the ATG family, during sperm development and in mature spermatozoa. Additionally, we have extended the insights into the autophagic flux and its autophagy-related proteins in the regulation of functional parameters such as motility, capacitation, acrosome reaction, mitochondrial function, and fertilization in different mammals such as humans, mice, horses, and swine. Furthermore, we discuss the emerging evidence showing how disruptions in autophagic protein homeostasis can impair sperm physiology and fertilization and contribute to male infertility, to answer the question of why spermatozoa would require autophagy?

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1. Introduction

After being ejaculated, sperm from mammals travels through the female genital tract accompanied by seminal fluid, which provides adequate substrates (e.g., fructose and citric acid) to supply the energetic demands to reach the egg and perform fertilization [1–3]. Sperm travels from the vagina to the oviducts, passing through the cervix, uterus, and utero-tubal junction [4]. Along this journey, spermatozoa experience a complex series of physiological changes called capacitation and the acquisition of a special motility pattern, known as hyperactivation, to become competent to fertilize the oocyte [5,6]; both events are considered prerequisites for the acrosome reaction [7].

After the acrosome reaction, the few sperm that reach the egg are ready to accomplish fertilization [8]. All these events require a high amount of energy, which is supplied by the sperm's energetic metabolism [9], through the oxidation of diverse substrates in the female reproductive tract (for example glucose, pyruvate, and lactate) [10] and hypothetically through autophagy [11]. Autophagy is a highly conserved catabolic process in eucaryotic cells essential to the maintenance of cell survival through the recycling/degradation of damaged biomolecules, organelles, and cytoplasmic components to provide metabolic intermediates and energy to support cellular homeostasis [12,13]. In somatic cells, there are three main types of autophagy depending on the cargo delivery pathway: macroautophagy, microautophagy, and chaperon-mediated autophagy [14]. Macroautophagy displays selective and non-selective processes characterized by a membrane elongation, resulting in the formation of a double membrane structure called an autophagosome, which sequesters a small portion of cytoplasm for further fusion to the lysosome to degrade its content [15]. On other hand, microautophagy is a process in which small cytoplasmic contents enter through invagination or membrane deformation into the lysosome [16]. Unlike other types of autophagy, in chaperone-mediated autophagy, the cargo proteins directly translocate across the lysosomal membrane without membrane reorganization [17]. Additionally, autophagy is divided into five stages: initiation, nucleation, elongation and maturation, fusion, and degradation [18] (Figure 1).

The initiation stage of autophagy involves several protein complexes, including Unc-51 Like Autophagy Activating Kinase 1/2 (ULK)-Autophagy-Related 1 (Atg1), class III Phosphoinositide 3-knase (PI3-K) complex, autophagy-related 9 (ATG9), Rab1, transport protein particle III (TRAPPIII), and the very well-known ubiquitin-like proteins, such as microtubule-associated protein 1A/1B-light chain 3 (LC3) [19]. This initial stage is defined by the assembly of the omegasome, which will serve as the starting point for the formation of the phagophore, the precursor of the autophagosome [20]. During the nucleation stage, the membrane starts to expand by attracting specific proteins and lipids, forming an initial double-membrane structure known as the phagophore. A key event in this phase is the assembly of the autophagy-related protein 14 (ATG14)-Class III/PI3-K complex, which generates phosphatidylinositol-3-phosphate (PI3-P), essential for macroautophagy. This complex also facilitates the recruitment of the vacuolar protein sorting proteins, vacuolar protein sorting 15 (VPS15) and vacuolar protein porting 34 (VPS34) and promotes the release of Beclin-1 and the activating molecule in BECN1-regulated autophagy protein 1 (AMBRA1) from their inhibitor (Bcl-2) [21]. During the elongation stage, the phagophore membrane expands and eventually encloses its cargo, forming a mature autophagosome. The key goal of this stage is to establish the site of LC3 lipidation, which is regulated by the activity of the elongation complex ATG5-12/16L1. Lipidation of LC3 (also known as LC3-II) is essential for its attachment to the autophagosomal

membrane. Membrane-bound LC3 not only facilitates the final formation of the autophagosome but also interacts with sequestosome-1 (p62/SQSTM) within the autophagosome to promote cargo recognition and degradation [22]. The closure and fusion stages involve the maturation of autophagosomes and their subsequent fusion with mature lysosomes, forming autolysosomes. This process is regulated by soluble N-ethylmaleimide-sensitive-factor attachment protein receptor (SNARE) proteins, particularly syntaxin-7 (STX7), which is found in the autophagosome. STX7 interacts with synaptosome-associated protein 29 (SNAP29) and the vesicle-associated membrane protein 8 (VAMP8) to mediate the full fusion between the autophagosome and lysosome. In the final degradation stage, lysosomal hydrolases break down the macromolecules within the autolysosome into monomeric components, which are then released back into the cytoplasm for reuse and recycling [23]. Additionally, there are organelle-specific autophagic processes that selectively target the degradation of damaged or unnecessary organelles, including mitophagy (mitochondria); peroxiphagy (peroxisomes); lysophagy (lysosomes); ribophagy (ribosomes); ER-phagy or reticulophagy (endoplasmic reticulum); nucleophagy (nucleus); and, in vegetal eucaryotic cells, chlorophagy [24,25].

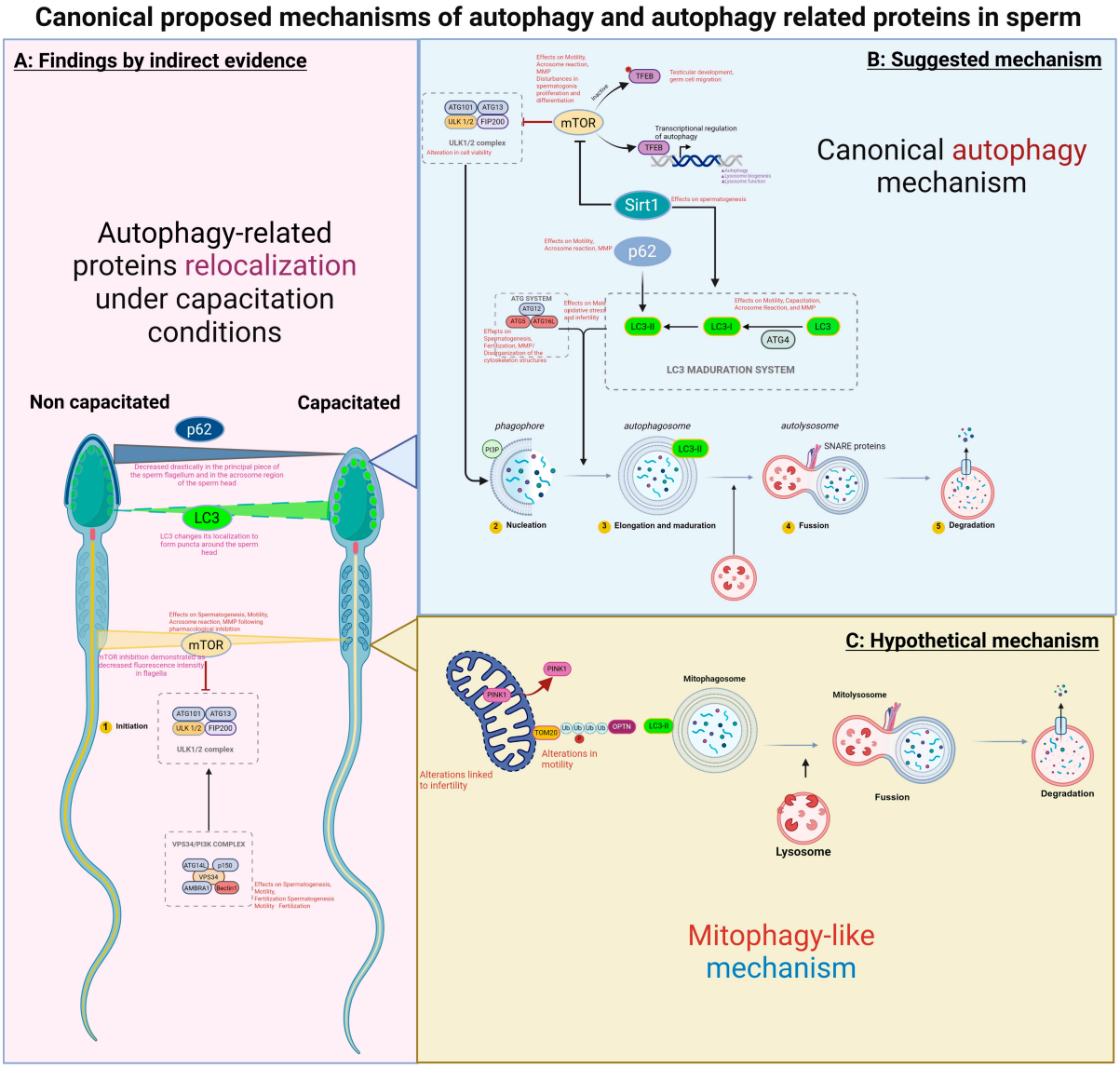


Figure 1. Schematic overview of the proposed canonical sperm autophagy and its major molecular machinery. (**A**) Indirect evidence of the presence of autophagy-related proteins (LC3, p62 and mTOR) in mature sperm and its relocalization under capacitation conditions. (**B**) Suggested mechanism of

canonical autophagy. The pathway is a multistep process divided into five sequential steps: (1) initiation, involving the activation of the ULK 1/2 complex (ULK 1/2, ATG13, FIP200, and ATG101); (2) nucleation, characterized by formation of the phagophore and recruitment of the VPS34/PI3K complex (VPS34, Beclin1, ATG14L, p150, and AMBRA1); (3) elongation and maturation, during which the phagophore expands and closes to form the autophagosome, involving the ATG12, ATG5 and ATG16L conjugation system and LC3 maturation; (4) fusion, in which the mature autophagosome fuses with the lysosome to form the autolysosome, involving SNARE proteins; and (5) degradation, during which lysosomal hydrolases degrade the autophagic cargo, and the resulting macromolecules are recycled into cytoplasm. LC3 is sequentially processed from its precursor from LC3-I and subsequently lipidated to generate LC3-II, which is associated with the autophagosomal membrane. (**C**) Hypothetical mechanism of sperm autophagy-like activity, suggested by the presence of mitophagy-related proteins (PINK1 and TOM20) and its correlation with alterations in motility and fertility.

Despite the recent description of autophagy-related proteins in mature ejaculated sperm from humans, stallion, and epididymal mouse sperm [26–28], early reports have outlined the important role of autophagy in different stages of spermatogenesis [29], as well as fertilization [30,31]. Currently, there is insufficient evidence to relate the activation of autophagy and autophagy flux to sperm development and fertility events or the detailed role of autophagy-related proteins in these events, considering motility, capacitation, and acrosome reaction. Given the small amount of literature regarding autophagy in mature sperm, we have focused our narrative review on the insights needed to synthesize the current literature regarding expression, localization, and participation of autophagy-related proteins in sperm physiology. Additionally, we discuss their role in spermatogenesis and viability, as well as on the kinematic parameters of sperm fertility, such as motility, capacitation, acrosome reaction, mitochondrial membrane potential and fertilization with the purpose of answering the question of why would spermatozoa require autophagy?

2. Materials and Methods

This review aims to synthesize and critically analyze the current and relevant evidence about the role of autophagy-related proteins in sperm physiology, highlighting the strengths, limitations, and perspectives of the recent advancements in this rising field.

2.1. Search Strategy Databases

The research was conducted across the databases available online: PubMed, Scopus and ResearchGate (as a literature-discovery platform). The research included only peer-reviewed articles and studies using sperm from mammals (human, mouse, stallion and swine), germ cells (development/spermatogenesis), ejaculated and epididymal sperm. Excluding criteria covered articles with no peer review (editorials, commentaries, letters to the editor and preprints), or articles with no full text available. Given the limited number of articles meeting these criteria, no restrictions were placed on the publication period regarding the inclusion or exclusion of papers.

2.2. Search Strategy Terms

The search strategy employed keywords related to sperm autophagy. Primary search terms included: “sperm autophagy”, “autophagy-related proteins”, “sperm mitophagy” and “autophagy spermatogenesis”. Boolean operator (“AND”) was used to refine the research: “autophagy AND sperm viability”, “autophagy AND sperm motility”, “autophagy AND sperm acrosome reaction” and “autophagy AND mitochondrial membrane potential”.

3. Autophagy-Related Proteins in Sperm Development

Role of Autophagy-Related Proteins in Spermatogenesis

Spermatogenesis is a complex process by which spermatozoa are produced in the seminiferous tubules perfectly rolled up into the testis [32]. The entire process is divided into four steps: (1) the proliferation of the primordial cells named spermatogonia, (2) differentiation of the spermatogonia into spermatocytes, (3) spermatocytes perform meiotic division to produce round spermatids, and (4) the round spermatids differentiate into spermatozoa through a highly specialized process known as spermiogenesis [33,34]. Spermiogenesis includes major changes involving the condensation of nuclear chromatin, cytoplasm removal, and acrosome biogenesis, as well as the development and implantation of the flagella [35]. During spermatogenesis, autophagy remains active; therefore, impaired autophagy may cause dysfunctional biogenesis of the flagella, abnormal sperm head shape, a reduction in sperm motility, abnormal acrosome biogenesis (globozoospermia), non-obstructive azoospermia, and defects in testosterone synthesis [36]. Additionally, the use of knockout mouse models has provided insights about the role of autophagic proteins in spermatogenesis.

Knockout of autophagy proteins 5 and 7 (ATG5, 7) in Sertoli cells ($Atg5^{-/-}$, $Atg7^{-/-}$) demonstrated their involvement in the ectoplasmic specialization, through the degradation of the negative cytoskeleton organization regulator (PDLIM1). Furthermore, Atg7 knockout in germinal cells revealed the involvement of this protein in acrosome and flagella biogenesis and cytoplasm removal [37–40]. This evidence suggests the particular role of ATG5 and ATG7 in spermatogenesis. Interestingly, evidence has also demonstrated that the transcriptional factor EB (TFEB), known as a key regulator for autophagy, increases rapidly its expression during testicular development after 7 days post-partum, locating primarily in the nucleus of spermatogonia from adult mice. In addition, TFEB was activated for nuclear translocation in the presence of retinoic acid, suggesting that TFEB is related to retinoic acid signaling [41]. Spermatogonia-specific deletion of TFEB ($tfeb^{-/-}$) in mouse induced the impairment of fertility, produced by a defective spermatogenesis and low sperm motility, also driven by metabolic hormonal dysregulation [42].

The negative regulator of autophagy, known as the mechanistic target of rapamycin complex-1 (mTOR1), is a key factor in the proliferation and differentiation of spermatogonia [43]. On one hand, the knockdown of mTORC1/C2 in rat primary Sertoli cells with small interfering RNA (siRNA) reduced the androgen-binding protein expression, producing a stimulation of autophagy flux [44]. On the other hand, conditional knockdown [45] in mouse Sertoli cells induced testicular atrophy, loss of pachytene spermatocytes and spermatids, loss of Sertoli cell polarity, and abnormalities [46]. Additionally, a conditional knockdown in mouse germ cells induced a reduction in the testis size, azoospermia, lack of spermatogonial proliferation, a decrease in pool and germ cell population, and the age-dependent perturbation of testicular development [45,47].

Another protein, ULK1, when knock-down, induced a decrease in autophagy flux in goat Sertoli cells. This observation was confirmed by a reduction in the expression of autophagy-related proteins LC3, Beclin1, ATG5, and ATG7. Moreover, the overexpression of ULK1 induced the activation of autophagy flux in presence of bisphenol A, suggesting that the activity of ULK1 plays an important role in the maintenance of viability [48]. Another member of the ULK family, ULK2, showed a similar role in Sertoli cells from swine [49]. Germ cell-specific knockout mice ($Sirt1^{F/F}$; Tnap-Cre) showed infertility, defects in acrosome biogenesis, globozoospermia-like, and disruption of ATG7 acetylation of LC3. As a result, LC3 changed its localization from the nucleus to the cytoplasm, suggesting abnormal autophagy flux in spermatids [50].

Knockout animals provide insight into the physiological role of proteins, in which the conditional knockout (cKO) of Raptor gene (mTORC1 component) and Rictor gene (mTORC2 component), induced infertility in germ and Sertoli cells and sterility in mouse germ cells, respectively [51,52]. Additionally, the use of rapamycin and the inhibition of mTOR exposed human male infertility as a side effect and its chronic administration in mice induced spermatogenic arrest and meiotic sex chromosome inactivation, which led to the identification of mTOR as a regulator of small RNA homeostasis in male germ cells [53,54].

Sirtuins, considered cellular stress sensors that regulate mitochondrial and nuclear activity, play a central role in the cell to adapt to diverse stress stimuli by performing deacetylation and ADP ribosylation of a wide number of substrates, using NAD^+ as a cofactor in the reaction [55]. Specifically, Sirtuin1 (Sirt1) is expressed in gonads and performs the removal of the acetyl group from histones. Through this activity, Sirt1 regulates several downstream pathways that include the metabolism of glucose and lipids, cell death, and autophagy [56]. Knockout mice for Sirt1 ($Sirt1^{-/-}$) revealed an important role of this protein in the spermatogenesis, where the individuals with this alteration suffered infertility, presenting small testis, reduced sperm count and motility, globozoospermia-like, and apoptotic germ cells; however, many individuals did not survive because of the role of this protein in vital organs [57].

Recent evidence has demonstrated that Beclin1/BECN1, a multidomain protein that dynamically interacts with diverse proteins to form the autophagosome, plays an important role in mouse spermatogenesis, as the cKO mouse ($Becn1^{flox/flox}$) showed partial failure in spermiation, acrosome malformation, low motility, and reduced fertility [58].

Diet is also an important factor that affects the autophagy process in spermatogenesis. A high-fat diet induced a disruption of spermatogenesis related to an overactivation of the autophagy process suggested by a significant increase in Beclin1 levels in a mouse model; moreover, the same increase in Beclin1 was observed in obese infertile patients [59]. Additionally, the incubation of sucralose in male mouse Leyding cells lines (TM3 and TM4) induced an increase in autophagy activation, where the p62/SQSTM levels decreased, followed by an increase in LC3-II levels; the same effect against autophagy was observed under in vivo conditions [60]. Other studies showed that curcumin ameliorates the aging-induced tight-junction damage induced by D-galactose through the AMPK-mTOR signaling pathway in Sertoli cells (TM4), where D-galactose decreased Beclin 1 and LC3 expression. However, the addition of curcumin restored the autophagy flux [61]. Finally, treatments with different concentrations of 5-Hydroxymethylfurfural induced increases in LC3-II and Beclin1 protein levels and inhibited the PI3K/AKT/mTOR signaling pathway in TM4 and type B spermatogonium (GC-1) cell lines, suggesting an activation of autophagy [62].

4. Autophagy-Related Proteins in Mature Sperm

4.1. Function of Autophagy-Related Proteins in Sperm Viability

A healthy sperm cell is required for successful fertilization. One of the most important characteristics evaluated in motile and non-motile sperm is viability [63], which is related to sperm membrane function, evidencing cells with an intact plasma membrane, and whether the sperm cell is dead or alive [64]. Under this scenario, could autophagy play a vital role in viability, determining whether a cell lives or dies. Therefore, the role of autophagy in cell death is classified into two different classes: autophagy-dependent cell death and autophagy-independent programmed cell death. On one hand, autophagy-dependent cell death is highly conditioned by the autophagy components [13]. On the other hand, in autophagy-independent cell death, the autophagosomes break down the cell matrix components with no apparent degradation of organelles [65].

The pharmacological intervention with chloroquine in human-ejaculated sperm did not affect viability in a significant manner. Moreover, the possible activation of autophagy using rapamycin did not affect sperm viability. However, both drugs induced changes in the LC3-II/I ratio, suggesting alterations in autophagy activity [26]. Furthermore, the induction of oxidative stress in human-ejaculated sperm produced an activation of autophagy to maintain sperm viability and lifespan; such activation was evidenced by the decrease in LC3-I and the accumulation of LC3-II, ATG5, ATG16, and pAMPK using ionomycin. This direct experimental evidence supports the hypothesis of autophagic flux [66]. In cryopreserved stallion spermatozoa, in which sperm viability decreases significantly, the hypothesis that autophagy plays an important role during storage as well as in apoptotic mechanisms is presented. The evidence showed an increase in LC3-II protein levels, suggesting sustained autophagic activity and a new possible mechanism for survival during short- and long-term storage [11,67]. In a rat model of varicocele, the levels of Atg7 and LC3-II increased, suggesting the hypothetical activation of autophagy. The findings of exacerbated autophagic activity have been considered as a preliminary step to switch towards apoptosis [68]. Currently, the pharmacological approach in sperm autophagy has limitations since there is not a specific drug for a protein target to show the real effect of the inhibition of autophagy in sperm physiology.

4.2. Role of Autophagy-Related Proteins in Sperm Motility

At the end of spermatogenesis, immature sperm rises from the lumen of the seminiferous tubules to the epididymis and changes to mature sperm. Among these changes, sperm acquires motility, which is essential to male fertility [69]. Sperm motility is defined as the propagation of transverse waves along the flagellum in a proximal–distal direction, producing an impulse that propels the spermatozoon [70]. In human-ejaculated sperm, the activation of autophagy through the inhibition of mTOR using rapamycin led to a significant increase in progressive and rapid motility, accompanied by a caspase 3/7 activation, a decreased expression of PTEN-induced kinase 1 (PINK1), and translocation of the outer mitochondrial membrane 20 (TOM20) [26]. However, the pharmacological intervention using chloroquine produced a significant decrease in progressive and rapid motility and an increase in PINK1 and TOM20 expression. This contradiction probably indicates that mTOR signaling also modulates mitochondrial turnover, inducing an increase in mitochondrial respiration when inhibited [11]. In epididymal mouse sperm, ATG5 is required for sperm motility, since the conditional knockout mouse ($Atg5^{F/F}$; *Stra8− iCre*) resulted in a low sperm count and low sperm motility due to defects in the middle piece of the flagella compared to the wild type. Additionally, Atg5 cKO mice showed disruption to male germ cells, abnormal acrosome formation, abnormal mitochondria distribution, defective nuclear structure, and a low fertilization rate [71]. Furthermore, in male patients diagnosed with asthenozoospermia (low sperm motility), immunochemistry staining showed the localization of LC3 mainly in the sperm's neck and overexpression of autophagy-related proteins, LC3, Atg5, Atg7 and Beclin1 [72]. Sperm from patients with the same diagnosis treated with granulocyte–macrophage colony stimulating factor showed a significant increase in motility and LC3-II and Atg7 expression, suggesting that the induction of autophagy enhances sperm motility [73]. Oligoasthenoteratozoospermia is characterized by the impairment of sperm parameters (low sperm count, low sperm motility, and abnormal sperm morphology), and patients diagnosed with this pathology showed a decrease in LC3-II and an increase in p62/SQSTM expression; in addition, sperm showed a decline in early embryonic development [74]. Male patients diagnosed with globozoospermia (round-headed sperm syndrome) and low sperm motility showed an increase in Atg7 levels, but the expression

of LC3-II remained unaltered, suggesting that autophagy initiated but did not completely produce sperm abnormalities [75]. This evidence provides insights into the involvement of autophagic proteins in achieving normal sperm motility parameters. On one hand, studies in mouse epididymal sperm demonstrated that the use of CQ, rapamycin, and K67 (an inhibitor of p62/SQSTM) significantly decreased the total and progressive motility after 90 min of incubation, suggesting the possible role of autophagy-related proteins in mouse sperm motility [28]. On the other hand, Atg5 cKO mice ($Atg5^{-/-}$) did not affect sperm motility. However, a drastic increase in the LC3-II and p62/SQSTM protein levels was observed, suggesting an impairment of autophagy activation [76].

This contradiction could be attributed to a low-specific effect on the pharmacological approach, where Atg5 cKO mice ($Atg5^{-/-}$) results appear to be more reliable; however, the pharmacological approach was performed under capacitation conditions, and the Atg5 cKO mice ($Atg5^{-/-}$) were recorded under non-capacitation conditions.

Finally, the toxic effects of cadmium in sperm from rats and its toxic effects were neutralized with melatonin and ginseng, as shown by the increase in sperm motility and probably regulation of autophagy, where the LC3 protein levels significantly decreased [77]. Overall, the evidence suggests that the maintenance of sperm motility could be modulated by the activation of autophagy-related proteins; therefore, these studies explore the functional dependence of sperm physiology on these mechanisms.

4.3. Activity of Autophagy-Related Proteins in Sperm Capacitation

Capacitation was first described as the capacity of the sperm to fertilize an egg after several hours of being injected into the pro-ovarian sac of mammals [78,79]. Currently, research advances have added other physiological changes related to the capacitation, including the rise in the intracellular concentrations of HCO_3^-, which promotes the alkalization of the pHi, which, in turn, leads to the activation of the Ca^{2+} and K^+ channels [80,81]. The increase in intracellular concentrations of Ca^{2+} and the alkalization of the pHi result in the activation of the soluble adenylyl cyclase [82] that synthesizes the cAMP needed for the subsequent activation of the protein kinase A (PKA) signal pathway, the hyperpolarization of the plasma membrane, the phosphorylation of tyrosine residues, and the acquisition of a special motility pattern named hyperactivation [83,84]. Under capacitation conditions, human-ejaculated sperm showed a hypothetical activation of autophagy-related proteins, where LC3 lipidation, showed a shift, as well as the relocalization of p62/SQSTM from the acrosome region and middle piece to the middle piece of the flagellum after 2 h of incubation under capacitation conditions, suggesting the presence and redistribution of autophagy markers under capacitation conditions [26]. In mouse epididymal sperm incubated under no capacitation conditions, LC3-I, p62/SQSTM, and mTOR showed a homogeneous distribution in the sperm head and the principal piece of flagellum. In contrast, under capacitation conditions, these autophagy-related proteins redistribute; in particular, LC3 appears as foci (showing puncta) around the acrosome region, and p62/SQSTM significantly decreased its fluorescence intensity. These changes suggest the hypothetical formation of autophagosomes [28]. Nevertheless, these hypothetical formation of autophagosomes under capacitation conditions suggests that the autophagy flux did not progress further than the elongation and maturation step. Although LC3-II and p62/SQSTM are considered important autophagy markers, the LC3-II amount during capacitation does not necessarily indicate the degree of autophagy flux [85], suggesting that the capacitation conditions do not support complete autophagy flux; hence, capacitation only supports the hypothetical activation of autophagy [28]. However, in fresh ejaculated porcine spermatozoa, autophagy could be active under capacitation conditions, showing a shift in LC3 lipidation and reduced p62/SQSTM protein levels. It is important to mention that more evidence and detection

methods are needed to determine whether the autophagy flux is active and its degree during sperm capacitation. Moreover, treatment with CQ (50 mM) increased the LC3 lipidation level and induced p62/SQSTM accumulation, which may reflect the hypothetical impairment of lysosomal degradation [86]. Finally, the presence of the autophagy-related proteins Atg16, Beclin1, and LC3 were detected mainly in the sperm head from stallion-ejaculated sperm. Additionally, LC3 was significantly processed, and Atg16 and Beclin1 underwent a redistribution from the post-acrosome region to the acrosome after 3 h of incubation under capacitation conditions [27]. Autophagy-related proteins from human and stallion sperm showed notable redistribution differences from mouse epididymal sperm, since they belong to sperm that has been in contact with seminal fluid; meanwhile, mouse epididymal sperm does not have these physiological stimuli. Note that observing the detection or modulation of autophagy-related proteins does not constitute direct evidence of functional canonical autophagy or complete autophagy flux in mature sperm under capacitation conditions.

4.4. Connection of Autophagy-Related Proteins in Acrosome Reaction

After capacitation, selected sperm undergo a highly regulated process called the acrosome reaction, which was first described in starfish and sea urchin [87,88]; this event comprises the fusion between acrosomal and plasma membranes, where there is a Ca^{2+}-dependent exocytic event [89]. The membrane fusion allows the release of the lytic acrosomal content to cause the enzymatic degradation of the egg's coat [90]. In ejaculated stallion sperm, the induction of the acrosome reaction is closely related to the processing of LC3, as it is redistributed to the sperm acrosome, followed by an LC3 fluorescence decrease, suggesting the hypothetical presence and fusion of autophagosome with lysosome; therefore, it is argued that autophagy flux continues [11,91]. Moreover, pharmacological intervention with chloroquine and E-64d resulted in a significant decrease in LC3 processing, suggesting that the acrosome reaction could be modulated by the proposed presence of the autophagy flux [27]. The existence of evidence has been demonstrated, thereby suggesting the presence of structures associated with the process of autophagy. Furthermore, in epididymal mouse sperm, the induction of the acrosome reaction with calcium ionophore A23187 was impeded significantly by the presence of chloroquine, rapamycin, and K67, suggesting that the hypothetical activation of autophagy-related proteins is associated with the induction of acrosome reaction [28], or we can hypothesize, since the acrosome structure is formed from Golgi apparatus, an intact acrosome may be required to maintain autophagy flux. LC3-II is considered an autophagosomal marker of canonical autophagy in mammalian sperm [74,75]; however, it is not clear if the lipidation of LC3 during the induction of acrosome reaction is a canonical or non-canonical autophagy event, since this event may differ from canonical degradative autophagy. Notably, the detection or modulation of autophagy-related proteins should not be interpreted as direct evidence of functional canonical autophagy or complete autophagic flux in mature spermatozoa during the induction of acrosome reaction.

4.5. Role of Autophagy-Related Proteins in Mitochondrial Activity and Mitochondrial Membrane Potential

Mitochondrial membrane potential is defined as the electron transfer through the respiratory chain complexes producing a pumping of the protons from the mitochondrial matrix into the inner mitochondrial membrane, generating an electric potential gradient across the inner mitochondrial membrane [92,93]. This membrane potential will be helpful for the synthesis of ATP, to produce reactive oxygen species (ROS) and oxidative stress. Additionally, changes in mitochondrial membrane potential influence Ca^{2+} homeostasis [26]. The mitochondrial function is closely related to the special type

of autophagy named mitophagy, which is classified as a macroautophagy where superfluous or unre-pairable mitochondria are removed one by one to maintain quality control [94]. In ejac-ulated human sperm, the mitochondrial activity was monitored using Mito Tracker Red CMXRos staining, where LC3 was also localized, suggesting a relation between mitochondrial activity and autophagy-like mechanisms [11]. PINK1 protein is considered a sensor of mitochondrial status and responsible for mitophagy in somatic cells [95] and TOM20 is an outer mitochondrial membrane protein used to evaluate mitochondrial mass during mitophagy [96], both were found in human-ejaculated sperm [59]. Treatment with chloroquine induced an increase in PINK1 and TOM20 protein levels and an increase in mitochondrial activity, whereas the treatment with rapamycin promoted a substantial decrease in PINK1 and TOM20 protein levels and also a decrease in mitochondrial activity, suggesting that activities of PINK1 and TOM20 hypothetically contribute to sperm mitophagy-like mechanism [11]. However, in epidydimal mouse sperm, the treatments with chloroquine, rapamycin, and K67 induced a decrease in the mitochondrial membrane potential [28]. Furthermore, in fresh ejaculated porcine sperm, the presence of chloroquine (50 μM) also induced a reduction in the mitochondrial membrane potential [86]. This evidence in both mouse and porcine sperm suggests that these drugs could hypothetically inhibit a mitophagy-like mechanism. Also, in fresh ejaculated and refrigerated stallion sperm, the evaluation of mitochondrial membrane potential showed no change after storage; however, these conditions induce an increase in LC3-I processing [67], suggesting the hypothetical presence of autophagy as a possible survival mechanism not linked to sperm mitophagy-like mechanisms. In male patients diagnosed with asthenozoospermia, ATP and mitochondrial membrane potential were reduced, and the protein levels of autophagy-related proteins LC3, Atg5, Atg7, and Beclin1 increased, suggesting two perspectives, namely, that the hypothetical presence of autophagy is associated with mitochondrial function [72], or the mitochondrial membrane potential may contribute to hypothetical mitophagy-like mechanism as well as to sperm motility; however, more evidence is needed to support the hypothesis of selective autophagy in mature sperm. Notably, identifying or altering of autophagy-related proteins should not be taken as direct evidence of functional mitophagy or complete autophagic flux regarding sperm mitochondial activity and membrane potential.

5. Autophagy-Related Proteins in Fertilization

5.1. Role of Autophagy-Related Proteins in Pre-Fertilization

Fertilization is a complex multistep process, where capacitated sperm reaches the oocyte and, through the acrosomal reaction, sperm crosses the cumulus oophorus and zona pellucida to fuse with the oolemma. Therefore, we conclude with the final fusion between the spermatozoa and the oocyte, blending genetic information from individuals and transferring genes to the next generation [2,97]. In various species, the mitochondrial genome comes from the maternal side; hence, the focus of many studies on the role of autophagy in fertilization is specifically related to mitophagy [98]. In mammals, sperm mitochondria are degraded early in the preimplantation embryo. However, the mechanism and timing by which the sperm mitophagy takes place will depend on the species [99].

5.2. Role of Autophagy-Related Proteins in Post-Fertilization

In pig and monkey resus, p62/SQSTM is associated with sperm mitochondria after fertilization, and the inhibition of this autophagy-related protein with proteosomal inhibitor MG132 delays the post-fertilization sperm mitophagy [100]. Nevertheless, evidence in mammals suggests that despite the presence of autophagy-related proteins LC3

and p62/SQSTM localized near mitochondria before the 2-cell stage, autophagy did not eliminate mitochondria and mitochondrial DNA [101]. Furthermore, embryos fertilized with spermatozoa from autophagy-related protein 5 knockout mice ($Atg5^{-/-}$) failed to progress to the four and eight-cell stages during early post-fertilization development. In contrast, normal embryonic development occurred when fertilization was performed using sperm from wild-type mice. Additionally, maternal Atg5 protein persisted in the cytoplasm, suggesting that autophagy plays a critical role during the post-fertilization stages of embryonic development [102].

6. Conclusions

Autophagy is a key process present during sperm development and recently described in mature sperm, which includes the participation of diverse proteins such as LC3, p62/SQSTM, mTOR, ATG5, Beclin1, ATG7, Sirtuin1, ATG16, PINK1, TOM20, TFEB, and ULK-1. A more robust body of evidence has emerged that substantiates the involvement of autophagy-related genes and pathways in germ-cell development and spermatogenesis in different mammals (human, mouse, stallion, and swine).

The absence of these autophagy-related proteins in mouse KO models does not necessarily indicate the presence of active autophagy in mature sperm; moreover, pharmacological approach generates moderate or indirect evidence which suggest a correlation between autophagy-related proteins and several functional parameters of mature sperm. Therefore, the question remains: Why would spermatozoa need autophagy? Evidence suggests that the role of autophagy in spermatogenesis is related to the biogenesis of the flagella, formation of the sperm head shape, sperm motility, acrosome biogenesis, cytoplasm removal, and even the disruption of spermatogenesis; accordingly, we can argue that the autophagy flux pertains to the biogenesis of the spermatozoa. Once the sperm is differentiated, this cell must achieve maturation in the epididymis to finally be stored in the *vas deferens*; upon ejaculation, sperm reaching the female reproductive tract will try to engage in capacitation, hypermotility, the acrosome reaction, mitochondrial membrane potential, and fertilization. All these events of sperm fertility have a high energy demand, which is supported by the sperm's energy metabolism or by substrates present in the genital reproductive tract; we can also hypothesize that this energy is obtained through the potential autophagy process. Nevertheless, in human and mouse sperm, the pharmacological intervention produced a decrease in sperm motility and impeded the induction of the acrosome reaction. Moreover, fertility-related abnormalities (oligozoospermia, asthenozoospermia, teratozoospermia, and azoospermia) are also associated with impairment of potential autophagy [103]. With this evidence, we can also hypothesize that the autophagy process is also needed to prevent the accumulation of damaged materials, biomolecules, and organelles, probably induced by starvation or stress conditions; therefore, sperm would need autophagy to overcome these events to perform fertilization. In addition, both the presence and physiological necessity of complete autophagic flux in mature sperm remains unclear. Supported by the current evidence, we can hypothesize that in sperm development and mature sperm, canonical macroautophagy is arguably present, since the presence of autophagy-related proteins was described (Table 1) and their correlation with the functional parameters of mature spermatozoa was indicated by indirect or associative evidence. The investigation of unresolved evidence concerning complete canonical autophagy flux, bona fide mitophagy, and their physiological necessity in mature spermatozoa is imperative.

Table 1. Autophagy-related proteins and their function in sperm fertility.

Species	Cell Type/Stage	Localization	Type of Scientific Evidence	Role in Sperm Fertility	Limitations of Study	Refs.
			LC3 			
Human	Mature sperm	Head Principal piece	Pharmacological evidence Use of the inhibitor CQ	Disruption of motility, capacitation, acrosome reaction and MMP	Research restricted only to pharmacological interventions that could have off target effects. Furthermore, researchers acknowledge that the described autophagy-related processes in sperm could be related to additional functions such as acrosome reaction.	[26]
Mouse	Mature sperm	Head Principal piece	Pharmacological evidence Use of the inhibitor CQ	Affects motility, capacitation, acrosome reaction and MMP	Research restricted only to pharmacological interventions that could have off target effects. Authors recognize that more evidence is required to confirm if capacitation conditions induce and maintain autophagy.	[28]
Stallion	Mature sperm	–	Pharmacological evidence Use of the inhibitor CQ	Disrupts survival	Research restricted to pharmacological interventions that could have off target effects. Moreover, authors admit that more studies are needed to clarify if autophagy is performed in equine sperm as in somatic cells.	[11,67]
			p62/SQSTM 			
Human	Mature sperm	–	Observational study	Infertility	Authors demonstrate that autophagy activity is impaired (including expressioin of p62) sperm of intertile men with oligoasthenoteratozoospermia	[74]
Mouse	Mature sperm	Head Principal piece	Pharmacological evidence Use of the inhibitor K67	Abnormal motility, acrosome reaction and MMP	Research restricted only to pharmacological interventions that could have off target effects. Authors recognize that more evidence is required to confirm if capacitation conditions induce and maintain autophagy.	[28]
Stallion	Mature sperm	Head Principal piece	Pharmacological evidence Use of the inhibitor CQ	Abnormal motility, acrosome reaction and MMP	Research restricted only to pharmacological interventions that could have off target effects	[26]

Table 1. *Cont.*

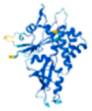
Species	Cell Type/Stage	Localization	Type of Scientific Evidence	Role in Sperm Fertility	Limitations of Study	Refs.
			mTOR 			
Mouse	Mature sperm	Head Principal piece	Pharmacological evidence Use of the inhibitor RA	Altered spermatogenesis, motility, acrosome reaction and MMP	Research restricted only to pharmacological interventions that could have off target effects. Authors recognize that more evidence is required to confirm if mTOR can induce and maintain autophagy under capacitation conditions.	[28]
Mouse	Spermatogonia	–	Genetic engineering evidence Stra8-Cre-induced mTOR deletion/mTOR KO mice	Disturbances in spermatogonia proliferation and differentiation	Authors mention that their study is limited due to the unspecific detection of mTOR in KO mice	[45,47]
			ATG5 			
Human	Mature sperm	Cytoplasm	Observational study	Alterations in motility, fertilization and MMP	Authors concluded that autophagy scavenge mROS to maintain sperm motility	[72]
Mouse	Mature sperm	–	Genetic engineering evidence *Atg5*$^{-/-}$ KO mice	Disorganization of the cytoskeleton structures	The scope is restricted due to the other possible mechanisms involved in the distribution of TBCs (tubulobulbar complexes)	[38]
			ATG6 (Beclin1) 			
Human	Mature sperm	–	Observational study in obese individuals	Impaired viability and motility	Authors demonstrate autophagy is overactivated (as evidenced by Beclin 1 increased expression) in obese and subfertile individuals. Additionally, semen parameters were lower than reference limits. The research is limited because they do not know which molecules from the HFD induces deregulation of autophagy.	[59]
Mouse	Mature sperm	–	Pharmacological evidence Use of 3-MA, CQ and PA (palmitic acid)	Abnormal viability and motility	Authors demonstrate that CQ and 3-MA attenuated abnormal sperm parameters caused by PA	

Table 1. *Cont.*

Species	Cell Type/Stage	Localization	Type of Scientific Evidence	Role in Sperm Fertility	Limitations of Study	Refs.
Mouse	Mature sperm	Head	Genetic engineering evidence *Becn1*$^{flox/flox}$ KO mice	Abnormalspermatogenesis, motility and fertilization	Researchers recognize that the limitation of their study is that KO mice exhibit diverse alterations and malformations yet retain partial fertility.	[58]
Mouse	Spermatogonium (GC-1 cell line)	Type B Spermatogonium	Pharmacological evidence Use of HMF (5-Hydroxymethylfurfural)	Altered spermatogenesis	Authors concluded that HMF disrupts cellular homeostasis and triggers the simultaneous induction of both apoptosis and autophagy	[62]
			ATG7 			
Human	Mature sperm	Cytoplasm	Pharmacological evidence Use of the inhibitor 3-MA	Altered spermatogenesis MMP	Research limited to off target effects of the 3-MA autophagy inhibitor	[72]
Mouse	Sertoli cells	Cytoplasm	Genetic engineering evidence *Atg7*$^{-/-}$ KO mice	Disorganization of the cytoskeleton structures Abnormal motility	Authors propose a new physiological role of autophagy in building a Sertoli cell specific structure (tubulobulbar complexes) to facilitate Sertoli-germ cell communication	[38]
			Sirt1 			
Human	Mature sperm	Head/neck junction (connecting piece)	Pharmacological evidence using YK 3-237	Suggested positive role in capacitation	Authors mention further evidence is needed to establish the exact mechanism of YK 3-237 (Sirtuin1 activator) on human sperm capacitation-related events	[104]
Mouse	Primitive spermatogenic stem cells	–	Genetic engineering evidence *Sirt1*$^{-/-}$ KO mice	Impaired spermatogenesis	Authors suggest the possible off-target effects of Sirt1 deficiency and compensation mechanisms of other sirtuins.	[57]

Table 1. *Cont.*

Species	Cell Type/Stage	Localization	Type of Scientific Evidence	Role in Sperm Fertility	Limitations of Study	Refs.
			ATG16 			
Human	Mature sperm	Head	Pharmacological evidence Use of the inhibitor CQ and treatment with H_2O_2	Male oxidative stress Impairment of sperm quality Sperm death	Authors mention the limitation of the study as the pharmacological and oxidative-stress experiments that could have off target effects	[66]
Stallion	Mature sperm	Head	Pharmacological evidence Use of the inhibitors CQ, E-64d, 3-MA, and STF-62247	Alterations in capacitation and acrosome reaction Infertility	Authors recognize the limitation of the off-target effects of pharmacological experiments and lack of evaluation of components of noncanonical autophagy	[27]
			PINK1 			
Human	Mature sperm	Midpiece (mitochondrial sheath)	Pharmacological evidence Use of the inhibitors CQ, BA1, and RP	Infertility	The research is limited to possible off-target effects of the pharmacological experiments. Additionally, authors could not reach a conclusion regarding mitophagy in sperm is similar to somatic cells.	[26]
			TOM20 			
Human	Mature sperm	Midpiece	Pharmacological evidence Use of the inhibitors CQ, BA1, and RP	Impaired motility	The study is limited to possible off-target effects of the pharmacological experiments. Furthermore, authors could not reach a conclusion regarding mitophagy in sperm is similar to somatic cells.	[26]

Table 1. *Cont.*

Species	Cell Type/Stage	Localization	Type of Scientific Evidence	Role in Sperm Fertility	Limitations of Study	Refs.
			TFEB 			
Mouse	Spermatogonia	Mitochondria	Genetic engineering evidence *TFEB*$^{-/-}$ KO mice	Defective spermatogenesis, motility	Authors address the possible off-target effects of TFEB deficiency and compensation in the knockout model	[42]
Mouse	Differentiating spermatogonia in mice/GC-1 cells	Mainly in nucleus	Pharmacological evidence Use of the inhibitor RA	Mouse testicular development, germ cell migration	The study is limited because the expression of TFEB in testis was insufficient to evaluate its role in spermatogonia, purification and in vitro studies of spermatogonia are necessary	[41]
			ULK1 			
Goat	Sertoli cells	Cytoplasm	Genetic engineering evidence ULK1 knockdown mice	Alteration in cell viability	Authors acknowledge the necessity to evaluate other pathways that fine-tube ULK-1	[48]
			ULK2 			
Swine	Sertoli cells	–	Genetic engineering evidence Expression of ULK2 was downregulated by miR-26a	Defective spermatogenesis	Authors mention that more evidence is needed to validate the specific target of miR-26a, ULK2 and its regulatory role in autophagy	[49]

CQ: Chloroquine, RP: Rapamycin, RA: Retinoic acid, 3-MA: 3-Methyladenine, BA1: Bafilomycin A1. LC3 (PDB ID: 1UGM); p62/SQSTM (PDB ID: 3B0F); mTOR (PDB ID: 4JSV); ATG5 (PDB ID: AF_AFA9UGY9F1); ATG6-Beclin1 (PDB ID: AF_AFQ4A190F1); ATG7 (PDB ID: 3VH2); Sirt1 (PDB ID: 5BTR); ATG16 (PDB ID: 6Y09); PINK1 (PDB ID: 5OAT); TOM20 (PDB ID: 2V1T); TFEB (PDB ID: 7Y62); ULK1 (PDB ID: 6QAS); ULK2 (PDB ID: 6QAV).

7. Perspectives

A significant amount of evidence presented in this work came from mouse KO models and pharmacological approaches; nevertheless, there is a lack of specific drugs to target autophagy-related proteins, since chloroquine, rapamycin, K67, E-64d, and even Bafilomycin A1 have non-specific protein targets and many secondary effects. Moreover, autophagy shows a series of methodological challenges; a major tool to directly evaluate autophagy flux is needed, since Western blotting and immunofluorescence localization have shown limitations. The final goal will be to resolve the inconsistencies in sperm development, discerning whether changes in autophagy-related proteins are a cause or consequence of sperm dysfunction and also to identify which ATG proteins are functionally required. In mature sperm, key challenges include elucidating interspecies differences in the autophagic process and the degree to which these mechanisms are conserved. Additionally, interpreting LC3-II and p62/SQSTM levels will be essential to determine whether complete canonical autophagy occurs in these cells and to explore the potential of autophagy-related pathways as biomarkers of sperm quality. Finally, more experimental approaches are required to differentiate canonical autophagy from non-canonical in LC3-associated processes and to distinguish the mitochondrial functional changes from direct evidence of mitophagy.

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Abbreviations

LC3	Microtubule-associated protein 1A/1B-light chain 3
p62/SQSTM1	Sequestosome 1
mTOR	Mammalian target of rapamycin
ATG5	Autophagy-related 5
ATG6	Autophagy-related 6
ATG7	Autophagy-related 7
ATG14	Autophagy-related 14
SIRT1	Sirtuin 1
ATG16	Autophagy-related 16
PINK1	PTEN-induced kinase 1
TOM20	Translocase of the outer mitochondrial membrane 20
TFEB	Transcription Factor EB
ULK1	Unc-51-like autophagy activating kinase 1
ULK2	Unc-51-like autophagy activating kinase 2
PKA	Protein kinase A
pHi	Intracellular pH
ROS	Reactive oxygen species

PI3-K	Phosphoinositide 3-kinase
PI3-P	Phosphatidylinositol-3-phosphate
cKO	Conditional knockout
TRAPPIII	Transport protein particle III
VPS15	Vacuolar protein sorting 15
VPS34	Vacuolar protein sorting 34
AMBRA1	Activating molecule in BECN1-regulated autophagy protein 1
SNARE	Soluble N-ethylmaleimide-sensitive-factor attachment protein receptor
STX7	Syntaxin-7
SNAP29	Synaptosome-associated protein 29
VAMP8	Vesicle-associated membrane protein 8

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