FYCO1 and autophagy control the integrity of the haploid male germ cell-specific RNP granules

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ABSTRACT

Ribonucleoprotein (RNP) granules play a major role in compartmentalizing cytoplasmic RNA regulation. Haploid round spermatids that have exceptionally diverse transcriptomes are characterized by a unique germ cell-specific RNP granule, the chromatoid body (CB). The CB shares many characteristics with somatic RNP granules but also has germline-specific features. The CB appears to be a central structure in PIWI-interacting RNA (piRNA)-targeted RNA regulation. Here, we identified a novel CB component, FYCO1, which is involved in the intracellular transport of autophagic vesicles in somatic cells. We demonstrated that the CB is associated with autophagic activity. Induction of autophagy leads to the recruitment of lysosomal vesicles onto the CB in a FYCO1-dependent manner as demonstrated by the analysis of a germ cell-specific FYCO1 conditional knockout mouse model. Furthermore, in the absence of FYCO1, the integrity of the CB was affected and the CB was fragmented. Our results suggest that RNP granule homeostasis is regulated by FYCO1-mediated autophagy.

Introduction

The genome of meiotic and postmeiotic male germ cells is expressed actively and broadly, which results in the production of a diverse transcriptome that has to be accurately regulated. These cells are faced with the responsibility of determining which transcripts should be eliminated and which ones directed for translation or other functions. Furthermore, due to the cessation of transcription in condensing elongating spermatids, long-term mRNA storage is required to supply necessary mRNAs for these transcriptionally inactive cells. To support the differentiation of round spermatids, the CB diminishes in size and forms a central structure in the CB that takes part in organizing the mitochondrial sheath of the midpiece. Leftover material from the CB is finally discarded with the rest of the cytoplasm in the residual body. In elongating spermatids, the CB is suggested to change its function, and this transformation is accompanied by the disappearance of typical CB components such as DDX4 and PIWI proteins and the appearance of testis-specific kinases TSSK1 and TSSK2 and their substrate TSKS (testis specific serine kinase substrate). Successful isolation of CBs from mouse testes has enabled extensive characterization of their molecular composition.

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CB contains several types of RNAs, including mRNAs, long non-coding RNAs, intergenic transcripts and PIWI-interacting RNAs (piRNAs), as well as a wide variety of RNA-binding proteins.12 The piRNA pathway is particularly prominent in the CB. The functions of piRNAs are diverse. In prospermatogonia, they play an important role in genome defense by silencing transposon expression.14-17 Postnatal pachytene piRNAs also direct meiotic and postmeiotic mRNAs and long noncoding RNAs for degradation.18-21 Pachytene piRNAs and PIWI proteins accumulate in the CB, and the current hypothesis is that RNA is targeted to the CB for piRNA-mediated degradation.

The CB is a dynamic structure that actively moves in the cytoplasm of round spermatids in a microtubule-dependent manner.22 It makes frequent contacts with the nuclear envelope and continuously sends and receives small particles.23,24 The CB is a nonmembrane bound organelle, but interestingly, it closely communicates with the cellular endomembrane system. It makes frequent contacts with the Golgi complex25 and it is always associated with multivesicular bodies and small vesicular structures that are often found embedded in the CB pockets.26

Recent reports have demonstrated the involvement of autophagy in the assembly and clearance of stress granules that are stress-responsive somatic RNP granules.27-29 In autophagy, part of the cytosol, including proteins or organelles, is sequestered into a double-membrane structure called a phagophore, which then closes upon itself to form an autophagosome. Autophagosomes subsequently fuse with late endosomes or directly with lysosomes, which leads to the degradation of the cargo by lysosomal proteases.30 FYCO1 (FYVE and coiled-coil domain containing 1) is a phosphatidylinositol 3-phosphate-binding protein that is involved in the plus end-directed transport of autophagosomes along microtubules.31 FYCO1 interacts with MAP1LC3/LC3 (microtubule-associated protein 1 light chain 3, LC3 hereafter) proteins31-33 that are lipidated to be anchored on both sides of the phagophore membrane, where they act in recruitment of cargo and other autophagic proteins to phagophores, as well as in facilitation of phagophore expansion.34 FYCO1 has also been implicated in the maturation of early phagosomes into late LAMP1 (lysosomal-associated membrane protein 1)-positive phagosomes,35 in the formation of tubular lysosomes in macrophage cell line upon lipopolysaccharide treatment36 and in the microtubule-dependent transport of late endosomes via endoplasmic reticulum-endosome contact sites to produce cell protrusions and neurite outgrowth.37 The intriguing connection between the nonmembrane-bound CB and cytoplasmic vesicles prompted us to investigate the role of autophagy in the CB function. In this study, we aimed to clarify the nature of the CB-associated vesicles and identify key factors mediating the communication between the CB and the autophagosome/lysosome system.

Results

FYCO1 is a novel CB component

We took advantage of the recently published CB proteome to explore novel players in CB function. We found that FYCO1 was consistently coprecipitated with the CB.17 (Fig. 1A). FYCO1 was shown to be a ubiquitous protein expressed in all studied tissues, with a relatively high expression level in the testes (Fig. 1B). Both Foxi mRNA and FYCO1 protein expression increased toward the latter time points during the first wave of spermatogenesis (Fig. 1C, D), indicating an increased expression in meiotic and especially postmeiotic cells in comparison to spermatogonia. Immunofluorescence analysis of adult (10 wk) testis sections confirmed that the FYCO1 protein was readily detected in late pachytene spermatocytes, round spermatids and elongated spermatids (Fig. 1E). FYCO1 localized in the cytoplasm, and the signal was concentrated in distinctive granules (Fig. 1E).

Costaining of FYCO1 with the CB markers DDX4, PIWIL1/MIWI (piwi-like RNA-mediated gene silencing 1) and DDX25 (DEAD [Asp-Glu-Ala-Asp] box polypeptide 25) validated that the FYCO1-positive granules in round spermatids represent CBs (Fig. 2A). Colocalization with DDX4 and DDX25 was detected already in the smaller cytoplasmic granules in late pachytene and diplotene spermatocytes that correspond to the CB precursors (Fig. 2B). Interestingly, costaining with TSKS demonstrated that in elongating spermatids, the FYCO1 signal remained in so-called late CBs that are devoid of round spermatid CB markers (Fig. 2A). These results revealed the association between FYCO1 and the CB throughout the existence of this structure, from the formation of CB precursor granules to the late CB stage in elongating spermatids.

Closer examination of the FYCO1 localization in round spermatids demonstrated that the FYCO1 signal did not completely overlap with the DDX4 or PIWIL1 signals. In contrast, FYCO1 appeared to localize in the peripheral region of the CB (Fig. 2C, upper row). Some FYCO1-positive small granules were also observed outside the main CB structure (Fig. 2C, bottom row). To better visualize the localization of FYCO1 in relation to the CB, we created a 3-dimensional model from confocal microscopy z stacks of round spermatids immunostained for FYCO1 and PIWIL2/MIWI (piwi-like RNA-mediated gene silencing 2), yet another CB marker (Fig. 2D). Confocal imaging revealed partial overlap of the FYCO1 signal with the PIWIL2 signal at the periphery of the CB. However, the FYCO1 signal also extended outside the PIWIL2-positive CB matrix (Fig. 2D, left panel). Size- and location-wise FYCO1-positive areas matched with the CB-associated vesicles as visualized by electron microscopy (EM; see Fig. 6 for an example). 3D imaging revealed the spreading of the FYCO1 signal at a specific surface of the CB (Fig. 2D, middle and right panels).

To further elucidate the possible membrane association of FYCO1, we performed a tubule culture experiment by incubating stage-specific (II-V) pieces of seminiferous tubules in the absence or presence of wortmannin, a well-characterized inhibitor of phosphatidylinositol 3-kinase.38 Phosphatidylinositol 3-kinase produces a pool of phosphatidylinositol 3-phosphate that is important for autophagosome formation and maturation.39-41 FYCO1 has been shown to bind phosphatidylinositol 3-phosphate via its FYVE domain.31 Interestingly, wortmannin treatment affected the appearance of the CB as detected by anti-DDX25 immunostaining. DDX25-positive CB appeared to form distinct domains, and FYCO1 signal was detected only in a specific CB domain while the other parts of the CB were FYCO1-negative (Fig. 3A to C).

FYCO1 interaction partners

To gain more insight into the role of FYCO1 in the CB function, we identified FYCO1-interacting proteins by
immunoprecipitating FYCO1 from testis cell lysate and subsequent mass spectrometric analysis. In contrast to the CB isolation protocol, anti-FYCO1 immunoprecipitation was performed without cross-linking, therefore enabling us to concentrate on proteins that form close complexes with FYCO1. Mass spectrometry analysis identified several interaction partners (Table S1). Some FYCO1-interacting proteins represented CB components, including DDX4, PIWIL2, and TDRD proteins, which reflects the strong association between FYCO1 and the CB. We validated the interaction of FYCO1 with PIWIL2 and another CB protein EIF4A3 (eukaryotic translation initiation factor 4A3), as well as 2 non-CB-associated
proteins RUVBL1 (RuvB-like protein 1) and RUVBL2 (RuvB-like protein 2) by anti-FYCO1 immuprecipitation followed by western blotting with specific antibodies (Fig. 4A).

Interestingly, PIWIL1 was not identified in the mass spectrometric analysis, nor was it detected in the anti-FYCO1 immuprecipitation by western blotting (Fig. 4A). The analysis of
FYCO1 localization in *piwil1* and *tdrd6* (tudor domain containing 6) knockout germ cells demonstrated that at least these 2 major CB components were not required for the CB-localization of FYCO1; even though CB morphology is greatly affected in the absence of PIWIL1 or TDRD6,42-44 FYCO1 still localized in the CB area in *piwil1* and *tdrd6* knockout spermatids (Fig. S1).

GO (gene ontology) term analysis demonstrated that the FYCO1-interacting proteins were enriched under the GO terms connected to microtubule- and vesicle-related processes (Table S1). The most significant GO terms included e.g., "microtubule-based process" and "macromolecule localization," but also male germ cell differentiation-related terms such as "spermatogenesis" (Fig. 4B). Closer examination of the proteins revealed that a number of kinesin motor proteins such as KIF5C, KIF3A and KIF3B that are involved in microtubule-mediated transport were identified as FYCO1-interacting proteins (Table S1). These results suggest that the somatic function of FYCO1 in microtubule-mediated vesicle transport31,37 is conserved in male germ cells. Altogether 20 of the FYCO1-interacting proteins were CB proteins, while 46 and 38 of them were associated with the GO terms "vesicle" and "microtubule-based process."
based process,” respectively (Fig. 4C). Interestingly, FYCO1-interacting proteins also included several proteins involved in the ubiquitin pathway, such as the E3 ubiquitin protein ligases HERC2 (HECT and RLD domain containing E3 ubiquitin protein ligase 2), TRIM36 (tripartite motif-containing 36) and UBR4 (ubiquitin protein ligase E3 component n-recongnin 4) as well as several CULLIN proteins (CUL9, CUL7, CUL4A), which are core components of the ubiquitin-protein ligase complexes (Table S1).

**CB is disintegrated in Fyc01 knockout round spermatids**

To elucidate the function of FYCO1 during spermatogenesis in vivo, we generated a conditional male germ cell-specific
Fyco1 knockout mouse model by crossing floxed Fyco1 mice with mice carrying Neurog3 promoter-driven Cre expression (Fyco1<sup>flox/flox</sup>;Neurog3 Cre<sup>−/−</sup>). These mice are called Fyco1 conditional knockout (cKO) in this study. As controls, we used mice carrying a floxed Fyco1 gene without Cre expression (Fyco1<sup>flox/flox</sup>;Neurog3 Cre<sup>−/−</sup>). The decreased expression of FYCO1 in the cKO testes was demonstrated by western blot analysis (Fig. 5A). Immunofluorescence analysis validated the absence of FYCO1 in Fyco1 cKO germ cells (Fig. 5B).

**Figure 5.** (For figure legend, see page 309).
Examination of CB in Fyco1 cKO round spermatids revealed that the CBs were clearly affected as detected by immunofluorescence staining using CB markers DDX4 and DDX25; CBs were smaller in size and several CBs were found in the cytoplasm of round spermatids compared with only one prominent structure in control cells (Fig. 5C). Quantification of the CB fragmentation revealed increased number of DDX4-positive granules per nuclei in early (stage I), mid (stage II-V) and late (stage VII-VIII) round spermatids (Fig. 5D). The fragmentation appeared to be more prominent in early stages of round spermatid differentiation. Furthermore, the size of individual DDX4-positive granules was reduced in Fyco1 cKO round spermatids, but, interestingly, the area occupied by DDX4-positive granules per nuclei was increased, suggesting a defective turnover of the CB material (Fig. 5D).

Similar CB fragmentation was detected after treatment of seminiferous tubules with nocodazole, which inhibits microtubule polymerization (Fig. 5E).22 However, nocodazole treatment did not further enhance the CB fragmentation caused by Fyco1 deletion (Fig. 5E). Considering the established function of Fyco1 in microtubule-mediated transport,31,37 these observations support a model in which Fyco1, by its interaction with the microtubule system, is involved in the correct assembly of the CB. The general organization of microtubule network appeared unaffected in Fyco1 cKO rounds spermatids (Fig. 5F).

Interestingly, Fyco1-dependent processes are clearly compensated by other mechanisms in the absence of Fyco1 since testis and epididymis weights in Fyco1 cKO mice appeared normal and we did not observe any abnormalities in spermatogenesis or in the morphology of epididymal spermatozoa (Figs. S2 and S3). Furthermore, Fyco1 cKO males produced a comparable number of pups to control males when mated with C57BL/6 females (Fig. S3).

**Transcriptomic profiling of Fyco1 cKO round spermatids**

To find out if the deletion of Fyco1 affects the CB-mediated regulation of the round spermatid transcriptome, we performed poly-A RNA profiling of Fyco1 cKO and control round spermatids by deep sequencing. The analysis of differentially expressed (DE) genes revealed that the majority of mRNAs were expressed at comparable levels in cKO and control round spermatids (Table S2). However, 322 genes were found to be significantly misregulated (P value ≤ 0.05) in Fyco1 cKO germ cells. Downregulation of Bloctsi6 (biogenesis of lysosomal organelles complex-1, subunit 6, pallidin) and Spg11 (spastic paraplegia 11), and upregulation of Rpap3 (RNA polymerase II associated protein 3) mRNAs in Fyco1 cKO round spermatids were validated by RT-qPCR (Fig. S4A). GO term analysis showed that the genes upregulated in Fyco1 cKO round spermatids were overrepresented under the GO terms connected to cell response to different stimuli, differentiation/morphogenesis and regulation of biological quality (Table S3).

In addition to mRNAs, the CB accumulates other transcripts such as piRNAs, piRNA precursors and nonannotated intergenic transcripts (termed CB-associated novel transcripts in this study).12 Mature piRNAs were not sequenced, but SYBR Gold staining of total testis RNA revealed that piRNA were detected at comparable levels in the control and Fyco1 cKO round spermatids (Fig. S4B). Out of 141 piRNA precursor transcripts detected in our RNA sequencing analysis, 6 were significantly misregulated (P value ≤ 0.05) (Table S2). When analyzing CB-associated novel transcripts, we detected 47 significantly misregulated ones in the absence of Fyco1 (Table S2). We also analyzed expressions of transcripts from nonannotated genomic regions predicted by Cufflinks. Out of 19,585 predicted transcripts we obtained 499 DE genes (P value ≤ 0.05) (Table S4). Interestingly, the analysis of chromosomal origins of these transcripts revealed a concentration on specific regions of chromosomes 8 and 12 (Fig. S4C). We did not detect any clear differences in the expressions of transposons in Fyco1 cKO round spermatids (Table S5).

**Fyco1 is required for the association of autophagosomes/autolysosomes with the CB**

Close examination of CB-associated vesicles by EM revealed that they often have a double-membrane c-shaped appearance and size varying between 100 to 200 nm (Fig. 6A).26 Although smaller in size than starvation induced phagophores in cultured cells (300 to 600 nm), these cup-shaped vesicles shared similar features with phagophores, including 2 limiting membranes that appear contrasting, which is typical for forming autophagosomes.45,46 While the exact nature of these vesicles remains to be characterized, the presence of LC3B-positive vesicles in the CB surroundings suggests that they could be involved in the autophagic pathway (Fig. 6C). In addition to the LC3B-positive vesicles that were interacting with the CB, a larger area of...
accumulated LC3B signal was consistently found in the proximity of the CB (Fig. 6C, bottom panel). This area could correspond to the accumulation of membrane structures found in the electron tomography slices, containing similar cup-shaped structures found in the CB, but also a larger phagophore-resembling structure with electron dense, CB-like material inside the lumen (Fig. S5).

FYCO1 has been reported to be a RAB7- and LC3-interacting protein involved in microtubule-dependent transport of autophagosomes and late endosomes in somatic cells.31,37 This

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**Figure 6.** (For figure legend, see page 311).
prompted us to explore the possible role of FYCO1 in the communication between the CB and autophagosomes/lysosomes. Coimmunofluorescence analysis of LC3B and the CB marker DDX25 showed that in Fyco1 cKO round spermatids, the association of CB fragments with LC3B signal was clearly reduced; 32% of the control CBs, but only 9% of cKO CBs, had overlapping LC3B signal (graph in Fig. 6D). Furthermore, LC3B-positive clouds that were consistently detected in the proximity of control CBs were not present in Fyco1 cKO spermatids (Fig. 6D). In the absence of FYCO1, LC3B vesicles were more evenly distributed in the cytoplasm, and no clear accumulation in the CB region was detected. Anti-FYCO1 immunoprecipitation from testicular vesicle-containing organelle fractions revealed that the somatic LC3B-binding activity of FYCO1 was conserved in male germ cells (Fig. 6E). This suggests that FYCO1 is directly involved in the recruitment of LC3B-positive vesicle into the CB. The lack of CB-associated vesicles in the absence of FYCO1 was also confirmed by electron microscopy (Fig. 6F).

**FYCO1 is required for the recruitment of lysosomal vesicles to the CB upon induction of autophagy**

To study the connection between the CB and autophagy more carefully, we cultured stage-specific (stage II-V) pieces of the seminiferous tubules in the absence and presence of autophagy modulators. We used LAMP1 as a marker to detect lysosomes and autolysosomes. Under control conditions (DMSO only), LAMP1-positive vesicles were detected widely in the cytoplasm of round spermatids (Fig. 7A). The LAMP1 signal was also concentrated in the peripheral areas of the CB, as shown earlier by immunoelectron microscopy. Treatment of cells with rapamycin, which inhibits the Ser/Thr protein kinase MTOR (mechanistic target of rapamycin) and induces autophagy, resulted in the strong accumulation of the LAMP1 signal on the CB (Fig. 7A). Incubation of the tubules with bafilomycin A1 (Baf), which inhibits the late phase of autophagy by preventing fusion between autophagosomes and lysosomes, resulted in the accumulation of LAMP1-positive vesicles next to the CB (Fig. 7A). In Fyco1 cKO seminiferous tubules, rapamycin did not induce accumulation of LAMP1-positive vesicles onto the CB. In addition, Baf did not have any effect on the LAMP1 localization pattern in Fyco1 cKO round spermatids (Fig. 7A). Quantification of colocalization using Manders coefficients showed that both rapamycin and Baf caused an increase in the overlap of LAMP1 and DDX25 signal in control round spermatids, while they did not enhance the colocalization in Fyco1 cKO CBs (Fig. 7B).

**FYCO1 regulates the expression level of PIWIL2**

We performed western blot analysis of total testis extracts to study the effects of FYCO1 inactivation on the expression levels of selected proteins. While the levels of other studied proteins were not affected, we revealed that the amount of PIWIL2 was significantly reduced in Fyco1 cKO testes compared with control testes (Fig. 8A). Interestingly, another PIWI protein PIWIL1 was not significantly affected, correlating with the fact that we did not detect an interaction between FYCO1 and PIWIL1 in coimmunoprecipitation experiments (Fig. 4A). Despite the downregulation of PIWIL2 expression, PIWIL2 was found to be correctly localized in the innermitochondrial cement of pachytene spermatocytes and fragmented CBs in step 1 round spermatids (Fig. 8B). The localization of PIWIL1 was also unaffected in Fyco1 cKO testes (Fig. 8B).

To study the effects of induced autophagy on PIWIL2 expression, we performed tubule culture experiments by incubating a batch of 30 segments of seminiferous tubules in the absence or presence of rapamycin and Baf. Rapamycin treatment caused significant downregulation of PIWIL2 levels in control tubules, but in contrast, it had no significant effect on PIWIL2 levels in Fyco1 cKO tubules (Fig. 8C). Baf did not affect PIWIL2 levels under the conditions used for the experiment (6 h incubation at 34°C). These results suggest that FYCO1 is required for the rapamycin-induced downregulation of PIWIL2 in male germ cells.

**Discussion**

Compartmentalized RNA regulation in the cytoplasmic RNP granules affords a powerful means for accurate control of the cellular transcriptome. The formation, function and homeostasis of RNP granules has to be strictly monitored, and increasing evidence supports the integral role of the vesicular transport system in their regulation. The interplay between RNP granules and the endomembrane system is beautifully exemplified by the haploid male germ cell-specific RNP granule, the CB, which is surrounded by small cytoplasmic vesicles and multivesicular bodies and dynamically communicates with the nuclear envelope and the Golgi complex. In this study, we revealed that CB-associated vesicles are involved in the autophagy-lysosomal pathway as suggested by the accumulation of LC3B and LAMP1 signal in the CB surroundings. Importantly, this interaction is dependent on FYCO1, as evidenced by the retention of CB-associated vesicles in Fyco1 cKO spermatids.
Figure 7. Lysosomal vesicles are not recruited to the CB in the absence of FYCO1. (A) Stage-specific (II-V) pieces of the control (CTRL) or Fyco1 cKO (cKO) seminiferous tubules were cultured in the presence of vehicle (DMSO), rapamycin or Baf. After cultures, squash preparations were made and immunostained with anti-DDX25 (red) and anti-LAMP1 (green) antibodies. Nuclei were stained with DAPI (blue). Arrows point to the LAMP1-signal recruited in the CB area after rapamycin treatment. Accumulations of LAMP1-positive vesicles next to the CB after Baf treatment are indicated by arrowheads. Scale bar: 10 μm. Intensity of LAMP1 and DDX25 signal was measured along the yellow lines to reveal the spatial localization of LAMP1-positive vesicles and the CB. (B) Colocalization of LAMP1 with DDX25 in control and cKO testis treated with rapamycin, Baf or the vector (DMSO) was measured using Manders coefficients. Error bars represent standard deviations.
Figure 8. PIWIL2 is downregulated in Fyco1 cKO testes. (A) Western blot images for expression of FYCO1, PIWIL2, PIWIL1, DDX4 and GAPDH in control and Fyco1 cKO testes (3 biological replicates per genotype). Quantification revealed significantly lowered amount of PIWIL2 in Fyco1 cKO testes (\( P \) value \( < 0.01 \); 2-tailed t test). The expression levels of PIWIL1 and DDX4 were unaltered. Anti-GAPDH signal was used for the normalization. Error bars represent s.e.m. (B) Immunofluorescence analysis of paraffin-embedded testis sections at stage II-V (PIWIL1: red, PIWIL2: green) and stage I (PIWIL2: green) in control (CTRL) and Fyco1 cKO (cKO) mice. Nuclei were stained with DAPI. PSpC, pachytene spermatocyte; RS, round spermatid; ES, elongating spermatid. The roman numbers in parentheses after RS or ES represent the step of spermatid differentiation. Scale bar: 10 \( \mu \)m. (C) Segments of seminiferous tubules of control and Fyco1 cKO mice were incubated with DMSO, rapamycin (Rap) or Baf (30 segments/treatment) and PIWIL2 was detected with a specific antibody. The western blotting image represents one of the 3 biological replicates that were used for quantification. Anti-GAPDH signal was used for normalization. The average of 3 biological replicates are shown. Error bars represent s.e.m. * \( P \) value \( \leq 0.05 \); 2-tailed t test.
we identified an LC3B-interacting protein FYCO1 as a novel CB component that mediates the association of the vesicles with the CB and controls the recruitment of lysosomes onto the CB when autophagy is induced.

The dramatic accumulation of LAMP1-positive lysosomes onto the CB upon induction of autophagy suggests a central role for the CB in the catabolic activity of haploid male germ cells. In somatic cells, lysosome position has been shown to coordinate autophagy via the MTOR pathway. Under nutrient-rich conditions, lysosomes localize in the cell periphery, while during starvation, they are clustered in the perinuclear area in a microtubule-dependent manner, which facilitates fusion of lysosomes with autophagosomes. We suggest that a similar process takes place in haploid male germ cells: the content of the autophagosomes formed in the vicinity of the CB is targeted to degradation by fusion with lysosomes, and the rate of autophagy is increased upon rapamycin treatment by active recruitment of lysosomes to the CB.

The analysis of Fyco1 cKO round spermatids revealed that FYCO1 functions as a docking platform for LC3 or LAMP1-positive membranes and mediates autophagosome and lysosome recruitment to the CB. Autophagy could provide a potential mechanism for the clearance of CB material and the maintenance of CB homeostasis. Electron dense material is indeed sometimes found inside CB-associated cup-shaped vesicles, and we have also detected larger phagophore-resembling structures with CB material in the lumen in the vicinity of the CB (Fig. S5). Although the total DDX4 level in the testis was not found to be significantly increased in Fyco1 cKO mice, the total volume of DDX4-positive granules per nuclei as detected by immunofluorescence analysis was increased, suggesting an increase in the CB material. We also revealed that the expression level of one of the PIWI proteins, PIWIL2, was compromised in the absence of FYCO1, and further investigation showed that PIWIL2 was targeted for degradation upon induction of autophagy in a FYCO1-dependent manner. Interestingly, several factors involved in the ubiquitin-proteasome pathway were identified as FYCO1-interacting proteins. Targets of selective autophagy are often ubiquitinated, which enables their recognition by receptor proteins and targeting to the LC3-containing autophagosomes. The complex formation between FYCO1 and ubiquitin E3 ligases suggests the involvement of protein ubiquitination in the CB-associated autophagy.

In addition to the digestion of bulk CB material, an intriguing option is that some specific RNA species are eliminated from the CB by FYCO1-mediated autophagy. It has recently been demonstrated that autophagy can protect genomic stability by degrading retrotransposon RNA. Similar processes could possibly take place in the CB, which is enriched with PIWI proteins and piRNAs that are known to target other RNA species for degradation. Indeed, our deep sequencing analysis revealed that the expression of a subset of mRNAs and CB-associated novel transcripts were misregulated in Fyco1 cKO round spermatids. Interestingly, FYCO1 was also found to be localized in the late CB in elongating spermatids, where the abundance of vesicles associated with the late CB is even more prominent. Therefore, the disruption of the CB-vesicle connection in Fyco1 cKO tests may have even more apparent effects on transcriptomic balance later in development in elongating spermatids.

Interestingly, the integrity of the CB was compromised, and the CB was fragmented in several pieces in the absence of FYCO1. It is currently unknown what causes the CB fragmentation, and one possibility is that detached fragments are normally degraded by FYCO1-mediated processes. However, FYCO1 may also augment the collection of smaller detached fragments back to the main CB structure. It is also possible that the FYCO1-mediated interplay between the CB and autophagosomes and lysosomes is involved in the maintenance of the CB integrity. The movements of the CB22 and the vesicle transport are dependent on microtubule network. The fact that the disruption of the microtubule network causes similar fragmentation of the CB as Fyco1 deletion (Fig. 5) suggests that microtubule-associated functions of FYCO1 are needed for the CB integrity. Identification of several kinesins as FYCO1-interacting proteins further highlights the importance of the microtubule network in FYCO1 function.

Because of the prominent molecular phenotype in Fyco1 cKO round spermatids, it was somewhat unanticipated that the conditional inactivation of Fyco1 gene in mice did not impair spermatogenesis and male fertility. In contrast, the deletion of any core components of the CB, such as PIWIL1, PIWIL2, DDX4, TDRD6 or TDRD7 (tudor domain containing 7), in mice results in spermatogenic failure and male infertility. FYCO1 is consistently found in the mass spectrometry analysis of isolated CBs. However, the current study showed that it appears not to be a core CB component, but localizes to the periphery of the CB and mediates the interaction of the CBs with vesicles and possibly with the microtubule network. Our results revealed that the presence of an intact single CB is not required for the progress of spermatogenesis but the RNA regulatory pathways remain functional when the CB is fragmented. This is in line with the studies on somatic RNP granules, the processing bodies (P-bodies), demonstrating that aggregation of P-body components into P-bodies is not required for their function but is instead a consequence of their activity.

It is possible that in the absence of FYCO1, the disrupted interplay between the CB and vesicle trafficking is compensated by other, yet unidentified processes or homologous proteins. Interestingly, cellular defects in the Fyco1 cKO germ cells were enhanced after challenging the cells in tubule culture conditions by autophagy inducers and inhibitors. Therefore, another option is that FYCO1-dependent processes are not required for the progress of spermatogenesis under physiological conditions, but become critical when the system is challenged by adverse conditions. Toxicological and aging-related mouse studies are in progress to clarify the potential role of FYCO1 in mediating stress-induced autophagy responses in spermatogenic cells.

Altogether, we revealed FYCO1 as a novel regulator of germ granules. Furthermore, our results provide the first molecular evidence on the factors that mediate the interplay between haploid male germ cell-specific RNP granules (CBs) and the autophagosome-lysosome system during spermatogenesis.

Materials and methods

Ethics statement

Mice were housed at the animal facility of the University of Turku, Finland, under controlled environmental conditions, following local laws and regulations (Finnish Act on the
Protection of Animals Used for Scientific or Educational Purposes [497/2013], Government Decree on the Protection of Animals Used for Scientific or Educational Purposes [564/2013]). Mice were sacrificed by CO₂ inhalation and cervical dislocation. The Laboratory Animal Care and Use Committee of the University of Turku approved all the animal experiments.

**Antibodies**

Primary antibodies used in this study were: ACTA2/α actin (sc-32251) and DDX25 (sc-51271) from Santa Cruz Biotechnology; TUBA/α tubulin (MS-S81-P1) from Thermo Fisher Scientific; DDX4 (ab13840), LAMP1 (ab25245), EIF4A3 (ab32485), RUVBL2 (ab36569) and DCP1A (ab47811) from Abcam; PIWIL1 (G82) and LC3B (2775) from Cell Signaling Technology; FYCO1 (H00079443-A01) from Abnova; FYCO1 (HPA035526 and SAB1400697) and RUVBL1 (HPA019948) from Sigma-Aldrich; GAPDH (5G4) from HyTest; PIWIL2 clone 13E-3 (MABE363) from Millipore. Rabbit polyclonal antibody against TSKS was a gift from Prof. J.A. Grootegoed, Department of Reproduction and Development, Erasmus MC - University Medical Center Rotterdam. Secondary antibodies conjugated with Alexa Fluor 488, 546, 594 and 647 made in donkey and streptavidin conjugated with Alexa Fluor 488 or 647 were purchased from Thermo Fisher Scientific (A-21202, A-21206, A-11055, A10036, A10040, A-11056, A-21203, A-21207, A-11058, A-31571, A-31573, A-21447, S23254, S23257). ECL anti-mouse IgG HRP-linked whole antibody made in sheep (NA931) and ECL anti-rabbit IgG HRP-linked made in donkey (NA934) were purchased from GE Healthcare Life Sciences.

**Electron microscopy and tomography**

Samples were prepared as described previously.²⁶ Briefly, testis samples were fixed in 5% glutaraldehyde and treated with a potassium ferrocyanide-osmium fixative. The samples were embedded in epoxy resin (Glycidether 100, Merck), sectioned, post-stained with 5% uranyl acetate and 5% lead citrate, and visualized on a JEOL 1400 Plus transmission electron microscope (JEOL Ltd., Tokyo, Japan). Electron tomography samples were prepared following the same protocol except that uranyl acetate en-bloc staining was performed before plastic embedding. Serial semithick 220 nm sections were cut and placed on single slot grids. Colloidal gold particles of 10 nm in diameter were placed on top and below the grids to serve as markers for alignment of the tilt series. Dual axis tilt series were acquired using SerialEM software (http://bio3b.colorado.edu/serialEM) running on a Tecnai FEG 20 microscope (FEI, the Netherlands) operating at 200 kV. Images from 3 consecutive sections were recorded at 1-degree intervals over a tilt range of ±62 degree. Tilt series were acquired using Ultrascan 4000 CCD camera (Gatan Corp., Pleasanton, CA, USA) at nominal magnification of 11.5 k providing a 2X binned pixel size of 1.94 nm. IMOD software (http://bio3d.colorado.edu/imod) was used to align the tilt series, and create 3D reconstructions. The images were segmented using Microscopy Image Browser, developed by the Electron Microscopy Unit, Institute of Biotechnology, University of Helsinki.³⁵ 3D rendering of selected cytoplasmic vesicles was performed with BioimageXD version 1.³⁶

**Chromatoid body isolation**

CB immunoprecipitation was performed as described previously.¹³ Briefly, germ cells were released from 4 testes of adult C57BL/6 mice by digestion in 50 mg/mL Collagenase Type I (CLS-1, Worthington Biochemical Corporation) 0.1% w/v glucose in PBS and fixed in 0.1% paraformaldehyde (PFA) solution (Electron Microscopy Sciences, 15714). After fixation cells were lysed by sonication (UCD-200, Diagenode; medium settings, 30 sec with 30 sec pause) in 1.5 mL of RIPA buffer (50 mM Tris-HCl, pH 7.5, 1% Triton X-100 [Sigma, 93443], 0.5% w/v sodium deoxycholate [Sigma, D6750], 0.05% w/v sodium dodecyl sulfate [Sigma, 436143], 1 mM EDTA, 150 mM NaCl, 1X complete protease inhibition cocktail [Roche, 04693116001], 0.2 mM PMSF, 1 mM DTT) and the CB-enriched pellet fraction was obtained by centrifugation at 300 × g for 10 min. The CBs were immunoprecipitated using Dynabead Protein G (Thermo Fisher Scientific, 10003D) coupled to either anti-DDX4 antibody (Abcam, ab13840), or rabbit IgG (Neomarkers, NC-100-P) at 4°C overnight with gentle mixing.

**Western blotting**

Tissue samples were homogenized in RIPA lysis buffer containing 1 mM DTT, 0.2 mM PMSF and 1X protease inhibitor cocktail, and the lysates were cleared by centrifugation at 14000 × g for 5 min. For ontogenesis studies protein concentration was measured using Pierce BCA protein assay kit (Life Technologies, 23227); absorbance was measured with a Victor2 plate reader (Wallac, Turku, Finland). Samples diluted in Laemmli buffer were incubated 5 min at 95°C before loading them on the gel. Samples were run at 100 V and then transferred to a PVDF membrane (Amersham, RP303F) with wet-blotting system (Bio-Rad) at 90 V for 1 h at 4°C. After blotting the PVDF membrane was incubated in 100% methanol for 15 seconds and air-dried for 30 min at 37°C or at room temperature overnight. The membrane was then incubated with primary antibody diluted in 5% skimmed-milk, 0.1% Triton X-100 (Sigma, 93443) in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4) (blocking solution) for 1 h at room temperature, washed 3 × 5 min with 0.1% Triton X-100 in PBS (PBST), incubated with secondary antibody diluted 1:1000 in blocking solution, washed 5 × 5 min in PBST, and incubated 1 min with Western Lightning ECL Pro (Perkin Elmer, NEL121001EA). The chemiluminescence signal was recorded using LAS4000 (Fujifilm) as 16 bit.TIFF files. Band intensity was measured using ImageJ software. The band intensities from actin and tubulin were used for normalization. The results represent the mean of biological duplicates.

**RT-qPCR**

RNA was isolated from FVB mouse testis at different time points postpartum or enriched populations of round spermatids with TRIsure (BIO-38033, Bioline) following the manufacturer’s instructions. RNA was resuspended in MilliQ water to a final concentration of 1 µg/µL. Before cDNA synthesis, 1 µg of RNA per sample was treated with DNase I (Sigma-Aldrich, AMPD1). cDNA was synthesized with DyNAmo cDNA Synthesis Kit (Thermo Scientific, F-470) following the manufacturer’s instructions using 1 µg of RNA as a template. The reverse transcription
reaction was then resuspended 1:20 in MilliQ water for qPCR. qPCR was performed with the DyNAmo HS SYBR Green qPCR kit (Thermo Scientific, F-410) following the manufacturer’s instructions. Ppia, Actb and Rplp0 (for FycO1 ontogenesis) or Ppia and Rplj19 (for sequencing validation) were used as reference genes. All reactions were performed on either a CFX96 or CFX384 Real Time qPCR detection system (Bio-Rad, Hercules, CA, USA). The geometric mean of the Ct values from the reference genes was used as a normalization factor for the calculation of the delta-Ct for each gene of interest at each sample. The delta-delta-Ct value was calculated using the 1-w time point (FycO1 ontogenesis) or control round spermatids (sequencing validation) as reference. Either 2 (sequencing validation) or 3 (FycO1 ontogenesis) biological replicates were analyzed. All RT-qPCR reactions and analysis were performed following MIQE guidelines. Further information (quality control of RNA template, validation of primers and reference genes, primer sequences) is available upon request.

Preparation of germ cells and tissues for immunostaining

Squash slides of stage specific sections of mouse seminiferous tubules were prepared as described earlier. Briefly, testes from FVB or C57BL/6 adult mice were decapsulated and sections representing specific stages of the seminiferous epithelium were isolated based on the light absorption pattern with the help of a stereomicroscope. The sections of the seminiferous tubules were then transferred to a glass slide with the use of a pipette and a glass coverslip deposited on top of the tubule section to allow the germ cells to spread out from the tubule. Once the germ cells formed a monolayer, the glass slide was snap-frozen in liquid nitrogen and, after quickly removing the coverslip, fixed in 100% ice-cold acetone for 10 min and air-dried overnight at room temperature. Slides were postfixed in 4% paraformaldehyde in PBS for 10 min, washed 5 min in PBS, incubated 5 min in 0.2% Triton X-100 in PBS and washed 3 × 5 min in PBS before starting with IF staining.

For paraffin embedding, testes collected from FVB or C57BL/6 adult mice were fixed in 4% PFA in PBS overnight at room temperature. Testes were washed in milliQ water for 2 h with repeated changes of fresh milliQ water, incubated 2 × 30 min in 50% ethanol and 2 × 30 min in 70% ethanol before embedding in paraffin. Paraffin-embedded testis sections were deparaffinized by incubation 3 × 5 min in xylene, 2 × 10 min in 100% ethanol, 2 × 10 min in 96% ethanol, 2 × 10 min in 70% ethanol and then washed in milliQ water 2 × 2 min. Antigen retrieval was performed by incubation in sodium citrate solution (10 mM sodium citrate, 0.05% Tween 20 [Sigma, P2287], pH 6.0) or in Tris-EDTA solution (10 mM Tris Base, 1 mM EDTA Solution, 0.05% Tween 20, pH 9.0) for 20 min, at 1 atmosphere at 120°C. After cooling down to room temperature for at least 2 h slides were washed 4 × 3 min in milliQ water and 5 min in PBS before starting with IF staining.

Immunofluorescence

Slides were incubated with 10% normal donkey serum (Jackson Immunoresearch, O17–000–121) and 3% bovine serum albumin (Sigma, A2153) in PBST (blocking solution) for 1 h. Primary antibody was diluted in blocking solution and incubation was performed for 1 h at room temperature or overnight at 4°C. Slides were washed 3 × 5 min with PBST. Secondary antibody was diluted 1:1000 in blocking solution and incubation performed for 1 h at room temperature. Blocking and incubation with antibody solutions were performed in a humidified environment protected from light. Slides were washed 3 × 5 min in PBST, incubated 5 min in DAPI (Sigma-Aldrich, D9542; 5 mg/mL stock) diluted 1:20,000 in PBS, washed 5 min in PBS followed by 5 min with milliQ water and finally mounted with Vectashield HardSet Mounting Medium (Vector Laboratories, H-1400) or Prolong Diamond Antifade Mountant (Life Technologies, P36970). Slides were left for 24 h at room temperature for the mounting medium to solidify.

Imaging

Widefield fluorescence images were acquired with Zeiss Axio Imager M1 microscope (Oberkochen, Germany) equipped with an AxioCam MRc camera using a 40X/0.75 DIC Plan-NeoFluar objective. Widefield images were acquired and processed for publication with Zeiss Zen 2011 software. Confocal images were acquired using either a Zeiss 510 META or Zeiss 780 laser scanning confocal microscope (Oberkochen, Germany) with 40X/1.2 Water or 100X/1.4 Oil DIC objective. Resolution in the 3 dimensions was set at optimal with Zeiss Zen 2011 software. Confocal images were analyzed and modified for publication (background subtraction, contrast and brightness adjustment) with BioimageXD version 1.056 or with ImageJ software. 3D model reconstruction was performed using Slidebook 6 reader, version 6.0.4 (24366) (31). All IF images represent confocal images unless otherwise stated in the figure legends.

Seminiferous tubule cultures

For immunofluorescence analysis rapamycin (Santa Cruz Biotechnology, sc-3504) and nocodazole (Sigma, M14004) were diluted 10 mg/mL in DMSO. Baf (Santa Cruz Biotechnology, sc-201550) was diluted 0.1 mg/mL in DMSO. Wortmannin (Sigma, W1628) was diluted to 0.12 mM in DMSO. Sections of the seminiferous tubules representing stage II-V of the seminiferous epithelial cycle were dissected as described above and cultured in 50 μL, final volume of DMEM with drug or vehicle alone diluted 1:100 for 6 h in a humidified incubator at 34°C, 5% CO2. After incubation, squashed slides were prepared, and samples were labeled for IF as described above. For protein expression quantification, sections of seminiferous tubules were cultured in 500 μM rapamycin, 5 μM Baf or vehicle alone for 6 h in a humidified incubator at 34°C, 5% CO2. Tubules were collected by centrifugation 500 × g, 5 min in 4°C, homogenized with a TissueLyser LT (Qiagen, Hilden, Germany) 50 Hz for 30 seconds in RIPA buffer. Cells were left to lyse 30 min on ice, centrifuged as before and supernatant analyzed by western blot analysis as described. Band intensity was measured using ImageJ software. GAPDH was used for normalization. The results represent the mean of biological triplicates with s.e.m and 2-tailed t test performed by GraphPad Prism 7.00 software.

Isolation of germ cells

Testes from 2 C57BL/6J adult mice were collected in PBS, transferred in 8 mL 1X KREBS buffer (25 mM NaHCO3, 1.2 mM
KH$_2$PO$_4$, 120 mM NaCl, 1.2 mM MgSO$_4$·7H$_2$O, 11.10 mM dextrose, 1.3 mM CaCl$_2$·2H$_2$O, 4.8 mM KCl, pH 7.4,[73] tunica albuginea was removed and seminiferous tubules were minced with scissors. The solution containing the seminiferous tubule fragments was divided into 2 tubes containing 25 mL collagenase solution (1X KREBS buffer with 22.5 mg collagenase type I [Worthington Biochemical Corporation, LS004194]) each prewarmed at 34°C. Cells in collagenase solution were incubated at 34°C for 10 min in rotation. Cell suspensions were centrifuged 2 min, 500 × g at room temperature, supernatant was discarded and each pellet (composed of germ cells and seminiferous tubule fragments) was resuspended in 25 mL trypsin solution (1X KREBS buffer with 15 mg trypsin [Worthington Biochemical Corporation, LS003708] and 5 μg DNase I [Sigma-Aldrich, DN25]) prewarmed at 34°C. Cell suspensions in trypsin solution were incubated at 34°C for 10 min in rotation. Cell suspensions were mixed 10 times with a wide bore pipette. 5 μg DNase I were added to each cell suspension and again incubated at 34°C for 10 min in rotation. After 2 min, 500 × g centrifugation at RT, the cell pellets were resuspended in 25 mL 1X KREBS buffer and filtered through 100 μm filter. Cell suspensions were pooled together and centrifuged 2 min, 500 × g at RT. Cell pellet was resuspended in 5 mL ice-cold 1X KREBS buffer. Cell suspension was loaded on the top of an ice-cold discontinuous BSA density gradient (1–2–3–4–5–6% BSA in 1X KREBS, 5 mL each) in a 50 mL tube. Cells were allowed to sediment for 1.5 h at 4°C. 1 mL fractions were collected starting from the top of the gradient, centrifuged 5 min at 500 × g, washed twice with ice-cold 1X KREBS buffer and stored on ice. 5 μL of each fraction were diluted in 10 μL fixing solution (4% PFA, 0.05% Triton X-100) with DAPI (Sigma-Aldrich, D9542; diluted 1:20,000 from 5 mg/mL stock solution). Each fraction was analyzed by DAPI staining and fluorescence microscopy for the enrichment in round spermatids.

**RNA sequencing and data analysis**

Total RNA was isolated from round spermatids using TRIzol (Bioline, BIO-38033) following the manufacturer’s instructions. RNA samples were further processed at the Finnish Microarray and Sequencing Center at the Turku Center for Biotechnology. Poly-A RNA was enriched using standard procedures and sequencing was performed using a HiSeq 3000 sequencing system (Illumina, San Diego, CA, USA). Two control and 2 cKO samples were submitted for sequencing. Transcript analysis was performed using the RSEM (Version 1.2.22) and count data was obtained with HTSeq (Version 0.6.1p1). Differential expression (DE) analysis was performed using the edgeR package.[59] We used piRNA precursor coordinates from Li et al.[60] and the coordinates for CB-associated novel transcripts were from Meikar et al. Nonexpressed genes were filtered from the data. Only genes with at least 2 counts per million mapped reads were considered for quantification analysis. TMM normalization was applied to account for the compositional bias. Transposons were quantified using the pIPes pipeline that uses the RepBase database for the transposon sequence information.[61] Cufflinks and Cuffmerge (Version 2.1.1)[62] were used to predict novel intergenic transcripts. Individual counts for the consensus loci were again obtained using HTSeq and the DE analysis was then performed with the edgeR package.

**Preparation of testicular vesicle fraction**

Testes from 3 C57Bl/6j adult mice were collected, tunica albuginea was removed and seminiferous tubules were minced with scissors. The solution containing the seminiferous tubule fragments was divided into 2 tubes containing 25 mL collagenase solution (0.1% glucose in PBS with 22.5 mg collagenase type I and 5 μg DNase I). Cells in collagenase solution were incubated at 34°C for 30 min with gentle shaking. Cells were centrifuged 5 min 500 × g at 4°C and the cell pellets washed twice with ice cold 0.1% glucose PBS. Cells were combined to one tube and after a final 500 × g at 4°C centrifugation, supernatant was discarded and 1,500 μL of HEPES buffer (0.25 M sucrose, 10 mM HEPES, pH 7.2, 1X complete protease inhibitor cocktail) was added. Cells were disrupted by nitrogen cavitation (Parr Instruments, 500 p.s.i, 5 min at room temperature). Cell lysate was centrifuged 5 min, 17,000 × g at 4°C. FYCO1 complexes were immunoprecipitated using Dynabead Protein G coupled to either rabbit anti-FYCO1 antibody (Sigma) or rabbit IgG at 4°C overnight.

**Cell culture**

HeLa cells (American Type Culture Collection, CCL-2) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM)/F12 media (Gibco) supplemented with 10% fetal calf serum (FCS; PromoCell), 50 IU/ml penicillin and 50 μg/ml streptomycin supplemented at 37°C in humidified atmosphere with 5% CO$_2$. To induce autophagy, cells were amino acid- and serum-starved for 1 h. Cells were collected, lysed with RIPA buffer and cell debris pelleted by centrifugation (17,000 × g, 5 min, 4°C). Supernatant diluted with Laemmli buffer was used in western blot analysis as described before.

**Immunoprecipitation**

Four testes from adult mice were collected in PBS. Seminiferous tubules were released from the tunica albuginea, quickly minced with scissors and incubated 60–90 min at RT in 50 mL of collagenase solution (0.5 mg/mL collagenase type I, 0.1% glucose in PBS) in rotation to release the germ cells from the seminiferous tubules. The cell suspension was centrifuged 5 min, 500 × g at 4°C. The pellet was resuspended in ice-cold 50 mL 0.1% glucose in PBS, filtered through a 100-μm filter to eliminate pieces of seminiferous tubules and centrifuged again. Cells were lysed on ice for 30 min in 1 mL is isotonic non-denaturing lysis buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 8.0, 1% Triton X-100, 1X complete mini mix [Roche, 469312401], 0.2 mM PMSF and 1 mM DTT). Samples were centrifuged 10 min, 500 × g at 4°C to eliminate cellular debris and intact chromatoid bodies. FYCO1 complexes were immunoprecipitated using Dynabead Protein G coupled to either rabbit anti-FYCO1 antibody (Sigma) or rabbit IgG at 4°C overnight.

**Mass spectrometry**

To remove Triton X-100, beads were washed 2 × 1 mL of 25 mM NH$_4$HCO$_3$ buffer and 2 × 200 μL of 6 M urea in
25 mM NH₄HCO₃ buffer. Samples were loaded on a Criterion XT Bis-Tris precast 12% SDS-PAGE gel (Bio-Rad) and run with constant 200 V for 9 min. MOPS buffer was used as a running buffer. Three pieces from the upper part of the SDS-PAGE gel were cut and samples were in-gel digested at the Turku Proteomics Facility according to the standard protocol. Digested peptides were dissolved in 1% formic acid (ctrl 11 μL and all the rest 15 μL). Samples (5 μL) were submitted to LC-ESI-MS/MS analysis. The LC-ESI-MS/MS analyses were performed on a nanoflow HPLC system (Easy-nLCII, Thermo Fisher Scientific, Waltham, MA, USA) coupled to the LTQ Orbitrap Velos Pro mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a nano-electrospray ionization source. Peptides were first loaded on a trapping column and subsequently separated inline on a 15-cm C18 column (75 μm x 15 cm, Magic 5 μm 200 A C18, Michrom BioResources Inc., Sacramento, CA, USA). The mobile phase consisted of water/acetonitrile (98:2 [v/v]) with 0.2% formic acid (solvent A) and acetonitrile/water (95:5 [v/v]) with 0.2% formic acid (solvent B). A linear 30 min gradient from 5% to 35% B was used to elute peptides. MS data was acquired automatically by using Thermo Xcalibur 3.0 software (Thermo Fisher Scientific). An information dependent acquisition method consisted of an Orbitrap MS survey scan of mass range 300 to 2000 m/z. The data files were searched for protein identification using Proteome Discoverer 1.4 software (Thermo Fisher Scientific) connected to an in-house Mascot server running the Mascot 2.4.1 software (Matrix Science). Data were searched against the SwissProt database (release 2014_08). The search parameters were used. Type of search: MS/MS Ion Search, Taxonomy: Mus musculus, Enzyme: Trypsin. Fixed modifications: Carbamidomethyl (C), Variable modifications: Oxidation (M), Mass values: Monoisotopic, Peptide Mass Tolerance: ± 5 ppm, Fragment, Mass Tolerance: ± 0.5 Da, Max Missed Cleavages: 1, Instrument type: ESI-TRAP. Results from ProteomeDiscoverer were exported and saved as Excel files. Only proteins assigned at least with 2 peptides were accepted.

**Generation of Fyco1 conditional knockout mice**

The genetic background of all the mice used in this study was mixed background with C57BL/6j and SV129. The construct for the generation of the *Fyco1* conditional knockout (MGI:1072777) was purchased from the International Mouse Phenotyping Consortium, and validity of construct was confirmed by restriction enzyme digestion and by sequencing. G4 embryonic stem cells (ES, derived from 129S6/C57BL/6Ncr mice) were cultured on neomycin-resistant primary embryonic fibroblast feeder layers, and 10⁶ cells were electroporated with 30 μg of linearized targeting construct. After electroporation, the cells were plated on 100 mm culture dishes and exposed to G418 (300 μg/ml; Sigma). Colonies were picked up after 7 to 9 d of selection, and grown on 96-well plates. In order to delete Neo cassette in the targeted ES cells, they were re-electroporated with plasmid pCAGGS-Cre and plated on 100 mm culture dishes. Colonies were picked up after 3 to 5 d growth and grown on 96-well plates. Targeted ES clones and ES clones with Neo deletion were used for blastocyst injection and for creation of chimera. Male chimeras were bred with wild-type females to determine the germline transmission. To achieve selective inactivation of *Fyco1* in germ cells, transgenic Neurog3 Cre mice were mated with homozygous Fyco1 floxed alleles, Fyco1(fx/fx);Neurog3 Cre+ and Fyco1(fx/wt);Neurog3 Cre− mice. These animals were then intercrossed to produce Fyco1(fx/fx);Neurog3 Cre+, and Fyco1(fx/wt);Neurog3 Cre− littermates. Cre-mediated recombination was detected and confirmed by PCR with different primer pairs. Further information (validation of primers, primer sequences) is available upon request.

**Histology and morphological analysis of spermatozoa**

For histological analyses, tissues were collected and directly fixed in 4% PFA or in Bouin fixative (4 to 20 h at room temperature). Tissues were then dehydrated in a series of ethanol washes as described above and embedded in paraffin. Paraffin-embedded tissues were cut and stained with hematoxylin and eosin (HE) or periodic acid-Schiff (PAS) according to standard protocols. Epididymal sperm was released in PBS from cauda epididymis and spread on glass slides, air-dried and stained with hematoxylin for morphological analysis.

**Statistical analyses**

For colocalization studies, Manders coefficients were calculated using BioimageXD and data analysis was done using MS Excel 2016. For other data analyses, the Graphpad Prism 7 (GraphPad Software Inc., LaJolla, CA, USA) was used. P values ≤ 0.05 were considered significant.

**Abbreviations**

- Baf: bafilomycin A1
- CB: chromatosid body
- cKO: conditional knockout
- DDX25: DEAD (Asp-Glu-Ala-Asp) box polypeptide 25
- DDX4: DEAD (Asp-Glu-Ala-Asp) box polypeptide 4
- DE: differentially expressed
- EM: electron microscopy
- FYCO1: FYVE and coiled-coil domain containing 1
- GO: gene ontology
- LAMP1: lysosomal-associated membrane protein 1
- LC3: microtubule-associated protein 1 light chain 3
- piRNA: PIWI-interacting RNA
- PIWI: P-element-induced wimpy testis
- PIWIL1: piwi-like RNA-mediated gene silencing 1
- PIWIL2: piwi-like RNA-mediated gene silencing 2
- RNP: ribonucleoprotein
- TDRD6: tudor domain containing 6
- TSKS: testis specific serine kinase substrate

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.
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References

[1] Soumillon M, Necsulea A, Weier M, Brawand D, Zhang X, Gu H, Barthes P, Kokkinaki M, Nef S, Ginerke A, et al. Cellular source and mechanisms of high transcripome complexity in the mammalian testis. Cell Rep 2013; 3:2179-90; PMID:23791531; http://dx.doi.org/10.1016/j.celrep.2013.05.031

[2] Chalmel F, Lardenois A, Evrard B, Rolland AD, Sallou O, Dumargne MC, Coffeic I, Collin O, Primig M, Jégou B. High-resolution profiling of novel transcribed regions during rat spermatogenesis. Biol Reprod [Internet] 2014 [cited 2015 Feb 25]; 91:5. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24740603

[3] Laiho A, Kotaja N, Gyenesei A, Sironen A. Transcriptome profiling of the murine testis during the first wave of spermatogenesis. PLoS One [Internet] 2013; 8:e61558. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3629203&tool=pmcentrez&rendertype=abstract; http://dx.doi.org/10.1371/journal.pone.0061558

[4] Idler RK, Yan W. Control of messenger RNA fate by RNA-binding proteins: an emphasis on mammalian spermatogenesis. J Androl 2012; 33:309-37; PMID:21757510; http://dx.doi.org/10.2164/jandrol.11014167

[5] Paronetto MP, Sette C. Role of RNA-binding proteins in mammalian spermatogenesis. Int J Androl 2010; 33:2-12; PMID:19281489; http://dx.doi.org/10.1111/j.1365-2609.2009.00959.x

[6] Gao M, Arkov AL. Next generation organelles: structure and role of germ granules in the germline. Mol Reprod Dev [Internet] 2013 [cited 2015 Jun 25]; 80:610-23. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3584238&tool=pmcentrez&rendertype=abstract; http://dx.doi.org/10.1002/mrd.22450

[7] Meikar O, Da Ros M, Korhonen H, Kotaja N. Chromatoid body and small RNAs in male germ cells. Reproduction 2011; 142:195-209; PMID:21652638; http://dx.doi.org/10.1583/11-0057

[8] Kotaja N, Sassone-Corsi P. The chromatoid body: a germ-cell-specific RNA-processing centre. Nat Rev Mol Cell Biol 2007; 8:85-90; PMID:17183363; http://dx.doi.org/10.1038/nrm2081

[9] Fawcett DW, Eddy EM, Phillips DM. Observations on the fine structure and relationships of the chromatoid body in mammalian spermatogenesis. Biol Reprod 1970; 2:129-53; PMID:4106274; http://dx.doi.org/10.1095/biolreprod.2.1.129

[10] Shang P, Baarens WM, Hoogerbrugge J, Ooms MP, van Cappellen WA, de Jong AAW, Dohle GR, van Eenennaam H, Gossen JA, Groote Joal. Functional transformation of the chromatoid body in mouse spermatids requires tests-specific serine/threonine kinases. J Cell Sci 2010; 123:331-9; PMID:20053632; http://dx.doi.org/10.1242/jcs.059949

[11] Meikar O, Da Ros M, Liljenbäck H, Toppari J, Kotaja N. Accumulation of piRNAs in the chromatoid bodies purified by a novel isolation protocol. Exp Cell Res 2010; 316:1567-75; PMID:20219458; http://dx.doi.org/10.1016/j.yexcr.2010.02.023
[27] Buchan JR, Kolaitis RM, Taylor JP, Parker R. Eukaryotic stress granules are cleared by autophagy and Cdc48/VCP function. Cell [Internet] 2013 [cited 2014 Jul 25]; 153:1461-74. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3760148/?tool=pmcentrez&rendertype=abstract; http://dx.doi.org/10.1016/j.cell.2013.05.037

[28] Ryu HH, Jun MH, Min KJ, Kang DJ, Lee YS, Kim HK, Lee JA. Autophagy regulates atrophymotous lacerol srosis-linked fused in sarcoma-positive stress granules in neurons. Neurobiol Aging [Internet] 2014 [cited 2015 Jun 9]; 35:2822-31. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2516585; http://dx.doi.org/10.1016/j.neurobiolaging.2014.07.026

[29] Seguin SJ, Morelli FF, Vinet J, Amore D, De Biasi S, Poletti A, Mizushima N, Komatsu M. Autophagy: renovation of cells and tissues. Cell [Internet] 2014; 159:264-76; PMID:25086568; http://dx.doi.org/10.1016/j.cell.2014.10.026

[30] Raiborg C, Wenzel EM, Pedersen NM, Aasland M, Løv H, Løvendahl P, Øvervatn A, Schmitz I, Lørgaard P. FYCO1 protein and regulates phagosome maturation and reactive oxygen species are cleared by autophagy and Cdc48/VCP function. Cell [Internet] 2013 [cited 2015 Apr 28]; 21:1838-51. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3502387; http://dx.doi.org/10.1016/j.cell.2013.04.036

[31] Olsvik HL, Lamark T, Takagi K, Larsen KB, Evjen G, Øvervatn A, Fujita H, Yokota S. Argonaute2 Protein in Rat Spermatogenic Cells Is Localized to Nuage Structures and LAMP2-positive stress granules in neurons. Neurobiol Aging [Internet] 2011 [cited 2016 May 27]; 32:147-58; PMID:21580003; http://dx.doi.org/10.1016/j.neurobiolaging.2011.04.022

[32] Cheng X, Wang Y, Gong Y, Li F, Guo Y, Hu S, Liu J, Pan L. Structural basis of FYCO1 and MAP1LC3A interaction reveals a novel binding mode for Atg8-family proteins. Autophagy [Internet] 2016 [cited 2016 Jun 5]; 12(8):1300-9. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4926427

[33] Shpilka T, Weidberg H, Pietrzkowski S, Elazar Z. Atg8: an autophagy-related ubiquitin-like protein family. Genome Biol [Internet] 2011 [cited 2016 May 14]; 12:226. Available from: http://www.pubmedcentral.nih.gov/pmc/articles/PMC3128822/?tool=pmcentrez&rendertype=abstract; http://dx.doi.org/10.1186/gb-2011-12-7-226

[34] Ma J, Becker C, Reyes C, Underhill DM. Cutting edge: FYCO1 recruitment to dectin-1 phagosomes is accelerated by light chain 3 protein and regulates phagosome maturation and reactive oxygen production. J Immunol [Internet] 2014 [cited 2016 Jun 5]; 192:1356-60. Available from: http://www.pubmedcentral.nih.gov/pmc/articles/_reader.fcgi?artid=3966112&tool=pmcentrez&rendertype=abstract; http://dx.doi.org/10.4049/jimmunol.1302835

[35] Mraakovic A, Kay JG, Furuya W, Brumell JH, Botelho RJ. Rab7 and Arl8 GTPases are necessary for lysosome tubulation in macrophages. J Cell Biol [Internet] 2012 [cited 2016 Jun 16]; 198:685-701. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3525538; http://dx.doi.org/10.1083/jcb.200803137

[36] Yla-Anttila P, Vihinen H, Jokitalo E, Eskelinen E-L. Monitoring autophagy by electron microscopy in Mammalian cells. Methods Enzymol [Internet] 2009 [cited 2016 Jun 16]; 452:143-64. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1920881

[37] Parvinen M. The chromatoid body in spermatogenesis. Int J Androl 2005; 28:189-201; PMID:16048630; http://dx.doi.org/10.1111/j.1365-2494.2005.tb01466.x

[38] Ajioka D, Lopera P, Ajioka D, Lopera P. Wortmannin inactivates phosphoinositide 3-kinase by covalent modification, requires the selective adaptor p62 and ubiquitin-dependent degradation of organelles in spermatogenic cells. J Histochem Cytochem 2005; 53:455-65; PMID:15805420; http://dx.doi.org/10.1369/jhc.4A6520.2005

[39] Haraguchi CM, Babuchi T, Hirata S, Shoda T, Hoshi K, Akasaka K, Yokota S. Chromatoid bodies: aggresome-like characteristics and degradation sites for organelles of spermatogenic cells. J Histochem Cytochem 2005; 53:455-65; PMID:15805420; http://dx.doi.org/10.1369/jhc.4A6520.2005

[40] Koory L, Mukherjee A, Chakraborty S, Chakraborty S, Chakraborty S, Chakraborty S, Chakraborty S. AUTOPHAGosomes in spermatogenic Cells Is Localized to Nuage Structures and LAMP2-positive stress granules in neurons. Neurobiol Aging [Internet] 2011 [cited 2015 Aug 6]; 32:147-58; PMID:21580003; http://dx.doi.org/10.1016/j.neurobiolaging.2011.04.022

[41] Svenning S, Johansen T. Selective autophagy. Essays Biochem [Internet] 2013 [cited 2015 Aug 14]; 55:79-92. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3907473; http://dx.doi.org/10.1042/bse0550079

[42] Lippai D, Lőw P. The role of the selective adaptor p62 and ubiquitin-like proteins in autophagy. Biomed Res Int [Internet] 2014 [cited 2015 Aug 6]; 2014:832704. Available from: http://www.pubmedcentral.nih.gov/pmc/articles/PMC4075091/?tool=pmcentrez&rendertype=abstract; http://dx.doi.org/10.1038/ncomms2204

[43] Dall'Ara M, Ceverino D, De Biasi S, Poletti A, Mizushima N, Komatsu M. Autophagy: renovation of cells and tissues. Cell [Internet] 2013 [cited 2015 May 30]; 3;523:8-9. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3566815; http://dx.doi.org/10.1038/ncomms2676

[44] Fujii Y, Onohara Y, Fujita H, Yokota S. Argonaute2 Protein in Rat Spermatogenic Cells Is Localized to Nuage Structures and LAMP2-positive stress granules in neurons. Neurobiol Aging [Internet] 2014 [cited 2015 Jun 7]; 35:2822-31. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2516585; http://dx.doi.org/10.1016/j.neurobiolaging.2014.07.026
Positive Vesicles Surrounding Chromatoid Bodies. J Histochem Cytochem [Internet] 2016 [cited 2016 Jun 8]; 64:268-79. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27029769; http://dx.doi.org/10.1369/0022155416638840

[54] Eulalio A, Behm-Ansmant I, Schweizer D, Izaurralde E. P-body formation is a consequence, not the cause, of RNA-mediated gene silencing. Mol Cell Biol [Internet] 2007 [cited 2016 May 4]; 27:3970-81. Available from: http://mcb.asm.org/content/27/11/3970; http://dx.doi.org/10.1128/MCB.00128-07

[55] Belevich I, Joensuu M, Kumar D, Vihinen H, Jokitalo E. Microscopy Image Browser: A platform for segmentation and analysis of multidimensional datasets. PLoS Biol 2015; 14:e1002340.in press

[56] Kankaanpää P, Paavolainen L, Tiitta S, Karjalainen M, Päivärinta J, Nieminen J, Marjomäki V, Heino J, White DJ. BiologImageXD: an open, general-purpose and high-throughput image-processing platform. Nat Methods 2012; 9:683-9; PMID:22743773; http://dx.doi.org/10.1038/nmeth.2047

[57] Kotaja N, Kimmins S, Brancorsini S, Hentsch D, Vonesch JL, Davidson I, Parmen M, Sassone-Corsi P. Preparation, isolation and characterization of stage-specific spermatogenic cells for cellular and molecular analysis. Nat Methods 2004; 1:249-54; PMID:16144087; http://dx.doi.org/10.1038/nmeth1204-249

[58] Bryant JM, Meyer-Ficca ML, Dang VM, Berger SL, Meyer RG. Separation of spermatogenic cell types using STA-PUT velocity sedimentation. J Vis Exp 2013; 80: e50648; PMID:24145866

[59] Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 2010; 26:139-40; PMID:19910308; http://dx.doi.org/10.1093/bioinformatics/btp616

[60] Li XZ, Roy CK, Dong X, Bolcun-Filas E, Wang J, Han BW, Xu J, Moore MJ, Schimenti JC, Weng Z, et al. An ancient transcription factor initiates the burst of piRNA production during early meiosis in mouse testes. Mol Cell [Internet] 2013 [cited 2014 Jan 24]; 50:67-81. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3671569&tool=pmcentrez&rendertype=abstract; http://dx.doi.org/10.1016/j.molcel.2013.02.016

[61] Han BW, Wang W, Zamore PD, Weng Z. piPipes: a set of pipelines for piRNA and transposon analysis via small RNA-seq, RNA-seq, degradome- and CAGE-seq, ChIP-seq and genomic DNA sequencing. Bioinformatics 2015; 31:593-5; PMID:25342065; http://dx.doi.org/10.1093/bioinformatics/btu647

[62] Trapnell C, Hendrickson DG, Sauvageau M, Goff L, Rinn JL, Pachter L. Differential analysis of gene regulation at transcript resolution with RNA-seq. Nat Biotechnol 2013; 31:46-53; PMID:23222703; http://dx.doi.org/10.1038/nbt.2450

[63] Korhonen HM, Meikar O, Yadav RP, Papaioannou MD, Romero Y, Da Ros M, Herrera PL, Toppari J, Nef S, Kotaja N, et al. Dicer is required for haploid male germ cell differentiation in mice. PLoS One 2011; 6:e24821; PMID:21949761; http://dx.doi.org/10.1371/journal.pone.0024821