Lose it or keep it: (how bivalves can provide) insights into mitochondrial inheritance mechanisms

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Lose it or keep it: (how bivalves can provide) insights into mitochondrial inheritance mechanisms.

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Abbreviated title: Paternal mitochondria degradation in clams

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Abstract

The strictly maternal inheritance (SMI) is a pattern of mitochondrial inheritance observed across the whole animal kingdom. However, some interesting exceptions are known for the class Bivalvia, in which several species show an unusual pattern called doubly uniparental inheritance (DUI) whose outcome is a heteroplasmic pool of mtDNA in males. Even if DUI has been studied for long, its molecular basis has not been established yet.
The aim of this work is to select classes of proteins known to be involved in the maintenance of SMI and to compare their features in two clam species differing for their mitochondrial inheritance mechanism, i.e. the SMI species *Ruditapes decussatus* and the DUI species *Ruditapes philippinarum.* Data have been obtained from the transcriptomes of male and female ripe gonads of both species. Our analysis focused on nucleases and polymerases, ubiquitination and ubiquitin-like modifier pathways, and proteins involved in autophagy and mitophagy. For each protein group of interest, transcription bias (male or female), annotation, and mitochondrial targeting (when appropriate) were assessed.
We did not find evidence supporting a role of nucleases/polymerases or autophagic machinery in the enforcement of SMI in *R. decussatus*. On the other hand, ubiquitinating enzymes with the expected features have been retrieved, providing us with two alternative testable models for mitochondrial inheritance mechanisms at the molecular level.

Research highlights

Studying transcriptomes in two clam species differing for their mitochondrial inheritance mechanism, we identified some candidate E3 ubiquitin ligases and proposed two alternative models describing their involvement in mitochondrial transmission.

Introduction

In animals, the mitochondrial genome (mtDNA) is usually transmitted to the progeny exclusively by the female parent. Despite strictly maternal inheritance (SMI) being nearly-ubiquitous across eukaryotes, its underlying molecular mechanism is widely variable, suggesting recurrent loss and restoration and/or several independent origins (Birky, 1995). Paternal inheritance can be prevented by mtDNA elimination by nucleases either during spermatogenesis or after fertilization; alternatively, paternal mitochondria can be selectively degraded after entering the oocyte through proteasomal action or mitophagy. In the fish *Oryzias latypes*, the copy number of nucleoids (i.e. mtDNA-protein complexes) decreases during spermatogenesis. Once the spermatozoon enters the oocyte, an unknown endonuclease degrades the remaining mtDNA molecules, leaving paternal mitochondria with no genomic content, yet morphologically intact (Nishimura et al., 2006). In spermatozoa of *Drosophila melanogaster*, the two mitochondria extend by the exceptionally long tail (1,800 μm); in this species, nucleoids are completely degraded during spermatogenesis in a proximal-distal way, from the neck to the end to the tail (DeLuca and O'Farrell, 2012). Endonuclease G (EndoG) was initially thought to be the main effector of this degradation; however, recent research revealed the essential role of the mitochondrial polymerase Tamas in nucleoid elimination (Yu et al., 2017). A second mechanism ensures the complete clearance of paternal nucleoids: during *D. melanogaster* spermatid individualization, an actin structure called 'investment cone' progresses along the sperm tail axoneme and collects trimmed nucleoids in a distal 'waste bag'. Subsequently, paternal mitochondria are degraded through autophagy soon after fertilization, between mitotic cycles 1 and 9 (Politi et al., 2014). The autophagic process involves the formation of a double-membrane vesicle that wraps the targeted structure and fuses with a lysosome, causing the degradation of the target. Autophagy has been extensively studied when occurring in response to starvation (Pfeifer and Scheller, 1975)—a process named also non-selective autophagy—but it performs a number of other selective tasks as well, such as pexophagy (i.e.: selective degradation of peroxisomes via autophagy; Oku and Sakai, 2016), and mitophagy (i.e.: mitochondrial autophagy; Lemasters, 2014).

The pioneering work of Sutovsky's research group highlighted the importance of the ubiquitination pathway in sperm mitochondria elimination in cows and pigs. Ubiquitin (Ub) is a highly conserved peptide of 76-amino acids that is linked to lysine residues of proteins (Ciehanover et al., 1978), determining their sorting, degradation, or signal transduction, depending on the ubiquitination pattern (Swatek and Komander, 2016). Ubiquitination occurs as a three-step process involving Ub-activating (E1), Ub-conjugating (E2) and Ub-ligating (E3) enzymes. Tag specificity and selectivity are

achieved by the high diversity of the E3 Ub-ligases (Hershko and Ciechanover, 1998). Ubiquitin moieties can be removed by a de-ubiquitinating enzyme (DUB), making ubiquitination a highly dynamic tagging system. During spermatogenesis in cows and pigs, the 30 kDa inner membrane protein prohibitin is di-ubiquitinated. After fertilization, mitochondrial membranes undergo a structural rearrangement that brings ubiquitinated prohibitins on the outer membrane, causing them to be exposed to recognition by zygotic/embryonic ubiquitination machinery. Such machinery, in turn, adds more ubiquitin moieties to prohibitin and marks the switch from the di-ubiquitin recognition signal to a poly-ubiquitin degradation one (Sutovsky et al., 2000). Subsequently, paternal mitochondria are targeted to proteolytic destruction by the conjoint action of proteasome and autophagy/lysosome system (Sutovsky et al., 2000, 2003; Rojansky et al., 2016). Further work by May-Panloup et al. (2003) and Luo et al. (2013) determined that vital sperm of mice and men has a very low nucleoid content, suggesting a process of mtDNA copy number reduction during spermatogenesis.
Lastly, autophagy and ubiquitination are the main processes responsible for the clearance of paternal mitochondria in *Caenorhabditis elegans* as well (Sato and Sato, 2011): upon entering the oocyte, sperm mitochondria and other structures of paternal origin called membranous organelles (MOs) are degraded through autophagy. MOs have been found to be ubiquitinated before and after fertilization, similarly to what happens in mammalian paternal mitochondria; however, no sign of ubiquitination has been detected on *C. elegans* paternal mitochondria.

The exception to SMI

The only known evolutionarily stable exception to the common SMI is represented so far by the doubly uniparental inheritance of mitochondria or DUI (Skibinski et al., 1994a; b; Zouros et al., 1994a; b). This mitochondrial inheritance mechanism has been found in ~100 species of bivalve molluscs (Gusman et al., 2016) and features two different mtDNAs, the F-type and the M-type, with high intraspecific divergence, and sex-specific inheritance. The distribution of the two mitochondrial genomes within an individual depends on its sex: females are homoplasmic for F-type mtDNA, whereas males carry the M-type mtDNA in the germline and both mitochondrial genomes in the soma, with varying proportions depending on species and tissue (Ghiselli et al., 2011; Obata et al., 2011; Milani et al., 2014a).
One of the most interesting peculiarities of DUI mtDNAs is that they contain a novel lineage-specific ORF (one in the F-type, one in the M-type) that, according to *in silico* prediction, might have had a viral origin (Milani et al., 2013, 2014b, 2016). Moreover, females of DUI species differ in offspring sex ratio, that can be either male-biased, female-biased or balanced, a feature that appears to be mostly dependent on the maternal genotype, but not immune to paternal influence (Saavedra et al., 1997; Kenchington et al., 2002; Ghiselli et al., 2012; Yusa et al., 2013). Observations in early

embryos of Mytilus and the venerid *Ruditapes philippinarum* (both with DUI) revealed that sperm mitochondria show two different distribution patterns across blastomeres: aggregated or dispersed (Cao et al., 2004; Milani et al., 2012). In Mytilus, the two patterns have been associated with male and female embryos, respectively. However, differences in the aggregation pattern cannot account completely for the aforementioned distribution of mtDNA in tissues, and additional active mechanisms such as paternal mitochondria degradation in females and preferential replication in males (i.e. meiotic drive) have been proposed (Ghiselli et al. 2011, Milani et al. 2015, 2016).

A further point of relevance concerns the evolutionary inception of DUI. It is not clear whether DUI had a single origin or arose several times throughout its evolutionary history. In the first case, DUI might be the result of a single event happened at the origin of the Autolamellibranchia superclass, more than 400 million years ago (Zouros, 2013). However, its distribution across the bivalve phylogenetic tree is not homogenous: for instance, within Pteriomorphia, mytilids have DUI, while ostreids and pectinids do not (Doucet-Beaupré et al., 2010), and among Veneridae the two lineage-specific mtDNAs have been found in *R. philippinarum* (Passamonti and Scali, 2001) and *Meretrix lamarckii* (Bettinazzi et al., 2016), while no evidence was found in *R. decussatus* (Ghiselli et al. 2017) and *Callista chione* (Plazzi et al., 2015). Besides being the result of incomplete sampling, this scattered distribution may also be imputed to false negatives due to the technical difficulties in the detection of the two different DUI mitochondrial genomes (see Theologidis et al., 2008 and Ghiselli et al., 2017 for a thorough discussion of this issue). In any case, if DUI had a single origin, several loss events have to be assumed to explain its scattered distribution across bivalves (Zouros, 2013). That said, a multiple-origin hypothesis might be more parsimonious. Recent works proposed that the mitochondrial lineage-specific ORFs found in several bivalve species may play a role in DUI emergence and establishment (Breton et al., 2011b; Milani et al., 2013, 2014b, 2015, 2016). According to this hypothesis, the endogenization of viral sequences in mtDNA might be the trigger for DUI evolution; such viral sequences might have provided the recipient mtDNA with the ability to invade the germ line (e.g. through meiotic drive), thus producing a selfish element (Milani et al., 2015, 2016). Although such ORFs share some common features, their alignments were possible only among sequences of closely related species (Breton et al., 2011a; Milani et al., 2013): this may be due either to their fast evolution making their homology undetectable, or to several independent endogenization events. As a matter of fact, a hypothesis featuring multiple viral origins of DUI may explain its scattered distribution across bivalves.

Being the only known stable exception to SMI, DUI provides a unique chance to study mitochondrial inheritance mechanisms by comparing two naturally occurring systems in two relatively close species. As mentioned before, it is well known that SMI maintenance, despite resulting in the same final outcome, is achieved through the most

diverse mechanisms (Birky, 1995, 2001; Sato and Sato, 2013). Similarly, it is conceivable that, at a molecular level, DUI relies on a machinery that differs from one taxon to another. So it seems legitimate to hypothesize that *R. philippinarum* may share a more similar machinery with a congeneric SMI species such as *R. decussatus,* rather than with other DUI species outside Veneroida. Of course, since the eventual mitochondrial distribution pattern between a SMI and a DUI species is completely distinct, there must be difference, but such difference can reside virtually in a single protein (Zouros, 2013).

Summarizing, the process of paternal mitochondria degradation in animals comprises two temporally distinct steps: degradation of sperm mtDNA and/or labeling of paternal mitochondria occurs during spermatogenesis, whereas degradation of nucleoids and/or recognition and degradation of paternal mitochondria happens after fertilization.

The sequences encoding the machinery for the first step have to be necessarily transcribed during spermatogenesis; the second step, instead, can comprehend sequences transcribed during oogenesis and accumulated into the oocyte, or by the zygote genome after maternal-zygotic transition, or both.

In order to uncover the molecular outline of mitochondrial inheritance*,* transcriptomic data from mature gonads of the SMI species *R. decussatus* and the DUI species *R. philippinarum* were analyzed, taking into account presence, transcription patterns, and mitochondrial targeting of all proteins belonging to pathways known to be involved in SMI achievement. Due to the nature of the available data, our research focused on the first step. Previous data (Ghiselli et al. 2012, Milani et al. 2013) show that, in *R. philippinarum* gonads, some sequences involved in the ubiquitination pathway are transcribed with a male bias, and *in situ* hybridization found some ubiquitin-related transcripts localized in gametogenic cells, hinting at a possible implication of ubiquitin system in DUI. A proteomic analysis on the DUI species *Mytilus edulis* (Diz et al., 2013) yielded similar results. Our analysis of transcripts belonging to nucleases/polymerases, autophagy and mitophagy, and ubiquitination pathway are consistent with pre-existing data, and allowed us to propose a model of SMI mechanism in *R. decussatus* and its modification in *R. philippinarum.*

Materials and Methods

Dataset

RNA-Seq libraries were prepared from ripe gonads of twelve individuals (six females and six males) of *R. philippinarum* from the Pacific coast of USA (Puget Sound, WA), and twelve individuals (six females and six males) of *R. decussatus* from the Northern Adriatic Sea (Goro, Italy), following the protocols of Mortazavi et al. (2008) with the modifications reported in Ghiselli et al. (2012). Raw reads and *de novo* assemblies of *R. philippinarum* and *R. decussatus* are available on NCBI (BioProjects PRJNA68513 and PRJNA170478, respectively). Details about sequencing, *de novo* assembly, and differential transcription analysis are described in Ghiselli et al. (2012), while statistics on the assemblies can be found in Supplementary materials S1. Differential transcription between males and females is expressed as the binary logarithm of the fold change of the transcription level [$\log_2$(FC)]; male-biased transcripts are defined as those for which $\log_2$(FC)<-1, whereas female-biased those for which $\log_2$(FC)>1.
In order to perform a comparative analysis of the two transcriptomes, the *de novo* assemblies were annotated with a transcriptome annotation pipeline for non-model organisms (Ghiselli et al., in preparation; detailed information, data and scripts can be found at the following link: https://osf.io/2gdqe/?view_only=f0b2cde926db43719f3d705012c4eeaa).
Mitochondrial targeting of all the sequences belonging to both transcriptomes was assessed with TargetP (Emanuelsson et al., 2007).

Data analysis

Following the literature on the subject, we narrowed our research to some “protein groups of interest” defined as follows: ubiquitin-proteasome system (UPS) and ubiquitin-like modifiers, mitophagy/autophagy, nucleases/DNA polymerases. FPKM data of the all the retrieved sequences can be found in Supplementary materials S2-S3.
Autophagy and mitophagy pathways rely on an evolutionarily conserved core machinery, and this has allowed us to compile lists of orthologs including all the proteins known to belong to these pathways. The sequences of the proteins included in such lists were used as queries in the searches against the transcriptomes of the two clam species. Conversely, proteins belonging to the groups of nucleases, DNA polymerases, and the UPS are part of multiple gene families varying in size and evolutionary history. As such, a gene-to-gene relationship with other species orthologs cannot be established. For this reason, we had to follow two different methods to retrieve loci of interest.

Orthologous sequences belonging to autophagy and mitophagy pathways in *H. sapiens* and in the oyster *Crassostrea gigas* (the only bivalve species available) were downloaded from the KEGG database (Kanehisa and Goto, 2000). In order to present the most comprehensive results possible, proteins involved in both autophagy and mitophagy were retained in both datasets. These sequences were used as queries in a BLASTP (Camacho et al., 2009) search against databases built from *R. decussatus* and *R. philippinarum* transcriptomes. We filtered out the hits with an E-value above 1E-50, and we checked the remaining sequences. If a sequence showed similarity for orthologs in both *C. gigas* and *H. sapiens*, it was retained only if the similarity with the bivalve species had a stronger support (i.e. a lower E-value). If a sequence showed similarity with a *C. gigas* sequence, but did not have any hit against human orthologs, it was kept as well; the opposite cases—similarity with *H. sapiens* but not with *C. gigas*—were regarded as possible contaminants and discarded. The KO (KEGG Orthology) identifier reported for the selected *C. gigas* and *H.sapiens* sequences was associated with each hit, so that exact correspondence with the KEGG reference pathways could be traced (Tables 2 and 3, supplementary materials S7-S10).
For UPS and nucleases/polymerases, instead, GO terms featuring the terms “ubiquitin”, “proteasome”, “nuclease” and “DNA polymerase” were selected from the GO database (Balakrishnan et al., 2013; downloaded on 12 october 2016) and manually curated (supplementary materials S4-S6). Sequences annotated with such GO terms were then extracted from the two transcriptomes (supplementary materials S11-S16). Additionally, prohibitin sequences belonging to *Caenorhabditis elegans*, *Xenopus tropicalis*, *Gallus gallus*, *Mus musculus*, *Rattus norvegicus*, *Bos taurus*, *Pongo abelli*, and *Homo sapiens* were downloaded from UniProtKB (The UniProt Consortium, 2017) and were used to perform a local BLASTP search, which unanimously retrieved the two evolutionarily conserved subunits of prohibitin in both species.

Results and Discussion

Nucleases and polymerases

We retrieved 277 sequences in *Ruditapes decussatus* and 230 sequences in *R. philippinarum* which were annotated with GO terms related to nuclease activity or polymerase activity (Table 1, supplementary materials S4-S5 and S11-S14). These sequences were mostly involved in DNA repair (GO:0006281, “DNA repair”, 56 occurrences in *R. decussatus* and 65 in *R. philippinarum*), but sequences involved in RNA retrotranscription were not uncommon (GO:0006278, “RNA-dependent DNA biosynthetic process”, 37 and 24 occurrences respectively), either annotated with transposon activity (according to BLASTP annotation, 23 and 14 respectively) or telomere maintenance (GO:0000723 “telomere maintenance” and child terms, 11 and 21 occurrences respectively). The biological functions uncovered by the annotation are expected, given the high proliferation activity of cells in gametogenic gonads—obviously requiring both polymerases and nucleases—and the physiological quality-check role of telomere maintenance in mitosis and meiosis. If any endonuclease or polymerase were to enter male mitochondria in order to reduce mitochondrial nucleotide content during *R. decussatus* spermatogenesis as it happens in *O. latypes*, we expect that the candidate sequence would have both a male biased transcription and a mitochondrial targeting presequence (Table 2). Regarding nucleases, several sequences possessing either one or the other feature have been retrieved, but none shows both (Figure 1a). As for polymerases, the great majority of sequences do not display a sex bias (Figure 1a), with only one female-biased contig per species and one strongly male-biased contig in *R. philippinarum* (-8.18397 $\log_2$(FC)), annotated as a “DNA polymerase nu-like”, an error-prone polymerase involved in DNA damage repair.
Our results are not consistent with a mechanism of nucleoid number reduction similar to that of *O. latypes* and some mammals, however it has to be noted that mitochondrial targeting assessment is especially prone to false negatives due to the presence of import signals other than presequences, or to transcript length biases. More extensive research has to be performed to rule out the involvement of endonucleases in SMI enforcement in *R. decussatus*.

Autophagy and mitophagy

Because of its high level of conservation across eukaryotes, autophagy is a particularly suitable pathway for transcriptomics studies in nonmodel species, so we were able to assess the completeness of autophagic supramolecular complexes by extracting autophagy-related orthologs from the two studied transcriptomes. The core components of autophagy are mostly present in both *R. decussatus* and *R. philippinarum*—for

instance GABARAP, an ortholog of yeast LC3, whose detection has been often used as a proxy for autophagy taking place (Kraft et al., 2010; Jin and Klionsky, 2014). Moreover, most of the functional annotation of the sequences involved in both autophagy and mitophagy is in common between the two clam species (Table 1).
Autophagy has been proved fundamental both for male and female gametogenesis, with roles ranging from regulation of signaling between follicle cells and oocytes in *Drosophila*, to correct acrosome formation in mouse spermatozoa (Barth et al., 2012; Kanninen et al., 2013; Wang et al., 2014; Agnello et al., 2016). In *R. philippinarum* transcriptome only two sequences out of 92 display a sex-biased transcription; in *R. decussatus*, instead, there are 22 sex-biased sequences out of 124, representing almost one-fifth of the total number of sequences involved in autophagy in this species (Figure 1b). These sequences code mainly for regulatory enzymes and display predominantly a female bias (16 female-biased vs 6 male-biased sequences; see Table 3 and supplementary materials S7-S8).
While these data suggest that autophagy-related genes are active at this stage in gonads, thus enabling the autophagy process, the same cannot be easily said for mitophagy: a core machinery for autophagy has been established with a wide consensus, whereas the molecular actors determining selective autophagy are more debated. A central mitophagic trigger mechanism revolves around the serine/threonine-protein kinase PINK1, which, upon attachment to the outer membrane of depolarized mitochondria, recruits the E3 ubiquitin ligase Parkin for their degradation through mitophagy (Durcan and Fon 2015). Other Parkin-independent pathways have been defined as well; for instance, hypoxia triggers mitophagy through activation of Nix/Fundc1 pathway (Campello et al., 2014; Georgakopoulos et al., 2017). Even if roughly half of the sequences involved in the mitophagy pathway are present in both species, most of the fundamental ones are missing in both species (i.e.: Parkin and the initiators of hypoxia-induced mitophagy FOXO3, Fundc1, Bnip3 and Bnip3L/Nix; see Table 4), while Ambra1, an effector of a hypothesized Parkin-independent mitophagy pathway, and PINK1 are present only in *R. philippinarum*. Moreover, a female-biased transcription of the retrieved mitophagy-associated genes in *R. decussatus*—even if weak (Figure 2)—point out to an inhibition of mitophagy rather than an activation (for a review on mitophagy regulation refer to Hamacher-Brady and Brady, 2016).
We can hypothesize at least two different mechanisms for SMI enforcement through mitophagy/autophagy (Table 2). On the one hand, mitophagy could have a role in reducing nucleoid number during spermatogenesis. As data do not point out male-biased transcription of any of the sequences, it appears that this mechanism is not put in place in *R. decussatus*. On the other hand, male mitochondria could be digested after fertilization, as in studied mammals and *C. elegans*. If this is the case, we could reasonably expect an accumulation of autophagy- and mitophagy-related transcripts in oocytes, resulting in a female bias. However, with the exception of the already

discussed bias regarding regulatory sequences, no other strong female bias has emerged. Still, this mechanism could take place after the maternal-zygotic transition and be due to zygotic transcripts (Schier, 2007), but in order to further elucidate this point, different developmental stages should be assessed for the presence of this pathway.

Ubiquitination and ubiquitin-like modifiers

We retrieved 778 and 728 ubiquitination-related sequences in *R. decussatus* and *R. philippinarum*, respectively (Table 1, supplementary materials S6 and S15-S16, Figure 1c). As the name of the pathway itself suggests, it is one of the most ubiquitous mechanism for routinely protein quality control within cells. As such, several E1, E2, E3 and deubiquitinating enzymes were retrieved (Table 5). Moreover, ubiquitination covers specialized roles during gametogenesis, especially in males (for thorough reviews see: Richburg et al., 2014; Suresh et al., 2016). In mammals, one of such roles is to provide sperm mitochondria with degradation signals by di-ubiquitinating the mitochondrial membrane protein prohibitin (Sutovsky et al., 2000). A similar pattern of prohibitin ubiquitination, even if with a slightly different timing, appears to extend to species outside the mammalian taxon: for instance, in the crayfish *Procambarus clarkii* prohibitin, ubiquitin, and mitochondria co-localize in late spermatogenesis (Dong et al., 2015).
Prohibitins have been retrieved in the analyzed clam species as well. Given the evolutionary conservation of ubiquitination of prohibitin during spermatogenesis, it is conceivable that they might play a role in paternal mitochondria recognition as in mammals.
Given the high substrate specificity of E3 ubiquitin ligases and their high recurrence in the two transcriptomes (258 in *R. decussatus* and 237 in *R. philippinarum* according to GO term annotation - see table 5), we expect the candidate sequences to show a strongly male-biased transcription level, if not a male-specific transcription (Table 2).
In order to explain the different mitochondrial inheritance outcomes between the two species investigated here, we propose two hypotheses (Figure 2). There is some speculation in such hypotheses, but they are all consistent with the available data and can be useful to guide future experiments and research by providing candidate targets for further investigation.
Hypothesis I
Effectiveness of degradation through ubiquitination relies on the recognition of ubiquitin moieties linked to the target. If the ubiquitination signal is persistent in both species, it has to be masked in *R. philippinarum* in order to achieve DUI. A candidate for this role is RPHM21, a protein encoded by a male-specific mitochondrial ORF transcribed and translated during spermatogenesis, localized in sperm mitochondria and nuclei, and in embryos (Milani et al., 2014b, 2015, 2016). Its main putative features are two transmembrane helices, a binding site for ubiquitin, and domains involved in

cytoskeleton interactions. As already hypothesized in Milani et al. (2014b), RPHM21 might prevent the recognition of the degradation signal on the male mitochondria by binding to ubiquitinated mitochondrial proteins (for instance, prohibitin dimers) through their ubiquitin binding site. Indeed, male mitochondria are not degraded before the 32-blastomere stage in all *R. philippinarum* embryos observed, irrespective of the aggregation pattern (Milani et al., 2014b), so RPHM21 protection mechanism could delay degradation of sperm mitochondria independently from the sex of the embryos. If this is the case, the E3 ubiquitin ligase, performing this task may be conserved in both species and show a male-biased transcription. Such features, indeed, apply to two sequences (identified as Locus_350 in *R. decussatus* and Locus_6979 in *R. philippinarum*, see figure 1c and figure 2-HP1) that belong to the same ortholog cluster, both undetectable in female gonads—designating them as male specific—and both annotated as “F-box only protein 39”, a substrate recognition component of the SCF (Skp1/Cullin/F-box) complex, a family of modular E3 ligases.

Hypothesis II

On the other hand, if the membrane protein carrying the male recognition signal is unmasked also in *R. philippinarum* (i.e.: no masking by RPHM21 or other factors), then the difference between the two species could lie instead in the ubiquitination pattern. Hence, ubiquitination in the SMI species could be performed by an E3 ubiquitin ligase whose ortholog is either absent or transcriptionally downregulated/silenced in *R. philippinarum*, resulting in a male-biased sequence in *R. decussatus* lacking an ortholog in the other species. This description delineates the characteristics of several *R. decussatus* male-biased sequences. While most of them are either involved in cell cycle maintenance or have a relatively weak male bias, the most transcriptionally biased one is a sequence (identified as Locus_14176, see Figure 1c) containing a mib/herc2 domain (a ubiquitin ligase domain; PF06701) also annotated with GO:0016020 “membrane” and GO:0016021 “integral component of membrane”. Studies suggest that transmembrane E3 substrates are preferentially transmembrane proteins themselves (Bauer et al., 2016). This E3 might ubiquitinate a male recognition protein on the mitochondrial outer membrane of the SMI species *R. decussatus* targeting sperm mitochondria for degradation.

Conclusions

We can detail the process of paternal mitochondria degradation in animals as composed of two steps: 1) during spermatogenesis - degradation of nucleoids and/or marking of paternal mitochondria as means to distinguish them from maternal ones; and 2) after fertilization - degradation of nucleoids or paternal mitochondria.

The sequences encoding the machinery for the first step have to be necessarily transcribed during spermatogenesis; the second step, instead, can comprehend sequences transcribed during oogenesis, or after maternal-zygotic transition, or both.

The transcriptomic data here analyzed, portraying late gametogenesis of the two bivalve species *R. decussatus* and *R. philippinarum*, allowed us to hypothesize which processes and genes might be involved in the first step, and which might be the molecular similarities and differences underlying the two different inheritance outcomes (Figure 2).
We propose two hypotheses (Figure 2). 1) the degradation signal present on the mitochondrial outer membrane (which could be represented by ubiquitinated prohibitins) is masked in the zygote (e.g. by RPHM21), so the enzyme responsible for such degradation labeling must be present in both *R. decussatus* and *R. philippinarum*. Two male-specific ortholog sequences annotated as “F-box only protein 39”, an E3 ubiquitin ligase, show characteristics which are compatible with this hypothesis; 2) the difference lies in the labeling pattern being absent or delayed in the DUI species. A transmembrane E3 ubiquitin ligase with a strong male bias, retrieved in *R. decussatus* and with no apparent ortholog in *R. philippinarum*, is a good candidate to perform this task.
As for the second step, that is degradation of paternal mitochondria after fertilization, may involve proteins transcribed after the maternal-zygotic transition, so further research involving developing embryos is needed to clarify this point.
Future perspectives include immunological analyses on sperm and zygotes of both species, and investigating localization and interaction among prohibitin/ubiquitin and the other suggested candidate proteins will help defining the described mechanisms.

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Tables

Table 1 – Overall sequences retrieved for each protein group of interest, comprehensive of transcription bias, orthology and mitochondrial target.

Table 2 - Summary of the assessed features of the proteins belonging to the pathways under study. Details in the main text.

Table 3 – Proteins involved in autophagy in *R. decussatus* (Rde) and *R. philippinarum* (Rph).

Table 4 – Proteins involved in mitophagy in *R. decussatus* (Rde) and *R. philippinarum* (Rph).

Table 5 - Estimate of the number of enzymes involved in the ubiquitination pathway according to the GO annotation.

Figure Legends

Figure 1. Distribution of *Ruditapes decussatus* and *Ruditapes philippinarum* loci according to the statistical significance and the transcriptional sex bias, expressed as the binary logarithm of the fold change of the transcription level. The horizontal gray line marks the significance threshold (p = 0.05), whereas the vertical gray lines mark the transcriptional sex bias threshold (see Materials and methods). a) Loci annotated as nucleases and polymerases; the loci represented with an empty square possess a mitochondrial presequence; b) Loci annotated as belonging to autophagy and/or mitophagy pathway; c) Loci annotated as belonging to the ubiquitination or ubiquitination-like pathways.

Figure 2. Representation of the ubiquitination state in mitochondria of spermatozoa in both clam species, according to the two hypotheses presented in this study.
Hypothesis 1 (HP1) – During spermatogenesis of both species, prohibitins or other proteins on the mitochondrial outer membrane are ubiquitinated by a “F-box only 39” E3. RPHM21 then masks the recognition/degradation signal in *R. philippinarum*, hindering mitochondria destruction after fertilization.
Hypothesis 2 (HP2) – RPHM21 is involved in processes other than masking the recognition signal, such as gonad differentiation or determination of the mitochondria aggregation pattern (Milani et al. 2014). The ubiquitinating enzyme is a transmembrane mib/herc E3 in *R. decussatus* and is absent in *R. philippinarum*.
Note: MOM: mitochondrial outer membrane; IMS: intermembrane space.

TABLE 1

	Rde	Rph			Rde	Rph	
Ubiquitination				**Autophagy** (total KO ids in KEGG: 100)			KO ids in common
total sequences	778	728		KO ids	62	50	45
female biased	48	18		total loci	124	92	
male biased	28	20		female biased	16	0	
orthologs	471 (394)	450 (387)	Clusters in common: 381	male biased	7	2	
mt target	38	42		orthologs	87 (59)	65 (59)	Clusters in common: 59
Nucleases				**Mitophagy** (total KO ids in KEGG: 57)			KO ids in common
total sequences	277	230		KO ids	27	24	22
female biased	25	12		total loci	46	35	
male biased	16	4		female biased	6	0	
orthologs	154 (127)	131 (113)	Clusters in common: 109	male biased	4	0	
mt target	24	13		orthologs	31 (29)	31 (29)	Clusters in common: 29
Polymerases							
total loci	284	266					
female biased	19	7					
male biased	14	6					
orthologs	173 (148)	147 (129)	Clusters in common: 128				
mt target	19	18					

Note: Orthologs = number of sequences that have one or more orthologs in the other species' transcriptome; in parentheses the number of sequences that have at least one ortholog with the same annotation and thus that belong to the clusters in common; clusters in common = ortholog clusters whose sequences have the same annotation in both species; KO ids: total KO identifiers with at least a corresponding sequence in the species – correspondence addressed in detail in tables 3 and 4; Rde = *R. decussatus*; Rph = *R. philippinarum*.

TABLE 2

	Endonucleases	**Polymerases**	**Autophagy**	**Mitophagy**	**Ubiquitination**
Proposed mode of action	Degrade mtDNA during spermatogenesis		Degrade mitochondria during spermatogenesis and/or after fertilization		Mark paternal mitochondria for degradation during spermatogenesis and/or after fertilization
Did we retrieve all the sequences necessary to enforce this pathway?	Yes	Yes	Almost all	Dubious	Yes
Is a transcriptional bias necessary? Male or female?	Yes - Male	Yes - Male	Yes – could be both	Yes – could be both	Yes - Male
Did we find sequences with such bias?	Yes, but lacking a mitochondrial presequence	Yes, but lacking a mitochondrial presequence	No	No	Yes
Does the resulting protein(s) have to enter the mitochondria (i.e. is a mitochondrial presequence necessary)?	Yes	Yes	No	No	No
Did we find sequences with the mitochondrial presequence?	Yes, but lacking a transcriptional bias	Yes, but lacking a transcriptional bias	N/A	N/A	N/A
Did we find sequences/groups of sequences with all the needed characteristics?	**No**	**No**	**No**	**No**	**Yes**

TABLE 3

KOid	Name	Rde	Rph	KOid	Name	Rde	Rph
K00914	PIK3C3	o	o	K08270	DDIT4	x	x
K00922	PIK3CA_B_D	o	x	K08331	ATG13	o	o
K01110	PTEN	o	o	K08333	PIK3R4	o	o
K01363	CTSB	o	o	K08334	BECN	o	o
K01365	CTSL	o	o	K08336	ATG12	o	x
K01379	CTSD	o	o	K08337	ATG7	o	o
K02158	BAD	x	x	K08339	ATG5	o	o
K02161	BCL2	x	x	K08341	GABARAP	o	o
K02649	PIK3R1_2_3	o	x	K08342	ATG4	o	o
K02833	HRAS	x	x	K08343	ATG3	o	o
K03175	TRAF6	o	o	K08491	STX17	x	x
K03237	EIF2S1	o	o	K08509	SNAP29	x	o
K04345	PKA	o	o	K08512	VAMP8	x	x
K04366	RAF1	x	x	K08803	DAPK	x	x
K04368	MAP2K1	o	o	K08852	ERN1	o	x
K04369	MAP2K2	x	x	K08860	EIF2AK3	o	x
K04371	MAPK1_3	o	o	K10802	HMGB1	x	x
K04382	PPP2C	o	o	K11248	SH3GLB1	o	x
K04427	MAP3K7	o	o	K15464	BNIP3	x	x
K04440	JNK	o	o	K16172	IRS1	x	x
K04456	AKT	o	o	K16184	AKT1S1	x	x
K04526	INS	x	x	K16185	RRAGA_B	x	o
K04570	BCL2L1	x	o	K16186	RRAGC_D	o	x
K04688	RPS6KB	o	o	K16196	EIF2AK4	o	o
K04724	CFLAR	x	x	K17445	IRS3	x	x
K04958	ITPR1	o	o	K17446	IRS4	x	x
K05087	IGF1R	x	x	K17589	RB1CC1	o	x
K06068	PRKCD	o	o	K17603	ZFYVE1	x	x
K06276	PDPK1	o	o	K17606	IGBP1	o	o
K06528	LAMP1_2	x	x	K17888	ATG10L	x	x
K07187	IRS2	x	x	K17889	ATG14L	o	x
K07198	PRKAA	o	o	K17890	ATG16L1	o	o
K07203	MTOR	o	o	K17906	ATG2	o	o
K07204	RAPTOR	o	o	K17907	ATG9	o	x
K07206	TSC1	o	o	K17908	WIPI	o	o
K07207	TSC2	o	x	K17985	AMBRA1	o	x
K07208	RHEB	o	o	K18052	PRKCQ	x	x
K07298	STK11	o	o	K18082	MTMR3_4	o	o
K07359	CAMKK2	x	x	K18086	MTMR14	o	x
K07827	KRAS	o	o	K19330	RUBCN	o	x
K07828	NRAS	x	x	K19730	ATG101	o	o
K07829	RRAS	x	x	K20402	DEPTOR	x	x
K07830	RRAS2	o	x	K20868	ATG16L2	x	x
K07831	MRAS	x	o	K21245	SUPT20H	o	x
K07897	RAB7A	o	o	K21246	NRBF2	x	o
K07898	RAB7B	x	x	K21247	TP53INP2	x	x
K07920	RAB33B	o	o	K21248	VMP1	o	o
K08266	MLST8	o	o	K21249	UVRAG	o	x
K08268	HIF1A	x	x	K21250	PRAP1	x	x
K08269	ULK2	o	o	K21357	ULK1	x	x

Note: KOids: KEGG ORTHOLOGY entries; Name: common name of the ortholog group; o = presence; x = absence.

TABLE 4

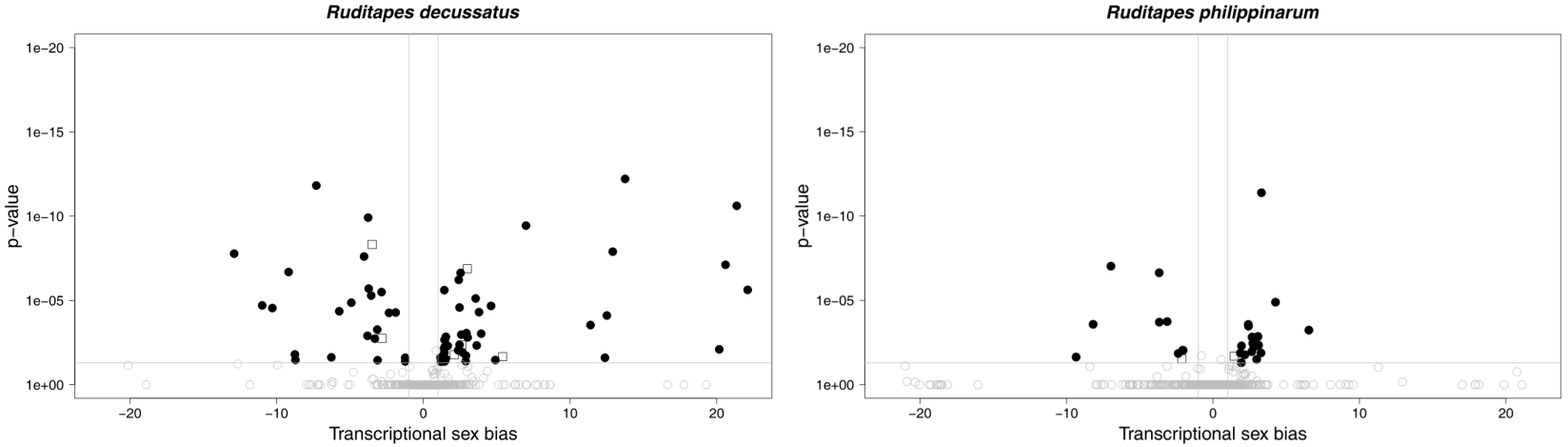
KOid	Name	Rde	Rph	KOid	Name	Rde	Rph
K02833	HRAS	x	x	K08341	GABARAP	o	o
K03097	CSNK2A	x	x	K08860	EIF2AK3	o	x
K03115	CSNK2B	o	o	K09105	TFE3	x	x
K04374	ATF4	x	x	K09455	MITF	o	o
K04440	JNK	o	o	K11839	USP8	o	o
K04448	JUN	x	o	K11851	USP30	o	o
K04451	TP53	x	x	K14381	SQSTM1	o	o
K04551	UBB	x	x	K15485	BCL2L13	x	x
K04570	BCL2L1	x	o	K15590	TFEB	x	x
K04684	SP1	x	x	K15637	PGAM5	o	o
K04735	RELA	x	x	K17454	E2F1	x	x
K05410	TBK1	o	o	K17771	TOM7	x	x
K05704	SRC	o	o	K17907	ATG9	o	x
K06030	MFN2	o	o	K17969	FIS1	o	x
K07827	KRAS	o	o	K17985	AMBRA1	o	x
K07828	NRAS	x	x	K17987	NBR1	o	o
K07829	RRAS	x	x	K19945	TBC1D17	x	x
K07830	RRAS2	o	o	K19946	OPTN	o	o
K07831	MRAS	x	x	K20168	TBC1D15	o	o
K07870	RHOT1	o	o	K21343	USP15	o	o
K07871	RHOT2	x	x	K21347	TAX1BP1	o	o
K07897	RAB7A	o	o	K21348	CALCOCO2	x	x
K07898	RAB7B	x	x	K21356	MFN1	x	x
K08268	HIF1A	x	x	K21357	ULK1	x	x
K08334	BECN	o	o	K21361	CITED2	x	x
K08339	ATG5	o	o				

Note: KOids: KEGG ORTHOLOGY entries; Name: common name of the ortholog group; o = presence; x = absence.

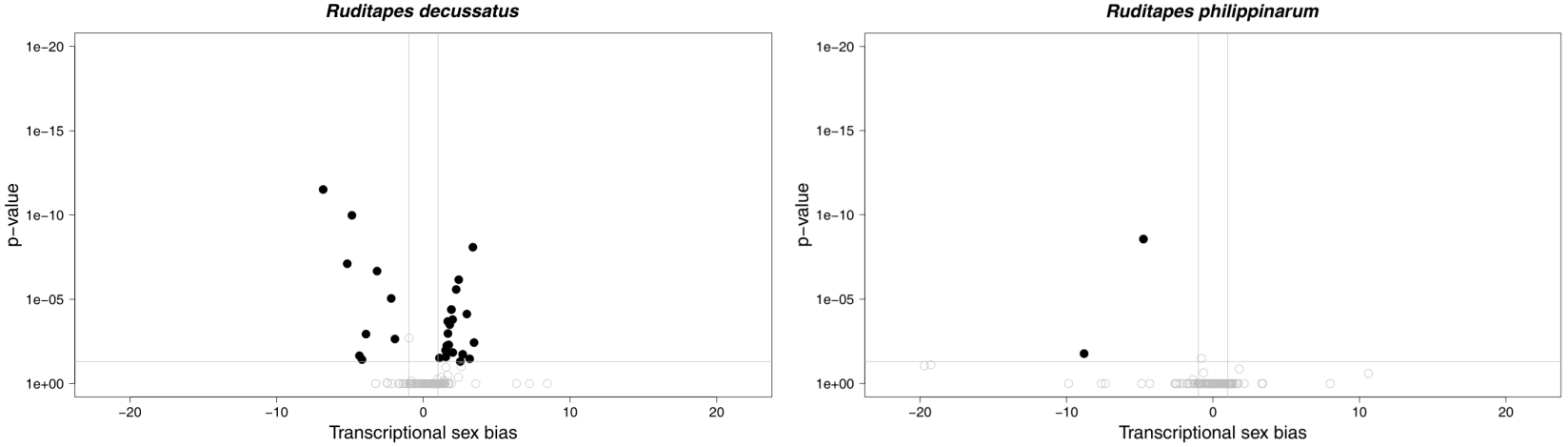
TABLE 5

	Ruditapes decussatus	*Ruditapes philippinarum*
E1 – Ub-activating enzymes	5	5
E2 – Ub-conjugating enzymes	7	7
E3 – Ub-ligases	258	237
De-ubiquitinating enzymes	57	61
Proteasome	153	144

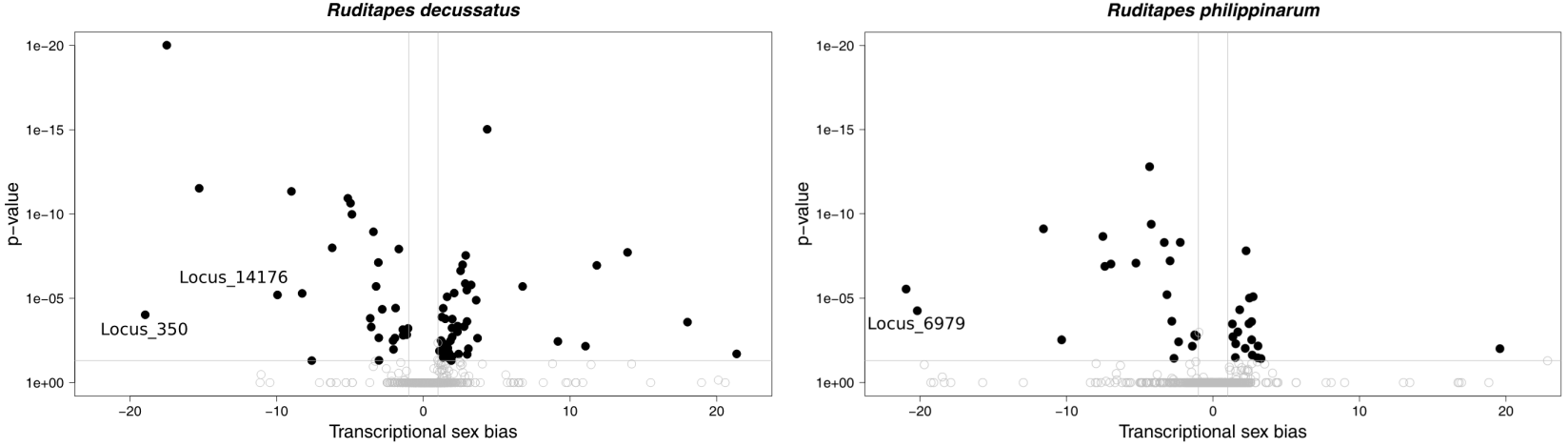
a) Nucleases and polymerases



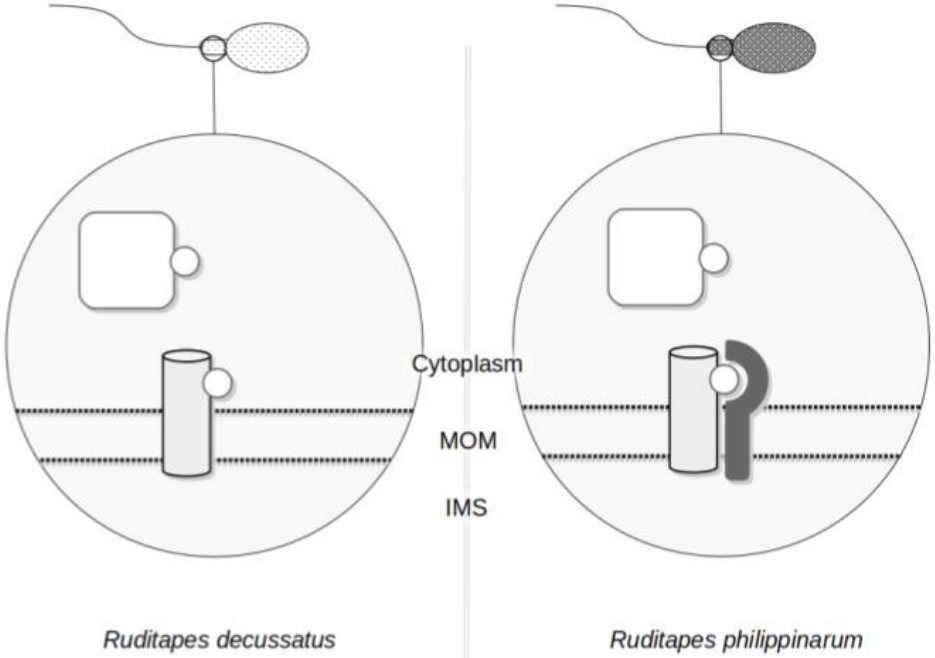
b) Autophagy and mitophagy



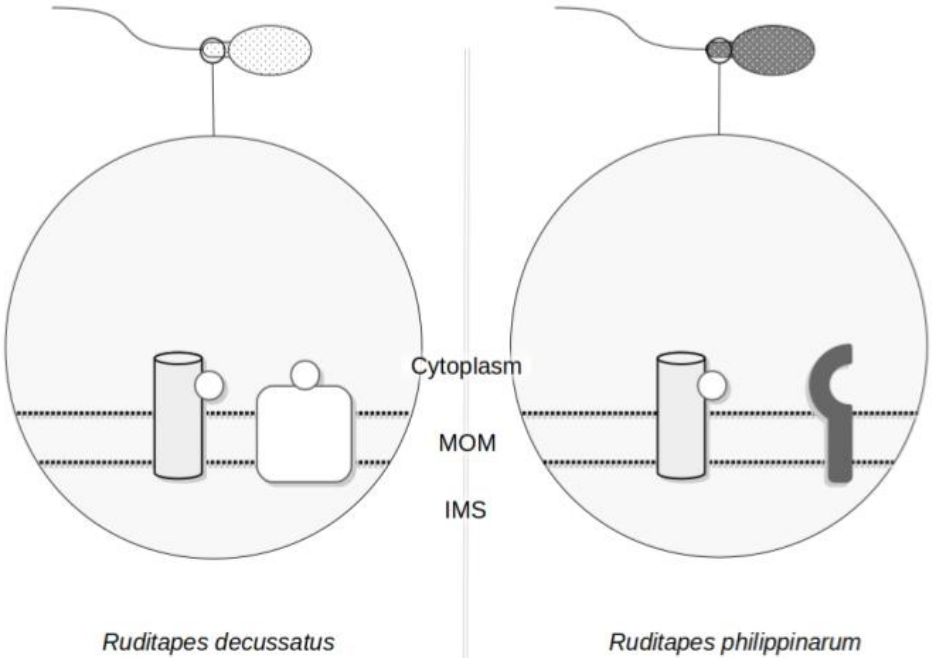
c) Ubiquitin and ubiquitin-like modifiers



HP1


Cytoplasm
MOM
IMS
Ruditapes decussatus
Ruditapes philippinarum

HP2


Cytoplasm
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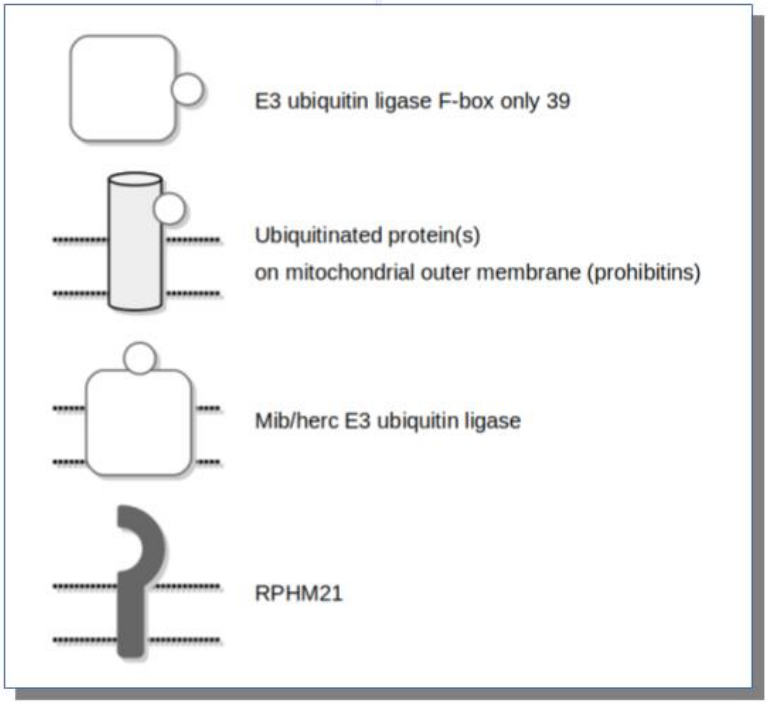
IMS
Ruditapes decussatus
Ruditapes philippinarum


E3 ubiquitin ligase F-box only 39
Ubiquitinated protein(s)
on mitochondrial outer membrane (prohibitins)
Mib/herc E3 ubiquitin ligase
RPHM21