On the origin of speciation

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Abstract

Charles Darwin proposed the theory of evolution that natural selection leads to the evolution of organisms in “On the Origin of Species.” However, he did not show how new species differentiate and fix. Speciation requires a system in which genes are not mixed by interspecific hybridization, and reproductive isolation, especially postmating reproductive isolation, is considered the most reliable guarantee. Haldane proposed that heterogametic sex was absent, rare, or sterile in interspecific hybrid F1. Dobzhansky and Muller predicted that postmating reproductive isolation occurs when mutations occurring at two or more interacting loci exhibit incompatibility in the hybrid. Genes that satisfy this observation and prediction are considered speciation genes. Here, the author would like to review the findings on reproductive isolation and speciation to consider the candidate conditions for the speciation genes and present the genes that fit these conditions.

Keywords: speciation, postmating reproductive isolation, Haldane's rule, hybrid sterility, hybrid inviability, Dobzhansky-Muller incompatibility, maternal mitochondrial DNA inheritance, SPAG1, Eri15, Xt-mir, mitochondrial quantity control, programmed mitophagy, mitoptosis, Warburg effect, meiotic arrest, mitotic arrest, *Wolbachia*

Introduction

In 1859, Charles Darwin presented the theory of evolution that natural selection leads to the evolution of organisms in “On the Origin of Species”(Darwin, 1859), which dramatically and revolutionarily advances the understanding of life. However, due to the limitations of genetics at the time, it was impossible to explain ‘the origin of speciation,’ which is how evolved populations are fixed and maintained as new species. There are many species definitions (Mallet, 1995), but it would be simplest and clearest to make it a population where genes are not mixed by mating with other populations. Reproductive isolation, especially postmating reproductive isolation, is considered the most reliable guarantee of gene flow blockage.

It is not uncommon to form hybrids by mating between species considered morphologically heterogeneous and maintain their hybrids (i.e., syngameon) (Seehausen, 2004). Furthermore, some species can be judged to be heterogeneous only after reproductive isolation is confirmed by crossing between those that were morphologically isogeny (i.e., cryptic species). The sympatric mix of cryptic species is believed to be far greater than currently reported (Trontelj, 2009). Morphological differences seem to have nothing to do with speciation. Haldane proposed that heterogametic sex (XY male or ZW female, etc.) was absent, rare, or sterile in interspecific hybrid F1 (i.e., Haldane's rule) (Haldane, 1922). In mammals, hybrid males develop spermatogenic deficiency (i.e., hybrid male sterility, HMS) or inviability (absent or rare). As a result, hybrid F2 does not occur, and the species is conserved. Dobzhansky and Muller predicted that reproductive isolation is caused by mutations occurring at two or more interacting loci, and the gene functionally diverges in each individual and shows incompatibility only in hybrid (i.e., Dobzhansky-Muller incompatibilities model, DMI model) (Dobzhansky, 1937; Muller, 1942). Haldane’s observation and Dobzhansky-Muller's prediction about postmating reproductive isolation seem very primitive and essential in considering the speciation genes. That is, in interspecific hybrid, mutations at two or more

loci cause incompatibility only in the heterogametic sex (two karyotypes, XY male type and ZW female type), and gene flow is blocked by two phenotypes (sterility or inviability) (HDM model、HDM system). HDM model genes can be said to be speciation genes. If HDM model genes are molecular evolutionarily neutral (Kimura, 1968) and not subject to selection pressure and speciation is established solely by that mutation, it will block the gene flow of a population with gene pools of precisely the same phenotype. Therefore, the two gene pools immediately after differentiation will be exactly the same except for HDM model genes. The subsequent accumulation of gene mutations in the two populations will change the phenotype and lead to natural selection, eventually making even mating difficult (i.e., premating reproductive isolation) (Safran, 2013). Therefore, even if the incompatibility genes detected between the two species are involved in fertility since a large proteome is involved in reproduction, it must be carefully considered whether they are speciation genes or mutations that occur after speciation. It is not easy to imagine that the system involved in the highly essential event of biological evolution differs for each taxon. There is a possibility that common genes and systems are working in taxa, where Haldane's rule is established in heterogametic sex and shows sterility and inviability. At the very least, the gene set is predicted to be preserved in a closely related species taxon, indicating incompatibility. Here, the author considered the conditions of the speciation genes by reviewing subsequent findings based on the HDM model. Many speciation genes have been reported so far, but none of them satisfy these conditions, so the author would like to present a hypothesis.

Conditions for speciation genes

Two karyotypes

Haldane showed that the phenomenon that causes reproductive isolation in hybrid is observed in heterogametic sex (two karyotypes, XY, XO male and ZW, ZO female) (Haldane, 1922). This phenomenon is not observed in homogametic sex (XX or ZZ), suggesting that the cause lies in the common system of single-copy genes on the X or Z chromosome. Moreover, the fact that the same phenomenon (hybrid sterility) is expressed in XY-type males and ZW-type females strongly suggests that the cause is not specific to spermatogenesis or oogenesis but is a mechanism common to the gametogenesis of both sexes.

Two phenotypes

Haldane showed two phenotypes of sterility or inviability (absent or rare) in the hybrid heterogametic sex (Haldane, 1922). Hybrid sterility is observed in mice with a meiotic arrest in primary spermatocytes rather than mitotic arrest in spermatogonia (Imai, 1981). Hybrid inviability is due to impaired embryogenesis, and Orr et al. have shown that it is caused by mitotic arrest in Drosophila (Orr et al., 1997/1). The two phenotypes are meiotic or mitotic arrest, suggesting cell division disorders cause it.

Orr recognizes inviability in hybrid XXY females by crossing females with attached two X chromosomes and heterogeneous males in two species of fruit flies that usually exhibit hybrid male sterility. For this reason, Orr argues that the genetic causes of Haldane's rule differ between sterility and inviability (Orr, 1993). It shows two sets (4 loci) of HDM model genes. However, when two gene sets showing two phenotypes due to incompatibility coexist in an interspecific hybrid, the sterility genes set observed after development would not be expressed because the inviability genes set is thought to be expressed at the developmental stage in normal hybrids. From Orr's observation, it cannot be denied that the HDM model genes are one set, showing two phenotypes depending on the expression time or cells. Furthermore, it is suggested that the heteromorphic chromosome contains factors that can control hybrid incompatibility.

Two genes

In the HDM model, at least two genes (loci) can be functionally diverged by mutations that do not cause incompatibility (Dobzhansky, 1937; Muller, 1942). When two mutated genes meet in a hybrid, the incompatibility occurs in heterogametic sex, causing sterility or inviability. This fact suggests that there is a loose tolerance for the interaction between the two genes such that individual mutations do not interfere with homeostasis, including fertility.

There are two possibilities for the results due to the incompatibility of two HDM model genes: 1) Loss of original function due to disruption of protein-protein interaction or incompatibility of epistatic and effector genes. 2) Disorders by misexpression (or overexpression) of harmful functions due to incompatibility of suppressive epistatic and effector genes. In the knockout verification of these genes in mice, sterility would not be seen in the effector gene of 2), but in other cases, it is predicted that sterility will occur only in males.

Large-X effect and large-Z effect

X chromosome replacement by backcrossing between heterogeneous has a more significant disproportionate effect on hybrid fitness than autosomal chromosomes (i.e., large-X effect) (Bhattacharyya, 2014). Haldane's rule predicts that even if a harmful mutation occurs in recessive X-linked allele, it is injurious in heterogametic hybrids. However, in homogametic hybrids, a harmless dominant allele masked it and no disorder appears (i.e., dominance theory) (Turelli et al., 1995). Furthermore, in the HDM model, two genes need to be expressed, that is, to be dominant, but in heterogametic sex, the genes on the X chromosome are single copies, so they are expressed regardless of dominant or recessive. For this reason, heterogametic sex is said to be affected by mutations (Dobzhansky, 1937). Both have shown the importance of the X chromosome in hybrid sterility. Furthermore, a large Z effect (Ellegren, 2009) has also been confirmed in ZW-type birds and butterflies that exhibit hybrid female sterility. These findings support Haldane's observation that the heterogametic sex, regardless of whether it is male or female, exhibits hybrid inviability and sterility.

Hybrid sterility 1 (Hst1) on chromosome 17 (Forejt et al., 1974) and hybrid sterility X2 (Hstx2) on chromosome X (Bhattacharyya et al., 2014; Heiden et al., 2009) are mapped as hybrid sterility loci by quantitative trait locus (QTL) analysis using consomic strain based on the sperm count and testis weight of mice as indicators. There are six protein-coding genes in the Hst1 locus, and meiotic histone H3 methyltransferase, Prdm9, is further identified as a hybrid sterility gene. Prdm9 caused meiotic arrest due to chromosomal synaptic failure by its defect and was considered the causative gene of hybrid sterility (Mihola et al., 2009). However, the Prdm9 knockout mouse shows infertility not only in males but also in females (Hayashi et al., 2005). Furthermore, it is shown that it is not essential for meiosis (Mihola et al., 2019), so it is hard to believe that Prdm9 is the effector gene of the HDM model.

Hstx2 contains ten protein-coding genes and 22 microRNAs (miRNAs), but the hybrid sterility gene has not yet been identified. Morimoto et al. confirm that knockout mice of six protein-coding genes, except for genes not to be involved in spermatogenesis (there is disagreement about Fmr1 as described later.), do not cause infertility (Morimoto et al. 2020). Therefore, the hybrid sterility gene of the Hstx2 locus is more likely to be microRNAs rather than the protein-coding genes. Hstx2 locus almost coincides with the human Xq27.3 region called the fragile-X region. The fragile-X region is composed of the protein-coding genes SLITRK2 and FMR1 and 22 microRNAs sandwiched between them and is located only on the X chromosome, and this composition is conserved in mammals (Zhang et al., 2019). This region is easily physically cleaved and was initially noted in studies of fragile sites abundant on chromosomes, demonstrating that the fragility lies in the FMR1 mutation (Garber et al., 2008). Recently, X-linked miRNA has been attracting attention for promoting evolution because it shows a fast evolution speed. The miRNAs (Fx-mir) in this fragile-X region (Hstx2) are composed of the hsa-miRNA888 cluster (miR888~892c, 7 genes, 10 mature miRNA) and the hsa-miR506 cluster (miR506~514b, 15 genes, 20 mature miRNA) in humans (Garber et al.,

2008) (Table S1). In mice, 22 genes (44 mature miRNA) are present as Fx-mir (Ramaiah et al., 2019) (Table S2). Some mouse Fx-mir are weakly expressed in various organs, including the ovary, but most are strongly expressed only in the testis (Ramaiah et al., 2019). MiRNAs are non-coding RNAs with a length of 20~25 nucleotides that mainly bind to mRNA 3'UTR and suppress gene expression. MiRNAs exist widely from fungi to plants and animals (Bartel, 2009). In the hybrid male sterility of Nematoda, the interaction between the X chromosome and the autosomal loci was shown to be essential (Bi et al., 2018), suggesting that the autosomal genes controlled by Fx-mir are potential effector genes in hybrid sterility. Furthermore, if the miRNA is an epistatic gene of HDM model genes, the effector gene is not expressed in normal gametogenesis, and its misexpression is presumed to cause disorders. Since miRNA and mRNA do not have a 1:1 relationship and a single miRNA typically downregulates target mRNA by only about 20~40%, multiple miRNAs are required to regulate more strongly (Ramaiah et al., 2019; Bartel, 2018). This fuzzy relationship between miRNA and mRNA seems to guarantee that individual mutations in HDM model genes can occur without functional impairment. More than half of the miRNA clusters usually have a paralogous cluster at different loci, but it has not been found in Fx-mir (Zhang et al., 2019). In the HDM model, it was expected that a single copy of the gene on the X (Z) chromosome of the heterogametic sex would guarantee incompatibility, and Fx-mir satisfies this condition.

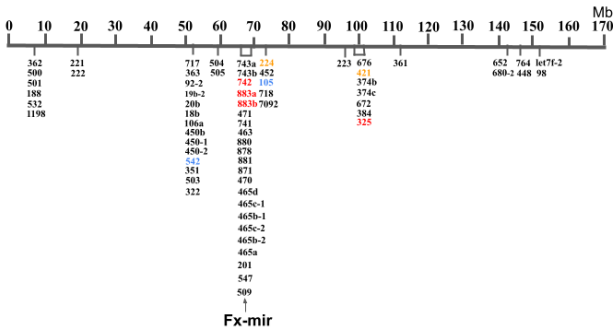
Faster-X effect and faster-Z effect

In general, X-linked genes, especially male-specific genes, are said to evolve faster than autosomal genes (i.e., faster-X effect and faster male effect) (Torgerson et al., 2003; Zhang et al., 2007; Orr, 1997/2). Since the genes on the male X chromosome are single copies, mutations are easily fixed, and intense selection pressure is applied to highly competitive sperm, it is suggested that their rapid evolution contributes to the speciation of mammals (i.e., sexual selection) (Zhang et al., 2007). However, this speculation breaks down since the faster-Z effect has been confirmed in birds (ZW female type) (Mank et al., 2007). As mentioned above, the genes for which mutations are always confirmed between species immediately after speciation may be only the HDM model genes. In addition, mutations in HDM model genes after species differentiation lead to the following differentiation, so regardless of the neutrality of molecular evolution (Kimura, 1968), HDM model genes will appear to evolve relatively faster than other genes. Evolution is not driven by fast evolution due to the selection pressure of X-linked genes, but they appear to be fast because it is directly involved in evolution. Because of the existence of the sympatric cryptic species, the sterility and inviability as a phenotype of HDM model genes are neither the result of natural selection nor undergoing selection pressure. In that sense, speciation may be considered an intrinsic, autonomous event, unlike the extrinsic, heteronomous evolution by natural selection.

The nucleotide substitution rate of miRNAs expressed mainly in the testis was 25 times higher on the X than on the autosomal chromosome, and there was no significant difference in the substitution rate of miRNAs not expressed in the testis between the X chromosome and the autosomal chromosome (Guo et al., 2009). That is, X-linked miRNAs expressed in the testis (X-linked testis miRNA, Xt-mir hereafter) evolves rapidly. In mice, 77 Xt-mirs, including Fx-mir, were detected and distributed over the entire X chromosome centering on Fx-mir (Fig.1). Xt-mir in the mouse testis was expressed partially in spermatogonia and mostly in round spermatids from spermatocytes, escaping meiotic sex chromosome inactivation (MSCI) (Song et al., 2009). Xt-mir, expressed in spermatocytes showing meiotic arrest in hybrid testis, is a likely candidate for the HDM model genes. Furthermore, since miRNAs and mRNAs are in a co-evolutionary relationship (Ramaiah et al., 2019), among the many target genes of Xt-mir, those corresponding to HDM model genes are predicted to evolve particularly rapidly. Among the HDM model genes, the evolution of the intermolecular action part, which is mainly related to incompatibility, seems faster than other parts. Of the nucleotide substitutions in primate random primary miRNAs (not including Fx-mir), 2.5% were found in mature miRNAs, compared with 19.5% in mature miRNAs of hsa-miR506 cluster (Berezikov et al., 2005).

HDM model genes should be the most fixed in the species because if mutated, they will be lost due to sterility or inviability or become new species. Hsa-miR509 has three copies and all three work and the number of copies was the same among races and firmly fixed within the species (Zhang et al., 2007). Fx-mir (or Xt-mir) clusters are very different from other miRNAs in evolution, and their involvement in speciation is strongly suspected, further strengthening the possibility of HDM model genes.

Figure 1. Xt-mir mapping on the mouse X chromosome Numbers: mouse X-linked miRNAs number expressed in the testis (Xt-mir). Colored numbers: Xt-mir targeting *Spag1-2* mRNA 3'UTR with 7-8mer matching. Red numbers: Xt-mir expressed mainly in spermatocytes~spermatids. Orange numbers: Xt-mir expressed in spermatocytes and organs other than the testis. Blue numbers: Xt-mir expressed in spermatogonia, spermatocytes, postnatal testis, and organs other than testis (Song et al., 2009).



Infertility

Azoospermia and oligospermia may result if reproductive dysfunction is caused by significant mutations or deletions in the HDM model genes that exceed the permissible range in germ cells. Furthermore, as a process of speciation, the son will be infertile if both partners are carriers of the incompatible gene. Investigating the causes of infertility in humans may provide clues to the speciation genes. 5% of males have infertility, of which 75% have idiopathic sperm dysfunction of unknown cause (Okada et al., 2008). The miR888 cluster was downregulated in the testis of non-obstructive azoospermia (NOA) patients compared to obstructive azoospermia patients (Piryaei et al., 2023). Misexpression of target genes due to Fx-mir downregulation is thought to impair spermatogenesis, and Fx-mir and its target genes may be candidates for HDM model genes.

Apoptosis or not?

Most reports refer to cell death due to the meiotic arrest of hybrid sterility as apoptosis. However, there are no characteristic histological findings of apoptosis, such as nuclear rupture or apoptotic body in hybrid testis (Kaku et al., 1995; Hayashida et al., 2009). Usually, in the histological proof of apoptosis, cells stained in response to ruptured nuclear DNA by TdT-mediated dUTP nick-end labeling (TUNEL) assay are judged as apoptotic cells, but they do not react to mitochondrial DNA (mtDNA). Therefore, apoptotic cells seem to be unconditionally determined only by positive staining. However, the cytosol of positive cells also appears to be stained in all reported micrographs. The spermatocyte has a minimal cytosol/nucleus ratio, so even if cytosol is stained, it is difficult to recognize it overlapping with counterstain in a standard light microscopic image. Hayashida et al.

showed by TUNEL assay using confocal fluorescence microscopic image that spermatocytes of mouse hybrid sterility testis have mtDNA disruption but nuclei (Hayashida et al. 2009). Recently, Yu et al. showed that the direct cause of hybrid sterility in scallops is cell cycle arrest due to ATP depletion in interspecific hybrid F1 gonads. Mutations, rearrangements, depletions, etc. of mtDNA are thought to cause mitochondrial dysfunction, but it is unclear why hybrid gonads cause structural changes (or disruption?) of mtDNA (Yu et al., 2022). The TUNEL assay of the Xenopus (frog) embryo, which exhibits hybrid inviability, shows a different image without nuclear staining and nuclear condensation compared to staining with apoptosis-inducing agents, suggesting cell death that is not apoptosis (it appears to be stained around the nucleus) (Gibeaux et al., 2018). Hybrid sterility and inviability may be caused by mtDNA depletion rather than apoptosis. The fact that the terminal image of hybrid sterility in very distant taxa, such as mammals and bivalves, was shown to be an unusual cell death that is not apoptosis suggests the possibility that the principle of speciation has a mechanism common to all organisms.

Summary of HDM model genes conditions

From the above, HDM model genes (speciation genes) conditions can be summarized as follows: 1) HDM model genes may be one set of Fx-mir (or Xt-mir) and effector genes on autosomes controlled by this. 2) Meiotic and mitotic arrest show sterility and inviability, respectively, due to the difference in expression time or cells of two genes incompatibility. 3) HDM model genes may involve cell division mechanisms common to meiosis and mitosis. 4) Hybrid incompatibility leads to cell death that is not apoptosis due to mtDNA destruction. 5) Effector genes impair gametogenesis or embryonic development due to their misexpression in the phase where their expression is usually suppressed in gonads and embryos of the heterogametic sex (both XY and ZW organisms). 6) HDM model genes are associated with mechanisms common to male and female gametogenesis. 7) There is a possibility that HDM model genes exist among the causative genes of human infertility of unknown etiology. 8) Not only the Xt-mir but also the target gene should evolve rapidly. 9) The genes and mechanisms of HDM model may be conserved across taxa.

Many speciation genes have been identified in yeast, thale cress, fruit fly, mouse, etc., and many are involved in transcriptional or translational regulation (Mack et al., 2018), but the underlying common mechanism remains unclear. Many hybrid sterility or inviability genes have been identified in fruit flies. Among them, the lethal hybrid rescue (Lhr) and the hybrid male rescue (Hmr) are reported as two distinct interacting genes (Brideau et al., 2006). However, both recognize few orthologs except Brachycera (fly) (ORTHOSCOPE, http://yurai.aori.u-tokyo.ac.jp/orthoscope/Actinopterygii.html). Below, the author would like to present a hypothesis that almost matches these conditions.

Proposing a candidate for speciation genes set

Maternal mitochondrial DNA inheritance and hybrid male sterility

Maternal mitochondrial DNA inheritance (MMI) system In most sexually reproducing eukaryotes, the mtDNA of one gamete is eliminated after mating between gametes (i.e., uniparental mtDNA inheritance, UMI) (Birky, 1995). In mammals, sperm mitochondria enter the egg together with the nucleus during fertilization, but sperm mtDNA is selectively eliminated from the egg, and mtDNA is inherited maternally (i.e., maternal mitochondrial DNA inheritance, MMI) (Szollosi, 1965). The MMI system is strict and is completely eliminated among allogeneic species (Birky, 1995). Based on speculation that this system is for the processing of sperm mtDNA damaged by reactive oxygen species (ROS), there are theories that the ubiquitin-proteasome system or autophagy of the fertilized egg selectively processes male sperm mitochondria (Sutovsky et al., 1999; Al Rawi et al., 2011). However, there is no guarantee that all male sperm mitochondria have deteriorated by the time of fertilization. Hepatocyte mitochondria were not eliminated by microinjection into an embryo (Shitara et al., 2000), but sperm mitochondria were eliminated by microinjection into

somatic cells (Manfredi et al., 1997). These facts mean that sperm mitochondria have a factor to be eliminated before being damaged, unlike somatic cell mitochondria, and somatic cells have a system that recognizes this factor and eliminates sperm mitochondria like eggs. Most of the mtDNA has been transferred to the nucleus in most eukaryotes. However, only a few genes remain in mitochondria, including genes essential for maintaining the function of mitochondria (ATP synthase, etc.) (Lang et al., 1999). In mice, a phenomenon that sperm mtDNA disappears before the mitochondrial membrane potential is lost was observed in the fertilized egg (Kaneda et al., 1995). Hayashida et al. considered that MMI is a purposeful programmed mitophagy by a system that controls mitochondria due to eliminating mtDNA essential for maintaining the function, and presented the following theory. The molecular chaperone cytosol type Spag1-isoform2/3 (Spag1-2/3, cSpag1) protein transports endogenous retroviral integrase 15kDa (Eri15) (new accession No. LC627956.1), which has endonuclease activity in the egg cytoplasm, to mitochondria. mitochondria type Spag1-isoform1 (Spag1-1, mSpag1) incorporated into the outer membrane of sperm mitochondria as a member of the translocase of the outer mitochondrial membrane (TOMM) 40 complex during spermatogenesis selectively taken in Eri15 into the matrix and destroys mtDNA. As a result, mitochondria that have lost their membrane potential are treated by the autophagy system (i.e., mitophagy) (Hayashida et al., 2005, 2008).

hybrid male sterility (HMS) system The MMI system is said to avoid competition with heterosexual mtDNA and parasites brought in by sperm mitochondria (Birky, 1995), but it is easily disrupted by intersubspecific and interspecific hybrid and paternal mtDNA is detected in the somatic cells of F1 individuals in mice (Kaneda et al., 1995). The intermolecular reactions involved in MMI can be said to be species-specific. For this reason, Hayashida et al. thought that this system may have evolved rapidly at the forefront of speciation (Hayashida et al., 2009). Spermatocytes of mutant mice lacking part of mtDNA have been shown to cause meiotic arrest (Nakada et al., 2006). Since Eri15 is also expressed in the spermatocytes (Hayashida et al., 2008), it is considered that *cSpag1* is suppressed by the epistatic gene so that the MMI system does not operate during spermatogenesis. Hayashida et al. showed that not only mSpag1 but also cSpag1 was expressed in the intersubspecific and interspecific hybrid testis in mice. Compared to the fact that nuclear and nuclear DNA ruptures were observed at all stages of the artificial cryptorchidism testis, the hybrid testis showed swelling of the spermatocyte mitochondria and cleavage of only mtDNA, causing meiotic arrest due to mitophagy-induced cell death (i.e., mitoptosis) which is not apoptosis (Hayashida et al., 2009). The evolutionary preservation of the MMI system is due to the need for speciation, and the residual mtDNA essential for maintaining function in mitochondria may be due to the functioning of the MMI and HMS systems.

Spag1 SPAG1 was discovered as one of the target proteins of anti-sperm antibodies in the seminal plasma of unexplained infertile males (Bohring et al., 2001). In mice, mSpag1 (114 kDa) is expressed only on the outer mitochondrial membrane of the testis. However, in epididymal sperm, it is post-translationally modified and detected at 166 kDa (Hayashida et al., 2005), suggesting that it has an important function even after maturation. The ortholog of *SPAG1* is widely recognized from fungi to plants and animals (ORTHOSCOPE). It has been shown that *Spag1* has a high synonymous substitution rate among sperm proteins that are said to have fast evolution (Torgerson et al., 2003). Spag1 may be target gene that have a molecular co-evolutionary relationship with Xt-mir (Ramaiah et al., 2019) as a HDM model gene.

Spag1-2 (64kDa) or Spag1-3 (75kDa) are widely expressed in the cytoplasm of organs other than the testis, and only one or both are expressed depending on the tissue, and it seems that they complement each other. Spag1-1 has three TPR domains, and Spag1-2 has two TPR domains (Hayashida et al., 2005, 2008). In humans, although three isoforms of 60 (or 50), 92 to 95, and 104 to 106 kDa have been detected, the intracellular localization, etc. have not been sufficiently investigated for each isoform. It is estimated that 92 to 95 and 104 to 106 kDa isoforms are cytoplasm type (SPAG1-3, SPAG1-2) and 60 (or 50) kDa isoform is mitochondria type (SPAG1-1), and the

molecular sizes are reversed in humans and mice (Neesse et al., 2007; Kanazawa et al., 2003; Smith et al., 2022). SPAG1 is expressed in both types in cancer cells and undifferentiated respiratory epithelial cells (Neesse et al., 2007; Smith et al., 2022), and only 50 kDa isoform in sperm [56] and is considered to be a cancer-testis antigen (CTA) (Siliņa et al., 2011). cSPAG1 provides a platform for quaternary protein folding of proteins via the TPR domain (Takaishi et al., 1999; Allan et al., 2011) that is involved in protein-protein interactions as a member of the R2SP complex (SPAG1, PIH1D2, RUVB1/2), which is a co-chaperone complex, and is involved in the assembly of protein complexes such as dynein arms (Smith, et al., 2022; Maurizy et al., 2018) (its mutation causes primary ciliary dyskinesia due to dysplasia of the axoneme dynein arm). The R2SP complex is identified as a ubiquitous R2TP (RPAP3, PIH1D1, RUVB1/2)-like chaperone, where RPAP3 is located at cSPAG1 of R2SP and is particularly strongly expressed in the testis. Its assembly function is most robust at the proper temperature of the testis, 32°C, and is optimized for the testis environment (Maurizy et al., 2018). This fact means that R2SP malfunctions in high-temperature environments. It has been observed that hybrid males of flower beetles exhibit malformations, and hybrid females also exhibit malformations in a high-temperature environment of 34°C (Wade et al., 1999). The Eri15/SPAG1 axis may also be related to hybrid inviability.

Translocase of outer mitochondrial membrane 34 (TOMM34) is a protein with the highest homology to SPAG1 (Hayashida et al., 2005). TOMM34 also has 2 TPR domains, is localized in the cytoplasm and outer mitochondrial membrane, and is mainly expressed in the testis (Faou et al., 2012). TOMM34 not only forms a large chaperone complex with Hsp70/Hsp90, etc., and provides a platform for protein folding but also shuttles mitochondrial precursor proteins to mitochondria and imports them into the matrix via TOMM34 on the outer mitochondrial membrane (Trcka et al., 2014). However, Tomm34 knockout mouse shows no obstacles (Terada et al., 2003). Some compensation functions probably worked. Tomm34 has a high similarity to Spag1 in terms of localization and functionality.

Eri15 In mice, Eri15 is present in the cytoplasm as a multimer with the cSpag1 protein in most somatic cells as well as in the ovary (Hayashida et al., 2008). This fact can explain the abovementioned observation that sperm mitochondria were eliminated by microinjection into somatic cells (Manfredi et al., 1997). In the mitochondria matrix, the pH is alkaline, the Mn^{2+} concentration is high (Hayashida et al., 2008), and the Zn^{2+} concentration is three orders of magnitude lower than that of the cytoplasm (Park et al., 2012). The activity of recombinant Eri15 is most vital at pH 8.5 and is enhanced by Mn^{2+}. Normal retroviral integrase has a Zn^{2+} binding site at its N-terminus and requires Zn^{2+} for its activity (Zheng et al., 1996), whereas Eri15 is truncated in this portion and endonuclease activity is preserved, but on the contrary, the activity is suppressed by Zn^{2+} (Hayashida et al., 2008). Eri15 is highly optimized for the environment in the mitochondria matrix. The ortholog of Eri15 is widely conserved in plants and animals (ORTHOSCOPE).

Hosts infected with the virus use apoptosis to remove infected cells and prevent the spread of the infection, but the virus avoids apoptosis via mitophagy in various ways (Vo et al., 2021). Herpes simplex uses amino terminally truncated isoform UL12.5 (which gives mitochondria directivity by truncation) of alkali endonuclease UL12 involved in replication to eliminate host mtDNA and prevent apoptosis (Safran et al., 2007). Xia et al. have shown that oncolytic measles virus-infected lung cancer cells induce mitophagy, suppress apoptosis through decreased cytochrome c release, and thus favor virus replication, and ultimately, cancer cells cause necrosis due to ATP depletion (Xia et al., 2014). This fact indicates that the final form of mitoptosis due to excessive mitophagy is necrosis rather than apoptosis. Spermatocytes of mutant mice with partial deletion of mtDNA exhibit meiotic arrest (Nakada et al., 2006). As mentioned above, interspecific hybrid F1 gonads of scallops indicate that cell cycle arrest due to ATP depletion is the direct cause of hybrid sterility (Yu et al., 2022). Dmc1 that causes meiotic arrest due to synaptic failure by the defect is ATP-dependent (Pittman et al., 1998). Therefore, the synaptic failure and subsequent meiotic arrest observed in hybrid sterility testis may be caused by ATP depletion due to excessive mitophagy resulting from disruption of mtDNA

by the Eri15/SPAG1 axis. The meiotic arrest of hybrid spermatocytes does not lead to apoptosis, probably because the apoptosis pathway is interrupted by programmed mitophagy for active quantity control, not passive quality control.

It is said that 8~10% of mammalian genomes are occupied by the endogenous retrovirus genes (Crowell et al., 2007), and some are used by the host, such as syncytin, which is involved in human placental formation (Mi et al., 2000). It is possible that Eri15, a protein derived from retrovirus, is used to eliminate mitochondria in the host.

Apoptosis and mitophagy

Apoptosis Eukaryotic cells are thought to have developed in the symbiosis of heterotrophic eubacteria in autotrophic archaea (Margulis,1971). Before the symbiosis, there seemed to be an attack and defense system for each other, and the system changed its shape and remained after the symbiosis. It is speculated that apoptosis is the control of archaea (later eukaryotic cells) by cytochrome c of eubacteria (later mitochondria), and preventing the spread of infected cells by apoptosis of virus-infected cells may be a protective reaction of mitochondria rather than a host. Furthermore, spermatogonia and cancer stem cells may use the Eri15/SPAG1 axis to escape from the control by apoptosis of eubacteria (mitochondria) just as the virus avoided apoptosis by its own endonuclease and ancestor return to archaea with infinite proliferating ability.

Since most of mtDNA has migrated to nuclear DNA (Lang et al., 1999), it cannot self-proliferate outside the cell, but it can do so inside the cell. Oncocytic tumors are benign, non-aggressive, and hypoproliferative lesions characterized by marked hyperplasia of mitochondria. As mtDNA mutations and mitochondrial structural changes are observed in oncocytic cells, there is a hypothesis that retrograde signals such as ROS due to respiratory chain disorder are transmitted from mitochondria to the nucleus, and signals that increase mtDNA copy number and mitochondrial proliferation are upregulated (Gasparre et al., 2011). Viewing oncocytic tumors from the perspective of a mitochondrial ancestor return to eubacteria may help elucidate their pathogenesis.

Mitophagy Recently, the association between many diseases and mitophagy has been pointed out (Pickles et al., 2018). However, most the research subjects are quality control of mitochondria, and little is understood about programmed mitophagy for quantity control. In oocytes, it was said that a temporary number reduction called the bottleneck effect was performed to homogenize mtDNA (Jansen et al., 1998). It was suggested that Spag1 is involved in mitochondria synthesis and dynamic regulation during meiosis in mouse oocytes (Huang et al., 2016). The copy number of mtDNA is actively reduced during spermatogenesis (Kasashima et al., 2014). In erythroblasts differentiated from hematopoietic stem cells, mtDNA degradation by ANKLE1 protein with endonuclease activity causes mitophagy to become mature erythrocytes. ANKLE1 is usually expressed only in erythroblasts but also in cancer and is associated with the risk of developing ovarian and breast cancers. Ectopic expression in mammary gland cells was shown to induce mitophagy through mtDNA degradation, shifting metabolism from oxidative phosphorylation to glycolysis (i.e., Warburg effect) (Warburg, 1956) and avoiding apoptosis (Przanowski et al., 2023).

Most stem cells, including cancer stem cells, have few mitochondria and exhibit the Warburg effect (Zhang et al., 2018). Aurrière et al. have identified and examined 70 mitochondria-related CTA out of 276 CTA since cancer cells in the hypoxic state in the center of the tumor and spermatogonia, primary spermatocytes outside the blood-testis barrier at the outer edge of the seminiferous tubule have a commonality of the metabolic shift to glycolysis. Only two, SPATA19 and COX6B2, were associated with mitochondria metabolism, and neither was found to have the effect of shifting metabolism to glycolysis (Aurrière et al., 2021). They included SPAG1 in the list of CTA searched but did not pick it up as a mitochondria-related CTA. Chromatin-remodeling complexes, which play an essential role in mitosis, are ATP-dependent (Vignali et al., 2000). The observation above by Xia et al. that ATP depletion by the measles virus in lung cancer cells led to necrosis (Xia et al. 2014) indicates that rapidly proliferating cells cannot survive by glycolysis alone. If the stem cells are depleted of ATP

by mitophagy at the stage of development, the mitotic arrest will occur, and the development will stop. The reason why the mitotic arrest is not seen in hybrid sterility testis (Kaku et al., 1995; Li et al., 2009) is that when the mitotic arrest is caused in spermatogonia, it becomes mitotic arrest in the whole stem cells at the same time. Its phenotype is not detected due to developmental failure. Not only meiotic arrest but also mitotic arrest in hybrids may be caused by ATP depletion due to excessive mitophagy.

As described above, mSPAG1 and cSPAG1 are expressed in cancer cells (Neesse et al., 2007). In lung adenocarcinoma cells, the overexpression of cSPAG1 increased autophagy and decreased cell proliferation and colony formation (Li et al., 2021). 94, 106 kDa SPAG1 isoform expression increases with the differentiation of human respiratory epithelial cells, whereas only 60 kDa isoform shows a constitutive expression (Smith et al., 2022). This fact may indicate expression in stem cells, which exist in a certain number of cultured cells. Multiple SPAG1 proteins of ~50 and ~100 kDa are expressed in iPS cells during differentiation induction (Horani et al., 2018). SPAG1 may be the CTA involved in the Warburg effect of stem cells. Cell division and proliferation may be associated with the risk of apoptosis by mitochondria. Stem cells may cause programmed mitophagy by Eri15/SPAG1 to avoid apoptosis and, as a result, eventually shift from oxidative phosphorylation to inefficient glycolysis (Heiden et al., 2009). From the above, it is possible to explain that mitotic or meiotic arrest is caused by cells expressing one set of HDM model genes and showing two phenotypes, inviability and sterility, respectively.

Spermatogonia and cancer stem cells have infinite proliferation potential like bacteria (spermatogonia are avoided from tumorization by excreting those out of the body as spermatocytes). Cancer cells can have amoeba-like movement using actin filaments, have chemotaxis, and move in the body (metastasis) (Roussos et al., 2011). Spermatocytes have the ability to migrate using flagella and exhibit chemotaxis (Lishko et al. 2011). Actin-Like Protein 8 (ACTL8), which is involved in the formation and maintenance of actin filaments, belongs to CTA, and the knockdown of *ACTL8* inhibits the mobility of oral squamous cell carcinoma cells (Wang et al., 2022). Archaea, the ancestors of eukaryotic cells, have the ability to migrate and chemotaxis using flagella and a cytoskeleton-like structure by actin-like proteins (Jarrell et al., 2008). As mentioned above, spermatogonia, spermatocytes, and cancer stem cells have very few mitochondria compared to oocytes and somatic cells. Just as viruses avoid the host's apoptosis by using their own endonuclease, spermatogonia and cancer stem cells may escape from apoptosis by mitochondria using the Eri15/ SPAG1 axis and return to archaea as ancestors.

Xt-mir and *SPAG1*

A single miRNA binds to mRNA in a 6 to 8 nucleotide (nt) match centered on a continuous seed sequence of 6 bases of 2 to 7nt on the 5'end (6nt match has a lower inhibitory effect than 7 to 8nt) (Bartel, 2018). The relationship between miRNA and mRNA is complex and uncertain, and it seems complicated to discuss the suppressive effect with only the match of the seed sequence, so experimental verification by gene knockout, etc., is required (Bartel, 2018). Since miRNA and miRNA cluster have evolved through genomic duplication events (Sun et al., 2013), and miRNA and mRNA are in a co-evolutionary relationship (Ramaiah et al., 2019), the number of target sites of miRNA for one mRNA 3'UTR and the resulting elongation of mRNA should be considered evolutionarily significant. In this paper, when discussing target gene candidates for miRNA, the author will basically use the number of miRNAs showing 6 to 8nt seed match (canonical sites) (Bartel, 2018) and the number of target sites in the 3'UTR as an index.

SPAG1 was estimated as the target of hsa-miR888 that is expressed in the human epididymis and is involved in forming the epididymis and sperm maturation (Li et al., 2010). According to TargetScan online software (https://www.targetscan.org/), five types (6 target sites on mRNA 3'UTR) for *cSpag1* and only 1 type (1 site) for *mSpag1* of hsa-Fx-mir is targeted (Table S1). In mice, five Xt-mirs other than the three Fx-mir were predicted as targets of *cSpag1* mRNA (Table S2). Its distribution on the

X chromosome (Fig. 1) is in the range of 52Mb around the Fx-mir, and it matches well with the QTL mapping according to the sperm count in the mouse hybrid shown by Bhattacharyya et al., including parts of other than the Hstx2 (Fx-mir) locus (Bhattacharyya et al., 2014).

Of the Fx-mir among mouse subspecies, the KO mouse of the mmu-miR743 with SNP and the mmu-miR465 cluster with copy number polymorphism did not become infertile (Pittman et al., 1998). Of these two miRNAs, miR743 has a 6nt seed match for *cSpag1* 3'UTR (Table S2). Ota et al. showed no histological abnormalities in the testes with individual KO mice of mmu-miR741, mmu-miR871, and mmu-miR880. However, with miR871+miR880 or all three KO mice, spermatogenesis is stopped in a part of the seminiferous tubule (Ota et al. 2019). Of these three miRNAs, only miR880 has a 6nt seed match to *cSpag1* 3'UTR (Table S2). However, Wang et al. reported that when 18 of the 21 Fx-mirs of the mice were knocked out simultaneously, the mice developed normally, and the testes were not histologically affected (Wang et al., 2020). If the results of Ota et al. and Wang et al. on the KO mouse of Fx-mir are correct, it is inferred that miRNA involved in the factor that suppresses the expression of cSpag1 is present in Fx-mir. Ramaiah et al. identified 11 Fx-mirs in mice (6 in humans) targeting *Fmr1* (Table S2), which are always present downstream of Fx-mir in the fragile X region in mammals, and confirmed that the induction of each of the four miRNAs suppresses the expression of Fmr1 protein (FMRP) (Ramaiah et al., 2019). Mutations in *FMR1* cause chromosomal fragility, and loss of FMRP causes fragile X syndrome (intellectual disability, giant testis, fragile X chromosome findings). FMRP is expressed in the central nervous system and testis (Sertoli cells, spermatogonia) and is considered involved in translational regulation as an RNA-binding protein (Garber et al., 2008; Feng et al., 2017). The *FMR1* orthologs are widely conserved in animals (ORTHOSCOPE). As mentioned above, Fx-mir does not have paralogous clusters (Zhang et al., 2019). The reason why Fx-mir cannot be compensated may be that the benefit (speciation) for selfish genes (Dawkins et al., 2017) is greater than the loss for the species. However, male sterility due to malfunction of the MMI system is indeed a loss for species preservation, and it seems that some defense system coexists. Fmr1 is involved in spermatogenesis, axoneme synthesis, and the Warburg effect (Zhang et al., 2004; Maddalena et al., 2020), and its function is very similar to that of SPAG1, so it may control cSpag1 expression as an RNA-binding translational regulatory protein. If the Eri15/cSpag1 axis runs out of control due to widespread loss of Fx-mir, it may be that the misexpressed FMRP suppresses the translation of cSpag1 mRNA. The two events in the testis by Eri15/SPAG1/Fx-mir axis and FMR1/Fx-mir axis may be the scene of the antinomy conflict of species evolution and conservation.

The relationship between miRNA and mRNA is many-to-many (Bartel, 2018). If the proteome is formed around miRNA cluster, attention should be paid to selecting miRNA to knockout and interpret the results. The HDM model shows quantitative traits that exhibit phenotypes at various stages of sperm and individual (absent~rare) count. Quantitative traits are usually the result of the combined action of many genes. The fuzzy relationship between mRNA and miRNA cluster will likely to be a candidate for the minimal unit of HDM model genes that allows for quantitative traits.

Most Fx-mir is downregulated in cancer cells except for some and is considered inhibitory for cancer growth and malignant transformation (Yoshida et al., 2021). Furthermore, Fx-mir is not expressed in spermatogonia, which shows the Warburg effect as well as cancer cells (Song et al., 2009). It can be said that the Eri15/SPAG1/Fx-mir axis suppresses apoptosis of stem cells, including cancer stem cells, by programmed mitophagy. This axis can be called an anti-apoptosis system.

Infertility and Eri15/SPAG1/Xt-mir axis

Examination of X-chromosome SNPs in non-obstructive azoospermia (NOA) males has detected SNPs associated with the onset of NOA near hsa-miR506/507 and hsa-miR510. SNPs near miR506/507 increased the risk of NOA, and SNPs near miR510 decreased (Ji et al., 2016). miR508 adjacent to miR506/507 and miR506 targets *cSPAG1* with 7nt and 6nt matching, respectively (Table S1). Many of the mechanisms of miRNA expression are unknown, but clusters are said to be collectively regulated (Yoshida et al., 2021). Therefore, miR506 and miR508 may be involved in

developing NOA via *cSPAG1*. miR510 targets not only *cSPAG1* but also *mSPAG1* with 7nt and 8nt matching, respectively (Table S1). The Fx-mir targeting *mSPAG1* is only miR510, and it seems that Fx-mir does not normally control *mSPAG1*, but it cannot be ruled out that the misexpression of miR510 may have an inhibitory effect on the onset of NOA through the suppression of *mSPAG1*. Therefore, these SNPs may be associated with the development of NOA by the action of SPAG1 via Fx-mir.

miR888 cluster is released into the peri-sperm fluid in epididymis via exosome, suggesting communication with mature sperm and downstream epithelial cells (Belleannée, 2015). An exosome is a small membrane vesicle surrounded by a lipid bilayer, which contains proteins, lipids, mRNAs, microRNAs, etc., and is released extracellularly and has been attracting attention as an intercellular communication medium in recent years (Raposo et al., 2013). Exosomes are released not only in blood but also in most body fluids (Belleannée, 2015; da Silveira et al., 2012; Griffiths et al., 2008). It has been confirmed that the protein in the exosome in the female reproductive fluid of mice is taken up by sperm (Griffiths et al., 2008). Of the 13 epididymis-derived miRNAs in the semen of patients with asthenozoospermia (AZS), only the miR888 cluster was downregulated, showing a positive correlation between the expression level of the miR888 cluster and sperm motility (Qing et al., 2017). Furthermore, there was no decrease in the amount of mitochondria in AZS patients, but the number of mtDNA was reduced to 9.7% of normal individuals (Kao et al., 2004). Usually, mitochondria depleted of mtDNA should lose their action potential and be eliminated during spermatogenesis. However, since they are incorporated into sperm, mtDNA will likely be eliminated during maturation in the epididymis. The male reproductive tract has a defense system using the miR888 cluster against sperm mtDNA depletion, and its target may be *cSPAG1*. It is suggested that disruption of this system leads to the expression of the cSPAG1 protein, which causes degradation of sperm mitochondrial DNA, resulting in ATP depletion, reduced sperm motility, and the development of AZS.

In an experiment in which the follicular fluid and sperm of a couple undergoing treatment of infertility with intracytoplasmic sperm injection (ICSI) etc. were used to observe sperm migration, a phenomenon that follicular fluid attracted sperm from a specific male was observed. The two are compatible, suggesting that the sperm may react to the chemical signal from the egg, and the egg may have selected sperm (Fitzpatrick et al., 2020). Since the reaction was different depending on the combination of follicular fluid and sperm, it cannot be denied that *cSPAG1* mRNA, which has an incompatibility relationship with Fx-miR in sperm, may be released to suppress sperm migration. Based on the above, some unexplained infertility may be caused by dysregulation or gene mutation of the Eri15/SPAG1/miRNA axis, and infertility patients may stand by the gateway to speciation. The encounter of compatible HDM model genes mutant, which was avoided by infertility of the degree of oligospermia and AZS, may be accelerated by treating infertility with ICSI, etc. It is possible that cryptic species already exist in humankind. From the above, it is suggested that Fx-mir may control the expression of cSPAG1.

Speciation system

Hybrid sterility There are many fragile sites on the chromosome (Feng et al., 2017), including the Xq27.3 region in which Fx-mir is located. In the process of evolution, if one of the chromosome pairs breaks at the fragile sites and the Fx-mir is deleted during gametogenesis, the Fx-mir without a paralogous cluster (Zhang et al., 2019) becomes a single copy. In fact, there is no miRNA on the Y chromosome (Yoshida et al., 2021). In this individual, if a mutation that cannot suppress cSpag1expression occurs in Fx-mir, it cannot be compensated, so mtDNA is eliminated, the energy supply is cut off, and gametes cease to mature. This mutation has no effect since mSpag1 is not expressed in somatic cells. Mutations in Fx-mir or cSpag1 in male germ cells result in loss of the mutated gene by meiotic arrest, whereas female germ cells that do not express Fx-mir mature without problems and can be carriers for the mutated genes. Gamete maturation is restored if a mutation matching the mutation occurs in the corresponding gene in the male primordial germ cell

that inherited the mutated gene from the mother. Even if there is a mutation in the cSpag1 coding sequence derived from the mother that cannot bind to mSpag1, the MMI system will work, and mtDNA is excluded if wild and mutant type mSpag1 is expressed in the mitochondria of F1 male spermatocyte. For this, SPAG1 needs to be co-dominant. In this way, post-reproductive isolation, called hybrid sterility, becomes possible, and new cryptic species covered with an invisible bubble that blocks gene flow is established.

Hybrid inviability As mentioned above, it was speculated that the Eri15/Spag1 axis is also involved in programmed mitophagy in stem cell mitosis, including spermatogonia. Two (mmu-miR105, 542) of the 11 Xt-mir that are strongly expressed in spermatogonia (Song et al., 2009) targeted *cSpag1* (Table S2, Fig.1). Since Xt-mir expressed in spermatogonia is often expressed in testis immediately after birth and in organs other than testis (Song et al., 2009) , it is highly possible that it is also expressed in stem cells other than spermatogonia. If incompatibility occurs between the Xt-mir and *cSpag1* mRNA, mitophagy will run away in the mitosis stage in stem cells. It seems that homogametic sex also uses the Eri15/Spag1 axis for programmed mitophagy in stem cells, including oocytes, but the control may be done by putting in and out of mSpag1. As mentioned above, according to Orr's observation that hybrid XXY females showed inviability upon introduction of the Y chromosome in attached X fruit flies (Orr, 1993), the genes on the Y chromosome seem to control the testis-specific expression of Xt-mir and mSpag1 in heterogametic sex (If HDM model can be explained by gonad-specific expression, the dominant theory would not be necessary). However, hybrid XXY females showed inviability rather than sterility, suggesting that the genes on the Y chromosome could not be expressed in the meiosis of spermatocytes due to MSCI and were involved in expressing mSpag1 in stem cells.

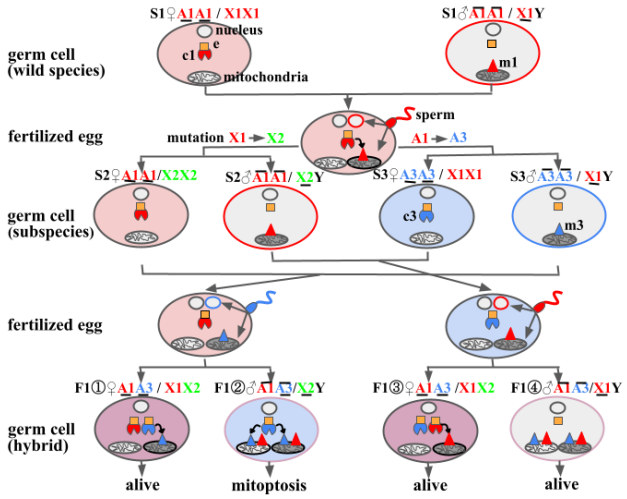
Schema of MMI system and hybrid sterility system

XY-type organisms (Fig. 2) It is considered that cSpag1 and mSpag1 interact with the TPR domain, and some of the multiple domains are the same due to the splicing variant (Hayashida et al., 2008; Maurizy et al., 2018). Therefore, it is predicted that it will not become unresponsive within the same species even if the domain is mutated. However, as mentioned above, Spag1 is a protein with rapid molecular evolution (Torgerson et al., 2003), and it is considered that the TPR domain mutation is progressing among the subspecies. So, it is assumed that the protein-protein interactions of cSpag1 protein (c) and mSpag1 protein (m) react only between the same species and not between subspecies. In the fertilized egg of S2♀xS3♂ mating, the relationship is c1 and m3, and in S3♀xS2♂, the relationship is c3 and m1, and mitophagy does not occur, so mtDNA cannot be eliminated and leaked to F1. S2♀xS3♂ produces F1 ①A1, A3/X1, X2, ②A1, A3/X2, Y. S3♀xS2♂ produces F1③A1, A3/X1, X2, ④A1, A3/X1, Y. Since Xt-mir genes targeting cSpag1 are not expressed in the eggs of F1①③♀, both c1 and c3 are expressed, but mitophagy does not occur because m1, m3 is not expressed (leaked mtDNA is eliminated, but only a few do not affect cell function). In F1④♂, both c1 and c3 are suppressed, so mitophagy does not occur. In the spermatocytes of F1②♂, c3 is expressed due to the relationship of A3/X2, so all mitochondria having m3 are excluded. Therefore, only the spermatogenesis of F1②♂ is impaired, and S2 and S3 have an incomplete reproductive isolation relationship. Furthermore, S2 and S3 give rise to subspecies S4 and S5 for each by the same mechanism, and S1, S4, and S5 become a heterogeneous relationship that is entirely hybrid male sterility. Eggs without organ-specific expression of Xt-mir and mSpag1 can coexist with A3/X2 in F1♀ and will be a source of incompatibility genes.

Then, is it possible that different species suddenly occur without passing the subspecies? Can heterogeneous A4, A4/X4, X4(Y) evolve from wild species A1, A1/X1, X1(Y), resulting in a reciprocal hybrid in which all males become sterile? For this purpose, A4 must be incompatible with X1, X4 must be incompatible with A1, and A4 and X4 must be compatible with each other simultaneously, so the probability is extremely low. Therefore, subspecies seems unavoidable as

a step leading to heterologous. Even if the wild species become rare, the gene is maintained by crossing with subspecies, leaving room to respond to circulating environmental changes. It can be said that the existence of subspecies is valuable because the diversity of species can be obtained via the subspecies. The reason why most species choose hybrid sterility, which produces subspecies after mating, rather than the incompatibility between gametes as a method to prevent gene flow is probably because it is advantageous for species conservation to leave room to maintain species diversity through subspecies.

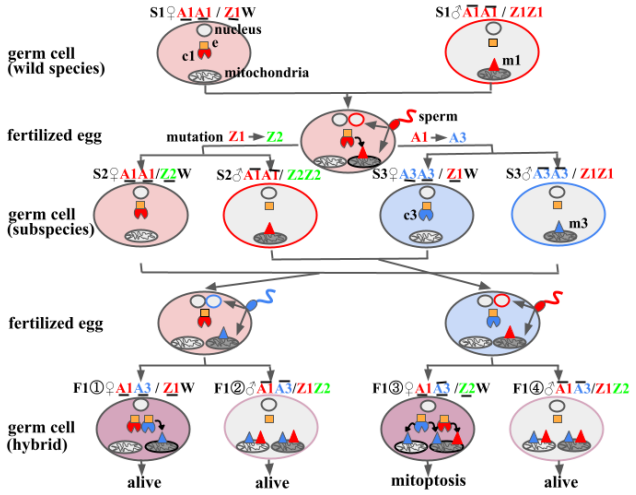
Figure 2. Maternal mtDNA inheritance and hybrid male sterility system in XY-type organisms S1: wild-type species. S2, S3: subspecies. F1: first filial generation. A: *Spag1* gene. X: Xt-mir genes targeting *cSpag1*. c: cytoplasm type Spag1(cSpag1) protein. m: mitochondria type Spag1(mSpag1) protein. e: Eri15 protein. Overlined A: mSpag1 (m) expression. Underlined A: cSpag1 (c) expression. Underlined X: Xt-mir expression. X and m are expressed only in the testis. X suppresses *cSpag1*. Allele A is co-dominant. X1 mutates to X2, which can suppress A1, and evolves into subspecies S2; A1, A1/X2, X2 (Y). Separately, A1 mutates to A3, which can be suppressed by X1, and evolves into subspecies S3; A3, A3/X1, X1 (Y). However, X2 cannot suppress A3 (*cSpag1*).



ZW-type organisms (Fig. 3) Mitochondria is also maternally inherited in ZW-type birds and butterflies (Berlin et al., 2004). Considering the same mechanism as the XY type, the HDM system and the MMI system break down. Even in ZW females, if the Z chromosome has Xt-mir (should it be Zo-mir?) (miRNAs also exist on the Z chromosome (Guo et al., 2009)) and suppresses *cSpag1*, male mitochondria cannot be eliminated. TOMM34 also hints at this problem. TOMM34 is the same molecule for both cytoplasm and mitochondria types (Faou et al., 2012). The molecular size of mSpag1 and cSpag1 is reversed in humans and mice. Therefore, the cytoplasm and mitochondria type of Spag1 are equivalent. Given that Xt-mir on the Z chromosome and the cSpag1 has organ (egg)-specific expression, and the mSpag1 is suppressed by Xt-mir (mSpag1 is expressed without being suppressed in spermatocytes), this problem will be resolved (genes on W chromosome may be controlling organ-specific expression).

Figure 3. Maternal mtDNA inheritance and hybrid female sterility system in ZW-type organisms S1: wild-type species. S2, S3: subspecies. F1: first filial generation. A: *Spag1* gene. Z: Xt-mir genes targeting *mSpag1*. c: cytoplasm type Spag1(cSpag1) protein. m: mitochondria type Spag1(mSpag1) protein. e: Eri15 protein. Overlined A: mSpag1 (m) expression. Underlined A: cSpag1 (c) expression. Underlined Z: Xt-mir expression. Z and c are expressed only in the eggs. Z suppresses mSpag1. Allele A is co-dominant. Z1 mutates to Z2, which can suppress A1, and evolves into subspecies S2; A1, A1/Z2, Z2 (Y). Separately, A1 mutates to A3, which can be suppressed by Z1, and evolves into

subspecies S3; A3, A3/Z1, Z1 (Y). However, Z2 cannot suppress A3 (*mSpag1*).



Sex-determination system

The relationship between the HDM system and the sex determination system Since hybrid male sterility is also observed in XO-type organisms (Wu et al., 1993), the responsible locus for sterility in the heterogametic sex of hybrid F1 lies on the X (or Z) chromosome. A similar composition is found in the sex-determination (SD) system. In the male heterotype, not only the XY type but also the XO type becomes male, and in the female heterotype, not only the ZW type but also the ZO type becomes female (Ellegren, 2011). Like the speciation system, the SD system seems to be caused by the X and Z single copy genes, which are not expressed in diploid but expressed in haploid only in the germline of heterogametic sex. Sex also exists in homozygous gametes of unicellular organisms. If sex is defined as a system that recognizes whether gametes can fuse, a SD factor may be a protein of the system that gametes recognize and fuse as the opposite sex or a gene that controls it. The primary sexual characteristics seen in multicellular organisms are thought to be merely subsequent changes in the gamete appendages, so primary sexual characteristics may also be controlled by the genes of the SD system of gametes. Since hybrids occur in crosses between closely related species, the SD system is not a species-specific reaction, and the speciation system (HDM system) may have utilized the existing SD system.

Liang et al. reported observing pupal developmental defects, including feminization (intersex), in a subset of F2 males produced by the backcross of F1 males resulting from interspecific hybridization between closely related mosquito species. They proposed that hybrid incompatibility causes a disruption of male SD, and further speculated that the expression of an uncharacterized gene in the SD pathway is implicated in this hybrid incompatibility (Liang et al., 2024). This fact indicates that the HDM system may be involved in the SD system of primary sexual characteristics. It is possible that the Xt-mir or even higher-level epistatic genes within the Eri15/SPAG1/Xt-mir axis, which functions as a common mechanism for both the MMI (UMI) and the HDM system, are also involved in the SD system. Temperature-sensitive R2SP complex containing Spag1 (Wade et al., 1999) may be involved in temperature-dependent SD, which is widely observed in reptiles (Bull, 1980). Xt-mir may be a material proof for "sex", a keyword common to MMI, HDM, and SD systems. Furthermore, since the organelle common to MMI system and HDM system is mitochondria, the SD system may also be brought about by mitochondria.

Convergence toward a sexual binary In true slime molds showing uniparental mitochondrial DNA

inheritance (UMI), the hierarchy of mitochondrial elimination is determined by the allele of one of the three mating type locus that determines many mating types (sex) (Meland et al., 1991). This observation suggests that the gamete SD system may also be involved in the UMI system. If the SPAG1 molecule is also expressed in the gamete plasma membrane, the relationship between the recognition of mating types in slime mold and the UMI hierarchy can be easily explained. SPAG1 was originally a molecule identified by an antibody in the seminal plasma that reacts with the sperm plasma membrane of infertile men (Bohring et al., 2001). The recombinant Spag1 antibody that Hayashida et al. used did not respond to the sperm plasma membrane. The mSpag1 protein undergoes post-translational modification at maturity in the epididymis (Hayashida et al., 2005). There may be differences in post-translational modifications on the outer mitochondrial membranes and plasma membranes. This difference may have changed the antigenicity and acquired a species-specific response to cSpag1 in MMI. The existence of multiple TPR domains formed by 2~3 TPR motifs in Spag1 protein (Hayashida et al., 2005) may ensure recognition among multiple sex gametes of slime mold. It will be necessary to revalidate with an antibody to post-translational modified Spag1 protein instead of recombinant Spag1 protein as an antigen.

At the stage before archaea and eubacteria begin symbiosis, it can be assumed that archaea use Eri15/cSpag1 axis to prevent the invasion of eubacteria, and the eubacteria that acquired Xt-mir enable symbiosis with archaea. Symbiosis with eubacteria that have multiple mSpag1 results in multiple sexes, and eukaryotic cells that have acquired multiple types of cSpag1 are ranked higher in the hierarchy, while eukaryotic cells that have acquired very few are ranked lower. Since cSpag1 is an infection-defense molecule, the lower the hierarchy, the stronger the negative selective pressure due to eubacterial infection. On the other hand, archaea that began symbiosis with eubacteria acquired sex, and the exchange of genes promoted diversity in response to environmental changes. The higher in the hierarchy, the more sexes there are and the more opportunities for hybridization, so the stronger the positive selection pressure. Eukaryotic cells, which started from a large number of sexes, may have ultimately converged into two sexes due to the above positive and negative selection pressures.

Wolbachia and the last boss

Similarity between *Wolbachia* and mitochondria The ancestor of mitochondria is thought to have been a *Rickettsia* belonging to the alpha-proteobacteria (Roger et al., 2017). Another member of the *Rickettsia*, *Wolbachia*, exists as an endosymbiont in arthropods and filarial nematodes. Approximately 100 years have passed since the discovery of Rickettsia-like symbionts in insects (Cowdry, 1923; Hertig et al., 1924), and although various intriguing ecological characteristics have been reported (Werren et al., 2008), the full nature remains unclear. *Wolbachia* exploits the intracellular environment constructed by mitochondria in eukaryotic cells—or alternatively competes or interferes with mitochondria—to influence its host. Comparing the intracellular dynamics of mitochondria and *Wolbachia* within eukaryotic cells is expected to provide important insights into their respective mechanisms of host interaction and into the symbiotic processes that shaped the evolution of mitochondria.

Wolbachia exhibits intracellular dynamics similar to those of mitochondria : (1) Maternal inheritance; *Wolbachia* can infect oocytes but is eliminated from sperm during maturation, resulting in maternal transmission. This phenomenon is reminiscent of mitochondrial quantity control during spermatogenesis and the elimination of sperm-derived mitochondria within the oocyte at fertilization. (2) Organelle-like intracellular trafficking; *Wolbachia* moves within host cells using dynein and kinesin motors, and during host cell division it utilizes the spindle apparatus to ensure equal distribution to daughter cells, behaving much like an intracellular organelle such as mitochondria (Pangou et al., 2021). (3) Morphological variation; *Wolbachia* displays diverse morphological forms inside host cells (Hertig et al., 1924; Bereiter-Hahn et al., 1994). (4) Lateral gene transfer; Portions of the *Wolbachia* genome have been inserted into many host genomes through lateral gene transfer and

are transcriptionally active (Hotopp et al., 2007). (5) Mutualism; The disruption of host oogenesis following antibiotic removal of *Wolbachia* indicates that the relationship between *Wolbachia* and its host is mutualistic (Dedeine et al., 2001). (6) Phylogenetic incongruence; The phylogenies of host species and *Wolbachia* do not match, and most *Wolbachia* infections do not persist following host speciation events (Baldo et al., 2006). This finding suggests a close relationship between *Wolbachia* and the mechanisms underlying host speciation.

Relationship between *Wolbachia* and mitochondria Additional observations further suggest parallels between *Wolbachia* and mitochondria: (1) Correlated fluctuations in abundance; the abundances of *Wolbachia* and mitochondria are correlated across the host cell cycle (Henry et al., 2018). (2) Modulation of ROS levels; *Wolbachia* infection influences the regulation of ROS levels in host cells (Mao et al., 2022; Zug et al., 2015). (3) Reduction in ATP levels; ATP levels decrease in *Wolbachia*-infected embryonic cells (Manokaran et al., 2020). This finding is reminiscent of the proposed ATP depletion mechanism in the HDM model.

Phenotypes of *Wolbachia*-infected insects *Wolbachia* exerts a variety of effects on host germ cells and embryonic cells (Kaur et al., 2021): (1) Cytoplasmic incompatibility; When *Wolbachia*-infected males (whose sperm lack *Wolbachia* due to elimination) mate with uninfected females, the eggs fail to develop (Tram et al., 2002). (2) Feminization and male killing; In crosses between *Wolbachia*-infected female moths (ZW-type) and uninfected males, the resulting *Wolbachia*-infected genetic males develop as phenotypic females and subsequently die, leaving only females to develop (Sugimoto et al., 2012). This phenomenon is reminiscent of hybrid male inviability observed in XY-type organisms and resembles the hybrid incompatibility (pupal abnormalities), accompanied by feminization, previously described in F2 males mosquitoes (Liang et al., 2024). (3) Masculinization and female killing; When larvae from *Wolbachia*-infected moths are cured with antibiotics and such females are crossed with uninfected males, the resulting *Wolbachia*-uninfected genetic females develop as phenotypic males and subsequently die, leaving only males to develop (Sugimoto et al., 2012). This phenomenon is reminiscent of hybrid female inviability observed in ZW-type organisms. Together, phenomena (2) and (3) suggest that the *Wolbachia* factors capable of manipulating the host SD system can also determine host inviability, implying that genes controlling the host SD system may also participate in programmed cell death. (4) Enhancement of hybrid male inviability; In mites that normally exhibit incomplete hybrid male inviability in F2 interstrain crosses, the presence of *Wolbachia* infection in males increases F2 male mortality (Vala et al., 2000). This indicates that *Wolbachia* interferes with the HDM system, suggesting in turn that mitochondria may also be involved in the HDM system. (5) Parthenogenesis; Female wasps infected with *Wolbachia* can reproduce without males, producing only female offspring, as males do not develop (Pannebakker et al., 2004). In insects exhibiting parthenogenesis, including wasps, the sex-determination system is haplodiploidy (2n/n): females arise from fertilized eggs and males from unfertilized eggs (Werren et al., 2008; Blackmon et al., 2017). In parthenogenesis, *Wolbachia* effectively assumes the functional role of sperm. This observation implies that among the structures present in sperm, mitochondria may exert some influence on early embryonic development. In early *Drosophila* embryos, mitochondrial ribosomal RNA (mt-rRNA) has been detected within the germ plasm, which specifies the differentiation of primordial germ cells (Kobayashi et al., 1993). The pathway by which mt-rRNA synthesized inside mitochondria is transported into the cytoplasm remains unknown. Hayashida et al. reported that in hybrid testes undergoing mitophagy, swollen mitochondria release fragmented mtDNA into the cytoplasm (Hayashida et al., 2009). During mitophagy of sperm mitochondria in fertilized eggs (Al Rawi, 2011), it is likewise possible that mitochondrial contents—including mt-rRNA—are released into the cytoplasm before they are processed by the proteasome–lysosome system. Just as *Wolbachia* infection can induce female

development in unfertilized eggs, sperm mitochondria may provide some form of signaling that contributes to the initiation of development in fertilized eggs. There may be a reason why many organisms favor the elimination of paternal mitochondria in the cytoplasm after fertilization rather than preventing the entry of heterologous mitochondria at the time of gamete fusion. Furthermore, programmed mitophagy observed not only during embryogenesis but also in stem cells, including cancer stem cells, can promote cell division, suggesting that mitochondria may have played a role in enabling multicellularity in eukaryotic cells.

Involvement in programmed mitophagy Fast et al. demonstrated via TUNEL assay and confocal imaging that programmed cell death occurring at checkpoints during germ cell development in *Drosophila* ovaries is apoptosis, and that *Wolbachia* infection reduces apoptosis while increasing the mitotic activity of germline stem cells (Fast et al., 2011). However, in the confocal images presented, the germline stem cells clearly show cytoplasmic rather than nuclear staining, indicating that the programmed cell death occurring during oogenesis is not apoptosis but mitophagy. Given that mitophagy might have been misinterpreted as apoptosis in past TUNEL assay studies, numerous instances of mitophagy were likely missed. Therefore, a re-evaluation utilizing confocal microscopy images is warranted. This finding indicates that what *Wolbachia* controls during the process of endosymbiosis is not the host's apoptosis system but rather the programmed mitophagy system. Furthermore, the fact that programmed mitophagy regulates checkpoints during oogenesis supports the author's hypothesis that the disruption of oogenesis in ZW-type hybrids results from dysregulated mitophagy, and it is also consistent with the mitoptosis-based explanation for embryonic failure in the heterogametic sex of hybrids. Moreover, cytoplasmic incompatibility has been shown to result from mitotic defects caused by disruptions of the cell cycle during early embryogenesis (Tram et al., 2002). This observation supports the speculation that HDM system results from mitotic or meiotic arrest induced by programmed mitophagy in stem cells or germline cells.

The host competitive endogenous RNA network As noted earlier in Orr's observations (Orr, 1993)—in which hybrid XY males are inviable in *Drosophila*, and hybrid XXY females generated using attached-XX females are likewise inviable—the testis-specific expression of Xt-mir appears to be controlled by genes on the Y chromosome. Although the Y chromosome does not encode miRNAs (Ji et al., 2016), it does contain long non-coding RNAs (lncRNAs), and knockout of Y-linked lncRNAs (Y-lncRNAs) has been shown to upregulate miRNAs and suppress apoptosis (Hao et al., 2021). LncRNAs are non-coding RNAs longer than 200 nucleotides that are widely present across diverse species, including viruses, prokaryotes, and eukaryotes. In animals, they often show tissue-specific expression, with particularly abundant expression in the testis. LncRNAs are considered regulatory molecules involved in genetic regulatory processes (Hong et al., 2018). Within the competitive endogenous RNA (ceRNA) network—comprising lncRNAs, miRNAs, and mRNAs—lncRNAs interact with miRNAs, reducing miRNA activity through a decoy effect, and they also modulate transcription by interacting with transcription factors, either activating or repressing gene expression (Paraskevopoulou et al., 2016). Xt-mir escapes meiotic sex chromosome inactivation (MSCI) and is expressed during meiosis (Song et al., 2009). Some lncRNAs participate in gene imprinting and X-chromosome inactivation (Sahlu et al., 2020), and knockout mice lacking these lncRNAs exhibit reduced sperm counts and a decreased proportion of male offspring (Hong et al., 2021).

Wolbachia exhibits host-dependent expression phenotypes (Werren et al., 2008). Moreover, it can either increase or decrease ROS levels depending on the host cell type. In *Wolbachia*-transinfected cell lines, ROS levels tend to be elevated, whereas in natural symbiotic systems they are generally unchanged or reduced (Zug et al., 2015). *Wolbachia* infecting *Drosophila* shows a strong antiviral effect (Hedges et al., 2008), which has been attributed to increased ROS production (Pan et al., 2012). In *Wolbachia*-infected *Aedes aegypti* mosquitoes, certain lncRNAs exhibit temporal expression changes. Among these, the upregulated aae-lnc-7598 induces antioxidant genes, whereas the downregulated aae-lnc-0165 increases miRNA levels through a ceRNA network, thereby

reducing intracellular mitochondrial ROS (mtROS) and ensuring stable endosymbiosis within the host (Mao et al., 2022). Damaged mitochondria, which release cytochrome c, mtROS, and mtDNA detrimental to cellular homeostasis, are eliminated by mitophagy (Ma et al., 2020). The findings on aae-lnc-0165 and the aforementioned Y-lncRNA imply the existence of miRNAs that suppress mitochondrial disruption, as well as Y-lncRNAs that regulate those miRNAs. It is therefore plausible that an lncRNA located on a heteromorphic chromosome operates upstream of the Spag1-mRNA/Xt-mir axis, forming a ceRNA network. Furthermore, *Wolbachia* may facilitate symbiosis by manipulating this network.

Two types of Y-lncRNAs can be envisioned: a mSpag1-lncRNA (mS-lncRNA$^-$) that suppresses mSpag1-Xt-mir expression, and a cSpag1-lncRNA (cS-lncRNA$^+$) that escapes MSCI and activates cSpag1-Xt-mir expression. Likewise, two types of Z-linked lncRNAs may exist: a cSpag1-lncRNA (cS-lncRNA$^-$) that suppresses cSpag1-Xt-mir expression, and a mSpag1 lncRNA (mS-lncRNA$^+$) that escapes MSCI and activates mSpag1-Xt-mir expression.

***Wolbachia* factors** *Wolbachia*-infected insects have sex chromosomes of the ZW, XO, 2n/n, and XY types. However, because the XY type of insects is mostly a unique type determined by the X-to-autosome ratio (Werren et al., 2008; Blackmon et al., 2016), the *Wolbachia* factors can be more appropriately examined in ZW-type species. Although *Wolbachia* does not have lncRNAs, it expresses small noncoding RNAs (snRNAs; 50–500 nt noncoding RNAs expressed in prokaryotes) (Mayoral et al., 2014), which crosstalk with the host ceRNA network and thereby facilitate symbiosis (Mao et al., 2022). In ZW-type insects, a *Wolbachia*-derived factor capable of inducing host-cell mitoptosis–mediated mitotic arrest, and thereby causing embryonic developmental failure, could be achieved by the simultaneous expression of cSpag1 and mSpag1 (i.e., concurrent suppression of cSpag1-Xt-mir and mSpag1-Xt-mir). Two such candidates can therefore be proposed: a cSpag1-targeting snRNA (cS-snRNA$^-$) that suppresses cSpag1-Xt-mir, and a mSpag1-targeting snRNA (mS-snRNA$^-$) that suppresses mSpag1-Xt-mir.

Re-transcription of transferred *Wolbachia* genes Although *Wolbachia* is eliminated from sperm during maturation, crosses between infected males and uninfected females exhibit cytoplasmic incompatibility. Because no developmental abnormalities occur when both sexes are infected, a toxin–antitoxin model involving two *Wolbachia*-derived proteins has been proposed (Porter et al., 2023). However, because developmental defects occur in females produced from crosses between antibiotic-cured *Wolbachia*-infected moth females and uninfected males (Sugimoto et al., 2012), it is unlikely that short-lived proteins or mRNAs are responsible for these two phenomena. A substantial portion of the *Wolbachia* genome has been shown to undergo lateral gene transfer and become integrated into many host genomes, where it can be transcribed even in eukaryotic cells lacking *Wolbachia* infection (Hotopp et al., 2007). In the testes of *Wolbachia*-infected hosts, ROS levels are elevated, leading to the induction of double-strand DNA breaks (DSBs) in the host genome (Brennan et al., 2012). Such DSBs may facilitate lateral gene transfer of *Wolbachia* genes into the host genome. The factors acting after bacterial elimination or removal are therefore likely to be re-transcripts of these transferred genes. Because the function of a gene can change when transferred and re-expressed in a new genomic context (Husnik et al., 2018), the resulting transcripts may exert effects distinct from those of the original *Wolbachia* genes. The absence of developmental failure in progeny from crosses in which both parents are *Wolbachia*-infected (Kaur et al., 2021) may be explained by the neutralizing interaction between *Wolbachia*-derived snRNAs and the re-transcribed RNAs (cS-snRNA* or mS-snRNA*) originating from transferred *Wolbachia* genes.

Sex reversal Although *Wolbachia* infects most host tissues (Pietri et al., 2016), as described above it has little effect on somatic cells and acts primarily on reproductive and developmental processes. Moreover, with the exception of cytoplasmic incompatibility, its effects are consistently sex-biased and often accompanied by sex reversal, suggesting involvement of the SD system. These observations closely resemble the HDM model of speciation and further imply that molecules participating

in the speciation system also play roles in the SD system. Regarding the mechanism of sex reversal, it is plausible to hypothesize that mS-lncRNA^{+} induces the expression of a masculinization factor-miRNA (Ma-miRNA) in ZW females. In *Wolbachia*-infected males that arise from infected females, mS-snRNA^{-} induces Ma-miRNA expression, resulting in feminization. In contrast, in antibiotic-cured females, the re-transcribed mS-snRNA* suppresses Ma-miRNA, thereby causing masculinization.

Based on these findings, the speciation gene set is likely the Eri15/Spag1/Xt-mir/Y(W)-lncRNA axis. If speciation is mediated by this axis, *Wolbachia*, which can survive in the host by manipulating this axis, would likely be unable to survive in a heterospecific host established by mutations in the axis genes. The lncRNAs on the heteromorphic sex chromosome may function as the "last boss" of the MMI, HDM, and SD systems.

Conclusions and future perspectives

Genes that match the Haldane-Dobzhansky-Muller model should be one set of an epistatic gene on the X (Z) chromosome and an effector gene on the autosome. The two genes cause incompatibility in gonads or stem cells in hybrid heterogametic sex and exhibit sterility and inviability due to ATP depletion by mitoptosis, respectively. The author proposed Xt-mir and SPAG1 as candidate genes and further advances a competitive endogenous RNA network mediated by Y(W)-linked lncRNAs as epistatic genes. The Eri15/SPAG1/Xt-mir/Y(W)-lncRNA axis is implicated not only in speciation but also in maternal mitochondrial DNA inheritance, infertility, the Warburg effect, and carcinogenesis, and may further provide insights into sex determination mechanisms and *Wolbachia*-based strategies for preventing mosquito-borne viral diseases. Moreover, this axis may contribute to the etiology of currently unexplained diseases involving mitophagy, warranting further investigation.

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Supplementary information

Table S1. Human Fx-mir and target genes (*SPAG1-1* and R2SP complex member) 8: 8mer matching. +7: 7mer-m8. -7: 7mer-A1. +6: 6mer. -6: offset 6mer (Bartel, 2018). 7 and 8mer searched by TargetScan. 6mer searched only *SPAG1* by Multiple sequence alignment (https://www.genome.jp/tools-bin/mafft). The total number indicates the miRNA type (mRNA 3' UTR target site) of 7 or 8mer matching (red numbers).

	SPAG1-2	*SPAG1-1*	*PIH1D2*	*RUVBL1*	*RUVBL2*
Fx-mir	1206 bp	261 bp	158 bp	971 bp	153 bp
hsa-mir-888-5p	7+				
hsa-mir-888-3p	6+				
hsa-mir-890					
hsa-mir-891a-5p					
hsa-mir-891a-3p					
hsa-mir-891b	8, 7-				
hsa-mir-892a		6-			
hsa-mir-892b		6+			
hsa-mir-892c-5p	7+				
hsa-mir-892c-3p	6+	6-			
hsa-mir-506-5p	6+				
hsa-mir-506-3p					
hsa-mir-507		6+			
hsa-mir-508-5p					
hsa-mir-508-3p	7+				
hsa-mir-509-5p					
hsa-mir-509-3p					
hsa-mir-509-3-5p					
hsa-mir-510-5p					
hsa-mir-510-3p	7-	8		7+	
hsa-mir-513a-5p	6-				
hsa-mir-513a-3p	6+, 6+				
hsa-mir-513b-5p			7+		
hsa-mir-513b-3p					
hsa-mir-513c-5p					7-
hsa-mir-513c-3p	6+, 6+				
hsa-mir-514a-5p					7+, 7+
hsa-mir-514a-3p					
hsa-mir-514b-5p					7-
hsa-mir-514b-3p					
Total (type/site)	5 (6)	1 (1)	1 (1)	1 (1)	3 (4)

Table S2. Mouse Fx-mir, Xt-mir, and target genes (*Spag1* and *Fmr1*) 8: 8mer matching. +7: 7mer-m8. -7: 7mer-A1. +6: 6mer. -6: offset 6mer (Bartel, 2018). 7 and 8mer searched by TargetScan. 6mer searched only *Spag1*/Fx-mir by Multiple sequence alignment. The total

number indicates the miRNA type (mRNA 3' UTR target site) of 7 or 8mer matching (red numbers). Xt-mir (except Fx-mir) is quoted from Song et al. (Song et al., 2009). The 3'UTRs of mouse *mSpag1* and *cSpag1* are almost the same.

	Spag1	*Fmr1*		*Spag1*	*Fmr1*
Fx-mir	**726bp**	**2299bp**	**Xt-mir**	**726bp**	**2299bp**
mmu-mir-743a-5p			mmu-mir-105	7 -	
mmu-mir-743a-3p	6-	7+	mmu-mir-106a		
mmu-mir-743b-5p			mmu-mir-1198		
mmu-mir-743b-3p	6-	7+	mmu-mir-18b		7 -
mmu-mir-742-5p	8		mmu-mir-188		7+
mmu-mir-742-3p	6-		mmu-mir-19b-2		8, 7-
mmu-mir-883a-5p		7-	mmu-mir-1906-2		
mmu-mir-883a-3p	7+, 6+, 6+		mmu-mir-20b		
mmu-mir-883b-5p			mmu-mir-2137		
mmu-mir-883b-3p	7+, 6+, 6+		mmu-mir-221		8
mmu-mir-471-5p			mmu-mir-222		8
mmu-mir-471-3p	6-		mmu-mir-223		
mmu-mir-741-5p			mmu-mir-224	7+, 7+	
mmu-mir-741-3p			mmu-mir-3112		
mmu-mir-463-5p			mmu-mir-322		7+
mmu-mir-463-3p	6-		mmu-mir-325	7-, 7-, 7-	8, 7+, 7-
mmu-mir-880-5p	6+	8	mmu-mir-3472		
mmu-mir-880-3p		7+	mmu-mir-3473a		
mmu-mir-878-5p	6-	7+	mmu-mir-351		7-
mmu-mir-878-3p			mmu-mir-361		
mmu-mir-881-5p		7+	mmu-mir-362		
mmu-mir-881-3p		7+	mmu-mir-363		8
mmu-mir-871-5p			mmu-mir-374b		7+
mmu-mir-871-3p			mmu-mir-374		
mmu-mir-470-5p			mmu-mir-384		7-
mmu-mir-470-3p			mmu-mir-421	7+	
mmu-mir-465d-5p			mmu-mir-448		7+, 7-, 7-
mmu-mir-465d-3p			mmu-mir-450a		
mmu-mir-465c-1-5p			mmu-mir-450a-2		8
mmu-mir-465c-1-3p			mmu-mir-450b		7+, 7-
mmu-mir-465b-1-5p			mmu-mir-452		8, 7+
mmu-mir-465b-1-3p			mmu-mir-500		
mmu-mir-465c-2-5p			mmu-mir-501		
mmu-mir-465c-2-3p			mmu-mir-503		7-, 7-
mmu-mir-465b-2-5p			mmu-mir-504		
mmu-mir-465b-2-3p			mmu-mir-506		
mmu-mir-465a-5p			mmu-mir-532		7-
mmu-mir-465a-3p			mmu-mir-542	7+	
mmu-mir-201-5p	6+	7-	mmu-mir-652		
mmu-mir-201-3p		8	mmu-mir-672		7-
mmu-mir-547-5p			mmu-mir-676		
mmu-mir-547-3p			mmu-mir-680-2		
mmu-mir-509-5p			mmu-mir-717		7+
mmu-mir-509-3p			mmu-mir-718		
			mmu-mir-764		
			mmu-let-7f-2		7+
			mmu-mir-92a-2		8
			mmu-mir-98		
Total (type/site)	**3 (3)**	**10 (10)**		**5 (8)**	**21 (29)**