AMX-1 is necessary for proper fertility and lack of its expression induced sterility in a p53/CEP-1 dependent manner by modulating piwi expression in *C. elegans*

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Abstract

Background

Histone methylation shapes the epigenetic configuration and adjusts multiple fundamental nuclear processes, including transcription, cell cycle control and DNA repair. The absence of histone demethylase LSD1/SPR-5 leads to progressive fertility defects as well as a reduction in brood size. Similarly, *C. elegans* LSD2 homolog AMX-1 has been implicated in regulating H3K4me2 and maintaining ICL susceptibility. However, the mechanisms of how lack of AMX-1 induces sterility have not been addressed so far.

Results

This study investigated the histone demethylase AMX-1 in *C. elegans* and uncovered how *amx-1* contributes to sterility in a p53/CEP-1 dependent manner. We show that while sterility in *spr-5* mutants exhibited progressive over generations, *amx-1* mutants displayed non-transgenerational fertility defects. Also, *amx-1* mutants exhibited a reduced number of sperms and produced low brood size (LBS) or sterile worms that retain neither sperms nor germline nuclei, suggesting that fertility defects originated from germline development failure. Surprisingly, sterility exhibited in *amx-1* was mediated by p53/CEP-1 in a DNA damage independent manner, indicating p53/CEP-1's roles beyond DNA damage repair. Consistent with this result, upregulation of Piwi expression in *amx-1* mutants suggested that AMX-1 is essential for germline development by regulating Piwi gene expressions.

Conclusions

We propose that AMX-1 is required for proper Piwi expression and transposon silencing in a p53/CEP-1 dependent manner; thus, the absence of AMX-1 expression leads to defective meiotic development and sterility. This study elucidates how LSD2/ AMX-1 contributes sterility for the first time, therefore, expanding the boundaries of histone demethylase function.

Background

Since Waddington first proposed the concept of epigenetics in 1942, epigenetic studies have been investigated to identify the mechanisms of how gene expression altered without modifying the nucleotide sequences [1, 2]. So far, various types of epigenetic progression have been discovered including methylation, acetylation phosphorylation, ubiquitination and sumoylation.

Chromatin is the complex made up of DNA, histones and associated proteins responsible for packaging DNA molecules thus fit into the nucleus [3, 4]. Histone modifications, including acetylation and methylation, alter chromatin structure and influence gene expression in an epigenetic manner. Histone

demethylases can be broadly classified into two families based on their enzymatic action: amine oxidase demethylases (LSD1/KDM1A and LSD2/KDM1B) and Jumonji C domain (JmjC) family, which is also known as the KDM3 family [5, 6].

Histone demethylase LSD1 has been related to numerous biological processes. For examples, studies have shown that lysine-specific histone demethylase LSD1 is required for proper germline maturation, DNA damage repair, chromosome segregation and aneuploidy [7–12]. Similarly, In *C. elegans* study, a lack of a mammalian LSD1 homolog SPR-5 exhibits inherited accumulation of the euchromatic H3K4me2 methylation mark along with a progressive decline in fertility [13]. Defective fertility becomes severer with generations, resulting in a sterile phenotype after multiple generations. In addition, *spr-5* mutants affect the lifespan of *C. elegans* and double-strand breaks repair [11, 12, 14]. Lastly, SPR-5 coordinate with FANCM/FNKM-1 to cope with replication stress and maintain H3K4me2 level [7]. These observations suggest histone demethylase's multiple roles and not restricted in maintaining H3H4me2 level.

C. elegans has three homologs of mammalian LSD1/2 histone demethylase: LSD1 homologs SPR-5 and LSD-1 and LSD2 homolog AMX-1. While most studies focused on LSD1 homolog SPR-5, the function of the LSD2 homolog AMX-1 was not very well understood until recently. Since all three histone demethylases belong to LSD1/2 homologs, which are involved in chromatin remodeling and modulating gene expressions, it has been considered that they may perform similar and redundant functions, thus compensating one another. Consistent with this idea, both AMX-1 and SPR-5 are implicated in fertility defect, sterility and lifespan [11, 13, 15], thus connecting fertility with histone methylation status. However, since SPR-5 and AMX-1 are implicated in DNA double-strand break repair (DSBR) and Interstrand Crosslink Repair (ICLR), respectively [15, 16], we postulate that phenotypes observed in two histone demethylases might originate from separate pathways.

Until now, systematic analysis of how *amx-1*, *per se*, contributes to defective fertility or sterility has not been investigated. Given that AMX-1 is requisite for proper fertility (Fig. 2A), we examined the mechanism of how AMX-1 contributes to fertility and sterility in this study. Here, we dissect the phenotypes presented in *amx-1* mutants to investigate how AMX-1 contributes to fertility defects. AMX-1 is necessary for normal fertility and the absence of its expression exhibits a decreased brood size. However, in contrast to mammalian LSD1 homolog SPR-5, AMX-1 is not essential for transgenerational fertility defects. In addition, *amx-1* mutants exhibit mild but significant sterility. *amx-1* mutants retained a reduced number of sperms and produced sterile worms that lack germline or sperms. Interestingly while p53/CEP-1 is dispensable for brood size reduction, the sterility in *amx-1* mutants requires p53/CEP-1 function. Also, the lack of gametes exhibited in *amx-1* mutants correlates with upregulation of Piwi expression, indicating that *amx-1* mutants are undergoing transposon desilencing. Altogether, our study demonstrates that lack of AMX-1 induced sterility in a p53/CEP-1 dependent manner.

Results

AMX-1 is required for proper fertility but dispensable for transgenerational fertility defect

Previously, both histone demethylases AMX-1 and SPR-5 exhibited brood size reduction [13, 15]. Consistent with these reports, *amx-1* and *spr-5* null mutants exhibit 21% and 28% reduction compared to wild type, respectively (Figure 1A, 100 vs. 79% and 100 vs. 72% in early generations). In addition, *spr-5* exhibited progressive fertility defect in which brood size decreases over generations (green dashed line; 72, 40, 27% in early, mid, late generations, $P = 0.0022$ in early and late generations of *spr-5*, [13]). On the contrary, *amx-1* did not alter fertility in either mid or late generations compared to early generations, suggesting that AMX-1 is dispensable for transgenerational fertility defects (Figure 1A, red dashed line; 79, 66, 76 in early, mid, late generations, $P = 0.5351$ in early and late generations of *amx-1*). Given that *amx-1* is required for ICL (intercrosslink) repair, we further analyze how AMX-1 contributes to defective fertility.

p53/CEP-1 is not required for fertility defects exhibited in *amx-1* mutants

The absence of AMX-1 expression leads to DNA damage apoptosis, which requires p53/CEP-1 [15]. Thus, we further tested whether the reduced brood size observed in *amx-1* requires p53/CEP-1. There was no distinct difference between wild-type and *cep-1* mutants (Figure 1B, 305 vs 276, $P = 0.1807$ by Mann-Whitney test). Also, *cep-1;amx-1* double mutants did not alter brood size significantly, indicating that reduced fertility in *amx-1* was independent of p53/CEP-1 pathway (201 in *cep-1;amx-1* vs 210 in *amx-1*, $P = 0.2770$).

amx-1 has been implicated in the activation of DNA damage and is required for proper ICL repair by modulating mismatch repair gene expression [15]. Thus, we examined whether CEP-1 is necessary for ICL sensitivity or not. We found that lack of p53/CEP-1 did not alter the level of embryonic lethality to nitrogen mustard, further supporting that CEP-1 is dispensable for fertility defects displayed in *amx-1* mutants (Figure 1C, 54.8 in *amx-1* vs 51.5 in *cep-1;amx-1*, $P = 0.3001$; 54.8 in *amx-1* vs 25.6 in wild type, $P = 0.0036$). Taken together, these observations suggest that lack of AMX-1 results in non-progressive fertility defects in a p53/CEP-1 independent manner.

The sterility of *amx-1* is not transgenerational

In addition to defective fertility, lack of SPR-5 expression exhibited the sterility phenotype that hermaphrodite worms produce no viable progeny [13]. Consistently with the report, lack of SPR-5 led to the sterility and became severer with generations (Fig 2A, 5.5 vs 7.4% in early and mid-late generations, $P = 0.0006$ and $P = 0.0036$ in early and mid-late generations of wild type and *spr-5* respectively).

Interestingly, *amx-1* mutants displayed a significantly higher sterility level than the control wild type. Specifically, early generations of *amx-1* exhibited 12.7-fold higher sterility than the wild type (Fig 2A, 0.07 vs 0.89 %, $P = 0.0104$ in early generations). Also, mid-late generations of *amx-1* exhibited an 11-fold induction of sterility compared to wild type supporting the idea that the sterility phenotype of *amx-1*

mutants is not transgenerational (0.12 vs 1.33%, $P=0.0251$ in wt and *amx-1*; 0.89 vs 1.33%, $P=0.5657$ in early vs mid-late generations of *amx-1*)

Besides sterility, *amx-1* mutants often produced ~100 or fewer progenies; hence we named it low brood size (LBS, ~6%). The LBS *amx-1* mutants did not maintain reduced fertility (Fig 2B, 26 vs 268 in P0 and F1 respectively, $P=0.0077$, Mann-Whitney test). The reduction of brood size in parental worms was recovered in their following generations, further confirming that the sterility of *amx-1* was not imprinted for the subsequent progenies.

p53/CEP-1 is required for sterility exhibited in *amx-1* mutants

Since AMX-1 functions in ICL damage repair [15], we tested whether a key regulator of DNA damage-induced checkpoint p53/CEP-1 is essential for sterility observed in the *amx-1* mutants.

cep-1 mutants exhibited no obvious sterility compared to wild-type control regardless of generations suggesting that CEP-1 is dispensable for sterility (Fig 2A, 0.07 vs 0.12% $P=0.8708$ in early generations and 0.12 vs 0.18% $P>0.9999$ in mid-late generations between wild type and *cep-1*). Interestingly, lack of p53/CEP-1 expression in *cep-1;amx-1* double mutants suppress sterility observed in the *amx-1* mutants in both early and mid-late generations, indicating that p53/CEP-1 is required for sterility exhibited in *amx-1* mutants (Fig 2A, 0.89 vs 0.3%, $P=0.0026$ in early generations. 1.33 vs 0.14%, $P=0.0423$ in mid-late generations).

We further examined whether *cep-1* suppresses sterility in *spr-5* mutants. On the contrary, unlike *amx-1*, sterility displayed in *spr-5* was not suppressed in the *cep-1;spr-5* double mutants suggesting that p53/CEP-1 is dispensable for its sterility (Fig 2A, 5.5 vs 4.9, $P>0.9999$ in early generation. 7.4 vs 5.9, $P=0.9360$ in late generations). Also, *spr-5;amx-1* double mutants were not considerably altered compared to *spr-5* single mutants implying that sterility phenotypes of two histone demethylases may stem from independent pathways (5.5 vs 5.3, $P=0.9383$ in early generations and 7.4 vs 7.3, $P=0.3359$ in late generations). Taken together, these observations suggest that lack of AMX-1 induces p53/CEP-1 dependent sterility.

Sterile worms exhibit a lack of germ cells

To further investigate and analyze the phenotypes of worms displaying sterility, we performed comparative morphological analysis using fluorescent staining. We utilized whole worms without dissection to avoid potential artifacts while preparing worms for slides. Wild type N2 worms enclosed germline nuclei, embryos and spermatheca as expected from healthy worms (Fig 3A). Similarly, nonsterile *amx-1* mutants contain embryos, germline nuclei and spermatheca.

Interestingly, in contrast, sterile *amx-1* mutants exhibit missing germline and/or spermatheca generally (95% and 100%, respectively). Rarely we observed worms containing severely defective embryonic developments and germline nuclei (5%, 1 out of 20), suggesting that the sterility originates from the inadequate gamete development. Nonsterile *spr-5* mutants, which exhibit ~28% reduction in brood size

(Fig 1A), contained germline, spermatheca and fertilized eggs. Also, we often found their embryos packed tightly inside the body (Fig 3A and 3B, 40%, 4 out of 10 worms), which was not observed in *amx-1* mutants. Sterile *spr-5* mutants lacked both sperms and oocytes (100%, 10 out of 10 worms). However, their gut cells frequently exhibited chromosome bridges (70%, 7 out of 10 worms). Also, *spr-5* mutants exhibit chromosome bridges and stacked embryos, not presented in the *amx-1* mutants.

Mating rescued the LBS phenotype

These observations suggest that the absence of germ cells leads to the sterility phenotype of *amx-1*. To further validate this idea, the low brood size (LBS) *amx-1* mutants that produced less than ~100 progenies were mated with wild type and monitored whether wild type sperms could rescue the brood size decrease (Fig 4A). No distinct difference in fertility was observed in control and mating groups before mating (75.7 vs 64.1 from L4 to 2 days after L4, $P = 0.4391$, M-H test). However, mating increased the brood size higher than the control group by 20 fold, indicating that fertility defects originate from defective sperms in *amx-1* (7.3 vs 151 in 3-4 days after L4, $P < 0.0001$ Mann-Whitney test, Fig 4B).

In line with this observation, we found a significant reduction in the number of sperms of *amx-1* mutants compared to control wild type (88 vs 113 in *amx-1* and wild type respectively, $P = 0.0077$ Mann-Whitney test, Fig 4C). Taken together, our genetic and cytological analysis demonstrated that defective sperm development contributes to the sterility of *amx-1* mutants.

***amx-1* mutants exhibit p53/CEP-1 dependent and DNA damage independent sterility**

p53/CEP-1 is known as a transcription factor of DNA damage-induced apoptosis and its signaling has been implicated in multiple pathways, including cell cycle checkpoint, senescent, angiogenesis and differentiation [17]. *C. elegans* has only one p53 gene CEP-1 [18, 19], thus the lack of CEP-1 leads to failure in DNA damage pathway [20, 21].

Given that p53/CEP-1 engaged in multiple pathways and lack of p53/CEP-1 suppresses the sterility phenotype observed in *amx-1* mutants (Fig 2A), we further tested whether the DNA damage pathway of p53/CEP-1 contributes to sterility. For this purpose, we exposed young adult hermaphrodites for ~20 hours in media containing DNA damaging agents and monitored the embryonic lethality as described previously [21, 22]. Consistent with the data presented in Fig 2A, *amx-1* mutants exhibited ~10 fold higher sterility compared to wild type control (Fig 5, 1.07 vs 0.09 % respectively, $P = 0.0034$).

Interestingly, wild type exposed in 50 μ M nitrogen mustard (HN2) did not induce sensitivity compared to control (Fig 5, 0.09 vs 0.08 in wt and wt + HN2, $P = 0.5000$) suggesting that ICL DNA damages may not affect sterility. Also, at a higher dose of HN2, where wild type worms displayed 24% embryonic lethality [15], similarly, both wild type and *amx-1* did not alter the level of sterility at 100 μ M HN2 (0.09 vs 0.08 in wt and wt + HN2, $P = 0.5000$; 1.07 vs 1.0 in *amx-1* and *amx-1*+HN2, $P = 0.1558$), indicating that ICL damages did not initiate sterility in *amx-1* mutants. Furthermore, hydroxyurea (HU), which induce DNA replication stress by depleting dNTP pools, did not alter sterility in either wild type or *amx-1* mutants,

further supporting the idea that DNA damage repair may be dispensable for sterility exhibited in *amx-1* (Fig 5, $P=0.4354$ in wild type and wild type + HU; $P=0.1966$ in *amx-1* and *amx-1*+HU).

Next, we examined whether p53/CEP-1 is required for sterility upon ICL damages. *cep-1* single mutant did not represent distinct changes over wild type (0.31 vs 0.09 in *cep-1* and wt, $P=0.2086$). *cep-1;amx-1* double mutants suppressed sterility of *amx-1* either in the presence or absence of HN2 treatment, suggesting that CEP-1 is required for the sterility of *amx-1* regardless of ICL DNA damage (Fig 5, 82% reduction, 1.07 vs 0.19 in *amx-1* and *cep-1;amx-1*, $P=0.0034$; 72% reduction; 1.0 vs 0.28 in *amx-1*+HN2 and *cep-1;amx-1*+HN2, $P=0.0442$). Taken together, these observations suggest that sterility of *amx-1* mutants is dispensable for ICL DNA damage and requires DNA damage independent roles of CEP-1.

p53/CEP-1 mediated upregulation of Piwi expression

Since sterility of *amx-1* mediated DNA damage independent p53/CEP-1 function, we further examined the possible cause of sterility. Transposon silencing is required for proper fertility [23]. Piwi genes function in germline stem cells, stem cell development, gametogenesis and RNA interferences in diverse organisms. Consistently, *C. elegans*, *piwi/prg-1* mutants exhibited various germline abnormalities including empty germline [24, 25]. The absence of germline phenotype presented in *amx-1* sterile mutants reminded us of *piwi*/PRGs genes, which are essential for normal germline development of various species. *C. elegans* *piwi*/PRG-1 are required for transposon silencing and the null mutants induced upregulation of transposons as well as transpositions [24].

Surprisingly, the mRNA expression level of *prg-1* or *prg-2* was considerably higher in *amx-1* mutants than wild-type (Fig 6, 1 vs 11 in *prg-1* expression, $P=0.0022$; 1 vs 6.6 in *prg-2* expression, $P=0.0033$). The induced level of *prg-1* or *prg-2* was suppressed in *cep-1;amx-1* double mutants significantly, suggesting the p53/CEP-1 mediated Piwi expression in *amx-1* mutants (11 vs 5.6 of *prg-1* expression, $P=0.0095$; 6.6 vs 2.2 in of *prg-2* expression, $P=0.0049$). *cep-1* single mutants did not alter expression significantly for both *prg-1* and *prg-2* ($P=0.1199$ and $P=0.6396$, respectively). Also, no distinct changes were observed between *cep-1* and *cep-1;amx-1* double mutants in both transposons ($P=0.1198$ in Tc3 and $P=0.1482$ in Tc1). Altogether, our mRNA analysis demonstrated that AMX-1 is required for the proper expression of Piwi genes in a CEP-1 dependent manner.

PRG-1 and PRG-2 are required for transposon silencing in *Drosophila* and *C. elegans* [24, 26, 27]. To examine transposon silencing in *amx-1* mutants, we analyze two major DNA transposons Tc1 and Tc3, using primers specific for 15 Tc1 and 20 Tc3 loci, as reported in Das et al.[24]. In line with the higher expression of both *prg-1* and *prg-2*, both Tc3 and Tc1 were significantly upregulated in *amx-1* mutants compared to the level of wild type suggesting that lack of AMX-1 leads to the sterility mediated by transposon desilencing (Fig 6, 1 vs 6.1 in Tc3 expression, $P=0.0002$; 1 vs 2.6 in Tc1 expression, $P=0.0358$). Also, consistent with Piwi expression data, *cep-1;amx-1* double mutants suppressed the increased Tc3 level presented in *amx-1* significantly, indicating that CEP-1 is required for Tc3 desilencing (2.6 vs 6.1, $P=0.0078$). However, no distinct suppression was observed in Tc1 expression, indicating that CEP-1 specific for Tc3 desilencing (1.6 vs 2.6, $P=0.0689$). Notably, low brood size *amx-1* mutants

exhibited a reduced level of Tc3 expression compared to *amx-1* mutants (2.2 vs 6.1, $P=0.0078$), suggesting that transposon silencing is restored to a normal level when *amx-1* mutants became LBS.

LBS worms do not alter H3K4me2 level

Given that *amx-1* mutants showed a higher level of H3K4me2 in embryos and germline nuclei and H3K4 methylation level contribute to transgenerational fertility, we further examined whether sterility is the outcome of the extra accumulation of dimethylation [13, 15, 28].

Dissected gonads from *amx-1* mutants exhibiting non-sterile (~200 brood size) or sterile age-matched adult hermaphrodite were probed for H3K4me2. In premeiotic tip nuclei, pachytene and diplotene nuclei, of sterile *amx-1* mutants did not alter signal intensities representing H3K4me2 compared to the control non-sterile worms, suggesting that sterility in *amx-1* was not the consequence of H3K4me2 accumulation (Fig 7 A and B, 100 vs 110 in PMT, $P=0.5732$; 100 vs 98 in pachytene, $P=0.9931$; 100 vs 100 in diplotene, $P=0.5849$). Since sterile *amx-1* either lacks eggs, or eggs undergoing severe degenerations, as shown in Fig 3, we employed LBS worms to measure the histone methylation level. Consistent with the germline nuclei results, no distinct difference in the methylation level between LBS and control groups was detected (in embryo, $P=0.8456$).

Discussion

***amx-1*'s sterility was not investigated**

A previous report found the transgenerational fertility defect in LSD1 homolog *spr-5* and *spr-5;amx-1* double mutants, where double mutants of histone demethylases exacerbate fertility in *spr-5* mutants [13]. Since all three belong to LSD1/2 histone demethylase homologs, it has been considered that they may perform similar functions. However, systematic analysis of how LSD2 homolog AMX-1, *per se*, contributes to defective fertility or sterility has not been investigated so far. Given that AMX-1 is requisite for proper fertility (Fig 2A), we examined the mechanism of how the lack of AMX-1 contributes to sterility in this study.

The redundant and non-redundant function of AMX-1

It has been assumed that both AMX-1 and SPR-5 contribute to fertility defects since both are LSD1/2 histone demethylases. However, a few recent reports support their non-redundant functions. Specifically, while SPR-5 plays a role in double-strand break repair, AMX-1 functions in ICL repair [6, 15]. Second, two histone demethylases exhibit tissue-specific expression profiles. For example, AMX-1 regulates the expression of H3K4me2 in the premeiotic tip, while SPR-5 does not. On the contrary, AMX-1 expression compensates for the lack of SPR-5 in the gut and embryonic cells, indicating their redundant functions [15].

AMX-1 is dispensable for transgenerational fertility defects

This study demonstrated that *amx-1* mutants reduced brood size compared to wild type throughout generations (Fig 1A). Also, unlike progressive fertility defect observed in *spr-5* mutants, reduction in *amx-1* was not significantly nor progressively altered in between generations indicating that AMX-1 is dispensable for transgenerational fertility defects and phenotypes in two histone demethylases may arise independently. Sterility exhibited in *amx-1* was not changed between early and late generations, supporting this idea (Fig 2A). Also, the LBS phenotype did not transduce to the following generations, further validating that both sterility and LBS are transient and not transgenerational (Fig 2B).

The sterility of two histone demethylases may originate separately

Early generations of *spr-5* mutants exhibited a mild reduction of brood size. The decline became severer along with generations and often led to sterility [13], suggesting that sterility may have risen from the accumulation of severe fertility defects in the absence of SPR-5. Likewise, *amx-1* displays both sterility and fertility defects. However, unlike *spr-5*, sterility exhibited in *amx-1* is not likely the outcome of the progress fertility defects since defective fertility (LBS) resets in the following generations and is not transmitted to the subsequent generations. Furthermore, generation independent sterility supports this idea (Supplemental Figure 1).

In line with these observations, it is worth noting that *amx-1* mutants displayed CEP-1/p53 dependent sterility (Fig 2A). However, reduced brood size shown in *amx-1* mutants do not require CEP-1/p53 (Fig 1B), further promoting the idea that sterility is not the consequence of reduced brood size; therefore, defective fertility (brood size reduction) and sterility (zero fertility) may originate from independent pathways (Fig 8). Consistent with faulty fertility phenotypes, morphological changes in two histone demethylase mutants displayed both similarity and non-similarity. Sterile worms of both mutants represented germlineless and spermless phenotypes. However, *amx-1* exhibited germline nuclei as well as degenerating embryos while *spr-5* did not. Instead, *spr-5* exhibited chromatin bridges between gut cells frequently.

Although sterility in *amx-1* mutants requires CEP-1/p53, unexpectedly, it is not induced upon exogenous DNA damages treatment suggesting that DNA damage is unnecessary for sterility. However, given that CEP-1/p53 is a multifaceted transcription factor that responds to a diverse biological response, it is not surprising that sterility requires DNA damage independent p53 role.

Lack of Piwi induces sterility

Piwis are argonaute proteins associated with Piwi interaction small RNAs (26-31 nucleotides, piRNAs) rich in germ cells [29, 30]. Piwi's functions include inhibition of the transposon and germline or stem cell renewal and lack of Piwi results in sterility in flies, Arabidopsis and vertebrates. Several studies proposed sterility observed in lack of piRNAs as a consequence of transposition [24] [31] [32]. Plus, epigenetic contribution to sterility has also been proposed as well. Simon *et al.* found that histone demethylase *spr-5* and *rbr-2* suppress sterility of *prg-1* indicating that epigenetic regulation is necessary for proper Piwi

functions [33]. Moore et al. reported *prg-1* dependent epigenetic inheritance of pathogenic avoidance [34]. However, its exact mechanism remains to be elucidated.

Interestingly, sterile *amx-1* mutants and Piwi mutants share several phenotypes in common. First, both worms displayed disorganized and empty germlines and the number of sperms was reduced. Second, both mutants exhibited misregulation of Tc3 transposon expression, which is the outcome of the inadequate Piwi expression. Lastly, worms displaying sterility phenotype presented no obvious telomere fusion but showed normal six bivalent chromosomes, thus phenotype is reversible (Supplemental Figure 2, [15, 33]).

AMX-1 modulates H3K4me2 level in germline and somatic cells and is required for proper mismatch repair MutL homolog expression [15, 16]. Likewise, the mammalian homolog of AMX-1 has been implicated in regulating gene transcription by modulating H3K4me2 [35]. It is worth noting that AMX-1 may target both *prg-1* and *prg-2* based on the significant changes of mRNA expression level (Fig 6). Thus, lack of AMX-1 leads to dysregulation of Piwis, resulting in defective germline development and sterility (Fig 8 Model). Indeed, this idea can explain how *amx-1* exhibited upregulation of Tc3 since Piwis are required for the proper expression of Tc3. Alternatively, the accumulation of H3K4me2, which induces active chromatin status, in worms lacking AMX-1 expression may induce upregulation of Piwis followed by desilencing of Tc3. These two ideas are may not exclusive of each other.

Two mutants exhibited nonidentical phenotypes as well. While *prg-1* mutants lead to a reduced level of Tc3 [24], *amx-1* mutants induced misregulation of both Tc3 and Tc1. However, given that AMX-1 has multiple roles, including histone demethylase and ICL repair, it is plausible that the lack of AMX-1 likely has broader impacts on multiple biological pathways. Indeed, Zhang *et al.* reported AMX-1 functions in spare roles in addition to the CEP-1/p53 mediated DNA damage-induced apoptosis [15]. Residual apoptosis found in *cep-1;amx-1* double mutants compared with *cep-1* single may advocate the additional function of AMX-1 in the *cep-1* independent pathway. Identifying the targets for AMX-1 would help understand how AMX-1 contributes to fertility and sterility. Taken together, Our findings define a function for AMX-1 in promoting fertility in a p53 dependent manner by modulating Piwi expression.

Conclusions

Histone methylation has been implicated in various human cancers via modulating chromatin status and gene expression. Although numerous studies focused on LSD1/SPR-5's roles in fertility, how LSD2/AMX-1 contributed to fertility has not been investigated so far. Our study demonstrated that mammalian LSD2 homolog lack of AMX-1 induced sterility in a p53/CEP-1 dependent manner by modulating Piwi expression. Our work may extend this topic into the context of a previously unknown link between histone demethylase and sterility.

Methods

Strains and alleles

C. elegans strains were cultured at 20°C under standard conditions as described in Brenner [36]. The N2 Bristol strain was used as the wild-type background. The following mutations and chromosome rearrangements were used in this study: LGI: *spr-5*(*by101*), *cep-1*(*lg12501*), *prg-1*(*n4357*), hT2[*bli-4*(*e937*) *let-?*(*q782*) *qls48*](I; III); LGIII: *amx-1* (*ok659*), *qC1*[*dpy-19*(*e1259*) *glp-1*(*q339*) *qls26*] (III),

The *amx-1* deletion mutant (*ok659*) was provided by the *C. elegans* Gene Knockout Project at the Oklahoma Medical Research Foundation, which was part of the International *C. elegans* Gene Knockout Consortium. *amx-1* mutant (*ok659*) carries a 2636 bp deletion encompassing the entire SWIRM domain and most of the amine oxidase domain [15]. *amx-1*(*ok659*) worms were outcrossed to the wild-type N2 and balanced with *qC1* with roller and GFP markers for maintenance.

Quantitative analysis

Statistical comparisons between genotypes were performed using the two-tailed Mann-Whitney test, 95% confidence interval (C.I.) unless otherwise specified.

DNA damage sensitivity

Young adult homozygous *amx-1* animals were picked from the progeny of *amx-1*/*qC1* parent animals. For HN2 sensitivity, 24 hours L4 young adult hermaphrodites were treated with 0, 150 µM of HN2 in M9 buffer containing *E. coli* OP50 with sharking at 50 rpm in dark for ~20 hours. After the DNA damage treatments, animals were washed with M9 containing Triton X100 (100 ml/L) [20, 21]

Hatching sensitivity (embryonic lethality) was examined in >24 animals 3-4 hours after drug treatment. For all other damage sensitivity experiments, >24 animals were plated, 7 per plate, and hatching was assessed for the period of 20–24 hours following treatment. Each assay was replicated at least twice in an independent experiment.

Sterility and fertility

Young adult homozygous *amx-1* animals were picked from the progeny of *amx-1*/*qC1* parent animals. To monitor fertility and sterility, brood size was scored corresponding to each worm's total number of eggs. For fertility analysis, at least 400 worms were analyzed for each genotype at each generation reported. For sterility analysis, at least 400 worms were analyzed for each genotype at each generation reported. We scored sterility when worms produced no viable progenies until 2 days after L4 stage. Worms produced ~100 or fewer progenies were counted as low brood size (LBS).

HN2 sensitivity was assessed by placing age-matched L4 hermaphrodites on seeded NGM plates containing 0, 50, or 100 μ M of HN2 (mechlorethamine hydrochloride). We scored fertility until 2 days after L4 stage. HU sensitivity was assessed by placing animals on seeded NGM plates containing either 0 or 2 mM HU. We scored fertility until 2 days after L4 stage [22]

Carnoy's fixation

To preserve internal organs and tissues, we prepared hermaphrodite worms with Carnoy's solution instead of dissecting them. In brief, worms were fixed on positively charged slides with Carnoy's solution (6ml ethanol, 3ml acetic acid and 1ml chloroform). Slides were air-dried at least 2 hours and stained with $\sim$ 2 μ g/ml of DAPI for >20 minutes before analysis.

Immunofluorescence staining

Whole-mount preparations of dissected gonads, fixation and immunostaining procedures were carried out as described in [32]. Primary antibody was used at the following dilutions: mouse anti-H3K4me2 (1:250, Millipore CMA-303). Secondary antibody used was: Cy3 anti-mouse (1:250) from Jackson ImmunoResearch.

Immunofluorescence images were collected at 0.2 μ m intervals with a Nikon Eclipse Ti2-E inverted microscope and a DSQi2 camera. Photos were taken with a 60x objective in combination with or without 1.5x auxiliary magnification. Partial projections of half-nuclei were shown. Images were subjected to deconvolution by using the NIS Elements software (Nikon).

To examine AMX-1 histone demethylase level *in vivo*, dissected gonads from *amx-1* age-matched adult hermaphrodites were probed for H3K4me2. To ensure that both control and mutant animals were processed and imaged under identical conditions, we dissected the both types of animals on the same slide as described in Zhang *et al* [15]. Fluorescent intensity signal is sampled from multiple nuclei from different gonads and quantitated by using ImageJ or GelQuantNet.

Quantitative real-time PCR (qPCR)

cDNA was produced from young hermaphrodite worm RNA extracts using the ABscript II First synthesis (ABclonal RK20400). Real-time quantitative PCR amplification for were carried out using ABclonal 2X SYBR Green Fast mix (Abclonal RK21200). Amplification was carried out in a LineGene 4800 (FQD48A BIOER) with initial polymerase activation at 95°C for 2 min, followed by 40 cycles of: 95°C for 15 sec denaturation, 60°C for 20 sec for annealing and elongation.

After 40 cycles, a melting curve analysis was carried out (60°C to 95°C) to verify the specificity of amplicons. Tubulin encoding *tba-1* gene was selected for a reference gene based on *C. elegans*

microarray expression data [37]. Primer sequences are listed in Supplemental Table 1. Each qPCR has been repeated independently at least two times.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data generated or analyzed for this study are included in this article.

Competing interests

We report that we have no competing interests.

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Authors' contributions

RXJ, TSS and MQH performed all the experiments and data analysis. HMK supervised the project and contributed to planning the project, interpreting results, and writing the manuscript.

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Figures

Figure 1

Histone demethylase AMX-1 is necessary for proper fertility: Defective fertility in *amx-1* mutants is neither transgenerational nor p53/CEP-1 DNA damage pathway-dependent.

(A) Brood size is scored among the progenies of indicated genotypes. *amx-1* mutants exhibit the reduced brood size compared to wild type in early (F2-F13, $P < 0.0001$), mid (F15-F21), $P < 0.0001$) or late generations (F25-27), $P < 0.0001$). No obvious transgenerational decline was observed in between early and late generations of *amx-1* mutants (dashed lines, $P = 0.5351$ in early vs. late generations of *amx-1* and $P = 0.0022$ in early vs. late generations of *spr-5*). Asterisks indicate statistically significant changes. Error bars represent standard errors of the mean. $n > 24$ for each genotype. (B) Brood size comparison between indicated genotypes. $P = 0.1807$ for wt and *cep-1*, $P = 0.2770$ for *amx-1* and *cep-1;amx-1*. (C) Relative hatching of wild type, *amx-1* and *cep-1;amx-1* mutants after treatment with the indicated dose of nitrogen mustard (HN2). Asterisk indicates statistical significance. N.S. indicates no statistical significance; P values calculated by the two-tailed Man-Whitney test, 95% C.I.

Figure 2

Lack of *amx-1* expression results in non-transgenerational sterility in a p53/CEP-1 dependent manner.

(A) Sterility is scored among the progenies of indicated genotypes. *amx-1* mutants exhibit the sterility (zero brood size) compared to wild type in early (F2-F13) or mid-late generations (F11-F32). No obvious transgenerational decline was observed in between early and mid-late generations of *amx-1* mutants ($P=0.0006$ in early vs. mid-late generations of *amx-1* and $P=0.0036$ in early vs. late generations of *spr-5*). Asterisks indicate statistically significant changes. Error bars represent standard errors of the mean. $n>24$ for each genotype. (B) Brood size is scored among the progenies of three independent LBS (low brood size) *amx-1* mutants. $P=0.0077$ in P0 and F1 by the two-tailed Man-Whitney test.

Figure 3

AMX-1 is necessary for germline development; Defective germline development in *amx-1* sterile worms

(A) Representative images of the worms exhibiting sterility. Wild type N2 and non-sterile *amx-1/spr-5* are shown as control. Scale bar 10 mM. (B) Quantification of the phenotypic defects observed in whole worms without dissection. Inset shows an example of chromatin bridge of gut cells. Arrows indicate internal compartments of hermaphrodites. n= 10 worms for all genotypes.

Figure 4

Mating rescues the LBS phenotype of *amx-1* which retains a reduced number of sperms.

(A) Schematic representation of assay for determining the number of sperms before and after mating. LBS animals have been mated with wild type 48 hours after L4. Quantification of the number of brood size before and after mating. Individual worms and mean values of brood size with or without mating are

shown. n=9 for control and n=8 for mating. Error bars represent standard errors of the mean. (B) Quantification of the number of hermaphrodite sperms in wild type and *amx-1*. Error bars represent standard errors of the mean. n=43 for wild type and n=48 for *amx-1*. Asterisks indicate statistically significant changes by the two-tailed Man-Whitney test, 95% C.I.

Figure 5

Sterility requires p53/CEP-1 but not DNA damage

Sterility was scored among the progenies of indicated genotypes. Worms were incubated with or without HN2 (nitrogen mustard) and HU (hydroxy urea) treatment at indicated doses. Asterisks indicate statistically significant changes. Blue colored asterisks or n.s. indicate statistic comparison over *amx-1* mutants. Error bars represent standard errors of the mean. At least three independent biological replicates have been performed. N= 1000-3624 worms for each analysis.

Figure 6

AMX-1 is required for the proper expression of Piwi and transposon silencing.

Lack of *amx-1* expression increases Piwi expression. Changes of relative mRNA expression levels of Piwis (*prg-1* and *prg-2*) and transposons (*Tc3* and *Tc1*). mRNA expression was monitored with qPCR primers spanning exon-exon junctions for each indicated genes. The data shown are the means with SEM analyzed employing an unpaired, two-tailed t-test. Assay repeated at least two independent sample preparations. Tubulin encoding *tba-1* was used as an endogenous reference. Asterisks indicate significant changes of mRNA expression.

Figure 7

Sterile worms do not exhibit significant alterations in H3K4me2 levels in both mitotic and meiotic germline nuclei.

(A) No significant changes in H3K4me2 signal intensity was detected in nuclei in the premeiotic tip (PMT), pachytene, diplotene and embryo (in PMT, $P=0.5732$; in pachytene, $P=0.9931$; in diplotene, $P=0.5849$ $P=0.9931$; in embryo, $P=0.8456$). Individual nuclei were sampled for fluorescence intensity using ImageJ. The bars represent the mean fluorescence intensity. $N=5$ worms (B) Representative images used for the analysis in A. Bars, 10 μm .

Figure 8

Schematic diagram of roles of AMX-1 in sterility in comparison with SPR-5

AMX-1 and SPR-5 function in both redundant and non-redundant manners. In wild-type animals, both AMX-1 and SPR-5 are necessary for proper fertility and sterility. However, sterility in *amx-1* mutants requires DNA damage independent p53/CEP-1 roles, while sterility in *spr-5* does not. AMX-1 regulates the adequate expression of Piwis in a CEP-1 dependent manner thus, the absence of AMX-1 expression results in sterility. Likewise, both AMX-1 and SPR-5 are required to maintain the level of H3K4me2. However, two histone demethylases function in separate DNA damage repair pathways.

Supplementary Files

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