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Catalytic-independent roles of UTX-1 in *C. elegans* development

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We recently analyzed the functional roles of UTX-1 during development. *utx-1* is an essential gene required for the correct embryonic and post-embryonic development of *C. elegans,* and it displays an H3K27me3 demethylase activity. Rescue experiments demonstrated that the enzymatic activity of UTX-1 is not relevant for its role in development. The phenotypes associated with loss of UTX-1 might, instead, be a result of compromised functions of an UTX-1-containing complex. Here we discuss the possible mechanisms by which UTX-1 contributes to normal development.

Keywords: development, epigenetics, chromatin, histone demethylase and histone methyltransferase, histone marks, transcription

Abbreviations: H3K27me3, trimethyl lysine 27 of histone H3; H3K4me3, trimethyl lysine 4 of histone H3; KDM, lysine demethylase; Jmj-C, jumonji-C domain

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Introduction

Although most cells in a multicellular organism contain the same genes, they are not all used in the same way in every cell. During development, genes are differentially expressed or repressed when needed, thus specifying features unique to each cell type. The activity of transcription factors in the regulation of gene expression patterns is well known, but in recent years the role of epigenetics in the regulation of transcription has become increasingly clear. The term "epigenetics" refers to heritable changes of gene expression or phenotypic alterations not related to changes in the DNA sequence, but resulting from modifications of the chromatin structure.[2] Epigenetic mechanisms include DNA methylation, histone modifications and non-coding RNAs.

In the nuclei of all eukaryotic cells, genomic DNA is tightly wrapped around core histones (H2A, H2B, H3 and H4) into a compacted structure called chromatin. Core histones have flexible tails of 25–40 amino acids, marked with a vast variety of post-translational modifications, such as methylation of lysines and arginines, phosphorylation of serines and threonines, and ubiquitylation and sumoylation of lysines. These modifications have been proposed to represent a combinatorial "histone code", that specifies the function of the affected genomic regions in terms of chromosome segregation, cell cycle progression, DNA replication/repair and transcriptional activity.[3,4] Thus, some of these modifications appear to play important roles in dividing the genome into transcriptionally active/relaxed and inactive/compacted regions. In addition to regulating chromatin compaction, modified nucleosomes provide binding sites for regulatory proteins that, in turn, might stabilize the chromatin signature and provide a platform to recruit additional factors.[5]

Methylation of the histone tails occurs at arginine and lysine residues and it is regulated by histone methyltransferases (HMTs) and histone demethylases (HDMs). Methylation on lysine 27 of histone 3 (H3K27) is considered a repressive marker and it is established by the EZH2-containing Polycomb Repressive Complex 2 (PRC),[6] the mes complex in *C. elegans,* and removed by the members of the KDM6 family, UTX (ubiquitously-transcribed TPR protein on the X chromosome) and JMJD3.[7-9] Another protein, UTY, located on the Y chromosome, is part of the KDM6 family and shares high homology with UTX, but it seems unable to remove the H3K27me3 mark, at least in vitro.[8,9] In *C. elegans* there are 3 homologs of JMJD3 *(jmjd-3.1–3)* and a single homolog of UTX/UTY, called *utx-1*.[1,7] UTX-1 protein contains four tetratricopeptide repeats (TPR) in its N-terminal region, which are predicted to be protein

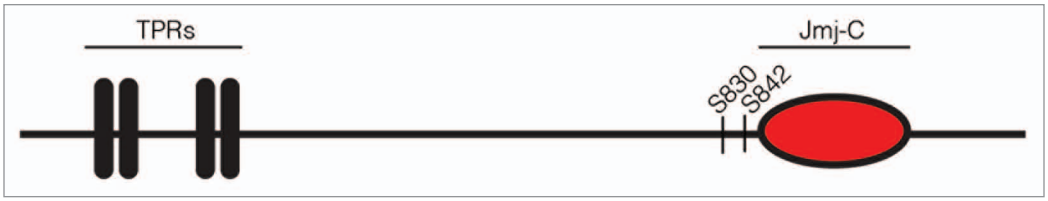

Figure 1. Schematic representation of UTX-1 protein. The tetratricopeptide repeats (TPRs) and the Jmj-C domain are in black and red, respectively. Phosphorylated serines are also indicated.

interaction motifs,[10] and a carboxyl-terminal Jmj-C domain, identified in proteins with demethylase activity (**Fig. 1**).[11] By RNA interference approaches, *utx-1* has been observed to be involved in vulva formation[12] and in longevity.[13,14]

Developmental Roles of UTX-1

The biological roles of UTX-1 during development were analyzed using *utx-1* null mutant alleles.[1] The phenotypes associated with UTX-1 loss are pleiotropic, suggesting that *utx-1* is an essential gene involved in several aspects of development. Homozygous *utx-1* animals with maternal contribution develop until adulthood and show somatic gonadal defects and sterility. Few eggs are produced with the majority failing to hatch. The escapers, lacking both maternal and zygotic contribution, develop as deformed animals and die at L1 stage, with prominent defects in body morphology. As its mammalian counterpart, UTX-1 is an H3K27me3 demethylase, and its loss results in a global increase of H3K27 trimethylation, indicating that UTX-1 actively removes a large portion of H3K27me3 in the animal.

One of the major findings in our manuscript is that the catalytic activity of UTX-1 is not required for the described phenotypes. Indeed, reintroduction of a catalytically dead UTX-1 (UTX-1DD) was able, similar to the wild-type form, to rescue the sterility, the embryonic lethality and the morphological defects of the null mutants. This implies that UTX-1 must have other functions beside its demethylase activity. A catalytic-independent function of the members of the KDM6 family has been previously hypothesized,[15] but never been demonstrated in vivo. However, recent results obtained in mice suggest that *Utx* also has a catalytic-independent role in this organism.[16] While female *Utx* KOs are embryonic lethal, hemizygous males that carry *Uty* on the Y chromosome, are born, suggesting that some developmental roles of mouse UTX can be fulfilled by UTY, despite its lack of catalytic activity. Taking together with our results, this shows that UTX has retained a catalytic-independent role during evolution.

Our analysis also suggests that the members of the KDM6 family serve different roles during nematode life. While *utx-1* is a broadly expressed gene required for viability, the JMJD3 homologs (*jmjd-3.1–3*) seem to have more specific and subtle roles. This is indicated by the restricted cells/tissue localization of these proteins and by the fact that single or triple mutant animals lacking the three *jmjd-3*-like genes are viable and fertile (Vandamme et al., unpublished).[7] Genetic analyses show that the three *jmjd-3* genes do not act redundantly with *utx-1*, thus further supporting that the members of KDM6 family play distinct roles in nematode. This is also in agreement with studies in mammals demonstrating specific roles for JMJD3 in the response to environmental cues.[17-19] The fact that in the nematode, JMJD3 is represented by 3 homolog genes and may reflect the necessity of the animal to adapt to several external stimuli. Notably, we previously reported that loss of *jmjd-3.1* results in aberrant gonadal migration and misshaped germline when the mutant animals are grown at a high temperature.[7] A detailed analysis of the other JMJD-3 homologs, in particular under stress conditions, will bring information on the specific role of these proteins in several aspect of nematode biology.

UTX-1 and Chromatin Complexes

Mass spectrometry analyses indicate that UTX-1 is part of a complex (that for brevity we call UTX-1/SET-16 complex) that includes WDR-5.1–2, ASH-2, F21H12.1, SET-16 and PIS-1 (**Fig. 2**). A similar complex has also been identified in mammalian cell.[20-22] While WDR-5, ASH-2 and F21H12.1 (the homolog of RBBP5) are in common with other complexes,[23-25] SET-16, the nematode homolog of MLL3/4 with H3K4 methyltransferase activity, and PIS-1, the homolog of PTIP, are specific components. The findings that the loss or reduction of any of the components of the complex gives rise to the phenotypes identified in the *utx-1* null mutant, together with the analyses of the genetic interactions between the members of the complex, indicates that the UTX-1/SET-16 complex plays a relevant role during development and that UTX-1 acts through the complex. By mass spectrometry, we also identified UTX-1 in association with other chromatin factors, such as HDA-1, LIN-53 and MIX-1, key components of the NuRD complex[26] and the Dosage Compensation Complex.[27] This finding suggests that UTX-1 is present in several chromatin-associated machineries and supports the notion that chromatin factors are components of large complexes, containing several enzymatic functions. Strikingly, histone demethylases are often found in association with histone methyltransferases[7,21,22,28-31] suggesting that the coordinated removal of histone modifications by HDMs and the addition of other modifications by HMTs may be required for the timely establishment of a chromatin environment required for a precise transcriptional output. Future studies will be directed toward the identification of UTX-1-containing complexes in specific tissues and at specific developmental stages.

One of the major questions in cell type specifications during development is how extracellular signals control the

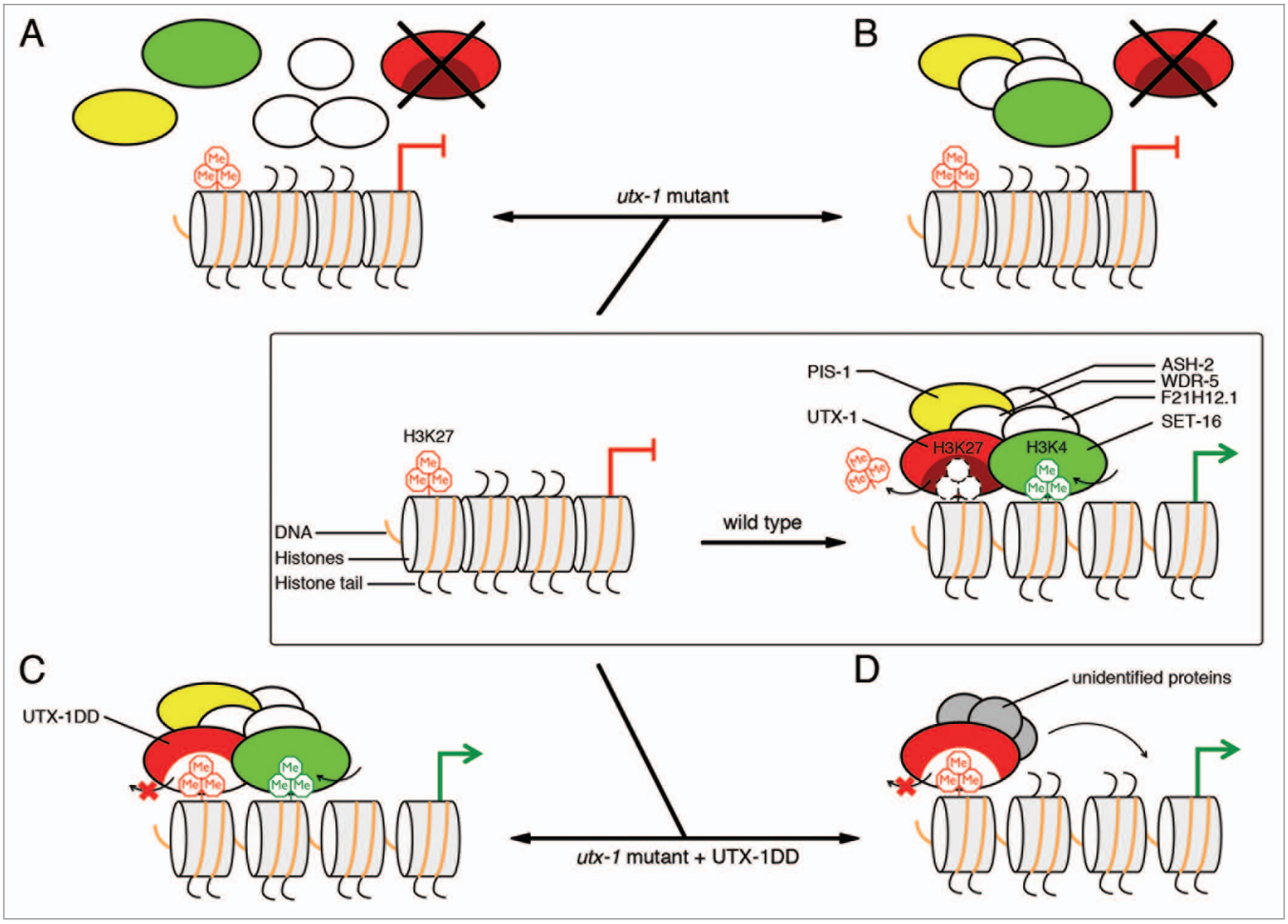

Figure 2. Schematic representation of possible UTX-1 functions. In wild-type conditions (central box), the UTX-1/SET-16 complex is recruited at the promoter of developmental genes. The combined action of UTX-1 (in red) and SET-16 (in green) results in loss of H3K27me3 and acquisition of H3K-4me3, leading to chromatin relaxation and proper transcription activation (green arrow). In *utx-1* null mutant (upper part of the drawing), the complex may be not assembled (A) or not properly targeted to the promoter regions (B). In both cases the transcription remains repressed (red line), leading to *utx-1* phenotypes. In the lower part of the drawing, possible functions of the catalytic inactive UTX-1 (UTX-1DD, indicated by the white portion in UTX-1 molecule) are represented. UTX-1DD is recruited at the complex where it may hide the H3K27me3 site (C), or recruit other chromatin-remodeling factors (in gray) (D). In both cases, the transcription is activated, even in the presence of H3K27me3, leading to correct development. Core components (WDR-5, ASH-2 and F21H12.1/RBBP5) of the complex are shown in white. PIS-1 is represented in yellow.

transcriptional programs and therefore the activity of epigenetic enzymes. Since posttranslational modifications are often required for correct protein localization, stability and activity, we took advantage of our mass spectrometry analysis to identify modifications associated to UTX-1. Interestingly, we found that UTX-1 is phosphorylated on serines 830 and 842, just upstream of the catalytic JmjC domain. While, we so far have not been able to detect any role of the phosphorylation sites during *C. elegans* development (Vandamme J et al., unpublished), their presence suggests that UTX-1 is subject to regulation by kinases and, possibly, phosphatases. Further studies in this direction may provide not only information on UTX-1 modulation, but also provides hints concerning how the epigenetic machineries integrate into signaling networks.

Mechanisms of UTX-1 Action

The evidences that UTX-1, a demethylase removing the repressive mark H3K27me3, associates with SET-16, a methyltransferase that deposits the activating mark H3K4me3, suggest that the UTX-1/SET-16 complex promotes transcriptional activation by modulating the chromatin environment (**Fig. 2**). However, the ability of the inactive UTX-1 to restore wild-type phenotypes strongly suggests that the catalytic function of UTX-1 is not absolutely required, at least during development, for the functionality of the complex. Here, taking in account our results, we briefly summarize possible models of UTX-1 action.

The fact that specific members of the complex are downregulated in *utx-1* mutants suggests that UTX-1 may be important to establish the complex by regulating the level of expression/availability of the components (**Fig. 2A**). Furthermore, the presence of UTX-1 may stabilize the complex by protein-protein interactions, mediated by the tetratricopeptide repeats, located at its N-terminal

region. Unfortunately, deletions of these regions destabilize or mislocalize UTX-1 (Vandamme, et al., unpublished), thus preventing further analysis in this direction. Point mutations in the TPRs, in vitro binding assays and reconstitution in vitro of the complex will help to identify the direct partners of UTX-1 and to clarify the dynamics of binding with the members of the complex. Alternatively, or in addition, UTX-1 may contribute to the localization of the complex in specific regions of the genome. The recruitment of UTX-1 to the genome, illustrated in **Figure 2B**, could be mediated by the H3K27me3 mark, recognized by UTX-1, or by unknown DNA-binding proteins and transcription factors (not shown). To test this hypothesis, chromatin immunoprecipitation should be performed in a *utx-1* null background, an experiment that is at the moment unfeasible due to the lethality of the mutants. Another possible explanation may rely on the ability of UTX-1 to counteract the recruitment of the PRC2/MES complex[32] responsible for H3K27me3 deposition, thus creating an unbalanced ratio of H3K27me3/H3K4me3 marks in favor of a higher level of H3K4me3, leading to transcriptional activation. However, no synthetic interactions with the components of the MES complex were identified, indicating that this scenario may not occur in the nematode.

Our results showing that the enzymatically inactive UTX-1 is able to rescue the developmental defects associated with the loss of *utx-1,* provide an additional framework to elaborate further on the possible mechanisms of function of UTX-1.

Mutagenesis analyses of the Jmj-C domain indicate that it has a critical role in the folding or localization of the protein. Indeed, deletion of 20 amino acids in the Jmj-C domain of UTX-1 mislocalizes UTX-1 to the cytoplasm (Vandamme, et al., unpublished). Nevertheless, the catalytically inactive mutant of UTX-1, that carries only two amino acid substitutions sufficient to impair the binding to iron ions required for the enzymatic activity, is nuclear and found in a large complex that includes WDR-5, thus suggesting that the catalytic activity is not required for the complex formation.

What are the mechanisms underlining the ability of UTX-1DD to fulfil the developmental functions of the wild-type protein?

A recent article suggests demethylase-independent roles of members of the JMJD6 family via their interaction with Brg1-containing Swi/Snf complexes and T-box proteins.[15] Despite the presence of several T-box genes in *C. elegans* and homologs of the Swi/Snf complex components, the mass spectrometry analysis failed to recover such proteins as UTX-1 partners, suggesting that this mechanism may be not relevant in *C. elegans*. Interestingly, it has been reported that phosphorylation of H3S28, in concomitance with H3K27me3, results in transcriptional activation due to the "hiding" of the repressive mark.[33] In light of our results, and considering that the point mutations in UTX-1DD do not impair the recognition of the substrate,[34] it is then conceivable that UTX-1DD, while failing to remove the H3K27me3, may be able to bind and hide this mark just as SET-16 is methylating H3K4, resulting in activation of gene transcription and rescue of *utx-1* phenotypes. This model is shown in **Figure 2C**. Alternatively, UTX-1DD may be able to recruit, alone or in the context of the complex, other unknown chromatin-remodeling factors that allow transcriptional activation, even in presence of H3K27me3 (represented in **Figure 2D**). To test this possibility, specific binding partners of UTX-1DD should be identified by mass spectrometry analysis.

Concluding Remarks

In general, our study provides indications that increased global levels of H3K27me3 are not deleterious. Indeed, *utx-1* animals rescued with UTX-1DD and animals lacking the 3 JMJD-3 homologs show a high level of global H3K27me3 and are viable and fertile. Our results, moreover, indicate that UTX-1 possesses functions beyond its catalytic activity. Similar findings are emerging also for other chromatin factors and in other model systems.[15,18,35-39] These outcomes should increase the caution of the researchers while establishing the roles of chromatin factors and emphasizing the need for specific experiments to directly assess the relevance of the enzymatic activities of the studied molecules. While not relevant for development, UTX-1 enzymatic activity may be important for other functions during nematode life. Indeed, recently, the catalytic activity of UTX-1 has been shown to modulate longevity.[13,14] Further analyses testing response to stresses or cell reprogramming, together with identification of target genes, may reveal other catalytic-dependent roles of UTX-1.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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