



MASTERWORK

Characterisation of KDM4A/JMJD2A as a histone
demethylase in *D.melanogaster*

Master of Science (MSc)

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Vienna, April 8th 2010

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I would love to thank Prof. Dr. Azorin for giving me the opportunity to be part of his team and prof. Schroeder for being my supervisor from Vienna allowing me to live this experience, to Olivera for her supervising and friendship, to Marta for her help and teaching, and the rest of the team for making a nice atmosphere and for being always available, pleasant and helpful.

INTRODUCTION

1. Chromatin and histone core post-translational modifications

In eukaryotic cells, DNA is a part of a nuclear structure called chromatin. Chromatin was found to contain both nucleic acids and a series of acid soluble proteins that were termed “histone” by Albert Kossel (Kossel, 1911). The histones and other chromosomal proteins are responsible for the proper packaging of the DNA into the chromosomes. In addition to its role in compacting DNA, chromatin structure also has an important function in regulating accessibility to DNA and therefore transcription and silencing of genes, recombination, DNA repair, replication, kinetochore and centromere formation and many more DNA-related processes (Li et al., 2007).

A single nucleosome is composed of 146 base pairs of DNA wrapped around the histone octamer (two copies each of H2A, H2B, H3 and H4) in a left-handed super-helix, and the linker histones referred to as H1 (Kornberg and Lorch, 1999). The DNA has 14 contact points with the core histone octamer which makes the nucleosome a very stable structure (Luger and Hansen, 2005).

Histones are among the most conserved proteins known in evolution, but are also among the most variable in covalent post-translation modification (PTMs) which include acetylation, methylation, ubiquitination, ADP-ribosylation and sumoylation (Kouzarides et al., 2007). These modifications take place in the globular domains of the histones and especially on the protruding, unstructured, basic N-terminal tails of the 8 core histones (Figure 1).

PTMs on histone tails, especially methylation of lysine residues, were hypothesised to play an important role in the storage of epigenetic information (Zhang and Reinberg, 2001).

There are over 60 amino acid (aa) residues in histones on which modifications have been detected so far (Kouzarides, 2007) and acetylation and methylation appear to be the most common. These modifications differ in two ways: histone acetylation results in a negative charge of the modified lysine residue, causing a decreased interaction between the histone and DNA that is generally associated with less condensed and transcriptionally active regions of the genome (euchromatin). In contrast, methylation of histones occurs at both arginine and

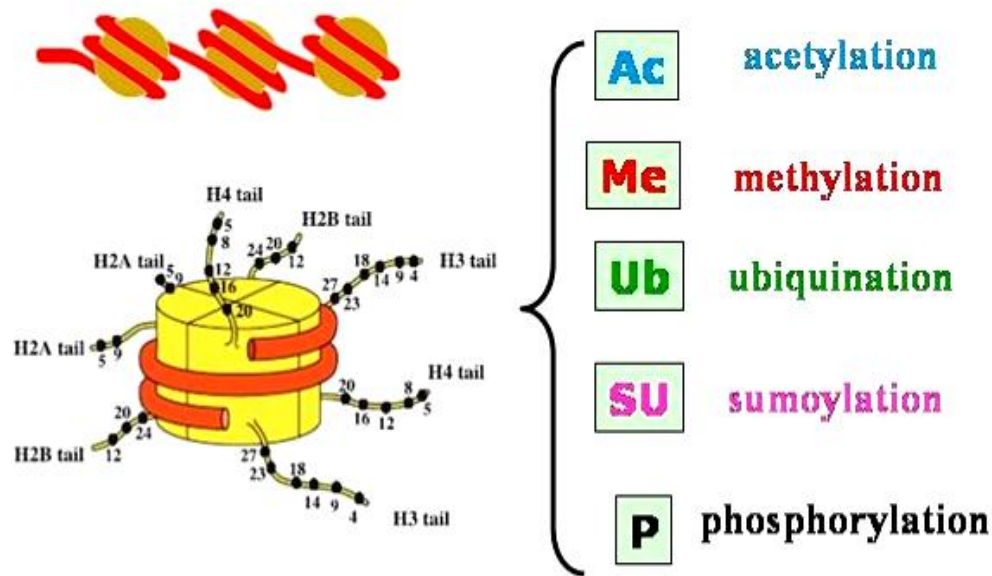


Figure 1: Core histone tails are subject to numerous post-translational modifications

lysine residues, and does not influence the net charge of the affected residues, and hence has no effect on DNA-histone interactions. Rather the effect of histone methylation impacts on the transcriptional activity of the underlying DNA by acting as a recognition template for effector proteins modifying the chromatin environment and leading to either repression or activation. Thus, histone methylation can be associated with either activation or repression of transcription depending on which effector protein is being recruited. Methylated lysine residues are specifically recognised and bound by several protein domains including the chromo, Tudor, WD40 repeat and PHD finger domains (Martin and Zhang, 2005). Therefore, proteins or protein complexes containing these domains can bind to specific chromatin domains indexed by histone lysine methylation and trigger a plethora of different effects. Depending on the particular lysine residue, the degree of methylation at the same lysine residue and the location of the methylated histone within a specific gene locus, histone methylation can induce either repression or activation (Lachner et al., 2002).

Generally, H2K4, H3K36 and H3K79 methylation are found in active regions of chromatin (Martin and Zhang, 2005; Schubeler et al., 2004), whereas methylation of H3K9, H3K27 and H4K20 is associated with silenced regions (Nielsen et al., 2001;

Reinberg et. al., 2004). Depending on its context though, the same modification can result in different effects (Kouzarides, 2007; Zhang and Reinberg, 2001).

Methylation is catalysed by histone methyltransferases (HTMTs) and affects transcriptional activation and repression as well as other fundamental processes such as X-chromosome inactivation, genomic imprinting and DNA repair (Lachner, 2002; Margueron et al., 2005; Zhang and Reinberg, 2001). Because it has been linked to a diverse set of biological processes, lysine methylation of histones has long been of particular interest (Margueron et al., 2005; Zhang and Reinberg, 2001).

Previously, methylation has been considered to constitute a permanent and irreversible histone modification that defined epigenetic programs in concert with DNA methylation. Only the discovery of lysine specific demethylases 1 (LSD1) (Shi et. al, 2004) and the family of JumonjiC (JmjC) domain containing histone demethylases (Tsukada et al., 2006) changed this dogma. Recently, however, a large number of enzymes have been discovered with the ability to demethylate methylated histone lysine residues.

2. JumonjiC (JmjC) family

The largest class of demethylase enzymes contain a JumonjiC (JmjC) domain and catalyse lysine demethylation of histones through an oxidative reaction that requires iron Fe(II) and α -ketoglutarate (α KG) as cofactors (Tsukada et al., 2006).

Unlike LSD1, which can only remove mono- and dimethyl lysine modifications, the JmjC-domain-containing histone demethylases (JHDMs) can remove all three histone lysine-methylation states. There are 27 different JmjC domain proteins within the human genome, of which 15 have been published to demethylate specific lysines or arginines in the H3 tail. Categorization based on JmjC-domain homology and protein domain architecture resulted in seven distinct JmjC-protein subfamilies (see Table 1). In general, it appears that each cluster has specificity for demethylating a certain histone mark. Currently, more than 11 000 sequence entries of JmjC domain containing proteins are present in Uniprot, PFAM Interpro and SMART, thus demonstrating the tremendous expansion of members in this protein superfamily (Hahn and Boese, 2008).

Enzymatic family	Subfamily	Enzyme(s)	Specific activity
PADI		PADI4	H3R2, R8, R17, R26 H4R3
Amine oxidase		LSD1	H3K4me2, me1
JmjC	JHDM1	JHDM1A, JHDM1B	H3K36me2, me1
	PHF2/PHF8	PHF2, PHF8	Unknown
	JARID	JARID1A/RBP2 JARID1B/PLU-1 JARID1C/ SMCX1 JARID1D/SMCY	H3K4me3, me2
	JHDM3/JMJD2	JMJD2A JMJD2B JMJD2C/GASC1 JMJD2D	H3K9me3/2, H3K36me2/3 H3K36me3, me2
	UTX/UTY	JMJD3 UTX	H3K27me3, me2
	JHDM2	JHDM2A JHDM2B JHDM2C	H3K9me3, me2
	JmjC only	MINA53 JMJD4 JMJD5	Unknown

Table 1: Enzymes that demethylate histones, subsequent subfamilies and specific substrates. *D. melanogaster* JMJD2A enzyme studied in this work is marked in red.

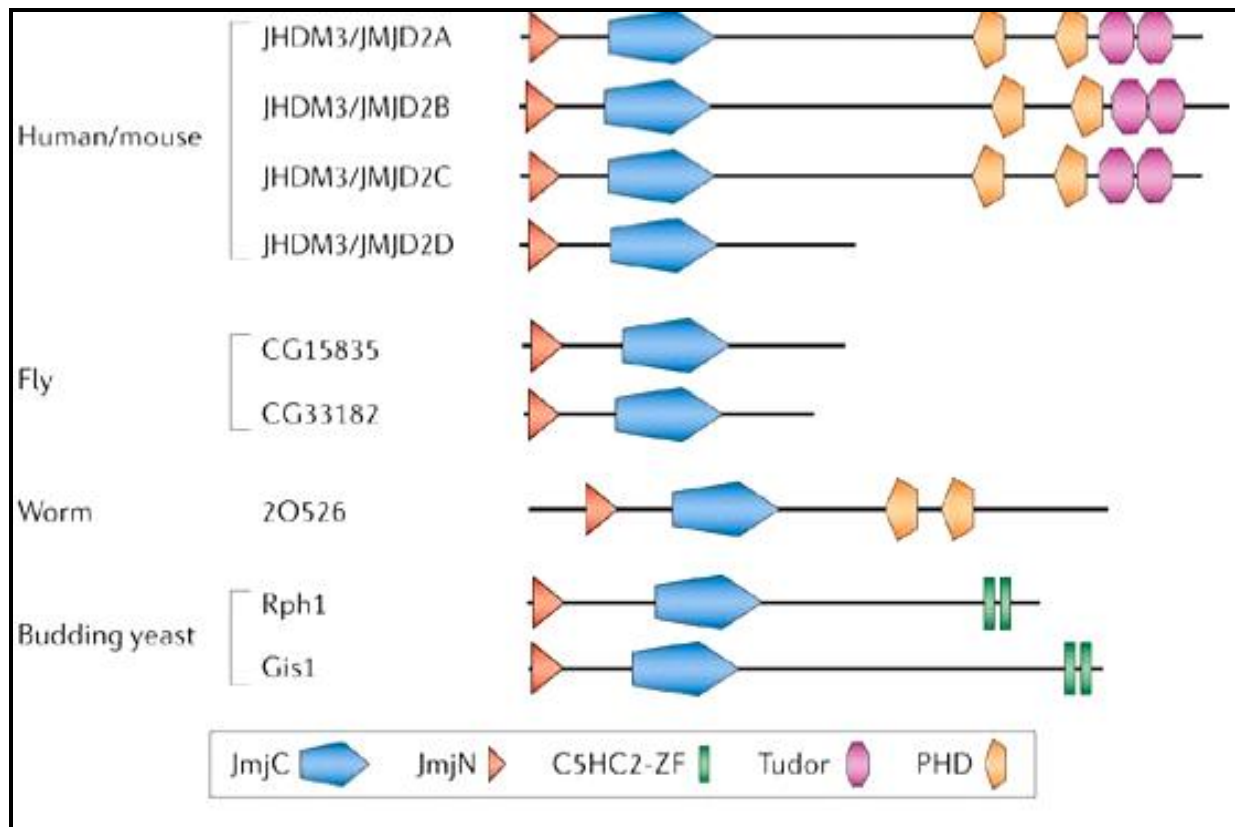


Figure 2: Schematic representation of the JMJD2 family of histone lysine demethylases. (Adopted from Klose et al., 2006)

3. JMJD2A

The first JmjC domain demethylase described was JMJD2A (also named: JHDM1, KDM4, CG15835, lysine (K)-specific demethylase 4A, Jumonji domain-containing protein 2A, JMJD2(1)), which was shown to specifically demethylate mono- and dimethylated H3K36 (Tsukada et al., 2006).

The JMJD2 cluster consists of four genes, JMJD2(A-D) in the human genome, each of which has orthologues from yeast to humans. In higher eukaryotes, proteins of this family contain JmjN, PHD and Tudor domains in addition to the JmjC domain (Fig. 2).

JMJD2A was purified and characterized by Yamane et al. in 2006. They demonstrated that the enzymatic activity of JMJD2A depends on an intact JmjC domain and requires cofactors Fe(II) and α -ketoglutarate (α -KG). JMJD2A is capable of demethylating mono and dimethyl-H3K9 in vitro and in vivo, but it fails to demethylate trimethyl-H3K9.

Lloret- Llinares et al. showed that they are capable of demethylating H3K9me3 and H3K36me3 when over-expressed in *D. melanogaster*.

Structural analysis of JMJD2A has shown that three distinct domains, in addition to the JmjC domain, are necessary for catalytic activity (Chen et al., 2006).

JMJD2A orthologues are found from yeast to human and all group members contain a JmjC (Jumonji C) and JmjN (Jumonji N) domain. Members of this group contain additional C-terminal domains, which are probably involved in protein targeting (PHD, plant homeobox domain) (Fig. 2). Tudor domains found in JMJD2A is the only functionally-characterized member of the mammalian JMJD2 family (Huang et al., 2006). Due to the homology of mammalian JMJD2A histone lysine demethylases and JMJD2A/CG15835 histone lysine demethylases in fly (Fig. 2) we investigated if there is a demethylation activity of mammalian JMJD2A homologue targeting H1K27me2 also in *Drosophila*.

3. Histone H1

As it was already mentioned, the histone octamer moiety of the core particle consists of two copies of each of the four histones H2A, H2b, H3 and H4. The fifth histone, termed H1 or linker histone, very lysine-rich protein, interacts with DNA entering and exiting the nucleosomal core particle. (Noll et al., 1977, Allan et al., 1980) (Fig. 3).

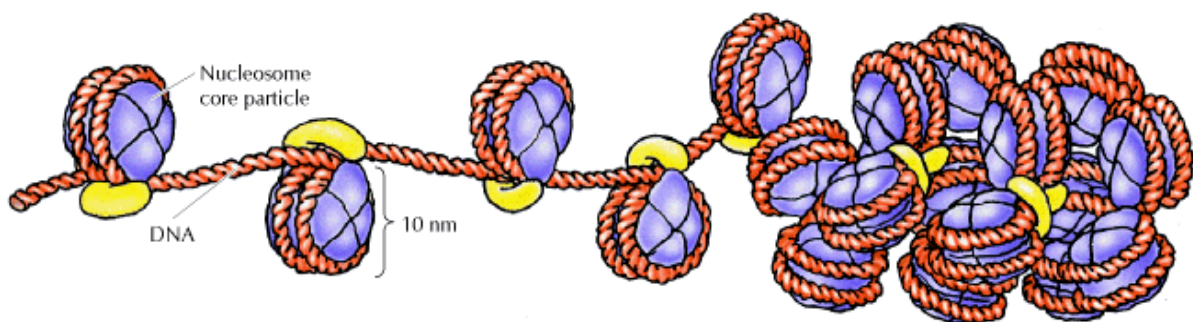


Figure 3. Molecular assembly of nucleosomes (picture taken from http://148.216.10.83/CELULA/4,2_chromosomes_and_chromatin.htm). The DNA (red) is wrapped around the histone octamer (blue) and both form the nucleosome core particle. This structure is locked in mammals by the linker histone H1 (yellow). The chromatin fiber is further folded into a thicker fiber, the so-called solenoid that is 30 nm in diameter.

The binding of the nucleosomal array promotes and stabilises the folding of the nucleosomal array into the 30nm fibre (Bednar et al., 1998) and prevents the unpeeling of DNA from the histone octamer. H1 can stabilise the position of nucleosomes by suppressing spontaneous transitions in the nucleosomal structure and ATP dependent chromatin remodeling (Ramachandran et al., 2003). Histone linker H1 has roles in many different processes in different organisms, such as gene expression regulation in mammals, position effect variegation in mouse, etc. (Fig. 4).

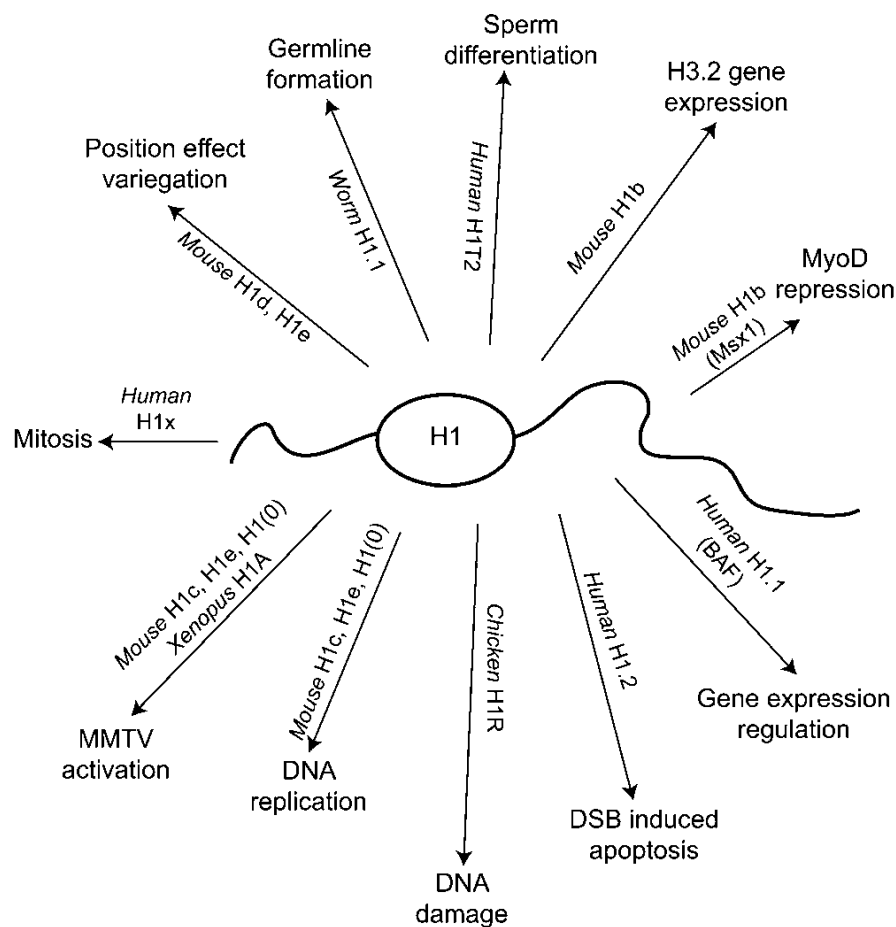


Figure 4. Schematic representation of roles of histone H1

The H1 family of linker histones is the most divergent class of histone proteins. Mammals express at least eleven H1 subtypes that can be grouped according to their temporal and spatial expression. The mammalian H1 variants are paralogs, i.e. their genes originated from gene duplication events. The expression of H1 varies between different cell types, with H1.2 and H1.4 being the predominant variants in most

human cells (Meergans et al., 1997). The existence of these variants in mouse or humans adds an additional level of complexity when studying H1 function.

To date, many knockouts of H1 in different organisms were done. The complete knockout done in unicellular organisms (*Tetrahymena thermophila* (Ramon et al., 2000), *Saccharomyces cerevisiae* (Patterson et al., 1998) and *Ascomyces nidulans* (Ramon et al., 2000) did not affect growth or viability. Although H1 is not essential for survival in unicellular organisms, distinct phenotypes were observed (Shen et al., 1996, Hellauer et al., 2001, Barra et al., 2000).

Inactivation of individual H1 variants in higher organisms was done on *Ceanorhabditis elegans* (Jedrusik et al., 2001), *Nicotiana tabacum* (Prymakowska-Bosak et al., 1999), *Xenopus tropicalis* (Crane-Robinson 1999; Bouvet et al., 1994; Kandolf, 1994; Steinbach et al., 1997) and mice (Fan et al., 2003).

Linker histones are essential for embryonic development in mice. The existence of multiple, non-allelic mouse H1 variant genes, it impeded to study the effects decreasing H1 expression in this species (Fan et al., 2005). To study the role of H1 *in vivo*, mouse embryonic stem cells depleted for three H1 isoforms were derived and were found to have 50% of the normal level of H1. H1 depletion caused dramatic chromatin structure changes, including decreased global nucleosome spacing, reduced local chromatin compaction and decreases in certain core histone modifications. (Fan et al., 2005).

Recently H1 mutant in *Drosophila* was made and it was seen that H1 linker histone is 1) an essential protein in *Drosophila*, 2) a major determinant of heterochromatin formation and function and 3) an important biochemical component of the machinery that maintains sister chromatid alignment in *Drosophila* polytene chromosomes (Xingwu Lu et al., 2009). In addition, ISWI chromatin-remodelling factors regulates higher-order chromatin structure by promoting the association of H1 with chromatin (Siriaco et al. 2009).

4. Posttranslational modifications of histone H1

H1 histones are, just as the core histones, targets of several posttranslational modifications (PTMs), including phosphorylation, lysine methylation and ADP-ribosylation.

Reversible phosphorylation of H1 histones has been discovered a long time ago (Balhorn et al., 1972) and is the most intensively studied modification of H1 histones (Villar-Garea and Imhof, 2008).

Most of the acetylated lysines identified in the globular domain of H1.4 have been considered to be directly involved in DNA binding (Goytisolo et al., 1996; Wisniewski et al., 2007). Deacetylation of the highly conserved lysine- 26 (K26) in H1 could be linked to the formation of facultative heterochromatin.

The first evidence for H1 lysine methylation was obtained from the analysis of the protozoan *Euglena gracilis* (Tuck et al., 1985). Later, the first mammalian H1 methylation site was identified on isoform 4 (also known as isotype H1b in human and H1e in mouse) at lysine 26 (H1.4K26) (Ohe et al., 1986).

H1.4 confers transcriptional repression and Lysine 26 methylation was found to be important in this context (Kuzmichev et al., 2004)

These are some of many evidences that also H1 histones contribute to the, so called, *Histone code*, combinatorial nature of histone amino- terminal modifications that many considerably extend the information potential of the genetic (DNA) code (Jenuwein and Allis, 2001), and just as in the case of core histones further studies are necessary to decipher this code and to identify the proteins that interact with the modified sites and thus are “reading” this code.

Recently, Trojer and colleagues discovered and showed that members of the JMJD2/KDM4 subfamily of jumonji-C type histone demethylases reduce mammalian trimethylated H1.4K26 to di- and monomethylated states. They showed for the first time that H1 can be demethylated in mammals (Trojer et al., 2009).

However, to date there is no report describing demethylation of histone H1 in *D. melanogaster*.

5. Objectives

In my project we investigated posttranslational modification (potential demethylation of lysine at position 27) of linker histone H1 and we used *Drosophila melanogaster* as a model organism.

Mass spectrometry mapping done in Ferran Azorin's laboratory revealed different posttranslational modification of Histone H1, such as Serine 10 phosphorylation, Serine 3 and/or 6 phosphorylation, N-terminal acetylation, ubiquitination, and the most important one for my work, dimethylation at Lysine 27 position (Figure 5. Taken from Olivera Vujatovic).

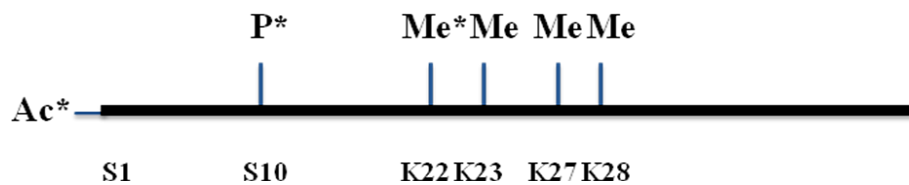


Figure 5. Schematic representation of posttranslational modifications of N terminus in histone H1 found by mass spectrometry in prof. Azorin's laboratory

Troyer and colleagues in 2009 identified members of the JMJD2A subfamily of JumonjiC histone demethylases as being responsible for the removal of H1.4K26methylation mark in mammals.

Taking in consideration homology between mammalian histone H1.4 sequence and histone H1 in fly (Fig. 6), and a homology of mammalian JMJD2A histone lysine demethylases and JMJD2A/CG15835 histone lysine demethylases in fly (Fig. 2), we wanted to investigate if there is a demethylation activity of mammalian JMJD2A homologue targeting H1K27me2 also in *Drosophila*.

To date there was no evidence of demethylation of histone H1 in *D. melanogaster*.

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H1.4      MSETAPAAPAA-----PAPAEKTPVKKKARKSAGAAKRKASG----PPVSELI TKAVAAS
His1_CG31617 MSDSAVATSASPVAAPPATVEKKVVQKKASGSAGTKAKKASATPSHPPTQQMVDASIKNL
          **: : * * : * : * : * : * : * : * : * : * : * : * : * : * : * :
          * : * : * : * : * : * : * : * : * : * : * : * : * : * : * :

H1.4      KERSGVSLAALKKALAAAG-YDVEKNNSRIKLGKSLVSKGTLVQTKGTGASGSFKLN--
His1_CG31617 KERGGSSLLAIKKYITATYKCD AQLAPFIKKY LKSAVVNGKLIQTGKGASGSFKLSAS
          *** * * * * : * : * : * : * : * : * : * : * : * : * : * : * :
          * : * : * : * : * : * : * : * : * : * : * : * : * : * : * :

H1.4      -KKAASGEAKPK-----AKKAGAAKAKKPAGAAKPKKATGAATPKKSA--
His1_CG31617 AKKEKDPKAKSKVLSAEKKVQSKKVASKKIGVSSKKTAVGAADKKPKAKKAVATKKTAEN
          ** . : * : * : * : * : * : * : * : * : * : * : * : * : * :
          * : * : * : * : * : * : * : * : * : * : * : * : * : * : * :

H1.4      KKT PPKAKKPAAAAG---AKKAKSPKKAKAAKPK---KAPKSPAKAKAVKPKAAKPKTA
His1_CG31617 KKTEKAKAKDAKKTGI I KSKPAATKAKVTAAKPKAVVAKASKAKPAVSAPKPKTVKKASV
          *** * * * * : * : * : * : * : * : * : * : * : * : * : * : * :
          * : * : * : * : * : * : * : * : * : * : * : * : * : * : * :

H1.4      KPKAAPK-KAAAKKK
His1_CG31617 SATAKKPKAKTTAAKK
          . . . * * * * : : * * *

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Figure 6: Here is shown alignment of the protein sequences of human H1.1 and histone H1 (accession no. His1_CG31617) of *D. melanogaster*. Sequences were allined using multiple sequence allinement program T-coffee Version_5.05 [http://www.tcoffee.org]. The two senquences are homologs. Symbols below the letters are denoting the degree of conservation observed in each column: " * " means that the residues or nucleotides in that column are identical in all sequeenes in the alignment. " : " means that conserved substitutions have been observed. "." means that semi-conserved substitutions are observed.

MATERIAL AND METHODS

Immunofluorescence in polytene chromosomes

Salivary glands of third-instar larvae were dissected in Cohens buffer (10mM MgCl₂, 25mM sodium glycerol 3P, 3mM CaCl₂, 10mM KH₂PO₂, 0.5% NP-40, 30mM KCl, 160mM sacarosa). They were further incubated with fix solution 1 (PBS supplemented with 18.5% formaldehyde) for 2min and fix solution 2 (50% acetic acid, 18.5% formaldehyde) for 3 min.

The salivary glands were then transferred onto cover glasses and mounted onto microscope slides. They were squashed as described (Paro, 2000). Before hybridisation with antibodies the microscope slides were washed three times 5min in PBS supplemented with 0.05% Tween®-20.

Primary antibodyds were diluted in PBS supplemented with Tween®-20 PBS (1:30 for α H1K27 and to 1:1000 for). A drop of 15µl primary antibody solution was placed

onto each microscope slides and covered with a cover glass. The slides were then left to incubate in a humid box for 1hr at RT and over night at 4°C.

The microscope slides were washed three times 5min in PBS supplemented with 0.05% Tween®-20.

The secondary Cy3-conjugated antibody (Jackson Laboratories) was diluted 1:400 in PBS supplemented with 0.05% Tween®-20. A drop of 15ul secondary antibody solution was placed onto each microscope slides and covered with a cover glass. The slides were then left to incubate in a humid box for 1.5hrs at RT without exposure to light.

Again, the microscope slides were washed three times 5min in PBS supplemented with 0.05% Tween®-20.

Cover slips were the mounted using 20µl Mowiol (Calbiochem-Novabiochem) containing 10% DAPI (2ng/µl) and the pictures were taken using a Leica SPE confocal microscope and formatted using GIMP software.

Immunofluorescence in impacted cells

Cells were detached from plates by pipetting and mixed with hypotonic medium MAC (50mM glycerol, 5mM KCl, 10mM NaCl, 0.8mM CaCl₂, 10mM sucrose) in a dilution of 2:5. They were incubated for 5min in order to inflate before adding 200µl to each cytocentrifuge tube. The cells were impacted onto microscope slide by centrifugation for at 500rpm for 10min at RT (ThermoShandon Cytospin 4). The slides were then dried 30-60min at RT before fixation of the cells with 4% paraformaldehyde for 10min at RT. After a single wash with PBS for 15min at RT the slides were dried and stored at 4°C. Before hybridisation with antibodies the slides were permeabilised two times 10min in PBS supplemented with 0.1% TritonX-100 and blocked 2 times with PBS supplemented with 0.1% TritonX-100 and 0.1% BSA.

The primary antibody was diluted (1:250 to 1:1000) in PBS supplemented with 0.1% TritonX-100 and 1% BSA. After drying the slides for 5-10min at RT, 10µl of primary antibody solution were added onto each slide. A cover glass was mounted and the slides were incubated in a wet chamber for 1hr at RT and over night at 4°C. After

incubation the slides were washed again two times 10min with PBS supplemented with 0.1% TritonX-100 and 1% BSA.

The secondary Cy3-conjugated antibody was diluted 1:400 in PBS supplemented with 0.1% TritonX-100 and 1% BSA. After drying the slides for 5-10min at RT, 10µl of primary antibody solution was added onto each slide. A cover glass was mounted and the slides were incubated in a wet chamber for 45min without exposure to light.

The slides were washed as described above with the addition of two final 10min washes in PBS only. They were left to dry for 10min at RT before adding mounting with 15µl Mowiol (Calbiochem-Novabiochem) containing 10% DAPI (2ng/µl).

The pictures were taken using a Leica SPE confocal microscope (High-Throughput image acquisition with 60x water objective and analysed using self-made ImageJ macro software.

Immunofluorescence in attached cells

Cells were diluted to a concentration of 10^6 cells/ml, seeded on concavalina coated cover glasses and left at 25°C for few hours (1-2h). The cells were washed 10min with PBS and fixed in 4% paraformaldehyde for 12 min at RT. After two washes with PBS and two further washes with PBS supplemented with 0.1% TritonX-100 and 0.1%BSA 200µl primary antibody solution (1:250 – 1:1500) was added to each well. The cells were incubated with gentle agitation at 4°C over night. The cells were washed two times with PBS supplemented with 0.1% TritonX-100 and 0.1%BSA before adding 200ul secondary antibody solution (1:400) to each well. They were incubated with gentle agitation and without exposure to light for 45min at RT. After the incubation with antibodies the cells were washed two further times with PBS supplemented with 0.1% TritonX-100 and 0.1%BSA and two times with PBS only. They cover slips were the mounted onto microscope slides using 4µl Mowiol containing 10% DAPI (2ng/µl).

The microscope slides were stored at 4°C before visualization using Leica SPE confocal microscope (High-Throughput image acquisition with 60x water objective) and analysed using self-made ImageJ macro software.

Antibodies

Antibodies against recombinant JMJD2A were raised in rats and antibodies against H1K27 and H1K27me2 were raised in rabbits. They were used in dilutions ranging from 1:250 to 1:1000. The α -unmodified antibody recognizes total H1 (predominantly H1K27 and also H1K27me2), while α -H1K27me2 recognise specifically H1K27me2. Purification of K27 and characterization of antibodies was done by Olivera Vujatovic in our laboratory (Figure 7.). Secondary antibody for IF experiments was Cy3/Cy2-conjugated anti-rabbit/anti-rat (Jackson Laboratories) which was always used at a dilution of 1:400.

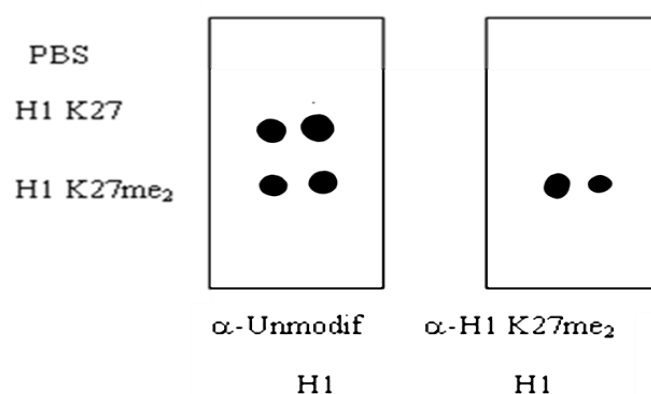


Figure 7. Characterisation of α -H1K27me2 and α -unmodified antibodies (work done by Olivera Vujatovic)

Overexpression in S2 cells

For overexpression in S2 cells, cDNAs were Flag-tagged at C-terminal, cloned into the *Drosophila* expression vector pActPPA, where expression is driven by the *actin5C* promoter, and transfected (15 μ g) into S2 cells by the calcium-phosphate method. Immunolocalization experiments were performed 48h after transfection according to standard procedures. using primary antibodies and were added at the same time than the secondary antibody against GFP. Cy3-conjugated anti-rat, Cy3-conjugated anti-rabbit and Cy2-conjugated anti-rabbit secondary antibodies were obtained from Jackson ImmunoResearch. The pictures were taken using a Leica

SPE confocal microscope (High-Throughput image acquisition with 60x water objective) and analysed using self-made ImageJ macro software.

Visualisation analysis by ImageJ macro software

As was mentioned above, the pictures of immunostained recombinant JMJD2A were taken using a Leica SPE confocal microscope (High-Throughput image acquisition with 60x water objective) and analysed using self-made ImageJ macro software. In average about 100 fields from each slide were visualized by the confocal microscope and analysed by ImageJ macro (Figure 8.). Fields showing no cells were excluded.

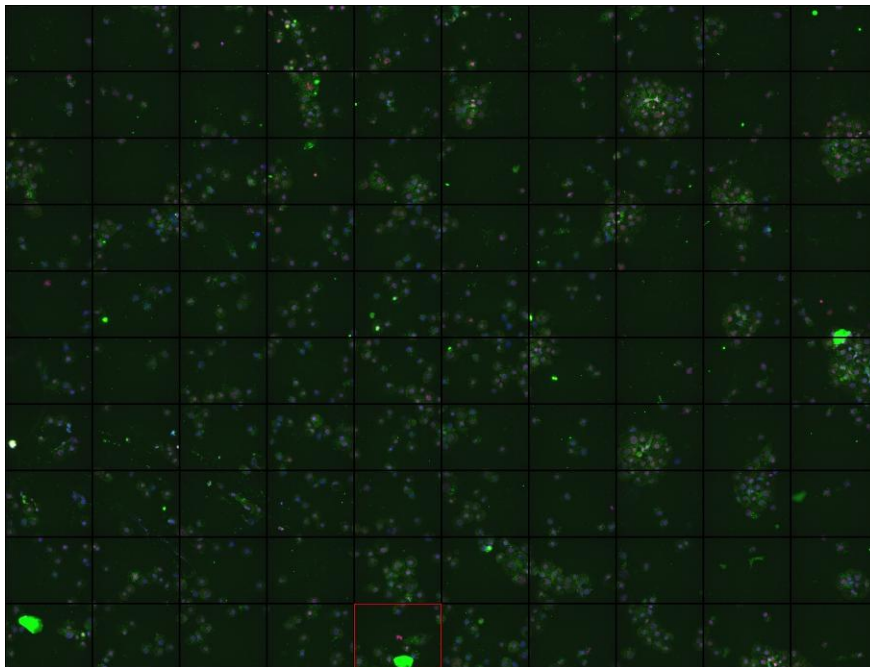


Figure 8. Immunostaining of JMJD2A-flag fusion protein overexpressed in Drosophila S2 cells. 100 fields visualized by ImageJ macro software.

The software was analysing marged images of DAPI, Cy3 and Cy2 stainings, than choosing the cells that were in good conditions (Figure 9a.) and calculating the intensity of their signal which would than be transformed in numbers and presented as “mean”. Mean1 was showing a mean grey value of Cy3 (secondary antibody used to stained JMJD2A), while mean2 was showing a mean grey value of Cy2 (used to stain H1K27me2 and H1K27.(Figure 9b).

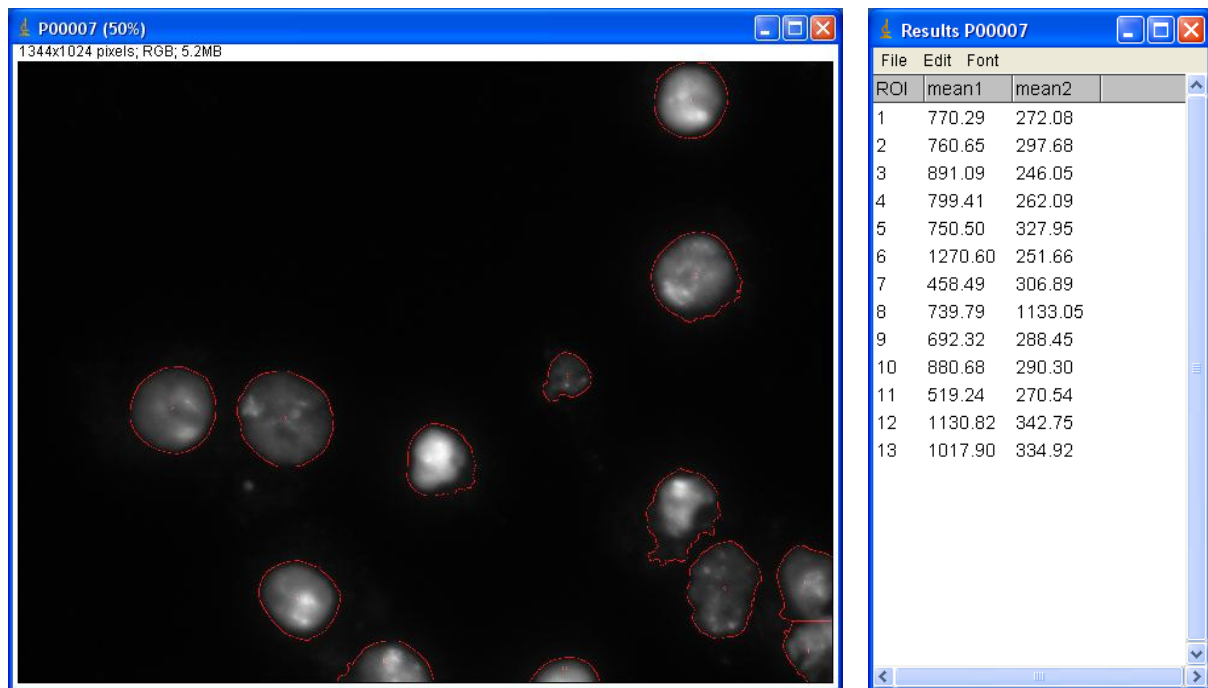


Figure 9. a) Merged image of DAPI staining (gray) and ROIs (cells in good conditions) found by Analyze particles plugin (red); b) Mean1 showing a mean grey value of Cy3 (secondary antibody used to stain JMJD2A), while mean2 was showing a mean grey value of Cy2 (used to stain H1K27me2 and H1K27).

RESULTS

In order to determine potential histone H1 demethylase activity of the *Drosophila* JMJD2A proteins, overexpression experiments in *Drosophila* cultured S2 cells were performed. We used a cell line stably harboring the JMJD2A-flag fusion on its genome: the JMJD2A-flag expression was induced by adding copper sulphate (CuSO₄). Then cells were stained with α -flag antibodies, to identify cells expressing the fusion protein, and with antibodies that recognize specifically H1K27me2 (α -H1K27me2), and those recognizing H1K27 (α -unmodified).

The cells were analysed by ImageJ macro software, as described previously in Material and methods. The intensity of each signal was represented as “mean” (Figure 9.). Since not all the immunostainings were done in the same experiment and the pictures could not have been taken with the same conditions, and ImageJ could not analyse the data with the same parameters in all the cases, it was necessary to normalize the data. A significant number of pictures was taken with Leica SPE confocal microscope (Figure 8.). Pictures containing a large number of cells were selected for ImageJ software analysis. Since ImageJ was used for the very first time, it was improved step by step until the point in which the software was capable to automatically eliminate false cells and avoid false signals not considering the overlapping cells. Intensity values of the cells for H1K27me2 and H1K27 stainings were uniform in all the experiments, specially in wild type cells. These cells were chosen randomly out of all the analyzed cells; such random choice was possible since the values detected were highly uniform from cell to cell (no significant standard deviation was observed). As mentioned previously, all the cells had a signal which was transformed by the software into a number. All the data were normalized at the first step of the analysis. In the next step it was decided which cells were transfected and which one were not. The cells with the strong signal for JMJD2A were clearly transfected while those with the weak signal were considered as not transfected.

Immunofluorescence staining revealed that overexpression of wild-type-flag- JMJD2A resulted in a decrease in the level of H1K27me2 (Fig. 10), while in control cells, which were not transfected, there was not observed any significant reduction of H1K27me2 levels.

Immunostaining of H1K27me2 in cells that are overexpressing JMJD2A revealed that 80% of transfected cells were having an decrease in H1K27me2 signal compared to the non transfected ones, (Figure 11. left chart, see appendix 1 for more informations). At contrary, the staining of H1K27 showed no decrease of the signal in transfected cells compared to non transfected cells (Figure 11. right graph, for more informations see appendix 2).

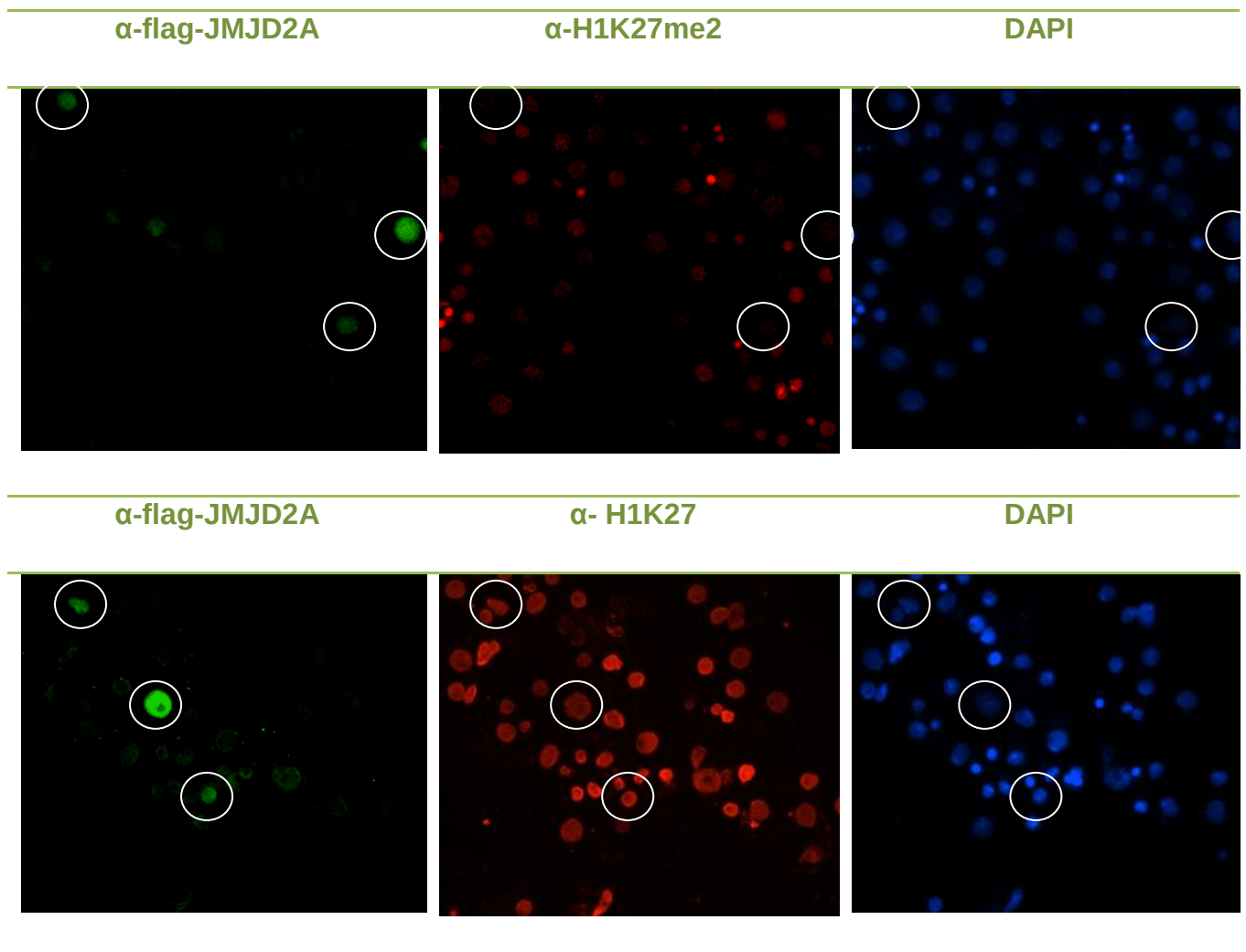


Figure 10. A JMJD2A-flag-fusion protein was overexpressed in Drosophila S2 cells and transfected cells were stained with α -flag (shown in green) and α H1K27 (shown in red). DNA was stained with DAPI. In the second row endogenous H1 was stained with α -unmodified antibody (shown in red). There is a decrease in the signal for H1K27me2 (first row), while no decrease in signal for H1K27 was observed with the antibody for H1K27 (second row). Cells labeled with the white circle are transfected.

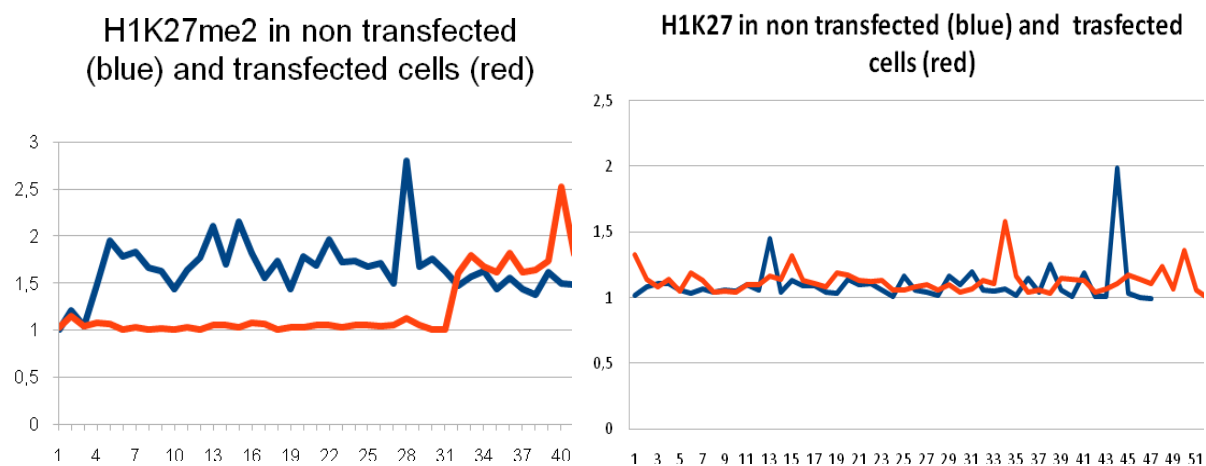


Figure 11. The charts show normalized mean values of H1K27me2 (left chart) and of H1K27 (right chart) stainings in 40 (in case of H1K27me2) and around 50 (in case of H1K27) randomly chosen cells that are overexpressing JMJD2A (blue) and about the same number of cells that were not transfected (control cells). The chart on the right shows no significant difference in mean values of H1K27 signal between transfected and non transfected cells. Y ordinate is showing normalized values of the grey signals. More informations about the data and analysis are in appendix 1 and appendix 2.

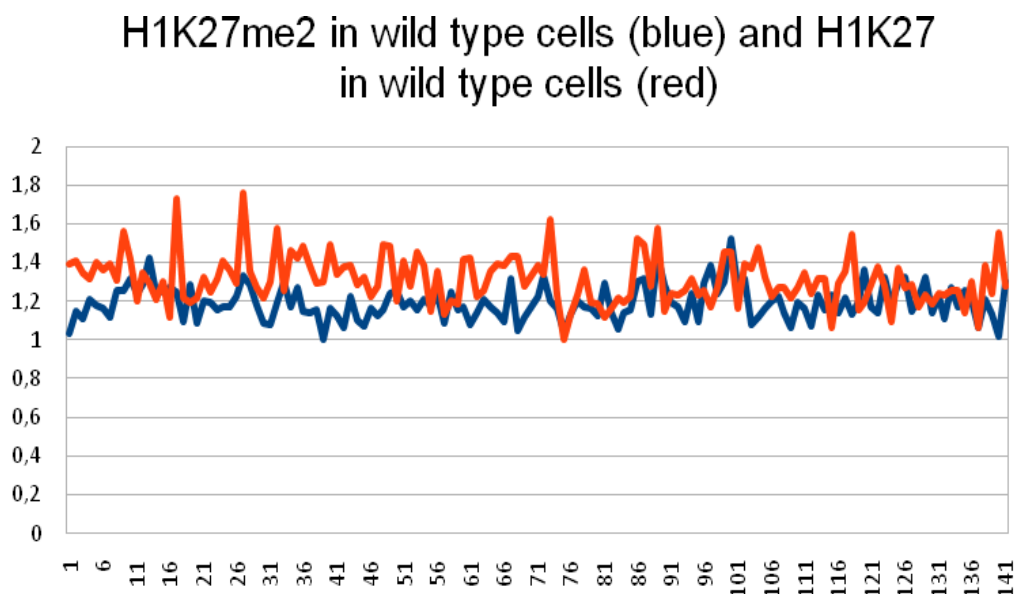


Figure 12. Immunostaining of H1K27 with unmodified antibody (red line) and of H1K27me2 with α -H1K27me2 in wild type S2 cells (1-141) (blue line) (showed is the sample of 141 cells). Y ordinate is showing normalized values of the signals. More informations about the data and analyses are in appendix 3.

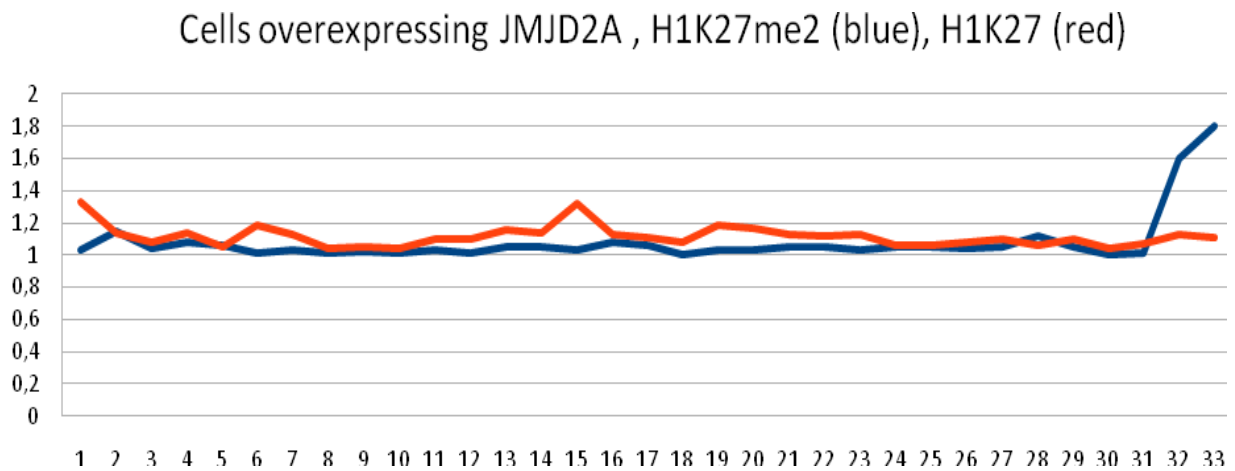


Figure 13. Transfected cells (1-33) stained with the antibody for H1K27me2 (blue line) and with the unmodified antibody for H1K27 (red line). Y ordinate is showing normalized values of the signals.

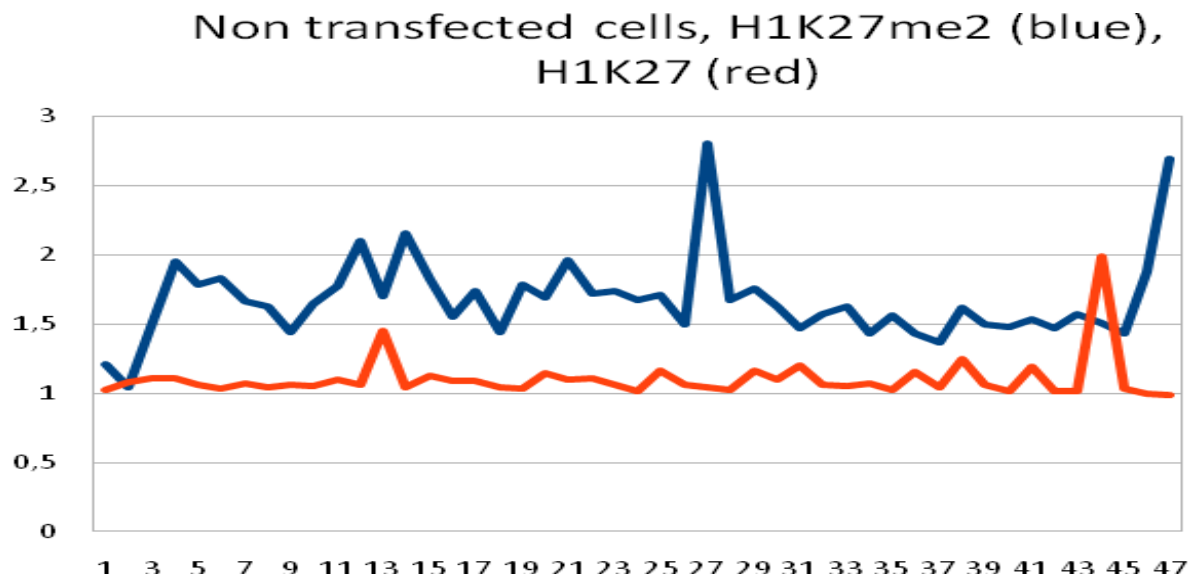


Figure 14. Non transfected cells (1-47) stained with the antibody for H1K27me2 (blue line) and with the unmodified antibody for H1K27 (red line). Y ordinate is showing normalized values of the grey signals.

Immunostaining results with both antibodies for H1K27me2 and unmodified for H1K27 in wild type cells, showed no significant difference between the two stainings (Figure 13., for more informations see appendix 3).

When the two stainings in transfected cells were compared, it was possible to observe that the staining with H1K27me2 showed a decrease in the signal compared to the transfected cells stained with H1K27, in 95% of cells (Figure 13.). On the other hand, comparing the two stainings in non transfected cells, in 98% of these cells was observed the higher signal for H1K27me2 than the signal for H1K27 (Figure 14.).

CONCLUSIONS

As mentioned previously, mass spectrometry mapping, done in Prof. Dr. Ferran Azorin' s laboratory, revealed dimethylation at Lysine 27 of histone H1, among other posttranslational modifications at the same histone.

In my project we investigated if JMJD2A proteins exhibit demethylase activity toward dimethylated H1K27, since few studies showed that the mammalian JMJD2A proteins are functional histone demethylases that target H3K9 and H3K36 (Cloose et al., 2006; Fodor et al., 2006; Kim et al., 2007; Klose et al., 2006; Whetstine et al., 2006), and recently was also shown that the same proteins demethylate mammalian homologues of the protein JMJD2A, which was the trigger for the idea of my project, also due to the sequence similarity between H1 in fly and mammalian H1.4, as mentioned above in the text.

Overexpression of JMJD2A in S2 cells and immunostaining with α -H1K27me2 (Figure 10. and 11.) showed a decrease of H1K27me2 signal in transfected cells in 80% of cells, compared to the staining with the same antibody in non transfected cells. To confirm the specificity of JMJD2A demethylase activity toward H1K27me2 and to exclude the possibility that this observed change in dimethylation level could be the consequence of the changes in the total H1K27 amount, we performed the immunostaining experiments with α -unmodified antibody (that recognizes total H1K27) in induced cells that are overexpressing JMJD2A, and we found no significant decrease of the signal in transfected cells compared to non transfected cells (Figure 11. right graph). This data are suggesting that the decrease observed in 80% of the cases in H1K27me2 in transfected cells could be due to demethylase activity of JMJD2A on lysine 27 of the linker histone H1.

Staining of the H1K27me2 and H1K27 in wild type cells (Figure 12.) revealed general higher signal of H1K27me2 than the signal of H1K27me2 observed in the transfected cells, which additionally confirms our hypothesis.

Comparing the two stainings with the different antibodies only in transfected cells, in 95% of the cells stained for H1K27me2 was observed a decrease in the signal, while no significant number of the cells with decreased signal was observed in the staining with H1K27. Thus, in non transfected cells was seen that almost no cell stained for H1K27me2 showed an decrease in signal (Figure 14.). All these data together are strongly suggesting that the JMJD2A could perform a demethylase activity toward histone H1 at Lysine 27 position.

To date there was no evidence of demethylation of histone H1 in *D. melanogaster*. Our data is indicating that there is probably existence of demethylase of histone H1 in *D. melanogaster* at Lysine27 position. However, additional controls and experiments in *D. melanogaster* tissues should be performed, to confirm this data.

APPENDIX 1: Data and analysis of H1K27me2 signals in induced cells

JMJD2A H1K27me2		ROI1:Normalized values of grey signal of JMJD2A		ROI2:Normalized values of grey signal of H1K27me2		Values of H1K27me2 of non transfected cells(ROI1<2)		Values of H1K27me2 of transfected cells (ROI1>2)	
Min	650.96	Min	246.27	2.15					
max	3202.14	max	688.51	normalized	normalized	<2 roi1 NT	>2 roi1 T		
ROI 1	ROI 2	roi1	roi2						
2296.94	253.78	3.55	1.03			1	1.03		
2447.9	282.03	3.78	1.15			1.21	1.15		
1113.01	247.45	1.72	1			1.04	1.04		
3080.29	255.4	4.76	1.04			1.48	1.08		
2526.16	265.76	3.9	1.08			1.95	1.06		
1874.7	261.65	2.9	1.06			1.78	1.01		
1378.21	249.96	2.13	1.01			1.83	1.03		
2034.93	254.2	3.14	1.03			1.66	1.01		
2317.71	248.77	3.58	1.01			1.63	1.02		
1990.42	251.14	3.08	1.02			1.44	1.01		
3202.14	248.03	4.95	1.01			1.64	1.03		
1427.23	252.98	2.21	1.03			1.77	1.01		
1021.29	298.4	1.58	1.21			2.1	1.05		
1166.82	256.55	1.8	1.04			1.7	1.05		
1786.48	248.02	2.76	1.01			2.15	1.03		
1629.7	257.65	2.52	1.05			1.82	1.08		
2022.11	258.37	3.12	1.05			1.55	1.06		
2246.14	253.9	3.47	1.03			1.74	1		
1993.31	265.06	3.08	1.08			1.44	1.03		
2069.28	261.55	3.2	1.06			1.78	1.03		
1621.04	246.27	2.51	1			1.69	1.05		
1519.96	253.3	2.35	1.03			1.96	1.05		
2880.79	254.76	4.45	1.03			1.72	1.03		
2760.99	259.56	4.27	1.05			1.74	1.05		
2298.04	259.3	3.55	1.05			1.67	1.05		
1837.99	252.63	2.84	1.03			1.71	1.04		

Each row represents one cell data, here is shown a representative number of cells, in total 105 randomly taken cells were analysed

APPENDIX 2: Data and analysis of H1K27 signals in induced cells

ROI1:Normalized values of grey signal of JMJD2A

ROI2:Normalized values of grey signal of H1K27

Values of H1K27 of non transfected cells (ROI1<1.4)

Values of H1K27 of transfected cells (ROI1>1.4)

JMJD2A	H1K27	1.7	NT	T	
ROI1	ROI2	normalized	normalized	<1.40	>1.40
		roi1	roi2		
350.21	247.95	1.18	1.02	1.02	1.33
327.86	262.35	1.1	1.08	1.08	1.14
376.5	268.67	1.27	1.11	1.11	1.08
699.7	268.64	2.35	1.11	1.11	1.14
382.62	256.67	1.29	1.06	1.06	1.05
398.81	248.88	1.34	1.03	1.03	1.19
386.13	258.69	1.3	1.07	1.07	1.13
632.41	322.87	2.13	1.33	1.04	1.04
470.88	276.31	1.58	1.14	1.06	1.05
488.6	261.01	1.64	1.08	1.05	1.04
1635.32	276.61	5.5	1.14	1.1	1.1
480.49	254.99	1.61	1.05	1.06	1.1
470.9	536.12	1.58	2.21	1.45	1.16
1402.3	288.8	4.71	1.19	1.04	1.14
465.94	273.33	1.57	1.13	1.13	1.32
378.46	252.77	1.27	1.04	1.09	1.13
374.57	255.5	1.26	1.06	1.09	1.11
747.97	252.67	2.51	1.04	1.04	1.08
594.77	255.19	2	1.05	1.03	1.19
401.78	266.37	1.35	1.1	1.14	1.17
403.97	257.16	1.36	1.06	1.1	1.13
467.29	495.94	1.57	2.05	1.11	1.12
411	349.86	1.38	1.45	1.06	1.13
345.05	250.74	1.16	1.04	1.01	1.06
449.09	253.89	1.51	1.05	1.16	1.06
561.12	490.37	1.89	2.03	1.06	1.08
503.55	252.37	1.69	1.04	1.04	1.1
347.54	274.26	1.17	1.13	1.02	1.06
378.54	263.54	1.27	1.09	1.16	1.1
357.33	264.8	1.2	1.09	1.1	1.04
331.31	251.11	1.11	1.04	1.2	1.07
514.58	266.03	1.73	1.1	1.06	1.13
439.4	265.67	1.48	1.1	1.05	1.11
502.03	281.42	1.69	1.16	1.07	1.58
483.2	276.78	1.62	1.14	1.02	1.16

Each row represents one cell data, here is shown a representative number of cells, in total 105 randomly taken cells were analysed

APPENDIX 3: Data and analysis of H1K27me2 and H1K27 signals in wild-type cells

325.11		328.26	
wt H1K27me2		wt unmodif	
334.89		457.32	
372.55		461.75	
361.54		442.67	
392.35		431.81	
384.27		459.6	
377.43		445.82	
362.74		457.71	
409.1		429.8	
408.88		512.98	
427.15		464.66	
401.85		394.51	
417.07		440.73	
463.78		423.31	
403.51		397.7	
408.83		427.55	
413.47		366.16	
405.75		567.62	
355.54		399.64	
418.72		392.09	
353.6		400.47	
390.34		435.42	
387.25		408.4	
375.89		431.93	
381.29		462.77	
381.64		447.18	
396.76		424.79	
431.59		577.75	
417.22		445.39	
382.77		416.24	
352.38		399.79	

Each row represents one cell data, here is shown a representative number of cells, in total 141 randomly taken cells were analysed

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