

**Characterization of the MYST histone acetyltransferase
Mof2 in *Drosophila melanogaster***

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Abbreviations

Beaf-32	boundary element associated factor of 32 kDa
ChIP	chromatin immunoprecipitation
CP190	centrosomal protein 190
dCTCF	<i>Drosophila</i> CCCTC-binding factor
dmMOF	males absent on the first
DRE	DNA replication-related element
DREF	DRE factor

DsRNA	double-stranded RNA
GSC	germ line stem cell
HAT	histone acetyltransferase
HP1	heterochromatin protein 1
IP	immunoprecipitation
MSL complex	males-specific-lethal complex
NCP	nuclear core particle
Non-LTR retrotransposon	non-long-terminal-repeat retrotransposon
NSL complex	non-specific lethal complex
Nurf-38	nucleosome remodeling factor-38 kDa
Su(Hw)	suppressor of hairy-wing
UAS	upstream activating sequence

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1 Introduction

1.1 Organisation of DNA in the eukaryotic nucleus

In eukaryotes, the DNA is mainly located within the nucleus and organised in higher order structures called chromatin. Depending on the degree of compaction, the DNA is roughly grouped into hetero- and euchromatic regions. This classification is due to the finding that DNA is not evenly stained but shows a banding pattern of differentially stained regions. In *Drosophila melanogaster*, this staining pattern can be easily examined in polytene chromosomes of salivary glands of L3 larvae (Fig. 1). The heterochromatic regions are mainly transcriptionally inactive whereas the euchromatic regions are active. In this way, the staining pattern reflects roughly the organisational structure of the DNA.

The proteins that are responsible for the skeletal structure of the chromatin are the histones. Along with the DNA, they form the nucleosome core particles (NCP), which are the basic repeat unit within the chromatin. The centre of a NCP consists of a histone octamer containing two copies of the four histones H2A, H2B, H3 and H4. About 146 bp of DNA are wrapped around these histones (Chakravarthy et al 2005). The N-termini of the core histones protrude from the nucleosome (reviewed by Lorch 1999) and contribute to the compaction of the chromatin by stabilising the interaction of different NCPs (Bertin et al 2004). Linker DNA separates the NCPs from each other. With the help of histone H1, the nucleosomes are more closely linked and the chromatin becomes more condensed (Lu et al 2009). There are also other proteins like the heterochromatin protein 1 (HP1) that contribute to the structure of the chromatin (Eissenberg et al 1990). HP1

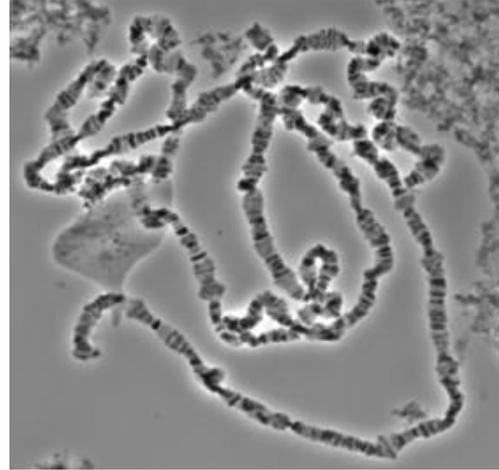


Figure 1: Polytene chromosomes from *Drosophila melanogaster*

The polytene chromosomes are derived from the salivary glands of wandering L3 larvae. Within the phase contrast picture, the typical banding pattern of acid-fixed polytene chromosomes is visible. The dark bands are referred to as heterochromatic regions whereas the light bands are referred to as euchromatic regions that contain less condensed DNA.

The picture was taken with 630x magnification.

is responsible for the formation of heterochromatic regions like the telomeres (Vermaark and Malik 2009).

1.2 Histone modifications

The tails of the core histones are object to a variety of modifications like methylation, acetylation, phosphorylation, ubiquitination and SUMOylation (Fig. 2). The most frequently studied modifications are the methylation (Desrosiers and Tanguay 1988) and the acetylation of lysine residues and the phosphorylation of histone H3S10 (Jin et al 1999).

A well-studied example for the link between a histone modification and its physiological function is the acetylation of histones by the acetyltransferase males absent on the first (dmMOF). dmMOF catalyses the hyperacetylation of histone H4K16 at the single male X chromosome. This leads to the dosage compensation via an upregulation of the transcription of genes that are located on the X chromosome in males.

The acetylation of histone H4K16 leads to a less condensed chromatin structure. This is the result of the weakened interactions between histone H4 of one NCP and the negatively charged patch of H2A of a neighbouring NCP (Shogren-Kaan et al 2006, Luger et al 1997). This leads to NCPs that are not as closely packed and the DNA is

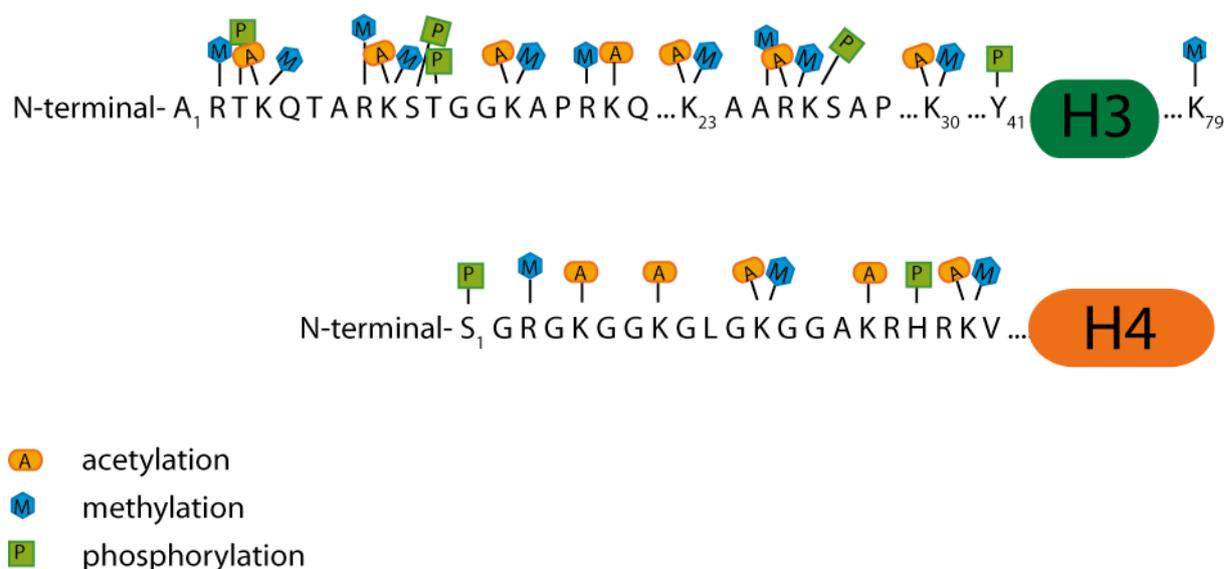


Figure 2: Posttranslational modifications of histone H3 and H4

The most studied posttranslational modifications of the core histones are acetylation, methylation and phosphorylation. Most of the modifications are found at the N-termini. For some lysine residues both methylation and acetylation was found. These modifications are antagonistic.

The figure was adapted from the work of Zu et al 2013.

more accessible to digestion. This change in the chromatin architecture correlates with an increased transcription (Lucchesi 2009).

Histone H4K16 acetylation is not only linked to transcription but also to DNA replication (Schwaiger et al 2009). A high content of histone H4K16 acetylated nucleosomes correlates with early DNA replication during the S-phase whereas heterochromatic regions are late replicating sequences (Gilbert 2002). Therefore, the single X chromosome of male flies is replicated mainly during the early S-phase whereas the female X chromosomes are late replicating.

However, acetylation does not lead to an increase in transcription and a more decondensed chromatin structure *per se*. Histone H3K4 acetylation resides in heterochromatin and this acetylation is required for the correct formation of pericentric heterochromatin (Xhemalce and Kouzarides 2010).

Like acetylation, methylation of lysines is linked to both transcriptional activation and inactivation and can antagonise acetylation at the same or neighbouring residues. One example for this is lysine 27 at histone H3. This residue can be trimethylated or acetylated. While the trimethylation at this residue is a hallmark for silenced genes, the acetylation at the same residue is a characteristic feature of transcribed genes and antagonises gene silencing by Polycomb group proteins (Tie et al 2014).

An example for both acetylation and methylation at the same gene is histone H3K9 acetylation at the promotor site and H3K4 methylation within the gene body. This combination of histone modifications is a hallmark of active transcription (Yin et al 2011).

The phosphorylation of histone H3S10 by JIL-1 is an example of a context-dependent function of histone modifications. In the absence of JIL-1, many genes show a change in transcription, and both up- and downregulation can be observed (Cai et al 2014). A closer analysis revealed that active genes become inactivated, whereas inactive genes become activated. At the same time, the repressive dimethylation of histone H3K9 increases at formerly active genes and decreases at formerly inactive genes. In this way, the histone H3S10 phosphorylation is thought to modify the chromatin architecture and modulate the histone H3K9me2 rather than having a direct effect on transcription.

1.3 Chromatin states

Studies on the classification of hetero- and euchromatic regions with the help of histone modifications and protein binding patterns reveal a considerably more complex organisation of these regions than it was expected from first studies. They show a complex cross-talk between overall chromatin structure, histone modifications and transcription (Schwartz et al 2010, Yasuhara and Wakimoto 2009). Studies by Filion and colleagues aimed at identifying general principles of the organisation of chromatin. To this end, they defined five different chromatin states in *Drosophila melanogaster* (Filion 2011, Filion et al 2010). These states are characterised by distinct histone modifications, proteins that bind to the chromatin and transcriptional activity (Fig. 3).

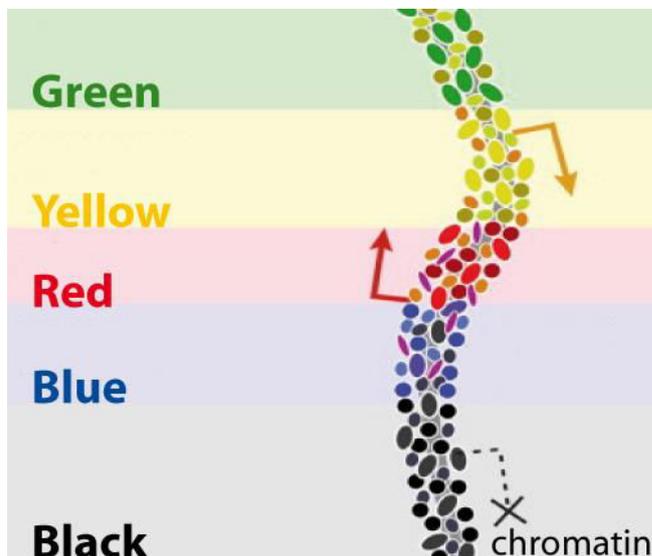


Figure 3: Different chromatin states in *Drosophila melanogaster*

The chromatin can be subdivided into transcriptionally active (yellow and red) and transcriptionally inactive (green, blue and black) regions. These regions differ in their protein binding and their histone modifications pattern. Further details are described within the text.

The figure was adapted from Filion et al 2010.

The heterochromatic regions are subdivided into “Green”, “Blue” and “Black” chromatin states. The “Green” chromatin is characterised by the binding of HP1 and H3K9me2/3 that is generated by SU(VAR)3-9. This kind of heterochromatin can be found at the telomeres and the pericentric regions.

Polycomb group proteins binding and H3K27me3 mark the “Blue” chromatin state. Although both chromatin states are repressive, they differ in their methylation by M.SssI. The M.SssI methylation is a hallmark of the accessibility of the chromatin for other proteins and reflects the degree of chromatin compaction. Thus, the “Blue” chromatin is poorly accessible to methylation as expected whereas the “Green” chromatin is still accessible to methylation (Bell et al 2010). The third

heterochromatic state, the “Black” one, lacks the marks typical of the two others, and there is little to no transcription activity.

The euchromatic regions are subdivided into two different groups. The “Red” state is characterised by the binding of trithorax proteins and H3K79me3. Mainly developmentally regulated genes constitute that kind of euchromatin. The “Yellow” state is characterised by histone H3K36me3. This mark is characteristic of house-keeping genes.

Besides these static states, there is also an exchange between different chromatin states possible. This is especially true for genes that are differentially expressed such as sex-specific genes.

1.4 Insulator proteins

Insulator proteins constitute a group of proteins that maintain the hetero- and euchromatic identity of the chromatin and act as boundary elements. In this way, they prevent the spreading of the repressive histone H3K27me3 into transcriptionally

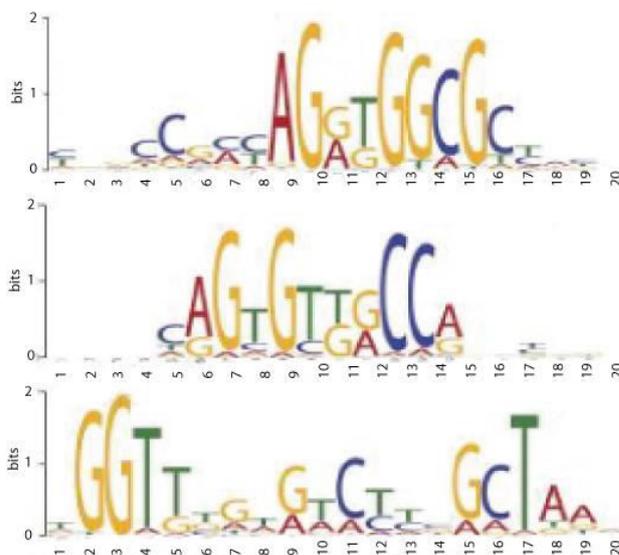


Figure 4: DNA-binding motifs of dCTCF

The binding sites of dCTCF are enriched for three DNA motifs. These motifs are similar but distinct and overlap with the binding sites of other insulator proteins. The DNA-binding sites are presented as weight matrices.

The figure is adapted from van Bortle et al 2012.

active regions. Additionally, they regulate the function of enhancers. Whereas older studies suggested a model in which the whole genome is partitioned by specialized boundary elements, recently studies suggests a more specialized function for insulator proteins (Schwartz et al 2012). Insulator proteins were found to have little effect on the genome-wide expression or spreading of histone H3K27me3 but rather have a specialized function for the regulation of selected genes e.g. a small number of Polycomb target regions. There, the insulator proteins prevent the spreading of repressive histone methylation of

adjacent genes that are transcriptionally inactive. Since many experiments concerning the general function of insulators were performed in cell culture the effect on the development of *Drosophila melanogaster* remains not completely understood. The most extensively characterised insulator proteins are the CCCTC-binding factor (dCTCF), the centrosomal protein 190 (CP190), the boundary element associated factor of 32 kDa (Beaf-32), suppressor of hairy-wing (Su(Hw)) and Mod(mdg4) with its splice variants. These proteins have both unique and shared binding sites in chromatin. In general, they act in varying combinations. For instance, CP190 and dCTCF have overlapping binding sites and interact *in vivo*, though not all CP190 binding sites include dCTCF like the gypsy chromatin insulator complex (Pai et al 2004). Also, the binding sites of dCTCF do not exclusively colocalise with CP190 (Mohan et al 2007). Approx. 40% of dCTCF sites align tightly with Su(Hw) and Beaf-32 without CP190. For dCTCF, not only different protein interactions are described, but also different DNA binding motifs (van Bortle et al 2012). These DNA motifs (Fig. 4) include multiple DNA binding sites for cofactors and increase the number of possible protein complexes. The different combination of insulator proteins and other cofactors may explain the regulation of enhancers and the constitution of transcriptional active and inactive regions and their change during development.

1.5 Histone acetyltransferase families

In addition to the methylation of histones that was used for the classification of the different chromatin states by Filion, the acetylation also plays a crucial role for the formation of different chromatin states.

The acetylation of histones is mediated by histone acetyltransferases. They transfer acetyl groups to different lysine residues of histones. Thus, the positive charge of this amino residue is neutralised, which changes the interactions between histones and DNA or different histones within the chromatin structure. This leads to a modified chromatin structure and the accessibility of the DNA to transcription factors and other chromatin binding proteins is altered. In addition, the acetylation of the N-terminus itself can serve as a recognition site (Lee et al 1993).

The different histone acetyltransferases (HATs) constitute a large group of chromatin modifying enzymes. They are subdivided into different groups based on their sequence similarities. There are five major groups: Gcn5-related HATs, p300/CBP

HATs, the general transcription factor HATs, nuclear hormone receptor-related HATs and the MYST family of HATs (Carrozza et al 2003, Sterner and Berger 2000).

The first group is named after Gcn5. DmGcn5 is known to be responsible for the acetylation of different lysines of core histones, namely histone H4 and H3. For this acetylation, the transcriptional cofactors dmAda2a or dmAda2b are needed (Ciurciu et al 2006).

The group of p300/CBP proteins acts as transcriptional coactivators and serves as a bridge between other cofactors and the basal transcription complex. These HATs are likely to play a role in the remodelling of the nucleosomes at the transcriptional start site via acetylation of histones (reviewed by Chan and La Thangue 2001).

The group of general transcription factor HATs binds directly to the DNA at the promotor or the enhancer sequence and acetylates histones and the nuclear hormone receptor HATs show a hormone dependent acetylation activity.

In addition to the acetylation of histones, the acetylation of non-histone proteins is described for all HAT groups.

1.6 MYST histone acetyltransferases

The MYST family is named by its founding members MOZ, Ybf2/Sas3, Sas2 and Tip60 in human and yeast (Fig. 5). The members of this family are closely related and can be found from flies to humans (Sanjuan and Marin 2001). In *Drosophila melanogaster*, there are five annotated MYST acetyltransferases. They are called dmMOF, Chameau, Enok, dmTip60 and the gene product of *cg1894* which is called Mof2 in this study due to its homology to dmMOF. DmMOF, Chameau, Enok, and dmTip60 are already characterised and effect the development of *Drosophila melanogaster* to a different extent.

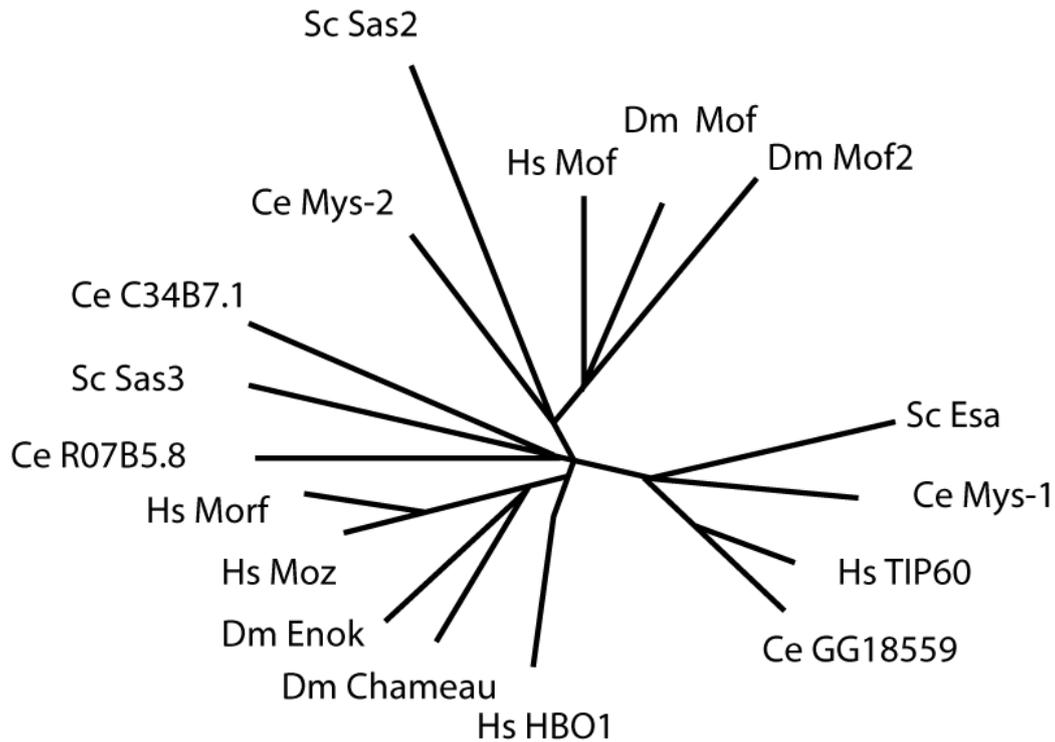


Figure 5: Phylogenetic tree of (annotated) MYST histone acetyltransferases

The phylogenetic tree of (annotated) MYST histone acetyltransferases includes proteins from *Drosophila melanogaster* (Dm), *Homo sapiens* (Hs), *Saccharomyces cerevisiae* (Sc) and *Caenorhabditis elegans* (Ce) and was adapted from the work of Sanjuan and Marin 2002.

DmMOF is the catalytically active subunit of the males-specific-lethal (MSL) complex. This complex is essential for the hyperacetylation of histone H4 at lysine 16 on the male X chromosome. The resulting hyperacetylation is detectable on polytene chromosomes in males and absent from the female X chromosomes (Turner et al 1992). It is assumed that the hyperacetylation of histone H4K16 leads to an increased transcription of the X chromosomal genes in males and therefore mediates dosage compensation in *Drosophila melanogaster*. A loss-of-function mutation in dmMOF leads to a severe loss of the acetylation on the male X chromosomes and results in lethality for male flies in different developmental stages but at the latest during early pupa stages. The viability of female larvae is largely unaffected (Hilfiker et al 1997). In summary, the histone H4K16 acetylation on the male X chromosome is a hallmark for 2-fold increased transcription in *Drosophila melanogaster*.

In addition to the MSL complex, dmMOF is also part of the non-specific lethal (NSL) complex. Within this complex, dmMOF still acts as histone H4K16 acetyltransferase, but the localisation differs from the MSL complex. While the MSL complex is mainly

located on the male X chromosome, the NSL complex is localised on the autosomes. There, the histone H4K16 acetylation is part of the transcriptional regulation of house keeping genes (Lam et al 2012).

Another MYST acetyltransferase in *Drosophila melanogaster* is Chameau. This HAT also shows a histone H4K16 activity *in vivo* and its function is linked to the formation of heterochromatin in yeast (Grienenberger et al 2002). In this way, the same histone modification results in a different chromatin state at least in yeast. The developmental function of Chameau is linked to the development of the adult thorax and the regulation of the maturing of progenitor cells that form sensory bristles (Hainaut et al 2012).

For Enok, the histone acetyltransferase activity is shown indirectly with the help of mutations of the predicted active centre. In contrast to dmMOF, Enok does not regulate many genes but has a more specific function in the formation of the mushroom body (Scott et al 2011) and the maintenance of female germ stem cells (Xin et al 2013).

DmTip60 is another MYST histone acetyltransferase in *Drosophila melanogaster*. It is essential for the development of both females and males. A strong knockdown of this protein is lethal during metamorphosis, whereas a slight reduction leads to viable flies with an additional wing vein (Schirling et al 2010, Zhu et al 2006). Further investigation of the function of dmTip60 showed a broad regulatory function on gene transcription (Schirling et al 2010). The regulation is both up- and down-regulating. Next to the regulatory function concerning gene transcription dmTip60 also plays a role in double strand DNA break repair by acetylating phospho-H2Av and simultaneously recruiting a chromatin remodeler complex to the repair site (Kusch et al 2004).

Like Enok, Chameau and dmMOF, dmTip60 has different functions and an influence on the proper development of *Drosophila melanogaster*. In *Drosophila melanogaster*, the MYST histone acetyltransferases and their relatives in other species show, as far as their enzymatic activity is known, a specificity for histone H4K16 acetylation.

1.7 Mof2

cg1894 is annotated as a protein coding gene in the *Drosophila* genome. Since it is most similar to dmMOF, it is named Mof2 in this study. It is a member of the MYST group of acetyltransferases. The bioinformatical search for different domains identifies only the catalytical domain of MYST acetyltransferases (Fig. 6). A more detailed sequence analysis showed that there is a high similarity to acetyltransferases with a specificity for histone H4K16 like dmMOF and Sas2 in yeast. High-throughput data from modENCODE revealed a low expression at all developmental stages and organs except in early embryos and testes of adult flies where a high expression level was detected.

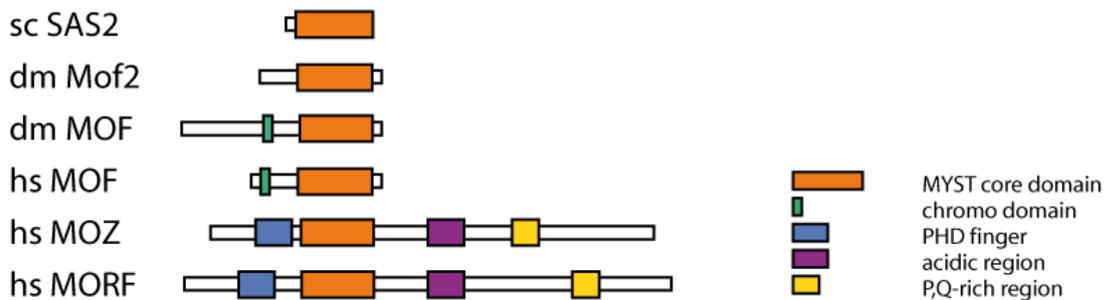


Figure 6: Domain structure of Mof2 and its homologs

In dmMof2, the MYST core domain is the only identified domain. Its closest relatives except Sas2 also contain other domains with functions in chromatin binding and gene regulation. The chromo domain mediates binding to chromatin and the PHD finger facilitates the binding to methylated histones, the P,Q-rich region of proteins stabilises protein-protein interactions and the acidic region is a characteristic of nuclear proteins and might interact with the positively charged histone tails.

The figure was modified from the work of Sanjuan and Marin 2002.

1.8 Development of sperm in *Drosophila melanogaster*

In the testes of *Drosophila melanogaster*, a special cell development with a complex change in the organisation of the nucleus takes place during the maturing of sperms (Fig. 7). The maturing of the male gametes starts with the development of pole cells during embryogenesis and ends with the mature sperm. The whole process of sperm maturing takes about two weeks and the appearance of mature sperm almost co-occurs with the hatching of the adult fly.

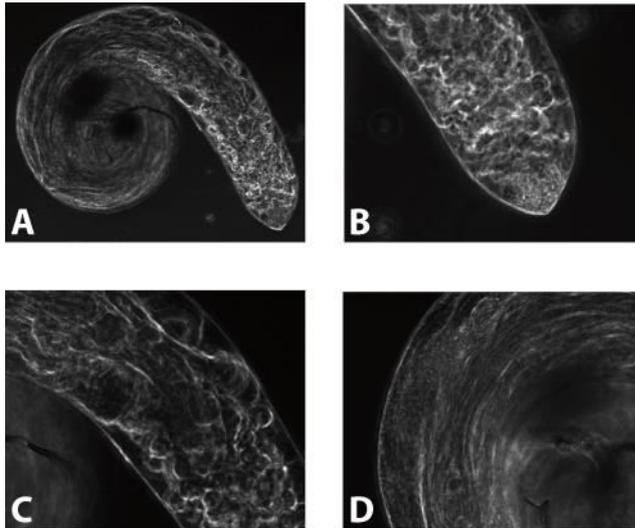


Figure 7: Testis of an adult male fly

(A) Within the testis, the sperm matures.

(B) At the tip of the testis, the stem cells and spermatogonial cells are located.

(C) Subsequently, late spermatocytes and spermatids are visible.

(D) The spermatids form long flagella that fill the remaining space of the testis.

The pictures were taken with a phase contrast filter with 100x **(A)** or 200x **(B,C,D)** magnification.

The pole cells are the precursor cells of the later germ line stem cells (GSC) within the testis (Dansereau and Lasko 2008). The GSCs are located at the tip of the testis within the stem cell niche and undergo an asymmetric mitotic cell division that results in a GSC and a primary spermatogonial cell. The primary spermatogonial cell is surrounded by two cyst cells and forms a cyst. The cyst moves laterally away from the tip of the testis, and the primary spermatogonial cell undergoes four mitotic divisions. The resulting 16 primary spermatocytes are still connected by cytoplasmic bridges. After a premeiotic S-phase, no synaptonemal complexes are formed, and therefore, no recombination of

homologous chromosomes occurs in male flies (McKee et al 2012). During the premeiotic phase, the primary spermatocytes are highly transcriptionally active and male specific genes whose products are needed for male fertility as well as genes that are necessary for the later development of sperms are transcribed. The transcripts are translationally repressed and stored as mRNAs (White-Cooper and Caporilli 2013).

After the meiosis, bundles of 64 connected spermatids are formed that are almost completely transcriptionally inactive, and only a small subset of genes is transcribed after meiosis II (Barreau et al 2008). At the beginning of their maturing, the spermatids have an almost round nucleus. During the following development, the nucleus elongates and becomes more sharpened. At the same time, the flagella of the spermatids are formed and elongated. At the end of their development, the spermatids are individualised and are stored as mature sperm.

During the maturation of sperms, the chromatin organisation changes from a histone-based to a protamine-based structure. This rearrangement starts in primary spermatogonial cells and is completed in mature sperm (reviewed in Rathke et al 2013). This major structural change is accomplished by a variety of modifications of the chromatin architecture. Hallmarks of the reorganisation are the reduction of histones that is accompanied by hyperacetylation of histone H4 and methylation of histones (Rathke et al 2012, Awe and Renkawitz-Pohl 2010). In addition, the incorporation of histone linker-like proteins like Don Juan and the transition protein Tpl94D can be observed. In the mature sperm, the histones are almost completely substituted by protamine A and B (Rathke et al 2010, White-Cooper and Caporilli 2010). During the maturing of sperm, the nucleus is greatly reduced in size and changes from a round form to a stretched and slim appearance. There are several mutants with a defect in the maturation of sperms known that cause male sterility or a reduced fertility (e. g. White-Cooper and Caporilli 2010).

1.9 Fecundity and longevity

In general, it is assumed that a high fecundity correlates with a reduced life span and *vice versa*. The reason for this are limited resources that can be used either for reproduction or somatic maintenance. These assumptions are based on studies with flies that are sterile or have a reduced fertility. One of the earliest observations that showed a correlation between fertility and life span was made for *Drosophila subobscura*. For these flies, a significantly increased life span could be observed in sterile flies compared to fertile wild type flies (Maynard and Smith 1958). In combination with other studies, the theory of a trade-off was established and refined on a molecular level (Tatar 2010, Tatar et al 2001). Thus, a change in the fecundity is likely to be linked to a change in life span and *vice versa*.

However, that was not the case for all studied mutant. There are also *Drosophila melanogaster* strains described that show both an at least comparable fecundity compared to control strains and an increased life span (Wit et al 2013). As far as the strains are characterised in more detail, the investigated strains show an overall improved metabolism compared to the initial strain.

1.10 Thermal stress

Another well-studied example for a considerable reorganisation of chromatin and a subsequently changed expression is the response to a heat shock. Under these conditions, heat shock proteins are highly expressed. They serve as chaperones and as such prevent proteins from being denatured. The heat shock response in *Drosophila melanogaster* leads to an almost complete shutdown of the expression of non-heat-shock genes (Lindquist 1984).

The DNA-dependent RNA polymerase II is the catalytic subunit of the transcription complex that is responsible for the transcription of protein-coding genes. The activity of this complex is regulated by the phosphorylation of the C-terminal region of the RNA polymerase II. The different states of phosphorylation present different distinct steps of transcription. The unphosphorylated RNA polymerase II can be found in preinitiation complexes at the promoter site. The phosphorylation on Ser2 can be found on promoter-paused RNA polymerase II and additional phosphorylation on Ser5 is characteristic for RNA polymerase II that elongates (Ivaldi et al 2007, Li et al 1996). In this way, a quick change from the paused to the active state of the transcription complex is possible.

Both the dramatically increased expression of heat shock genes and the decreased expression of other genes can be monitored by the binding of RNA polymerase II to polytene chromosomes. There, the RNA polymerase II binding to the heat shock loci is increased, whereas the binding to other sites is greatly diminished after a heat shock. At the same time, a change of the binding of transcription factors like TFIIB can be observed, and co-activators like the heat shock factors are recruited to the heat shock loci (Lebedeva et al 2005). The enhanced transcriptional activity at the heat-shock loci leads to the formation of so-called puffs. At these regions, the DNA appears decondensed and hazy.

In addition to an acute heat shock, there is also a stable adaptation to permanent thermal stress. For high temperatures, a substantial change in the translation of different groups of proteins is observed (Colinet et al 2013). This change in translation affects heat shock proteins like Hsp70 and Hsp22 as well as energy-metabolism associated proteins like alcohol dehydrogenase. Furthermore, proteins that are involved in chromatin organisation like the nucleosome remodeling factor-38kD (Nurf-38) were found to be upregulated and reflect the reorganisation of the

chromatin during thermal stress (Tetievsky and Horowitz 2010). In this way, heat shock proteins are not only expressed as a response to a heat shock, but also as an adaptation to high non-lethal temperatures.

The heat shock response in the salivary glands of L3 larvae is a special case of the expression of heat shock genes. The expression of heat shock genes is neither exclusively linked to the heat shock response, nor is it detectable during all developmental stages or tissues. The heat shock proteins are also expressed at different stress conditions and at different developmental stages and organs (Lakhotia and Prasanth 2002). Different expression patterns before and after induction can be found for the well-studied heat shock protein Hsp70 and vary from no induction in early embryos and the malpighian tubules to high expression in salivary glands of L3 larvae (Vazquez 1991, Pauli et al 1988). In addition, especially for the small heat shock proteins, functions besides the heat shock response and during the normal development could be observed. For example, the small heat shock protein sHS23 was found in the testes exclusively in spermatocytes without thermal stress (Joanisse et al 1999).

1.11 Telomeres in *Drosophila melanogaster*

Another special chromatin organisation can be found at the telomeres of *Drosophila melanogaster*. As in other eukaryotes, the DNA in *Drosophila melanogaster* is organised in linear chromosomes. This leads to severe problems concerning the genome integrity.

One structural problem is the result of the unidirectional specificity of the DNA dependent DNA polymerase, which leads to an incomplete replication of the end of the newly synthesised DNA strand. That results in a shortening of the chromosomes with each DNA replication cycle.

The DNA repair machinery causes another problem that arises with linear chromosomes. The responsible protein complexes recognise chromosome ends as broken DNA strands. This leads to an activation of different DNA damage response pathways and can cause chromosome end fusions and other chromosome modifications. In *Drosophila melanogaster*, the end replication problem and the problem of free chromosome ends are solved independently (Biessmann and Mason, 1988).

The protection of the ends of chromosomes is sequence-independent and is likely to be determined in an epigenetic way (Oikemus et al 2006).

The end replication problem is solved by the introduction of telomeres, which are specialised DNA sequences at the end of chromosomes (Fig. 8). In contrast to many other eukaryotes, in *Drosophila melanogaster* these DNA sequences are neither repeats of short GC-rich motives nor synthesised by a telomerase. Instead, the telomeres are generated by three highly specialised non-long-terminal-repeat (non-LTR) retrotransposons, which are called HeT-A, TART and TAHRE (Pardue et al 2005, Abad et al 2004). These retrotransposons are only found at the telomeric sequences and not within other heterochromatic regions (George et al 2006). HeT-A and TART have been characterised in more detail than TAHRE and are likely to have complementary features. While the TART retrotransposon codes for both the Gag-like protein and the reverse transcriptase which are essential for the retrotransposition, HeT-A only codes for a Gag-like protein. The Gag-like protein is responsible for the recognition of the RNA of the retrotransposons in the cytoplasm, the import into the nucleus and the correct localisation of the RNA to the telomeres. The Gag-like protein of HeT-A can translocate both the RNA of the TART and the HeT-A retrotransposon. The reverse transcriptase of the TART element can act on the RNA of HeT-A and TART and has two different functions. First, it recognises the RNA and distinguishes between the RNA of HeT-A and TART or other retrotransposons and then, it rewrites the RNA sequence in a DNA sequence.

The timing of the retrotransposition of the retrotransposons is controlled by mechanisms that are only poorly understood. With the help of the so-called Gaiano



Figure 8: Structure of the telomeres in *Drosophila melanogaster*

The telomere of *Drosophila melanogaster* is formed by the HTT array which is composed of the retrotransposons HeT-A (light orange), TART (orange) and TAHRE (red). The retrotransposons are attached to the DNA by their polyadenosine tail at their 3'-end (indicated by the A between the different retrotransposons). At the end of the telomere, the terminin complex protects the chromosome from non-homologous end joining. The telomere associated sequence (TAS) is a transition zone between the telomere and the unique chromosome sequence.

The figure was adapted from Capkova Frydrychova et al 2008.

strain a genetic factor that controls the length of the telomeres could be identified. The Gaiano strain is special compared to other laboratory wild type strains. Although this strain does not show any phenotypical abnormalities, the flies have remarkably long Het-A/TART arrays at their telomeres (Siriaco et al 2002). At these extraordinarily long telomeres, the binding of different factors can be investigated more easily due to a reduced loss of Het-A/TART arrays during the preparation of polytene chromosomes

In this way, two proteins were identified at the telomeres by immunostaining that also play a role in the overall regulation of chromatin structure and the regulation of transcription, namely the DNA replication-related element factor (DREF) and Z4 (Takács et al 2012, Andreyeva et al 2005).

1.12 Function of the transcription factor DREF

DREF is part of a multi protein complex that includes remodelers and other chromatin associated proteins like Z4/Putzig (Kugler and Nagel 2007). Within this complex, DREF is involved in the regulation of the cell cycle and cell growth. It binds to a subset of core promoters that contain the DNA replication-related element (DRE) sequence and acts as a positive regulator (Hochheimer et al 2002). In that way and amongst others, DREF is responsible for the correct proliferation of cells. It regulates the expression of replication factors like Orc2 and E2F (Okudaira et al 2005). If these factors are not or not sufficiently expressed, the cells cannot complete the S-phase properly (Hyun et al 2005).

DREF also shares some binding sites with MSL-1 especially on the X chromosome. Since MSL-1 is part of the MSL-complex, which is responsible for dosage compensation of the male X chromosome, DREF might be involved in the correct binding of the MSL-complex to promoters (Legube et al 2006) and might play a role in the correct positioning of other factors.

In addition to its function within the cell cycle, DREF is also involved in the regulation of the expression of the retrotransposons in the HTT arrays at the telomeres. There, DREF binding motifs were identified within the TART element, and DREF regulates the expression of this retrotransposon. It also regulates the expression of the Het-A element in an indirect way and acts as a repressor of its transcription (Silva-Sousa et al 2013).

1.13 Function of the chromatin binding protein Z4/Putzig

Z4/Putzig (also known as p160) is a chromatin-associated protein that exhibits a prominent interband-specific binding to polytene chromosomes (Fig. 9). In addition, Z4 can be found at the telomeres of the left arm of chromosome 2 and the X chromosome. A lack of Z4 has a severe impact on the overall structure of the chromatin and the structure appears more decondensed. This suggests a function in the correct condensation of heterochromatin and implies a role as a boundary factor although the mechanism remains unclear (Eggert et al 2004).

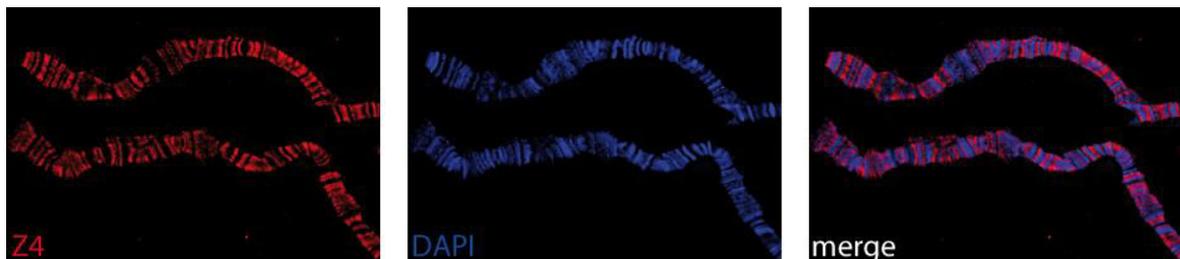


Figure 9: Binding sites of Z4 on polytene chromosomes

The DNA of polytene chromosomes exhibits a reproducible banding pattern for DAPI. The more condensed regions are referred to as bands and represent heterochromatic regions. The less condensed regions between bands are referred to as interbands and represent euchromatic regions. Z binds specifically to interbands and the telomeres of the left arm of chromosome 3 and the X chromosome. Z serves as a modulator of the overall chromatin architecture.

The picture was taken with technical help of Dr. T. Zielke.

The function of Z4 at the telomeres is only partially understood. Although Z4 and DREF interact in general (Hochheimer et al 2002), it remains unclear if that is also true for the telomeric HTT arrays. So far, it seems more likely that there is no interaction at the telomeres (Silva-Sousa et al 2013, Takács et al 2012). Instead, Z4 interacts with JIL-1 and the HeT-A Gag-like protein. These proteins form a complex that is essential for stable telomeres. Z4 and DREF share some but not all binding sites at the chromatin and are also part of different complexes (Silva-Sousa et al 2012).

1.14 Outline of the thesis

The aim of this study was to gain insight into the function of Mof2 in flies. For this purpose its impact on development was investigated with a knock-down strain using the Gal4/UAS system and a hypomorph strain of Mof2 which showed only a relatively

mild effect on development. In addition, the localisation of Mof2 was of interest. Since Mof2 was supposed to be a member of the MYST histone acetyltransferases with a specificity for histone H4K16 DNA-binding properties were expected and tested on polytene chromosomes and in different developmental stages. On polytene chromosomes Mof2 was located within the interbands. To characterise the binding sites of Mof2 on DNA in more detail, a genome-wide analysis was performed. This analysis revealed multiple binding sites for Mof2 that were enriched at highly transcribed genes and a colocalisation with the transcription factor DREF and the insulator proteins CP190 and dCTCF. This suggested a function for Mof2 in the regulation of chromatin structure.

2 Material and Methods

2.1 Organisms and cell lines

2.1.1 *E. coli* strains

Table 1: *E. coli* strains used in this study

BL21 (DE3)	F- ompT hsdS(rB -mB-), dcm+ Tetr gal l(DE3) endA Hte [argU ileY leuW Camr]
Rosetta (DE3) pLysS	F- ompT hsdSB(RB- mB-) gal dcm λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5]) pLysSRARE (CamR)
TOP 10	F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araΔ139 Δ(ara-leu)7697 galU galK rpsL (StrR) endA1 nupG
DH5α	F- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17(rK- mK+), λ-
DH10EMBacY	DH10 derivat with the baculoviral genome as Bac, blue-white selection possible (TetracyclineR, GentamycinR, KanaR)

The BL21 (DE3) strain was used for recombinant protein expression.

The Rosetta (DE3) pLysS strain contains a plasmid coding for rare tRNAs in *E. coli*. It was used if protein expression in BL21 (DE3) was weak or absent.

The TOP 10 and the DH5α strain were used for plasmid amplification. For the amplification of the acceptor plasmid pFL, the TOP 10 strain was used exclusively.

The DH10EMBacY strain was used for the *in vivo* integration of the coding sequence of *mof2* into the baculovirus genome.

2.1.2 *Drosophila* stocks

Table 2: *Drosophila* strains used in this study

Name	source	Special features/Application
Oregon R	AG Ehrenhofer-Murray	wild type strain
w1118	BestGene	used for control crosses

Mof-1F knock-down	Corinna Schirling (isolate 1F)	inducible knock-down of Mof
Mof2 knock-down 1	this work	inducible knock-down of Mof2, isolate 1
Mof2 knock-down 2	this work	inducible knock-down of Mof2, isolate 2
Mof2 knock-down 3	this work	inducible knock-down of Mof2, isolate 3
Mof2 knock-down Wien	Vienna Drosophila RNAi Centre	inducible knock-down of Mof2
Da-Gal4	Prof. H. Saumweber	early and ubiquitous Gal4 expression under the control of the <i>daughterless</i> promotor
T80-Gal4/CyO	Prof. H. Saumweber	early (but later than Da-Gal4) and ubiquitous Gal4 expression
SG 58	Prof. H. Saumweber	Gal4 expression in larval salivary glands
Gaiano-I	Prof. G. Cenci	Elongated telomeres at the X-chromosomes
Gaiano-II	Prof. G. Cenci	Elongated telomeres at the second chromosome
PBac(WH) cg1894 ^{f06204} /TM6B, Tb ¹	Bloomington Stock Centre	heterozygous strain with an piggyBac insertion within the promotor site of Mof2
PBac(WH) cg1894 ^{f06204}	this work	homozygous strain of PBac(WH) cg1894 ^{f06204}

2.1.3 Schneider cell line 2

In this work, Schneider cell line 2 (S2 cells) was used for ChIP experiments and the RNAi approach (Schneider 1972). This cell line is derived from late embryonic developmental stages. S2 cells are mainly tetraploid and show both female and male

characteristics. The karyotype is described to be female but there is no expression of *Sxl* detectable. Therefore, expression of MSL2 is possible and the binding of the male specific lethal (MSL) complex as a typical characteristic of male X chromosomes is found at the X chromosomes (Bashaw and Baker 1997).

The cells were cultured at 26 °C in InsectExpress Sf9-S2 Medium (PAA, Gerbu) in flasks. The medium was supplemented with 10 % heat-inactivated fetal calf serum. The cell viability was assayed with trypan blue solution (0.4 %, Sigma) and cells were counted in a Neubauer counting chamber.

2.1.4 Sf9 cells

Sf9 cells were used for the overexpression of the full-length Mof2 protein. The cell line was derived from a cell line of ovarian cells of *Spodoptera frugiperda* pupae (Vaughn et al 1977). In contrast to proteins that are expressed in *E. coli*, proteins from Sf9 cells show posttranslational modifications similar to other eukaryotes.

The cells were maintained at 27 °C in SF900 III medium. They were cultured in Erlenmeyer flasks and agitated at 115 rpm.

2.2 Media and growth conditions

2.2.1 *E. coli* media and growth conditions

E. coli strains used for plasmid amplification and expression of recombinant proteins were cultured according to standard procedures (Sambrook et al 1989) at 37 °C in Luria-Bertani (LB) medium (10g/l caseinpeptone, 5 g/l yeast extract, 5 g/l NaCl) if not indicated otherwise. Depending on the transformed plasmids, the medium was supplemented with either 100 µg/ml ampicillin or 50 µg/ml kanamycin. If the Rosetta strain was used, the medium was additionally supplemented with 34 µg/ml chloramphenicol. For the DH10EMBacY cells, 10 µg/ml tetracycline, 7 µl/ml gentamycin and 50 µg/ml kanamycin were used. For transformation, SOC medium (2 g/l tryptone, 500 mg/l yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose) was used.

For plates, 20 g/l agar was added to liquid LB media.

2.2.2 *Drosophila* husbandry

The flies were maintained on instant Formula 24-4 (Carolina Biological Supply Company) and supplemented with yeast in vials with foam plugs. The flies were set on fresh media at the latest after three weeks at room temperature.

2.3 Test for lethality of the heterozygous PBac(w⁺)*cg1894*^{f06204} strain

The annotated genotype of the heterozygous knock-out of *mof2* is

$w^{1118}, \frac{PBac [w^+] cg1894^{f06204}}{TM6B, Tb^1}$. The insertion is a modified piggyBac element and causes the disruption of the promoter region of *mof2*. *Cg1894* codes for the protein Mof2 and is located on the third chromosome.

F₀ generation

$$w^{1118}, \frac{PBac [w^+] cg1894^{f06204}}{TM6B, Tb^1} \times w^{1118}, \frac{cg1894}{cg1984}$$

F₁ generation

$$\underbrace{w^{1118}, \frac{PBac [w^+] cg1894^{f06204}}{cg1894}}_{red\text{-}eyed} \text{ or } \underbrace{w^{1118}, \frac{cg1894}{TM6B, Tb^1}}_{white\text{-}eyed}$$

The next cross was performed only with the male red-eyed littermates which are heterozygous for PBac[w⁺]*cg1894*^{f06204} and heterozygous $w^{1118}, \frac{PBac [w^+] cg1894^{f06204}}{TM6B, Tb^1}$ females that are balanced with TM6B.

F₂ generation

$$\underbrace{w^{1118}, \frac{PBac[w^+]cg1894^{f06204}}{PBac[w^+]cg1894^{f06204}}}_{red\text{-}eyed} \quad \text{or} \quad \underbrace{w^{1118}, \frac{PBac[w^+]cg1894^{f06204}}{cg1894}}_{red\text{-}eyed} \quad \text{or} \quad \underbrace{w^{1118}, \frac{cg1894}{TM6B, Tb^1}}_{white\text{-}eyed, tubby} \quad \text{or} \\
 \underbrace{w^{1118}, \frac{PBac[w^+]cg1894^{f06204}}{TM6B, Tb^1}}_{red\text{-}eyed, tubby}$$

The flies with the balancer chromosome can be distinguished from the others by the tubby appearance of the larvae.

The next cross was performed as single-cross to differentiate between flies that are heterozygous or homozygous for $PBac[w^+]cg1894^{f06204}$ and have no balancer chromosome. To this end, red-eyed male flies were crossed to $w^{1118}, \frac{PBac[w^+]cg1894^{f06204}}{TM6B, Tb^1}$ females. Two different cases can be distinguished.

F_{3a} generation (with homozygous father)

$$\underbrace{w^{1118}, \frac{PBac[w^+]cg1894^{f06204}}{PBac[w^+]cg1894^{f06204}}}_{red\text{-}eyed} \quad \text{or} \quad \underbrace{w^{1118}, \frac{PBac[w^+]cg1894^{f06204}}{TM6B, Tb^1}}_{red\text{-}eyed, tubby}$$

If the tested fly is homozygous for $PBac[w^+]cg1894^{f06204}$ all of the littermates are red-eyed.

F_{3b} generation (with heterozygous father)

$$\underbrace{w^{1118}, \frac{PBac[w^+]cg1894^{f06204}}{cg1894}}_{red\text{-}eyed} \quad \text{or} \quad \underbrace{w^{1118}, \frac{PBac[w^+]cg1894^{f06204}}{PBac[w^+]cg1894^{f06204}}}_{red\text{-}eyed} \quad \text{or} \\
 \underbrace{w^{1118}, \frac{cg1894}{TM6B, Tb^1}}_{white\text{-}eyed, tubby} \quad \text{or} \quad \underbrace{w^{1118}, \frac{PBac[w^+]cg1894^{f06204}}{TM6B, Tb^1}}_{red\text{-}eyed, tubby}$$

If the tested fly is heterozygous for $PBac[w^+]cg1894^{f06204}$, the tubby littermates are red- and white-eyed at a ratio of 1:1.

No difference between males and females was expected.

2.4 Molecular cloning

2.4.1 Vectors

For the cloning of different PCR products, the pGEMT-T (Promega) vector system was used according to the manufacturer's specifications. The vectors with the PCR fragment of interest were sequenced (GATC biotech). For the expression of recombinant proteins, the vector was digested and the DNA of interest was cloned directly into pET15b or pET41 (modified by the group of Beyer at the ZMB at the University of Essen). For the construction of the pUAST-Mof2 vector two different fragments were subsequently inserted into the pUAST-vector.

2.4.2 PCR

PCR was performed according to standard procedures (Mullis and Faloona 1987). If not mentioned otherwise, Vent (NEB) or Phusion polymerase was used for the PCR according to the manufacturer's specifications and purified by gel extraction (Qiagen). If the PCR product was to be ligated into the pGEM-T easy vector (Promega), the PCR products were incubated with *Taq* polymerase (NEB) for 10 min at 72 °C.

2.4.3 Oligonucleotides

Table 3: Oligonucleotides used in this study

Oligonucleotides	Sequence (5'→ 3' direction)	Application
<i>mof2</i> -ab-NdeI-fw	cgtcatatgaaggaatcctccgcgactcg	cloning of <i>mof2</i> in pET-15b for antibody generation
<i>mof2</i> -ab-BamHI-rv	tagggatccgtttccagaaagagctttgaca ttaggc	cloning of <i>mof2</i> in pET-15b for antibody generation
<i>mof2</i> -2 nd -exon-NdeI-fw	acgccatggctaatgccaaggaatcctccg	cloning of the 2 nd exon of <i>mof2</i> and the core domain of Mof2 in pET41
<i>mof2</i> -2 nd -exon-NotI-rv	actgcggccgcgggtgtcttccaggagaggc	cloning of the 2 nd exon of <i>mof2</i> in pET41
<i>mof2</i> -core-NotI-rv	actgcggccgccttgcgagagatctcgta	cloning of the core domain of

		Mof2 in pET41
<i>mof2</i> -full-NdeI-fw	cagcatatgatggacaaggaaaacagatcaggtga	cloning of the full-length DNA sequence of Mof2 in pGEMT-easy
<i>mof2</i> -full-BamH1-rv	tagggatccctactttcgtcctgt	cloning of the full-length DNA sequence of Mof2 in pGEMT-easy
<i>mof2</i> -full-BamHI-fw	catggatccatgcatcaccatcaccatcacatggacaaggaaaacagatcaggtga	cloning of the full length DNA sequence of Mof2 in pFL
<i>mof2</i> -full-Sall-rv	catgtcgacttatcgtcctgtgggtgtctcc	cloning of the full length DNA sequence of Mof2 in pFL
dsRNA-1,2-rv	taatacgaactcactatagggggccatcgacctcgtagata	RNAi construct 1 and 2 for S2 cells
dsRNA-2-fw	taatacgaactcactataggggaagtccgaaatggggaaatc	RNAi construct 2 for S2 cells
dsRNA-3-rv	taatacgaactcactataggacattaggcagaggcactgg	RNAi construct 3 for S2 cells
dsRNA-1,3-fw	taatacgaactcactatagggtaaagtatcagccgttgggc	RNAi construct 1 and 3 for S2 cells
dsRNA-4-rv	taatacgaactcactatagggactttcgtcctgtgggtgt	RNAi construct 4 for S2 cells
dsRNA-4-fw	taatacgaactcactatagggccctatatgcccgaaggata	RNAi construct 4 for S2 cells
RNAi1-EcoRI-fw	catgaattcaaggaatcctccgactcg	first fragment of <i>mof2</i> that was cloned into pUAST for RNAi in flies
RNAi1-NotI-rv	gtagcggccgcaagacgtggctcgcgatctcgttaa	first fragment of <i>mof2</i> that was cloned into pUAST for RNAi in flies
RNAi2-KpnI-fw	ctaggtaccaaggaatcctccgactcg	second fragment of <i>mof2</i> that was cloned into pUAST

		for RNAi in flies
RNAi2-NotI-rv	taggcggccgcattcacaacgtaaattggtg gtcgc	second fragment of <i>mof2</i> that was cloned into pUAST for RNAi in flies
<i>actin</i> -rtPCR-fw	gctcctcgttgagaagtctacgagc	quantification of <i>actin</i> transcripts
<i>actin</i> -rtPCR-rv	gtacttgcgctctggcggggc	quantification of <i>actin</i> transcripts
<i>mof2</i> -rtPCR-fw	gacgggataagttcgaaattgccg	quantification of <i>mof2</i> transcripts
<i>mof2</i> -rtPCR-rv	cttgagcttcgatgcacgatcc	quantification of <i>mof2</i> transcripts
<i>cg6479</i> -fw	gtgtctccgctaaggtatcttgagc	ChIP-Seq verification
<i>cg6479</i> -rv	tagaattcagaaaattaacctgaatgctc	ChIP-Seq verification
<i>Rca1</i> -fw	ggagtctacgattaactgtcagattcca	ChIP-Seq verification
<i>Rca1</i> -rv	Ctattatcggcgcgcggc	ChIP-Seq verification
<i>Orc2</i> -fw	taattcccgcacttctgag	ChIP-Seq verification
<i>Orc2</i> -rv	Gattcactgcattccaac	ChIP-Seq verification
<i>CycA</i> -fw	Cgtcatggttctctttgtcg	ChIP-Seq verification
<i>CycA</i> -rv	Ttctgttccttcagcaggt	ChIP-Seq verification
<i>Cg5966</i> -fw	Gagatattcggctccacca	ChIP-Seq verification
<i>Cg5966</i> -rv	tcgaaaccgtaaccgaaatc	ChIP-Seq verification
<i>E2F</i> -fw	ggcaacttttcgaaggcagt	ChIP-Seq verification
<i>E2F</i> -rv	aacttggacatttcgctgcc	ChIP-Seq verification
<i>Osa</i> -fw	tgttggtattggtgctgc	ChIP-Seq verification
<i>Osa</i> -rv	accaacaacaacaacgctga	ChIP-Seq verification
<i>Wts</i> -fw	ctgtgctgtgcgagtgttag	ChIP-Seq verification
<i>Wts</i> -rv	accaggaagaacaaccca	ChIP-Seq verification
background-fw	caatgatcctgcattgtaacca	ChIP-Seq verification
background-rv	tgatggcaattgaaaccatc	ChIP-Seq verification

2.4.4 Plasmids

Table 4: Plasmids used in this study

Name	Source	Special features/Application
pGEM-T easy	Promega	T/A end cloning, blue-white selection, Amp resistance
pGEM-T-Mof2	This work	Full length sequence of Mof2 derived from cDNA and subsequent PCR, NdeI and BamHI restriction sites were introduced
pET15-b	AG Ehrenhofer-Murray	Expression vector, IPTG inducible protein expression, Amp resistance, N-terminal His-tag
pET15-b-Mof2-ab	This work	<i>Mof2</i> sequence corresponds to aa 39-221 of Mof2, used for the overexpression of Mof2 for antibody generation
pET41	AG Bayer	Expression vector, C-terminal GST-Tag, N-terminal His-Tag, Kanamycin resistance
pET41-Mof2-2-exon	This work	<i>Mof2</i> sequence corresponds to aa 36-418 of Mof2, used for the overexpression of the second exon of Mof2, used for the acetylation assay
pET41-Mof2-core	This work	<i>Mof2</i> sequence corresponds to aa 36-308 of Mof2, used for the overexpression of the core domain of Mof2, used for the acetylation assay
pUAST	AG Ehrenhofer-Murray	Modified P-element with Gal4/UAS binding site and mini <i>white</i> gene, Amp resistance
pUAST-Mof2	This work	Inverted repeats of 189-552 bp

		of <i>mof2</i> , used for the generation of fly strains with inducible knock-down of Mof2
pFL	AG Meyer	Mini Tn7 transposon, Amp resistance, two MCSs
pFL-Mof2	This work	Derived from pGEM-T-Mof2, used for the integration of the Mof2 sequence into the genome of the baculovirus

The primers and restriction sites used for the generation of the plasmids are listed in chap. 2.4.3 “Oligonucleotides”.

2.5 DNA and RNA

2.5.1 Isolation of genomic DNA

The isolation of genomic DNA from flies was performed to the protocol of Sullivan and colleagues (Sullivan et al 2000). Thirty anesthetized flies were ground in 400 µl extraction buffer (100 mM Tris-HCl, 100 mM EDTA, 100 mM NaCl, 0.5 % SDS, pH 7.5) until only cuticles were visible. The samples were incubated for 30 min at 65 °C. Then, 800 µl precipitation buffer (5 M potassium acetate, 6 M lithium chloride mixed 2:5) was added, carefully mixed and incubated for at least 10 min at -20 °C. The samples were centrifuged and the supernatant was mixed with the 0.6 fold volume of isopropanol. The precipitated DNA was pelleted and washed with 70 % ethanol. The pellet was solved in 150 µl sterile water.

2.5.2 ChIP-Seq

The preparation of DNA fragments that were obtained by a conventional ChIP for sequencing was performed with the NEXTflex ChIP-Seq Kit (Bioo life science) according to the manufacture’s instructions.

For this purpose, several steps of DNA treatment were necessary. First, the DNA ends were filled-up with a PCR in order to obtain completely double-stranded DNA. Second, the adenylation of the 3’ end of the DNA was performed. Third, adaptors containing unique barcode sequences were ligated to the different samples. Fourth,

the DNA was amplified via PCR. The DNA was analysed on an Agilent HS chip (Biochip lab at the Universitätsklinikum Essen) and sequenced on an Illumina platform.

2.5.3 RNA purification from fly tissue or cells

Total RNA from flies was isolated with peqGOLD TriFast (peqlab). For the isolation, 0-24 h old flies were used. They were frozen in liquid nitrogen and ground either in an Eppendorf tube or in a mortar, depending on the amount of flies. The powder was mixed with peqGOLD TriFast, and the RNA was isolated according to the manufacturer's instructions. The RNA was precipitated with the 0.5-fold volume of isopropanol at -20 °C over night. The RNA was washed with 75 % ethanol, air-dried and dissolved in RNase-free water.

If RNA was isolated from small amounts of fly tissue or cells, the column-based kit of Macherey Nagel was used according to the manufacturer's instructions.

2.5.4 cDNA synthesis

Before cDNA synthesis, the RNA was treated with DNase (NEB) according to the manufacturer's recommendations and inactivated for 10 min at 70 °C. To avoid RNA degradation, EDTA was added to a final concentration of 5 mM. The RNA was tested for persisting contamination with genomic DNA with a conventional PCR for *actin* genes.

The cDNA-synthesis was performed with the superscript III first-strand synthesis system for RT-PCR kit (Invitrogen) with random hexamers. A conventional PCR was performed to estimate the quality and quantity of the cDNA. The possible residual contamination with genomic DNA was estimated with the primers that were used for the quantification of *mof2* transcripts. These primers hybridize to the two different exons of *mof2*, and therefore, the PCR product derived from genomic DNA is 70 bp longer than that of the cDNA.

2.5.5 Real-time PCR

Real-time PCR was used for the semiquantitative analysis of transcripts or binding sites of Mof2 on the DNA.

The relative quantification of Mof2 in male and female flies was normalised to the expression of *actin*. The time point of RNA isolation was between 0 to 24 hours after eclosion in order to avoid effects due to changes in *actin* expression during

development. For the analysis of Mof2 binding sites a ChIP with the preimmune serum was performed as negative control and a standard curve with the input of the ChIP was used.

The qPCR was performed with the perfecta sybr green mix (Quanta Bioscience) in a Rotor Gene 3000 (Corbett). The primers were chosen such that the resulting PCR product had a size between 150 bp and 300 bp. The samples were cycled 45 times for 15 sec at 94 °C, 30 sec at 60 °C and 40 sec at 68 °C.

2.6 Antibodies

2.6.1 Antibody generation

The antibodies against Mof2 were raised against the recombinant protein Mof2-ab. Mof2-ab corresponds to the amino acids 39-288 of the annotated full-length protein (www.flybase.org). The resulting recombinant protein was expressed and purified as described. After purification under denaturing conditions, the protein was dialysed against the lysis buffer with 50 % (v/v) glycerine over night at 4 °C. The buffered protein solution was used for immunization and polyclonal Mof2-specific antibodies were raised in two different rabbits (Pineda).

2.6.2 Affinity purification of antibodies

To reduce the background staining for embryos the antibodies were affinity purified using a membrane-based approach. For this purpose, the recombinantly expressed fragment Mof2-ab of Mof2 was used. The fragment was expressed and purified as described. The lysate was separated on a SDS-PAGE and transferred to a nitrocellulose membrane. The part of the membran carrying the Mof2-fragment was excised and incubated with blocking solution (2 % milk powder in the TBS-T) for 1 h at room temperature. The membrane was preeluted once with glycine buffer (5 mM glycine, 500 mM NaCl, 0.01% BSA, 0.05% Tween-20) and once with 50 mM Triethylamine. Between the elution steps, the membrane was washed with TBS-T. The membrane was incubated with blocking solution for 30 min at room temperature. After washing with TBS-T, the membrane was incubated with approx. 2 mg of the antiserum over night at 4 °C. The protein concentration of the antiserum was measured with a Bradford solution according to the manufacturer's instructions (Sigma).

Before the elution of the antibody, the membrane was washed with distilled water. The antibody was eluted with 5 ml glycine buffer twice for 20 s. The eluate was immediately neutralised with Tris-HCl pH 8.2. Thereafter, the elution was performed with 50 mM Triethylamine. The eluates were neutralised with Tris pH 6.8 and HCl. The eluates were concentrated with Centricons (Millipore) and rebuffered with PBS according to the manufacturer's instructions.

2.6.3 Antibodies

Table 5: Plasmids used in this study

Name	Source/host	Application
Mof2-1	This work, polyclonal, rabbit	Western blot (1:10000 to 1:25000), IP, ChIP
Mof2-2	This work, polyclonal, rabbit	Western blot (1:10000 to 1:25000), IP, ChIP, staining of salivary glands (1:1000), embryos (1:1500), testes (1:1500)
PI-Mof2-1	This work, preimmune serum, rabbit	Control for Mof2-1 in ChIP and the IP
PI-Mof2-2	This work, preimmune serum, rabbit	Control for Mof2-2 in ChIP and immunostainings
DREF	Kindly provided by Prof. V. Corces (published in Gurudatta et al 2013), rabbit	Co-IP with Mof2 (1:1000)
Z4	Kindly provided by Prof. H. Saumweber (published in Saumweber et al 1980), monoclonal, mouse	Costaining with Mof2-2 on polytene chromosomes (used without diluting)
Poly-His	Sigma (Catalogue No. H1029), monoclonal, clone His-1, mouse	Western blots with recombinantly expressed Mof2 (1:3000)
RNA-Polymerase II	Millipore (Catalogue No.	Costaining with Mof2-2 on

	CBL221), monoclonal antibody, clone ARNA-3, mouse	polytene chromosomes (1:200)
Tubulin	Abcam (Catalogue No. ab7291), monoclonal, clone DM1A, mouse	Loading control in Western blots (1:8000)

The secondary antibodies for Western blots were derived from goat, raised against mouse and rabbit, coupled with horseradish peroxidase and used in a dilution of 1:10000. The secondary antibodies for immunostainings were coupled with Alexa 555 or Alexa 488 and used in a dilution of 1:1000.

2.7 Protein expression and purification

2.7.1 Protein expression and purification in *E.coli*

If not mentioned otherwise, the protein expression in *E. coli* was induced at $OD_{600nm} \approx 1.0$ with 1 mM IPTG for 1 h to 3 h at 37 °C. For the purification of proteins with a his-tag, Ni²⁺-NTA beads were used (Invitrogen). The Mof2-ab protein was purified under denaturing conditions with urea according to the manufacturer's instructions. The main fraction of the protein was eluted at pH 4.5.

The expression of the Mof2-core-domain and Mof2-2nd-exon was induced at $OD_{600nm} \approx 0.8$ with 1 mM IPTG for 1.5 h – 2 h at 30 °C. The purification was done under native conditions at 4 °C according to the manufacturer's instructions. The lysate was incubated with the beads in the lysate buffer containing 20 mM imidazole. The beads were washed with the lysing buffer that contained 60 mM imidazole. The elution of the protein was performed with 150 mM imidazole.

2.7.2 Integration of *mof2* into the baculovirus genome and purification of genomic DNA from *E. coli*

The Mof2 sequence was integrated into the baculovirus genome via a transposition of a mini Tn7 transposon. For this purpose, the EMBacY cells contain the baculovirus genome as a bac and a helper plasmid that encodes for the Tn7 transposase. The bac contains an attTn7 sequence, which is the acceptor sequence of the Tn7 transposase. The Tn7 transposase recognises the Tn7L and Tn7R sites of the mini Tn7 transposon

that is encoded on the pFL vector and integrates these sites into the bac sequence (Bieniossek et al 2012; Fitzgerald et al 2006).

The pGEMT-Mof2 vector with the whole coding sequence of Mof2 was used as a template to generate the N-terminal His-tagged variant in the pFL vector. The tag, the translational start and stop codon and restriction sites were introduced by PCR. The pFL vector and the constructed pFL-Mof2-NT vector were exclusively amplified in the Top 10 *E. coli* strain. The transformation of the pFL-Mof2-NT vector into the EMBacY cells was performed by a heat shock. Then, the cells were incubated for 6 h at 37 °C and slow shaking. The cells were plated on agar plates containing the appropriate antibiotics and 40µg/ml IPTG with 100µg/ml X-Gal for the blue-white selection of clones with the Mof2-NT sequence integrated into the bac. The cells were incubated for 24 h at 37 °C until white and blue colonies could be distinguished. The genomic DNA was isolated from positive clones. For this purpose, the cells were resuspended in suspension buffer (250 µl of 50 mM Tris-HCl, 10 mM EDTA, 100 µg/mL RNase A, pH 8.0), lysed (250 µl of 1% SDS, 0.2 M NaOH) and the proteins were precipitated (300 µl of 3.0 M potassium acetate, pH 5.5). The genomic DNA with the bac was precipitated with 700 µl isopropanol at -20 °C for at least 48 h. The precipitated DNA was dissolved in sterile TE buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA) and kept at 4 °C.

2.7.3 Protein expression in Sf9 cells

The Sf9 cells were maintained at 27 °C in SF900 III medium. For the transfection with the baculovirus, 10⁶ cells per ml/well were used in a six well plate. The cells were incubated with 200 µl medium mixed with 20 µl of the genomic DNA with the Bac that contains the baculovirus genome with the integrated coding sequence of Mof2 and 5 µl FuGene HD transfection reagent. The plate was incubated for 4 days at 27 °C. Afterwards, the cells and the supernatant were mixed with 10 ml medium with 10⁶ cells/ml and incubated for 4 days at 27 °C for initial virus amplification. The supernatant with the baculovirus generation V₀ was supplemented with 10 % FCS and stored at 4 °C in the dark.

The V₁ generation of the baculovirus was performed in 50 ml with 10⁶ cells/ml and 2 ml of the V₀ generation of the baculovirus in a 500 ml flask. The cells were incubated at 27 °C and 115 rpm. The supernatant with the baculovirus generation V₁ was supplemented with 10 % FCS and stored at 4 °C in the dark.

With the V₁ generation of the baculovirus, again 50 ml with 10⁶ cells/ml and 2 ml of the V₁ generation of the baculovirus were incubated in a 500 ml flask for 4 days at 27 °C and 115 rpm. The infected cells were tested for protein expression with a pull-down experiment.

2.7.4 Protein expression test in Sf9 cells

The Sf9 cells were pelleted and resuspended in cold PBS and pelleted again (5 min, 1000 x g, 4 °C). The cells were resuspended in purification buffer (50 mM HEPES, 150 mM KCl, 2 mM MgCl₂, 20 mM imidazole, 5 % glycerol, pH 8.0) and sonicated with 20 pulses at 40 % intensity. The lysate was cleared by centrifugation (15 min, 15000 x g, 4 °C) and incubated with Ni²⁺-NTA beads (Qiagen) for 30 min at 4 °C. The beads were washed five times with 5 ml of the purification buffer. The protein is eluted two times with elution buffer (purification buffer with 300 mM imidazole). The eluates were tested for protein expression with a SDS-PAGE and Western blotting.

2.7.5 Protein purification from Sf9 cells

The 300 ml Sf9 cells were pelleted, washed with cold PBS and resuspended in 20 ml purification buffer (50 mM HEPES, 150 mM KCl, 2 mM MgCl₂, 20 mM imidazole, 5 % glycerol, 0.5 mM DTT, pH 8.0). The cells were sonicated with 10 pulses at 40 % intensity and the lysate was cleared by centrifugation (15 min, 15000 x g, 4 °C). Prior to affinity chromatography with Ni²⁺-beads, the lysate was filtrated. The lysate flow rate was approx. 1.5 ml/min. The column was washed with 600 ml purification buffer with a flow rate of approx. 3 ml/min. The protein was eluted with 20 ml elution buffer (purification buffer with 300 mM imidazole) and concentrated with Centricons (Millipore) with a nominal molecular weight limit of 10 kDa.

2.7.6 SDS-PAGE and Western blot

For the separation of proteins, SDS-PAGE with a Tris-glycine buffer was used as described by Shapiro and colleagues (Shapiro et al 1967). The concentration of acrylamide varied between 8 % and 15 % depending on the protein of interest. The proteins were visualised by staining the SDS-PAGE with a coomassie blue solution (50 % methanol, 10 % glacial acetic acid, 0.1 % Coomassie Brilliant Blue R-250) and subsequently removing the unspecific staining (50 % methanol, 10 % glacial acetic acid).

The transfer of proteins to nitrocellulose membranes was accomplished by blotting with the BIO-RAD Tank Transfer System in transfer buffer (39 mM glycine, 48 mM Tris base, 0.037 % SDS, 20 % Methanol). The membranes were subsequently incubated with blocking solution (TBS-T with 5 % milk powder) for 1 h at room temperature or over night at 4 °C, primary antibody solution (blocking solution with antibody in the indicated dilution) over night at 4 °C, twice with TBS-T for at least 10 min, secondary antibody solution (TBS-T with the appropriate antibody that was coupled with horse radish peroxidase) and twice with TBS-T. For signal detection, Amersham ECL Western Blotting Analysis System (GE Healthcare) and Amersham Hyperfilm ECL chemiluminescence films (GE Healthcare) were used.

Antibodies on a nitrocellulose membrane can be removed in different ways. A quite harsh method is the incubation with 10 % acetic acid for 10 min at room temperature and subsequent intensive washing with PBS. This method was used if a strong signal for the second antibody was expected. A less harsh method was performed with 2-mercaptoethanol. For this purpose, the membrane was incubated with stripping buffer (100 mM 2-Mercaptoethanol, 2 % SDS, 62.5 mM Tris-HCl pH 6.7) for 30 min at 50 °C. Then, the membrane was washed carefully with PBS. The stripped blots were reprobated as described for Western blots above.

2.7.7 Immunoprecipitation (IP)

The physical interaction of DREF and Mof2 was analysed by an IP. If not mentioned otherwise, all steps were performed on ice or at 4 °C.

Therefore, 100 pairs of salivary glands of L3 larvae were dissected for each sample in PBS-PI (PBS with Roche complete proteinase inhibitor, EDTA-free), lysed for 10 min by addition of Triton X-100 to a final concentration of 0.1 % and sonicated five times (10 sec ON, 30 sec OFF). After a brief centrifugation, BSA was added to a final concentration of 1 mg/ml and incubated with protein A sepharose beads for 2 h. The precleared lysates were incubated with the indicated serum overnight. Then, the lysates were incubated with protein A sepharose for 4 h at 4 °C and agitation. The beads were washed five times with PBS-PI and incubated with 2x SDS-loading buffer (200 mM DTT, 4 % SDS, 20 % glycerol, 0.2 % bromophenol blue, 100 mM Tris-Cl pH 6.8) for 5 min at 95 °C. The samples were analysed by SDS-PAGE and Western blot analysis with the α -DREF antibody.

2.8 Chromatin immunoprecipitation (ChIP)

The ChIP with antibodies against Mof2 was performed in order to identify potential binding sites of this protein on DNA in S2 cells. It was essentially performed as described (Wu et al 2003) with some modifications. After cross-linking of the DNA-binding proteins and the DNA for 10 min at room temperature, the cells were lysed (1 % SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1) for 10 min at 4 °C. Then, the lysate was sonicated until the DNA had the desired size. The DNA fragments had either a size of around 500 bp for subsequent qPCR experiments or of around 200 bp for subsequent ChIP-Seq experiments. The lysates were diluted 10-fold with immunoprecipitation buffer (0.01 % SDS, 1.1 % Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.1, 16.7 mM NaCl, Roche complete proteinase inhibitor) and precleared with 20 µl of 50 % prewashed protein A sepharose beads for 1.5 h. The precleared lysate was incubated with the indicated non-purified or concentrated serum at 4 °C over night. Protein A sepharose beads were added and incubated for 5 h at 4 °C. The beads were washed five times first with low salt buffer (0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, 150 mM NaCl, pH 8.1) followed by a high salt buffer (0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, 500 mM NaCl, pH 8.1) and a lithium buffer (0.25 M LiCl, 1 % NP-40, 1 % sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1). The last two wash steps were performed with TE buffer. After the washing steps, the beads were incubated with elution buffer (1 % SDS, 0.1 M NaHCO₃) twice at room temperature for 15 min to elute the chromatin. The cross-linking of the DNA and the proteins was reversed under high salt conditions (NaCl added to a final concentration of 200 mM) at 65 °C over night. Contaminating RNA was removed by RNase treatment for 1 h at 37 °C. The remaining proteins were degraded by proteinase K after adjusting the appropriate buffer conditions (10 mM EDTA, 40 mM Tris-HCl, pH 6.5) and incubated at 42 °C for 1 h. The DNA was isolated either with phenol/chloroform extraction according to standart procedures and a subsequent ethanol precipitation or with a column based approach (Machery Nagel) according to the manufacturer's instructions. The purified DNA was dissolved in water for further experiments.

2.9 Histone acetylation assay

The Mof2 protein is annotated as a histone H4 acetyltransferase with a specificity for lysine 16. The acetylation activity of the recombinantly expressed protein was tested in an acetylation assay. For this purpose, the purified Mof2 fragment or full-length protein was incubated with histone H4 that was purified from *E. coli* (Upstate) and ^3H -acetyl-coenzyme A (American radiolabeled chemicals). One reaction with a total volume of 25 μl contained 1 μg histone H4, 0.5 μCi ^3H -acetyl-coenzyme A and up to 2 μg purified protein in the reaction buffer (100 mM NaCl, 50 mM Tris-HCl, pH 7.4). The reaction was incubated for 45 min at 30 °C. Subsequently, the solution was spotted on p81 paper (Upstate). The dried filters were washed 3 times with 0.1 mM NaHCO_3 at pH 9.0 for 5 min and once with acetone. Radioactivity was counted in scintillation fluid for 10 min. Recombinant human PCAF (Upstate) was used as a positive control for the assay and used in similar concentrations as purified Mof2. Human PCAF is a known acetyltransferase that acetylates histone H3 and to a lesser extent histone H4 at different lysine residues (Galvez et al 2011).

2.10 Immunohistochemistry

2.10.1 Preparation and fluorescent staining of polytene chromosome staining

The staining of polytene chromosomes was educed in the lab of Prof. H. Saumweber from his former work (Saumweber et al 1980). According to this preparation, both formaldehyde and acetic acid are used. In this manner, the proteins are well fixed and the chromosomes can still be well squashed. The polytene chromosomes were obtained from salivary glands of wandering 3rd instar larvae. The glands were dissected in preparation solution (15 mM Tris-HCl, 60 mM KCl, 15 mM NaCl, 0.5 mM spermidine, 0.15 mM spermine, pH 7.4) with 1 % triton on a slide. Subsequently, there were two fixation steps. First, the glands were incubated in the fixation solution (1 % Triton-X 100, 3.7 % formaldehyde in preparation solution) for 30 sec. Second, they were transferred to the squash solution (3.7 % formaldehyde, 50 % acetic acid) and incubated for 3 to 4 min. Then, the glands were squashed and tapered with a blunt forceps. The quality of the squashes was judged under the microscope with a phase contrast filter. The squashed glands were frozen in liquid nitrogen and stored in ethanol at -20 °C. The storage did not exceed 10 days.

The preparations were rehydrated in preparation solution two times for 15 min at room temperature. Then, the squashes were incubated with the primary antibody in RF-20 which is a protein-rich calf serum or 3 % BSA in preparation solution over night at 4 °C. The squashes were washed with the preparation solution at least twice. The fluorescent secondary antibody was diluted with RF-20 or 3 % BSA in preparation solution and incubated for 4 h at room temperature. After washing with preparation solution DAPI staining was performed. Therefore, the squashes were incubated with 0.04 µg/ml DAPI and mounted with Moviol.

2.10.2 Preparation and fluorescent staining of *Drosophila* embryos

Embryos were collected at the indicated times after laying and washed off the collecting plates (apple juice with 2.5 % bacto agar and freshly added yeast paste) with distilled water and washed. Then, the embryos were dechorionated with 2.8 % sodium hypochlorite for 3 min and fixed in 5 ml fixation buffer (4 % formaldehyde, 1 % Tween 20 in PBS) with equal amounts of heptane for 25-30 min with agitation. The aqueous phase was removed and 5 ml methanol was added. The embryos were mixed vigorously for 30 sec. The devitellinized embryos sank to the bottom of the container and were transferred to a new container. Then, embryos were washed with methanol at least three times in order to remove residual heptane and stored at -20 °C in methanol.

The stored embryos were rehydrated in PBS-T (0.3 % Triton-X 100 in PBS) and blocked with 5 % BSA in PBS-T for 1 h. Then, the antiserum was added at the desired dilution and incubated over night at 4 °C. Then, the embryos were washed four times with PBS-T. The secondary antibody was added and incubated for one hour at room temperature. After washing for four times with PBS-T, DAPI at a concentration of 0.04 µg/ml was added. The stained embryos were stored in 70 % glycerol at -20 °C.

2.10.3 Preparation and fluorescent staining of *Drosophila* testes

The staining was adapted to the work of Hime (Hime et al 1996). The testes of wandering L3 larvae and young adult flies (0-24 h) were isolated in PBS and carefully squashed on a polylysine-coated slide. The squashes were dehydrated in pure ethanol at -20 °C for at least 10 min. The testes were fixed in PBS-F (3.7 % formaldehyde in

PBS) for 7 min to 10 min. The squashes were permalised and incubated with PBS-TD (0.3 % Triton X-100 and 0.3 % sodium deoxycholate in PBS) two times for at least 15 min. Then the testes were washed with PBT (0.1 % Tween 20 in PBS). Subsequently, unspecific binding sites were blocked with PBT-BSA (3 % BSA in PBT) and incubated with the desired serum concentration in PBT-BSA over night at 4 °C. The preparations were washed two times with PBT-BSA and incubated with the secondary antibody that was coupled with an AlexaFluor Dye. The testes were washed with PBT-BSA twice and incubated with Hoechst or DAPI in PBT-BSA for 20 min and washed twice with PBT-BSA.

2.11 Knock-down of *mof2*

2.11.1 *Mof2* knock-down in S2 cells

The knock-down of Mof2 in S2 cells was performed as described (Clemens et al 2000). To establish a knock-down of Mof2 in S2 cell culture, four double-stranded RNAs (dsRNA) corresponding to different parts of Mof2 were synthesized. As a negative control, dsRNA for eGFP was used. The DNA template for the RNA synthesis was generated by PCR. The primers that were used contained T7 transcription start sites. In this way, the 5' and 3' end of the PCR fragment had the T7 polymerase binding site. Therefore, transcription of these DNA templates by the T7 polymerase led to the formation of dsRNA. The RNA synthesis was done according to the manufacturer's instructions (MegaScript, Ambion). After dsRNA synthesis, the DNA template was digested with TurboDNase (Ambion) for 15 min at 37 °C. The obtained dsRNA was precipitated with LiCl, washed with 70 % ethanol and solved in nuclease-free water.

10⁶ SL2 cells were incubated with 10 µg dsRNA in 1 ml serum-free medium with Pen/Strep (50 U/ml) with agitation at room temperature for 10 min and for 50 min without agitation at 26 °C. 2 ml medium with Pen/Strep and FCS to a final concentration of 10 % were added.

2.11.2 *Mof2* knock-down in flies

The knock-down of Mof2 in flies was performed according to Brand and Perrimon (Brand and Perrimon 1993). They established a system in flies for a tissue-specific induction of gene expression. For this purpose, they used the yeast protein Gal4

whose expression is regulated by *Drosophila*-specific promoters. The tissue or/and developmental specific expression of the Gal4 protein can bind to an upstream activating sequence (UAS) and activate the transcription of downstream DNA sequences.

For the knock-down of Mof2 in flies, a modified P-element was used. The P-element contains the UAS and a DNA-sequence with inverted repeats of a part of the DNA sequence of Mof2. When the inverted repeats are transcribed double-stranded RNA is generated and recognised by the RNAi machinery of *Drosophila*. As a consequence the mRNA of *mof2* is degraded and the amount of the Mof2 protein is reduced.

The P-element was randomly inserted into the genome of w1118 flies (BestGene). The mini-white gene served as a marker for a successful integration. Flies that were homozygous for the P-element insertion were crossed with Gal4 expressing strains. Because of the temperature dependent expression of Gal4, the crosses were performed at 23 °C and 29 °C.

2.12 Bioinformatics

The bioinformatical analysis was performed by A. Ehrenhofer-Murray.

The ChIP-seq data were obtained from two independently derived antibodies against Mof2. They were aligned to the *Drosophila* genome with Bowtie 0.12.8 (Langmead et al 2009). Repetitive sequences were not included, and therefore, genome regions like the telomeres were not included in the further analysis.

Peaks were identified with MACS 1.4.2 (Zhang et al 2008). The peaks of the antibodies were compared to the input control. The p-value cut-off was defined as 10^{-5} . Peaks that were present for both antibodies were classified as high-confidence peaks and were used for further analysis.

A *de novo* motif search was performed with the Bioconductor package rGADEM (Droit et al 2014). For this purpose, the motif match algorithm of MotIV with 652 position weight matrices from the FlyFactorSurvey database (Zhu et al, NAR 2011) was used.

Gene ontology was analysed with topGO (Alexa and Rahnenfuhrer 2010).

The binding of Mof2 was compared to binding sites of other known DNA-binding factors and analysed using DiffBind (Stark and Brown 2011).

Table 6: Publicly available data that was used for the bioinformatic analysis of the ChIP-Seq data of Mof2

BEAF-32	modEncode 3745
Chriz	modEncode 278
CP190	modEncode 3748
dCTCF	modEncode 2638
DREF (in Kc cells)	GEO GSE39664 (Gurudatta et al 2013)
Mod(mdg4)	modEncode 3789
Pho-like (in embryos)	Schuettengruber et al 2009
Su(Hw)	modEncode 330

3 Results

3.1 Knockdown of *mof2* in *Drosophila melanogaster*

Mof2 is annotated as a putative MYST histone acetyltransferase. Most of the known MYST histone acetyltransferases have a severe impact on the development that often causes lethality in strains lacking the respective protein. To test a possible effect of Mof2 on the development of *Drosophila melanogaster*, strains with an inducible knockdown of *mof2* were generated. In contrast to a knockout, a knockdown can be performed for different developmental stages and tissues, and to a certain extent, the strength of the knockdown can be varied. For this purpose, the Gal4/UAS system was used as described by Brand and Perrimon.

In this system two different strains are used. One strain contains a modified P-element with the Gal4 upstream activating sequence (UAS) and the genetic element of interest. The second strain is a Gal4 driver line and expresses the yeast specific Gal4 transcriptional activator protein under the control of a *Drosophila* specific promoter. The F₁ generation of a cross of a UAS line and a Gal4 line expresses the genetic element of interest of the UAS line. The exact temporal and spatial expression depends on the chosen Gal4 driver line. The expression can be varied to a certain extent because the expression of the Gal4 transcriptional activator protein increases with temperature with 29 °C as highest temperature without severe effects like male sterility caused by thermal stress.

For the present study, the Gal4/UAS system was used for a knockdown of *mof2*. For this purpose, the genetic element of interest in the UAS line contained inverted repeats of a part of *mof2*. These inverted repeats form a double-stranded RNA (dsRNA) after transcription that is recognised by the natural antiviral response of *Drosophila melanogaster*. The enzyme Dicer subsequently degrades the dsRNA. The resulting RNA fragments bind the RNA-induced silencing complex (RISC) that serves as recognition complex for complementary single stranded mRNA and mediates its degradation by the enzyme Slicer (Kingsolver et al 2013). The degradation of the mRNA is assumed to lead to a reduced protein level.

In order to characterise the knockdown of Mof2 in flies, different Mof2-RNAi lines using the modified P-element for the Gal4/UAS system were generated as described

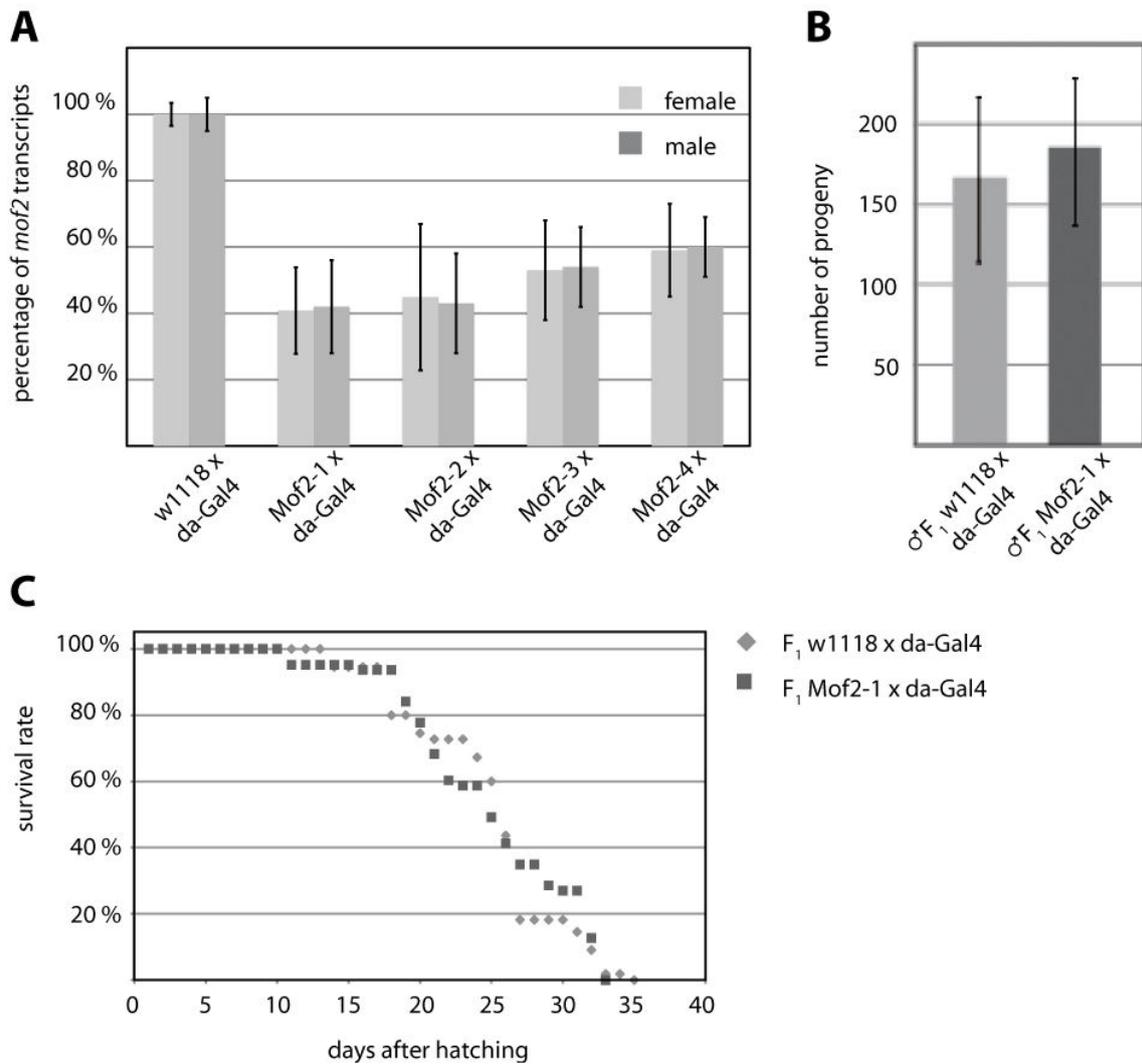


Figure 10: Characterisation of the knockdown of *mof2*

(A) Four different lines with an inducible knockdown of *mof2* were tested for their knockdown efficiency with the da-Gal4 driver line at 29 °C. For males and females, the knockdown efficiency was similar and varied between 40 % and 60 %.

(B) The fertility of males with a knockdown of *mof2* was tested. For this purpose, the males were crossed with three wild type virgins and the progeny was counted. Males without a knockdown served as control. No significant difference in the number of progeny was observed.

(C) The viability of flies with a knockdown was compared to the viability of flies without a knockdown of *mof2* at 29 °C. Neither the life span nor the average life expectancy changed significantly.

in material and methods. Four chosen lines showed no phenotype concerning viability, fertility or development. For further characterisation, the flies were crossed with the da-Gal4 driver line at 29 °C. The da-Gal4 driver line exhibits a ubiquitous and early expression of Gal4 under the control of the daughterless promoter (Caudy et al 1988). The crossing of the da-Gal4 driver line with w1118 served as control because

the expression of the Gal4 transcription activator protein itself can have an effect on behaviour or cell death (Rezaval et al 2007).

The efficiency of the knockdown was quantified with quantitative real time PCR on cDNA, normalised to *actin* transcripts and compared to the control cross (Fig. 10, A). The efficiency of the knockdown was similar for females and males and varied from approx. 40 % for the insertion line Mof2-1 to 60 % for the insertion line Mof2-4. For further experiments the insertion line Mof2-1 was used. For all insertion lines, the knockdown of *mof2* did not show a visible phenotype.

High-throughput data from modENCODE revealed a relatively high transcription level of *mof2* in the testes of adult male flies. Therefore, a knockdown of *mof2* might affect the fertility and subsequently the viability (Maynard and Smith 1958). The fertility of the male flies with a knockdown of *mof2* was tested with crosses with three virgin female wild type flies at 29 °C (Fig. 10, B). No significant difference in the number of progeny was observed. Also the viability of flies with a *mof2* knockdown was comparable to the flies of a control cross (Fig. 10, C).

3.2 Characterisation of the putative knock-out strain of *mof2*

During the course of this study, a not further characterised heterozygous knockout strain of *mof2* became available from the Bloomington stock centre. This strain contains a piggyBac insertion within the promoter site of *mof2* and was reported to be homozygous lethal. Here, the lethality was tested by crossing out the balancer chromosome and crossing the heterozygous males without the balancer chromosome with heterozygous females with the balancer chromosome in order to avoid recombination within the female germ line. If the homozygous genotype is lethal for early developmental stages, approx. 1/4 of the larvae is expected not to hatch. However, crosses at 23 °C and 29 °C exhibit no significant difference of the rate of hatched larvae compared to a control cross with w1118 males indicating that the piggyBac insertion at the promoter site was not lethal.

Due to the finding that the homozygous genotype is not lethal at least during early developmental stages, further crosses were performed as described (methods and material). The homozygous *mof2* knockout strain was viable. In females, and to a lesser extent also in male flies, a cleft on the back was observed (Fig. 11, A). This indicates a developmental defect during the substitution of larval tissue when metamorphosis takes place (Ninov et al 2007).

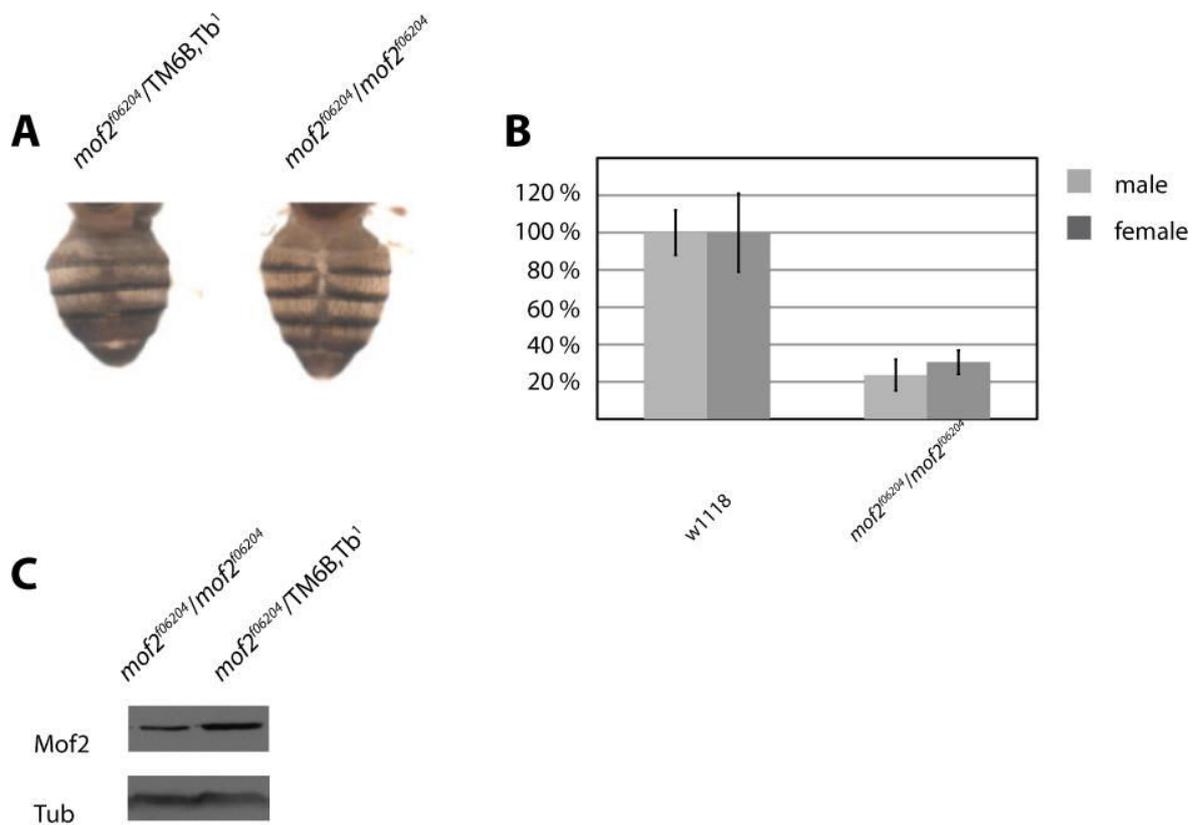


Figure 11: Characterisation of the piggyBac insertion in the promoter of *mof2*

(A) The heterozygous strain with the piggyBac insertion in the promoter of *mof2* is maintained with the homozygous lethal TM6B balancer chromosome. Tubby served as dominant marker of the balancer chromosome. The homozygous strain exhibits a cleft on the back.

(B) The quantitative real time PCR analysis of the transcripts of cDNA from whole RNA extracts revealed a substantial but strongly reduced amount of remaining *mof2* transcripts in the homozygous strain. The transcripts were normalised to *actin*.

(C) The Western blot analysis showed a reduced but still detectable protein level of Mof2 within the homozygous strain. Tubulin served as loading control.

An analysis of the transcripts of *mof2* in the homozygous knockout strain indicated that there was remaining *mof2* transcript (Fig. 11, B). A Western blot analysis of the homozygous strain with an antibody against Mof2 that was generated during this study revealed that the putative knockout strain still expressed Mof2, albeit at a strongly reduced but detectable level (Fig. 11, C). This indicated that the annotated Mof2 knockout strain was not a true knockout strain but rather a hypomorph strain. Therefore, it remains unclear if Mof2 is essential for the viability of *Drosophila melanogaster*.

3.3 Generation of antibodies against Mof2

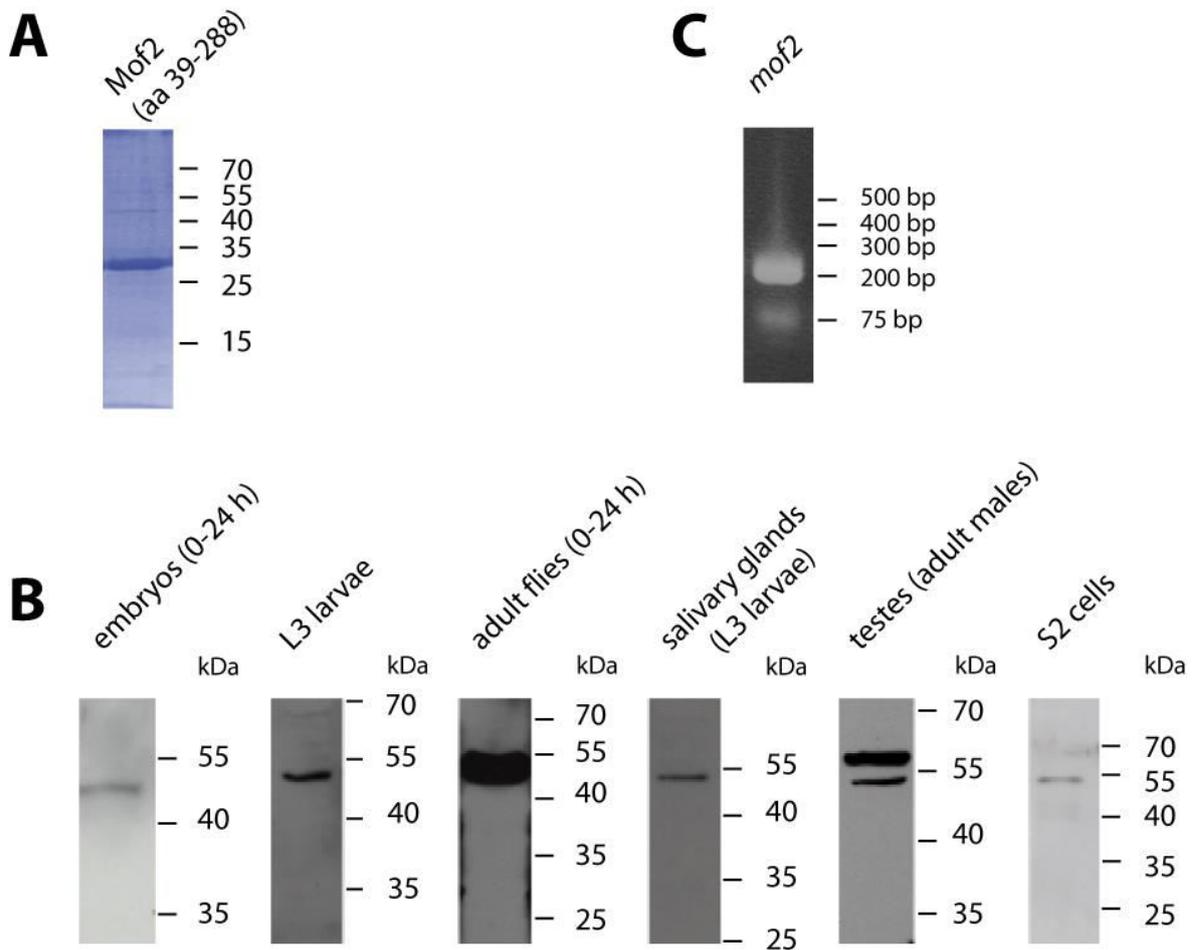


Figure 12: Specificity of the antibody against Mof2 for different developmental stages and tissues

(A) The recombinantly expressed Mof2 fragment (aa 39-288) was purified under denaturing conditions. Its purity was tested on a SDS-PAGE and subsequent staining with Coomassie blue. The calculated molecular weight of the fragment was approx. 28 kDa.

(B) The Western blot analysis indicated that the antiserum raised against Mof2 recognized Mof2 in different developmental stages, in larval salivary glands, testes and Schneider cells line 2 (S2 cells).

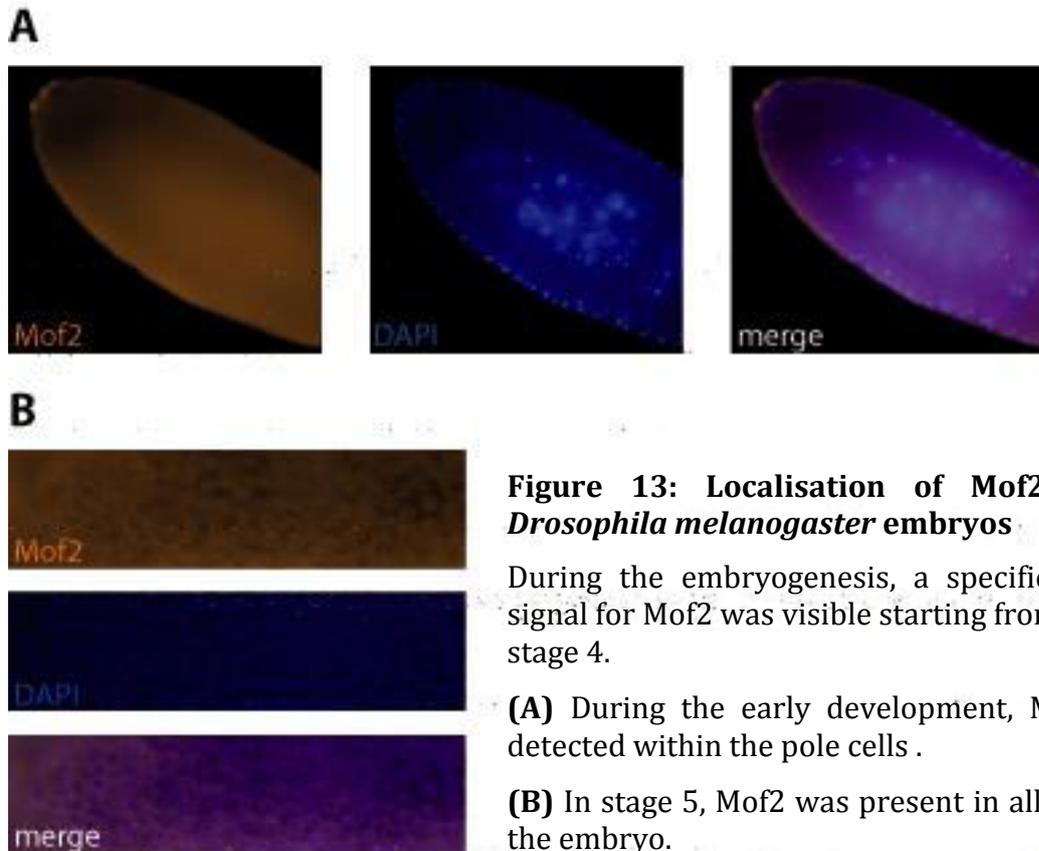
(C) A part of *mof2* without the intron was amplified from DNA derived from mRNA.

For a more precise investigation with regard to the localisation of Mof2 within *Drosophila melanogaster* and possible DNA-binding sites of Mof2, antibodies against a fragment of Mof2 were raised in two different rabbits. The fragment that was used for the immunisation spanned the amino acids 39-288 of the annotated full-length

protein. The protein was recombinantly expressed in *E. coli* and purified under denaturing conditions (Fig. 12, A). The obtained antisera were tested without further purification or concentration with Western blotting for their specificity for different developmental stages and tissues of *Drosophila melanogaster* and the *Drosophila* cell line S2 (Fig. 12, B).

In extracts of embryos, wandering L3 larvae and adult flies, a single specific signal at the expected molecular weight of approx. 49 kDa was detected (Fig. 12, B). This was also seen in salivary glands of wandering L3 larvae and for S2 cells. So far, S2 cells have been reported not to express Mof2. Therefore, the presence of *mof2* transcripts was tested with conventional PCR on cDNA of whole RNA extracts and showed a signal at the expected size (Fig. 12, C). For the testes of adult flies, a relatively weak signal at the expected molecular weight of approx. 49 kDa was detected. In addition, a prominent band at a molecular weight around 57 kDa was seen (Fig. 12, B). The corresponding preimmune serum showed no signal for all tested samples and served as a negative control (not shown).

3.4 Nuclear localisation of Mof2 in embryos



The finding that Mof2 is present in embryos (Fig. 12, B) led to the question from which time point on Mof2 was detectable in flies. To this end, whole embryos were analysed at different early developmental stages for Mof2 expression.

There, starting at stage 4, a specific pattern of Mof2 staining was observed (Fig. 13, A). At stage 4, Mof2 was located prominently within the pole cells. The pole cells are the progenitor cells of the later germ cells. At later stages, Mof2 was also located in almost all nuclei of the embryo (Fig. 13, B). The striking signal in pole cells suggests a function within germ cell development or the development of the gonads in general, whereas the ubiquitous nuclear localisation at later time points suggests a more general function of Mof2 within development. Therefore, Mof2 might have distinct functions.

3.5 Localisation of Mof2 to the interbands of polytene chromosomes

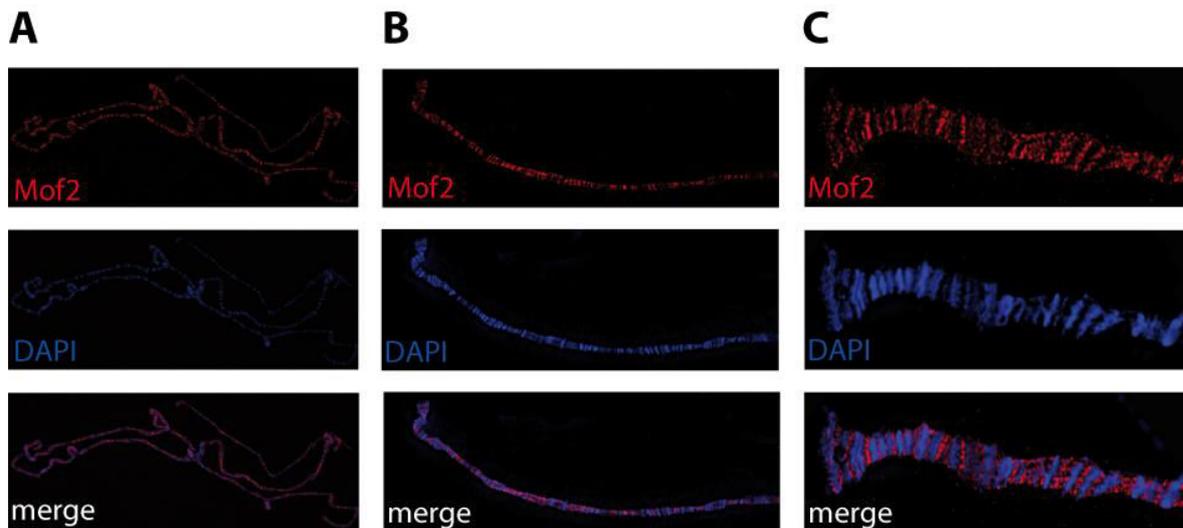


Figure 14: Localisation of Mof2 to the interbands of polytene chromosomes

(A) Mof2 localised to multiple sites on polytene chromosomes of salivary glands of L3 larvae.

(B, C) The binding was observed in interbands and almost completely absent in bands. The pictures were taken with technical help of Prof. H. Saumweber **(A)** and Dr. T. Zielke **(B, C)**. The pictures were taken with 630x **(A and B)** or 1000x magnification **(C)**.

Due to sequence similarities, Mof2 is classified as a putative HAT of the MYST family. These HATs show a prominent nuclear localisation and exhibit binding to DNA. In this way, most of the known MYST HATs in *Drosophila melanogaster* have a severe impact on the regulation of transcription. For Mof2, a nuclear localisation was found for embryos (Fig. 13). For further investigation of the DNA-binding sites of Mof2, the binding of Mof2 to DNA was tested on polytene chromosomes of salivary glands of wandering L3 larvae. There, the polytene chromosomes are highly pronounced due to up to 10 endoreplications. Since the homologous chromatids remain together after endoreplication, a characteristic banding pattern of the DNA is visible as a result of differentially compacted chromatin. The strongly stained bands contain the more compacted heterochromatin, while the less densely stained interbands contain the euchromatin. The euchromatin harbours most of the transcribed genes.

The staining of polytene chromosomes with the antibody against Mof2 showed multiple distinct binding sites for Mof2 (Fig. 14). These binding sites differed in their intensity. Some were found to be sharp and to form a clear band whereas others had a

more blurred appearance. However, all of them were found within the interbands and covered more than 80 % of all interbands (Fig. 14, A). There was no distinct binding within the bands visible. The clear localisation of Mof2 to interbands and therefore to euchromatic regions suggested a general role of Mof2 in gene regulation.

3.6 Telomeric localisation of Mof2 on polytene chromosomes

The binding of Mof2 to interbands on polytene chromosomes was seen reproducibly in intensity and banding pattern. Mof2 binds to euchromatic interbands, which represent a more open chromatin structure with transcribed genes. In addition to the interband binding, there was also a distinct binding to telomeres (Fig. 15, A). At the telomere of the left arm of chromosome 2, a very prominent signal for Mof2 was detected. For the X chromosome, the signal at the telomere was remarkably less robust and not in all preparations of polytene chromosomes of w1118 or Oregon R larvae visible. This may be due to the preparation of the nucleus in that the squashing of the nucleus can lead to a disruption of the telomeres that are tethered to the nuclear envelope (Dernburg et al 1995).

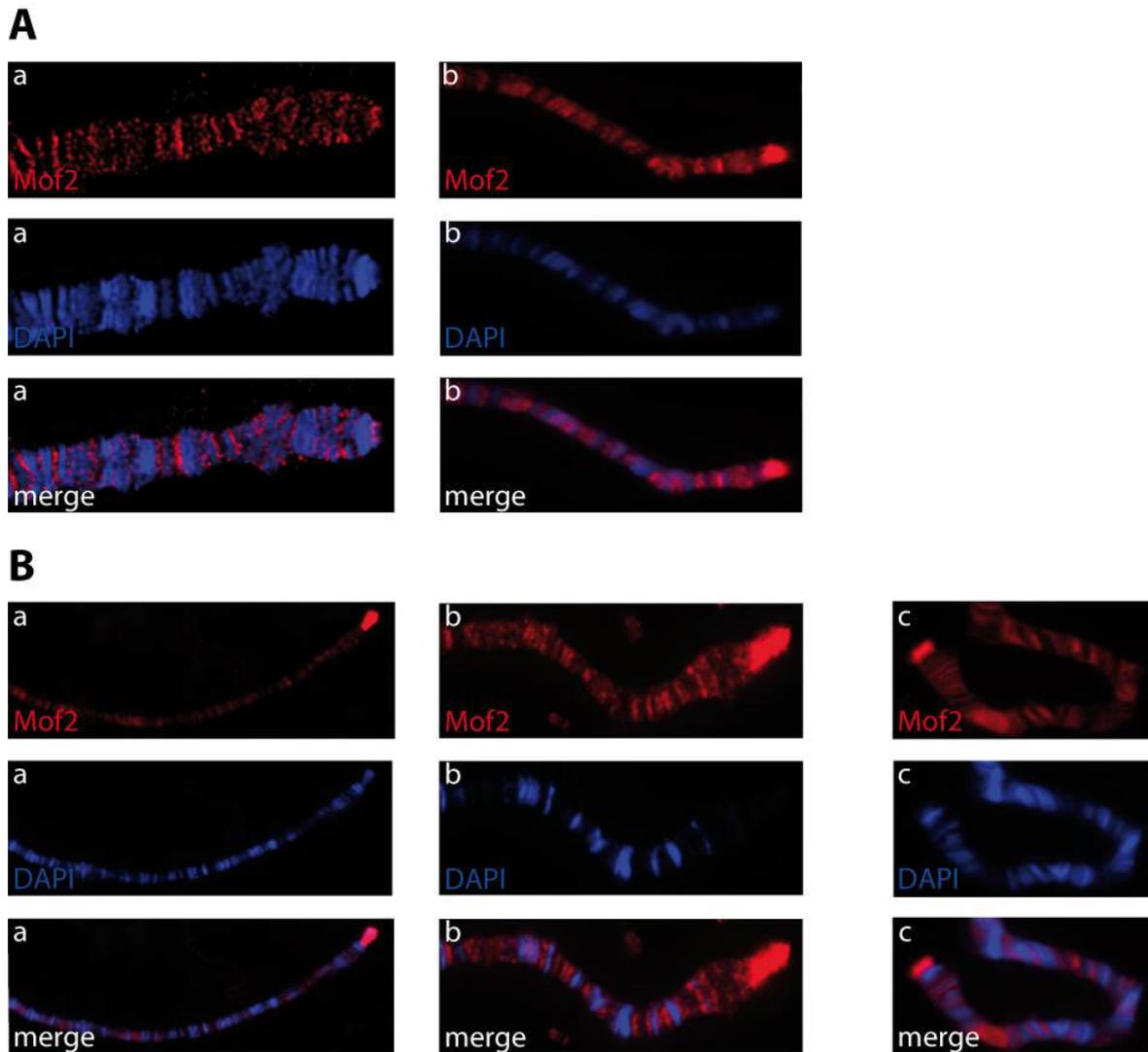


Figure 15: Localisation of Mof2 at the telomeres

(A) For Mof2, a telomeric localisation was observed at the X chromosome **(A,a)** and the left arm of chromosome 2 **(A,b)** on polytene chromosomes of w1118 and Oregon R larvae.

(B) The staining was repeated with Gaiano-I and Gaiano-II larvae. The Gaiano-I strain was used for the picture **(B,a)** and the Gaiano-II strain for the pictures **(B, b and c)**. An additional telomeric binding site for Mof2 at the right arm of chromosome 2 was detected **(B,c)**.

The picture **(A,a)** was taken with technical help of Dr. T. Zielke.

To test if the signal at the telomeres was a common feature of Mof2 or whether it depended on distinct conditions like temperature or sex, the staining was repeated with the Gaiano-I and the Gaiano-II strain. These strains contain extraordinarily long telomeric HTT arrays that are composed of the three retrotransposons HeT-A, TART and TAHRE. These retrotransposons are exclusively found at the telomeres of *Drosophila melanogaster* and their retrotransposition determines the length of the

telomeres. In the Gaiano strains the HTT array is extended at the X chromosome (Gaiano-I) or the second chromosome (Gaiano-II).

The staining of these polytene chromosomes revealed a very prominent signal for both the X chromosome and the left arm of chromosome 2 for Mof2 (Fig. 15, B, a and B, b). In addition, a striking binding of Mof2 to the right arm of chromosome 2 was observed (Fig. 15, B, c). Therefore, the weak signal at the telomere of the X chromosome and the absent signal at the right arm of chromosome 2 in strains with an average HTT array length were very likely due to the squashing preparation, and it can be assumed that Mof2 generally localised to the HTT arrays of the telomeres.

The prominent localisation of Mof2 to telomeres was surprising because these regions are heterochromatic and characterised by little transcriptional activity. Their main function is to protect the ends of the chromosomes from degradation and fusion with other chromosomes. Therefore, the telomeres differ functionally and structurally very distinctly from interbands. Thus, Mof2 might not only have a function concerning gene transcription as indicated by its binding to a great number of interbands but also in chromosome end protection or maintaining or regulating the telomeric sequence.

3.7 Colocalisation of Mof2 with Z4/Putzig

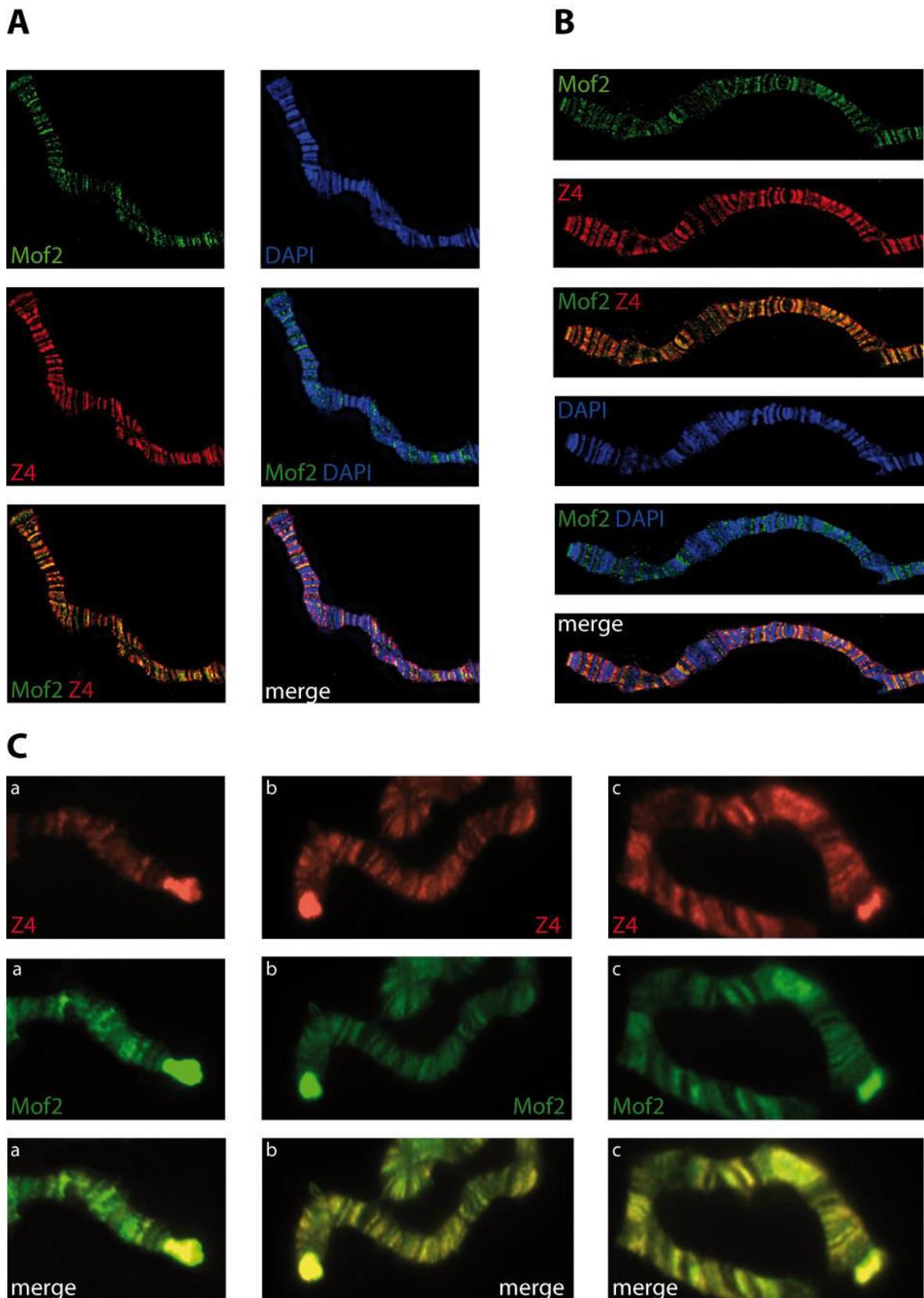


Figure 16: Colocalisation of Mof2 with Z4/Putzig at the interbands and at the telomeres (legend on the next site)

(A and B) Both, Mof2 and Z4 showed an interband-specific banding pattern. Costaining of Mof2 and Z4 revealed a substantial overlap of the binding sites of Mof2 (green) and Z4 (red). Oregon R larvae were used.

(C) Z4 and Mof2 showed a prominent staining at the telomers and colocalised at the telomeres of the X- chromosome **(C,a)** and the left **(C,b)** and the right **(C,c)** arm of chromosome 2.

Gaiano-I **(C,a)** and Gaiano-II **(C;b,c)** larvae were used.

The pictures **(A and B)** were taken with technical help of Dr. T. Zielke.

The characterised MYST histone acetyltransferases dmMOF and dmTip60 in *Drosophila melanogaster* are the catalytic subunit of multiprotein complexes and co-localise with other components of the complex on polytene chromosomes (Lam et al 2012, Kusch et al 2004, Hilfiker et al 1997). Therefore, the question arose if there are other chromatin-associated proteins that might interact with Mof2. A possible candidate was Z4/Putzig. Z4/Putzig binds to a number of interbands and also shows prominent telomeric binding like Mof2 (Silva-Sousa et al 2013, Eggert et al 2004). Therefore, a possible colocalisation of Mof2 and Z4/Putzig was tested.

For this purpose, a co-staining of these two proteins was performed on polytene chromosomes (Fig. 16). The banding pattern revealed a substantial, but not complete, colocalisation of Mof2 and Z4 within the interbands (Fig. 16, A and B). The telomeric binding was tested with the Gaiano-I (Fig. 16, C, a) and Gaiano-II (Fig. 16, C, b and c) strains. There, for both Z4 and Mof2, a remarkably strong signal at the telomere of the X chromosome and the telomeres of chromosome 2 was visible and colocalised well.

Therefore, Mof2 and Z4 might act at least for some binding sites within the interbands in the same complex. In addition, they might have a shared function at the telomeric regions that likely differs from their function within interbands.

3.8 Temperature-dependent transcription of *mof2*

The analysis of *mof2* transcripts in knockdown flies that were cultured at different temperatures revealed a temperature-dependent transcription of *mof2* for the control crosses. To quantify the temperature dependence, young adult (0 h – 24 h after eclosion) w1118 flies and wandering L3 w1118 larvae were used (Fig. 17). For both developmental stages, a relatively high amount of *mof2* cDNA was detectable for males at 23 °C whereas in females considerably less *mof2* transcripts were detectable. The amount of *mof2* was approx. 10x higher in male larvae compared to females and approx. 5x increased in adult males compared to adult females. At 29 °C, the amount

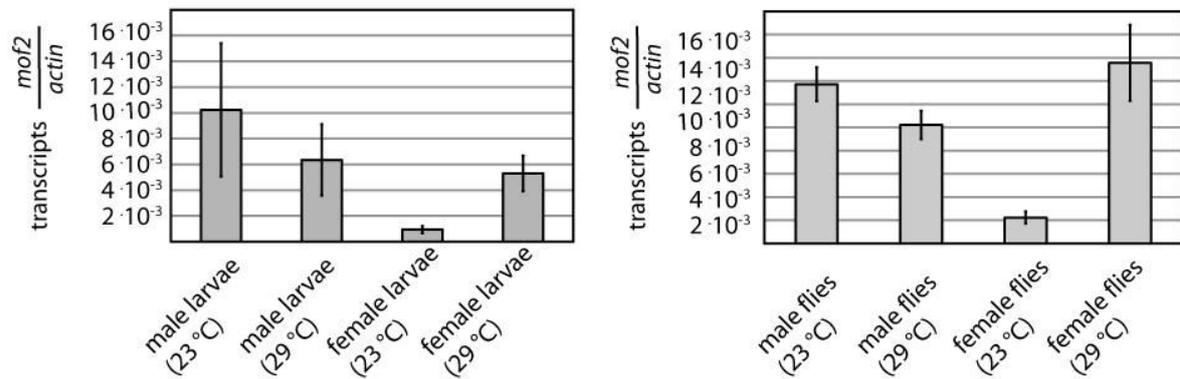
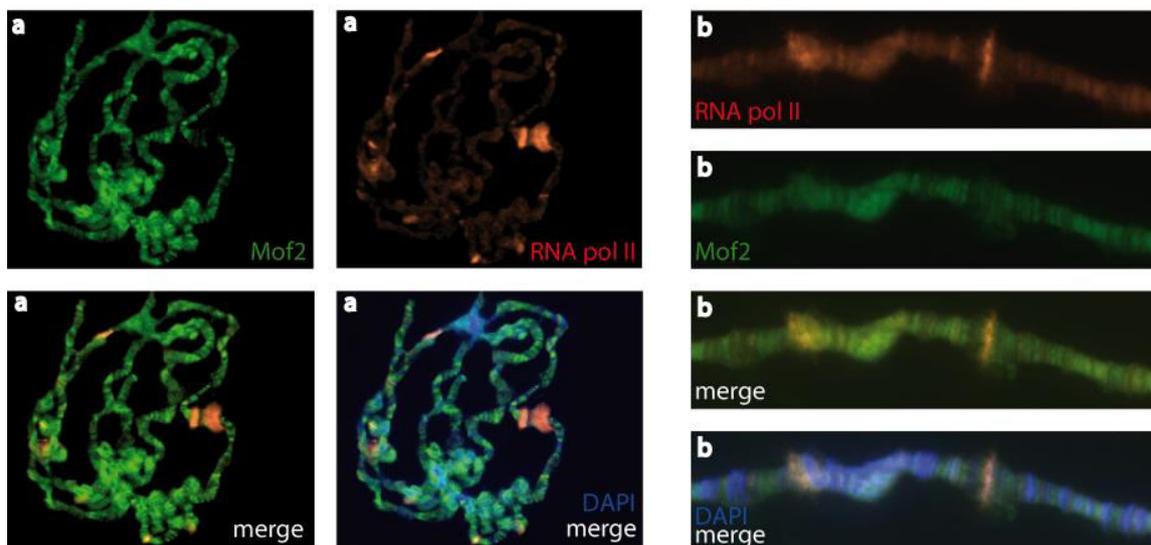
A**B**

Figure 17: Temperature-dependent transcription of *mof2* and heat shock independent binding of Mof2 on polytene chromosomes

(A) The transcription of *mof2* is sex- and temperature-dependent. At 23 °C, the amount of *mof2* transcripts was increased in males compared to female larvae or adult flies. At 29 °C, female adults and larvae showed an increased transcription compared to males or females at 23 °C. The quantification was done for biological triplicates with cDNA of whole RNA extracts. The error bars represent the standard deviation.

(B) After a heat shock at 37 °C for 30 min, the localisation of Mof2 (green) on polytene chromosomes in females remained unchanged whereas the RNA polymerase II (red) showed the expected reduced overall binding and an increased binding to heat shock loci **(B,a)**. The banding pattern for the RNA polymerase II became blurred whereas the banding pattern of Mof2 remained distinct **(B,b)**.

of transcripts was slightly decreased in males but strikingly increased in females compared to the finding at 23 °C. This observation suggests a function for Mof2 in heat adaptation in females.

Since several proteins that are involved in heat adaptation are also involved in the heat shock response (Colinet et al 2013, Tetievsky and Horowitz 2010), it was tested whether Mof2 is part of the heat shock response. To this end, polytene chromosomes of salivary glands of female L3 larvae were stained against Mof2 and RNA polymerase II after heat shock (Fig. 17, B). There, the localisation of Mof2 showed no obvious changes while the RNA polymerase II showed the expected, greatly increased binding to heat shock loci. Since the change of the localisation to the heat shock loci is a hallmark of proteins that are directly involved in the transcriptional heat shock response, this indicated that Mof2 was not directly involved in this response.

3.9 Different expression of Mof2 in males and females

The approx. 5x higher amount of *mof2* transcripts in adult males compared to adult females at 23 °C (Fig. 17, A) led to the question whether this also reflected a different protein level of Mof2 in males and females. To investigate this, a Western blot analysis was performed (Fig. 18, A). An at least 3x increased amount of Mof2 was found in adult males compared to females. Therefore, the transcription levels of *mof2* roughly correlated with the expression level of the Mof2 protein.

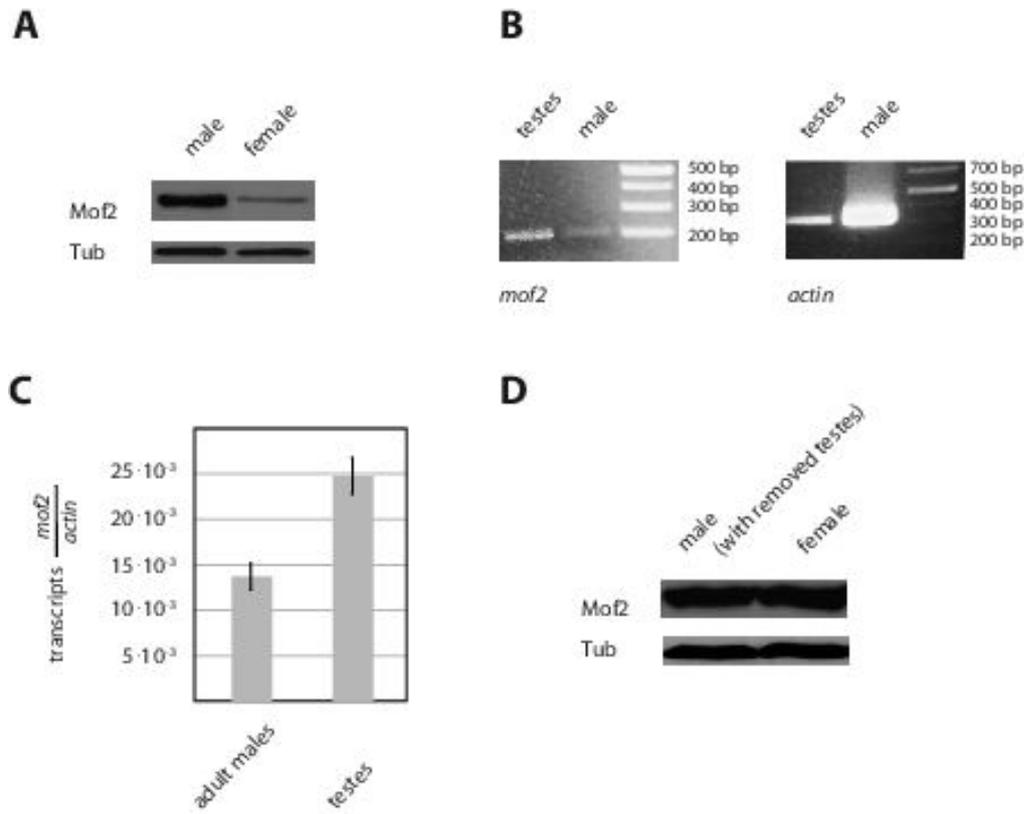


Figure 18: The expression of Mof2 in the testes is responsible for the different expression of Mof2 in female and male flies

(A) A comparative Western blot analysis of Mof2 in males and females revealed an increased amount of Mof2 in males. Tubulin served as loading control.

(B) The conventional PCR for *mof2* on the cDNA of male flies and testes showed an enrichment of *mof2* transcripts within the testes compared to the whole fly. Actin transcripts served as loading control.

(C) Quantitative real time PCR on cDNA of whole RNA extracts showed an approx. 1.7x enrichment of *mof2* in testes compared to the whole fly. The amount of transcripts was normalised to *actin*.

(D) The Western blot analysis of Mof2 in females and males with mechanically removed testes showed no difference. Tubulin served as loading control.

This observation raised the question whether the observed different expression was caused by an overall increased expression in males or whether there is a specific organ that is responsible for the increased expression. In high-throughput data from modENCODE, *mof2* is reported to have a relatively high transcription within the testes of adult males. This is supported by the localisation of Mof2 within the pole cells during early embryonic development (Fig. 13), which indicates that Mof2 is involved in germ cell or gonad development. To compare the amount of *mof2* transcripts in testes to *mof2* transcripts in the whole fly, the cDNA of whole RNA extracts from testes and male adult flies was analysed with conventional PCR and quantified with quantitative real time PCR and normalised to *actin* (Fig. 18, B and C). In this way, an increased transcription level within testes of adult males was found. Compared to the whole adult male fly, the testes show an approx. 1.7x enrichment of *mof2* transcripts. Since the transcripts of *mof2* correlate with the expression of Mof2, a relatively high level of Mof2 protein within the testes can be assumed.

Since the testes are a relatively small organ compared to whole flies, it was tested whether the high expression of Mof2 within testes explained the different expression of Mof2 in male and female flies. To this end, a Western blot analysis of females and males whose testes were mechanically removed was performed. This analysis revealed a similar amount of Mof2 for both males and females (Fig. 18, D). Therefore this indicates that a substantial proportion of Mof2 in males was located within the testes, which explained the difference of the Mof2 levels between males and females.

3.10 Localisation of Mof2 within the testes

The different expression of Mof2 in males and females indicated that Mof2 is highly expressed within the testes. To determine its localisation within this tissue, squashes of larval and adult testes were prepared and stained with an antibody against Mof2. In larval testes, only the four premeiotic stages of sperm maturation are present, whereas in newly hatched male flies, all sperm stages except the individualised sperms are observed. The maturing sperm cells are surrounded by two cyst cells and are interconnected via cytoplasmic bridges and form a cyst. The size of the cyst increases during the four mitotic divisions prior to meiosis.

In larval and in adult testes, Mof2 exhibited a prominent signal in intact cysts of late spermatogoniae and spermatocytes (Fig. 19, A and B). Mof2 was distinctly located within the cytoplasm of these developmental stages of sperm and was absent in the

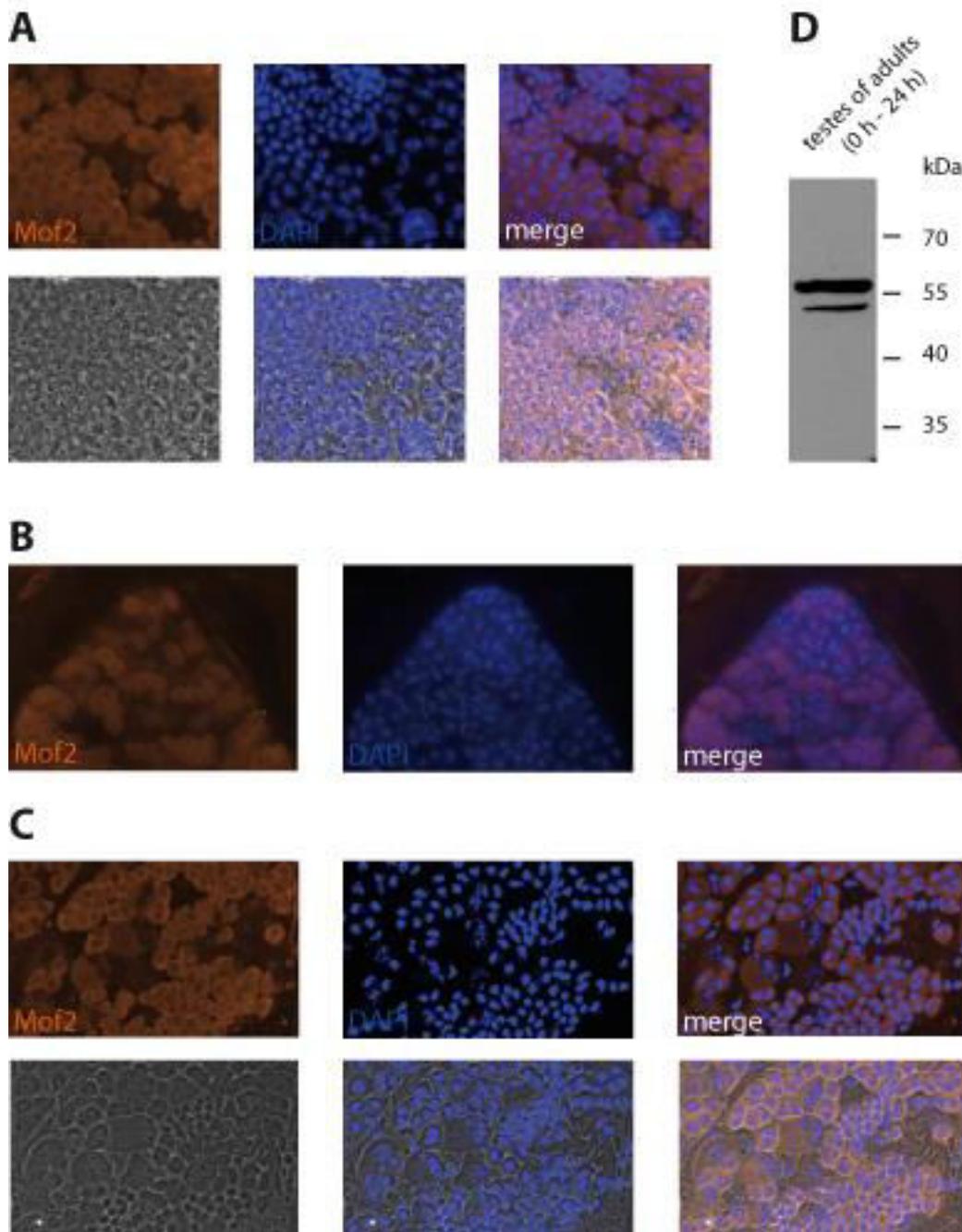


Figure 19: Cytoplasmic localisation of Mof2 within the testes

In both larvae (**A**) and adult (**B, C**) testes squashes, Mof2 (red) was detectable in late spermatogonia and early and to a lesser extent also in late spermatocytes. Mof2 was mainly located within the cytoplasm and not detectable in other stages of sperm maturation.

The first picture of the second row of (**A**) and (**C**) was taken with a phase contrast filter.

(**D**) The Western blot analysis of testes of adult males showed a signal at the expected molecular weight of approx. 49 kDa and an additional signal at a higher molecular weight.

nuclei. This was surprising because in embryos as well as in polytene nuclei of salivary glands of L3 larvae, a clear nuclear localisation was detected (e.g. Fig. 13 and Fig. 14). The different localisation of Mof2 within the testes might be due to posttranslational modifications as one might speculate because of the finding that the Western blot analysis of testes showed an additional signal at a higher molecular weight than expected (Fig. 19, C).

3.11 Histone acetyltransferase activity of Mof2

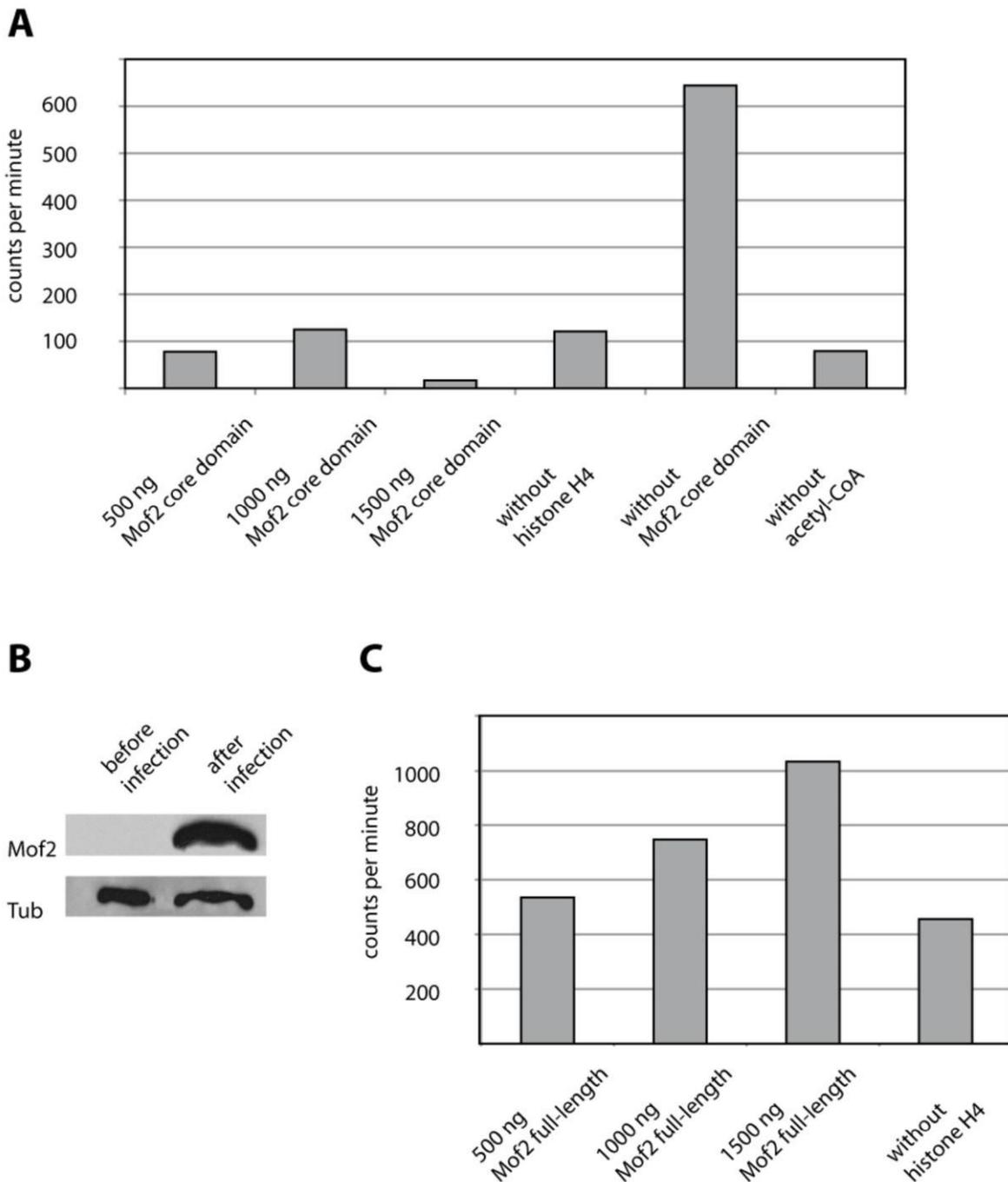


Figure 20: The *in vitro* acetylation assay showed no acetylation activity for the core domain of Mof2 but for the full-length Mof2

The *in vitro* acetylation assay was performed with 3H-labelled acetyl-CoA, the recombinantly in *E. coli* expressed Mof2 core domain with its predicted catalytically active domain or the in Sf9 cells expressed full-length protein and commercial available recombinant histone H4. 500 ng of human P/CAF served as positive control with 12560 counts per minute (not shown).

(A) For the Mof2 core domain, the signal was similar to the background signal without acetyl-CoA. Histone H4 without the Mof2 core domain exhibited autoacetylation activity that was inhibited when the Mof2 core domain was added.

(B) The Sf9 cells expressed Mof2 after infection with the V2-generation of the baculovirus. Tubulin served as loading control.

(C) The full-length Mof2 protein exhibited a concentration depending acetylation activity on histone H4.

The sequence of the predicted catalytically active core domain of Mof2 is similar to the sequence of proteins that exhibit a histone H4K16 acetyltransferase activity. Thus, a radioactive *in vitro* assay was performed to test the enzymatic specificity of the Mof2 core domain. For this purpose, the core domain of Mof2 was recombinantly expressed in *E. coli* and purified. The Mof2 core domain was incubated with histone H4 and H³-labelled Acetyl-CoA. The mixture was spotted on p31 paper, washed and the radioactivity was counted with a scintillation counter. Human P/CAF was used as positive control. Due to the high number of counts per minute compared to the other samples it was not included in the figure (Fig. 20). For histone H4, a weak autoacetylation activity was detected that was inhibited when the Mof2 core domain was added. The Mof2 core domain showed no acetylation activity on histone H4 and the detected radioactivity was similar to the background activity when no H³-Acetyl-CoA was added.

The absence of acetylation activity could have different reasons. First, the Mof2 core domain might be inactive without the remaining parts of the protein. Secondly, Mof2 might be expressed without essential modifications in *E. coli*. Thus, the full-length protein was expressed in Sf9 insect cells. At the end of this study, first initial experiments were performed.

Sf9 cells were transfected with a baculovirus that contained the complete coding sequence of *mof2* and an N-terminal 6x-his-tag. For the expression of Mof2, the V₂-generation of the virus was used and the efficiency of the transfection was monitored with the help of YFP. YFP is a yellow fluorescent protein and its sequence is integrated into the baculovirus' genome of the DH10EMBacY cells that were used in this study. Successfully transfected cells have a prominent yellow-orange colour while untransfected cells appear grey-brownish.

The expression of Mof2 was tested with a Western blot analysis of cell lysates (Fig. 20, B). While no signal for Mof2 was detectable for cells that were not transfected, the transfected cells had a clear signal for Mof2. Mof2 that was expressed in Sf9 cells had an N-terminal his-tag and was affinity purified with a Ni²⁺-column. The subsequent acetylation assay exhibited a concentration dependent acetylation activity of Mof2 on histone H4 (Fig. 20, C). In addition, an autoacetylation activity of Mof2 was detected.

To gain further insight into the enzymatic activity of Mof2, the acetylation assay was performed with peptides that consisted of the 20 N-terminal amino acids of histone

H4 and had an acetylated lysine at position 8, 12 or 16. For all of these peptides, there was no concentration dependent acetylation activity detectable and the acetylation activity was greatly reduced compared to the acetylation activity on the full-length histone H4 (data not shown). This indicates that Mof2 regains full-length histone H4 for its acetylation activity and is inactive on the much shorter histone H4 peptide. This might cause fewer interactions between Mof2 and the peptides and reduce the efficiency of the acetylation activity of Mof2. Another possible explanation might be that Mof2 only acts on unacetylated histones and has no or a greatly reduced activity on acetylated histone H4 and truncated variants.

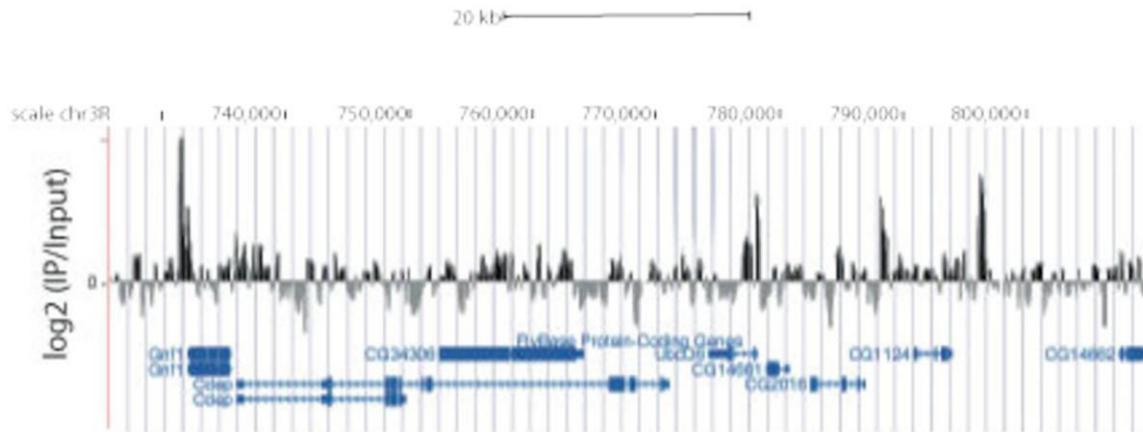
3.12 Genome-wide binding sites of Mof2

The staining of Mof2 on polytene chromosomes revealed a large number of binding sites (Fig. 13). To gain a better insight into the binding of Mof2 to the DNA, a ChIP-Seq experiment with S2 cells and two different antibodies against Mof2 was performed. The sequencing was performed on an Illumina platform. The alignment of the achieved data to the published genome of *Drosophila melanogaster* exhibited a clear enrichment of certain DNA sequences compared with the input (Fig. 20, A). Peaks were identified with MACS 1.4 for both antibodies against Mof2. In this way, 2114 peaks were identified for the first antibody and 5322 peaks for the second. Though the two antibodies showed similar but not completely identical peaks only the 1863 peaks, which were present for both antibodies, were used for further analysis. In addition, repetitive sequences and sequences that could not be aligned to the published genome were excluded from further analysis. The correct alignment of ChIP-Seq data is a special problem for telomeres because of the great variation of the composition of the HTT arrays (Abad et al 2004) and therefore these sequences could not be analysed, too.

To test the viability of the ChIP-Seq data, a conventional ChIP was performed with both antibodies that were used for the ChIP-Seq experiment and seven randomly chosen peaks of the ChIP-Seq data were tested with quantitative real time PCR for their enrichment (Fig. 20, B). The corresponding preimmune sera of the antibodies served as control for the specificity of the enrichment and a region with no Mof2 binding served as control for the background. All of the tested peaks showed an at least 2x enrichment with the antibody against Mof2 compared to the corresponding

preimmune serum. Therefore, the ChIP-Seq is at least for the tested regions likely to be reliable.

A



B

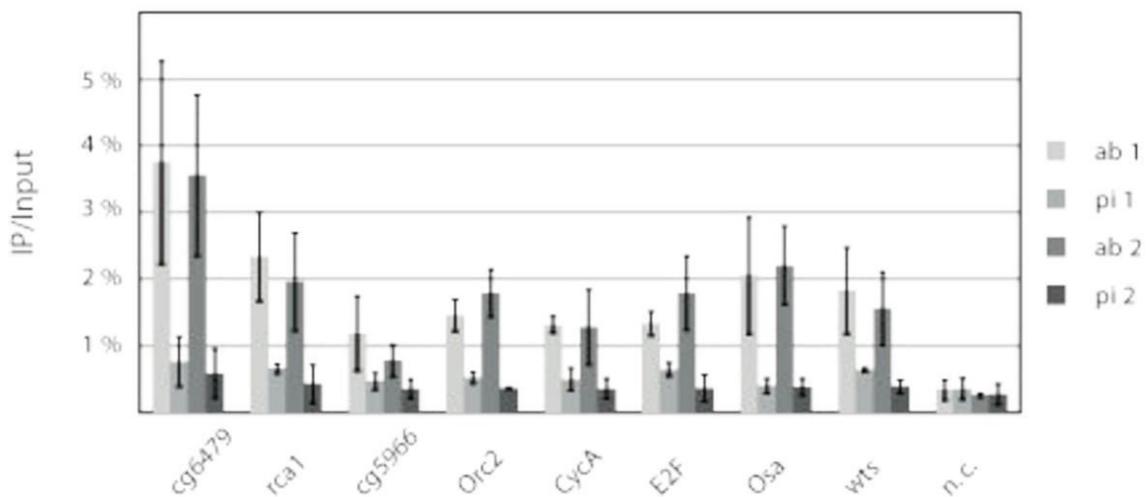


Figure 21: Genome-wide binding sites of Mof2

The ChIP-Seq was performed with S2 cells and two different antibodies.

(A) The analysis of genome Mof2 binding exhibited sites with a clear enrichment compared to the input. A Genome Browser snapshot of a part of chromosome 3 is shown and shows typical binding sites that were found for Mof2. The genes that correspond to the genome sequence are annotated at Flybase and are shown at the bottom. The bioinformatical analysis was done by A. Ehrenhofer-Murray.

(B) The binding of Mof2 was confirmed for randomly chosen binding sites with quantitative real time PCR (qPCR) for both antibodies (ab) and their corresponding preimmune serum (pi). The qPCR was performed for three biological samples and the error bars represent the standard deviation. The negative control (n.c.) corresponds to a region where no Mof2 was bound.

3.13 Characterisation of binding sites of Mof2

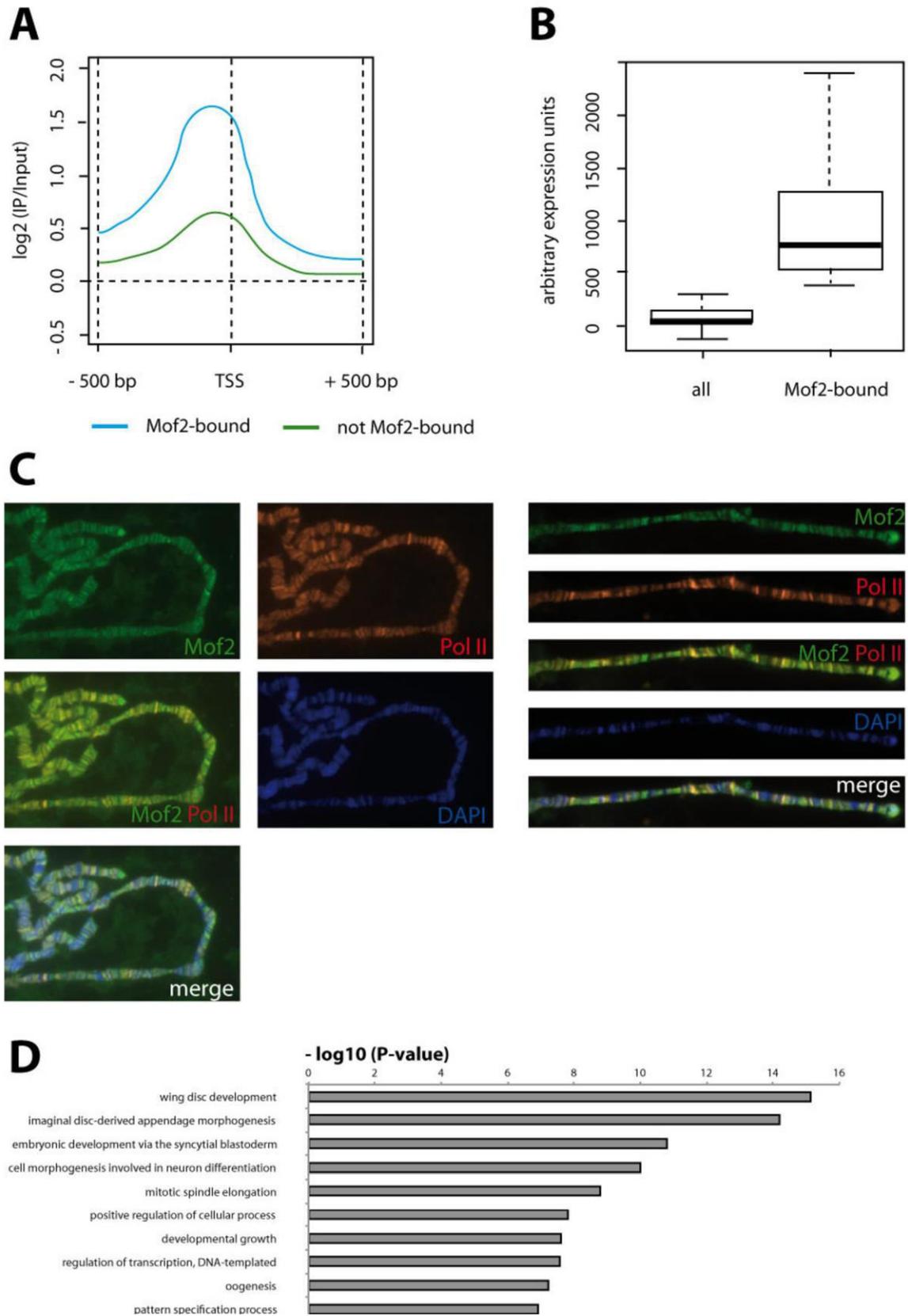


Figure 22: Mof2 binding sites are enriched at promoter sites of strongly expressed genes that are involved in embryogenesis (legend on the next site)

(A) 61.6 % of the peaks of Mof2 were located within +/- 500 bp relative to the transcription start site (TSS). Within this region an enrichment at approx. 50 bp upstream the TSS was detected.

(B) Mof2 is preferentially bound at the promoter sites of genes that have high expression levels in S2 cells.

(C) Mof2 (green) and the RNA polymerase II (Pol II, red) colocalised at several but not all binding sites.

(D) The GO annotations showed an enrichment of genes that are involved in embryogenesis and cell differentiation.

Both, the binding of Mof2 on polytene chromosomes (Fig. 14) and the ChIP-Seq data (Fig. 20) revealed multiple binding sites of Mof2 within the genome. The interband-specific binding of Mof2 suggested a function in gene regulation. Therefore, the summits of the peaks of Mof2 were analysed with regard to their relative position to the transcription start site (TSS) (Fig. 21). 61.6 % of 1862 peaks were located within +/- 500 bp of the TSS and 69.7 % were found +/- 1000 bp relative to the TSS (Fig. 21, A). The peaks were not equally distributed but showed a clear enrichment approx. 50 bp upstream the TSS. Although also regions that are not Mof2-bound showed a mild bias for the TSSs Mof2-bound regions showed a clear enrichment. Therefore, the binding of Mof2 at the promoter sites was very likely not due to experimental bias of certain DNA-sequences. All in all, these findings support the idea that Mof2 might be directly involved in the regulation of transcription.

A more detailed analysis of the genes at whose promoter sites Mof2 was bound revealed a prominent enrichment of genes that showed a strong expression in S2 cells (Fig. 21, B). Together with the enriched binding upstream but near the TSS of Mof2 that led to the question whether Mof2 also colocalised with the RNA polymerase II (Fig. 21, C). The co-staining of polytene chromosomes with antibodies against Mof2 and the RNA polymerase II showed several signals that colocalised but there was also a substantial amount of binding sites that did not colocalise. This observation on the polytene chromosomes reflects the findings of the bioinformatical analysis. Although the binding of Mof2 is enriched at approx. 50 bp upstream of the TSS this only z true for approx. 20 % of all binding sites of Mof2. In addition, the RNA polymerase II is generally located at the core promoter that is located within approx. 50 bp upstream and approx. 30 bp downstream of the TSS.

3.14 Knock-down of *mof2*

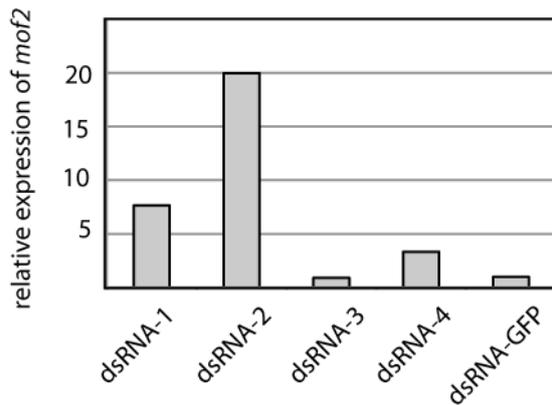


Figure 23: Induction of a knock-down of *mof2* in S2 cells led to an upregulation of *mof2*

Four different dsRNA constructs were used for the induction of a knock-down of *mof2*. In three out of four treatments an upregulation of *mof2* transcripts compared to the control (dsRNA-GFP) was detected.

The ChIP-Seq data revealed numerous binding sites at the promoter of genes and further bioinformatical analysis showed that these genes are mainly strongly expressed. To test whether Mof2 directly regulates the expression of these genes, a knockdown in S2 cells was performed. For this purpose, dsRNA was added to the medium and incubated with the cells. This led to the induction of the RNAi and the cDNA of whole RNA extracts was analysed with quantitative real time PCR. For the chosen dsRNA constructs, which varied in size and sequence, this resulted for three out of four treatments in an upregulation of *mof2* compared to the control (Fig. 23). This indicates either the induction of a strong off-target effect that results in the upregulation of *mof2* or that *mof2* might be involved in the RNAi pathway. In this study, this question was not further investigated.

3.15 DNA-binding motifs

Mof2 is an annotated member of the MYST family of HATs. As far as their activity is further characterised, these HATs are recruited to the chromatin by the complexes they are part of. Therefore, if proteins share the same binding sites with Mof2, they might also physically interact with Mof2. To identify such proteins, the peak summits +/- 50 bp of Mof2 were analysed. For this purpose, a *de novo* motif search was performed and nine different motifs were identified (Fig. 15). The achieved motifs were compared to DNA-binding sequences of proteins from the FlyFactorSurvey database. Most of the data that is included in this database is derived from bacterial one-hybrid assays (Zhu et al 2011). The binding motifs m1 and m3 of Mof2 exhibited a great similarity to binding motifs of ftz-f1 and CG7928. And motif m2 is similar to the binding motif of pleiohomeotic like (Pho-like, phol). The identified motifs m4 and

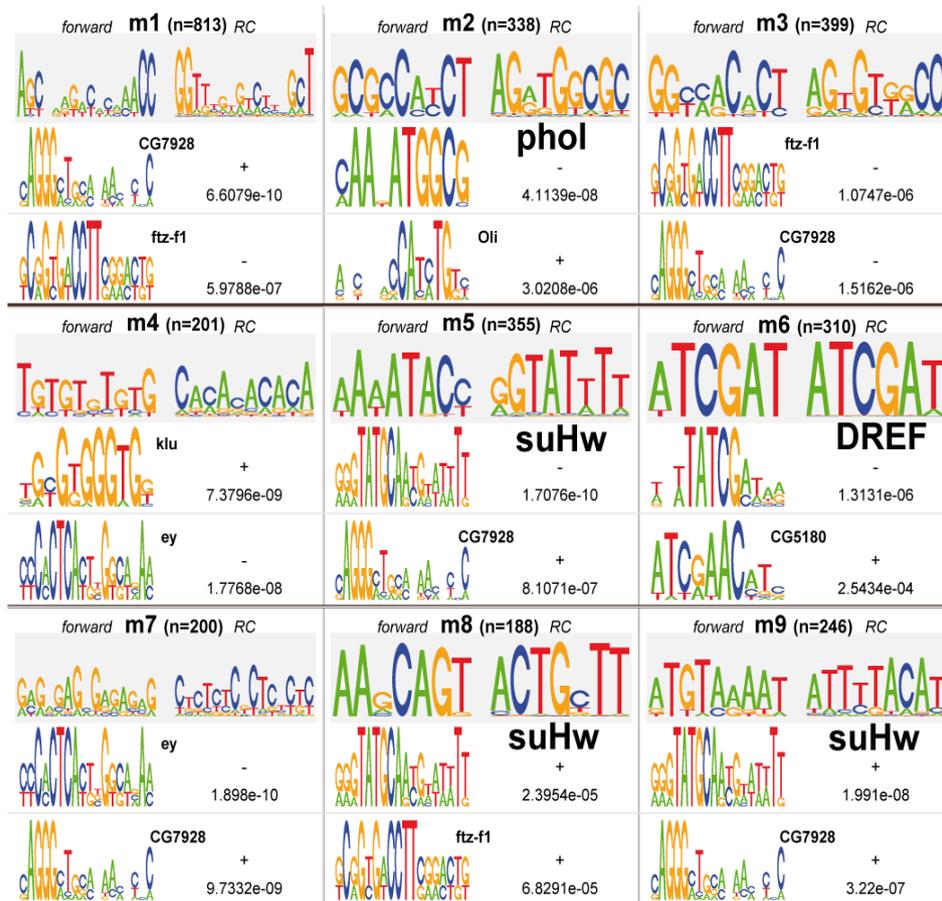


Figure 24: The *de novo* DNA-binding motif search for Mof2 identified nine different motifs

The nine DNA-binding motifs of Mof2 are presented as weight matrices. The forward and the reverse orientation are shown and the discovery number of a motif within the peaks of Mof2. The motifs were compared to known DNA-binding motifs and those with the highest similarity are shown below with the corresponding DNA-binding protein and their p-value.

The bioinformatical analysis was performed by A. Ehrenhofer-Murray.

m7 showed striking similarity to the binding sites of eyeless (ey). Motif m4 also showed resemblance to the binding motif of klumpfuss (klu). Furthermore, m5, m8 and m9 showed a similarity to the binding site of Suppressor of hairy wing Su(Hw), and m6 was similar to the consensus site of DREF. The remarkably similarity of the binding motifs of Mof2 to the binding motifs of other DNA-binding proteins indicates that these proteins might also interact with Mof2 and recruit it to the chromatin.

3.16 Colocalisation of Mof2 with other DNA-binding proteins

The analysis of the identified DNA-binding motifs of Mof2 revealed several proteins that shared at least one DNA-binding motif with Mof2 (Fig. 24). To test whether this

correlates with a substantial overlap of genome-wide binding sites, the published binding sites of the DNA-binding proteins and Mof2 were compared (Fig. 25, A). Since only for Pho-like, Su(Hw) and DREF published data with a sufficient quality were available, the other DNA-binding proteins were not included in further analysis. BEAF-32 was included because of its known interband-specific binding pattern that showed some similarities to the binding pattern of Mof2 on polytene chromosomes. For BEAF-32 and Su(Hw), the data for the binding sites was generated from S2 cells, whereas the data for DREF was derived from Kc cells, and for Pho-like, early embryos (4-8 hours) were used. The strongest correlation was found for the binding profile of DREF. DREF shared 44.8 % of its binding sites with Mof2 and Mof2 shared 46.5 % of its binding sites with DREF. Thus, Mof2 and DREF colocalised at approx. half of their binding sites.

This prominent colocalisation led to the question whether DREF and Mof2 also

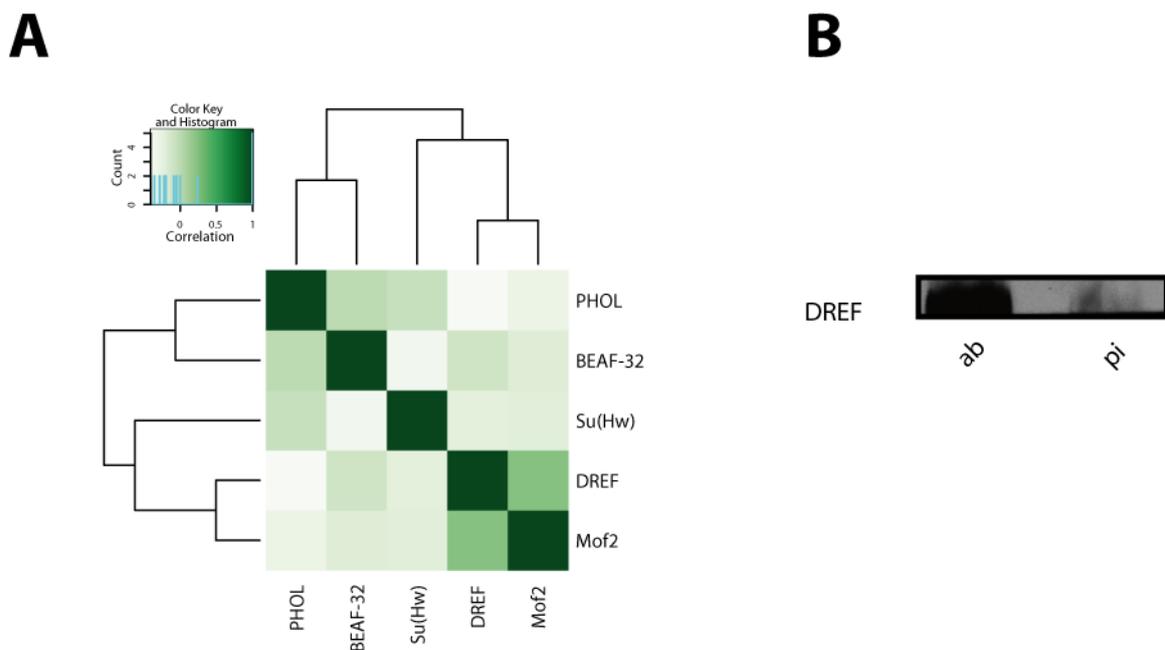


Figure 25: Mof2 and DREF shared genome-wide binding sites and interacted physically

(A) A correlation heat map of genome-wide binding sites of Mof2 and other DNA-binding proteins revealed DREF as the protein that shared most of its binding sites with Mof2 and vice versa. The bioinformatical analysis was performed by A. Ehrenhofer-Murray.

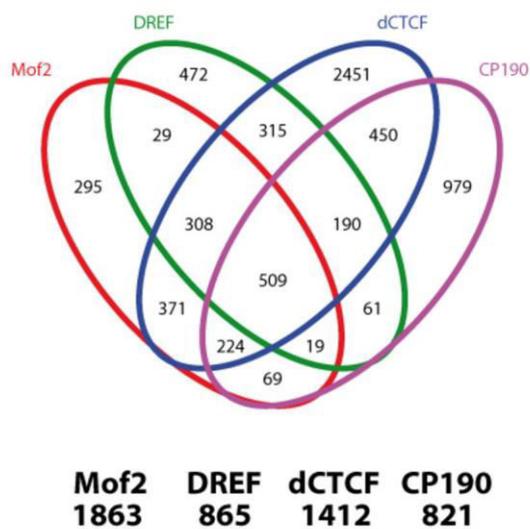
(B) An IP with salivary glands extracts with an antibody (ab) against Mof2 or the corresponding preimmune serum (pi) revealed a physical interaction between Mof2 and DREF.

interact physically. To test this, an IP with extracts of salivary glands against Mof2 was performed and analysed with an antibody against DREF (Fig. 25, B). The comparison with the corresponding preimmune serum of the antibody of Mof2 showed a strikingly enrichment of DREF for the antibody. Thus, Mof2 and DREF might also interact directly *in vivo* at several binding sites.

3.17 Colocalisation with insulator proteins

Mof2 has a large number of binding sites that colocalised with DREF. Since DREF is

A



B

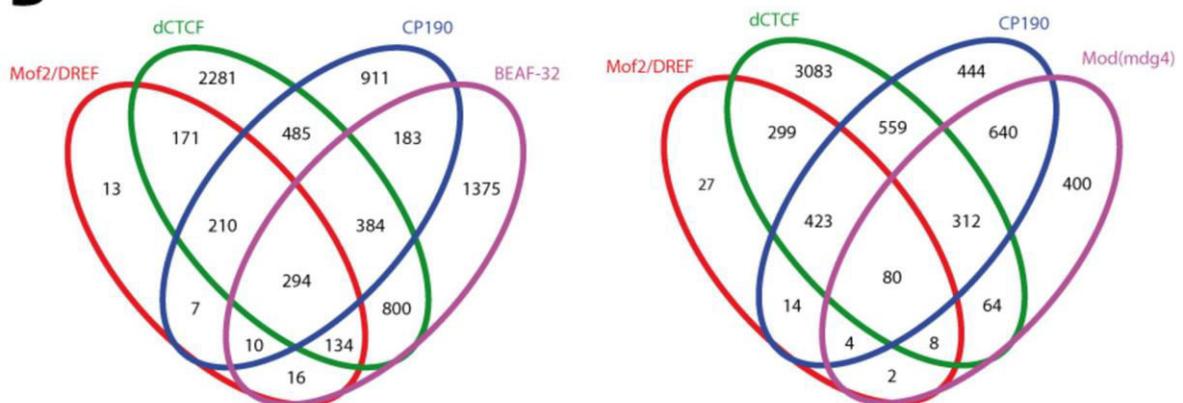


Figure 26: Mof2 binding sites colocalised with DREF, dCTCF and CP190

(A) The Venn diagram showed overlapping binding sites of Mof2 with the binding sites of DREF and the insulator proteins dCTCF and CP190. The number of colocalising binding sites with Mof2 is shown below.

(B) The binding sites at which Mof2 and DREF colocalised showed a substantial overlap with binding sites of dCTCF and CP190 and to a lesser extent also with BEAF-32 and Mod(mdg4). The bioinformatical analysis was performed by A. Ehrenhofer-Murray.

known to colocalise with insulator proteins like dCTCF and CP190 (Gurudatta et al 2013), it was tested if this is also true for Mof2 (Fig. 26, A). 29.3 % of the dCTCF binding sites and 32.8 % of the CP190 binding sites colocalised with Mof2 binding sites. To test whether the binding sites that are shared by DREF and Mof2 also colocalised with binding sites of insulator proteins or if DREF and Mof2 shared insulator-independent binding sites, the overlapping binding sites of DREF and Mof2 were compared to the binding sites of dCTCF, CP190, BEAF-32, and Mod(mgd4). 94 % of the Mof2/DREF binding sites intersect with dCTCF or CP190, whereas the other insulator proteins showed a substantial overlap of binding sites. Taken together, Mof2 alone and together with DREF exhibited a substantial colocalisation with insulator proteins especially with CP190 and dCTCF. These findings indicate a function of Mof2 within boundary formation together with the insulator proteins.

3.18 Colocalisation of Mof2 and Z4/Putzig binding

The staining of polytene chromosomes revealed a substantial overlap of Mof2 binding sites with Z4/Putzig (Fig. 16). Since there are no genome-wide sequencing data available for Z4/Putzig this could not be verified with a direct bioinformatical approach. Because Z4/Putzig extensively colocalises with Chriz on interbands of polytene chromosomes (Eggert et al 2004) the genome-wide binding sites of Chriz were compared to Mof2 (Fig. 27). The analysis revealed that 73.3 % of the binding sites of Mof2 overlap with Chriz. This indicates that Mof2 also colocalised with Chriz and Z4/Putzig at several binding sites as expected from the staining of polytene chromosomes and might share their function in maintaining the overall chromatin structure.

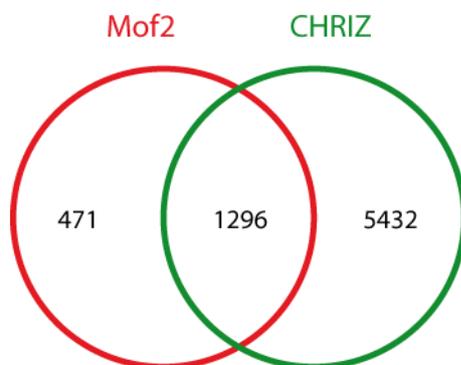


Figure 27: Mof2 binding sites colocalised with CHRIZ binding sites

The Venn diagram shows a substantial overlap of binding sites of Mof2 and CHRIZ. Since CHRIZ extensively colocalises with Z4/Putzig also a colocalisation of binding sites of Mof2 and Z4/Putzig can be assumed.

The bioinformatical analysis was performed by A. Ehrenhofer-Murray.

4 Discussion

In this study, the putative MYST histone acetyltransferase Mof2 was characterised with respect to its biological and enzymatic function. A hypomorph strain of Mof2 showed a developmental defect that caused a cleft on the back of adult flies, whereas a moderate knock-down of Mof2 by RNA_i had no phenotype. Mof2 was found to be present in the nucleus of embryos and localised to about 80 % of interbands on polytene chromosomes. Genome-wide binding analysis showed that Mof2 was enriched at promoter sites of highly transcribed genes and at genes that are involved in embryogenesis. Further analysis of Mof2 binding sites indicated that Mof2 might constitute a new subclass of insulator complexes together with DREF and CP190 or dCTCF. Concerning the enzymatic function of Mof2, a histone H4 acetyltransferase activity was shown *in vitro*.

4.1 Localisation of Mof2 on polytene chromosomes

The localisation of DNA-binding proteins can be investigated on polytene chromosomes. Next to other tissues, polytene chromosomes in *Drosophila melanogaster* are easily accessible within the salivary glands of third instar larvae. There, up to 1000 chromatids that are attached to each other are found in a single cell. The polytene chromosomes are organized in bands and interbands that are visible with a conventional light microscope. Roughly, the bands represent heterochromatic and transcriptionally inactive regions, whereas the interbands correspond to regions with transcriptionally active sites.

Mof2 localises to approx. 80 % of the interbands. The binding sites of Mof2 are found at the border of bands and interbands as well as within the interbands. While proteins at the border of bands and interbands are described to prevent spreading of heterochromatin into euchromatin and act as boundary elements that establish or maintain chromatin domains (Dorman et al 2007), proteins within the interbands have more diverse functions like regulation of transcription. Therefore, the localisation of Mof2 on polytene chromosomes very likely reflects different functions of Mof2.

The findings concerning possible functions of Mof2 are also reflected in the bioinformatical findings in S2 cells in this study. There, a partial overlap of binding

sites with the insulator factors dCTCF and CP190 was found. This colocalisation suggests a function of Mof2 in the formation of chromatin domains. In addition, Mof2 binding sites are enriched upstream of transcription start sites. This suggests a function in gene regulation. These two functions need not to be exclusive since insulators like SF1 also have a gene-specific regulatory function (Belozherov et al 2003).

4.2 Function of Mof2 within the testes

Mof2 is present in early developmental stages. During stage four of the embryogenesis, the formation of the pole cells takes place. At this stage, Mof2 is present in the nuclei of both somatic and pole cells. The pole cells are the progenitor cells of the later gonads in larvae and adult flies. In larval and adult testes, Mof2 has changed from a nuclear to a distinct cytoplasmic localisation.

For the expression of some genes that are expressed in somatic cells and in testes of *Drosophila melanogaster*, substantial differences in testes-specific cells compared to somatic cells have been described. The usage of alternative transcription start sites as well as alternative splicing is observed (Kearse et al 2010; Madigan et al 1996). This can lead to completely different enzymatic activities of the resulting protein and therefore alter the function and/or the localisation of a protein. Also, a switch from a nuclear to a cytoplasmic localisation in testes is described for some proteins (Cheng et al 1998).

These findings may explain the distinct cytoplasmic localisation of Mof2 within the germ cells, which is in striking contrast to the prominent nuclear localisation in somatic cells. In addition, the main fraction of Mof2 in testes has a higher molecular weight than Mof2 in somatic cells. This may be due to testes-specific posttranslational modifications or testes-specific gene expression and RNA processing.

The question of the function of Mof2 was addressed with a knockdown of Mof2 with the Gal4-UAS system. The knockdown of proteins in the germ line of testes is described to work less efficiently than in other tissues (White-Cooper 2012). The main reason for this is the different regulation and timing of transcription in the germ line of testes. There, the expression of most genes is restricted to early mitotic stages of spermatogenesis especially to primary spermatocytes and regulated by testes-specific transcription factors (White-Cooper 2010). Therefore, the expression driven by ubiquitously active promoters is very often reduced compared to other tissues.

The knockdown of Mof2 that was performed here with a Gal4-driver line with the ubiquitously active promoter of *daughterless* was very likely even less efficient in testes than for the whole fly. Since the most efficient knockdown of Mof2 on the RNA level was around 40 % the knockdown only led to a relatively mild reduction and had no effect on the fertility of the tested males. Yet, whether Mof2 is essential for the fertility of male flies cannot be excluded.

Also the enzymatic activity of Mof2 within the testes remains subject to speculations since a cytoplasmic localisation is in contrast to the predicted chromatin modifying activity Mof2. Nevertheless, Mof2 in testes may exhibit the same acetylation activity on histone H4 that has been found for recombinantly expressed Mof2, since in testes modifications of histones within the cytoplasm are known (Hennig and Weyrich, 2013).

4.3 Function of Mof2 at the telomeres

The organisation of telomeres in *Drosophila melanogaster* differs substantially from that in mammals. In *Drosophila melanogaster*, the telomeres are elongated and maintained by the specialised retrotransposons HeT-A, TART and TAHRE that form the HTT array and are characterised by a specialised form of heterochromatin (Filion et al 2010, George et al 2006). To establish the heterochromatic structure and regulate the expression of the retrotransposons at the telomeric sites, different proteins assemble at HTT arrays. In addition, proteins that protect the ends of the telomeres from non-homologous end joining and other activities of the DNA damage machinery are present at the ends of the telomeres and form the so-called terminin complex (Raffa et al 2009). This complex is specific for *Drosophila*, and it is functionally analogous but not homologous to the shelterin complex that is found at the ends of the telomeres in mammals. At the telomeric sites, a broad variety of proteins is found (Raffa et al 2011). Two of these proteins are Z4/Putzig and DREF.

At the telomeres, DREF acts as a regulator of transcription of the retrotransposon TART and HeT-A and is assumed to regulate the retrotransposition of these retrotransposons in an indirect way (Silva-Sousa et al 2013). Since a physical interaction between DREF and Mof2 has been shown in this study, Mof2 may also play a role in transcription regulation at the telomeres. DREF binds in a cell cycle depending manner to chromatin and thus there may also be a cell cycle dependent binding to the telomeres and complex formation with Mof2.

On the polytene chromosomes, Z4/Putzig and Mof2 showed an almost complete colocalisation at the telomeres. Since other studies have shown that Z4/Putzig likely does not interact with DREF but with JIL-1 at the telomeres (Silva-Sousa et al 2013, Takács et al 2012) Mof2 may be part of two distinct complexes at the telomeres with one containing DREF and one containing Z4/Putzig and JIL-1.

For all tested elongated telomeres of different Gaiano strains, Mof2 was found to be present at the whole telomeric site. Thus, it is possible that Mof2 plays a general role at the telomeres. Since Mof2 localised to the whole telomeric sites and not only to the end, a function in the protection of the end of the telomeres within the terminin complex is not very likely but cannot be excluded.

4.4 Function of Mof2 and CHRIZ/Chromator

On polytene chromosomes, Mof2 strongly colocalised with Z4/Putzig. Z4/Putzig is ubiquitous expressed in *Drosophila melanogaster* and regulates the overall structure of chromatin (Eggert et al 2004). Since no ChIP-Seq data for Z4/Putzig were available at the time of this study, the data for CHRIZ/Chromator was used instead. Z4/Putzig and CHRIZ/Chromator physically interact in coimmunoprecipitation experiments and show an extensive overlap of binding sites on polytene chromosomes (Eggert et al 2004). CHRIZ/Chromator exhibits a cell-cycle dependent binding to chromosomes. This binding reflects different functions of CHRIZ/Chromator. It is suggested to play a role in the correct spindle matrix formation during mitosis and in the correct chromatin formation during the interphase (Rath et al 2006; Rath et al 2004).

Bioinformatical comparism of binding sites of Mof2 and CHRIZ/Chromator revealed a significant colocalisation. CHRIZ/Chromator shared approx. 70 % of the binding sites of Mof2. The colocalisation of Mof2 with Z4/Putzig on the polytene chromosomes and with CHRIZ/Chromator based on bioinformatical approaches strongly suggests a function of Mof2 in modulating the chromatin structure and that Mof2 may form a complex with Z4/Putzig or CHRIZ/Chromator or both of these proteins at the same time.

Although Z4/Putzig and CHRIZ/Chromator show a clear colocalisation on polytene chromosomes and interact physically, their binding to the chromatin appears to be independent of each other *in vivo* (Gortchakov et al 2005). This suggests that they bind independently to the same complex that consists of still unknown components. Both proteins have a severe but remarkably different and rather opposing impact on

the chromatin structure. While a reduction or loss-of-function of Z4 leads to decondensed polytene chromosomes that have lost their clear band and interband organisation, a loss of CHRIZ/Chromator results in a coiling and shortening of the chromatin structure. Mof2 may be the catalytic part of a Z4/Putzig and CHRIZ/Chromator containing complex and be regulated by Z4/Putzig and/or CHRIZ/Chromator. Since the bioinformatical data for both Mof2 and CHRIZ are derived from S2 cells that were not synchronised, neither a cell cycle nor a development dependent complex formation can be excluded.

4.5 Possible insulator formation of Mof2 and DREF

The bioinformatical analysis of the ChIP-Seq data revealed a substantial overlap of binding sites of Mof2 and DREF. DREF is a DNA-binding protein that acts as a positive regulator of transcription (Hochheimer et al 2002). The majority of its binding sites are shared by Mod(mdg4) (Gurudatta et al 2013). During interphase more than 62 % of DREF binding sites colocalise with Mod(mdg4) binding sites. Mod(mdg4) has no DNA-binding properties on its own but binds to other DNA-binding proteins like the insulator binding proteins Su(Hw) and CP190 (Pai et al 2004). The binding and homodimerisation of Mod(mdg4) are essential for the correct formation of chromatin loops. Whether DREF in combination with Mod(mdg4) also mediates intrachromosomal contacts is still subject to investigation but current studies support this idea (Gurudatta et al 2013).

There is little colocalisation found between Mof2/DREF and Mod(mdg4) binding sites. Only 10.8 % of these binding sites colocalise. Therefore, the binding of Mof2 and Mod(mdg4) at DREF binding sites is mainly exclusive and suggests different functions for Mof2 and Mod(mdg4) in combination with DREF.

In addition, more than 82 % of Mof2 binding sites alone and approx. 95 % of Mof2/DREF binding sites are shared either by the insulator binding protein dCTCF or CP190. Since Mof2 has no predicted DNA-binding domains (Sanjuan and Marin 2001) and interacts physically with DREF together with dCTCF or CP190 it may constitute a new subclass of insulator binding complexes.

4.6 Colocalisation of binding sites of Mof2/DREF and BEAF-32

DREF and BEAF-32 are both DNA-binding proteins that share several of their binding sites. Their binding to the DNA is very likely not synergistic but antagonistic and has a

regulatory function with respect to transcriptional changes during development (Hart et al 1999). BEAF-32 functionally separates neighbouring genes and facilitates an independent regulation of these genes (Yang et al 2012). During the course of development, proteins that regulate transcription like DREF can substitute BEAF-32 and in this way the transcription of genes can be altered.

Due to the different functions of BEAF-32 and DREF, the colocalisation of binding sites of Mof2/DREF and BEAF-32 is probably not an indication for a common function within boundary formation but rather reflects distinct chromatin states. Since the data for binding sites of the three proteins Mof2, DREF and BEAF-32 are derived from non-synchronised S2 cells, the binding of Mof2/DREF and BEAF-32 at the same sites very likely reflects different phases of the cell cycle. The finding that DREF regulates cell cycle progression and exhibits a cell cycle dependent binding to DNA supports this (Gurudatta et al 2013, Hyun et al 2005).

4.7 Impact of Mof2 on the development

The genes at which Mof2 binding sites in S2 cells were found were analysed with the help of a Gene Ontology enrichment analysis. For this analysis, all more or less extensively characterised genes are grouped in at least one out of the three main ontologies “molecular function”, “biological process” or “cellular component” that are further subdivided into different groups (Gene Ontology consortium 2001). This analysis revealed that target genes of Mof2 show an enrichment of genes that are involved in the biological processes “wing disc development”, “morphogenesis of appendages” and “embryonic development”. Together with the finding that Mof2 has been found in the nucleus of embryos this suggests a role for Mof2 in regulating developmental genes during embryogenesis.

Also the phenotype of the hypomorph strain of Mof2 suggests a function for Mof2 within development since the cleft on the back that was observed in adult flies is probably the result of a defect of the substitution of larval tissue by adult tissue (Sekyrova et al 2010).

The hypomorph strain of Mof2 was derived from a heterozygous strain that was described to be a knockout strain that is homozygous lethal (Bloomington stock centre). Further analysis in this study revealed that the strain was neither homozygous lethal nor was Mof2 absent in the homozygous strain. Instead, the strain

showed a severe reduction of Mof2 but still Mof2 was detectable on protein level. Thus, Mof2 has an effect on the development of *Drosophila melanogaster* but since the described knockout strain is a hypomorph strain it is not clear whether Mof2 is essential for the viability of *Drosophila melanogaster*.

4.8 Regulation of transcription by Mof2

The bioinformatical analysis of the binding sites of Mof2 revealed that Mof2 preferentially binds at the promoter site of highly transcribed genes. Partially, this binding that was found for S2 cells is supported by the binding of Mof2 on polytene chromosomes. There, Mof2 colocalised with the RNA polymerase II at some sites.

The bioinformatical analysis also revealed a substantial colocalisation of binding sites for Mof2 and DREF. DREF is known to be part of a multi protein complex and regulates the expression of replication factors (Kugler and Nagel 2007, Hochheimer et al 2002). In addition, a function in the correct positioning of other promoter binding factors is discussed (Legube et al 2006). For DREF, a subset of binding sites colocalises with the TBP-related factor 2 (TRF2) dependent genes (Hochheimer et al 2002). These genes mainly have TATA-box-less promoters and in general they are not bound by TBP and TAFs. These two different classes of promoters contribute to differential expression patterns of neighbouring genes (Isogai et al 2007). Since TRF2 does not bind to the DNA on its own, other binding partners like DREF are responsible for the correct positioning of TRF2. These factors contribute to the regulation of transcription. Since a physical interaction between Mof2 and DREF has been described in this study, this suggests a function in the regulation genes without a TATA-box within the promoter site for Mof2.

Although Mof2 was found to be associated with highly transcribed genes and showed a temperature dependent expression in females, it was not linked to the heat shock response in polytene chromosomes of salivary glands of third instar larvae. There, the transcription factors are recruited to the heat shock loci together with the RNA polymerase II and a change in the localisation of these factors can be found. For Mof2, no change in the localisation on polytene chromosomes after heat shock was observed.

In addition to the temperature-dependent expression, there was also a sex-specific expression of *mof2*, and males exhibited an increased expression of *mof2* at room

temperature compared to females. Since the main difference in the expression of *mof2* in males and females can be explained by the increased expression of *mof2* within the testes and Mof2 in the testes is localised in the cytoplasm, it is very likely that Mof2 has no sex-specific effect on transcription.

The bioinformatical data for Mof2 is derived from S2 cells. Since different cell lines exhibit different binding sites for DNA-binding or DNA-associated factors a single cell line is likely to reflect only a specialised chromatin state (Cherbas et al 2011). In addition, the chromatin changes during the course of development and differentiation of cells. Therefore, this data can neither reflect the dynamic of DNA-binding during development nor within different tissues.

4.9 Acetylation activity of Mof2

The sequence of Mof2 shows a high similarity to the sequence of MYST histone acetyltransferases with a predicted specificity for histone H4K16 acetylation (Sanjuan and Marin 2001). However, the recombinant MYST core domain of Mof2 which presumably is responsible for the enzymatic activity of Mof2 expressed in *E. coli* has no detectable acetylation activity *in vitro*. This can be caused by the lack of essential parts of Mof2 or by missing or inappropriate posttranslational modifications, since proteins in *E. coli* are processed differently compared to eukaryotes. In addition, some eukaryotic proteins are not folded properly in a prokaryotic system and remain inactive.

When expressed recombinantly in eukaryotic Sf9 cells, full-length Mof2 is expected to have the same posttranslational modifications as native Mof2 since the cells are derived from an insect (Vaughn et al 1977). The full-length Mof2 exhibited a concentration dependent acetylation activity on histone H4. This activity is distinct from the background signal. At least *in vitro*, Mof2 is an active histone acetyltransferase that acts on histone H4 without further cofactors than acetyl-CoA or proteins. This *in vitro* activity has also been described for the well-characterised histone acetyltransferase dmMOF (Akhtar and Becker 2001) where it is representative of the catalytic activity of dmMOF *in vivo*.

The specificity of the acetylation activity of Mof2 remains unclear because of the reduced activity of Mof2 on acetylated peptides of histone H4. Since it is unclear if this is due to a reduced activity on truncated histone H4, this question could in principle be addressed with a non-radioactive assay with unacetylated full-length histone H4

and subsequent mass spectroscopy since further radio-activity based assays could not be performed by me because of my pregnancy.

The enzymatic activity of Mof2 *in vivo* is not known so far and as for other histone acetyltransferases like dmMOF or dmTip60 it is likely that it is a part of a multi protein complex that regulates both localisation and enzymatic activity of Mof2. The finding that Mof2 colocalises with other DNA-binding proteins like CTCF, CP190 and DREF for which also a physical interaction with Mof2 has been shown in this study supports this idea.

In addition to its acetylation activity on histone H4, Mof2 exhibits an autoacetylation activity. An autoacetylation activity has been described for other MYST histone acetyltransferases, namely the human MOF and Esa1 and SAS-I from yeast. These proteins exhibit an acetylation of a lysine residue within the catalytic core. This autoacetylation is essential for their activity *in vitro* and *in vivo* (Yuan et al 2012) and due to sequence similarities Mof2 may also have a functionally relevant autoacetylation activity.

4.10 Outlook

This study showed that Mof2 is present in the nucleus and in different developmental stages and tissues in *Drosophila melanogaster*. Since no real knock-out strain of Mof2 was available during this study, it is not clear whether Mof2 is essential for the viability of flies or not. Therefore, knock-out strain would help to gain further insight into the function of Mof2 concerning development and function within different tissues like the testes. In the testes, Mof2 was found in the cytoplasm in contrast to other findings. To test whether this is linked to tested-specific modifications of Mof2, Mof2 from testes could be analysed with mass spectroscopy.

The analysis of genome-wide binding sites of revealed that Mof2 was bound at the promoter sites of highly transcribed genes. A genome-wide analysis of the transcription compared to the transcription in S2 cells with a severe knock-down of Mof2 could answer the question whether Mof2 is also involved in the regulation of these genes. The colocalisation of Mof2 with DREF, CP190, dCTCF, Z4/Putzig and CHRIZ/Chromator raised the question whether Mof2 is part of a new subgroup of insulator complexes and regulates the chromatin structure and whether these proteins interact also physically and form a complex. To answer this question, an analysis of proteins that are coprecipitated with Mof2 could be performed.

5 Abstract

Members of the MYST histone acetyltransferases are widespread throughout eukaryotes and found from yeast to *Drosophila melanogaster*. They catalyse the acetylation of lysine residue of histones and in this way they alter the structure of the chromatin and regulate biological processes like transcription or DNA repair. Due to sequence similarities, Mof2 was supposed to be a MYST histone acetyltransferase with a specificity for histone H4K16. At the beginning of this study, little was known about Mof2 except from its DNA sequence and high throughput data concerning the expression of *mof2*.

A moderate reduction of Mof2 with RNA_i had no effect on the viability or fertility of flies. A greater reduction of Mof2 in a hypomorph strain led to a cleft on the back of adult flies what suggests a developmental defect. This study showed that Mof2 is present in embryos, larvae, adult flies and testes of larvae and adult males. In contrast to former reports, Mof2 was also present in S2 cells. A nuclear localisation of Mof2 was found in embryos and on polytene chromosomes, whereas Mof2 had a surprising cytoplasmic localisation in the testes of larval and adult flies. On polytene chromosomes, Mof2 localised to approx. 80 % of the interbands. There, Mof2 colocalised with Z4/Putzig. Together with the bioinformatically found colocalisation of binding sites of Mof2 with the binding sites of the transcription factor DREF and the insulator proteins CP190 and dCTCF this suggests a function for Mof2 within the regulation of the structure of chromatin. The colocalisation of Mof2/DREF and BEAF-32 was very likely antagonistic and reflects different chromatin states during the cell cycle.

Genome-wide analysis of binding sites revealed an enrichment of Mof2 at the promoter sites of genes with a high transcription level. Although also a temperature-dependent expression of Mof2 was found Mof2 is not involved in the regulation of the heat shock response in salivary glands. A more detailed classification of the binding sites of Mof2 with a gene ontology enrichment analysis showed that most of these genes are involved within embryogenesis.

The enzymatic activity of Mof2 was investigated with an in vitro assay and showed that Mof2 had a histone H4 activity in vitro, yet its specificity is still unknown. In

summary, it was found that the MYST histone acetyltransferase Mof2 might be involved in the regulation transcription and the formation of chromatin domains.

6 Zusammenfassung

MYST Histoneacetyltransferasen sind bei Eukaryoten weit verbreitet und finden sich von Hefe bis zu *Drosophila melanogaster*. Sie katalysieren die Übertragung von Acetylgruppen auf die Lysine von Histonen und bewirken dadurch die Veränderung der Chromatinstruktur und biologischer Prozesse wie Transkription oder DNA Reparatur. Auf Grund von Sequenzähnlichkeiten wurde Mof2 den MYST Histoneacetyltransferasen mit einer Spezifität für die Acetylierung von Histon H4K16 zugeordnet. Zu Beginn der Arbeit war außer der DNA Sequenz und Hochdurchsatzdaten zur Expression von *mof2* nichts über Mof2 bekannt.

Ein moderater knock-down von Mof2 durch RNA_i hatte keinen Effekt auf die Lebensfähigkeit oder Fertilität von Fliegen. Eine stärkere Reduktion von Mof2 in einem hypomorphen Stamm führte zu einer Lücke auf dem Rücken von adulten Fliegen und hat seine Ursachen wahrscheinlich in einem Entwicklungsdefekt. Diese Arbeit zeigte, dass Mof2 in Embryonen, Larven, adulten Fliegen und den Hoden von Larven und adulten Fliegen vorhanden ist. Im Gegensatz zu vorherigen Daten ist Mof2 auch in S2 Zellen vorhanden. In Embryonen und polytären Chromosomen ist Mof2 im Zellkern lokalisiert, während es in den Hoden im Zytoplasma nachgewiesen werden kann. Auf den polytären Chromosomen findet sich Mof2 in ca. 80 % der Interbanden und kolokalisiert mit Z4/Putzig. Zusammen mit der durch bioinformatische Auswertungen gefundene Kolokalisierung von Bindestellen von Mof2 mit den Bindestellen von dem Transkriptionsfaktor DREF und den Insulatorproteinen CP190 und dCTCF wäre eine Funktion von Mof2 bei der Regulation der Chromatinstruktur möglich. Bei der ebenfalls gefundenen Kolokalisierung von Bindestellen von Mof2/DREF mit BEAF-32 handelt es sich wahrscheinlich um eine antagonistische Bindung, die verschiedene Chromatinzustände abbildet.

Genomweite Untersuchungen der Bindestellen von Mof2 zeigten eine Anreicherung von Mof2 an Promotoren von Genen mit einer hohen Transkriptions. Obwohl auch eine temperaturabhängige Expression von *mof2* nachgewiesen werden konnte, ist Mof2 nicht an der Hitzeschockantwort in den Speicheldrüsen beteiligt. Eine genauere Klassifizierung der Bindestellen von Mof2 durch eine GO Analyse zeigte, dass Mof2 bevorzugt an Promotern von Genen bindet, die an der Embryogenese beteiligt sind.

Die Enzymaktivität von Mof2 wurde in einem *in vitro* Assay untersucht. Dabei zeigte Mof2 eine Acetylierungsaktivität für Histon H4 mit unbekannter Spezifität.

In dieser Arbeit konnte gezeigt werden, dass die MYST Histonacetyltransferase Mof2 wahrscheinlich an der Regulation der Transkription und der Chromatinstruktur beteiligt ist.

7 References

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10 Veröffentlichungen

Heseding C, Saumweber H and Ehrenhofer-Murray AE

Identification of the novel MYST histone acetyltransferase Mof2 in *Drosophila* (in Vorbereitung)

Schirling C, **Heseding C**, Heise F, Kesper D, Klebes A, Klein-Hitpass L, Vortkamp A, Hoffmann D, Saumweber H, Ehrenhofer-Murray AE

Widespread regulation of gene expression in the *Drosophila* genome by the histone acetyltransferase dTip60, *Chromosoma* (2010)

Wagner S, **Heseding C**, Szlachta K, True JR, Prinz H, Hovemann BT

Drosophila photoreceptors express cysteine peptidase tan, *J Comp Neurol.* (2007)