On the histone acetyltransferase hMOF

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ABBREVIATIONS

CBP	CREB-binding protein
DCC	dosage compensation complex
DSB	double-stranded break
GCN5	glucose non-fermenting
GlcNAc	N-acetylglucosamine
kDa	kilodalton
KSHV	Kaposi's sarcoma-associated herpesvirus
HAT	histone acetyltransferase
HCF-1	host cell factor 1
HDAC	histone deacetylase
HMT	histone methyltransferase
HP1	heterochromatin protein 1
IC50	50% inhibitory concentration
ILF-1	interleukin 2 enhancer binding factor 1
ISWI	imitation switch
LC	liquid chromatography
MALDI-TOF	matrix-assisted laser desorption ionization time-of-flight
MCRS1	microspherule protein 1
MLE	maleless
MOF	males absent on the first
MOZ	monocytic leukemia zinc finger protein
MORF	MRG-related factor
MS	mass spectrometry
MSL	male-specific lethal
MYST	MOZ/YBF2/SAS2/TIP60
NER	nucleotide excision repair
NHEJ	non-homologous end joining
NSL	non-specific lethal
OGT	O-linked β-N-acetylglucosaminetransferase
ORC	origin recognition complex
PCAF	p300/CBP associated factor
PHF20	PhD finger protein 20
PHF20L1	PHF20-like 1
RHA	RNA helicase A
RSTS	Rubinstein-Taybi syndrome
SAS	something about silencing 2
SIR	silent information regulator
Su(var)3-9	Suppressor of variegation 3-9
TIP60	Tat-interacting protein 60
TPR	translocated promoter region
TSA	trichostatin A
WDR5	WD40 repeat protein 5
WGA	wheat germ agglutinin

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications and manuscripts. In the text, they are referred to by Roman numerals. In addition, some unpublished results will be presented and discussed.

I

Taipale, M., S. Rea, K. Richter, A. Vilar, P. Lichter, A. Imhof, and A. Akhtar (2005). hMOF histone acetyltransferase is required for histone H4 lysine 16 acetylation in mammalian cells. *Mol. Cell. Biol.* 25:6798-6810

II

Taipale, M.*, S. Pfister*, S. Rea*, F. Mendrzyk, B. Straub, M. Vermeulen, M. Schelder, P. Gebhardt, S. Durrinck, H.P. Sinn, M. Wilm, H. Stunnenberg, P. Lichter, and A. Akhtar (2005). Concomitant loss of hMOF and histone H4 lysine 16 acetylation by hMOF complexes is a common feature in breast tumors and medulloblastomas. *Manuscript*

III

Buscaino, A., T. Kocher, J.H. Kind, H. Holz, **M. Taipale**, K. Wagner, M. Wilm, and A. Akhtar (2003). MOF-regulated acetylation of MSL-3 in the *Drosophila* dosage compensation complex. *Mol. Cell.* 11:1265-127

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ABSTRACT

The eukaryotic genome is organized in the nucleus as long chromatin fibers, or chromosomes, that are composed of DNA and associated proteins, mostly histones. Histones together with DNA constitute the basic repeating unit of chromatin, the nucleosome. They pack the DNA such that during cell division chromosomes are about 50 000 times shorter than their extended length would be. Yet, chromatin is unwrapped and packed again during the lifetime of a cell in a highly regulated manner.

All processes in the nucleus involving DNA have to deal with the condensed and mostly inaccessible chromatin structure. DNA can be rendered more or less accessible by several mechanisms. Chromatin remodeling enzymes can spatially move nucleosomes and create new arrangements of the chromatin fiber. Canonical histones can also be replaced by histone variants that subtly change the properties of chromatin. Post-translational modifications of histones such as acetylation, on the other hand, both create docking sites for effector proteins and physically change chromatin structure by various mechanisms.

This thesis presents the functional characterization of the hMOF histone acetyltransferase and the multiprotein complexes containing hMOF. hMOF is the most specific histone acetyltransferase characterized so far in human cells. It specifically acetylates histone H4 lysine K16, a modification implicated in transcriptional activation in the fruit fly *Drosophila melanogaster*. Depletion of hMOF in cultured cells leads to a dramatic reduction in H4K16 acetylation. In addition, the cells show reduced ability to repair damaged DNA, proliferation defects, and nuclear morphology aberrations.

Purification of the human hMOF complexes revealed that it resides in two evolutionary conserved multiprotein complexes. The MSL complex is required for dosage compensation in male flies. The novel NSL complex, which was previously uncharacterized, contains transcriptional co-activators, a known oncogene, a glycosyltransferase, a methyllysine-binding protein, and previously unknown proteins.

Finally, analysis of breast cancer and medulloblastoma samples revealed that loss of hMOF expression and H4K16 acetylation is a very common feature of these cancers, suggesting a role for global histone modifications in tumorigenesis. hMOF is the first protein linked to global histone acetylation patterns in normal tissues and tumors.

TIIVISTELMÄ

Aitotumallisten eliöiden DNA muodostaa soluissa pitkiä kromatiinirihmoja eli kromosomeja. Kromatiini on helminauhamainen rakenne, jonka perusyksikkö on nukleosomi. Nukleosomi koostuu kahdeksasta histoniproteiinista, joiden ympärille 246 emäsparia DNA:ta tiiviisti kietoutuu. Nukleosomit pakkautuvat tiiviisti toisiinsa siten, että solun jakautuessa kromosomin pituus on vain yksi viideskymmenestuhannesosa sen alkuperäisestä pituudesta. Solun elinaikana kromatiini avautuu ja taas kietoutuu jatkuvasti. Koska tiiviisti kietoutunut kromatiini voi estää säätelyproteiinien sitoutumisen DNA:han, kromatiinin rakennetta on voitava muuttaa kontrolloidusti.

Solut ovat kehittäneet useita tapoja säädellä kromatiinin kietoutumista. Jotkut proteiinit voivat liu'uttaa nukleosomeja DNA-ketjua pitkin muokaten täten kromatiinirihmaa. Histonit voidaan myös korvata ns. histonivarianteilla, jotka muuttavat nukleosomin rakennetta hienovaraisesti. Histoneita muokataan myös proteiinisynteesin jälkeen liittämällä niiden aminohappotähteisiin kovalentisti esimerkiksi asetyyli-, metyyli-, tai fosfaattiryhmiä. Tällaiset muutokset voivat joko suoraan vaikuttaa kromatiinin rakenteeseen tai luoda erityisiä sitoutumispintoja säätelyproteiineille.

Tämän väitöskirjatyön aiheena on histoniasetyylitransferaasi hMOF ja sen tehtävä ihmissoluissa. hMOF asetyloi lysiini K16:n histonista H4 *in vitro*. hMOF-proteiinin poisto ihmissoluista vähensi selvästi H4K16:n asetylaatiota, mikä osoittaa että hMOF on tärkein ellei ainoa H4K16-spesifinen asetyylitransferaasi ihmissoluissa. hMOF-proteiinin poiston jälkeen solut eivät enää kasvaneet normaalisti, niiden tuman muoto vaihtui, eivätkä ne pystyneet korjaamaan vaurioitunutta DNA:ta normaalisti.

hMOF-proteiinikompleksin biokemiallinen puhdistus paljasti, että hMOF on kahden erillisen proteiinikompleksin (MSL ja NSL) katalyyttinen osa. MSL-kompleksin tehtävä banaanikärpäsessä on annoskompensaatio eli koiraan X-kromosomin geenien hyperaktivoiminen. NSL-proteiinikompleksia ei ole ennen tätä työtä tunnettu. NSL sisältää mm. transkriptiotekijöitä, tunnetun onkogeenin, ja useita tuntemattomia proteiineja.

hMOF-proteiinin analysointi rintasyöpä- ja medulloblastoomanäytteissä paljasti että sen ilmentyminen, ja siten myös H4K16:n asetylaatio, on vähentynyt suuressa osassa näistä syövistä. hMOF on täten ensimmäinen proteiini, joka on yhdistetty laajoihin histoniasetylaation muutoksiin syöpäkudoksissa.

REVIEW OF THE LITERATURE

A single human cell contains six billion base pairs of DNA, which equals about 2 meters in length. All this must be packaged into the nucleus, whose diameter varies between 5 and 20 micrometers, or millionths of a meter. DNA, however, is only half the story – indeed, in many ways – for chromosomes consist of both DNA and proteins. In the nucleus, DNA is wrapped around histones that are small positively charged proteins that tightly bind to DNA to form a repeating structure called the nucleosome. Nucleosomes decorate our DNA like beads on a string, and pack the long DNA molecule inside the cell nucleus to build our chromosomes.

STRUCTURE OF CHROMATIN

There are five canonical histones in all eukaryotes: H1, H2A, H2B, H3, and H4. Histones are small positively charged proteins rich in arginine and lysine, and their amino acid sequence is remarkably well conserved among eukaryotes (Sullivan and Landsman, 2003). The nucleosome consists of two copies of each H2A, H2B, H3, and H4 (Kornberg, 1974; Kornberg and Thomas, 1974; Oudet et al., 1975), which are wrapped around by 146 base pairs of DNA. In physiological ionic strength, histones H3 and H4 form a stable tetramer, whereas H2A and H2B form two dimers in absence of DNA. Together with DNA, histones form the typical octamer structure, and DNA wraps around it 1.65 superhelical turns (Luger et al., 1997). The nucleosome core particle is thus the first step of DNA compaction (Figure 1).

The second step of compaction is the addition of the linker histone H1, which can protect another 20 base pairs of DNA from nuclease digestion at one edge of the nucleosome, although its exact position is not known, perhaps because it can vary between cells and tissues (Gilbert et al., 2005; Thomas, 1999). The core nucleosome together with histone H1 is sometimes referred to as the chromatosome. This structure is the repeating unit of all chromosomes in higher eukaryotes: about 166 base pairs of DNA covered by the nucleosome and histone H1, together with linker DNA of variable length between adjacent nucleosomes. The role of histone H1 is still somewhat unclear, but it appears to function in the stabilization

and folding of the 30 nanometer fiber (Gilbert et al., 2005; Harvey and Downs, 2004; Thomas, 1999).

Although the structure of the nucleosome core particle is, apart from the N-terminal tails, well-characterized (Chakravarthy et al., 2005; Luger et al., 1997; Richmond and Davey, 2003; White et al., 2001), the nature of the 30 nm fiber is still poorly understood. This is both because of inaccessibility of the fiber to experimental manipulation *in vivo* and the technical obstacles preventing high-resolution structure analysis *in vitro*. Recent experiments have shown that the traditional solenoid model, where 6-8 nucleosomes form a superhelical turn and H1 molecules reside inside of the filament, is probably wrong. The nucleosomes appear to organize themselves, at least *in vitro*, in a so-called two-start fiber, where two stacks of helically arranged nucleosomes are connected by the linker DNA and H1, such that they are on the surface of the fiber (Dorigo et al., 2004; Khorasanizadeh, 2004; Schalch et al., 2005). 30 nm fiber is still further compacted to form tightly coiled or extended chromonema fibers that are 100-130 nanometers in diameter, at least in the G1 phase of the cell cycle before DNA replication (Belmont and Bruce, 1994). The chromatin fibers are further condensed during cell division by cohesins and condensins, such that mitotic chromosomes are about 50.000 times shorter than their extended length (Nasmyth and Haering, 2005).

Now that the 2 meters of DNA are tightly packed together with the histones inside the nucleus, another difficulty emerges. How can regulatory proteins access the DNA if it is buried under so many levels of organization? In cells, chromatin exists in two distinct states, euchromatin and heterochromatin. Heterochromatin is further divided to facultative and constitutive heterochromatin. Euchromatin is generally considered the open chromatin structure, whereas heterochromatin is tightly packed, closed chromatin. Heterochromatin stains brightly with DNA-binding dyes and is composed of more electron-dense material than euchromatin, supporting this idea (Gilbert et al., 2005). Also the protein components and their post-translational modifications differ in heterochromatin and euchromatin (reviewed in Richards and Elgin, 2002). Furthermore, the physicochemical properties of heterochromatin imply that it is indeed tightly packed, most likely as a continuous 30 nm fiber (Ghirlando et al., 2004; Gilbert and Allan, 2001). In euchromatin, on the other hand, this fiber is interspersed with more relaxed structures (Gilbert and Allan, 2001).

Here lies the fundamental question of chromatin structure: What causes the difference between active and inactive chromatin domains, between condensed and decondensed regions? The issue is complicated by the fact that active genes can be found in tightly condensed chromatin, and inactive genes can be surrounded by large open chromatin

structures (Dimitri et al., 2005; Gilbert et al., 2004). As constitutive heterochromatin bears some hallmark sequence features, such as paucity of genes and high number of repetitive DNA elements, it could be that these sequences are directly responsible for the organization of the heterochromatin domains. However, it is clear that DNA sequence itself is not enough to determine the state of chromatin. For example, human centromeres, as a classical example of constitutive heterochromatin, have very few or no genes and they are composed mainly of repetitive elements, but studies on neocentromeres have shown that DNA sequences that bear no similarity to normal centromeres can acquire centromere identity *de novo* and maintain it through multiple generations (Cleveland et al., 2003).

Facultative heterochromatin, also referred to as silenced chromatin, is another example of the plasticity of chromatin. In this case the chromatin state can vary between individuals, tissues, and/or cells despite identical DNA sequences. For example, in female mammals one X chromosome is inactivated in a random manner, forming a heterochromatic structure called the Barr body at the nuclear periphery (Avner and Heard, 2001). It is again clear that we must be able to explain the structural and functional difference between the active and the inactive X chromosome independently of the underlying DNA sequence.

Epigenetics is the field that studies such phenomena. Conrad Waddington coined the term “epigenetics” to describe the interaction between environment, developmental processes (*epigenesis*), and genes (*genetics*) during embryogenesis (see Van Speybroeck, 2002) and references therein). Currently, epigenetics refers more restrictedly to heritable changes in chromatin organization without changes in DNA sequence. In the past 15 years, the field of epigenetics has vastly expanded through identification and characterization of the biochemical processes associated with distinct chromatin states. However, despite extensive research there is still a clear gap in our knowledge in connecting these biochemical changes to alterations in higher order chromatin structure.

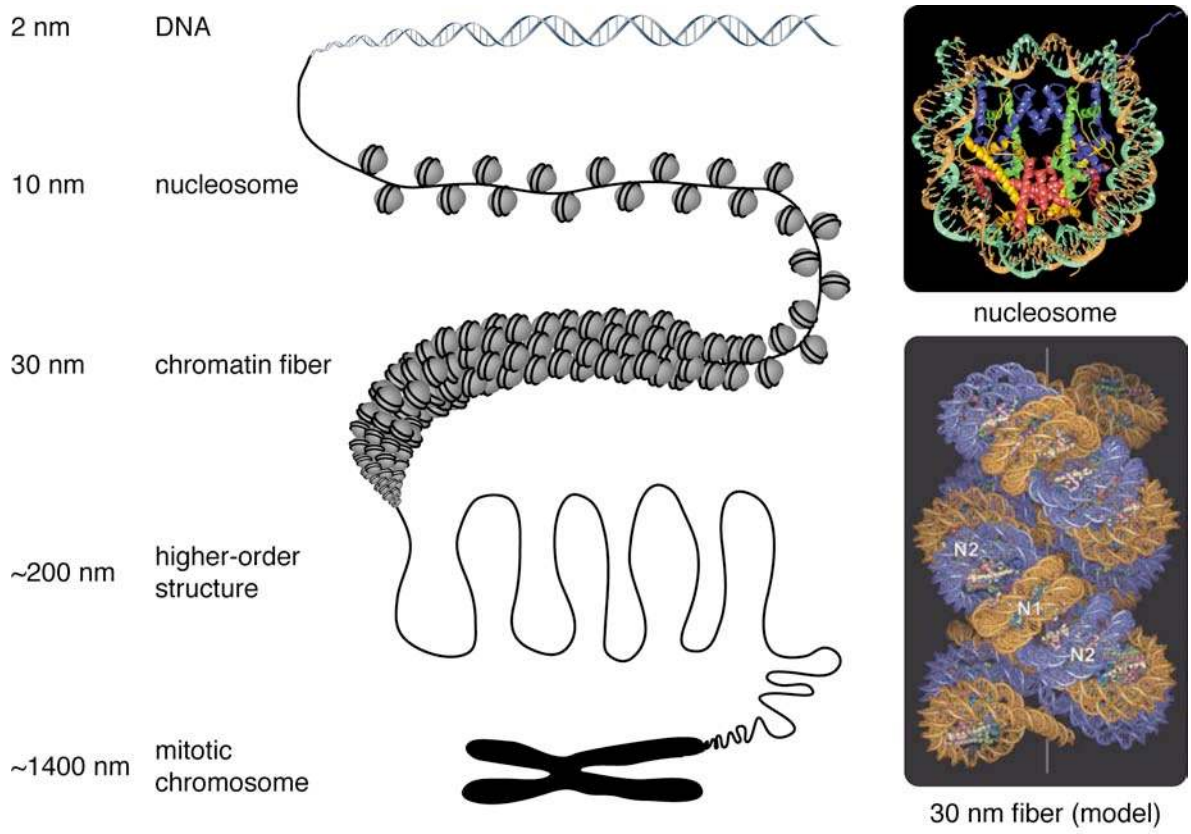


Figure 1. Organization of DNA in the eukaryotic nucleus. The crystal structure of the mononucleosomes is adapted from Luger et al. (1997), and the suggested 30 nm fiber structure from Schalch et al. (2005).

BIOCHEMISTRY OF CHROMATIN

Biochemical activities that epigenetically modify chromatin structure are usually divided into three classes. Chromatin remodeling enzymes are components of multiprotein complexes that can alter the chromatin structure by physically moving nucleosomes. Another group of enzymes modify histones post-translationally and thus influence the structure of the surrounding chromatin or, alternatively, facilitate the binding of other effectors to chromatin. Enzymes that methylate DNA can also be considered members of this group. In addition to modifications, canonical histones can be replaced by slightly different histone variants. Even though these variants can be highly homologous to the canonical histones, they may impose very distinct outcomes.

As the main topic of this thesis is histone acetylation, the role of chromatin remodeling and histone variants in chromatin organization will be only briefly discussed.

CHROMATIN REMODELING ENZYMES

Nucleosome positions on chromosomes are usually well-conserved in eukaryotes, implicating that they either prefer certain DNA sequences or that there are other factors influencing the positioning (Yuan et al., 2005). Clearly, nucleosomes need to be moved, as an immobilized nucleosome can very effectively prevent transcription (Gottesfeld et al., 2002). This applies also to many other cellular processes, such as DNA replication, repair, and recombination (Fyodorov and Kadonaga, 2001; Tsukiyama, 2002).

Chromatin remodeling enzymes use ATP as an energy source to move nucleosomes along the DNA or to modify histone-DNA contacts. All characterized ATP-dependent remodeling enzymes have an ATPase domain that belongs to the Swi2/Snf2 helicase superfamily. They often also associate with large multiprotein complexes that can be even 2 MDa in size (Smith and Peterson, 2005). The complexes are further subdivided to four classes based on the ATPase subunit identity, namely SWI2/SNF2, Mi-2/CHD, ISWI, and INO80 (Tsukiyama, 2002).

Although the exact biochemical mechanism as to how the complexes remodel nucleosomes is still debated (see Flaus and Owen-Hughes, 2004 for discussion), these complexes can catalytically move nucleosomes in an ATP-dependent manner *in vitro* (Hamiche et al., 1999; Langst et al., 1999; Whitehouse et al., 1999). Associated factors can also modify the properties of the ATPase. For example, ISWI ATPase is associated with three different complexes. ACF and CHRAC complexes can create regularly spaced nucleosomes on a DNA template *in vitro*, whereas the NURF complex disrupts a regular array (Langst and Becker, 2001).

In vivo, nucleosome remodeling complexes have various functions. ISWI is associated with regions with little RNA polymerase II, suggesting that it is a general repressor of transcription (Deuring et al., 2000). Mi-2/CHD complexes are also involved in repression, as substantiated by the observation that efficient repression of homeotic genes in flies requires intact Mi-2 function (Kehle et al., 1998). In contrast, SWI2/SNF2 is required for activation of several genes, both in yeast and higher eukaryotes (Smith and Peterson, 2005).

Chromatin remodeling complexes cooperate with histone- and DNA-modifying enzymes in gene regulation. Generally, chromatin remodeling is needed to render chromatin more or less accessible to DNA-binding transcription factors and/or RNA polymerase II

(Narlikar et al., 2002). In addition to local changes on promoters, they can also regulate global chromatin structure. *ISWI* mutant male flies have grossly puffed X chromosomes (Deuring et al., 2000), and DDM1 ATPase is required for genomic cytosine methylation in *Arabidopsis thaliana* (Jeddeloh et al., 1999).

HISTONE VARIANTS

In addition to the five canonical histones, most if not all eukaryotes have genes coding for variants that belong to the histone protein family. Most histone variants contain a conserved histone-fold domain, which is flanked by more variable sequences, or even additional domains (Sarma and Reinberg, 2005; Sullivan and Landsman, 2003). With the exception of histone H4, there are sequence variants for each core histone and H1. Most variants are expressed during all stages of the cell cycle, whereas major histones are expressed only during S phase to ensure their proper deposition only to newly synthesized DNA (Sarma and Reinberg, 2005).

Even very small differences in amino acid sequence can have drastic consequences. For example, histone variant H3.3 differs from H3 by only four amino acid residues, but they display very different characteristics. H3.3 is deposited into DNA in a replication-independent manner, whereas H3 needs DNA replication to be incorporated (Ahmad and Henikoff, 2002; Tagami et al., 2004). Furthermore, H3.3 is enriched in histone modifications associated with active chromatin, such as methylation of lysine K4 and lysine K79 (McKittrick et al., 2004). Therefore, it seems that H3.3 is specifically incorporated into active chromatin, perhaps in conjunction with transcription.

Another histone variant, macroH2A, is associated with inactive chromatin, and in particular, with the inactive X chromosome of female mammals (Costanzi and Pehrson, 1998). MacroH2A contains a macro domain in addition to its globular histone fold domain. The macro domain is found in diverse proteins in eukaryotes, and its function has been elusive (Allen et al., 2003; Ladurner, 2003). *In vitro*, macroH2A-containing nucleosomes are refractory to remodeling by SWI/SNF and can inhibit binding of NF-κB on nucleosomal arrays, suggesting that macroH2A functions at least partly by steric hindrance (Angelov et al., 2003). Recently, macro domains were shown to bind ADP-ribose or O-acetyl-ADP-ribose, revealing an interesting connection between histone variants and metabolism (Karras et al., 2005; Kustatscher et al., 2005).

Of course, transcription is not the only process that histone variants mediate. Phosphorylation of histone H2AX at a C-terminal serine (S139 in mammals) is a mediator of DNA repair response in many organisms. H2AX gets rapidly phosphorylated in its SQ(E/D)ϕ consensus sequence by PIKK family kinases ATM, ATR, and DNA-PK following DNA damage (Burma et al., 2001; Stiff et al., 2004; Ward and Chen, 2001). In addition, H2AX has a role in meiotic silencing of the XY body in the male germline and maintenance of genomic stability (Celeste et al., 2003; Celeste et al., 2002; Fernandez-Capetillo et al., 2003).

How all these histone variants change chromatin structure is still poorly understood. Crystallization experiments have shown that the nucleosome can accommodate significant variation in the amino acid sequence of the variants and still retain most of its structure (Suto et al., 2000). However, physicochemical properties of these nucleosomes are often different, as illustrated by analysis of H2AZ-containing chromatin *in vitro* (Abbott et al., 2001; Fan et al., 2002; Fan et al., 2004).

To ensure proper distribution of the variant histones to chromatin during the cell cycle and differentiation, deposition of these variants must be carefully controlled in the cell. As previously mentioned, variants are mostly expressed throughout the cell cycle, while major histones are expressed only as a short pulse during DNA synthesis. Similarly, in contrast to major histones, many variants can be deposited into chromatin regardless of DNA replication (Kamakaka and Biggins, 2005). Biochemical purification of histone variant containing protein complexes has recently shed light on the mechanistic aspects of deposition (Kobor et al., 2004; Krogan et al., 2003; Kusch et al., 2004; Mizuguchi et al., 2004; Tagami et al., 2004). These studies have shown that at least histone variants H2AZ in *S. cerevisiae*, H2Av in *D. melanogaster*, and H3.3 in human cells are deposited by chromatin remodeling complexes that are different from the CAF-1 chromatin assembly factor utilized by the major histones (Loyola and Almouzni, 2004; Tagami et al., 2004). Thus, histone variants can regulate genome structure both by temporal and structural means.

It is to some degree misleading to discuss histone ‘variants’, for in many cases they represent a significant fraction of all histones. For example, H3.3 constitutes about 25% of all H3 in *Drosophila* cells (Ahmad and Henikoff, 2002), and this can increase up to 80% in some postmitotic cells (Pina and Suau, 1987), as the major H3 species is only synthesized during the S phase. Likewise, H2AX represents about 10% of all H2A in mammals. Moreover, from a yeast cell’s perspective H3.3 or H2AX are not variants, since they are the only histone genes in yeast encoding histones H3 and H2A, respectively (Malik and Henikoff, 2003).

POST-TRANSLATIONAL MODIFICATIONS

Histone modifications have arguably received the most attention in chromatin research in the past 5-10 years. Histone methylation and acetylation were known already 30 years ago, and acetylation was shown to be associated with gene activity (Allfrey et al., 1964). In addition, studies in budding yeast, *Tetrahymena*, and fruit fly suggested that the modifications were highly specific (Grunstein, 1997). Hallmark studies characterizing the first acetyltransferases (Brownell et al., 1996; Kleff et al., 1995; Parthun et al., 1996), deacetylase (Taunton et al., 1996), and methyltransferases (Chen et al., 1999; Rea et al., 2000) finally allowed experimental manipulation of the responsible enzymes and protein complexes. Research in recent years has created an impressive catalog of different histone modifications and their varying roles in chromatin-related processes. In addition to methylation and acetylation, histones can be phosphorylated, ubiquitinated, SUMOylated, ADP-ribosylated, and biotinylated (Margueron et al., 2005). Again, as this thesis is mainly concerned with histone acetylation, other modifications will be discussed rather briefly.

Most post-translational modifications of histones occur in their well-conserved N-terminal tails. In particular, histones H3 and H4 are subject to extensive modifications. Figure 2 illustrates the current state of post-translational histone tail modifications, as of July 2005. In addition to those listed here, mass spectrometric analysis of endogenous histones has revealed numerous other sites that are subject to modifications (reviewed in Cosgrove et al., 2004).

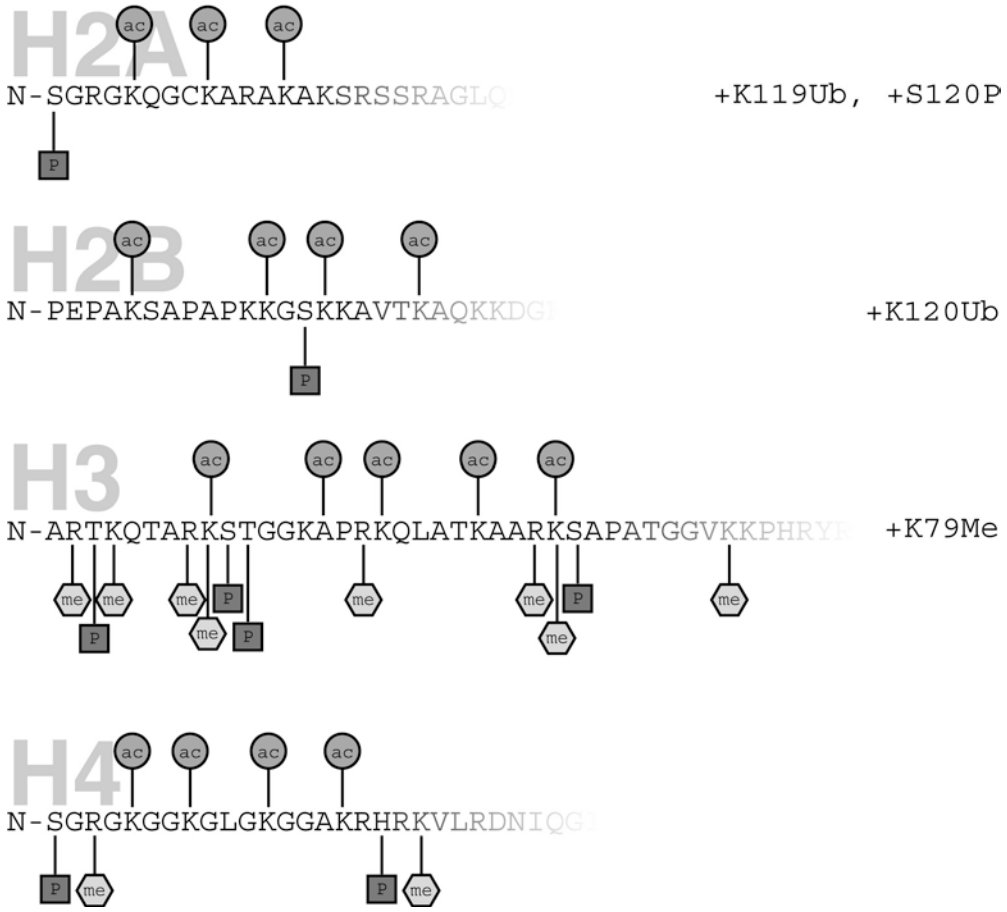


Figure 2. Post-translational modifications of histones in human cells. Ac, acetylation; Me, methylation; Ub, ubiquitination; P, phosphorylation. Adapted from Margueron et al. (2005).

Several theories have been put forth to explain the function of histone modifications. The charge hypothesis asserts that neutralization of positively charged lysine residues and the negative charge brought about by phosphorylated serine residues would functionally influence the interaction surface of the histones with DNA (Grunstein, 1997). This has also been extended to post-translation modifications of the histone core domains (Cosgrove et al., 2004). On the other hand, different modifications could constitute a "histone code", so that they would act sequentially and combinatorially to create distinct outputs that could not necessarily be predicted from single modifications (Jenuwein and Allis, 2001; Strahl and Allis, 2000; Turner, 1993; Turner, 2000; Turner, 2002). Finally, Schreiber and Bernstein (2002) proposed that signaling through histone modifications resembles what is seen in, for example, receptor tyrosine kinase signal transduction. Although these models need not be mutually exclusive, they do contain certain predictions that can be and have been tested.

Acetylation-deacetylation as a charge switch

According to the charge neutralization model, histone acetylation and/or phosphorylation patterns should have largely redundant roles. Furthermore, replacing an acetylatable lysine with an arginine, a structurally similar and positively charged amino acid, should mimic the deacetylated state, while lysine-to-glutamine substitution should mimic acetylation. Indeed, some structural and biochemical data support the charge neutralization model. In the crystal structure of the nucleosome, most histone tails are not structured, and therefore it is not possible to obtain clear information about their positions (Luger et al., 1997). However, the positively charged amino acids in the N-terminal region K16-R23 of H4 make hydrogen bonds and salt bridges to acidic residues on H2A and H2B (Luger et al., 1997). It is thus conceivable that neutralization of H4K16 (and possibly other three lysine residues K5, K8, and K12) by acetylation have an effect on internucleosomal H2A-H4 interaction. This would influence the higher-order chromatin structure but not the structure of a single nucleosome.

Histone hyperacetylation has been shown to increase the accessibility of DNA to DNA-modifying enzymes and transcription factors *in vitro* (Anderson et al., 2001; Lee et al., 1993; Vettese-Dadey et al., 1994) and also to destabilize nucleosomes (reviewed in Wolffe and Hayes, 1999). Furthermore, it has been shown in budding yeast that a deletion of a single lysine has only a weak effect on gene silencing and sporulation (Park and Szostak, 1990), cell cycle (Megee et al., 1990; Megee et al., 1995), DNA repair (Bird et al., 2002), or transcription (Dion et al., 2005). Conversely, addition of a single lysine, even to an ectopic location, in a quadruple lysine-to-arginine mutant H4 tail can restore DNA repair response (Bird et al., 2002) and proper G2/M progression (Megee et al., 1995). In further support of the charge hypothesis, gene expression profiling of H4K5, H4K8, and H4K12 mutants and combinations thereof show that the acetylation effects are largely cumulative, not specific (Dion et al., 2005).

Histones are also modified in their globular domains, yet the role of these modifications is poorly understood. Mutations in some of the histone H3 and H4 core domain residues reported to be acetylated affect gene silencing in the budding yeast (Park et al., 2002a). Cosgrove and colleagues (2004) proposed that these modifications alter nucleosome dynamics by disrupting histone-DNA contacts by chemical interference, such as charge neutralization. In contrast to histone tail modifications, they would mostly be specific and not

redundant in function, because each residue has a specific contact to the DNA backbone (Cosgrove et al., 2004).

But not all tail lysine residues are created equal – lysine 16 seems to be an outlier on the H4 tail. First, mutations in K16 cause phenotypes that are distinct from other tail lysine mutations (Megee et al., 1990; Megee et al., 1995; Park and Szostak, 1990). Secondly, the gene expression profile of K16R yeast mutant strain is also distinct from those from the strains with mutations in the other H4 tail lysine residues (Dion et al., 2005). Finally, specific acetylation of K16 has been shown to mediate spreading of heterochromatin in yeast (Kimura et al., 2002; Suka et al., 2002), and it is a hallmark of hyperactive chromatin in the male X chromosome of *Drosophila* (Turner et al., 1992). These data would argue against a simple charge effect of acetylation. Furthermore, as lysine methylation does not influence the charge, there must also be other mechanisms interpreting these marks. This brings us to the histone code hypothesis.

Is there a histone code?

Pioneering studies on the fruit fly 15 years ago illustrated that there are specific patterns of histone acetylation and that acetylated lysine residues differ in their localization in the nucleus (Turner et al., 1992). This led to the histone code hypothesis, which states that (i) different histone modifications exert distinct outcomes, (ii) modifications act sequentially and combinatorially, and that (iii) there are specific binding modules that recognize these marks and combinations, thus 'translating' the code, which leads to a specific output, e.g. transcriptional activation (Jenuwein and Allis, 2001; Strahl and Allis, 2000; Turner, 1993; Turner, 2000; Turner, 2002).

Indeed, the number of different combinations is impressive. Solely with the currently known modifications of the histone H3 tail, H3 could theoretically form almost a thousand different combinations of three modified residues, without taking di- and tri-methylation into account. However, many of the modifications are mutually exclusive, reducing the real number of combinations *in vivo*. It will be of major importance to functionally decipher the meaning of these modifications and combinations. On the other hand, one is left to wonder if there really is a need for a combinatorial code, if there are already 44 single modification possibilities, if also mono-, di-, and trimethylation are considered.

It is clear that histone modifications can have very distinct outcomes. Perhaps the best understood modifications are methylation of histone H3 at lysine K4 and lysine K9. H3K4 methylation, and trimethylation in particular, is associated with active genes both in yeast and metazoa (Bernstein et al., 2002; Bernstein et al., 2005; Santos-Rosa et al., 2002; Schneider et al., 2004; Strahl et al., 1999). In contrast, H3K9 methylation is a repressive histone modification, associated mainly with heterochromatin (Nakayama et al., 2001; Peters et al., 2001). Tethering Suv39h1, an H3K9-specific methyltransferase, to chromatin represses transcription, which suggests that H3K9 methylation does not merely correlate with repression, but is actively involved in establishing it (Snowden et al., 2002). H3K4 and H3K9 methylation are largely mutually exclusive, corresponding to their opposing roles in maintaining and establishing chromatin structure.

Other modifications have also been implicated in various cellular processes. Other activating histone marks are H3S10 phosphorylation, H3R17, H3K79, H3K36, and H4R3 methylation as well as H2BK123 ubiquitination, whereas H3K27 and H4K20 methylation are associated with repressed chromatin (reviewed in Peterson and Laniel, 2004). Beyond transcription, phosphorylation of H3S10 also correlates with mitosis in many organisms (Hsu et al., 2000), and is required for proper mitosis at least in *Tetrahymena* (Wei et al., 1999), while phosphorylation of H2BS14 is a marker for apoptosis in vertebrates (Cheung et al., 2003).

What about proteins that read and translate the marks? Again, there is a great deal of data showing that there are protein domains that recognize modified histone tails. Chromodomains have been shown to specifically bind methylated lysine tails. The chromodomain of HP1 binds H3K9 methyllysine (Bannister et al., 2001; Jacobs and Khorasanizadeh, 2002; Lachner et al., 2001; Nielsen et al., 2002), and Polycomb chromodomain H3K27 methyllysine (Fischle et al., 2003). Methylation of H3K9 by Suv39h1 and Suv39h2 is required for proper localization of HP1 to pericentric heterochromatin *in vivo* (Lachner et al., 2001). Recently, WD40-domain containing protein WDR5 was shown to bind dimethylated H3K4 *in vitro*, adding a new domain to methyllysine binding motifs (Wysocka et al., 2005). Similarly, acetylated lysines can be recognized by bromodomains (Dhalluin et al., 1999; Jacobson et al., 2000; Kasten et al., 2004). The unifying theme is that methyllysine- or acetyllysine-binding proteins are often associated with enzymes that catalyze these reactions (such as Su(var)3-9 and HP1) or even carry the enzymatic activity themselves, as is the case with the histone acetyltransferase PCAF (Dhalluin et al., 1999).

In some cases, a modification masks a binding site instead of creating one. The bromodomain factor Bdf1 binds acetylated histone tails (Ladurner et al., 2003), but preferentially only when H4K16 is deacetylated (Kurdistani et al., 2004). Similarly, Sir3 protein, which regulates telomeric silencing, interacts with H4 only when lysine K16 is deacetylated, both *in vitro* and *in vivo* (Kimura et al., 2002; Suka et al., 2002). Furthermore, binding of a repressive ISWI ATPase to histone H4 tail is blocked by H4K16 acetylation (Corona et al., 2002).

Sequential order of different modifications has been addressed in cell culture and *in vitro*. For example, during activation of the human IFNβ gene, GCN5 acetylates first H3K9 and H4K8 on a specific nucleosome at the promoter, after which an unknown kinase phosphorylates H3S10. These modifications, in turn, are required for GCN5-dependent H3K14 acetylation and binding of SWI/SNF and TFIID complexes to the promoter, a requirement for transcription initiation (Agalioti et al., 2002).

Thus, overwhelming evidence shows that histone modifications can be specific, temporally and spatially regulated, and recognized by effector proteins. However, there is less evidence that these modifications work combinatorially rather than, for example, in a reinforcing manner. Active histone modifications (H3K79met, H3K4dimet, H3K4trimet, H3ac, and H4ac) correlate very significantly on a genome-wide scale (Bernstein et al., 2005; Schubeler et al., 2004), implicating that there are fewer combinations *in vivo* than would be expected. In addition, there is only limited evidence so far that combinations of modifications create an output that is qualitatively different from single modifications. An interesting example is the DNA methyltransferase CHROMOMETHYLASE3 (CMT3) in *Arabidopsis thaliana*. It contains a chromodomain that binds H3 tails only when both K9 and K27 are trimethylated (Lindroth et al., 2004). Thus, it is conceivable that it is targeted to the correct loci by two different histone methyltransferases, and both modifications are required for a binary output, i.e. DNA methylation.

Histone modifications as signal transductors

The histone code hypothesis has received criticism based on the lack of evidence for combinatorial, predictable outputs (see for example Henikoff, 2005; Kurdistani et al., 2004). Therefore, alternative models have been proposed. Schreiber and Bernstein (2002) noted the similarities between histone modifications and signal transduction. In both cases,

modifications would be to some extent redundant in order to confer network properties, such as robustness, to signaling events. Creating docking sites by post-translational modifications can increase the local concentration of effector proteins, and provided that they are connected by feedback loops, it could promote bistability (Schreiber and Bernstein, 2002). Histone modifications could, in principle, be divided into two categories. First class would be responsible for recruiting effector proteins such as HP1, and the second class would function in downstream signaling. Cosgrove et al. (2004) propose that histone tail modifications belong primarily to the former group, and core modifications form the latter group. However, this distinction does not account for the fact that there are relatively few modifications in the histone tails that have been demonstrated to function in the recruitment step.

Regardless of the hypotheses, one of the greatest challenges is the abundance of modifications. Some modifications are highly abundant in the cell, very much like the histone variants discussed earlier. About 50% of histone H4 tails are acetylated in human cells, of which 90% (45% of the total H4 tail acetylation) is on lysine 16 (Turner et al., 1989). This means that at any given moment, almost half of the nucleosomes are acetylated at H4K16, and this only if nucleosomes are symmetrically modified. In yeast, a nucleosome carries, on average, 13 acetyl groups (Waterborg, 2000). Similarly, 90% of histone H3 is methylated at K79 in yeast (van Leeuwen et al., 2002), and 35% of *Tetrahymena* H3 is methylated at K4 (Strahl et al., 1999). How to gain specificity if the modifications are so abundant? It is conceivable that there is a significant amount of global acetylation and methylation background in the cell, and gene activation or repression is a result of a local, relative change in the modification patterns (see below).

HISTONE ACETYLTRANSFERASES

Since cloning of the first histone acetyltransferases 10 years ago (Brownell et al., 1996; Kleff et al., 1995; Parthun et al., 1996), extensive studies have characterized their biological functions, mainly in budding yeast, fruit fly, and mammalian cells. It has become clear that HATs participate in most, if not all, biological processes involving chromatin.

STRUCTURE OF HISTONE ACETYLTRANSFERASES

Histone acetyltransferases catalyze the transfer of an acetyl group to the ε-amino group of lysine residues. In the reaction, acetyl coenzyme A serves as the acetyl group donor, and the final products are acetyl-lysine and CoA. Histone acetyltransferases share sequence and structural homology to bacterial aminoglycoside N-acetyltransferases, suggesting a common evolutionary origin for these enzymes, and many other enzymes catalyzing similar reactions (Wolf et al., 1998). In particular, they share an invariant Arg/Gln-X-X-Gly-X-Gly motif, which regulates acetyl-CoA recognition (Dutnall et al., 1998; Wolf et al., 1998). The catalytic core of HATs consists of two substructures, one with three β-strands and the other with a β-strand followed by a loop and an α-helix. Acetyl-CoA and the histone tail are accommodated between these domains. The specificity of HATs seems to be provided by less-conserved residues outside the catalytic region, or by other interacting factors (Roth et al., 2001).

Interestingly, although HATs share significant structural homology, their mechanism of catalysis can differ. PCAF and Gcn5 form a ternary protein-cofactor-substrate complex using a conserved glutamate residue, and the reaction proceeds via a nucleophilic attack of the substrate on acetyl-CoA (Clements et al., 1999; Rojas et al., 1999; Trievel et al., 1999). Esa1p, which belongs to another subfamily of HATs (see below), utilizes a ping-pong mechanism, where acetyl-CoA forms a covalent intermediate with a conserved cysteine residue, which in turn transfers the acetyl group to the ε-amino group of a target lysine (Yan et al., 2002). Also p300 appears to catalyze histone acetylation with kinetics compatible with a ping-pong mechanism (Thompson et al., 2001).

HATs can also have autoregulatory functions. Thompson et al. (2004) showed that p300 contains an unstructured loop that is highly acetylated *in vitro* and *in vivo*. Hyperacetylation of the loop enhances the HAT activity of p300 both *in vivo* and *in vitro*, suggesting that HATs may be regulated like some kinases with an autoinhibitory loop (Thompson et al., 2004).

HAT FAMILIES

The human genome contains about 30 histone acetyltransferases. However, it should be noted that HAT might be a misnomer in some cases. Many HATs have been shown to acetylate also non-histone targets, and some of them might not acetylate histones at all in more physiological conditions (Roth et al., 2001). A number of acetylated non-histone proteins, such as transcription factors, importins, and tubulin, have been characterized in recent years (Roth et al., 2001). It is conceivable that acetylation also participates in, for example, signal transduction – perhaps it does not have as pervasive a role as protein kinases do, but nevertheless a substantial one (Kouzarides, 2000).

Classically, histone acetyltransferases were divided into two groups based on their subcellular localization. Nuclear A-type HATs acetylate histones in a chromatin context, whereas B-type HATs are cytoplasmic, and they acetylate histones prior to their deposition to chromatin. However, this distinction is somewhat unclear, as HATs can shuttle between nucleus and the cytoplasm (Ai and Parthun, 2004; Poveda et al., 2004).

After initial correlative observations (Allfrey et al., 1964), the connection between gene activity and histone acetylation gained further support by experiments showing that known transcriptional co-activators had intrinsic HAT activity (Bannister and Kouzarides, 1996; Brownell et al., 1996; Mizzen et al., 1996; Ogryzko et al., 1996; Yang et al., 1996). Consequently, acetylation of histone tails as a mechanism for transcriptional activation has become a paradigm in molecular biology, although some counterexamples show that a causal link from acetylation to gene activation is sometimes too simplified a model (Kurdistani et al., 2004; Wang et al., 2002).

B-HATs

During histone synthesis, histone H4 is acetylated in the cytoplasm at lysines K5 and K12 in wide variety of organisms (Sobel et al., 1995). A conserved enzyme, Hat1, catalyzes this acetylation. An intriguing observation is that neither acetylation of H4 nor Hat1 is required for histone deposition, although the acetylation pattern, the enzyme, and its associated factor are evolutionary conserved. Thus, the function of acetylation of newly synthesized histones has remained elusive (reviewed in Mello and Almouzni, 2001). Deletion of the N-terminal tails of histones H3 and H4 has no effect on histone deposition *in vitro* (Shibahara et al., 2000). *Hat1* mutant strains are also viable (Kleff et al., 1995; Parthun et al., 1996). It has been shown in yeast that Hat1p has a role in repressing telomeric genes, implicating that pre-deposition histone acetylation may function in maintenance of chromatin domains (Kelly et al., 2000). However, this effect was seen only when combined with multiple lysine substitutions in the histone H3 tail, suggesting significant redundancy in the process (Kelly et al., 2000). As a result, although Hat1 was the first acetyltransferase to be molecularly characterized, its role in the cell is still poorly understood.

Prior to histone deposition, histone H4 is also acetylated at lysine K91. However, Hat1 is not required for this modification, suggesting that there are also other B-type HATs encoded by the yeast genome (Ye et al., 2005). A yeast strain where lysine K91 has been replaced by alanine or arginine is hypersensitive to DNA-damaging agents. Moreover, these mutants genetically interact with chromatin assembly factors, suggesting that pre-deposition H4K91 acetylation is regulating chromatin remodeling after DNA damage (Ye et al., 2005).

A-HATs

A-HATs are usually divided into two subgroups. GCN5-related N-acetyltransferase (GNAT) family is the largest group of HATs in the human genome, whereas the MYST (MOZ/YBF2/SAS2/TIP60) family has five members. In addition, there are some divergent HATs that do not clearly belong to either group, such as $TAF_{II}250$, CBP/p300, and Elp3 (Yang, 2004).

Most if not all HATs are associated with multiprotein complexes that often are evolutionary well-conserved (Ogryzko, 2001). Each complex has a distinct substrate

specificity, brought about by either domains adjacent to the catalytic core or associated factors. Indeed, many HATs show very little specificity *in vitro* (Ikura et al., 2000; Kimura and Horikoshi, 1998), or have low activity without associated factors (Morales et al., 2004). Specificity also changes depending on whether the substrate is a histone tail peptide, histone octamers, or nucleosomes.

Complexes containing GNAT family HATs typically acetylate histone H3. In addition, p300 and CBP, two closely related HATs, acetylate mostly H3 and H2A tails *in vitro* and *in vivo* (Ogryzko et al., 1998; Schiltz et al., 1999). On the other hand, MYST family HATs are usually more specific for histone H4. *Drosophila* MOF specifically acetylates histone H4 lysine K16 (Akhtar and Becker, 2000; Smith et al., 2000), whereas closely related Tip60 acetylates both H4 and H2A (Ikura et al., 2000). Table 1 summarizes currently characterized HAT complexes, their evolutionary links, and their substrate specificity.

Table 1. Histone acetyltransferase complexes and their specificity in humans, flies, and the budding yeast. Catalytic subunits are indicated in italics. Adapted from Yang, 2004. For a complete list of lysine acetyltransferases, see Yang, 2004.

Humans	**Fruit flies**	**Budding yeast**	**Specificity**	**Family**
TAFII250 TFIID	*TAFII230* TFIID	*TAFII130* TFIID	H3, H4	Divergent
?? Mediator	*??* Mediator	*Nut1p* Mediator	H3, H4	Divergent
Elp3 Elongator	*Elp3* Elongator	*Elp3p* Elongator	H3, H4	Divergent
GCN5, PCAF STAGA, TFTC, PCAF	*GCN5* ??	*Gcn5p* SAGA, ADA, SLIK	H3	GNAT
HBO1 ??	*Chameau* ??	*Sas3p* NuA3	H3, H4	MYST
TIP60 TIP60	*dTIP60* dTIP60	*Esa1p* NuA4, picNuA4	H2A, H4	MYST
hMOF MSL, NSL	*dMOF* MSL, NSL	*Sas2p* SAS2	H4K16	MYST

Parallels between different HAT complexes were nicely illustrated by the purification of GCN5- and PCAF-containing complexes from human cells (Ogryzko et al., 1998). Both complexes show remarkable similarity in subunit composition to the TFIID core complex. All complexes contain histone H3-specific HAT activity, histone-like TAFs (TFIID-associated factors), and bromodomain proteins (Ogryzko, 2001). Furthermore, purification of the human TIP60 complex revealed that, although subunit composition was very different from GCN5 and PCAF, they all contained PAF400/TRRAP, an ATM-related enzymatically inactive kinase, which is a cofactor for c-Myc and E2F oncogenes (Ikura et al., 2000; McMahon et al., 1998). Another feature of HAT complexes is that they can also possess other enzymatic activities. The TIP60 complex has also ATPase activity (Ikura et al., 2000; Kusch et al., 2004), whereas Ubp8 in the yeast SAGA complex can deubiquitinate histone H2B (Henry et al., 2003).

BIOLOGICAL FUNCTIONS OF HATS

HISTONE ACETYLATION IN GENE REGULATION

Initially, HATs were appreciated mostly as local regulators of gene expression, for several reasons. First identified HATs, such as Gcn5, CBP, and p300, were already previously characterized co-activators with a role in the regulation of a specific set of genes. Moreover, the purification of HAT complexes revealed that they did not contain DNA-binding proteins, further supporting the notion that they act as co-activators.

However, histone acetylation participates, perhaps surprisingly, both in genome-wide and gene-specific regulation. Despite very high basal level of histone acetylation in the cell, HATs can still function as gene-specific activators. It is currently poorly understood why and how these roles are separated. Some experimental data suggest that basal acetylation acts as a balance, so that both gene repression and gene activation can be temporally and spatially regulated more accurately (Vogelauer et al., 2000). Another possibility is that abundant histone modifications function as an exclusion mechanism by limiting the binding of promiscuous silencing-inducing factors (Deuring et al., 2000; van Leeuwen and Gottschling, 2002). Pulse-chase labeling and kinetic analysis of acetylated histones in various organisms has suggested that there are two pools of acetylated histones (reviewed in Waterborg, 2002). It is possible that the slow turnover fraction corresponds to global acetylation and the fraction

with fast turnover to gene-specific regulation. However, experiments have not directly addressed this issue.

Thinking globally

The role of HATs in global chromatin regulation has mostly been studied in yeast and the fruit fly. It is thought that global acetylation is mediated by non-specific interactions of HATs with chromatin, in contrast to local acetylation, which is regulated by sequence specific DNA-binding proteins. For example, the histone deacetylase Rpd3 is a global regulator of gene expression in yeast, but it is also targeted to specific promoters by DNA-binding factors such as Ume6 (Kurdistani et al., 2002; Robyr et al., 2002). Deletion of Ume6 affects the acetylation pattern of only a subset of Rpd3-regulated genes and has no effect on acetylation beyond specific promoters (Kurdistani et al., 2002), illustrating that local and global effects can be genetically separated. Biochemical evidence supporting this idea is provided by the purification of Esa1-containing complexes in yeast. Esa1 is the catalytic subunit of a large HAT complex (NuA4) and a smaller subcomplex, picNuA4. Global effects on histone acetylation by Esa1 are provided by picNuA4, whereas the larger NuA4 complex most probably regulates only a subset of yeast genes (Boudreault et al., 2003).

Telomeric silencing is another area of research where yeast has proven valuable. Silencing of chromatin near telomeres is mechanistically perhaps the best-understood process involving global acetylation. In *S. cerevisiae*, a complex consisting of DNA-binding protein Rap1 and silent information regulators Sir2, Sir3, and Sir4 silences telomere-proximal chromatin (Kurdistani and Grunstein, 2003). Sir2 is an H4K16-specific deacetylase whose activity is antagonized by the H4K16-speficic acetyltransferase Sas2. Deletion of Sas2 allows the SIR complex to spread further from the telomere and cause ectopic silencing, whereas deletion of Sir2 produces the opposite effect (Kimura et al., 2002; Suka et al., 2002). Thus, the balance of Sir2/Sas2, and therefore the extent of H4K16 acetylation, regulates the spreading of the silent chromatin state in yeast.

In *Drosophila*, the paradigm for global acetylation is dosage compensation. The H4K16-specific histone acetyltransferase MOF associates with the male X chromosome and acetylates it across its whole length. The majority of H4K16 acetylation in *Drosophila* males resides on the X chromosome, where it correlates with an approximately two-fold upregulation of gene expression (Bone et al., 1994; Gu et al., 1998). It is currently not known

whether H4K16 acetylation is a cause or a consequence of this upregulation. The former hypothesis is supported by the observation that MOF can activate transcription both *in vitro* and *in vivo* (Akhtar and Becker, 2000). However, MOF can also be targeted to an ectopic site on the X chromosome by activating transcription from a UAS-transgene with the GAL4 transcriptional activator (Sass et al., 2003). In other words, MOF appears to be recruited to the ectopic site after artificial activation. In addition, H4K16 acetylation is associated with coding regions rather than promoters, supporting the idea that acetylation merely enhances transcription (Smith et al., 2001). It is possible that MOF-mediated H4K16 acetylation modifies the acetylation/deacetylation balance such that X-linked genes are less prone to ubiquitous repressive effects of deacetylases and other factors. Interestingly, mutation in the repressive ISWI chromatin remodeling factor results in male-specific puffing of the X chromosome, as though the acetylation/deacetylation balance was severely biased towards activation (Deuring et al., 2000). H4K16 acetylation by MOF is both necessary and sufficient for X chromosome puffing in ISWI mutant flies, and *in vitro* H4K16 acetylation reduces the ATPase activity of ISWI (Corona et al., 2002; Deuring et al., 2000). Drawing parallels with yeast, the limited experimental evidence on global acetylation suggests that it indeed fine-tunes gene expression.

The role of global histone acetylation in mammals is not very well understood. Previous studies have not addressed the effect of individual HATs or HDACs on acetylation of specific residues *in vivo*. Most studies have been performed with histone deacetylase inhibitors, such as trichostatin A (Taddei et al., 2005). Treatment of cultured cells with TSA leads to a reversible delocalization of HP1α protein and histone H3 lysine K9 methylation from pericentric heterochromatin, implicating need for balanced acetylation in this process (Maison et al., 2002; Taddei et al., 2001). It was also reported that TSA treatment leads to reversible relocalization of centromeres to the nuclear periphery (Taddei et al., 2001), but this was not seen in another study (Gilchrist et al., 2004). There is also some evidence for a histone acetylation dependent telomere position effect in human cells, suggesting that similar mechanisms regulate mammalian telomeres (Baur et al., 2001).

The inherent problem in the analysis of global acetylation with current HDAC inhibitors is their relative unspecificity. TSA inhibits many HDACs, which makes the dissection of the responsible molecules complicated. Additionally, as HDACs have many other cellular targets besides histones, it is not possible to exclude their effect. It should be noted, however, that TSA and other HDAC inhibitors have a surprisingly limited effect on gene expression profiles (Gius et al., 2004; Glaser et al., 2003; Van Lint et al., 1996),

suggesting that the effect of TSA on mammalian heterochromatin is not indirectly caused by changes in gene expression.

Acting locally

The role of HATs in transcriptional activation has been extensively studied. It is clear that HATs co-operate with other factors. In yeast, Esa1 is the only essential acetyltransferase; other HAT mutants showing only modest phenotypes. For example, both Gcn5 (HAT) and Swi2/Snf2 (ATPase) mutant strains are viable, but the double mutant is not, and they coordinately regulate gene expression during mitosis (Krebs et al., 2000). The general view has been that DNA-binding transcription factors "recruit" multiprotein complexes with HAT activity to promoters, and that together with chromatin remodeling complexes they allow the assembly of the RNA polymerase pre-initiation complex (PIC), which leads to transcription initiation. The actual order of events, however, depends strongly on the promoter being analyzed (Cosma, 2002; Ptashne and Gann, 2002).

The diversity of transcriptional activation can be appreciated by comparing a few well-characterized promoters. The yeast HO gene is activated by a sequence-specific transcription factor Swi5. It recruits the Swi/Snf remodeling complex, which in turn is required for the association of the Gcn5-containing SAGA complex with the promoter. SAGA and Swi/Snf together facilitate the binding of another transcription factor, SBF, which then induces transcription initiation (Cosma et al., 1999).

During the induction of the human IFNβ gene, in contrast, three transcription factors (NF-κB, IRF, ATF-2/c-Jun), referred to as the enhanceosome, first recruit the GCN5 complex, which acetylates H3K9 and H3K14 of two nucleosomes (I and II) at the promoter. This facilitates the arrival of the TFIID holoenzyme complex together with CBP, another HAT. Acetylation of H4K8 by GCN5 allows the binding of SWI/SNF complex, which remodels the nucleosome II adjacent to the TATA box, thus allowing transcription initiation to take place (Agalioti et al., 2002; Agalioti et al., 2000).

The human α_1 antitrypsin gene (α_1-AT) promoter is the third example. Here, the pre-initiation complex is assembled and RNA polymerase II is phosphorylated long before activation. Only after several days can CBP and PCAF acetyltransferases and the SWI/SNF complex be detected on the promoter, coincident with nucleosome remodeling and

transcription initiation. Thus, the association of PIC with the promoter is not always preceded by histone acetylation and/or chromatin remodeling (Soutoglou and Talianidis, 2002).

These three examples illustrate how genes can have different requirements for histone acetylation and chromatin remodeling. Lomvardas and Thanos (2002) provided an elegant example of plasticity of a single promoter. They rendered the IFNβ core promoter accessible by artificially delivering a nucleosome II to the position where it is usually moved by SWI/SNF activity. This resulted in a GCN5- and SWI/SNF-independent induction of the gene, although it was still dependent on the proper inductive signal, i.e. viral infection (Lomvardas and Thanos, 2002). These results provide some mechanistic ideas to how the local chromatin structure can impose requirements for histone acetylation in transcription.

In addition to transcription activation, histone acetylation has been implicated in the elongation phase. Nucleosomes can inhibit transcription *in vitro*, but *in vivo* transcription through chromatin occurs very rapidly. Histone acetylation significantly increases transcription elongation rate *in vitro* (Protacio et al., 2000). *In vivo*, transcription elongation is facilitated by factors that associate with RNA polymerase II and help it overcome the nucleosome obstacle (Svejstrup, 2002). The Elp3p subunit of the multiprotein Elongator protein complex is a histone acetyltransferase that acetylates H3K14 and H4K8 and regulates global H3 acetylation (Winkler et al., 2002). Although the *Elp3* mutant yeast strain is viable, *Elp3* interacts genetically with *Gcn5*, implying that activation- and elongation-associated histone modifications are functionally connected (Wittschieben et al., 2000).

Nucleosomes are often considered a repressive structure that the basal transcription machinery needs to circumvent during gene activation. However, it has been shown in yeast that depletion of histone H4 affects the transcription of only about 25% of all genes, with telomere-proximal genes being more sensitive to histone loss (Wyrick et al., 1999). This supports the model that genes respond differently and individually to the chromatin environment, thus explaining their varying requirements for histone acetylation.

DNA REPAIR

Many mechanisms have evolved to protect DNA from different kinds of damage, such as double-stranded breaks or thymine dimers. Undetected, they could lead to aberrant gene expression, cell death, or hyperproliferation. DNA repair is as a multistep process, involving initial recognition of damage by sensor proteins, signaling to downstream effector

proteins, and finally, repair of DNA by these effectors and other factors. The DNA repair machinery faces the same challenge as the RNA polymerase discussed above; DNA has to be accessible for various effectors. Thus, it is not surprising that histone acetylation is linked to efficient DNA repair (reviewed in Gong et al., 2005; Koundrioukoff et al., 2004).

Rather than being a static structure during DNA damage response, chromatin appears to actively participate in the process. Treating cells with TSA, chloroquine, or hypotonic salt, which alter chromatin structure without causing DNA damage, can activate the major DSB checkpoint protein ATM (Bakkenist and Kastan, 2003). Furthermore, inhibition of HDACs can also enhance DNA synthesis after UV-induced damage (Gong et al., 2005).

Further evidence for the active role of chromatin in signaling DNA damage was recently provided by two studies in fission yeast and human cells. 53BP1 is a sensor protein that accumulates very early at DSB sites (Schultz et al., 2000). The crystal structure of 53BP1 revealed that it contains a tandem Tudor domain, which interacts with methylated lysine K79 of histone H3 *in vitro*. Consistently, knock-down of the H3K79-specific enzyme hDOT1 reduced accumulation of 53BP1 at the DSB sites (Huyen et al., 2004). Similarly, a homologous *S. pombe* protein Crb2 is directed to DSBs in an H4K20-methylation dependent manner (Sanders et al., 2004). The interesting aspect of these findings is that both H3K79 and H4K20 methylation are very abundant modifications, and normally buried inside the nucleosome core. Therefore, it is possible that damage sensors detect aberrations in chromatin structure rather than DNA damage *per se*, although evidence for the hypothesis remains indirect (Huyen et al., 2004; Sanders et al., 2004).

Histone acetylation was first linked to DNA repair in genetic studies performed in yeast. Certain yeast strains with mutations in the conserved lysine residues of histone H4 were shown to accumulate in G2/M phase of the cell cycle. This phenotype could be suppressed with a mutation in *rad9*, which suggested that it was due to activation of the G2/M checkpoint (Megee et al., 1995). These studies provided only indirect evidence, but it was later shown that an *esa1* mutant defective in acetyltransferase activity similarly arrested in G2/M in a RAD9-dependent manner (Clarke et al., 1999). Indeed, *esa1* mutants are also hypersensitive to DNA-damaging agents camptothecin (CPT) and methyl methane sulphonate (MMS), identical to the histone H4 lysine substitution mutants (Bird et al., 2002). Esa1 HAT activity is also required for efficient non-homologous end joining (NHEJ) in yeast (Bird et al., 2002). Another NuA4 complex subunit, Arp4, interacts with phosphorylated histone H2A, a hallmark of DSB repair, and recruits the complex to DSB sites (Downs et al., 2004). Furthermore, the NuA4 complex is particularly active on linear nucleosomal arrays,

suggesting a mechanism for spatially limited histone acetylation at the site of damage (Bird et al., 2002).

Rapidly after induction of a DSB, chromatin in the vicinity of the break is modified by both remodeling and acetyltransferase complexes (Downs et al., 2004; Morrison et al., 2004). H4K16 acetylation levels have been reported to be modestly decreased near the break, whereas H4K8 acetylation increases (Downs et al., 2004; Jazayeri et al., 2004), when DNA is repaired via NHEJ in yeast. Tamburini and Tyler (2005) systematically measured changes in histone acetylation after DSB induction and during homologous recombination. Generally, histones were deacetylated after DSB induction, but acetylation increased significantly during homologous recombination. The authors suggested that deacetylation is a response to damage, whereas subsequent acetylation serves as a signal that repair is complete (Tamburini and Tyler, 2005).

Another histone modification linked to DNA repair is acetylation of histone H4 lysine K56. While abundant during the S phase, H4K56 gets deacetylated in G2. However, in the presence of DNA damage, H4K56 acetylation is retained near the DSBs in a checkpoint-dependent manner. Since this modification is in the nucleosome core, it is possible that its function is to retain a favorable chromatin structure for efficient DNA repair (Masumoto et al., 2005). This is comparable to the phenotype seen in yeast cells mutant for H4K91, another nucleosome core modification (Ye et al., 2005). Furthermore, both modifications also regulate gene expression, illustrating that they are used in multiple contexts (Xu et al., 2005; Ye et al., 2005).

The function of HATs in DNA repair in Metazoa is not as well understood. The purification of the mammalian Tip60 complex, orthologous to yeast NuA4, provided an indication that it could be involved in DNA repair. The complex contains helicases that are homologous to the bacterial repair enzyme ruvB (Ikura et al., 2000). Expression of a dominant-negative version of Tip60 inhibited γ-irradiation induced apoptosis and delayed the kinetics of DSB repair (Ikura et al., 2000). Kusch and colleagues (2004) elegantly demonstrated a molecular mechanism for Tip60's function in DSB repair. They showed that *Drosophila* Tip60 preferentially acetylates the phosphorylated form of H2Av, which leads to the replacement of this histone with the canonical H2A by the Domino/p400 ATPase subunit of the complex (Kusch et al., 2004). Even though these results do not explain how the dominant-negative Tip60 cells accumulate DSBs, they do demonstrate how phospho-H2Av can be cleared from the damaged area during or after repair.

p300 was suggested to have a function in DNA repair based on its association with PCNA (Hasan et al., 2001), a protein mediating nucleotide excision repair (NER). However, there is no evidence that this interaction modulates NER. Another HAT linked to NER is GCN5. It is the catalytic subunit of the TFTC complex (TBP-free TAFII complex), which preferentially binds and acetylates nucleosomes that contain UV-damaged DNA (Brand et al., 2001). Histone acetylation is also increased in HeLa cells and yeast cells upon UV irradiation (Brand et al., 2001; Yu et al., 2005), and *gcn5* mutant yeast is sensitive to UV (Teng et al., 2002).

DNA REPLICATION

DNA replication initiates at elements called origins of replication that are distributed all over the genome. In contrast to the budding yeast, where the origin is a rather well defined DNA sequence, higher eukaryotes do not seem to have clear *cis*-acting sequences directing replication initiation (Bell and Dutta, 2002). Chromatin environment is clearly a contributing factor, as origin identity can change during development (Aggarwal and Calvi, 2004). Furthermore, heterochromatic regions are often associated with late origins (Bell and Dutta, 2002).

Early studies on the timing of histone acetylation during the cell cycle showed that it peaks during S phase (Weiss and Puschendorf, 1988). *In vitro*, hyperacetylation of histones can enhance the efficiency of replication elongation (Alexiadis et al., 1997). Furthermore, TSA-treated cells can complete the S phase of the cell cycle more rapidly than untreated cells, and the treatment affects the distribution of replication initiation in the human β-globin locus and in *Drosophila* follicle cells (Aggarwal and Calvi, 2004; Kemp et al., 2005). Thus, quite expectedly, histone acetylation appears to enhance DNA replication.

Mammalian HATs PCAF and GCN5 can stimulate replication initiation when tethered to a polyomavirus origin of replication, whereas p300 and CBP have no effect (Xie et al., 2002). However, HBO1 seems to be the HAT involved in general replication. HBO1 belongs to the MYST family of HATs, and has been shown to interact with origin recognition complex (ORC) proteins ORC1 and MCM2 (Burke et al., 2001; Iizuka and Stillman, 1999). It also associates with the Kaposi's sarcoma-associated herpesvirus (KSHV) origin of replication and its knock-down results in reduced KSHV replication *in vivo* (Stedman et al., 2004). In *Drosophila*, the HBO1 ortholog Chameau regulates origin activity in the follicle

cells. Treating follicle cells with TSA or tethering Chameau to an origin of replication significantly increases re-replication, whereas the HDAC Rpd3 has the opposite effect (Aggarwal and Calvi, 2004). The timing of acetylation of histones H3 and H4 at the origin is coincident with ORC binding, suggesting that chromatin structure regulates an early step in replication, i.e. origin recognition.

DNA replication presents another, unrelated challenge for histone acetylation. During replication, new histones must be deposited on the newly synthesized DNA strands. There are two questions that emerge – how are the parental histones divided between daughter strands, and how can the established modification patterns be maintained through DNA replication? The latter is a fundamental question in epigenetics. The simplest way to preserve the epigenetic patterns would be semiconservative replication, where histone octamers are split during replication and each daughter strand inherits half of the old octamer, followed by restoration of the original pattern by maintenance methyl- and acetyltransferases. Although histones H3 and H4 form dimers during deposition (Tagami et al., 2004), most evidence suggests that replication is, nevertheless, conservative (Annunziato, 2005; Henikoff et al., 2004). That is, the daughter strands inherit either the old tetramer or a newly synthesized one. One possibility is that the transcription machinery itself regulates G2 phase histone modifications so that active genes re-establish correct histone modifications during next rounds of transcription (Henikoff et al., 2004). Thus, transcription would feed back to histone modifications that were establishing the original transcriptional state.

RECOMBINATION

Meiotic recombination events are nonrandomly distributed in the genome. Most recombination occurs in hotspots, whereas the rest of the genome shows very little recombination activity (Kauppi et al., 2004). Yet, the molecular mechanisms as to why some regions are more prone to recombine are poorly understood, because hotspots share no sequence similarity. Even in closely relates species, hotspots do not appear to be conserved (Ptak et al., 2005; Ptak et al., 2004). Thus, chromatin has been suggested to play a role in determining recombination frequency (Petes, 2001).

Unfortunately, very little is known about epigenetic regulators participating in meiotic recombination, even though the role of chromatin structure in the process was revealed over 10 years ago (Wu and Lichten, 1994). So far, histone acetylation, methylation and

ubiquitination have been linked to different steps in recombination. Deletion of the Set1 methyltransferase was shown to reduce meiotic double-stranded breaks (DSBs) in the fission yeast (Sollier et al., 2004), and histone methylation has also been indirectly implicated in recombination in *Caenorhabditis elegans* (Reddy and Villeneuve, 2004). It was also recently shown that Rad6-mediated H2B ubiquitination is important for recombination in some hotspots in the budding yeast (Yamashita et al., 2004). Yamada and colleagues (2004) addressed the role of histone acetylation in the well-characterized *M26* hotspot in the fission yeast. They showed that histone H3 and H4 are specifically hyperacetylated at the hotspot, and that *SpGcn5* deletion reduces both H3 acetylation and recombination frequency at the locus. The effects were enhanced in a *swi/snf* mutant background, again illustrating the interplay between chromatin remodeling factors and histone acetylation (Yamada et al., 2004). It is therefore likely that histone acetylation participates in generating the open chromatin structure at a recombination hotspot.

Not surprisingly, a similar theme is seen in recombination of immunoglobulin and T-cell receptor loci in humans. Histone acetylation enhances V(D)J recombination (McBlane and Boyes, 2000; McMurry and Krangel, 2000) and is also correlated with class switch recombination (Nambu et al., 2003). It is likely that acetylation enhances the access of the RAG1 and RAG2 recombinases to DNA during V(D)J recombination (Bergman et al., 2003). Again, histone acetylation seems to act in concert with Swi/Snf remodeling activity in V(D)J recombination (Kwon et al., 2000). Acetylation has also been implicated in correct targeting of the activation-induced cytidine deaminase (AID) to the variable V region in the immunoglobulin gene (Li et al., 2004). As AID is the enzyme responsible for somatic hypermutation of the V chain, its targeting must be precisely regulated. This appears to happen at the level of chromatin. However, studies have not yet addressed which HATs are regulating somatic recombination and AID targeting.

HATs IN DEVELOPMENT AND DISEASE

LESSONS FROM MICE

Mouse models have provided valuable information about the function of histone acetyltransferases during normal development. In contrast to budding yeast, where most HAT mutants are viable, mice deficient for HATs have proven lethal, with the sole exception of PCAF.

p300-deficient mice die between embryonic days e9 and e11.5 with incomplete neural closure and abnormal heart development (Yao et al., 1998). $p300^{-/-}$ embryonic fibroblasts also proliferate poorly. These defects are partly dosage dependent, as heterozygous mice are born in sub-mendelian ratios and show similar, albeit weaker, embryonic defects than homozygous mice (Yao et al., 1998). Furthermore, p300 acetyltransferase activity is required for muscle differentiation (Roth et al., 2003).

p300 is highly related to the CBP acetyltransferase, and they are often referred commonly to as p300/CBP. $p300^{+/-}$ $CBP^{+/-}$ compound heterozygotes are not viable, illustrating that they do indeed have partly overlapping, dosage dependent functions. However, although CBP deficiency results in embryonic lethality, $CBP^{-/-}$ embryos have distinct hematopoietic defects and can develop tumors in a dosage dependent manner (Kang-Decker et al., 2004; Kung et al., 2000; Oike et al., 1999b; Rebel et al., 2002). The function of p300/CBP appears to be largely dependent on the acetyltransferase activity, as point mutations abolishing HAT activity have a partial dominant-negative effect (Shikama et al., 2003).

GCN5 and PCAF are another pair of closely related acetyltransferases, and they reside in almost identical protein complexes (Ogryzko et al., 1998; Xu et al., 1998). However, whereas PCAF-deficient mice develop normally, GCN5 nullizygous mice die *in utero* (Yamauchi et al., 2000). GCN5 is required for the formation of dorsal mesoderm lineages during embryogenesis (Xu et al., 2000). Like p300 and CBP, $PCAF^{-/-}$ $GCN5^{-/-}$ mice have a more severe phenotype than $PCAF^{+/-}$ $GCN5^{-/-}$,(Xu et al., 2000; Yamauchi et al., 2000). GCN5 also shows synthetic effects with p300, illustrating extensive overlap between acetyltransferase functions *in vivo* (Phan et al., 2005).

Hypomorphic MOZ mutant mice were recovered from a genetic screen for regulators of neurogenesis in the cerebral cortex (Thomas et al., 2000). *querkopf* mutants are born with

craniofacial abnormalities and low weight, and most mice die shortly after birth. Furthermore, mutant mice have been reported to have a reduced number of nestin-positive, PNA-binding and HSA-negative neural stem cells (Rietze et al., 2001). MOZ was also found in a zebrafish screen for genes required for segmental identity in the pharyngeal arches. *bimandibular* mutants had a partial transformation of the second arch cartilages, which coincided with aberrant expression of Hox1-4 genes (Miller et al., 2004). The results suggest that MOZ participates in maintenance of Hox expression domains, and thus behaves like a Trithorax group gene (Miller et al., 2004).

CHROMATIN AND DISEASE

The role of epigenetics in tumorigenesis has received much attention since the first report connecting global DNA hypomethylation to tumor samples (Feinberg and Vogelstein, 1983). The malignant state appears to be a combination of specific gene mutations, genetic instability, and epigenetic factors. The interplay of point mutations, loss of heterozygosity, and genomic instability is a well-established concept in cancer research, although details as to the importance of each step continue to be debated (Cahill et al., 1999; Rajagopalan and Lengauer, 2004). The contribution of epigenetics to tumorigenesis, however, is rather poorly understood, despite extensive research (Feinberg, 2004; Feinberg and Tycko, 2004).

Given that histone acetylation is participating in all the above-mentioned chromatin-related processes, it is hardly surprising that HATs are also involved in disease. Epigenetic changes in the genome, and thus changes in gene expression patterns, can occur by mutations in chromatin-modifying enzymes or by environmental influences. For example, DNA methylation and histone acetylation patterns of identical twins tend to diverge with age, and this is correlated with differences in gene expression (Fraga et al., 2005a). On the other hand, histone acetylation has been shown to contribute to epigenetic canalization, i.e. the production of varying phenotypes in highly inbred organisms (Sollars et al., 2003). These states can also be transgenerational (Cavalli and Paro, 1998; Cavalli and Paro, 1999; Morgan et al., 1999). It is thus very likely that chromatin has a significant role in many quantitative traits that so far have eluded traditional DNA-based approaches (Bjornsson et al., 2004).

Beyond purely circumstantial evidence, HATs have also been shown to directly contribute to disease. Mutations in CBP or p300, or chromosomal rearrangements involving the genes, underlie Rubinstein-Taybi syndrome (RSTS), whose clinical features are mental and growth retardation and facial dysmorphology (Petrij et al., 1995; Roelfsema et al., 2005).

Analysis of point mutations in RSTS patients indicates that loss of CBP HAT activity is sufficient to cause the disease. Targeted mutation in mouse CBP leads to RSTS-like phenotypes and suggests that RSTS is caused by dominant negative CBP mutations (Oike et al., 1999a). In addition to the previously mentioned clinical features, RSTS patients also have higher incidence of cancer (Kalkhoven, 2004). As the syndrome is dominantly inherited, CBP and p300 seem to function as classical tumor suppressors. Indeed, mutations in p300 have been found in primary tumors and cell lines, albeit with a low frequency (Gayther et al., 2000; Ozdag et al., 2002). Consistent with this observation, reintroduction of p300 to a p300-deficient cell line suppresses growth (Suganuma et al., 2002).

Further evidence for the direct role of HATs in tumorigenesis is their recurrence in malignant translocations. p300 and CBP are common translocation partners with the MLL histone methyltransferase in acute myeloid leukemia (Ida et al., 1997; Sobulo et al., 1997; Taki et al., 1997). Furthermore, CBP and p300 genes have also been shown to translocate to MORF and MOZ genes in AML (Borrow et al., 1996; Chaffanet et al., 2000; Panagopoulos et al., 2001). This is an interesting connection, because both MORF and MOZ are MYST family histone acetyltransferases, and the fusion proteins retain both CBP/p300 and MORF/MOZ enzymatic domains. MOZ also has another fusion partner, the transcriptional coactivator TIF2 (Carapeti et al., 1998). To close the circle, MOZ-TIF2 fusion protein interacts with CBP, and this interaction is necessary for transformation (Deguchi et al., 2003).

Hence, aberrant histone acetylation can lead to cell transformation. What, then, is the mechanism? On the one hand, CBP and p300 appear to act as a tumor suppressors in Rubinstein-Taybi syndrome, and CBP also in mouse models (Kang-Decker et al., 2004; Kung et al., 2000; Rebel et al., 2002). The MOZ-TIF2 fusion protein has also been reported to have a dominant-negative effect on CBP function (Kindle et al., 2005). However, CBP fusion proteins could also function as oncogenes. The MLL-CBP fusion protein upregulates Hoxa9 expression, and this is required for bone marrow progenitor cell expansion (Wang et al., 2005). In addition, MOZ-TIF2 fusion appears to exert its effect partly via tethering CBP to MOZ target genes, suggesting that CBP gain-of-function regulates leukemogenesis (Deguchi et al., 2003).

Systematic analysis of histone modifications by mass spectrometry and immunohistochemistry has demonstrated that also global patterns of acetylation and methylation are aberrant in tumors (Fraga et al., 2005b; Seligson et al., 2005). Fraga and colleagues (2005) analyzed multiple primary tumors and cancer cell lines by mass spectrometry and demonstrated that histone H4 lysine K16 acetylation and lysine K20

trimethylation were markedly reduced in many tumors, whereas other modifications remained unchanged. Changes in post-translational modifications can also have predictive value, as shown by analysis of H3K9, H3K18, and H4K12 acetylation as well as H4R3 and H3K4 dimethylation in prostate cancer samples (Seligson et al., 2005). By grouping tumor samples based on similar patterns of modifications, the authors could identify cancer subtypes and predict tumor recurrence (Seligson et al., 2005).

Targeting histone acetylation in cancer therapy has received extensive attention in recent years. Studies revealing that compounds with known antiproliferative effects, such as TSA, sodium butyrate, and valproic acid, increased histone acetylation by inhibiting deacetylation raised interest in HDACs as therapeutic targets (Candido et al., 1978; Phiel et al., 2001; Yoshida et al., 1990). Several HDAC inhibitors have been shown to be well tolerated and functional against different kinds of tumors, and some compounds have now entered phase I and phase II trials (Drummond et al., 2005).

DOSAGE COMPENSATION AS A MODEL FOR CHROMATIN REGULATION

In many animal species, sex is determined by specialized chromosomes. In mammals, females have two X chromosomes while males have one X and one Y chromosome. Thus, male is the heterogametic sex. The opposite is true in snakes and birds, for males are ZZ and females ZW. Fruit flies again are like mammals (males XY, females XX), whereas the nematode *Caenorhabditis elegans* lacks the Y chromosome altogether. Therefore, XX individuals are hermaphrodites and those with XO chromosome composition are males. In all cases, sexes have different number of X or Z chromosomes.

Even though the evolutionary paths leading to diverged sex chromosomes are separate, all species with diverged sex-determining chromosomes face a similar problem. Losing or gaining a single chromosome (aneuploidy) almost invariably leads to developmental abnormalities or miscarriage. Turner syndrome patients with only one X chromosome and Down syndrome patients with an extra chromosome 21 are examples of aneuploidy in humans. Because X and Z chromosomes are no different in that sense from other chromosomes, evolution must have come up with a solution – dosage compensation. This means that despite a different number of sex chromosomes, males and females express

the same amount of sex chromosome linked genes. The Y and W chromosomes are the only exceptions, for they carry genes that are essential only for the heterogametic sex (Charlesworth, 1996).

Although sex determination is a fundamental biological phenomenon, it evolves surprisingly quickly (Marin and Baker, 1998). The fruit fly, nematode, and mammalian sex chromosomes have divergent evolutionary origins, and this is also reflected in different mechanisms of dosage compensation in these species.

MAMMALS: X = X(X)

Mammalian dosage compensation occurs by X inactivation in females. During early female embryogenesis, one X chromosome is randomly chosen to become epigenetically inactivated (Lyon, 1961). Inactivation persists through cell division, so that all progeny of a given cell will have the same X chromosome inactive. In humans, the inactive X chromosome can be visualized in the nuclear periphery as a condensed chromatin structure referred to as the Barr body (Latham, 2005).

Mammalian dosage compensation is regulated by the non-coding RNA Xist, which is both necessary and sufficient for the initiation of inactivation (Brown et al., 1991; Lee and Jaenisch, 1997; Penny et al., 1996). The *Xist* gene resides on the X chromosome, and is only expressed from the inactive X. The Xist RNA spreads *in cis* and coats the inactive X chromosome. By a yet-unknown mechanism it recruits the Polycomb complexes PRC1 and PRC2 to the inactive X to facilitate the maintenance of inactivation. The PRC1 complex has H3K27-specific methyltransferase activity, whereas PRC2 carries ubiquitin ligase activity (Muller et al., 2002; Wang et al., 2004).

Consequently, trimethylation of H3K27 and ubiquitination of histone H2A are hallmarks of the inactive X chromosome (de Napoles et al., 2004; Hernandez-Munoz et al., 2005; Plath et al., 2003; Silva et al., 2003). Other epigenetic marks on the inactive X are the relative deacetylation of histone H4 and the accumulation of the histone variant macroH2A (Costanzi and Pehrson, 1998; Jeppesen and Turner, 1993). Thus, the cytogenetically visible Barr body is also a distinct chromatin domain, specified by a precise pattern of histone modifications.

NEMATODES: X = ½XX

C. elegans hermaphrodites equalize the dose of X-linked gene products with the males by downregulating global X chromosomal expression by half. This is brought about by a protein complex that shares similarity with the 13S condensin complex implicated in mitotic chromosome compaction (Hagstrom and Meyer, 2003). It is therefore likely that chromosome condensation is directly responsible for the downregulation of the hermaphrodite X chromosomes.

The nematode dosage compensation complex (DCC) is composed of eight subunits. SDC-2 acts as the master switch that integrates upstream signals, i.e. somatic sex determination, to the assembly of the complex on the X chromosomes (Dawes et al., 1999). MIX1, DPY-26, DPY-27, and DPY-28 proteins are homologous to condensin subunits, and they function in fine-tuning X chromosomal gene expression, probably by altering the global chromatin structure (Hagstrom and Meyer, 2003).

In contrast to the mammalian dosage compensation where inactivation occurs *in cis* with the *Xist* locus, the nematode DCC has to be specifically targeted to the X chromosome. Therefore, the X chromosome contains multiple sequence elements that are able to bind the DCC, thus limiting dosage compensation strictly to the X chromosome (Csankovszki et al., 2004). It is possible that the complex then spreads *in cis* from the limited number of targeting sites to the flanking chromatin to cover the whole X chromosome.

Very little is known about the mechanisms by which the DCC can precisely downregulate X-linked genes by 50% in hermaphrodites. The DCC is surprisingly adaptable, for it can specifically repress transcription of a single gene (*her-1*) twenty-fold and simultaneously regulate global X chromosome repression (Chu et al., 2002).

FRUIT FLIES: 2X = XX

Dosage compensation in *Drosophila* species bears similarities to both mammals and nematodes. Analogous to *Xist* RNA in mice, the *Drosophila* DCC has non-coding RNAs as functional components, but the X chromosome also contains elements that ensure the correct targeting of the complex. However, in contrast to the other two model organisms, dosage

compensation in fruit flies occurs in males. Compared to females, the male X chromosome is approximately two-fold more active (Belote and Lucchesi, 1980).

Extensive genetic screens have recovered five genes (*msl-1, msl-2, msl-3, mof, mle*) essential for dosage compensation based on their requirement for male viability while having no visible phenotype in females (Bashaw and Baker, 1995; Gorman et al., 1995; Hilfiker et al., 1997; Kelley et al., 1995; Kuroda et al., 1991; Palmer et al., 1993; Zhou et al., 1995). These genes are collectively referred to as male-specific lethals or MSLs (Taipale and Akhtar, 2005). Furthermore, two non-coding RNAs, roX1 and roX2, associating with the complex were found in unrelated screens (Amrein and Axel, 1997; Meller et al., 1997). Immunofluorescence studies have confirmed that roX RNAs and MSL proteins associate with the whole X chromosome in male cells.

Like SDC-2 in *C. elegans*, MSL-2 is the link between sex determination and dosage compensation in *Drosophila*. The master sex-determining gene Sex-lethal (Sxl) inhibits the translation of MSL-2 mRNA in females, and thus MSL-2 protein is only found in males (Bashaw and Baker, 1997; Kelley et al., 1997). MSL-2, in turn, regulates the assembly of the MSL complex and participates in targeting of the complex to the X chromosome.

The distinguishing feature of the male X chromosome is the hyperacetylation of histone H4 at lysine K16. This is a very specific modification, as other histone H4 lysine residues are not hyperacetylated on the male X (Turner et al., 1992). The enzyme responsible for this specific acetylation is the MYST family acetyltransferase MOF. It is an H4K16-specific enzyme *in vitro* and as a component of the MSL complex (Akhtar and Becker, 2000; Smith et al., 2000), and *mof* mutant males have no H4K16 acetylation on the X chromosome (Hilfiker et al., 1997).

How the dosage compensation complex is targeted to the X chromosome is poorly understood. Earlier studies suggested that there is a limited number of specific sequence elements, “chromatin entry sites”, that recruit the complex, and the DCC subsequently spreads *in cis* to cover the whole X chromosome (Kelley et al., 1999; Lyman et al., 1997). However, chromosomal regions without the putative entry sites can still attract the complex when translocated to autosomes, implicating that the targeting is not only due to these high affinity sites (Fagegaltier and Baker, 2004). Nevertheless, it is clear that the DCC can spread to flanking chromatin domains *in cis*, as it can be seen in large chromatin domains surrounding a high affinity site inserted in an autosome (Kelley et al., 1999; Park et al., 2002b).

OTHER ORGANISMS: ??

Since research in molecular biology is usually confined to a few model organisms, very little is known about dosage compensation, or even sex determination, in other species. Of animals with ZW/ZZ sex determination, very limited evidence suggests that butterflies do not compensate for the difference in Z-chromosomal doses (Suzuki et al., 1998; Suzuki et al., 1999), whereas birds appear to have dosage compensation (McQueen et al., 2001). The molecular mechanism in birds is still unknown (Ellegren, 2002). Whether other species, for example fish, have dosage compensation remains a mystery. On the other hand, in some cases there is clearly no need for dosage compensation. Many species have homomorphic sex chromosomes (such as the house fly *Musca domestica*), or sex is determined by environmental factors (reptiles, some insects), and they therefore also lack dosage compensation (Charlesworth, 1996).

An interesting question is how the different dosage compensation mechanisms have evolved. Evolution seems to favor co-option, that is, old components are recruited to perform new functions (Carroll et al., 2001). That is clearly the case in *C. elegans*, where proteins usually involved in mitotic chromatin compaction are re-used in lowering the gene expression level on the hermaphrodite X chromosome. The mammalian polycomb complexes implicated in X inactivation are orthologous to the complexes in fruit flies, where they repress homeotic genes during development. The *Drosophila* MSL complex regulates only X chromosomal genes in males, and appears to have no function in females.

Where did the MSL complex come from? Does it have a function in mammals, and if yes, what might it be?

That is the subject of this thesis.

AIMS OF THE STUDY

The initial interest in the project came from the observation that orthologs for three of the *Drosophila* dosage compensation complex members, namely MLE, MSL-3, and MOF, were identified in mammals. It was a reasonable assumption that the proteins would also form an orthologous complex in mammals. But what would the function be?

Sequence conservation usually implies functional conservation. However, dosage compensation in mammals occurs in a very different way from the fruit fly – repression instead of activation. Could these proteins be involved in X inactivation in mammals? Perhaps not, given the independent evolutionary origins and fundamental differences in the mechanisms of dosage compensation in the two species. Maybe they are involved in general chromatin regulation in both sexes, instead?

The aim of the project was to characterize the role of MSL protein orthologs in mammals, with emphasis on hMOF and hMSL3. The first approach was mostly biochemical, an initial characterization of the proteins *in vitro* and in cell culture. The second work approached the question from two different angles; what do the hMOF and hMSL3 protein complexes consist of, and whether H4K16 acetylation by hMOF is associated with diseases.

MATERIALS AND METHODS

PUBLISHED RESULTS

Methods for the published results can be found in the Materials and Methods section of the articles. For references, see the list of original publications, page 6.

UNPUBLISHED RESULTS

ANALYSIS OF IN VITRO *ACETYLATED HISTONES BY MASS SPECTROMETRY*

Coomassie-stained gel bands were excised, washed, reduced, and alkylated and then digested with trypsin overnight as previously described (Shevchenko et al., 1996). The peptide mixture was extracted twice from the gel matrix and dried in a vacuum centrifuge. The dried peptide mixture was dissolved, desalted on reversed phase material using pulled glass capillaries and eluted directly into the spraying needle as described before (Shevchenko et al., 1996). Mass spectrometry experiments were performed on an API III triple-quadrupole instrument (PE-Sciex, Canada) with a nanoelectrospray source installed. Acetylated peptides were detected with precursor ion scanning (Wilm et al., 1996) for the immonium ion derived ion m/z 126.1 of acetylated lysine. The detected potentially acetylated peptides were sequenced with tandem mass spectrometry using manually optimised collision energies. Fragment spectra were interpreted manually and the acetylated lysine residues were assigned to the sequence of the protein.

FLY CROSSES AND POLYTENE SQUASHES

Full-length hMOF, FLAG-hMSL3, HA-dMOF, and dMSL3 were cloned in the pUAST vector (Brand and Perrimon, 1993). Expression of UAS-hMOF and UAS-dMOF was driven with armadillo-Gal4 as follows: *w cv mof*^{1}/FM7 x w;UAS-hMOF (or dMOF)/CyO;arm-Gal4/+. Rescue efficiency was measured by counting male offspring with both UAS-hMOF (or UAS-dMOF) and arm-Gal4, and either FM7 (control) or *w cv mof*^{1}

(rescue) chromosome. UAS-hMSL3, UAS-dMSL3, and tubulin-Gal4 were recombined onto the *msl3* chromosome, and used in rescue cross as follows: w;tub-Gal4 *msl3*/TM6C x w;UAS-hMSL3 (or dMSL3) *msl3*, and rescue was measured by the ratio of male offspring receiving tub-Gal4 *msl3* chromosome (rescue) to those with the TM6C balancer (control). The mof^1 mutation is a single amino acid substitution (G691E) in the conserved catalytic core, thus rendering the protein enzymatically inactive (Hilfiker et al., 1997). The *msl3* mutation is a substitution (R154Q) that probably renders the protein unstable, since mutant embryos do not appear to express any MSL-3 protein (A. Buscaino, personal communication). Polytene chromosome squashes were performed using standard protocols.

SEQUENCE ALIGNMENTS

Sequences of orthologous proteins were obtained from ENSEMBL (www.ensembl.org), and aligned with ClustalX 1.83 (Chenna et al., 2003). Alignments were edited further with the Jalview software (Clamp et al., 2004). Proteins containing the NSL2 CTCH domain were identified with PSI-BLAST (www.ncbi.nlm.nih.gov), ScanProsite (www.expasy.ch), and HMMer (bio.ifom-firc.it), aligned with ClustalX 1.83, and edited with Jalview.

RESULTS AND DISCUSSION

HISTONE ACETYLTRANSFERASE ACTIVITY OF HMOF

hMOF protein consists of 458 amino acids. It has a non-canonical chromodomain, or a chromo-barrel domain, at its N-terminus (Nielsen et al., 2005). In the *Drosophila* MOF protein, it is required for RNA binding, although without the adjacent linker region this domain is not sufficient for binding (Akhtar et al., 2000; Nielsen et al., 2005). The C-terminus of hMOF contains the domain characteristic for MYST family acetyltransferases. It consists of a putative acetyl-CoA binding pocket and a C_2HC-type zinc finger.

To functionally characterize the hMOF protein, full-length hMOF and its deletion derivatives were expressed as GST fusion proteins and used in filter-binding histone acetyltransferase assays. As expected, hMOF is a potent histone acetyltransferase (Publication **I:**Figure 1C). The intact MYST homology domain is both necessary and sufficient for HAT activity, since the chromo-barrel domain alone or the MYST domain without the zinc finger had no activity (**I:**Figure 1C). This is consistent with the observation that the zinc finger in dMOF is required for substrate recognition (Akhtar and Becker, 2001).

In vitro, recombinant hMOF acetylates both H3 and H4 on histone octamers. However, in contrast to *Drosophila* MOF that acetylates only histone H4 lysine K16 on nucleosomes (Akhtar and Becker, 2000; Smith et al., 2000), hMOF does not appear to be more specific when mono- or oligonucleosomes are used a substrate, as it acetylates both H3 and H4, albeit with a lower efficiency (**I:**Figure 1D)

We mapped the sites of acetylation on nucleosomal histones by mass spectrometry. Nucleosomes were assembled on a linearized DNA template bound to paramagnetic beads and used as a substrate for acetylation by hMOF *in vitro*. The acetylated histones H3 and H4 were in-gel digested and analyzed by mass spectrometry (Shevchenko et al., 1996). Even though hMOF acetylation is slightly more specific on nucleosomes in comparison to the octamer substrate (data not shown), hMOF acetylates all four lysines (K5, K8, K12 and K16) on the histone H4 tail, and lysines 14 and 23 of the histone H3 tail (Figure 3). *In vitro*, hMOF has no preference for a specific H4 lysine residue, because all acetylated peptides carried multiple acetyl-lysines. If hMOF first acetylated preferentially one lysine residue, and only

subsequently other residues, this would have been detected as singly acetylated peptides in mass spectrometry.

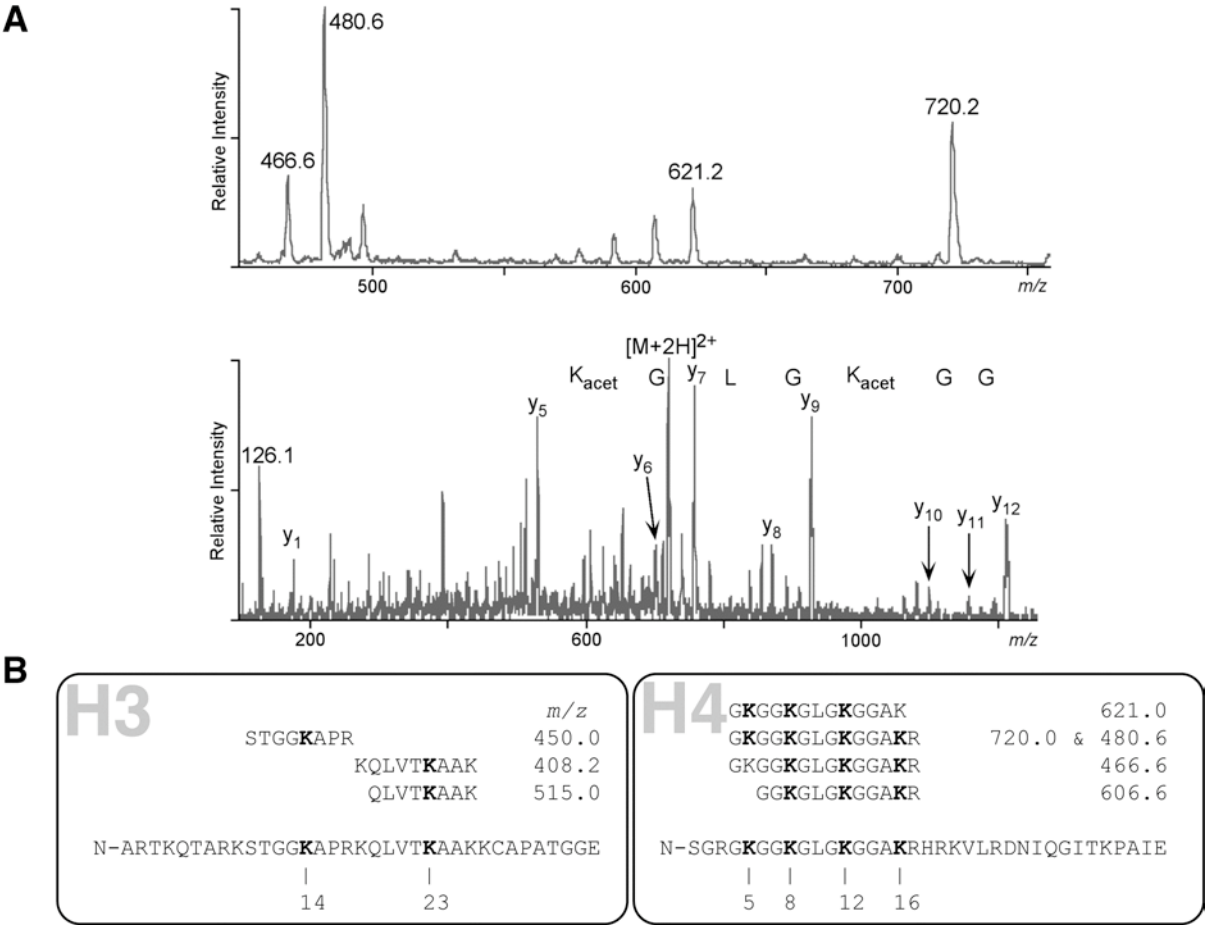


Figure 3. (A) Top, precursor ion scanning for the reporter ion *m/z* 126.1, corresponding to the immonium ion of acetylated lysine after the loss of ammonia. Acetylated peptides are specifically detected in the tryptic peptide mixture. The most prominent peaks are indicated with their *m/z* values. Bottom, determination of the acetylation site in the tryptic peptide *m/z* 720.2 with tandem mass spectrometry: The product ion spectrum of the peptide G[Kacet]GG[Kacet]GLG[Kacet]GGA[Kacet]R is shown. The quadruply acetylated peptide was detected both as a doubly and as a triply charged species (*m/z* 720.2 and *m/z* 480.6, respectively) using precursor ion scanning for *m/z* 126.1. The doubly charged precursor ion was chosen for fragmentation using the same instrument. The C-terminal y-ion series identified the peptide and some of the acetylation sites within the peptide. The prominent y-ions, the corresponding amino acids, the selected precursor ion and the immonium ion of acetylated lysine are indicated. (B) Acetylated peptides detected by mass spectrometry. *m/z* values are indicated on the right. Left, acetylated peptides on histone H3; right, acetylated histone H4 peptides. Acetylated lysines are indicated in bold.

Associated proteins can regulate the activity and specificity of many HATs. For example, recombinant Tip60 acetylates all four lysine residues on H4, K14 of H3, and K5 of H2A, while Tip60 complex preferably acetylates H2A and H4 (Ikura et al., 2000; Kimura and

Horikoshi, 1998). The Tip60 complex is also more active on nucleosomal substrate than the recombinant protein (Ikura et al., 2000). This is also true of *Drosophila* MOF, whose activity is enhanced 30-fold by the associated factors MSL-1 and MSL-3 (Morales et al., 2004). Also hMOF showed more specificity when it was associated with the complex. The immunoprecipitated HA-2xFLAG-hMOF complex was specific for H4K16 *in vitro*, both when bound to α-FLAG beads (**I:**Figure 1E) and when in solution after FLAG peptide elution (**II:**Figure 2B).

These results demonstrate that hMOF is, like the *Drosophila* ortholog, an H4K16-specific acetyltransferase. This is hardly surprising, as hMOF is very conserved evolutionary. hMOF is 52% identical and 69% similar to the dMOF protein. Between mouse and human, there are only eight differences, of which seven map to the N-terminus with no known domains, and the remaining substitution is a conservative tyrosine-to-phenylalanine change (data not shown).

The distribution of acetylated H4K16 in human and *Drosophila* cells suggests that this modification has a different role in these species. In human, about 90% of all histone H4 acetylation is on K16, whereas in the fruit fly acetyl-K16 is less abundant than other acetyl-lysines on H4 (Munks et al., 1991; Turner et al., 1989). This could reflect the fact that H4K16 acetylation is found almost exclusively on the male X chromosome in *Drosophila*, while in human cells it is equally distributed among all chromosomes (Jeppesen and Turner, 1993; Turner et al., 1992). This indirectly implies that hMOF has a general role in global chromatin regulation in human cells.

DIVERGED FUNCTION OF HMOF AND HMSL3

To directly test for a conserved function of hMOF and hMSL3, the genes were expressed in transgenic flies. In order to avoid possible lethality caused solely by expression of ectopic proteins, I used the inducible UAS-Gal4 system (Brand and Perrimon, 1993). Corresponding *Drosophila* genes were used as a control in the experiment.

The binary UAS-Gal4 expression system is based on the yeast Gal4 transactivator and its sequence-specific interaction with UAS (upstream activation sequence) sites. The gene of interest can be cloned to a vector with multiple UAS sites, and the resulting vector used for the creation of transgenic fly lines. The gene will not be expressed unless another component,

the Gal4 transactivator, is present. Gal4 expression can be regulated temporally and spatially with different promoters, thus restricting the expression of the target gene to a specific tissue or developmental stage (Brand and Perrimon, 1993).

Since inducing expression of hMOF in flies with strong tubulin-Gal4 or actin-Gal4 drivers caused embryonic lethality in both males and females (data not shown), I opted for a weaker armadillo-Gal4 driver. hMSL3 was expressed by crossing UAS-hMSL3 flies to a fly strain expressing Gal4 under the tubulin promoter. Interestingly, both hMOF and hMSL3 transgenes were unable to rescue *mof* and *msl3* mutant flies, respectively, even though in the control lines expression of dMOF and dMSL3 rescued the male-specific lethal phenotype (Table 2).

Table 2. Human orthologs of dMOF and dMSL3 fail to rescue mof^1 and *msl-3* mutant flies from male-specific lethality. Rescue efficiency was controlled by transmission of control (balancer, *FM7* or *TM6C*) chromosome versus mutant chromosome (mof^1 or *msl-3*). Combined results from several independent transgenic lines are shown.

UAS-transgene	**Number of male offspring with UAS-transgene, Gal4, and**		
	control chromosome (*FM7*)	mutant chromosome (mof^1)	% rescue
dMOF (2 lines)	40	41	103
hMOF (4 lines)	396	0	0
	control chromosome (*TM6C*)	mutant chromosome (*msl-3*)	
dMSL3 (3 lines)	412	468	114
hMSL3 (4 lines)	502	0	0

There could be several explanations why hMOF and hMSL3 could not rescue the corresponding fly mutants. The proteins may have been unable to integrate into the *Drosophila* dosage compensation complex or, alternatively, they might be unable to function properly despite correct integration. To test these alternatives, localization of hMOF and hMSL3 on polytene chromosomes of 3rd instar male larvae expressing the transgenes was examined by immunofluorescence.

While *Drosophila* MOF is localized almost exclusively to the X chromosome, human MOF bound chromatin in a nonspecific manner. Interestingly, the staining of hMOF and dMOF was almost mutually exclusive (Figure 4A-D). Similarly, hMSL3 was also localized to all chromosomes with very little overlap with the endogenous MSL-3 protein (Figure 4E-H).

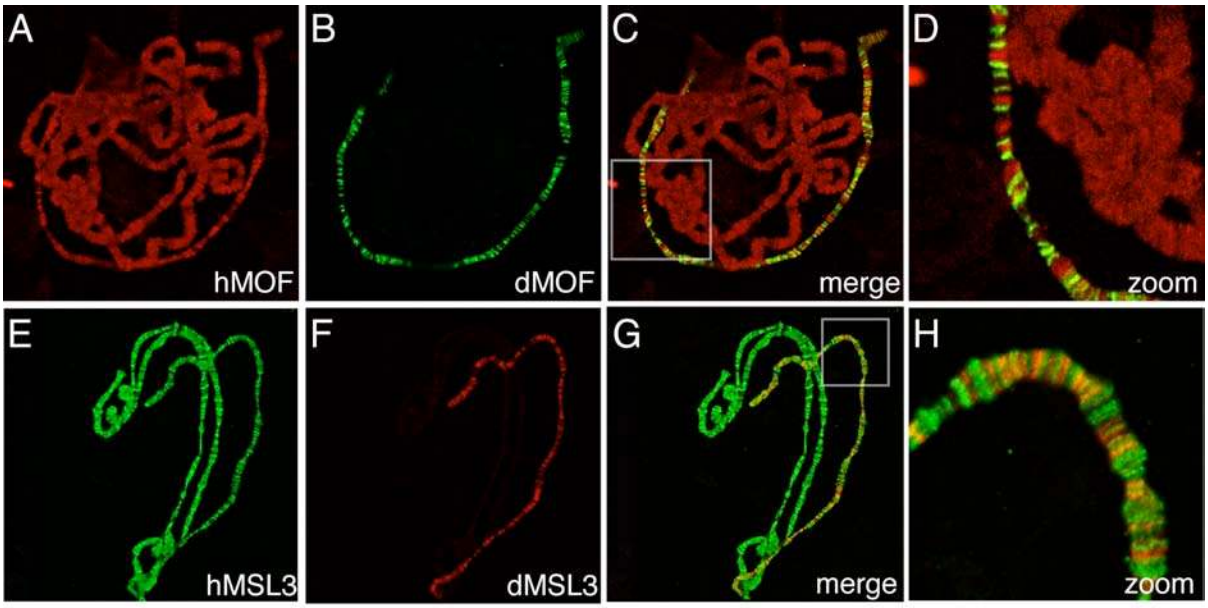


Figure 4. hMOF and hMSL3 expressed in flies fail to localize correctly to the male X chromosome. (A-D) Confocal images of polytene chromosomes from a male 3rd instar larva expressing UAS-hMOF with an armadillo-Gal4 driver. (E-H) Confocal images of a male polytene chromosome. UAS-hMSL3 was expressed with a tubulin-Gal4 driver. Zoomed areas are illustrated with grey boxes.

It is likely that the human orthologs cannot integrate into the *Drosophila* DCC and target similar regions on the male X chromosome, although both proteins are evolutionary well-conserved. hMSL3 protein is 26% identical and 42% similar to the *Drosophila* MSL3. Both proteins have preserved the same domain structure during evolution, and this conservation extends beyond known domains (Marin and Baker, 2000; Sanjuan and Marin, 2001).

Chromatin-associated proteins such as mouse polycomb (M33) have been shown to rescue the corresponding mutation in the *Drosophila* ortholog (Muller et al., 1995), even though they are not as well conserved as hMOF and hMSL3. Similarly, human MGN can functionally integrate to the *Drosophila* protein complex and replace its ortholog mago nashi (Forler et al., 2003). Thus, it is fairly unexpected that hMOF and hMSL3 are not functional orthologs of *Drosophila* MOF and MSL-3. A possible explanation for these results is that this sequence conservation is not sufficient to replace the endogenous proteins with their mammalian ortholog. It also remains possible that the two proteins have evolved to interact differentially with other protein substrates and that these interactions differ between the two species. An interesting example is fem-3 and tra-2, two sex-determining genes in nematodes.

These two proteins evolve exceptionally fast but still retain a species-specific interaction (Haag et al., 2002). Similarly, the mouse Intersex gene cannot complement mutations in the *Drosophila Intersex* ortholog (Siegal and Baker, 2005). Sex determination pathways are known to evolve rapidly (Marin and Baker, 1998), and since dosage compensation is intimately linked to sex determination (in *Drosophila*, via the interaction between Sex-lethal and MSL-2), it is plausible that proteins involved in dosage compensation are also subject to rapid evolutionary change. It is therefore likely that MOF and MSL3 have evolved to perform a divergent function either in *Drosophila* or in mammals.

In *Drosophila* cells, MOF not only acetylates histones but also MSL-3 (Buscaino et al., 2003). MSL-3 is acetylated *in vitro* by MOF at lysine 116, and it is also acetylated *in vivo* by MOF (**III**:Figure 3B and **III**:Figure 3C). Acetylation leads to reduced affinity of MSL-3 for roX2 RNA *in vitro* while having no influence on its affinity for nonspecific Y14 RNA (**III**:Figure 4D). In addition, acetylation of MSL-3 reduced its chromatin-binding affinity moderately but reproducibly (**III**:Figure 4F), whereas there was no effect on DNA binding (**III**:Figure 4D and **III**: Figure 4D). Consistently, treating SL-2 cells with TSA reduced the amount of roX2 RNA co-immunoprecipitating with MSL-3 (**III**: Figure 4B).

These data show that acetylation of MSL-3 by MOF regulates its affinity for at least roX2 RNA. Since acetylated MSL-3 also has a reduced affinity for chromatin, it is postulated that MSL-3 acetylation regulates the spreading of the DCC along the male X chromosome. DCC with an acetylated MSL-3 might bind chromatin and roX2 with reduced affinity and diffuse in the nucleoplasm until deacetylation by RPD3 (**III**:Figure 5). Such a mechanism could partly explain how DCC spreads to flanking chromatin *in cis* (**III**:Figure 6). The model where the DCC initially recognizes specific sequences or chromatin structures on the X chromosome and subsequently spreads to the flanking chromatin has recently been disputed (Fagegaltier and Baker, 2004). However, DCC components are capable of epigenetic diffusion from either a targeting sequence (roX1 or roX2 genomic sequence) inserted on the autosome (Kelley et al., 1999) or from the endogenous *roX1* or *roX2* loci (Oh et al., 2003).

The results above led us to test whether hMOF can similarly acetylate hMSL3 (Figure 5). However, I could not detect any acetylation of hMSL3 by hMOF (lane 1), even though hMOF acetylated *Drosophila* MSL3 with a similar efficiency to dMOF (compare lanes 2 and 4). Consistent with this observation, sequence analysis has shown that the target lysine residue, K116, of *Drosophila* MSL3 is not conserved across species, and is replaced by an arginine in other species (Kelley, 2004). It is thus possible that acetylation of MSL-3 is a *Drosophila*-specific modulation evolved for dosage compensation purposes.

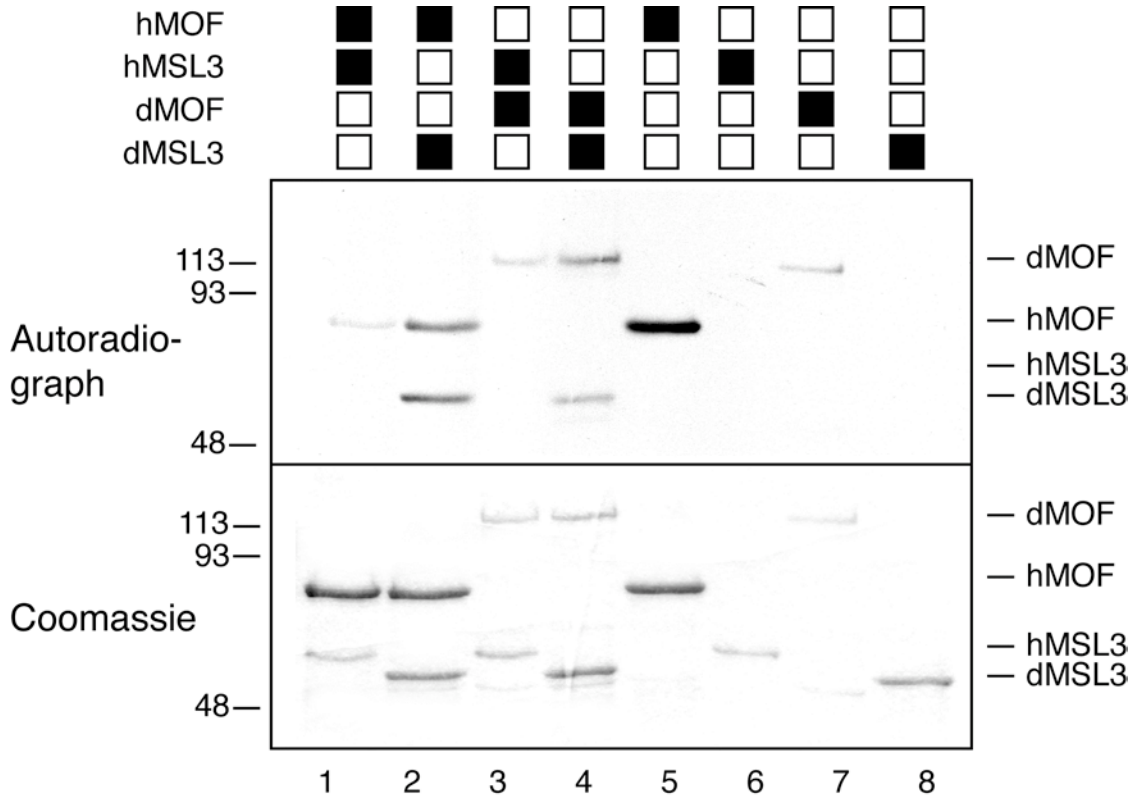


Figure 5. hMSL3 is not acetylated by hMOF. *In vitro* acetylation assay using hMSL3 (lanes 1, 3) and dMSL3 (lanes 2, 4) as substrates for acetylation with either hMOF (lanes 1 and 2) or dMOF (lanes 3 and 4). Lanes 5-8 show incubation of each protein alone in the acetylation reaction. Position of each protein on the Coomassie (bottom panel) and on the autoradiograph is indicated. Black boxes indicate the proteins included in the reaction.

FUNCTION OF HMOF IN MAMMALIAN CELLS

ACETYLATION OF HISTONE H4 LYSINE K16

To study the function of hMOF in mammals *in vivo*, hMOF protein was depleted from HeLa cells with specific siRNAs. Since siRNAs can have non-specific effects due to partial sequence similarity (Bridge et al., 2003; Jackson et al., 2003; Sledz et al., 2003), three independent siRNAs against hMOF were used, and only results consistent with all three siRNAs were considered. Furthermore, an siRNA without genomic targets was used as a control in all experiments.

After siRNA transfection, hMOF protein was depleted to approximately 10% of the wild-type levels (**I:**Figure 2A). Global histone acetylation was first analyzed by western

blotting with specific antibodies. Whereas there was no significant effect on H3K14, H3K23, or H4K12 acetylation, global H4K16 acetylation was severely reduced in hMOF-depleted cells (**I:**Figure 2A). The reduction in H4K16 acetylation corresponded to the reduction in hMOF protein levels, suggesting that hMOF is the major, if not the only, acetyltransferase targeting H4K16 in HeLa cells. A similar decrease in H4K16 acetylation upon hMOF knockdown could be detected in HepG2 cells, ruling out a cell-specific effect (data not shown).

Initial western blotting results were confirmed and extended by mass spectrometric analysis. Consistent with the previous observation, the amount of histone H4 lysine K16 acetylation was reduced to approximately 15% in hMOF-depleted cells when compared to control siRNA-transfected cells (**I:**Figure 2E). There was no reduction in the acetylation of other lysine residues. On the contrary, the acetylation of K5, K8, and K12 increased in response to hMOF knockdown. This explains why the effect of hMOF depletion on total H4 monoacetylation is modest compared to its effect on K16 acetylation (**I:**Figure 2C, 2E).

These data bring about two conclusions. Firstly, hMOF is a global regulator of H4K16 acetylation in mammalian cells. Significantly, no HATs have previously been linked to global acetylation of any lysine residues in mammals. The role of hMOF in global regulation of acetylation is further supported by the observation that it can non-specifically bind chromatin *in vitro*, whereas it does not have affinity for DNA (Figure 6). It is therefore possible that hMOF acetylates histones *in vivo* without any DNA- or chromatin-binding co-factors.

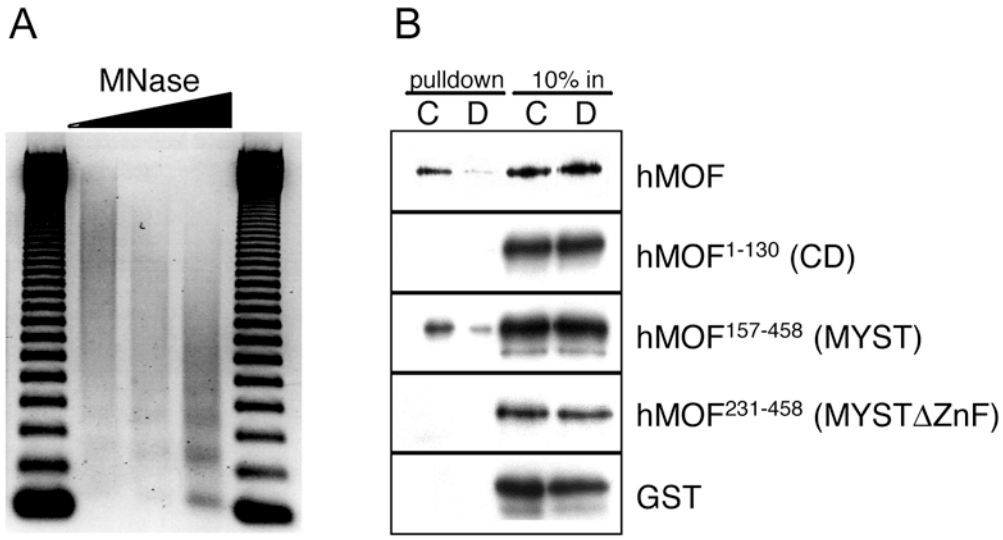


Figure 6. hMOF is a chromatin-binding protein. Chromatin was assembled on a linearized DNA immobilized on magnetic beads. Untreated DNA was used as a control. (A) Micrococcal nuclease digestion of the chromatinized template. (B) hMOF binds to chromatin via its MYST homology domain. C, chromatin; D, DNA.

Several studies have detected H4K16 acetylation at various genomic regions in mammalian cells, but so far no enzymes have been linked to the modification (Grandjean et al., 2001; Gregory et al., 2002; Johnson et al., 1998; Wan et al., 2001). Considering the data above, it is likely that hMOF is responsible for this modification on these sites, suggesting more biological functions for hMOF.

The second implication of the data considers the order of acetylation in mammals. It has been proposed that acetylation of histone H4 proceeds in a sequential fashion, such that lysine K16 acetylation is required for subsequent modifications on K5, K8, and K12 (Turner et al., 1989; Zhang et al., 2002). However, this is inconsistent with the results of hMOF knockdown experiments. For the model to be correct, loss of H4K16 acetylation should lead to a significant reduction of K5, K8, and K12 acetylation due to the action of histone deacetylases. In contrast, acetylation of these sites increased in hMOF-depleted cells, demonstrating that K16 acetylation cannot be a prerequisite for further modifications. Furthermore, as the order of acetylation differs between species (Chicoine et al., 1986; Munks et al., 1991; Thorne et al., 1990), it is an unlikely scenario that prerequisite modifications would similarly change during evolution. Therefore, although histone H4 acetylation proceeds in a sequential manner in human cells, it is not due to implicit restrictions in the order.

MAINTENANCE OF NUCLEAR STRUCTURE

Another striking feature of hMOF-depleted cells was their nuclear morphology. Most HeLa cell nuclei acquired invaginations and protrusions, and a significant fraction of the nuclei appeared polylobulated (**I:**Figure 4B). Similar nuclear abnormalities are often seen in cancer cells *in situ* (Zink et al., 2004). HepG2 cells showed less pronounced deformities, but they were nevertheless significantly more common in the hMOF-depleted cells than in the control cells. FACS analysis revealed that polylobulation was not due to an incomplete cytokinesis resulting in polyploidy. Furthermore, staining with the monoclonal antibody mAB414 that recognizes several nucleoporins showed that the nuclear envelope was intact in these cells (**I:**Figure 4D).

A typical feature of the polylobulated nuclei was the concentration of lamin A/C and lamin B2 in the central constriction (**I:**Figure 4D). Together with other proteins, lamins constitute the nuclear lamina that coordinates nuclear architecture in cells (Gruenbaum et al., 2005). Electron microscopy revealed that the polylobulated nuclei had a thickened electron-dense layer at the constriction, further supporting the immunofluorescence data with lamin staining. In addition, it implicated the role of the cytoplasm in the process, as there was a significant structural reorganization on the cytoplasmic side of the folds (**I:**Figure 4E). The appearance of deformed nuclei was further studied in a stable HeLa cell line expressing GFP-tagged histone H2B. In all cases, cells acquired multiple lobes directly after mitosis in late telophase or early G1, suggesting that the phenotype is linked to nuclear envelope reassembly (**I:**Figure 4C).

These data are consistent with previous studies. Firstly, a similar role for lamins and the cytoskeleton have been observed in other cell lines. The leukemia cell line HL-60 can be differentiated to granulocytes with all-*trans*-retinoic acid (ATRA) treatment. The granulocyte nuclei have a typical polylobular appearance, and this correlates with lamin B1 redistribution and cytoskeletal polarization (Olins et al., 1998; Olins et al., 2000). Mutations in lamin A cause Hutchinson-Gilford progeria syndrome, which is characterized by an abnormal nuclear shape (De Sandre-Giovannoli et al., 2003; Eriksson et al., 2003). A similar phenotype is seen in Pelger-Huet anomaly, which results from mutations in the lamin B receptor gene (Hoffmann et al., 2002). Moreover, overexpression of wild type or dominant negative versions of lamins results in aberrations in nuclear structure, demonstrating that lamins are

required for the maintenance of proper nuclear architecture (Bechert et al., 2003; Izumi et al., 2000; Schirmer et al., 2001).

The nuclear shape is not only determined by nuclear factors – the cytoskeleton also contributes to it. The lobulation of differentiating HL-60 cells can be efficiently suppressed by treating the cells with nocodazole, a drug that inhibits microtubule polymerization (Olins and Olins, 2004). Nocodazole also counteracts nuclear multilobulation caused by the mislocalization of Chk kinase (Nakayama and Yamaguchi, 2005). Similarly, treatment of cells with taxol, another microtubule destabilizing drug, can induce changes in the nuclear shape without differentiation (Olins and Olins, 2004; Theodoropoulos et al., 1999). Thus, the nuclear lamina and the cytoskeleton coordinately maintain proper nuclear structure in cells.

It is therefore likely that the depletion of hMOF causes a change in the balance between forces of the nuclear lamina and the cytoskeleton. However, it remained possible that the result was independent of histone acetylation by hMOF. Therefore, hMOF-depleted cells were treated with TSA for 72 hours to counteract deacetylation. With all siRNAs tested, TSA could suppress the nuclear phenotype in hMOF-depleted cells (**I:**Figure 5B). Furthermore, a stable cell line expressing an enzymatically inactive hMOF showed similar morphological defects, whereas expression of the wild-type hMOF had no effect. This indicates that acetylation by hMOF is involved in the process. Recenntly, another HAT p300 has been shown to modulate nuclear structure in prostate cancer cells, although the effects were rather modest (Debes et al., 2005).

It is likely that global H4K16 acetylation by hMOF is not directly implicated in the polylobulation, because a similar phenotype was seen in hMSL3-depleted cells without any loss of global H4K16 acetylation. In addition, TSA treatment did not increase global H4K16 acetylation in hMOF-depleted cells, and the known HDACs that can deacetylate lysine 16 belong to the TSA-insensitive SIR family of deacetylases (Imai et al., 2000; Vaquero et al., 2004). It is possible that TSA exerts its effects by other means. For example, TSA-sensitive HDAC6 deacetylates microtubules, and this is correlated with their destabilization (Hubbert et al., 2002; Matsuyama et al., 2002). Stabilization of microtubules could, for example, cancel out the partial strengthening of the nuclear lamina in hMOF-depleted cells.

Upon hMOF siRNA transfection, HeLa cells displayed proliferation defects (**I**:Figure 6A). Trypan blue staining of the cells indicated that this was not due to apoptosis or necrosis (Figure 7). TSA treatment improved the growth rate of hMOF-depleted cells, suggesting that hMOF acetyltransferase activity is required for HeLa cell proliferation. Interestingly, control cells treated with TSA grew more poorly than hMOF-siRNA transfected cells with a similar treatment (**I:**Figure 5A). This suggests that the growth suppressive effect of TSA on HeLa cells is partly due to misregulation of hMOF target genes or proteins. That is, the HDACs inhibited by TSA deacetylate the same residues that hMOF acetylates. FACS analysis indicated that hMOF-depleted cells accumulated in both G2 and M phases of the cell cycle (**I**:Figure 6B and Figure 8).

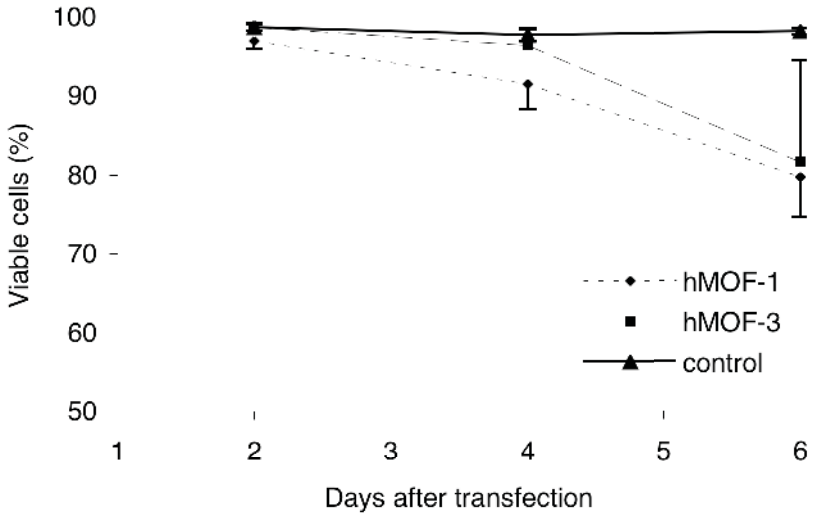


Figure 7. Cell death in hMOF-depleted cells and control cells. Cells were stained with Trypan blue that stains only inviable cells. Error bars indicate standard error of mean.

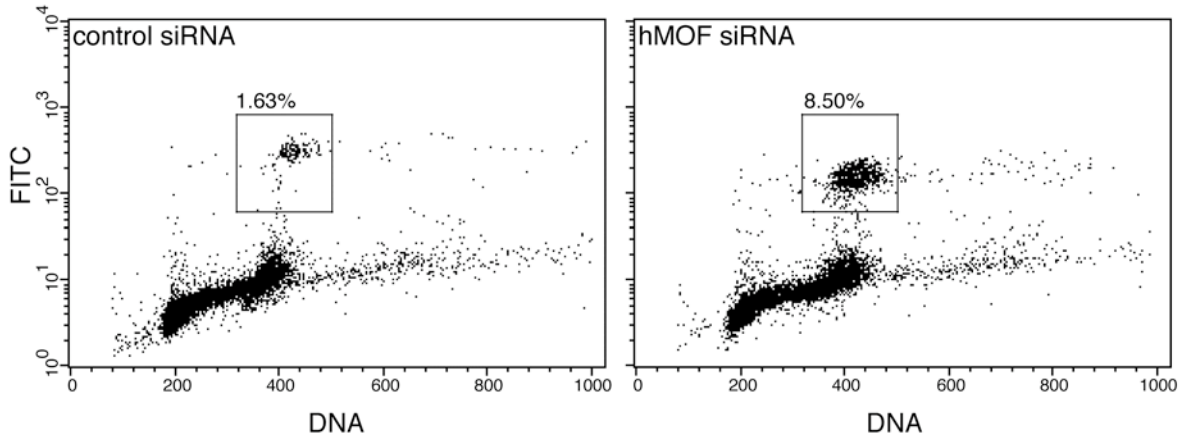


Figure 8. hMOF-depleted cells accumulate in mitosis. Mitotic population was separated by staining cells with an antibody against histone H3 phosphorylated at serine S10.

Cells have evolved multiple mechanisms to protect DNA from damage. One of them is to temporarily halt cell cycle progression, in order to give the DNA repair machinery more time to repair the acquired damage. Otherwise cells could accumulate mutations, which could in turn lead to overproliferation and cancer (Kastan and Bartek, 2004). Several different checkpoints that monitor DNA integrity operate during the cell cycle. Mammalian cells have distinct G1/S, intra-S, and G2/M checkpoints. The G1/S checkpoint prevents cells from entering S phase in the presence of damaged DNA, while the intra-S checkpoint monitors the progression of DNA replication (Sancar et al., 2004).

The G2/M checkpoint is elicited through two PIKK-like kinases ATM and ATR. They initiate a kinase cascade that results in phosphorylation and inactivation of the Cdc25C phosphatase, whose activity is required for entry into mitosis (Sancar et al., 2004). In addition, there is a cytoplasmic p38-dependent checkpoint that functions through phosphorylation of Cdc25B (Bulavin et al., 2001). ATM activation by autophosphorylation is the main pathway regulating cell cycle arrest following DNA double-stranded breaks (Bakkenist and Kastan, 2003), whereas ATR and p38 pathways respond to UV-induced DNA damage (Bulavin et al., 2001; Tibbetts et al., 1999).

I studied the role of the G2/M checkpoint in hMOF-depleted cells by treating the cells with two inhibitors of PIKK family kinases, wortmannin and caffeine. Caffeine inhibits both ATM and ATR, but the IC50 of caffeine for ATM over five-fold lower than for ATR (Sarkaria et al., 1999). Wortmannin is even more selective for ATM (Sarkaria et al., 1998). I used caffeine at 2 mM and wortmannin at 2 μM concentration to distinguish between ATR- and ATM-mediated responses. Both wortmannin and caffeine treatment (24 hours) restored

the cell cycle profile of the hMOF-depleted cells to almost that of control cells (**I**: Figure 6C, and data not shown), suggesting that the cell cycle arrest observed was dependent on ATM kinase activity. It should be noted that caffeine and wortmannin can also inhibit DNA-PK, a related kinase of the PIKK family (Block et al., 2004; Sarkaria et al., 1999; Sarkaria et al., 1998).

To address the possible role of ATM in the hMOF-depleted cells more directly, cells were stained with antibodies against the phosphorylated form of ATM, which is a common marker for ATM activation and DNA double-stranded breaks (Bakkenist and Kastan, 2003). In control HeLa cells, about 20% of the cells had visible phospho-ATM foci. This was to be expected, as many cancer cell lines have a constitutively active DSB checkpoint (DiTullio et al., 2002; Gorgoulis et al., 2005). In contrast, almost 40% of hMOF-depleted cells had ATMp foci, suggesting that they had accumulated more spontaneous DNA damage (**I**:Figure 6D).

ATM can also be activated by changes in chromatin structure and acetylation without DNA damage. For example, TSA treatment activates ATM (Bakkenist and Kastan, 2003). However, in this case ATM activation was not visible in discrete foci but as evenly distributed in the nucleoplasm. It is therefore likely that hMOF depletion induced foci are true DSBs. There could be two scenarios for how hMOF knockdown causes more DNA damage. First, hypoacetylated chromatin could be more susceptible to double-stranded breaks. Alternatively, hMOF-depleted cells could accumulate the same amount of DNA damage but fail to repair it swiftly.

To distinguish between these two scenarios, cells were irradiated with X-rays. After irradiation, both control and hMOF siRNA treated cells have approximately the same number of DSBs, thus canceling out the initial difference in the number of DSBs. If hMOF-depleted cells were more prone to DNA damage but not deficient in DNA repair, it would be expected that the repair kinetics are the same in control and hMOF siRNA-treated cells. However, cells depleted of endogenous hMOF showed significantly lower kinetics of DSB repair as measured by clearance of phospho-ATM and γH2AX foci (**I**:Figure 4E).

Recently, it was reported that hMOF interacts with ATM *in vitro* and *in vivo* and influences ATM activation in response to DSBs (Gupta et al., 2005). Consistent with the results above, Gupta and colleagues (2005) found that 293 cells expressing a mutant version of hMOF were less efficient in DSB repair following irradiation than control cells expressing wild type hMOF. In addition, they reported that hMOF-depleted cells show decreased viability after irradiation and spontaneous genomic instability (Gupta et al., 2005).

Considering the results of Gupta et al. together with the data presented above, it is plausible that hMOF is involved in DNA repair and maintenance of genome stability.

However, Gupta et al. (2005) had data implicating that hMOF is involved in ATM activation. This would be inconsistent with our observation that hMOF depletion causes activation of ATM. However, the apparent inconsistency could be explained in several ways. Firstly, ATM is still partially activated in hMOF-depleted cells (Gupta et al., 2005). Detecting a small change in staining intensity in immunofluorescence is very difficult, and we could have missed this in our assays. Partial activation of ATM could still initiate the G2/M checkpoint, also given that hMOF depletion delays DNA repair. Secondly, the results obtained by Gupta and colleagues could also be explained by altered kinetics of ATM activation. They used earlier time points compared to our experiments, and thus they might have missed full activation of ATM, should it have occurred after their last time point (60 minutes). Both results could be reconciled if hMOF depletion merely delayed the activation of ATM. Third, HeLa cells have a constitutively active DSB checkpoint, whereas 293 cells do not (DiTullio et al., 2002; Gorgoulis et al., 2005) and data not shown). This means that the two cell lines have different initial state of DSB checkpoints, which could influence the results. It should also be noted that we failed to detect an interaction between hMOF and ATM in HeLa cells, despite using several different antibodies against hMOF and ATM under conditions where hMOF robustly interacts with hMSL3 (data not shown).

Histone acetylation has also previously been linked to DNA repair. Yeast cells mutant for all H4 tail lysine residues accumulate in G2/M in a RAD9-dependent manner (Megee et al., 1995). RAD9 is the major checkpoint protein in the budding yeast that halts cell cycle progression in case of DNA damage (Weinert and Hartwell, 1988). Esa1p HAT is required for efficient DSB repair in the budding yeast (Bird et al., 2002), and the NuA4 complex accumulates at the sites of DNA damage (Downs et al., 2004). The orthologous Tip60 complex in the fruit fly is required for the exchange of phosphorylated H2Av with H2A after DSB induction (Kusch et al., 2004). The human Tip60 has also been implicated in DNA repair (Ikura et al., 2000).

In budding yeast, H4K16 is deacetylated in the vicinity of the double-stranded break (Jazayeri et al., 2004), whereas there is very little detectable γH2A within 1-2 kilobases of the break (Shroff et al., 2004). Interestingly, histone acetylation increases the activity of DNA-PK on H2AX *in vitro* (Park et al., 2003). If these aspects of DSB repair were conserved in mammals, it would suggest a potential role for H4K16 acetylation in regulation of H2AX

phosphorylation. Nevertheless, the molecular mechanism for the role of hMOF during DNA damage needs to be addressed more directly.

It is still possible that hMOF is only indirectly involved in DNA repair. hMOF could transcriptionally regulate genes required for DSB response. However, we have not seen significant changes in the expression of genes involved in DNA repair upon hMOF knockdown in HeLa cells (S. Rea, personal communication). The only downregulated gene implicated in DNA repair in hMOF-depleted cells was DNA ligase IV, which is required for non-homologous end joining (NHEJ) after DSB induction in eukaryotes (Wilson et al., 1997). However, the NHEJ pathway is mainly utilized in G0, G1, and early S phase, which would not explain the accumulation of the hMOF-depleted cells in G2/M (Lieber et al., 2003).

hMOF-CONTAINING PROTEIN COMPLEXES

Most HATs are components of multiprotein complexes (Ogryzko, 2001). *Drosophila* MOF is the enzymatic core of the dosage compensation complex that has been reported to consist of at least five proteins and two non-coding RNAs (Copps et al., 1998). Since all the dosage compensation complex proteins have mammalian orthologs (Marin, 2003; Marin and Baker, 2000; Sanjuan and Marin, 2001) (Prakash et al., 1999), it was a fair assumption that there would be an orthologous mammalian complex. To be able to study the function of the hMOF protein, I decided to purify the hMOF-containing complexes from HeLa cells. I opted for affinity purification with haemagglutinin (HA) and FLAG epitope tags for several reasons. Firstly, they have previously been shown to be efficient in purification of multiprotein complexes from mammalian cells (see for example Groisman et al., 2003; Ikura et al., 2000; Tagami et al., 2004). Secondly, both epitopes are very small, only eight amino acid residues, compared to other affinity tags such as TAP (Rigaut et al., 1999). In parallel to the hMOF complex purification, the *Drosophila* MOF and MSL3 complexes were purified in the laboratory, thus allowing comparison of the two complexes and cross-species functional studies.

PURIFICATION OF HMOF-CONTAINING COMPLEXES

The hMOF complex was purified from HeLa cells stably expressing HA-2xFLAG epitope tagged hMOF. The expression level of the tagged protein was slightly higher than hMOF expression in HeLa cells, but in the tagged cell line the expression of endogenous hMOF was downregulated (data not shown). A mock purification was performed from the parental HeLa cell line to control for non-specific binding of proteins to the α-FLAG and α-HA affinity matrices.

Silver staining of co-purifying proteins revealed that hMOF is associated with several polypeptides in HeLa cells. The pattern of co-purifying proteins was very consistent between independent purifications (Figure 9). In the mock purification, no proteins were detected by silver staining apart from carryover immunoglobulin G heavy and light chains from the α-HA beads.

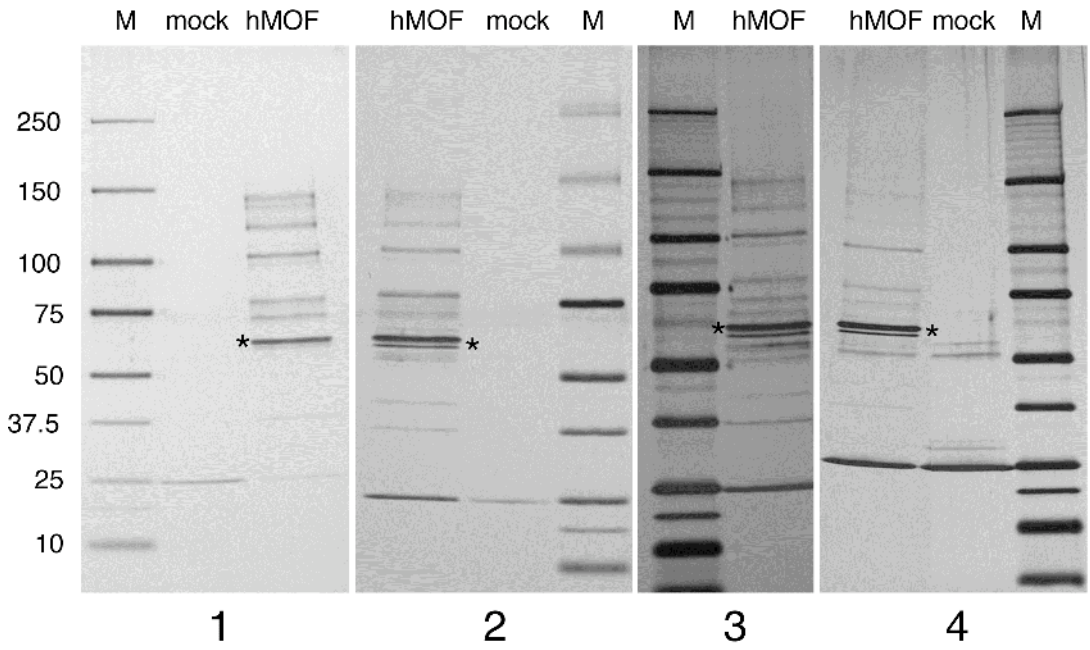


Figure 9. Purification of hMOF-containing protein complexes. Comparison of four purifications from independent experiments reveals extensive consistency of co-purifying proteins. HA-2xFLAG-hMOF used as a bait is indicated as an asterisk. M, molecular weight marker.

Co-purifying proteins were identified by mass spectrometry. In order to avoid losing any interacting proteins, we used two different approaches. Silver-stained bands were

excised, in-gel digested, and analyzed by MALDI-TOF and nano-electrospray mass spectrometry (Shevchenko et al., 1996). In parallel, proteins were analyzed with nano-LC-MS/MS without gel separation. Mass spectrometry revealed reproducible purification of 15 polypeptides with hMOF (Figure 10, **II**:Figure 1A, and **II**:Table 1).

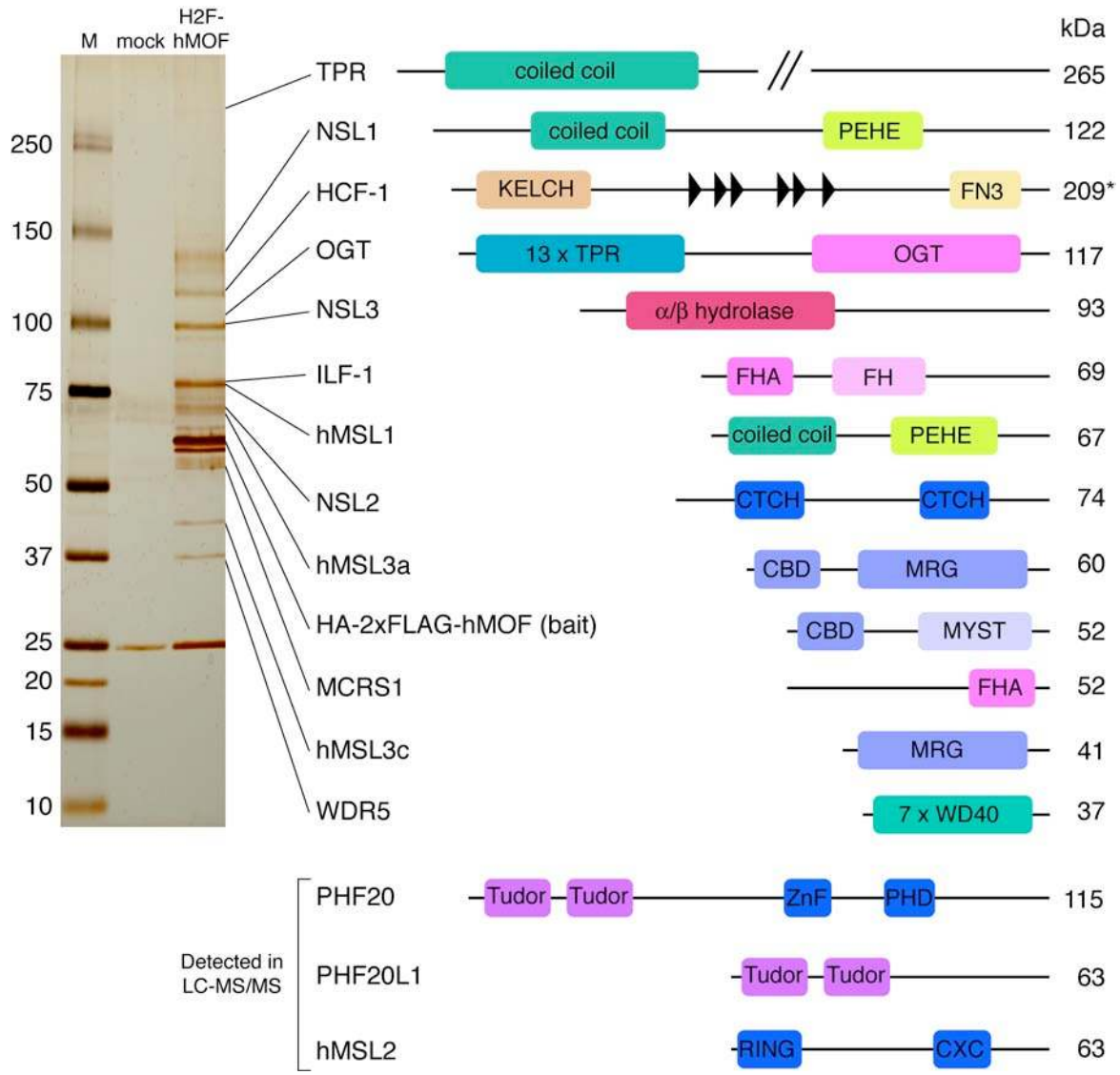


Figure 10. Proteins co-purifying with HA-2xFLAG-hMOF in HeLa cells. Vertical lines illustrate the positions of the proteins. Domain structure is illustrated next to the identified protein. Note that HCF-1 is proteolytically cleaved (black triangles) to produce smaller N- and C-terminal fragments. FN3, fibronectin 3 homology domain; TPR, tetratricopeptide repeat domain; FH, forkhead domain; FHA, forkhead associated domain; CBD, chromo-barrel domain.

DROSOPHILA *DCC PROTEIN ORTHOLOGS*

With the exception of MLE, all the human orthologs of the previously documented *Drosophila* dosage compensation complex proteins co-purified with hMOF. hMSL1 and hMSL3 were found in both nano-electrospray and nano-LC-MS/MS analyses, while hMSL2 was only found by nano-LC-MS/MS. The absence of RNA helicase A, the ortholog of *Drosophila* MLE is not surprising. The bulk of MLE is not associated with the DCC, and its interaction with other components is very sensitive to salt concentration (Buscaino et al., 2003; Copps et al., 1998). Neither is it a stable component of biochemically purified *Drosophila* DCC (Mendjan et al., 2005). Furthermore, MLE/RHA has additional, DCC-independent roles in both fruit flies and mammals (Nakajima et al., 1997; Reenan et al., 2000).

hMSL1 and hMSL2 contain the same domain architecture as the *Drosophila* orthologs. hMSL1 has a N-terminal coiled-coil domain, or a putative leucine zipper motif, and a novel PEHE domain in the C-terminus (Marin, 2003). hMSL2 has a RING finger and a CXC motif that is found in Enhancer of zeste [E(z)] orthologs (Marin, 2003).

hMSL3 is present in two splicing isoforms, hMSL3a and hMSL3c. hMSL3a is a full-length isoform that contains both the N-terminal chromo-barrel domain and the C-terminal MRG homology domain. hMSL3c is an alternative splicing isoform, that lacks the first exon and the first 166 amino acids including the chromo-barrel domain (Figure 10). Interaction of hMOF with both isoforms was verified also by co-immunoprecipitation of the endogenous proteins from HeLa cells (**I**:Figure 3A). The presence of the shorter isoform in the complex is consistent with the recent observation that MSL-3 MRG domain, which interacts with MSL1, is sufficient for *Drosophila* DCC assembly (Morales et al., 2005). It is therefore very likely that hMSL3 similarly interacts with the human MSL complex with its MRG domain.

These data prove that there is a mammalian complex orthologous to the *Drosophila* DCC. Its existence has been postulated before (Marin, 2003; Marin et al., 2000; Ogryzko, 2001), but there has been no biochemical data prior to this purification. The data implicate that *Drosophila melanogaster* has co-opted an ancient chromatin-modifying complex to maintain dosage compensation, as this complex must predate the Cambrian explosion about 600 million years ago. Interestingly, also mammals and nematodes have adopted old protein complexes for dosage compensation, suggesting that it is a common theme in the evolution of different dosage compensation mechanisms.

In addition to the known MSLs, a number of previously uncharacterized proteins associated with hMOF. We named the unknown proteins NSLs (non-specific lethal), because P-element insertions in the fly orthologs were homozygous lethal in both males and females (data not shown), in contrast to male-specific lethal genes.

NSL1

The NSL1 protein consists of 1105 amino acids. It has also been known as KIAA1267 or LOC284058. The protein sequence provides little clue as to its function. Interestingly, it contains a putative coiled-coil domain in the C-terminus and an N-terminal PEHE domain, like hMSL1 (Marin, 2003). *Drosophila* MSL-1 has been shown to interact directly with MOF through its PEHE domain, suggesting that this domain is a general MOF-interacting motif (Morales et al., 2004). To confirm the interaction of NSL1 with hMOF *in vivo*, antibodies against NSL1 were produced in rats. Affinity-purified antibodies detected NSL1 in the purified complex (**II**:Figure 1B). In addition, NSL1 co-immunoprecipitated with hMOF from HeLa cell nuclear extracts (**II**:Figure 1B).

NSL1 is an evolutionary conserved protein. It shares several regions of homology with its *Drosophila* ortholog, CG4699 or dNSL1 (Figure 11). The same regions are also conserved between NSL1 and its close paralog FLJ23861/LOC151050, suggesting that these regions form globular folds (Figure 11, next page).

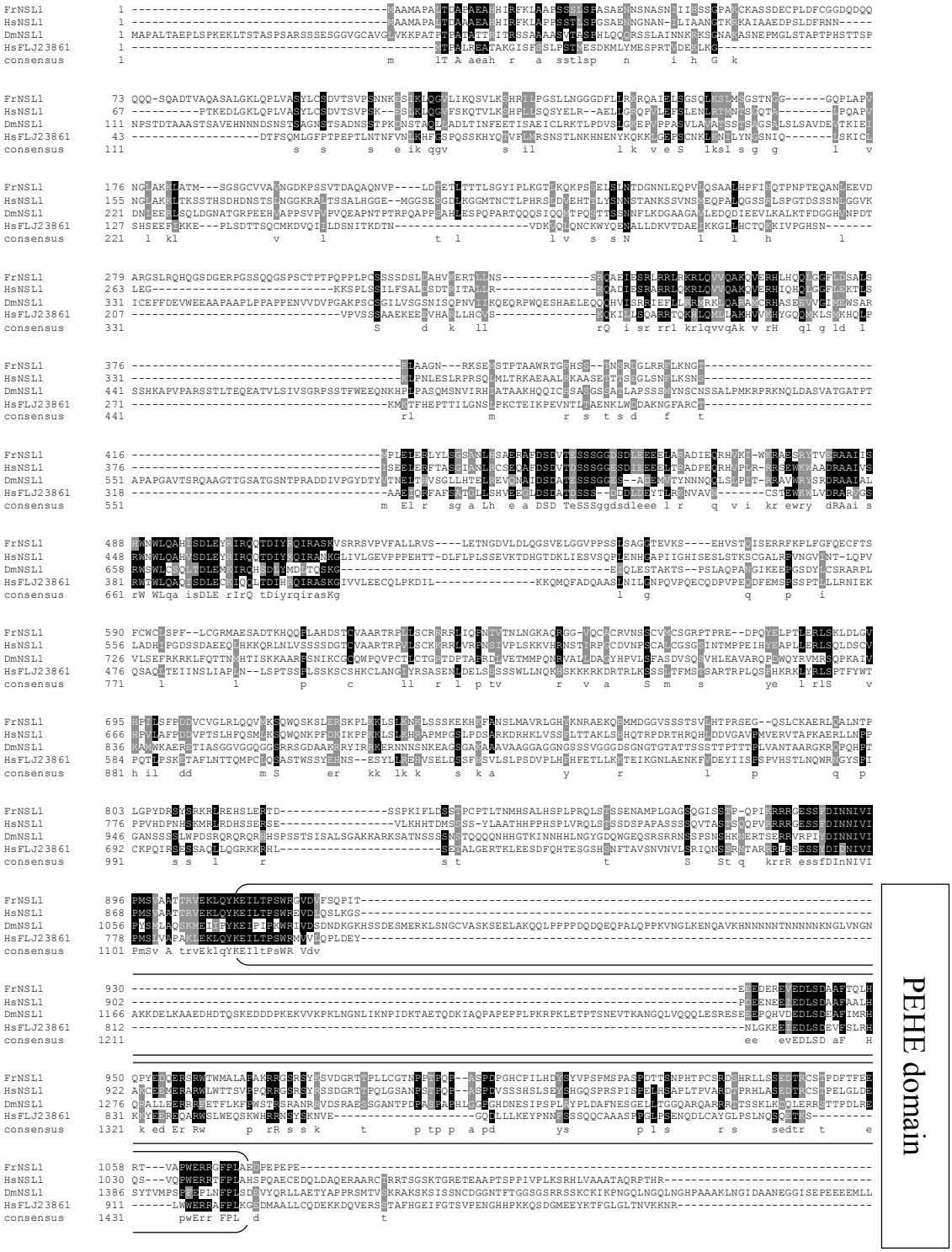


Figure 11. Alignment of human NSL1 orthologs and its paralog FLJ23861. Position of the previously identified PEHE domain is indicated. Hs, *Homo sapiens*; Fr, *Fugu rubripes*; Dm, *Drosophila melanogaster*.

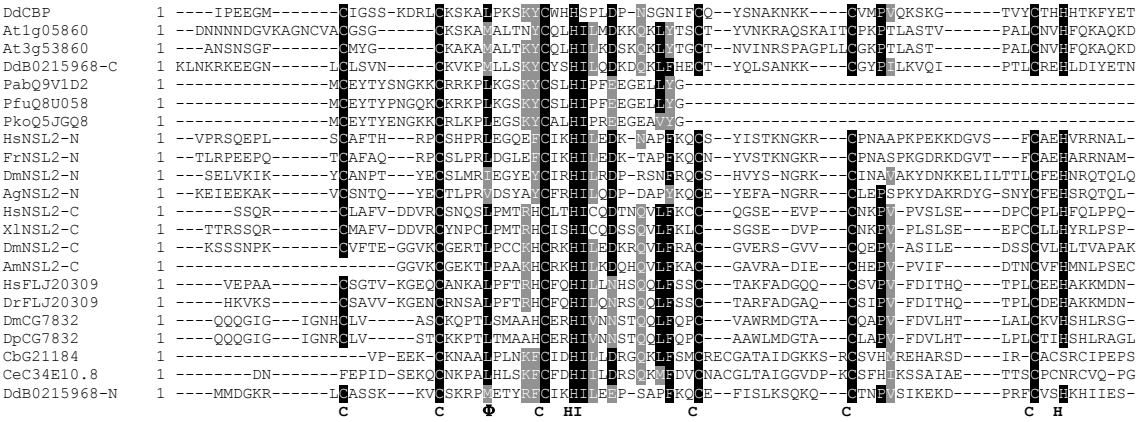
NSL2

Polyclonal antibodies against a C-terminal fragment of NSL2 were produced in rats. Western blotting confirmed that NSL2 is present in the hMOF complex. Most likely only a fraction of NSL2 associates with hMOF, because only very little NSL2 could be detected in the purified hMOF complex **(II**:Figure 1B**)**.

NSL2 was previously deposited into Genbank with the name FLJ20436. NSL2 is expressed ubiquitously in mice and goats, and it has several putative splicing isoforms (Mata et al., 2003). In *Xenopus laevis* embryos, the NSL2 ortholog 4G2 is expressed in the animal pole from the stage IV onwards (Shim et al., 2000). In addition to a bipartite nuclear localization signal, Shim et al. (2000) reported that NSL2 and its orthologs have RGD and LDV motifs that have been shown to regulate integrin binding (Arnaout et al., 2002). The function of the fly NSL2 ortholog is still unknown.

Although conventional domain-predicting software failed to find any domains in NSL2, careful sequence analysis revealed it does contain two copies of a previously uncharacterized domain rich in cysteine and histidine. I named the domain CTCH (C-ten-C-H) because of the conserved spacing between two cysteines and a histidine. PSI-BLAST, HMMer, and ProfileScan searches identified a number of proteins from vertebrates, insects, nematodes, plants, slime molds, and archaebacteria containing the CTCH domain, illustrating that it is an evolutionary ancient motif. The core motif consists of the sequence **C**-X_4-Φ-X_4-[FHY]-**C**-X_2-**H**-**I** (Φ denotes a hydrophobic residue, X any residue), where the spacing between conserved residues is absolutely conserved (Figure 12A). Most proteins also contain a C-terminal cysteine, but the spacing varies between three and eight residues (Figure 12A). The eukaryotic proteins also contain an N-terminal extension of the domain with the consensus [DEN]-X_{3-7}-[FY]-X_2-**C**-X_{7-14}-**C**-X_{11-18}-**C**-X2-**H** (Figure 12A). All NSL2 orthologs, and the putative *Dictyostelium discoideum* protein DDB0215968, have two CTCH domains, whereas the other proteins only contain a single CTCH motif. Phylogenetic clustering revealed similarities of the new CTCH motifs with both the C-terminal and the N-terminal CTCH of NSL2 (Figure 12B).

A



B

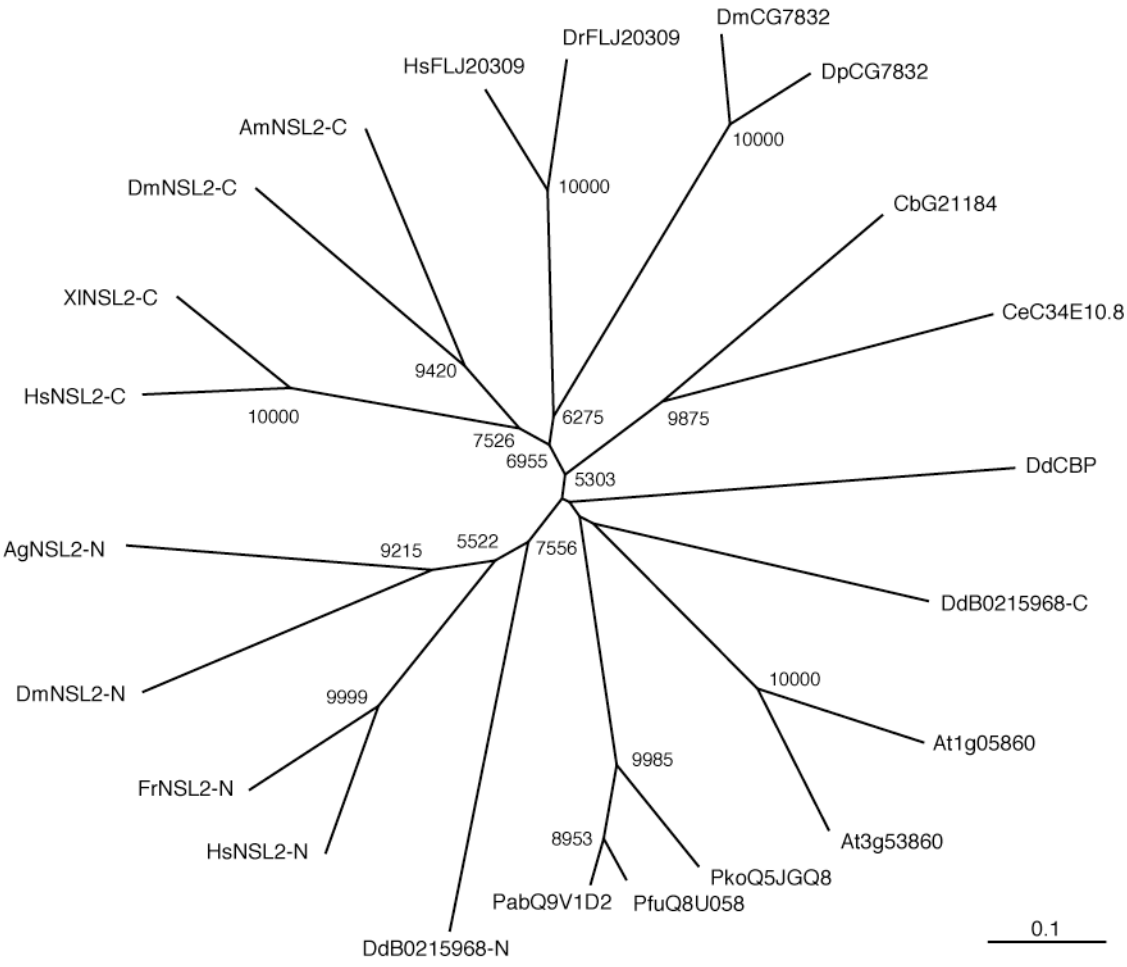


Figure 12. The CTCH domain. A, Alignment of CTCH domains. Consensus sequence is indicated below the alignment. B, Neighbor-joining tree of metazoan CTCH domains. Bootstrap values from 10000 replications are indicated. Dp, *D. pseudoobscura*; Ag, *Anopheles gambiae*; Cb, *Caenorhabditis briggsae*; Ce, *C. elegans*; At, *Arabidopsis thaliana*; Dd, *Dictyostelium discoideum*; Xl, *Xenopus laevis*; Am, *Apis mellifera*; Dr, *Danio rerio*, Pfu, *Pyrococcus furiosus*; Pab, *P. abyssii*; Pho. *P. horikoshii*; Pko, *P. kodakaraensis*.

NSL3

We produced rabbit polyclonal antibodies against NSL3 and confirmed its association with the hMOF complex by western blotting (**II**:Figure 1B). NSL3 (or alternatively FLJ10081, KIAA1310, or LOC55683) is an 878-residue protein encoded by a gene on the human chromosome 2p12-p11.2. It contains a domain similar to α/β-hydrolases. α/β-hydrolases constitute one of the largest, oldest, and most plastic structural superfamilies (Heikinheimo et al., 1999; Nardini and Dijkstra, 1999). It includes enzymes like esterases, peptidases, and lipases, and despite low sequence similarity they all share a common fold and a catalytic triad. The catalytic triad is composed of a nucleophile (serine, cysteine, or aspartic acid), an acid, and an invariable histidine residue. Interestingly, although NSL3 has very typical features of an α/β-hydrolase fold, the catalytic triad is not conserved (Figure 13). In particular, the histidine residue that is absolutely required for enzymatic activity is substituted for an aspartic acid in NSL3. This argues very compellingly that NSL3 carries no enzymatic activity.

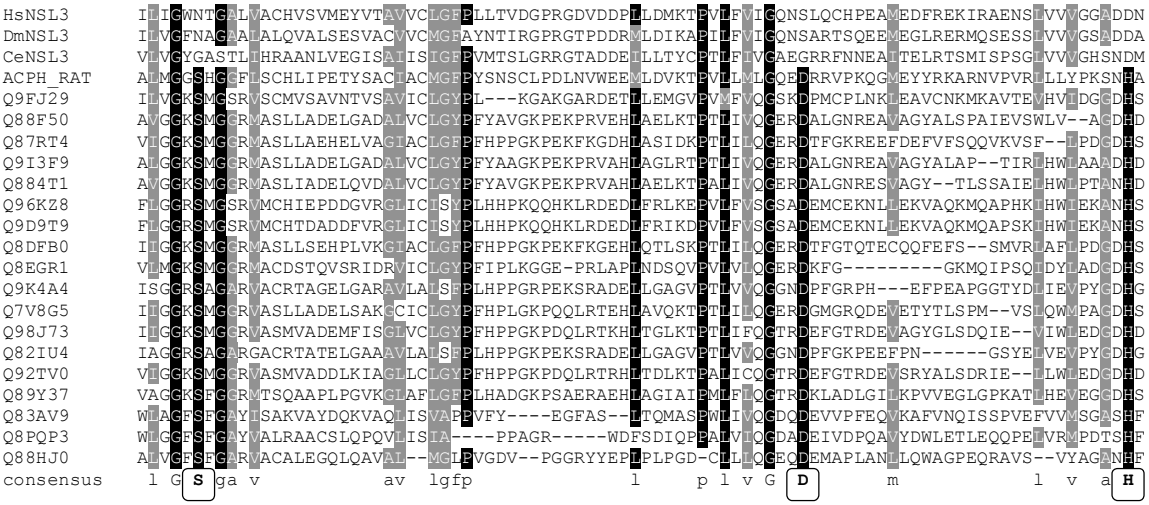


Figure 13. Alignment of NSL3 orthologs with several α/β hydrolase fold domains from prokaryotes and eukaryotes. The residues contributing to the catalytic triad are indicated as boxes. ACPH, acylpeptide hydrolase.

What, then, is the function of the inactive α/β-hydrolase fold? Perhaps instead of catalyzing a reaction, NSL3 could bind the substrate. The hydrolase fold would thus function as a binding domain. Often domains that have lost their enzymatic activity still retain the ability to bind the substrate (Todd et al., 2002). For example, heparin-binding protein (HBP)

has a serine proteinase fold, which belongs to the α/β-hydrolase superfamily. However, the catalytic triad is mutated, and as the name of the protein implies, it only binds heparin and lipopolysaccharides (Iversen et al., 1997).

It is tempting to speculate that NSL3 binds small molecules or peptides. The most obvious candidate, for many reasons, would be the histone H4 tail acetylated at lysine K16. Firstly, chromatin-modifying protein complexes often contain not only enzymes but also proteins that bind the modified residues. The best-characterized example is Su(var)3-9 and HP1, where Su(var)3-9 methylates histone H3 lysine K9, which creates a binding site for the HP1 chromodomain (Bannister et al., 2001; Lachner et al., 2001; Rea et al., 2000). Secondly, several different, unrelated domains can recognize a particular modification. The unrelated SH2, PTB, and C2 domains can all recognize phosphotyrosine residues (Sondermann and Kuriyan, 2005). Thus, it is conceivable that there are other domains besides bromodomains that are able to recognize acetyl-lysine residues. Third, many α/β-hydrolases, such as acylpeptidehydrolases, are deacetylases, illustrating that acetyl groups can act as substrates for these enzymes. Needless to say, further structural and biochemical work is needed to determine whether the NSL3 hydrolase domain really is a binding module and to discover putative targets.

Another noteworthy sequence feature of NSL3 is that the C-terminal region bears similarity to many glycosylated proteins, most notably HCF-1 that is also associated with the complex (data not shown). Given that HCF-1 is glycosylated, and that also the OGT glycosyltransferase is in the complex, it is possible that NSL3 is similarly a nuclear glycoprotein.

HCF-1

HCF-1 was isolated as a protein forming a complex with the transcription factor Oct-1 and the viral transactivator VP16 upon herpes simplex virus infection (Wilson et al., 1993). This complex activates viral immediate early genes to initiate the lytic cycle, hence the name "host cell factor 1" (Wysocka and Herr, 2003). HCF-1 is a large 2035-amino-acid glycoprotein that is proteolytically cleaved to N- and C-terminal subunits, which nevertheless associate non-covalently with each other (Wilson et al., 1993).

HCF-1 regulates cell growth and proliferation. A temperature-sensitive allele of HCF-1 causes cytokinesis defects and a G0/G1 arrest at a restrictive temperature (Goto et al., 1997;

Reilly and Herr, 2002). Cytokinesis defects appear to be due to aberrant H4K20 dimethylation in mitosis. HCF-1 knockdown cells have abnormally high levels of H4K20 dimethylation during mitosis, and depletion of the H4K20-specific methyltransferase PR-Set7 in HeLa cells suppresses the cytokinesis defects (Julien and Herr, 2004).

HCF-1 associates with both HDAC and histone methyltransferase complexes in human cells. It interacts with both the repressive Sin3A/HDAC1/HDAC2 complex and the activating Set1/Ash2/WDR5 complex, suggesting that it can tether opposing chromatin-modifying activities to target promoters (Wysocka et al., 2003). Interestingly, OGT and WDR5 co-purify with HCF-1, which supports our data that all three proteins are *bona fide* members of the hMOF complex (Wysocka et al., 2003). Nevertheless, it is clear that only a minor fraction of cellular HCF-1 associates with hMOF, since only very little HCF-1 co-immunoprecipitates with hMOF and is present in the complex (**II**:Figure 1B and **II**:Figure 1C).

OGT

O-linked β-N-acetylglucosaminetransferase, OGT, is the second protein with enzymatic activity in the hMOF complex. OGT transfers an N-acetylglucosamine (GlcNAc) moiety to serine or threonine residues in target proteins (Vosseller et al., 2002). It is an abundant nuclear and cytoplasmic protein that is required for cell viability and mammalian development (O'Donnell et al., 2004; Shafi et al., 2000). It is thought that O-GlcNAc counteracts protein phosphorylation, as they both can target the same residues.

OGT modifies many nuclear proteins, such as RNA polymerase II large subunit, c-Jun, c-Fos, and Sp1 (Zachara and Hart, 2004). Generally, O-GlcNAc modification appears to be linked to gene repression. Fluorescently labeled wheat germ agglutinin (WGA), which selectively binds O-GlcNAc residues, mostly stains inactive regions on the *Drosophila* polytene chromosomes (Kelly and Hart, 1989). When tethered to promoters, OGT can repress transcription (Yang et al., 2002). In addition, the enzyme that removes O-GlcNAc residues, NCOAT, possesses intrinsic HAT activity, suggesting that O-GlcNAc removal is linked to gene activation (Toleman et al., 2004).

However, we have not yet tested whether the hMOF complex has O-glycosylation activity.

WDR5

WDR5, or BIG-3, was originally identified as a BMP-2-induced gene in a prechondroblastic cell line (Gori et al., 2001). It contains seven WD40 repeats but no other known domains. WDR5 and its yeast ortholog Swd3p have been purified as subunits of several H3K4-specific methyltransferase complexes (Hughes et al., 2004; Miller et al., 2001; Nagy et al., 2002; Wysocka et al., 2003; Yokoyama et al., 2004). In humans, it is associated with histone methyltransferases hSET1, MLL1, and MLL2.

Recently, Wysocka and colleagues (2005) offered an explanation for why WDR5 is associated with many different methyltransferase complexes with similar specificities. They showed that WDR5 binds histone H3 dimethylated at lysine K4, and functions as a transcriptional activator (Wysocka et al., 2005). Knockdown of WDR5 reduces global H3K4 mono-, di-, and trimethylation in mammalian cells, and H3K4 mono- and trimethylation in Xenopus embryos. Morpholino-induced downregulation of WDR5 in Xenopus embryos results in misregulation of HoxC8 and HoxA9 genes and developmental abnormalities (Wysocka et al., 2005). The authors suggest that WDR5 functions as a binding module that helps MLL-like complexes convert dimethylated H3K4 to the trimethylated state.

Recently, the WDR5 protein complex was purified from HeLa cells and found to contain hMOF and several other proteins identified also here (Dou et al. 2005; see also discussion below). In particular, MLL1 methyltransferase was part of the same complex. Even though we could not detect MLL1 in our complex, it is plausible that the complex described here collaborates with other chromatin-modifying activities, including H3K4-specific methyltransferases.

In the human HepG2 cell line, H3K4 dimethylation is mainly found near or at gene loci, but not on gene promoters (Bernstein et al., 2005). Nevertheless, these sites are not enriched in the phosphorylated form of RNA polymerase II, suggesting that they are not actively transcribed. It is possible that WDR5 tethers hMOF to genes that are poised for transcription, and H4K16 acetylation by hMOF further enhances transcriptional activity. Consistent with this idea, H4K16 acetylation on the *Drosophila* X chromosome is mainly found at the coding regions of genes, not on promoters (Smith et al., 2001).

TPR

Although TPR (translocated promoter region) was mostly detected in MALDI-TOF as degradation products in multiple excised bands, it is likely to be a true hMOF-interacting protein. Firstly, full-length TPR is degraded very rapidly in HeLa cells during extract preparation (V. Cordes, personal communication). Secondly, the fly ortholog of TPR, Megator, co-purifies with the *Drosophila* MOF complex and regulates dosage compensation (Mendjan et al., 2005).

TPR is a component of the nuclear pore complex and it is localized to the nuclear basket facing the nucleoplasm (Frosst et al., 2002; Krull et al., 2004). It oligomerizes through its coiled-coil domain, which is the mechanism by which it constitutively activates oncogenic protein kinases, such as MET, RAF, and TRK, in chromosomal translocations (Park et al., 1986; Rodrigues and Park, 1993) (Greco et al., 1992). In normal cells, TPR and its orthologs appear to have a role in nuclear export of RNA (Bangs et al., 1998; Cornett et al., 2005; Frosst et al., 2002; Galy et al., 2004). In addition to RNA export, TPR has been shown to regulate spatial organization of the genome and gene expression. Deletion of the budding yeast TPR orthologs, Mlp1p and Mlp2p, leads to derepression of the silent telomeric domains (Galy et al., 2000). On the other hand, Mlp1p is also linked to active chromatin; induction of gene expression by the mating pheromone α factor causes a rapid association of Mlp1p with the activated genes (Casolari et al., 2005).

It has become evident in recent years that spatial positioning of genes in the nucleus contributes to gene expression (Misteli, 2004). The *Drosophila* dosage compensation complex is normally localized to the nuclear periphery (Akhtar et al., 2000). However, depletion of TPR in *Drosophila* Schneider cells leads to mislocalization of the DCC to the nucleoplasm and to subsequent loss of dosage compensation (Mendjan et al., 2005). It is therefore possible that TPR contributes to hMOF-regulated gene expression in mammalian cells, perhaps by tethering activated genes to the nuclear periphery.

MCRS1

MCRS1, also known as MCRS2, p78, and MSP58, contains an FHA domain, which has been implicated in phosphopeptide binding (Okumura et al., 2005). Expression of

MCRS1 can transform chicken fibroblasts (Bader et al., 2001). MCRS1 can also transform $PTEN^{-/-}$ mouse fibroblasts (Okumura et al., 2005). The FHA domain interacts directly with the C-terminus of PTEN in a phosphorylation-dependent manner, and this interaction is sufficient to suppress transformation caused by the overexpression of MCRS1 (Okumura et al., 2005).

MCRS1 has also been reported to functionally interact with the transcriptional repressor Daxx, MCRS1 relieves the repressor activity of Daxx, possibly by sequestering it to the nucleolus (Lin and Shih, 2002). Overexpression of MCRS1 has also been reported to reduce the telomere length in cell culture (Song et al., 2004). It is likely that MCRS1 interacts promiscuously with many proteins in the cell, since it has been found in multiple yeast two-hybrid screens (Bruni and Roizman, 1998; Ivanova et al., 2005; Lin and Shih, 2002; Okumura et al., 2005; Ren et al., 1998; Song et al., 2004).

The interaction of hMOF with MCRS1 was confirmed by western blotting analysis of the hMOF complex (**II**:Figure 1B**)**. Furthermore, the fruit fly ortholog of MCRS1, CG1135, co-purified with *Drosophila* MOF. This interaction is most likely direct, because CG1135 was found in a yeast two-hybrid screen with MOF as the bait (D. Slocum and A. Akhtar, personal communication). These data indicate that MCRS1 interacts with hMOF in human and *Drosophila* cells.

PHF20 AND PHF20L1

PHF20, or GLEA2, is protein of 1012 amino acids containing a tandem Tudor-domain, a C_2H_2-type zinc finger and a PHD-finger. PHF20L1 is a related but shorter protein that only contains the N-terminal Tudor domains. Very little is known about the proteins. PHF20 has been shown to elicit immune responses in glioblastoma and medulloblastoma patients, and the immune response appears to correlate with a good prognosis in glioblastoma (Behrends et al., 2003; Fischer et al., 2001; Pallasch et al., 2005)

An interesting sequence feature of PHF20 becomes evident when it is compared to its fruit fly ortholog, MBD-R2. MBD-R2 has all three domains of PHF20, but it has two additional domains. In the extended N-terminus it contains a THAP domain, which is a sequence-specific DNA-binding domain (Clouaire et al., 2005; Roussigne et al., 2003). More interestingly, it has a methyl-DNA binding domain between the Tudor domains and the zinc finger (Figure 14). This is rather unexpected, as there is very little methylated DNA in

Drosophila and its role is gene regulation is still unclear, in contrast to mammalian DNA methylation whose role in repression is well-established (Freitag and Selker, 2005; Lyko et al., 2000; Tweedie et al., 1999).

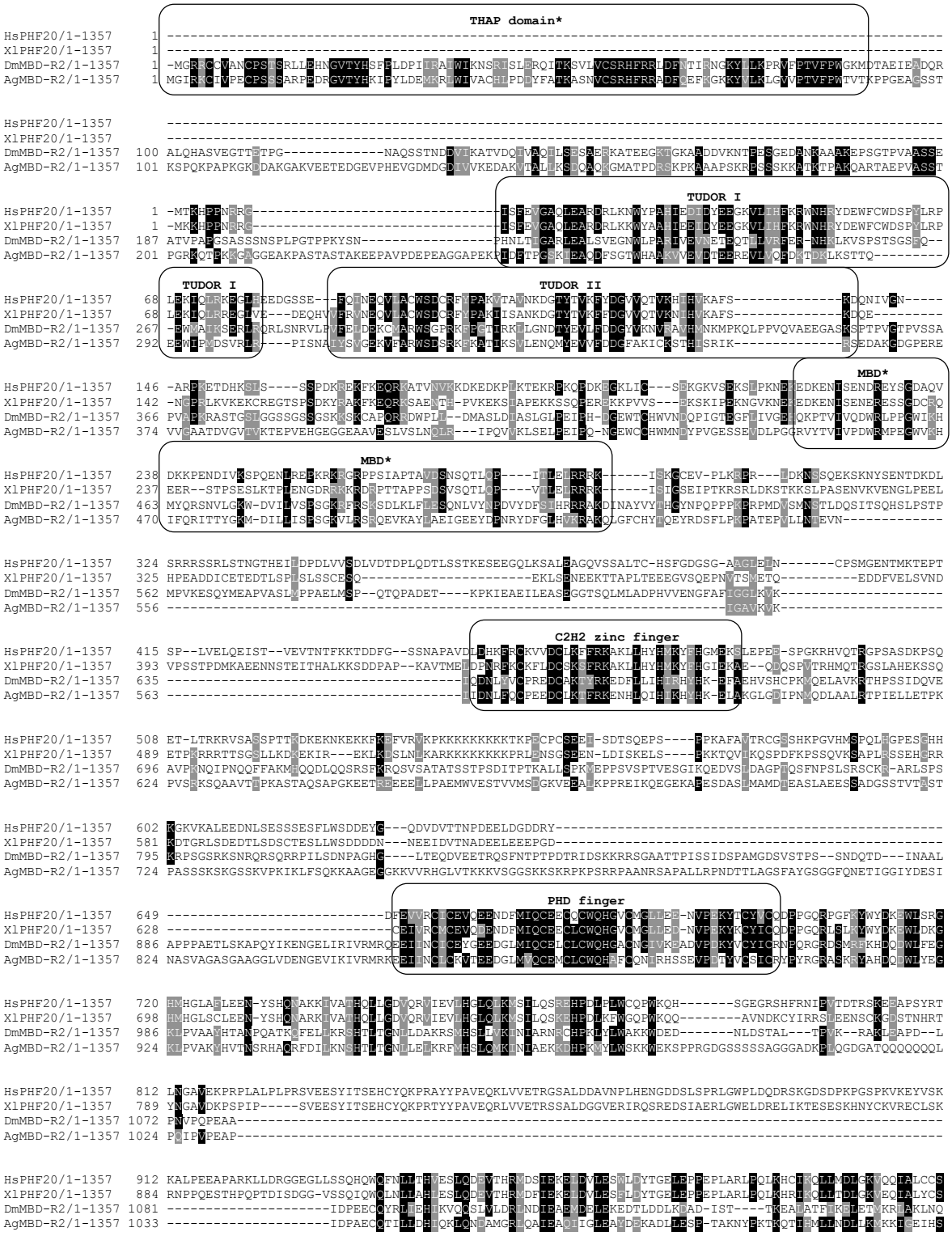


Figure 14. Evolutionary conservation of PHF20/MBD-R2. Functional domains are indicated as boxes. Note that the vertebrate PHF20 proteins lack the N-terminal THAP domain and a recognizable MBD domain.

ILF1

ILF1 (interleukin enhancer binding factor 1), or FOXK2, was cloned as a factor that binds human immunodeficiency virus long terminal repeats and interleukin 2 enhancer *in vitro* (Li et al., 1991). It binds constitutively the interleukin 2 enhancer that contains the ILF consensus sequence TGTTTAC, even in the absence of an inductive signal (Nirula et al., 1997). However, the biological function of ILF is still unknown.

CONSERVATION OF THE HMOF-CONTAINING COMPLEXES

Simultaneous purification of the *Drosophila* MOF complex uncovered remarkable evolutionary conservation. Of the 14 hMOF-associated proteins, 10 were found also in the *Drosophila* complex. ILF1 and PHF20L1 have no clear orthologs in the fruit fly, whereas HCF-1 and OGT are conserved, but nevertheless they were not associated with MOF. It is possible that they have different functions in the fruit fly. Of the *Drosophila* complex, all but three proteins were also found in the hMOF complex. Chromator and Z4 appear to be *Drosophila*-specific genes, as no clear orthologs could be found by PSI-BLAST (data not shown). Neither was the nucleoporin Nup153 present in the human complex. This could reflect the lower amount of TPR in the mammalian complex as detected by mass spectrometry. TPR interacts directly with and is tethered to the nuclear pore by Nup153 (Hase and Cordes, 2003). Hence, Nup153 could be present in the *Drosophila* complex via its interaction with TPR, and since less TPR was detected in the human complex, the amount of Nup153 might be below the detection limit.

NSL AND MSL – SEPARATE COMPLEXES?

Characterization of the proteins associated with HA-2xFLAG-hMOF could not address whether there were several independent complexes, each containing hMOF as the enzymatic core. Multiprotein complexes in eukaryotes form an extensive network, whose nodes are connected by proteins associating with more than one complex (Gavin et al., 2002). Therefore, we created a stable HeLa cell line expressing HA-3xFLAG-tagged hMSL3.

Epitope-tagged hMSL3 was expressed in slightly higher levels than the endogenous protein (data not shown). Nuclear extract from HA-3xFLAG-hMSL3 cell line was incubated with α-FLAG affinity beads, followed by elution with excess FLAG peptide and another immunoprecipitation with α-HA agarose beads. Silver staining of the proteins bound to α-HA affinity matrix revealed that only a fraction of hMOF-associated proteins interacted with hMSL3 (Figure 15A).

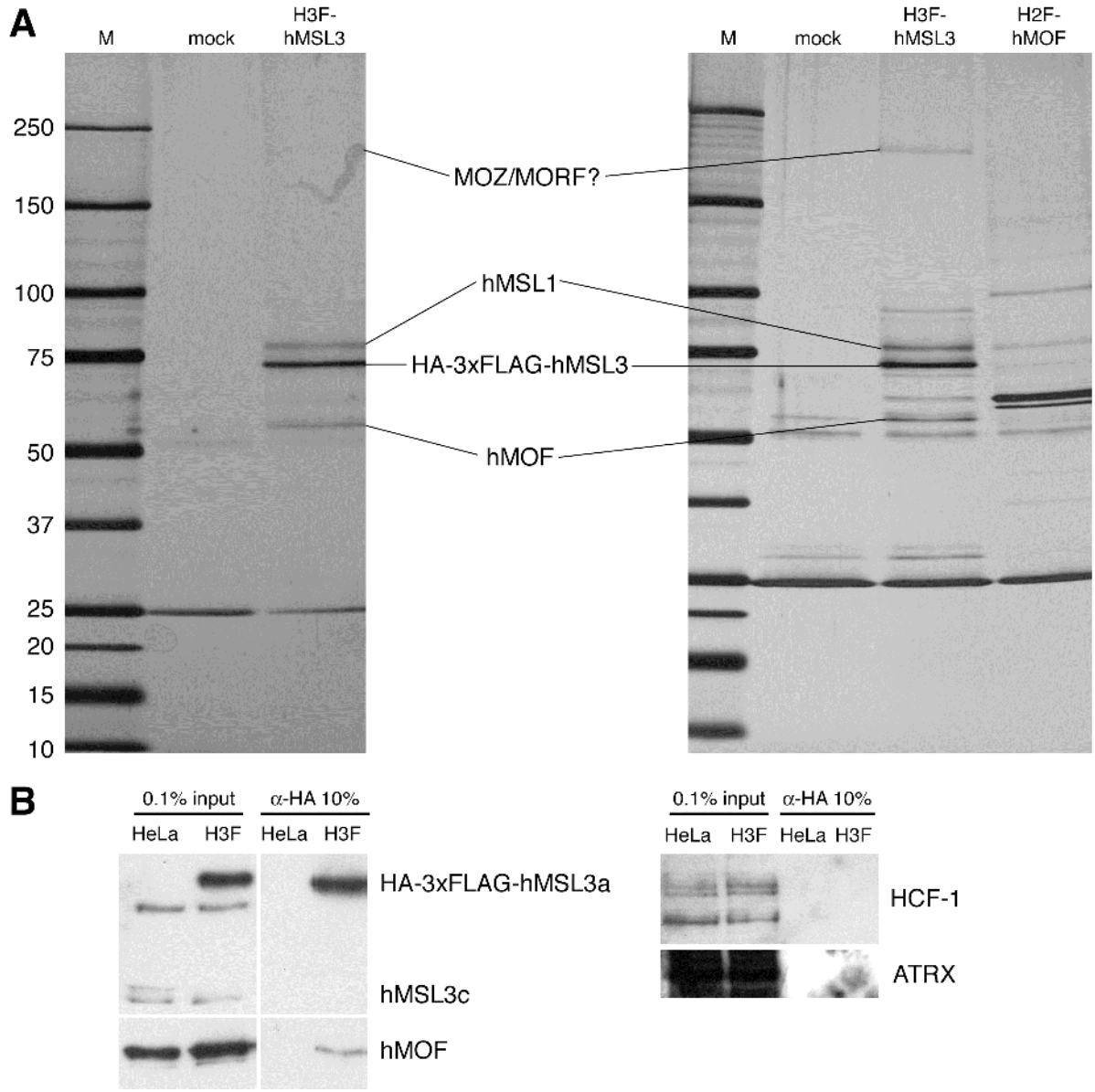


Figure 15. The hMSL3 complex. A, silver-stained gels after two independent purifications. As a comparison, hMOF complex was run next to the hMSL3 complex after the second purification. B, Western analysis of the hMSL3 complex with antibodies against hMSL3, hMOF, HCF-1, and ATRX.

MALDI-TOF analysis of excised bands confirmed that only hMSL1 and hMOF were present in the hMSL3 complex. The presence of hMOF was also confirmed with western blotting. In contrast, NSL1 that robustly interacts with hMOF, or HCF-1 could not be detected in the hMSL3 complex (Figure 15B and data not shown). The identity of the other bands could not be verified by mass spectrometry. However, there are a number of reasons to believe that hMSL2 is also present in the complex. Firstly, it is a subunit of the *Drosophila* MSL-3 complex. Secondly, the detection of hMSL2 and *Drosophila* MSL-2 in mass spectrometry has been difficult due to unknown reasons. Thirdly, the alternative explanation that hMSL2 is a subunit of the other complex is less parsimonious, given that MSL-1 and MSL-2 interact robustly *in vitro* (Copps et al., 1998; Lyman et al., 1997).

We detected several peptides from MOZ, MORF, and ATRX in mass spectrometry. However, ATRX could not be detected in the complex by western blot (Figure 15). The presence of MOZ or MORF has not been verified by western blot or co-immunoprecipitation. However, it is plausible that hMSL3 interacts also with MOZ and/or MORF, as they belong to the same MYST family of acetyltransferases with hMOF.

To further characterize the hMOF and hMSL3 complexes, HeLa nuclear extract was fractionated by 20-50% glycerol gradient centrifugation, and the fractions probed with antibodies against hMOF, HCF-1, NSL1, NSL3, and hMSL3. hMOF was present in fractions corresponding to the size range 50-400 kDa, further illustrating that it is a member of multiprotein complexes. However, hMSL3 sedimented very differently from NSL1 and NSL3 in the glycerol gradient. NSL1 and NSL3 were in a large ~400 kDa complex, while hMSL3 was present only in a small molecular weight complex of ~100-200 kDa (**II**:Figure 3A). HCF-1 was present in multiple fractions ranging from 100 kDa to 600 kDa, corroborating an earlier report (Wysocka et al., 2003). These data indicate that NSL1 and NSL3 are predominantly associated with only one complex in the cell, most likely the one with hMOF. In addition, the bulk of HCF-1 is not bound to hMOF in the cell.

The above evidence thus suggests that hMOF (and *Drosophila* MOF) is present in two separate multiprotein complexes. Firstly, hMSL3 associates only with a subset of hMOF-interacting proteins. Secondly, *Drosophila* MSL-1 interacts only with the other MSLs in co-immunoprecipitation experiments, while NSL1 interacts with a non-overlapping set of proteins (**II:**Figure 3B**)**. Finally, the WDR5-MLL1 complex purified from HeLa cells contains hMOF, NSL1, HCF-1, MCRS1, and PHF20 but no MSLs (Dou et al., 2005).

Morales and colleagues (2004) provided a clue as to how the two complexes are assembled. *In vitro*, *Drosophila* MSL-1 interacts with MOF with its PEHE domain, and this

interaction is required for a stable association of MSL-3 with MOF (Morales et al., 2004). Interestingly, there are only two proteins with a PEHE domain in the *Drosophila* genome – MSL-1 and NSL1. This immediately suggests that PEHE is an hMOF-interacting domain. Indeed, NSL1 PEHE domain interacts directly with full-length hMOF in a GST pulldown assay, whereas *Drosophila* ESC as a negative control shows no interaction (**II**:Figure 3C).

Taken together, hMOF is a subunit of at least two protein complexes. The MSL complex contains hMSL1, hMSL2, hMSL3, and hMOF. The larger NSL complex is composed of hMOF, NSL1, NSL2, NSL3, MCRS1, WDR5, PHF20, and HCF-1. TPR likely belongs to the MSL complex, suggested by its functional link to dosage compensation and its co-purification with both MOF and MSL-3 in *Drosophila*. The hMOF complexes might be bifurcated by hMSL1 and NSL1, as their interaction with hMOF is almost certainly mutually exclusive. Also hMSL3 and MCRS1 directly interact with hMOF, which might contribute to further stabilization of the interactions (Figure 16).

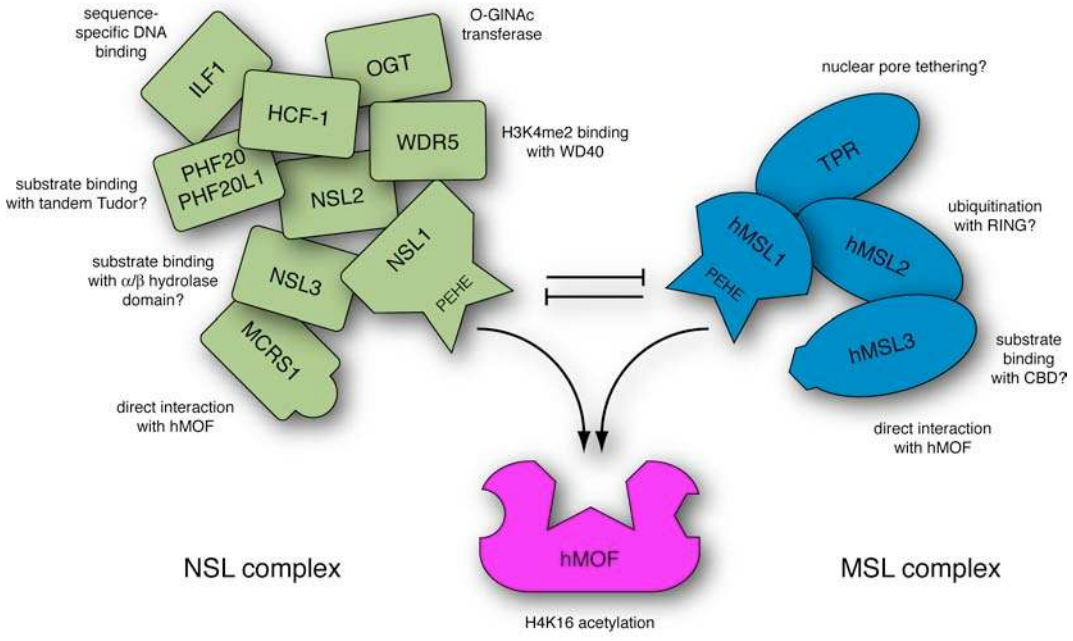


Figure 16. Schematic representation of the hMOF complexes in human cells.

In addition to these two complexes, hMOF is also in the MLL1 complex (Dou et al., 2005). It is apparently an extension of the NSL complex, because they share many subunits and no MSLs were found in the purification (Dou et al., 2005). How the NSL and MLL1 complexes are related and why no other MLL1 complex components were identified in our purifications remains to be seen.

Do the different complexes modulate hMOF activity or specificity? It is unlikely; the MSL complex in flies is H4K16-specific, as is the human MLL1-complex. Furthermore, hMOF depletion affects only H4K16 acetylation *in vivo*. A more plausible explanation, therefore, is that the complexes are involved in gene regulation by H4K16 acetylation in different contexts.

IMPLICATIONS TO DROSOPHILA *DOSAGE COMPENSATION*

The data presented here provides new insights into the evolution of dosage compensation. It appears that Drosophilid flies have co-opted only one MOF-containing complex for dosage compensation. This also explains why MOF is expressed in both male and female flies, in contrast to MSL-1 and MSL-2, which are expressed only in males. Initial analysis of P elements disrupting *nsl1*, *nsl2*, *nsl3*, and *mcrs1* genes suggests that they are required for both male and female viability (hence, non-specific lethal; data not shown). It is therefore likely that the NSL complex performs an essential, general function in the fruit fly development. Consistently, NSL1 is localized to all chromosomes in both female and male flies, and colocalizes with MOF to the same chromosomal sites (P. Gebhardt, personal communication).

Why is MOF then required only for male viability, while NSLs are essential for both sexes? It could be that NSLs are components of other protein complexes besides the one with MOF, or that they have complex-independent functions as monomers. An interesting possibility is that there is another protein replacing MOF in the complex. MOF has an uncharacterized autosomal paralog in the *Drosophila* genome. CG1894 contains the MYST homology domain but lacks the N-terminal chromodomain. Perhaps the CG1894 protein can substitute for MOF in the NSL complex, thus rendering MOF redundant in females. This could be addressed, for example, by purification of CG1894 or NSL1 protein complexes from female flies.

hMOF AND H4K16 ACETYLATION IN TUMORS

It was recently reported that loss of H4K16 acetylation is a common feature of human tumors (Fraga et al., 2005b). In principle, this could result from either overexpression of histone deacetylases or downregulation of histone acetyltransferases. The observation that hMOF is the major histone H4 lysine K16 acetyltransferase in human cells prompted us to study the expression pattern of hMOF in tumor samples and healthy tissues. We assessed the level of H4K16 acetylation by immunohistochemistry in 298 breast carcinomas, 281 brain tumors (mostly medulloblastomas and ependymomas), and 50 colon adenocarcinomas. For the breast tumors, 54 healthy tissue sections and 32 mastopathies were used as controls. In addition, single carcinoma samples of various origins were also analyzed.

Corroborating the findings of Fraga and colleagues (2005), we detected loss or reduction of H4K16Ac-specific staining in a significant fraction of the samples (**II**:Figure 5). These data show that reduction of H4K16 acetylation in tumor samples can also be detected by immunohistochemistry, in addition to mass spectrometry and western blotting analysis.

To determine the role of hMOF in these samples, we stained adjacent sections with two different antibodies against hMOF. Both antibodies showed a similar staining pattern (data not shown). Loss of or reduction in hMOF staining was detected in 21% (49/280) of breast carcinomas, 40% (66/166) of medulloblastomas, and in 27% (19/71) of ependymomas. Level of hMOF staining correlated very significantly with the level of acetylated H4K16 in the breast tumors (Spearman's correlation coefficient ρ=0.521, 95% CI 0.422-0.619), brain tumors (ρ=0.420, 95% CI 0.277-0.544), and colon carcinomas (ρ=0.706, 95% CI 0.517-0.830), suggesting that hMOF is the global H4K16 acetyltransferase also in the tissues examined. Quantitative real-time PCR verified the reduction in hMOF expression in both breast tumors and medulloblastomas (**II**:Supplementary Figure S3). Furthermore, hMOF expression could be used as a prognostic marker for survival in medulloblastoma patients (**II**:Figure 6).

These data pinpoint hMOF as the first protein linked to global loss of histone acetylation in human tumors. Previous studies have only addressed changes in the histone modification patterns without implicating any specific enzymes in the process (Fraga et al., 2005b; Seligson et al., 2005).

What could be the function of H4K16 hypoacetylation in tumors? Obviously, with little functional data most discussion inevitably remains speculative. Nevertheless, based on

the available data, there are several possible explanations. Fraga et al. (2005) reported that the H4K16 acetylation is mainly lost in repetitive regions of the genome, whereas promoter regions of known tumor suppressors were not affected. This is hardly surprising, since loss of H4K16 acetylation only at specific promoter elements would not explain a global loss of H4K16 acetylation. Fraga and colleagues (2005) also showed that hMOF is associated with the non-satellite D4Z4 repeat in normal lymphocytes but not in HL-60 cancer cell line, and this correlates with reduction of H4K16 acetylation.

In normal cells, a repressive protein complex binds the subtelomeric D4Z4 repeat (Gabellini et al., 2002). Reduction in the number of repeats leads to activation of genes several megabases away from the repeats, with more proximal genes being more sensitive (Gabellini et al., 2002). This is reminiscent of telomeric silencing in the budding yeast, where the hMOF ortholog Sas2p regulates the spreading of heterochromatin (Kimura et al., 2002; Suka et al., 2002). Interestingly, TSA-dependent telomere position effect has also been reported in human cells (Baur et al., 2001). Thus, loss of hMOF and H4K16 acetylation could lead to ectopic spreading of heterochromatin and silencing of adjacent genes.

It is likely that hMOF acts in concert with other chromatin-modifying activities. Arguably the best-characterized epigenetic modification in cancer is DNA methylation. It is intimately linked to histone modifications and chromatin remodeling in eukaryotes (Freitag and Selker, 2005). Epigenetic inactivation of a tumor suppressor by CpG island methylation has been documented in numerous cases (reviewed in Feinberg and Tycko, 2004). However, whether DNA methylation is the cause or the consequence of gene silencing is not clear. Mice mutant for some DNA methyltransferases and methyl-DNA binding proteins appear to be less cancer-prone than their wild type siblings (Laird et al., 1995; Sansom et al., 2003), while DNMT1-deficient ES cells show genomic instability (Chen et al., 1998). In cell culture, gene silencing precedes DNA methylation, suggesting that it is a late event (Bachman et al., 2003), but inactivation of DNMT1 and DNMT3b can lead to derepression of tumor suppressors (Rhee et al., 2002).

Histone modifications and DNA methylation act together to establish chromatin domains, and it has been proposed that they form a “slippery slope” via feedback mechanisms to maintain the established states (Jaenisch and Bird, 2003). HDAC inhibitors function synergistically with drugs inhibiting DNA methylation in gene activation, and DNA methylation and histone deacetylase machineries interact *in vivo* (Ng et al., 1999). Loss of H4K16 acetylation could tip the balance in favor of silencing, thus rendering tumor suppressor genes more susceptible to DNA methylation and other repressive modifications.

hMOF knockdown or expression of its dominant negative mutant in cell lines was reported to lead to genomic instability, which is a hallmark of human cancers (Gupta et al., 2005; Rajagopalan and Lengauer, 2004). Maybe genomic H4K16 hypoacetylation could lead to chromatin reorganization and instability, similar to what has been reported with histone hypomethylation. Disruption of the major H3K9 methyltransferases Suv39h1 and Suv39h2 causes a loss of heterochromatin organization, genomic instability, and B-cell lymphomas (Peters et al., 2001), illustrating the role of chromatin organization in tumorigenesis.

Aberrant checkpoint activation has been implicated in tumorigenesis, and many of the proteins involved are also tumor suppressors (Kastan and Bartek, 2004). ATM and its downstream targets are phosphorylated in many precancerous lesions but not in normal tissues, suggesting that activation of checkpoint pathways is an early event in tumorigenesis (Bartkova et al., 2005; Gorgoulis et al., 2005). Defects in DNA repair and checkpoint activation, as seen in hMOF knockdown cells (Gupta et al., 2005; Taipale et al., 2005), could promote genomic instability and tumor progression.

It is clear that future studies need to address whether the loss of hMOF is a causative event in cancer, and by which mechanism(s) it might contribute to it. Regardless of the mechanism, it is also evident that global histone modifications are an important factor in malignant conditions (Fraga et al., 2005b; Seligson et al., 2005). It could be said that research on global histone modifications in tumorigenesis stands currently where DNA methylation research was 20 years ago. Loss of DNA methylation in tumors had been documented (Feinberg and Vogelstein, 1983), but the enzymes responsible for the modification were still unknown. Hopefully the characterization of hMOF as the major H4K16 acetyltransferase will facilitate further advances in this field.

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Now that you've read all the acknowledgements, how about starting from page one? It's a nice thesis. I promise.

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