Functional characterization of CDY family proteins and their role in recognition of the heterochromatic histone H3K9me3 modification

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Henriette Franz
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Thesis Supervisor:
Dr. Wolfgang Fischle

Doctoral Committee:

Dr. Wolfgang Fischle (1st Referee)	*Chromatin Biochemisty, Max-Planck-Institute for biophysical Chemistry, Göttingen*
Prof. Dr. Herbert Jäckle (2nd Referee)	*Molecular developmental Biology, Max-Planck-Institute for biophysical Chemistry, Göttingen*
Prof. Dr. Sigrid Hoyer-Fender	*Developmental Biology, Georg-August-University, Göttingen*

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Affidavit

I hereby ensure that the thesis has been written independently and with no other sources and aids than quoted.

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List of publications

Franz H, Mosch K, Soeroes S, Urlaub H, Fischle W (2009) Multimerization and H3K9me3 binding is required for CDYL1b heterochromatin association. J Biol Chem. Epub Oct 5

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Fischle W*, Franz H*, Jacobs SA, Allis CD, Khorasanizadeh S (2008) Specificity of the chromodomain Y chromosome family of chromodomains for lysine-methylated ARK(S/T) motifs. J Biol Chem. Jul 11;283(28):19626-35 Epub May 1

Somel M, Creely H, Franz H, Mueller U, Lachmann M, Khaitovich P, Pääbo S (2008) Human and chimpanzee gene expression differences replicated in mice fed different diets. PLoS ONE. Jan 30;3(1):e1504

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Abstract

In the nucleus of eukaryotic cells the DNA is wrapped around octamers of histone proteins containing two H2A-H2B dimers and one $(H3\text{-}H4)_2$ tetramer. This entity represents the basic repeating unit of chromatin called the nucleosome. N- and C-terminal regions of all four histones are protruding out of the nucleosome and are therefore available for many different posttranslational modifications (PTMs). A huge diversity of histone PTMs like methylation, acetylation and phosphorylation regulate DNA-templated processes like transcription, replication and DNA repair.

One of these modifications trimethylation of histone H3 lysine 9 (H3K9me3) is a hallmark of heterochromatin, which is densely packed, mostly transcriptionally silent and late replicating during S-phase. We characterized a new group of proteins, the CDY family (chromodomain on the Y) as H3K9me3 binding proteins. The human genome encodes three CDY family genes, two autosomal genes CDYL1, CDYL2 and the Y-chromosomal CDY gene. CDY family proteins contain a N-terminal chromodomain, a known methyllysine recognition module, and a C-terminal enoyl-CoA-hydratase (ECH) domain. Interestingly, the CDYL1 gene has three different splicing variants CDYL1a, CDYL1b and CDYL1c. Due to splicing CDYL1c contains no chromodomain at all.

Using *in vivo* and *in vitro* approaches we delineated the specificity of the CDY family chromodomains for methyllysine recognition. We show that CDY as well as CDYL1b exhibit specific binding to H3K9me3, whereas CDYL2 binds with comparable strength to different methyllysines embedded in ARK(S/T) motifs. Subtle amino acid changes in the CDYL1a chromodomain prohibit H3K9me3 interaction *in vitro* and *in vivo*. This deficient binding could be rescued by mutation of specific amino acids residues. The results elucidate essential elements of chromodomains and indicate that intact chromodomains are necessary for association with H3K9me3. However, additional experiments showed that chromodomains are not sufficient for heterochromatin association of CDY proteins *in vivo*. We demonstrated that multimerization of CDYL1b via the ECH-like domain is essential for efficient heterochromatin localization. In agreement, CDYL1c overexpression could displace CDYL1b from heterochromatin. Based on these results we speculate that homomeric CDYL1b complexes are implicated in directing higher order chromatin structures by crossbridging different regions of H3K9me3 chromatin.

CDYL1 is able to repress transcription via a C-terminal domain most likely due to interaction with HDAC1, HDAC2 and the repressor complex CoREST. We could show that PRMT5 is a

new CDYL1-modifier, which methylates an arginine embedded in an ARKQ motif *in vitro*. The ARKQ motif is also a target of G9a that methylates the neighboring lysine residue. Surrounding serine residues are phosphorylated in a mitosis-dependent manner. It is possible that this highly modified region is implicated in regulation of CDYL1-mediated interactions. Lastly, *Xenopus laevis* knockdown and overexpression experiments suggest that CDYL1b is highly important for developmental processes.

Altogether this work represents *in vivo* and *in vitro* results indicating that members of the CDY family are basic heterochromatin proteins involved in translation of the H3K9me3 modification. Our studies point toward an important function of CDY family proteins in diverse epigenetic pathways.

Abbreviations

ac	Acetylation
ADP	Adenosine triphosphate
ar	ADP-ribosylation
BD	Bromodomain
BPTF	Bromodomain PHD finger transcription
BSA	Bovine serum albumine
CARM1	Co-activator associated arginine methyltransferase 1
CD	Chromodomain
cDNA	complementary DNA
chromo	Chromatin modifier
Cit	Citrulline
CMV	Cytomegalovirus
CtBP	C-terminal binding protein
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxid
DNA	Desoxyribonucleic acid
DNMT1	DNA methyltransferase 1
dNTPs	Desoxyribonucleotide triphosphate
DTT	DL-1,4-dithiothreitol
E.coli	Escherichias coli
ECH	Enoyl-CoA-hydratase
ECHM	mitochondrial ECH
ECHP	peroxisomal ECH
EDTA	Ethylenediaminetetraacetic acid
EST	Expressed sequence tags
Fl	Flag tag
h	Hour
H3K9me1	Histone H3 lysine 9 monomethylation
H3K9me2	Histone H3 lysine 9 dimethylation
H3K9me3	Histone H3 lysine 9 trimethylation
H3K9me3S10ph	H3K9me3 phosphorylated on serine 10

HA	Hemagglutinin tag
HAT	Histone acetyltransferase
hCDY	Human chromodomain on the Y
hCDYL	Human chromodomain on the Y-like
HDAC	Histone deacetylase
HEK293	Human embryonic kidney 293 cell line
HeLaS3	Human cervix epithel carcinoma cell line
HEPES	2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid
His	Histidine
HMT	Histone methyltransferase
HP1	Heterochromatin protein 1
hPRMT5	Human protein arginine methyltransferase 5
HRP	Horse radish peroxidase
IF	Immunofluorescence
Ig	Immunoglobulin
IKZF3	Ikaros family of zink fingers
ING1	Inhibitor of growth 1
IP	Immunoprecipitation
IPTG	Isopropylthio-b-D-galactoside
K_D	Dissociation constant
kDa	Kilo Dalton
MBD	methyl CpG-binding domain
MBP	Maltose binding protein
me	Methylation
MEF	Mouse embryonic fibroblast cell line
min	Minutes
NURD	Nucleosome remodeling and deacetylase complex
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
ph	Phosphorylation
Pisom	isomerized Proline

PRMT1	Protein arginine methyltransferase 1
PRMT5	Protein arginine methyltransferase 5
PTM	Post translational modifications
REST	RE-1 silencing transcription factor
RNA	Ribonucleic acid
rpm	Rounds per minute
RSC	remodels the structure of chromatin
RT	Room temperature
RT-PCR	Reverse transcriptase PCR
s.e.m	Standard error of mean
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium docecyl sulfate polyacrylamide gel electrophoresis
sec	Seconds
Sir3	Silent information regulator 3
su	Sumoylation
Su(var)	suppressor of variegation
TK	Tyrosine kinase
TSS	Transcriptional start site
UAS	Upstream activator sequences
ub	Ubiquitylation
UTR	Untranslated regions
UV	Ultraviolet
WDR5	WD repeat domain 5
WIZ	Widely interspaced zinc finger motifs
wt	wild type, original sequence without mutations
xlCDYL1	*Xenopus laevis* CDYL1

1 Introduction

1.1 Chromatin

DNA as genetic information of eukaryotic cells is stored in the cell nucleus [1]. The genome of a eukaryotic cell contains up to and in some cases over 3 billions of base pairs [2]. For this reason DNA reaches a length of about two meters but it has to be packaged into the nucleus, which is only a few micrometers in diameter. To manage the dimensions of the eukaryotic genome the DNA has to be significantly compacted. This condensation is accomplished by association of the DNA with a set of nuclear proteins resulting in a structure called *chromatin*. Chromatin as a complex of DNA and proteins controls gene activity and the inheritance of traits [3].

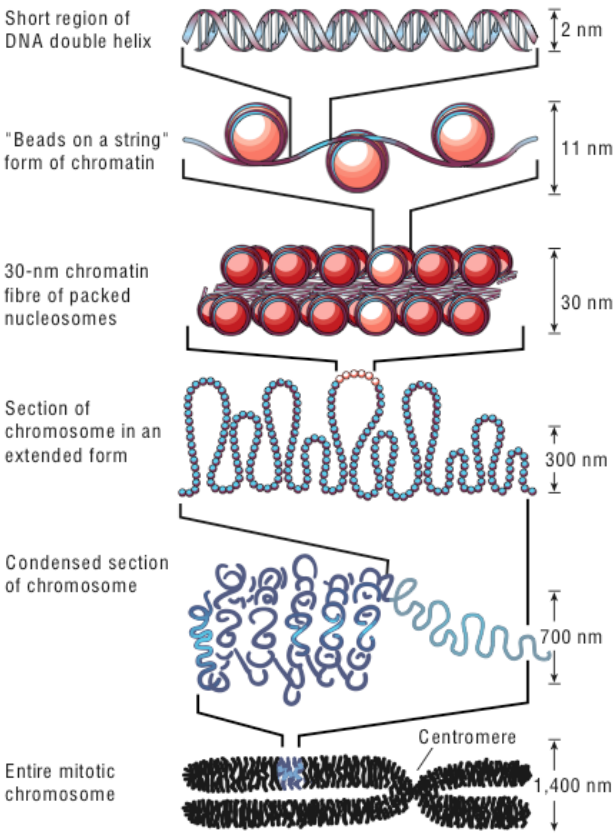

Figure 1-1 DNA organization and chromatin structure within the cell.

The DNA is wrapped around nucleosomes in regular intervals forming the ‘beads on the string’ type of chromatin. 30 nm diameter fibers and further higher order structures are folded by currently not-well known mechanism. During mitosis the DNA is than compacted more than 10000 fold (adapted from [3])

On a physiological level, firstly shown by Heitz et al, two different forms of chromatin exist called *euchromatin* and *heterochromatin* [4]. Euchromatin has an open accessible

conformation, is early replicating during S-phase and contains the majority of active genes [5]. In contrast, heterochromatin has a condensed structure, is replicating late during S-phase and is relatively gene-poor [5] (compare also with Table 1-1).

Table 1-1 Summary of euchromatin and heterochromatin characteristics.

Listed characteristics will be discussed in more detail in following chapters. BPTF: bromodomain PHD finger transcription factor, WDR5: WD repeat domain 5, HP1: heterochromatin protein 1. Table was combined from [3, 5-7].

	Euchromatin	**Heterochromain**
Conformation during S-phase	decondensed	condensed
Gene density	high	low
Replication	mainly early	late
Levels of Histone acetylation	high	low
Specific modifications	H3K4me H3K36me	H3K9me H3K27me H4K20me
Effector proteins	e.g. BPTF, WDR5	e.g. HP1, Polycomb
Histone variants	H3.3	MacroH2A
Levels of DNA methylation	low	high

Some parts of the genome including centromeres, pericentric and telomeric regions are condensed in structure and not actively transcribed at all times. Therefore these parts are known as *constitutive heterochromatin* [8]. Other heterochromatic regions can change their status during development or differentiation and are able to respond to cellular signals. Thus they have been called *facultative heterochromatin* [8].

1.1.1 Histones and nucleosomes

The repetitive unit of chromatin consists of 146 bp of DNA wrapped around an octamer of histones and is called the *nucleosome*. The octamer of histones includes two histone H2A/histone H2B dimers flanking one tetramer built of two copies of histone H3/histone H4 ([9] and Figure 1-2).

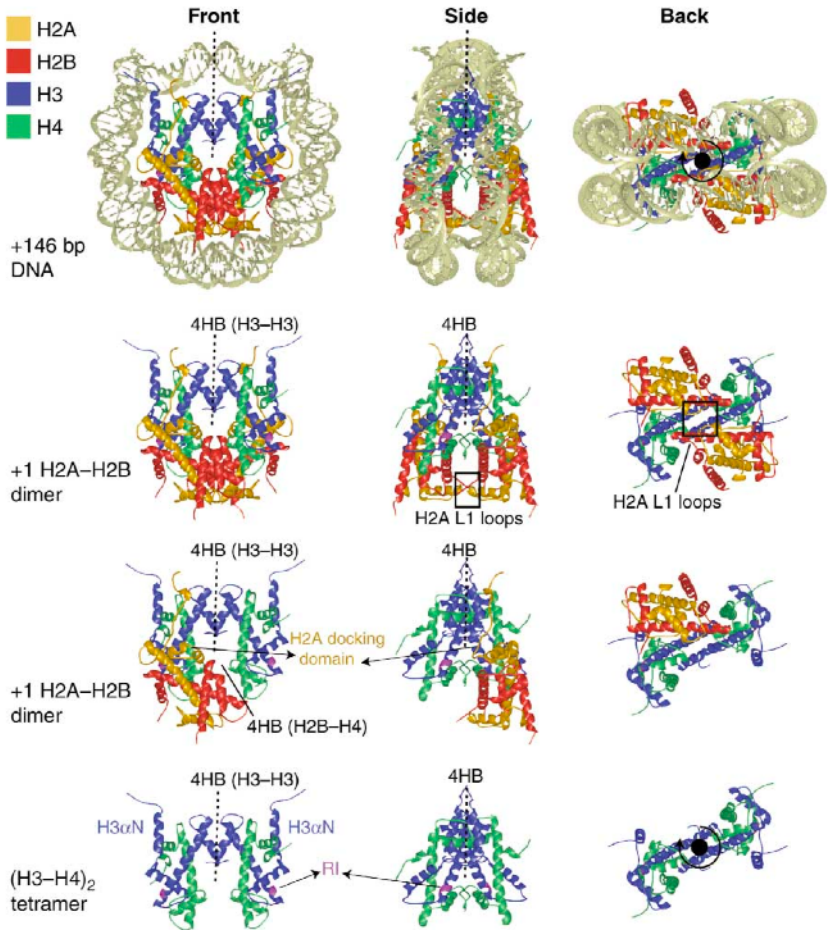

Figure 1-2 Structure of the nucleosome

One $(H3\text{-}H4)_2$ tetramer and two H2A-H2B dimers form an octamer around which 146 bp of DNA is wound in a left-handed superhelix to form the fundamental unit of chromatin, the nucleosome. In these pictures the different layers of histone and DNA interaction are shown starting with the $(H3\text{-}H4)_2$ tetramer followed by adding the two H2A-H2B dimers and in the end the DNA (adapted from [10]). The dyad axis for the histone core is marked by a dot and a clockwise arrow. The location of the H3 αN helix, the H2A docking domain and the H3-H3 (marked as 4HB) four helix bundle are shown. H2B-H4 four helix bundle is indicated as 4HB (H2B-H4). The interaction surface between the two H2A L1 loops is boxed. Residues in H3 that have been identified to maintain selectivity between different assembly pathways are shown in magenta ('RI'). H2A: yellow, H2B: red, H3: blue, H4: green.

A fifth histone, H1 interacts with the nucleosomal core as well as with the linker DNA between two nucleosomes and is therefore able to compact chromatin to higher order structures [11].

The histone family proteins are small and contain a substantial amount of lysine and arginine residues, which lead to an isoelectric point at a very basic pH. The core histones H2A, H2B, H3 and H4 contain a ~65 amino acid motif called the histone fold. The histone fold is evolutionarily very conserved and reaches up to 100% identity between plants and humans [12, 13]. In a histone dimer, three connected helices of the paired histone fold interdigitate in a head to tail fashion and form a compact structure [14]. The final association of these dimers

to an octamer provides a globular domain around which the DNA is supercoiled, mainly interacting with its negatively charged backbone phosphate groups [9].
In specific cases nucleosomes contain different histone variants. Histone variants (e.g H3.3 or MacroH2A) have high homology to canonical core histones but are generally not as ubiquitous and have specific function in DNA replication, DNA repair and chromosome segregation [15].
In addition, nucleosomes can differ from one another by covalent modifications. The N- and C-terminal regions as relatively unstructured parts of the histones protrude out of the nucleosomal entity [9] and carry many different posttranslational modifications, which are important for regulation of higher-order DNA packing and therefore transcriptional activity [7].

1.1.2 Chromatin as genomic regulator

Chromatin makes it possible to store the genetic information encoded in the DNA sequence in the cellular nucleus. But chromatin is not only the protective and constant scaffold of the DNA. Dynamic changes in chromatin structure regulate important cellular processes like transcription, replication, mitotic chromosome condensation, recombination, apoptosis and DNA repair [3, 16, 17].
Structural changes of chromatin can be accomplished by chromatin remodeling complexes, by integration of different histone variants or by posttranslational modifications (PTMs) on the histone tails [18-20]. Variations in chromatin structure lead to a more or less accessible DNA and therefore influence on processes, which require the admission to the genetic information. In addition, nuclear factors are targeted to specific regions of chromatin by PTMs on histones [21, 22]. Different combinations of the covalent modifications on histones recruit distinct mediator proteins for downstream functions [7]. Therefore, by directing the accessibility and readout of different genomic regions, chromatin acts a key player in multiple fundamental DNA-template based pathways.

1.1.3 Chromatin modifications

As described in chapter 1.1.2; chromatin can influence important cellular processes directly by changes in structure or indirectly by recruitment of effector proteins. Both processes can be maintained by posttranslational modifications mainly on histone N- or C-terminal tails or by DNA methylation. Histones can carry PTMs at many sites such as acetylation on lysine

residues, phorphorylation on serine, threonine or tyrosine residues, methylation on lysine or arginine residues, sumoylation on lysine residues, ubiquitylation on lysine residues, deimination of arginine residues, ADP-ribosylation of glutamates or isomerization of proline residues as defined by mass spectrometry and specific antibodies [6, 23]. Lysines can carry mono-, di- or trimethylation and arginine residues can be mono- or dimethylated (symmetric/asymmetric). This complexity of histone modifications gives an enormous potential for functional responses.

Table 1-2 Excerpt of chromatin modifications in mammals

Observed transcriptional role of chromatin modifications in mammals (adapted from [6, 23-26])

Mark	**Relevant site**	**Transcriptional role**
DNA methylation		
Methylated cytosine (meC)	CpG islands	Repression
Histone PTMs		
Acetylated lysine (Kac)	H3 (9, 14, 18, 56) H4 (5, 8, 13, 16) H2A H2B	Activation
Phosphorylated serine/threonine/tyrosine (S/T/Yph)	H3 (3, 10, 28, 41) H2A H2B	Activation
Methylated lysine (Kme)	H3 (4, 36, 79)	Activation
	H3 (9, 27) H4 (20)	Repression
Methylated arginine (Rme)	H3 (17, 23) H4 (3)	Activation
	H3 (8)	Repression
Ubiquitylated lysine (Kub)	H2A (119)	Repression
	H2B (120)	Activation
Sumoylated lysine (Ksu)	H2A (126) H2B (6, 7)	Repression
Isomerized proline	H3 (30-38)	Activation/Repression

(Pisom)		
Deimination (R>Cit)	H3 H4	Repression
ADP-ribosylation (Ear1)	H2B (2)	Indirect Activation

Histones PTMs and also DNA methylation correlate with the transcriptional status of a gene or a genomic region (Table 1-2).

Additionally, PTMs such as acetylation, methylation of lysines, phosphorylation and ubiquitylation are implicated in DNA repair [23]. The condensation status of chromatin is influenced by phosphorylation as well as by acetylation [27, 28] and only acetylation is up to now known to be required for S phase initiation and fixing of replication origins [29, 30].

But recent publications show that PTMs do not have only one role but cover many functions, which are sometimes conflicting. Most of the PTMs act in a dynamic way rather than with static on/off switches, resulting in many combinations and functional possibilities. These observations raised the idea of the 'histone code'. According to this theory the combination of different histone modifications can create synergistic or antagonistic interaction affinities for chromatin-associated proteins. The combinatorial nature reveals a 'histone code', which could extend the information of the genetic code. Therefore it was proposed that the histone modifications regulate most chromatin-templated processes by recruitment or displacement of chromatin-associated proteins [7].

1.1.4 Histone modifying enzymes

Histone modifications are established by enzymes, which set a mark on a specific sequence position ('writers'). Histone acetyltransferase (HATs) [31], histone kinases [32], histone methyltransferases (HMTs) [33] and enzymes that mediate histone ubiquitylation [34], sumoylation [35], ADP-ribosylation [36], deimination [37] and proline isomerization have been identified [38].

Several enzymes, that remove histone modifications have been described ('erasers') such as histone deacetylases (HDACs) [39], specific histone phosphatases [32] and histone lysine demethylases [40]. Arginine methylations are reversed by arginine demethylases or by deimination [41]. In addition, clipping of the histone tail [42] and exchange of the histone itself can remove or change histone modifications [43]. Histone methyltransferases and kinases are the most sequence specific histone-modifying enzymes known to date [23]. But it

is possible that interaction partners, surrounding modifications and their methylation status influence histone-modifying enzymes and maintain a specific establishment of a histone PTM [44, 45].

1.1.5 Translating a histone mark

Histone modifications can either influence the chromatin directly by structural changes ('cis') or more indirectly by recruitment of DNA and accordingly chromatin manipulating factors ('trans') (compare with Figure 1-3).

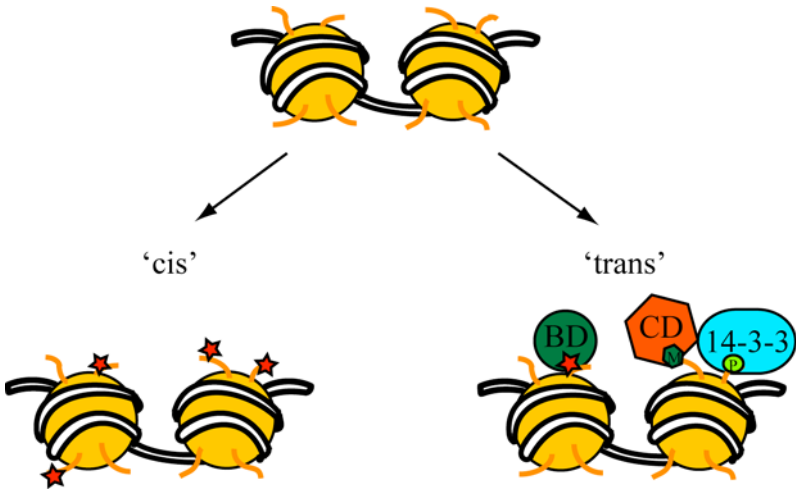

Figure 1-3 Translation of histone modifications

Histone modifications can fulfill their function via two general not mutually exclusive mechanisms. They can either influence the charge of chromatin, which might affect inter/intra nucleosomal contacts and results in structural changes of the chromatin fiber ('cis'). Or histone modification can recruit histone PTM recognizing proteins, which mediate downstream mechanism ('trans'). Red star: acetylation; M: methylation; P: phosphorylation, BD: bromodomain; CD: chromodomain, 14-3-3: 14-3-3 proteins

Direct influence on chromatin ('cis')

Histone modifications may influence the chromatin structure directly by disruption or establishment of nucleosomal contacts to unwind or to compact chromatin. An argument for such an action in cis is that the whole histone tail is important for the regulation of higher order chromatin structure [46]. The most potential for a direct effect on chromatin structure is accredited to histone acetylation since acetylation neutralizes the basic charge of the lysine and could affect the interaction of basic histone proteins and the negatively charged DNA. *In vitro* studies have shown that chromatin condensation is blocked by a certain amount of histone acetylation in general [47, 48]. This observation was recently verified *in vitro* by showing that subtraction of H4K16 acetylation by histone deacetylases facilitates compaction

of chromatin [28]. Vice versa H4K16ac has a negative effect on the formation of 30 nm fibers and higher-order packaging [49]. Due to its charge effects, phosphorylation of histones may also mediate a decompacting effect on chromatin structure [50].

Indirect effect on chromatin - readout of histone modifications ('trans')

Effector proteins containing specific domains can recognize histone PTMs depending on both modification state and position within a histone sequence ('readers') and can thereby modulate chromatin function ('trans' action).

Table 1-3 Histone PTMs with their binding modules

Histone modifications and their associated binding domains are shown (adapted from [22]). Italic font indicates domains belonging to the royal family [22, 51].

Reader module	**PTM mark**
Bromodomain	Many histone Kac
Chromodomain	H3K9me2/3, H3K27me2/3
Double chromodomain	H3K4me1/2/3
Chromo barrel	H3K36me2/3
Tudor	(Rme2s)
Double/tandem tudor	H3K4me3, H4K20me1/2/3
MBT	H4K20me1/2, H1K26me1/2, H3K4me1, H3K9me1/2
PHD finger	H3K4me0/3, H3K9me3, H3K36me3
WD40 repeat	H3R2/K4me2
14-3-3	H3S10ph, H3S28ph
BRCT	H2AX S139ph

Histone PTMs and their corresponding binding domains are listed in Table 1-3. Bromodomains, for example, are binding modules for acetylated lysines. Bromodomains are found in transcription factors or chromatin remodeling complexes [52]. Recruitment of these effector proteins to promotor regions induce transcriptional activation as shown in several model systems [53].

Chromodomains recognize methylated lysines. Important examples harboring this binding module are heterochromatic protein 1 (HP1), which recognizes H3K9me2/3 and Polycomb, which binds to H3K27me3 [54]. By binding of HP1 to methylated lysines it mediates gene

silencing and heterochromatinization of genetic loci [55]. Targeting of Polycomb proteins leads to transcriptional repression [56].

Interestingly, some effector proteins contain more than one type of the described histone PTM binding module. Examples are the Ubiquitin-E3-ligase ICBP90, the transcription factor TAFII250 and the methyltransferase MLL1. Besides its putative enzymatic region the ICBP90 protein contains a PHD finger, a tandem tudordomain, and an SRA domain, and is able to bind to methylated histones as well as to hemi-methylated DNA [57, 58]. TAFII250 contains two bromodomains recognizing acetylated lysine residues and the MLL1 factor contains a bromodomain and several PHD fingers providing several binding opportunities [59]. Different combinations of reading, writing and even erasing modalities are established by interactions of proteins in large chromatin complexes. One of these complexes is the Mi-2/nucleosome remodeling and deacetylase (NURD) complex. The complex contains among others the ATP-dependent chromatin remodeling factor Mi-2, which combines conserved PHD fingers and chromodomains, HDACs, which deacetylate histones, and the MBD protein (methyl CpG-binding domain), which specifically binds to methylated DNA [60].

The RSC complex (remodels the structure of chromatin), a chromatin remodeling and DNA repair complex, also contains 15 subunits with altogether eight bromodomains [61]. Another example is the CtBP co-repressor complex, which contains beside the repressor protein CtBP, the chromodomain containing protein CDYL1, histone H3 lysine 9 methyltransferase G9a, the histone H3 lysine 4 demethylase LSD1 and histone deacetylases HDAC1 and 2 [62]. The CtBP co-repressor complex with its many functionalities has been connected to several important processes such as developmental control and mitosis [63, 64].

1.1.6 Histone modification distribution along genomic regions

As described before, histone PTMs occur in many different combinations and can be translated by their appropriate binding partners. Mapping approaches such as chromatin immunoprecipitaion (ChIP) followed by microarray analysis or high throughput sequencing revealed some general patterns.

The histone PTMs can generally be divided in two groups: (a), broad domains of modified nucleosomes covering several kb of the DNA or (b), peaks occurring within 1 kb of DNA as highly localized modified nucleosomes (Figure 1-4).

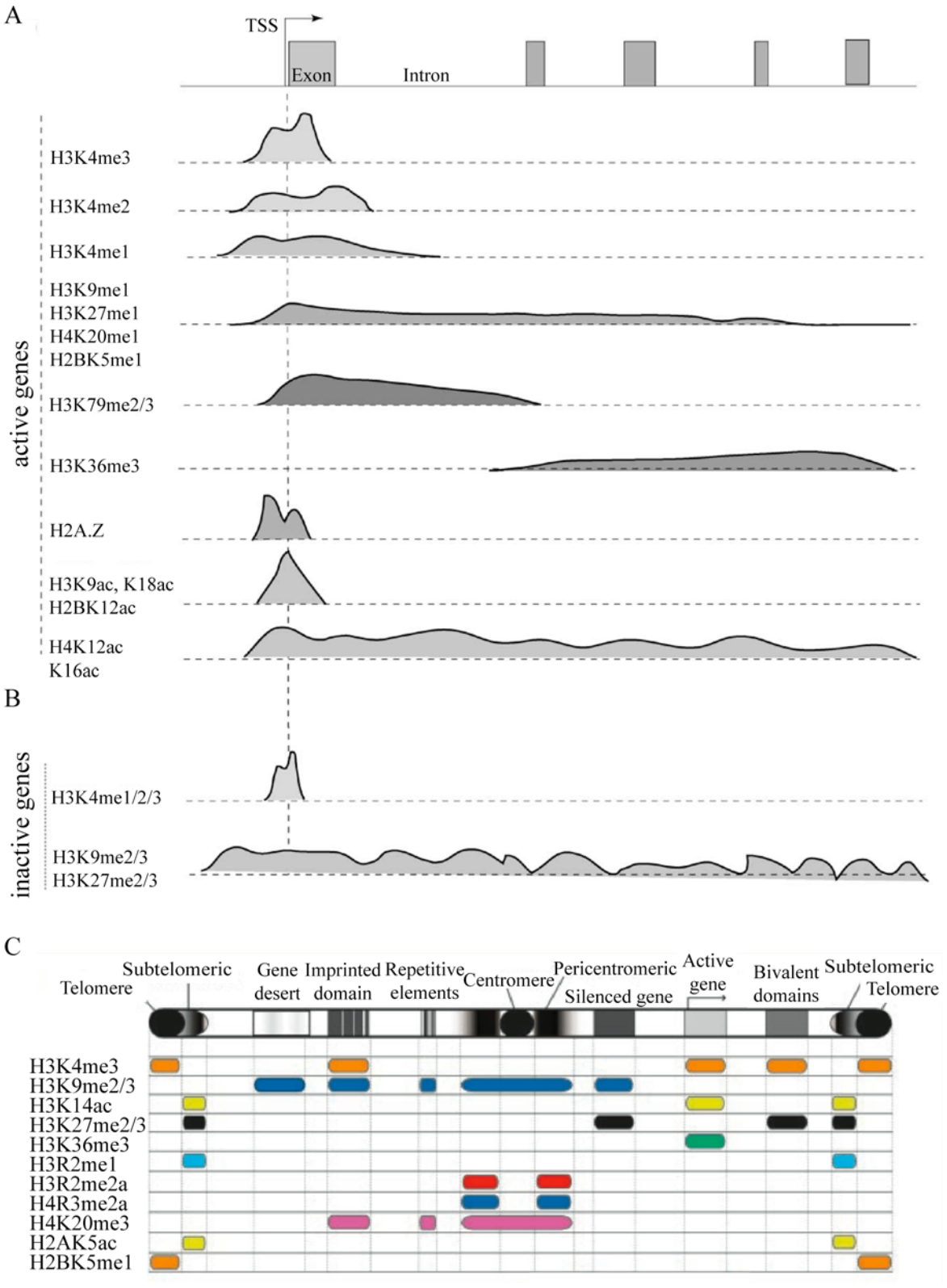

Figure 1-4 Distribution of histone modifications along chromatin regions.

A, Distribution of histone PTMs along active promotor regions. **B,** Inactive gene promoters with according histone PTMs. **C,** Histone PTMs patterning along a typical mammalian chromosome. Figure was adapted from [65, 66].

Special histone PTMs cluster in transcriptionally active parts of the genome (compare with chapter 1.1) such as acetylation of lysines or methylation on histone H3 lysine 4 or 36. Di- and trimethylated lysine 9 of histone H3 or lysine 20 of histone H4 are largely associated with permanently silenced regions such as constitutive heterochromatin. Interestingly, mostly H3K27me2/3 as well as H3K4me3 cover facultative heterochromatic regions with bivalent behavior [65, 66]. Bivalent domains are silent domains, which are poised for gene activation during development [67].

Along active genes H3K4me, H3K9ac and H3K18ac are closely distributed around the transcriptional start site (TSS) of genes. In contrast, H3K36me3 covers the body of genes excluding the TSS completely. H4K12ac as well as H4K16ac decorate the whole gene region independent from promotor or intron/exon structure. Inactive genes include mainly nucleosomes methylated at H3K9 or H3K27, but interestingly they also contain a discrete peak of H3K4me at the TSS.

1.1.7 Cross-talk between histone modifications

As highlighted before, the high density and variation in histone modifications raised the idea of the ‘histone code’. Different combinations of histone marks plus DNA methylation lead to diverse possibilities of inter-modificational influences (cross-talk) [68]. Histone modifications might affect each other positively or negatively in a direct or in an effector-mediated way.

The simplest possibility for the direct influence is by blocking a modification by another preexisting mark. For example H3K9 methylation can be avoided by an existing H3K9 acetylation or vice versa. Histone modifications on neighboring sites might also block the binding sites for certain enzymes, as shown for the histone methyltransferase of histone H3 lysine 9 Suv39h, which recruitment is suppressed by a neighboring phosphorylation mark on histone H3 serine 10 [69].

It is also likely that different histone PTMs enhance the binding of recruitment factors as suggested for BPTF, the PHD finger-linked bromodomain [22]. The PHD finger binding to H3K4me3 and the bromodomain recognizing hyperacetylated histone H4 are separated by a fixed distance, which defines the relative orientations of their binding pockets. Therefore it is possible that both domains are simultaneously used to engage both H3K4me3 and an acetylated lysine.

Existing histone modifications might also act via recruiting enzymes, which set other additional marks, as was demonstrated for H3K9me3 and Dim-2, which methylates DNA [70].

Another possibility is that enzymes are activated or suppressed by the interaction of a present histone mark. Enzymes such as Dot1 histone methyltransferase are stimulated by an ubiquitin-mark on H2A [71]. In contrast, acetylation of histones seems to be repressed by sumoylation of histones [72]. These examples make clear that cross-talk between histone modifications is a general regulation mechanism of chromatin function.

1.1.8 Histone H3 lysine 9 methylation

Establishment and removal of the H3K9 methylation mark

In mammals the histone methyltransferases G9a and G9a-like protein GLP monomethylate lysine 9 of histone H3 [73]. G9a is implicated in downregulation of euchromatic gene regions most probably by methylation of H3K9 [74]. However, G9a expressed in cell-culture is distributed to heterochromatic regions [75]. In *Drosophila* G9a has a suppression effect in position-effect variagation experiments and is required for gene silencing [76]. Therefore it is likely that G9a has an influence on H3K9 monomethylation of heterochromatin.

Suv39h1/h2 as well as ESET/SETDB1 histone methyltransferase mediate di- or trimethylation of histone H3 lysine 9 [23, 77]. These mechanisms include interaction of the histone methyltransferases with DNA-binding proteins as well as with small RNAs [78]. Suv39-like enzymes are mainly located at heterochromatin and are obviously responsible for heterochromatic-specific H3K9 marks in animals [79, 80]. Heterochromatic foci of human double null *Suv39h1*$^{-}$*/Suv39h2*$^{-}$ mice failed to show H3K9 trimethylation [81] and in *Drosophila* a gain-of-function mutation of Su(var)3-9 lead to ectopic heterochromatinization [82].

In contrast, SETDB1 was mainly found in euchromatic regions, where it participates in gene silencing [83]. Without SETDB1, the relative concentration of H3K9 methylation at heterochromatic regions remain unchanged [84]. Therefore it is likely that G9a as well as Suv39h1/h2 are the main HMTs establishing H3K9 methylation at heterochromatic regions.

Several demethylating enzyme ‘erasers’ of the H3K9 methylation have been described. For example, in a complex with the androgen receptor LSD1 demethylates H3K9me and activates transcription [44]. H3K9 can also be demethylated by the jumonji proteins JHDM2A, JMJD2A/JHDM3A, JMJD2B, JMJD2C/GASC1 and JMJD2D [23] but the mechanism and occurrences of these events are not well understood so far.

Localization of H3K9 methylation

H3K9me1 occurs at insulator or enhancer regions of genes. Additionally H3K9me1 can be detected at active gene regions predominantly in close proximity to the transcriptional start site and the 5' coding region [65]. H3K9me2/3 is a classic mark of constitutive heterochromatin associating with gene deserts, imprinted domains, repetitive elements, centromeric and pericentromeric regions as shown in Figure 1-4C. Nevertheless, H3K9me3 can also be found in active gene regions [85].

Thus, three localizations of H3K9me exist, which (a) define heterochromatic regions, (b) are involved in silencing of euchromatic loci by modification of the promotor regions or (c) might take part in repression of unintentional transcription inside of active transcriptional units [86].

The H3K9 metylation seem to be a very ancient mark of heterochromatin. Evolutionary, three of the five basal groups of eukaryotes (unikonts, plants, chromalveolates) show heterochromatin-associated H3K9 methylation [86]. In contrast, other heterochromatic methylation marks such as H4K20me3, which is used only in animals to establish heterochromatin, seem to be more or less lineage-specific [87].

Biology of H3K9 methylation

Besides DNA methylation, H3K9 methylation seems to be an ancient feature of heterochromatic regions of most eukaryotes. In *Drosophila* the knockout of the H3K9 histone methyltransferase Su(var)3-9 leads to defects in differentiation and to impaired heterochromatin stability [88, 89]. These data are consistent with observations of knockouts of the homologous *Suv39h1/h2* genes in mice. Suv39h1/h2 deficient mice have an impaired viability and severely reduced genome stability [90]. Therefore H3K9 methylation might play not only a role in heterochromatin formation and maintenance but might also function in cellular memory and epigenetic pathways [27, 57, 87, 91].

Epigenetic changes are changes in phenotype or gene expression caused by mechanisms other than modulation of the DNA sequence. Importantly, these changes are heritable. Stable propagation of DNA methylation was directly demonstrated [92]. But the mechanism of transmission of H3K9me from one cell generation to the next is still under debate. During replication the assembly of the nucleosomal core particle seems to include two steps. The $(H3\text{-}H4)_2$ tetramer is deposited on DNA followed by H2A-H2B dimer association. Recently it was suggested that instead of $(H3\text{-}H4)_2$ tetramer one newly synthesized H3-H4 dimer are paired with H3-H4 dimer from the mother strand, which would lead to an even segregation of parental nucleosomes [93]. Nevertheless, both pathways could provide the inheritance of

histone modification marks. Interestingly, the propagation of the silencing H3K9 methylation mark is dependent on the RNAi machinery and DNA recognition factors [94]. In addition it was shown, that H3K9 methylation could direct DNA methylation [87]. Thus, H3K9 methylation is implicated in the epigenetic pathways of the cell.

Regulation of H3K9me3 mark

Lysine 9 of histone H3 is embedded in an ARKS motif, which occurs also in other histone and non-histone proteins [95]. Interestingly PTMs of the neighboring arginine (methylation) as well as the neighboring serine (phosphorylation) has been described [96, 97]. These additional PTMs establish another layer of regulation as shown for phosphorylation of serine 10 and HP1. At the onset of mitosis Aurora B phosphorylates serine 10. This phosphorylation destroys the interaction of the HP1 chromodomain with H3K9me3 due to blocking of an essential hydrogen bond (compare also with 1.2.3/chromodomains) and displaces HP1 from heterochromatin [27]. This PTM correlates strongly with the initial condensation of chromatin during mitosis and has recruitment potential for chromosomal condensation factors [50].

H3K9me2/3 readout

In higher eukaryotes heterochromatin protein 1 isoforms HP1α, HP1β and HP1γ bind to the H3K9 methylation mark [55, 98] with their chromodomains and mediate the heterochromatinization of genetic regions in *Drosophila* [99]. The chromodomain of Chp1, a protein of *S. pomb*e, also recognizes H3K9me3 and is critical for efficient establishment of centromeric heterochromatin [100]. The ankyrin repeats of G9a were also shown to interact with H3K9me1/2 at least *in vitro* [73]. Recently also ICBP90, a ubiquitin-E3-ligase, interacting with the DNA-methyltransferase DNMT1 has been described as H3K9 methylation binding protein [57, 58].

Because of the many different functions of H3K9 methylation it is likely that also other heterochromatin effector proteins recognize H3K9me2/me3. Recently, a new protein family harboring a putative histone methylation-binding module, a chromodomain, was identified, the CDY family (chromodomain on the Y). Several findings such as its association with the CoREST complex or interaction with HDAC1 and HDAC2 indicate a heterochromatin association and function [62, 101, 102]. But until now, it is not known if and how CDY family proteins interact and function on heterochromatin.

1.2 CDY family of proteins

The CDY family (chromodomain on the Y) has three members: the Y chromosomal multicopy gene CDY and the two autosomal genes CDYL1 and CDYL2.

1.2.1 Identification of the CDY family

The CDY family of proteins was first identified in 1997 using a human testis library. CDNA clones of multiple copies of the *CDY* gene on the human Y chromosome were isolated and sequenced [103]. Interestingly, *CDY* has not only a Y chromosomal localization but has also testis specific mRNA expression. This observation of a functional coherence of testis expression and localization of the genes disproved the theory of a Y chromosomal wasteland. Instead it was predicted that there is a functional connection of Y deletions and male infertility. Later on this theory was verified by PCR-assays for the presence or absence important Y chromosomal landmarks. The examination of 48 male individuals by these assays validated that Y-chromosomal deletion of the region containing the *CDY* genes lead to spermatogenic failure [104].

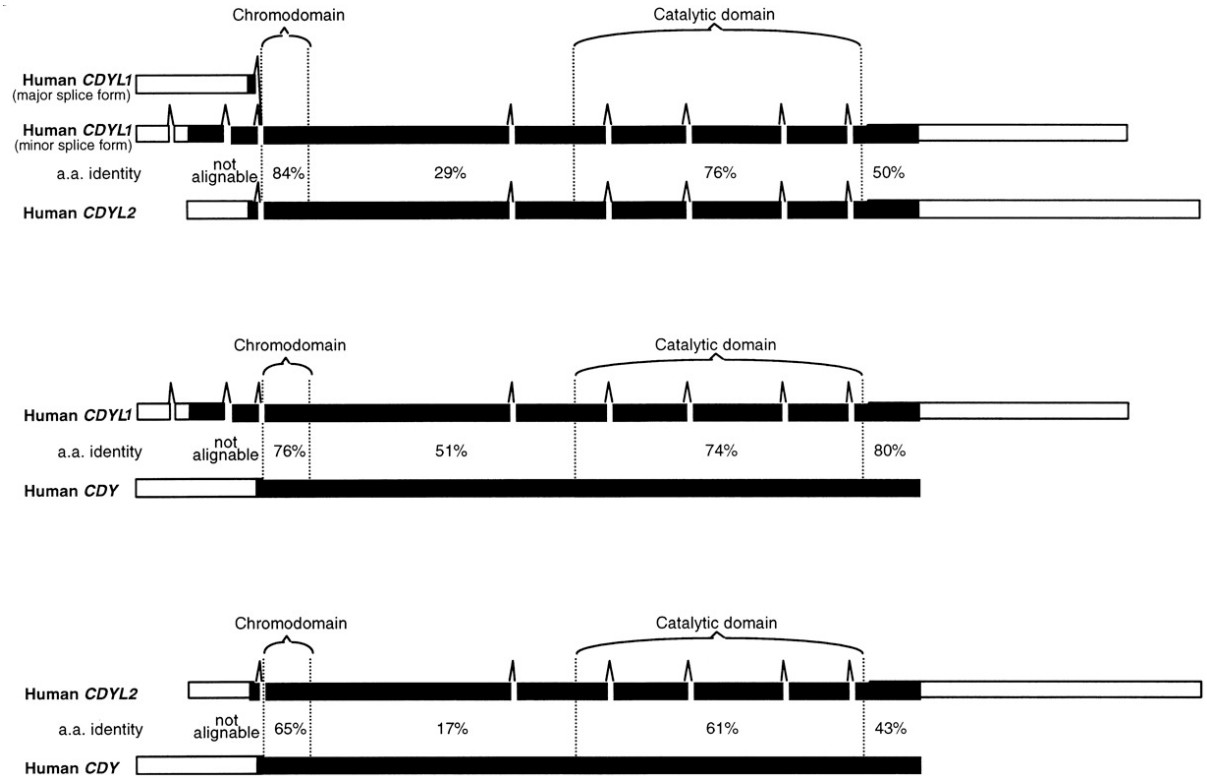

Figure 1-5 Alignment of CDY family members.

Exon/intron structure of *CDY*, *CDYL1* and *CDYL2* genes is shown. *CDYL1* and *CDYL2* are multiple exon genes in contrast to *CDY*, which harbors only one exon. Amino acid identity (a.a. identity) of amino acid sequence of the different exons is given in percent. Figure is adapted from [105].

In 1999 a second gene located on the autosomal chromosome 6 could be assigned to the CDY family: *CDYL1* (chromodomain on the Y like) [106]. The study provides evidence that *CDYL1* has two transcripts. One of the transcripts is ubiquitously transcribed and the other has a testis-specific expression. Comparison of *CDYL1* and *CDY* revealed that *CDYL1* has an equal exonic sequence compared to *CDY* but has additional introns [106]. Thus *CDY* is a single-exon gene whereas *CDYL1* is a multi-exon gene.

The third member of the CDY family, *CDYL2*, was identified in 2003 and is located on the human chromosome 16. *CDYL2* is expressed ubiquitously at low levels but has prevalence in the spleen, prostate, testis and leukocytes [105]. *CDYL1* and *CDYL2* have a similar but not identical intron/exon structure and are quite divergent from each other in their amino acid sequence [105]. Overview alignments (amino acid identities) between CDY, CDYL1 and CDYL2 are schematically presented in Figure 1-5.

1.2.2 Evolutionary aspects of the CDY family

A common ancestor of the CDY gene family arose in the chordate and deuterostomia *ciona savignyi*. CDY family proteins contain two recognizable domains: a chromodomain and an enoyl-CoA-hydratase (ECH) domain. Chromodomains are present in almost all eukaryotes and enoyl-CoA-hydratases are even more ancient, as they are found also in prokaryotic organisms.

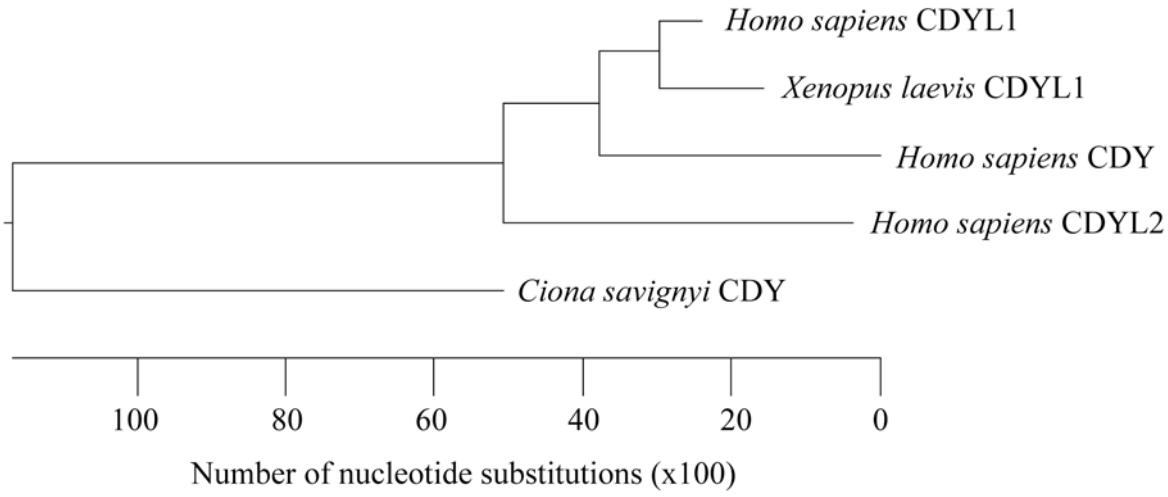

Figure 1-6 Evolution of CDY family

CDYL1 and *CDYL2* evolved from a common ancestor by an ancient duplication. Later a processed CDYL1 mRNA was retro-transposed to the Y chromosome and created the single-exon *CDY* gene. *CDY* underwent the most nucleotide substitutions and was therefore predicted to be under positive selection.

Therefore, the fist progenitor of CDY family members must have emerged *de novo* by domain accretion on the chordate lineage. Mechanistically it is likely that the progenitor evolved from exon shuffling [107]. Thereby the chromodomain exons could be juxtaposed in front of the exons encoding the ECH domain via genomic rearrangement such as translocation or transposition [105]. From the phylogenetic tree represented in Figure 1-6 it can be concluded that *CDYL1* and *2* derived from one common ancestor gene by an ancient duplication. *CDY* derived much more recently from the *CDYL1* gene. This event could have been a retro-transposition of a processed CDYL1 mRNA, followed by several rounds of amplification resulting in a multi-copy *CDY* gene on the Y chromosome [106].

Interestingly, CDY can only be found in simian primates [105] and has a higher amino acid substitution rate than CDYL1 and CDYL2 [105]. These observations lead to the hypothesis that CDY evolved under positive selection pressure. Accordingly it was predicted that the *CDY* gene underwent a neofunctionalization, which resulted in a testis-specific expression with a function in spermatogenesis [105].

1.2.3 Domain structure of CDY family proteins

All CDY family members consist of a chromodomain, a connecting hinge region, an enoyl-CoA-hydratase domain and a short C-terminal part (compare with Figure 1-7). The chromodomain consists of 55 amino acids and the enoyl-CoA-hydratase domain has about 173 amino acids.

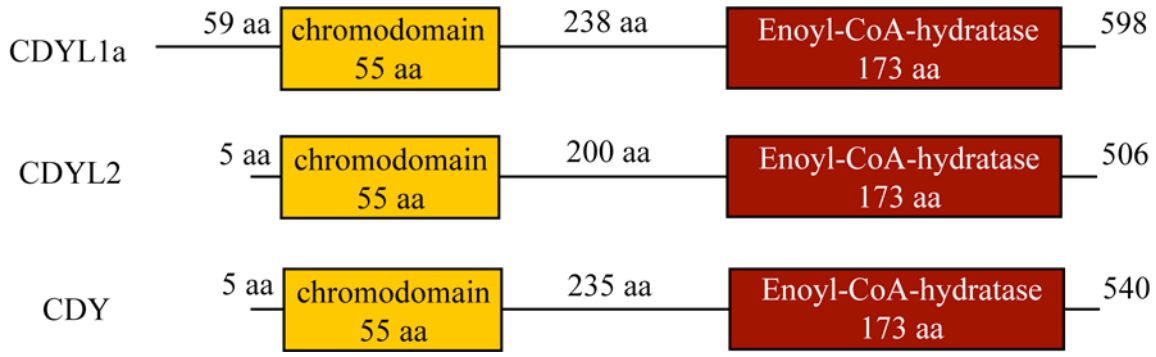

Figure 1-7 Domain structure of CDY family members

Chromodomain

Chromodomains are evolutionarily highly conserved regions of about 40 amino acids, which are present in various proteins involved in chromatin organization and gene regulation [108].

The domain folds into a globular conformation of about 30 Å in diameter. It consists of an antiparallel three-stranded β-sheet packed against an α-helix in the carboxy-terminal segment of the domain (Figure 1-8). Overall the β-sheets have a negative net charge and form a hydrophobic groove on one side, which is composed of conserved residues.

Connected to these observations the first defined molecular function could be assigned to the HP1 chromodomain. Primarily it was suggested that chromodomains are RNA-binding modules [109]. But then several groups showed that the main function of the HP1 chromodomain is the binding of the histone H3 tail methylated on lysine 9 [98, 110]. Accordingly, it was demonstrated that canonical chromodomains in general recognize methylated H3.

The binding of the HP1 chromodomain to methylated lysine 9 of histone H3 is maintained by its hydrophobic pocket by providing an appropriate environment for docking onto the histone H3 tail. The H3 tail adopts a β-sheet conformation and builds together with two antiparallel β-sheets of the chromodomain a three β-sheet bundle.

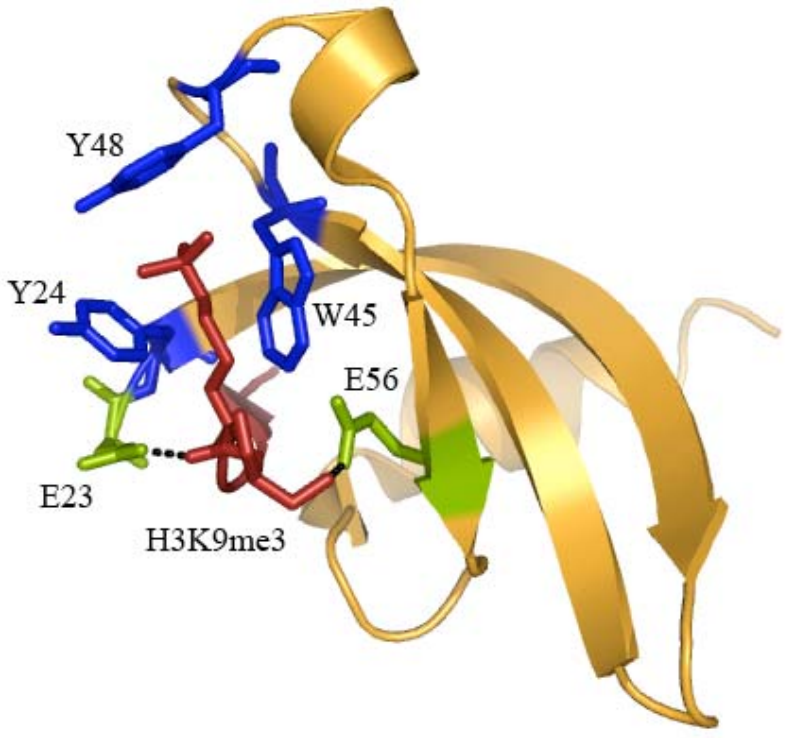

Figure 1-8 HP1 chromodomain binding to trimethylated lysine 3 of histone H3.

HP1 chromodomain (orange) binds to the H3 peptide carrying a trimethylation on lysine 9 (red). The three blue residues Y24, W45 and Y48 build a tri-aromatic cage binding the methylation. The glutamate 23 (green) makes a hydrogen bond to the peptide backbone, whereas glutamate 56 (green) contacts serine 10 of the histone H3 tails. Figure was adapted from PDB: 1kne and [110].

Additionally, three aromatic residues (Figure 1-8) cage the methylammonium group of histone H3 methylated on lysine 9. Glutamate 23 of the chromodomain makes a hydrogen bond to the H3 peptide backbone and glutamate 56 interacts with the serine 10 adjacent to the methylated lysine 9 (Figure 1-8).

Methylations of lysines occur also on other sites within histones (compare with Table 1-2) but interestingly chromodomains studied to this point can only recognize lysines embedded in ARKS motifs (e.g. H3K9me, H3K27me) or ARTKQ motifs (e.g. H3K4me). The chromodomain of the Polycomb protein binds specifically to methylated lysine 27 of histone H3 [111]. The overall folds of the HP1 and the Polycomb chromodomain are similar, but their peptide-binding grooves show distinct features that provide the discrimination between the two marks [111].

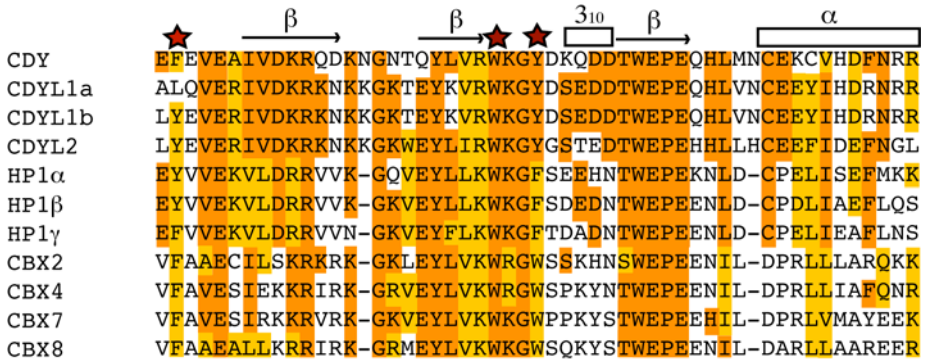

Figure 1-9 Alignment of chromodomains.

Alignment of chromodomains from the human CDY family CDY, CDYL1a, CDYL1b and CDYL2 with human HP1α, HP1β and HP1γ as well as with CBX2, CBX4, CBX7 and CBX8 the Polycomb homologs of *Homo sapiens*. Red stars indicate the three residues forming the tri-aromatic cage for histone methylation binding.

The Polycomb chromodomain interacts with more amino acids of the histone tail surrounding the methylation mark and the HP1 chromodomain recognizes the residues in close vicinity of the PTM in a more precise manner. An alignment of the CDY family of chromodomains with the chromodomains of the three human isoforms of HP1 (α, β, γ) and with the chromodomain of the human Polycomb proteins CBX2, CBX4, CBX7 and CBX8 is presented in Figure 1-9. CDY family chromodomains show a high homology to the HP1 and Polycomb chromodomains. The three aromatic residues described as important for the function are present in the CDY family chromodomains, exept for in the CDYL1a splicing variant (see also 1.2.4). A superposition of the CDYL2 chromodomain with the HP1 chromodomain bound to the H3 peptide carrying a methylation on lysine 9 shows that CDYL2 and by homology also CDY and CDYL1 may have a tertiary structure similar to HP1 (Figure 1-10).

Therefore it is possible that the chromodomain of CDY family proteins may interact with histones methylated on certain lysine residues.

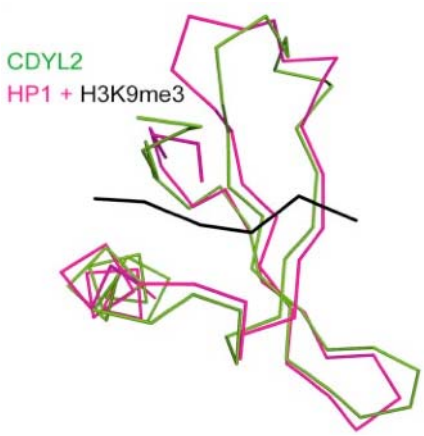

Figure 1-10 Alignment of HP1 and the human CDYL2 chromodomain.

HP1 (PDB: 1kne) chromodomain (pink) is bound to a histone H3 peptide carrying a trimethylation on lysine 9 (black). The overlayed CDYL2 (PDB: 2dnt) chromodomain is shown in green. Figure was adapted from [95].

Enoyl-CoA-hydratase domain

Enoyl-CoA-hydratases (also termed as crotonases) can act as isomerases, dehydrogenases and hydratases [112].

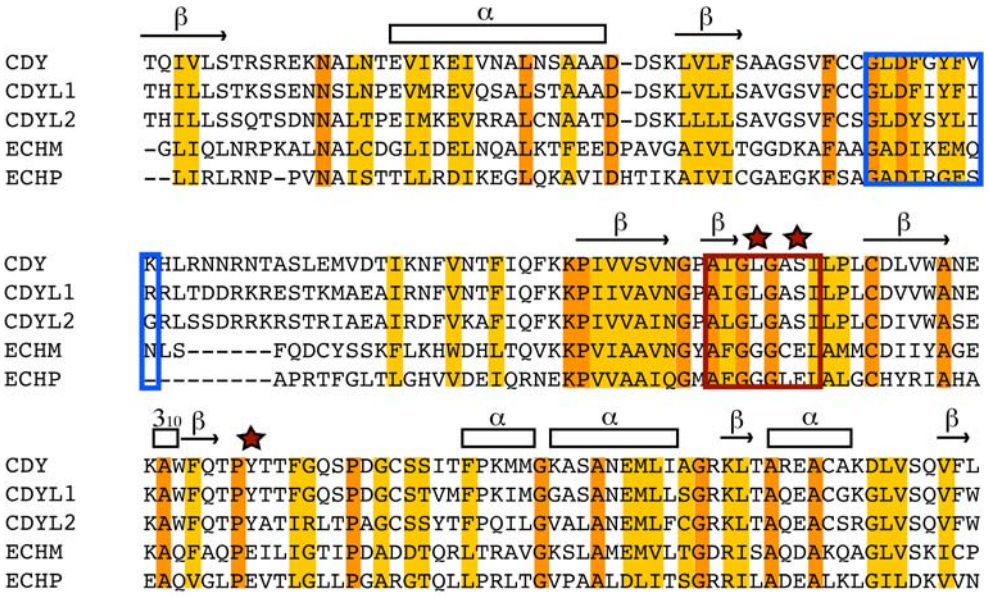

Figure 1-11 Alignment of the Enoyl-CoA-hydratase domains of the CDY family.

Dark orange indicates identical residues, whereas light orange labels homologous residues. Red stars label the three important enzymatic residues in enoyl-CoA-hydratases. ECHP: human peroxisomal ECH, ECHM: human mitochondrial ECH. Blue box: Adenine binding site, Red box: active loop.

The substrates of crotonases are mainly double bonds of unsaturated fatty acids during β-oxidation in mitochondria (short fatty acids) as well as in peroxisomes (very long fatty acids). An alignment of the CDY family enoyl-CoA-hydratase with the mitochondrial and peroxisomal enzymatic domains reveal only a moderate overall homology (Figure 1-11). The adenine binding pocket interacting with Acetyl-CoA in enoyl-CoA-hydratases shows a slightly higher degree of homology. In agreement, it was demonstrated that CDYL1 ECH domain interacts with coenzyme A [101].

Despite a good consistence of amino acids within the active loop, the three important residues (one glycine and two glutamates see Figure 1-11) maintaining the enzymatic reaction of enoyl-CoA-hydratases are exchanged in the ECH domain of the CDY family.

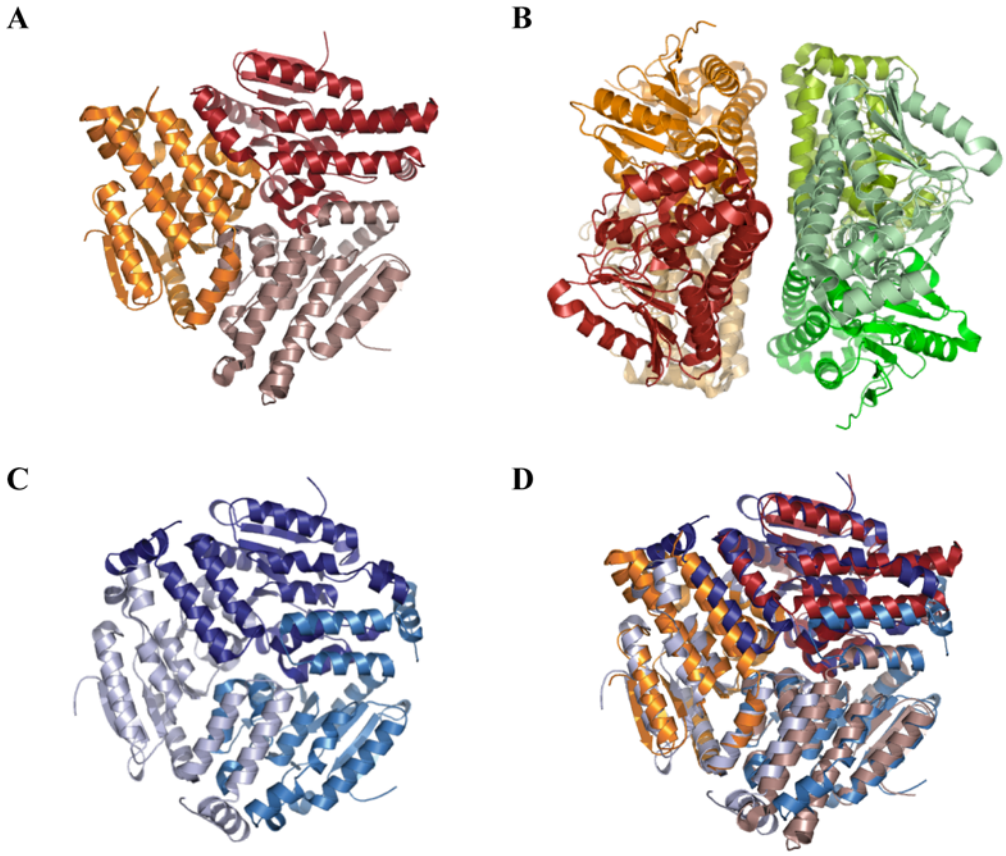

Figure 1-12 CDY family enoyl-CoA hydratase domains build trimeric structures

A, trimeric fold of the CDYL1 ECH domain (PDB: 1gtr). Each monomer has a different color. **B,** hexameric fold of CDY ECH domain (PDB: 2fw2). **C,** rat peroxisomal enoyl-CoA hydratase (PDB: 1dub). **D,** superposition of A and C.

Nevertheless, the ECH domains of the CDY family fold into the typical arrangement of enoyl-CoA-hydratase enzymes (Figure 1-12). Figure 1-12A shows the compact trimeric

structure formed by three CDY family proteins. Two of the homotrimers are able to form loose dimers (Figure 1-12B).

Figure 1-12C and D represent a superposition of the CDY-family ECH domain with the rat enoyl-CoA-hydratase enzyme and reveal that at least the crotonase-like fold of the ECH domain is conserved.

1.2.4 Splicing variants of CDYL1

According to NCBI, the mRNA of the transcribed CDYL1 gene can be formed into at least three distinct splicing variants (a, b and c). CDYL1a and b differ in their N-termini, so that the first aromatic cage residue of the CDYL1a variant is missing. Therefore it is likely that the chromodomain of the CDYL1a splicing variant is not functional (compare also with the chromodomain alignment of Figure 1-9). The CDYL1c variant includes the ECH domain and a few additional N- and C-terminal residues, but has no chromodomain at all.

Due to the differences in domain structure, the splicing variants might harbor distinct functions.

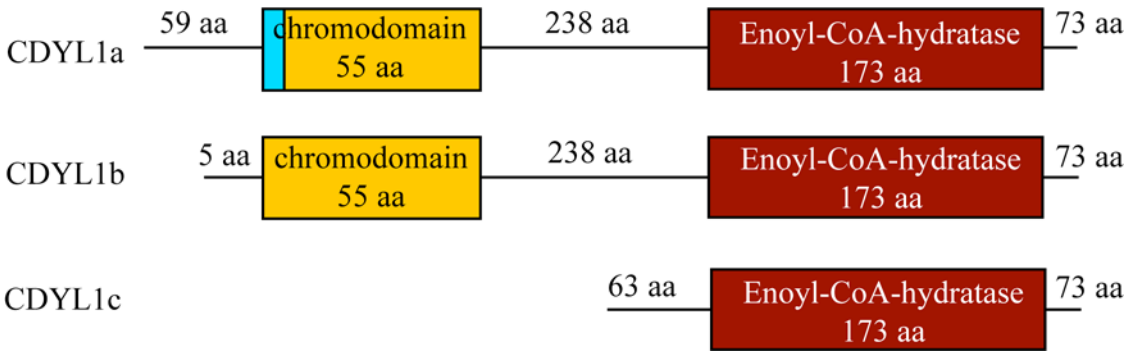

Figure 1-13 Splicing variants of CDYL1

CDYL1a, b and c are splicing variants of the transcribed CDYL1 gene. CDYL1a has thereby a different N-terminal region. CDYL1c consists mainly of the ECH domain.

1.2.5 Interaction partners of CDY family proteins

Over the last few years several proteins interacting with CDYL1 have been described. These include the histone deacetylases HDAC1 and HDAC2, the histone methyltransferase G9a, the repressor REST (repressor element 1-silencing transcription factor) and WIZ (widely interspaced zinc finger motifs) [101, 102, 113].

The histone deacetylases HDAC1 and HDAC2 interact with the C-terminal part of CDYL1 containing the ECH domain [101]. Binding of HDAC1 to CDYL1 reduces CDYL1 affinity to coenzyme a [101].

Secondly, it was demonstrated that CDYL1 has the ability to bridge G9a and REST to target the histone methyltransferase G9a to conserved REST responsive sites to repress transcription [102].

In addition, CDYL can interact with Wiz, which binds to the C-terminal binding protein CtBP, a member of the CoREST complex, with the help of its PLDLS motif [113]. Interestingly, it was also shown that WIZ is an interaction partner of G9a [113].

Due to its physical interactions with the described proteins, CDYL1 can be part of at least two high molecular weight complexes. One of them is the CoREST complex containing the co-repressor CoREST, the histone demethylase LSD1, G9a, HDAC1 and 2 and the C-terminal binding protein CtBP and about 15 more proteins [62]. Secondly CDYL1 can be part of the 'CDYL co-repressor complex'. Besides HDAC1 and 2, G9a and about ten other proteins this complex contains also WIZ and the repressor protein REST [102]. Therefore CDYL1 obviously conducts part of its functions via different multiprotein complexes. But interestingly not all CDYL1 of the cell is associated with the described proteins. Cellular fractionation experiments conducted via ultracentrifugation revealed that a major part of CDYL1 does not co-fractionate with HDAC1 and 2, G9a and REST [102] suggesting further interaction partners and functions of the CDYL1 protein.

1.2.6 Biological impact of CDY family proteins

The first hints of a probable CDY family function came from systematic Y chromosome analysis. Deletion of a region containing among others the CDY gene locus was correlated to spermatogenic failure in 48 patients [104]. These findings were supported by data showing that CDY and CDYL1 expression correlates with histone H4 hyperacetylation during spermatogenesis and that both proteins locate to the nucleus of maturing spermatids where the H4 hyperacetylation takes place. Additionally, it was demonstrated that CDY as well as CDYL1 exhibit histone acetyltransferase activity *in vitro* [114].

In contrast, Caron et al demonstrated that CDYL1 could act as a transcriptional co-repressor when targeted to a reporter gene. Interestingly, the chromodomain is not involved in this repressive function. These observations were connected with the CDYL1 ability to interact with HDAC1 and 2, which could mediate the repression of the reporter gene [101]. CDYL1 is also involved in repression of the E-cadherin gene as part of the CoREST complex. Knock

down of CtBP, one of the key players of the CoREST complex, led to a relief of the CDYL1-mediated repression [101].

These results disagreed partially with former observations presenting CDY family proteins as histone acetyltransferases, because acetylation of histones is known to be linked to transcriptional activation [115]. But Caron et al also demonstrate an interaction of coenzyme A and CDYL1. Acetyl-coenzyme A is on one hand a co-substrate of acetyltransferase reactions because it delivers the transferred acetyl-group. But on the other hand CoA is also a substrate of crotonases, which act on unsaturated fatty acids.

Due to these conflicting results the function of CDY family proteins is not well understood so far. But these data and the facts that CDY family genes are very conserved in chordates and are specifically expressed in tissues (compare with chapter 1.2.2), point to a function of CDY family proteins in more general processes such as development.

1.3 Open questions

In CDY family proteins a chromodomain is connected to an enoyl-CoA-hydratase domain with the help of more flexible hinge region. It is known that chromodomains are located in the nucleus and that they are implicated in chromatin organization and regulation [108]. As described, a very different function can be assigned to enoyl-CoA-hydratase enzymes. They are located in peroxisomes or mitochondria and are involved in water addition of β-oxidation process [112].

Expression profile, chromosome location, splicing events, interaction partners and the conservation of both the chromodomain fold and the ECH domain trimeric structure, point to a very specific function of CDY family members (compare with chapter 1.2) probably on heterochromatic regions. CDY family proteins can be part of repressive heterochromatic complexes (e.g. CoREST) and are, if targeted to a promotor, able to repress the transcription of a reporter gene [101, 102, 113]. The deletion of the Y chromosomal part including the CDY locus leads to spermatogenic failures in *Homo sapiens*.

It is likely that the chromodomain as well as the ECH domain are conducting essential functions in these processes. However, detailed information about the biological role of the chromodomain and the ECH domain of the CDY family proteins are rather limited. So far it is not known if CDY family proteins are able to bind to heterochromatin or heterochromatin specific histone modifications and how this is connected to the ECH domain.

Therefore it is necessary to carefully examine the interaction profile of CDY family chromodomains *in vitro*, compare them to binding properties of known chromodomains and

connect them to *in vivo* localization and function of CDY family proteins. Further it is important to analyze differences between the family members as well as between splicing variants to shed light on the main role of CDY family proteins in different cell types and organisms. In addition, it is necessary to understand how CDY family proteins mediate their probable heterochromatin functions and how they are regulated during these activities.

2 List of publications

The thesis is based on the following original papers published in the Journal of Biological Chemistry:

Chapter 3 Fischle W*, Franz H*, Jacobs SA, Allis CD, Khorasanizadeh S (2008) Specificity of the chromodomain Y chromosome family of chromodomains for lysine-methylated ARK(S/T) motifs. J Biol Chem. Jul 11;283(28):19626-35 Epub May 1
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* equally contributed

Chapter 4 Franz H, Mosch K, Soeroes S, Urlaub H, Fischle W (2009) Multimerization and H3K9me3 binding is required for CDYL1b heterochromatin association. J Biol Chem. 2009 Dec 11;284(50):35049-59. Epub 2009 Oct 5
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Chapter 5 presents experiments not mentioned in the described original publications.

3 Specificity of the chromodomain Y chromosome family of chromodomains for lysine methylated ARK(S/T) motifs

Citation

Fischle W*, Franz H*, Jacobs SA, Allis CD, Khorasanizadeh S (2008) Specificity of the chromodomain Y chromosome family of chromodomains for lysine-methylated ARK(S/T) motifs. J Biol Chem. Jul 11;283(28):19626-35 Epub May 1
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* equally contributed

Original contribution

Preparation and accomplishment of biological experiments presented in Figure 2, 4 and 5 and the Supplement Figure S1, i.e. cloning and purification of CDY and CDYL2, fluorescence polarization experiments, transfection and immunostaining of NIH3T3 cells, classification and quantification of observed phenotypes; writing of the according method section in the manuscript.

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Specificity of the Chromodomain Y Chromosome Family of Chromodomains for Lysine-methylated ARK(S/T) Motifs*[S]

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Wolfgang Fischle[‡§¶1,2], **Henriette Franz**[¶1], **Steven A. Jacobs**[‡3], **C. David Allis**[‡§], **and Sepideh Khorasanizadeh**[‡4]

From the [‡]*Department of Biochemistry and Molecular Genetics, University of Virginia Health System, Charlottesville, Virginia 22908-0733,* [§]*Laboratory of Chromatin Biology, The Rockefeller University, New York, New York 10021, and* [¶]*Laboratory of Chromatin Biochemistry, Max Planck Institute for Biophysical Chemistry, 37077 Göttingen, Germany*

Previous studies have shown two homologous chromodomain modules in the HP1 and Polycomb proteins exhibit discriminatory binding to related methyllysine residues (embedded in ARKS motifs) of the histone H3 tail. Methylated ARK(S/T) motifs have recently been identified in other chromatin factors (*e.g.* linker histone H1.4 and lysine methyltransferase G9a). These are thought to function as peripheral docking sites for the HP1 chromodomain. In vertebrates, HP1-like chromodomains are also present in the chromodomain Y chromosome (CDY) family of proteins adjacent to a putative catalytic motif. The human genome encodes three CDY family proteins, CDY, CDYL, and CDYL2. These have putative functions ranging from establishment of histone H4 acetylation during spermiogenesis to regulation of transcription co-repressor complexes. To delineate the biochemical functions of the CDY family chromodomains, we analyzed their specificity of methyllysine recognition. We detected substantial differences among these factors. The CDY chromodomain exhibits discriminatory binding to lysine-methylated ARK(S/T) motifs, whereas the CDYL2 chromodomain binds with comparable strength to multiple ARK(S/T) motifs. Interestingly, subtle amino acid changes in the CDYL chromodomain prohibit such binding interactions *in vitro* and *in vivo*. However, point mutations can rescue binding. In support of the *in vitro* binding properties of the chromodomains, the full-length CDY family proteins exhibit substantial variability in chromatin localization. Our studies underscore the significance of subtle sequence differences in a conserved signaling module for diverse epigenetic regulatory pathways.

The human Y chromosome has been thoroughly sequenced and compared with partially sequenced Y chromosomes of chimpanzee and mouse (1–3). The Y chromosomes are believed to be enriched in genes essential for spermatogenesis and testis development. Interstitial Y chromosome deletions are associated with spermatogenic failure and male infertility (1, 4, 5). One gene that is present in multiple copies on the human Y chromosome is CDY,[5] which exhibits testis-specific expression (1). Interestingly, the mouse Y chromosome does not encode CDY, suggesting a developmentally advanced usage of CDY in primates (3).

The human CDY gene seems to be derived from the autosomal homologs CDYL or CDYL2 (Fig. 1, *A* and *B*) (6). CDYL is ubiquitously expressed, whereas CDYL2 exhibits selective expression in tissues of testis, prostate, spleen, and leukocytes (6). The mouse genome also encodes related CDYL and CDYL2 genes (Fig. 1*B*). The presence of CDY-like genes appears to be a hallmark of echinoderm and vertebrate genomes. In sea urchin and chicken genomes we found only one CDY-like gene that corresponds to mammalian CDYL2 (Fig. 1*B*).

CDY family proteins have two conserved domains implicated in histone modification and recognition; that is, a chromodomain followed by an enoyl-coenzyme A hydratase/isomerase (*ECH*) putative catalytic domain (Fig. 1*A*). Previously, it was shown that the chromodomain of human CDY interacts with methylated lysine 9 of the histone H3 tail (H3K9me) (7). The ECH domain has been implicated in conflicting chromatin modification processes. One function of this domain is acetylation of germ line histone H4 (8). Another function is direct recruitment of histone deacetylases to sites within somatic cells (9).

Epigenetic control of gene expression hinges on effecter recognition modules that help establish appropriate methyllysine-dependent interactions with chromatin (for review, see Ref. 10). The HP1 and Polycomb chromodomains (Fig. 1*B*), which are similar to the chromodomains of CDY family proteins, distinguish two methylated lysine residues within ARKS motifs in the H3 tail (H3K9me and H3K27me) (11–16). Sequences immediately preceding the ARK(S/T) motif impact on the specificity of chromodomain interactions. HP1 chromodomains are subject to a binary methyl-phos switch as they are prohibited from interaction with H3K9me3 upon phosphorylation of the adjacent serine 10 in the H3 tail (H3S10ph) (17–20).

Additional complexity in epigenetic control arises from usage of histone variants. For example, substitutions of histone H3 with variants results in indexing of chromatin for transcriptional activation or repression (22, 23), and an H3 barcode

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[1] These authors contributed equally to this work.
[2] A Robert Black fellow of the Damon Runyon Cancer Research Foundation. To whom correspondence may be addressed. E-mail: wfischl@gwdg.de.
[3] Present address: Centocor Research & Development, Radnor, PA, 19087.
[4] To whom correspondence may be addressed. E-mail: khorasan@virginia.edu.

[5] The abbreviations used are: CDY, chromodomain Y chromosome; ECH, enoyl-coenzyme A hydratase/isomerase; PBS, phosphate-buffered saline; DAPI, 4′,6-diamidino-2-phenylindole; MBP, maltose-binding protein.

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hypothesis has been proposed (24). In human testis, three somatic histone H3 variants (H3.1, H3.2, and H3.3) are encoded together with a testis-specific subtype (H3t) (25). These variants have no amino acid changes N-terminal to the ARKS motifs, except in the H3t subtype. Although mass spectrometry has identified *in vivo* methylation of H3tK27 (26), an effector binding module has not yet been reported. Another lysine-methylated ARKS motif is found in the N-terminal tail of a linker histone H1 subtype in humans (H1.4K26) (27, 28).

An ARKT mimic of H3K9me has also been identified in the lysine methyltransferase G9a (G9aKme), suggesting a broader usage of ARK(S/T) motifs in human epigenetic signaling (29, 30). The G9aKme is targeted by the HP1 chromodomain in one transcriptional co-repressor complex (30). Interestingly, the human CDYL protein was also found associated with G9aKme in pulldown experiments from cellular extracts.

Because methylation of lysine residues within numerous ARK(S/T) motifs appears to orchestrate complex epigenetic pathways (for review, see Refs. 29, 32–34), determining the specificity of effectors may be a paradigm for understanding epigenetic signaling. Using a series of *in vitro* and cell-based assays, we studied the biochemical specificity of the chromodomains in the CDY family proteins. Our studies reveal a surprising variability in discriminatory interactions of CDY and CDYL2 chromodomains with methylated ARK(S/T) motifs.

EXPERIMENTAL PROCEDURES

Antibodies—Polyclonal antibodies specific for H3K9me3 were a gift from Dr. Thomas Jenuwein (IMP Vienna). The monoclonal antibody against mouse HP1α was obtained from Chemicon, and the monoclonal anti-FLAG antibody was purchased from Sigma.

Peptide Preparation—The sequences of the synthetic peptides corresponding to histone tails are listed below. A non-native tyrosine residue at the C terminus of each peptide was used for concentration determination by UV absorption measurements. Peptides were labeled with fluorescein as previously described (13):

H3K9me1, H3K9me2, H3K9me3: NH_2-ARTKQTARK-(me)STGGKAY-COOH; H3K27me, H3K27me2, H3K27me3: NH_2-APRKQLATQAARK(me)SAPSTY-COOH; H3tK27me3: NH_2-APRKQLATQVARK(me)SAPSTY-COOH; H1.4K26-me3: NH_2-TPVKKKARK(me)SAGAAKY-COOH; H3K9me3-S10ph: NH_2-ARTKQTARK(me)S(ph)TGGKAY-COOH; H3K27-me3S28ph: NH_2-APRKQLATQAARK(me)S(ph)APSTY-COOH; H3K9ac: NH_2-ARTKQTARK(ac)STGGKAY-COOH; H3K4me3, NH_2-ARTK(me3)QTARKSTGGKAY-COOH; H3K4me2K9me2: NH_2-ARTK(me2)QTARK(me2)STGGKAY-COOH; G9aKme3, G9aKme1: NH_2-QPKVHRARK(me)TMSKPGY-COOH; unmodified H3(1–15): NH_2-ARTKQTARKSTGGKAY-COOH; unmodified H3(15–32): NH_2-APRKQLATQVARKSAPSTY-COOH; unmodified H1.4(18–32): NH_2-TPVKKKARKSAGAAKY-COOH; unmodified G9a, NH_2-QPKVHRARKTMSKPGY-COOH.

Molecular Biology—For binding studies, the chromodomains of human CDY (GenBank^{TM} AF000981; residues 1–78), human CDYL (GenBank^{TM} AF081259; residues 1–133, 58–133, or 60–133), mouse CDYL (GenBank^{TM} AF081261; residues 1–128 or 51–128), and mouse CDYL2 (GenBank^{TM} AK015452; residues 1–75) were amplified by PCR and cloned into the BamHI/NdeI sites of the pET16b vector (Novagen). Full-length CDY and CDYL2 were cloned into a modified pMAL vector (New England Biolabs) for expression as MBP-His fusion proteins (details are available upon request). To generate C-terminal epitope-tagged constructs for the transient expression of full-length human CDY and human CDYL, we used PCR amplification with reverse primers containing the sequence encoding for the FLAG and hemagglutinin peptides (for details, see Ref. 47). cDNAs were cut with the appropriate restriction enzymes and cloned into the BamHI/XbaI sites of the pcDNA3.1+ vector (Invitrogen). Site-directed mutagenesis was performed according to the QuikChange protocol (Stratagene).

Fluorescence Polarization Binding Assays—Fusion proteins with the N-terminal His tag were expressed in *Escherichia coli* strain BL21(DE3) (Novagen) and purified by Ni^{2+}-affinity chromatography (Qiagen) and gel filtration chromatography (Superdex 75 resin, GE Healthcare). N-terminal MBP-His fusion proteins of full-length CDY and CDYL2 were expressed in BL21 RIL (Novagen) and purified consecutively on maltose and nickel-nitrilotriacetic acid resins. Protein concentrations were determined by absorbance spectroscopy using predicted extinction coefficients (for CDY chromodomain ϵ_{280} = 19,750 $M^{-1}cm^{-1}$; for MBP-His-CDY ϵ_{280} = 113,000 $M^{-1}cm^{-1}$; for CDYL chromodomain ϵ_{280} = 15,220 $M^{-1}cm^{-1}$; for CDYL2 chromodomain ϵ_{280} = 20,910 $M^{-1}cm^{-1}$; for MBP-His-CDYL2 ϵ_{280} = 126,000 $M^{-1}cm^{-1}$). Peptide concentrations were determined using absorbance spectroscopy (extinction coefficient for tyrosine, ϵ_{280} = 1280 $M^{-1}cm^{-1}$; extinction coefficient for fluoresceinated peptides ϵ_{492} = 68,000 $M^{-1}cm^{-1}$). Fluorescence polarization binding assays were performed under conditions of 20 mM imidazole, pH 8.0, 25 mM NaCl, 2 mM dithiothreitol and in the presence of 100 nM fluorescein-labeled peptide following a previously described protocol (13). Data were obtained using a Teacan Polarion 96-well plate reader or a Hidex Chameleon II plate reader (100 flashes). Sample plates were kept on ice until fluorescence reading at room temperature. Titration binding curves were analyzed using KaleidaGraph (Synergy Software) as previously described (13).

Isothermal Titration Calorimetry—Isothermal titration calorimetry (ITC) experiments were carried out as described previously using a VP-ITC instrument (MicroCal) (13). Chromodomains of CDY or HP1 were dialyzed against 50 mM sodium phosphate, pH 8.0, 25 mM NaCl, and 2 mM dithiothreitol. Peptides were purified by gel filtration in water (G10 resin, GE Healthcare), lyophilized, and dissolved in the chromodomain dialysis buffer. Exothermic heats of reaction (μcal/s) were measured at the indicated temperatures by automated sequencing of 30 injections of the H3 peptides (750 μM), each 10 μl, spaced at 2-min intervals, into 1.41 ml of chromodomain (CDY at 60 μM; HP1 at 70 μM). Binding curves were analyzed by non-linear least squares fitting of the data using the Origin (MicroCal) software package.

Cell Transfection—NIH3T3 (American Cell Culture Collection) and mouse embryonic fibroblast (a kind gift of Dr. Thomas Jenuwein, IMP Vienna) cells were grown at 37 °C in a humidified atmosphere, 5% CO_2 using Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal

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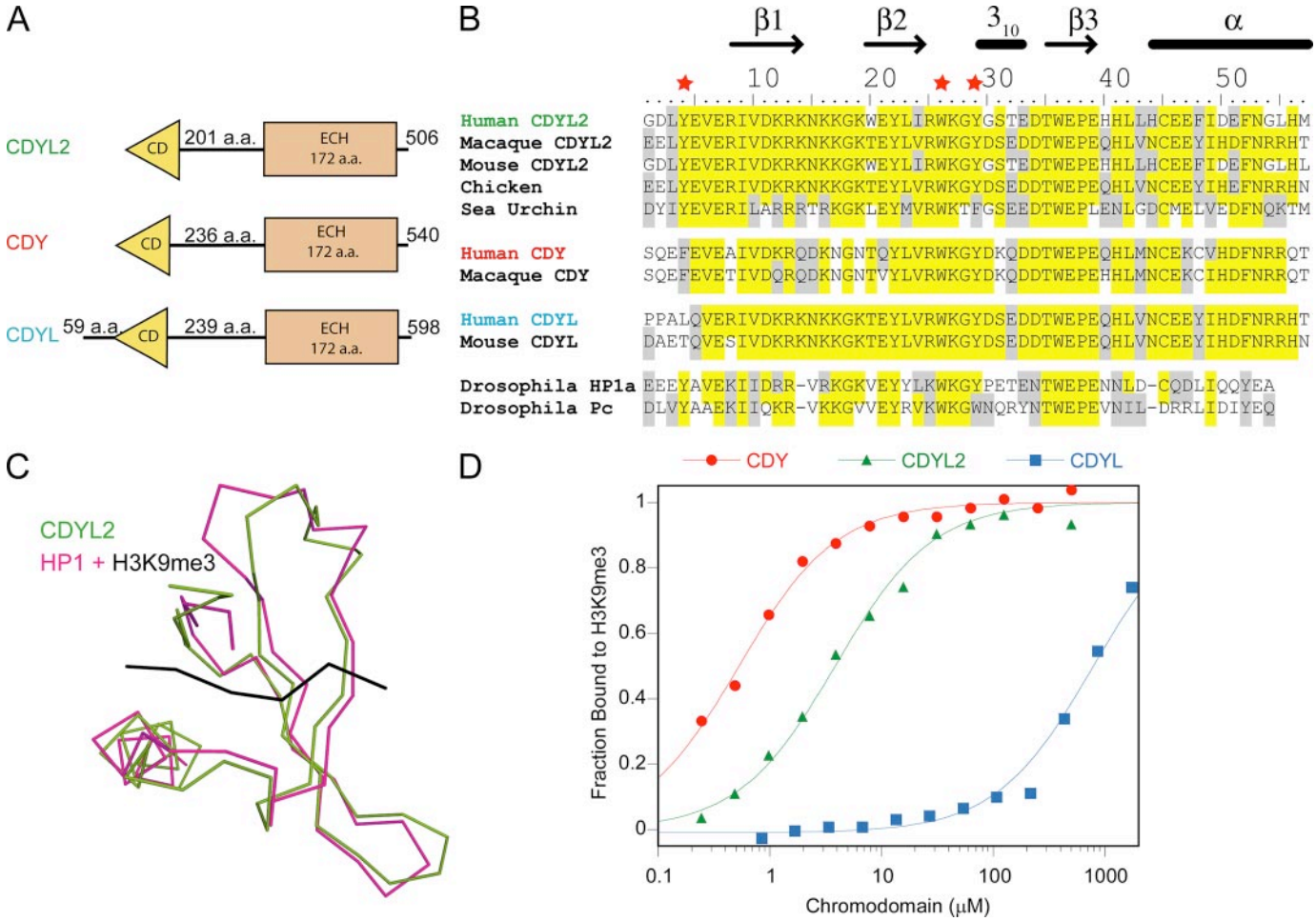

FIGURE 1. **The CDY protein family and their chromodomains.** *A*, schematic representation of the primary structures of CDYL, CDYL2, and CDY. *a.a.*, amino acids. *B*, sequence alignment of CDY family chromodomains, which are related to HP1 and Polycomb chromodomains. *Three stars above* the *sequence mark* the positions of the aromatic cage residues. Secondary structure elements above the sequence correspond to that deduced for CDYL2 (from PDB code 2dnt). The accession codes are: human CDY, Q9Y6F8; Macaque CDY, AJ31484;, human CDYL, Q9Y232; mouse CDYL, Q9WTK2; human CDYL2, AK096185; Macaque CDYL2, AY271718; mouse CDYL2, AK015452; chicken CDYL2, XP_418964; sea urchin CDYL2, XP 781347. *C*, backbone superposition of the three-dimensional structure of the human CDYL2 chromodomain (*green*, from PDB code 2dnt) on the structure of the *Drosophila* HP1 (from PDB code 1kne) chromodomain (*pink*) bound to an H3K9me3 peptide (*black*). *D*, interaction of recombinant human CDY family chromodomains with an H3K9me3 peptide as measured by fluorescence polarization. Averages from at least three independent measurements are plotted. See Table 1 for dissociation constants.

bovine serum, 2 mM glutamine. Cells were transfected using Lipofectamine 2000 as instructed by the manufacturer (Invitrogen).

Immunofluorescence—For immunofluorescence staining, cells were grown and transfected on glass coverslips. 48 h post-transfection, cells were fixed in solution I (1× PBS, 3.7% formaldehyde, 1% Triton X-100, 2% Nonidet P-40) for 10 min and then washed in 1× PBST (PBS, 1% Triton X-100) for 3 × 10 min. Slides were blocked for 1 h (1× PBST, 5% goat serum, 2% bovine serum albumin) and incubated with the indicated primary antibodies overnight in a humidified atmosphere. Dilutions for primary antibodies were anti-H3K9me3 (1:1500), anti-HP1α (1:2000), and anti-FLAG (1:500). Slides were washed in 1×PBST for 3 × 10 min and incubated with the appropriate secondary antibodies (Molecular Probes, Jackson ImmunoResearch) for 2 h in a humidified atmosphere. After washing in 1× PBST, DNA was stained with DAPI (1 μg/ml) for 10 s. Pictures were taken on a Leica SP5 confocal microscope or a Zeiss Axiopod II both equipped with 60× lenses.

RESULTS

Variability in Binding to H3 Methylated on Lys-9—The CDY class of chromodomains exhibits high homology to HP1 and Polycomb chromodomains (Fig. 1*B*). Three aromatic residues were shown to be necessary for assembling a methyllysine binding aromatic cage in HP1 and Polycomb chromodomains (11, 12, 16). Interestingly, the chromodomains of the CDYL proteins do not have the first aromatic cage residue, suggesting substantial difference in the function of this factor. A three-dimensional structure corresponding to the chromodomain of CDYL2 has been deposited in the protein data bank (PDB accession code 2dnt). We prepared a superposition of the CDYL2 structure with the peptide-bound structure of the HP1 chromodomain involving an H3K9me3 peptide (11) (Fig. 1*C*). This comparison established that CDYL2 and, by homology, CDY and CDYL chromodomains, despite a moderate sequence identity (~42%), have tertiary structures similar to HP1. Whereas HP1 and Polycomb chromodomains are 50-residue modules, the CDY family chromodomains are 55-residue mod-

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ules containing a longer C-terminal α-helix (Fig. 1*B*). Previously, binding of the CDY chromodomain to H3K9me2 and H3K9me3 peptides was identified through protein microarray analysis (7).

To determine the specificity and affinity for methylated histone lysine residues among the three CDY family members, recombinant chromodomains of CDY, CDYL, and CDYL2 were expressed in bacteria and purified. Because the CDYL chromodomain has an incomplete aromatic cage plus an N-terminal extension (Fig. 1, *A* and B), we prepared a recombinant construct that included the N terminus of the protein together with the chromodomain module. Recombinant proteins were used in fluorescence polarization assays to measure equilibrium binding to fluoresceinated synthetic peptides corresponding to the histone H3 tail. The binding data are shown in Fig. 1*D*, and the dissociation constants are listed in Table 1. In accordance with sequence prediction and the presence of an incomplete aromatic cage, we measured no binding of the CDYL chromodomain to H3K9me3 (K_D > 500 μM). Surprisingly, we measured a substantial difference in the dissociation constants associated with CDY and CDYL2. The interaction of the CDY chromodomain with the H3K9me3 peptide is 8-fold stronger than that of CDYL2. The results confirm subtle sequence differences among CDY family chromodomains, impacting on their interaction with methylated histone tails. The binding studies potentially implicate each CDY family member as part of distinct chromatin modification pathways.

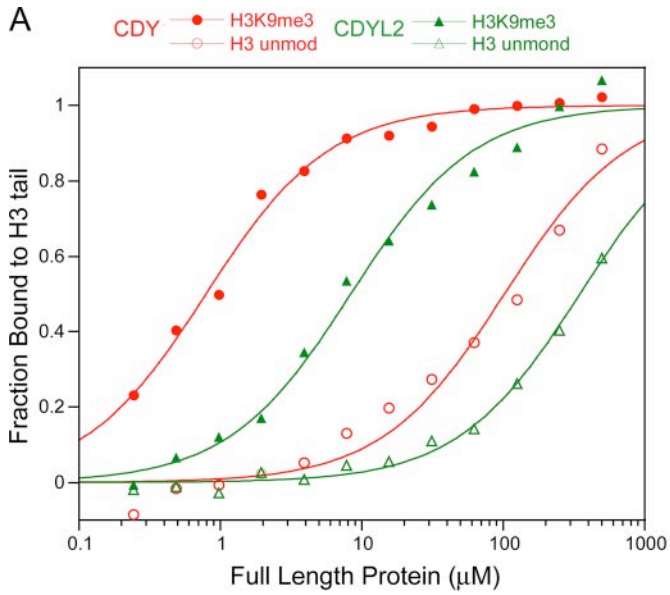
TABLE 1
Dissociation constants (μM) measured by fluorescence polarization for CDY family chromodomains interacting with synthetic peptides as described under "Experimental Procedures"

Peptide	CDY	CDYL	CDYL2
H3 Lys-9			
Unmodified	~300	>500	>500
H3K4me3	~300	>500	>500
H3K9ac	~300	>500	>500
H3K9me1	3.4 ± 0.5	>500	67 ± 10
H3K9me2	0.7 ± 0.1	>500	8.9 ± 1.1
H3K9me3	0.5 ± 0.1	452 ± 81	3.9 ± 0.5
H3K4me2K9me2	0.9 ± 0.2	~500	11.0 ± 2.1
H3K9me3S10ph	38 ± 4	>500	>500
H3 Lys-27			
Unmodified	>500	>500	>500
H3K27ac	>500	>500	>500
H3K27me1	~300		113 ± 15
H3K27me2	119 ± 37		18.4 ± 3.1
H3K27me3	76 ± 11	>500	12.4 ± 1.2
H3K27me3S28ph	>500		>500
Testis H3 Lys-27			
H3tK27me3	7.5 ± 0.8	~500	2.6 ± 0.4
H1.4 Lys-26			
Unmodified	>500	>500	>500
H1.4K26me3	10 ± 1	~500	2.2 ± 0.4
G9a Lys-185			
Unmodified	185 ± 17	>500	>500
G9a-K185me1	36 ± 9	>500	108 ± 27
G9a-K185me3	0.6 ± 0.2	~500	4.9 ± 1.2

To compare the binding affinity of the chromodomain constructs with that of the full-length proteins, we prepared recombinant full-length CDY and CDYL2 (Fig. 2) and measured binding to the H3K9me3 peptide by fluorescence polarization (Fig. 2). As observed for the chromodomain fragments, the full-length CDY protein displayed a significantly stronger binding (by 10-fold) compared with the full-length CDYL2 protein. These results reveal the chromodomain of CDY and CDYL2 are able to bind to methylated ARK(S/T) motifs autonomously.

Distinct in Vivo Distribution of CDY, CDYL, and CDYL2 Proteins—Given the CDYL chromodomain does not bind to the H3K9me3 peptide and the CDY chromodomain binds more avidly than the CDYL2 chromodomain, we investigated the *in vivo* localization of these proteins in relation to heterochromatin in mammalian systems. We made use of mouse fibroblast cells (NIH3T3 and MEF cells) that show a distinct pattern of DNA-dense, pericentromeric heterochromatin regions in the nucleus. These coincide with local enrichment of H3K9me3 modification. The distribution of CDY family proteins in these cells was compared using transiently expressed fusion constructs that encode their full-length polypeptides with a C-terminal FLAG tag. As Fig. 3 shows, all three fusion proteins displayed an exclusively nuclear distribution (see also supplemental Fig. S1).

In all cells inspected, we detected the transiently expressed CDY protein excluded from the nucleoli and enriched at the many large DAPI-dense regions reminiscent of the endogenous HP1α protein (17) (supplemental Fig. S2). Previously, Kim *et al.* (7) also showed CDY and



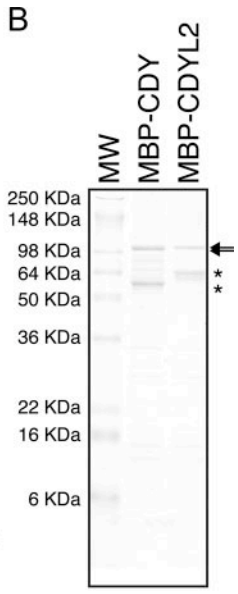


FIGURE 2. **Interaction with methylated lysine residues is an intrinsic property of CDY family proteins.** *A*, interaction of recombinant full-length human CDY and mouse CDYL2 proteins fused to MBP with an H3K9me3 or the corresponding unmodified peptide as measured by fluorescence polarization. Averages from at least three independent measurements are plotted. *B*, recombinant MBP fusion proteins used for the measurements in *panel A* were run on SDS-PAGE gels and stained with Coomassie Blue. *Arrows* indicate the MBP-CDY and MBP-CDYL2 recombinant proteins. Major degradation products co-purifying with the recombinant proteins are indicated. Molecular weight (*MW*) markers are shown on the *left*.

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HP1β exhibit overlapping distributions in chromatin with the H3K9me3 modification. Although the transiently expressed CDYL protein was also excluded from the nucleoli, CDYL showed a diffuse distribution pattern that never overlapped with regions of H3K9me3 (Figs. 3*B* and supplemental Fig. S2*B*). Interestingly, the CDYL2 protein was found throughout the nucleus in a punctate distribution pattern with pronounced cell-to-cell variation. Whereas some nuclei displayed granular staining of CDYL2, others showed larger areas of CDYL2 enrichment (Figs. 3*C* and supplemental Fig. S3*C*). Although CDYL2 enrichment at DAPI dense regions was less pronounced as compared with CDY, all cells analyzed exhibited some CDYL2 co-localized with regions of H3K9me3. Together, these observations underscore substantial differences in chromatin recognition and binding of the CDY family proteins.

CDY and CDYL2 Are Differentially Sensitive to the Degree of Lysine Methylation—The level of methylation (mono-, di-, or trimethylation) of lysine residues in histone tails is important for chromatin regulation (10, 35). We, therefore, measured the binding affinities of CDY and CDYL2 as a function of the methylation level on H3K9. Previous studies as well as data listed in Table 2 indicate that the HP1 chromodomain exhibits 2–3-fold reduced interaction with H3K9me2 peptide as compared with H3K9me3 peptide, whereas the binding to H3K9me1 peptide is substantially weaker. We found CDYL2 to resemble these features in HP1 (Table 1). However, the CDY chromodomain showed 20-fold stronger binding to H3K9me1 as compared with CDYL2. Interestingly, the binding of CDY to the H3K9me1 peptide is as efficient as the binding of CDYL2 or HP1 to the H3K9me3 peptide (Tables 1 and 2).

To determine the thermodynamic basis for the unusually strong binding of CDY to the methylated H3 tail, we performed isothermal titration calorimetry. We studied the chromodomains of CDY and *Drosophila* HP1 proteins under identical solution conditions (Table 2). These measurements showed CDY binds the H3K9me3 peptide 8-fold stronger than HP1. The free energy of binding to the H3 tail is more favorable for CDY by 1.2 kcal/mol. Isothermal titration calorimetry measurements of CDY binding to the H3K9me3 peptide at five different temperatures allowed us to estimate the heat capacity of binding ($\Delta C_p = -0.22$ kcal/mol·K). The small ΔC_p suggests negligible conformational changes occur in the CDY chromodomain upon binding to the H3 tail, a result in close agreement with that found for *Drosophila* HP1 (11, 36).

Using calorimetry, we found the enthalpy of binding to the H3K9me3 peptide is substantially more favorable for CDY than for HP1. This finding emphasizes the

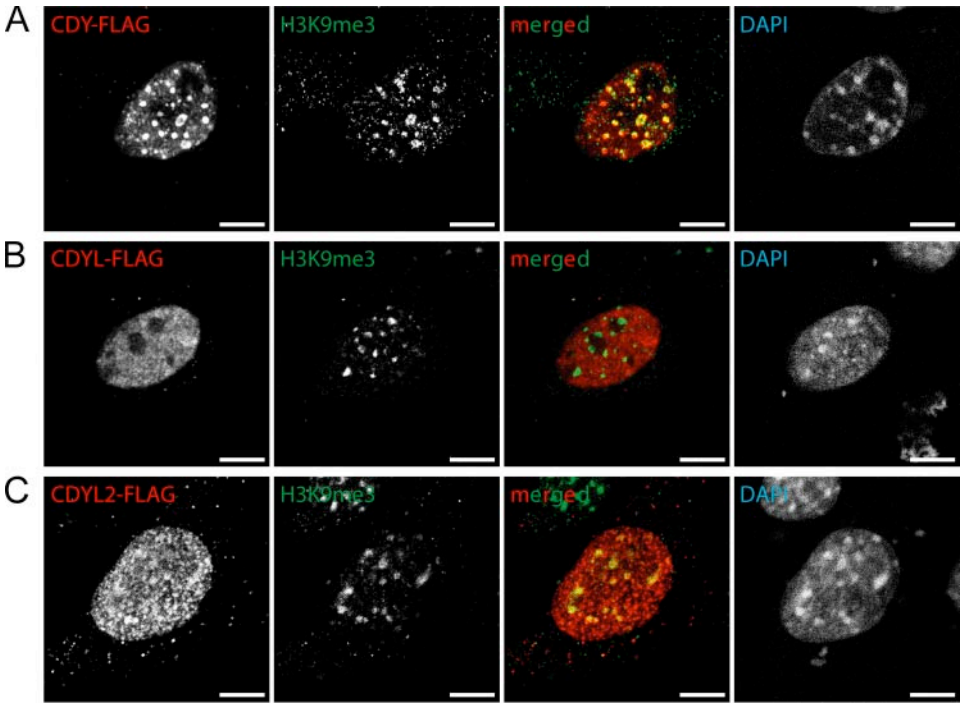

FIGURE 3. **Differential nuclear distribution of CDY family proteins.** FLAG-tagged human CDY (*A*), human CDYL (*B*), or mouse CDYL2 (*C*) were transiently expressed in NIH3T3 cells. Immunostaining with anti-FLAG-specific antibodies (*green*) and anti-H3K9me3 (*red*)-specific antibodies was analyzed by confocal microscopy. The merged image corresponds to the overlay of the two color channels. *Yellow areas* indicate colocalization sites for CDY family proteins with H3K9me3 modification. Cells of medium expression level representative for the nuclear distribution of the CDY family proteins are shown (see supplemental Fig. S1 for more images). DNA inside the cell nucleus was stained with DAPI and defines areas of high DNA density that are presumed to be heterochromatic. *Scale bar*, 10 μm.

TABLE 2

Thermodynamic parameters for binding to H3 peptides methylated on Lys-9

Isothermal titration calorimetry analysis of the human CDY chromodomain is compared with the *Drosophila* HP1α chromodomain under identical solution conditions.

Protein	Peptide	T	K_D	ΔH	N	ΔG	$T\Delta S$
		°C	μM	kcal/mol		kcal/mol	kcal/mol
CDY	H3K9me3	5	0.11 ± 0.03	−11.83 ± 0.04	0.97	−8.61	−3.22
CDY	H3K9me3	10	0.14 ± 0.05	−12.62 ± 0.03	0.96	−8.54	−4.08
CDY	H3K9me3	15	0.30 ± 0.07	−13.69 ± 0.03	1.00	−8.59	−5.10
CDY	H3K9me3	20	0.35 ± 0.11	−14.99 ± 0.04	0.99	−8.57	−6.42
CDY	H3K9me3	25	0.73 ± 0.17	−16.19 ± 0.04	1.02	−8.53	−7.66
CDY	H3K9me2	15	0.86 ± 0.16	−17.21 ± 0.04	1.03	−8.23	−8.98
CDY	H3K9me1	15	7.86 ± 1.1	−17.70 ± 0.04	1.18	−6.38	−11.32
HP1	H3K9me3	16	2.3 ± 0.1	−9.71 ± 0.04	1.02	−7.36	−2.46
HP1	H3K9me2	16	6.9 ± 0.2	−9.47 ± 0.04	1.02	−6.78	−2.69
HP1	H3K9me1	16	31.3 ± 0.5	−7.75 ± 0.03	1.04	−5.92	−1.81

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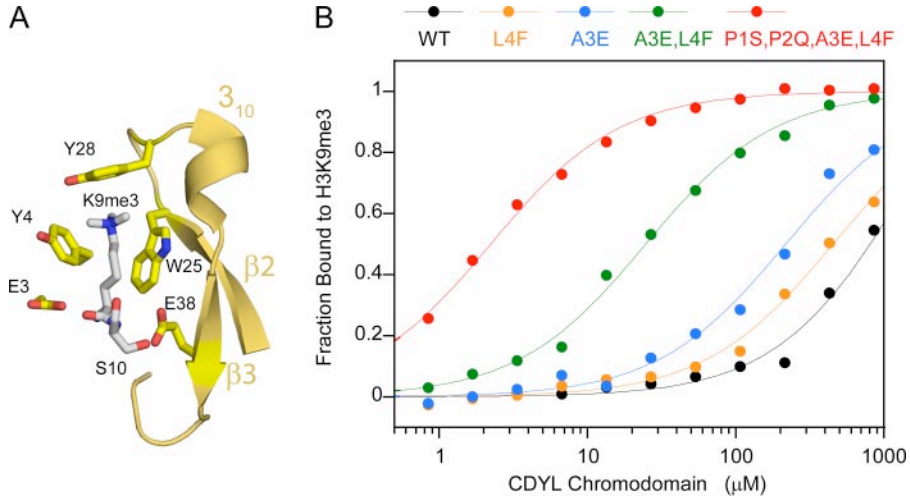

FIGURE 4. **Point mutations rescue CDYL H3K9me3 binding.** *A*, close-up view of the aromatic cage and surrounding secondary structure elements of the HP1 chromodomain interacting with H3K9me3 (PDB code 1kne). The side chains of E3 and E38 form hydrogen bonds with the backbone of the H3 tail and are solvent-exposed. Residues are numbered according to the sequence alignment in Fig. 1*B*. *B*, fluorescence polarization analysis of wild-type (*WT*) and mutant human CDYL chromodomains interacting with an H3K9me3 peptide. Averages from at least three independent measurements are plotted.

ability of CDY to establish more favorable polar interactions with the H3 tail than those used by HP1. Previous studies have shown that recognition of methyllysine by the HP1 chromodomain is driven by cation-π interactions between the methylated ammonium group of methylated H3K9 and the side chains of three aromatic residues (11, 16). The magnitude of the cation-π interaction depends on the electron density of the aromatic ring, which can substantially vary from a tyrosine to a phenylalanine as well as the degree of solvent exposure in the interaction site (37, 38). Whereas both CDYL2 and HP1 have a tyrosine at the first position of the aromatic cage, this position in CDY is substituted with a phenylalanine residue (Fig. 1*B*). Interestingly, this phenylalanine residue in CDY is further positioned between two polar (glutamic acid) residues that appear to improve solvent exposure of the aromatic cage. Together, these observations suggest that subtle amino acid changes surrounding the aromatic cage can lead to substantial differences in the strength of the cation-π interactions between chromodomains and methylated lysine residues.

CDY and CDYL2 Respond to a Binary Switch—A binary switch mechanism has been associated with the simultaneous presence of the H3K9me3 and H3S10ph modifications on the same histone tail (H3K9me3S10ph) (19, 20). Human HP1 variants are unable to interact with chromatin during mitosis because of the overwhelming presence of serine 10 phosphorylation (17, 18). In addition to its relevance to mitosis, H3S10ph is associated with transcriptionally active chromatin (10). To investigate the response of CDY and CDYL2 to such a binary switch, we performed additional fluorescence polarization binding assays. We found that both CDY and CDYL2 chromodomains are sensitive to the presence of H3S10ph. The binding to the H3K9me3S10ph peptide is 76-fold weaker for CDY and 100-fold weaker for CDYL2 as compared with binding to the H3K9me3 peptide (Table 1).

CDY and CDYL2 Chromodomains Bind to a Similar Fragment of the H3 Tail—The comparison of the structure of the CDYL2 chromodomain with that of HP1 suggests that H3 tail residues 5 through 10 should be sufficient for binding to CDY and CDYL2 chromodomains (11) (Fig. 1*C*). The interaction of CDY and CDYL2 chromodomains with the H3 tail involves one residue C-terminal to the methyllysine, serine 10. As discussed above, serine 10 phosphorylation has a major impact on binding affinity. To determine to what extent residues N-terminal to the methyllysine could impact on CDY and CDYL2 interaction with the H3 tail, we tested the influence of an adjacent modification on binding affinity, the presence of methylation at H3K4. We performed additional fluorescence binding assays using a peptide with simultaneous dimethylations at H3K4 and H3K9 (H3K4me2K9me2 peptide). As listed in Table 1, binding of CDY and CDYL2 chromodomains to the H3K4me2K9me2 peptide is nearly the same as interaction with H3K9me2, ~1.2-fold weaker. This finding suggests residue 4 of the H3 tail (position n-5) does not impact on the binding of CDY and CDYL2 to the H3 tail, similar to what has been observed for the interaction of *Drosophila* HP1 with the H3 tail (11).

Point Mutations Rescue CDYL Binding to the H3 Tail—Table 2 shows that we could not measure appreciable binding of the CDYL chromodomain to any methylated H3K9 peptide or the unmethylated peptide. We speculated that the critical contributing factor might be the absence of one of the residues in the aromatic cage (Fig. 1*B*). Given the 70% sequence identity between CDYL and CDY, we asked whether the H3 tail could bind to CDYL using alternate histone H3 modifications. We concentrated on methylated H3K4 (H3K4me3) and acetylated H3K9 (H3K9ac) modifications, as both modifications are found associated with the histone H3.3 variant within transcriptionally active chromatin. As shown in Table 1, we found no appreciable binding of CDYL to either of these peptides. We then asked whether the N-terminal extension of the chromodomain in CDYL could interfere with binding interactions. By preparing a separate recombinant construct of human CDYL that excluded the N-terminal extension, we measured the affinity for methylated and unmethylated H3 peptides again and observed no substantial differences (data not shown). The corresponding recombinant chromodomain of the mouse CDYL also did not bind to the H3K9me3 peptide (data not shown).

We then asked whether we could establish efficient binding of the CDYL chromodomain to the H3K9me3 peptide by using site-directed mutagenesis. We used the CDY sequence as a guide to prepare point mutations in CDYL. To restore the aromatic cage, we prepared a Leu-61 to Phe mutant (position 4 in the chromodomain; Figs. 1*B* and 4*A*). This mutant exhibits negligible improvement in binding affinity for the H3K9me3 peptide (Fig. 4*B* and Table 3). In the structure of the complex of HP1 with the H3 tail, the side chain of the residue preceding the

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TABLE 3

Dissociation constants (μM) measured by fluorescence polarization for recombinant mutant CDYL chromodomains interacting with synthetic peptides as described under "Experimental Procedures"

Peptide	Wild type	A3E	L4F	A3E,L4F	P1S,P2Q,A3E,L4F
H3 Lys-9					
Unmodified	>500	>500	>500	>500	>500
H3K4me3	>500	>500	>500	>500	>500
H3K9ac	>500	>500	>500	>500	>500
H3K9me1	>500	240 ± 60	>500	55 ± 10	13 ± 2
H3K9me2	>500	186 ± 24	~500	33 ± 8	3.4 ± 0.5
H3K9me3	>500	216 ± 31	452 ± 81	25 ± 11	2.2 ± 0.3
H3K4me2K9me2	>500	177 ± 33	~500	41 ± 7	3.5 ± 0.4
H3K9me3S10ph	>500	>500	>500	>500	>500
H3 Lys-27					
Unmodified	>500	>500	>500	>500	>500
H3K27ac	>500	>500	>500	>500	>500
H3K27me3	>500	323 ± 41	>500	101 ± 17	23 ± 5
Testis H3 Lys-27					
H3TK27me3	>500	225 ± 22	~500	38 ± 8	13 ± 3
H1.4 Lys-26					
Unmodified	>500	>500	>500	>500	100 ± 24
H1.4K26me3	>500	500 ± 71	~500	81 ± 12	6.5 ± 1.9
G9a Lys-185					
Unmodified	>500	>500	>500	>500	>500
G9aK185me3	>500	182 ± 31	~500	37 ± 7	40 ± 10
G9aK185me1	>500	271 ± 53	>500	52 ± 19	19 ± 3

first aromatic residue is a Glu (position 3 in the chromodomain), which forms intermolecular hydrogen bond with the backbone of Lys 9 (Fig. 4*A*). Glu-3 also improves solvent exposure at the binding pocket of the HP1 chromodomain. Because this residue is substituted with an Ala in human CDYL, we tested the effect of an Ala to Glu mutation for binding to the H3K9me3 peptide and measured a small but noticeable improvement in binding (factor of 3) (Fig. 4*B*). Subsequently, we prepared a double mutant of CDYL with substitutions at both positions 3 and 4 of the chromodomain and found a synergistic effect (30-fold improvement in binding) (Fig. 4*B*, Table 3).

Because the mutation of the CDYL chromodomain residue 3 exhibited a synergistic effect with the addition of the aromatic residue 4, we hypothesized mutations at residues 1 and 2 may further improve solvent exposure of the peptide binding pocket, impacting cation-π interactions. Therefore, we prepared four simultaneous mutations in CDYL to convert residues 1–4 to those of the CDY chromodomain (P1S,P2Q,A3E,L4F, Fig. 1*B*). This mutant CDYL exhibited a further 10-fold improvement in binding to the H3K9me3 peptide (Fig. 4*B*, Table 3). As compared with CDY, this mutant CDYL bound only 4-fold weaker to the H3K9me3 peptide, yet the binding was 2-fold stronger than that measured for CDYL2.

To test the affinity of these CDYL mutants for H3K9me3 in a cell-based assay, we transiently expressed full-length CDYL polypeptides bearing the A3E,L4F and P1S,P2Q,A3E,L4F mutations in NIH3T3 cells. As Fig. 5 shows, both mutant proteins were exclusively localized to the cell nucleus and were excluded from the nucleoli as was the wild-type CDYL protein (Fig. 3*B*). Interestingly, the nuclear distribution of the mutant proteins in relation to the DAPI dense areas and regions of pericentric heterochromatin with H3K9me3 varied among different cells. Three types of localization patterns for CDYL mutants could be observed (Fig. 5*C*). In a substantial number of cells, both mutants showed diffuse staining (type I) that was excluded from pericentromeric heterochromatin and is reminiscent of the wild-type CDYL protein (Figs. 3*B* and 5). Interestingly, in some cells the A3E,L4F mutant CDYL showed some overlap with DAPI dense regions and H3K9me3 modification as indicated by the yellow areas in the merged images (type II). Although the P1S,P2Q,A3E,L4F mutant CDYL exhibited both type I and type II localization patterns, it also showed punctate overlap with pericentromeric heterochromatin (type III), reminiscent of the CDY protein (Fig. 3*A*). Overall, the gradual changes in subnuclear localization observed support the biochemical function of these point mutations.

Other Potential Binding Sites for CDY and CDYL2 in Chromatin—In addition to H3K9me3, other lysine-methylated ARK(S/T) motifs depicted in Fig. 6*A* may recruit CDY family chromodomains. Therefore, we measured dissociation constants for the binding of CDY, CDYL2, wild-type CDYL, and the mutant CDYL chromodomains to four additional peptides (Fig. 6*B*, Tables 1 and 3). We found the binding of the CDY chromodomain to H3K27me3 and H3tK27me3 peptides to be 150- and 15-fold weaker, respectively, than binding to the H3K9me3 peptide. Interaction of CDY chromodomain with the H1.4K26me3 peptide was also found to be 20-fold weaker than binding to the H3K9me3 peptide. Surprisingly, the CDY chromodomain bound to the G9a-K185me3 peptide with dissociation constant similar to that found for the H3K9me3 peptide (Fig. 6*B*). When we compared binding differences among monomethylated peptides, we found the dissociation constant for binding of CDY to H3K9me1 and G9a-K185me1 peptides are significantly different, 3.4 and 36 μM, respectively. Together, these results suggest the CDY chromodomain is capable of discriminating between various lysine-methylated ARK(S/T) motifs.

Interestingly, the CDYL2 and the mutant CDYL chromodomains are substantially less selective than the CDY chromodomain in binding to different ARK(S/T) motifs (Fig. 6*B*). Among the peptides tested, the H3K27me3 peptide showed the weakest

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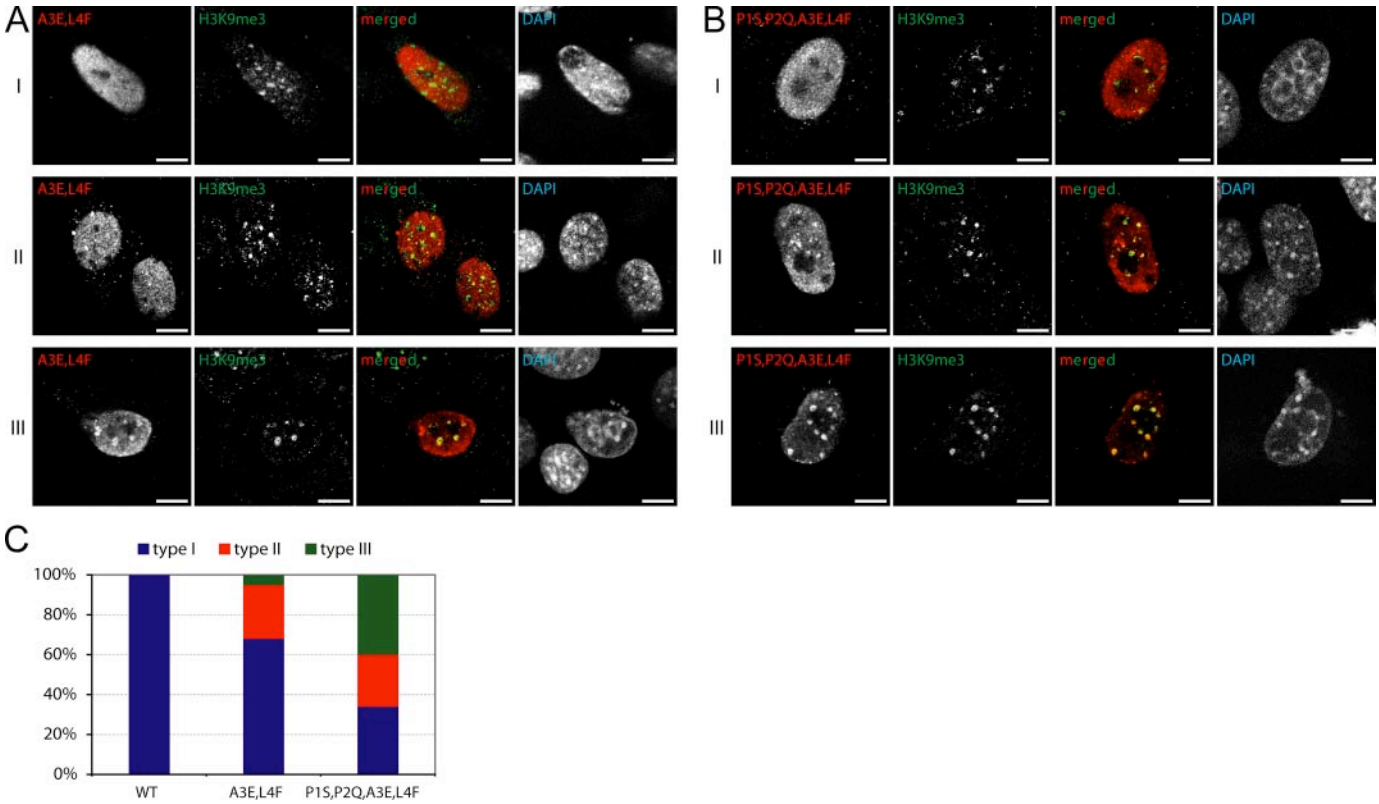

FIGURE 5. **CDYL mutant chromodomain H3K9me3 interaction is sufficient for localization to pericentromeric heterochromatin.** FLAG-tagged human CDYL chromodomain mutants A3E,L4F (*A*), and P1S,P2Q,A3E,L4F (*B*) were transiently expressed in NIH3T3 cells. Cells were immunostained and analyzed as described in Fig. 3. Cells of medium expression level representative for three different types (I-III) of nuclear distribution found for both mutants are shown. The *scale bar* is 10 μm. *C*, the occurrence of type I, II, or III nuclear distribution in each cell for wild-type CDYL (*WT*), CDYL A3E,L4F, and CDYL P1S,P2Q,A3E,L4F were statistically analyzed. Frequency plots resulting from the evaluation of at least 40 cells each from different transfection experiments are shown.

interaction for all CDY family chromodomains. Although the CDYL2 chromodomain exhibits similar binding to G9a-K185me3, H1.4K26me3, H3tK27me3, and H3K9me3 peptides, the mutant CDYL chromodomain is able to discriminate and bind strongly to the H3K9me3 peptide. The variability in dissociation constants listed in Tables 1 and 3 underscores the role of sequence differences in CDY family chromodomains for the specificity of their interactions with modified histone tails.

DISCUSSION

The expansion of HP1-like chromodomains in diverse proteins of higher eukaryotes suggests an increase in complexity of epigenetic regulatory pathways from lower to higher eukaryotes. The human genome contains the largest number of HP1-like chromodomains that are encoded by 16 different genes, and these encode proteins belonging to 5 different families, HP1, Suv91, Polycomb, CDY, and MPP8 (supplemental Fig. S3). Among these factors, HP1 and Suv91 are typically found associated with H3K9me3 regions of chromatin. The CDY and MPP8 families appear to be hallmarks of vertebrate genomes. The origin of their expansion into multiple variants is not well known. Previously, an evolutionary tree was delineated for the human CDY family genes, which suggested CDYL is the ancestor of CDY and CDYL2 (6). This would suggest that evolutionary forces first abolished methyllysine binding in an HP1-like chromodomain to formulate CDYL; subsequently methyllysine binding was gained in CDYL2 and CDY. This order of events is unlikely because the genomes of simple vertebrates like sea urchin or chicken encode only one CDY family gene whose sequence suggests full capability of binding to lysine-methylated ARK(S/T) motifs (Fig. 1*B*). Therefore, we assume CDYL2 is the ancestor of CDY and CDYL proteins.

The CDYL chromodomain is unable to bind to any lysine-methylated ARK(S/T) motif. We identified mutations in the CDYL chromodomain that can impact the interaction of the full-length protein with H3K9me3 regions of chromatin, suggesting its chromodomain is significant for cellular localization. Previous studies have implicated CDYL in transcriptional co-repressor complexes containing multiple chromatin modification enzymes and docking factors (39). Interestingly, the CDYL protein co-purified with the methylated G9a protein in human cell extracts (30). Together these results suggest the combined action of another factor could make up for the missing portion of the CDYL binding pocket. This may be related to a study on the yeast histone deacetylase complex Rpd3S, where the combined action of a PHD finger and a chromodomain from two different polypeptides has been suggested to regulate binding to H3K36me3 modification (40).

Although human CDY and CDYL2 chromodomains are able to recognize a variety of lysine-methylated ARK(S/T) motifs, there are substantial differences in their binding specificities. We found that the CDY chromodomain preferentially binds to the H3K9me3 peptide, whereas CDYL2 can equally interact

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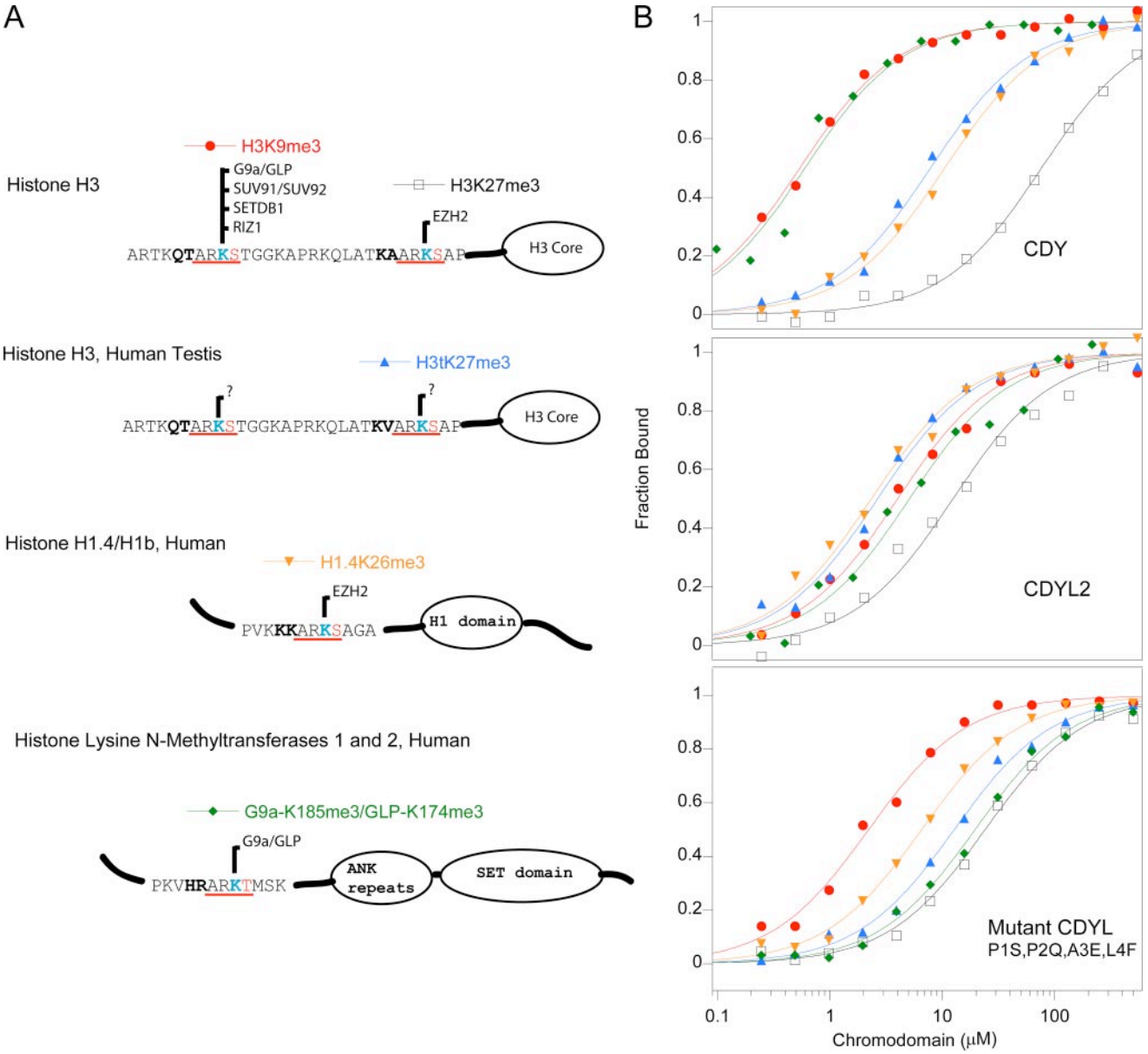

FIGURE 6. **Interaction of CDY family chromodomains with methyllysines embedded in ARK(S/T) motifs.** *A*, the ARK(S/T) motifs in chromatin that are potential binding sites of CDY family chromodomains are specified. The various methyltransferases that are known to establish the different methylations are listed *above each lysine residue*. *B*, fluorescence polarization binding assays were used to determine the specificities of the CDY, CDYL2, and CDYL P1S,P2Q,A3E,L4F chromodomains to each of these peptides containing a trimethyllysine. Averages from at least three independent measurements are plotted.

with H3K9me3, H3tK27me3, and H1.4K26me3 peptides. Studies on mouse polycomb (Pc) variants also showed substantial diversity in binding selectivity toward H3K9me3 and H3K27me3 (21). For example, Pc1 (Cbx2) showed equal binding to H3K9me3 and H3K27me3, whereas Pc2 (Cbx4) showed a 3-fold stronger binding to H3K9me3 (21). The mammalian Pc proteins are believed to contribute to chromatin architecture on the inactive X chromosome and reflect assembly of facultative heterochromatin using H3K9me3 and H3K27me3 modifications (21). The same chromatin modifications also seem to contribute to chromatin regulation during spermatogenesis (41, 42). Additional studies are required to determine how variability in methyllysine recognition by CDY, CDYL, and CDYL2 could impact on their functions in chromatin regulation in testis. Acetylation of histone H4 during spermatogenesis is suggested to hinge on the expression of CDY and CDYL, and these polypeptides are unique for their ability to link a repressive modification like H3K9me3 with that of histone H4 acetylation (8). Enrichment of CDY variants in testis might contribute to genomic imprinting, which leads to appropriate transcription of paternally derived genes (for review, see Refs. 31, 32, and 43). Unfortunately, at present only limited knowledge is available on the features of chromatin in human male germ cells.

Recently, we reported substantial evolutionary differences in the binding abilities of CHD double chromodomains that correlate with limited amino acid changes (44). These observations are consistent with complex control mechanisms associated with signaling enzymatic function in higher eukaryotes (45). In

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both the CDY and CHD families of chromodomains, catalytic activity is physically separable from an N-terminal chromodomain that appears to link input or output signals associated with epigenetic regulation. Another role of the chromodomain may be direct participation in enzymatic activity. For example, studies on Suv91 (supplemental Fig. S3) showed deletion of the chromodomain or point mutations in the aromatic cage impaired enzyme activity (*i.e.* trimethylation of H3K9) despite the presence of an intact catalytic SET domain (46). Additional studies are necessary to further dissect the catalytic function of CDY family ECH domains and subsequently investigate whether their chromodomains directly participate in enzymatic activity and/or serve to link with input/output signaling networks.

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Supplementary Material

SPECIFICITY OF THE CDY FAMILY OF CHROMODOMAINS FOR LYSINE-METHYLATED ARKS/T MOTIFS*

Wolfgang Fischle[1,2,3,4], Henriette Franz[3,4], Steven A. Jacobs[1,5], C. David Allis[1,2] & Sepideh Khorasanizadeh[1]

From Department of Biochemistry and Molecular Genetics[1], University of Virginia Health System, Charlottesville, VA 22908-0733, USA

Laboratory of Chromatin Biology[2], The Rockefeller University, New York, NY 10021, USA

Laboratory of Chromatin Biochemistry[3], Max Planck Institute for Biophysical Chemistry, 37077 Göttingen, Germany

[4]These authors contributed equally to this work

[5]Current address: Johnson & Johnson BIO, Radnor PA, USA

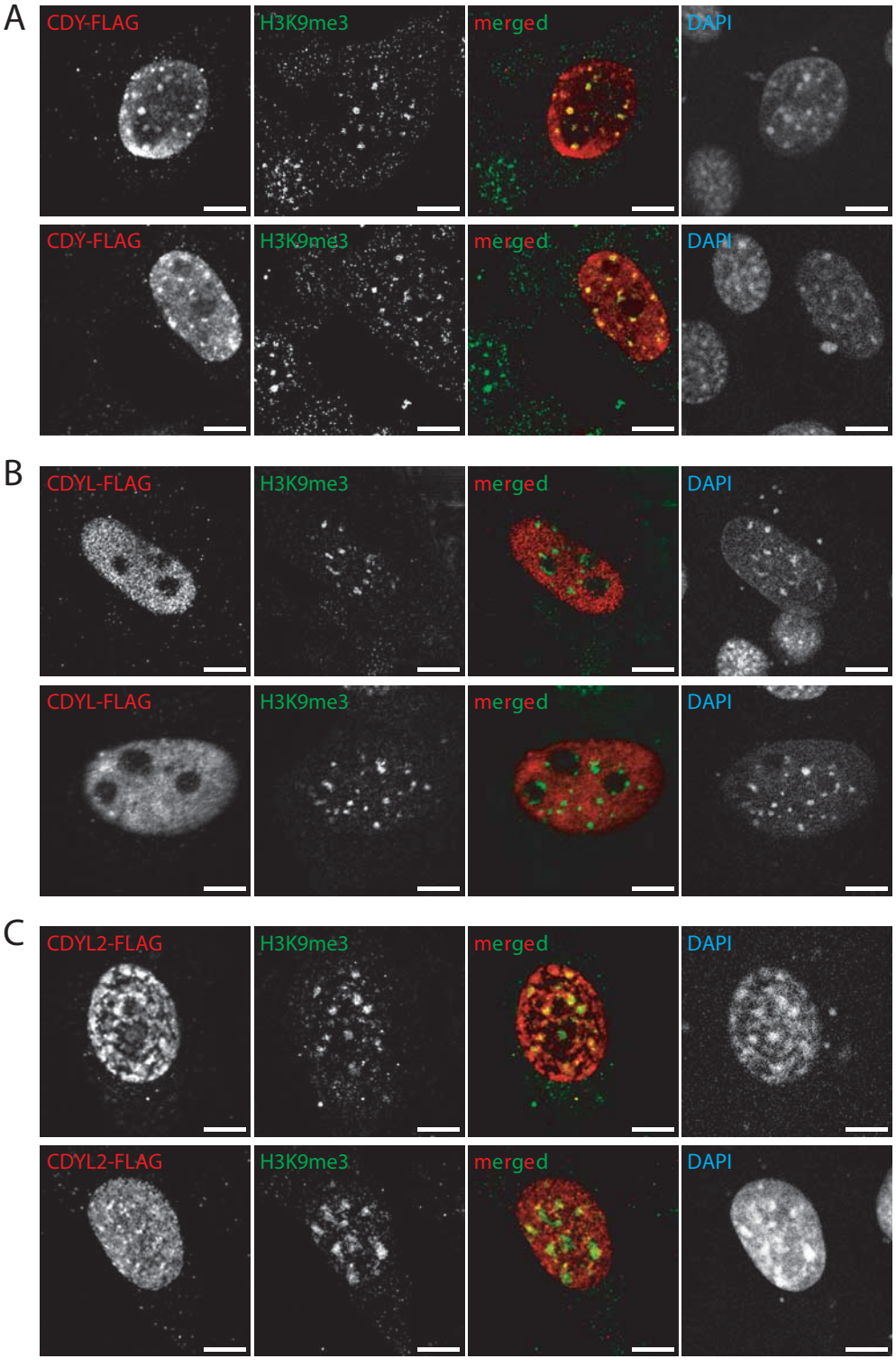
Running head: methyllysine recognition by CDY family of chromodomains

Address correspondence to Sepideh Khorasanizadeh or Wolfgang Fischle; E-mail: khorasan@virginia.edu or wfischl@gwdg.de

Supplementary Figure S1. CDY family proteins display distinct nuclear distribution. FLAG-tagged hCDY (**A**), hCDYL (**B**), or mCDYL2 (**C**) were transiently expressed in NIH3T3 cells. Immunostaining with anti-FLAG specific antibodies (green) and anti-H3K9me3 (red) specific antibodies is shown. The merged image corresponds to the overlay of the two color channels. Different cells of medium expression level are shown. DNA inside the cell nucleus was stained with DAPI. Whereas hCDY and hCDYL showed similar distribution in all cells analyzed, the localization of CDYL2 varied from cell to cell. Images representative of the different distributions observed are shown (see also Figure 3C). Scale bar, 10 μm.

Supplementary Figure S2. CDY but not CDYL localizes to pericentromeric heterochromatin enriched in H3K9me3. A. Immunostaining of MEF cells with anti-HP1α (green) and anti-H3K9me3 (red) specific antibodies shows co-localization to pericentromeric regions inside the cell nucleus. DNA inside the cell nucleus was stained with DAPI and defines areas of high DNA density that are presumed to be heterochromatic. FLAG-tagged hCDY (**B**) or hCDY (**C**) were transiently expressed in MEF cells. Immunostaining with anti-FLAG specific antibodies (green) shows co-localization of hCDY, but not hCDYL, with regions enriched in H3K9me3 (red). Note that the subnuclear, DAPI-dense, dot-like structures are not an artifact of the transient overexpression of the proteins but are also observed in non-transfected cells (see untransfected cells in (A), the lower half of (B), and the upper half of (C)).

Supplementary Figure S3. A. Sequence alignments of the chromodomains of all 16 human proteins with potential to recognize lysine-methylated ARKS/T motifs. Sequence identity in each protein family is highlighted in yellow. Residues of the aromatic cage are marked with red stars above the sequences. **B.** Schematic representation of the conserved domains in the five distinct protein families aligned in panel A. The chromodomain is represented by a yellow triangle. Two polypeptide families, Suv91 and CDY, contain (putative) catalytic modules.



Supplementary Figure S1

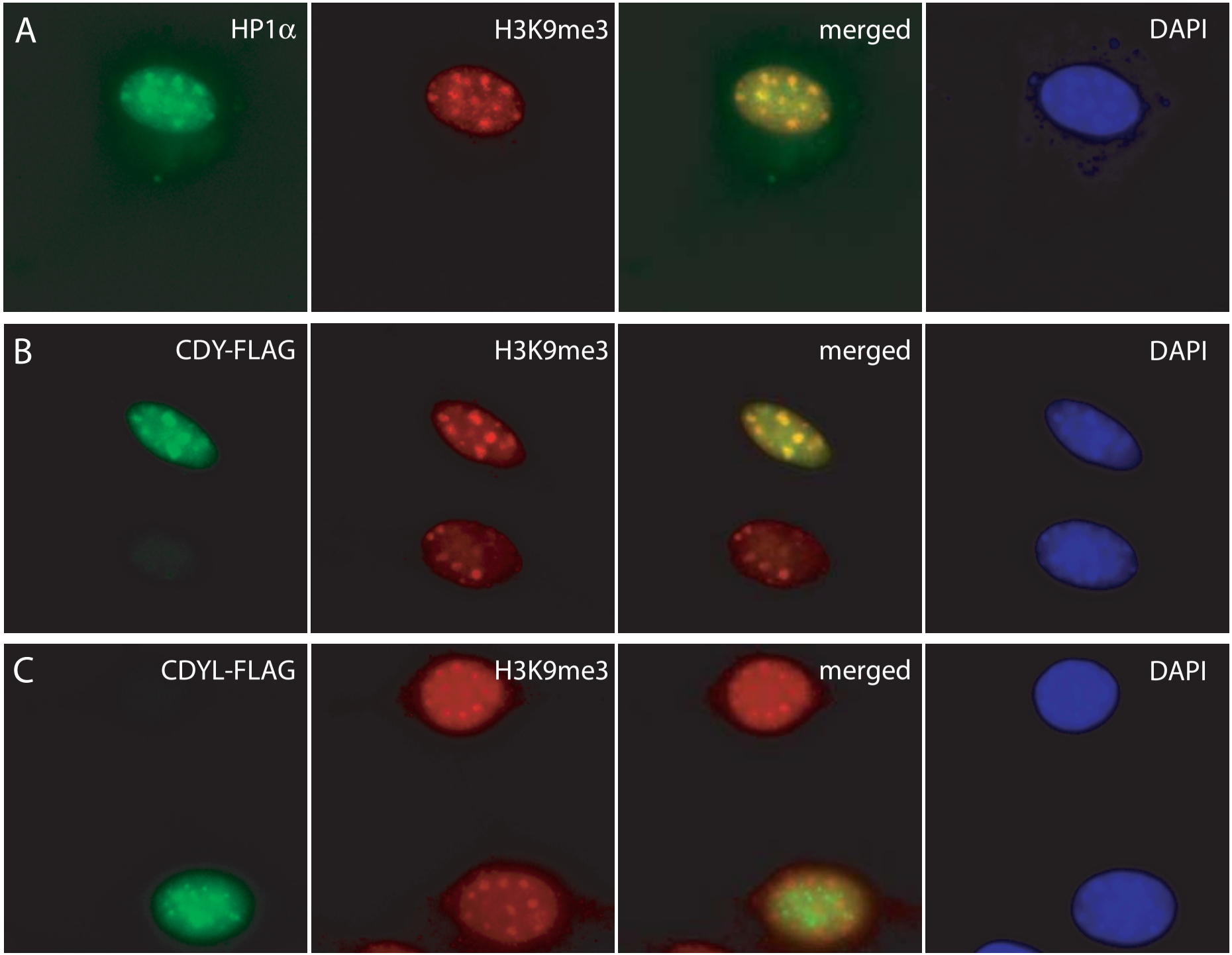

A
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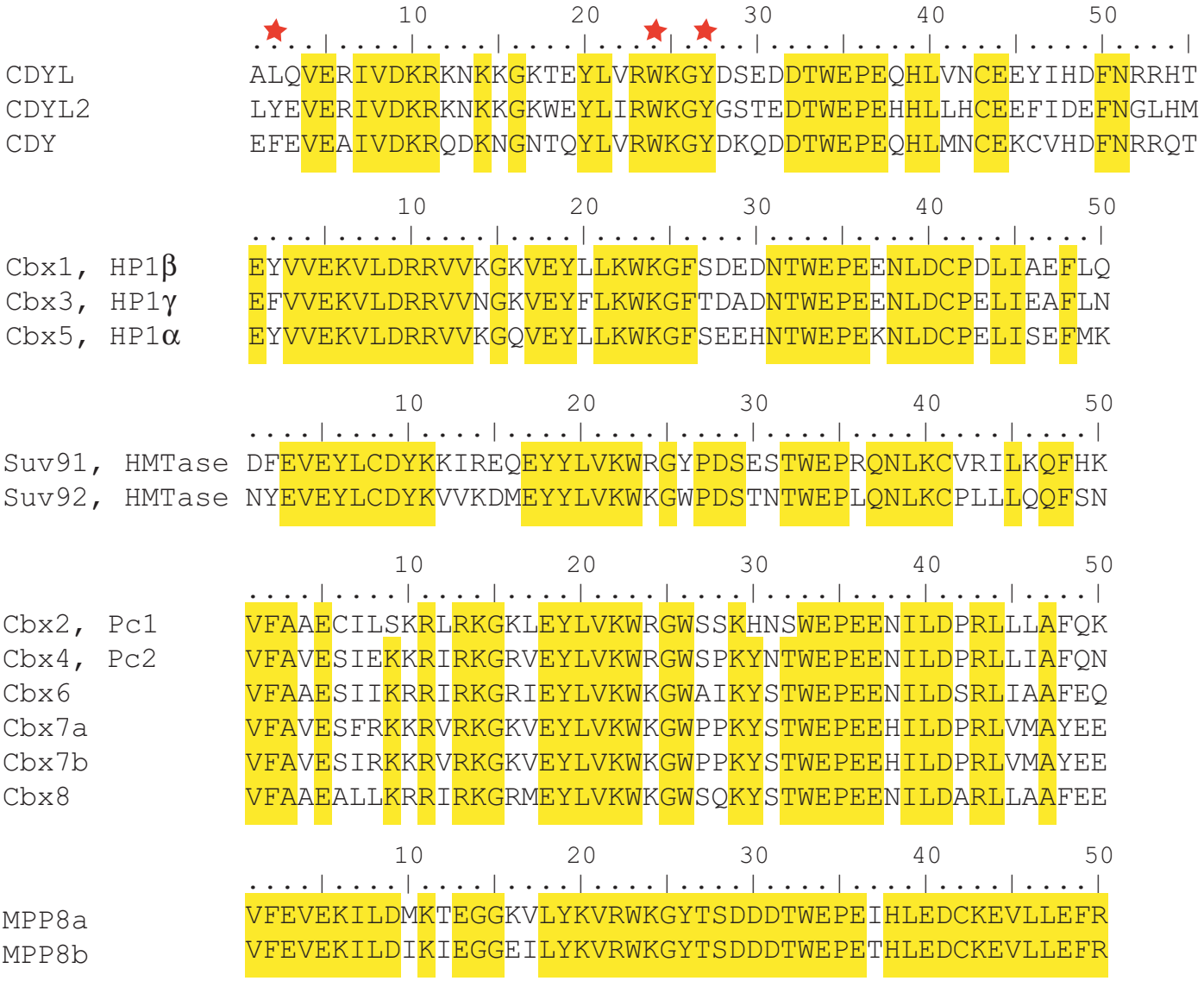
Figure S2

A



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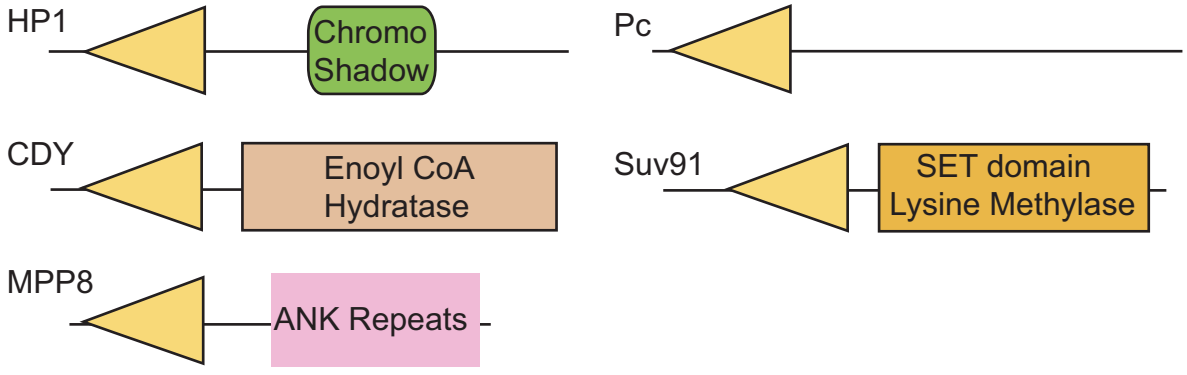

Supplementary Figure S3

4 Multimerization and H3K9me3 binding is required for CDYL1b heterochromatin association

Citation

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Original Contribution

Preparation and accomplishment of the biological experiments presented in Figure 1, 2, 3 (except panel D), 4, 5, 6 and 7 and supplemental Figures 1, 5 and 6, i.e. cloning of described plasmids, real-time PCR, pulldown experiments, chromatin precipitation, purification of CDYL1 chromodomain, fluorescence polarization assays, multimerization assay, transfection and immunostaining of described constructs; writing of the manuscript (large parts of introduction, methods and results section).

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Multimerization and H3K9me3 Binding Are Required for CDYL1b Heterochromatin Association*[S]

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Henriette Franz[‡], Kerstin Mosch[‡1], Szabolcs Soeroes[‡1,2], Henning Urlaub[§], and Wolfgang Fischle[‡3]

From the [‡]Laboratory of Chromatin Biochemistry and the [§]Bioanalytical Mass Spectrometry Group, Max Planck Institute for Biophysical Chemistry, 37077 Göttingen, Germany

Proteins containing defined recognition modules mediate readout and translation of histone modifications. These factors are thought to initiate downstream signaling events regulating chromatin structure and function. We identified CDYL1 as an interaction partner of histone H3 trimethylated on lysine 9 (H3K9me3). CDYL1 belongs to a family of chromodomain factors found in vertebrates. We show that three different splicing variants of CDYL1, a, b, and c, are differentially expressed in various tissues with CDYL1b being the most abundant variant. Although all three splicing variants share a common C-terminal enoyl-CoA hydratase-like domain, only CDYL1b contains a functional chromodomain implicated in H3K9me3 binding. A splicing event introducing an N-terminal extension right at the beginning of the chromodomain of CDYL1a inactivates its chromodomain. CDYL1c does not contain a chromodomain at all. Although CDYL1b displays binding affinity to methyl-lysine residues in different sequence context similar to chromodomains in other chromatin factors, we demonstrate that the CDYL1b chromodomain/H3K9me3 interaction is necessary but not sufficient for association of the factor with heterochromatin. Indeed, multimerization of the protein via the enoyl-CoA hydratase-like domain is essential for H3K9me3 chromatin binding *in vitro* and heterochromatin localization *in vivo*. In agreement, overexpression of CDYL1c that can multimerize, but does not interact with H3K9me3 can displace CDYL1b from heterochromatin. Our results imply that multimeric binding to H3K9me3 by CDYL1b homomeric complexes is essential for efficient chromatin targeting. We suggest that similar multivalent binding stably anchors other histone modification binding factors on their target chromatin regions.

For packaging the chromosomes of an eukaryotic cell into the nucleus the negatively charged DNA is wrapped around a positively charged octamer of histone proteins consisting of two H2A–H2B dimers and one $(H3–H4)_2$ tetramer. 147 bp of DNA are wound around one histone octamer forming the fundamental repeating unit of chromatin, the nucleosome. N- and C-terminal tails of the histones are protruding out of this structural entity.

Histones are subject to a plethora of post-translational modifications (1–3). Among these methylation of lysine residues plays a special role. Specific sites of lysine methylation as well as distinct stages of lysine methylation are associated with different nuclear processes (4, 5). For example, trimethylation of H3 lysine 4 (H3K4me3) is found in euchromatic structures, which are open for transcription and are early replicating during S-phase. In contrast trimethylation of lysine 9 of histone 3 (H3K9me3) is accumulated at heterochromatic regions. These are densely packed, mostly transcriptional silent and late replicating in S-phase (6, 7).

Whereas histone modifications might directly affect chromatin structure (8, 9), a number of protein domains have been identified that specifically bind certain histone modifications (10, 11). These factors are thought to read-out and translate the effects of individual histone modifications or combinations thereof. Different proteins containing chromodomains have been implicated in binding methylated histone lysine residues preferentially when in higher (tri- and dimethylated) states. For example, heterochromatin protein 1 (HP1)[4] was shown to recognize H3K9me, whereas Polycomb binds H3K27me (12). Structural analysis of a number of binding domains has identified aromatic cages of at least three residues as central elements in histone methyl-lysine binding (10). Although the interaction of different protein domains with histone modifications has been well studied *in vitro* using isolated histone tail peptides, the exact parameters by which these proteins are recruited to their target sites on chromatin have not been worked out.

Recently, a new chromodomain containing group of proteins was described (13). The *cdy* (chromodomain on the Y) family represents a set of related genes in higher eukaryotes. In humans the *CDY* family comprises two autosomal genes, namely *CDYL1* and *CDYL2* as well as multiple gene copies of *CDY* on the Y chromosome (14). Evolutionary, a common ancestor of the *cdyl* gene appeared first in deuteriostomia. This predecessor was later duplicated to yield the *cdyl1* and *cdyl2* genes. A very recent multiplication of the *cdyl1* gene in the primate lineage lead to multiple *cdy* copies on the Y chromosome (14–16).

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S6 and "Materials and Methods."
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[2] Supported by a predoctoral fellowship of the Boehringer Ingelheim Foundation.
[3] To whom correspondence should be addressed. Tel.: 49-551-2011340; Fax: 49-551-2011337; E-mail: wfischl@gwdg.de.

[4] The abbreviations used are: HP1, heterochromatin protein 1; CDYL, chromodomain on the Y chromosome; WT, wild type; MEF, mouse embryonic fibroblast; NLS, nuclear localization signal; aa, amino acid; ECH, enoyl-CoA hydratase; HA, hemagglutinin; MS, mass spectrometry; YFP, yellow fluorescent protein; PBS, phosphate-buffered saline; PDB, Protein data bank; HEK, human embryonic kidney.

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CDY family proteins have a N-terminal chromodomain, a central hinge region, and a C-terminal enoyl-CoA hydratase-like (ECH) domain in common (see Fig. 1*A*). Recently, we could show that the chromodomains of CDY and CDYL2, which have high sequence similarities with HP1 and Polycomb chromodomains, can recognize di- or trimethylated histone and non-histone lysine residues. The strongest interaction was found for the H3K9me3 modification (17). In contrast, CDYL1 was not able to recognize H3K9me3 because of subtle sequence differences at the very beginning of the chromodomain affecting the first of three aromatic cage residues. Mutagenesis of a few residues at the N terminus of the CDYL1 chromodomain lead to restored binding activity to H3K9me3 peptides *in vitro* and partial relocalization to heterochromatic regions *in vivo*. Other studies indicated that CDYL1 binding to H3K9me3 might be increased after methylation by the histone methyltransferase G9a (18). Interestingly, biochemical pull-down experiments identified CDYL1 as the binding protein of automethylated G9a (19). The exact reasons for the different methyl-lysine binding behavior of CDYL1 are not understood.

Biochemically, CDYL1 was found in the CoREST complex where it bridges the repressor REST and the histone methyltransferase G9a (20–22). The ECH-like domain of CDYL1 was shown to interact with histone deacetylases HDAC1 and HDAC2 likely via CoREST association thereby acting as corepressor during transcriptional repression (23). Conflicting results have suggested that the ECH-like domain of CDY and CDYL1 might constitute a histone acetyltransferase activity in elongating spermatids during hyperacetylation and replacement of histones (24). In peroxisomes and mitochondria, trimeric enoyl-CoA hydratases accomplish the hydration of the double bond of fatty acids during β-oxidation (25, 26). The functional impact of putative multimerization of ECH-like domains onto the CDY family protein function has not yet been investigated.

In this study we present results that clarify the different binding abilities reported for CDYL1 to methylated lysine residues. CDYL1 exists in three different splicing variants, a, b, and c. The b form is not only the most abundant splicing variant but is also exclusively able to recognize H3K9me3 *in vitro* and *in vivo*. We further demonstrate that the chromodomain of CDYL1 alone is not sufficient to stably bind to H3K9me3 chromatin *in vitro* and to mediate localization to DNA-dense heterochromatic regions *in vivo*. Besides a functional chromodomain multimerization of the ECH-like domain is necessary to bring CDYL1b to heterochromatin.

EXPERIMENTAL PROCEDURES

Plasmids—Plasmids for expression of hCDYL1a were described elsewhere (17). cDNAs corresponding to the open reading frame of hCDYL1b or hCDYL1c were amplified from an EST clone (IMAGE: 6140263) using PCR and cloned into a derivative pcDNA3.1 vector (Invitrogen) generating C-terminal fusion to a 2× FLAG-2× HA epitope tag. The GenBank™ accession numbers of the cDNAs used are as follows: hCDYL1a, AF081259; hCDYL1b, BC108725; hCDYL1c, BC119682. For expression in *Escherichia coli* cDNAs corresponding to the open reading frame of hCDYL1a, hCDYL1b, and hCDYL1c were subcloned into pET11a (Novagen). The hCDYL1b chromodomain (1–78 aa) was cloned into pET16b (Novagen) for expression with a His_{10} tag. hCDYL1c (309 aa) and hCDYL1cΔCterm (1–236 aa) were cloned into pcDNA3.1myc-His (Invitrogen). cDNAs corresponding to the CDYL1b fragments described in Fig. 5*C* were PCR amplified from *Xenopus laevis* EST clone XL213m10 and cloned into the pCS2+ vector (RZPD) generating fusion to a C-terminal 1× FLAG epitope: chromo, 1–64 aa; chromohingeΔNLS, 1-233 aa; chromohinge, 1–295 aa; hingeECH, 64–541 aa; CDYL1cΔC-term, 233–541 aa; CDYL1cΔNLS, 295–541 aa; ECH, 295–463 aa; chromoCDYL1c, 1–64 aa fused to 233–541 aa; and chromoCDYL1cΔC-term, 1–64 fused to 233–463 aa.

Real Time PCR—Total RNA of human tissues was a gift from Dr. Thomas Giger (Max Planck Institute for Evolutionary Anthropology, Leipzig). For real time PCR total RNA was isolated according to the TRIzol protocol (Invitrogen). DNA was digested with a DNA free kit (Ambion). cDNA was made using random hexamers and the First Strand Synthesis kit (Invitrogen). cDNA was used for real time PCR using IQ SYBR Green Supermix on a MJ Research DNA engine Opticon (Bio-Rad). The following primers were used for real time PCR detection: hCDYL1a forward, 5′-GGTCAGCCTGGGGAAAAAGC-3′; hCDYL1a reverse, 5′-CGGGAGGCTGCTGTGCC-3′; hCDYL1b forward, 5′-CTTCCGAGGAGCTGTACGAGGTTG-3′; hCDYL1b reverse, 5′-TCTCCGTGTGGCGTCTGTTGAA-3′; hCDYL1c forward, 5′-GCTTCCGAGGAGCTGTACGAGTACATCTC-3′; hCDYL1c reverse, 5′-CAAAAGGCTGGTCTCTTCTGTCGTCAAT-3′.

Western Blotting—For Western blot analysis primary antibodies were used as follows: anti-CDYL (Abcam), 1:1,000; anti-H3, 1:40,000 (Abcam); anti-HP1α and anti-HP1β (Upstate), 1:2,000; anti-FLAG (Sigma), 1:1,000; anti-H3 (Upstate), 1:10,000; anti-green fluorescent protein (Roche), 1:10,000.

Recombinant Chromatin—Expression and purification of WT *X. laevis* histones was performed as described (27). H3K9me3 was generated by native protein ligation (28). In short, the coding sequence for *X. laevis* H3Δ1–20,C21A was amplified by PCR and cloned into the pET3d expression vector. The truncated H3 protein was expressed and purified like the WT histones. The H3 N-terminal peptide containing residues 1–20 and trimethylated lysine 9 was synthesized using Fmoc (*N*-(9-fluorenyl)methoxycarbonyl)-based solid-phase synthesis and activated at the C terminus by thioesterification. Ligation of the activated H3 peptide to the truncated H3 histone and purification of the ligation product was performed as described (28). Identity and purity of histones was verified by SDS-PAGE as well as by mass spectrometry (supplemental Fig. S2).

Assembly of histone octamers containing H3unmod and H3K9me3 as well as reconstitution of recombinant oligonucleosomes was performed by salt dialysis as described using the 12 × 200 × 601 template (27, 29). Briefly, octamers were reconstituted using H3unmod or H3K9me3 and purified by gel filtration on Superdex 200 (GE Healthcare). Scavenger DNA corresponding to a 148-bp length fragment PCR amplified from pUC18 was used in all reconstitutions. Assembly reactions were titrated at different octamer:DNA ratios. Reproducibly, an octamer:DNA ratio of 1.1:1 resulted in saturated nucleosomal arrays. Assembly reactions were analyzed by native gel electro-

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phoresis and analytical ultracentrifugation. To further control the saturation level of the oligonucleosomal templates, assembled material was analyzed after digestion with the restriction enzyme HhaI, which cuts within every 601 repeat that is not protected by histone octamers in the 12-mer template. Also, material was analyzed by scanning force microscopy after mild fixation with glutaraldehyde and fixation on mica support (see supplemental Fig. S3) (30). Last, responsiveness of reconstituted material to higher order compaction by Mg^{2+} titration for chromatin templates containing H3unmod and H3K9me3 was compared (supplemental "Materials and Methods" and Fig. S4). Reconstituted material was used for all measurements without further purification after extensive dialysis against 10 mM triethanolamine, 0.1 mM EDTA, pH 7.5.

Peptide and Chromatin Pulldowns—Peptides used for pulldown studies carried a biotinylated lysine residue at the C terminus: H3unmodified, ARTKQTARKSTGGKAPRKQLK-biotin; H3K9me3, ARTKQTARK(me3)STGGKAPRKQLK-biotin. For peptide pulldown 10 μg of biotinylated histone peptide was bound to 40 μl of prewashed strepatvidin-coated magnetic beads (Pierce) for 3 h at room temperature. 1 ml of precleared HeLa S3 nuclear extract (5 mg/ml) prepared as described was incubated with the peptide-bound magnetic beads overnight at 4 °C. Beads were washed six times with 1 ml of PD150 (20 mM HEPES, pH 7.9, 150 mM KCl, 0.2% (v/v) Triton X-100, 20% (v/v) glycerol) supplemented with Protease Inhibitor Complete EDTA-free (Roche Applied Science). After boiling the beads in loading buffer recovered proteins were separated on 4–20% gradient SDS-polyacrylamide gels (Invitrogen). Proteins were either detected by Western blot procedures or analyzed by mass spectrometry (MS). Chromatin pulldowns were carried out accordingly using 2 μg of recombinant chromatin assembled on biotinylated DNA templates.

Chromatin Precipitation—Proteins were translated *in vitro* using the T7 TnT kit (Promega). 1.75 μg of chromatin and 45 μl of the TnT reaction were mixed in 500 μl of end volume of CP buffer (20 mM Tris-HCl, 0.2% (v/v) Triton X-100, 150 mM NaCl, 1 mM dithiothreitol, protease inhibitor EDTA free) and incubated 2 h at 4 °C. $MgCl_2$ was added to 5 mM final concentration and the reactions were incubated for 15 min at 4 °C. Precipitated chromatin was collected at 13,000 × *g* for 30 min at 4 °C. Chromatin pellets were washed once with CP buffer supplemented with 5 mM $MgCl_2$. The supernatant was removed and the pellet dissolved by boiling (5 min) in SDS gel loading buffer.

Mass Spectrometry—SDS-PAGE gels were stained with Coomassie Blue, and the entire gel lanes were cut into 23 slices of equal size. Proteins within the slices were digested according to Shevchenko *et al.* (31). Peptides were extracted and analyzed by LC-coupled tandem MS on an Orbitrap Xl mass spectrometer (Thermo Fisher Scientific). CID fragment spectra were searched against the NCBInr data base using the MASCOT search engine.

Fluorescence Polarization Measurements—Peptides used for fluorescence polarization measurements were as described (17). Fluorescence polarization assays were essentially carried out and analyzed as described (32). A titration series of 10-μl volumes in 384-well plates were read multiple times on a Plate Chameleon II plate reader (HIDEX Oy). Multiple readings and the independent titration series were averaged after data normalization.

Cell Transfection and Immunofluorescence—Mouse embryonic fibroblast (MEF, gift of Dr. Thomas Jenuwein, IMP, Vienna) cells were grown at 37 °C in a humidified atmosphere, 5% CO_2 using Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, and 1× penicillin/streptomycin (Invitrogen). Transfection was carried out using JetPei (PolyPlusTransfections). For immunofluorescence, cells were grown and transfected on glass coverslips. 24 h post-transfection cells were fixed with 3% paraformaldehyde in PBS for 15 min at 37 °C. After three washing steps with PBST (1× PBS, 0.2% Triton X-100) and a 10-min permeabilization (PBST, 0.2% Nonidet P-40) at room temperature, cells were blocked for 1 h at room temperature (1× PBS, 5% goat serum, 2% bovine serum albumin, 0.2% Triton X-100). Coverslips were incubated with the indicated primary antibodies diluted in blocking buffer for 1 h at room temperature. Dilutions of primary antibodies were as follows: anti-H3K9me3 (Upstate), 1:1,000; anti-FLAG and anti-HA (Sigma), 1:1,000; anti-myc (Millipore), 1:1,000. Coverslips were washed three times with PBST and then incubated with the appropriate secondary antibodies (anti-mouse Alexa555, 1:2,000 or anti-rabbit Alexa488, 1:2,000; Molecular Probes) for 1 h at room temperature. After washing (3 × 5 min, 1× PBST) cells were mounted with MOWIOL (Calbiochem) containing 50 μg/ml of 4′,6-diamidino-2-phenylindole. Pictures were captured on a Leica SP5 confocal microscope (×60 objective).

Multimerization Assay—15 μg of anti-FLAG antibody per 40 μl of goat anti-mouse IgG magnetic beads (Invitrogen) were incubated 2 h in 1 ml of PBS at 4 °C under constant rotation. YFP-CDYL1b and FLAG-tagged CDYL1b constructs were expressed using the SP6 TnT kit (Promega). 50 μl of the reactions were incubated with the anti-FLAG-coated beads in 1 ml of PD150 overnight at 4 °C under constant rotation. Beads were washed six times in PD150 and boiled in SDS gel loading buffer (5 min).

RESULTS

Identification of CDYL1 as H3K9me3-binding Protein—To gain insight into translation of the H3K9me3 histone modification for regulating heterochromatin formation and maintenance we used H3 peptides trimethylated on lysine 9 for pulldown experiments out of HeLa S3 nuclear extracts. Compared with a mock (beads only) and the unmodified H3 peptide control we identified 25 protein factors specifically bound to the H3K9me3 peptide as analyzed by SDS-PAGE and following MS analysis (Fig. 1*A*). Specific proteins were assigned with an arbitrary cut-off of at least three sequenced peptides per protein where at least two peptides had to be unique in sequence (not shown). According to gene ontology analysis, out of the 25 factors 18 have functions in nucleobase, nucleoside, nucleotide, and nucleic acid metabolic processes. Among these 18 proteins 10 are involved in chromatin maintenance and architecture. The identified chromatin-related factors include the three mammalian HP1 isoforms (α, β, γ), CAF1, and surprisingly CDYL1. A total of 12 peptides covering 45.6% of the amino acid sequence of CDYL1 could be identified (Fig. 1*B*). None of the

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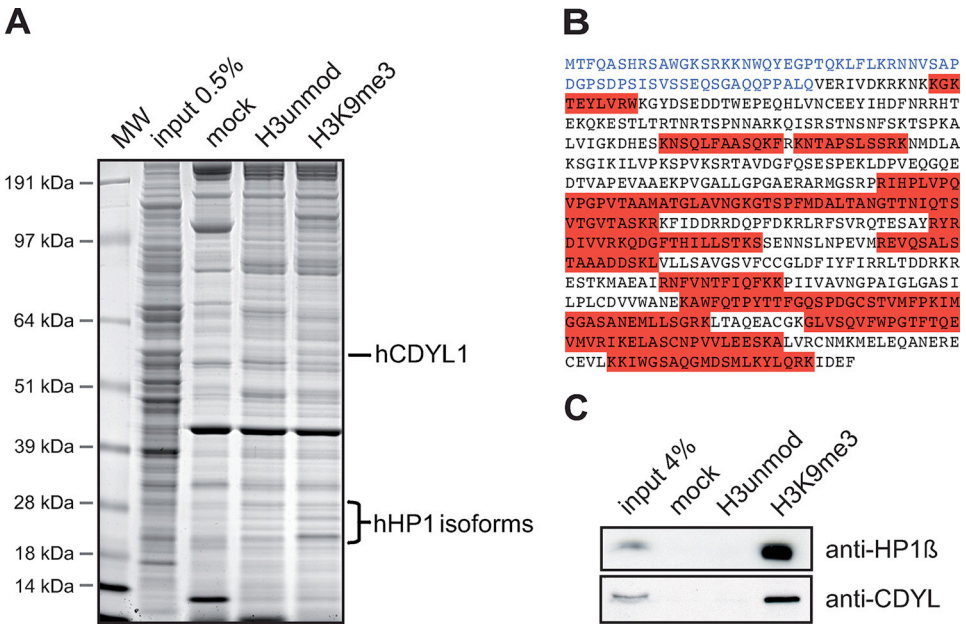

FIGURE 1. **CDYL1 is an H3K9me3 binding factor.** *A*, the indicated histone H3 peptides were used in pulldown experiments of HeLa S3 cell nuclear extract. Beads without coupled peptides were used as control (*mock*). Specifically recovered proteins were run on SDS-PAGE and stained with Coomassie Blue. Proteins were identified by MS/MS analysis. The positions of CDYL1 and HP1 proteins are indicated on the *right*. Molecular weight (*MW*) markers are indicated on the *left*. *B*, peptide sequence of CDYL1. The highlighted (*red*) peptides were identified by MS/MS analysis. The N terminus of CDYL1 is shown in *blue*. *C*, Western blot analysis of a histone H3 peptide pulldown experiment of HeLa S3 cell nuclear extract as in *A* using the indicated antibodies.

sequenced peptides could be assigned to the very N-terminal region of the CDYL1 polypeptide (originally described by Lahn *et al.*) (*blue* sequence in Fig. 1*B*) (15, 24). Also, no precursor could be detected in the MS analysis of this particular molecular weight region of the SDS-PAGE gels that matched any tryptic peptide derived from the very N-terminal region. Recent studies have reported a new exon acquisition in the mammalian *cdyl1* gene (33). Also, we reported that the chromodomain of the originally described CDYL1 polypeptide sequence does not bind to H3K9me3 (17). These observations lead us to hypothesize that alternative splicing might generate different CDYL1 protein species with different functionality.

To independently confirm the results of the MS analysis, we repeated the H3 peptide pulldowns from HeLa S3 nuclear extracts. Using antibodies specific for CDYL1 we could confirm specific enrichment of CDYL1 exclusively on the H3K9me3 matrix compared with the unmodified control peptide and the mock control in Western blots (Fig. 1*C*). Similarly, HP1β was found highly enriched in the H3K9me3-associated protein fraction. From these results we conclude that CDYL1, in contrast to former results, can associate with the heterochromatic H3K9me3 modification.

CDYL1 Has Three Splicing Variants and CDYL1b Is the Most Abundant Splicing Variant—To clarify whether differential splicing could indeed explain the distinctive H3K9me3 binding behavior of CDYL1 in different experimental settings, we performed data base searches. *In silico* analysis defined three putatively alternative spliced mRNAs (a, b, and c) transcribed from the *CDYL1* locus on chromosome 6 (supplemental Fig. S1). The *CDYL1* gene locus contains 10 exons. The previously known CDYL1 mRNA is generated by splicing the first three to the last six exons. From here on, we will refer to this mRNA as CDYL1a as also suggested by Li *et al.* (33). The second splice variant CDYL1b mRNA emerges from exons 4–10 and a third variant originates from splicing of exon 4 to exons 6–10 of the *CDYL* gene locus (CDYL1c).

Sequence analysis and domain comparison indicate that the polypeptides corresponding to CDYL1 variants a and b both connect a N-terminal chromodomain via a 238-aa long linker region to a C-terminal ECH-like domain (Fig. 2*A*). Due to the described splicing events the CDYL1a protein contains a prolonged N-terminal part. Sequence comparison of the chromodomain of CDYL1 splicing variants a and b with the canonical chromodomain of HP1β reveals high sequence homology (Fig. 2*D*). However, splicing eliminates the first of three aromatic cage residues from the CDYL1a sequence, as the splicing site is located to the very N terminus of the CDYL1 chromodomain (Fig. 2*D*). Mutagenesis of any of the three HP1 aromatic cage residues in the *Drosophila* HP1α protein results in loss of H3K9me interaction (34). Mutation of the CDYL1a residues at this position to the corresponding residues in CDY restores H3K9me3 interaction (17). We therefore conclude that alternative splicing of CDYL1 generates two proteins containing a chromodomain signature with differential H3K9me3 interaction potential. In contrast, CDYL1c lacks the chromodomain region and a 175-aa long part of the linker region altogether.

Real time PCR analysis of cDNAs of two human cell lines (HeLa S3 and HEK293) and four human tissues (brain, liver, kidney, and testis) revealed that CDYL1b is the major splicing variant of the *CDYL1* gene (Fig. 2*B*). In agreement with former results CDYL1a mRNA levels in testis were found elevated and reaching CDYL1b expression suggesting a specific role for this splicing variant in this tissue (14). Also, Western blot analysis of HEK293 human cell nuclear extracts using an antibody specific for CDYL1 showed the highest abundance of CDYL1b (Fig. 2*C*). Our observations indicate that transcription of the CDYL1 locus results in three splicing variants with different expression levels and different capabilities to bind to H3K9me3.

Only CDYL1b Recognizes Methyl-lysines—To find out whether CDYL1b is indeed a CDYL1 splicing variant that recognizes H3K9me3 we *in vitro* transcribed and translated FLAG-tagged CDYL1 variants a, b, and c. We then tested binding to H3K9me3 in peptide pulldown experiments. HP1β was used as positive control. As Fig. 3*A* shows, CDYL1a and -c displayed no preferential binding to the H3 tail trimethylated on lysine 9. In contrast, CDYL1b was clearly enriched in the H3K9me3 peptide-bound fraction.

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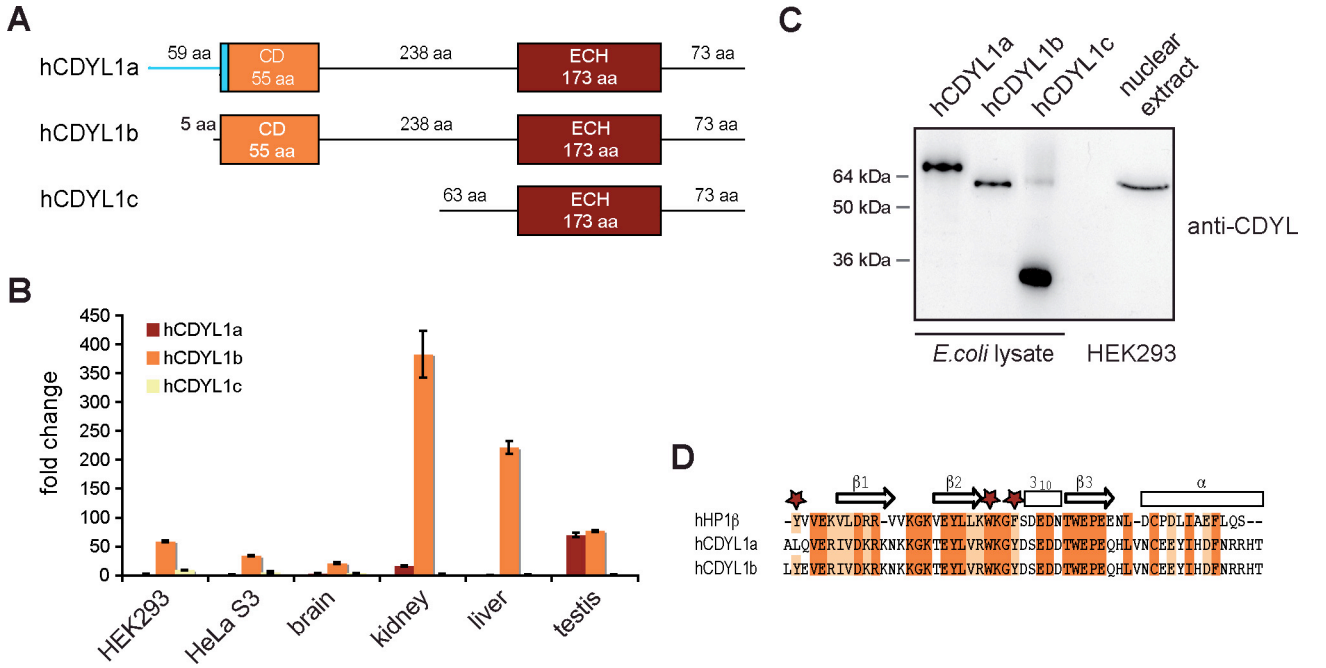

FIGURE 2. **CDYL1b is the major splicing variant.** *A,* schematic representation of the protein domain structure of CDYL1 splicing variants a, b, and c. *CD,* chromodomain. *B,* mRNA levels of CDYL1 splicing variants a, b, and c in human cell lines HEK293 and HeLa S3 as well as in the indicated human tissues were analyzed using real time PCR. Relative expression levels were normalized to the lowest expression (hCDYL1a in HEK293), which was set arbitrarily to 1. *Error bars* represent standard deviation of three independent measurements. *C,* Western blot analysis of *E. coli* cells expressing recombinant CDYL1a, -b, or -c and nuclear extracts of HEK293 cells using anti-CDYL antibodies. *D,* sequence alignment of chromodomains of CDYL1a and -b with the chromodomain of HP1β. Identical residues are highlighted in *red;* homolog residues are highlighted in *orange.* Secondary structure elements of HP1β are indicated on the *top*. *Stars* mark aromatic amino acids forming an aromatic cage for methyl-lysine recognition.

Fluorescence polarization measurements were used to quantify interaction of the CDYL1a and CDYL1b chromodomains to differentially modified histone and non-histone peptides. Fig. 3*B* shows the binding curves to H3 peptides with or without trimethylation on lysine 9. Although the CDYL1a chromodomain had very little affinity for the H3K9me3 peptide over the unmodified counterpart, the CDYL1b chromodomain displayed significant affinity to H3K9me3 but discriminated against the unmodified H3 peptide. In agreement with the results from the pulldown assays, the chromodomain of CDYL1b was found to interact the strongest with the H3K9me3 peptide (see Fig. 3*C* for a summary of all binding data). The measured K_d of 2 μM was highly similar to that reported for the HP1/H3K9me3 interaction (12, 34). Similar to HP1, weaker interaction with the dimethylated (1.5-fold) and monomethylated (4-fold) forms of this site as well as with the H3K27 methylation marks was seen. The chromodomain of CDYL1b recognized H3K4me3 and H4K20me3 peptides with much lower affinity.

A phosphate on serine 10 (H3S10) next to H3K9me3 abolished the affinity of the CDYL1b chromodomain for this modification. H3S10 is phosphorylated at the onset of mitosis by the Aurora B kinase (35). We and others (36, 37) have shown previously that this phosphorylation event delocalizes HP1 from heterochromatin during M-phase. In agreement with a putative regulation of CDYL1b chromatin association by a "methyl-phos switch" (38), immunofluorescence analysis of CDYL1b during mitosis showed the absence of the protein from condensed chromatin during M-phase (data not shown).

Previous studies had identified CDYL1 as the binding partner of automethylation of the G9a histone methyltransferase on lysine 185. However, *in vitro* interaction with the available CDYL1 protein could not be verified (19). In fluorescence polarization assays we found the CDYL1b chromodomain to bind a peptide corresponding to G9aK185me3 with 8 μM affinity. In contrast, the chromodomain of CDYL1a showed no interaction (Fig. 3*C*). Altogether, our analysis demonstrates that only the chromodomain of CDYL1b but not that of CDYL1a can bind differentially methylated lysine residues in distinct histone and non-histone sequence context.

CDYL1b Recognizes H3K9me3 in Chromatin Context—Reduced binding of recombinant HP1 to chromatin templates compared with isolated histone peptides has been reported (39). Because 15–20-aa long peptides do not represent the targeting and binding situation of CDYL1 in the cell, we wanted to analyze H3K9me3 binding of CDYL1b in the context of chromatin. Therefore, we established an *in vitro* chromatin reconstitution system that makes use of uniformly K9me3-modified H3 obtained by native protein ligation (supplemental Fig. S2) (28, 40). Oligonucleosomal arrays on a 12 × 200 × 601 sequence were assembled under template saturating conditions and contained 10–12 nucleosomes in average (supplemental Fig. S3). No differences in the biophysical properties between arrays reconstituted with unmodified H3 or H3K9me3 could be detected (supplemental Fig. S4 and data not shown) (27, 29, 41). To analyze the CDYL1 interaction biotinylated recombinant chromatin with or without the H3K9me3 modification was bound to streptavidin-coated beads. This matrix was used to pull out the binding proteins of HeLa S3 nuclear extracts. After

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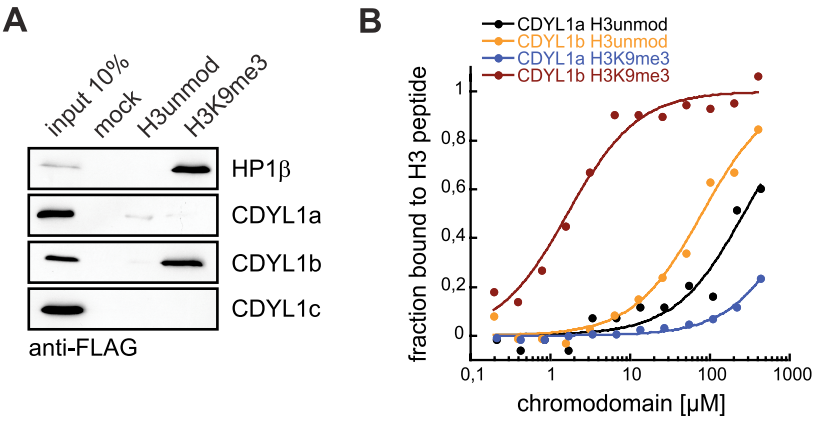

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		CDYL1a K_d (4°C, μM)	CDYL1b K_d (4°C, μM)
H3 Lys 9	unmodified	> 400	87 ± 39
	H3K9me1	—	8 ± 2.5
	H3K9me2	—	3 ± 0.9
	H3K9me3	> 400	2 ± 0.3
	H3K9me3S10ph	—	> 400
H3 Lys 4	H3K4me3	> 400	37 ± 2
H3 Lys 27	unmodified	> 400	> 400
	H3K27me1	—	56 ± 9
	H3K27me2	—	14 ± 3
	H3K27me3	> 400	8 ± 2.3
H4 Lys 20	unmodified	—	368 ± 129
	H4K20me3	—	146 ± 34
G9a Lys 185	unmodified	> 400	> 400
	G9aK185me3	> 400	8 ± 3.2

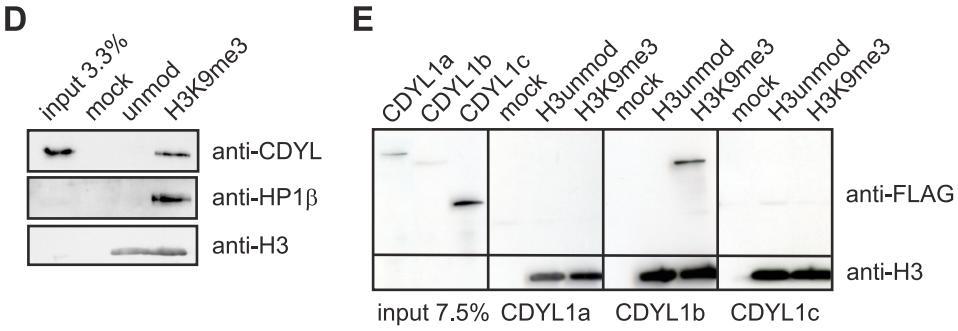

FIGURE 3. **CDYL1b specifically recognizes H3K9me3 in the context of chromatin.** *A,* the indicated FLAG-tagged proteins were produced by *in vitro* translation and tested for interaction with differentially modified H3 peptides using pulldown assays. Beads without coupled peptides were used as control (*mock*). Western blot analysis of the input and bound fractions using anti-FLAG antibodies is shown. *B,* fluorescence polarization binding experiments using the indicated H3 peptides and the recombinant chromodomains of CDYL1a and CDYL1b. The average bound fraction of H3 peptides from three independent experiments is blotted. *C,* summary of K_d values of interaction of recombinant CDYL1a and CDYL1b chromodomains with the indicated peptides as measured by fluorescence polarization binding assays. The average of at least three independent experiments including standard deviation is given. *D,* recombinant 12-mer oligonucleosomal arrays reconstituted with the indicated H3 species were immobilized on agarose beads and incubated with HEK293 cell nuclear extract. Beads without coupled chromatin were used as control (*mock*). Western blot analysis of the input and bound fractions using the indicated antibodies is shown. *E,* CDYL1a, -b, and -c were produced by *in vitro* translation and incubated with recombinant 12-mer oligonucleosomal arrays reconstituted with the indicated H3 species. Recombinant chromatin was precipitated by addition of Mg^{2+} as described under "Experimental Procedures." Western blot analysis of input recombinant proteins and material recovered after centrifugation using the indicated antibodies is shown. *Mock*, precipitation in the absence of added recombinant chromatin.

extensive washing the bound fractions were analyzed by Western blotting. As Fig. 3*D* shows, HP1β and CDYL1 were clearly enriched on the H3K9me3 chromatin template compared with the unmodified control or the mock (beads only) reaction.

To further analyze chromatin binding of CDYL1, we *in vitro* transcribed and translated CDYL1 splicing variants a, b, and c and repeated the analysis using recombinant proteins. Although CDYL1b was able to bind to the H3K9me3 chromatin template, no interaction could be detected for CDYL1a or CDYL1c (Fig. 3*E*).

Heterochromatic Localization of CDYL1b Is Dependent on H3K9me3—Next, we asked whether CDYL1 splicing variants localize to heterochromatic, H3K9me3-enriched loci *in vivo*. Because the commercially available antibodies as well as our specifically raised antisera recognizing CDYL1 failed to detect the protein in immunofluorescence experiments, we transiently expressed FLAG-tagged CDYL1 a, b, or c in MEF WT cells. Immunofluorescence analysis was carried out using antibodies against the FLAG tag and compared with the distribution of H3K9me3 and DNA (Fig. 4*A*). Whereas CDYL1a and CDYL1c were found diffusely distributed throughout the whole cell nucleus only CDYL1b showed a dotted distribution pattern. The regions enriched in CDYL1b-FLAG co-localized with the DNA-dense regions of pericentric heterochromatin as well as with the spots highly enriched in the H3K9me3 modification.

To find out if this localization to areas of heterochromatin is indeed dependent on H3K9me3 modification, we used MEF Suv3–9h1/h2 (−/−) double knock-out cells. Suv3–9h1 and Suv3–9h2 are known as main enzymes establishing the H3K9me3 modification (42). Therefore, MEF Suv3–9h1/h2 (−/−) double knock-out cells show a strongly reduced amount of H3K9me3. The cells nevertheless, sustain the dotted structure of DNA-dense regions

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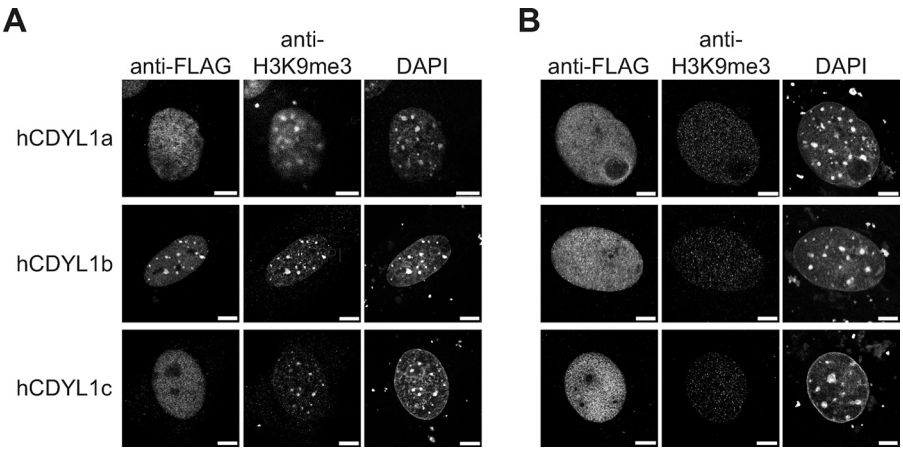

FIGURE 4. **CDYL1b subnuclear localization is dependent on H3K9me3.** *A,* MEF WT cells were transfected with FLAG-tagged CDYL1a, -b, or -c and stained with the indicated antibodies. DNA was visualized using 4′,6-diamidino-2-phenylindole (*DAPI*). *Bars* represent 7.5 μm. *B,* MEF Suv3–9h1/h2 (−/−) double knock-out cells were transfected and analyzed as in *A*.

corresponding to pericentric heterochromatin (43). We transiently expressed the different CDYL1 splicing variants in MEF Suv3–9h1/h2 (−/−) cells (Fig. 4*B*). Although the absence of Suv3–9h1/h2 neither impacted CDYL1a nor CDYL1c subnuclear localization, distribution of CDYL1b was significantly different compared with the wild type situation. CDYL1b did not localize to the DNA-dense heterochromatic areas, but showed a diffuse appearance in the nucleus. According to these observations, CDYL1b is a heterochromatin factor whose localization is dependent on the presence of H3K9me3.

CDYL1b Is Forming Multimers—An identical ECH-like domain is at the C terminus of all CDYL1 splicing variants. Enoyl-CoA hydratase enzymes are known to have besides hydratase activity also dehydrogenase and isomerase activity. During β-oxidation in mitochondria and peroxisomes enoyl-CoA hydratases add water to unsaturated acyl-CoA at the position between the second and third carbon atom within the fatty acid chain (25, 44). Sequence comparison of the CDYL1 ECH-like domain with the human peroxisomal (ECHP) and mitochondrial (ECHM) enoyl-CoA hydratase proteins revealed a good overall homology (Fig. 5*A*). BLAST alignment resulted in 39% identity, 58% homology to hECHM, and 24% identity, 47% homology to hECHP over a stretch of 167 aa. Three residues, one glycine and two glutamines are essential for ECH enzymatic activity (26, 45). In CDYL1 these three residues are exchanged against leucine, serine, and tyrosine, respectively, suggesting a different role for the CDYL1 ECH-like domain. In agreement, closer inspection of the structure of rat ECH with a bound acetoacetyl-CoA (Protein Data Bank code 1dub), which is a potent inhibitor of ECH enzymes (45), revealed that it is unlikely that the ECH-like domain of CDYL1 can catalyze a hydratase reaction (supplemental Fig. S5). Despite these amino acid exchanges, structural superposition of the CDYL1 ECH-like domain (PDB 1gtr) and the rat peroxisomal ECH (PDB 1dub) show that the overall trimeric-fold of these proteins remains unchanged (Fig. 5*B*).

We confirmed multimerization of CDYL1. Anti-FLAG immunoprecipitation of *in vitro* translated FLAG-tagged CDYL1b mixed with *in vitro* translated YFP-CDYL1b contained YFP-CDYL1b as analyzed by Western blotting using anti-green fluorescent protein antibodies (Fig. 5*D*). We then mapped the CDYL1 interaction regions using a series of CDYL1b-FLAG deletion constructs (see Fig. 5*C*). As Fig. 5*D* shows CDYL1 multimerization was depending on the C-terminal part of CDYL1b that contains the ECH-like domain. This region is identical in sequence to CDYL1c (Fig. 5*D*, *CDYL1c*). Multimerization of this 309-aa long region of CDYL1 (see also Fig. 2*A*) was abolished when the 73 aa C-terminal to the predicted ECH-like domain were missing (Fig. 5*D*, *CDYL1cΔCterm*). The isolated ECH-like region as determined by sequence alignment and domain prediction programs were not sufficient for CDYL1 multimerization (Fig. 5*D*, *ECH*). Additional self-binding of CDYL1b was mapped to the protein part covering a fragment of the hinge region and the chromodomain (Fig. 5*D*, *chromohinge*). We conclude that CDYL1 can interact with itself via a C-terminal region containing the ECH-like domain and an N-terminal part of the protein.

Multimerization Is Required for CDYL1b H3K9me3 Chromatin Binding—It has been suggested that simultaneous, multivalent histone modification binding is required to anchor chromatin proteins on their target sites (46, 47). Because CDYL1 shows a strong tendency to multimerize via a C-terminal region containing the ECH-like domain, we reasoned that multimeric presentation of the H3K9me3-binding N-terminal chromodomain might be a prerequisite for stable chromatin association. Reticulocyte extracts programmed to express CDYL1b and different deletion mutants (see Fig. 5*C*) were incubated with oligonucleosomal arrays reconstituted with unmodified H3 or H3 uniformly bearing the K9me3 modification. As Fig. 6 shows only mutant CDYL1 protein chromoCDYL1c, which contains a chromodomain directly linked to the C-terminal region containing the ECH-like domain, interacted robustly with H3K9me3 chromatin, like WT CDYL1b. The H3K9me3 interacting chromodomain is required for this binding as the multimerizing C-terminal region containing the ECH-like domain when linked to the hinge region showed no interaction (Fig. 6, *hingeECH*). Similarly, the chromodomain when linked to the C-terminal region containing the ECH-like domain that misses the very C-terminal part required for multimerization did not bind H3K9me3 chromatin in this assay (Fig. 6, *chromoCDYL1cΔCterm*). In agreement with the multimerization potential of the chromohinge domain mutant, this protein showed weak chromatin interaction (Fig. 6, *chromohinge*). From these experiments we conclude that multimerization is required for stable CDYL1 chromatin binding.

Multimerization Is Necessary for H3K9me3 Localization—To investigate if multimerization has any effect on localization of CDYL1, the subnuclear distribution of different FLAG-tagged

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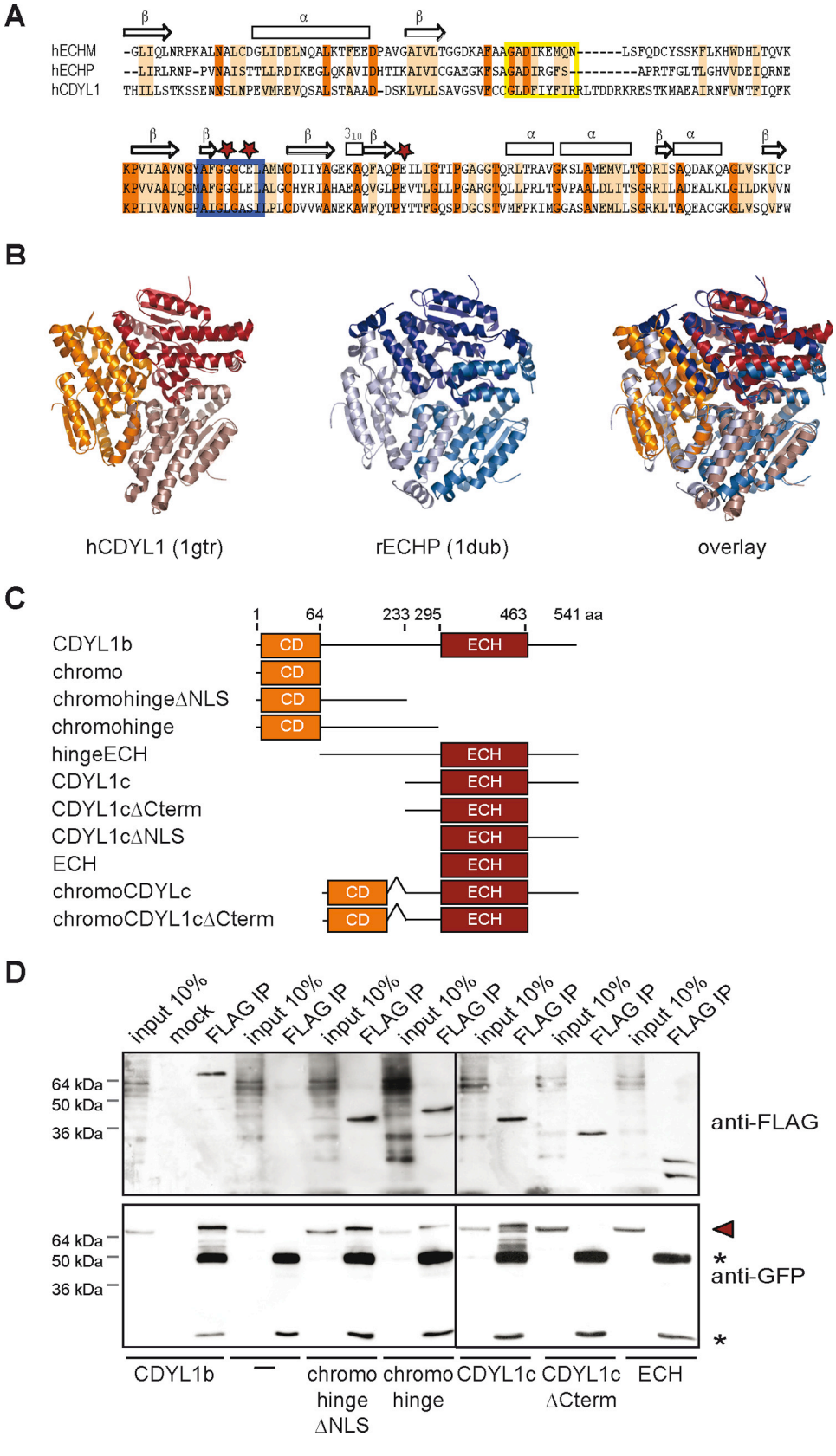
truncated variants of CDYL1b were analyzed in MEF WT cells (Fig. 7*A*, for constructs, see Fig. 5*C*). With the exception of the ECH-like domain alone all mutant proteins showed accumulation in the cell nucleus. Although the chromodomain might accumulate in the nucleus indirectly, the results indicate a putative NLS at the end of the hinge region. Indeed, additional experiments verified a bona fide NLS at residues 235–298 of CDYL1b (supplemental Fig. S6).

Despite the interaction with H3K9me3, immunofluorescence analysis showed that the CDYL1b chromodomain alone is not able to localize to the regions containing pericentric heterochromatin. Also, the chromodomain connected to the following hinge region of the CDYL1 protein (chromohinge) showed no overlap with the DNA-dense regions enriched in H3K9me3. As predicted due to the absence of an H3K9me3 binding domain, the CDYL1b construct missing the chromodomain (hingeECH) showed no preferential localization to heterochromatin. In contrast, the chromodomain connected to the CDYL1c splicing variant (chromoCDYL1c) displayed a dotted localization within the nucleus overlapping with H3K9me3-dense areas. When this chromoCDYL1c construct lacks the very C-terminal part, the spotted distribution is abolished. This 73-aa region at the very C terminus of CDYL1c is necessary for multimerization (see Fig. 5*D*). From this analysis we conclude that the chromodomain of CDYL1b is necessary but not sufficient to localize the protein to H3K9me3 chromatin. Additionally, multimerization of the protein is required to anchor CDYL1b to heterochromatic regions.

Overexpression of CDYL1c Can Displace CDYL1b from H3K9me3 Heterochromatin—As shown in Figs. 5*D* and 7*A*, CDYL1c can interact with itself forming multimers but is not able to localize to H3K9me3 chromatin *in vivo*. We reasoned that CDYL1c could form complexes with CDYL1b thereby negatively regulating CDYL1b chromatin association. Therefore, we transfected myc-tagged CDYL1c and HA-tagged CDYL1b into MEF WT cells at different plasmid ratios. As Fig. 7*B* shows coexpression of CDYL1c at a 1:1 ratio did not impair CDYL1b



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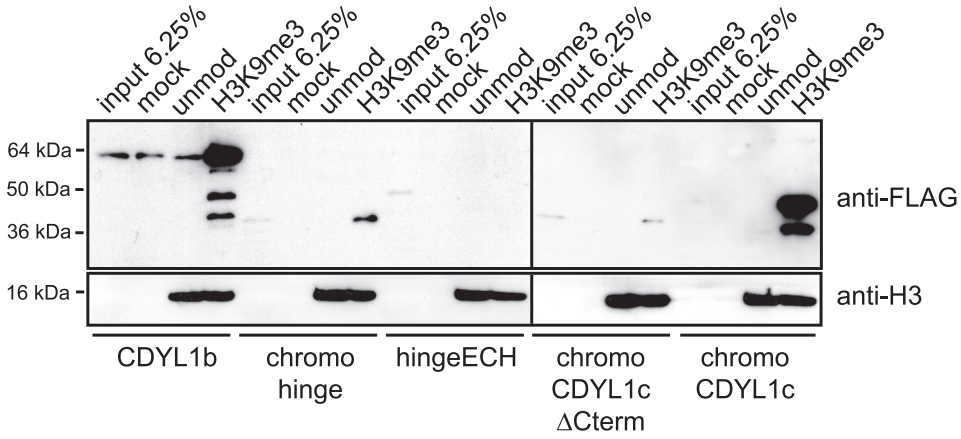

FIGURE 6. **CDYL1b multimerization is required for H3K9me3 chromatin interaction.** The indicated FLAG-tagged XlCDYL1 proteins were produced by *in vitro* translation and incubated with recombinant 12-mer oligonucleosomal arrays reconstituted with the indicated H3 species. Recombinant chromatin was precipitated by addition of Mg^{2+} as described under "Experimental Procedures." Western blot analysis of *in vitro* translated recombinant proteins (*input*) and material recovered after centrifugation using the indicated antibodies is shown. *Mock*, precipitation in the absence of added recombinant chromatin.

localization to the DNA-dense foci of pericentric heterochromatin. In contrast, 80–90% of MEF cells transfected with CDYL1c:CDYL1b at a 100:1 plasmid ratio showed delocalization of CDYL1b from heterochromatin displaying a diffuse CDYL1b signal in the nucleoplasm. Importantly, coexpression of CDYL1cΔCterm, which is neither able to localize to H3K9me3 nor to multimerize with full-length CDYL1b (see Fig. 5*D*), did not have a similar negative effect on CDYL1b localization to heterochromatin. These experiments further sustain the interpretation that an intact chromodomain and multimerization is required for CDYL1b heterochromatin localization.

DISCUSSION

Our detailed analysis of CDYL1 function in the context of H3K9me binding has the following implications: (i) different splicing variants a, b, and c of CDYL1 bind differentially to methylated lysine residues in histones and other proteins. (ii) CDYL1b chromodomain/H3K9me3 interaction is not sufficient for association of the factor with heterochromatin. (iii) Multimerization of CDYL1b is necessary for stable H3K9me3 chromatin association *in vitro* and for H3K9 heterochromatin localization *in vivo*.

The originally described CDYL1 polypeptide corresponds to the less abundant CDYL1a splicing variant (15, 33). CDYL1 was identified and described in different experiments interacting with methylated lysine residues in histones and G9a (18, 19, 48). Although the CDYL1 splicing variant was not specified, interaction in the case of methylated G9a could not be verified using recombinant proteins (19). We think that it is likely that CDYL1a and not CDYL1b was used in these studies. Indeed, the chromodomains of both CDYL1b and CDYL1a after restoration of the aromatic cage by mutagenesis bind a methylated G9a peptide with affinity comparable with methylated histone peptides *in vitro* (this study and see Ref. 17). At present, it is unclear whether the N-terminal extension of CDYL1a compared with CDYL1b brings additional functionality to the protein, potentially establishing a different role for the methyl-lysine binding deficient chromodomain.

Biochemical evidence has been provided that places CDYL1 as a bridge between G9a and REST in a WIZ and histone modifying enzymes containing corepressor complex (21, 22). Although direct interaction of CDYL1 and G9a could be demonstrated using recombinant proteins, it is tempting to speculate that this interaction is *in vivo* further controlled by G9a automethylation and CDYL1b chromodomain binding. Methylation of CDYL1 might also play a role because *in vitro* G9a can target CDYL1 for methylation at a lysine residue C-terminal to the chromodomain. It has been suggested that this post-translational modification might negatively regulate the CDYL1/H3K9me3 interaction (18). However, as the observed effect was only 2-fold and as CDYL1a was used for these studies, the exact relation of G9a, CDYL1, and their methylation within the REST-CoREST complex needs further investigation. In any case, it has to be noted that a large fraction of CDYL1b exists outside of the REST-CoREST complex, likely targeting other methyl-lysine residues (see Ref. 22).[5]

Besides the chromodomain the ECH-like domain is likely central to CDYL1 and CDY protein family function. These regions show a very high degree of conservation, whereas the linking hinge region is more variable. It was originally suggested that the ECH-like domain of CDY and CDYL1 might have intrinsic histone acetyltransferase activity (24). However, structural analysis and modeling makes it unlikely that acetyl-CoA is indeed a substrate of the CDYL1 ECH-like domain (49). Indeed, we have failed to reproduce histone acetyltransferase activity of CDY family proteins.[5] Nevertheless, it was found that the CDYL1 ECH-like domain interacts with CoA. Although CoA can indeed be modeled onto the structure of CDYL1 it remains

[5] H. Franz and W. Fischle, unpublished observations.

FIGURE 5. **CDYL1b multimerizes via its ECH-like domain.** *A,* sequence alignment of the human CDYL1 ECH-like domain with mitochondrial (*ECHM*) and peroxisomal enoyl-CoA hydratases (*ECHP*). Identical residues are highlighted in *red*; homolog residues are highlighted in *orange*. Secondary structure elements of ECH enzymes are indicated on the *top*. *Stars* mark residues directly involved in ECH catalysis. *Blue box,* catalytic center; *yellow box*, adenine binding site. *B,* tertiary and quaternary structures of human CDYL1 (1gtr, *red*) and rat ECHP (1dub, *blue*) as determined by x-ray crystallography are shown. The image on the *right* represents an overlay of hCDYL1 and rECHP structures. *Different color shading* indicates different polypeptides. *C,* schematic representation of XlCDYL1 constructs used for immunoprecipitation and immunofluorescence experiments (see *D*, and Figs. 6 and 7*A*). Domain boundaries of the mutant proteins are as indicated by the aa positions. *D,* the FLAG-tagged CDYL1 proteins indicated on the *bottom* were produced by *in vitro* translation together with full-length YFP-CDYL1b. Expressed proteins (input) as well as material bound to beads (mock) or anti-FLAG antibodies (*FLAG IP*) were analyzed by Western blotting using the indicated antibodies. *Arrowhead* marks position of YFP-CDYL1b; *asterisks* mark antibody heavy and light chain.

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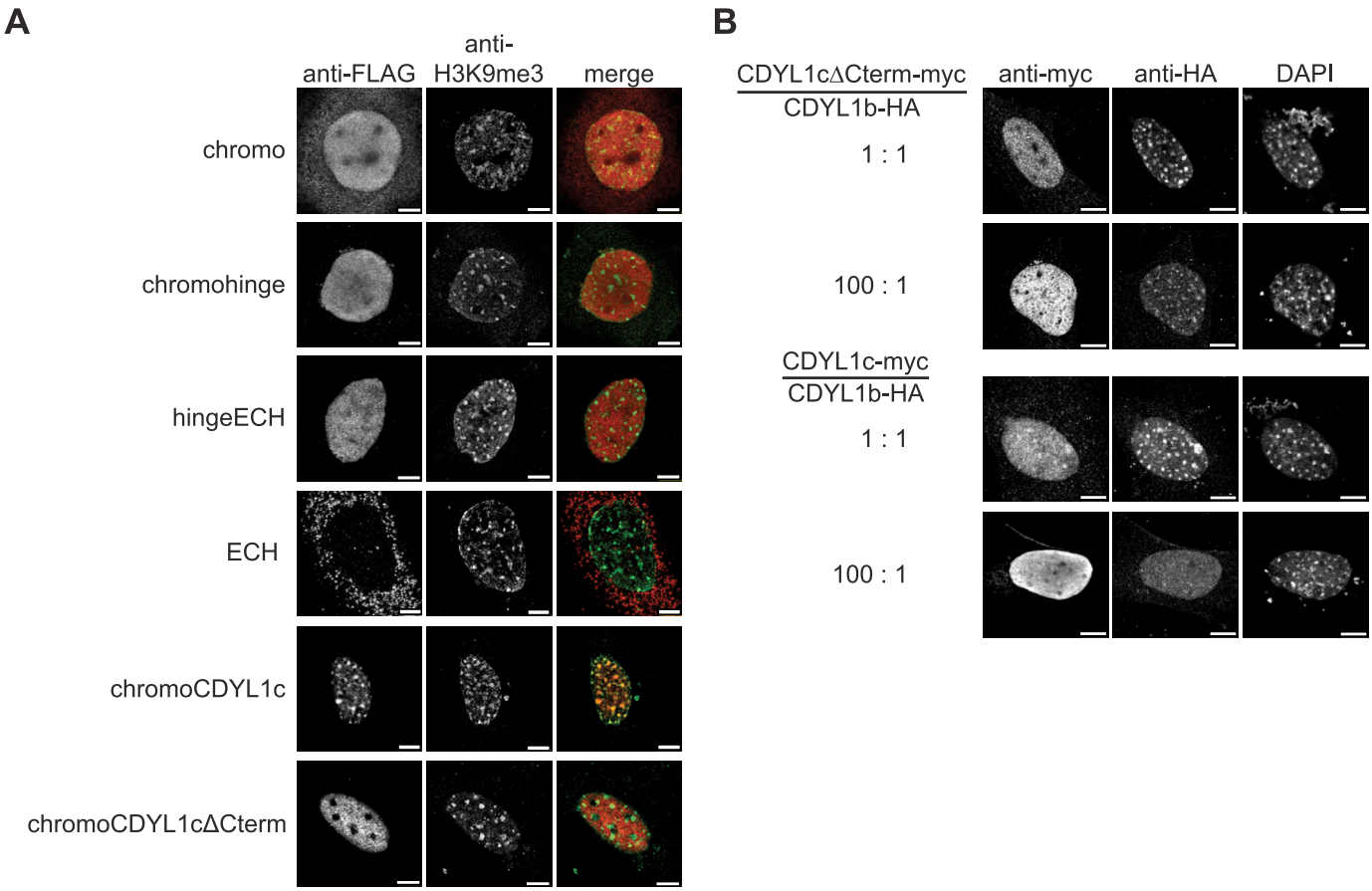

FIGURE 7. **CDYL1b subnuclear localization is dependent on multimerization.** *A,* the FLAG-tagged CDYL1 proteins indicated on the *left* were transiently expressed in MEF WT cells. Immunofluorescence analysis was carried out using the indicated antibodies. Images representative of a large number of analyzed cells are shown. *B,* MEF WT cells were transfected using the indicated ratios of CDYL1cΔNLS-myc and CDYL1b-HA or CDYL1c-myc and CDYL1b-HA plasmids. Immunofluorescence analysis was carried out using the indicated antibodies. DNA was visualized using 4′,6-diamidino-2-phenylindole (*DAPI*). *Bars* represent 7.5 μm.

to be determined whether CoA is a substrate, substrate carrier, or possibly an allosteric regulator.

Our studies indicate that a major function of the ECH-like domain resides in multimerization. Indeed, only CDYL1 that can interact with itself is directed to H3K9me3 heterochromatin, but a functional chromodomain on itself is not capable of this targeting. Although the CDYL1b chromodomain/H3K9me3 interaction at 2 μM is comparable with other chromodomain methylhistone peptide interactions (12, 34), it is nevertheless, weak. We hypothesize that CDYL1b protein multimerization generates a multivalent binding mode that significantly enhances interaction (46). Similar behavior has been suggested for HP1, which dimerizes via a C-terminal chromoshadow domain. Mutations that affect HP1 dimerization as well as overexpression of the chromoshadow domain alone cause dissociation of HP1 from heterochromatin (50, 51). Further studies are required to directly analyze and quantify the impact of ECH-like domain multimerization onto CDYL1b/H3K9me3 chromatin binding.

Our results also show that multimerization of CDYL1 might not be limited to the ECH-like domain, but could also be mediated by additional regions of the protein. Although it is currently unclear how many CDYL1 polypeptides do associate to form chromatin binding complexes, structural analysis of the homologous CDY has found evidence for hexamerization beyond trimerization (PDB 2fw2) (49). Obviously, stronger chromatin interaction will result from the presence of extra H3K9me3 binding sites if additional associations are sterically possible. Indeed, multiple histone modification binding domains in complexes or multimerization of individual chromatin readout factors could be key in reading and translating histone modifications by establishing robust interaction. Enhancement of binding via multimerization could possibly explain the obvious strong targeting of CDYL1b to sites of H3K9me3 *in vivo* compared with not much weaker binding to H3K27me3 *in vitro*.

Another aspect of CDYL1b multimerization besides generating binding strength could be affecting chromatin structure and behavior by binding multiple regions simultaneously. Although multivalent binding of chromatin factors to histone modifications has not yet been demonstrated and whereas effects of multiple binding events have not been analyzed, we wonder whether such interaction could be involved in establishing or maintaining higher order chromatin structures. In this sense CDYL1 might act as a chromatin architectural protein enhancing the transition of 10-nm fibers to 30-nm structures and higher order chromatin arrangements (52).

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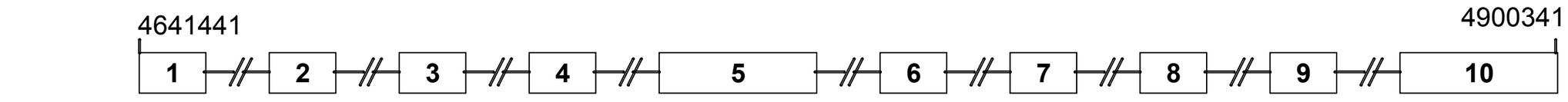
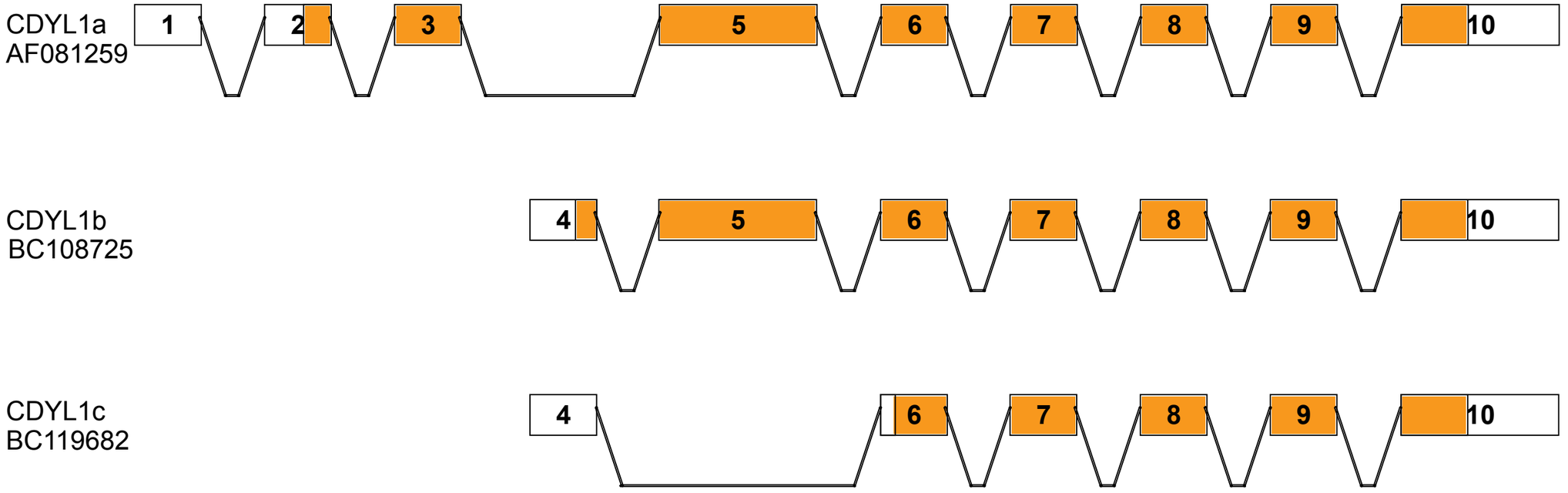
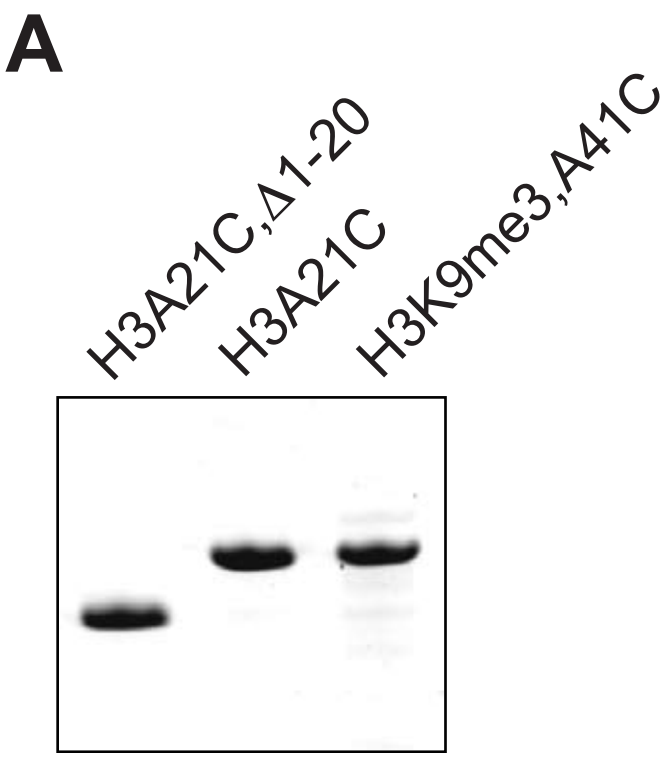
Homo sapiens Chromosome 6
CDYL locus

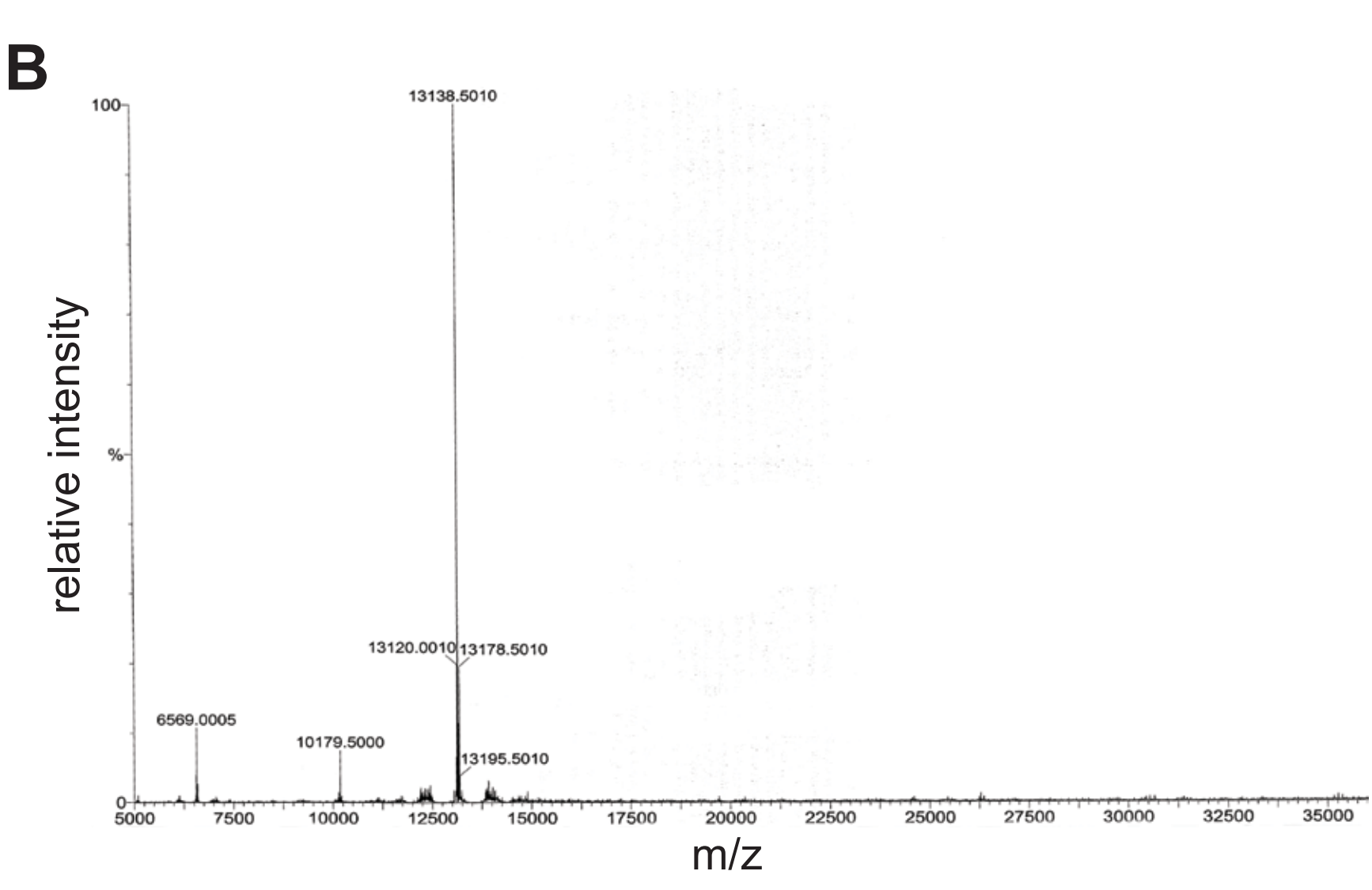


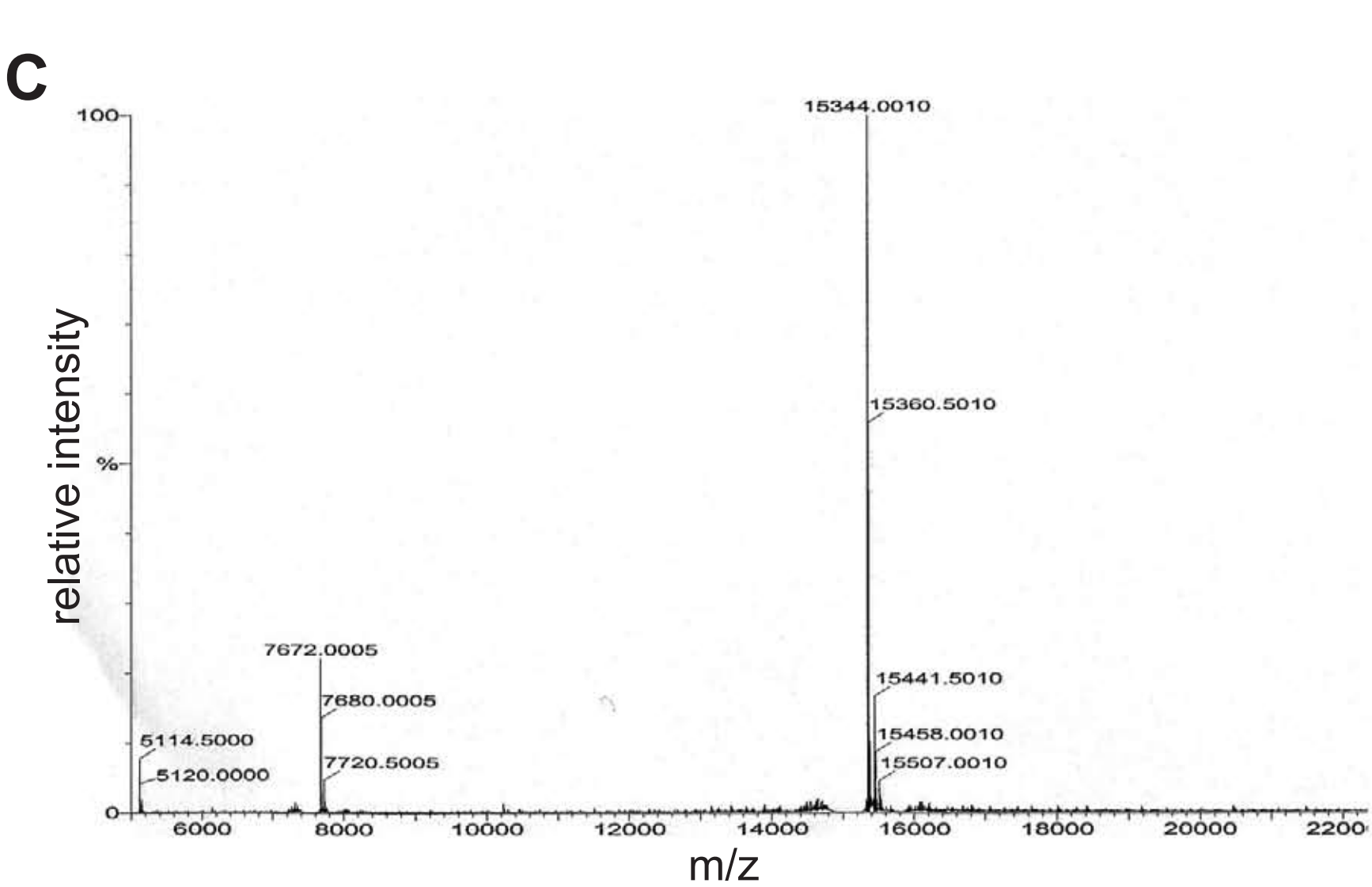
spliced mRNA



Franz et al. Supplementary Figure 2







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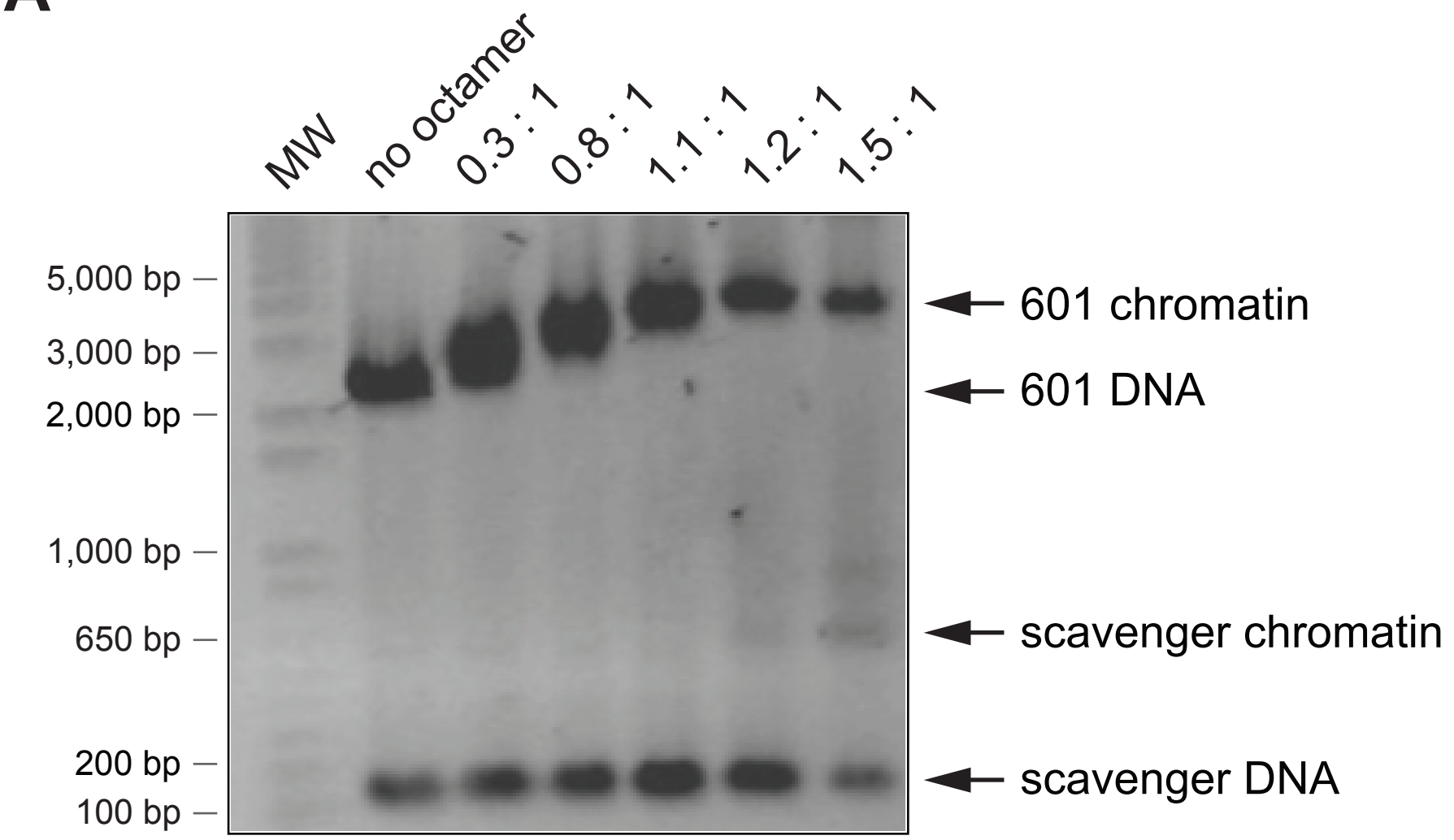
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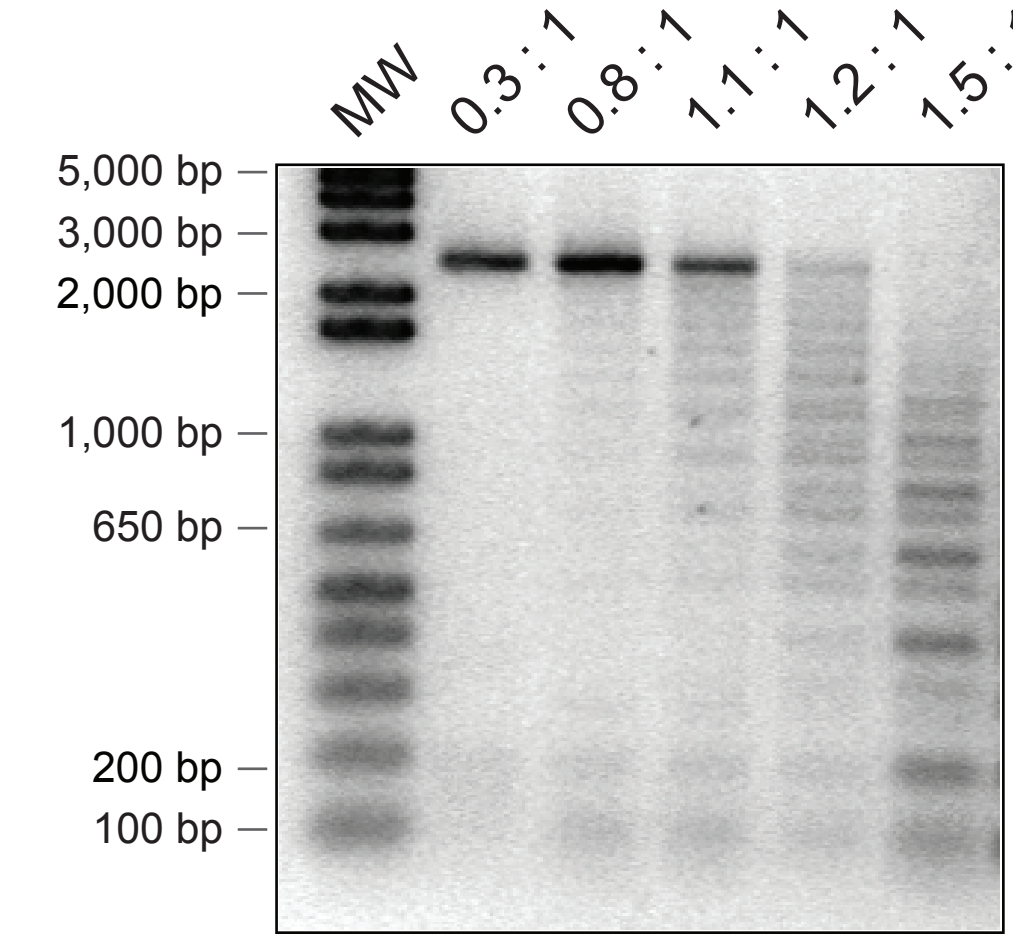
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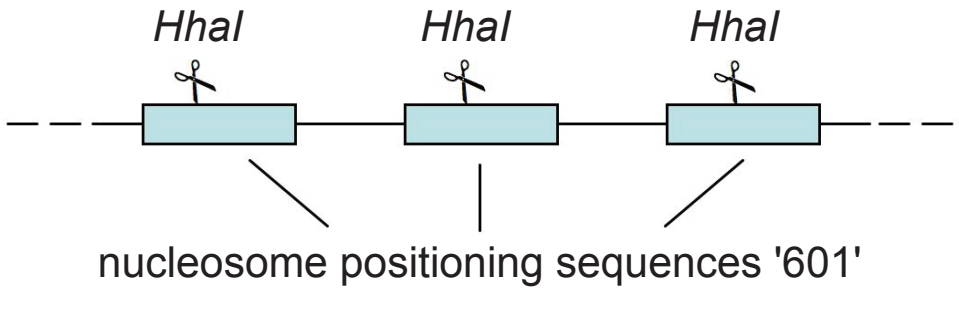
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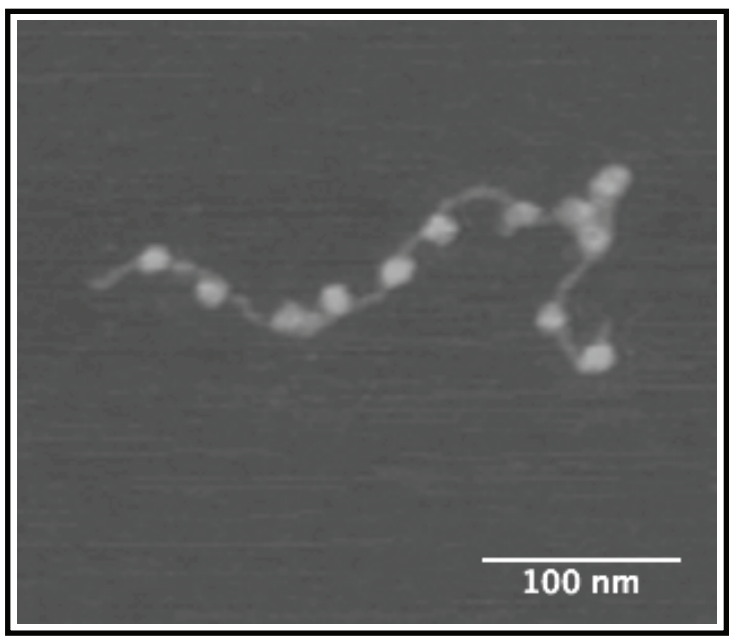
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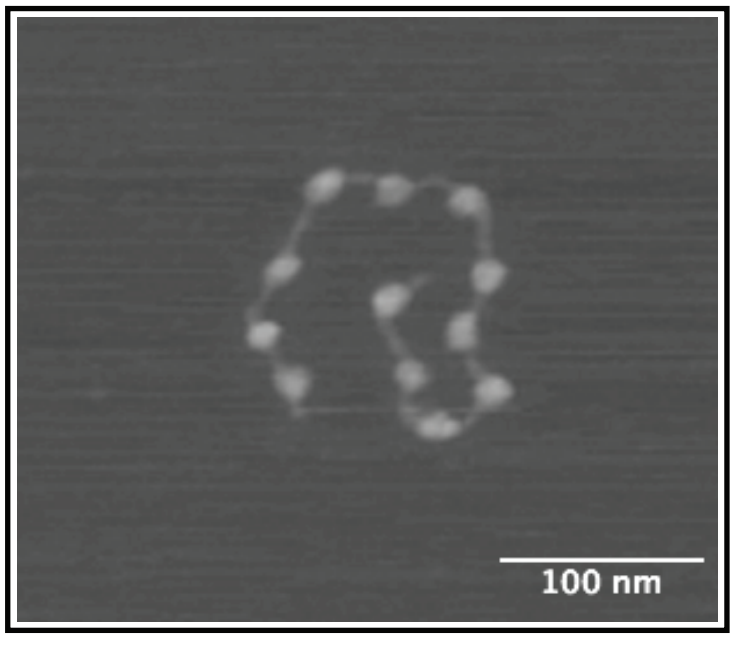
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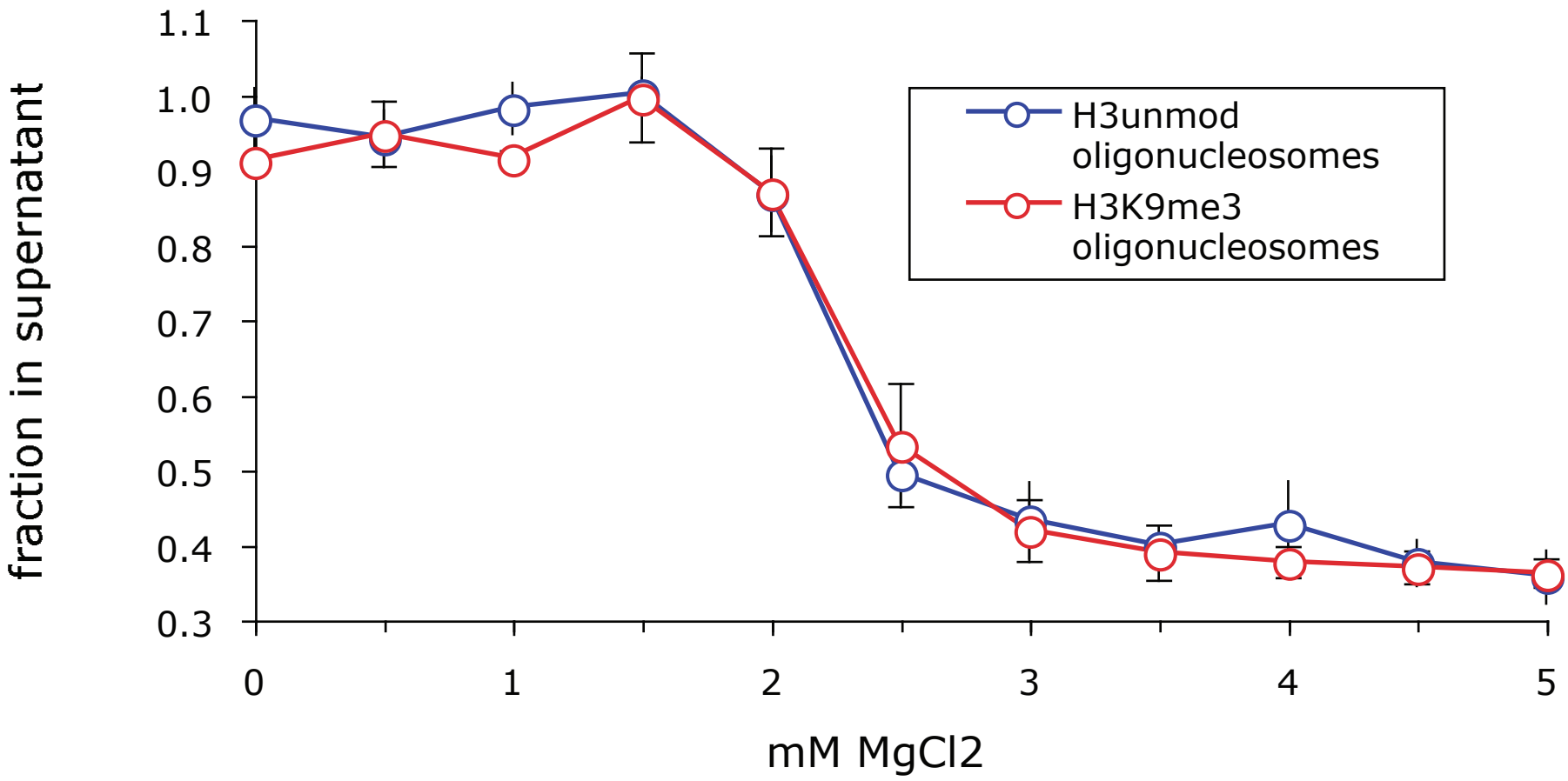
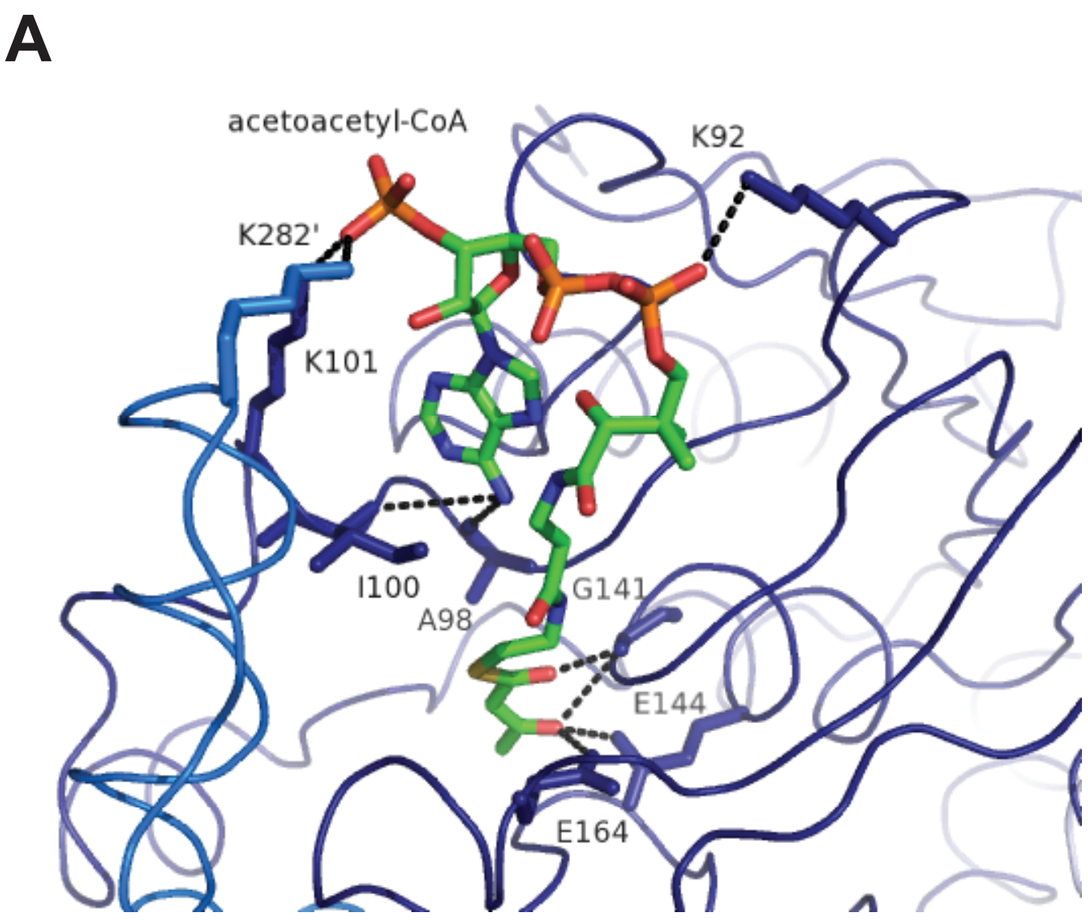
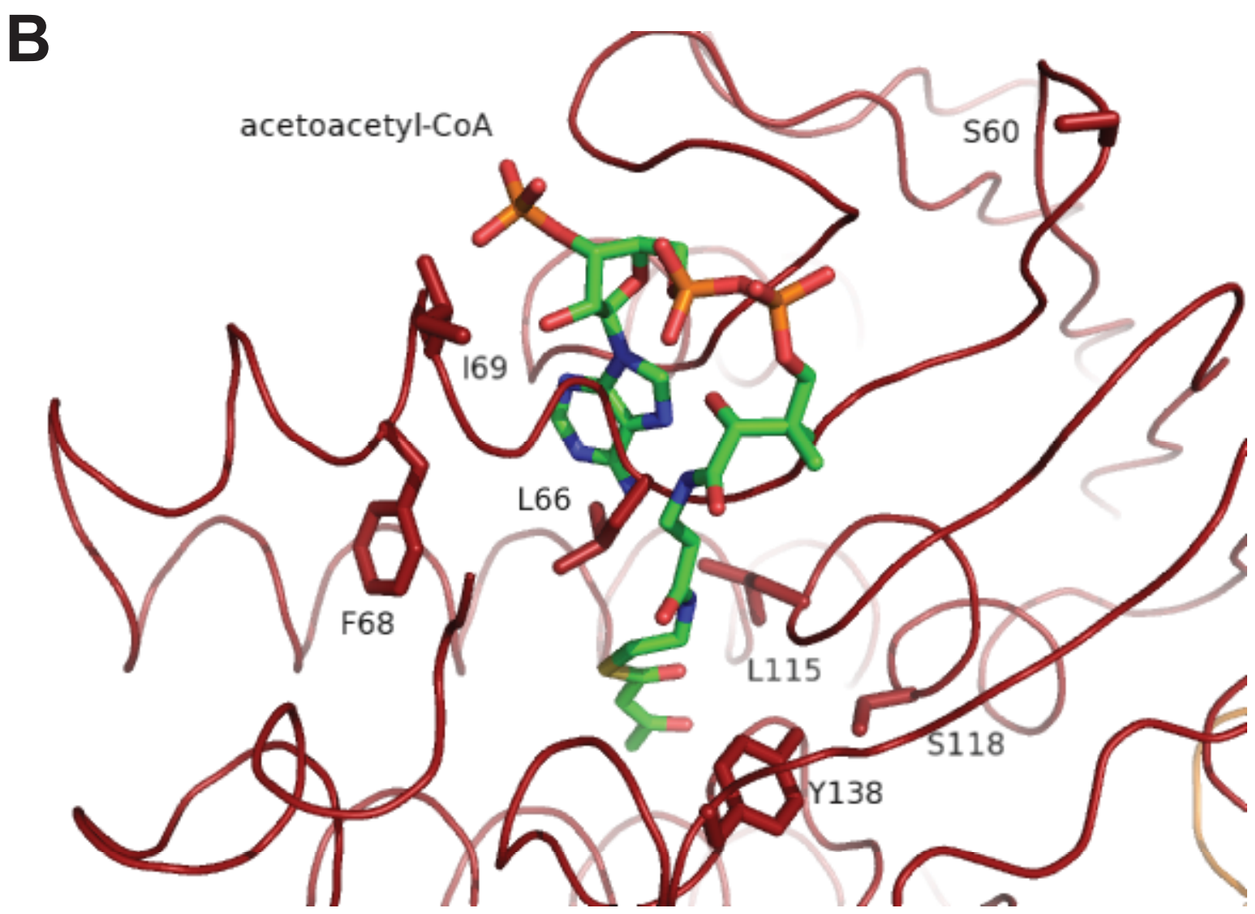
Franz et al. Supplementary Figure 4

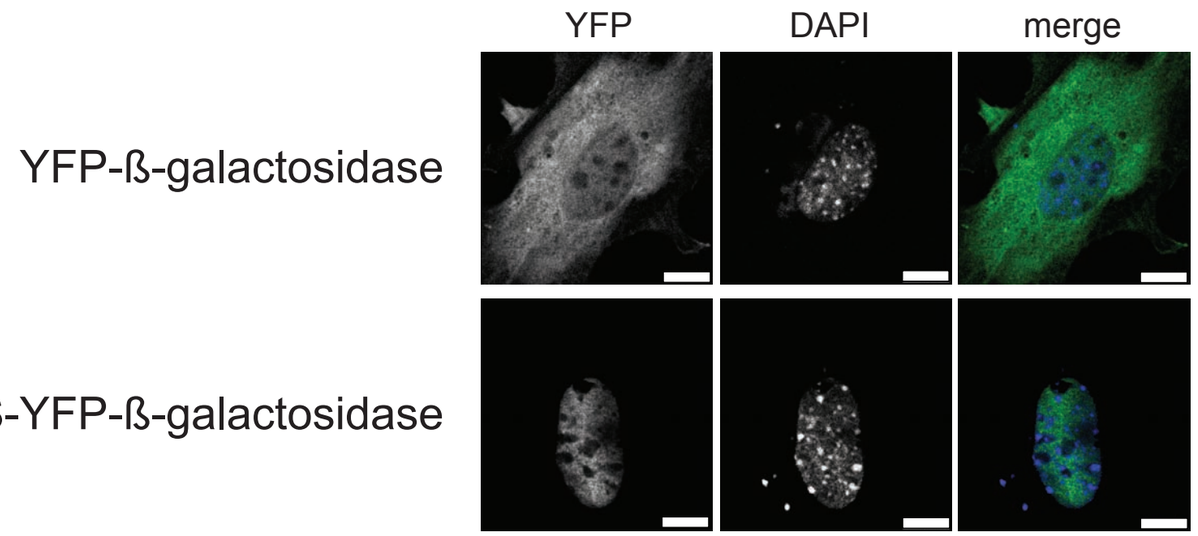


Franz et al. Supplementary Figure 5





Franz et al. Supplementary Figure 6



5 Additional results

5.1 CDYL1b function at heterochromatin

Proteins of the CDY family were shown to function as histone acetyltransferases in human spermatogenesis [114]. Expression of histone acetyltransferases in cells lead to an enrichment of the overall acetylation status of chromatin [116]. To test if CDYL1b is also a histone transferase in somatic-like cells, overexpression experiments in MEF cells were carried out.
In addition chromatin effector proteins such as histone deacetylases HDAC1 or HDAC2, histone methyltransferase G9a and histone demethylase LSD1 were found to interact with CDYL1 directly or within a complex [62, 101, 102]. Histone deacetylases reduce the acetylation status of histone 3 [117, 118], G9a is able to methylate the lysine 9 of histone 3 [119, 120] and LSD1 removes the active mark methylation of lysine 4 of histone 3 [121]. These findings lead to the second hypothesis that CDYL1 might function as recruitment platform within heterochromatic regions. Thus different amounts of CDYL1b on heterochromatic regions could result in changes in the overall modification status of histones.
Thirdly, one former study revealed that CDYL1 is able to repress transcription [101] with its C-terminal domain. To verify these results and to map the repressive regions in CDYL1 proteins, luciferase assays were accomplished.

5.1.1 CDYL1b expression has no influence on histone modifications

To test if CDYL1b is a histone acetyltransferase and acts as recruitment platform for heterochromatic factors, FLAG-tagged CDYL1b was expressed in MEF cells. CDYL1b was stained with an antibody against its FLAG tag and the DNA with DAPI. Several histone modifications were detected with specific antibodies. The level of histone acetylation, H3K4me1, H3K4me2, H3K4me3, H3K9me1, H3K9me2 and H3K9me3 was analyzed on a single cell level (Figure 5-1). Representative pictures are shown and reveal that the variability of the inspected histone modification status is not dependent on CDYL1b expression.
Although it is still possible that CDYL1b acts as histone acetyltransferase or as recruitment factor for chromatin modifying enzymes in special cases, these experiments show that the modulation of histone modification level is not changed by CDYL1b expression.

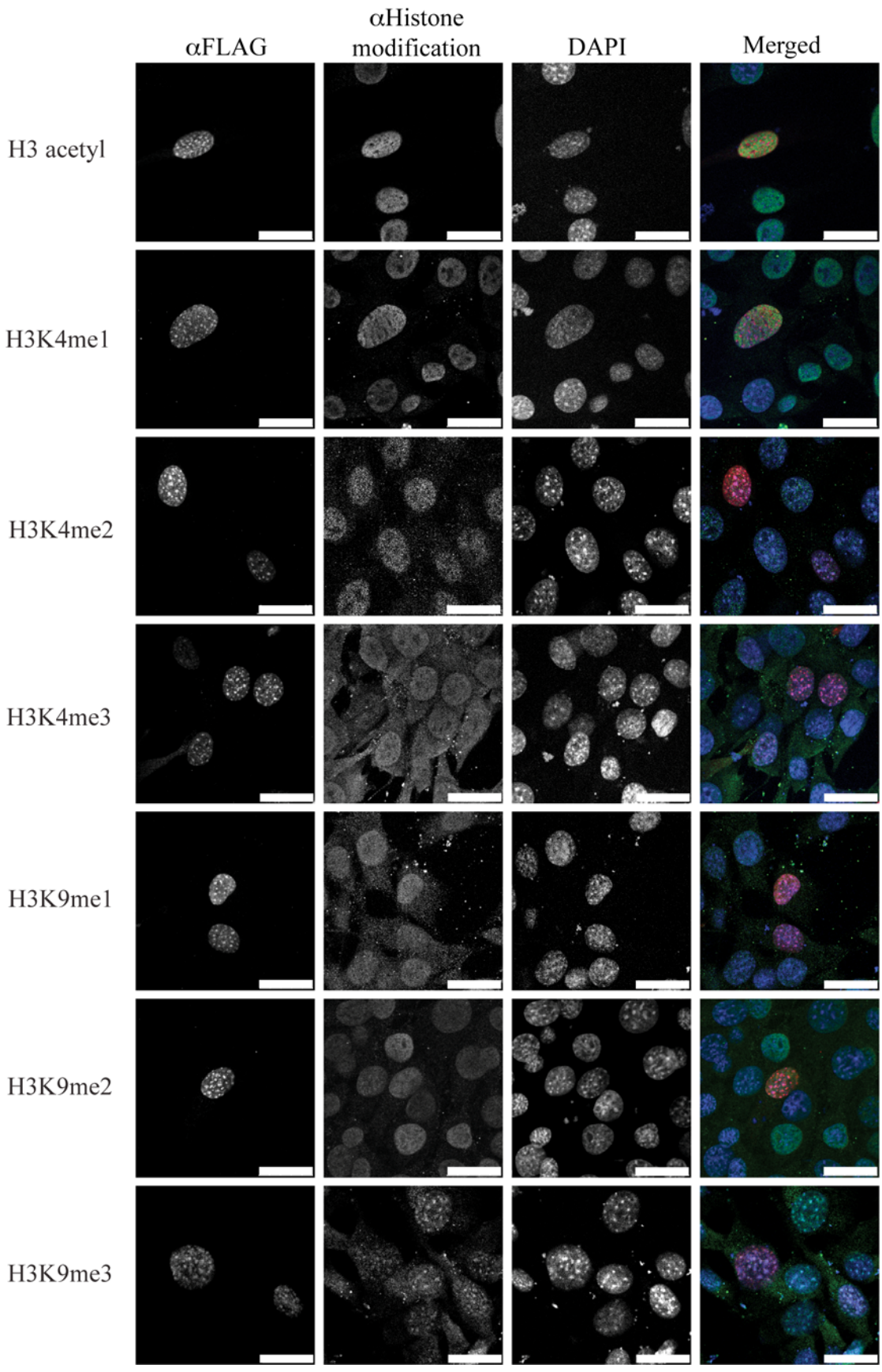


Figure 5-1 CDYL1b overexpression does not influence overall histone modification level

NIH3T3 cells were transfected with FLAG-tagged CDYL1b and stained with αFLAG, antibodies against the represented histone modifications and DAPI as DNA-coloring dye. Representative pictures for each histone modification are shown. Bar = 25µm.

5.1.2 CDYL1 has at least two repressive activities

Previous studies revealed that CDYL1 is able to repress transcription [101] with its C-terminal domain. To map the repressive activity of the protein, different GAL4-tagged constructs of CDYL1 were cloned (compare with chapter 7.2.2). These constructs were transfected into HEK293 cells together with a plasmid containing the GAL4-binding sites UAS in front of the tyrosine kinase promotor and following the firefly luciferase gene. A plasmid containing a renilla luciferase gene controlled by a CMV- promotor was used as an intrinsic transfection efficiency measurement. The luciferase expression was determined by substrate turnover, which produces countable luminosity. The firefly luminosity was then normalized to the renilla signal as well as to untransfected controls, determining the background signal.

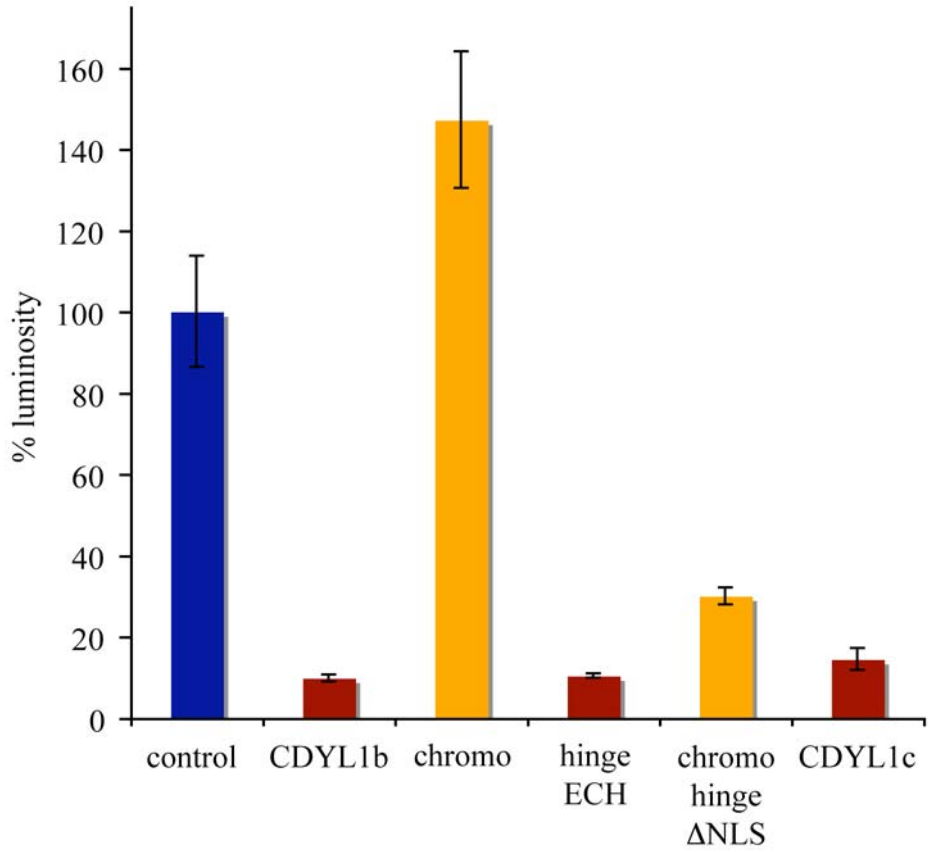


Figure 5-2 CDYL1b harbors two repressive activities.

Dual luciferase assay with GAL4-tagged CDYL1b constructs cloned from the *Xenopus laevis* CDYL1b (compare also with 7.2.2 and 7.4.6). Error bars represent s.e.m. from three independent experiments.

This dual luciferase assay showed clearly (Figure 5-2) that GAL4-tagged CDYL1b is able to repress firefly luciferase transcription compared to the control. The chromodomain alone

improves the transcription of the reporter about 1.4 fold in contrast to the hingeECH, which combines the whole repressive activity of the protein. Interestingly, two different constructs, one containing the chromodomain and a part of the hinge region and the other one harboring the whole part of the CDYL1c variant, were both able to repress the firefly luciferase transcription. These observations reveal that CDYL1b has at least two transcriptional repressor activities.

5.2 CDYL1b regulation

At the onset of mitosis Aurora B phosphorylates serine 10 to induce phosphorylation dependent chromatin changes [122]. This serine 10 phosphorylation mark prevents the heterochromatic protein HP1 from binding to H3K9me3 modification [27, 123].
Fluorescence polarization experiments described in chapter 4 show that the chromodomain of CDYL1b binds with a K_d of 2 μM to a histone H3 peptide (aa 1-20) carrying a trimethylation on lysine 9. Phosphorylation of the neighboring serine 10 abolishes this interaction (K_d value of above 400 μM). To test if serine phosporylation also inhibits CDYL1b localization to H3K9me3 enriched loci in cell culture, immunofluorescence experiments were performed.
In addition, it was shown that heterochromatic regions are sequestered to the nuclear periphery and that the localization of the chromatin correlates with the transcriptional activity of the region [124]. Thus repressed sections of the DNA are found close to the nuclear membrane whereas transcribed regions are located within the nucleoplasm in much closer proximity to the center of the nucleus. The targeting of CDYL1b to the promotor of a luciferase leads to repression of its transcription (Figure 5-2). Thus it is possible that also CDYL1 acts as a sequestering factor for genes that are actively repressed.

5.2.1 CDYL1b is displaced from chromatin during mitosis

To test if histone H3 serine 10 phosphorylation regulates CDYL1b localization, MEF cells were transfected with FLAG-tagged hCDYL1b and stained with αFLAG and αH3K9me3S10ph. Cells in different phases during mitosis such as G2/M transistion, metaphase, anaphase and telophase as defined by DNA staining were imaged by confocal microscopy (Figure 5-3). Thus CDYL1b is displaced from chromatin when serine 10 is phosphorylated at the beginning of mitosis (G2/M transition). Dephosphorylation of histone H3 serine 10 in telophase is correlated with the relocalization of CDYL1b to chromatin.

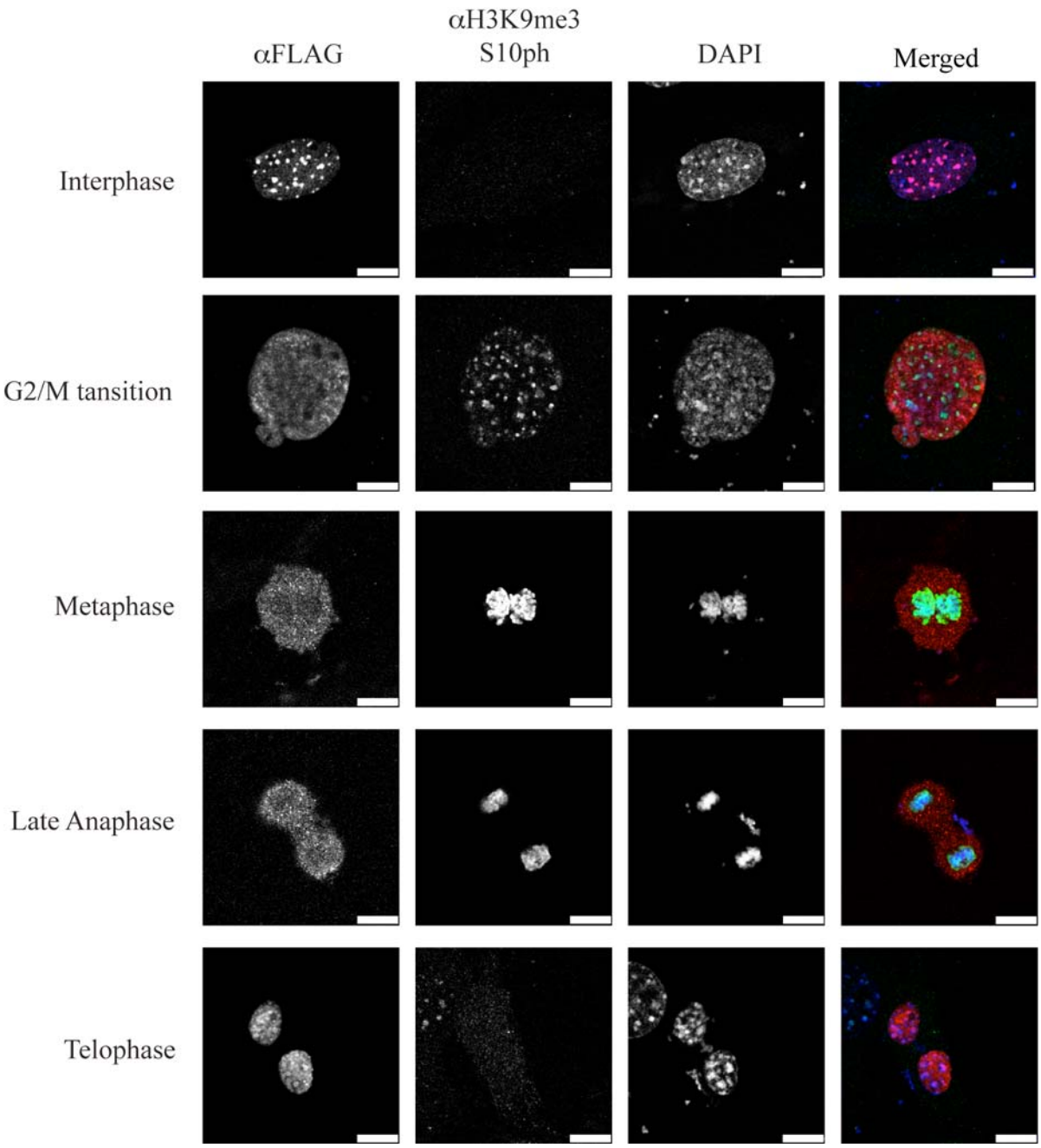


Figure 5-3 CDYL1b delocalizes from chromatin during mitosis

MEF cells were transfected with FLAG-tagged hCDYL1b and stained with antibodies against the FLAG tag and the histone H3 carrying a tri-methylation on lysine 9 and a phosphorylation on the neighboring serine 10. The DNA was stained with DAPI. Representative cells in different stages during mitosis are shown.

Together with the findings that CDYL1b chromodomain bind to H3K9me3 peptides but not to H3K9me3S10ph peptides desribed in chapter 4, these results provide evidence that CDYL1b binding to heterochromatin is regulated by histone H3 serine 10 phosphorylation.

5.2.2 CDYL1b associates with the nuclear membrane/matrix

To test if CDYL1 is sequestered to a special nuclear compartment, fractionation experiments were carried out. Membranes belong to the insoluble fraction in a classical nuclear extraction experiment (Figure 5-4). In contrast to Lamin B, CDYL1b was extracted from the insoluble fraction with a buffer containing 420 mM salt.

To answer the question if CDYL1 might be associated with membranes, a membrane extraction experiment was performed. For this experiment the prepared HeLaS3 nuclei were treated with the DNA/RNA digesting enzyme Benzonase. A centrifugation step pelleted the membrane and matrix proteins whereas chromatin or DNA-only associated proteins remained in the supernatant. Therefore proteins such as the TATA-box binding protein TBP are mainly solubilized and can be detected in the nuclear soluble fraction. The core histone H2A was found within the nuclear soluble fraction as well as associated with membranes.

Interestingly, CDYL1 was not separated from the membranes by this treatment. CDYL1 stayed associated with the Lamin B containing portion of the cell. To test at which salt concentration the association of CDYL1 to membranes is disrupted, the membranes were incubated with different salt concentrations. Lamin B as well as histone H3 were associated with the isolated membranes until a concentration of 500 mM salt.

In contrast to that the heterochromatic protein LSD1, lysine-specific demethylase 1, and CDYL1 were partially washed off the membranes at 300 mM salt. Above 400 mM CDYL1 was completely detached from membranes. The observation that the CDYL1-membrane association was dependent on salt concentration excludes hydrophobic interactions with the membrane. Nevertheless it is possible that CDYL1 binds to membrane associated factors or nuclear matrix proteins via ionic bonds, which are highly sensitive to salt concentrations.

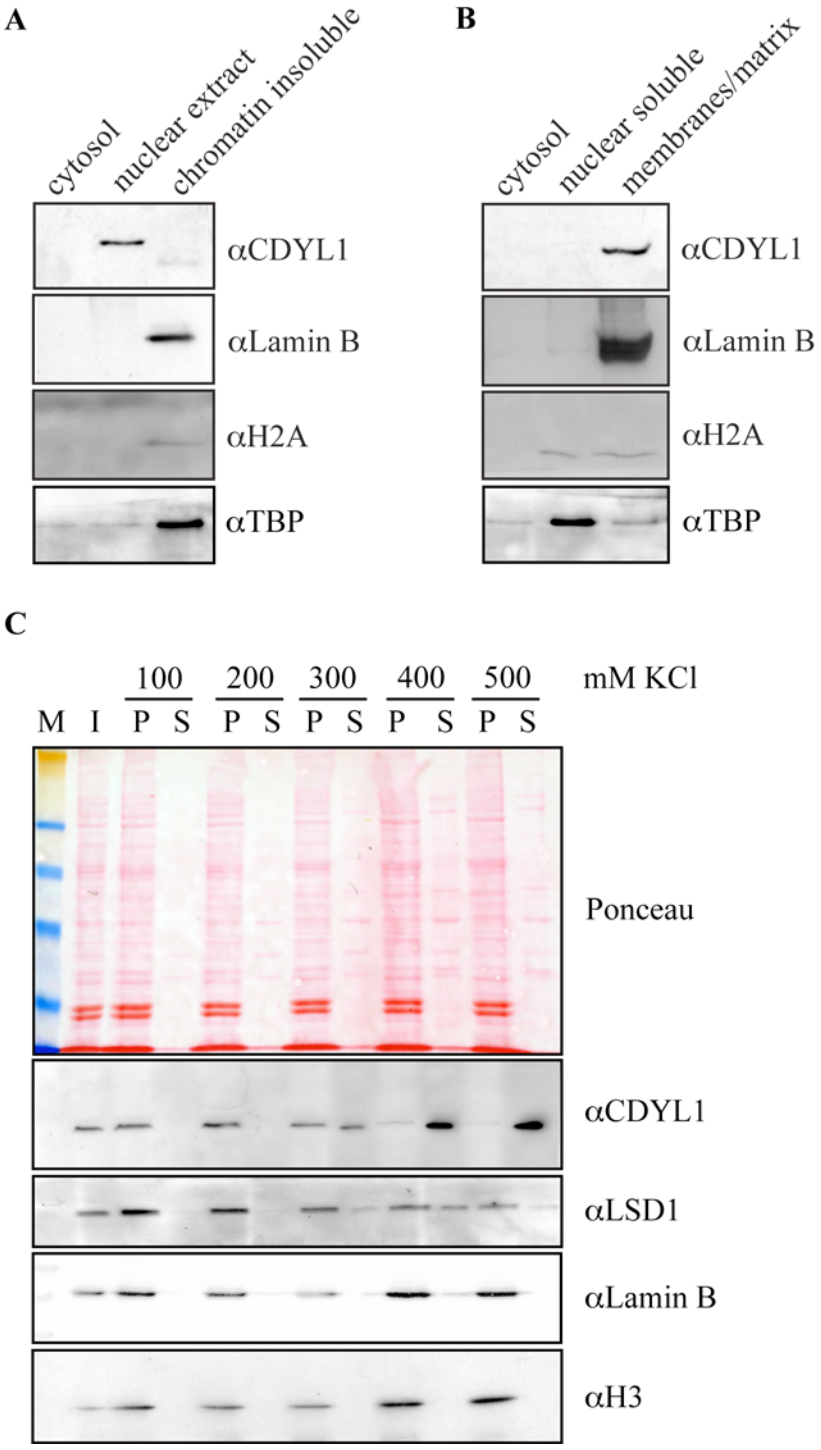


Figure 5-4 CDYL1 associates with nuclear membranes

A, nuclear extraction of HeLaS3 cells. Western Blot of the cytosol, nuclear proteins extracted with 420 mM salt (nuclear extract) and the insoluble chromatin fraction is shown examined with antibodies against CDYL1, Lamin B, TATA-box binding protein TBP and histone H2A. **B,** membrane extraction of HeLaS3 cells. Cytosol, soluble nuclear protein extracted using Benzonase digestion, and the nuclear membrane/matrix fraction were blotted and stained with the same antibodies as in A. **C,** membrane treatment with different salt concentrations. Membranes were extracted with increasing amounts of salt. From each extraction step pellet and soluble fraction are probed. Western Blots of CDYL1, LSD1, Lamin B and histone H3 were performed. Ponceau stain of the Western Blot membrane inidicated equal loading of the gel. M: marker, I: 20% input.

To map the region of CDY that associates with membranes, the FLAG-tagged splicing variants CDYL1a, b, c were transfected into HEK293 cells and were used for membrane isolation experiments. As presented in Figure 5-5 CDYL1a and b showed no differences in membrane/matrix association. Interestingly, the CDYL1c splicing variant was not enriched in the membrane but in the nuclear soluble fraction. This leads to the conclusion that the chromohinge region of the CDYL1 protein might be necessary to establish an interaction to the nuclear membrane or with the nuclear matrix.

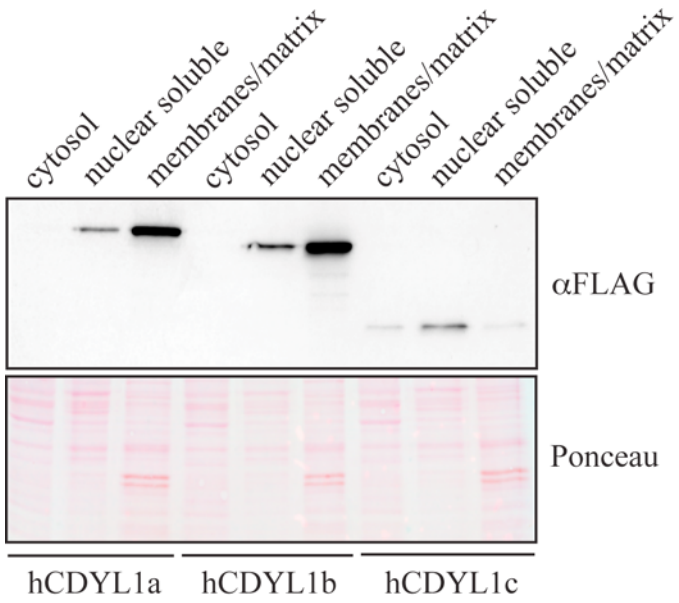


Figure 5-5 CDYL1a and b are membrane associated

HEK293 cells were transfected with FLAG-tagged CDYL1a, b or c. The nuclei of the cells were extracted and the cytosol was saved. The chromatin was digested with Benzonase. The nuclear remnants such as membranes and matrices were pelleted via centrifugation. The supernatant contained the soluble nuclear proteins.

5.3 CDYL1b is a substrate of PRMT5

As described in chapter 1.2.5, fractionation experiments of cellular extracts revealed that CDYL1 is not only a part of multiprotein complexes such as CoREST. A large portion of CDYL1 is not co-fractionated with the complex proteins. This observation leads to the hypothesis that CDYL1 has other functions than acting in the CoREST complex and possibly has also other interaction partners. It is also likely that these interaction partners support or even mediate the transcriptional repression seen with CDYL1 targeting. To test this hypothesis different strategies of finding new interaction partners of CDYL1 were conducted.

5.3.1 Identification of PRMT5 as interaction partner of CDYL1b

To identify new interaction partners of CDYL1b, two different strategies an indirect pulldown and an immunoprecipitation (IP) were carried out. First, pulldown experiments were performed using bead-coupled histone H3 peptides in HeLaS3 nuclear extracts. Peptides unmodified or trimethylated on lysine 9 were used. Beads without coupled peptide served as a negative control.

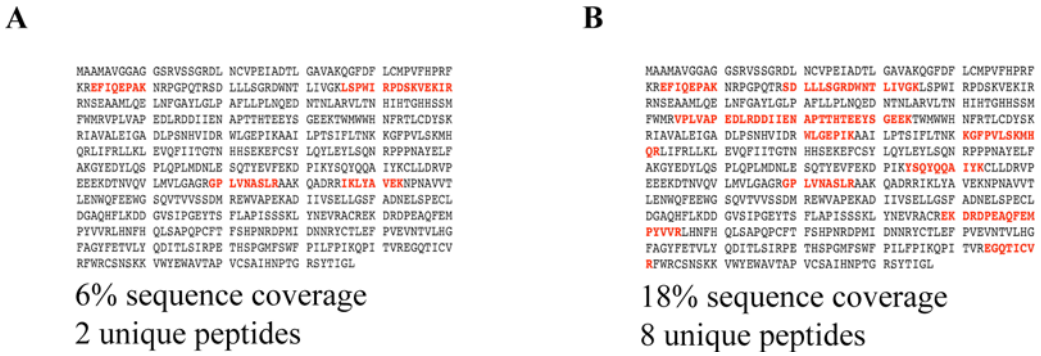


Figure 5-6 Identification of PRMT5 as an interaction partner of CDYL1b

A, Pulldown experiment with mock control, unmodified or lysine 9 trimethylated histone H3 peptide (1-20) out of HeLaS3 nuclear extract. Fractions bound to the peptides were loaded on a SDS-PAGE and were analyzed via mass spectrometry. Factors exclusively bound to H3K9me3 were extracted from mass spectrometry results employing R comparisons with the proteins bound to mock control and unmodified peptide. An arbitrary cut-off of at least 2 unique peptides was applied. Sequence coverage and unique peptides of the H3K9me3 enriched factor PRMT5 are shown. **B,** Immunoprecipitation of FLAG-tagged CDYL1b out of HEK293 cells was performed with following mass spectrometry analysis. Interaction factors of CDYL1b were extracted by R comparison to mock contol (cut-off: unique peptides≥2). Sequence coverage and the number of unique peptides of the identified PRMT5 are shown.

The protein fraction bound to the corresponding peptide was loaded on a SDS-PAGE and analyzed via mass spectrometry. Proteins only appearing in the H3K9me3 bound fraction identified with at least two unique peptides were extracted from the data set using the

statistical program R (for R protocol see chapter 8.4). Among 37 specifically identified proteins CDYL1 was found with 12 identified peptides (for complete results see 8.5.1). PRMT5, a arginine-methyltransferase modifying histones [125] and other nuclear proteins [126], was discovered with 2 unique peptides (Figure 5-6A). Therefore PRMT5 is a factor interacting directly or indirectly via another protein with the heterochromatic modification H3K9me3 (for further description of the experiments see chapter 4/Figure 1A).

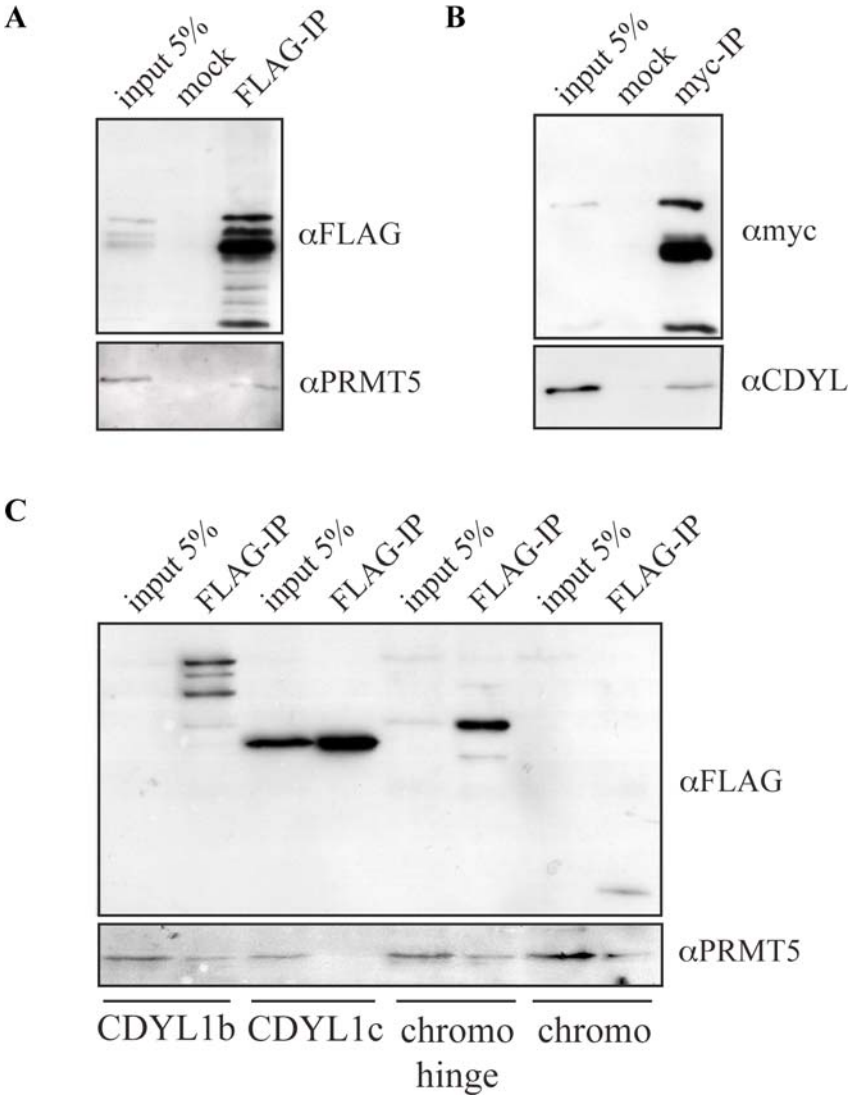


Figure 5-7 Verification of PRMT5 as interaction partner of CDYL1b

A, Immunoprecipitation of FLAG-tagged CDYL1b out of HEK293 nuclear extract. Input, mock control and CDYL1b-FLAG-IP were loaded on an SDS-gel with following Western Blot. The blotted proteins were detected with the antibodies αFLAG and αPRMT5. **B,** Immunoprecipitation of transfected myc-tagged PRMT5 out of HEK293 nuclear extracts. The samples were loaded on a SDS-gel and were then analyzed via Western Blot. The membrane was stained with αmyc and αCDYL antibodies. **C,** Indicated FLAG-tagged CDYL1b constructs were transfected into HEK293 cells, immunoprecipitation and Western Blot analysis was performed as described in A.

Secondly, HEK293 cells were transfected with FLAG-tagged CDYL1b and nuclear extracts were prepared. The extracts containing FLAG-tagged CDYL1b were used to find interaction partners of CDYL1b by immunoprecipitation. Untransfected HEK293 cells were used as negative control. Proteins only found in the CDYL1b-FLAG-IP above an arbitrary cut-off of at least two unique peptides were extracted from the mass spectrometry data via R (see 8.4 for R protocol, see 8.5.2 for complete results). Among 98 identified factors binding to CDYL1b, PRMT5 was identified with 8 unique peptides (Figure 5-6B). For this reason PRMT5 was within the group of 13 factors with the highest number of identified peptides and have a high probability of interacting with CDYL1b.

To verify these results independent IPs were carried out. FLAG-tagged CDYL1b was transfected into HEK293 cells and immunoprecipitated via its FLAG-tag. The immunoprecipitation results were analyzed via Western Blot (Figure 5-7A). The blotted proteins were detected with an antibody against the FLAG-tag of CDYL1b and an antibody against PRMT5. Compared to the control lane, CDYL1b is able to co-precipitate nuclear PRMT5. These results confirm the mass spectrometry results of Figure 5-6. In a reverse experiment of immunoprecipitation of transfected myc-tagged PRMT5 followed by analysis via SDS-PAGE and Western Blot (detection of proteins with αmyc and αCDYL1) a co-precipitation of endogenous CDYL1 was detected (Figure 5-7B). PRMT5 was therefore able to interact with CDYL1b.

To map the interaction surface of PRMT5, different FLAG-tagged deletion constructs of CDYL1b were transfected into HEK293 cells. The truncated versions of CDYL1b were immunoprecipitated from nuclear extracts and examined by Western Blot with αFLAG and αPRMT5 antibodies (Figure 5-7C). The immunoprecipitaions revealed that the chromodomain as well as the chromodomain connected to the hinge region were able to co-precipitate PRMT5. In contrast, the construct covering the whole C-terminal region of the protein (CDYL1c) was not able to co-precipitate PRMT5. CDYL1b therefore interacts with PRMT5 with its chromodomain and its hinge region.

5.3.2 PRMT5 methylates CDYL1b R80 *in vitro*

PRMT5 is a type II arginine methyltransferase mediating symmetric dimethylation of arginine residues. PRMT5 is implicated in various cytoplasmic processes including modulation of signaling cascades and biogenesis of small nuclear ribonucleotide proteins [127, 128]. PRMT5 also has nuclear functions that are not yet clearly understood. For example PRMT5 methylates histone H3R8 and histone H4R3 and thereby downregulates transcription [24, 125,

129]. In addition non-histone proteins such as p53 or the elongation factor SPT5 are regulated by arginine methylation mediated by PRMT5 [130, 131].

As seen in Figure 5-7, the interaction between PRMT5 and CDYL1b appeared to be rather weak. Therefore it is possible that PRMT5 binds to CDYL1b in order to modify it and to regulate CDYL1b mediated transcriptional repression.

To test this hypothesis an *in vitro* methyltransferase assay for a PRMT5-mediated reaction was established. Myc-tagged PRMT5 was transfected into HEK293 cells and was immunoprecipitated with a αmyc antibody bound to magnetic beads. As a control, beads without αmyc antibody were used for the immunoprecipitation (mock-IP). The beads with or without the PRMT5 protein were incubated with recombinant CDYL1b constructs expressed in *E.coli* in the presence of ^{3}H-S-adenosyl-methionine.

A Coomassie stained gel and autoradiograph of the reaction are shown in Figure 5-8A. Lanes one and two of this gel and its corresponding autoradiograph demonstrated that PRMT5 was able to modify CDYL1b full-length protein.

The modified residues were lying within the hinge region of the CDYL1b protein because in contrast to the hinge region (lane seven and eight) CDYL1c and the chromodomain alone were not modified by PRMT5.

The hinge region of CDYL1b contains an ARKS-like motif as shown in Figure 5-8B. PRMT5 is able to methylate arginine residues of histones within the ARKS motif. Thus it is likely that PRMT5 modifies CDYL1b within this amino acid sequence. To test this hypothesis the arginine 80 residue of CDYL1b was mutated to an alanine. Wild type CDYL1b and the mutant CDYL1b R80A were expressed in *E.coli* and used for methyltransferase assays as described. Figure 5-8C reveals that the PRMT5 methylation reaction on CDYL1b was abolished by the mutation of arginine 80. Therefore arginine 80 lying within the hinge region of CDYL1b was the target of PRMT5-mediated methylation.

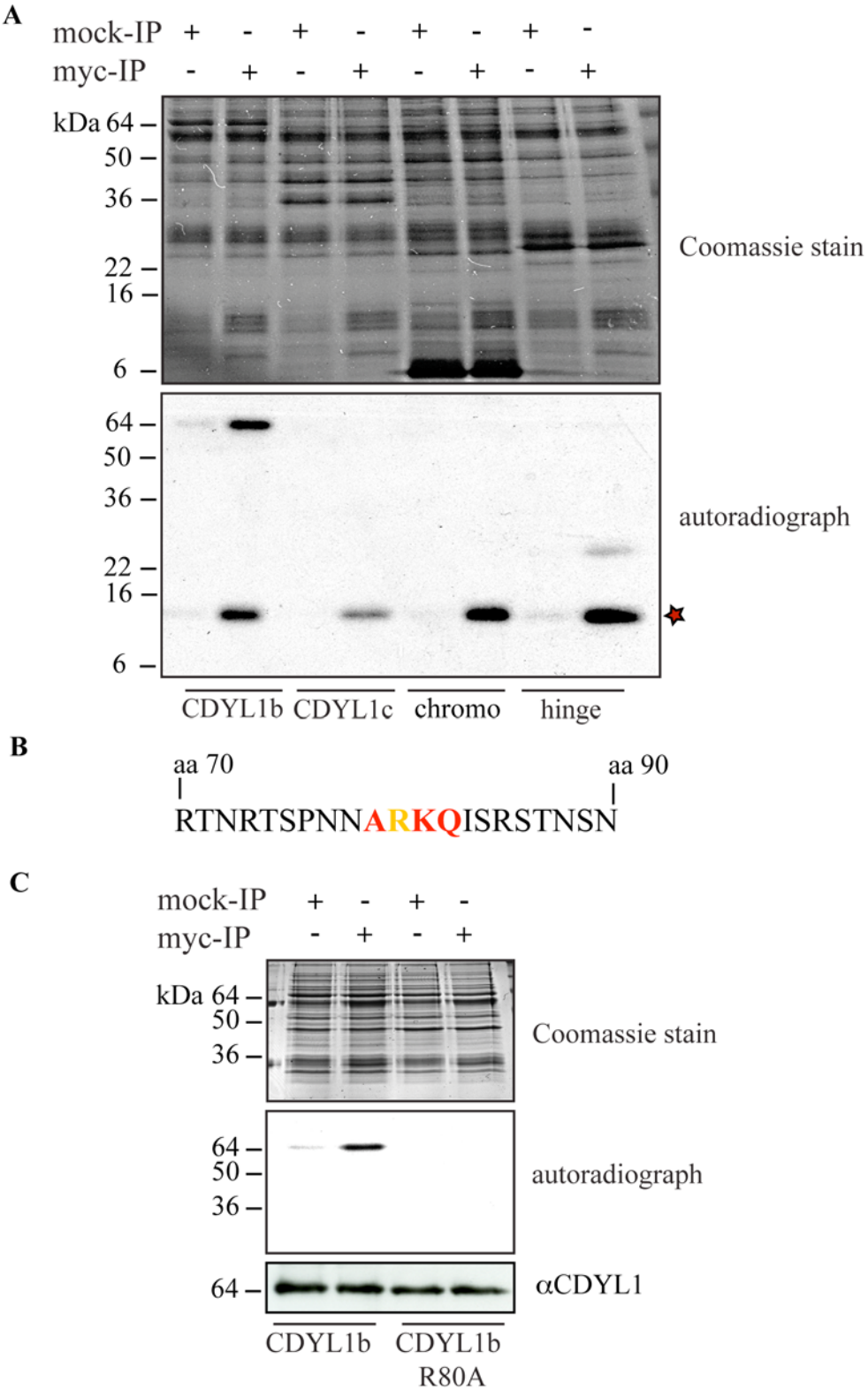


Figure 5-8 PRMT5 modifies R80 of CDYL1b *in vitro*

A, Methyltransferase assay with immunoprecipitated myc-tagged PRMT5 or with beads alone (mock-IP) and recombinant CDYL1b constructs (CDYL1b: 60 kDa, CDYL1c: 35 kDa, chromodomain: 8 kDa, hingeregion: 18 kDa). Coomassie stain and autoradiograph of the experiment are shown. **B,** sequence of aa 70-90 of the CDYL1b hinge region with marked ARKQ motif. Orange arginine residue was mutated to an alanine. **C,** Methyltransferase assay as described in A with wt CDYL1b and CDYL1b R80A. In addition, Western Blot of recombinant proteins is shown.

5.3.3 CDYL1b R80 methylation has only a minor effect on transcriptional repression

As shown in Figure 5-2 CDYL1b represses transcription. CDYL1b was methylated by the arginine methyltransferase PRMT5 at arginine 80 *in vitro*. Arginine methylation of factors such as the transcription elongation factor SPT5 lead to transcriptional repression [126].

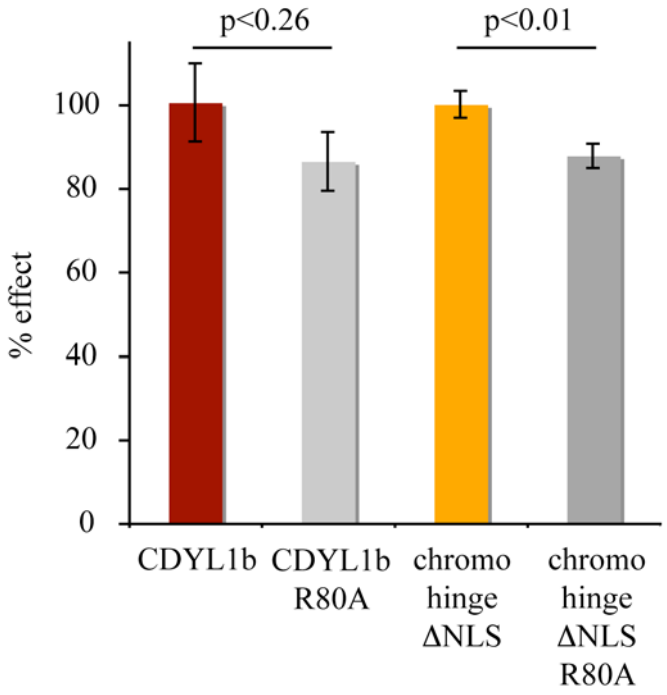


Figure 5-9 CDYL1b R80 methylation has a minor effect on transcriptional repression

Dual luciferase assay of the Gal4-tagged *Xenopus laevis* constructs CDYL1b, CDYL1b R80A, chromohingeΔNLS and chromohingeΔNLS R80A. Percentage of the effect relative to wild type CDYL1b is plotted. Error bars represent s.e.m of three independent experiments. p-values: calculated with a standard Student t-test.

It is also possible that CDYL1b arginine methylation leads to transcriptional repression. A mutation of the methylated arginine residue might therefore affect the CDYL1b mediated transcriptional repression. To test this hypothesis, the aginine 80 residues of the Gal4-tagged CDYL1b and the Gal4-tagged chromohingeΔNLS were mutated to alanine. The mutated constructs were tested in a dual luciferase assay in comparison to the wt proteins. As shown in Figure 5-9, the mutation of the arginine residue had only a minor effect on the repressive abilities of CDYL1b. The repression of CDYL1b R80A and chromohingeΔNLS R80A was about 13% reduced. P-values were determined with a two-tailed, paired, standard Students t-test. The difference between chromohingeΔNLS and the corresponding mutant was significant.

According to these results, it is not likely that arginine methylation of CDYL1b hinge mediates the whole repressive effect on transcription.

5.4 Function of CDYL1b during *Xenpous laevis* development

The first common ancestor of the CDY protein family appears in chordates (1.2.2). CDYL1 as well as CDYL2 are very conserved in the vertebrate lineage. Database analysis revealed that the *CDYL1* gene is expressed in *Xenopus laevis* in oocytes, during gastrula and during neurula stages. Interestingly, mouse *in situ* hybridizations published on genepaint.org showed an expression of CDYL1 during embryogenesis. Therefore, it is likely that the evolution and the expression of CDYL1 are coincident with the acquisition of new abilities within the chordate lineage. It is possible that this function is connected to the CDYL1 function to bind to heterochromatic regions enriched in H3K9me3. To determine whether CDYL1 has a function in development, the model organism *Xenopus laevis* was used for initial CDYL1 knock down and overexpression experiments.

5.4.1 Expression of CDYL1b during *Xenopus laevis* development

To test the hypothesis of a putative CDYL1b function during development, CDYL1b expression was examined during *Xenopus laevis* maturity. First, the expression of xlCDYL1b was profiled on mRNA level.

As shown in Figure 5-10A the xlCDYL1b mRNA levels did not change within the stages 10.5 to 40 compared to the ornithine decarboxylase, a house keeping gene, which is permanently expressed. Next, *in situ* hybridizations with a probe against the mRNA of xlCDYL1b were performed to identify regions of increased xlCDYL1b expression (Figure 5-10B). Therefore embryos of different stages were stained. As presented xlCDYL1b mRNA is not enriched in specialized regions until stage 29/30. At that point xlCDYL1b was highly enriched in the eye placode as well as within the ear placode. In stage 32, neural tissues neighboring ear and eye placode showed increased xlCDYL1b expression. The other tissues all had an equally low amount of xlCDYL1b mRNA expression.

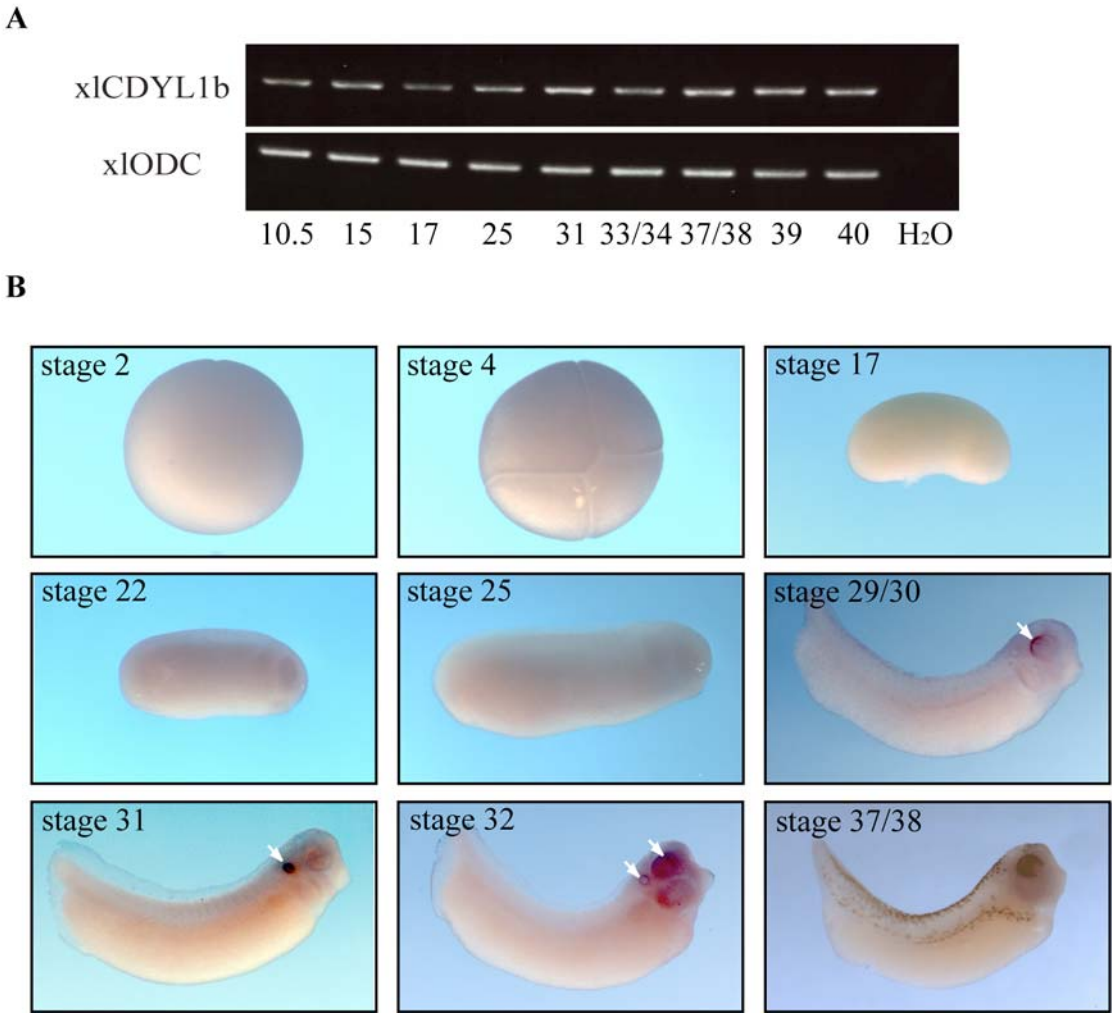


Figure 5-10 Expression of CDYL1b during *Xenopus laevis* development

A, RNA of *Xenopus laevis* embryos was extracted, reverse transcribed and used for PCR reactions with following gel electrophoresis clarifying the mRNA expression status of the *CDYL1* gene. The non-cycling ornithine decarboxylase was used as a control. **B,** Whole mount *in situ* hybridizations of *Xenopus laevis* embryos. Antisense probe obtained from the xlCDYL1b plasmid XL007a18 was used. Representative embryos are shown. Arrows indicate eye (stage 29/30) and ear placode (stage 31).

5.4.2 CDYL1b overexpression in *Xenopus laevis* embryos

The xlCDYL1b gene was transcribed during *Xenopus laevis* early development in comparable amounts. It is likely that the amount of xlCDYL1b mRNA in the cells is tightly regulated because of a very specialized function during development. Overexpression of the protein might lead therefore to a deregulated development. To shed light into this hypothesis, one-cell-stage embryos were injected with different amounts of *in vitro* synthesized xlCDYL1b mRNA.

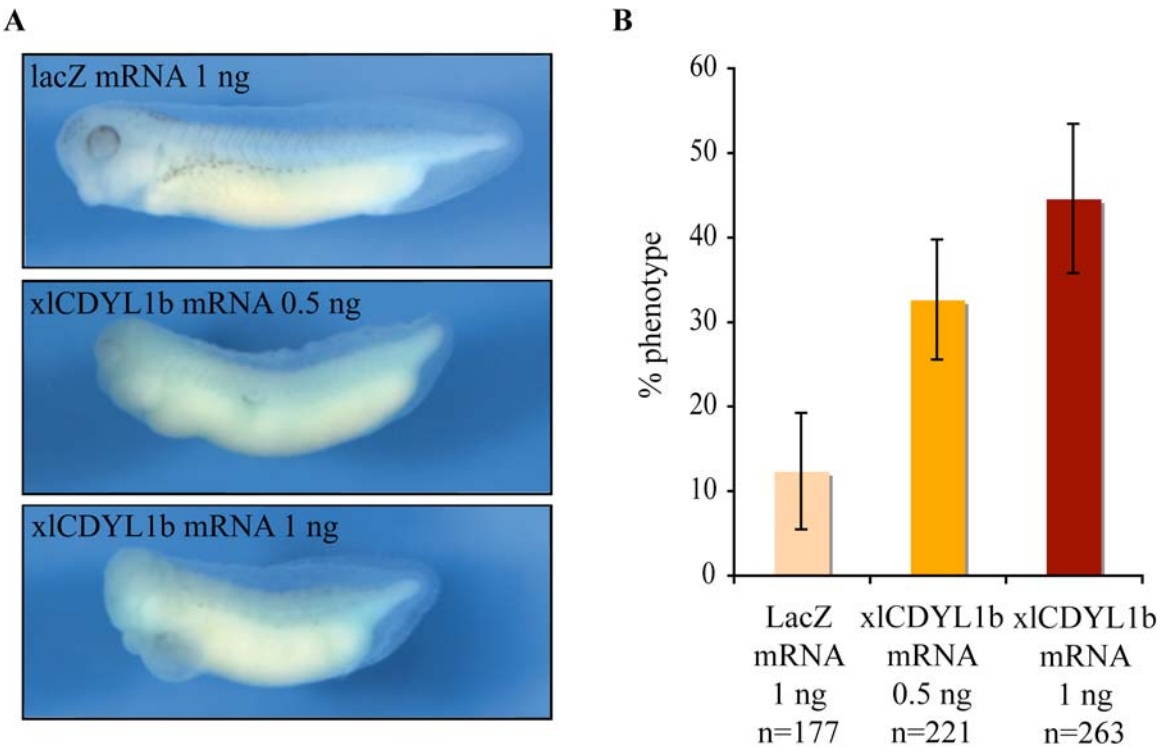


Figure 5-11 CDYL1b overexpression leads to developmental defects

A and B, β-galactosidase (lacZ) or xlCDYL1b sense mRNA were injected into one-cell-stage embryos in the indicated amounts. **A,** representative pictures of the main phenotypes in the injection experiments are shown. **B,** quantification of the *Xenopus laevis* phenotype prevalence. Counted embryos are given by n. S.e.m. was calculated from at least three independent experiments.

As control β-galactosidase mRNA was used. Figure 5-11 shows that xlCDYL1b overexpression led to a deregulated development of *Xenopus laevis* embryos. Increasing amounts of xlCDYL1b resulted in a shortening of the embryo at the dorsal site. Injection of 0.5 ng of xlCDYL1b mRNA led to 30% phenotype within the population. Doubling the amount of xlCDYL1b mRNA increased this value to about 45%.

Therefore it is likely that *Xenopus laevis* development is dependent on a normal xlCDYL1b level.

5.4.3 Knock down of CDYL1b during development

Verification of the overexpression phenotype of xlCDYL1b was achieved by a knock down experiment. If *Xenopus laevis* development might depend on a tight regulation frame of CDYL1b also lower amounts of the mRNA and therefore of the protein would result in developmental defects.

To test this hypothesis, Morpholino oligomers targeted to the 5'UTR region of xlCDYL1b were synthesized (M). The oligomers were tested in comparison to controls (Control M) by

adding them to a normal *in vitro* transcription/translation experiment of a plasmid containing xlCDYL1b as well as a part of the 5‘UTR region.

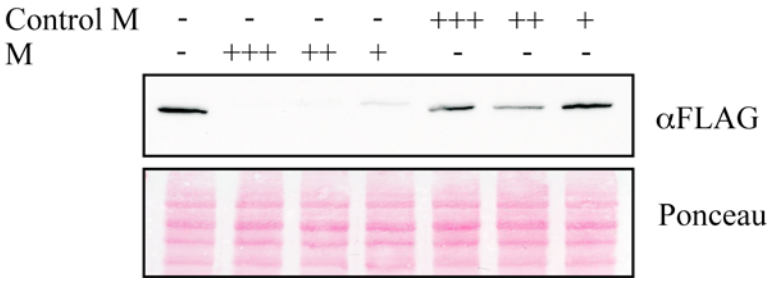


Figure 5-12 CDYL1b Morpholino efficiency

Morpholinos were tested with *in vitro* transcription and translation experiments. A 5’UTR containing plasmid of xlCDYL1b-FLAG was transcribed/translated in presence of control Morpholinos (Control M) or xlCDYL1b Morpholino (M) in decreasing concentrations. Results were analyzed by SDS-PAGE and Western Blot using an antibody against the FLAG-tag of the protein.

Increasing amounts of Morpholino oligomer targeted against xlCDYL1b led to a decrease of xlCDYL1b production in comparison to the control (Figure 5-12). Next, 20 ng of the described oligomers were injected into one-cell-stage *Xenopus laevis* embryos. The embryos were grown as described and then examined in the tadpole stage.

Embryos with a lower amount of xlCDYL1b developed a prolonged dorsal region with a smaller head size in comparison to the controls (shown in Figure 5-13A). This phenotype was seen in about 65% of knockdown CDYL1b embryos. Importantly, this phenotype was rescued by co-injection of different amounts of xlCDYL1b mRNA.

250 pg of xCDYL1b mRNA reduced the prevalence of the phenotype within *Xenopus laevis* population to 35% (compare with Figure 5-13B). These experiments reveal that xlCDYL1b needs to be tightly regulated during embyogenesis. Up- or downregulation of the xlCDYL1b mRNA levels resulted in severe developmental defects.

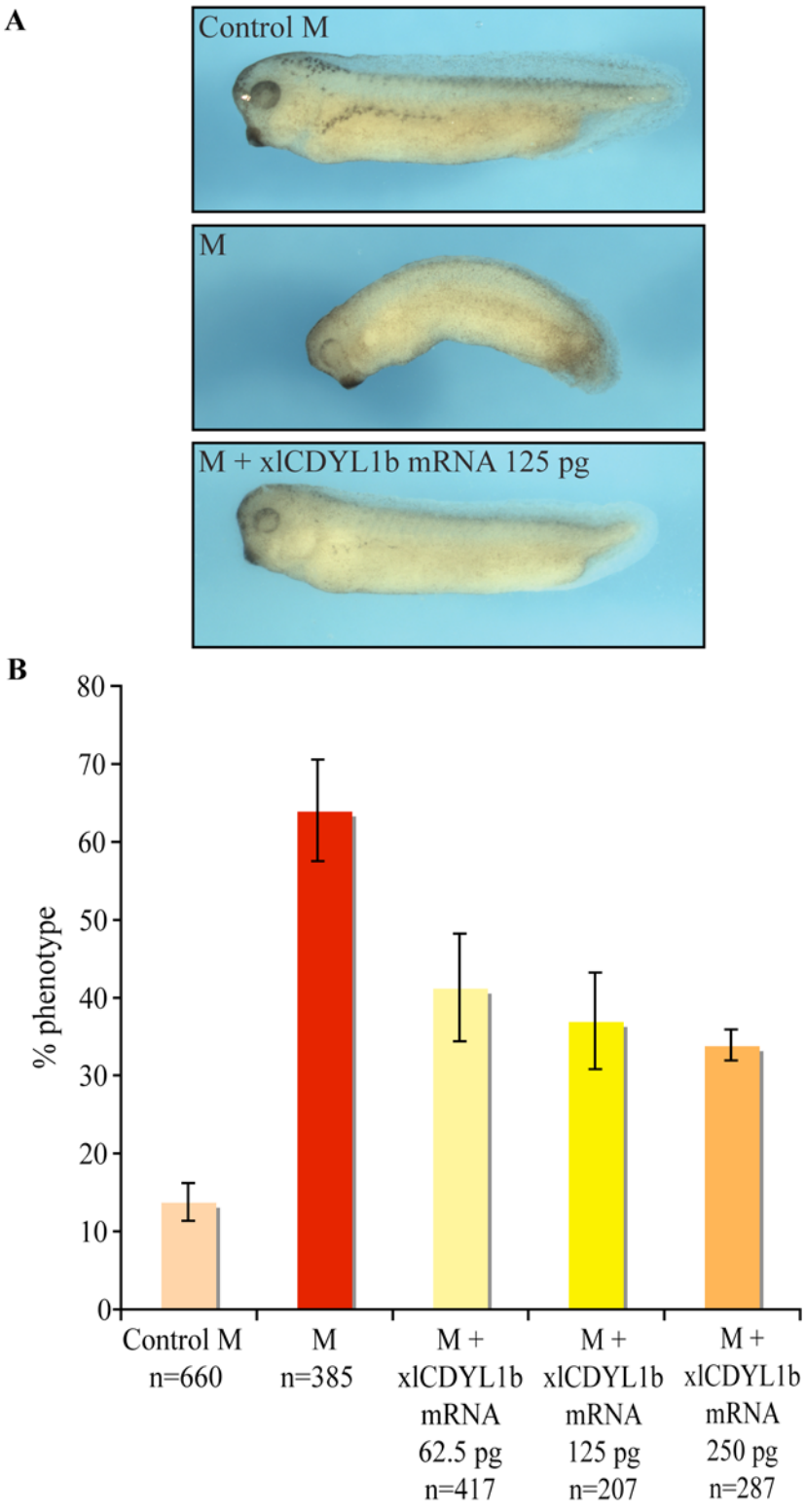


Figure 5-13 Knock down of xlCDYL1b can be rescued with xlCDYL1b mRNA

A and B, 20 ng of Control Morpholinos (Control M) or xlCDYL1b Morpholinos (M) lying within the 5' region of the xlCDYL1b gene were injected into one-cell-stage *Xenopus laevis* embyos. For rescue sense RNA of xlCDYL1b was co-injected in indicated amounts (62.5-250 pg). **A,** representative pictures of main phenotype occurring in controls, xlCDYL1b knock downs or rescued *Xenopus laevis* embryos. **B,** quantification of the phenotype prevalence in injection experiments. n displays the number of embryos injected. S.e.m of at least three independent experiments is shown.

6 General discussion

6.1 Chromodomains of CDY family members

6.1.1 Chromodomains of CDY family proteins bind to H3K9me3

CDY family proteins contain a chromodomain, a connecting hinge region, and an ECH domain (compare with 1.2.3). Chromodomains are found in several nuclear proteins and they have been connected to chromatin organization and gene regulation [108]. Proteins such as HP1, Polycomb or CHD1 (Chromain helicase DNA-binding 1) contain chromodomains, which bind to methylated histones. Small amino acid exchanges within the chromodomains lead to specificity differences as shown for HP1 and Polycomb [111]. The preferred target of HP1 chromodomain is H3K9me3 and for Polycomb it is H3K27me3, both heterochromatic PTMs. In contrast, CHD1 can recognize H3K4me3, a hallmark of euchromatic regions, with its two neighboring chromodomains.

As shown in chapter 3 and 4, chromodomains of the CDY family bind also to methylated lysine marks. From fourteen analyzed peptides, the major targets of CDY and of the CDYL1 splicing variant CDYL1b chromodomains are H3K9me2 and with a slightly lower binding constant, and therefore better binding, H3K9me3 (chapter 3/Table 1 and chapter 4/Figure 3).

CDYL2 binds to H3K9me2/me3 with a K_d comparable to the other family members but surprisingly it has an even higher affinity to H1K26me3 and testis H3K27me3.

The binding behaviour to methylated histones is also reflected in the nuclear localization of the proteins. Whereas CDY and CDYL1b clearly colocalize with the heterochromatic regions enriched in H3K9me3, the CDYL2 distribution overlaps only partially with this modification (chapter 3/Figure 3 and chapter 4/Figure 4). A large proportion of the protein is also localized in the nucleoplasm with a difuse distribution or with a dotted-like structure without overlapping with H3K9me3. This and the evolutionary observations on a DNA level that CDYL1 and 2 diverged from each other long time ago and that CDY arose from a retro-transposition event of a processed CDYL1 mRNA (1.2.2 and [103, 106, 114]), point to different and maybe very specific tasks of CDY family members within the cell nucleus. In addition, the very distinct expression patterns of the CDY proteins argue also for functional specifications within different tissues or cell types. Whereas CDYL1b mRNA is ubiquitously expressed at high levels (chapter 4/Figure 2), the CDY locus has a testis-specific gene expression [106]. In contrast the CDYL2 gene is transcribed at very low levels in all tested cell types or tissues and is upregulated in the spleen, prostate, testis and in leukocytes [114].

Taken together, the CDY family members CDY and CDYL1b but not CDYL2 are newly defined heterochromatic proteins, which might affect heterochromatin establishment and maintenance.

6.1.2 Chromodomain differences within splicing variants

Interestingly, not all CDY family members recognize histone modifications or methylated lysines. Splicing variants of the CDYL1 gene CDYL1a and also CDYL1c are not able to bind to H3K9me2/3 for different reasons. CDYL1c is lacking the whole chromodomain whereas CDYL1a harbors a non-functional chromodomain and a prolonged N-terminal part (compare with chapter 4/Figure 2A/Supplementary Figure 1). Both proteins are therefore not located at heterochromatin regions (chapter 4/Figure 4A). In contrast, CDYL1b distribution within the nucleus clearly overlaps with H3K9me3 enriched loci. This localization is dependent on Suv39h1/h2, the H3K9me3 methyltransferase. Knock out of the two isoforms lead to displacement of CDYL1b from heterochromatin (chapter 4/Figure 4).

Presented in Figure 1-9 and also in chapter 4/Figure 2D, CDYL1a has only a few amino acid exchanges within the chromdomain compared to CDYL1b. The CDYL1a chromodomain lacks the first aromatic residue of the tri-aromatic cage and a neighboring glutamate, which establishes hydrogen bonds to the peptide backbone. Mutation of these two residues to corresponding amino acids in CDY improves the binding to H3K9me3 and leads to localization to H3K9me3 mark in 30% of the analyzed cells (chapter 3/Table 3 and Figure 5).

Two proline residues are located in front of the chromodomain of CDYL1a. Additional mutations of these two proline residues within the CDYL1a chromodomain to amino acids found in CDY at this position restored the CDYL1a binding to H3K9 methylation. The localization to H3K9me3 enriched loci within the nucleus was rescued in 70% of all cases (chapter 3/Table 3 and Figure 5).

Thus, a functional chromodomain is necessary for targeting CDYL1 to the heterochromatic mark H3K9me3.

A further conclusion of these experiments is that splicing events (i.e. CDYL1a, CDYL1b, CDYL1c) of the CDYL1 mRNA can actively influence the ability of the protein to bind to H3K9me3. Interestingly, under normal conditions the mRNA of the CDYL1a splice variants is upregulated in testis and the CDYL1c mRNA occurs only at very low levels in all examined tissues and cell lines (compare with chapter 4B/Figure 2). Theoretically these splicing events could occur in developmental stages, specific cell types or in response to DNA

damage or oxidative stress to regulate if CDYL1 proteins are located at H3K9me3 dense heterochromatin or not.

6.1.3 Chromodomain binding to non-histone targets

As shown in chapter 3/Table 1 and Table 2 and chapter 4/Figure 3 CDY family proteins exept CDYL1a and CDYL1c recognize methylated lysines of non-histone proteins such as the automethylation site of the histone methyltransferase G9a. Earlier studies demonstrated a direct interaction between CDYL1b (referred as mRNA isoform 2 in the original publications) and G9a using recombinant proteins and within the CoREST complex [62, 102]. It is possible that this contact is stabilized or even established by the CDYL1b chromodomain interaction with methylated G9a on lysine 185. An argument for this hypothesis is that pulldown assays carried out with the CDYL1a splicing variant failed to demonstrate a CDYL1a/G9a interaction *in vitro* [132].

Because CDYL1b chromodomain in contrast to the CDYL1a chromodomain is able to bind G9aK185me3 it is likely that the G9a/CDYL1 contact is mediated by the trimethylation mark (compare also with Figure 6-2).

6.1.4 Regulation of CDY family chromodomain binding to H3K9me2/me3

It is possible that CDY family proteins are not permanently associated with heterochromatin regions. One hint is supplied by the chromatin displacement of CDYL1b during mitosis (shown in Figure 5-3). The delocalization of the protein is correlated with the appearance of the phosphorylation of histone 3 serine 10 set by the kinase Aurora B [122]. The removal of the phorphorylation mark occurs at a time coincident with CDYL1b relocalization to H3K9me3 enriched regions. Thus it is possible that the CDYL1b localization to heterochromatin is regulated by the H3K9me3S10ph mark. This hypothesis is supported by *in vitro* binding studies, which reveal that CDYL1b chromodomain binding to H3K9me3 is abolished if the neighboring serine is phosphorylated (chapter 4/Figure 3C). Therefore it is likely that CDY family chromodomain binding to H3K9me3 is regulated by additional histone PTMs such as phosphorylation. This regulatory possibility was already shown for the heterochromatin protein 1 (HP1) [27] and indicates that this might be a mechanism for heterochromatin binding proteins in general or chromodomain containing proteins in particular.

A second regulation mechanism for chromodomains of CDY family proteins was recently suggested [133]. CDYL1 can be methylated by the histone methyltransferase G9a *in vitro.* The methylated lysine residue of CDYL1 is located C-terminal to the chromodomain but in very close proximity. Methylation of this CDYL1 lysine residue leads to a about 2-fold reduced CDYL1/H3K9me3 interaction. Thus G9a mediated methylation within CDYL1 might negatively regulate the H3K9me3 binding. But interestingly, CDYL1a instead of CDYL1b was used for the study. The CDYL1a affinity to H3K9me3 is about 200-fold less than the affinity of CDYL1b and could have had a major influence on the represented results.
A third regulation mechanism of the CDY protein chromodomain binding to H3K9me3 can be splicing events (compare with 6.1.2). The only splicing events known to date regard CDYL1. Splicing of the first three exons (exons 1, 2, 3) to the six last exons (exons 5, 6, 7, 8, 9, 10) (chapter 4/Supplementary Figure 1) lead to a non-functional chromodomain in splicing variant CDYL1a. Alternatively exon 4 is spliced to the six 3' exons resulting in CDYL1b having a functional chromodomain. Third, only the last six exons are spliced together producing the mRNA for CDYL1c, which lacks the chromodomain. It is likely that these splicing events are regulated since CDYL1a transcript is only expressed in testis, but further investigations are needed to provide detailed mechanism for CDYL1 splicing variant expression.

6.2 ECH domains of CDY family proteins

6.2.1 Functionality of the ECH domain of CDY family proteins

The chromodomain of CDY family proteins is connected to – via a more flexible hinge region – an effector-like enoyl-CoA-hydratase domain. Previous studies showed that CDY and CDYL1 had histone acetyltransferase activity *in vitro* [114]. This activity was assigned to their ECH domain. Still not verified, it was demonstrated that CDYL1 directly interacts with coenzyme A [101]. In contrast to these results recent structural comparisons revealed no overlap of the ECH domain of the CDY family with the known histone acetyltransferases [134]. Rather CDY family proteins are similar to enzymes with an enoyl-CoA-hydratase fold. Enoyl-CoA-hydratases are thought to be involved in enzymatic reactions of the β-oxidation within the peroxisome and the mitochondria. During these reactions enoyl-CoA-hydratases act as dehydrogenases, isomerases and/or hydratases on unsaturated CoA-coupled fatty acids [135]. From database searches, CDY family proteins seem to have the highest similiarity to enoyl-CoA-hydratases catalyzing isomerase reactions [134].

Interestingly, enoyl-CoA-hydratases are arranged in a propeller-like structure composed of three independent monomers. In addition, two trimers are able to interact to form a hexamer. Structurally, enoyl-CoA-hydratases can be divided into three groups according to the position of their C-terminal helix [134] and [136]. The first group contains enoyl-CoA-hydratases in which the C-terminus protrudes out of the monomeric unit and covers the active site of the neighboring monomer of the same trimer. Within the second group the C-terminus folds back to cover the active site of its monomer and C-termini of the third group of enoyl-CoA-hydratases cross the trimer-trimer border and envelop the active site of an opposing trimer. As seen from structural comparisons (see chapter 4/Figure 5/Supplemental Figure 5 and [134]) the ECH domain of CDY family proteins belong to the second group of enoyl-CoA-hydratases.

Due to these structural and functional observations it is not likely that CDY family members are histone acetyltransferases. Rather it is possible that CDY family proteins have a function similar to the described enoyl-CoA-hydratases.

However, closer inspections reveal that within CDY family proteins the three active residues of enoyl-CoA-hydratases (chapter 4/Figure5A) are exchanged. In addition, no amino acids forming the active pocket of enoyl-CoA-hydratases are present in the ECH domain of the CDY family (chapter 4/Supplementary Figure 5). Therefore it is likely that although CDY family members harbor a typical enoyl-CoA-hydratase fold they are not able to catalyze a typical enoyl-CoA-hydratase enzymatic reaction.

Fascinatingly the ECH domain of CDY family members is the only known enoyl-CoA-hydratase localizing to the nucleus. Unsaturated fatty acids as targets of enoyl-CoA-hydratases can be mainly found as posttranslational modifications (e.g. myristoylation, prenylation) or integrated into the nuclear membrane (e.g. phospholipids and glycolipids) [137-139].

Thus it is possible that the ECH domain of CDY family proteins has a very different newly evolved nuclear function, or more likely that it has no enzymatic activity and just maintained the enoyl-CoA-hydratase-like fold because of its multimerization.

6.2.2 Multimerization of ECH domain

As described in the previous section it is possible that the ECH domain of CDY family proteins kept the enoyl-CoA-hydratase-like-fold because of its multimerization. Several experiments described in chapter 4 demonstrate the importance of multimerization for the CDY family proteins on the example CDYL1b.

Although the chromodomain of CDYL1b binds clearly to the H3K9me3 modification *in vitro*, cell-based experiments reveal that the chromodomain alone is not sufficient to localize to H3K9me3 enriched loci *in vivo* (chapter 4/Figure 7). In contrast, full length CDYL1b localizes to DAPI-dense and H3K9me3 stained regions within the nucleus whereas the splicing variant CDYL1a - a natural chromodomain mutant – shows no overlap with these heterochromatic areas (chapter 4/Figure 4). Thus another region of CDY family proteins is needed for targeting to H3K9me3.

Interestingly, the chromodomain directly connected to the CDYL1c, a CDYL1 splicing variant harboring mainly the ECH domain, is confined to H3K9me3 dense regions. The same construct lacking the C-terminal part has a diffuse distribution within the nucleus (chapter 4/Figure 7).

Recent structural observations predict that the C-terminus of the ECH containing CDYL1c is important for forming homomultimers [134]. Multimerization assays reveal that the C-terminus of the CDYL1c variant is necessary to form ECH domain mediated oligomers (chapter 4/Figure 4). In addition, it was proven that the multimerization ability highly increases the CDYL1b association to H3K9me3 modified chromatin *in vitro* (chapter 4/Figure 6). Therefore chromodomain and ECH domain mediated multimerization are essential for localization to H3K9me3 enriched heterochromatin.

Interestingly, the connection between multimerization and localization is not only a CDY family specific pattern. Several other heterochromatin proteins are also able to multimerize. These include for example HP1, the DNA methyltransferase DNMT1 or the silencing protein Sir3 [140-142]. For HP1 it was shown that a single-point mutation within the dimerizing chromoshadow domain prevents the protein from localizing to H3K9me3 [143]. Thus multimerization might be a general prerequisite of heterochromatin-localized proteins.

6.3 Regulation of CDY family proteins

6.3.1 Regulation by expression, splicing events or by splicing variants

As described, CDY family proteins evolved very different expression patterns (1.2.2 and chapter 4/Figure 2). Whereas CDYL1 has a ubiquitous expression pattern, CDY is only expressed in testis [106]. CDYL2 is also consistently transcribed and translated but at very low levels. In spleen, prostate, testis and in leukocytes the expression of the CDYL2 locus is upregulated [114]. The CDY expression was correlated with hyperacetylation of histones during spermatogenesis, but its detailed function at this time point remains unclear [114].

In addition, splicing events regulate the CDYL1 mRNA expression (i.e. CDYL1a, b and c compare with chapter 4/Figure 2). As described only CDYL1b exhibits heterochromatic distribution. Interestingly artificial overexpression of CDYL1c in cell lines displaces CDYL1b from heterochromatin (chapter 4/Figure 7). This effect is not observed when CDYL1c lacks the C-terminus, which mediates multimerization. A similar effect was observed for HP1. Overexpression of the dimerizing chromoshadow domain led to the displacement of endogenous HP1 from heterochromatin [144].

It is conceivable that the displacement of CDYL1b from heterochromatin by its splicing variants is a general regulation mechanism. It has been reported that CDYL1c has a very low expression level (chapter 4/Figure 2). However, CDYL1c expression levels were only determined in cell lines at mixed cell cycle stages and in fully differentiated tissues. Could it be possible that CDYL1c is upregulated at different stages in cell cycle or during development? If this were the case CDYL1c could regulate CDYL1b heterochromatin localization. Further CDYL1a has a multimerizing ECH domain. Upregulation of CDYL1a in testis could theoretically influence CDYL1b localization to the H3K9me3 modification. Interestingly, CDYL1a gradually alleviates the repression activity of CDYL1b as shown with luciferase reporter assays [145].

Regulation of chromatin compounds by alternatively spliced variants has been reported. It was shown that the function of heterochromatin proteins like the ING1 (inhibitor of growth 1) or the HDAC associated transcription factor IKZF3 (Ikaros family of zink fingers 3) are influenced by their splicing variants [146, 147]. ING1a/INGb ratios are important for senescence in human diploid fibroblasts and different IKZF3 splicing variant combinations influence the IKZF3 interaction abilities with Ikaros and HDAC-containing complexes.

In this context, it might indeed be possible that combinatorial effects of its splicing variants influence also the CDYL1b interaction with H3K9me3 enriched heterochromatin regions. This would be one important mechanism to regulate CDYL1b function at heterochromatin.

6.3.2 Sequestration to nuclear membranes

The experiments shown in Chapter 5.2.2 demonstrate that endogenous CDYL1 can be associated with nuclear membranes. Washing the membranes with buffers containing increasing amounts of salt influences this association. Salt concentrations above 400 mM detached CDYL1 from membranes completely. Thus, it is likely that the CDYL1 membrane association is mediated by ionic bonds to membrane localized proteins rather than hydrophobic interactions. So far interactions between lamin or lamin-interacting proteins and

CDYL1 were not described. But it is known that CDYL1 interacts with HDAC1 and HDAC2 [101]. HDAC1 is able to associate with membranes [148] and could therefore serve as CDYL1 anchoring protein. It also was shown that also the heterochromatin proteins 1 is dynamically associated with membranes in dependence of histone deacetylase activity [149].
As shown by mutant analysis membrane association is mediated by the chromohinge region of CDYL1 (Figure 5-5). The nuclear envelope associated part of HP1 was as well mapped to its N-terminal region including the chromodomain [149]. Even so, immunofluorescence experiments (chapter 3 and 4) staining of transfected FLAG-tagged CDYL1 do not show membrane association. Thus it is possible that the membrane association of CDYL1 seen in fractionation experiments is a secondary effect of its multimerization attribute and its heterochromatic localization.
Recent genome-wide studies show that silenced regions of chromatin, i.e. heterochromatin, locate close to the nuclear lamina in Drosophila Kc cells [150] as well as in human fibroblasts [151]. CDYL1b is located at heterochromatin regions enriched in H3K9me3 modification mark (chapter 4/Figure 4). It is known that heterochromatin in general is closely localized to the nuclear periphery [152]. Additionally, CDYL1b is able to built multimeric structures up to hexamers, which might influence its solubility in membrane extraction experiments. It is possible that this characteristic is even more enhanced in transfected cells overexpressing CDYL1b.
Therefore it is likely that the nuclear lamina association of CDYL1b is a secondary effect, rather than a regulatory pathway, but further experiments are needed to solve that issue.

6.3.3 Posttranslational modifications

PTMs such as arginine methylation are known to regulate the function of proteins [153]. PRMT5 mono- or synchronously dimethylate arginine residues of histones and other proteins [154]. Arginine methylation plays important roles in transcriptional regulation by influencing protein-protein, protein-DNA or protein-RNA interactions [126]. For example, histone H3 arginine 8 and histone H4 arginine R3 are modified by PRMT5 and are known to be implicated in transcriptional repression. The transcriptional elongation factor SPT5 is regulated by PRMT5 methylation as well. Mutation of the targeting sites leads to increased interaction with RNA polymerase II stimulated transcriptional elongation *in vitro* [130].
The experiments shown in chapter 5.3 demonstrate that the CDY family member CDYL1b is modified by the arginine methyltransferase PRMT5 at arginine 80 *in vitro.* Arginine 80 is embedded in a ARKQ motif, which is related to ARKS motifs found in histone and non-

histone proteins. ARKS motifs are targeting sites for modifying enzymes as well as binding partners recognizing PTMs [95, 133].

As determined by luciferase reporter assays, mutation of arginine 80 to alanine led to a very minor decrease of transcriptional repression (Figure 5-9). Since CDYL1b has at least two repressive modalities and additionally interacts with repressive factors such as HDACs or G9a, it is possible that one mutation will not abolish the whole effect on transcription. Rather than influencing all CDYL1b function it is likely that the modification mediates or inhibits the contact to one interacting partner.

G9a was shown to modify CDYL1 *in vitro* [133] at a lysine 81 directly neighboring arginine 80. A modification on the residue next to the lysine residue might play a role in establishment or prevention of CDYL1 lysine methylation. The lysine 81 methylation can be removed by JMJD2A-C *in vitro* [155].

Interestingly, mass spectrometry analysis revealed that CDYL1 is phosphorylated on serines (serine 86 and serine 88) in close proximity to the described modified amino acids during the cell cycle [156]. According to this study serine 86 phosphorylation appears only during mitosis. Phosphorylation is known to introduce negative charge in the modified amino acid sequence. Thus, a phosphorylation would definitely influence interaction partners that bind to the positively charged arginine and/or lysine residues.

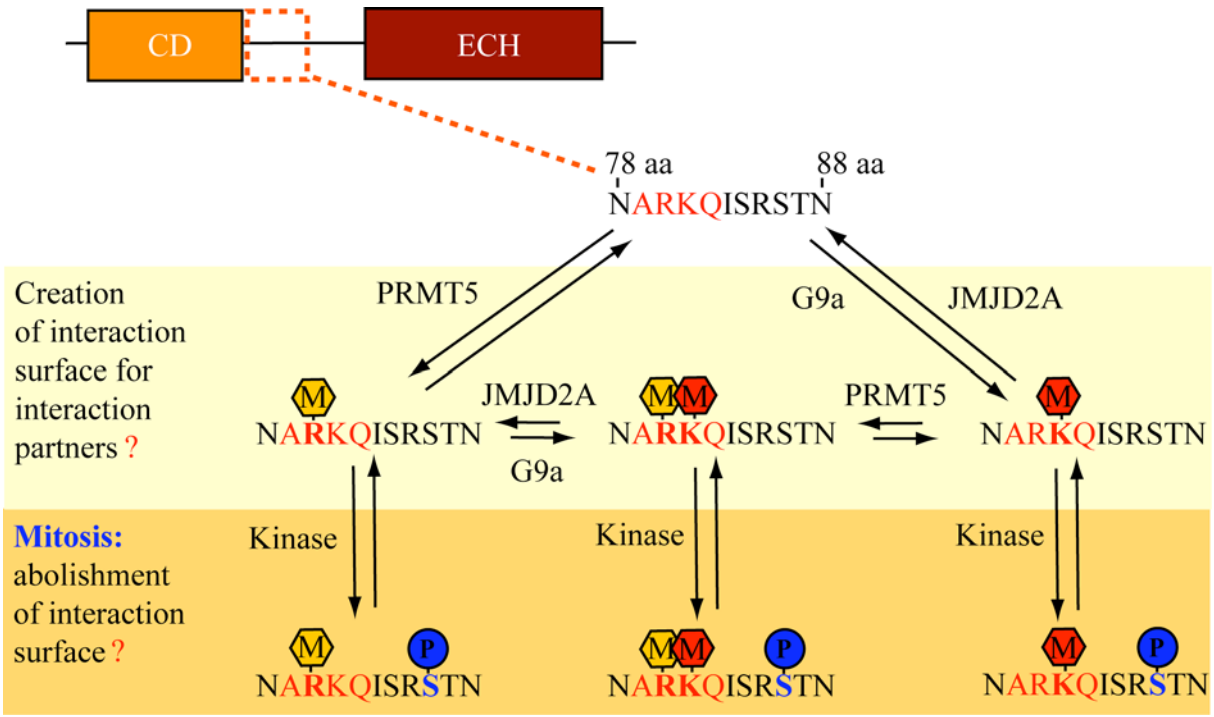


Figure 6-1 Possible model of CDYL1b interaction regulation by posttranslational modifications

CD: chromodomain, ECH: enoyl-CoA-hydratase domain, M: methylation, P: phosphorylation.

Although lysine and arginine methylation of CDYL1 have still to be verified *in vivo* the ARKQ motif and its surrounding amino acids seem to be diversly modified. Interestingly, it was shown that CDYL1 interacting partners such as HDAC1 and HDAC2 bind to the ARKQ-motif-containing region of CDYL1 [101]. HDACs are known to be associated with heterochromatin. During mitosis they are displaced from heterochromatin by an unknown mechanism [157].

Interestingly, a comparable diversly modified region of thirty amino acids regulates the tumor suppressor p53 [158]. Lysine methylation within this area enhances or suppresses the p53 transcriptional activity. Furthermore, demethylation by LSD1 prevents p53 interaction with its co-activator 53BP1 [158]. Protein arginine methyltransferases like PRMT1 or CARM1 co-activate p53 and facilitate p53 mediated transcription [158]. Additionally, p53 is phosphorylated within this region, which promotes its degradation and in response to UV-radiation [159, 160].

Therefore it is likely that the ARKQ-motif-containing region is involved in regulating CDYL1b association with its interaction partners. A possible model is shown in Figure 6-1. CDYL1b modifications by PRMT5 or G9a would create a binding surface for other interaction partners. During mitosis the interaction surface would be disturbed by phosphorylation. Several experiments are still necessary to confirm this hypothesis. First, the modifications R80me and K81me need to be verified *in vivo* and second, a modification-dependent interaction between CDYL1b and its partners need to be demonstrated.

6.4 Function of CDY family proteins

6.4.1 Repressive function

The detailed function of CDY family proteins remains unclear so far. Luciferase assays showed that CDYL1 targeted to a reporter gene repressed transcription [101]. The repressive activity is located in the C-terminal region of the protein (Figure 5-2). Targeting of the very N-terminal chromodomain to the reporter gene did not lead to transcriptional repression. Interestingly, the CDYL1 protein has at least two repressive domains, the hinge region as well as ECH domain.

It is likely that CDYL1 mediates its function as co-repressor by recruiting tanscription regulating factors. CDYL1 interacts with HDACs as well as with G9a [101, 102]. Deacetylation of histones by HDACs and methylation by G9a are implicated in silencing of genetic loci [6, 31] and could therefore cause transcriptional repression. Additionally, it was

shown that CDYL1 is part of at least two partially overlapping repressive complexes named CoREST complex and CDYL repressor complex [62, 102]. Components of the complex such as the histone demethylase LSD1, which removes the active mark H3K4me3 [44, 121], the RE-DNA-element binding repressor REST or the C-terminal binding protein CtBP, which is recruited by more than thirty transcription factors for transcriptional repression [161], may enhance or mediate CDYL1 caused downregulation of transcription. Expression of CDYL1 in cell lines leads to no change of histone modifications like acetylation, H3K9me3 and H3K4me3 (Figure 5-1). The CDYL1b localization to H3K9me3 does not seem to be the rate limiting step of changes of these histone modifications. It is likely that the expression of CDYL1b together with its interaction partners HDAC1/2, LSD1 or G9a would reveal more details. But it is also possible that the results are influenced by overexpression of CDYL1b in transfected cells and therefore do not represent the native situation in the nucleus.

Interestingly, not all CDYL1 is associated with different complex partners as shown by ultracentrifugation experiments [102]. It is possible that CDYL1 interacts also with other heterochromatin proteins, which might mediate the repressive activity of CDYL1 or other heterochromatin downstream effects.

Thus CDYL1 is involved most likely indirectly in transcriptional repression and directly in crosslinking of complex components. It remains unclear if the other CDY family proteins might also have repressive functions.

6.4.2 Putative developmental function of CDYL1b

CDYL1b is expressed in oocytes, during the gastrula and the neurula state of *Xenopus laevis* development and during mouse embryogenesis (see chapter 5.4). Therefore it is possible that CDYL1 is implicated in so far unknown developmental processes.

In absence of a cellular system initial experiments addressing this question were carried out in *Xenopus laevis*. *In situ* hybridizations show no specific enrichment of the xlCDYL1b gene in the embryos. The staining was not comparable to *Xenopus laevis* expression of the complex partner xlCoREST, which is localizing to neurogenic regions [162]. As published in xenbase.org, which combines information of *in situ* hybridizations and cDNA libraries, xlHDAC1, xlSuv39h as well as xlHP1 are expressed in the whole organism. No complete overlapping expression patterns with xlCDYL1b could be found.

Overexpression of CDYL1b in different concentrations leads to severe developmental defects in *Xenopus laevis* in about 45% of the population. The embryos experienced impaired gastrula

and neurula, which resulted in head deformations and a decreased length of the dorsal region (see 5.4.2).

Knock down experiments of CDYL1b were accomplished using Morpholino oligomers. The downregulation of the CDYL1b transcription caused a phenotype with a prolonged dorsal region and a smaller head size in about 65% of the examined embryos. This phenotype could be rescued by injecting CDYL1b mRNA. CDYL1b mRNA injected embryos showed a decreased phenotype prevalence of about 35%. Thus, the phenotype seems to be CDYL1b specific.

Interestingly, HDAC1 null mutations in zebrafish embryos lead to multiple developmental defects. Prolonged dorsal area (curled down tail), reduced brain size as well as an absent jaw were clearly seen in HDAC1 mutants [163, 164]. Therefore it is possible that CDYL1b and HDAC1 phenotypes are correlated.

To clearly understand the phenotype caused by CDYL1b further experiments are needed. It is possible that the up- or downregulation of CDYL1b expression leads to downregulation of downstream effectors important for a normal grastrula and neurula. Therefore the mRNA expression of factors orchestrating different developmental stages needs to be measured.

As described for most eukaryotes H3K9me3 is also a hallmark of heterochromatin in *Xenopus laevis* [165, 166]. Theoretically different levels of CDYL1b expression could therefore lead to a decreased or increased occupancy of H3K9me2/3 containing heterochromatic regions. This possibility could also result in the observed developmental abnormalities.

Although further experiments solving these questions are definitely required, the results presented here allow the conclusion that the CDYL1b levels have to be well balanced during development. Tight regulation could be a hint for an important developmental function, which needs to be further investigated.

6.4.3 Hypothetical function of CDYL1b in establishment and maintenance of heterochromatin

As shown in chapters 3, 4 and 5 CDYL1b chromodomain recognizes H3K9me2/3 methylation *in vivo* and *in vitro*. It localizes to heterochromatin in cell culture and its nuclear distribution is dependent on the H3K9me3 mark. CDYL1b is implicated in transcriptional repression (5.1.2) and interacts with some heterochromatic factors (5.3). These attributes are characteristic for heterochromatin proteins.

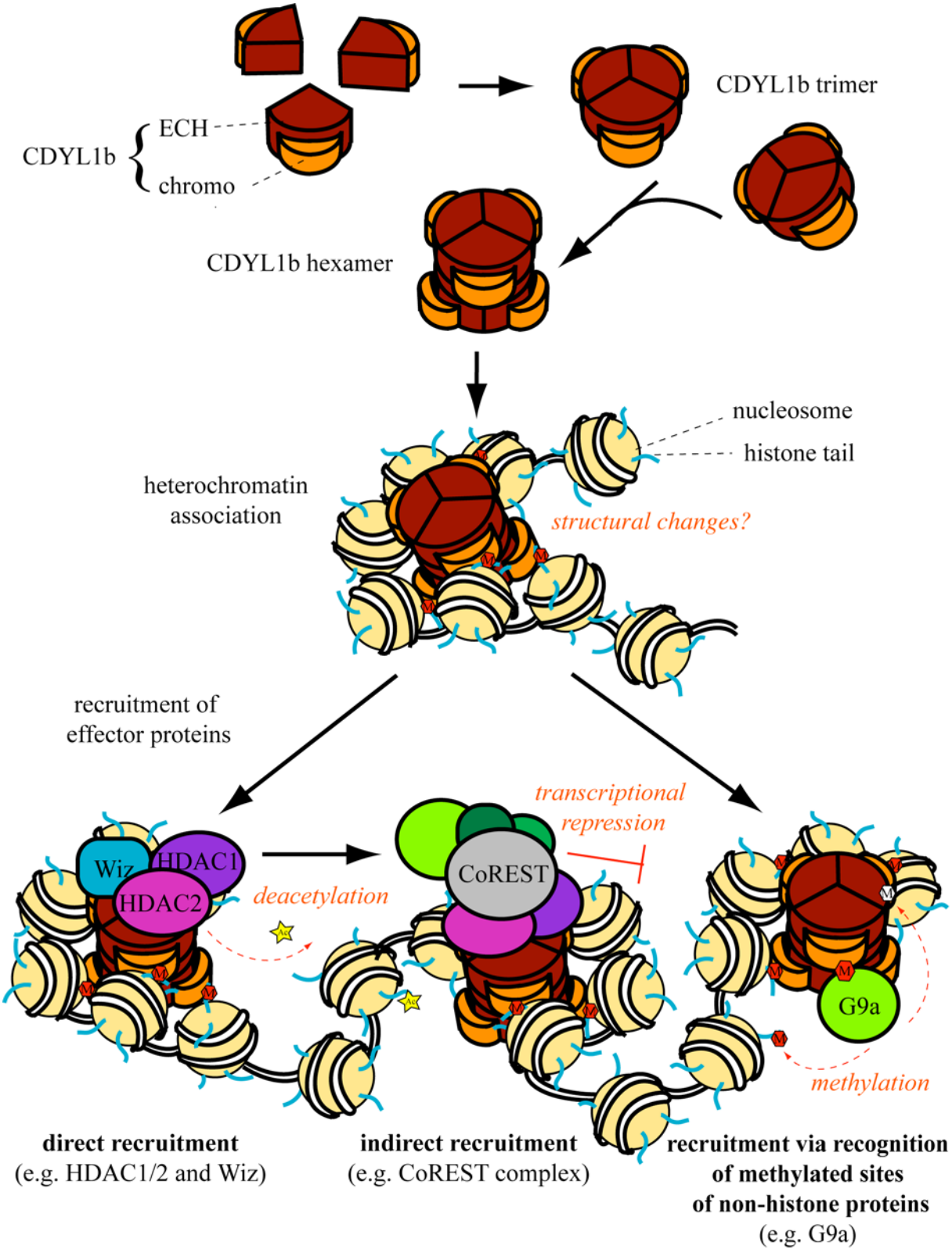


Figure 6-2 Model of CDYL1b function at heterochromatin

CDYL1b binds to the heterochromatin H3K9me3 mark via its chromodomain. The binding is dramatically enhanced by multimerization of the ECH domain. It is likely that the binding of CDYL1b induces structural changes in chromatin. In addition, CDYL1b might act as recruitment factor for indicated partners, which mediate downstream effects. M: methylation, Ac: acetylation.

In contrast to other CDY family members, CDYL1b is ubiquitously expressed, which might point to a general function in cellular maintenance (chapter 4). Besides the chromodomain, CDYL1b has an ECH domain. This domain is able to form trimers (chapter 4 and chapter 1.2.3). Two trimers are thought to interact resulting in a loose hexamers. Multimerization is required for heterochromatin association *in vitro* and *in vivo* (chapter 4). The combination of six chromodomains in heterochromatin could cross-connect six histone tails carrying the H3K9me2/3 mark. This connection could therefore result in higher order structures (compare with Figure 6-2) found in heterochromatin [167].

The putative developmental function as well as the putative regulation of CDYL1b localization to heterochromatin during mitosis and by its splicing variants (chapter 5) might further sustain to the hypothesis that CDYL1b might be an important factor in heterochromatin maintenance or even in heterochromatin establishment. It is possible that the CDYL1b interactions with heterochromatic factors like HDAC1/2, Wiz, CoREST components and G9a are implicated in these processes (compare also with Figure 6-2).

For verification of this hypothesis further experiments examining the CDYL1b function *in vitro* and importantly *in vivo* are needed. Structural changes of chromatin by CDYL1b could be analyzed *in vitro* by compaction assays, ultracentrifugation and atomic force microscopy with recombinant CDYL1b and recombinant chromatin. The CDYL1b influence on development and possible heterochromatin establishment could be further analyzed in *Xenopus laevis* embryogenesis and with CDYL1 knockout mice.

7 Supplemental material and methods to chapter 5

7.1 Materials

7.1.1 Chemicals

2-Mercaptoethanol	Sigma, Steinheim
4,6-Diamidino-2-phenylindole (DAPI)	Sigma, Steinheim
Acetic acid	Merck, Mannheim
Adenosine 5'-triphosphate (ATP)	Sigma, Steinheim
Agar	Roth, Karlsruhe
Agarose	Serva, Heidelberg
Albumin, Bovine (BSA)	New England Biolabs, Ibswich (USA)
Ampicillin	AppliChem GmbH, Darmstadt
Bromophenol blue	Serva, Heidelberg
Calcium chloride ($CaCl_2$)	Roth, Karlsruhe
Chorionic gonadotropin (human)	Sigma, Steinheim
Coelenterazine	Invitrogen, Karlsruhe
Coenzyme A	Invitrogen, Karlsruhe
Colorless GoTaq Buffer (5x)	Promega, Madison (USA)
Coomassie Brilliant Blue	BIO-RAD, Muenchen
Cysteine chloride	Sigma, Steinheim
DL-Dithiothreitol (DTT)	Alexis Biochemicals, Loerrach
Dimethylsulfoxid (DMSO)	Sigma, Steinheim
DMEM	Sigma, Steinheim
DMEM GlutaMAX II [-Pyruvate]	Gibco, Muenchen
dNTPs	Invitrogen, Karlsruhe
ECL plus ™	GE Healthcare, Buckinghamshire (UK)
Ethanol	Merck, Mannheim
Ethidiumbromide	Roth, Karlsruhe
Ethylene glycol-bis (β-aminoethylether) N,N,N',N' tetraacetic acid (EGTA)	Roth, Karlsruhe
Ethylenediaminetetraacetate (EDTA)	Roth, Karlsruhe
Fetal calf serum 10x (FCS)	Sigma, Steinheim
Ficoll	Sigma, Steinheim

Glycerol	Merck, Mannheim
Glycine	Merck, Mannheim
Glycogen	Fermentas, St Leon-Rot
Glycylglycine	AppliChem GmbH, Darmstadt
Hydrochloric acid (HCl)	Merck, Mannheim
Isopropyl ß-D-thiogalactopyranoside (IPTG)	AppliChem GmbH, Darmstadt
JetPei	PolyPlusTransfection, New York (USA)
L-Glutamine 100 x	Gibco, Muenchen
Lipofectamine 2000	Invitrogen, Karlsruhe
Luciferin	PJK, Rehovot (Israel)
Magnesium chloride ($MgCl_2$)	Merck, Mannheim
Magnesium sulfate ($MgSO_4$)	Roth, Karlsruhe
Methanol	Sigma, Steinheim
Milk powder	regilait, Saint-Martin-Belle-Roche (France)
Mowiol	Calbiochem, Darmstadt
N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid (Hepes-NaOH)	Merck, Mannheim
Non essential amino acids	Gibco, Muenchen
Nonidet P-40 (NP-40)	Roche, Penzberg
Normal goat serum	Bioscience International, Maine (USA)
Paraformaldehyde	Sigma, Steinheim
Penicillin Streptomycin 100x (PenStrep)	Gibco, Muenchen
Peptone	Roth, Karlsruhe
Pfu Polymerase II reaction buffer	New England Biolabs, Ibswich (USA)
Phenol/Chloroform/Isoamyl alcohol	Roth, Karlsruhe
Phenylmethanesulphonylfluoride (PMSF)	Serva, Heidelberg
PLB buffer	Promega, Madison (USA)
Ponceau S	Sigma, Steinheim
Potassium chloride (KCl)	Roth, Karlsruhe
Potassium monohydrogen phosphate	Merck, Mannheim

(K_2HPO_4)	
Potassium nitrate (KNO_3)	Merck, Mannheim
Protease Inhibitor	Roche, Penzberg
Random Hexamers	Invitrogen, Karlsruhe
RNase Inhibitor	Ambion, Austin (USA)
S-[^{3}H]adenosylmethionine	GE Healthcare, Buckinghamshire (UK)
Sodium acetate	Roth, Karlsruhe
Sodium azide (NaN_3)	Alfar Aesor, Karlsruhe
Sodium chloride (NaCl)	Merck, Mannheim,
Sodium dodecyl sulfate (SDS)	VWR, Poole (UK)
Sodium hydrogen phosphate (Na_2HPO_4)	Merck, Mannheim
Sodium pyruvate 100x	Gibco, Muenchen
Sodium tetraborate	Amresco, Ohio (USA)
Sodiumhydrogencarbonate ($NaHCO_3$)	Merck, Mannheim
Streptavidin-coated beads	Promega, Madison (USA)
Succrose	Serva, Heidelberg
Triethanolamine	VWR, Poole (UK)
Tris (hydroxymethyl) aminoethane (Tris)	Roth, Karlsruhe
Triton X100	Merck, Mannheim
Trypan Blue	Gibco, Muenchen
Trypsin solution	Gibco, Muenchen
Tween 20	Sigma, Steinheim
Yeast extract	MOBIO, Hamburg

7.1.2 Buffers and media

Blocking solution: 1x PBS, 2% BSA, 0.2% Triton X100 (v/v), 5% Normal goat serum (v/v)

Coomassie solution: 2.5% Coomassie Brilliant Blue, 10% acetic acid, 50% methanol

Destaining solution: 10% acetic acid (v/v), 7.5% methanol (v/v)

DMEM cell culture medium: 1x DMEM GlutaMAX II [-Pyruvate], 1x L-Glutamine, 1x Pencillin Steptomycin, 1x FCS

Fixation solution: 3% Paraformaldehyde (w/v) in 1x PBS

Freezing medium: DMEM cell culture medium, 10% (v/v) DMSO

Laemmli buffer 1x: Tris 63 mM (pH 6.8), 0.1% 2-Mercaptoethanol (v/v), 0.0005% Bromophenol blue (w/v), 10% glycerol (v/v), 2% SDS (w/v)

LB agar: 1.5 % (w/v) Agar in LB medium

LB Ampicillin medium/agar: LB medium or agar with 100 µg/ml Ampicillin

LB medium: 1 % (w/v) Peptone, 0.5 % (w/v) Yeast extract, 0.5 % NaCl % (w/v), pH 7,5, autoclaved

Low-ionic-strength suspension medium (LISM): 1 mM PMSF, 0.1 mM $MgCl_2$

Luciferase assay reagent "Firefly": 25 mM Glycylglycine, 15 mM K_2HPO_4 (pH 8.0), 4 mM EGTA, 15 mM $MgSO_4$, 4 mM ATP pH 7.0, 1.25 mM DTT, 0.1 mM Coenzyme A, 80 µM Luciferin

Luciferase assay reagent "Renilla": 1.1 M NaCl, 2.2 mM Na_2EDTA, 0.22 M K_2HPO_4 (pH 5.1), 0.5 mg/ml BSA, 1.5 mM NaN_3, 1.5 µM Coelenterazine

Lysis buffer: 50 mM Tris (pH 7.5), 5 mM EDTA, 0.5% SDS (w/v), 50 mM NaCl, Proteinase K (20µg/ml)

MAB 2x: 50 mM Tris (pH 8.5), 20 mM $MgCl_2$, 300 mM KCl, 500 mM Succrose

MBS 1x: 10 mM Hepes-NaOH (pH 7.0), 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.41 mM $CaCl_2$, 0.66 mM KNO_3

MEF cell culture medium: 1x DMEM, 1x L-glutamine, 1x PentStrep, 1x FCS, 1 ml β mercaptoethanol, 1x Non essential amino acids, 1x Sodium pyuvate, steril filtered

Mounting medium: Mowiol including 50 µg/ml DAPI

NE buffer A: 10 mM Hepes-NaOH (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl

NE buffer B: NE Buffer A including 0.1% NP-40 (v/v)

NE buffer C: 20 mM Hepes-NaOH (pH 7.9), 25% glycerol (v/v), 420 mM NaCl, 0.2 mM EDTA, 1.5 mM $MgCl_2$

NE buffer D: 20 mM Hepes-NaOH (pH 7.9), 20% glycerol (v/v), 50 mM KCl, 0.2 mM EDTA

Nuclear membrane storage medium (NMSM): 20% glycerol (v/v), 1 mM EDTA, 1 mM PMSF, 10 mM Tris (pH 7.5)

PBS 1x: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 (pH 7.4), 1.47 mM KH_2PO_4

PBST: PBS including 0,1% of Tween 20 (v/v)

PD150: 20 mM Hepes NaOH (pH 7.9), 20% glycerol, 150 mM KCl, 0.2% Triton X100 (v/v), 1x Protease Inhibitor

PD300: 20 mM Hepes NaOH (pH 7.9), 20% glycerol (v/v), 300 mM KCl, 0.2%

Triton X100 (v/v), 1x Protease Inhibitor

Permibilization solution: 1xPBS, 0.2% Trition X100 (v/v), 0.2% NP-40 (v/v)

Ponceau: 5% acetic acid (v/v), 0.1% ponceau S (w/v)

SDS-Running buffer: 25 mM Tris, 200 mM glycine. 0.1% SDS (w/v)

Sucrose buffer pH 7.4 (SB 7.4): 0.3 M Sucrose, 0.1 mM $MgCl_2$, 5 mM 2-Mercaptoethanol, 1 mM PMSF, 10 mM Triethanolamine, pH adjusted to 7.4 (HCl)

Sucrose buffer pH 8.5 (SB 8.5): as Sucrose buffer pH 7.4 but pH 8.5

TBE: 90 mM Tris, 90 mM Sodiumtetraborate, 2 mM EDTA (pH 8.0)

Transferbuffer: 200 mM glycine, 25 mM Tris-HCl, 0,04% SDS (w/v), 20% Methanol (v/v)

Wash buffer: 1x PBS, 0.1% Triton X100 (v/v)

7.1.3 Antibodies

Used antibody dilutions are given in the according method sections (i.e. for Western Blot chapter 7.3.1 and for immunofluorescence chapter 7.4.7).

αFLAG	M2 clone, lab intern
αH3 acetyl	Millipore, Billerica (USA)
αH3K4me1	Abcam, Cambridge (UK)
αH3K4me2	Abcam, Cambridge (UK)
αH3K9me1	Millipore, Billerica (USA)
αH3K9me2	Millipore, Billerica (USA)
αH3K9me3	Millipore, Billerica (USA)
αH3K9me3S10phos	Millipore, Billerica (USA)
αH4K3me3	Abcam, Cambridge (UK)
αLamin B	ImmuQuest, North Yorkshire (UK)
αmouse-alexa-555	Invitrogen, Karlsruhe
αmouse-HRP	DakoCytomation, Hamburg
αmouse-IgG-magnetic beads	DYNAL Biotech, Oslo (Norway)
αmyc	Millipore, Billerica (USA)
αPRMT5	Millipore, Billerica (USA)
αrabbit-alexa-488	Invitrogen, Karlsruhe
αrabbit-HRP	DakoCytomation, Hamburg
αTBP	Santa Cruz, Heidelberg

7.1.4 Kits

$CaPO_4$ transfection Kit	Clontech, Mountain View (USA)
Dig-RNA-labeling Kit	Roche, Penzberg
DNA purification Kit	Qiagen, Hilden
MiniPrep	Qiagen, Hilden
mMessage/Machine	Ambion, Austin (USA)
PCR purification Kit	Qiagen, Hilden
RNeasy purification Kit	Qiagen, Hilden
TNT Quick Coupled Transcription/Translation System	Promega, Madison (USA)

7.1.5 Enzymes

Antarctic phosphatase	New England Biolabs, Ibswich (USA)
Benzonase	Merck, Darmstadt
Digestion enzymes	New England Biolabs, Ibswich (USA)
DNase, 10x DNase buffer	New England Biolabs, Ibswich (USA)
Dpn1	New England Biolabs, Ibswich (USA)
MuLV reverse transcriptase	Applied Biosystems, Darmstadt
Pfu Polymerase II	Stratagene, La Jolla (USA)
Proteinase K	Invitrogen, Karlsruhe
T4-DNA-ligase and 10x buffer	New England Biolabs, Ibswich (USA)

7.1.6 Primers and other oligomers

Oligonucleotides sequences were designed with the help of DNASTAR Lasergene 7 and were ordered from MWG, Ebersberg. The primer sequences used are listed in 7.2.2 or described when needed. Morpholino oligomers were obtained from GeneTools, LLC, Philomath (USA).

7.1.7 Plasmids obtained from different sources

For detailed informations plasmid cards are available at 8.3 except plasmids described in indicated publications or obtained from indicated people.

DBD Gal4 null	Judd Rice [168]
pBluescript XL007a18	NIBB, Okazaki
pBluescript XL213m10	NIBB, Okazaki
pcDNA3.1 (-) myc His	Invitrogen, Karlsruhe
pcDNA3.1 (+) Flag HA	Nora Koester-Eiserfunke [169]

pcDNA3.1 (+) hCDYL1a Flag HA	Wolfgang Fischle [95]
pcDNA3.1(+)	Invitrogen, Karlsruhe
pCMV Renilla	Judd Rice [168]
pCS2+	Imagene (RZPD), Berlin
pCS2+ lacZ	Annette Borchers [170]
pET11a	New England Biolabs, Ibswich (USA)
UAS TK	Judd Rice [168]

7.1.8 Bacteria

Escherichia coli BL21 (DE3) RIL (Stratagene, La Jolla) und DH5α (Invitrogen, Karlsruhe).

7.1.9 Cell lines

	Origin	Organism	Medium	Reference
HEK293	Embryonic Kidney	Human	DMEM	Wolfgang Fischle
HeLaS3	Cervical cancer	Human	DMEM	Wolfgang Fischle
MEF	Embryonic Fibroblasts	Mouse	MEF	Thomas Jenuwein
NIH3T3	Fibroblasts	Mouse	DMEM	Wolfgang Fischle

7.1.10 Peptides

Peptides were obtained from the Griesinger department of the Max Planck Institute of biophysical Chemistry.

Name	Histone (aa)	Peptide sequence	Modification	Label
H3unmod	H3 1-20	MARTKQTARKSTGGKAPRKQ	-	Biotin C-term
H3K9me3	H3 1-20	MARTKQTARKSTGGKAPRKQ	K9me3	Biotin C-term

7.1.11 Software

KaleidaGraph Version 4.0	Synergy Software, Reading (USA)
Lasergene 7	DNASTAR, Madison (USA)
Microsoft Office	Microsoft Cooperation (USA)
MicroWin	MSE, Muenster
R a language for statistival computing	R Foundation for Statistical Computing, Vienna (Austria)

7.1.12 Others

10 cm cell culture dish	Greiner, Solingen
10 cm Petri dish	Greiner, Solingen
15 cm cell culture dish	Sarstedt, Sarstedt
6-well, 12-well, 24-well plates	Greiner, Solingen
Amersham ECL Hyperfilms	GE Healthcare, Buckinghamshire (UK)
Bioruptor	Diogenode, Liége (Belgium)
Centrifuge 5415 R	Eppendorf, Hamburg
Centrifuge 5810 R	Eppendorf, Hamburg
Coverslips d=1 cm	VWR, Poole (UK)
Cryo Freezing controller	Nalgene, Ohio (USA)
Cryotubes	Greiner, Solingen
Freezer -150°C	Thermo Scientific, Braunschweig
Freezer -80°C	Thermo Scientific, Braunschweig
Gel Doc 2000	BIO-RAD, Muenchen
Gel dryer Model 583	BIO-RAD, Muenchen
Hereaus Heracell 240 Incubator	Thermo Scientific, Braunschweig
Hereaus Kelvitron® Incubator	Thermo Scientific, Braunschweig
Homogenizer 2 ml (DOUNCE)	VWR, Darmstadt
Kodak X OMAT 2000 proccessor	Carestream Health, New York (USA)
Leica TCS SP5	Leica, Wetzlar
Microinjector (Picospritzer)	Parker, Cleveland (USA)
Microscale	Ted Pella Inc., Redding (USA)
Microscope Axiovert 40CFL	Zeiss, Jena
Mini-PROTEAN 3 Cells	BIO-RAD, Muenchen
MiniTrans-Blot®	BIO-RAD, Muenchen

Multitron shaker (Bacteria)	HT Infors, Braunschweig
Nanodrop ND-1000	Peqlab, Erlangen
Neubauer chamber	BRAND, Wertheim
Nitrocellulose membrane	BIO-RAD, Muenchen
Optiplates	Perkin Elmer, Shelton (USA)
Orbitrap Xl mass spectrometer	Thermo Scientific, Braunschweig
PCR machine epgradient S	Eppendorf, Hamburg
PlateChameleon	Hidex, Turku (Finnland)
Scanner Perfection V750 PRO	Epson, Meerbusch
Stuart Gyrorocker SSL3	Sigma, Steinheim
Sub-Cell-GT® Agarose gel electrophoresis	BIO-RAD, Muenchen
Superfrost Ultra Plus slides	Thermo Scientific, Braunschweig
Thermomixer comfort	Eppendorf, Hamburg

7.2 Molecular biological methods

7.2.1 Polymerase chain reaction (PCR)

hCDYL1b, CDYL1c, xlCDYL1b and hPRMT5 were amplified from EST clones (hCDYL1b Image clone: IMAGE:6140263, xlCDYL1b EST-clone: XL213m10 and hPRMT5 Image full length cDNA clone: IRAUp969D1078D) with the indicated primers (Table 7-1). For detailed sequences see chapter 8.1. The 50 µl reaction volume contained 0.5 µg template, 1xPfu Polymerase reaction buffer, 200 µM dNTPs, 1 mM of each primer pair and 1µl of Pfu Polymerase II. The mixture was incubated in the PCR machine with the following protocol: 2 min 94ºC; 5x (30 sec 94ºC, 1 min 55ºC, 2.5 min 72ºC); 25x (30 sec 94ºC, 1 min 60ºC, 2.5 min 72ºC); 10 min 72ºC.

7.2.2 Cloning

Target DNA was amplified by PCR with indicated primers (Table 7-1). PCR products as well as target vectors were digested with indicated enzymes according to NEB protocols [171]. Then the digested vectors were dephosphorylated with Antarctic Phosphatase (NEB protocols [171]). The digested products were purified by PCR purification Kit and the concentration was determined by Nanodrop measurements at 260 nm. Then the DNA was ligated with DNA-ligase according to NEB protocols [171]. *E. coli* DH5α bacteria cells were transformed with the ligated DNA [172] and plated on ampicillin agar plates. The agar plates were incubated overnight at 37ºC. Single colonies were picked and cultured in 5 ml LB medium at 37ºC overnight. Plasmid DNA was extracted by MiniPrep Kit and 500 ng were digested by the cloning enzymes [171]. The digest was loaded on a 1% agarose gel containing 0.01% ethidiumbromide (v/v) separated by electrophoresis in TBE buffer (20 min, 100V) using the Sub-Cell-GT® system. Gels were visualized with Gel Doc 2000. Plasmids showing the right insert sizes were sequenced by MWG (Ebersberg) or Seqlab (Göttingen) using company provided primers.

Table 7-1 Cloned plasmids

Indicated inserts were cloned into corresponding vector. Plasmids were used for production of recombinat proteins in *E.coli* (EC), transfection in cell culture (CC), Luciferase assays (LC), Morpholino tests (MT) and microinjection in *Xenopus laevis* embryos (MI).

	Use	Vector	Primers (Sequence 5'-3')	Cloning sites 5'-3'
5'UTR xlCDYL1b FLAG	MT	pCS2+	xlCDYL1b_5UTR_for ttttgcaggatccggaggaggccgagcacac xlCDYL1b_rev tccaggctcgagttacttgtcatcgtcgtccttctagtcgaac	BamHI XhoI

			tcatcaatttttctttg	
hCDYL1b	EC	pET11a	hCDYL1b_for aaggttcatatggcttccgaggagctgtacg hCDYL1b_rev ggccattggatccttagaactcatcgatcttcctctg	NdeI BamHI
hCDYL1b chromo	EC	pET11a	hCDYL1b _for aaggttcatatggcttccgaggagctgtacg hCDYL1b_chromo_rev ggccattggatccttacttctgcttctccgtgtggcgtc	NdeI BamHI
hCDYL1b chromo Flag tagged	CC	pcDNA3.1 (+) Flag HA	hCDYL1b_chromo_Fl_for attgcgctagcAtggcttccgaggagctgtacga hCDYL1b_chromo_Fl_rev gcgcatgcggccgccttctgcttctccgtgtggcgt	NheI NotI
hCDYL1b chromohinge Flag tagged	CC	pcDNA3.1 (+) Flag HA	hCDYL1b_chromo_Fl_for attgcgctagcAtggcttccgaggagctgtacga hCDYL1b_chromohinge_Fl_rev Gcgcatgcggccgcgaagccatcctgcttcctgac	NheI NotI
hCDYL1b Flag HA tagged	CC	pcDNA3.1 (+) Flag HA	hCDYL1bFlHA_for tatccgcggccgcgccaccatggcttccgaggagctgta hCDYL1bFlHA_rev ggtatgcggccgcgaactcatcgatcttc	NotI NotI
hCDYL1b hinge	EC	pET11a	hCDYL1b_hinge_for aaggttcatatggagagcacattgaccagaacaaac hCDYL1b_hinge_rev ggccattggatccttagaacggagatgtacctttcccg	NdeI BamHI
hCDYL1c	EC	pET11a	hCDYL1b_for aaggttcatatggatgcattaacagccaatgg hCDYL1c_rev ggccattggatccttagaactcatcgatcttcctctg	NdeI BamHI
hCDYL1c FLAG HA tagged	CC	pcDNA3.1 Flag HA	hCDYL1cFlHA_for gatcctcttcctcaccatggatgcattaacagccaatg hCDYL1bFlHA_rev ggtatgcggccgcgaactcatcgatcttc	NotI NotI
hPRMT5 myc-tagged	CC	pcDNA3.1 (-) myc His	PRMT5myc_for agcccgctcgagatggcggcgatggcggtcggg PRMT5myc_rev gcttccggatccgaggccaatggtatatgag	XhoI BamHI
xlCDYL1b chromohinge Gal4 tagged	CC LA	DBD Gal4 null	xCDYL1b_Gal4_for cctggaggatccatggcttcagaggaactctac xCDYL1b_ch_Gal4_rev gcgcatgcggccgctcagaaaccatcttgctttctgac	BamHI NotI
xlCDYL1b chromo Gal4 tagged	CC LA	DBD Gal4 null	xCDYL1b_Gal4_for cctggaggatccatggcttcagaggaactctac xCDYL1b_c_Gal4_rev gcgcatgcggccgctcatttaggcttctcactgtgtctt	BamHI NotI
xlCDYL1b chromohinge ΔNLS Gal4 tagged	CC LA	DBD Gal4 null	xCDYL1b_Gal4_for cctggaggatccatggcttcagaggaactctac xCDYL1b_chΔNLS _Gal4_rev gcgcatgcggccgctcataaagtagaagtacctttacc	BamHI NotI
xlCDYL1b FLAG	MI	pCS2+	xlCDYL1b_for cctggaggatccatggcttcagaggaactctac xlCDYL1b_rev tccaggctcgagttacttgtcatcgtcgtccttctagtcgaac tcatcaatttttctttg	BamHI XhoI
xlCDYL1b Gal4 tagged	CC LA	DBD Gal4 null	xCDYL1b_Gal4_for cctggaggatccatggcttcagaggaactctac	BamHI NotI

xlCDYL1b hingeECH Gal4 tagged	CC LA	DBD Gal4 null	xCDYL1b_Gal4_rev tccagggcggccgcttagaactcatcaatttttctttg xCDYL1b_hinECH_Gal4_for cctggaggatccatgcacagtgagaagccraaaga xCDYL1b_Gal4_rev tccagggcggccgcttagaactcatcaatttttctttg	BamHI NotI
xlCDYL1c Gal4 tagged	CC LA	DBD Gal4 null	xCDYL1c_Gal4_for cctggaggatccatggaggctttgacacagctagt xCDYL1b_Gal4_rev tccagggcggccgcttagaactcatcaatttttctttg	BamHI NotI

7.2.3 Mutagenesis

The target plasmid was amplified with the indicated primers (Table 7-2) with the same reaction mix as described in 7.2.1. The reaction was incubated in the PCR machine with the following protocol: 2 min 94ºC; 3x (30 sec 94ºC, 1 min 57ºC, 2 min 72ºC); 17x (30 sec 94ºC, 1 min 63ºC, 2 min 72ºC); 10 min 72ºC. The products was purified with the PCR purification kit and digested with Dpn1 using the NEB protocol [171]. Next the DNA was transformed into *E.coli* DH5α, plated and analyzed as described before (7.2.2).

Table 7-2 Primers for mutagenesis

Target	Mutagenesis primer (Sequence 5'-3')
hCDYL1b R80A	hCDYL1b_R80A_for: cctctcccaacaatgctgcaaaacaaatctccagatccacc hCDYL1b_R80A_rev: ggatctggagatttgttttgcagcattgttgggagaggtcctg
xlCDYL1b R80A	xCDYL1b_R80A_for: gacatctccaaataatgcagcaaaacaaatttccaggtcaac xCDYL1b_R80A_rev: gacctggaaatttgttttgctgcattatttggagatgtccg

7.3 Biochemical methods

7.3.1 SDS-PAGE and Western Blot

Acrylamide gels were poured as described [173] and were used for gel electrophoresis with the Mini-PROTEAN 3 Cells. Gels were run in SDS running buffer for 30 min, 35 mA. Then the gels were blotted on nitrocellulose membranes with MiniTrans-Blot® using Transferbuffer (1 h, 100V) analyzed by mass spectrometry or stained with Coomassie (compare with 7.3.2). The membranes were stained with Ponceau solution (5 min, RT). For removal of the Ponceau excess the membrane was washed 3x with PBST. Next the membrane was blocked for 1 h with PBST including 5% fat free milk powder at RT. Afterwards the membrane was incubated with PBST (5% milk) including αFLAG, αmyc, αLamin B, αTBP, αCDYL or αPRMT5 in a 1:1000 dilution for 1 h, RT. Then the membrane was washed 3x with PBST, 5 min each followed by incubation with the secondary antibodies αmouse or

αrabbit conjugated to HRP in a 1:2000 dilution in PBST (5% milk) for 1 h, RT. Washing steps (3x, 10 min) with PBST removed excess of antibody solution. The membrane was developed with ECL plus and the signals detected with Amersham ECL Hyperfilms. The film was developed with the Kodak X OMAT.

7.3.2 Coomassie staining

To stain acrylamide gels the stacking gel was removed and the separating gel was stained for 10 min in Coomassie solution under constant luffing. To remove the backround the gel was then placed in Destaining solution for at least 1 h. Afterwards the gel was scanned with the Epson Scanner.

7.3.3 Mass spectrometry and analysis of the results

The mass spectrometry analysis was performed by the mass spectrometry facility of Dr. Henning Urlaub at the Max Planck Institute of biophysical Chemistry. SDS PAGE gels were stained with Coommassie and entire gel lanes were cut into 23 slices of equal size. Proteins within the slices were digested according to Shevchenko et al. [174]. Peptides were extracted and analyzed by LC-coupled tandem MS on an Orbitrap Xl mass spectrometer (Thermo Fisher Scientific). CID fragment spectra were searched against NCBInr database using MASCOT as search engine. Output files were subtracted according to the gi-numbers (NCBI) of the found proteins with the help of the statistical program R (for details of programming compare with 8.4). Arbitrary cut-off values of at least 2 unique found peptides per protein were used.

7.3.4 Protein expression

Plasmids containing the DNA of the protein of interest were transformed into *E.coli* BL21 RIL as described [172] and plated on ampicillin agar plates for incubation overnight at 37°C. Single colonies were picked and cultured in 5 ml LB media at 37°C, 150 rpm until an OD of 0.5 determined by Nanodrop was reached. The adding of 0.2 mM IPTG induced the expression of the protein. After incubation time (4 h at 37°C) the bacteria were pelleted by cenrifugation (5 min, 4000 rpm). For methyltransferase assays the pellet was dissolved in 500 µl of MAB 2x and the solution was sonicated for 30 min (30 sec pulse, 30 sec pause) with the Diogenode Bioruptor. Then the solution was centrifuged for 10 min, 16000 rpm. The resulting pellet was discarded and the supernatant was used as reaction educts for methyltransferase assays.

7.4 Cell-based methods

7.4.1 Cell culture

Thawing of cells – Cryotubes were thawed in a 37°C water bath and 1 ml of 37°C prewarmed cell line specific medium was added (7.1.9). Then cells were transferred to a 10 cm plate with 10 ml of 37°C prewarmed CO_2–equilibrated medium. Afterwards the cells were incubated for overnight at 37°C in a 5% CO_2 saturated atmosphere (Hereaus Heracell incubator). At the next day the medium was exchanged and the cells were cultivated until 80% confluence.

Maintenance of cells – For maintenance of cells the confluence level of the cell line was determined by the Microscope Axiovert with a 20x amplification lens. A confluence level of about 80% required cell splitting. For that the cells were washed once with 1x PBS. Then 0.5-2 ml (0.5 ml for a well of a 6-well plate, 1ml for 10 cm cell culture dish and 2 ml for a 15 cm cell culture dish) of Trypsin solution was added and incubated for 5 min 37°C. The detached cells were then diluted with 3 ml, 10 ml or 15 ml medium depending on the dish size respectively. Approximately 1/10 for HEK293 and HeLaS3 or 1/5 for MEF and NIH3T3 of the cells were seeded into new cell culture dishes.

Freezing of cells – For freezing the cells were grown to a confluence of about 70%. Then the cells were trypsinised as described in the former paragraph and an aliquot of 20 µl were diluted with 20 µl of Trypan Blue solution to stain dead cells. The living cells were counted with the help of a Neubauer chamber. The rest of the cells was centrifuged at 300xg, 5 min, RT. The cellular pellet was then resuspended in ice-cold Freezing medium ($2x10^6$ cells/ml) and aliquoted in 1 ml cryotubes. The tubes rested in a precooled Cryo Freezing Controller at -80°C overnight and were then stored at -150°C.

7.4.2 Nuclear extraction

For nuclear extraction all buffers were used at 4°C and were supplemented with 1x protease inhibitor, 1 mM DTT and 1 mM PMSF. $4x10^7$ HEK293 or HelaS3 cells were scraped of the dish and centrifuged at 300xg, 5 min, 4°C. The cell pellet was washed 2x with PBS followed by centrifugation (300xg, 5 min, 4°C). Cells were resuspended in 500 µl NE buffer A and transferred to a 1,5 ml tube. The resuspension was centrifuged (600xg, 5 min, 4°C) and the pellet was washed again with 500 µl NE buffer A. Afterwards the pellet was resuspended in 500 µl NE buffer B and incubated for 10 min on ice. The solution was mixed briefly after 5 and 10 min during incubation time. Next the isolated nuclei were pelleted with 600xg, 5 min, 4°C. Afterwards the supernatant was removed and the nuclear pellet was resuspended in 400 µl of NE buffer C and was transferred to a dounce homogenisator. The resuspension was

dounced every 5 min for 20 times, the 15 min incubation time was followed by an centrifugation of 10 min, 20000xg at 4°C. The nuclear extract was then transferred to a new tube and was diluted with 600 µl of NE buffer C. Diluted extract was then used for immunoprecipitation. For detecting interaction partners of hCDYL1b FLAG and hPRMT5 myc the nuclear extract was diluted with 600 µl NE buffer D.

7.4.3 Immunoprecipitation

For immunoprecipitation HEK293 cells were transfected with the plasmid of interest using the $CaPO_4$ transfection Kit. After two days of incubation on 37°C the proteins of the cells were extracted by the Nuclear extraction protocol. 5 µl of the immunoprecipitating antibody (αmyc or αFlag) was coupled to 40 µl of αmouse-IgG-magnetic beads for 3 h, RT under constant rotation. After washing (3x PBS) the beads were added to 1 ml of nuclear extract and were incubated 4 h or overnight at 4°C. Afterwards the beads were washed 6x with 1 ml PD150 at 4°C. For more stringency, especially for immunoprecipitated hPRMT5 myc protein used for methyltransferase assays, the six washing steps were performed with PD300. After washing the beads were either stripped with 1x Laemmli (5 min, 95°C) or resuspended in PD150.

7.4.4 Methyltransferase assay

For methyltransferase assays 5µl beads covered with immunoprecipitated hPRMT5 myc (7.4.3) were incubated with E.coli expressed recombinant proteins (7.3.4) in a reaction mixture containing MAB 1x and 2 µCi S-[^{3}H]adenosylmethionine (SAM) for 1,5 h at 30°C, 1200 rpm. The products were loaded on a SDS-page. The resulting gel was dried for 2 h, 80°C with the help of the gel dryer and exposed to an Amersham ECL Hyperfilm. The film was developed with the Kodak X OMAT.

7.4.5 Pulldown

To detect binding of proteins to modified histone peptides, 1 µg of the biotinylated peptides (7.1.10) were bound to 40 µl streptavidin coated beads for 3 h at RT, 1400 rpm. After binding the beads were washed 3x with PBS to get rid of the peptide excess. 1 ml of nuclear extract or 50 µl of a *in vitro* transcription and translation reaction (TNT Quick coupled Transcription/Translation System) were incubated with 40 µl peptide-bound beads overnight at 4°C under constant rotation. On the next day the beads were washed 6x with 1 ml PD150 at 4°C. Then the beads were stripped with 20 µl 1x Laemmli (5 min, 95°C) and loaded on a SDS-PAGE followed by mass spectrometry analysis or Western Blot analysis.

7.4.6 Dual luciferase assay

HEK293 cells were seeded into standard 12-well plates 24 h prior treatment at a concentration of $1.5x10^5$ cells/well. On the next day cells were transfected with a total amount of 1.2 µg DNA per well. The total DNA contained 100 ng of the targeting plasmid UAS TK, which containes Gal4 binding sites and a tyrosine kinase promotor, 2 ng of CMV Renilla, a transfection control under CMV promotor control, the Gal4-tagged protein of interest in different amounts (2ng-50ng), and emty pcDNA3.1 vector to reach 1.2 µg in total. The DNA was mixed with 100 µl Serum-free DMEM GlutaMAX II. Per well 3,6 µl Lipofectamine were added to 100 µl Serum-free DMEM GlutaMAX II. The Lipofectamine solution was incubated for 5 min at RT and was then combined with the DNA solution followed by an incubation of 20 min, RT. The Lipofectamine/DNA complexes were then added to the HEK293 cells and were incubated for 24 h at 37°C/ 5% CO_2. Next day the cells were scraped off in medium and transferred to 1.5 ml Eppendorf tubes. Cells were pelleted by centrifugation of 5 min, 3000 rpm, RT. The supernatant was discarded. The cells were resuspended in 100 µl of 1x PLB buffer and were incubated for 15 min, RT under constant agitation 1400 rpm. The lysed cells were centrifuged 1 min, 14000 rpm and 20 µl of the supernatant was pipetted into one well of a 96-well Optiplate. 100 µl of the "Firefly" solution and afterwards 100 µl "Renilla" solution were then added to each well by the PlateChameleon. After each step the luminosity was counted. The measured values were transferred into Microsoft Exel and normalized against the control transfected cells and the Renilla signal.

7.4.7 Immunofluorescence

Cells were grown in 24 well plates on coverslips (d = 1 cm). If necessary the cells were transfected at 30% confluency with JetPei as described [175]. The immunstaining was then performed two days post transfection. First the cells were washed 2 times with PBS, covered with Fixation solution and incubated at 37°C for 15 min. Afterwards Fixation solution was removed and the cells were washed 1x with the Wash buffer. 500 µl of the Permeabilization solution was added to the cells followed by an incubation for 10 min at RT. Next the the cells were incubated for 1 h at RT in Blocking solution. Then, primary antibodies were added directly on the coverslip diluted in a total volume of 65 µl Blocking solution (αH3 acetylated, αH3K4me1, αH3K4me2, αH3K9me1, αH3K9me2 and αH3K9me3S10phos were used in a 1:200 dilution, αH3K4me3, αH3K9me3, αLamin and αFLAG in a 1:1000 dilution) and incubated again for 1h, RT. This step was followed by 3x washing with 500 µl of the Wash buffer and adding of the secondary antibodies diluted in 65 µl total volume in Blocking

solution (αrabbit-alexa-488 and/or αmouse-alexa-555 1:2000). Excess of antibodies was removed with 3x washing with Wash Buffer. Then coverslips were dipped into water to remove other salt contaminations and were mounted with the mounting medium Mowiol to Superfrost Ultra Plus slides. Slides were dried overnight at RT and analysed with the confocal microscope Leica TCS SP5.

7.4.8 Membrane isolation

Nuclei were prepared as described for nuclear extraction (7.4.2). A sample of the cytosolic fraction was saved. The nuclei from $2x10^7$ cells were resuspended in 200 µl LISM. 500 U Benzonase and 800 µl of SB 8.5 were added. This resuspension was incubated under gentle stirring for 15 min at RT, and then 1 ml of ice-cold distilled water was added. After a centrifugation step of 30 min 16000xg the supernatant I was saved and the pellet resolved in 800 µl of SB 7.4. 250 U of Benzonase was added and incubated for 15 min, RT under constant rotation. After centrifugation of 30 min, 16000xg the supernatant II was removed and the pelleted membranes were dissolved in 20µl NMSM. The supernatant I was combined with the supernatant II and was analysed together with the membranes. For salt treatment indicated amounts (100 mM to 500 mM) of KCl were added to NMSM. The membranes were pelleted (5 min, max speed, 4°C), supernatant was removed and 100 µl of NMSM containing 100 mM, 200 mM, 300 mM, 400 mM or 500 mM KCl was added for 10 min, RT. Then membranes were pelleted again and the supernatant as well as the washed membranes were analyzed by Western Blot.

7.5 *Xenopus laevis* methods

7.5.1 Production and culturing of Xenopus laevis embryos

Female frogs were stimulated the evening prior egg collection by injection of 50 U of human chorionic gonadotropin (hCG) into the dorsal lymph sac. Injected frogs were kept at room temperature overnight and the eggs were collected approximately 14 h later. For testis preparation of males the frogs were placed on ice for 30 min, decapitated and then the belly was opened. Testis was removed, cleaned from fat tissue, placed in 1x MBS containing 1x PenStrep and stored at 4°C. For in vitro fertilization approximately one quarter of a testis was macerated in 1.5 ml 1x MBS. The eggs of a female frog were collected in a 10 cm Petri dish and 100 µl of the testis mixture diluted in with 900 µl of water was added. After 15 min incubation at room temperature 0.1x MBS was added until the Petri dish was full. The success of the fertilization was visualized by turning of the eggs with their animal pigmented

hemisphere located upwards. This process normally occurred 15-30 min after addition of the testis mixture. 30-60 min after fertilization the eggs were separated from the Petri dish and were transferred to a beaker. The buffer was removed and replaced with 0.1x MBS containing 2% (w/v) cysteine chloride, pH 8.0. Under gentle rotation the embryos were dejellyed for 2-4 min, then washed for 5 times with 0.1x MBS and placed in a new Petri dish. Unhealthy eggs were removed and the remaining were incubated at 14-20°C until the desired stages were reached or were injected directly.

7.5.2 In vitro synthesis of mRNA and obtainment of Morpholino oligomers

Messenger RNA was prepared from the plasmids lacZ pCS2+ [170] and CDYL1b pCS2+ with the help of the mMessage/Machine Kit. Morpholino oligomers were obtained from GeneTools, LLC (Philomath). The xlCDYL1b morpholino is located in the 5-UTR of the xlCDYL1b gene and has the sequence 5'-CCGGGCTGAGGAGATTACTTTCTTT-3'. For control Standard Control oligos were used. Morpholinos were tested with TNT kit of Promega by spiking the TNT reaction of pCS2+ 5UTR xlCDYL1b-FLAG plasmid with 2-200 µM of xlCDYL1b Morpholino or control. Resulted transcription was analyed via SDS PAGE and Western Blot.

7.5.3 Microinjection of mRNA and Morpholinos

After dejellying, the fertilized eggs were transferred to 1x MBS /1% Ficoll (w/v) prior to injection. The injection needle was back loaded with the mRNA and the drop size was calibrated using a microscale. 4 nl of the solution containing 10-1000 pg of the *in vitro* prepared mRNA and/or 20 ng of indicated morpholinos was injected. For injection approximately 50 eggs were transferred to a glas slide and the buffer was removed. Then eggs were injected in all orientations to exclude positioning effects of mRNA using a microinjector. Injected eggs rested for 1 h at RT in 1x MBS/1% Ficoll solution. Then they were transferred to 0.1x MBS and incubated until the desired stage at 14-18°C.

7.5.4 RNA isolation and Reverse Transcription PCR

To isolate the RNA embryos of the desired stage were collected in a Eppendorf tube (1 embryo/tube) and 200 µl lysis buffer was added. The embryos were homogenized by pipetting up and down and incubated at 42°C for 30 min. 200 µl of Phenol/Chloroform/Isoamyl alcohol was added and centrifuged for 3 min, 13000xg, RT in a table top centrifuge. The aqueous top layer was transferred into a new tube containing 20 µl 3 M sodium acetate, 1 µl glycogen (20mg/ml) and 500 µl of ethanol. After centrifugation (as described before) 500 µl of 70%

ethanol (v/v) was added to wash the pellet followed by another centrifugation step, removal of the ethanol and drying of the pellet. The dried pellet was resuspended in 15 µl of water. The DNA of the resuspension was removed with DNase treatment like described in the NEB protocol [171]. Afterwards the RNA was purified by Phenol/Chloroform/Isoamyl alcohol extraction. This time the pellet was dissolved in 20 µl of water. 2 µl of random hexamers were added and incubated for 4 min, 65°C. For reaction 4 µl of Colorless GoTaq Buffer (5x including $MgCL_2$), 1 µl of 20 mM DTT, 0.5 µl RNase inhibitor, 1 µl dNTP mix, 1.5 µl water and 1 µl of the MuLV reverse transcriptase were added to 11 µl of the RNA/hexamer mixture to a total volume of 20 µl. As control an additional reaction was performed replacing the reverse transcriptase with 1 µl of water. The mix was incubated for 30 min at 42°C. 1 µl of this reaction was then used for a PCR reaction as described with an annealing temperature of 60°C for all cycles, 27 cycles using the following primers xlCDYL1b for 5'-CAGGAGTAACAGGCAAGCGAAGAT-3', xlCDYL1b rev 5'-GGCAGGTCCATTTACAG CAACAA-3' producing a 428 bp product or ornithine decarboxylase standard primers for 5'-AGACCTTCGTGCAGGCAATC-3' and rev 5'-AGGAAAGCCACCGCCAATAT-3'. The products were analyzed by gel electrophoresis described in the Cloning chapter (7.2.2).

7.5.5 Whole mount in situ hybridization

To prepare Digoxigenin-labeled antisense RNA the plasmids pBluescript XL213m10 and XL007a18 (7.1.7) were digested with BstXI or SacI for linearization due to NEB protocols [171]. The DNA was purified using a DNA-purification Kit and the concentration was determined by using the Nanodrop. Up to 1 µg of the purified plasmid DNA were taken for preparing antisense RNA using the DIG-RNA-labeling Kit. The Digoxigenin-labeled RNA was purified with the RNeasy Kit and 1 µl was loaded on a 1% agarose gel (see 7.2.2). The RNA could be stored at -80°C. Whole mount *in situ* hybridization was performed as described previously [170].

8 Appendix

8.1 DNA sequences of used proteins

hCDYL1a:

ATGACATTTCAGGCAAGCCACAGGTCAGCCTGGGGAAAAAGCAGGAAGAAAAACTGGCAATACGAGGGCCCAACCCAAAAG
TTATTCCTGAAGAGAAACAACGTGTCAGCACCAGATGGGCCTTCAGACCCCAGCATCTCCGTGAGCAGTGAGCAAAGCGGGG
CACAGCAGCCTCCCGCTTTACAGGTTGAAAGGATTGTTGACAAAAGGAAAAATAAAAAAGGGAAGACAGAGTATTTGGTTCG
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AAACTGGACCCCGTCGAGCAGGGTCAGGAGGACACAGTGGCACCCGAAGTGGCAGCGGAAAAGCCGGTCGGAGCTTTATTG
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TTGCGTTTCAGCGTGAGGCAAACAGAAAGTGCCTACAGATACAGAGATATTGTGGTCAGGAAGCAGGATGGCTTCACCCACA
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AAGAAGCCCATTATTGTAGCAGTCAATGGCCCAGCCATTGGTCTAGGAGCATCTATATTGCCTCTTTGCGATGTGGTTTGGGC
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hCDYL1b:

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GAGAAACTGGACCCCGTCGAGCAGGGTCAGGAGGACACAGTGGCACCCGAAGTGGCAGCGGAAAAGCCGGTCGGAGCTTTA
TTGGGCCCCGGTGCCGAGAGGGCCAGGATGGGGAGCAGGCCCAGGATACACCCACTAGTGCCTCAGGTGCCCGGCCCTGTGA
CTGCAGCCATGGCCACAGGCTTAGCTGTTAACGGGAAAGGTACATCTCCGTTCATGGATGCATTAACAGCCAATGGGACAAC
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TGCTTGAGGAATCCAAAGCCCTCGTGCGCTGCAACATGAAGATGGAGCTGGAGCAGGCCAACGAGAGGGAGTGTGAGGTGC
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hCDYL1c:

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GCAGAGGAAGATCGATGAGTTC

xlCDYL1b/XL213m10:

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CTGCAGCATCTGCATTAACTGTAAATGGTAAAGGTACTTCTACTTTAATGGAGGCTTTGACAGCTAGTGTTACTACCAATATA
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TCCACAAGGCATGGATTCCATGTTAAAATATTTGCAAAGAAAAATTGATGAGTTCTAA

hPRMT5:
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ACACACTAGGGGCTGTGGCCAAGCAGGGGTTTGATTTCCTCTGCATGCCTGTCTTCCATCCGCGTTTCAAGAGGGAGTTCATT
CAGGAACCTGCTAAGAATCGGCCCGGTCCCCAGACACGATCAGACCTACTGCTGTCAGGAAGGGACTGGAATACGCTAATTG
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GCTGAATTTTGGTGCATATTTGGGTCTTCCAGCTTTCCTGCTGCCCCTTAATCAGGAAGATAACACCAACCTGGCCAGAGTTTT
GACCAACCACATCCACACTGGCCATCACTCTTCCATGTTCTGGATGCGGGTACCCTTGGTGGCACCAGAGGACCTGAGAGATG
ATATAATTGAGAATGCACCAACTACACACACAGAGGAGTACAGTGGGGAGGAGAAAACGTGGATGTGGTGGCACAACTTCC
GGACTTTGTGTGACTATAGTAAGAGGATTGCAGTGGCTCTTGAAATTGGGGCTGACCTCCCATCTAATCATGTCATTGATCGC
TGGCTTGGGGAGCCCATCAAAGCAGCCATTCTCCCCACTAGCATTTTCCTGACCAATAAGAAGGGATTTCCTGTTCTTTCTAA
GATGCACCAGAGGCTCATCTTCCGGCTCCTCAAGTTGGAGGTGCAGTTCATCATCACAGGCACCAACCACCACTCAGAGAAG
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CCATCAAATACTCTCAGTACCAGCAGGCCATCTATAAATGTCTGCTAGACCGAGTACCAGAAGAGGAGAAGGATACCAATGT
CCAGGTACTGATGGTGCTGGGAGCAGGACGGGGACCCCTGGTGAACGCTTCCCTGCGGGCAGCCAAGCAGGCCGACCGGCG
GATAAAGCTGTATGCTGTGGAGAAAAACCCAAATGCCGTGGTGACGCTAGAGAACTGGCAGTTTGAAGAATGGGGAAGCCA
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AGGCCCAGTTTGAGATGCCTTATGTGGTACGGCTGCACAACTTCCACCAGCTCTCTGCACCCCAGCCCTGTTTCACCTTCAGCC
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TTTGCCGGCTACTTTGAGACTGTGCTTTATCAGGACATCACTCTGAGTATCCGTCCAGAGACTCACTCTCCTGGGATGTTCTCA
TGGTTTCCCATCCTCTTCCCTATTAAGCAGCCCATAACGGTACGTGAAGGCCAAACCATCTGTGTGCGTTTCTGGCGATGCAG
CAATTCCAAGAAGGTGTGGTATGAGTGGGCTGTGACAGCACCAGTCTGTTCTGCTATTCATAACCCCACAGGCCGCTCATATA
CCATTGGCCTTGGTAA

8.2 Amino acid sequences of used proteins

hCDYL1a:
MTFQASHRSAWGKSRKKNWQYEGPTQKLFLKRNNVSAPDGPSDPSISVSSEQSGAQQPPALQVERIVDKRKNKKGKTEYLVRWK
GYDSEDDTWEPEQHLVNCEEYIHDFNRRHTEKQKESTLTRTNRTSPNNARKQISRSTNSNFSKTSPKALVIGKDHESKNSQLFAASQ
KFRKNTAPSLSSRKNMDLAKSGIKILVPKSPVKSRTAVDGFQSESPEKLDPVEQGQEDTVAPEVAAEKPVGALLGPGAERARMGSR
PRIHPLVPQVPGPVTAAMATGLAVNGKGTSPFMDALTANGTTNIQTSVTGVTASKRKFIDDRRDQPFDKRLRFSVRQTESAYRYRD
IVVRKQDGFTHILLSTKSSENNSLNPEVMREVQSALSTAAADDSKLVLLSAVGSVFCCGLDFIYFIRRLTDDRKRESTKMAEAIRNFV
NTFIQFKKPIIVAVNGPAIGLGASILPLCDVVWANEKAWFQTPYTTFGQSPDGCSTVMFPKIMGGASANEMLLSGRKLTAQEACGK
GLVSQVFWPGTFTQEVMVRIKELASCNPVVLEESKALVRCNMKMELEQANERECEVLKKIWGSAQGMDSMLKYLQRKIDEF

hCDYL1b:
MASEELYEVERIVDKRKNKKGKTEYLVRWKGYDSEDDTWEPEQHLVNCEEYIHDFNRRHTEKQKESTLTRTNRTSPNNARKQISR
STNSNFSKTSPKALVIGKDHESKNSQLFAASQKFRKNTAPSLSSRKNMDLAKSGIKILVPKSPVKSRTAVDGFQSESPEKLDPVEQGQ
EDTVAPEVAAEKPVGALLGPGAERARMGSRPRIHPLVPQVPGPVTAAMATGLAVNGKGTSPFMDALTANGTTNIQTSVTGVTASK
RKFIDDRRDQPFDKRLRFSVRQTESAYRYRDIVVRKQDGFTHILLSTKSSENNSLNPEVMREVQSALSTAAADDSKLVLLSAVGSVF
CCGLDFIYFIRRLTDDRKRESTKMAEAIRNFVNTFIQFKKPIIVAVNGPAIGLGASILPLCDVVWANEKAWFQTPYTTFGQSPDGCST
VMFPKIMGGASANEMLLSGRKLTAQEACGKGLVSQVFWPGTFTQEVMVRIKELASCNPVVLEESKALVRCNMKMELEQANEREC
EVLKKIWGSAQGMDSMLKYLQRKIDEF

hCDYL1c:
MDALTANGTTNIQTSVTGVTASKRKFIDDRRDQPFDKRLRFSVRQTESAYRYRDIVVRKQDGFTHILLSTKSSENNSLNPEVMREV
QSALSTAAADDSKLVLLSAVGSVFCCGLDFIYFIRRLTDDRKRESTKMAEAIRNFVNTFIQFKKPIIVAVNGPAIGLGASILPLCDVV
WANEKAWFQTPYTTFGQSPDGCSTVMFPKIMGGASANEMLLSGRKLTAQEACGKGLVSQVFWPGTFTQEVMVRIKELASCNPVV
LEESKALVRCNMKMELEQANERECEVLKKIWGSAQGMDSMLKYLQRKIDEF

xlCDYL1b/XL213m10:
MASEELYEVEQIVDKRKNKKGKVEYLVHWKGYDSGDDTWEPEQHLVNCEEYIHEFNRRHSEKPKDNSTIRSNRTSPNNARKQISR
STNSAITRTSPKSVSLSKESDSKNNQLLHSSPKLRRSPSQAIPSRKNIDLSRSGIKILVPKSPMKSRTTVDGFQNESTEKLNHLEQEQDT
TAPEVTAEKPVGALLGPGAERARMGSRPRPQSLVPQIQMPLTVTAASALTVNGKGTSTLMEALTASVTTNIQTSAGVTGKRRFIDE

RRDQPFDKRLRFSVRQTESAYRYRDIVVRKQDGFTHILLSTKSSENNSLNPEVMKEVQGALNTAAADDSKLVLFSAVGSVFCCGLD
FIYFIRRLTDDRKRESIKMAEAIRTFVNTFIQFKKPIIVAVNGPAIGLGASILPLCDVVWANEKAWFQTPYTTFGQSPDGCSSLMFPKI
MGLASANEMLFSGRKLTAQEACAKGLVSQVFWPGTFTQEVMVRIKELVTCNPVVLEESKGLVRNVMRGDLEQTNERECEVLKKI
WGSPQGMDSMLKYLQRKIDEF

hPRMT5:

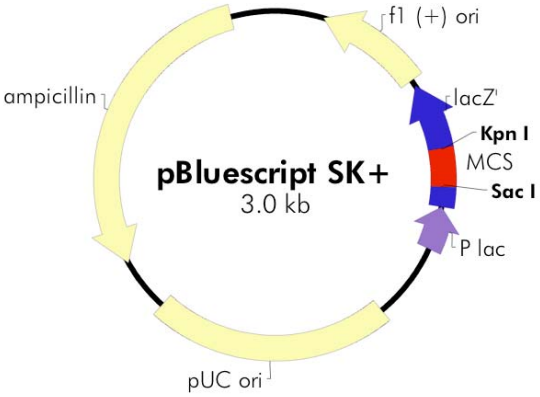
MAAMAVGGAGGSRVSSGRDLNCVPEIADTLGAVAKQGFDFLCMPVFHPRFKREFIQEPAKNRPGPQTRSDLLLSGRDWNTLIVGK
LSPWIRPDSKVEKIRRNSEAAMLQELNFGAYLGLPAFLLPLNQEDNTNLARVLTNHIHTGHHSSMFWMRVPLVAPEDLRDDIIENAP
TTHTEEYSGEEKTWMWWHNFRTLCDYSKRIAVALEIGADLPSNHVIDRWLGEPIKAAILPTSIFLTNKKGFPVLSKMHQRLIFRLLK
LEVQFIITGTNHHSEKEFCSYLQYLEYLSQNRPPPNAYELFAKGYEDYLQSPLQPLMDNLESQTYEVFEKDPIKYSQYQQAIYKCLL
DRVPEEEKDTNVQVLMVLGAGRGPLVNASLRAAKQADRRIKLYAVEKNPNAVVTLENWQFEEWGSQVTVVSSDMREWVAPEK
ADIIVSELLGSFADNELSPECLDGAQHFLKDDGVSIPGEYTSFLAPISSSKLYNEVRACREKDRDPEAQFEMPYVVRLHNFHQLSAPQ
PCFTFSHPNRDPMIDNNRYCTLEFPVEVNTVLHGFAGYFETVLYQDITLSIRPETHSPGMFSWFPILFPIKQPITVREGQTICVRFWRCS
NSKKVWYEWAVTAPVCSAIHNPTGRSYTIGL

8.3 Maps of used plasmids

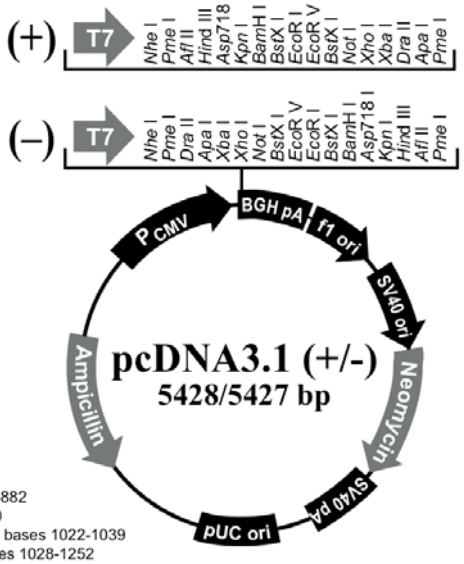
Plasmid maps are obtained from indicated companies.

pBluescript (NIBB)

f1 (+) origin 138–444
β-galactosidase α-fragment 463–816
multiple cloning site 653–760
***lac* promoter 817–938**
pUC origin 1158–1825
ampicillin resistance (*bla*) ORF 1976–2833



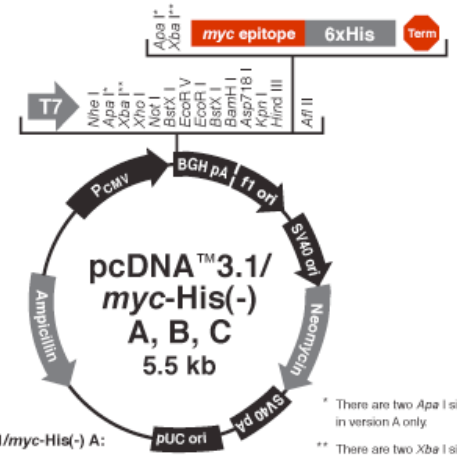
pcDNA3.1(+) (Invitrogen)



Comments for pcDNA3.1 (+)
5428 nucleotides

CMV promoter: bases 232-819
T7 promoter/priming site: bases 863-882
Multiple cloning site: bases 895-1010
pcDNA3.1/BGH reverse priming site: bases 1022-1039
BGH polyadenylation sequence: bases 1028-1252
f1 origin: bases 1298-1726
SV40 early promoter and origin: bases 1731-2074
Neomycin resistance gene (ORF): bases 2136-2930
SV40 early polyadenylation signal: bases 3104-3234
pUC origin: bases 3617-4287 (complementary strand)
Ampicillin resistance gene (*bla*): bases 4432-5428 (complementary strand)
ORF: bases 4432-5292 (complementary strand)
Ribosome binding site: bases 5300-5304 (complementary strand)
bla promoter (P3): bases 5327-5333 (complementary strand)

pcDNA3.1(+) myc His (Invitrogen)



Comments for pcDNA™3.1/*myc*-His(-) A: 5522 nucleotides

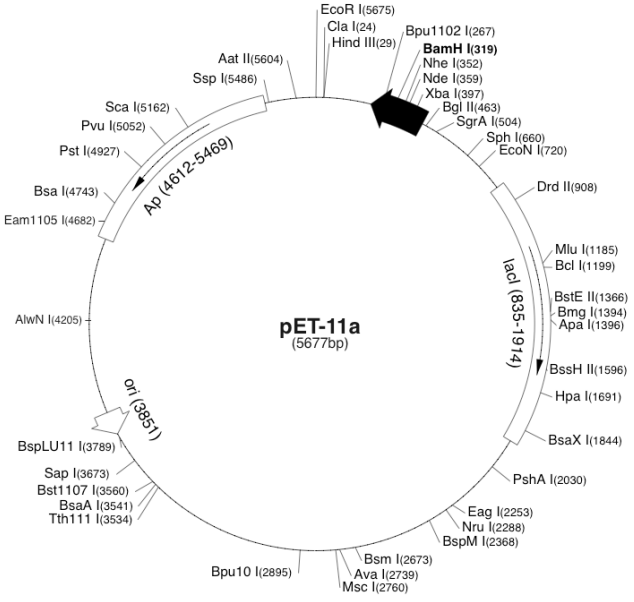
CMV promoter: bases 209-863
T7 promoter/priming site: bases 863-882
Multiple cloning site: bases 895-1006
myc epitope: bases 1007-1036
Polyhistidine tag: bases 1052-1069
BGH reverse priming site: bases 1113-1130
BGH polyadenylation signal: bases 1116-1343
f1 origin: bases 1389-1817
SV40 promoter and origin: bases 1844-2152
Neomycin resistance gene: bases 2227-3021
SV40 polyadenylation signal: bases 3195-3325
pUC origin: bases 3708-4381
Ampicillin resistance gene: bases 4526-5386 (complementary strand)

pet11a (Novagen)

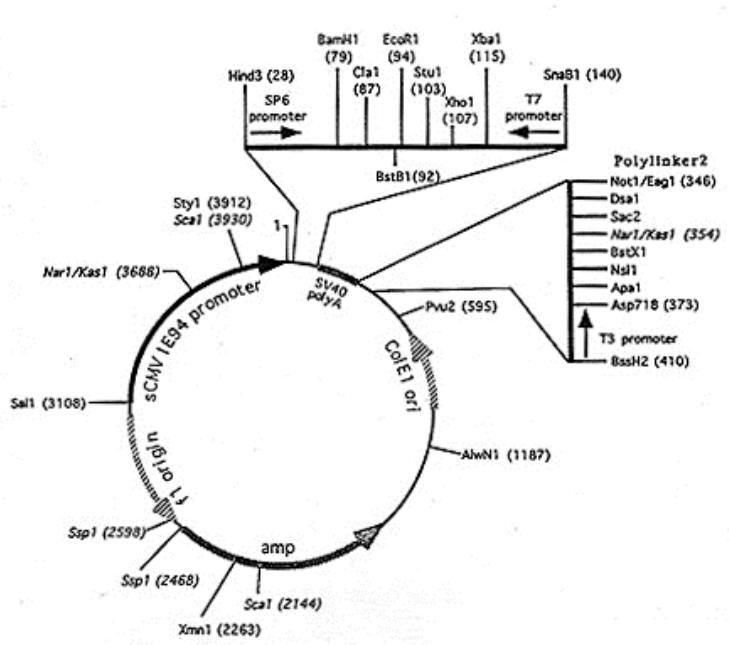
pET-11a sequence landmarks

T7 promoter	432-448
T7 transcription start	431
T7•Tag coding sequence	328-360
T7 terminator	213-259
lacI coding sequence	835-1914
pBR322 origin	3851
bla coding sequence	4612-5469

The maps for pET-11b, pET-11c and pET-11d are the same as pET-11a (shown) with the following exceptions: pET-11b is a 5676bp plasmid; subtract 1bp from each site beyond *Bam*H I at 319. pET-11c is a 5675bp plasmid; subtract 2bp from each site beyond *Bam*H I at 319. pET-11d is a 5674bp plasmid; the *Bam*H I site is in the same reading frame as in pET-11c. An *Nco* I site is substituted for the *Nde* I site with a net 1bp deletion at position 359 of pET-11c. As a result, *Nco* I cuts pET-11d at 355. For the rest of the sites, subtract 3bp from each site beyond position 360 in pET-11a. *Nde* I does not cut pET-11d.



pCS2+ (RZPD)



8.4 R programming for mass spectrometry analysis

open “R” (D1=moc, D2=unmod, D3=me1, D4=me3)
D1=read.table("/Users/hfranz/Desktop/S-Soros-250407-S2L1_dros.txt", sep="\t", header=T) ####### *when that’s not working try “read.csv”*
D2=read.table("/Users/hfranz/Desktop/S-Soros-250407-S2L2_dros.txt", sep="\t", header=T)
D3=read.table("/Users/hfranz/Desktop/S-Soros-250407-S2L3_dros.txt", sep="\t", header=T)
D4=read.table("/Users/hfranz/Desktop/S-Soros-250407-S2L4_dros.txt", sep="\t", header=T)
####### *remove moc control (D1) from each lane*
D1_D2=merge(D1,D2, by.x=1, by.y=1) ####### *combining tables by GI number*
D1_D3=merge(D1,D3, by.x=1, by.y=1)
D1_D4=merge(D1,D4, by.x=1, by.y=1)
dim(D1_D2) ####### *showing dimensions of table*
dim(D1_D3)
dim(D1_D4)
D2_unique=subset(D2,is.element(db_id,D1_D2$db_id)==FALSE) ####### *building a subset of GIs of all which are not in the combined table D1_D2 but in D2*
dim(D2_unique)
D3_unique=subset(D3,is.element(db_id,D1_D3$db_id)==FALSE)
dim(D3_unique)
D4_unique=subset(D4,is.element(db_id,D1_D4$db_id)==FALSE)
dim(D4_unique)
####### *remove unmod control (D2) from each lane*
D2_unique_D3_unique=merge(D2_unique,D3_unique, by.x=1, by.y=1)
dim(D2_unique_D3_unique)
D3minusD2=subset(D3_unique,is.element(db_id,D2_unique_D3_unique$db_id)==FALSE)
dim(D3minusD2)
D2_unique_D4_unique=merge(D2_unique,D4_unique, by.x=1, by.y=1)
D4minusD2=subset(D4_unique,is.element(db_id,D2_unique_D4_unique$db_id)==FALSE)
dim(D4minusD2)
write.table(D3minusD2, file="/Users/hfranz/Desktop/D3minusD2.txt", sep="\t")
write.table(D4minusD2, file="/Users/hfranz/Desktop/D4minusD2.txt", sep="\t")
####### *use only proteins which have more than 1 unique peptide*

```
D4minusD2_gr1= D4minusD2[D4minusD2[,8]>1,]
dim(D4minusD2_gr1)
D3minusD2_gr1= D3minusD2[D3minusD2[,8]>1,]
dim(D3minusD2_gr1)
write.table(D3minusD2_gr1,  file="/Users/hfranz/Desktop/D3minusD2_gr1.txt",  sep="\t")  #######
save table where you want
write.table(D4minusD2_gr1, file="/Users/hfranz/Desktop/D4minusD2_gr1.txt", sep="\t")
####### proteins which are in L3 and L4 or in L3 or L4 only
D3andD4=merge(D3minusD2,D4minusD2, by.x=1, by.y=1)
write.table(D3andD4, file="/Users/hfranz/Desktop/D3andD4.txt", sep="\t")
dim(D3andD4)
D4only=subset(D4minusD2,is.element(db_id,D3andD4$db_id)==FALSE)
dim(D4only)
D3only=subset(D3minusD2,is.element(db_id,D3andD4$db_id)==FALSE)
dim(D3only)
D3minusD2_gr1= D3minusD2[D3minusD2[,8]>1,]
dim(D3minusD2_gr1)
D3only_gr1= D3only[D3only[,8]>1,]
dim(D3only_gr1)
D4only_gr1= D4only[D4only[,8]>1,]
dim(D4only_gr1)
write.table(D3minusD2_gr1, file="/Users/hfranz/Desktop/D3minusD2_gr1.txt", sep="\t")
write.table(D3only, file="/Users/hfranz/Desktop/D3minusD2andD4.txt", sep="\t")
write.table(D3only_gr1, file="/Users/hfranz/Desktop/D3minusD2andD4_gr1.txt", sep="\t")
write.table(D4only_gr1, file="/Users/hfranz/Desktop/D4minusD2andD3_gr1.txt", sep="\t")
write.table(D4only, file="/Users/hfranz/Desktop/D4minusD2andD3.txt", sep="\t")
```

8.5 Mass spectrometry results

8.5.1 H3K9me3 bound fraction

Row.names	proteinname	occurence	unique_peptides	Sum_of_peptides
gi|12229217	activity-dependent neuroprotector [Homo sapiens]	1	31	56
gi|5032179	tripartite motif-containing 28 protein [Homo sapiens]	2	20	28
gi|12025678	actinin, alpha 4 [Homo sapiens]	1	17	17
gi|15082258	chromobox homolog 3 [Homo sapiens]	8	16	27
gi|50513245	chromatin assembly factor 1, subunit A (p150) [Homo sapiens]	1	15	15
gi|6912292	chromobox homolog 5 (HP1 alpha homolog, Drosophila) [Homo sapiens]	1	14	18
gi|89886458	hypothetical protein LOC64423 isoform 1 [Homo sapiens]	1	13	16
gi|25777617	chromodomain protein, Y chromosome-like isoform a [Homo sapiens]	2	12	18
gi|15431323	keratin, hair, basic, 3 [Homo sapiens]	1	8	8
gi|14141161	heterogeneous nuclear ribonucleoprotein U isoform b [Homo sapiens]	1	6	6
gi|4885379	H1 histone family, member 4 [Homo sapiens]	1	5	5
gi|4504447	heterogeneous nuclear ribonucleoprotein A2/B1 isoform A2 [Homo sapiens]	1	5	5
gi|41281612	hypothetical protein LOC283489 [Homo sapiens]	1	5	5
gi|16507204	ubiquitin-like, containing PHD and RING finger domains, 1 [Homo sapiens]	1	5	5
gi|7661920	DEAD (Asp-Glu-Ala-Asp) box polypeptide 48 [Homo sapiens]	1	4	4
gi|5031699	flotillin 1 [Homo sapiens]	1	4	4
gi|4757718	actin-like 6A isoform 1 [Homo sapiens]	1	4	4
gi|46397390	pogo transposable element with ZNF domain isoform 1 [Homo sapiens]	1	4	4
gi|4507123	small nuclear ribonucleoprotein polypeptide B'' [Homo sapiens]	1	4	4
gi|4504257	H2B histone family, member A [Homo sapiens]	2	4	7
gi|41281917	polybromo 1 isoform 4 [Homo sapiens]	1	4	4
gi|21237805	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin c2 isoform a [Homo	2	4	5
gi|50726970	hypothetical protein LOC23383 [Homo sapiens]	1	3	3
gi|5031923	meiotic recombination 11 homolog A isoform 1 [Homo sapiens]	1	3	3
gi|4507131	small nuclear ribonucleoprotein polypeptide F [Homo sapiens]	1	3	3
gi|4504279	H3 histone, family 3A [Homo sapiens]	2	3	4
gi|21040371	DEAD (Asp-Glu-Ala-Asp) box polypeptide 39 isoform 1 [Homo sapiens]	1	3	3
gi|18104948	ribosomal protein L21 [Homo sapiens]	1	3	3
gi|16306580	F-box and leucine-rich repeat protein 11 [Homo sapiens]	1	3	3
gi|15431310	keratin 14 [Homo sapiens]	1	3	3
gi|10337581	type I hair keratin 3B [Homo sapiens]	1	3	3
gi|89044285	PREDICTED: structural maintenance of chromosomes flexible hinge domain containing 1 [Homo	1	2	2
gi|89031563	PREDICTED: similar to ribosomal protein L13a isoform 2 [Homo sapiens]	1	2	2
gi|81295404	acyl-Coenzyme A thioesterase 2, mitochondrial isoform b [Homo sapiens]	1	2	2
gi|7661968	enthoprotin [Homo sapiens]	1	2	2
gi|73623028	carnitine palmitoyltransferase 1A isoform 2 [Homo sapiens]	1	2	2
gi|5803076	chromobox homolog 1 (HP1 beta homolog Drosophila) [Homo sapiens]	2	2	4
gi|5454064	RNA binding motif protein 14 [Homo sapiens]	1	2	2
gi|53759151	stearoyl-CoA desaturase [Homo sapiens]	1	2	2
gi|4758410	fragile X mental retardation syndrome related protein 2 [Homo sapiens]	1	2	3
gi|4758394	flotillin 2 [Homo sapiens]	1	2	2
gi|47419909	transcriptional intermediary factor 1 alpha isoform b [Homo sapiens]	1	2	2
gi|46195723	ribosomal L1 domain containing 1 [Homo sapiens]	1	2	2
gi|4507869	vasodilator-stimulated phosphoprotein isoform 1 [Homo sapiens]	1	2	2
gi|4506715	ribosomal protein S28 [Homo sapiens]	1	2	2
gi|4504239	H2A histone family, member C [Homo sapiens]	2	2	3
gi|25188179	voltage-dependent anion channel 3 [Homo sapiens]	1	2	2
gi|21359951	hypothetical protein LOC79171 [Homo sapiens]	1	2	2
gi|20070220	protein arginine methyltransferase 5 isoform a [Homo sapiens]	1	2	2
gi|15431303	ribosomal protein L9 [Homo sapiens]	1	2	2
gi|14670350	general transcription factor II, i isoform 1 [Homo sapiens]	1	2	2

8.5.2 Co-precipitated factors of CDYL1b IP

db_id	proteinname	occurence	unique_peptides	Sum_of_peptides
gi|119579690	RNA binding motif protein 10, isoform CRA_d [Homo sapiens]	1	28	30
gi|547754	Keratin, type II cytoskeletal 2 epidermal (Cytokeratin-2e) (K2e) (CK 2e) (keratin-2)	1	20	99
gi|122937227	U2-associated SR140 protein [Homo sapiens]	1	18	20
gi|62897625	beta actin variant [Homo sapiens]	1	16	20
gi|189306	nucleolin	1	14	14
gi|4505591	peroxiredoxin 1 [Homo sapiens]	1	10	10
gi|119577231	heterogeneous nuclear ribonucleoprotein L, isoform CRA_b [Homo sapiens]	1	9	10
gi|119589356	ELAV (embryonic lethal, abnormal vision, Drosophila)-like 1 (Hu antigen R), isoform CRA_b [Homo sapiens]	2	9	16
gi|306875	C protein	2	9	12
gi|119574084	guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1, isoform CRA_h [Homo sapiens]	1	8	8
gi|20070220	protein arginine methyltransferase 5 isoform a [Homo sapiens]	1	8	8
gi|45219787	Ribosomal protein S3A [Homo sapiens]	2	8	10
gi|4558756	testis-specific chromodomain Y-like protein [Homo sapiens]	4	8	21
gi|47115317	VIM [Homo sapiens]	1	8	8
gi|119874201	furry-like [Homo sapiens]	1	7	9
gi|45827771	autoantigen RCD8 [Homo sapiens]	1	7	7
gi|62896589	eukaryotic translation elongation factor 1 alpha 1 variant [Homo sapiens]	1	7	7
gi|12804561	Ribosomal protein S15a [Homo sapiens]	1	6	7
gi|21410970	Ribosomal protein L6 [Homo sapiens]	1	6	6
gi|25992622	SR-related CTD associated factor 6 [Homo sapiens]	1	6	6
gi|30268331	hypothetical protein [Homo sapiens]	1	6	6
gi|4506619	ribosomal protein L24 [Homo sapiens]	1	6	6
gi|4826686	DEAD (Asp-Glu-Ala-Asp) box polypeptide 1 [Homo sapiens]	1	6	6
gi|4885379	histone cluster 1, H1e [Homo sapiens]	1	6	10
gi|10433990	unnamed protein product [Homo sapiens]	1	5	5
gi|109114529	PREDICTED: clathrin heavy chain 1 isoform 6 [Macaca mulatta]	1	5	5
gi|119590969	X-ray repair complementing defective repair in Chinese hamster cells 5, isoform CRA_b [Homo sapiens]	1	5	5
gi|337580	ribosomal protein L3	1	5	5
gi|4759098	splicing factor, arginine/serine-rich 10 [Homo sapiens]	4	5	11
gi|51476390	hypothetical protein [Homo sapiens]	5	5	13
gi|83318444	HSP90AA1 protein [Homo sapiens]	1	5	5
gi|119585237	DEAH (Asp-Glu-Ala-His) box polypeptide 30, isoform CRA_a [Homo sapiens]	1	4	4
gi|19879661	eukaryotic initiation factor 2C2 [Homo sapiens]	1	4	4
gi|2498733	Caprin-1 (Cytoplasmic activation- and proliferation-associated protein 1)	1	4	4
gi|28875797	hypothetical protein LOC26097 [Homo sapiens]	1	4	4
gi|31645	glyceraldehyde-3-phosphate dehydrogenase [Homo sapiens]	1	4	4
gi|37547120	PREDICTED: similar to 40S ribosomal protein S7 (S8) [Homo sapiens]	1	4	4
gi|4506613	ribosomal protein L22 proprotein [Homo sapiens]	1	4	5
gi|11415026	ribosomal protein L18a [Homo sapiens]	1	3	3
gi|119580772	trinucleotide repeat containing 6B, isoform CRA_b [Homo sapiens]	1	3	3
gi|119581557	polypyrimidine tract binding protein 1, isoform CRA_b [Homo sapiens]	2	3	5
gi|119609849	splicing factor, arginine/serine-rich 2, isoform CRA_d [Homo sapiens]	2	3	6
gi|14249398	PHD-finger 5A [Homo sapiens]	1	3	3
gi|27151474	Ribosome biogenesis protein BMS1 homolog	1	3	3
gi|30354619	YWHAZ protein [Homo sapiens]	1	3	3
gi|33874520	SYNCRIP protein [Homo sapiens]	3	3	8
gi|386772	histone H3	2	3	4
gi|4506663	ribosomal protein L8 [Homo sapiens]	1	3	3
gi|4506711	ribosomal protein S27 [Homo sapiens]	1	3	3
gi|4506901	splicing factor, arginine/serine-rich 3 [Homo sapiens]	1	3	6
gi|4929587	CGI-59 protein [Homo sapiens]	1	3	3
gi|53690156	Sjogren syndrome antigen A1 [Homo sapiens]	1	3	3
gi|553640	ribosomal protein S13 [Homo sapiens]	2	3	4
gi|55743159	WD repeat domain 33 isoform 3 [Homo sapiens]	1	3	3
gi|58082083	transketolase-like 1 [Homo sapiens]	1	3	3
gi|6005860	ribosomal protein L35 [Homo sapiens]	1	3	3
gi|7263962	OTTHUMP00000045488 [Homo sapiens]	1	3	3
gi|76779245	RPL7 protein [Homo sapiens]	1	3	3
gi|11067747	CDC5-like [Homo sapiens]	1	2	2
gi|113421727	PREDICTED: similar to peptidylprolyl isomerase A isoform 1 [Homo sapiens]	1	2	2
gi|119592597	PRP31 pre-mRNA processing factor 31 homolog (yeast), isoform CRA_b [Homo sapiens]	1	2	2
gi|119601492	RNA binding motif protein 25, isoform CRA_a [Homo sapiens]	1	2	2
gi|119610575	eukaryotic translation initiation factor 4A, isoform 1, isoform CRA_c [Homo sapiens]	1	2	2
gi|119611307	BAT2 domain containing 1, isoform CRA_d [Homo sapiens]	1	2	2
gi|119612797	signal recognition particle 14kDa (homologous Alu RNA binding protein), isoform CRA_b [Homo sapiens]	1	2	2
gi|1244508	assembly protein 50	1	2	2
gi|12653415	Heat shock 70kDa protein 9 (mortalin) [Homo sapiens]	1	2	2
gi|12711674	UPF3 regulator of nonsense transcripts homolog B isoform 2 [Homo sapiens]	1	2	2
gi|12804225	Unknown (protein for IMAGE:3543711) [Homo sapiens]	1	2	2
gi|13277568	Similar to RIKEN cDNA 3930401K13 gene [Homo sapiens]	1	2	2
gi|13385036	ribosomal protein L15 [Mus musculus]	1	2	2
gi|13904866	ribosomal protein L28 [Homo sapiens]	1	2	2
gi|14133213	KIAA0732 protein [Homo sapiens]	1	2	2
gi|147900596	hypothetical protein LOC644907 [Homo sapiens]	1	2	2
gi|1665723	RPD3 protein [Homo sapiens]	1	2	2
gi|18860916	5'-3' exoribonuclease 2 [Homo sapiens]	1	2	2
gi|1942977	Chain A, Macrophage Migration Inhibitory Factor (Mif)	1	2	2
gi|24432016	pre-mRNA cleavage factor I, 59 kDa subunit [Homo sapiens]	1	2	2
gi|27477136	zinc finger antiviral protein isoform 1 [Homo sapiens]	1	2	2
gi|27529734	KIAA0727 protein [Homo sapiens]	1	2	2
gi|3088341	ribosomal protein S21 [Homo sapiens]	1	2	2
gi|32140872	FIP1L1/PDGFRA fusion protein; Rhe-PDGFRA [Homo sapiens]	1	2	3
gi|33875203	ARS2 protein [Homo sapiens]	1	2	2
gi|34201	unnamed protein product [Homo sapiens]	1	2	2
gi|34789	muscle-specific enolase [Homo sapiens]	1	2	2
gi|39777586	DEAH (Asp-Glu-Ala-Asp/His) box polypeptide 57 [Homo sapiens]	1	2	2
gi|4176369	similar to 60S ribosomal protein L7; similar to P18124 (PID:d133021) [Homo sapiens]	1	2	2
gi|4506643	ribosomal protein L37a [Homo sapiens]	1	2	2
gi|4557032	lactate dehydrogenase B [Homo sapiens]	1	2	2
gi|5031635	cofilin 1 (non-muscle) [Homo sapiens]	1	2	2
gi|5803225	tyrosine 3/tryptophan 5 -monooxygenase activation protein, epsilon polypeptide [Homo sapiens]	1	2	2
gi|5803227	tyrosine 3/tryptophan 5 -monooxygenase activation protein, theta polypeptide [Homo sapiens]	1	2	2
gi|62087384	fusion (involved in t(12;16) in malignant liposarcoma) isoform a variant [Homo sapiens]	1	2	2
gi|62897593	squamous cell carcinoma antigen recognized by T cells 1 variant [Homo sapiens]	1	2	2
gi|6919894	Rho/Rac guanine nucleotide exchange factor 2 (GEF-H1 protein) (Proliferating cell nucleolar antigen p40)	1	2	2
gi|693933	2-phosphopyruvate-hydratase alpha-enolase; carbonate dehydratase [Homo sapiens]	1	2	2
gi|7243239	KIAA1429 protein [Homo sapiens]	2	2	3
gi|72534750	splicing factor YT521-B isoform 1 [Homo sapiens]	1	2	2
gi|7546189	Chain A, Crystal Structure Of Mutant Human Lysozyme With Four Extra Residues (Eaea) At The N-Terminal	1	2	2
gi|755746	p85Mcm protein [Homo sapiens]	1	2	2

9 Curriculum vitae

Personal data

Name:	Henriette Franz
Date of birth:	08.02.1982
Place of birth:	Jena, Germany
Nationality:	German

Schooling

1988 – 1992	10. Grundschule Jena (elementary school)
1992 – 2000	Ernst-Haeckel-Gymnasium Jena
06/2000	Ernst-Haeckel-Gymnasium Jena Abitur Majors: German, Chemistry

Studies

10/2000-03/2005	Biochemistry University of Leipzig Diploma in Biochemistry Diploma thesis: “Systematic analysis of the gene expression profile of the human brain”
since 06/2006	Molecular Biology of Development and Interaction between Organisms Georg-August-University Göttingen PhD studies Thesis: “Functional characterization of CDY family proteins and their role in recognition of the heterochromatic histone H3K9me3 modification”

Stipends

2006-2008	Max-Planck-Institute stipend

Work Experience

02/2003-03/2003	Practical work Paul-Flechsig-Institute of brain research, Leipzig
07/2003-09/2003	Project work Centre of Neuroscience/Kings College London
02/2004-04/2004	Student assistant Institute of Biochemistry/University of Leipzig
05/2004-03/2005	Student assistant Max-Planck-Institute of evolutionary Anthropologies, Leipzig
04/2005-02/2006	Technical assistant Max-Planck-Institute of evolutionary Anthropologies, Leipzig

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