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The cohesin protein NIPBL recruits histone deacetylases to mediate local chromatin modifications

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The Cohesin loading factor NIPBL recruits histone deacetylases to mediate local chromatin modifications.

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1. Introduction

1.1 The cohesion complex

During the mitotic cell cycle, the sister chromatids products of DNA replication are held together from the time of replication in S phase until their separation at the onset of anaphase. This process is crucial for the correct segregation of chromosomes during mitosis. As expected from this essential function in mitosis, cohesin, a protein complex that keeps sister chromatids together, is highly conserved among eukaryotes. In proliferating cells, the cohesin core complex consists of two very long protein molecules known as SMC1 and SMC3 (structural maintenance of chromosome) and two smaller subunits, the kleisin family protein SCC1 (RAD21) and Stromalin SCC3 (that exists in different isoforms, called SA1 or SA2, in somatic vertebrate cells, all information in human, there are even three different SCC3 orthologs described as STAG-1,-2,-3, see figure 1) (Codde and Heck, 2000; Lee JY and Orr-Weaver, 2001). These protein form an unusually large ring-shaped structure of about 30-40 nm in diameter. Like other SMC protein, SMC1 and SMC3 fold back on themselves to form antiparallel coiled coils, with their N- and C- termini forming ATPase head domains (Hirano, 2002). Their hinge regions interact to form the SMC1-SMC3 heterodimer. The N- and C- termini of RAD21 interact with the head domains of SMC3 and SMC1 respectively to form a ring-like structure (Fig.1). The SMC3 arm has a kink, which forms a more open structure than seen in other SMC complexes (Anderson et al., 2002). Structural and functional studies suggest that the cohesin ring can encircle DNA. Current evidence strongly support the idea that the cohesin complex hold sister chromatids together, but the precise mechanism is not completely established. It is possible that cohesin could mediate sister chromatid cohesion by multiple mechanism, and the mechanisms may depend on chromosome location and other chromosomal proteins (Ivanov and Nasmyth., 2005; Huang et al., 2005; Chang et al., 2005).

The activity of cohesin core complex is regulated by various regulatory factors: including the SCC2/SCC4 loading complex that is required for deposition of cohesin onto the sister chromatids; the ECO1 (Ctf7,Eso1,ESCO2) family of protein acetyltransferase enzymes; the Pds5 protein that associates with cohesin to chromosome; cohesin protector Sgo1 and cohesin-binding protein Wapl (Hartman et al., 2000; Tanaka et al, 2000; Nasmyth and Haering, 2005; Losada et al., 2005;Gandhi et al., 2006)(Tab.1). SCC2 belongs to the family of chromosomal adherins. Orthologs of SCC2 have also been identified in fission yeast (Mis4), *Drosophila* (Nipped-B), *Xenopus* (XSc2), and human (NIPBL/ DELANGIN) (Fig.1).

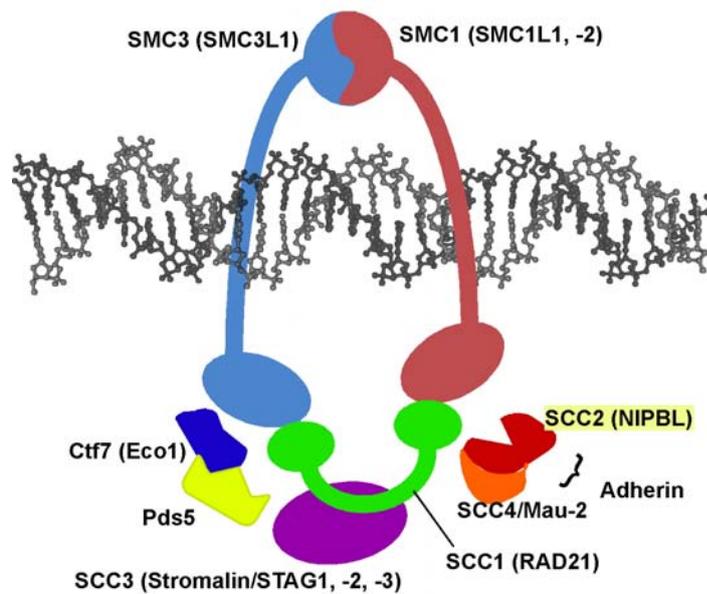


Figure 1. Structure of cohesin complex.

The cohesin core complex consists of two very long proteins known as SMC1 and SMC3, and two smaller subunits termed SCC1 and SCC3. Together, these proteins form a large ring-shaped structure of about 30-40 nm in diameter which can encircle DNA. And the cohesin core complex interacts with several other proteins, including SCC2/SCC4 loading complex; acetyltransferase enzyme- ECO1; the cohesin associated protein Pds5; cohesin protector Sgo1 and cohesin-binding protein Wapl and others perhaps still undetected so far.

	<i>S.cerevisiae</i>	<i>S.pombe</i>	<i>C.elegans</i>	<i>Drosophila</i>	Human
Cohesin					
<i>Smc1</i>	Smc1	Psm1	Him-1	Smc1	SMC1
<i>Smc3</i>	Smc3	Psm3	Smc-3	Smc3/Cap Smc3	SMC3
<i>Rad21</i>	Mcd1/Scs1	Rad21	Coh-2/Scs-1	Rad21	SCC1/Rad21
<i>Stromalin</i>	Scs3	Psc3	Scs-3	Stromalin/SA	SCC3(STAG-1,-2,-3)
Adherin					
<i>Scs2</i>	Scs2	Mis4	Pqn-85	Nipped-B	DELANGIN/NIPBL
<i>Scs4</i>	Scs4	Ssl3	Mau-2	Mau-2	MAU-2/SCC4
<i>Pds5</i>	Pds5	Pds5	Evl-14/Pds-5	Pds5	PDS5A, PDS5B
<i>Eco1</i>	Ctf7/Eco1	Eso1		Eco/Deco	ESCO1, ESCO2

Dorsett, 2007

Table 1. Major components of the mitotic sister chromatid cohesion complex

1.2 Cornelia de Lange syndrome

It was recently discovered that certain human syndromes are caused by mutations in genes encoding components of the sister chromatid cohesion complex. The Roberts-SC phocomelia syndrome is caused by mutations in ESCO2, one of the human orthologs of *Ctf7/Eco1* (Vega et al., 2005). Two independent groups found that patients with Cornelia de Lange syndrome (CdLS) carry a heterozygous mutation in the *NIPBL* gene localized on chromosome 5p13.2 (Krantz et al., 2004; Tonkin et al., 2004), up to now, several studies have identified a variety of *NIPBL* mutations in about half of the patients with CdLS (Borck et al., 2004; Bhuiyan et al., 2005; Miyake et al., 2005). Recently, mutations in the *SMC1L1* (*SMC1A*) gene on chromosome X have been identified in about 5% of mild cases of CdLS (Musio et al., 2006). In addition, one patient with CdLS is currently known carrying a mutation in *SMC3* with predominant mental retardation (Deardorff et al., 2007).

Cornelia de Lange syndrome (CdLS) is a rare but well-characterized multiple congenital anomaly/mental retardation disease with estimated prevalence of 1/10,000 to 1/30,000 (Jackson et al., 1993; Ireland et al., 1993). Affected individuals are typically identified as sporadic cases, but several reports have documented families with multiple affected members. In some multicase pedigrees there is strong evidence for autosomal

dominant inheritance; in others, germinal mosaicism can account for unaffected parents having more than one affected child (Russell et al., 2001).

Patients with CdLS display significant deficits in both physical and mental development, show a typical phenotype characterized by a distinctive facial appearance: arched eyebrows with synophrys, long eyelashes, ptosis, depressed nasal bridge with anteverted nares, long philtrum with thin upper lip and micrognathia (Fig.2). Limb deficiency: upper extremity abnormalities that mainly consist of small hands, but may also involve various severe limb reduction defects. Major malformations of various organs are occasionally associated: gastroesophageal dysfunction, cardiac, ophthalmologic and genitourinary anomalies; hirsutism. Genital anomalies, pyloric stenosis, congenital diaphragmatic hernias, cardiac septal defects, hearing loss and autistic and self-injurious tendencies also frequently occur (Tab.2). The deficits of CdLS begin prenatally and continue after birth, the remarkable feature is the diversity of developmental effect, and patients display slow growth and are small.



Krantz 2004

Figure 2.Characteristic features of Cornelia de Lange syndrome.

(A) microcephaly, notable ptosis, long eyelashes, depressed nasal bridge with upturned nasal tip, thin upper lip with down-turned corners, small chin, posterior-rotated ears, (B)absence of forelimbs, (C)clinodactyly and syndactyly.

Features of the CdLS phenotype	
Faculty or developmental system	characteristics
Growth	Retarded both pre-natal and post-natal
Neurodevelopment and communication	Delayed psychomotor development; difficulties with learning and speech; autism; seizures; self-injurious behaviour
Craniofacial development	Microcephaly; broad and/or depressed nasal bridge; anteverted nares; thin lips; small jaw; palate often with high arch and significant frequency of clefting
Limb development	Occasionally grossly malformed upper limbs(absence of forelimbs; oligodactyly); small hands and short digits; proximally placed thumbs; clinodactyly; small feet and short toes; syndactyly of second and third toes
Gastrointestinal and respiratory systems	Gastroesophageal reflux; twisted bowels; gut duplication; esophageal stenosis; pyloric stenosis; congenital diaphragmatic hernia
Hearing	Sensorineural hearing loss; external auditory canal atresia
Ocular development	Especially myopia and nystagmus
Kidney development	Primarily ureteric reflux
Heart development	Ventricular and atrial septal defects; pulmonic stenoses; tetralogy of Fallot
Genitourinary system	Undescended testes; hypoplastic male genitalia
Blood cells	Occasionally abnormal platelet development
Hair development	Excessive body and facial hair; long eyelashes

Strachan, 2005

Table 2. Features of the CdLS phenotype

1.3 Various functions of cohesion complex

The essential role of cohesin in sister chromatid cohesion is well established, but evidence obtained in yeast and different animal species implies that cohesion also contributes to gene regulation, chromatin structure and development. The first indication came from experiment in *Drosophila*, it was shown that cohesin “helps” to establish the boundaries that define the ends of a silenced chromatin domains. Nipped-B, the ortholog of the cohesin-loading component Scc2, is required for long-range activation of the *cut* and *Ultrabithorax* homeobox protein genes that play critical roles in development (Donze et al., 1999; Rollins et al., 1999). The *Bacillus subtilis* cohesin-like protein complex SMC-ScpA-ScpB could recruit the AddAB helicase/nuclease to act in post-replicative repair and form a complex with the DegS sensor kinase that inhibits its kinase activity

(Dervyn et al., 2004). And two cohesin core complex subunits, Rad21 and Smc3 control *Runx* gene expression in zebrafish (Horsfield et al., 2007). Mice lacking *Pds5b* exhibit multiple developmental abnormalities (Bin et al., 2007). Furthermore, certain human diseases have been linked to hypomorphic mutations in cohesins, and in proteins that are associated with cohesin. For example, Cornelia de Lange syndrome (CdLS), and Roberts phocomelia syndrome (RBS). But, surprisingly, most of these mutations do not cause obvious defects in cell proliferation, even partial reduction of Nipped-B by RNAi, sufficient to cause lethality had no detectable effect on cohesion (Rollins et al., 2004), these evidences imply that the resulting developmental abnormalities are not caused by defects in cohesion but reflect a distinct, yet unknown, function of cohesion proteins. The mechanisms that link cohesin to developmental gene regulation remain unclear.

1.4 Identification of human NIPBL binding proteins

The human *NIPBL* gene product, delangin, has two major isoforms, delangin-A (2804 amino acid) and delangin-B (2697 amino acid), the first 2683 amino acid residues of two isoforms are in common, just the carboxy- terminal tails are different (Tonkin et al., 2004). A bipartite nuclear localization sequence (NLS) is predicted at residues 1108-1124. A major highly conserved region spans from approximate residues 1150-2650, including five conserved HEAT (Huntington-elongation-A subunit-TOR) repeat region spans from approximately residues 1750-2350.

In order to get new insights in the molecular mechanisms underlying CdLS, first, Dr. Frank J. Kaiser of our group used the yeast-two-hybrid technique to identify binding proteins of NIPBL. Because of the protein size, they could not detect expression of a full-length constructs of *NIPBL* in yeast cells, two overlapping fragments were created: fragment N and fragment C, that representing the C-terminal half of *NIPBL*, which is highly conserved during evolution (Fig.3).

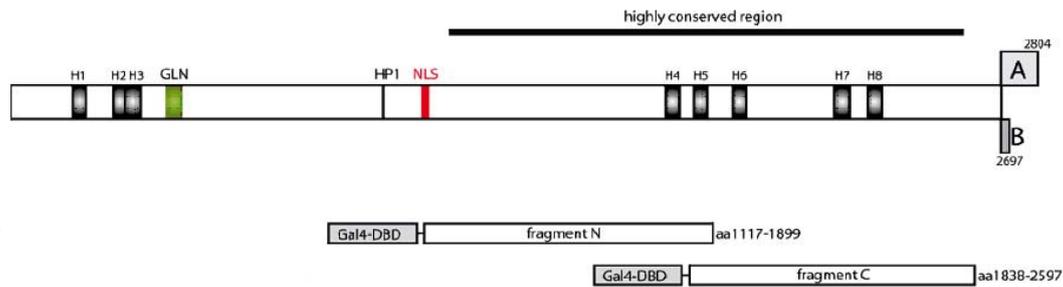


Figure 3. the predicted structure of NIPBL protein and the fragments used for yeast-two-hybrid assay.

The human NIPBL has two major isoforms, Delangin-A(2804 amino acid) and Delangin-B(2697 amino acid), differ only in the very C-terminal part of the protein. A major highly conserved region spans from approximately residues 1150-2650. The domains predicted by *in silico* analyses are indicated, including the 8 HEAT repeats (H1: 167-203; H2: 276-312; H3: 313-349; H4: 1767-1805; H5:1843–1881; H6:1945–1984; H7: 2227–2267; H8: 2313–2351), the Glutamine (Gln)-rich region (418-462), the bipartite nuclear localisation signal (NLS; 1108–1124 and the HP1-interacting motif (995-999). Fragments “N” and “C” were fused to the GAL4-DNA-binding domain and used as baits in yeast-two-hybrid assays.

Both fragments were used to screen a human chondrocyte and a human ovary library, respectively. By this, 248 clones encoding 56 different proteins were obtained. The proteins identified show a variety of functions, but interestingly about 35% are described as (co-)regulators of transcription or chromatin-remodeling or –associated factors, such as histone deacetylases (HDACs). Using fragment C as bait, five clones, three of those were independent, coded for the histone deacetylase 1(HDAC1) and three clones, two independent, encoded the histone deacetylase 3(HDAC3).

To confirm our yeast-two-hybrid results and to narrow down the interaction region of HDAC1 and HDAC3 within NIPBL, liquid β -galactosidase assays were performed. For this purpose, fragment C and five additional constructs (fragment 4-8) encoding overlapping part of fragment C were used as baits (Fig. 5). As prey constructs, the full-length open reading frames of *HDAC1* and *HDAC3* were fused to the GAL4-activating domain (GAL4-AD). Fragment 4(encoding aa 1838-2000) and fragment 5(encoding aa 1838-2380) interact with HDAC1 as well as HDAC3, whereas no interactions with fragment 6-8 (fragment 6 encodes aa 2000-2380; fragment 7, aa 2000-2598 and fragment 8, aa 2200-2597) were detectable, thus, the HDAC-interacting

region of the NIPBL protein could be narrowed down to a stretch of 163 aa (1838-2000). This domain includes the highly conserved HEAT repeat 5 and 6 (H5, H6). As additional control we analyzed the interaction of NIPBL with HDAC6, which was not found in our yeast-two-hybrid screens. Proper expressions of all fusion-constructs were verified by Western Blot analysis (Fig. 4)

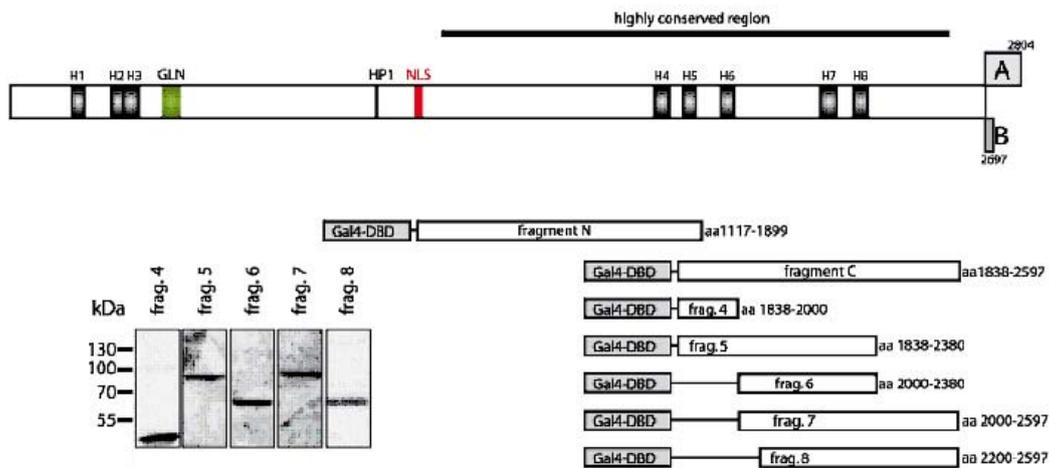


Figure 4. Fragments used in the β -galactosidase assays

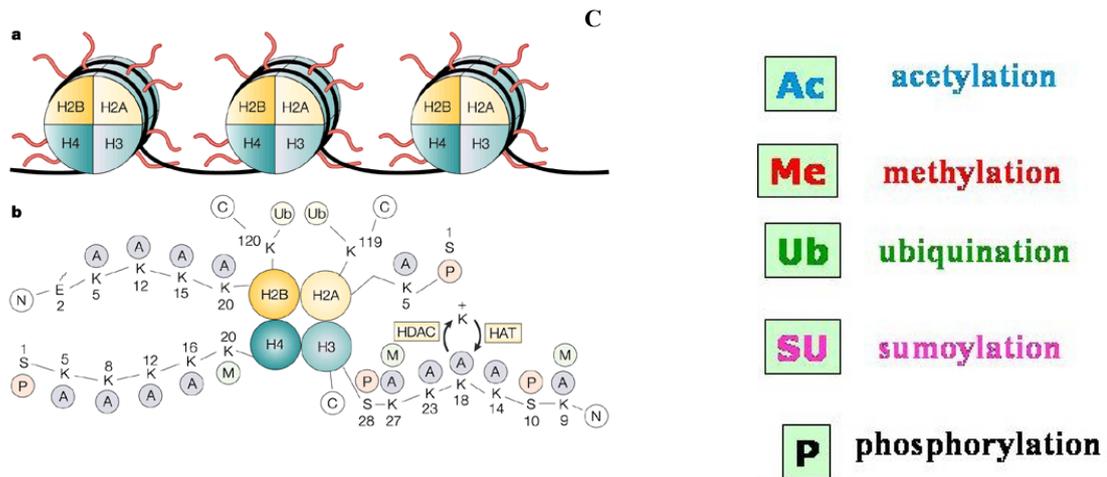
Fragments 4 – 8 represent overlapping sub-fragments of fragment C and were used in β -galactosidase assays to narrow down the smallest region for interaction of NIPBL with HDAC1 and HDAC3. The expression of all constructs was verified by the use of an anti GAL4-DBD antibody.

1.5 Histone modification and histone deacetylases (HDACs)

The organization of chromatin is of great importance for the regulation of eukaryotic gene expression. During recent years, it has become more and more evident that chromatin is a rather dynamic entity which changes between structurally accessible (transcriptionally active states) and structurally condensed (transcriptionally repressive states) temporally and spatially. Regulation occurs on the level of the basic repeating unit of chromatin-- the nucleosome. The 147 bp of super helical DNA is wrapped around the histone octamer which comprises a central histone H3/H4 tetramer and two H2A/H2B dimers (Luger et al., 1997), and both form the nucleosome core particle. All histones are highly basic proteins. Their N-termini are unstructured domains that protrude out of the nucleosomes,

called histone tail, which contains different amino acid residues and vary in length, characteristic for the respective histone. The purpose of the tails are not totally clear at present, but they appear to contribute to the stability of the nucleosome as well as serve as docking sites for other proteins (Margueron et al., 2005).

It has been known for over 40 years now that the histones can be post-translationally modified by enzymes that covalently add or remove a number of different chemical modifications, including acetyl-, phosphor-, methyl- groups and others (Fig.5). Increasing evidence indicates that major post-translational modification processes play essential role in gene expression regulation. The post-translationally modified histones serve as an extremely selective binding platform for specific regulatory proteins (Sternier and Berger, 2000; Zhang and Reinberg, 2001; Chen et al., 2007). Since these modifications occur only on specific amino acid residues on specific histones in various eukaryotic organisms, these observations strongly linked the modifications' involvement in some processes performed in nucleus. For example, up to now, there is abundant evidence indicating that lysine acetylation in histone (Hong et al., 1993) and non-histone proteins (Bergel et al., 2000) modulate local chromatin structure and particularly changes in histone-DNA, histone-histone, and histone-nonhistone protein interactions. In general, the level of acetylation is related to transcription activity: acetylation activity is correlated with transcriptional activation, whereas deacetylation activity is accompanied by transcriptional repression (Grunstein, 1997; Wade et al., 1997; Peterson, 2002). The precise mechanism by which histone hyperacetylation facilitates transcriptional activation has remained elusive.



chemistry.gsu.edu/faculty/Zheng/nucleosome.jpg

Figure 5. The sketch of histone tail and major post-translational modification

(A) The nucleosome particle consists of 147 bp of super helical DNA wrapped around a histone octamer. (B) The end of each histone protein contains a tail of amino acid residues of different lengths, characteristic of that histone. And the amino acids of histone tail can be modified by chemical, called post-translational modification. (C) The major post-translational modifications including acetylation, methylation, ubiquitination, sumoylation and phosphorylation. It has been hypothesized that histone modifications acting alone, or in combination represent a ‘histone code’ that is involved in the regulation of gene expression (Jenuwein and Allis, 2001).

Histone deacetylases (HDACs) are enzymes that influence transcription by selectively deacetylating the ϵ -amino groups of lysines located near the amino termini of core histone proteins. At least 18 members of HDAC have been identified and categorized in four classes (Table 3). Classes 1, 2 and 4 are closely related zinc-dependent enzymes, whereas class 3, the SIR2 protein family, is NAD^+ -dependent. In general, class 1 enzymes are ubiquitously expressed and primarily located in the nucleus (De Ruijter et al., 2003), and many class 1 enzymes are well-known transcriptional corepressor. Class 2 enzymes contain several conserved sequence motifs in catalytic domain which are significantly different from those of class 1 enzymes (De Ruijter et al., 2003; Gregoretta et al., 2004). Currently it is thought that HDACs of class 1 are expressed in most cell types, whereas the expression pattern of class 2 HDACs is more restricted, suggesting that they might be involved in cellular differentiation and developmental processes (Buggy et al., 2000; Galasinski et al., 2002).

Class	Homology in yeast	Enzyme	Amino acid	Catalytic domain	Mechanism	Subcellular localization
1	Rpd3	HDAC1	482	1	Zn ²⁺ -dependent	Nuclear
	Rpd3	HDAC2	488	1	Zn ²⁺ -dependent	Nuclear
	Rpd3	HDAC3	428	1	Zn ²⁺ -dependent	Nuclear/cytopl.
	Rpd3	HDAC8	377	1	Zn ²⁺ -dependent	Nuclear
2	HDA1	HDAC4	1,084	1	Zn ²⁺ -dependent	Nuclear/cytopl.
	HDA1	HDAC5	1,122	1	Zn ²⁺ -dependent	Nuclear/cytopl.
	HDA1	HDAC7	855	1	Zn ²⁺ -dependent	Nuclear/cytopl.
	HDA1	HDAC9	1,011	1	Zn ²⁺ -dependent	Nuclear
	HDA1	HDAC6	1,215	2	Zn ²⁺ -dependent	Nuclear/cytopl.
	HDA1	HDAC10	699	2	Zn ²⁺ -dependent	Nuclear/cytopl.
3	SIR2	SIRT 1-7	310-747	1	NAD ⁺ -dependent	Nuclear/cytopl.
4	rpd3/HDA1	HDAC11	373	1	Zn ²⁺ -dependent	Nuclear

De ruijter, 2003

Table 3. Human histone deacetylases

Even there is increasing evidence indicating that sister-chromatid cohesion is correlated with chromatin remodeling processes, these above results of the group Dr. Frank J Kaiser shown for the very first time a direct interaction of histone deacetylating enzymes with the cohesion protein NIPBL. The aims of my work were to verify the interaction of endogenous HDACs with NIPBL in native mammalian systems, and to analyze if the NIPBL recruits HDACs to mediate chromatin modification.

2. Material and method

2.1 chemical materials, enzymes, kits, antibodies, vectors, mediums, buffers and instruments

2.1.1 Chemical materials

Acrylamide Rotiphorese Gel 30	Carl Roth GmbH & Co, Germany
Acetic acid	Merck KGaA, Germany
-Ade/-His/-Leu/-Trp	
Dropout Solution Supplement	Clontech, USA
Agar	Sigma-Aldrich chemie GmbH, Germany
Alpha-D-Glucose	Merck KGaA, Germany
Ammonium sulfate (APS)	Carl Roth GmbH & Co, Germany
Antibiotic/Antimycotic	PAA Laboratories GmbH, Germany
Anti-FLAG M2-agarose from mouse	Sigma-Aldrich chemie GmbH, Germany
Bacto™ Yeast Extract	Becton, Dickinson and Company Sparks, USA
Beta-Mercaptoethanol	Merck KGaA, Germany
Bisacrylamide Rotiphorese Gel 30	Carl Roth GmbH & Co, Germany
Boric acid	MP Biomedicals, Germany
Bromphenolblue	Sigma-Aldrich chemie GmbH, Germany
Chloroform	Merck KGaA, Germany
DMEM (Dulbeccos Modified Eagle Medium)	Invitrogen, USA
D-Sorbitol	Sigma-Aldrich chemie GmbH, Germany
DTT	Merck KGaA, Germany

EDTA	Haupthaus, Germany
EGTA	Sigma-Aldrich chemie GmbH, Germany
Ethanol	Merck KGaA, Germany
FKS (Fetal bovine serum)	Invitrogen, USA
FuGENE HD Transfection Reagent	Roche, Germany
Gene Pulser Electroporation Buffer	Bio-Rad, USA
Glycerol about 87%	Merck KGaA, Germany
Glycerine	Sigma-Aldrich chemie GmbH, Germany
-His/-Leu Dropout Solution Supplement	Clontech, USA
HEPES	Sigma-Aldrich chemie GmbH, Germany
KCl	Fluka BioChemika GmbH, CH
KH ₂ PO ₄	Merck KGaA, Germany
Lipofectamine2000	Invitrogen, USA
Methanol	Merck KGaA, Germany
Na ₂ HPO ₄	Sigma-Aldrich chemie GmbH, Germany
NaCl	Merck KGaA, Germany
NaHCO ₃	Sigma-Aldrich chemie GmbH, Germany
NaOH	Sigma-Aldrich chemie GmbH, Germany
o-nitrophenyl-β-D-galactopyranoside (ONPG)	Sigma-Aldrich chemie GmbH, Germany
Peptone	Life Technologies, Scotland
Potassium acetate	Sigma-Aldrich chemie GmbH, Germany
Proteinase Inhibitor Cocktail (PIC)	Roche, Germany
Protein G PLUS/ Protein A-Agar Suspension	Calbiochem, USA
Protein A Sepharose 4 Fast Flow	Amersham, Sweden
Skim milk powder	Merck KGaA, Germany
Sodium butyrate	Sigma, Germany
TEMED (C ₆ H ₁₆ N ₂)	Carl Roth GmbH & Co, Germany
Tris-Acetate	MP Biomedicals, Germany
Tris-HCl	MP Biomedicals, Germany

Triton X-100, sigmaultra	Sigma-Aldrich chemie GmbH, Germany
Trypsin 1:250	SERVA Electrophoresis GmbH, Germany
Tween 20	Sigma-Aldrich chemie GmbH, Germany
Yeast-Nitrogen base w/o amino acid and	Becton, Dickinson and Company Sparks,
X-gal(5-Bromo-4-chloro-3-indolyl	Sigma-Aldrich chemie GmbH, Germany
β -D-galactopyranoside)	
x-ray developer	TETENAL, Germany
x-ray fixing solution	TETENAL, Germany

2.1.2 Enzymes

BamHI	New England Biolabs (NEB), USA
EcoRI	NEB, USA
Hind III	NEB, USA
NcoI	NEB, USA
NotI	NEB, USA
KpnI	Roche, Germany
SacI	NEB, USA
SalI	NEB, USA
SmaI	Roche, Germany
XbaI	NEB, USA
T4 DNA Ligase	Roche, Germany
pfu DNA polymerase	Fermentas, USA

2.1.3 Kits

Dual Luciferase Reporter Assay System	Promega, Germany
ECL Western Blot Detection	USB, USA
Expand Lang Range, dNTPack	Roche, Germany
Gal4 Two-Hybrid System 3	Clontech-Takara, France
GC RICH PCR System	NEB Roche, Germany

Human Fetal Brain Marathon-ready cDNA	Clontech-Takara, USA
Micron Centrifugal Filter Devices	Millipore, USA
Platinum Taq DNA Polymerase High Fidelity	Invitrogen, USA
OIAquick PCR Purification Kit	QIAGEN, Germany
QIA Gel Extraction Kit	QIAGEN, Germany
QIAfilter Midi Kit	QIAGEN, Germany
QuickChange Site-Directed Mutagenesis Kit	STRATAGENE, USA
Taq CORE kit 10	Q Biogene, USA

2.1.4 Size markers

DNA-size marker: HyperLadder I 200-10000 bp	Bioline, Germany
Protein-size marker: pre-stained SDS-PAGE Standard PageRuler, 10- 250 kDa	Fermentas, USA

2.1.5 Antibodys

anti-Acetyl-Histon 3 acetyl. K9+ K14	Upstate Biotechnology
anti-c-myc HRP	Roche, Germany
anti-Delangin	Absea, China
anti-FLAG	Sigma, Germany
Anti- Gal4 DBD	Santa Cruz, USA
Anti- Gal4 AD	Santa Cruz, USA
anti- HDAC 1	Santa Cruz, USA
anti- HDAC 3	Santa Cruz, USA
anti-FLAG M2 Affinity Gel Freeaer-safe (agarose)	Sigma, USA
Second anti-mouse antibody	USB, USA
Second anti-rabbit antibody	USB, USA
Second anti-rat antibody	PIERCE, USA

2.1.6 Vectors

pcDNA3.1/myc-His B	Invitrogen, USA
pGEM –T and PGEM- T Easy Vector System	Promega, USA
Topo PCR Cloning Kit	Invitrogen, USA
pFLAG-N3	Base on pEGFP-N3, Clontech, USA
pGADT7 (Prey)	Clontech-Takara, France
pGBKT7 (Bait)	Clontech-Takara, France
phRG-TK Renilla Luciferase Expression vector	Promega, Germany

2.1.7 Medium and Buffer

LB-Medium:	1 %	NaCl
	1 %	Bacto-Tryptone
	0.5 %	Hefe-Extrakt
LB-Agar:	15 g/l	Bacto-Agar in LB-Medium
		Ampicillin: final concentration 100 µg/ml
		Kanamycin: final concentration 35 µg/ml
SD- Medium:	6.7g/l	Yeast nitrogen base without amino acids
	20g/l	Agar (for plates only)
	10%	Appropriate sterile 10×Dropout solution
	2%	Dextrose (glucose)(after medium to cool to 55°C)
YPD Medium	10g/l	yeast extract
	20g/l	peptone
	10g/l	dextrose; autoclave separatly
10×TE	0.1 M	Trish; pH 7.5 filter sterilized
	0.01M	EDTA; pH7.5 filter sterilized

10×LiAc	1M	LiAc, pH7.5 adjusted with acetic acid filter sterilize
50%PEG4000	500g/l	PEG4000, autoclave
Z Buffer	16.1 g/l	Na ₂ HPO ₄ ·7H ₂ O
	5.5 g/l	NaH ₂ PO ₄ ·H ₂ O
	0.75 g/l	KCl
	0.246 g/l	MgSO ₄ ·7H ₂ O
		Adjust pH to 7
ONPG stock solution	4 mg/mL	in Z buffer, adjust to pH 7.0 and mix well
Z buffer withβ -mercaptoethanol	2.7ml/l	In Z buffer
Buffer 1 for Mini preparation	10mM	EDTA
	50mM	Tris- HCL, pH 8.0
	100μg/ml	RAase A
Buffer 2 for Mini preparation	0.2M	NaOH
	1%	SDS
Buffer 3 for Mini preparation	3M	CH ₃ COOK
Incubation buffer (Lysis buffer)	20mM	HEPES pH 7.9
	75mM	KCl
	2.5mM	MgCl ₂
	1mM	DTT
	0.1%	NP-40
	0.5mM	phenylmethylsulfonyl fluoride
	1mM	Na ₃ VO ₄
10% APS solution	10%	Ammoniumpersulfate
Separating buffer for Western blot	1.5M	Tris- HCL, pH 8.8
	0.4%	SDS
Collecting buffer for Western blot	0.5M	Tris- HCL, pH 6.8
	0.4%	SDS

10×SDS buffer	1.25M	Tris- Base
	2M	Glycine
	1%	SDS
4×SDS protein loading buffer	62mM	Tris- HCL, pH 6.8
	10%	Glycine
	2%	SDS
	5%	DTT
	0.025%	Bromphenolblue
	5%	β-Mercaptoethanol (add when use)
Transfer buffer	5.82g/l	Tris- Base
	2.93g/l	Glycine
	3.75ml/l	10%SDS
	200ml	methanol

2.1.8 Instruments

1. Tube and Plate

Micro Tube 1.5 ml	SARSTEDT, Germany
Tube 15ml	SARSTEDT, Germany
Tube 50ml	SARSTEDT, Germany
96 half area well Plate	Corning, Germany
OIAquick 96 plate	QIAGEN, Germany
Single/loose 1.1ml micro tubes in microrack	National Scientific Supper Co, USA
225cm ² Easy Flask	Nunclon Surface, Denmark
Cell culture plate	Nunclon Surface, Denmark

2. Pipette

pipettes	Eppendorf, Germany
Easypet	Eppendorf, Germany
Finnpipette	Thermo labsystem, USA

3. Centrifuge

Omnifuge 2.0RS	Heraeus Sepatech, Germany
centrifuge, 5804	Eppendorf, Germany
centrifuge, 5417R	Eppendorf, Germany
Biofuge 93	Heraeus, Germany
Centrifuge 5415D	Eppendorf, Germany
Thermomixer comfort	Eppendorf, Germany
Thermomixer compact	Eppendorf, Germany

4. Incubator

Incubator for yeast	Heraeus, Germany
Incubator for bacteria	Heraeus, Germany
Incubator for cell	Köttermann, Germany
Shaking incubator inova 4300	New Brunswick Scientific, Germany
Shaking incubator	Certomat H, Germany

5. Cycler and Sequencer

T gradient Cycler	Biometra, Germany
Cycler (PCR system 2700)	Applied Biosystems, USA
PCR Cycler UnoII	Biometra, Germany
2720 Thermal Cycler	Applied Biosystems, USA
Sequencer (3130x/Genetic Analyzer)	Applied Biosystems-HITACHI, USA

6. others

Autoclave	WEBECO, Germany
Bio photometer	Eppendorf, Germany
Bench	Heraeus Sepatech, Germany
Cell Lifter	Corning Incorporated, Mexico
GeniosPro Luminometer	Tecan, Germany
Film developer (FUJIFILM FPM800A)	FUJI photo Film Co, Dermank
Microprocessor pH meter	WTW, Germany
Transilluminator Chroma 43	Vetter GmbH, Germany

Trans-blot SD Cell	BIO RAD, USA
Power Pac Basic	BIO RAD, USA
Ultrasonic generator	BANDELIN electronic GmbH, Germany
Universal Vacuum System Plus (Savant)	Werner Hassa, Germany
Speed Vac Plus (savant)	Werner Hassa, Germany
Vortex Top Mixer	Fischer Bioblock Scientific
Water bath	GFL, Germany
Water bath	Köttermann, Germany
Westernblot SemiDry Trans-Blot SD Semi-Dry	Bio-Rad, Hercules, USA
Transfer Cell	
Western blot power supply	Bio-Rad, Hercules, USA

2.2 Methods

2.2.1 Co-immunoprecipitation (Co-IP) assay

prepare sepharose: Add 400 μ l protein A/G sepharose and 1100 μ l PBS solution to 1.5ml microcentrifuge, 4°C, incubate at least 2hours. Use PBS wash sepharose at least 4 times, 3500rpm, 3min, 4°C. Resuspend the sepharose with 200 μ l lysis buffer (with Triton-X), store at 4°C.

prepare HeLa cells: culture HeLa cell line till cells full of the plate. Transfer the medium, wash by PBS. Add 1ml lysis buffer (with Triton-X) and 4 μ l PIC (250:1), harvest cells using cell scraper. Collect cells to 1.5ml microcentrifuge tube, put on ice for 5min, full speed centrifuge for 2min at 4°C. Transfer supernatant to a fresh 1.5ml microcentrifuge tube.

Immunoprecipitation: Add 20 μ l sepharose to above supernatant, 4°C, up-side-down shaking for 1h. centrifuging 3500rpm for 2 min at 4°C. Transfer supernatant to a fresh 1.5ml microcentrifuge tube, add 10 μ l anti-delangin antibody, up-side-down shaking for 2h at 4°C. Add 180 μ l sepharose, up-side-down shaking overnight at 4°C. Centrifuge the overnight tube 1000rpm, 1min, 4°C. Transfer supernatant to a fresh 1.5ml microcentrifuge

tube, put on ice. Use 500µl lysis buffer (without Triton-X) with 2µl PIC wash the pellet 3-5times, centrifuge with 1500rpm for 1min, 4°C, discard the supernatant.

Western blot analysis: Add 100µl 4x SDS-loading-buffer (freshly prepared) to pellet. add 50µl 4x SDS-loading-buffer to 100µl supernatant. Denature at 95°C for 5min. 25µl samples for western blot analysis. Using 12% gel. Run the gel for 1-1.5h at 80mV, membrane transfer for 2h at 70mA. Using 4% Skin milk powder solution for blocking, 0.5h. Add 5µl anti-delangin antibody, anti-HDAC1 antibody and anti-HDAC3 antibody respectively for overnight primary antibody incubation. Use PBS to wash membranes 3 times. Add 10µl anti-rabbit antibody for anti-delangin; anti-goat antibody for anti-HDAC1 and anti-rabbit antibody for anti-HDAC3 antibody respectively for secondary antibody incubation for 2h. Use PBS to wash membranes 3 times. Detect the proteins.

Gel prepared for western blot:

<u>Collecting gel</u>	<u>for two gels</u>
Collecting buffer	2.5ml
Acrylamide Rotiphorese Gel 30 (AA/BA)	1.3ml
Water	6.2ml
10% APS	150µl
TEMED	15µl

<u>Separating gel</u>	<u>for two gels</u>	<u>8%</u>	<u>10%</u>	<u>12%</u>	<u>15%</u>
Separating buffer				2.5ml	
AA/BA		2.8ml	3.3ml	3.9ml	5ml
Water		4.6ml	4.1ml	3.5ml	2.3ml
10% APS				150µl	
TEMED				15µl	

2.2.2 β -Galactosidase Activity Assay (Liquid culture assay using ONPG as substrate)

Liquid β -galactosidase assays were performed as described in the Matchmaker 3 handbook (Clontech). The truncated NIPBL-fragments 4 was inserted into the pGBKT7 plasmid. The full length open reading frames of HDAC1, HDAC3 and HDAC6 were amplified and specific restriction sites were added by PCR, using human fetal brain cDNA (HDAC1) or full length ORFs containing plasmids (InvivoGen, USA) and inserted into the pGADT7 plasmid. Yeast cells (AH109) were co-transfected with the NIPBL-fragments 4 and all HDAC containing plasmids, respectively, and proper expression was verified by Western blotting using specific anti-GAL4-DBD and anti-GAL4-AD antibodies.

Protocol in brief: Prepare 2-3ml two-day's culture in liquid SD (-trp/-leu) selection medium. On the day of the experiment, dissolve ONPG AT 4mg/ml in Z buffer with shaking for 1-2hr. Vortex the overnight culture tube for 0.5-1 min to disperse cell clumps, immediately transfer 2ml of the overnight culture to 8ml YPD medium. Incubate the fresh culture at 30°C for 3-5hr with shaking (230-250rpm) until the cells are in mid-log phase. Record the exact OD₆₀₀ when we harvest the cells, try to get all OD₆₀₀ to be between 0.5-0.8. Place 1.5ml of culture into each of three 1.5ml microcentrifuge tubes. Centrifuge at 14,000 rpm for 30 sec. Remove supernatants carefully. Add 1.5ml of Z buffer to each tube and vortex until cells are resuspended. Centrifuge cells again and remove supernatants. Resuspend each pellet in 300 μ l of Z buffer. Thus, the concentration factor is 1.5/0.3= 5-fold. Transfer 0.1 ml of the cell suspension to a fresh microcentrifuge tube. Place tubes in liquid nitrogen until the cells are frozen (0.5-1 min). Place frozen tubes in a 37°C water bath for 0.5-1 min to thaw. Repeat the freeze/thaw cycle four times to ensure that the cells have broken open. Set up a blank tube with 100 μ l of Z buffer. Add 0.7ml of Z buffer+ β - mercaptoethanol to the reaction and blank tubes. Do not add Z buffer prior to freezing samples. Start timer. Immediately add 160 μ l of ONPG in Z buffer to the reaction and blank tubes. Place tubes in a 30°C incubates. After the yellow color develops, add 0.4ml of 1 M Na₂CO₃ to the reaction and blank tubes. Record elapsed time in minutes.

Centrifuge reaction tubes for 10min at 14,000 rpm to pellet cell debris. Carefully transfer supernatant to clean cuvettes. Calibrate the spectrophotometer against the blank at A_{420} and measure the OD_{420} of the samples relative to the blank. The ODs should be between 0.02-1.0 to be within the linear range of the assay. Calculate β -galactosidase units. 1 unit of β -galactosidase is defined as the amount which hydrolyzes 1 μ mol of ONPG to o-nitrophenol and D-galactose per min per cell:

Use the following equation to calculate units of enzyme activity:

$$\beta\text{-galactosidase units} = 1000 \times OD_{420} / (t \times V \times OD_{600}).$$

T = time of the reaction in minutes.

V = volume of culture used in the assay in mLs.

$OD_{600} = A_{600}$ of 1ml of culture

2.2.3 Reporter gene assays

NIPBL fragment 4 was inserted into a pcDNA-GAL4-DBD expression plasmid to obtain the GAL4-NIPBL fusion construct. Full length ORF of HDAC1 was amplified as described above and inserted into pcDNA3.1. HDAC3 and HDAC6 expression plasmids were obtained from InvivoGen. The GAL4-TATA-Luc and the GAL4-*tk*-Luc reporters have been well described.

Transient transfection assays of COS-7 and CHO cells were performed in 96 half area well plates with Lipofectamine2000 reagent according to the manufacturer's instruction. The phRG-TK Renilla luciferase expression vector was used as a transfection control. Activity of Firefly and Renilla luciferase was measured after 48 h incubation with the Dual Luciferase Reporter Assay System in a GeniosPro Luminometer. All measurements were performed in triplicates. Relative luciferase activity was determined as rate of the average firefly : renilla luciferase activity. To inhibit Histone deacetylases, the cells were treated with 5 μ M Sodium butyrate, respectively for 24 h prior to measurement. DMSO was used as negative control for Sodium butyrate treated cells. All assays were done at least as triplicates.

Protocol in brief: Predispense 100 μ l of LAR II into the appropriate number of

luminometer tubes to complete the desired number of DLR. Assays. Program the luminometer to perform a 2-second premeasurement delay, followed by a 10-second measurement period for each reporter assay. Carefully transfer up to 20µl of cell lysate into the luminometer tube containing LAR II; mix by pipetting 2 or 3 times. Do not vortex. Place the tube in the luminometer and initiate reading. If the luminometer is not connected to a printer or computer, record the firefly luciferase activity measurement. If available, use a reagent injector to dispense 100µl of Stop & Glo® Reagent. If using a manual luminometer, remove the sample tube from the luminometer, add 100µl of Stop & Glo® Reagent and vortex briefly to mix. Replace the sample in the luminometer, and initiate reading. If the luminometer is not connected to a printer or computer, record the *Renilla* luciferase activity measurement. Discard the reaction tube, and proceed to the next DLR. Assay.

2.2.4 Chromatin immunoprecipitation (ChIP) assay

This assay was adapted from Wei et al: COS7 cells were transfected with GAL4-*tk*-reporter and either the NIPBL-fragment4 GAL4-DBD or the empty GAL4-DBD expression plasmids as described.

Material

2.5M glycine solution

Liquid nitrogen

Crosslinking solution:	11%	Formaldehyd
	0.1M	NaCl
	2.7 mM	KCl
	1 mM	EDTA
	0.5 mM	EGTA
	50 mM	HEPES, pH 8,0
PBS:	137 mM	NaCl
	10 mM	Na ₂ HPO ₄

	2 mM	KH ₂ PO ₄
	2.7 mM	KCl
Lysis buffer 1:	50m M	HEPES (KOH), pH 7,5
	0.14 M	NaCl
	1 μM	EDTA
	10%	Glycerin
	0.5%	NP40
	0.25%	TritonX-100
		PIC*
		*PIC (Proteinase Inhibitor Cocktail) have to be added 250:1 volume immediately before use
Lysis buffer 2:	0.2 M	NaCl
	1 μM	EDTA
	0.5 μM	EGTA
	10 μM	Tris, pH 8,0
		PIC*
Lysis buffer 3	1 μM	EDTA
	0.5 μM	EGTA
	10 μM	Tris, pH 8,0
		PIC*
sarkosyl(sodium lauryl sarcosine)		
RIPA buffer:	50mM	HEPES (pH 7.6)
	1mM	EDTA
	1%	NP40
	0.5M	LiCl
		PIC*
TE buffer		
Elution buffer	50mM	Tris

	10mM	EDTA
	1% SDS	SDS
Reverse-crosslink buffer	0.3M	NaCl
	50mM	NaHCO ₃
	1%	SDS

A. Co-transfection in COS-7 cell line

COS-7 cells were co-transfected with GAL4-tK- reporter and either GAL4-BD fusing NIPBL fragment 4 or empty GAL4-BD expression plasmids.

B. Preparation of chromatin

1. Crosslinking

- 1) harvest cells in 40ml aliquots to 50ml Sarstedt tubes (10^9 cells).
- 2) Incubate 10min on ice.
- 3) Add 4ml of cold crosslinking-solution, incubate on ice for 10min.
- 4) Add 2.2ml (1/20 of volume) of 2.5M glycine solution for stopping the crosslinking reaction.
- 5) Centrifugation 2.000g, 10min, 4°C.
- 6) Wash 2 times in cold PBS.
- 7) Shock-frost the cell pellet in liquid nitrogen, store at -80°C.

2. Extraction of chromatin

- 1) Resuspend cell pellet in 30ml of lysis buffer 1 (PIC*)
- 2) Incubate by slightly shaking, 10min, 4°C. Centrifugation 2,000g, 10min, 4°C.
- 3) Resuspend pellet in 24ml lysis buffer 2 (PIC*), slightly shaking, 10min, room temperature (RT). Centrifugation 2,000g, 10min, 4°C.
- 4) Resuspend in 10ml lysis buffer 3 (PIC*).

3. Sonication: fragmentation of the chromatin

- 1) aliquot the samples to 2 x 5ml in small 15ml Sarstedt tubes. (samples must be hold at 4°C, ice all the following procedure)
- 2) put small sonicator tip into the chromatin solution (on ice).
- 3) The parameters of sonication are:

amplitude (strength)	25%
time	20 seconds
times	13-15

MS 72

Pulsation off

Between each step the samples must be cool down on ice for at least 1min.

- 4) Finally add sarkosyl (sodium lauryl sarcosine) up to total concentration of 0.5%. RT, gently shaking for 10min. Centrifugation 10,000g for 10min, 4°C.
- 5) Carefully transfer each 1ml supernatant (chromatin solution) to 1.5ml microcentrifuge tube. Store at -80 °C.
- 6) Check the chromatin fragments after sonication:

take 50µl sample add 2% glycogen and 2µl of 20 mg/ml proteinase K, incubation for 2h at 37°C. Check on 1% agarose gel. The available fragments of chromatin are between 1,000bp to 2,000 bp.

C. Chromatin immunoprecipitation

1. Prepare sepharose

- 1) Add 400µl protein A/G sepharose and 1100µl PBS solution to 1.5ml microcentrifuge, 4°C, incubate at least 2hours.
- 2) Use PBS wash sepharose at least 4 times, 3500rpm, 3min, 4°C.
- 3) Resuspend the sepharose with 200µl lysis buffer 3, store at 4°C.

2. Immunoprecipitation

- 1) 1-2mg of chromatin solution first add to 0.1% TritonX-100 (destroy the weak interaction of proteins with chromatins); 1mM PMSF (proteinase inhibitor) and 20µl sepharose, up-side-down shaking for 1h, 4°C.
- 2) Centrifugation 3,500rpm, 2min, 4°C.
- 3) Carefully transfer the supernatant to a fresh 1.5ml microcentrifuge tube, add 10µl anti-acetyl H3 antibody. Up-side-down shaking for 2h, 4°C.
- 4) Add 180µl sepharose to above supernatant, up-side-down shaking, overnight, 4 °C.
- 5) Centrifugation 2,000g 1min, 4°C. Discard the supernatant.

reverse: ttaagcgggtcgctgcag

PCR reaction mixes:

template	1 μ l
10 x buffer	5 μ l
Primer F	1 μ l
Primer R	1 μ l
dNTP	1 μ l
Taq polymerase	0.5 μ l
Water	40.5 μ l

total: 50 μ l

Thermal cycling:

	Temperature	Time	Cycles
Initial denaturation	95°C	2min30sec	1
Denaturation	95°C	30sec	30
Annealing	50°C	30sec	
Elongation	72°C	30sec	
Final elongation	72°C	5min	1
Cooling	4°C	unlimited time	

F. Detection of amplified fragment

Amplified fragment of tK promoter region and SV40 promoter region are analyzed on a 2% agarose gel.

3. Results

3.1 NIPBL forms stable complexes with HDAC1 and HDAC3 in mammalian cells

To confirm our data obtained in yeast cells only expressing fragments of NIPBL we set out to analyze if the endogenous NIPBL protein interacts with HDAC1 and HDAC3 in a native mammalian cell line, For this, extracts of HeLa cells, which were shown to express detectable amounts of NIPBL protein (Seitan et al, 2006), were used to perform co-immunoprecipitation analysis. First, protein A/G sepharose was loaded with an anti-delangin specific antibody to immunoprecipitate NIPBL. After several washing steps, the precipitates were analyzed by SDS-PAGE and Western blot detection using the anti-delangin, anti-HDAC1 and anti-HDAC3 antibody. As a control, an anti-IG antibody was used for immunoprecipitation.

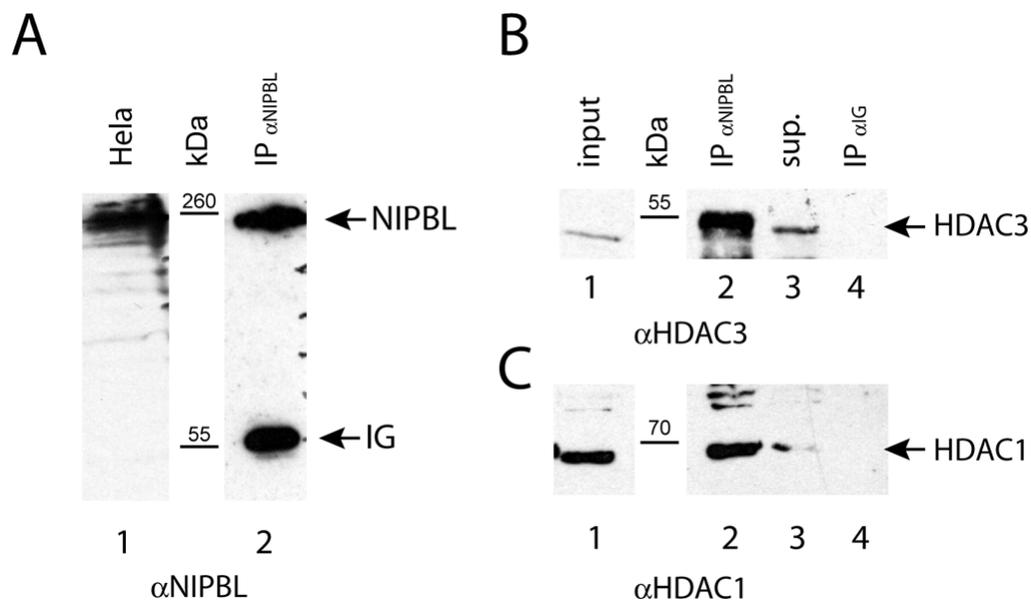


Figure 6. NIPBL co-immunoprecipitates with HDAC1 and HDAC3 in HeLa cells

(A) Expression of *NIPBL* was detected with an anti-delangin antibody in extracts of HeLa cells (lane 1), and specific precipitation of NIPBL was monitored (lane 2). (B) HDAC3 was shown with an anti-HDAC3 antibody in HeLa cell extracts (lane 1). And HDAC3 was found to be co-precipitated with NIPBL (lane 2), whereas no HDAC3-specific signal could be detected in the control IP (lane 4). None co-precipitated HDAC3 was visible in the supernatant (lane 3). (C) Expression of HDAC1 was monitored in HeLa cell extracts (lane 1). HDAC1 could only be identified in the anti-NIPBL precipitates (lane 2), while no HDAC1-specific signal was detectable in the IPs using the anti-IG antibody (lane 4). None-precipitated HDAC1 could be found in the supernatants of the IPs (lane 3).

Proper expression of *NIPBL* and *HDAC1*, *HDAC3* in HeLa cells were monitored. HDAC1 and HDAC3 could only be identified in the anti-NIPBL precipitates, while no HDAC1- or HDAC3-specific signal could be detected in the controls using the anti-IG antibody for precipitation. None-co-precipitated HDAC1 could be found in the supernatants of the IPs. Taken together, our results support that NIPBL could form stable complexes with HDAC1 and HDAC3 in mammalian cells

3.2 NIPBL recruits HDACs for trans-repressional activity

To analyze if the association of NIPBL with histone deacetylases has any functional effect on promoter activities we performed luciferase reporter gene assay.

The minimal HDAC-interacting region of NIPBL, fragment 4, was fused to the GAL4-DNA-binding domain (GAL4-DBD), to obtain a GAL4-DBD-NIPBL fusion construct. Different mammalian cells (COS7 and Cho) were transiently transfected with different reporter plasmids containing multiple GAL4-binding sites 5' to the *luciferase* cDNA under the control of the *TATA* box (TATA-Luc) or the *tk* promoter, respectively. According to our hypothesis, NIPBL should recruit HDACs to the respective promoter sequences, which would result in different promoter activities and reporter gene expression (Fig. 7).

The luciferase activities of cells transfected with empty GAL4 and LUC reporters were arbitrarily set as 100% (Fig. 8A, column 1). Transfections with increasing amounts of the NIPBL construct result in a dose-dependent decrease of the reporter activity (Fig. 8A, columns 2 – 4). Moreover, co-transfection with HDAC1 and HDAC3 expression

plasmids support these effects and induce an enhanced reduction of the reporter activities--approximately 5-6 folds decreased. Whereas co-transfection with HDAC6, which was excluded as NIPBL binding protein, did not show any functional effect (Fig. 8B). Here the reporter gene expression of HDAC6 co-transfected cells were similar with these cells just transfect with NIPBL-fragment 4. Additionally, we could exclude that over-expression of the respective histone deacetylase (HDAC-1, -3 or -6) in the absence of the GAL4-DBD-NIPBL construct significantly influences the reporter activities (Fig. 6B, columns 4, 6 + 8).

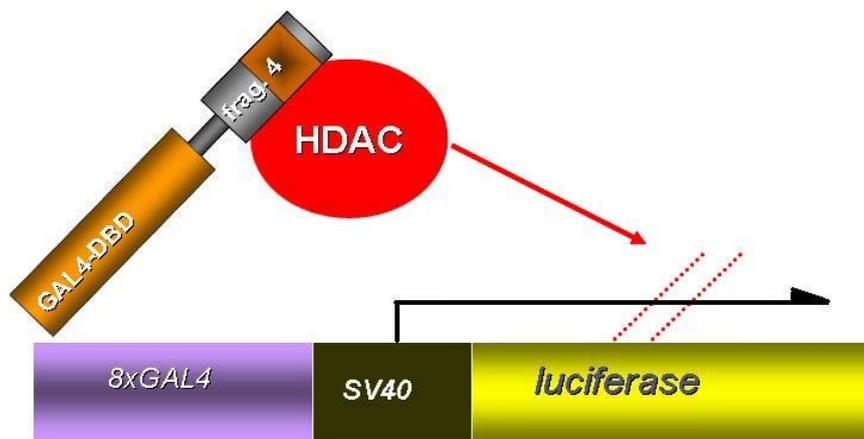


Figure 7. Model of the luciferase reporter gene assay. The HDAC-interacting region of NIPBL, fragment 4, was fused to GAL4-DBD to obtain a GAL4-DBD-NIPBL-frag4 construct (NIPBL-frag4). Cells carrying a luciferase reporter with GAL4-consensus binding sequences 5' to a *LUC* reporter gene were transfected with of NIPBL-frag4.

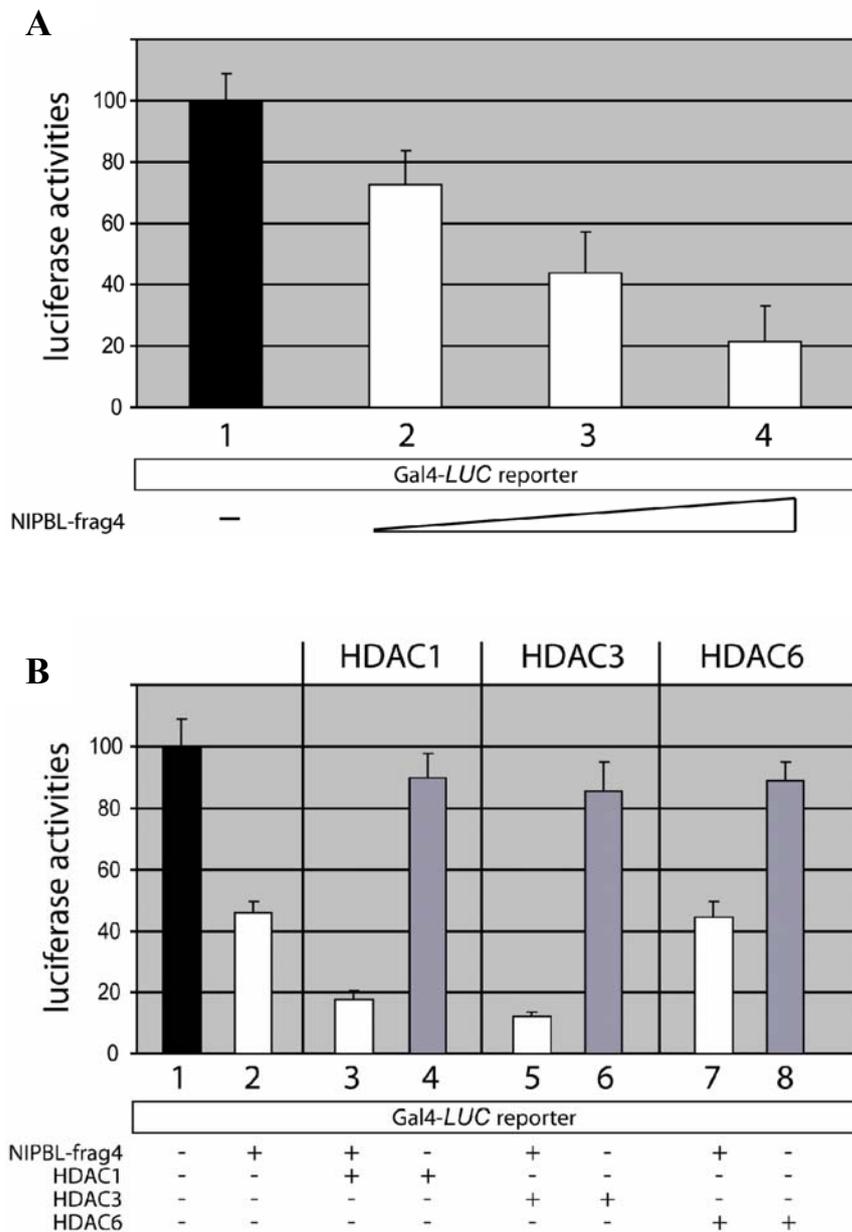


Figure 8. NIPBL induces trans-repressional activity

(A) Cells carrying a luciferase reporter gene were transfected with increasing amounts of NIPBL-frag4. The luciferase activity of cells transfected with an empty GAL4-DBD plasmid was set as 100% (column 1). NIPBL-frag4 reduces the reporter activity in a concentration dependent manner (compare columns 1 with 2-4). (B) Co-transfections with HDAC1- as well as HDAC3-encoding plasmids enhance the transcriptional repression of NIPBL-frag4 (columns 3+5), while transfection with HDAC6 do not alter the activity (column 7). As control, the effects of the individual HDAC on the reporter (HDAC1+3+6, columns 4+6+8) were monitored in the absence of NIPBL.

3.3 The trans-repressional activity of NIPBL-mediated recruitment of HDACs is sensitive to chemical inhibition of HDACs

The specific interaction of NIPBL with HDAC1 and HDAC3, as well as the increase of NIPBL-mediated repression related with the increased expression of HDAC1 and HDAC3, strongly indicate a correlation of NIPBL with histone deacetylation mechanisms. We further set out to analyse if the inhibition of histone deacetylases result in a decreasing trans-repressional activity of NIPBL. Sodium-butyrate (SoBu) was used for chemical inhibition of histone deacetylases. SoBu is known to cause hyperacetylation of the histones H3 and H4, which results in enhanced promoter activities (Han et al., 2007). The effect of sodium-butyrate (SoBu) on the trans-repressive activity of the NIPBL-frag4 construct was assessed in COS7 cells. By this, we could find that the trans-repressional activity could be almost completely abolished (Fig. 9). This result show that histone deacetylation may play a mayor role in the trans-repressional activity mediated by the GAL4-DBD-NIPBL, and further indicate, a directed connection between the NIPBL-mediated repression and the histone deacetylating processes. Additional experiments, using the empty GAL4-DBD construct as control, show that SoBu-treatment only slightly increase the reporter gene activity.

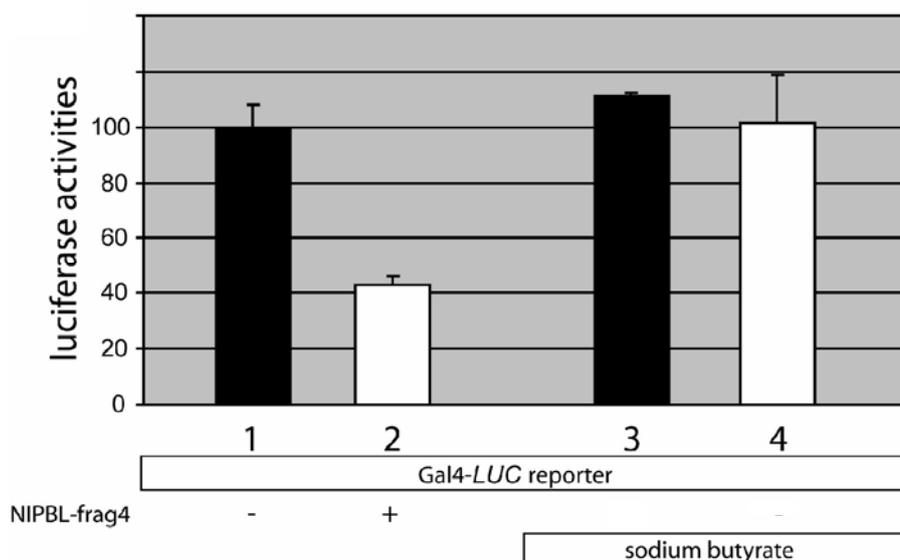


Figure 9. Repressional activity mediated by NIPBL is sensitive to sodium-butyrate (SoBu).

Column 1, 3 shows the activities of cells transfected with the empty GAL4-DBD plasmid. Columns 2,4 represent the results of cells transfected with the NIPBL-construct. While the transfection with NIPBL-frag4 decreases reporter gene activity down to 50% in nontreated cells (column 2), treatments with SoBu, almost completely abolish the trans-repressional effect of NIPBL (columns 4). Experiments were done at least in triplicates and the standard deviations are indicated by error bars.

3.4 The NIPBL-mediated recruitment of HDACs induces histone deacetylation

To test the hypothesis that histone deacetylation may play a role in the trans-repressional activity of the GAL4-DBD-NIPBL, we used chromatin-immunoprecipitation (ChIP) assays to examine the histone deacetylation of its target promoter in COS-7 cells. In this assay, COS-7 cells were co-transfected with GAL4-*tK*-reporter and either GAL4-BD fusing NIPBL fragment 4 or empty GAL4-BD expression plasmids. Acetylated chromatin is precipitated with anti-acetyl histone 3 antibody which could specifically recognizes acetylated lysines 9 and 14 of histone 3. The precipitated acetylated *tK* promoter DNA fragments were amplified in specific PCR reactions, and analyzed in agarose gel electrophoresis. Conversely, if the lysines 9 and 14 of histone 3 in *tK* promoter region are deacetylated, that deacetylated *tK* promoter could not be precipitated, therefore, no *tK* promoter DNA fragments would be precipitated and amplified by PCR reaction.

As shown in the lower panel of figure 11, an expected 130 bp *tK* promoter specific fragment can be amplified from chromatin prepared from cells transfected with the control GAL4-BD plasmid(lane 6), but not from precipitates of cells transfected with the GAL4-DBD-NIPBL construct (lane 5). Figure 11, lane 7 and 8 show the negative controls where a nonspecific anti-IG antibody was used. Lanes 3 and 4 are positive controls using input DNA before precipitation, lane 1 shows a negative control of water and lane 2 is a positive control of plasmid DNA. This result clearly indicates that the expression of GAL4-BD-NIPBL fragment 4 renders histone deacetylation on the GAL4 target. In absence of the NIPBL fragment 4, histone remains acetylated on this promoter despite its binding by GAL4-BD. For an internal control of this assay, the acetylation status of the *SV40* promoter, regulating the expression of the NIPBL construct, was monitored in parallel experiment (shown in the lower panel of Fig. 11). Because no GAL4-binding sites

are present in this vector, this “active” promoter was acetylated, and therefore amplified, regardless of the presence or absence of the GAL4-DBD-NIPBL construct (lanes 4 and 5). This result verifies the specificity of deacetylation on the promoter containing the GAL4 binding sites, as a result of GAL4-BD-NIPBL fragment 4 expression.

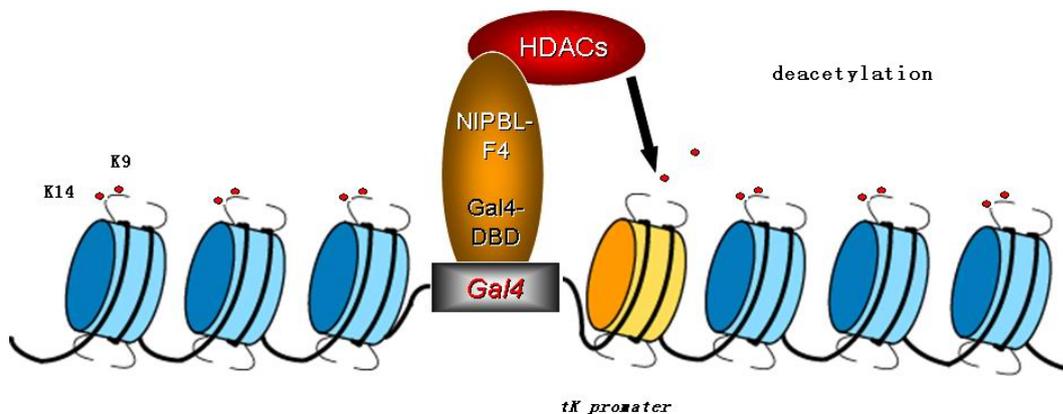


Figure 10. Mechanism of the proposed chromatin immunoprecipitation assay.

COS-7 cells were co-transfected with GAL4-*tK*- reporter and either GAL4-BD fusing NIPBL fragment 4 or empty GAL4-BD expression plasmids. In this assay, acetylated *tK* promoter is precipitated with anti-acetyl histone 3 antibody which could specifically recognizes acetylated lysines 9 and 14 of histone 3. And the precipitated *tK* promoter DNA fragments are amplified in PCR reactions. Conversely, if the lysines 9 and 14 of histone 3 in *tK* promoter region are deacetylated, that deacetylated *tK* promoter could not be precipitated, therefore, no *tK* promoter DNA fragments are precipitated and amplified after PCR reaction. This assay could clearly show the specificity of deacetylation on the *tK* promoter region.

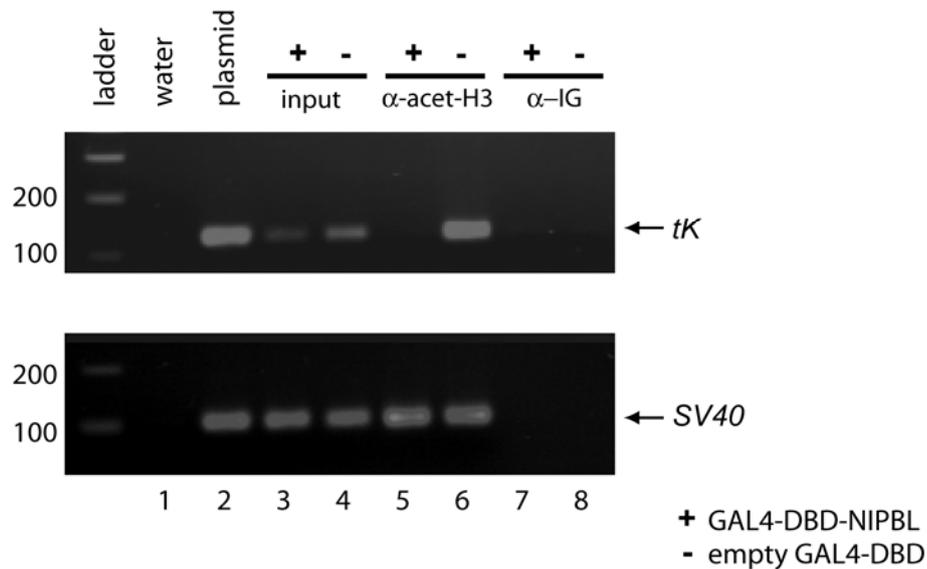


Figure 11. Chromatin-immunoprecipitation (ChIP) assays were used to demonstrate histone deacetylation. The histone deacetylation occurs as the result of the GAL4-DBD-NIPBL (+) expression (lane 5), but not if the GAL4-DBD is expressed alone (-) (lane 6). The upper panel of the figure shows a GAL4-*tK* promoter specific fragment of approximately 130 bps. In the bottom part a 120 bps PCR product indicating acetylation of the SV40 promoter is shown. Water (lane 1), plasmid DNA (lane 2) as well as input DNA before immunoprecipitation (lanes 3 + 4) were used as controls. As additional control a nonspecific antibody was used for precipitation (α -IG; lanes 7 + 8).

3.5 Missense mutations identified in patients with CdLS affect the interaction of NIPBL with HDACS, and affect the NIPBL-mediated repression

In our ongoing attempts to augment the spectrum of *NIPBL* mutations we could identify a new missense mutation (R1985T) resulting in an amino acid exchange within the critical region for the interaction of NIPBL with HDAC1 and HDAC3 (Fig. 12). The patient shows a classical CdLS phenotype. A second missense mutation (c.5566A>G; p.R1856G) within this part of the protein was described recently by Selicorni *et al.* (2007). The patient shows a severe CdLS phenotype: face dysmorphisms; pre- and post-natal growth retardation; severe mental retardation; no speech, but without any limb anomalies. From

62 CdLS patients analyzed, this patient was the one and only who showed severe phenotype with a missense mutation in the *NIPBL* gene. Almost all missense mutations in *NIPBL* gene cause a mild-moderate CDLS-phenotype, whereas the patients with truncating mutations of *NIPBL* are frequently severely affected (Selicorni et al., 2007).

To analyze whether the two missense mutations R1985T and R1856G within the HDAC-interacting region of NIPBL have any effect on the interaction with NIPBL and HDAC1 or HDAC3, we used site-directed mutagenesis to create NIPBL-expression plasmids including the amino acid exchanges R1985T and R1856G, respectively. To test whether these mutations have any influence on the binding capacity of NIPBL to the histone deacetylases 1 and 3 we performed β -galactosidase assays. While both amino acid exchanges do not alter the binding of NIPBL to HDAC1 significantly, compared to the wild-type, only 50% of β -galactosidase activity were detected, indicating a extensive reduction of the interaction capacities to HDAC3 (Fig. 12A).

We next examined if these two missense mutations affect the NIPBL-mediated repression. First, we used site directed mutagenesis to generate the mutant constructs GAL4-DBD-R1985T and GAL4-DBD-R1856G. These constructs were used to transfect different cell lines carrying the TATA-*Luc* or *-tK* promoter containing reporter plasmid. While the mutation R1985G only slightly decreases the NIPBL-mediated repression (55.4% to 64.9 %), mutation R1856G severely reduces the repressional activity of the NIPBL construct (55.5% to 83.8 %) (Fig.12B). These results suggest our hypothesis that the trans-repressional activity of NIPBL correlates with the specific recruitment of histone deacetylating enzymes.

Equal expression of all NIPBL constructs was verified by analyzing appropriate cell extracts in Western blotting assays using an anti-GAL4-DBD antibody (Fig. 12B).

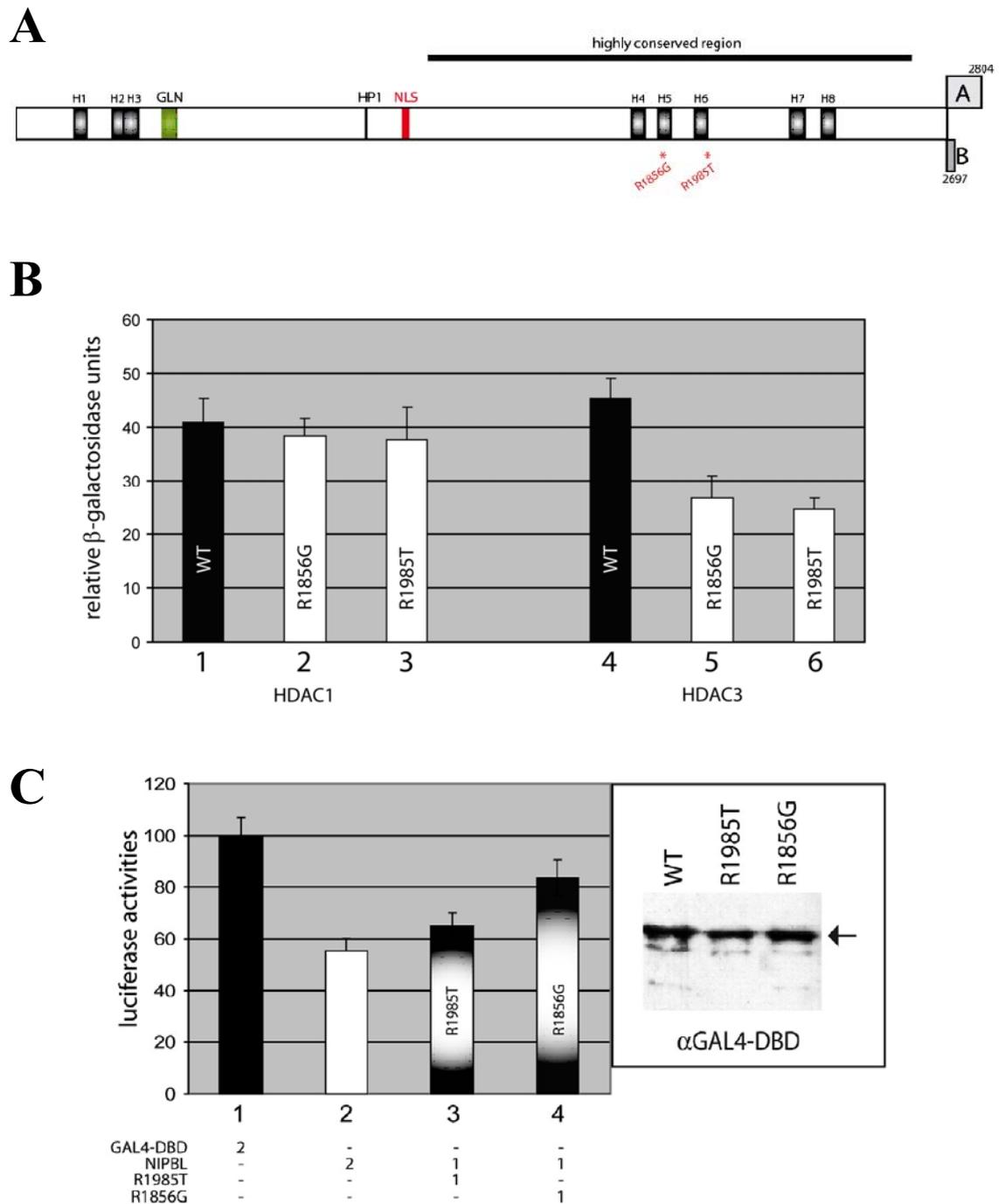


Figure 12. Two missense mutations R1985T and R1856G within the HDAC-interacting region of NIPBL affect the NIPBL-mediated repression.

(A) Localization of two missense mutations R1985T and R1856G on NIPBL. (B) The two human missense mutations R1856G and R1985T were generated by site-directed mutagenesis and the binding capacities to HDAC1 and HDAC3 were analyzed and compared to the wildtype protein, respectively. While both amino acid substitutions do not alter the binding capacities of NIPBL to HDAC1 (compare columns 1 with 2 + 3), the interaction with HDAC3 resulted in a reduction of β -galactosidase activity

to 50% for both mutations compared to the wildtype construct (columns 4-6). Experiments were done at least in triplicates and the standard deviations are indicated by error bars. (C) Whereas missense mutation R1985T only slightly decreases the trans-repressional activity of NIPBL (55.4% to 64.9 %, column 3), mutation R1856G severely reduces the activity (55.5% to 83.8 %, column 4). Equal expression of wild-type and mutant constructs was monitored by the use of an anti-GAL4-DBD antibody in western blots.

4. Discussion

By the using of yeast-two hybrid assay, using two overlapping fragments representing the highly conserved region of NIPBL as baits, the group of Dr. Kaiser could identify more than 250 clones coding for 56 putative NIPBL-binding proteins, which are currently under investigations. Interestingly, most of these proteins are known as regulators of transcription or as chromatin associated proteins. Two of these identified proteins are the histone deacetylases 1(HDAC1) and HDAC3, which are described as important cofactors in regulating gene transcription by acetylating histone proteins. To confirm these yeast-two-hybrid assay results, I used extracts of native HeLa cell, to perform Co-immunoprecipitation analysis. By this, the specific interaction of the endogenous NIPBL with endogenous HDAC1 and HDAC3 in mammalian cell could be verified.

Through the use of truncated NIPBL constructs we could narrow down the critical region for the interaction of NIPBL with HDAC1 and HDAC3 to a stretch of 163 aa (1838-2000), containing two of the five conserved HEAT repeats (see figure 3). Screening the *NIPBL* gene in patients with CdLS we could identify a new missense mutation (R1985T) affecting the critical region for the interaction of NIPBL with HDAC1 and HDAC3. Although we do not have detailed clinical data of this patient, he is described as having a classical CdLS phenotype without limb abnormalities. Recently, Selicorni et al.(2007) described a severely affected patient with a new missense mutation (R1856G) which also lied inside this HDAC-intracting domain of NIPBL. In quantitative protein-protein interaction assays we could show that both amino acids exchanges result in a decreased binding capacity of NIPBL to HDAC3, whereas the interaction with HDAC1 seems to be almost unaffected. The two mutations are spaced by a stretch of more than 100 amino acids and are localized within distinct HEAT-repeats. It might be possible that both HEAT repeats act as independent motifs for the interaction of NIPBL with HDACs or co-contribute to form one or parts of one HDAC-binding domain, which are critically important for interaction of HDAC3 with NIPBL protein, but not for

HDAC1 binding to NIPBL, although HDAC1 and HDAC3 belong to same class of HDACs and have similar catalytic domain. These results show the very first interaction of NIPBL with other proteins via the HEAT repeats which are described as protein-protein interaction motifs (Neuwald and Hirano, 2000).

In luciferase reporter gene assays we show that this region (1838-2000 amino acids) of NIPBL exerts trans-repressional effects if it is recruited to a promoter. This repressional activity could be enhanced by co-transfection with HDAC1 and HDAC3, respectively. Whereas HDAC6, which could be excluded to be directly associated with the NIPBL fragment, does not modify the reporter gene expression. While the two missense mutations, R1985T and R1856G have similar effects on the binding affinities of NIPBL to HDAC1 and HDAC3, they result in dissimilar trans-repressional activities. Mutation R1985T only slightly alters the function, whereas mutant R1856G significantly decreases the NIPBL-mediated repression. In addition, chemical inhibition of histone deacetylation by treatment of the cell cultures with SoBu almost completely abolishes the NIPBL-mediated repression. Our results clearly indicate that the trans-repressional effect of NIPBL correlates with the specific recruitment of histone deacetylating enzymes.

4.1 Affinity of adherin and cohesin complex

Human NIPBL protein and its orthologs (Mis4, Nipped-B, Xscc2), are described as adherins (Michaelis et al., 1997; Furuya et al., 1998; Gillespie and Hirano, 2004; Rollins et al., 2004; Kaur et al., 2005; Seitan et al., 2006). The predicted protein sequence for the long (2804 amino acids) and short (2697 amino acids) NIPBL isoforms reveals only a few features that provide clues to function. NIPBL contains a putative bipartite nuclear localization signal, a glutamine-rich region, an HP1-interacting motif (Lechner et al, 2005) and several HEAT repeats, HEAT repeats are bihelical folds that likely to be sites of interaction with other proteins (Neuwald and Hirano, 2000). These HEAT repeats are located within the C-terminal part of NIPBL, spanning from approximately residue 1750-2350, within a highly conserved region which bestrides the amino acids 1150-2650. The vast majority of reported CdLS-associated missense mutations map to this region.

Another adherin is Scc4, which have been shown to interact with Scc2 orthologs in *Drosophila*, *C. elegans* and human cells (Toth et al., 1999; Ciosk et al., 2000; Watrin et al., 2006). The Scc2/Scc4 complex is essential for the recruitment of cohesin to chromatin (Ciosk et al., 2000; Watrin et al, 2006; Seitan et al., 2006).

Cohesin association with yeast and human chromosomes has been studied in the last decade, the loss of cohesin binding is likely responsible for the cohesion defects caused by adherin mutant, but the defining characteristics of cohesin binding to chromosomes has remained vague. Watrin et al (2006) found the human Scc4 depletion causes precocious loss of sister-chromatid cohesion and prometaphase arrest. In a *Xenopus* cell-free system, licensing of the replication origins is required for the Xscc2 adherin to bind to chromatin, which is then followed by binding of cohesin (Gillespie and Hirano, 2004). This suggests that early in the cell cycle, long before replication occurs, adherin plays a critical role in coordination of chromatid cohesion with DNA replication. In the high resolution analyses of cohesin's association along budding yeast chromosomes III-VI, it was found that cohesin is loaded onto chromosomes at Scc2/Scc4 binding sites, and thereafter relocates to more permanent places. This suggests that cohesin provides chromosomes with an unexpectedly flexible architecture (Lengronne et al., 2004). In contrast, very recently similar analyses in three different *Drosophila* cell lines indicate that the pattern of cohesin on *Drosophila* chromosomes is very different from that in yeast, whereby most cohesin remains close to its loader, implying that the two components do not dissociate after loading of cohesin onto DNA (Misulovin et al., 2008).

The adherins are large proteins with several HEAT repeats, which are implicated in protein-protein interactions. Scc2/Scc4 complex has been suggested to stimulate cohesin's ATPase activity, required to open the cohesin ring to allow passage of the chromosome into the ring (Arumugam et al., 2003; Weitzer et al., 2003). It is also possible that the adherins facilitate assembly of cohesin on chromosomes.

4.2 Diverse roles of the sister chromatid cohesion factors

Accumulating evidences obtained in yeast and different animal species implies that the

sister chromatid cohesion factors are not only essential for chromosome segregation but also contributes to gene regulation, DNA repair, chromatin structure and development. Furthermore, mutations of human sister chromatid cohesion factors cause severe developmental disorders, including Cornelia de Lange syndrome and Roberts syndrome.

4.2.1 Gene regulation

Recently, a mass of evidences show that the sister chromatid cohesion factors are involved in the regulation of gene expression. This function is conserved from yeast to mammals. The first evidence was arose from study in *Drosophila*, Rollins and colleagues (1999) could show that the *Drosophila* Nipped-B protein is required for long-range activation of two homebox protein genes, by facilitating long- distance interactions between enhancers and promoters. In gene expression studies in the budding yeast, cohesin was identified as a part of a chromosomal boundary, which consists of molecular elements that separate a region of gene repression from regions of active expression (Donze et al., 1999). Very recently, Misulovin et al. (2008) reported that cohesin shows a broad distribution with preference for transcription units of the *Drosophila melanogaster* genome, particularly at intronic sequences. Moreover, cohesin associates with the genes when they are expressed, and deplete from inactive genes. This data is in striking contrast to the data obtained in yeast cells. Detailed mapping by high resolution analysis in budding yeast chromosomes III-VI revealed that almost all cohesin association sites identified lie at intergenic regions between converging genes. Interestingly, the relocation of cohesin is dependent on transcription. At several inactive genes, cohesin is spread along the transcription unit, but it shifts to the downstream regions of these genes upon activation of transcription (Lengronne et al., 2004). The study focuses on fission yeast *S.pombe* found deletion of Scc1/Rad21 caused a dramatic increase in readthrough transcription of the convergent genes during G2, suggest cohesin play a role in the control of transcription. These data indicate that very different targeting mechanisms may govern the locations of cohesin in yeast and flies, which may be related to different roles in gene regulation (Hagstrom and Meyer, 2003; Peric-Hupkes and Steensel, 2008).

New evidence from data obtained in *Drosophila* indicate that cohesin can also control transcription in non-cycling cells – cells that no longer proliferate, so have no sister chromatids and lack cohesion in their nuclei (Pauli et al., 2008; Schuldner et al., 2008). It was found that loss of SMC1 causes decreased expression of the ecdysone receptor gene *EcR-B1* in postmitotic neurons, and over-expression of EcR-B1 protein can partially rescue the neuronal defects. These observations clearly indicate that cohesin acts independently of sister chromatid cohesion, in many cases, it contributes to gene regulation (Uhlmann, 2008).

Three recent ChIP analyses of mouse and human cohesin reveal another targeting mechanism and a surprising regulatory role for cohesin in mammals (Parelho et al., 2008; Stedman et al., 2008; Wendt et al., 2008). A striking co-localization between cohesin and a well-know gene regulator, CTCF, is reported. CTCF is a sequence-specific DNA-binding protein that has been implicated as a transcriptional regulator, insulator and organizer of higher-order chromatin structure (Barski et al., 2007; Xie et al., 2007). Knockdown of *Scc1/Rad21* or SMC3 resulted reduced insulator activity of CTCF in reporter assays, and knockdown of CTCF substantially also reduced the association of the cohesin complex with its genomic target loci. These results show that CTCF recruits cohesin, and in several instances, cohesin appears to play an essential role in CTCF's insulator function.

Taken together, the data from yeast, flies and mammalian cells reveal a striking evolutionary plasticity in the molecular mechanisms of cohesin in gene regulation. Cohesin has been highly conserved in eukaryotes and related complex exist in bacteria, the targeting mechanisms appears vastly different in these three branches of the evolutionary tree. It is possible that cohesin might adopted different way to perform it's function during evolution, CTCF has only been described in vertebrates and *Drosophila*, cohesin might therefore perform insulator function late in evolution.

4.2.2 DNA repair

Cohesin are also described as a key structural component of chromosomes involved in DNA damage, it act a conserved role in DNA repair is suggested by observations from

yeast and mammals. The first indication was the discovery that mammalian SMC1 and SMC3 are part of a biochemically purified recombinational repair complex, RC-1, which promotes the repair of DNA gaps and deletions (Jessberger et al., 1996). In budding yeast, sister chromatids are the preferred partners for mitotic DSB repair in diploid cells (Kadyk and Hartwell, 1992). Mutations of *S. cerevisiae* *Sccl*, *Sccl3*, *Smc3* or *Smc1* have decreased viability in response to γ -irradiation (Heo et al., 1998; Sonoda et al., 2001). Furthermore, inactivation of cohesin in S or M phase leads to a reduced efficiency of post-replicative DSB repair. This defect has been attributed to lack of cohesion (Sjogren and Nasmyth, 2001). Because sister chromatid cohesion is established during S phase, the cohesin loaded along the chromosome arms during S phase is thought to be necessary and sufficient for proper post-replicative repair. Using budding yeast, it is found that cohesion is generated by an Eco1-dependent but replication-independent mechanism in response to DSBs in G/M phase (Unal et al., 2007).

Cytological studies in mammalian cells indicate that cohesin is recruited to regions of DNA damage (Kim et al., 2002). This suggests that efficient DNA repair in mammalian cells may require cohesin at the site of DNA damage. It is likely that cohesin facilitates repair by promoting cohesion between sisters, so ensuring that the correct sequence is used as a donor template for repair. And it is found that cohesin accumulation at the damage site also requires Sccl2/Sccl4 complex, which indicate the loading mechanism is similar to that for the genome-wide cohesin binding following mitosis (Strom et al., 2004; Unal et al., 2004).

Although the damage-induced cohesin binding is intriguing, the mechanisms by which these complexes or their subunits promote repair are unknown. Recently, it was reported that cells derived from CdLS patients, both with and without detectable *NIPBL* mutations, have an increased sensitivity for DNA damage. Exposure of CdLS fibroblast to MMC (mitomycin) and x-ray lead to increased numbers of chromosomal aberrations (Vrouwe et al., 2007), this imply that mutation in *NIPBL* might also influence the DNA repair.

4.2.3 Development

Study in *Drosophila* showed that the Nipped-B facilitate a dosage dependent long-range activation of two homeobox protein genes –the *cut* gene and *Ultrabithorax (Ubx)* gene that play critical roles in development by distant transcriptional enhancers (Rollins et al., 1999). Later, the subsequent work revealed that opposite to the effect of Nipped-B, the Scc3 component of cohesin inhibits long-range activities of *cut* (Rollins et al., 2004). This group further found that cohesin binds between a remote wing margin enhancer and the promoter at the *cut* locus in cultured cells, and that reducing the dosage of the Smc1 cohesin subunit increases *cut* expression in the development wing margin. In addition, *cut* expression is increased by a unique *Pds5* gene mutation that reduces the binding of cohesin to chromosomes (Dorsett et al., 2005). The *cut* gene is a regulator gene that is important for wing and limb development. Insufficiency of activate *cut* in wing-margin cells produces a ‘cut wing’ phenotype. *Ubx* suppresses limb formation in the fly abdomen by repressing *DLL*, a gene required for distal limb development (Gebelein et al., 2002). A number of recently reports show the mammalian *DLL* and *cut* homologs are involved in multiple developmental processes, including limb and branchial arch patterning, neurogenesis and hematopoiesis (Ellis et al., 2001; Panganiban and Rubenstein, 2002; Iulianella et al., 2003).

Runx proteins are multi-lineage transcriptional regulators with roles in both proliferation and differentiation, maintaining balanced levels of Runx proteins is crucial, as deregulated expression leads to cancers and developmental disorders. Genetic screen in zebrafish indicate that lacking Rad21 or Smc3, zebrafish embryos fail to express *runx3* and lose hematopoietic *runx1* expression in early embryonic development (Horsfield et al., 2007). Study on *Caenorhabditis elegans* revealed that the depletion of COH-1, the homolog of the cohesin subunit Scc1/Rad21, could arrested these worms at either the late embryonic or the larval stage, with no indication of mitotic dysfunction (Mito et al., 2003). PDS5 is a regulatory component contribute to cohesion dynamics, observing the phenotype of *Pds5B* knockout mice found that loss of *Pds5B* results in a number of

developmental defects, such as dysmorphic face, cleft palate, skeletal patterning and bone development defects, cardiac malformation, abnormal autonomic nervous system formation, and depletion of primordial germ cells, of which are similar to abnormalities found in human with CdLS (Zhang et al., 2007). These results suggest that cohesins also have essential functions in early development of multi-cellular organisms, perhaps as regulators of multiple aspects in organogenesis.

4.2.4 Human developmental deficit syndromes

It was recently discovered that certain human developmental disorders are associated with mutations in genes encoding cohesins. The Cornelia de Lange syndrome (CdLS), displays a dominantly genetic multisystem developmental deficiencies, including diagnostic facial characteristics, slow growth, upper limb abnormalities, cardiac malformations, gastroesophageal defects and mental retardation. Spontaneous loss-of-function mutations in one copy of the human *NIPBL* gene, the human ortholog of Nipped-B and Scc2, are responsible for ~50% of the cases of CdLS. Mutations have been shown to cause the full spectrum from mild to severe forms of CdLS. Interestingly, many of the milder cases are associated with mutations that cause amino acid substitutions, whereas severe forms are associated with truncating mutations (Gillis et al., 2004; Bhuiyan et al., 2005; Yan et al., 2006). Only a few missense mutations, like mutation (R1856G) of *NIPBL* gene described by Selicorni *et al.* (2007), result in a severe CdLS phenotype. Recently, mutations in *SMC3* and *SMC1A* (also called *SMC1L1*) were shown to cause a mild variant of CdLS with predominant mental retardation, giving an overall prevalence of ~5% among CdLS patients (Musio et al, 2006; Deardorff et al., 2007). In the data presented here, we could identify a new missense mutation (R1985T) of *NIPBL* gene in a patient with a classical CdLS phenotype.

As described above, another syndrome caused by mutations in a cohesin gene is Roberts syndrome (RBS), RBS is a recessive multiple malformation syndrome that resembles CdLS in many aspects, like slow growth, mental retardation, and limb defects. RBS is caused by mutations in the *Esco2/Efo2* homolog of *Ctf7/Eco1* which is located at

8p21.1, and encodes a protein that functions in sister chromatid cohesion and post-replicative double-strand DNA repair (Vega et al., 2005; Schule et al., 2005). The *S.pombe* Eso1 protein interact with Pds5, and genetic evidence suggests that Pds5 inhibits establishment of cohesion in the absence of Eso1 (Tanaka et al., 2001). As mentioned above, mice lacking Pds5B exhibit developmental abnormalities reminiscent of CdLS (Zhang et al., 2007).

A key distinguishing feature of Roberts syndrome is the presence of chromosomal and mitotic defects (German, 1979; Tomkin and Siskin, 1984; Jabs et al, 1991). CdLS patients show developmental deficits similar to those in RBS but without the same chromosomal defects. Although, effects on sister chromatid cohesion are seen in homozygous *Nipped-B* mutant, no effects on cohesion could be observed in heterozygote mutants. Even partial reduction of *Nipped-B* by RNAi sufficient to cause lethality had no detectable effect on sister chromatid cohesion (Rollins et al., 2004).

Although we have gained some exciting insights of molecular mechanisms of sister chromatid cohesion factors function, many questions remain to be answered. Such as, how the adherin recruits cohesin complex loading to certain sites on chromosome? Whether this peculiar position of cohesin binding may have additional character? The cohesin localization to preferred chromosome sites is found in absence of sequence specificity (Lengronne et al., 2004), suggest that the underlying chromatin structurally states might play a critical role in cohesion binding.

4.3 chromatin structure modification is associated with the functions of cohesion complex

Over the last two decades, a growing number of publications indicate that the chromatin structures does not only serve as essential structural elements to preserve genetic information, it but also play an active roles in governing the accessibility of DNA sequences to transcription factors and the general transcription machinery. It has become evident that chromatin is a highly flexible environment, wherein spatially and temporally

coordinated changes between transcriptionally repressive (structurally condensed states), and transcriptionally active (structurally accessible states) regulate gene expression. Chromosomes are capable of remarkable structural adaptability that enables their diverse functions. Two known mechanisms that regulate chromatin structure are ATP-dependent chromatin remodelling, and covalent chromatin modifications. ATP-dependent chromatin remodelling is performed by using the energy from ATP hydrolysis to weaken the interactions between histones and the surrounding DNA (Vignali et al., 2000; Fyodorov and Kadonaga, 2001). Covalent chromatin modification can occur on either DNA or histones. Whereas DNA is modified by cytosine methylation, histones are targets for post-translational modification, such as acetylation, methylation and so on (Vignali et al., 2000; Jenuwein and Allis, 2001; Fyodorov and Kadonaga, 2001).

Chromatin remodelling plays an important role in determining whether and where cohesin bind to chromosomes. In human cells, a remodelling complex containing the ATPase SNF2h has been co-purified with the SCC1 and SMC subunits, presumably due to a direct interaction between cohesin and SNF2h complex (Hakimi et al., 2002). Furthermore, the absence of RSC reduces the interaction of cohesin with its binding sites on chromosomal arms (Baetz et al., 2004; Huang et al., 2004). RSC (remodels the structure of chromatin) is an evolutionarily conserved and abundant chromatin remodelling complex in yeast, several subunits of which have sequence homology to components of human SNF2h complex. Although the precise mechanism by which RSC participates in cohesion is still unknown, it is tempting to speculate that RSC might restructure the nucleosomes to facilitate cohesin loading, or play a part in direct recruitment of cohesin to its binding sites. Recently, it was reported that a loss of INO80, which is a 12-subunit ATP-dependent chromatin remodelling complex in yeast, perturbs the recruitment of cohesion establishment proteins to replication forks. This implicated the INO80 complex functions in cohesion establishment rather than cohesin recruitment (Ogiwara et al., 2007).

Histone modifications play pivotal roles in conferring structural diversity to chromosomes in eukaryotic cells (Margueron et al., 2005). It is already known that histone modifications affect the recruitment of cohesin to particular chromosomal loci.

However, the exact functional relationship between cohesin and heterchromatin (repressive chromatin) is not yet understood in detail. Several indications have shown that the heterchromatin is necessary to maintain cohesion, whereas the cohesin also influences establishment of the heterchromatin state. In fission yeast, one function of centromeric heterochromatin is to attract cohesin, thereby ensuring sister chromatid cohesion and proper chromosome segregation (Bernard et al., 2001). Also in yeast, artificial depletion of Scc1 result in earlier “chromatin silencing” (Chang et al., 2005). In general, acetylation of histone H3 on lysines 9 and 14 are known to be associated with euchromatin (active chromatin), while methylation of histone H3 lysines are linked with heterchromatin. In our ChIP analyses we could show that the region span from amino acid 1838 to 2000 of NIPBL could recruit HDAC1 and HDAC3 to initiate the deacetylation of lysine 9 of histone 3 (H3K9), which would allow its methylation and may create a platform to recruit other chromatin remodelling protein, such as the heterchromatin binding protein 1 (HP1). HP1 recognizes only methylated lysine 9 on histone H3 (H3K9), and it could enhance the recruitment of cohesin (Bernard et al., 2001). Interestingly, HP1 was also described as NIPBL-binding protein (Lechner et al., 2005). In fission yeast, cohesin is recruited to heterochromatin by interaction between the Scc3 cohesin subunit and Swi6, the yeast ortholog of the mammalian HP1 (Nonaka et al., 2002). Furthermore, the persistence of acetylated histones on mitotic chromosomes interferes with chromosome segregation in mitosis. Sister chromatids could not separate when cells entered mitosis with hyperacetylated histones. Thus, the presence of acetylated histones in mitosis induces both aberrant chromosome numbers (i.e. aneuploidy) and defects in chromosome structure (Cimini et al., 2003). Very recently, Kimata and colleagues (2008) found that diminishing HDACs by drugs or mutations could promote normal or abnormal sister chromatid separation by affecting adherin function in the fission yeast.

In summary, all the data and results presented here have give insights into the molecular mechanism of cohesion factors’s functions. Cohesin does not only act as a significant structural component of chromosomes during interphase, but also participated in the regulatory mechanisms of gene expression. It is plausible that most CdLS

developmental deficits reflect changes in gene expression instead of in sister chromatid cohesion, probable are caused by gene expression changes similar to those in *Drosophila* and zebrafish. Indeed, effects on homeotic gene expression similar to those caused by partial loss of *Drosophila Nipped-B* might explain many features of CdLS, such as upper limb abnormalities. CdLS patients show neuro-developmental, gastrointestinal and skeletal abnormalities. The development of each of these systems depends on the proper regulation of Runx proteins, which are themselves dose-sensitive in function (Blyth et al., 2005).

Association of cohesin and adherin with many genes suggests that the diversity of CdLS phenotypes stems from effects on multiple genes. Many of the genes bound by cohesin encode transcription factors and receptors that control different system development (Misulovin et al., 2008). *Nipped-B* has been shown to interact with some genes in the Notch signalling pathway that is known to be important in wing development (Rollins et al., 1999). Our data shown that even most of the NIPBL-binding proteins, represent evolutionarily conserved transcription factors and chromatin remodeling factors. Mutations and defects of cohesin or adherin probably disturb the exploration of further gene regulatory function. We speculate that the effects of cohesin on developmental gene expression are likely related to the etiology of CdLS.

A variety of recent studies has also offered glimpses into the important roles of chromatin structure states and histone modification in relation with the functions of cohesion factors. Taken together, it could be speculated that the change of chromatin structure states, which induced by histone modification, could directly or indirectly affect the regulation functions performed by cohesion factors. Our data verified the interaction of the endogenous NIPBL with endogenous HDAC1 and HDAC3 in mammalian cell, and shown that mutations R1985T and R1856G identified in patients with CdLS, which localize on HDACs binding domain of NIPBL, significantly alter the NIPBL-mediated trans-repressional activity. Our result further contributes to the hypothesis that changes on chromatin structure and effects on gene expression represent the molecular mechanisms causing CdLS.

Our data show for the very first time a direct interaction of adherins with histone

modifying enzymes and directly combines the chromatin remodeling pathways with the function of the cohesion associated protein NIPBL. The next challenges of us will be to identify genes which are “misregulated” in patients with CdLS. In further analyses we hope to find a direct or indirect effect of these NIBPL-mediated and HDAC-catalyzed chromatin remodeling processes to the expression of these target genes.

5. Summary

Cornelia de Lange Syndrome (CdLS) is a rare congenital malformation disorder, characterized by mental retardation, distinctive facial feature and limb reduction defects. About half of the patients with CdLS carry mutations in the *NIPBL* gene. *NIPBL* encodes a homolog of the *Drosophila* Nipped-B or the fungal Scc2-type protein and is associated with the chromatid cohesin complex. Recent studies show that cohesin have additional functions independent of its role in sister chromatid cohesion, and that chromatin-remodeling complexes could directly or indirectly affect the cohesin's functions.

By co-immunoprecipitation analysis, we show the interaction of the endogenous NIPBL with HDAC1 and HDAC3 in mammalian cells. Through the use of truncated NIPBL constructs we narrowed down the critical region for the interaction of NIPBL with HDAC1 and HDAC3 to a stretch of 163 aa (1838-2000), containing two of the five conserved HEAT repeats. Screening the *NIPBL* gene in patients with CdLS we identified a new missense mutation (R1985T) affecting the critical region for the interaction of NIPBL with HDAC1 and HDAC3. In quantitative protein-protein interaction assays we could show this R1985T amino acid change and another recently reported missense mutation R1856G, which associated with severe CdLS phenotype, result in a decreased binding capacity of NIPBL to HDAC3. In luciferase reporter gene assays we show that this HDAC1 and HDAC3 binding region of NIPBL exerts trans-repressional effects, which could be enhanced by co-transfection with HDAC1 and HDAC3, respectively, whereas both missense mutations decrease the NIPBL-mediated trans-repressional activities. In addition, chemical inhibition of HDACs by treatment of the cell cultures with SoBu almost completely abolishes the NIPBL-mediated repression. Our results clearly indicate that the trans-repressional effect of NIPBL correlates with the specific recruitment of histone deacetylating enzymes.

Taken together, this is the first time discussed the interaction of histone deacetylating enzymes with the cohesion protein NIPBL. Our result support the hypothesis that changes on chromatin structure and effects on gene expression could be link to the etiology of the CdLS syndrome.

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