Aus dem Institut für Molekularbiologie und Tumorforschung
des Fachbereichs Medizin der Philipps-Universität Marburg
Geschäftsführender Direktor: Prof. Dr. Rolf Müller

ATP - dependent chromatin remodelers

- Analysis of expression patterns and impact on gene regulation

Inaugural-Dissertation
zur Erlangung des Doktorgrades der gesamten Humanmedizin
(Dr. med.)

Dem Fachbereich vorgelegt von
Nina Rosa Neuendorff aus Braunschweig
Marburg, 2013

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Meinem Vater in liebevoller Erinnerung.

„Es gibt zwei menschliche Hauptsünden, aus welchen sich alle andern ableiten: Ungeduld und Lässigkeit. Wegen der Ungeduld sind sie aus dem Paradiese vertrieben worden, wegen der Lässigkeit kehren sie nicht zurück.
Vielleicht aber gibt es nur eine Hauptsünde: die Ungeduld. Wegen der Ungeduld sind sie vertrieben worden, wegen der Ungeduld kehren sie nicht zurück."

Franz Kafka

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1. Summary

ATP-dependent chromatin remodelers are enzymes which use the energy from ATP hydrolysis to alter the chromatin structure. Thus, they play a key role in the transcriptional control of many important cellular processes such as proliferation, senescence and differentiation.

The first part of this study focused on CHD5, a novel chromatin remodeling enzyme. To gain further insight into its expression profile and biological function, polyclonal peptide antisera were, together with a commercially available antiserum, successfully established for use in western blot, immunoprecipitation and immunofluorescence staining.

ATP-dependent chromatin remodelers are expressed by very different patterns ranging from ubiquitous expression to an expression restricted to specific cell populations. So far, CHD5 expression was thought to be restricted to neural-related tissues. Here, it was shown for the first time that CHD5 expression is not restricted to neural tissues but additionally expressed in rodent testes. Furthermore, several cell lines were tested for CHD5 expression on transcript and protein level. CHD5 expression was not detected in any of the tested neuroblastoma and glioblastoma cell lines. In neuroblastoma cell lines this was expected due to the suggested function as tumor suppressor. Furthermore, a primary astrocyte culture was established. No CHD5 could be detected in protein lysates from astrocytes but was found in those from murine neural stem cells. This suggests that CHD5 expression in brain is restricted to neural stem cells and probably also to neurons.

In the second part of this thesis, the impact of the chromatin remodelers CHD4 and BRG1 on a TNFα-induced inflammatory response was investigated. BRG1 and CHD4 expression was disrupted by a siRNA-mediated knockdown in HEK293 cells. After stimulations with TNFα for one to four hours, gene induction of typical inflammatory NF-κB target genes was determined by RT-qPCR. BRG1 and CHD4 were both required for the efficient induction of all tested target genes exclusively after one hour but not after four hours of TNFα treatment. Expression of the housekeeping gene ß-actin was unaffected. Surprisingly, although CHD4 is mainly known to be involved in transcriptional repression, a requirement for CHD4 in active transcription during this

inflammatory response was revealed. To gain further insight into the mechanism of involvement, co-immunoprecipitations of the NF-κB subunit p65 with BRG1 and CHD4, respectively, were performed upon TNFα stimulation. No robust interactions between these proteins could be observed. Furthermore, ChIP experiments with the NF-κB subunit p50, histone H3 and CHD4 were carried out on the model target gene CXCL2. p50 was recruited to the promoter of CXCL2 rapidly after TNFα was added. Histone H3 binding to the promoter and the open reading frame of the gene strongly decreased within the first hour of TNFα stimulation, indicating that chromatin remodeling took place during the early period of gene induction. Recruitment of CHD4 was non-conclusive. Thus, the mechanism of how BRG1 and CHD4 act during TNFα response needs to be investigated further.

In summary, a major influence of the chromatin remodelers BRG1 and CHD4 on the TNFα-induced gene activation during its early phase in HEK293 cells was demonstrated.

1. Zusammenfassung

ATP-abhängige Chromatin Remodeler sind eine Gruppe von Enzymen, welche die Energie aus der ATP-Hydrolyse nutzen, um die Chromatinstruktur zu verändern. Damit spielen sie eine bedeutende Rolle für die Transkriptionskontrolle wichtiger zellulärer Prozesse, wie beispielsweise Proliferation, Seneszenz und Differenzierung.

Der erste Teil dieser Arbeit konzentriert sich auf CHD5, ein neu identifiziertes Mitglied der ATP-abhängigen Chromatin Remodeler. Um wichtige biologische Funktionen dieses Proteins studieren zu können, wurden polyklonale Peptid-Antiseren, zusammen mit einem später kommerziell erhältlichen Antikörper, für den Gebrauch in Western Blot, Immunpräzipitation und Immunfluoreszenzfärbungen etabliert.

ATP-abhängige Chromatin Remodeler zeigen vollkommen unterschiedliche Expressionsmuster. Diese reichen von einer ubiquitären Präsenz bis hin zu einer auf spezielle Zellpopulationen beschränkten Expression. Bislang ging man davon aus, dass CHD5 nur in neuronalen Geweben exprimiert wird. In dieser Arbeit konnte erstmalig gezeigt werden, dass dies nicht zutrifft, da das CHD5 Protein in Hoden-Lysaten von Nagetieren detektiert werden konnte.

Weiterhin wurden RNA- und Proteinextrakte diverser Zelllinien auf ihre CHD5-Expression hin untersucht. Keine der untersuchten Neuroblastom- und Glioblastom-Zelllinien zeigte eine signifikante CHD5-Expression. Zumindest in den Neuroblastom-Zelllinien steht dies in Einklang mit der vermuteten Rolle als Tumorsuppressor in dieser Tumorentität. Außerdem konnte eine primäre Astrozytenkultur etabliert werden. Proteinlysate dieser Kultur waren negativ in Bezug auf eine CHD5-Expression, jedoch konnte das Protein in Extrakten muriner neuronaler Stammzellen nachgewiesen werden. Daraus ergibt sich die Schlussfolgerung, dass eine CHD5-Expression im Hirn auf bestimmte Zellpopulationen beschränkt ist, nämlich auf neuronale Stammzellen und vermutlich zusätzlich auf Neurone, jedoch nicht in Astrozyten exprimiert wird.

Im zweiten Teil dieser Arbeit wurde der Einfluss der Chromatin Remodeler BRG1 und CHD4 auf die TNFα-vermittelte Induktion von NF-κB Targetgenen untersucht. Die Expression von BRG1 bzw. CHD4 wurde mit Hilfe von spezifischen siRNAs in HEK293 Zellen herunter reguliert. Anschließend wurden die Zellen für ein bis vier Stunden mit

TNFα stimuliert. Die Induktion von typischen NF-κB Targetgenen wurde mit Hilfe einer RT-qPCR untersucht. Dabei ergab sich, dass beide Proteine für die Induktion aller untersuchter Targetgene nach einer Stunde benötigt werden. Die Expression der Targetgene nach vier Stunden war nicht beeinträchtigt, ebenso unverändert war die des Haushaltsgens ß-Actin. Dies war überraschend, denn CHD4 ist hauptsächlich als Repressor bekannt. Hier konnte jedoch ein Stellenwert innerhalb der aktiven Transkription gezeigt werden.

Um einen Einblick zu erhalten, in welcher Form CHD4 und BRG1 an der untersuchten Targetgen-Induktion beteiligt sind, wurden Ko-Immunpräzipitationen der NF-κB Untereinheit RelA mit CHD4 bzw. BRG1 nach einer Inkubation mit TNFα durchgeführt. Es konnten keine robusten Interaktionen der Proteine gezeigt werden. Weiterhin wurden ChIP-Experimente mit der NF-κB Untereinheit p50, Histon H3 und CHD4 am Beispiel des Targetgens CXCL2 nach einer TNFα-Induktion durchgeführt. Nach einer kurzen Inkubationszeit mit TNFα wurde p50 zum CXCL2-Promoter rekrutiert. Die Dichte von Histone H3 im Bereich des Promoters und, weniger ausgeprägt, auch des Open-Reading-Frame sank ebenfalls innerhalb der ersten Stunde. Dies symbolisiert eine stattgehabte Aktion von Chromatin Remodelern. Eine Rekrutierung von CHD4 blieb jedoch unklar aufgrund technischer Details. Daher bleibt der Mechanismus der beobachteten Effekte während dieser Transkriptionsaktivierung unbekannt. Zusammengefasst wurde eine wichtige aktivierende Funktion der beiden Chromatin Remodeler BRG1 und CHD4 während der frühen Phase der TNFα-Antwort gezeigt.

2. Introduction

2.1. General introduction

Nearly every higher eukaryotic cell contains within its DNA the complete genomic information of the whole organism. This condition has two major implications: firstly, the total length of a single cell's chromosomal DNA would extend to over two meters. Thus, the DNA has to be compacted to fit into the small nucleus. This compaction is achieved by wrapping the acidic DNA around basic proteins, the histones. Such a polymer of DNA and histones together with some non-histone proteins is called *chromatin*. Secondly, only a small part of a cell's whole genome needs to be actively transcribed at the same time. That enables different cells to take over independent functions in the formation of a higher organism. These two implications in conjunction additionally require a cell to have a fine-tuned dynamic chromatin structure which can regulate access to the transcription machinery depending on the cells current metabolic activity and overall function.[1]

In summary, the dynamics of higher-order chromatin structures serve as one key regulatory element of transcriptional control.

Although so many intriguing questions are still unsolved in the field of chromatin research, the importance in pathology and also in the treatment of various diseases is increasing, resulting even in the approval of drugs modifying epigenetic mechanisms. As one example, 5-azacytidine, a drug modifying the DNA methylation pattern, was approved for the treatment of Myelodysplastic syndrome in Europe in 2009.

2.2 Chromatin structure

The term chromatin, as defined by the complex of DNA and associated proteins, was coined by the German cell biologist Walther Fleming at around 1880. He found a strongly basophile stainable sub-cellular structure which he called chromatin after the ancient Greek word for color (χρῶμα, chroma). He wrote "*the word chromatin may stand until its chemical nature is known, and meanwhile stands for that substance in*

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[1] Allis DC, Jenuwein T, Reinberg D; Overview and Concepts; in: Allis CD, Jenuwein T, Reinberg D; Epigenetics, Cold Spring Harbor Laboratory Press, New York (2007).

the cell nucleus which is readily stained."[2] After these words it took over 90 years until a more thorough understanding of the chromatin structure was achieved[3] by the ground-breaking work of Woodcock,[4] the Olins[5] and Kornberg[6]. Finally, Luger et al[7] could resolve a detailed structure of the chromatin key subunit, the nucleosome, by X-ray crystallography in 1997.

The repeating key subunit of chromatin is the nucleosome and provides the first level of higher-order packaging of chromosomal DNA by histones. It consists of 147 base pairs of DNA wrapped around a histone octamer in 1.65 superhelical turns resulting in a 5 – 10 fold compaction of DNA. The histone octamer includes dimers of the core histones H2A, H2B, H3 and H4. All core histones have a similar basic structure with a globular domain for histone-DNA as well as histone-histone interactions within the nucleosome and the protruding N-terminal and C-terminal tails. These tails as well as the globular domains are rich in the basic amino-acids lysine and arginine. Within the tails, the basic amino-acids provide a site for common post-translational regulatory modifications such as methylation and acetylation.[8]

Between the nucleosomes, linker DNA of variable length is interposed, producing the characteristic appearance of 'beads on a string' (10 - nm fibre). This string of nucleosomes is further compacted into a 30 - nm fibre fixed by the linker histone H1, resulting in an approximately fifty fold compaction of chromatin. The 30 - nm fibre is additionally capable of condensing into secondary and tertiary chromatin structures. The mechanisms contributing to these higher order structures are yet unknown.

The mitotic or meiotic metaphase chromosome displays a highly condensed structure with a 10 000 fold compaction. Remarkably, the condensation of the Barr body is even higher. [9]

For an overview of different levels of chromatin compaction see Fig. 2.1.

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[2] Cited after Olins DE, Olins AL; Chromatin history: Our view from the bridge. Nat Rev Mol Cell Biol (2003).

[3] Olins DE, Olins AL; Chromatin history: Our view from the bridge. Nat Rev Mol Cell Biol (2003).

[4] Woodcock CL et al, Structural repeating units in chromatin. I. Evidence for their general occurrence. Exp Cell Res (1976).

[5] Olins AL and Olins DE, Spheroid chromatin units (v bodies). Science (1974).

[6] Kornberg RD, Chromatin structure: a repeating unit of histones and DNA. Science (1974).

[7] Luger K et al, Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature (1997).

[8] Horn PJ and Peterson CL, Molecular biology. Chromatin higher order folding: wrapping up transcription. Science (2002).

Luger K and Hansen JC, Nucleosome and chromatin fiber dynamics. Curr Opin Struct Biol (2005).

[9] Felsenfeld G and Groudine M, Controlling the double helix. Nature (2003).
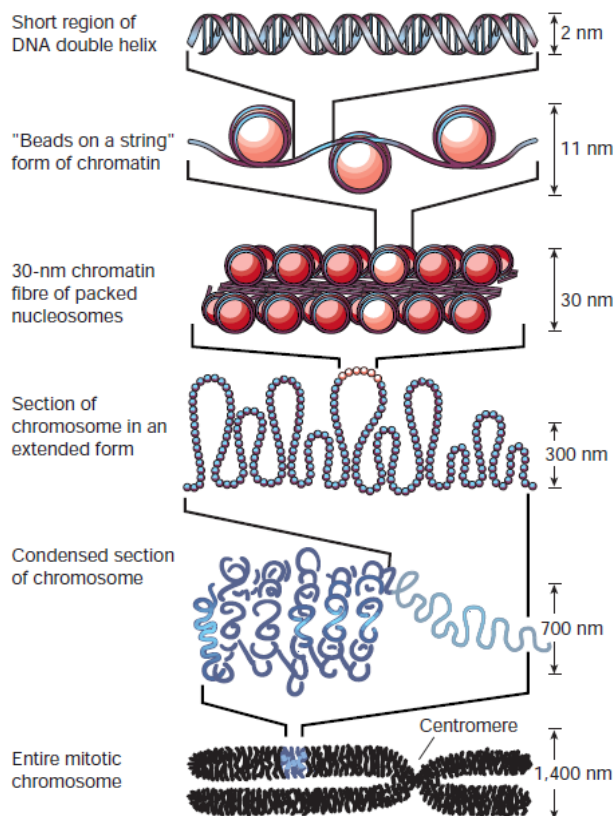
Fig. 2.1: Multiple levels of chromatin folding
(from: Felsenfeld G, Groudine M, Controlling the double helix. Nature 2003)

According to its nuclear staining pattern, chromatin is further classified into euchromatin as an 'active' form and heterochromatin as a more compacted and 'inactive' form. Euchromatin is usually gene-rich, although it is not necessarily always transcriptionally active. Conversely, heterochromatin remains packaged in interphase and consists mostly of non-coding and repetitive DNA. Specific markers of heterochromatin include a general histone hypoacetylation, the presence of histone H3K9 methylation and cytosine hypermethylation in DNA. This leads to a recruitment of heterochromatin-associated proteins (such as HP1) in order to further stabilize the chromatin compaction.[10]

Moreover, constitutive heterochromatin is distinguished from facultative heterochromatin. Constitutive heterochromatin is commonly found around centromeres and telomeres. It displays the same condensed packaging in nearly all somatic cell types of an organism under physiological conditions. In contrast, facultative heterochromatin is only compacted in some cell types where the inactivation is dependent on developmental decisions.[11]

[10] Elgin SC, Grewal SI, Heterochromatin: silence is golden. Curr Biol (2003).
[11] Elgin SC, Grewal SI, Heterochromatin: silence is golden. Curr Biol (2003).

2.3 Chromatin modifying enzymes, histone modifications and DNA methylation

Regulating the degree of chromatin compaction is one major regulatory element in transcription, DNA repair and replication. Strong compaction of chromatin impairs the accessibility of DNA towards DNA binding factors that are involved in the above mentioned processes. Thus, an extra machinery to dynamically alter the local chromatin state is essential.

Basically, three major groups of enzymes are responsible for structural changes of chromatin: histone modifying enzymes, DNA methyltransferases and ATP-dependent chromatin remodelers. All three groups are crucial for a dynamic interplay between each other. They are described in the following part. Because this thesis focuses on ATP-dependent chromatin remodelers, these are discussed more broadly in a separate chapter (see 2.4.).

2.3.1 Histone modifications

The usually regular distribution of nucleosomes along the 10 - nm fibre can be changed by covalently modified histone tails.

Vincent Allfrey was the first to discover the influence of histone acetylation and methylation on transcriptional activity in 1964.[12]

Histones can be modified by attachment of small chemical groups (e.g. acetyl- and methyl-groups) or larger peptides (e.g. SUMO).

The effects of the histone modifications on the chromatin structure are divided into *cis-* and *trans*-effects. *Cis*-effects generate alterations in the physical properties of the covalently modified histone tails. As the prime example, histone acetylation neutralizes positive charges of the basic histone tails, bringing about localized stretching of the chromatin fibre, thus allowing a better access of the transcription machinery to the DNA.[13] *Trans*-effects can be described as the recognition of histone modifications by specific protein domains and the subsequent recruitment of protein complexes for further remodeling of the chromatin structure. As example, methylated lysine residues of histone tails might be recognized by protein domains of the Royal family (including chromo, tudor and MBT domains), acetylated lysine-residues by bromodomains and

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[12] Allfrey VG et al, Acetylation and Methylation of histones and their possible role in the regulation of RNA synthesis. Proc Natl Acad Sci USA (1964).

[13] Allis DC, Jenuwein T, Reinberg D; Overview and Concepts; in: Allis CD, Jenuwein T, Reinberg D; Epigenetics, Cold Spring Harbor Laboratory Press, New York (2007).

phosphorylations by 14-3-3 domains.[14] Although a specific modification is often said to be linked to a more repressive or more activating outcome, most likely many modifications can in principle contribute to both, activation and repression, depending on the context and the position of the nucleosome along the DNA.[15]

In conclusion, histone modifications are via their *cis*-effects as well as via *trans*-effects an important part of the control over the chromatin accessibility. Their occurrence can be transient, stable or even propagated from one cell generation to the next.

For some of the histone modifying enzymes pharmacological inhibitors are already in clinical use like the HDAC inhibitor vorinostat for the treatment of cutaneous T-cell lymphomas. It is noteworthy that, only seven years after the discovery of the histone demethylase LSD1 [16] specific inhibitors are already under investigation. This demonstrates both that the field is rapidly developing and of great clinical relevance.

2.3.2 DNA methylation

In DNA methylation the fifth carbon position of cytosine is covalently modified. That methylation is associated with gene silencing and is involved in important developmental processes such as genomic imprinting and X chromosome inactivation. It occurs to various degrees in all higher eukaryotes with the exception of yeast. In mammals, CpG dinucleotides are modified, but different patterns occur in N. crassa and plants.

CpG dinucleotides are not equally distributed across the genome. Rather, there are CpG islands and CpG-poor regions. [17]

A CpG island is defined as a genomic region longer than 500 base pairs, C+G content greater than 55 % and an "observed CpG/expected CpG" ratio of more than 0.65.[18]

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[14] Kouzarides T, Chromatin modifications and their function. Cell (2007).
Allis DC, Jenuwein T, Reinberg D; Overview and Concepts; in: Allis CD, Jenuwein T, Reinberg D; Epigenetics, Cold Spring Harbor Laboratory Press, New York (2007).
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Nakayama J et al, Role of histone H3 lysine 9 methylation in epigenetic control of heterochromatin assembly. Science (2001).
[15] Kouzarides T, Chromatin modifications and their function. Cell (2007).
[16] Shi Y et al, Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. Cell (2004).
[17] Miranda TB, Jones PA. DNA methylation: the nuts and bolts of repression. J Cell Physiol (2007).
Allis DC, Jenuwein T, Reinberg D; Overview and Concepts; in: Allis CD, Jenuwein T, Reinberg D; Epigenetics, Cold Spring Harbor Laboratory Press, New York (2007).

About 40 % of genes contain CpG islands. They are mostly located in the 5' region of a gene, usually within the promoter, the 5' untranslated region and first exon. In normal cells a CpG island is most likely hypomethylated. The CpG poor regions are usually methylated. During the development of cancer CpG islands can become hypermethylated resulting in the silencing of tumor suppressor genes. Furthermore, loss of methylation at CpG poor regions can lead to genomic instability.[19]

Hypermethylated CpG islands and histone tail modifications together are often described as 'epigenetic marks'.

In mammals, DNA methyltransferases (DNMTs) are categorized into three major families (DNMT1-3).[20] Basically, DNA methylation is divided into *de novo* and maintenance methylation. In early embryogenesis, developmental processes and carcinogenesis the methyltransferases DNMT3a and DNMT3b establish *de novo* methylation patterns.[21] For maintenance of methylation patterns, especially during replication, DNMT1 is thought to be the main factor.

DNMTs catalyse the methyl transfer using S – adenosyl - methionine as the methyl donor.[22]

The biological role of DNMT2 is less well defined in comparison to DNMT1 and DNMT3. Recent evidence suggests that it works as a RNA methyltransferase specifically methylating tRNA^Asp.[23]

DNA methylation contributes in many ways to gene silencing. One possibility is that the binding of transcription factors is directly disturbed by the presence of the methyl group in the major groove of the DNA. As an example, Prendergast et al[24] showed that CpG methylation of the Myc recognition site inhibits its binding. Another mechanism is the recruitment of Methyl-CpG-binding proteins like MeCP1[25] and the methyl-CpG-

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[18] Takai D, Jones PA. Comprehensive analysis of CpG islands in human chromosomes 21 and 22. Proc Natl Acad Sci USA (2002).

[19] Miranda TB, Jones PA. DNA methylation: the nuts and bolts of repression. J Cell Physiol (2007).

[20] Xu F et al, Molecular and Enzymatic Profiles of Mammalian DNA Methyltransferases: Structures and Targets for Drugs. Curr Med Chem (2010).

[21] Okano M et al, Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. Nat Genet (1998).

Allis CD, Jenuwein T, Reinberg D; Epigenetics, Cold Spring Harbor Laboratory Press, New York (2007), p.345.

[22] Miranda TB, Jones PA. DNA methylation: the nuts and bolts of repression. J Cell Physiol (2007).

[23] Goll MG et al, Methylation of tRNAAsp by the DNA methyltransferase homolog Dnmt2. Science (2006).

[24] Prendergast GC et al, Association of Myn, the murine homolog of max, with c-Myc stimulates methylation-sensitive DNA binding and ras cotransformation. Cell (1991).

[25] Meehan RR et al, Identification of a mammalian protein that binds specifically to DNA containing methylated CpGs. Cell (1989).

binding domain (MBD) family.[26] These proteins are known to associate with different co-repressor complexes like the NuRD complex. This constitutes a link between DNA methylation and ATP-dependent chromatin remodeling as discussed in greater detail in the next chapter.

2.4 ATP-dependent chromatin remodelers

2.4.1 ATP-dependent chromatin remodelers – overview and mode of action

ATP-dependent chromatin remodelers utilize the energy from ATP hydrolysis to alter the chromatin structure in a non-covalent manner, e.g. to remove or exchange histones, slide nucleosomes along DNA and disrupt DNA - nucleosome contacts (see Fig. 2.2 for an overview).
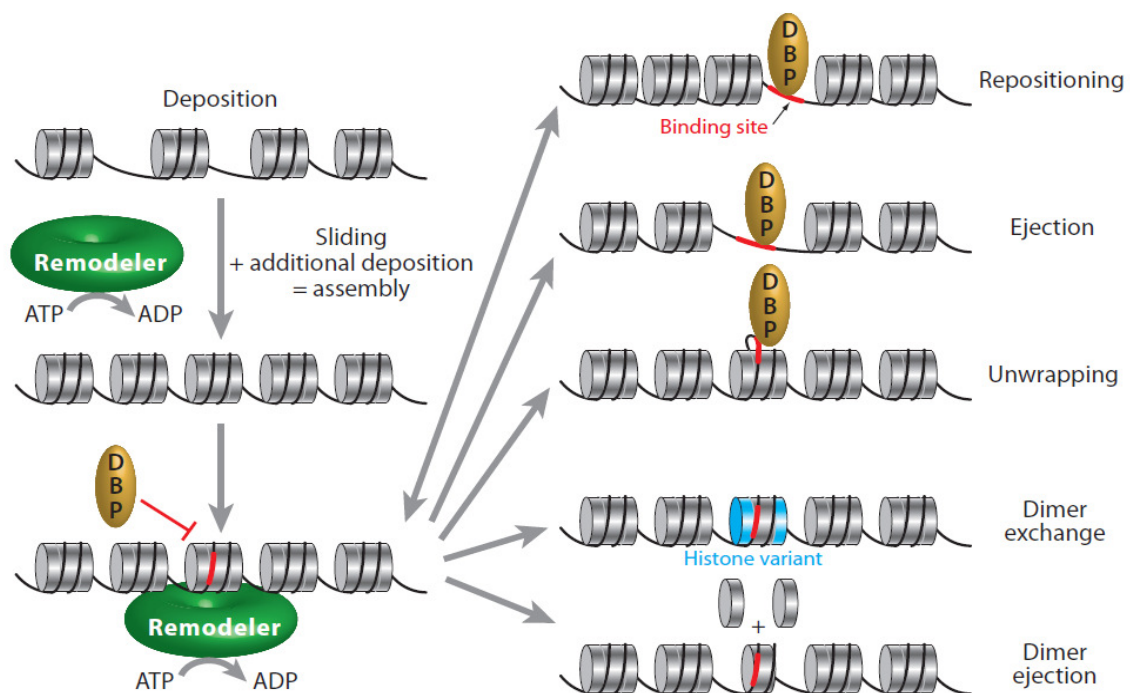
Fig. 2.2: Alterations of chromatin structure by chromatin remodeling enzymes
Chromatin remodelers can assist in chromatin assembly by moving already deposited histone octamers, thus generating space for additional deposition. Other modes of action include repositioning, ejection and unwrapping of nucleosomes as well as histone dimer exchange or dimer ejection.
(modified after: Clapier CR and Cairns BR, The biology of chromatin remodeling complexes. Annu Rev Biochem. 2009)

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[26] Bird AP and Wolffe AP, Methylation-induced repression - belts, braces, and chromatin. Cell (1999).

The exact mechanism how the hydrolysis of ATP is linked to nucleosome sliding is still a matter of investigation. One hypothesis was the 'twist-diffusion-model'. It assumed that Brownian energy fluctuations are enough to rotate the DNA helix at one edge of a nucleosome leading to a replacement of current DNA-histone interactions by neighboring DNA base pairs. By spreading of that twist around the histone octamer surface, the translational and rotational position of the nucleosome would change. Several findings argue against this model.[27]

An alternative, more favoured model is the 'loop-recapture-model'. It postulates that energy from ATP hydrolysis leads to the separation of a DNA segment from the histone octamer at the entrance or exit of the nucleosome forming a small DNA loop. The propagation of this loop around the nucleosome would change the translational position of the nucleosome. The detached DNA of the loop could then interact with transcription factors.[28]

All nucleosome remodeling ATPases belong to the SNF2-like family of ATPases characterized by their similarity of their ATPase/helicase domain to the yeast Snf(sucrose non-fermenting)2p domain. The Snf2-like family members can be further divided into several subfamilies according to the presence of additional protein domains.[29]

Eukaryotes have four main families of chromatin remodelers namely SNF2H/ISWI, INO80/SWR1, SWI/SNF and CHD/Mi-2.[30]

Most of the ATP-dependent chromatin remodelers are part of large multi-protein complexes.

2.4.2 The SNF2H/ISWI family

The ATPase ISWI (=imitation switch) was first identified in *Drosophila melanogaster*.[31]

At the C-terminus of the ISWI protein two characteristic domains reside, the SANT (switching-defective protein 3, adaptor 2, nuclear receptor co-repressor, transcription

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[27] Längst G and Becker PB, Nucleosome remodeling: one mechanism, many phenomena? Biochim Biophys Acta (2004).

[28] Längst G and Becker PB, Nucleosome remodeling: one mechanism, many phenomena? Biochim Biophys Acta (2004).

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Lusser A et al, Chromatin remodeling by ATP-dependent molecular machines. Bioessays (2003).

[30] Marfella CG and Imbalzano AN, The Chd family of chromatin remodelers. Mutat Res (2007).

[31] Tsukiyama T et al, ISWI, a member of the SWI2/SNF2 ATPase family, encodes the 140 kDa subunit of the nucleosome remodeling factor. Cell (1995).

factor IIIB) domain for binding of unmodified histone tails and the SLIDE (SANT-like ISWI) domain for interactions with nucleosomal DNA.[32]

In Drosophila, three ISWI-containing complexes are known, dNURF (nucleosome remodeling factor), dCHRAC (chromatin accessibility complex) and dACF (ATP-utilizing chromatin assembly and remodeling factor). All three are capable of inducing nucleosome sliding to the next DNA segments *in vitro,* thereby possibly facilitating DNA access to interacting factors *in vivo*.[33]

Mammals possess two ISWI homologues, SNF2L within the NURF and CERF complexes, and SNF2H in CHRAF, NoRC, ACF and others.[34]

In mouse brain, ovaries and testes, SNF2H was found to be predominantly expressed in proliferating cell populations during development whereas SNF2L was mainly expressed in terminally differentiated cells after birth and in adult animals, suggesting that the different ISWI homologues own distinct functions during development.[35]

Interestingly, the RNA levels of SMARCA5 (SNF2H) were found to be upregulated in CD34$^+$ hematopoietic progenitor cells of AML patients. After complete hematologic remission SMARCA5 levels decreased, suggesting a major role in hematopoietic differentiation.[36]

Moreover, protein complexes containing ISWI have been implicated in a huge number of other biological functions such as DNA replication, heterochromatin formation and transcriptional repression. [37]

2.4.3 The INO80 family

INO80 (inositol requiring 80) was first identified in a genetic screen for mutants interfering with the inositol biosynthesis in yeast.[38] Subsequently, orthologues in

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[32] Saha A et al, Chromatin remodelling: the industrial revolution of DNA around histones. Nat Rev Mol Cell Biol (2006).

[33] Becker PB and Hörz W, ATP-dependent nucleosome remodeling. Annu Rev Biochem (2002).

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[36] Stopka T et al, Chromatin remodeling gene SMARCA5 is dysregulated in primitive hematopoietic cells of acute leukemia. Leukemia (2000).

[37] Hargreaves DC and Crabtree GR, ATP-dependent chromatin remodeling: genetics, genomics and mechanisms. Cell Res (2011).

[38] Ebbert R et al, The product of the SNF2/SWI2 paralogue INO80 of Saccharomyces cerevisiae required for efficient expression of various yeast structural genes is part of a high-molecular-weight protein complex. Mol Microbiol (1999).

higher metazoan like the human INO80[39] and hSRCAP (SNF2-related CREB-activator protein) [40] were identified. The INO80 and SWR1 family shares the property that their conserved ATPase domain is split by a long insert.[41]

A broad variety of functions have been described for INO80/SWR1 complexes so far. The SWR1 complex is able to exchange histone H2A with H2A variants like H2AZ within one nucleosome.[42] Furthermore, a major role for INO80 complexes in DNA repair has been suggested. For example, the INO80 null mutant is hypersensitive to DNA-damaging agents[43] and γ-H2AX is required for recruitment of INO80 to sites of double-strand breaks[44] where it was shown to regulate the DNA accessibility around these double-strand breaks.[45]

2.4.4 The CHD/Mi-2 family

The CHD (chromodomain, helicase, DNA binding domain) family of remodelers has as its most characteristic feature two tandemly arranged chromodomains at the N-terminus in addition to the ATPase domain.

The term 'chromodomain' was first used by Paro and Hogness[46] describing a region of similarity in *Drosophila* Polycomb and HP1 protein. They assumed a function in chromatin regulation, so 'chromo' stands for chromatin organization modifier. The prototype chromodomain consists of around 50 amino acids and is folded in three-stranded antiparallel ß-sheets and an α-helix running across the sheet.[47] By direct interaction with RNA, DNA and methylated histone tails chromodomains are involved in chromatin regulation.[48]

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[41] Bao Y et al, INO80 subfamily of chromatin remodeling complexes. Mutat Res (2007).

[42] Mizuguchi G et al, ATP-driven exchange of histone H2AZ variant catalyzed by SWR1 chromatin remodeling complex. Science (2004).

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[44] Bao Y et al, INO80 subfamily of chromatin remodeling complexes. Mutat Res (2007).

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[47] Brehm A et al, The many colours of chromodomains. Bioessays (2004).

[48] Eissenberg JC, Molecular biology of the chromo domain: an ancient chromatin module comes of age. Gene (2001).

In yeast, only one CHD protein, namely CHD1, was identified.[49] Drosophila has four CHD proteins and nine family members (CHD1-9) are known in mammals which are further divided into three subfamilies according to the presence of additional domains besides the ATPase domain.

Subfamily I: CHD1 and CHD2

Subfamily I includes CHD1 and CHD2 from higher eukaryotes. They contain a C-terminal DNA - binding domain that is known to interact with AT-rich DNA motifs.[50]

CHD1 is thought to play an important role in the maintenance of an open, active chromatin state.[51] Drosophila CHD1 localizes to the interbands and puffs of polytene chromosomes at transcriptionally active sites. Chromodomains of human CHD1 were shown to selectively bind to H3K4me3[52] which is associated with the 5' end of actively transcribed genes indicating that the mammalian CHD1 is targeted to open, active chromatin. This view is furthermore supported by the findings that CHD1 downregulation leads to an accumulation of heterochromatin and that its presence is required for maintenance of mouse ES cell pluripotency.[53]

A homozygous CHD2 mutant mouse model showed embryonic and perinatal lethality.[54] In CHD2 heterozygous mutant mice an increased number of megakaryocytes and a defective differentiation of hematopoietic stem cells into the erythroid lineage were found. In addition, mice were prone to develop T-cell lymphomas and had defects in the DNA damage-induced γ-H2AX response.[55]

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[49] Tran HG et al, The chromo domain protein chd1p from budding yeast is an ATP-dependent chromatin-modifying factor. EMBO J (2000).

[50] Marfella CG and Imbalzano AN, The Chd family of chromatin remodelers. Mutat Res (2007).

[51] Persson J and Ekwall K, Chd1 remodelers maintain open chromatin and regulate the epigenetics of differentiation. Exp Cell Res (2010).

[52] Flanagan JF et al, Double chromodomains cooperate to recognize the methylated histone H3 tail. Nature (2005).

[53] Gaspar-Maia A et al, Chd1 regulates open chromatin and pluripotency of embryonic stem cells. Nature (2009).

[54] Marfella CG et al, Mutation of the SNF2 family member Chd2 affects mouse development and survival. J Cell Physiol (2006).
Nagarajan P et al, Role of chromodomain helicase DNA-binding protein 2 in DNA damage response signaling and tumorigenesis. Oncogene (2009).

[55] Nagarajan P et al, Role of chromodomain helicase DNA-binding protein 2 in DNA damage response signaling and tumorigenesis. Oncogene (2009).

Subfamily II: The NuRD complex and CHD5

Subfamily II includes CHD3 (Mi-2α) and CHD4 (Mi-2ß). They are characterized by N-terminal paired PHD (<u>p</u>lant <u>h</u>omeo <u>d</u>omain) zinc-finger-like domains absent in subfamily I.[56] In addition, CHD5 can be placed into this family because of its large similarity to CHD4 and the presence of two PHD domains (see chapter 2.5.2 for details) but this classification is controversially discussed. Some authors place CHD5 into the third subfamily because of a strikingly unique region of fifty residues at the C-terminus that is significantly diverging from CHD3/4.[57]

PHD fingers are commonly found in a variety of nuclear proteins. They span around 50 to 80 residues and have a characteristic Cys_4-His-Cys_3 motif.[58] They are proposed to function as 'histone code readers' but the exact function in most CHD proteins is unknown so far.[59]

The PHD zinc fingers of Mi2ß are required to bind HDAC-1 within the NuRD complex.[60] Furthermore, the first PHD finger of CHD4 was found to interact with the N-terminus of H3.[61] The second PHD finger of CHD4 was shown to bind to the N-terminus of H3 facilitated by methylation or acetylation of Lys9 (H3K9me and H3K9ac, respectively) and this binding was inhibited by methylation of Lys4 (H3K4me).[62] Recently, it was demonstrated that this concomitant interaction of the tandem PHD domains of CHD4 with two histone H3 tails is required for the repressive role of CHD4.[63]

Since my thesis focuses mainly on the chromatin remodelers Mi2ß/NuRD complex and CHD5 I will describe these proteins in greater detail.

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[57] Thompson PM et al, CHD5, a new member of the chromodomain gene family, is preferentially expressed in the nervous system. Oncogene (2003).

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[60] Zhang Y et al, The dermatomyositis-specific autoantigen Mi2 is a component of a complex containing histone deacetylase and nucleosome remodeling activities. Cell (1998).

[61] Mansfield RE et al, Plant homeodomain (PHD) fingers of CHD4 are histone H3-binding modules with preference for unmodified H3K4 and methylated H3K9. J Biol Chem (2011).

[62] Musselman CA et al, Binding of the CHD4 PHD2 finger to histone H3 is modulated by covalent modifications. Biochem J (2009).

[63] Musselman CA et al, Bivalent recognition of nucleosomes by the tandem PHD fingers of the CHD4 ATPase is required for CHD4-mediated repression. Proc Natl Acad Sci USA (2012).

CHD3 (Mi2α) and CHD4 (Mi2ß) were first described as autoantigens in dermatomyositis.[64] Patients with this autoimmune disease are prone to develop cancers.[65]

The Mi2 protein exists in many species including *Drosophila*, *Caenorhabditis elegans* and *Xenopus laevis* but is absent in yeast. It has ATP-dependent chromatin remodeling activity. ATPase activity of *Drosophila* Mi2 is stimulated by nucleosomes but not by free DNA or core histones.[66] In contrast, ATPase activity of human Mi2ß was shown to increase in presence of DNA as well as nucleosomes.[67]

Human CHD3 and CHD4 are the core subunits of the hNuRD (Nucleosome Remodeling and Deacetylase) complex. This complex is considered a classical repressor complex and uniquely links three fundamental activities in regulation of chromatin structure, namely chromatin remodeling, recognition of methylated CpG dinucleotides and histone deacetylation. The chromatin remodeling activity is performed by CHD3 and CHD4, the histone deacetylation by HDAC-1 and/or HDAC-2. Other non-enzymatic components of this complex include metastasis - associated proteins (MTA) 1/2/3, methyl CpG binding domain (MBD) proteins 2/3, p66α/ß and histone-binding/retinoblastoma-binding proteins (RBBP7/RbAp46 and RBBP4/RbAp48). [68]

Recently, the histone demethylase LSD1 was described to be associated with the human NuRD complex.[69] But whether LSD1 associates with NuRD ubiquitously and permanently is still a matter of debate.

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[64] Seelig HP et al, The major dermatomyositis-specific Mi-2 autoantigen is a presumed helicase involved in transcriptional activation. Arthritis Rheu (1995).

[65] Sigurgeirsson B et al, Risk of cancer in patients with dermatomyositis or polymyositis. A population-based study. N Engl J Med (1992).

[66] Brehm A et al, dMi-2 and ISWI chromatin remodelling factors have distinct nucleosome binding and mobilization properties. EMBO J (2000).

[67] Wang HB and Zhang Y, Mi2, an auto-antigen for dermatomyositis, is an ATP-dependent nucleosome remodeling factor. Nucleic Acids Res (2001).

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[68] Xue Y et al, NURD, a novel complex with both ATP-dependent chromatin-remodeling and histone deacetylase activities. Mol Cell (1998).

Wang HB and Zhang Y, Mi2, an auto-antigen for dermatomyositis, is an ATP-dependent nucleosome remodeling factor. Nucleic Acids Res (2001).

Zhang Y et al, The dermatomyositis-specific autoantigen Mi2 is a component of a complex containing histone deacetylase and nucleosome remodeling activities. Cell (1998).

[69] Wang Y et al, LSD1 is a subunit of the NuRD complex and targets the metastasis programs in breast cancer. Cell (2009).

Whyte WA et al, Enhancer decommissioning by LSD1 during embryonic stem cell differentiation. Nature (2012).

Drosophila Mi2 resides in a NuRD-like complex containing the histone deacetylase dRPD3, dp66, dp55 (as homologue to RbAp46/48) and dMBD2/3.[70] Recently, a second dMi2 containing complex, dMec (Drosophila MEP-1 containing complex), was purified that does not rely on histone deacetylation to effect transcriptional repression.[71]

The composition of human NuRD complex can vary dependent on the context and function.

The integral catalytic deacetylase subunits of NuRD are HDAC-1 and/or HDAC-2.[72] In addition to NuRD, HDAC-1/2 were found in different co-repressor complexes such as CoREST[73] and mSin3.[74] In this context, it is still far from clear whether different HDAC-1/2 containing complexes can repress synergistically their targets or whether the different complexes are specifically targeted to different regions of the genome.[75]

Besides the two catalytic subunits, NuRD includes some important proteins mainly regulating interactions. The two methyl CpG-binding domain proteins MBD2 and MBD3 are alternative subunits.[76] But only MBD2 is able to interact with methylated DNA,[77] MBD3 seems to have lost this ability during vertebrate evolution.[78]

MBD2 was recently found to preferentially bind to methylated transcriptional start sites. [79]

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[70] Brehm A et al, dMi-2 and ISWI chromatin remodelling factors have distinct nucleosome binding and mobilization properties. EMBO J (2000).

Marhold J et al, The Drosophila methyl-DNA binding protein MBD2/3 interacts with the NuRD complex via p55 and MI-2. BMC Mol Biol (2004).

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[71] Kunert N et al, dMec: a novel Mi-2 chromatin remodelling complex involved in transcriptional repression. EMBO J (2009).

[72] Denslow SA and Wade PA, The human Mi-2/NuRD complex and gene regulation. Oncogene (2007).

[73] Humphrey GW et al, Stable histone deacetylase complexes distinguished by the presence of SANT domain proteins CoREST/kiaa0071 and Mta-L1. Biol Chem (2001).

[74] Hassig CA et al, Histone deacetylase activity is required for full transcriptional repression by mSin3A. Cell (1997).

Zhang Y et al, Histone deacetylases and SAP18, a novel polypeptide, are components of a human Sin3 complex. Cell (1997).

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[76] Le Guezennec X et al, MBD2/NuRD and MBD3/NuRD, two distinct complexes with different biochemical and functional properties. Mol Cell Biol (2006).

[77] Hendrich B and Bird A, Identification and characterization of a family of mammalian methyl-CpG binding proteins. Mol Cell Biol (1998).

[78] Hendrich B and Tweedie S, The methyl-CpG binding domain and the evolving role of DNA methylation in animals. Trends Genet (2003).

[79] Chatagnon A et al, Preferential binding of the methyl-CpG binding domain protein 2 at methylated transcriptional start site regions. Epigenetics (2011).

MBD3 was linked to the establishment of altered epigenetic marks in acute promyelocytic leukemia (APL). The key mediator of APL, the t(15;17) fusion protein PML-RARα, recruited Mi2/NuRD to target genes like the important tumor suppressor RARß2. Upon RNAi-mediated depletion of MBD3, leukemia cells displayed a more matured morphology.[80] However, it is unclear whether that effect of MBD3 depletion is due to a destabilization of NuRD complex or to a so far unknown property of MBD3.

The family of metastasis-associated proteins (MTA) was found based on the discovery of MTA1 in a model for metastatic growth.[81] Several other members of this family, including different isoforms, have been identified so far. NuRD seems to be able to constitute specialized complexes with the different MTA proteins since many different functional roles are reported for MTA1, 2 and 3 up to now.

MTA1 is upregulated in several cancers. In gastric and colorectal cancers an overexpression correlated with an advanced stage.[82] In breast cancer cells MTA1 is a potent co-repressor of estrogen-receptor element (ERE)-driven transcription, thus it blocks the ability of estradiol to stimulate ER-mediated transcription.[83] This finding provides a partial explanation for the clinical data in which the highest expression of MTA1 in untreated node-negative breast cancer was associated with increased relapse risk. Furthermore, Tamoxifen and/or anthracyclene-based chemotherapies eliminated all MTA1 associations with clinical outcome.[84]

Whether the described MTA1 effects on carcinogenesis are due to an increase of MTA1-containing NuRD, or whether it acts as a monomer or even in a different complex is far from clear.

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[80] Morey L et al, MBD3, a component of the NuRD complex, facilitates chromatin alteration and deposition of epigenetic marks. Mol Cell Biol (2008).
[81] Pencil SD et al, Candidate metastasis-associated genes of the rat 13762NF mammary adenocarcinoma. Breast Cancer Res Treat (1993).
Toy Y et al, A novel candidate metastasis-associated gene, mta1, differentially expressed in highly metastatic mammary adenocarcinoma cell lines. J Biol Chem (1994).
[82] Toh Y, et al, Overexpression of the MTA1 gene in gastrointestinal carcinomas: correlation with invasion and metastasis. Int J Cancer (1997).
[83] Mazumdar A et al, Transcriptional repression of oestrogen receptor by metastasis-associated protein 1 corepressor. Nat Cell Biol (2001).
[84] Martin MD et al, Breast tumors that overexpress nuclear metastasis-associated 1 (MTA1) protein have high recurrence risks but enhanced responses to systemic therapies. Breast Cancer Res Treat (2006).

In a MTA2 knock-out mouse abnormal T-cell activation upon stimulation and lupus-like autoimmune disease were observed.[85]

MTA3 is also linked to carcinogenesis. The absence of MTA3 or estrogen receptor resulted in aberrant expression of the transcriptional repressor Snail, a master regulator of epithelial to mesenchymal transition (EMT). Aberrant Snail expression reduced the expression of the cell adhesion molecule E-cadherin, an event associated with changes in epithelial architecture and invasive growth.[86] Interestingly, transient overexpression of MTA1 leads to dramatic decrease in MTA2 and MTA3 on protein level but not on transcript level.[87]

The additional subunits RbAp48 (RBBP7) and RbAP48 (RBBP4) were originally identified based on their interactions with Retinoblastoma protein.[88] They are also able to interact with histone H4 to mediate further recruitment of proteins to chromatin.[89]

The subunits p66α/ß are both able to interact with MBD2 as well as MBD3.[90] Both proteins interact with the tails of all core histones *in vitro*.[91]

As already mentioned, the NuRD complex is thought to be a classical repressor complex as it uniquely combines histone deacetylation with chromatin remodeling. One mechanistic model for this repression is that the binding of methylated DNA by MBD2 recruits other NuRD components. Of these, HDAC-1/2 are then responsible for deacetylation of histones and CHD3/4 use their ATPase-dependent remodeling property to further compact the chromatin.[92] An alternative model suggests that CHD3/4 remodel nucleosomes to facilitate access of HDAC-1/2 to histone tails.[93]

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[85] Lu X et al, Inactivation of NuRD component Mta2 causes abnormal T cell activation and lupus-like autoimmune disease in mice. J Biol Chem (2008).

[86] Fujita N et al, MTA3, a Mi-2/NuRD complex subunit, regulates an invasive growth pathway in breast cancer. Cell (2003).

[87] Fujita N et al, Hormonal regulation of metastasis-associated protein 3 transcription in breast cancer cells. Mol Endocrinol (2004).

[88] Qian YW et al, A retinoblastoma-binding protein related to a negative regulator of Ras in yeast. Nature (1993).

[89] Verreault A et al, Nucleosomal DNA regulates the core-histone-binding subunit of the human Hat1 acetyltransferase. Curr Biol (1998).

[90] Brackertz M et al, Two highly related p66 proteins comprise a new family of potent transcriptional repressors interacting with MBD2 and MBD3. J Biol Chem (2002).

[91] Brackertz M et al, p66alpha and p66beta of the Mi-2/NuRD complex mediate MBD2 and histone interaction. Nucleic Acids Res (2006).

[92] Denslow SA and Wade PA, The human Mi-2/NuRD complex and gene regulation. Oncogene (2007).

[93] Zhang Y et al, The dermatomyositis-specific autoantigen Mi2 is a component of a complex containing histone deacetylase and nucleosome remodeling activities. Cell (1998).

An alternative recruitment by co-repressors is also possible: Mi2α is able to interact with co-repressor KAP-1, thereby recruiting the NuRD complex to target genes of KRAB- (Krüppel-associated-box) zinc-finger proteins.[94]

Another possibility to recruit NuRD is the interaction with DNA-binding proteins, for example the DNA-binding protein Ikaros recruits Mi2/NuRD to regions of heterochromatin upon T-cell activation.[95]

Within this repressive role, the NuRD complex can also function as a barrier to complexes known as transcriptional activators like SWI/SNF family members. Such antagonistic roles were shown for Mi2ß and SWI/SNF remodeling complexes during the activation of inflammatory genes in response to LPS stimulation. BRG1 was required for activation and Mi2ß for limitation of this response.[96] Similar opposing effects were observed for Mi2/NuRD and SWI/SNF in reprogramming of genes by EBF (early B-cell factor) and Pax5 during B-cell differentiation.[97]

CHD5 is a new member of the CHD family. A more detailed introduction to CHD5 is given in chapter 2.5.2 in the context of Neuroblastoma.

Subfamily III: CHD6-9

Subfamily III contains CHD6-9. This family is the most variable of all three because they are characterized by different additional domains in the C-terminal area, including paired BRK (Brahma and Kismet) domains, a SANT-like domain, CR (conserved region) domains and DNA-binding domains.[98] These DNA-binding domains are similar to those in subfamily I although a DNA binding activity has only been shown for CHD9 so far.[99]

CHD6 is ubiquitously expressed in mouse tissues and it co-localizes with RNA polymerase II engaged in transcription pre-initiation and elongation.[100] The location of

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[94] Schultz DC et al, Targeting histone deacetylase complexes via KRAB-zinc finger proteins: the PHD and bromodomains of KAP-1 form a cooperative unit that recruits a novel isoform of the Mi-2alpha subunit of NuRD. Genes Dev (2001).

[95] Kim J et al, Ikaros DNA-binding proteins direct formation of chromatin remodeling complexes in lymphocytes. Immunity (1999).

[96] Ramirez-Carrozzi VR et al, Selective and antagonistic functions of SWI/SNF and Mi-2beta nucleosome remodeling complexes during an inflammatory response. Genes Dev (2006).

[97] Gao H et al, Opposing effects of SWI/SNF and Mi-2/NuRD chromatin remodeling complexes on epigenetic reprogramming by EBF and Pax5. Proc Natl Acad Sci USA (2009).

[98] Marfella CG and Imbalzano AN, The Chd family of chromatin remodelers. Mutat Res (2007).

[99] Shur I and Benayahu D, Characterization and functional analysis of CReMM, a novel chromodomain helicase DNA-binding protein. J Mol Bio (2005).

[100] Lutz T et al, CHD6 is a DNA-dependent ATPase and localizes at nuclear sites of mRNA synthesis. FEBS Lett (2006).

CHD6 on human chromosome 20q12 is a locus commonly deleted in human ataxias. Interestingly, in a mouse model with deletion of CHD6 exon 12 (located within the ATPase domain) impaired motor coordination was observed.[101]

CHD7 encodes a protein mutated or deleted in most patients with CHARGE syndrome. CHARGE is an acronym for coloboma of the eye, heart defects, atresia of nasal choanae, retardation of growth and/or development, genital and/or urinary abnormalities, ear abnormalities and deafness. [102]

CHD7 is highly expressed in ES cells but its expression becomes restricted to tissue specific progenitor populations during development. It is noteworthy that, these progenitors give rise to specific cell types in different organs that are affected in CHARGE syndrome, including neural crest derivatives, ear, eye, heart and kidney.[103]

CHD7 binds several PBAF specific subunits in human neural crest-like cells[104] but forms different complexes in other cell types and developmental stages.[105]

CHD8 was identified to be a negative regulator of the Wnt-/β-catenin pathway that binds directly to β-catenin. Thus, it suppresses its transactivation activity.[106] Recently, CHD8 was found to be involved in regulation of HOX2A gene expression.[107]

The last family member, CHD9, was shown to take part in osteogenic cell differentiation.[108]

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[101] Lathrop MJ et al, Deletion of the Chd6 exon 12 affects motor coordination. Mamm Genome (2010).

[102] Bergman JE et al, CHD7 mutations and CHARGE syndrome: the clinical implications of an expanding phenotype. J Med Genet (2011).

[103] Martin DM, Chromatin remodeling in development and disease: focus on CHD7. PLoS Genet (2010).

[104] Bajpai R et al, CHD7 cooperates with PBAF to control multipotent neural crest formation. Nature (2010).

[105] Takada I et al, A histone lysine methyltransferase activated by non-canonical Wnt signalling suppresses PPAR-gamma transactivation. Nat Cell Biol (2007).

[106] Thompson BA et al, CHD8 is an ATP-dependent chromatin remodeling factor that regulates beta-catenin target genes. Mol Cell Biol (2008).

[107] Yates JA et al, Regulation of HOXA2 gene expression by the ATP-dependent chromatin remodeling enzyme CHD8. FEBS Lett (2010).

[108] Shur I et al, Dynamic interactions of chromatin-related mesenchymal modulator, a chromodomain helicase-DNA-binding protein, with promoters in osteoprogenitors. Stem Cells (2006).

2.4.5 The SWI/SNF family

Initially, the family of SWI/SNF chromatin remodelers was discovered in two genetic screens in yeast.[109] The first screen revealed genes involved in mating-type switching (switch (SWI) mutants)[110] and the second identified genes required for SUC2 expression in sucrose metabolism (sucrose non-fermenting (SNF) mutants).[111]

The central catalytic subunits of SWI/SNF complexes in mammals are hBRG1 (Brm-related gene 1; also known as SMARCA4) and hBRM (human Brahma; or SMARCA2); but presumably no individual SWI/SNF complex contains both. Additionally, nine to twelve subunits which are referred to as BRG1/Brm-related factors (BAFs) constitute such a complex. Core subunits are BAF155, BAF170 and SNF5 (also known as INI1 or BAF47). Among the accessory subunits are BAF180 and BAF250. Depending on their presence, the BRG1-containing complexes are further divided into BRG1/BAF (containing BAF250) and BRG1/PBAF (with BAF180)[112] but several other BRG1-containing complexes like WINAC[113] or NUMAC[114] were identified.

BRG1 and Brm share approximately 75 % amino acid identity. However, they seem to take over some different functions, as a homozygous Brm knockout mouse was viable[115] whereas homozygous BRG1 deficiency resulted in embryonic lethality.[116] Underscoring these differences, distinctive expression patterns were observed in adult tissues: BRG1 was predominantly expressed in cell types constantly undergoing proliferation or self-renewal and Brm mainly seen in brain, liver, fibromuscular stroma, and endothelial cell types.[117]

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[109] Wilson BG and Roberts CW, SWI/SNF nucleosome remodellers and cancer. Nat Rev Cancer (2011).

[110] Stern M et al, Five SWI genes are required for expression of the HO gene in yeast. J Mol Biol (1984).

[111] Neigeborn L and Carlson M, Genes affecting the regulation of SUC2 gene expression by glucose repression in Saccharomyces cerevisiae. Genetics (1984).

[112] Reisman D et al, The SWI/SNF complex and cancer. Oncogene (2009).

[113] Kitagawa H et al, The chromatin-remodeling complex WINAC targets a nuclear receptor to promoters and is impaired in Williams syndrome. Cell (2003).

[114] Xu W et al, A methylation-mediator complex in hormone signaling. Genes Dev (2004).

[115] Reyes JC et al, Altered control of cellular proliferation in the absence of mammalian brahma (SNF2alpha). EMBO J (1998).

[116] Bultmann S et al, A Brg1 null mutation in the mouse reveals functional differences among mammalian SWI/SNF complexes. Mol Cell (2000).

[117] Reisman DN et al, The expression of the SWI/SNF ATPase subunits BRG1 and BRM in normal human tissues. Appl Immunohistochem Mol Morphol (2005).

Both proteins were shown to have chromatin remodeling activity by themselves and this activity was further stimulated by presence of the core subunits *in vitro*.[118]

A hallmark of BRG1 and Brm is the conserved C-terminal bromodomain known to be a typical recognition module for acetylated lysine residues of histones. The yeast BRG1/Brm homologue Swi2/Snf2 requires that domain for a stable association with chromatin *in vitro*. [119]

Although SWI/SNF complexes are considered as characteristic transcriptional activators, some evidence suggests that they can also function as repressors. For example, in embryonic stem cells BRG1 was shown to be required for repression of genes involved in differentiation while activating genes for maintenance of pluripotency.[120]

Emerging evidence has shown that various SWI/SNF subunits are involved in the development of cancer. BRG1 or Brm interfere with a couple of key regulatory proteins like p53, [121] BRCA1, [122] Rb [123] and the ß-catenin/Wnt pathway. [124] Furthermore, reintroduction of BRG1 into cells lacking its expression was sufficient to reverse their transformed phenotype and induce growth arrest as well as a senescent phenotype.[125]

The most prominent example for the relevance of SWI/SNF complexes in cancer is the core subunit SNF5 that turned out to be a bona fide tumor suppressor in malign rhabdoid tumors.[126]

BRG1 was shown to be mutated or deleted in many cell lines and primary samples from non-small cell lung cancer (NSCLC). [127] Loss of BRG1/Brm occurred in around 10 %

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[118] Phelan ML et al, Reconstitution of a core chromatin remodeling complex from SWI/SNF subunits. Mol Cell (1999).

[119] Hassan AH et al, Function and selectivity of bromodomains in anchoring chromatin-modifying complexes to promoter nucleosomes. Cell (2002).

[120] Ho L et al, An embryonic stem cell chromatin remodeling complex, esBAF, is an essential component of the core pluripotency transcriptional network. Proc Natl Acad Sci USA (2009).

[121] Bochar DA et al, BRCA1 is associated with a human SWI/SNF-related complex: linking chromatin remodeling to breast cancer. Cell (2000).

[122] Bochar DA et al, BRCA1 is associated with a human SWI/SNF-related complex: linking chromatin remodeling to breast cancer. Cell (2000).

[123] Bartlett C et al, BRG1 mutations found in human cancer cell lines inactivate Rb-mediated cell-cycle arrest. J Cell Physiol (2011).

[124] Griffin CT et al, The chromatin-remodeling enzyme BRG1 modulates vascular Wnt signaling at two levels. Proc Natl Acad Sci USA (2011).

[125] Wong AK et al, BRG1, a component of the SWI-SNF complex, is mutated in multiple human tumor cell lines. Cancer Res (2000).

[126] Versteege I et al, Truncating mutations of hSNF5/INI1 in aggressive paediatric cancer. Nature (1998).

of NSCLC and was, independent of the stage, significantly associated with a worse patient survival in comparison with BRG1/Brm expressing tumors.[128] In a lung-specific conditional knockout of BRG1 using an ethyl carbamate lung carcinogenesis mouse model, the heterozygous inactivation of BRG1 resulted in an increased number and size of tumors whereas the homozygous loss was associated with increased apoptosis in lung tissue without tumor formation.[129] However, BRG1 resides on the short arm of chromosome 19, only around 10 Mb from LKB1, another candidate tumor suppressor in NSCLC.[130] Since loss of both proteins in cancer is mostly due to larger deletions, a concomitant loss of both seems likely. Unfortunately, in none of the publications with clinical data a multivariate analysis was performed showing that the prognostic significance of either protein is independent of the other. In conclusion, although the role of BRG1 in NSCLC is suggestive I would take these data with great caution.

2.5 Neuroblastoma and CHD5

2.5.1 Neuroblastoma

The neuroblastoma is the most common extracranial solid tumor during childhood. It arises from the sympaticoadrenal lineage of the neural crest. Therefore it can occur everywhere in the sympathetic nervous system; even though it is usually located paraspinally in the abdomen or the chest.[131]

A clinical hallmark is the amazing variability of this cancer, differing from spontaneous regression without any therapy to extended disease upon aggressive multimodal treatment. [132] Based on the tumor's ability to secrete catecholamines, a Neuroblastoma screening for raised urine catecholamines at the age of one year was performed in two independent population-based controlled trials in Germany and North America in the 1990s. Both trials resulted in an increased incidence (more

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[127] Medina PP et al, Frequent BRG1/SMARCA4-inactivating mutations in human lung cancer cell lines. Hum Mutat (2008).
Medina PP et al, Genetic and epigenetic screening for gene alterations of the chromatin-remodeling factor, SMARCA4/BRG1, in lung tumors. Genes Chromosomes Cancer (2004).
[128] Reisman DN et al, Loss of BRG1/BRM in human lung cancer cell lines and primary lung cancers: correlation with poor prognosis. Cancer Res (2003).
[129] Glaros S et al, Targeted Knockout of BRG1 Potentiates Lung Cancer Development. Cancer Res (2008).
[130] Rodriguez-Nieto S and Sanchez-Cespedes M, BRG1 and LKB1: tales of two tumor suppressor genes on chromosome 19p and lung cancer. Carcinogenesis (2009).
[131] Maris JM, Hogarty MD; Neuroblastoma. Lancet (2007).
[132] Maris JM, Hogarty MD; Neuroblastoma. Lancet (2007).

children suffering from this disease detected by screening) without any change in mortality caused by neuroblastomas. [133]

Typical features of neoplasia such as mutations of TP53 and Ras as well as deletions of the INK4a locus are rarely reported in primary neuroblastomas, indicating that there might be different events which influence major tumor promoting pathways like aberrant cell cycle control and apoptosis. [134]

Since the neuroblastoma is genetically one of the best characterized tumors, there are several clinical features and molecular markers allowing a prediction of the clinical course although there remain cases in which the established prediction factors show limited clinical utility:[135]

A major prognostic factor is the age. In general, infants have a much better prognosis than children older than one year.[136] In addition, the expression of the NGF receptor TrkA is highly correlated with favourable outcome.[137]

Roughly 20 % of primary tumors have an amplification of N-Myc which is associated with a poor outcome, advanced stage of the disease (according to the International Neuroblastoma Staging System, INSS) and treatment failure. Furthermore, the biological impact of this amplification is indicated by the fact that also patients with otherwise favourable disease have a poor prognosis.[138] 25 - 35 % of neuroblastomas show an allelic loss of the short arm of chromosome 1, of which the smallest region of deletion could be mapped to 1p36. This LOH occurs commonly in patients with an advanced stage of the disease and seems to predict the disease progression and not the overall survival, although this has been controversially discussed. Most tumors with amplified N-Myc additionally own a LOH 1p, but not every tumor with LOH 1p has an amplified N-Myc. This deletion is characterized by whole chromosome gains lacking rearrangements suggesting that this deletion might precede the amplification.[139] Furthermore, a microcell-mediated chromosome transfer of 1p into neuroblastoma

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[133]Schilling FH et al, Neuroblastoma Screening at One Year of Age. N Engl J Med (2002).
 Woods WG et al, Screening of Infants and Mortality Due to Neuroblastoma. N Engl J Med (2002).
[134] Maris JM, Hogarty MD; Neuroblastoma. Lancet (2007).
[135] Garcia I et al, Expression of the neuron-specific protein CHD5 is an independent marker of outcome in neuroblastoma. Mol Cancer (2010).
[136] Simon T, Neuroblastom. Urologe A (2005).
[137] Brodeur GM, Neuroblastoma: Biological insight into a clinical enigma. Nat Rev Cancer (2003).
[138] Maris JM, Hogarty MD; Neuroblastoma. Lancet (2007).
[139] Brodeur GM, Neuroblastoma: Biological insight into a clinical enigma. Nat Rev Cancer (2003).

cells suppresses transformation and tumorigenity.[140] All these data suggest that there might reside one or more potent tumor suppressors on 1p36, but despite intensive investigations a defined 1p tumor suppressor could not be identified so far. Recently Chromodomain Helicase DNA binding domain 5 (CHD5) was proposed as a candidate tumor suppressor gene. CHD5 was absent in neuroblastoma cell lines and a large percentage of primary tumors.[141] In a group of 99 neuroblastoma patients, CHD5 expression correlated with the event-free survival independently from a deletion of the short arm of chromosome 1 (1p) but correlated with amplified N-Myc and age.[142]

The prognostic impact of CHD5 is further supported by a recent publication of Garcia et al[143], demonstrating low or absent CHD5 expression in high-risk neuroblastoma and intense nuclear CHD5 staining in low-risk tumors. These findings suggest a possible clinical utility to distinguish between low-risk stage 4s and high-risk stage 4 tumors in infants. According to the International Neuroblastoma Staging System (INSS), stage 4 neuroblastomas include metastasized tumors, usually with an unfavourable prognosis. In contrast, Stage 4S denotes a specific metastatic stage of neuroblastomas in children younger than one year with small primary tumors and metastases in liver, skin, or bone marrow that spontaneously regress in most cases.[144] To distinguish between stage 4 and stage 4S is important for a treatment decision (ranging from aggressive multimodal treatment to watch-and-wait strategy) but can be difficult. Thus, a molecular marker that improves the differentiation between these two stages would be of great clinical advantage.

In addition, high CHD5 expression was found to be associated with an increased event-free and overall survival independently of INSS stage, patient age and amplification of MYC-N. In addition, a reactivation of CHD5 expression was related to a complete or very good response towards cytotoxic induction therapy in high-risk neuroblastoma.[145] [A complete response is defined by the absence of tumor in CT or MRI scan; a

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[140] Bader SA et al, Dissociation of suppression of tumorigenicity and differentiation in vitro effected by transfer of single human chromosomes into human neuroblastoma cells. Cell Growth Differ (1991).

[141] Thompson PM et al, CHD5, a new member of the chromodomain gene family, is preferentially expressed in the nervous system. Oncogene (2003).

[142] Fujita T et al, CHD5, a Tumor Suppressor Gene Deleted From 1p36.31 in Neuroblastomas. J Natl Cancer Inst (2008).

[143] Garcia I et al, Expression of the neuron-specific protein CHD5 is an independent marker of outcome in neuroblastoma. Mol Cancer (2010).

[144] Maris JM, Hogarty MD; Neuroblastoma. Lancet (2007).

[145] Garcia I et al, Expression of the neuron-specific protein CHD5 is an independent marker of outcome in neuroblastoma. Mol Cancer (2010).

classification as very good partial response includes tumors with > 90 % reduction in 3D tumor volume reconstructed by CT/MRI].[146]

2.5.2 Chromodomain Helicase DNA-binding domain 5 (CHD5)

The human CHD5 gene consists of 42 exons coding for a 223 kDa protein. Predicted from its protein sequence it contains two zinc-fingers of the PHD class, two chromodomains and a Helicase/ATPase domain (see Fig 2.3).[147]

NH$_3$ ——— ◇ ◇ ——— ⬭ ⬭ ——— ▮ ——— COOH

2 PHD zink fingers   2 chromodomains   Helicase/ATPase domain
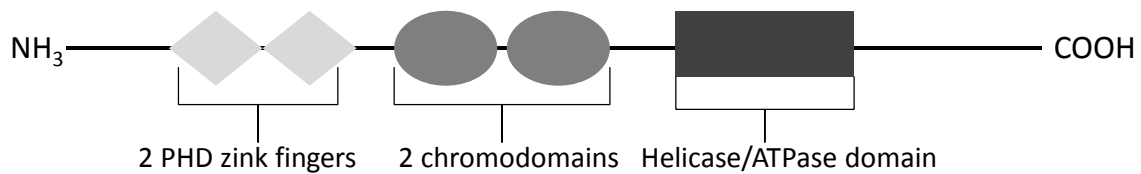
Fig. 2.3: Predicted structural motifs of CHD5

(modified after *Thompson PM et al, Oncogene 2003*)

Thompson et al[148] described a CpG island within the CHD5 gene starting 630 base pairs 5' to the transcriptional start site, containing the complete exon one and the first 680 base pairs of intron one. The presence of a CpG island suggests a possible transcriptional regulation by methylation.

CHD5 is a vertebrate-specific gene. Sequence homology between vertebrate CHD5 proteins is very high as shown in table 2.1. Notably, not all vertebrates have a CHD5-like protein encoded in their genome according to their predicted transcripts or to a blast search for a CHD5-specific region of its C-terminal part. Interestingly, the *NCBI*[149] database suggests only very few orthologues. In contrast, many predicted orthologues are listed in the *Ensembl*[150] database. However, both databases provide only a prediction of CHD5-like protein sequences. It has not yet tested whether the predicted CHD5-like proteins really exist and if they are expressed in the different species.

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[146] Baker DL et al, Outcome after reduced chemotherapy for intermediate-risk neuroblastoma. N Engl J Med (2010).

[147] Thompson PM et al, CHD5, a new member of the chromodomain gene family, is preferentially expressed in the nervous system. Oncogene (2003).

[148] Thompson PM et al, CHD5, a new member of the chromodomain gene family, is preferentially expressed in the nervous system. Oncogene (2003).

[149] Internet source: www.ncbi.nlm.nih.gov.

[150] Internet source: www.ebi.ac.uk.

A

species	% protein identity	species	% protein identity
gorilla	99	horse	94
marcaque	99	cow	93
orangutan	99	marmoset	92
chimpanzee	98	mouse	92
pig	97	guinea pig	91
dog	96	tasmanian devil	89
elephant	96	chicken	87
gibbon	96	kangaroo rat	87
panda	96	turkey	87
dolphin	95	anole lizard	82
rat	95	cat	82
bushbaby	94		

B

species	% protein identity
chimpanzee	96
horse	96
rat	95
dog	95
cow	95
mouse	94

Table 2.1: Conservation of CHD5 between vertebrates expressed as % protein identity after alignment of predicted CHD5 protein sequences according to Ensembl (A) and NCBI database (B) with ClustalW.
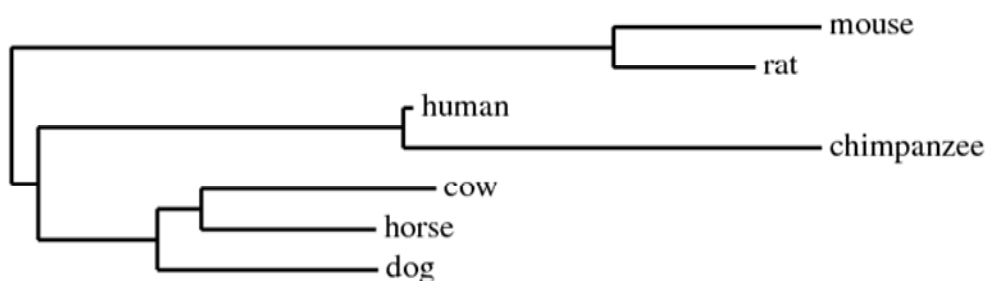
Fig. 2.4: Phylogenetic tree of CHD5 in selected species
The phylogenetic tree was constructed with the "one-click" method (www.phylogeny.fr.). Sequences from NCBI were used.

In addition, hCHD5 shows an identity of 70 % with hCHD3 and 75.5 % with hCHD4.[151]

In comparison to CHD4 which is ubiquitiously expressed in all tissues, CHD5 expression seems to be restricted to neural-derived tissues like brain and adrenal gland. CHD5 mRNA could not be detected in liver, placenta, spleen, bone marrow, thyroid, stomach, pancreas, small intestine, colon and prostate.[152]

Recently, CHD5 was identified as a tumor suppressor controlling proliferation, senescence and apoptosis. Bagchi et al[153] used chromosome engineering to generate different mouse models with gain and loss of a region on mouse chromosome 4 that corresponds to human 1p36.

A gain resulted in perinatal lethality caused by developmental abnormalities within the nervous system; MEFs harvested during late gestation showed reduced proliferation, increased apoptosis and senescence.

Loss of this region was tolerated during embryogenesis; in the corresponding MEFs an enhanced proliferation, facilitation of Ras-induced transformation and spontaneous immortalization were found. Furthermore, mice with a heterozygous deficiency of the (human) 1p36 locus were prone to develop several non-neural tumors (lymphoma, squamous cell carcinoma and hibernoma).

Within this region, expression of the candidate tumor suppressor genes was sequentially disrupted by specific shRNAs. Of these, CHD5 was identified to cause the observed phenotype. Further experiments revealed that CHD5 positively regulate p53 presumably via p14/p19[ARF].[154] The exact molecular mechanisms for these effects could not be defined.

The high sequence homology between CHD4 and CHD5 suggests that CHD5 might be also a part of a multi-protein complex like NuRD. Recently, Potts RC et al[155] identified CHD5 in a NuRD-like complex in brain containing MTA3, p66ß, MBD3, RbAp46 and HDAC-2. Furthermore, shRNA-mediated CHD5 depletion in primary rat neurons altered neuronal expression of genes implicated in aging, Alzheimer's disease and neuronal

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[151] Thompson PM et al, CHD5, a new member of the chromodomain gene family, is preferentially expressed in the nervous system. Oncogene (2003).
[152] Thompson PM et al, CHD5, a new member of the chromodomain gene family, is preferentially expressed in the nervous system. Oncogene (2003).
[153] Bagchi A et al, CHD5 is a Tumorsuppressor at Human 1p36. Cell (2007).
[154]Bagchi A et al, CHD5 is a Tumorsuppressor at Human 1p36. Cell (2007).
[155] Potts RC et al, CHD5, a Brain-Specific Paralog of Mi2 Chromatin Remodeling Enzymes, regulates Expression of Neuronal Genes. PLoS One (2011).

function. Besides that, CHD5 was found to bind to its target genes, thus suggesting a direct regulation.

2.6 Malignant Glioma and 1p deletions

2.6.1 Malignant Glioma

Malignant gliomas are a spectrum of tumors displaying various glial cell differentiation and malignancy grades. The major histopathological entities include oligodendroglioma, ependymoma and astrocytoma, but they also exist as a continuous spectrum.[156] The glioblastoma is the most aggressive form of an astrocytoma and the most common primary brain tumor in adults although it is a relatively rare event with an annual incidence of about 2/100 000 in Europe.[157] The prognosis is almost always fatal with a median survival of 4.7 months after diagnosis despite an overall improvement of multimodal cancer therapy. [158] Histopathologic hallmarks of glioblastomas are diffuse infiltration, large necroses and brisk glomeroid microvascular proliferation.

Although malignant gliomas (including glioblastomas) present many cancer typical features in their biology like oncogene and tumor suppressor alterations, they exhibit an otherwise unique biology: Most solid tumors expand first into areas of soft tissue where their growth is not physically limited, e.g. colon cancer into the gut lumen. The expansion of brain tumors necessitates active tissue destruction nearly right from the start because only a small part of the brain is occupied by the ventricular system as a possible space for tumor expansion without tissue destruction. Indeed, apoptotic cell death in tumor surrounding neurons can be observed.[159] A second unique finding is the mode of tumor cell spread. Glioma cells usually do not form metastases by passive circulation in the blood flow and the development of metastases itself is an extremely rare phenomenon. Infiltration of the brain takes place by active migration of tumor cells through the extracellular spaces in the brain.[160]

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[156] Louis DN, Molecular Pathology of Malignant Gliomas. Annu Rev Pathol Mech Dis (2006).
[157] Brandes AA et al, Glioblastoma in adults. Crit Rev Oncol Hematol (2008).
[158] Ohgaki H, Kleinhues P; Genetic Pathways to Primary and Secondary Glioblastoma. Am J Pathol (2007).
[159] Sontheimer H, Malignant gliomas: perverting glutamate and ion homeostasis for selective advantage. Trends Neurosci (2003).
[160] Sontheimer H, Malignant gliomas: perverting glutamate and ion homeostasis for selective advantage. Trends Neurosci (2003).

Besides these observations epigenetic alterations might contribute extensively to glioblastoma formation as suggested by mainly two findings:

Hypermethylation of the DNA repair gene O^6 Methylguanine DNA transferase (MGMT) promoter seems to be associated with a substantially better response to the alkylating agent temozolomide.[161] Data from preclinical experiments (e.g. Papi A et al[162], Svechnikova I et al*[163])* and case reports (e.g. Witt O et al[164]) suggest HDAC inhibition as a promising part of future glioblastoma therapy but clinical trials are discouraging so far.[165]

2.6.2 Loss of the short arm of chromosome 1 in malignant glioma

Loss of 1p occurs in primary and secondary glioblastomas with the same frequency (~15 %).[166] The combined loss of 1p and 19q is a distinctive genetic feature of oligodendrogliomas and associated with a better response to chemotherapy and prolonged survival.[167]

Two different types of 1p deletions are reported in general: Firstly, the complete loss of chromosome 1p associated with loss of 19q in oligodendroglial phenotypes. Secondly, the partial loss of usually 1p36 is mainly found in astrocytic gliomas. In oligodendrogliomas and low-grade astrocytomas LOH 1p /19q and p53 mutations are rarely detected in the same tumor indicating that these alterations lead to distinct molecular subtypes[168] or that the same molecular effect is achieved by these alternating events.

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[161] Hegi ME et al, MGMT gene silencing and benefit from temozolomide in glioblastoma. N Engl J Med (2005).

[162] Papi A et al, Epigenetic modifiers as anticancer drugs: effectiveness of valproic acid in neural crest- derived tumor cells. Anticancer Res (2010).

[163] Svechnikova I et al , HDAC inhibitors effectively induce cell type-specific differentiation in human glioblastoma cell lines of different origin. Int J Oncol (2008).

[164] Witt O et al, Valproic acid treatment of glioblastoma multiforme in a child. Pediatr Blood Cancer (2004).

[165] Friday BB et al, Phase II trial of vorinostat in combination with bortezomib in recurrent glioblastoma: a north central cancer treatment group study. Neuro Oncol (2011).
Iwamoto FM et al, A phase I/II trial of the histone deacetylase inhibitor romidepsin for adults with recurrent malignant glioma: North American Brain Tumor Consortium Study 03-03. Neuro Oncol (2011).

[166] Ohgaki H, Kleinhues P, Genetic Pathways to Primary and Secondary Glioblastoma. Am J Pathol (2007).

[167] Wernersbach Pinto L et al, Glioblastomas: correlation between oligodendroglial components, genetic abnormalities and prognosis. Virchows Arc (2008).

[168] Ohgaki H, Kleinhues P, Population-based studies on Incidence, Survival Rates, and Genetic Alterations in Astrocytic and Oligodendroglial Gliomas. J Neuropathol Exp Neurol (2005).

A recent study by Wernersbach Pinto et al[169] could not detect a different outcome of glioblastoma patients regarding their 1p status whereas Ichimura et al[170] found a significantly longer survival in patients with total 1p deletions.

A tumor suppressor mapped on chromosome 1p36 is not yet identified, but data from Ichimura et al[171] found CHD5 mostly included in the deleted area of 1p36 suggesting a possible function as a tumor suppressor. This is supported by the findings of Bagchi et al[172] showing absent expression of CHD5 in glioma samples in comparison to normal brain.

2.7 Inflammation and cancer

Chronic inflammatory and infectious conditions can trigger a variety of cancers. Thus, as prime examples the relation between Helicobacter pylori infection and gastric cancer[173] and gastric MALT lymphoma[174], respectively, chronic inflammatory bowel disease and colon cancer[175] as well as cervical cancer and human papilloma virus (HPV) infection[176] are omnipresent. The characteristics of cancer-related inflammation include the infiltration of the tumor and its environment with inflammatory cells and the presence of tumor-promoting chemokines and cytokines like TNFα and IL1ß. On the sub-cellular level, inflammation often operates on the same signalling pathways as oncogenic processes (like ras or myc). One of the key mediators of these is NF-κB. [177]

2.8 NF-κB, a key regulator of inflammation

The NF-κB family of transcription factors includes a number of structurally-related proteins that constitute homodimers and heterodimers. In mammals, it contains

[169] Wernersbach Pinto L et al, Glioblastomas: correlation between oligodendroglial components, genetic abnormalities and prognosis. Virchows Arch (2008).
[170] Ichimura K et al, 1p36 is a preferential target of chromosome 1 deletions in astrocytic tumors and homozygously deleted in a subset of glioblastoma. Oncogene (2008).
[171] Ichimura K et al, 1p36 is a preferential target of chromosome 1 deletions in astrocytic tumors and homozygously deleted in a subset of glioblastoma. Oncogene (2008).
[172] Bagchi A et al, CHD5 is a Tumorsuppressor at Human 1p36. Cell (2007).
[173] Reviewed in: Rathbone M and Rathbone B, Helicobacter pylori and gastric cancer. Recent Results Cancer Res (2011).
[174] Reviewed in: Sagaert X et al, Gastric MALT lymphoma: a model of chronic inflammation-induced tumor development. Nat Rev Gastroenterol Hepatol (2010).
[175] Reviewed in: Rizzo A et al, Intestinal inflammation and colorectal cancer: a double-edged sword? World J Gastroenterol (2011).
[176] Reviewed in: Zur Hausen H, Papillomaviruses in the causation of human cancers - a brief historical account. Virology (2009).
[177] Reviewed in: Mantovani A et al, Cancer-related inflammation. Nature (2008).

p50/p105, p52/p100, p65 (RelA), c-Rel and RelB. All these proteins share a N-terminal Rel homology (RH) domain that organizes DNA-binding, dimerization and IκB-binding. At their C-terminus RelA, RelB and c-Rel have transcriptional activation domains. In contrast, p105 and p100 possess inhibitory domains. p50 and p52 are the shortened and active forms of p105 and p100, respectively. The most abundant heterodimer is formed by p50/p65. When inactive, the heterodimer is kept in the cytoplasm by direct binding of the IκB (inhibitor of κB) protein. Upon stimulation by various signals NF-κB is released from its inhibitor and translocated into the nucleus.[178] As a transcription factor, the heterodimer binds specifically to its consensus site, the κB-site. This DNA-binding site spans 9 – 11 base pairs and is highly degenerated. It is usually represented as $G_{-5}\ G_{-4}\ G_{-3}\ R_{-2}\ N_{-1}\ N_0\ Y_{+1}\ Y_{+2}\ C_{+3}\ C_{+4}$ where R represents purine, N any nucleotide, Y pyrimidine and the subscribed number gives the relation to the assumed centre of the structure.[179]

Aberrant regulation of NF-κB plays an important role in many cancer entities. It can affect all six major hallmarks of cancer, namely cell proliferation, suppression of apoptosis, angiogenesis, acquisition of limitless replication, metastasis and inflammation.[180] However, the NF-κB network displays a confusing complexity: Depending on the context, it was also shown to promote apoptosis[181] and even a tumor suppressor-like role was suggested.[182]

2.9 Tumor necrosis factor alpha (TNFα)

Since I use the TNFα and TLR-9 pathway to study the impact of ATP-dependent chromatin remodelers on gene activation, I briefly describe these molecules and their pathways.

The history of tumor necrosis factor began with an amazing observation of the New Yorker surgeon William Coley. At the end of the 19th century, he started to treat sarcoma patients with filtrated bacterial cultures, the so-called 'Coley's mixed toxins',

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[178] Reviewed in: Rayet B and Gélinas C, Aberrant rel/nfkb genes and activity in human cancer. Oncogene (1999).
[179] Natoli G et al, Interactions of NF-kappaB with chromatin: the art of being at the right place at the right time. Nat Immunol (2005).
[180] Baud V and Karin M, Is NF-kappaB a good target for cancer therapy? Hopes and pitfalls. Nat Rev Drug Discov (2009).
[181] Ryan KM et al, Role of NF-kappaB in p53-mediated programmed cell death. Nature (2000).
[182] Keller U et al, Myc suppression of Nfkb2 accelerates lymphomagenesis. BMC Cancer (2010).

and achieved a hemorrhagic necrosis of this otherwise devastating disease. Although his work was controversial and poorly reproducible, the role of TNFα in cancer and inflammation as a currently major research topic was initiated by Coley's experiments.[183]

Despite the tumor-destructive role of TNFα, today used only for some rare indications, it has a paradoxical tumor-promoting activity. These pro-tumor actions are not fully understood but they are most likely related to its pro-inflammatory properties within the cancer microenvironment. [184]

Two human TNFα receptors are known: TNFR1 and TNFR2. TNFR1 is ubiquitously expressed and belongs to the death receptor family that possess the ability to induce apoptotic cell death. TNFR1 is activated by soluble TNFα. Binding of TNFα to TNFR1 leads to an activation of the NF-κB, MAPK pathway and AP-1 (see Fig. 2.5). The TNFR2 expression is restricted to hematopoietic cells and does not harbour a death domain like TNFR1. Its pathway has been less well studied.**[185]**

Both TNF receptors seem to participate in tumor promotion by TNFα. Within tumors, TNFR1 in mainly found on tumor and stroma cells whereas TNFR2 mainly resides on tumor-infiltrating immune cells. Mice deficient in TNFR1 exhibit an attenuated development of cancer in mouse models for cancer.[186] Interestingly, TNFR2 is highly expressed on $CD4^+$ $FoxP3^+$ regulatory T cells. Its activation contributes to activation, proliferative expansion and survival of Treg.[187] Several subsets of regulatory T cells were found to inhibit the antitumor immunity, thus, promoting tumor growth and metastasis.[188]

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[183] Balkwill F, Tumour necrosis factor and cancer. Nat Rev Cancer (2009).
[184] Balkwill F, Tumour necrosis factor and cancer. Nat Rev Cancer (2009).
[185] Wang X and Lin Y, Tumor necrosis factor and cancer, buddies or foes? Acta Pharmacol Sin (2008).
[186] Balkwill F, Tumour necrosis factor and cancer. Nat Rev Cancer (2009).
[187] Chen X and Oppenheim JJ, TNF-alpha: an activator of CD4+FoxP3+TNFR2+ regulatory T cells. Curr Dir Autoimmun. (2010).
[188] Byrne WL et al, Targeting regulatory T cells in cancer. Cancer Res. (2011).
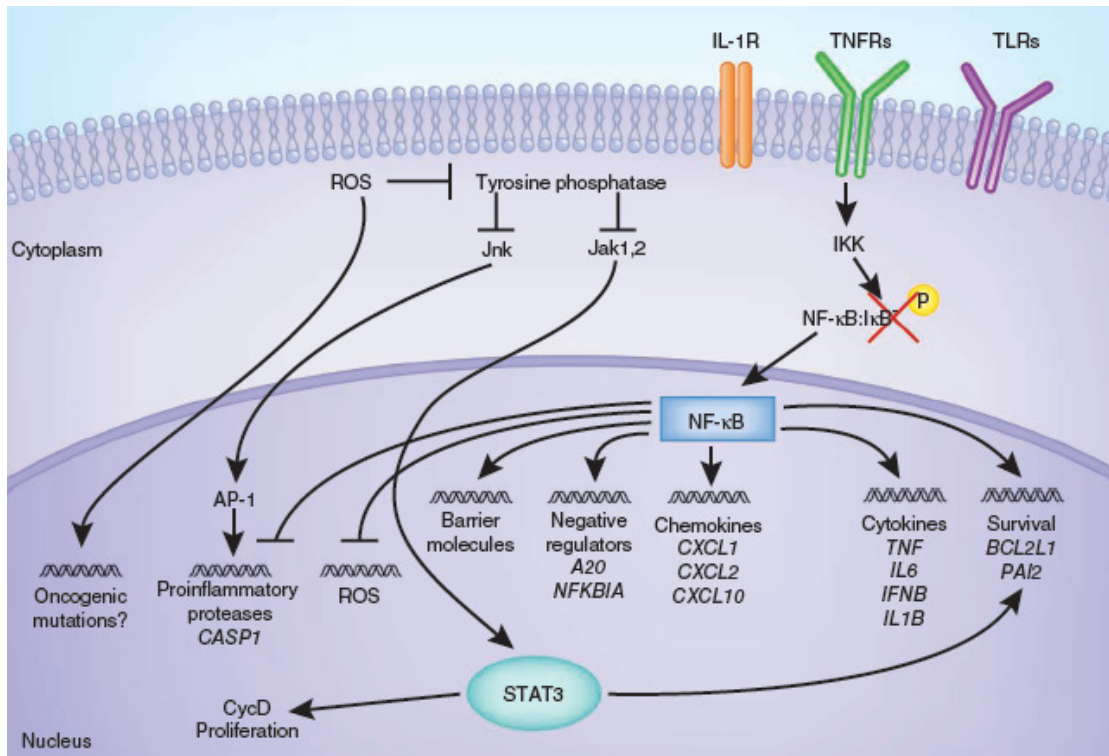
Fig. 2.5: Activation of NF-κB downstream of TNFR resulting in pro- and anti-inflammatory functions (modified after *Ben-Neriah Y and Karin M, Nat Immunol. 2011*)

2.10 Toll-like receptor 9 (TLR-9)

The mammalian family of Toll-like receptors (TLRs) is named after the *Drosophila melanogaster* gene *Toll*. That receptor is involved in Drosophila development and plays an important role in the defence against infections in the adult fly.[189]

In mammals, TLRs belong to the group of pathogen-associated molecular pattern (PAMP) recognition receptors that are important for cells of the innate immunity system to sense microbial structures. They recognize conserved molecules like lipopolysaccharide from Gram-negative bacteria (TLR-4), flagellin (TLR-5) or ssRNA (TLR-7). TLR-9 is located in the endosomal compartment and recognizes unmethylated CpG motifs that are a hallmark of bacterial DNA. Its activation results in an activation of the NF-κB pathway.[190]

TLR9 agonists are currently under investigation for cancer treatment. One of these so-called *immune modulatory oligo nucleotides* (IMOs) stimulating TLR-9 signalling,

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[189] Reviewed in: Chtarbanova S and Imler JL, Microbial sensing by Toll receptors: a historical perspective. Arterioscler Thromb Vasc Biol (2011).
[190] Kawai T and Akira S, The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol (2010).

IMO-2055, was demonstrated to possess anticancer activity in a mouse model. This anticancer activity was even amplified when it was used in combination with chemotherapeutic agents but the mechanism of action remains unclear.[191]

2.11 Objectives

The two main research objectives of this thesis focus both on ATP-dependent chromatin remodelers but are otherwise independent projects.

1. Analysis of expression pattern and function of the ATP-dependent chromatin remodeler CHD5.

2. Impact of different ATP-dependent chromatin remodelers on NF-κB induced transcriptional activation.

1.) Recently, the novel chromatin remodeler CHD5 has been identified (see chapter 2.5.2). So far, little is known about its distribution, function and biochemical characteristics. Therefore, we decided to generate polyclonal antisera against CHD5 for further studies on this protein. One main goal of this thesis is the characterization of these and commercially available antisera.

CHD proteins are in general expressed by very different patterns, for example, CHD4 and CHD6 are ubiquitously expressed whereas the expression of CHD7 becomes restricted to progenitor cells during development (see chapter 2.4.4). To gain insight into the expression pattern of CHD5, a detailed analysis is carried out. At the beginning of this thesis, CHD5 was thought to be restricted to neural-related tissues[192] but further evidence was required. Thereby, particular attention is paid to its expression in glioblastoma cell lines because CHD5 resides on 1p36, a locus commonly deleted in glioblastomas (see 2.6.2). This suggests a possible role as tumor suppressor in this cancer entity.

Furthermore, CHD5 has been implicated in senescence, cell cycle control and apoptosis.[193] In order to study its function, a suitable cell system is explored and some key experiments on its biological role are carried out.

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[191] Hennessy EJ et al, Targeting Toll-like receptors: emerging therapeutics? Nat Rev Drug Discov. (2010).
[192] Thompson PM et al, CHD5, a new member of the chromodomain gene family, is preferentially expressed in the nervous system. Oncogene (2003).
[193] Bagchi A et al, CHD5 is a Tumorsuppressor at Human 1p36. Cell (2007).

2.) Upon NF-κB activation by various stimuli, chromatin structure has to be opened up in order to allow the transcriptional machinery to get full access to the DNA. One major group of enzymes involved in these changes is the family of ATP-dependent chromatin remodelers. To study the impact of two typical family members, CHD4 and BRG1, on NF-κB induced transcriptional regulation, two pathways leading to NF-κB activation are chosen as models. The TNFα pathway is chosen to study the direct impact of chromatin remodelers on a subset of NF-κB target genes in HEK293 cells; the Toll-like receptor 9 (TLR-9) pathway is used in a NF-κB reporter cell system to get information about the influence of CHD4 and BRG1 on overall NF-κB activity.

Recently published data suggest an opposing role for BRG1 and CHD4 in macrophages upon LPS stimulation whereas BRG1 was required for activation of NF-κB induced transcription and CHD4 for the termination of it.[194] Whether CHD4 and BRG1 are also involved in TNFα and TLR-9-induced NF-κB activity and whether an opposing role of CHD4 and BRG1 represents an universal model in immunologic pathways needs to be determined.

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[194] Ramirez-Carrozzi VR et al, Selective and antagonistic functions of SWI/SNF and Mi-2beta nucleosome remodeling complexes during an inflammatory response. Genes Dev (2006).

3. Material and Methods | 39

3. Material and methods

3.1 Material

Unless otherwise stated, all common chemicals were ordered from Applichem, Biomol, Calbiochem, Fluka, Roth, SERVA and Sigma-Aldrich. Tubes, pipettes and any plastic ware were purchased from Greiner Bio-One, Sarstedt and Eppendorf. Enzymes were purchased from Fermentas, New England Biolabs, Roche and Promega.

3.1.1 Standard solutions

Stock solutions and buffers were prepared according to standard protocols. The most common solutions are listed below:

Phosphate Buffered Saline (PBS)
140 mM NaCl
2.7 mM KCl
8.1 mM Na_2HPO_4
1.5 mM KH_2PO_4
- pH adjusted to 7.4 with HCl

TAE buffer
40 mM Tris-Acetat
1mM EDTA

LB medium
1 % (w/v) tryptone
0.5 % (w/v) yeast extract
177 mM NaCl
- pH 7.0

LB agar plates
1.5 % agar
LB medium

SDS-PAGE running buffer
192 mM glycine
25 mM Tris
0, 1 % (w/v) SDS

5x SDS loading buffer
250 mM Tris-HCl pH 6.8
10 % (w/v) SDS
50 % (w/v) glycerol
0.5 % (w/v) bromphenolblue
500 mM DTT

3.2 Mammalian tissue culture

Material	Source
DMEM (with high glucose and L-glutamine)	PAA
RPMI 1640 + GlutaMax	Invitrogen
IMDM + GlutaMax	Invitrogen
Dulbecco's PBS without Ca^{2+} and Mg^{2+} (1x)	PAA
Trypsine (1x, 10x)	PAA
Sodium-pyruvate (100mM)	PAA
ß-mercaptoethanol	Invitrogen
Penicilline/Streptomycin	PAA
DMSO	Roth
Fetal Bovine Serum	Sigma-Aldrich
Horse Serum	Sigma-Aldrich
Poly-L-Lysin, 0.01 %	Sigma-Aldrich
OPTI-MEM + GlutaMax	Invitrogen
Oligofectamine Reagent	Invitrogen
Nanofect Reagent	Quiagen
HBSS (1x, without Ca^{2+} and Mg^{2+})	PAA
Dnase I	Sigma-Aldrich
Polyethylenimin (PEI)	Sigma-Aldrich
Puromycin	PAA
NGF (2.5 S) from murine submaxillary glands	Sigma-Aldrich
recombinant hTNFa	ImmunoTools
PTO 1668	Gift from A. Kaufmann
Diester 1668	Gift from A. Kaufmann
5-Azacytidine (50x)	Sigma-Aldrich
cell culture flasks, dishes, pipettes	Greiner

3.2.1 General cell line maintenance

All used cell lines, their growth medium and their source are listed below. Cells were cultured in tissue culture flasks in a humidified incubator at 37°C with 5 % CO_2. Tissue culture work was done under sterile conditions. Fresh medium was added at least twice per week regarding the half life-time of the used antibiotics. When cells have reached a confluence of 80-90 % they were trypsinized and splitted in a ratio between 1:2 to 1:10 depending on their growth behaviour and further use.

The cell number was determined using an improved Neubauer chamber. A Mycoplasma test was performed several times.

cell line	description	source	culture medium
Gl261	glioblastoma, murine	A. Pagenstecher, Dept. of Neuropathology, University Hospital Marburg	DMEM, 10 % FBS, 1 % PS
A172	glioblastoma, human	Chris van Bree, Academic Medical Center, Amsterdam	DMEM, 10 % FBS, 1 % PS
U373	glioblastoma, human	Chris van Bree, Academic Medical Center, Amsterdam	DMEM, 10 % FBS, 1 % PS
U87	glioblastoma, human	Chris van Bree, Academic Medical Center, Amsterdam	DMEM, 10 % FBS, 1 % PS
T98G	glioblastoma, human	Chris van Bree, Academic Medical Center, Amsterdam	IMDM, 10 % FBS, 1 % PS
U251	glioblastoma, human	Chris van Bree, Academic Medical Center, Amsterdam	DMEM, 10 % FBS, 1 % PS
SH-EP	neuroblastoma, human	Tobias Otto, IMT Marburg	RPMI, 10 % FBS, 1 % PS
SH-SY-5Y	neuroblastoma, human	Tobias Otto, IMT Marburg	RPMI, 10 % FBS, 1 % PS
SMS-KCN	neuroblastoma, human	Tobias Otto, IMT Marburg	RPMI, 10 % FBS, 1 % PS
IMR-32	neuroblastoma, human	Tobias Otto, IMT Marburg	RPMI, 10 % FBS, 1 % PS
Ht22	hippocampal neurons, murine	C. Culmsee, Dept. of Pharmacy, University of Marburg	DMEM, 10 % FBS, 1mM sodium -pyruvate, 2mM stable L-glutamate, 1 % PS
Pc12	pheochromocytoma, rat	M. Schäfer, Dept. of Anatomy, University of Marburg	RPMI, 10 % HS, 5 % FBS, 1 % PS
Hela	cervical carcinoma, human	G. Suske, IMT Marburg	DMEM, 10 % FBS, 1 % PS
HEK293T	human embryonic kidney	U.M. Bauer, IMT Marburg	DMEM, 10 % FBS, 1 % PS
SAOS-2	osteosarcoma, human	M. Eilers, Würzburg	DMEM, 10 % FBS, 1 % PS
HEK293-TLR9 -NF-κB- reporter	HEK293, stably expressing mTLR9 and NF-κB reporter	Andreas Kaufmann, Institute for Immunology, University of Marburg	DMEM, 10 % FBS, 1 % PS 50 µM ß-mercapto- ethanol

3.2.2 Mycoplasma Test

Primers were synthesized by MWG:

Name	Sequence
Myco3	5'-actcctacgggaggcagcagta-3'
Myco 5	5'-tgcaccatctgtcactctgttaacctc-3'

Mycoplasma is a small bacterium without a cellular wall. Its infection of cultured cells can disturb cell culture maintenance, growth behaviour and tranfectability leading to uncontrolled biased results. Infected cells are not detectable by light microscopy certainly; thus a regular exclusion of mycoplasma infection has to be performed. Therefore, 100 µl of the cell culture supernatant was cooked at 95 ° C for 5 minutes in an Eppendorf tube, followed by a brief centrifugation at 13 000 rpm in a *Hereus Biofuge pico*. The supernatant was transferred to a new tube and DNA precipitation was achieved by addition of 10 µl 10 mM TRIS/HCl, pH 8.8. After a centrifugation at 13 000 rpm the supernatant was transferred to a new tube and stored at 4°C. 1 µl was used as template in the PCR (25 µl total PCR volume). In a positive mycoplasma test a band at around 500 base pairs can be observed on agarose gel.

PCR program:

94° C for 3 min.

94° C 30 sec.

55° C 30 sec. } 38 cycles

72° C 30 sec.

72° C 5 min.

3.2.3 Kryoconservation

Medium I:
60 % DMEM/RPMI
40 % FBS

Medium II:
80 % DMEM/RPMI
20 % DMSO

Confluent cells from a 10 cm dish were washed with PBS and trypsinized with 3 ml trypsine-EDTA. After addition of 10 ml DMEM with 10 % FBS this mixture was transferred to a 15 ml tube and centrifuged at 1000 rpm for 5 minutes. The

supernatant was removed and the remaining cell pellet re-suspended in 500 µl Medium I followed by addition of 500 µl Medium II and transfer to a kryovial which was stored in 7 ml isopropanol at -80°C.

3.2.4 Thawing cells

Cells were thawed at room temperature and immediately seeded. After re-attachment of the cells to the culture flask bottom, the medium was removed to stop DMSO induced growth arrest, cells washed twice with PBS and fresh medium was added.

3.2.5 Primary astrocyte culture: Coating cell culture flasks with Poly-L-Lysin

To improve the attachment of the primary cells to the surface of the tissue culture flask the flasks were coated with Poly-L-Lysin. Therefore, the flasks were incubated with 2-5 ml of Poly-L-Lysin (MW > 300 000) overnight at 4° C. Afterwards the Poly-L-Lysin was removed and the flasks were washed twice with ice-cold PBS. Prepared flasks were kept at 4° C until use.

3.2.6 Primary astrocyte culture: Preparation and maintenance

Dnase I solution:
Dnase I 0.05 % (w/v)
0.25 % glucose (w/v)
10 mM HEPES
DMEM (+2 mM L-Glutamine)
10 % Horse Serum (HS)
1 % Penicillin/Streptomycin (PS)

In order to keep primary astrocytes into culture, 3 day old pups from C57BL/6 mice were decapitated; their forebrain cortices were harvested and freed of meninges and blood vessels. The extracted forebrains were washed with ice-cold Hank's balanced salt solution (HBSS) and incubated for 15 minutes at room-temperature in 1 % (w/v) trypsine solution. 7 ml HBSS were added and centrifuged for 8 minutes at 600 g at 4° C. The sediment was incubated for several minutes on ice in Dnase I solution and centrifuged again. The sediment was re-suspended in DMEM with 10 % HS and 1 % Penicillin/Streptomycin and cultured in Poly-L-Lysin coated 25 cm^2 flasks (one brain per flask). 6 hours later, non-viable and floating cells were removed, attached cells were washed with PBS and fresh medium was added. Afterwards, the medium was refreshed daily. The cells were kept until they reached confluence. Then, they were

thoroughly expanded. In the general maintenance of these cells there is no major difference to the used cancer cell lines regarding splitting procedures, freezing and thawing.[195]

It was assumed that any neurons and oligodendrocytes naturally present in the culture after the dissection of the brain are killed by the neurotoxicity of glutamate in the culture medium. A small percentage of microglial cells cannot be excluded but reduced by a gentle washing step with trypsine before splitting. Astrocyte presence in the culture was supported by their typical morphology.[196] The astrocyte culture was kept over several passages.

A   **B**
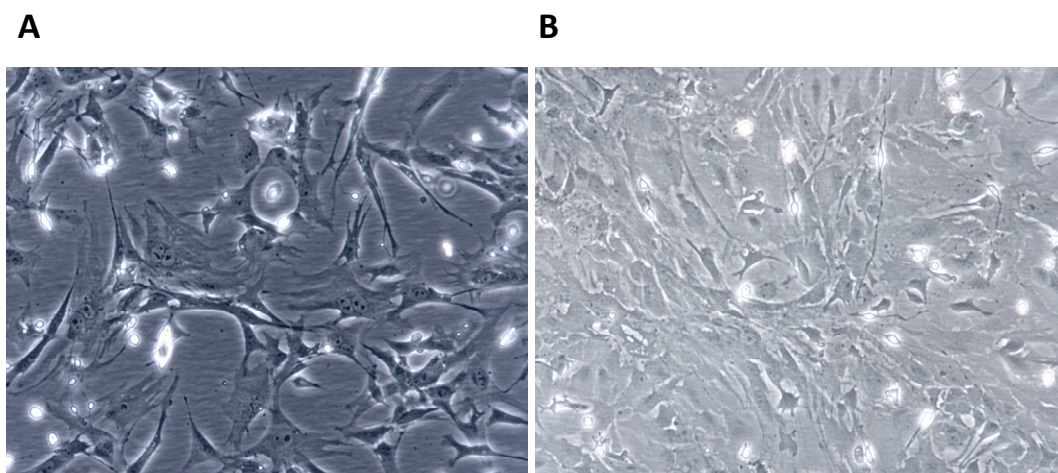
Fig. 3.1: Primary astrocytes in culture

Microscopic view of primary astrocytes in culture. A: after 5 DIV, B: after 8 DIV.

Pictures were taken with the IMT-2 light microscope (Olympus) with 100 fold magnification showing the typical astrocyte cell morphology.

3.2.7 Transient DNA-transfection of human cell lines

Plasmid	Source
pcDNA3.1/V5-His-TOPO/LacZ	Invitrogen
pcDNA3.1/V5-His-TOPO-hCHD5	Judith Bergs
pCMV-hRb(379-928)	A. Brehm
pcDNA3.1-hp53	Th. Stiewe, IMT Marburg
pcDNA3-hMi2beta-Flag	Bauer group, IMT Marburg

Cells were transfected with Nanofect, according to the manufacturer's instructions.

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[195] Protocol modified after N. Weiler, Inaugural-Dissertation, Medizinischen Fakultät der Rheinischen Friedrich-Wilhelms-Universität Bonn (2007).

[196] Nagasawa K et al, Astrocyte cultures exhibit P2X7 receptor channel opening in the absence of exogenous ligands. Glia, (2009).

3.2.8 Tranfection of HEK293 cells with siRNA

siRNA	sequence	source
siBRG1/Brm	gcuggagaagcagcagaag	Huuskonen J et al (2005)[197]
siCHD3	cguaugagcugaucaccau	Dharmacon, D-023015-01
siCHD4.1	ccaaggaccugaaugauga	Dharmacon, D-009774-01
siCHD4.2	caaaggugcugcugaugua	Dharmacon, D-009774-02
siNon-targeting#2	uaaggcuaugaagagauac	Dharmacon, pre-designed

On the day before transfection cells were seeded at a low densitiy. 4-6 hours prior to transfection cells were washed and the medium was exchanged to antibiotic-free medium. siRNAs and Oligofectamine were separately solved in OPTI-MEM (used amounts according to the dish size, see following table) and incubated for 5 minutes at room-temperature. Both mixtures were gently mixed and incubated for 20 minutes at room-temperature. In the meantime cells were washed and medium without serum and antibiotics was added. The siRNA-Oligofectamine-solution was added to the cells dropwise. 4 hours after transfection the doubled amount of the beforehand added medium with 20 % FBS was provided to the cells.

dish	siRNA	OPTI-MEM	Oligofectamine	OPTI-MEM	medium on cells
6-well-plate	10 µl	175 µl	3 µl	15 µl	800 µl
6 cm dish	20 µl	350 µl	7 µl	30 µl	1600 µl

3.2.9 Flat cell assay

PEI stock solution:
450 µl 10 % PEI in 50 ml H_2O, pH 7.0

A mixture of DNA with PEI leads to a DNA/PEI complex formation resulting in the formation of compact colloids which are taken up by the cell via endocytosis[198].

For PEI transfection 1×10^7 cells were seeded into a 25 cm² flask the day before transfection. For transfection 214 µl PBS and 6 µg DNA were mixted (3 µg vector coding for LacZ + 2 µg Rb, 3 µg CHD5 + 2 µg LacZ, 3 µg CHD5 + 2 µg Rb, each +1 µg pBABE) and a solution of 214 µl PBS with 9.8 µl PEI was added, briefly vortexed and incubated for 15 minutes at room-temperature. The cells were washed twice with PBS,

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[197] Huuskonen J et al, Activation of ATP-binding cassette transporter A1 transcription by chromatin remodeling complex. Arterioscler Thromb Vasc Biol. (2005).
[198] Godbey WT et al, Improved packing of poly(ethylenimine)/DNA complexes increases transfection efficiency, Gene Ther. (1999).

4 ml of DMEM with 2 % FBS were supplied and the DNA solution was added dropwise. 4 hours after transfection cells were washed again and 4 ml of DMEM with 10 % medium added.

48 hours post-transfection cells were trypsinized and 100 000 cells seeded on a 10 cm dish. 24 hours later puromycin was added at a concentration of 2µg/ml. Puromycin was refreshed after 48 hours. On day 4 of selection, cells were washed twice with ice-cold PBS, fixed with Methanol (-20°C) for 10 min, again washed with PBS and stained with crystal violett for 4 hours (2% crystal violett, 20 % Methanol) at 4°C on a shaker. Cells were washed with PBS. Around 200 cells (10 views) were counted under a light microscope (100 fold magnification). The experiment was done in triplicates and percentage of flat cells was calculated.

3.2.10 Treatment of cells with 5-Azacytidine

Cells were incubated with their normal growth medium including 1 or 10 µM 5-Azacytidine for 3 days. RNA and protein was extracted.

3.2.11 Differentiation of Pc12 cells with NGF (2.5 S) from murine submaxillary glands

Pc12 cells were seeded on Poly-L-Lysin coated 6 cm dishes in DMEM with 10 % HS, 5 % FBS and 1 % penicilline/streptomycin. 24 hrs later cells were washed twice with PBS and 3 ml of fresh medium (DMEM + 1 % FBS) with mNGF at a concentration of 10 ng/ml were added. Cells were kept over 7 days, medium and NGF were refreshed every second day.

3.2.12 NF-κB activation by stimulation with TLR-9 agonists and TNFα

For stimulation of HEK293 cells with TNFα at a concentration of 20 ng/ml TNFα was supplied in DMEM medium with 0.1 % FBS and cells were kept for 1-18 hrs under normal growth conditions.

For stimulation of TLR-9 cells were incubated in DMEM with 10 % FBS and 1 µM 1668 PTO/Diester for 2-18 hours under normal growth conditions.

3.3 Bioinformatics

DNA and protein sequences were taken from NCBI[199] and Ensembl.[200] Alignments were performed with ClustalW.[201]

Primers were designed by hand under calculation of melting temperature and exclusion of disturbing secondary structures by Oligo Analyser 3.0[202], with Roche Universal Probe Library Assay Design[203] and Primer3.[204]

Prediction of transcription factor binding was performed with the UCSC genome browser.[205]

Phylogenetic trees were generated using the "one-click"-method.[206]

3.4 Molecular biology

Standard procedures in molecular biology, including preparation of competent bacteria, transformation of chemically-competent bacteria with DNA, amplification of plasmid DNA in bacteria, purification, concentration determination, restriction enzyme digestion, analysis of DNA on agarose gels and amplification of DNA by polymerase chain reaction (PCR) were performed according to standard protocols.

In addition, plasmid DNA was prepared using plasmid purification kits (Qiagen).

3.4.1 Quantification of mRNA by RT-qPCR (real-time PCR)

The quantitative reverse transcription polymerase chain reaction is the currently most sensitive method for detection and quantification of mRNA. Compared to Northern blot and RNAse protection assay it allows a detection of smaller RNA amounts. As RNA cannot serve as template in PCR, the first step of a RT-PCR is the reverse transcription of the RNA into cDNA followed by the PCR itself. All currently available chemistries for real-time PCR allow the detection of the PCR products by generation of a fluorescent signal. One of them is SYBR Green, a fluorogenic dye. It exhibits little fluorescence in

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[199] www.ncbi.nlm.nih.gov
[200] www.ebi.ac.uk
[201] http://vit-embnet.unil.ch/software/ClustalW.html
Chenna R et al, Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Res (2003).
[202] Integrated DNA Technologies: www.eu.idtdna.com.
[203] www.roche-applied-science.com
[204] www.fokker.wi.mit.edu/primer3/input.htm
[205] http://genome.ucsc.edu
[206] www.phylogeny.fr; Dereeper A et al, Phylogeny.fr: robust phylogenetic analysis for the non-specialist. Nucleic Acids Res. (2008).

solution and emits a strong signal upon binding to the minor groove of double-stranded DNA. This fluorescent signal is measured after each PCR elongation step and increases when the PCR product accumulates.[207]

For relative quantification the target RT-PCR product is normalized to an internal control which is usually an abundantly expressed gene, called "housekeeping gene". This internal standard should be unaffected by the experimental procedure and expressed constantly.

As SYBR Green binds to any double-stranded DNA, also to primer dimers and unspecific products, leading to an overestimation of the target concentration, there is a strong need to exclude such disturbances by a precise melting curve analysis and a gel electrophoresis of the resulting PCR products. A melting curve is created at the end of the PCR by a slow increase of the temperature above the melting temperature of the amplicon while measuring the fluorescence which is then plotted as a function of the temperature. A specific product should result in a single peak at the amplicon's melting temperature.[208]

3.4.2 RNA isolation

Material	Source
peqGOLD Total RNA Kit, Safety Line	PeqLab
Injection water	B.Braun
Trizol	Invitrogen
Chloroform-Isoamylalcohol, 49:1	Fluka

RNA from human cell lines was extracted using PeqGold total RNA kit according to the manufacturer's instructions which allows a purification of RNA with a size of greater than 200 bases.

The RNA was finally eluted with RNase-free water; the concentration was measured by *ND-1000 Nanodrop* from PeqLab.

RNA from tissue, rat or mouse cell lines was extracted by Trizol (Invitrogen) using the manufacturer's protocol. RNA with an OD 260/280 between 1.8 and 2.18 was estimated to have a sufficient pureness. The RNA was stored at -80°C.

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[207] Pfaffl MW et al, Real-time RT-PCR: Neue Ansätze zur exakten mRNA Quantifizierung. BIOspektrum (2004).
[208] Bustin SA, Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J Mol Endocrinol. (2000).
Bustin SA, Nolan T. Pitfalls of quantitative real-time reverse-transcription polymerase chain reaction. J Biomol Tech.(2004).

3.4.3 cDNA synthesis

Material	Source
M-MLV Reverse Transcriptase	Invitrogen
dNTPs	Fermentas
oligo (dT)	MWG
random primers	Invitrogen
Aqua ad iniectabilia Braun	B.Braun
Ribolock	Fermentas

1-1.5 µg of total RNA, 1 µl oligo(dT) (500 µg/ml) and 1 µl 10 mM dNTP Mix (10 mM each dATP, dGTP, dCTP and dTTP at pH 7.0) were pipetted in an Eppendorf tube and filled up to 12 µl with RNAse-free distilled water. To open the secondary structure of the RNA this mixture was heated up at 70° C for 5 minutes and chilled on ice for 1 minute. The contents of the tube were collected by a brief centrifugation, then 4 µl 5X First Strand Buffer, 2 µl 0.1 M DTT and 1 µl RNAse-Inhibitor (RNAsin) were added and pre-warmed at 37°C for 2 minutes. After addition of 1 µl (200 units) M-MLV RT the mixture was incubated at 37°C for one hour. The enzyme activity was inactivated by heating for 15 minutes at 70°C. The cDNA pool was diluted (1:10) and stored at -20°C.

When cDNA synthesis was performed with random primers, 150 ng of these primers were used alternatively to oligo (dT) primers.

When RNA was isolated with Trizol, 2.0 µg as starting material for cDNA synthesis were used.

3.4.4 SYBR Green real-time PCR

Material	Source
Absolute QPCR SYBR Green Mix	Thermo Scientific
Thermo-Fast® 96 (0.2ml non-skirted 96-well PCR plates)	Thermo Scientific
Adhesive PCR Film	Thermo Scientific
Aqua ad iniectabilia Braun	B. Braun
Stratagene Mx3000P	Agilent Technologies

All listed primers were synthesized by MWG:

Name	Sequence	Description	Source
qCHD5-f	5'-agctggagctgtaccaca-3'	Human CHD5	
qCHD5-r	5'-tcttgtcaaagctatggttcagga-3'		
CHD5/2-F	5'-cctctgggaaaatctacgaca-3'	Human CHD5, located closed to 3'-end	
CHD5/2-R	5'-taccgtgggtcattctgga-3'		
hRPS14-fwd	5'-ggcagaccgagatgaatcctca-3'	Human RPS14	Eilers group
hRPS14-rev	5'-caggtccaggggtcttggtcc-3'		
CHD5-Maus-F	5'-aatgctgtcctgcacaagg-3'	Mouse CHD5	
CHD5-Maus-R	5'-tgctttcatgtcactcagca-3'		
ß-actin-f	5'-gatctggcaccacaccttct-3'	Mouse ß-actin	Bagchi et al[209]
ß-actin-r	5'-ggggtgttgaaggtctcaaa-3'		
rRPS14-F	5'-tcatcagccttggacctca-3'	Rat RPS14	
rRPS14-R	5'-ttactcggcaaatggtttcc-3'		
rCHD5-F	5'ctaccacttgcactgcctca-3'	Rat CHD5	
rCHD5-R	5'-cccttcagtgggggggacat-3'		
BRG1-F	5'-ccttcctcatcatcgtgcctc-3'	Human BRG1	Huuskonen J et al, 2005[210]
BRG1-R	5'-cggtgaccttcgtccacaatc-3'		
hBrm-F	5'-agaaaggctcacctgtgaaga-3'	Human Brm	
hBrm-R	5'-ccaaattgccgtcttcgat-3'		
CHD4-F	5'-tctgtccccgttgtacgtg-3'	Human CHD4	
CHD4-R	5'-ttgggatcagcatctggag-3'		
CHD3-F	5'-ggggcaagactgagaagga-3'	Human CHD3	
CHD3-R	5'-catcggcaaaggtttcagag-3'		
IL8-RT-F	5'-actgagagtgattgagagtggac-3'	Human IL-8	
IL8-RT-R	5'-aaccctctgcacccagtttc-3'		
CXCL2-F	5'-catcgaaaagatgctgaaaaatg-3'	Human CXCL2	Bauer group, IMT
CXCL2-R	5'-ttcaggaacagccaccaata-3'		
CCL2-F	5'-agtctctgccgcccttct-3'	Human CCL2	Bauer group, IMT
CCL2-R	5'- gtgactggggcattgattg-3'		
TNFa-F	5'- gtgactggggcattgattg-3'	Human TNFa	Bauer group, IMT
TNFa-R	5'-gccagagggctgattagaga-3'		
IRF1-F	5'-gagctgggccattcacac-3'	Human IRF-1	Bauer group, IMT
IRF1-R	5'- ttggccttccacgtcttg-3'		
IkB-F	5'cttgggtgctgatgtcaatg-3'	Human IkB	Bauer group, IMT
IkB-R	5'- acaccaggtcaggattttgc-3'		
CXCL10-F	5'-gaaagcagttagcaaggaaaggt-3'	Human CXCL10	Bauer group, IMT
CXCL10-R	5'-gacatatactccatgtagggaagtga-3'		

The primers for the gene of interest and the housekeeping gene should have a similar primer efficacy to avoid accumulating errors. Therefore, a dilution series for qCHD5

[209]Bagchi A et al, CHD5 is a Tumorsuppressor at Human 1p36. Cell (2007).
[210]Huuskonen J et al, Activation of ATP-binding cassette transporter A1 transcription by chromatin remodeling complex. Arterioscler Thromb Vasc Biol (2005).

and RPS14 (human) in the range from 1:5 to 1:1000 followed by the calculation of the primer efficacy was performed.

For QPCR, a reaction volume of 25 µl per well was used consisting of 6 µl cDNA and 19 µl PCR master mix (8 µl water, 0.5 µl of each primer at a concentration of 70 nM, 12 µl Sybr Green).

QPCR program:

step	temperature	time	cycles
enzyme activation	95°	15'	1x
denaturation	95°	15''	
annealing	60°	30''	40x
elongation	72°	30''	

Each experiment was done thrice in technical duplicates or triplicates.

Relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ method.[211]

The mean threshold cycle (Ct) of duplicates or triplicates was computed for each sample. The sample mean Ct for the housekeeping gene (ribosomal protein RPS14 in human system and rat samples, ß-actin for mouse) was subtracted from the sample mean Ct of the respective gene of interest (ΔCt). The mean ΔCt of the control (e.g. untreated sample, not-expressing sample) was selected as a calibrator and subtracted from the mean ΔCt of each experimental sample (ΔΔCt). The several fold change in gene expression (normalized to the housekeeping gene and relative to the control sample) was expressed as $2^{-\Delta\Delta Ct}$.

Product specificity was tested by generation of a melting curve after QPCR and an agarose gel electrophoresis (2%) on resulting QPCR products.

Result reliability for the CHD5 expression analysis was tested by a second CHD5-specific primer pair (CHD5/2) and the comparison of oligo (dT)-generated cDNAs with random-primers generated cDNA as a qPCR template.

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[211] Pfaffl MW, A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. (2001).

3.4.5 RT-PCR

All PCR reagents were purchased from Fermentas.

Primers used in RT-PCR:

Name	Sequence
mouse-iso 1-f	5'-cgtggaaggcatgatgt-3'
mouse-iso1-r	5'-ctttgttatcgcctggc-3'
mouse-iso2-f	5'-gtggaaggcgataacaaag-3'
mouse-iso 2-r	5'-aggtactcgttcatgttct-3'

PCR mix (30 µl):
MgCl$_2$: 3 µl
10x PCR buffer (-Mg Cl$_2$, +KCl): 3 µl
10 mM dNTPs: 2 µl
4 µM forward primers: 1 µl
4 µM reverse primers: 1 µl
Taq polymerase: 0.3 µl
H$_2$O: 15.7 µl
cDNA (1:10 dilution): 4 µl

PCR program:

step	temperature	time	cycles
enzyme activation	95°	5'	1x
denaturation	95°	30''	
annealing	55°	30''	35x
elongation	72°	30''	

PCR products were separated on a 1.5 % agarose gel, DNA was stained with ethidium-bromide and visualization was achieved with the *intas imager 2009 gel documentation system*.

3.5 Analysis of Proteins

3.5.1 Preparation of whole cell extract (WCE)

Lysis Buffer:
50 mM Tris pH 8.0
300 mM NaCl
10 mM MgCl$_2$
0.4 % NP-40
protease inhibitors (Leupeptin/Pepstatin/Aprotinin 1 µg/ml, PMSF (1 mM))
DTT (1mM)

<u>Preparation from adherent growing cell lines:</u>

Cells were washed once with ice-cold PBS. An appropriate amount of Lysis Buffer was added. After harvesting the cells with a cell-scraper, the lysate was transferred to a pre-cooled tube, incubated for 15 min on ice followed by additional 10 minutes in an ultrasound bath and finally centrifuged at 13 000 rpm and 4°C for 15 min in a table-top centrifuge. The supernatant was transferred to fresh tubes, frozen in liquid nitrogen and stored at -80°C. The protein concentration was determined by Bradford assay.

<u>Preparation from suspension cells:</u>

Cells were centrifuged at 1000 rpm for 3 minutes at room-temperature in a *Hereus Megafuge 1.0*. Medium was removed and the cell pellet was washed once in ice-cold PBS and again centrifuged at 1000 rpm for 3 minutes. After removal of PBS, the cell pellet was re-suspended in an appropriate amount of lysis buffer followed by the above described procedure.

3.5.2 Preparation of whole cell extract (WCE) from mouse/rat tissue

The tissue of interest was dissected and washed several times in ice-cold 0.9 % sodium-chloride solution to remove as much blood as possible. A part of the tissue was cut into small pieces, subsequently transferred into a Lysis Buffer filled and pre-cooled Eppendorf tube and incubated for 20-30 minutes on a rotation-wheel at 4° C, followed by the described procedure.

3.5.3 Preparation of nuclear extract (NE)

<u>Buffer A:</u>
10 mM Hepes/KOH pH 7.9
1.5 mM $MgCl_2$
10 mM KCl
- proteinase inhibitors and DTT were added freshly

<u>Buffer B:</u>
20 mM Hepes/KOH pH 7.6
1.5 mM $MgCl_2$
420 mM NaCl
0.2 mM EDTA
25 % glycerol
- proteinase inhibitors and DTT were added freshly

Cells were washed twice with ice-cold PBS. An appropriate amount of Buffer A (low salt buffer) was added. Cells were harvested with a cell scraper and transferred to an Eppendorf tube. After incubation on ice for 10 minutes, the lysate was briefly vortexed and centrifuged at 13 000 rpm for 10 seconds. The supernatant was kept representing the cytoplasmic fraction. The pellet was re-suspended in Buffer B (high salt buffer), incubated for 20 minutes on ice and centrifuged at 13 000 rpm for 10 minutes at 4°C. The supernatant was transferred to a fresh tube representing the nuclear fraction.

3.5.4 Determination of the protein concentration by Bradford Assay

Protein concentration was determined using the colorimetric assay as described by Bradford.[212] The Bradford reagent contains the dye 'Coomassie Brillant Blue' which changes its maximal absorption from 465 nm to 595 nm in presence of proteins. It is thought to be the most sensitive protein quantification based on a dye.[213]

3.5.5 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Denaturising SDS-PAGE[214] allows a separation of proteins according to their molecular weight irrespective of their electrical charge. [215]

Material	Source
Gel-Cassettes, 1.0 mm	Invitrogen
Novex Mini-Cell gel chamber	Invitrogen
PageRuler Prestained Protein Ladder	Fermentas
Rotiphorese® Gel 30	Roth
TEMED	Roth

SDS-polyacrylamide gel electrophoresis was performed using the Invitrogen "Novex Mini-Cell" system and pre-assembled gel cassettes. Running and stacking gels were poured according to standard protocols. Before electrophoresis, protein samples were mixed with 5x SDS-PAGE sample buffer and denatured by heating up to 95°C for 5 min. Proteins were separated at 24 mA (constant)/gel until the dye front had run out of the gel. The molecular weight of proteins was estimated by loading 5 µl of PageRuler pre-stained protein marker next to the protein samples. Following electrophoresis, proteins were stained with PageBlue or subjected to Western blotting.

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[212] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. (1976).

[213] Lottspeich F, Engels JW (eds.), Bioanalytik, München (2006)[2.]

[214] Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. (1970).

[215] Lottspeich F, Engels JW (eds.), Bioanalytik, München (2006)[2]

3.5.6 Coomassie Blue/PageBlue staining of protein gels

Material	Source
PageBlue Protein Staining Solution	Fermentas
Whatman paper, 1mm	Roth

Proteins on a SDS-PAGE gel can be visualized by Coomassie staining. Here, PageBlue staining was used alternatively. Therefore, the gel was washed with ddH$_2$O three times for 10 minutes on a shaker. The staining solution was added and incubated on a shaker overnight at room-temperature. Afterwards, the gel was again washed in ddH$_2$O three times. For documentation the gel was placed on a Whatman paper and dried for 2 hours at 60°C in a *Biorad gel dryer, Model 583*.

3.5.7 Western Blotting

Material	Source
Mini Trans-Blot Cell	BioRad
Immobilon Western Kit	Millipore
Hyperfilm ECL	GE-Healthcare
Roti PVDF	Roth

Transfer buffer:
25 mM Tris
192 mM glycine
0.005 % SDS
20% methanol (v/v)

Proteins were separated by SDS-PAGE as described followed by transfer to PVDF membranes using the *BioRad Mini Trans-Blot Cell*, a wet blot system. The PVDF membrane was activated in Methanol for 15 seconds and placed onto the gel and sandwiched by Whatman paper and sponges in a gel holder. The proteins were then transferred onto the membrane for 1 hour (proteins < 100 kDA) or 2 hours (>100kDa) at 400 mA (constantly). The transfer reaction was cooled by surrounding the gel chamber with ice and the addition of an ice block into the transfer chamber. To reduce the non-specific background, the PVDF membranes were incubated for 1-2 hr in blocking solution (PBS + 0.1% Tween-20 + 5-10 % dried milk) after transfer. The membranes were then incubated overnight on a horizontal shaker at 4°C with an appropriate dilution of the primary antibody (see following table).

Antibody	Dilution	Specificity	Species	Source
α-CHD5-K	1:1000	CHD5, Aa 661-676	rabbit, polyclonal	Ina Wanandi, Jakarta
α-CHD5-S	1:200	CHD5, Aa 46-61	rabbit, polyclonal	Ina Wanandi, Jakarta
α-CHD5-R	1:2000	CHD5, Aa 1386-1405	rabbit, polyclonal	Ina Wanandi, Jakarta
α-CHD5-K,S (pre-immune serum)	1:1000	pre-immune serum	rabbit	Ina Wanandi, Jakarta
α-CHD5 (M-182)	1:200-1:400	CHD5 from human, mouse and rat origin	rabbit, polyclonal	sc-68389, Santa Cruz
α-Mi2 (H-242)	1:300	Mi2	rabbit, polyclonal	sc-11378, Santa Cruz
α-BRG1 (H-88)	1:1000	BRG1	rabbit, polyclonal	sc-10768
α-p53 (DO-1)	1:1000	p53	mouse, monoclonal	sc-126
α-p53 (FL-293)	1:300	p53	rabbit, polyclonal	sc-6243, Santa Cruz
α-Flag	1:4000	Flag-Tag	mouse, monoclonal	Sigma
α-p65 (F-6)	1:300	RelA	rabbit, polyclonal	sc-109, Santa Cruz
α-ß-Tubulin	1:15 000	ß-Tubulin	mouse, monoclonal	MAB 3408, Chemikon
α-mouse ECL IgG	1:30 000	HRP-coupled secondary antibogy against mouse IgG/IgM	sheep, polyclonal	Amersham
α-rabbit ECL IgG	1:30 000	HRP-coupled secondary antibody against rabbit IgG/IgM	donkey, polyclonal	Amersham

PVDF membranes were washed five to six times in PBS + 0.1 % Tween-20 for 8-10 min and incubated for 50 minutes at room temperature with horseradish-peroxidase-coupled secondary antibodies. After five additional washes (7 min each, in PBS + 0.1 % Tween-20) antigen-antibody complexes were detected using the Immobilon™ Western Kit and autoradiography according to the manufacturer´s instructions.

3.5.8 Stripping of Western blot membranes

Stripping Buffer:
100 mM ß-mercaptoethanol
2% SDS
62.5 mM Tris-HCl pH 6.8
ddH$_2$O

To re-use a PVDF membrane, a disruption of the antigen-antibody binding is necessary. Thus, the membrane was incubated with 50 ml stripping buffer in a water bath at 50°C for 30 minutes and washed in PBS + 0.1 % Tween-20 twice for 10 minutes, followed by normal western blot procedure starting with the blocking step.

3.5.9 Immunoprecipitation

Material	Source
ProteinG beads	GE Health Care
ProteinA beads	GE Health Care
Low-binding Eppendorf tubes (1.5 ml)	Biozym

Flag IP Buffer:
25 mM Hepes pH 7.6
12.5mM $MgCl_2$
0.1 mM EDTA pH 8.0
10 % glycerol
0.1 % NP-40
150 mM NaCl
+ proteinase inhibitors and DTT

400-2000 µg of protein were diluted to a total volume of 600 µl with ice-cold PBS in a low-binding (siliconized) Eppendorf tube and incubated overnight on a rotation wheel at 4°C with an appropriate amount of antisera (for dilution see following table).

Antibody	dilution	specificity	species	source
α-CHD5-K	1:2-1:200	CHD5, Aa 661-676	rabbit, polyclonal	Ina Wanandi, Jakarta
α-CHD5-S	1:2-1:200	CHD5, Aa 46-61	rabbit, polyclonal	Ina Wanandi, Jakarta
α-CHD5-R	1:2-1:200	CHD5, Aa 1386-1405	rabbit, polyclonal	Ina Wanandi, Jakarta
α-CHD5 (M-182)	1µg antibody/500µg protein	CHD5 from human, mouse and rat origin	rabbit, polyclonal	sc-68389, Santa Cruz
α-Mi2 (H-242)	1µg antibody/500µg protein	Mi2	rabbit, polyclonal	sc-11378, Santa Cruz
α-BRG1 (H-88)	1µg antibody/500µg protein	BRG1	rabbit, polyclonal	sc-10768, Santa Cruz
α-p53 (DO-1)	1µg antibody/500µg protein	p53	mouse, monoclonal	sc-126, Santa Cruz
α-p65 (F-6)	1µg antibody/500µg protein	RelA	rabbit, polyclonal	sc-109, Santa Cruz

Protein A or G beads were washed 3 x 5 minutes in 1 ml of ice-cold Flag-IP buffer on a rotation wheel, centrifuged at 2000 rpm for 2 minutes at 4°C and pre-blocked in 1:1 1 % BSA/Flag-IP buffer overnight at 4 °C. The next day, 20 μl of this 1:1 slurry was pipetted to the protein-antisera-mixture and incubated on a rotation wheel at 4°C for 60 minutes. Afterwards, the mixtures were centrifuged at 2000 rpm for 2 minutes at 4°C and washed three times with 600 μl of ice-cold Flag-IP buffer. After the last washing step the supernatant was thoroughly removed and the beads were re-suspended in 40 μl of 2xSDS-loading buffer. This mixture was boiled at 92°C for 5 minutes and centrifuged at 2000 rpm for 2 minutes at room-temperature. 20 μl of the supernatant were loaded onto a SDS-PAGE and subjected to western blotting.

3.5.10 αFlag-Co-immunoprecipitation

Material	Source
Anti-Flag M2 agarose from mouse	Sigma-Aldrich

Flag IP Buffer:
25 mM Hepes pH 7.6
12.5 mM $MgCl_2$
0.1 mM EDTA pH 8.0
10 % glycerol
0.1 % NP-40
150 mM NaCl
+ proteinase inhibitors and DTT

αFlag M2 beads were equilibrated in Flag-IP buffer and 20 μl of a 1:1 bead slurry were added to 500 μg whole cell extract. This extract including the 1:1 slurry was incubated in Flag-IP buffer for 3 hours to overnight at 4°C on a rotating wheel. Afterwards, the beads were washed three times with Flag-IP buffer. After washing, beads were re-suspended in 40 μl of 2xSDS-loading buffer. This mixture was boiled at 92°C for 5 minutes and centrifuged at 2000 rpm for 2 minutes at room-temperature. 20 μl of the supernatant were loaded onto a SDS-PAGE and subjected to western blotting.

3.5.11 Co-immunoprecipitation

Protein G beads were equilibrated in Flag-IP buffer. 500 μg of nuclear extract were pre-incubated with benzonase for 2 hrs at 4° C. Then the extracts were incubated with the respective antibody overnight at 4°C on a rotating wheel and after addition of 20 μl of

protein G beads for another hour. The beads were washed three times with Flag-IP Buffer, re-suspended in 2 x SDS-PAGE loading buffer and analyzed by Western Blot.

3.5.12 Immunofluorescence staining (IF)

Material	Source
Microscope Slides 76x26mm	Thermo Scientific
Cover Slides 18x18mm	Roth
10% formaldehyde (methanol-free), UltraPure EM grade	Polysciences
Bovine serum albumine (BSA)	Sigma-Aldrich

Glass slides were washed with Methanol and air-dried before the cells were cultivated on them at the bottom of a 6-well-plate. For immunofluorescence staining cells were washed twice with ice-cold PBS, fixed in 1-2 ml 3.7 % formaldehyde/PBS for 10 minutes and again washed in PBS twice. The cells were permeabilized by incubation in 1 ml PBS/0.1 % Triton-X-100 for 15 minutes. After removal of the permeabilizing reagent by two washes with ice-cold PBS, unspecific binding was blocked by incubation with PBS/3% BSA at room-temperature for 30 minutes. Incubation with the antibody against the protein of interest was performed on parafilm in 100 μl of PBS/3 % BSA with an appropriate dilution of the antibody (see following table) in a humid chamber overnight at 4° C.

antibody	dilution	specificity	species	source
α-CHD5 (M-182)	1:100	CHD5 from human, mouse and rat origin	rabbit, polyclonal	sc-68389, Santa Cruz
α-p50 (H-119)	1:100	NF-κB subunit p50	rabbit, polyclonal	Sc-7178, Santa Cruz
α-p65 (F-6)	1:100	RelA	rabbit, polyclonal	sc-109, Santa Cruz
Alexa Fluor 488 goat α-rabbit IgG (H+L)	1:200	rabbit IgG, heavy and light chain	goat, polyclonal	invitrogen

The following day the glass slides were washed three times with PBS/0.1 % Triton-X-100/3 % BSA and incubated with a suitable secondary antibody at 4° C for 1 hour. Glass slides were again washed in ice-cold PBS three times.

Cellular nuclei were visualized by staining with 50 μl DAPI [1 μg/ml] for 1 minute. Glass slides were rinsed several times in PBS followed by washing in PBS three times for 5 minutes in the dark. Finally the slides were mounted with adherent cells upside down

and sealed with nail polish. Pictures were taken with the 020-519.010 fluoerescence microscope from Leica. Merging of pictures was performed with Photoshop.

3.5.13 Concentration of antisera by Protein G affinitychromatography

Material	Source
Poly-Prep® Chromatography Columns	BioRad
ProteinG beads	GE Health Care

Binding Buffer: 20 mM sodiumdihydrogenphosphate, pH 7.0 at 4°C

Elution Buffer: 0.1 M Glycin-HCl, pH 2.5 at 4° C

Neutralization Buffer: 1 M Tris-HCl, pH 9.0 at 4° C

To prepare the column, 3x 600 µl Protein G-Sepharose were washed in low-binding tubes with 1 ml of Binding Buffer for 5 minutes on a rotation wheel and centrifuged at 2000 rpm for 2 minutes. The supernatant was removed and the washing repeated twice. After the last washing step Binding Buffer was added in a ratio of 1:1. This slurry was loaded onto the column. After settling of the beads the column was connected to a peristaltic pump and washed with 10 ml of Binding Buffer, followed by 10 ml Elution Buffer and again 10 ml Binding Buffer. 1.5 ml of Antiserum was diluted with 8 ml Binding Buffer and loaded onto the column endless running through the pump overnight (flow rate always 1 ml/min).

Before the elution the column was washed with 30 ml of Binding Buffer. 4 ml of Elution Buffer were loaded onto the column and the flow through collected in eight fraction of 400-500 µl subsequently mixed with 150 µl Neutralization Buffer. Finally the column was washed with 30 ml Binding Buffer, 10 ml Elution Buffer and again 10 ml Binding Buffer and stored in 20 % Ethanol at 4°C.

To control the success of the procedure a sample of each step/fraction was taken and used for SDS Gel electrophoresis. The fractions of purified antiserum were stored at 4°C.

3.5.14 Chromatin-Immunoprecipitation (ChIP)

The protocol was modified after Upstate.

SDS lysis buffer
1% SDS
10 mM EDTA
50 mM Tris pH 8.0

IP buffer
0.01% SDS
1.1% Triton-X-100
1.2 mM EDTA
16.7 mM Tris-HCl pH 8.0
1 mM PMSF
1 µg/ml aprotinin
1 µg/ml pepstatin A

Low salt wash buffer
0.1% SDS
2 mM EDTA ph 8.0
20 mM Tris-HCl pH 8.0
150 mM NaCl
1% Triton-X-100

High salt wash buffer
0.1% SDS
1% Triton-X-100
2 mM EDTA pH 8.0
20 mM Tris-HCl pH 8.0
500 mM NaCl

LiCl wash buffer
0.25 M LiCl
1% NP-40
1% sodium-deoxycholate
1mM EDTA pH 8.0
10 mM Tris-Hcl pH 8.0

TE buffer
10 mM Tris-HCl pH 8.0
1 mM EDTA pH 8.0

Elution buffer
1% SDS
0.1% $NaHCO_3$

Material	Source
PeqGOLD Cycle-Pure Kit	PeqLab
10% formaldehyde (methanol-free), UltraPure EM grade	Polysciences

All listed primers were synthesized by MWG:

name	sequence	amplicon
CXCL2_0.4kbds-F	5'-ggcattcgaagtcacaggat-3'	region 0.4 kb downstream of CXCL2
CXCL2_0.4kbds-R	5'-ttggctttggtgggtttttag-3'	
CXCL2_Prom-F	5'-cgggagttacgcaagacagt-3'	CXCL2 promotor
CXCL2_Prom-R	5'-atcccgagttcggaagga-3'	
CXCL2-ChIP_prom2-F	5'-tcgccttccttccgaactc-3'	CXCL2 promotor
CXCL2-ChIP_prom2-R	5'-cgaacccctttatgcatggt-3'	
CXCL2_ex1-F	5'-ctggagctccgggaattt-3'	CXCL2 exon 1
CXCL2_ex1-R	5'-acccctttatgcatggttg-3'	
CXCL2_ex2-F	5'-gcagggaattcacctcaaga-3'	CXCL2 exon 2
CXCL2_ex2-R	5'-agcgatgggcgagacttac-3'	
CXCL2_intr3-F	5'-agggttagctgcaggactga-3'	CXCL2 intron 3
CXCL2_intr3-R	5'-gggcactgggaatatcctct-3'	
CXCL2_ex4-F	5'-ggggaaactgcattcagaaa-3''	CXCL2 exon 4
CXCL2_ex4-R	5'-gaaaatgggtgccctgagta-3'	
ß-actin_prom-F	5'-gaggggagaggggggtaaaa-3'	ß-actin promotor, source: Gromak N et al, 2006[216]
ß-actin_prom-R	5'- agccataaaaggcaactttcg-3'	

$3-4 \times 10^6$ HEK293 cells were seeded in 10 cm dishes on the day before chromatin preparation. Cells were stimulated with TNFα at a concentration of 20 ng/ml for 40 and 60 minutes or left untreated.

Formaldehyde was added to a final concentration of 1 % (1 ml). After 10 minutes incubation at room-temperature 1.3 ml 2 M glycine was added. Cells were incubated for 5 minutes at room-temperature, dislodged from the plate with a cell scraper, transferred to a 50 ml tube and centrifuged at 1800 rpm in a *Hereus Labufuge 400R* at 4°C for 10 min. The supernatant was removed, the pellet washed once in ice-cold PBS and again centrifuged at 1800 rpm at 4°C for 10 min. The cell pellet was then re-suspended in 210 µl/IP SDS lysis buffer and incubated on ice for 10 min, intermittently agitated. The lysate was transferred to a 15 ml tube and sonicated with

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[216] Gromak N et al, Pause Sites Promote Transcriptional Termination of Mammalian RNA Polymerase II. Molecular and Cellular Biology (2006).

30 cycles (10 sec. on, 30 sec. off) to achieve a fragmentation of the DNA to approximately 500 bp. The lysate was transferred to a 1.5 ml tube and centrifuged at 13 000 rpm at 4° C for 10 min in a *Hereus BiofugeA* and the supernatant was diluted 10 fold in IP buffer. The solution was pre-cleared by addition of 80 μl 50 % pre-blocked Protein G beads/1% BSA/TE buffer per IP for 40 minutes on a rotation wheel in the cold room. To remove the beads, the solution was centrifuged at 1500 rpm for 10 min at 4° C in a *Hereus Labufuge 400R*. 40 μl of the pre-cleared solution was kept as input at 4° C. 2 ml were used for each IP and incubated with a suitable amount of antibodies on the rotation wheel overnight in the cold room. The next day 35 μl of 50 % Protein G beads which were pre-blocked with 1 % BSA overnight were added to the IP and incubated for 2h on a rotation wheel at 4° C. Beads were collected by a 5 min centrifugation at 6400 rpm in a *Hereus Biofuge pico* at room-temperature and the supernatant was removed. The beads were re-suspended in 1 ml of low salt wash buffer, transferred to a siliconized 1.5 ml tube and centrifuged at 3000 rpm for 5 min. at 4° C in a *Hereus BiofugeA*. The immunoprecipitates were then washed once with low salt wash buffer, once with high salt wash buffer, once with LiCl wash buffer and twice with TE buffer. After the last washing the supernatant was removed carefully and immunoprecipitated DNA-protein complexes were eluted by addition of 250 μl of elution buffer. After 15 min of incubation at room-temperature, beads were spun down and the supernatant was transferred to a fresh tube. The elution procedure was repeated once and eluates were combined.

The input samples were diluted by addition of 460 μl elution buffer. 20 ml of 5 M NaCl were added to the eluates and inputs and incubated overnight at 65° C for de-crosslinking of the immunoprecipitated DNA-protein complexes. The next day, 10 μl of 0.5 M EDTA, 20 μl of 1 M Tris-HCl, pH 6.8 and 2 μl of 10 mg/ml proteinase K were added to each sample, followed by an incubation of 1 h at 45° C.

DNA was finally extracted with the PeqGOLD Cycle-Pure Kit, according to the manufacturer's instructions, eluting the DNA to a final volume of 30 μl.

For QPCR 6 μl of a 1:5 DNA dilution was used for input DNA and ChIP DNA.

The % input and the standard deviation were calculated as followed:

$$\Delta Ct = Ct_{Input} - Ct_{sample}$$

$$\% \text{ Input} = 2^{\Delta Ct(sample)}$$

$$S = \ln(2) \times \% \text{ input} \sqrt{(S_{Input}^2 + S_{sample}^2)}$$

3.5.15 Luciferase-Reporter Assay

Material	Source
Dual-Luciferase Reporter 1000 Assay System	Promega
AutoLumat LB953	EG&G Berthold

HEK293 cells stably transfected with the murine TLR9 and a NF-κB reporter were used to measure the NF-κB activity after different stimuli using the *Dual-Luciferase Reporter 1000 Assay System* from Promega according to the manufacturer's instructions and the luminometer *AutoLumat LB953* from EG&G Berthold. Experiments were performed in technical duplicats.
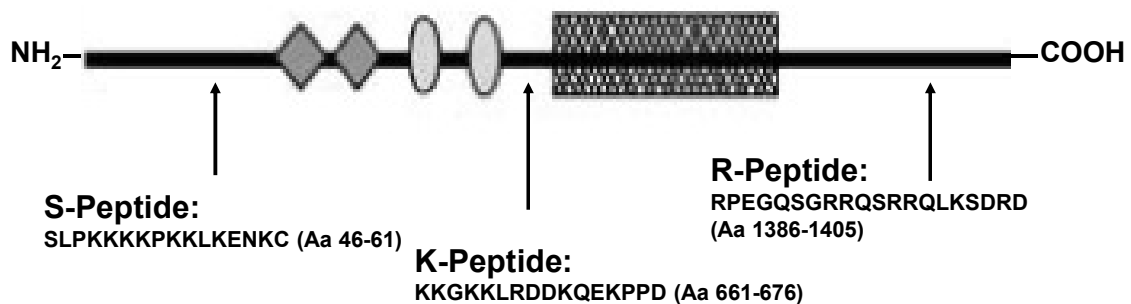
4. Results

4.1.1 Establishment of polyclonal peptide antibodies directed against three different regions of CHD5 protein

In order to detect and further biochemically characterize CHD5 protein, three polyclonal peptide antibodies were raised against different regions of human CHD5 (see Fig. 4.1).

These antisera were tested for their use in western blot, immunoprecipitation and immunofluorescence. Later during the study, a commercial CHD5 antibody (M-182) became available and was included in the characterization of our antisera for comparison.

The peptide sequences (R-, K- and S-peptide) that were specific to human CHD5 (and not present in related CHD family members as determined by blast search[217]) were chosen for immunization of rabbits. Of these, only the R-peptide matches additionally the corresponding sequences of dog, horse, cow, mouse, rat and chimpanzee CHD5, suggesting that the corresponding antibody could possibly recognize CHD5 protein in all of these species (see Fig. 4.2).

NH₂–   –COOH

R-Peptide:
RPEGQSGRRQSRRQLKSDRD
(Aa 1386-1405)

S-Peptide:
SLPKKKKPKKLKENKC (Aa 46-61)

K-Peptide:
KKGKKLRDDKQEKPPD (Aa 661-676)

Fig. 4.1 Scheme of peptide positions within the CHD5 protein

◆ = PHD zink finger   ⬭ = chromodomain   ▦ = Helicase/ATPase domain

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[217] Internet source: http://blast.ncbi.nlm.nih.gov.

S-peptide:

dog	GLPKKKKSKKLKENRS
horse	SLPKKKKSKKLKENRS
cow	SLPKKKKSKKLKENRS
mouse	SLPKKK-PKKLKESKS
rat	SLPKKK-PKKLKESKS
human	SLPKKKKPKKLKENKC
chimpanzee	SLPKKKKPKKLKENKC

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K-peptide:

dog	KKGKKLKDDKQEKPPD'
horse	KKGKKLKDDKQEKPPD'
cow	KKGKKLKDDKQEKPPD'
mouse	KKGKKLKDDKQEKPPD'
rat	KKGKKLKDDKQEKPPD'
human	KKGKKLRDDKQEKPPD'
chimpanzee	KKGKKLRDDKQEKPPD'

*****:********

R-peptide:

dog	RPEGQSGRRQSRRQLKSDRD
horse	RPEGQSGRRQSRRQLKSDRD
cow	RPEGQSGRRQSRRQLKSDRD
mouse	RPEGQSGRRQSRRQLKSDRD
rat	RPEGQSGRRQSRRQLKSDRD
human	RPEGQSGRRQSRRQLKSDRD
chimpanzee	RPEGQSGRRQSRRQLKSDRD

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Fig. 4.2: Alignment of peptide sequences used for immunization
(www.ebi.ac.uk/Tools/clustalw2/index.html)

The three peptides were synthesized by Peptide Speciality Laboratories, Heidelberg, Germany, and coupled to KLH (Keyhole Limpet Hämocyanine). Immunization of three rabbits (one per peptide) was carried out by Ina Wanandi, Jakarta/Indonesia.

The specificity of the antisera was tested by direct comparison of the pre-immune serum and the crude antiserum in western blot using purified recombinant Flag-tagged CHD5, protein extracts from HEK293 cells transiently transfected with pcDNA3.1/V5-His-TOPO-hCHD5 (Flag-tagged CHD5) and protein extract from untransfected HEK293 cells (negative control). In addition, mouse brain extract was analysed to determine whether the antisera were capable of detecting endogenous CHD5.

Immune serum S strongly recognized a protein with an apparent molecular mass of greater than 170 kDa when baculovirus-expressed recombinant CHD5 protein was analyzed (Fig. 4.3, lane 5). This protein did not significantly react with the corresponding pre-immune serum (see Fig. 4.3, lane 1 in comparison to lane 5). Moreover, immune serum S detected a protein with the same electrophoretic mobility in extract from HEK293 cells expressing recombinant CHD5 protein (Fig. 4.3, lane 7) but not in extract from control HEK293 cells (lane 8).

Again, the pre-immune serum failed to detect a corresponding protein in neither HEK293 extract (Fig. 4.3, lanes 3 and 4). The electrophoretic mobility of the protein recognized by serum S is in agreement with the theoretical molecular mass of CHD5 (223 kDa). These results demonstrate that immune serum S is capable of reacting with recombinant CHD5 in Western blot.

By contrast, serum S did not detect a protein with the same electrophoretic mobility in mouse brain extract (Fig. 4.3, lane 6). Given that the mouse brain extract contains CHD5 (see also Fig. 4.5 A, lane 2; Fig. 4.5 B, lane 2), this indicates that the crude serum S is not able to detect endogenous levels of CHD5 in *Mus musculus.* Predicted by

sequence alignment of CHD5 with S-peptide, immune serum S is specific for *Homo sapiens* and possibly *Pan troglodytes* (not tested; see Fig. 4.2).
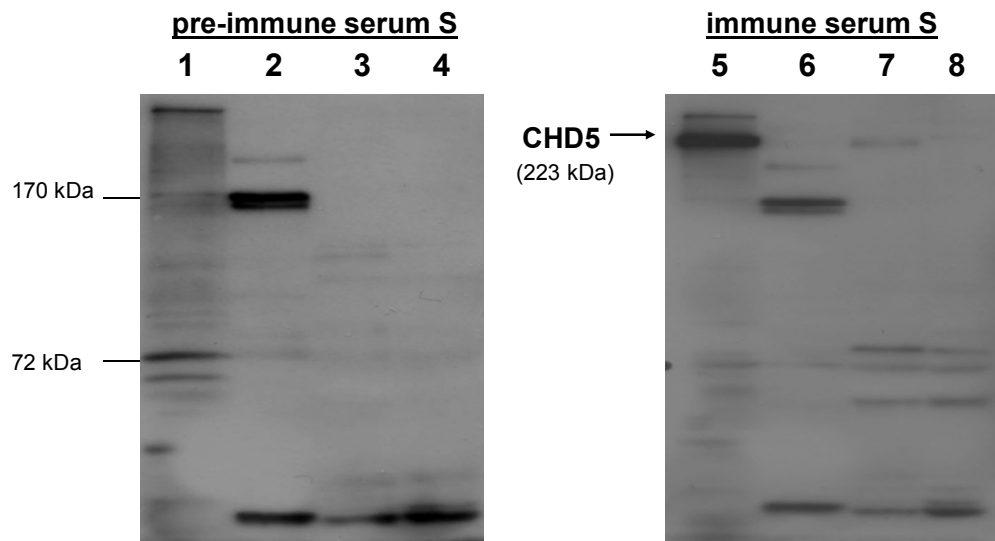
Fig. 4.3: **Generation of a polyclonal antiserum against the N-terminal part of CHD5 (α-CHD5-S).**

The reactivity of the pre-immune serum and the unpurified immune serum was tested in western blot using purified recombinant CHD5 (lanes 1 and 5), mouse brain protein extract (lanes 2 and 6), WCE from HEK293 cells transiently transfected with CHD5-Flag (lanes 3 and 7) and from untransfected HEK293 cells as a negative control (lanes 4 and 8).

Immune serum K detected a protein with an apparent molecular mass of more than 170 kDa which is consistent with the estimated molecular mass of CHD5 when purified recombinant CHD5 was used in Western blot (see Fig. 4.4, lane 5). In agreement, antiserum K recognized a protein with the same electrophoretic mobility in extract of HEK293 cells transiently transfected with Flag-tagged CHD5 (Fig. 4.4, lane 7). The pre-immune serum neither recognized a corresponding protein (see Fig. 4.4, lanes 1 and 3) nor was a corresponding band detected by serum K in HEK293 control extract (Fig. 4.4, lane 8). In summary, immune serum K is able to detect recombinant CHD5 in Western blot.

Immune serum K does not react with CHD5 in mouse brain extract (see Fig. 4.4, lane 6) as expected because the peptide sequence mismatches the CHD5 sequence from *Mus musculus*. According to the peptide sequence immune serum K is regarded to be specific for *Homo sapiens* and possibly *Pan troglodytes* (not tested; see Fig. 4.2). No human tissue was available for a further assessment of endogenous CHD5.
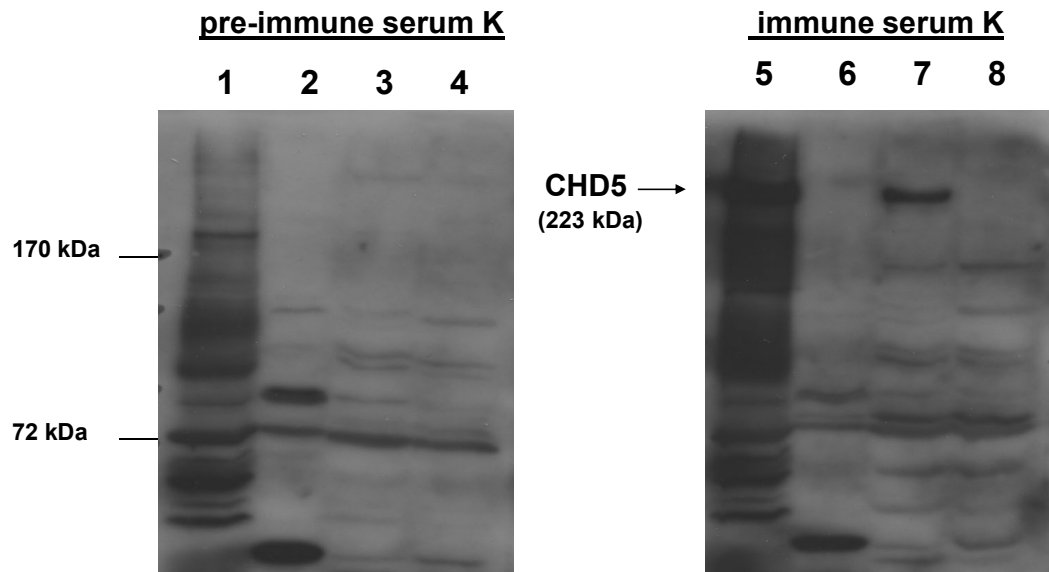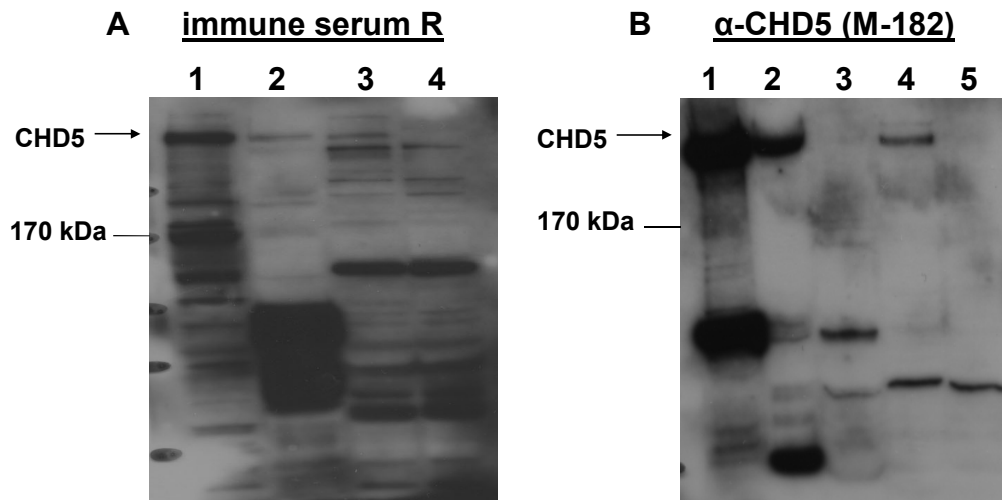
Fig. 4.4: Generation of a polyclonal antiserum: α-CHD5-K.

The reactivity of the pre-immune serum and the unpurified immune serum was tested in western blot using purified recombinant CHD5 (lanes 1 and 5), mouse brain protein extract (lanes 2 and 6), WCE from HEK293 cells transiently transfected with CHD5-Flag (lanes 3 and 7) and from untransfected HEK293 cells as a negative control (lanes 4 and   8).

When recombinant CHD5 was analyzed in Western blot probed with immune serum R, this antiserum recognized a protein with similar electrophoretic mobility as the protein detected by both other antisera (see Fig. 4.5 A, lanes 1 and 3 in comparison with Fig. 4.3, 4.4). No corresponding band appeared in extract from untransfected HEK293 cells (negative control) (see Fig. 4.5 A, lane 4). No pre-immune serum was available from the rabbit immunized with R-peptide; therefore a comparison could not be carried out. Antiserum R does additionally detect a corresponding protein in mouse brain extract (Fig. 4.5 A, lane 2). This indicates that it is capable of detecting endogenous levels of CHD5 in extracts from *Mus musculus*.

In conclusion R-peptide immune serum recognizes recombinant CHD5 and endogenous CHD5 from mouse brain in Western blot, whereas no corresponding signal is detected in extract from control HEK293 cells.

In addition to recombinant CHD5, all three antisera also react with a number of other proteins, several of which are also recognized by the pre-immune sera or that are detected in both CHD5-expressing and non-expressing HEK293 cell extracts. Some of these Western blot signals are likely due to cross reactivity of the antibodies.

Fig. 4.5: Immune serum R (against the C-terminus of CHD5) and the CHD5 antibody M-182

A: Generation of a polyclonal antiserum against the C-terminal part of CHD5 (α-CHD5-R), 3rd bleed. Antiserum reactivity was tested with purified recombinant CHD5 (lane 1), protein extract from mouse brain (lane 2), WCE from transiently with CHD5-Flag transfected HEK293 cells (lane 3) and WCE from untransfected HEK293 cells (lane 4) as a negative control.

B: Polyclonal CHD5 antibody (M-182, sc-68389), dilution 1:300.
Antiserum reactivity was tested with purified recombinant CHD5 (lane 1), protein extract from mouse brain (lane 2), WCE from mouse glioblastoma cell line Gl261 (lane 3), WCE from transiently with CHD5-Flag transfected HEK293 cells (lane 4) and WCE from untransfected HEK293 cells (lane 5) as a negative control.

When the polyclonal CHD5 antibody M-182 became available, it was included in the analysis. M-182 recognized a protein above 170 kDa with the same electrophoretic mobility as that one recognized by our antisera using purified recombinant CHD5 and extract from HEK293 cells expressing recombinant CHD5 (Fig. 4.5 B, lanes 1 and 4). Besides, M-182 detected a strong band in mouse brain extract (Fig. 4.5 B, lane 2). No corresponding band appeared when extract from control HEK293 cells or from mouse glioblastoma cell line Gl261 (not CHD5 expressing) was loaded (Fig. 4.5 B, lanes 5 and 3, respectively).

To further confirm that the detected band above 170 kDa is CHD5, a Western blot using extract from HEK293 cells expressing recombinant Flag-tagged CHD5 was probed with α-Flag antibody in comparison with α–CHD5 (M-182). The α-Flag antibody stained a directly corresponding band to that one detected by α–CHD5 (see Fig. 4.6, compare lanes 1 and 7) indicating that both detected proteins are indeed identical.
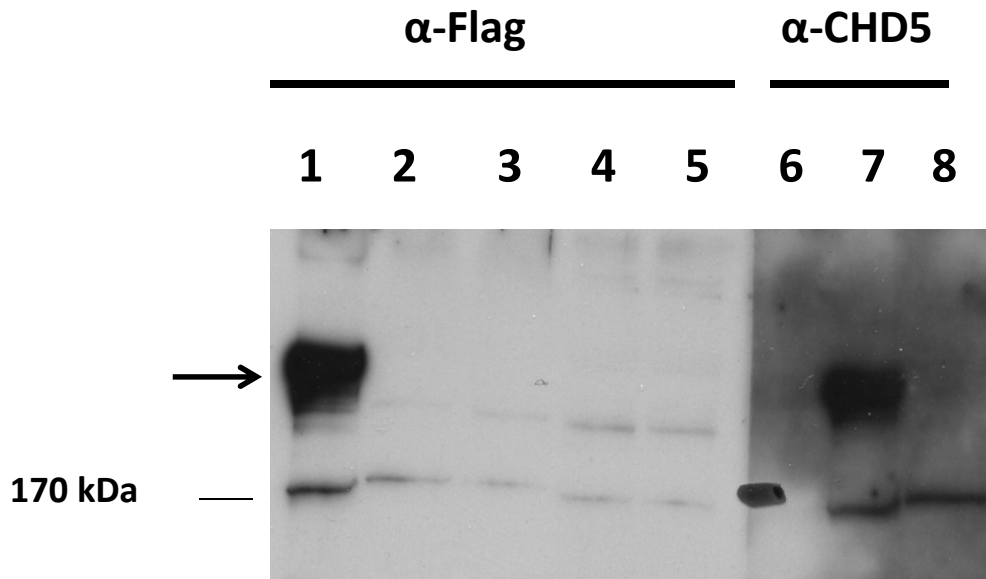
Fig. 4.6: α-Flag recognizes a protein with the same molecular weight as α-CHD5 (M-182)
To confirm the band (→) above 170 kDa detected by M-182 as CHD5, a Western blot with WCE from HEK293 cells transiently transfected with CHD5-Flag (lanes 1 and 7) was probed with α-Flag antibody in comparison to α-CHD5 (M-182). Both antibodies recognized a protein with the same electrophoretic mobility.
Lanes 2-5, 8: WCE from cell line A172 (of no further interest). Lane 6: molecular weight marker.

CHD5 protein shows a high sequence homology to other members of the CHD protein family, especially to Mi2ß (see Introduction). Although the CHD5 peptide sequences used for antiserum generation do not show any sequence homology to other CHD proteins, an extract of Mi2ß-overexpressing HEK293 cells (transfected with pcDNA3-hMi2beta-Flag) was tested in western blot probed with the CHD5 antisera to exclude any cross reactivity to Mi-2ß. Regarding the electrophoretic mobility, Flag-tagged Mi2ß is expected to run slightly above Flag-tagged CHD5. A faint band at this level was observed for S-peptide antiserum (see Fig. 4.7, lane 2). Anyhow, this band appears similarly in extract from Mi2ß-overexpressing cells as well as from untransfected HEK293 cells without a difference in intensity (Fig. 4.7, compare lanes 2 and 3). Additionally – as already mentioned – S-peptide does not show any sequence homology to Mi2ß. Hence, cross reactivity with Mi2ß cannot be excluded but is still very unlikely.

For K-peptide antiserum and α-CHD5 (M-182) there was no hint for a cross reactivity with Mi2ß because no further band could be detected (see Fig. 4.7, lanes 4-9).
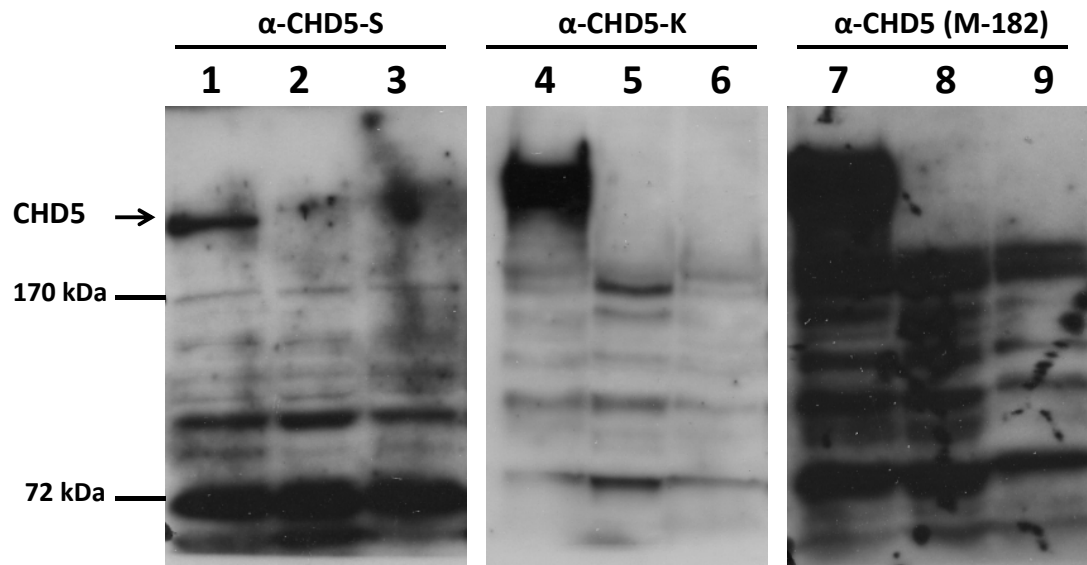
Fig. 4.7: Cross reactivity of CHD5 antibodies with CHD4

To exclude a cross reactivity of CHD5 antisera with CHD4, HEK293 cells were transiently transfected with CHD5-Flag (lane 1, 4 and 7) in comparison with Mi2ß-Flag (lanes 2, 5 and 8). Untransfected HEK293 cells were used as control (lanes 3, 6 and 9). Mi2ß is expected to run slightly higher than CHD5. A faint band appeared at that level in lane 2 and 3. Thus, a cross reactivity cannot be excluded for α-CHD5-S. No band was observed above CHD5 in lanes 5 and 8 (α-CHD5-K and M-182), making a cross reactivity for α-CHD5-K and M-182 unlikely.

Using the crude antisera in Western blot, the imbalance between weak specific bands and strong background signals can impede their application. Therefore, a concentration of the antibody might be quite helpful.

For concentration of antibodies the crude antisera were purified by affinity chromatography with Protein G-Sepharose columns.

Protein G is a part of the cellular wall of Group C/G Streptococci which is characterized by its strong pH dependent affinity for the Fc region of immunoglobulin G making it suitable for IgG purification. The dramatic decrease of this affinity by lowering the pH is the basis for the purification. But it has to be mentioned that the natural spectrum of antibodies occurring in rabbits as well as antibodies against the carrier substance are not removed, so it is unlikely that any cross reactivity of the antibodies decreases.[218] As a minor effect non-IgG proteins like albumin are also removed.

The success of the concentration was demonstrated by SDS-PAGE loading samples from every different step of the purification (as example see antiserum K purification,

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[218] Harlow E and Lane D, Using Antibodies: A laboratory Manual; New York (1999), p.74-76. Internet source: www.nunc.de.

Fig. 4.8). The heavy and the light chain appear nearly exclusively in the third to eighth elution fraction as expected.

After concentration α-CHD5-R did not detect recombinant CHD5 in western blot any longer (data not shown). Probably, this might be due to the changes in pH during the elution. Thus, its further application was limited and M-182 was the preferred antibody when material from mice was analysed.

For antisera K and S the concentration facilitated its use and no decrease in affinity was observed.
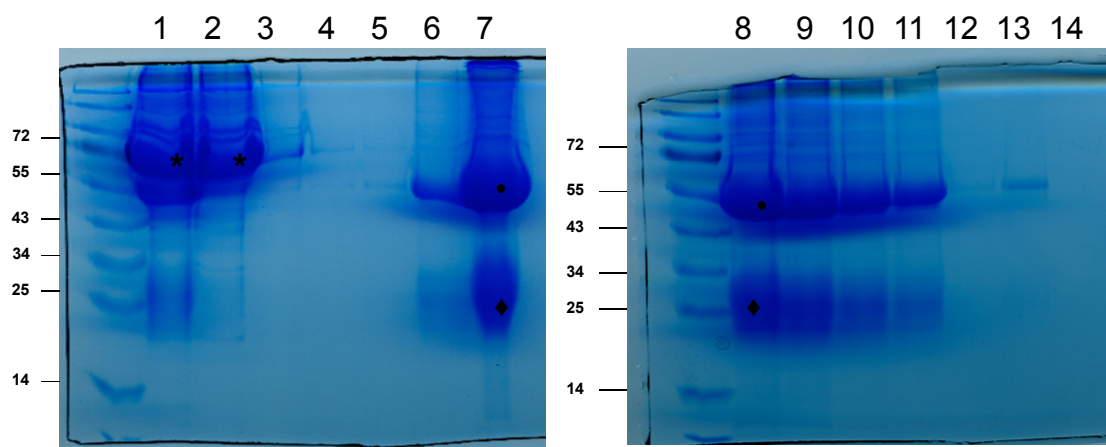
Fig. 4.8: Concentration of crude CHD5-K antiserum by Protein G affinity chromatography

**Samples from each step of the purification process were loaded on a 12.5 % SDS-PAGE.**

Lane 1: input, lane 2: flow-through, lane 3: washing step, lane 4-11: fractions 1-8, lane 12: wash step with binding buffer, lane 13: wash step with elution buffer, lane 14: wash step 2 with binding buffer.

● = antibody heavy chain   * = albumine   ♦ = antibody light chain

The different antisera as well as M-182 antibody were tested for their ability to immunoprecipitate CHD5. Unfortunately, all three antisera (K, S and R) were not able to immunoprecipitate recombinant CHD5 as well as endogenous CHD5 from mouse brain extract although very high concentrations of antibody were tested (up to a dilution of 1:2; see Fig. 4.9 for immune serum S as example).

Only M-182 successfully immunoprecipitated recombinant CHD5 as well as endogenous CHD5 from mouse whole cell extracts (Fig. 4.10).
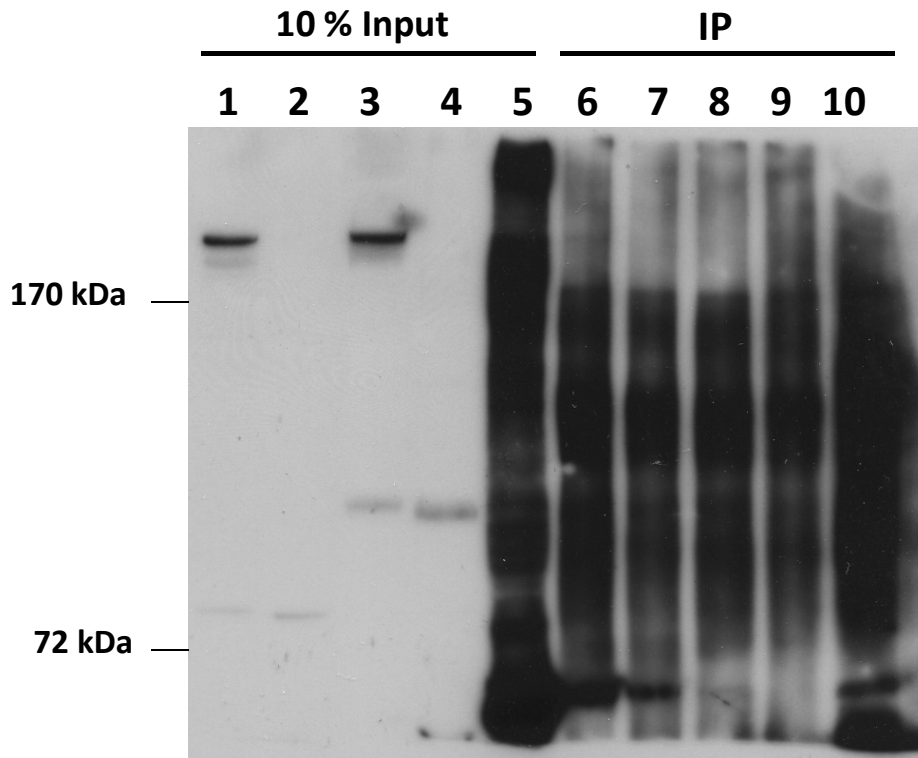
Fig. 4.9: S-peptide antiserum does not immunoprecipitate CHD5

The S-peptide antiserum was tested for its ability to immunoprecipitate CHD5. The S-antiserum was used in a dilution of 1:100 for IP, M-182 probed the following western blot. WCE from HEK293 cells transiently transfected with CHD5-Flag was used in lanes 1 and 6, WCE from HEK293 in lanes 2 and 7, from mouse brain in 3 and 8, from mouse glioblastoma cell line Gl261 in 4 and 8 and from astrocytes in lane 5 and 10.
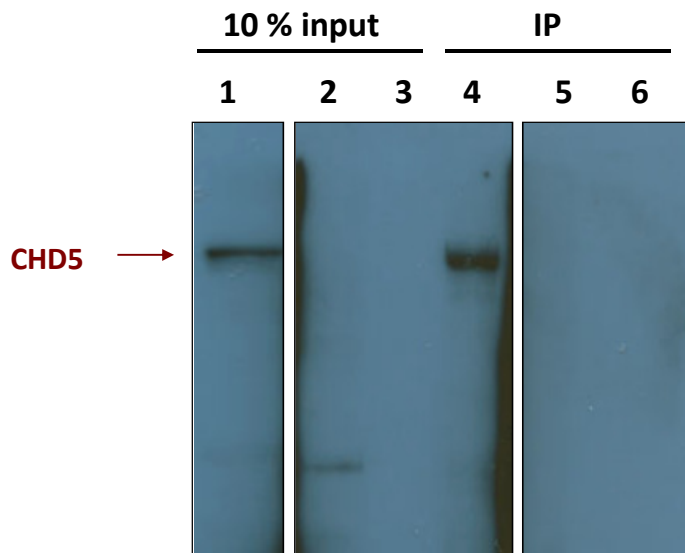

Fig. 4.10: M-182 does immunoprecipitate CHD5 from mouse brain extract

The M-182 antibody was tested for its ability to immunoprecipitate CHD5 from mouse brain WCE (lanes 1 and 4). The mouse glioblastoma cell line Gl261 (lanes 2 and 5) and PBS (lanes 3 and 6) were taken as negative controls. The antibody was used in a dilution of 1:200 in IP and 1:300 in the following western blot.

The different antisera as well as the M-182 antibody were tested in immunofluorescence for detection of overexpressed CHD5 in HEK293 cells.

Our antisera turned out to be inapplicable for immunofluorescence. Antisera K and S produced a diffuse unspecific staining (pictures not shown). Immune-serum R gave a homogenous nuclear pattern in CHD5-overexpressing cells as well as in control HEK293 cells in equal intensity (see Fig. 4.11). Therefore, it was considered as unspecific staining.

CHD5 - antibody (M-182) gave no apparent background and some bright nuclear signals. Assuming a mean transfection efficacy these few signals were reasonable. No staining was observed in HEK293 control cells. Therefore, this immunofluorescence staining was thought to be specific (see Fig. 4.12).
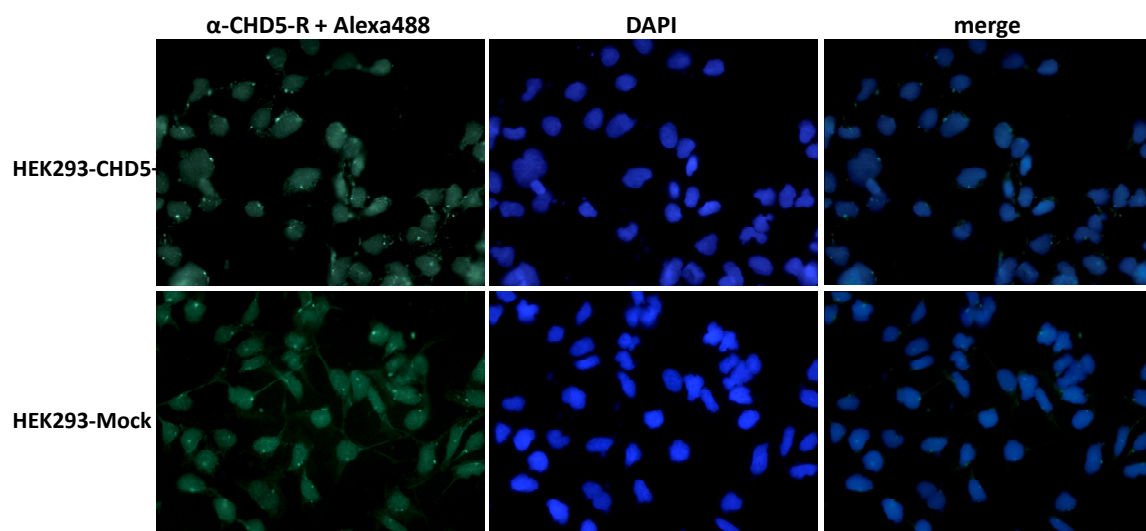
Fig. 4.11: Immuneserum R stains HEK293 cell nuclei unspecifically
The immuneserum R was tested for its use in immunofluorescence. Therefore, HEK293 cells were transiently transfected with pcDNA3.1/V5-His-TOPO-hCHD5 or left untransfected and stained with immuneserum R in a dilution of 1:10 or DAPI. As secondary antibody Alexa Fluor 488 goat α-rabbit IgG was used. In both CHD5 overexpressing and not-expressing cells the nuclei are brightly stained. Thus, it was considered to be unspecific.

| α-CHD5 (M-182) + Alexa488 | DAPI | merge |
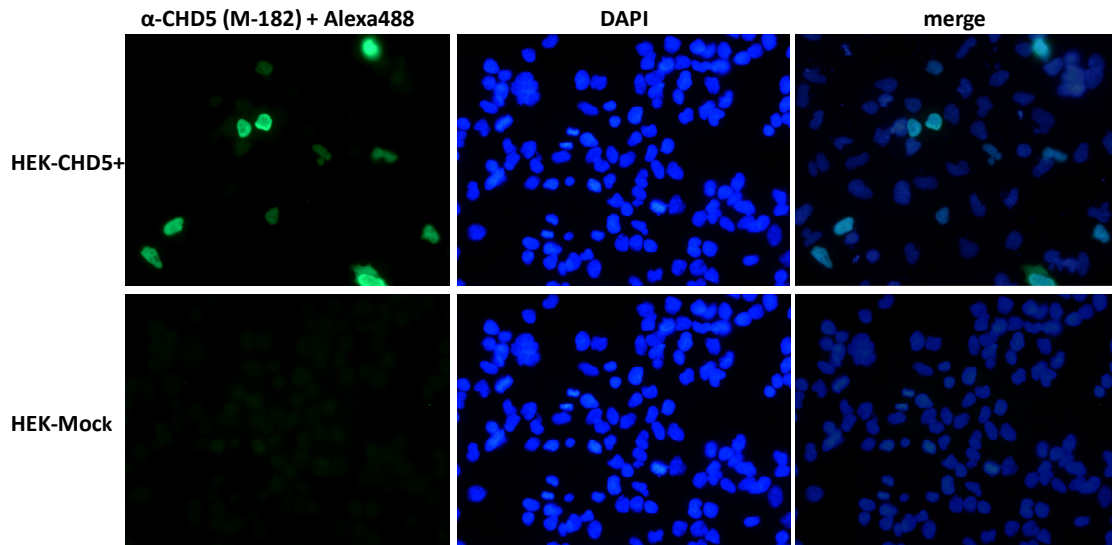
Fig. 4.12: M-182 is capable of detecting recombinant CHD5 in immunofluorescense

The CHD5 antibody M-182 was tested for its use in immunofluorescence. Therefore, HEK293 cells were transiently transfected with pcDNA3.1/V5-His-TOPO-hCHD5 or left untransfected and stained with M-182 in a dilution of 1:50 or DAPI. As secondary antibody Alexa Fluor 488 goat α-rabbit IgG was used. In CHD5 overexpressing cells some nuclei are brightly stained, whereas no apparent background was visible HEK293-Mock cells. Thus, the staining was considered to be specific.

In synopsis, all antisera generated in this study are able to specifically react with recombinant CHD5 protein in western blot. However, antisera S and K failed to detect endogenous CHD5 in mouse brain extract, even after concentration on a Protein G column. That seems to be comprehensible considering the mismatch of peptide sequences with mouse CHD5 protein sequence (see Fig. 4.2). Antiserum R was able to detect endogenous CHD5 in mouse brain extract but lost its activity during the concentration process on the Protein G column. Without purification it gave a strong background in western blot hindering its further application. In addition, the antisera turned out to be inapplicable in IP and IF experiments. It is possible that the performance of these antisera could be improved after affinity purification on a column containing immobilized peptides. However, in the interest of time, I continued my investigations using the M-182 CHD5 antibody. This CHD5 antibody efficiently detected both recombinant and endogenous CHD5, was able to immunoprecipitate CHD5 and allowed detection of CHD5 by immunofluorescence.

4.1.2 CHD5 overexpression does not induce senescence in SAOS-2 cells

CHD5 was found to interfere with the p16-pRb-pathway for promotion of cellular senescence in MEFs.[219]

The molecular mechanism of how CHD5 can influence senescence is not understood. In order to gain mechanistic insight into the effects of CHD5 on Rb-induced senescence I decided to use the so-called 'flat cell assay'[220]. This is an established experimental system to study senescence in SAOS-2 cells.

The human osteosarcoma cell line SAOS-2 lacks functional pRb. Instead of the full-length nuclear localized Rb a mutant 90 kDa fragment is produced which is localized to the cytoplasm.[221] A transfection of the wild-type Rb gene or its large pocket domain (Aa 379-928) together with a plasmid encoding a selection marker leads to an arrest in the G1-phase of the cell cycle, driving the cells into senescence.[222] These cells undergo dramatic morphological changes which result in the formation of large, flat cells with cessation of cell division. Flat cells are still alive (the dye Trypan blue is excluded) and metabolically active. They can be easily identified and quantified under the light microscope; thereby the proportion of senescent cells can be determined.

Co-expression of proteins in addition to pRb can be used to study the effect of these proteins in pRb-mediated senescence.[223]

Firstly, it was necessary to determine whether CHD5 interferes with pRb-mediated senescence in this experimental system.

Here, four different transfections in SAOS-2 cells were performed as follows:

1. with pcDNA3.1/V5-His-TOPO/LacZ as Mock control

2. with pCMV-hRb(379-928) as positive control known to induce senescence in SAOS-2 cells

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[219] Bagchi A et al, CHD5 is a Tumorsuppressor at Human 1p36. Cell (2007).

[220] Hinds PW et al, Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. Cell (1992).

[221] Shew JY et al, C-terminal truncation of the retinoblastoma gene product leads to functional inactivation. Proc Natl Acad Sci USA (1990).

[222] Welch PJ and Wang JY, Disruption of retinoblastoma protein function by coexpression of its C pocket fragment. Genes Dev (1995).

[223] Hinds PW et al, Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. Cell (1992).

3. with pcDNA3.1/V5-His-TOPO-hCHD5 to test if CHD5 overexpression alone has an influence on cellular senescence within this system and

4. with pcDNA3.1/V5-His-TOPO-hCHD5 and pCMV-hRb(379-928) together to test whether CHD5 modulates pRb-induced senescence or even co-induces it in presence of pRb (large pocket domain).

To each transfection pBABE was added, a plasmid containing a puromycin resistence. After transfection, cells were selected with puromycin for 4 days and stained with crystal violet for a better visualization of the flat cell morphology. Depending on the cell density at least 100 to 300 cells were counted using a light microscope.

LacZ overexpression resulted in 4 % of flat cells as a kind of baseline senescence. pRb(large pocket domain) transfection led to an average of 68 % of flat cells indicating that this assay worked in my hands as published (see above for references).

CHD5 overexpression alone produced 5 % flat cells which was comparable to the baseline senescence in the LacZ control. So this overexpression alone did not drive SAOS-2 cells into senescence. This result allowed then to assess whether Chd5 modulates pRb-induced senescence when co-expressed.

Upon co-tranfection of pRB and CHD5 72 % of cells developed a flat cell morphology. In comparison with the pRb overexpression the number of flat cells rose by 4 % in average. Taking into account that this assay is based on counting a limited number of cells, 4 % of difference in average does not indicate significance.

It has to be mentioned that overexpression of proteins was not controlled by western blot because the selection process resulted in a reduced number of cells complicating protein extract preparation. Efficient transfection procedures were assumed by the visible effect of pRb transfections leading to flat cell formation.

In conclusion, this demonstrates that CHD5 does not affect pRb-mediated senescence in this experimental system (see Fig. 4.13).
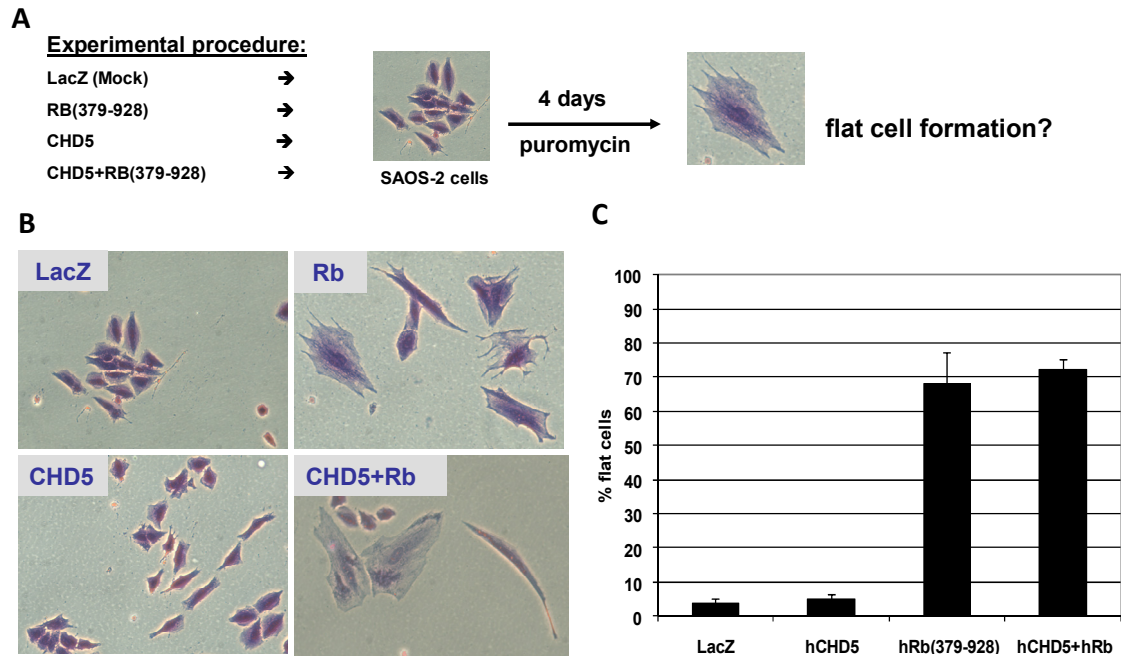
Fig. 4.13: CHD5 overexpression does not influence flat cell formation in SAOS-2 cells
A Scheme of experimental procedure: SAOS-2 cells were transfected either with an expression vector coding for LacZ (as Mock control), CHD5, Rb (large pocket domain) or RB and CHD5. All transfections included the puromycin resistence plasmid pBabe. Cells were selected with puromycin for 4 days, and the resulting flat (G1 arrested) cells were counted.
B Lighmicroscopy at 100-fold magnification after crystal violet staining of transfected cells
C Percentage of flat cells was calculated after crystal violet staining.

4.1.3 Overexpressed CHD5 does not directly interact with endogenous p53

It has been proposed that CHD5 regulates the p53 level via the p19^{ARF} pathway.[224] However, an alternative and so far unexplored possibility is a direct interaction between CHD5 or a CHD5-containing complex and p53. A similar model was suggested for a MTA2-containing NuRD complex regulating the p53-mediated transactivation by modulating the p53 acetylation status.[225]

The classical approach to overexpress candidate interaction partners and perform co-immunoprecipitations failed because neither CHD5 nor any other protein besides ARF could be co-expressed together with wild-type p53 in HEK293 cells, even though expression of the individual proteins was successful (data not shown). The explanation for this effect remains unclear.

Since HEK293 cells express robust levels of functionally inactive p53, we tested if it interacted with Flag-tagged CHD5. Therefore, HEK293 cells were transiently transfected with expression vectors for LacZ as a negative control, Flag-tagged CHD5 or

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[224] Bagchi A et al, CHD5 is a Tumorsuppressor at Human 1p36. Cell (2007).
[225] Luo J et al, Deacetylation of p53 modulates its effects on cell growth and apoptosis. Nature (2000).

wild-type p53. Whole cell extracts were subjected to immunoprecipitation using α-Flag M2 beads and α-p53-DO1, respectively. The precipitates were analysed in western blot by staining with α-Flag and α-p53 (FL-393). CHD5-Flag was successfully expressed (Fig. 4.14, compare lanes 1 and 2) and efficiently precipitated with Flag antibody (Fig. 4.14, lane 5). In addition, endogenous and overexpressed p53 were successfully immunoprecipitated by DO-1 antibody (Fig. 4.14, lanes 7, 8 and 10).

However, no co-immunoprecipitation of Flag-tagged CHD5 with endogenous p53 could be achieved, suggesting there is no direct interaction between overexpressed CHD5 and endogenous p53 or that the interaction is not strong enough for a robust co-immunoprecipitation.
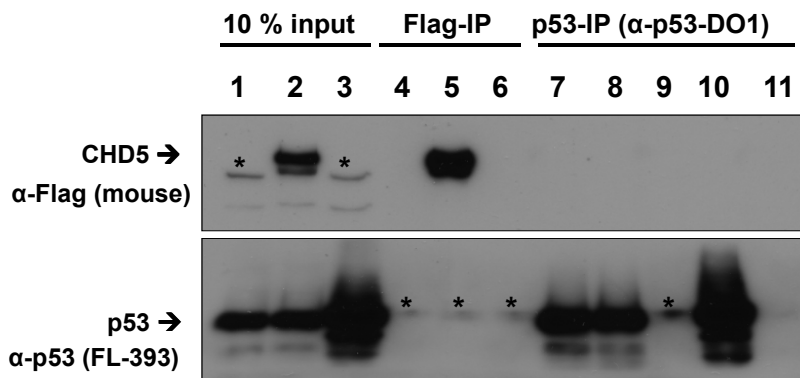
Fig. 4.14: Co-immunoprecipitation of CHD5 and p53
HEK293 cells were transiently transfected with expression vectors for LacZ (lanes 1, 4 and 7), CHD5 (lanes 2, 5, 8 and 9) and wt-p53 (lanes 3, 6, 10 and 11). IP from WCE was performed with α-Flag M2 beads or α-p53-DO1, western blot analysis of inputs and precipitates with α-Flag antibodies and α-p53 (FL-393). Lanes 9 and 11: control with addition of beads without Do-1.
 * unspecific bands, unlikely to be the DO1 heavy chain because DO1 and FL-393 are generated in different species.

4.1.4 CHD5 expression profile in tissues and cell lines

So far, little is known about the biological function of CHD5. To gain further insight into that, a detailed expression analysis of CHD5 in various tissues and cell lines on transcript and protein level was carried out in order to establish conditions and a cell system in which CHD5 is inducible or even expressed at robust levels for further functional assays.

Recently, it was published that CHD5 is predominantly expressed in tissues from neural origin (brain and adrenal gland). A testicular expression was predicted by SAGE but a further validation on transcript and protein level was not carried out so far.[226]

CHD5 expression analysis in primary tissue samples from rat and mouse

A variety of tissue samples from rat and mouse were analyzed for CHD5 expression on RNA and protein level.

CHD5 was highly expressed in rat brain and testes on transcript level in RT-qPCR (see Fig. 4.15 B), indicating that CHD5 expression is restricted to brain and testes within the panel of analyzed tissues. In muscle, liver, heart and lung CHD5 was only detectable at levels of background transcription. Background transcription was defined by very high threshold cycles (close to water control Cts) and a multi-peaked melting curve in some cases indicating that non-specific PCR products have contributed to the threshold cycle.

Also on protein level CHD5 could be robustly detected in WCEs from rat brain and testes of an adult male rat. Neither muscle nor liver showed CHD5 expression (see Fig. 4.15 A). Equal amounts of protein were carefully loaded but the loading control ß-tubulin showed redundantly major differences between the different tissues (see Fig. 4.15 A, 4.16 A). I assume that this reflects most likely the different expression levels of ß-tubulin in various tissues so that it was an inappropriate loading control. An alternative loading control was not tried; considering the high molecular weight of CHD5 (223 kDa), other typical loading controls like ß-actin are inapplicable because of their small size. Instead, protein amounts on the western blot membrane were randomly controlled by Ponceau staining. However, a statement on CHD5 expression based on these western blots remains critically but since the RNA and protein data completely coincide I would assume that the western blot reflects realistic expression levels even in liver and muscle.

Extracts from lung and heart gave a non-acceptable smeary background (data not shown). Therefore these extracts were excluded from reasonable protein analysis.

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[226] Thompson PM et al, CHD5, a new member of the chromodomain gene family, is preferentially expressed in the nervous system. Oncogene (2003).

Next, protein extracts from different tissues of an adult male mouse were tested for CHD5 expression in western blot to exclude a species specific expression pattern. Like in rat CHD5 expression was found to be restricted to brain and testes; lung, spleen, liver, kidney and gut were CHD5-negative (see Fig.4.16). Again, differences in the loading controls limit that conclusion.
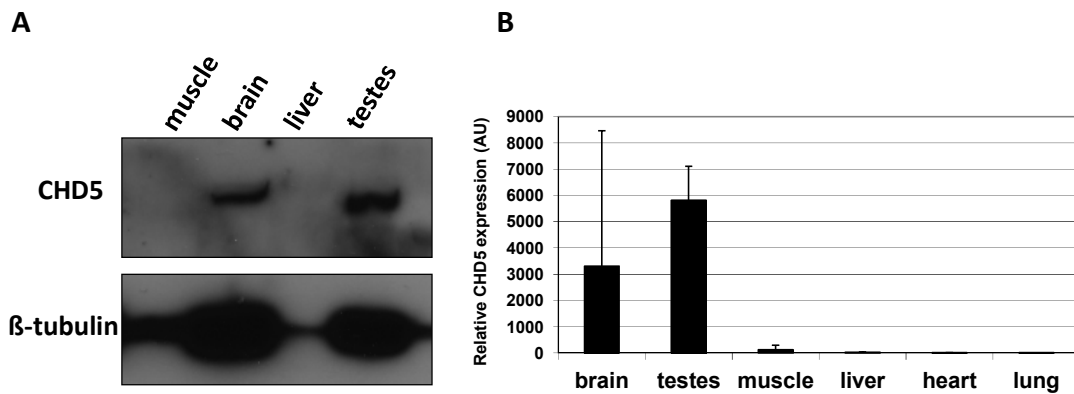
Fig. 4.15: CHD5 expression in different rat tissues
A In order to detect CHD5 protein in various tissues WCEs from different rat tissues (80 µg each) were subjected to western blot (7.5 % SDS-PAGE), ß-tubulin was used as loading control.
B For verification of western blot results RNA was extracted from different rat tissues and used for mRNA quantification in RT-qPCR; the error bars represent the standard deviation between three different RNA isolations.
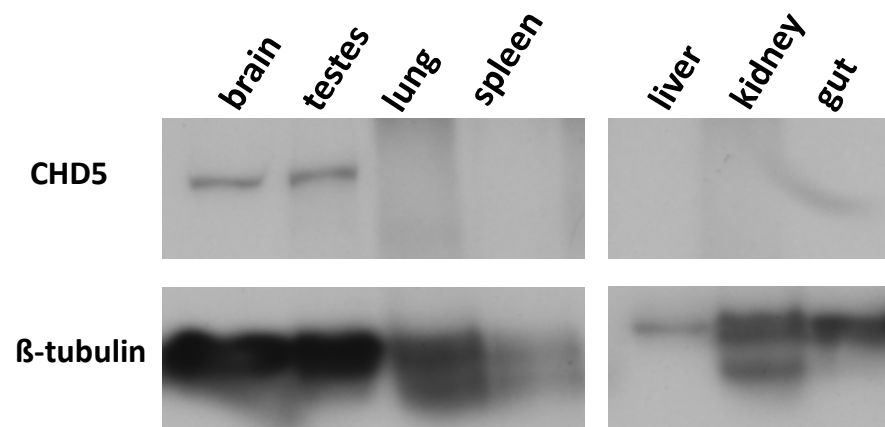
Fig. 4.16 CHD5 expression in different mouse tissues
WCEs from different mouse tissues were prepared and analyzed in western blot for CHD5 expression, ß-tubulin was used as a loading control

CHD5 expression analysis in cell lines

In order to find a CHD5 expressing cell system for further functional studies 14 mostly neural-related cell lines were screened for CHD5 expression on transcript and protein level.

The neuroblastoma cell lines SH-SY-5Y, SH-EP, SMS-KCN and IMR-32 were tested for CHD5 expression in western blot and RT-qPCR. In none of the cell lines CHD5 protein could be detected (see Fig. 4.17 A). On transcript level all cell lines showed expression levels comparable with those from HEK293 cells which were taken as negative controls because of their high threshold cycle in RT-qPCR and the absent protein expression (see 4.17 B). Thus, all tested neuroblastoma cell lines were assumed to be CHD5-negative.

There are several explanations for this absence of CHD5 expression. One is a homozygous deletion of the short arm of chromosome 1 (1p) where CHD5 is located. Second, a biallelic hypermethylation of the CHD5 promoter leads to the silencing of the gene (see introduction). Furthermore, both explanations can be combined if one allele is deleted and the promoter of the second is hypermethylated (loss of heterozygosity, LOH). Another possibility to silence the second allele is a mutation resulting in a truncated or functionally inactive protein.

For two of the tested cell lines cytogenetic data regarding chromosome 1 are available: A deletion of one 1p allele is known in IMR-32 cells;[227] SH-EP cells have a completely intact chromosome 1.[228]

The hypothesis that the CHD5 expression of the intact allele is repressed by promoter hypermethylation in IMR-32 cells was further evaluated by treatment of these cells with the DNMT inhibitor 5-azacytidine. In case of a hypermethylated promoter such a demethylating treatment is supposed to restore CHD5 expression.

However, 5-azacytidine treatment did not restore CHD5 protein expression in IMR-32 cells (see Fig. 4.17 A, lane 8).

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[227] Schleiermacher G et al, Combined 24-color karyotyping and comparative genomic hybridization analysis indicates predominant rearrangements of early replicating chromosome regions in neuroblastoma. Cancer Genet Cytogenet (2003).
[228] Carr J et al, High-resolution analysis of allelic imbalance in neuroblastoma cell lines by single nucleotide polymorphism arrays. Cancer Genet Cytogenet (2007).
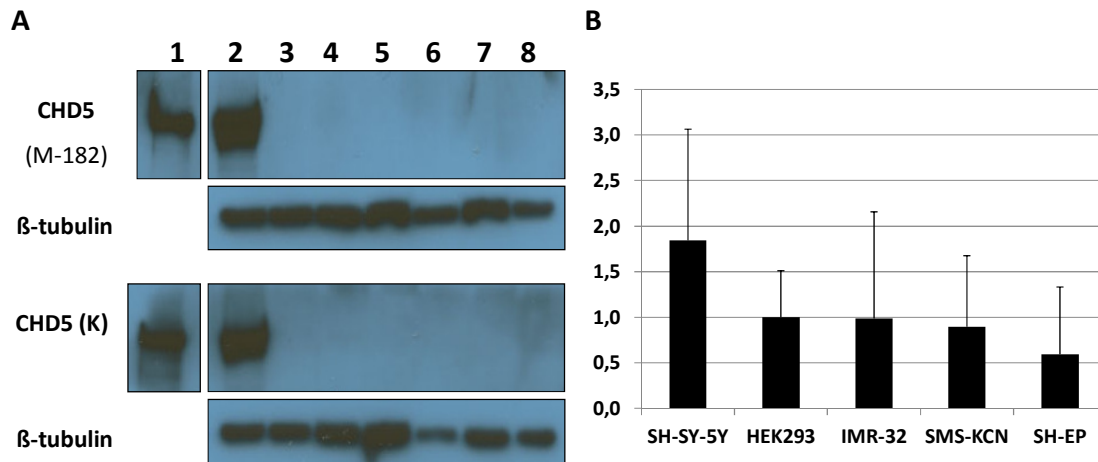
Fig. 4.17: CHD5 is not expressed in neuroblastoma cell lines
A 80 µg of WCE from different neuroblastoma cell lines was loaded on SDS-PAGE and further subjected to western blot using two different antibodies against CHD5; ß-tubulin was used as a loading control; recombinant CHD5 (lane 1) and W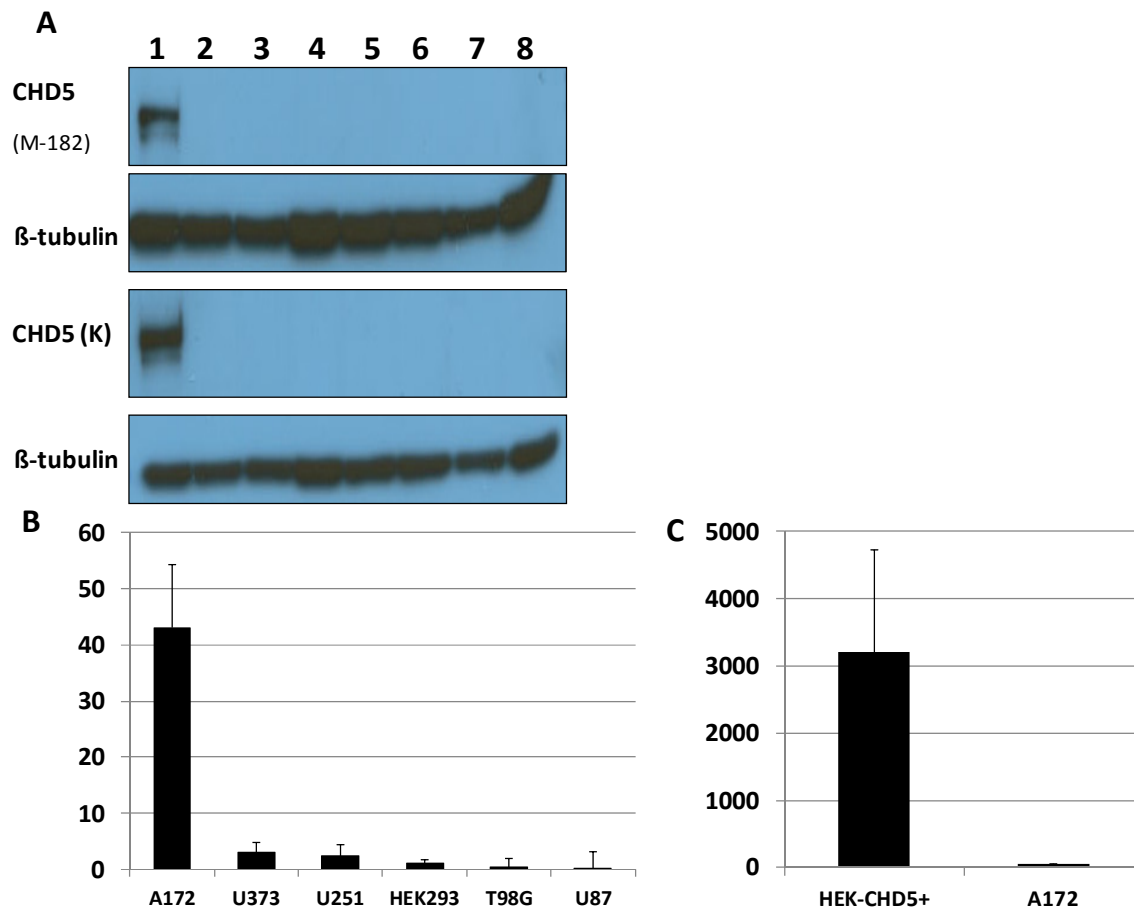CE from transiently CHD5-transfected HEK293 cells (lane 2) were used as positive controls, from HEK293 cells (lane 3) as negative control. Neuroblastoma cell line extracts: SH-SY-5Y (lane 4), SMS-KCN (lane 5), SH-EP (lane 6), IMR-32 (lane 7) and IMR-32 treated with 10 µM 5-azacytidine for 3 days (lane 8).
B RNA from different neuroblastoma cell lines was extracted and analysed by RT-qPCR, confirming the western blot results. Expression levels were calculated according to the $2^{-\Delta\Delta Ct}$ method; RPS14 was used as housekeeping gene and ΔCt(HEK293) was set as calibrator. Error bars represent the standard deviation between three different RNA isolations.

Like neuroblastoma cell lines, the lymphocytic MOLT-4 cell line (data not shown), the glioblastoma cell lines U251, U373, U87, T98G (all from human origin, see Fig. 4.18) and Gl261 (from mouse, see Fig. 4.19 B and 4.20 B) do not express CHD5 at more than background levels. The glioblastoma cell line A172 showed an approximately forty fold higher expression of CHD5 than HEK293 cells in RT-qPCR although no protein was detectable in western blot (see Fig. 4.18, A and B). Transcript levels in HEK293 cells transiently transfected with CHD5 were in comparison around sixty fold higher than in A172 cells (see Fig. 4.18 C). This order of magnitude was similar to the difference between testicular and muscular expression in rat where muscle was not assumed to express functional CHD5. In order to further verify the absent CHD5 expression in A172 cells, a large amount of nuclear extract was prepared and subjected to western blot. No signal was observed for A172 assuming an absent expression (see Fig. 4.20, A).

Although all these data from cell lines were highly reproducible in different experiments, the results were additionally verified by the use of a second primer pair for CHD5 which amplifies a region closed to the 3' end of the mRNA and a repetition of the cDNA synthesis with random primers. Using these primers confirmed the data from earlier experiments showing absent CHD5 expression (data not shown).

A

B

C

Fig. 4.18: CHD5 is not expressed in glioblastoma cell lines
A 80 µg of WCE from different glioblastoma cell lines was loaded on SDS-PAGE and further subjected to western blot using two different antibodies against CHD5; ß-tubulin was used as a loading control; WCE from transiently CHD5 transfected HEK293 cells (lane 1) was used as positive control, WCE from HEK293 cells (lane 2) and Hela cells (lane 3) as negative controls. Glioblastoma cell line extracts: A172 (lane 4), U251 (lane 5), U373 (lane 6), U87 (lane 7) and T98G (lane 8).
B RNA from different neuroblastoma cell lines was extracted and analysed by RT-qPCR, confirming the western blot results. Expression levels were calculated according to the $2^{-\Delta\Delta Ct}$ method; RPS14 was used as housekeeping gene and ΔCt(HEK293) was set as calibrator. Error bars represent the standard deviation between three different RNA isolations.
C Comparison of CHD5 RNA levels in transiently transfected HEK293 cells and A172 cells.

An explanation for the absence of CHD5 in glioblastoma cell lines besides a role as tumor suppressor might be the astrocytic origin of this tumor.

There are 1.4 astrocytes per neuron in the human cortex but only 0.4 in mouse and rat cortex.[229] Although it is likely that the robust western blot signal from brain is at least partially caused by such an abundant cell type like the astrocyte, a restriction to neurons or oligodendrocytes could not be excluded. Such a restriction might be

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[229] Nedergaard M et al, New roles for astrocytes: Redefining the functional architecture of the brain. Trends Neurosci (2003).

supported by the data of *The Allen Mouse Brain Atlas,*[230] a large collection of in situ hybridization data from mouse brain, suggesting a preferential expression of CHD5 in specific areas of the brain like the hippocampus. Therefore, a mouse cell line derived from hippocampus neurons, Ht 22, was tested in western blot, but no CHD5 protein could be detected (see Fig. 4.19 A).

In order to exclude a repression of CHD5 during proliferation or immortalization, especially in cell culture, primary cells were tested. Primary astrocyte cultures are broadly used for a variety of approaches and are not difficult to keep in culture. Therefore, such a culture was established (see Methods, Primary astrocyte culture) and tested in early and late passages for CHD5 expression. No significant CHD5 expression could be detected (see Fig. 4.19). Because no obvious difference between the early and late passage could be observed in different experiments, the shown RT-qPCR data represent an average of both.

A late passage (P19) from an ARF-deficient strain of a MEF culture was tested in western blot with no evidence for CHD5 expression (see Fig. 4.20 B).

A nuclear extract from murine neural stem cells was also tested in western blot for CHD5 expression and gave a signal in western blot as the only material besides tissue extracts (see Fig. 4.20 A). Because a huge amount of nuclear extract (100µg) were used, an equal amount of NE from the glioblastoma cell line A172 was loaded in comparison without any evidence of CHD5 expression in this cell line as already shown in Fig. 4.18.

In summary, analysis of primary rat and mouse tissues revealed robust levels of CHD5 only in brain and testes. None of the tested cell lines showed significant CHD5 expression. The only cell system in which CHD5 is expressed at detectable levels are murine neural stem cells and HEK293 cells transiently transfected with an expression vector for Flag-tagged CHD5. The absence of CHD5 expression in cells of neural origin like Ht22 or astrocytes requires further investigation.

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[230] Internet source: www.mouse.brain-map.org.

A

B
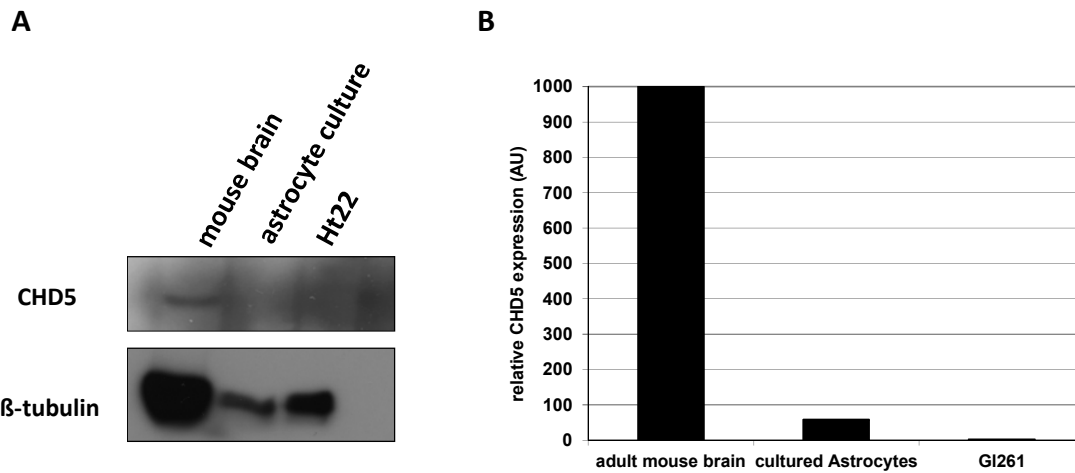
**Fig. 4.19: CHD5 expression in cultured astrocytes**
A 60 µg of whole cell extracts from mouse brain, an astrocyte culture and Ht22 cells (a cell line from mouse hippocampal neurons) in western blot probed with the α-CHD5 antibody M-182
B RT-qPCR amplifying CHD5 in cDNA from mouse brain, astrocytes and the mouse glioblastoma cell line GI261. ß-actin was used as housekeeping gene, CHD5 mRNA levels were normalized to mouse brain.

A

B
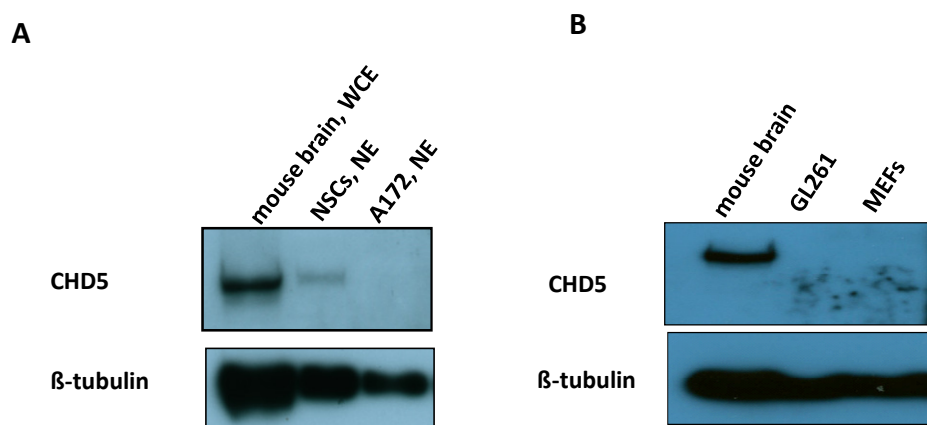
Fig. 4.20 CHD5 expression in primary cells from mice
A 100µg Nuclear extract from neuronal stem cells were analysed in western blot, mouse brain WCE was used as a positive control, NE from A172 as negative control.
B WCE from a MEF culture, GI261 as negative control.

4.1.5 Both isoforms of CHD5 are expressed in mouse brain and testes

According to the NCBI database[231] CHD5 is predicted to exist in two isoforms in *Mus musculus.* So far, the existence of more than one isoform is not suggested for any other organism besides the mouse.

The second isoform lacks an in-frame exon (no 24) compared to isoform 1 resulting in a smaller protein. The corresponding area in the protein is localized close to the

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[231] Internet source: www.ncbi.nlm.nih.gov/gene.

C-terminus (see Fig. 4.21 B). Remarkably, the corresponding part of this exon 24 is also absent in CHD4, although both proteins show a high overall similarity.
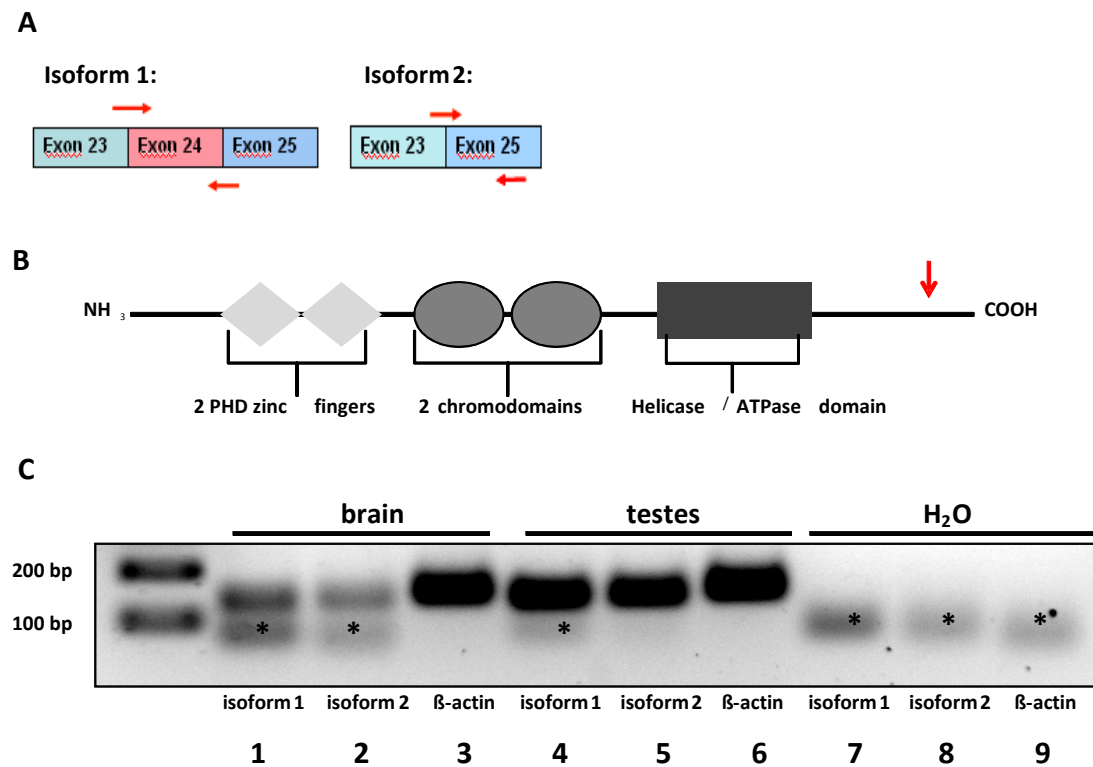
Here, it was tested, whether both isoforms are expressed on RNA level in mouse brain and testes. Therefore, primers were designed that bridge specifically the corresponding exon borders (exon 23 to 24 for isoform 1, exon 23 to 25 for isoform 2); thus they were supposed to amplify exclusively one isoform (see Fig. 4.21 A).

RNA from both tissues was extracted, reversely transcribed, analyzed in RT-PCR and separated on a 1.5% agarose gel. A water control was included in RT-PCR.

To assure overall RT-PCR reliability ß-actin as a typical housekeeping gene was included and gave bright signals in brain and testes and no apparent band in water control (see Fig. 4.21 C; lanes 3, 6 and 9).

A band between 100 and 200 base pairs was visualized for both isoform specific primers in brain and testes (see Fig. 4.21 C; lanes 1, 2, 4 and 5) that were absent in the water control (see Fig. 4.21 C; lanes 7 and 8). Considering the expected size of the amplicons (131 bp each) these bands were assumed to represent isoform 1 and isoform 2, respectively. At around 100 bp a second band was frequently observed (see Fig, 4.21 C; lanes 1, 2, 4, 7, 8 and 9). Most likely these unspecific bands represent primer dimers.

In summary, transcripts of both CHD5 isoforms are detectable in brain and testes. No additional statement on their relative distribution can be made because RT-PCR is a semi-quantitative approach. To further test whether both isoforms are expressed on protein level a generation of isoform-specific antibodies would be necessary. Especially in the context of a CHD5-containing NuRD-like complex that might be interesting.

A

Isoform 1:   Isoform 2:

Exon 23 Exon 24 Exon 25   Exon 23 Exon 25

B

NH₃ — ... — COOH

2 PHD zinc   fingers   2 chromodomains   Helicase / ATPase domain

C

| | brain | | | testes | | | H₂O | | |

200 bp
100 bp

isoform 1 isoform 2 ß-actin   isoform 1 isoform 2 ß-actin   isoform 1 isoform 2 ß-actin
   1   2   3   4   5   6   7   8   9

Fig. 4.21: Isoform expression in mouse tissue
A Scheme of exon structure and position of isoform-specific primers
B Position of the alternative exon in CHD5 protein
C cDNA from mouse brain and testes was analyzed with isoform-specific primers by RT-PCR, followed by 1.5 % agarose gel.
* primer dimers

4.1.6 CHD5 expression is not upregulated upon differentiation of Pc12 cells

The absence of CHD5 expression in tumor cell lines and its involvement in proliferation control as reported by Bagchi et al[232] might suggest that CHD5 expression is restricted to non-proliferating cells e.g. terminally differentiated cells.

The rat pheochromocytoma cell line Pc12 is a broadly used model to study neural differentiation in cell culture because they can be easily differentiated in presence of recombinant NGF for several days.[233]

In order to test the hypothesis that CHD5 expression is upregulated during differentiation in a potentially CHD5 expressing cell line (because of its neural origin) we used Pc12 cells in differentiation.

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[232] Bagchi A et al, CHD5 is a Tumorsuppressor at Human 1p36. Cell (2007).
[233] Brynczka C et al, NGF-mediated transcriptional targets of p53 in Pc12 neuronal differentiation. BMC Genomics (2007).

Therefore, Pc12 cells were treated with 10 ng mNGF for 1, 4 and 7 days under low-serum conditions (1 % FBS). During the first day, Pc12 cells started to loose their round shape and outgrowth of neurites could be observed under the light microscope. On day 7 the cell bodies were star-shaped and surrounded by a dense network of neurites (see Fig. 4.22 A). This morphology is characteristic for nerve cells and indicated the success of the differentiation process.

Protein samples were taken on day 1, 4 and 7 after mNGF treatment and from undifferentiated Pc12 cells (day 0) for comparison. RNA was extracted on day 0 and day 7 after mNGF treatment.

CHD5 was not detectable in western blot neither before differentiation nor after it (see Fig. 4.22 B). In addition, CHD5 mRNA was only expressed at very low levels compared to its robust expression in rat brain. No significant difference in transcript levels could be observed during differentiation.

In summary, even though Pc12 cells are from neural origin and a CHD5 expression seemed reasonable, neither significant CHD5 transcripts in RT-qPCR nor protein in western blot could be detected. There was no upregulation of CHD5 during differentiation as assumed.
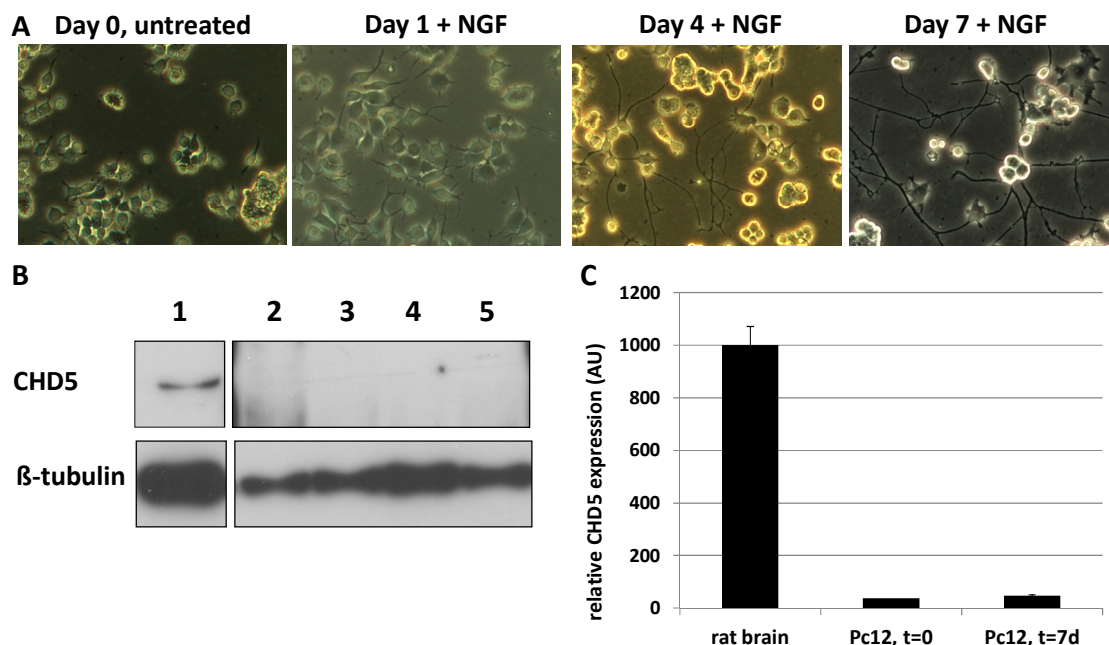
Fig. 4.22: CHD5 expression upon Pc12 differentiation
A Morphologic changes upon differentiation of Pc12 cells in light microscopy, 100x magnification.
B Pc12 cells were differentiated by addition of mNGF for 7 days, WCE was extracted on day 0 (lane 5), 1 (lane 2), 4 (lane 3) and 7 (lane 4) and analysed in western blot for CHD5 expression. WCE from rat brain was used as positive control for CHD5 (lane 1), ß-tubulin was used as loading control.
C RNA was isolated from undifferentiated (t=0) and differentiated (t=7d) Pc12 cells, CHD5 mRNA was subjected to RT-qPCR and normalized to rat brain.

4.2.1 NF-κB is activated upon TNFα stimulation in HEK293 cells

In the absence of NF-κB pathway activation the most abundant NF-κB heterodimer p50/p65 is kept in the cytoplasm by interaction with IκB molecules. Upon activation the heterodimer is released, translocated into the nucleus and directed to its target genes.

To confirm NF-κB nuclear re-localization in the cell line used for these studies, HEK293 cells were incubated with TNFα at a concentration of 20 ng/ml for one hour. The NF-κB subunits p50 and p65 were visualized by immunocytochemistry. In untreated HEK293 cells p50/p65 staining was preferentially cytoplasmic. Upon stimulation the staining pattern switched to a predominantly nuclear staining (see fig. 4.23). Both observations are consistent with an activation of the NF-κB pathway by TNFα in HEK293 cells.
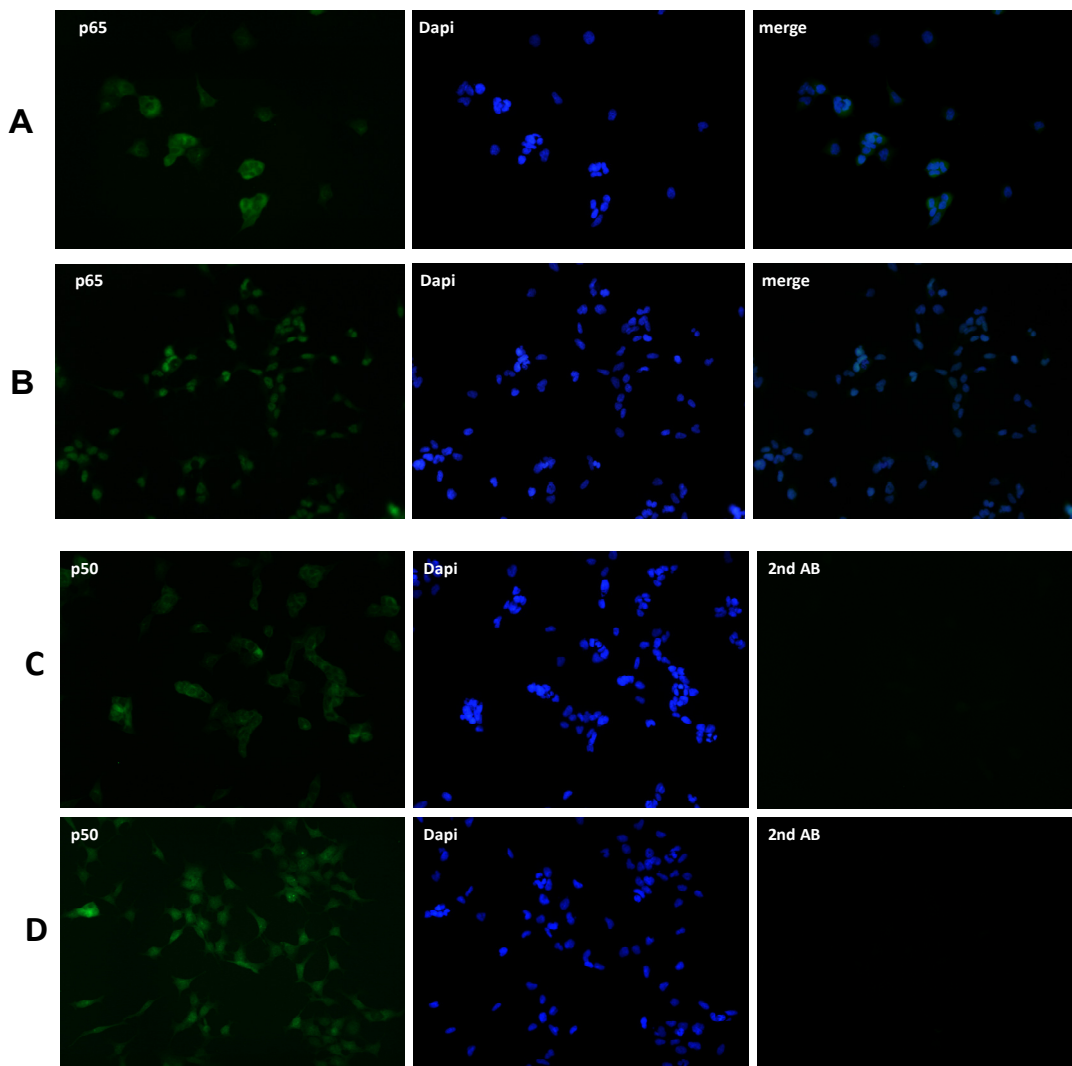
Fig. 4.23: NF-κB is translocated to the nucleus upon TNFα stimulation in HEK293
For visualization of NF-κB translocation HEK293 cells were stimulated with TNFα at a concentration of 20 ng/ml for one hour (B, D) or left untreated (A, C). Immunofluorescence staining was performed with α-p65 (A, B) and α-p50 (C, D). Nuclei were visualized by DAPI staining. As secondary antibody Alexa Fluor 488 α-rabbit was used.

4.2.2 Activation of NF-κB upon TNFα and PTO stimulation in a luciferase reporter system

To assess the general impact of different chromatin remodelers on NF-κB - induced transcriptional activation, we first used 293-mTLR9-luc cells.

These HEK293 cells stably express the murine Toll-like-receptor 9 (TLR-9) and a 6-fold NF-κB luciferase reporter plasmid. These cells respond to different agents stimulating TLR-9 with a nuclear translocation of NF-κB[234] (see figure 4.24 A).

To establish the use of this cell system in our laboratory, Luciferase activity was determined at different points in time after treatment with TNFα or the TLR-9 agonist PTO. After TNFα stimulation, a 1.25 fold increase in luciferase activity was detectable after 2 hours. Luciferase activity steadily increased at later points in time and reached a maximum of 25 fold after 18 hours (see fig. 4.24 B). After PTO stimulation, luciferase activity rose by six fold after 2 hours and increased to a maximum of 118 fold after 18 hours (see Fig. 4.24 C). These results confirm that NF-κB activity can be activated in these HEK293 cells by different stimuli.
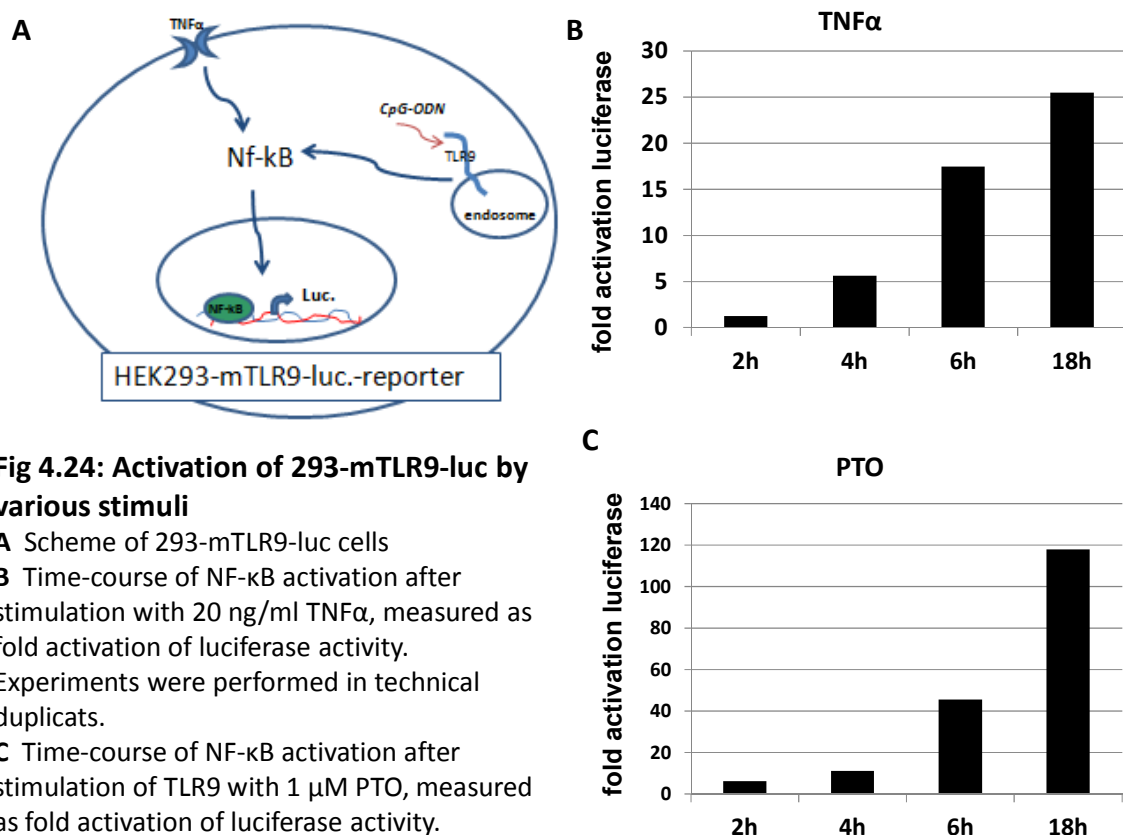
Fig 4.24: Activation of 293-mTLR9-luc by various stimuli
A Scheme of 293-mTLR9-luc cells
B Time-course of NF-κB activation after stimulation with 20 ng/ml TNFα, measured as fold activation of luciferase activity. Experiments were performed in technical duplicats.
C Time-course of NF-κB activation after stimulation of TLR9 with 1 µM PTO, measured as fold activation of luciferase activity.

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[234] Bauer S et al, Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. Proc Natl Acad Sci USA (2001).

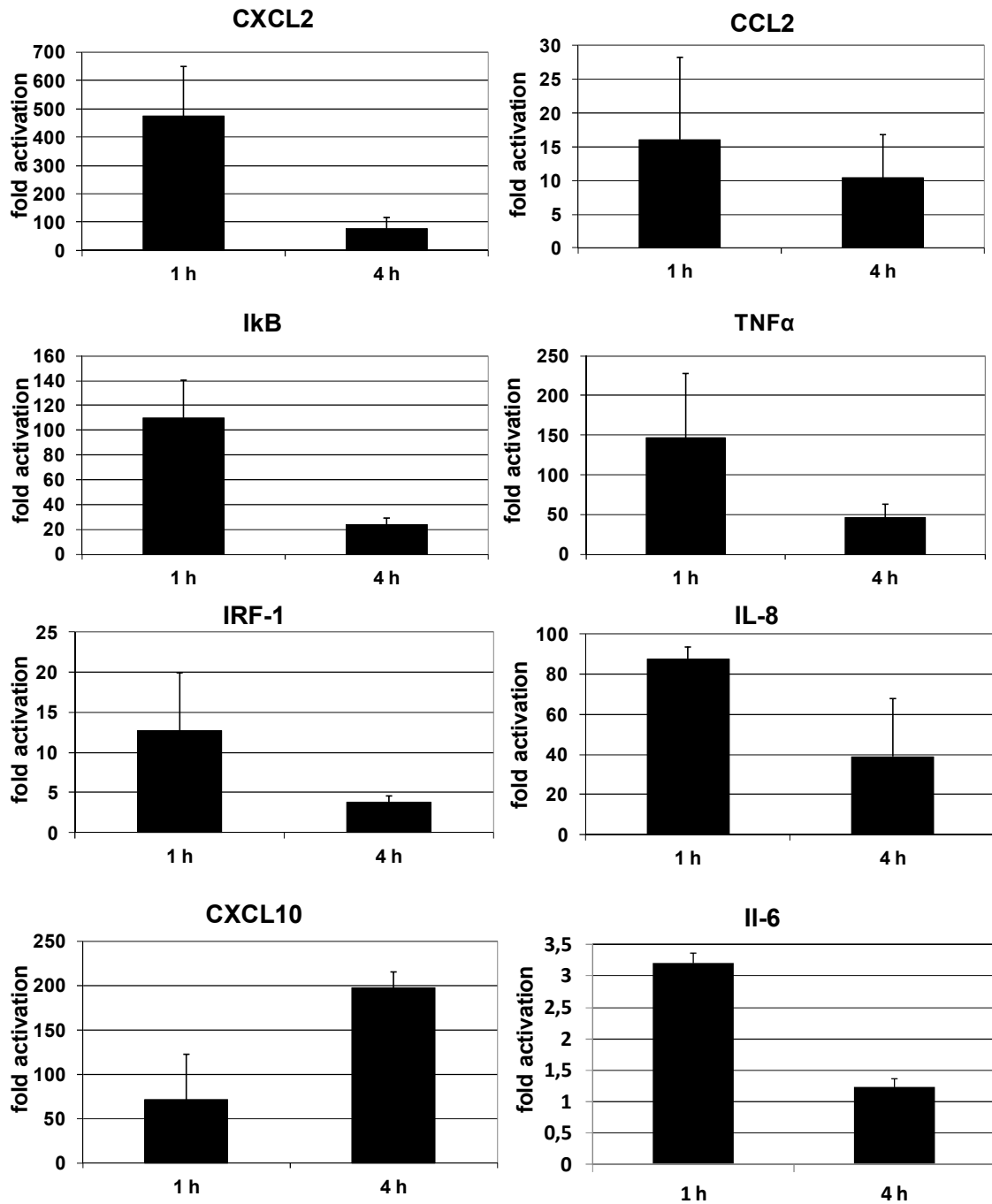### 4.2.3 Induction of different target genes upon TNFα stimulation in HEK293 cells

Having established that the NF-κB pathway is in principle intact in HEK293 cells, I investigated the activation of endogenous NF-κB target genes. The classical NF-κB target genes CXCL2 (C-X-C motif ligand 2; also called MIP-2α, GRO-ß), CCL2 (C-C motif ligand 2, also named MCP-1), CXCL10 (C-X-C motif ligand 10), IκB, TNFα, Il-8 (interleukin 8), IRF-1 (Interferon regulatory factor 1) and IL-6 (interleukin 6) were chosen.

To determine gene activity during early and late phases of the NF-κB response, RNA levels were quantified by RT-qPCR one and four hours after TNFα treatment. With the exception of IL-6, all tested genes were activated by at least 10 fold at one or both points in time following TNFα stimulation (see Fig. 4.25). The analysed genes showed kinetic differences with respect to expression levels during early and late points in time. CXCL2, CCL2, IκB, TNFα, IRF-1 and IL-8 were strongly induced after one hour of TNFα stimulation. By 4 hours gene activity had decreased to values between 20 % (CXCL2) and 60 % (CCL2) of the activity after one hour. Thus, this group of genes exhibits strongest expression during the early phase of the NF-κB response.

By contrast, CXCL10 showed a seventy fold induction after one hour and further increased to nearly 200 fold by 4 hours (see fig. 4.25). This demonstrates that CXCL10 is most strongly expressed during later stages of NF-κB response.

Il-6 showed a maximum induction by three fold after one hour (see Fig. 4.25). This was regarded as very mild upregulation. Thus, Il-6 was not included in further studies.

The induction of endogenous target genes was much more pronounced than most of the activation levels achieved in the 293-mTLR9-luc system. RT-qPCR is a very sensitive method to detect even small changes in gene activation that could be missed in a Luciferase assay. Taking into account that chromatin remodeling is just one influencing factor on gene activation and knockdown efficacy is always below 100 %, I did not expect tremendous effects in the further studies. Thus, to avoid less pronounced effects to be hidden I focused on the endogenous target gene activation for further studies and did not include experiments with 293-mTLR9-luc cells.

Fig.4.25 : Activation profiles of different NF-κB target genes upon TNFα stimulation. HEK293 cells were stimulated with recombinant TNFα (20 ng/ml). RNA was isolated after 1 and 4 hours. NF-κB target genes were normalized to RSP14 as housekeeping gene. Error bars represent the standard error.

4.2.4 siRNA-mediated knockdown of Mi-2ß, CHD3 and BRG1/Brm in HEK293 cells

To study the role of chromatin remodeling factors during the activation of NF-κB target genes, I established siRNA-mediated knockdowns of CHD3, CHD4 and BRG1/Brm in HEK293 cells. A time-course was performed for every siRNA to determine the most efficient knockdown.

Two siRNAs against CHD4 were tested (siCHD4#1 and siCHD4#2), either alone or in combination. Both were capable of reducing the level of CHD4 protein after 2 and 3 days (see Fig. 4.26, compare lanes 1-3 with 4-5 and 6-8 with 9-10, respectively). The strongest effect was observed for siCHD4#1 after 4 days (Fig. 4.26, lane 11). The use of both siRNAs together did neither augment the knockdown efficiency nor resulted in a diminished knockdown. On RNA level both siRNAs alone and in combination achieved a reduction of transcripts to less than 30 % at all tested points in time. Treatment with siCHD4#1 resulted in the strongest suppression of transcripts after 3 days (see Fig. 4.27).
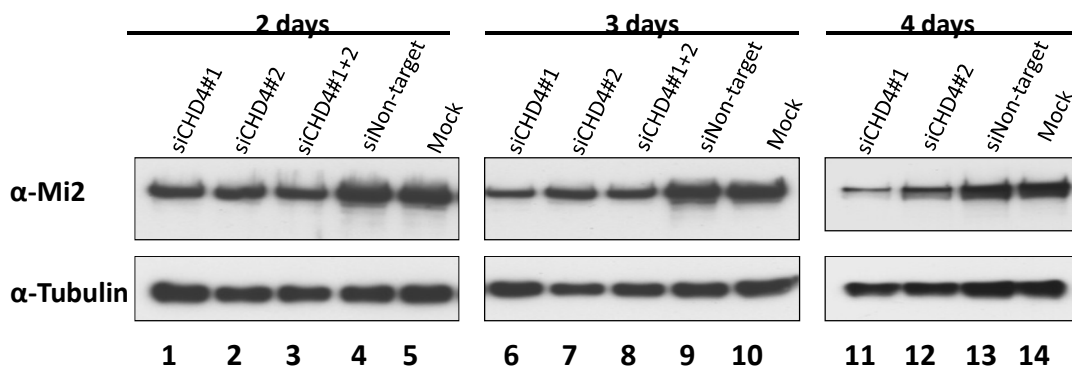
Figure 4.26: Efficiency of Mi2ß protein knockdown in HEK293 cells after 2, 3 and 4 days.

In order to determine the most efficient Mi2ß knockdown a time-course was performed. 50 µg of WCEs were used for western blotting comparing two different siRNA against CHD4 alone or together with a non-targeting siRNA (siNon-target) or a Mock control.
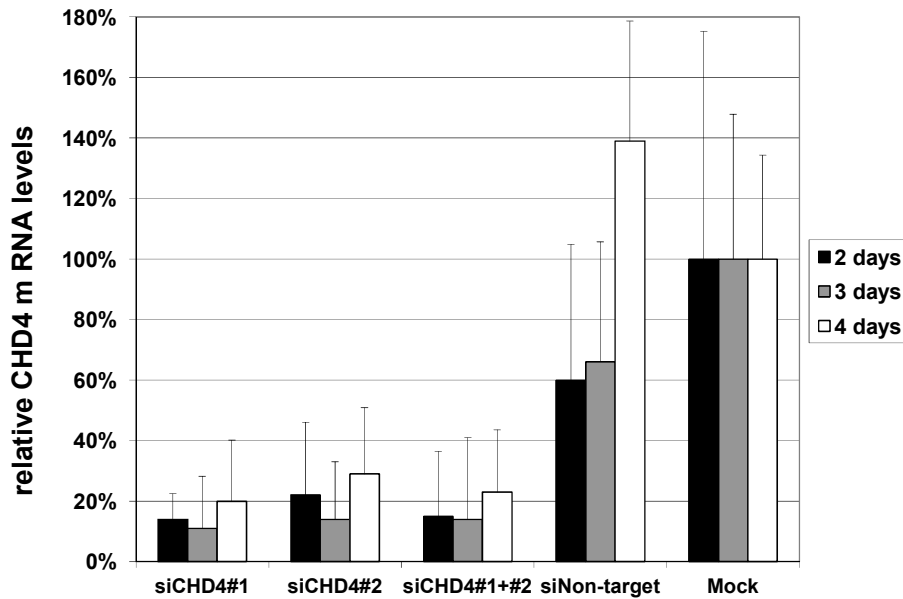
Fig. 4.27: Efficiency of Mi2ß knockdown on transcript level.

HEK293 cells were incubated with 2 different siRNAs against CHD4 (siCHD4#1, siCHD4#2) for two to four days. Both siRNAs were tested alone and in combination. RNA was extracted, reversely transcribed and subjected to RT-qPCR. CHD4 mRNA levels were normalized to RPS14 as housekeeping gene. Error bars indicate the standard error.

BRG1 and Brm are supposed to be partially redundant and compensatory. The used siRNA disrupting BRG1 expression (siBRG1/Brm) also targets Brm although it is expressed to a much lesser extent in HEK293 cells. Whether Brm is functionally relevant in HEK293 cells at this low expression level is unclear. Thus, this study will focus on BRG1. BRG1 protein was reduced by siBRG1/Brm after 2, 3 and 4 days but the highest reduction was observed after 4 days (see Fig. 4.28). On RNA level BRG1 was strongest repressed after 2 days but transcripts remained below 40 % on day 3 and 4 (see Fig. 4.29). Brm RNA levels were kept below 50 % (see Fig. 4.30).

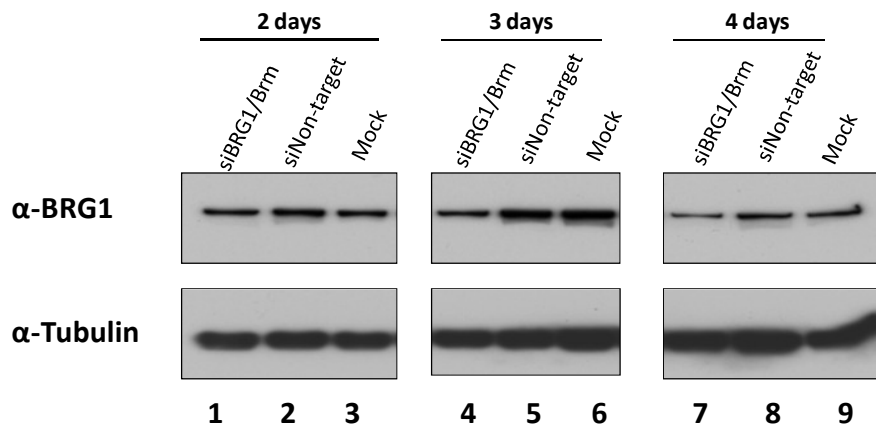**Fig. 4.28: Efficiency of BRG1/Brm knockdown in HEK293 cells after 2, 3 and 4 days.**

In order to determine the most efficient BRG1/Brm knockdown a time-course was performed. 50µg of WCEs were used for western blotting comparing a BRG1/Brm knockdowns with siNon-target (non-targeting siRNA) and Mock control.
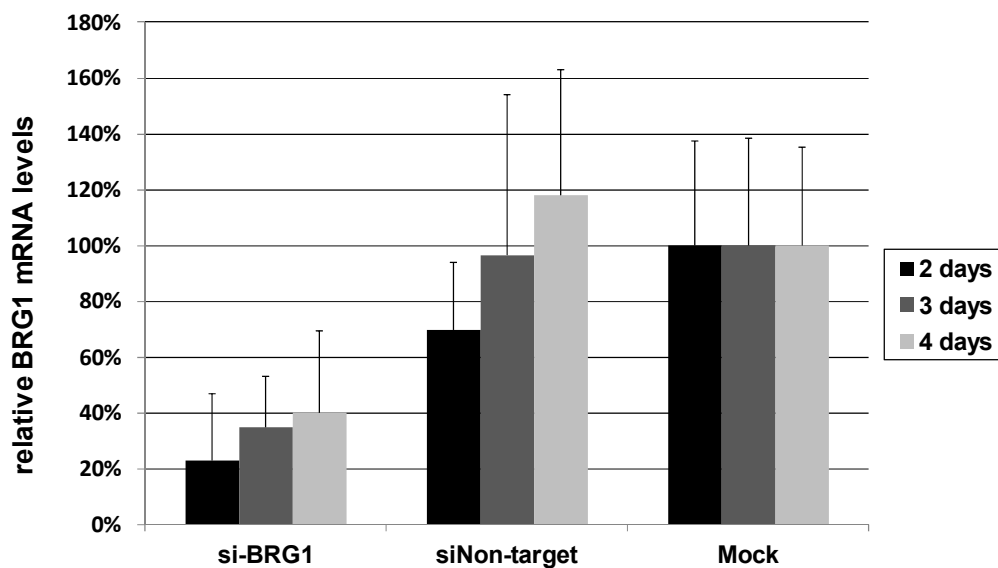
Fig. 4.29: Efficiency of BRG1 knockdown on transcript level.

HEK293 cells were treated with a siRNA against BRG1/Brm for two to four days. RNA was extracted, reversely transcribed and subjected to RT-qPCR. BRG1 mRNA levels were normalized to RPS14 as housekeeping gene. Error bars represent the standard error.
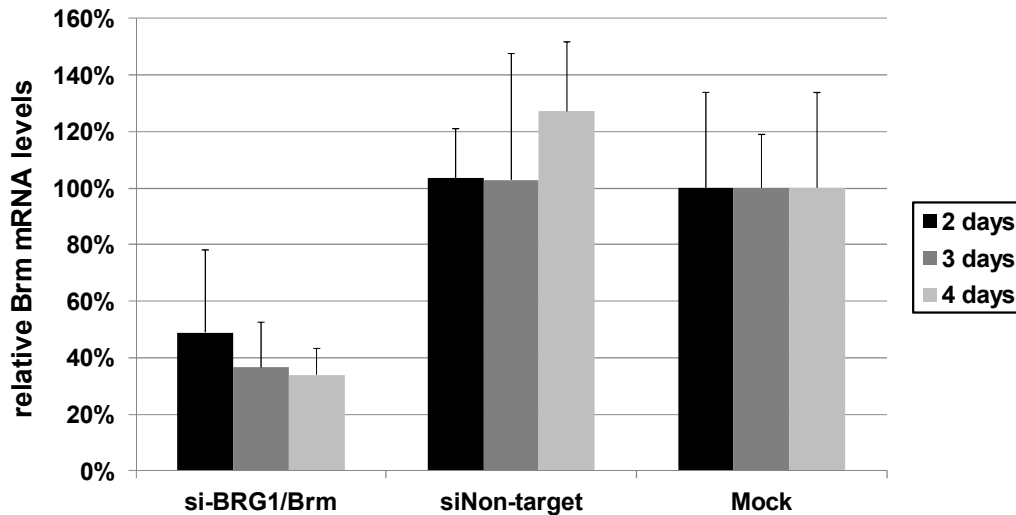
Fig. 4.30: Efficiency of Brm knockdown after 2-4 days

HEK293 cells were treated with siRNA against BRG1/Brm for two to four days. RNA was extracted, reversely transcribed and subjected to RT-qPCR. Brm mRNA levels were normalized to RPS14 as housekeeping gene. Error bars represent the standard error.

Because CHD4 and BRG1 proteins showed the highest reduction after 4 days, this point in time was chosen for further experiment. At this stage transcript levels were still below 40 % assuming a sufficient knockdown.

A combination of siCHD4#1 and siCHD4#2 was of no advantage regarding the knockdown efficiency (see above).

Because the use of siNon-target resulted also in a reduction of CHD4 and BRG1/Brm transcripts (see Fig. 4.27, 4.29), a siRNA against CHD3 (siCHD3) was additionally used as a second 'non-targeting' control because CHD3 expression is not detectable on protein level in HEK293 cells (Gundula Streubel, personal communication). CHD3 RNA level was reduced by > 50 % after 4 days of knockdown (see Fig. 4.31).

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Fig. 4.31: Efficiency of CHD3 knockdown after 4 days
HEK293 cells were treated with a siRNA against CHD3 (siCHD3) for four days. RNA was isolated, reversely transcribed and subjected to RT-qPCR. CHD3 mRNA levels were normalized to RPS14 as housekeeping gene. Error bars represent the standard error.

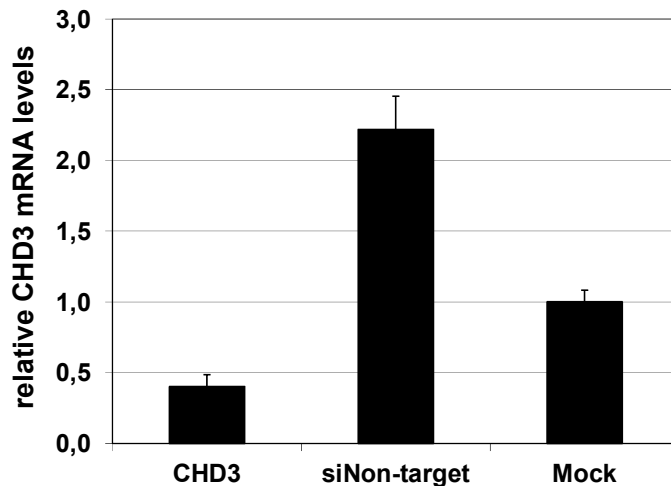To test whether CHD3 knockdown had any influence on CHD4, WCEs from CHD3 knockdown and Mock were compared in western blot (see Fig. 4.32). The Mi2 antibody used for that blot (H-242) is a rabbit polyclonal antibody raised against amino acids 1671-1912 of human CHD4 but also recognizes CHD3.[235] A significant difference in Mi2 protein levels between the siCHD3 treated sample and the Mock control could not be observed (Fig. 4.32, compare lanes 1 and 5). Thus, siCHD3 was assumed to be a suitable second non-targeting control for further experiments.
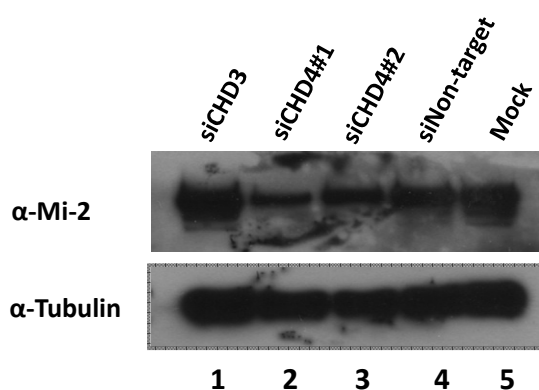
Fig. 4.32: CHD3 knockdown does not significantly influence Mi-2 protein level
To ensure that CHD3 siRNA does not reduce CHD4 protein levels, 50 µg of protein was used for western blot comparing Mi-2 levels after a four-day CHD3 or CHD4 knockdown with the Mock control and non-targeting siRNA treated samples.

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[235] Internet source: www.scbt.com/datasheet-11378-mi2-h-242-antibody.html.

4.2.5 Knockdown of CHD4 and BRG1 impairs induction of NF-κB target genes

In order to assess the impact of CHD4 and BRG1 for the induction of different TNFα-induced NF-κB target genes, a siRNA-mediated knockdown of both proteins was performed in HEK293 cells. Cells were stimulated with TNFα for one or four hours, RNA was extracted, reversely transcribed and subjected to RT-qPCR. A siRNA against CHD3 (siCHD3) was taken as a second unspecific control (as described in 4.2.4).

BRG1 knockdown resulted in a reduced induction of all tested NF-κB target genes by app. 50 % after one hour of TNFα stimulation (see Fig. 4.33 and 4.34). Surprisingly, CHD4 knockdown affected similarly the induction of the tested genes. With the exception of IRF-1 and Il-8, no difference in target gene induction impairment of greater than 10 % was observed between siCHD4#1 and siCHD4#2. These differences might reflect different knockdown efficiencies or siRNA-specific off-target effects. Interestingly, the induction after four hours was not impaired for either siRNA (Fig. 4.33, 4.34). Thus, the observed effects are assumed to be dependent on the kinetics of the activation.

A
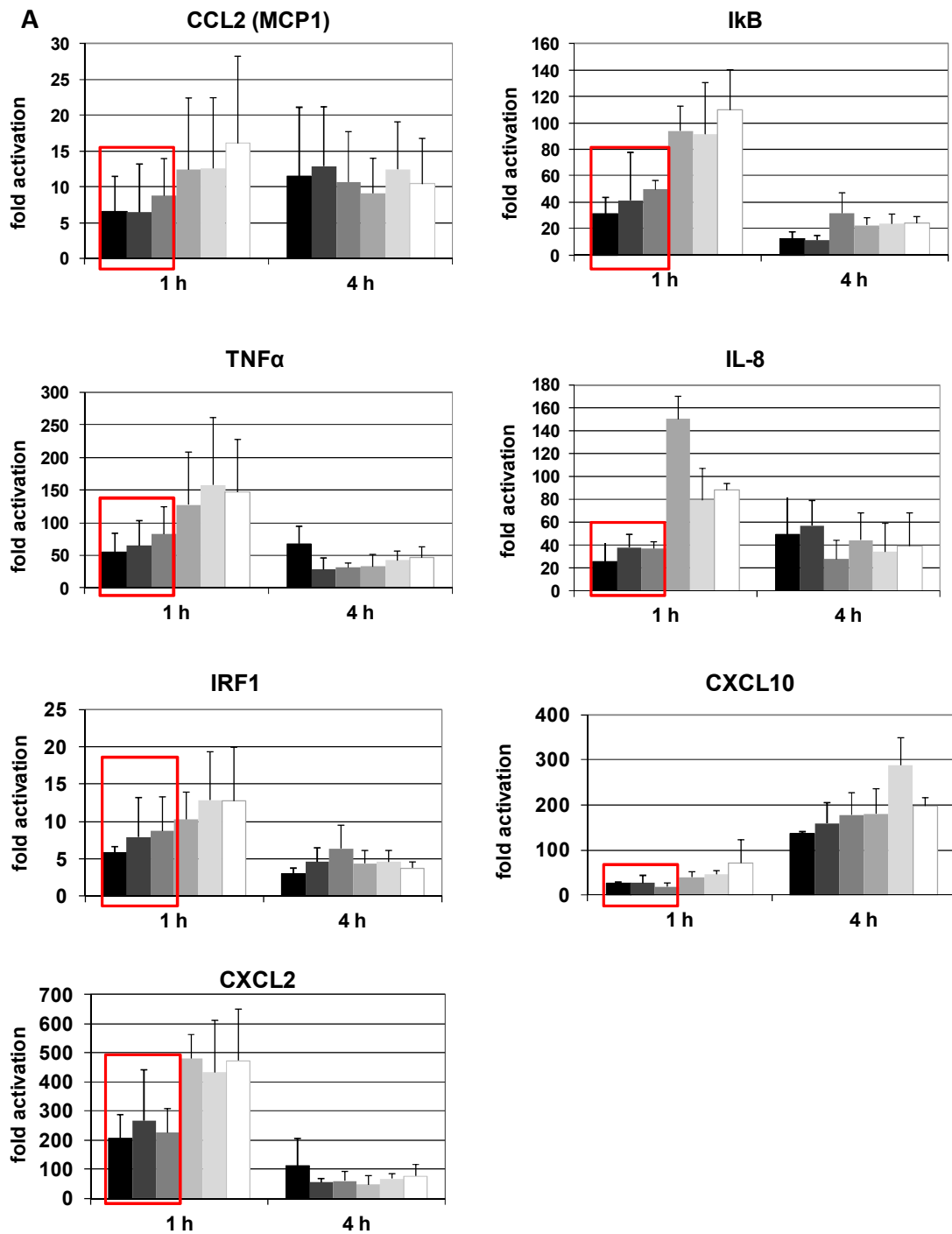
Fig. 4.33: Induction of TNFα induced NF-κB target genes is impaired by knockdown of CHD4 and BRG1 (1)

CHD4 and BRG1 protein levels were reduced by siRNA-mediated knockdown for 4 days in HEK293 cells. NF-κB was activated by stimulation with TNFα (20ng/ml). RNA was extracted after one and four hours, reversely transcribed and subjected to RT-qPCR. Induction of target genes was calculated as n-fold activation normalized to Mock. Error bars indicate the standard deviation between 3 independent experiments.

■ siCHD4#1   ■ siCHD4#2   ■ siBRG1/Brm   ■ siCHD3   ■ si-nt   □ Mock
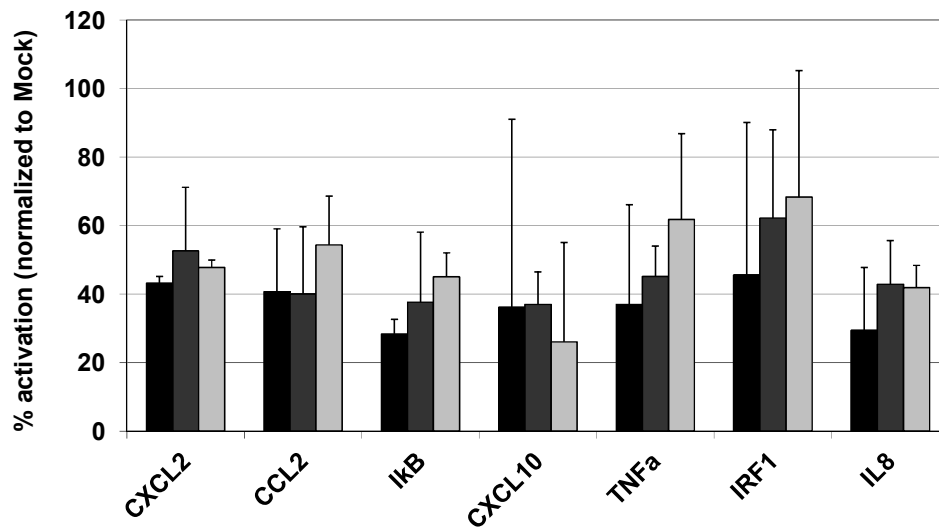
Fig. 4.34: Induction of TNFα induced NF-κB target genes is impaired by knockdown of BRG1 and CHD4 (2)
Summary of impaired NF- κB target gene induction after CHD4 or BRG1 knockdown. The target gene activation after TNFα treatment for 1 hour is presented as % induction of Mock. Error bars represent the standard deviation between three independent experiments.

■siCHD4#1   ■siCHD4#2   □siBRG1

To rule out an unspecific effect of CHD4 or BRG1 knockdown on transcription in general, the expression of the housekeeping gene ß-actin was measured. No alteration of ß-actin by knockdown or TNFα stimulation could be observed (see Fig. 4.35). Thus, the observed effects are specific for the tested target genes.
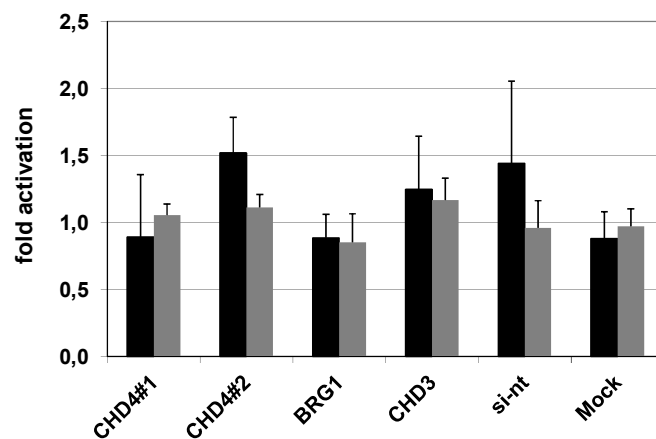
Fig. 4.35: CHD4 and BRG1 knockdown does not influence ß-actin expression
To test whether CHD4 and BRG1 knockdowns influence the expression of the housekeeping gene ß-actin, its expression level was determined by RT-qPCR. ß-actin was neither induced by TNFα stimulation nor significantly altered by any knockdown. Error bars indicate the standard deviation between three independent experiments.

■1 h   ■4 h

Treatment with siCHD3 or non-targeting siRNA had in most cases no significant effect on gene expression compared to untreated cells (Mock). However, the expression of two genes was significantly affected in control siRNAs experiments: Firstly, siCHD3 treatment resulted in a strong increase of Il-8 (150 fold versus 90 fold in Mock) one hour after TNFα addition (see Fig. 4.33). Secondly, treatment with non-targeting siRNA led to stronger increase in CXCL10 expression compared to Mock (290 fold versus 200 fold) after 4 hours of TNFα incubation (see Fig. 4.33). Since these deviations were limited to single genes, it is conceivable that they represent gene-specific off-target effects of the control siRNAs.

4.2.6 Mechanism of impairment

In HEK293 cells, a knockdown of BRG1 and CHD4 significantly impaired the induction of NF-κB target genes after TNFα stimulation. In order to assess the mechanism of this impairment, ChIP experiments were performed to determine whether the studied chromatin remodelers are directly associated with the target genes. CXCL2 was chosen for these experiments because the observed effects on this gene were strong and robust. CXCL2 is an inducible chemokine involved in attraction of neutrophils to sites of infection.[236] Five primer pairs were designed for CXCL2, amplifying the promoter region, a part of exon 2, intron 3, exon 4 and a region 0.4 kb downstream of the gene (Fig. 4.36). As a second non-specific control besides the downstream region of CXCL2, the ß-actin promoter was chosen, because it was already shown that TNFα stimulation neither induces its transcription nor that any of the tested chromatin remodeler knockdowns impair it.
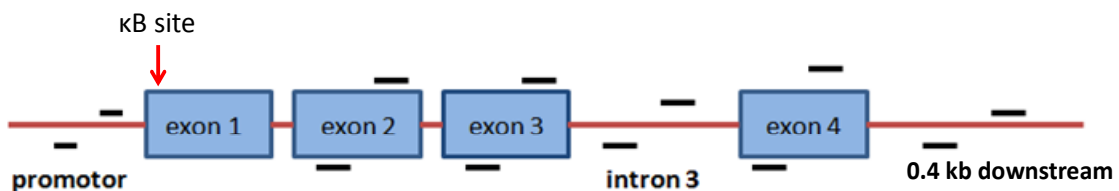
Fig. 4.36: Localization of ChIP primers within the CXCL2 gene
Scheme of ChIP primer positions. κB site (predicted NF-κB binding site) is indicated by the red arrow.

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[236] Ramirez-Carrozzi VR et al, Selective and antagonistic functions of SWI/SNF and Mi-2beta nucleosome remodeling complexes during an inflammatory response. Genes Dev (2006).

To assess any background binding, a sample with the exclusive addition of beads without antibody was run resulting in acceptable low signals (less than 0.003 % input, see Fig. 4.37).

Recruitment of NF-κB was tested by ChIP experiments using an antibody against the NF-κB subunit p50. A predicted binding site of NF-κB (κB site) is located at the beginning of exon one (chromosome 4: 74964991-74964980; "CGGGAATTTCCC",[237] only few base pairs apart from the reverse promoter primer, see Fig. 4.36). The p50 ChIP revealed a strong recruitment to the CXCL2 promoter after 40 minutes (0.04 % input) and a further increase after 60 minutes (0.06 % input) of TNFα stimulation. Recruitment of p50 to the other tested regions of the gene and the ß-actin promoter was only slightly increased (less than 0.03 % input).

Histone H3 ChIPs showed a strong decrease of binding within the promoter region after 40 minutes (from 0.2 % to 0.1 % input) and even less (0.05 % input) after 60 minutes (see Fig. 4.37). At exon 2 a similar decrease was notable after 60 minutes, indicating a more open state of the chromatin. A decrease of H3 binding was also present at the ß-actin promoter (ranging from 0.01% to 0.05 % input). Because the overall binding of H3 at this gene was also much lower (presumably due to its housekeeper role), any relevance of the latter observation remained elusive.

The depletion of histone H3 within the promoter region of CXCL2 suggested that chromatin remodeling took place. Thus, a recruitment of CHD4 and BRG1 especially to the promoter was conceivable and ChIP experiments for both were carried out.

Mi2 ChIPs produced a weak constitutive signal but no increased binding after TNFα stimulation was observed (see Fig. 4.37). One explanation for this result could be a constitutive low binding of Mi-2 to CXCL2. However, the Mi2-antibody has not been used in our laboratory for ChIP experiments so far. Thus, it remained unclear whether CHD4 is not recruited to the CXCL2 gene or the Mi2-antibody does not work in ChIP experiments. Such a failure to detect recruitment of CHD4 to NF-κB-bound CXCL2 promoter by ChIP could be due to the inability of the CHD4 antibody to precipitate CHD4 in the context of cross-linked chromatin. Thus, it would be useful to test another Mi-2 antibody in these experiments.

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[237]Internet resource: UCSC Genome browser: http://genome.ucsc.edu.

BRG1 ChIPs produced unspecific signals for all amplified regions. Thus, I assumed that the used antibody was not suitable for ChIP experiments (data not shown).

However, although the depletion of histone H3 suggested the involvement of chromatin remodelers and the knockdown experiments revealed a role for CHD4 and BRG1 in the TNFα-induced NF-κB activation, a direct recruitment of both remodelers to the model target gene, CXCL2, could not be confirmed. It might be necessary to try different CHD4 and BRG1 antibodies and other target genes for this ChIP experiment in future.
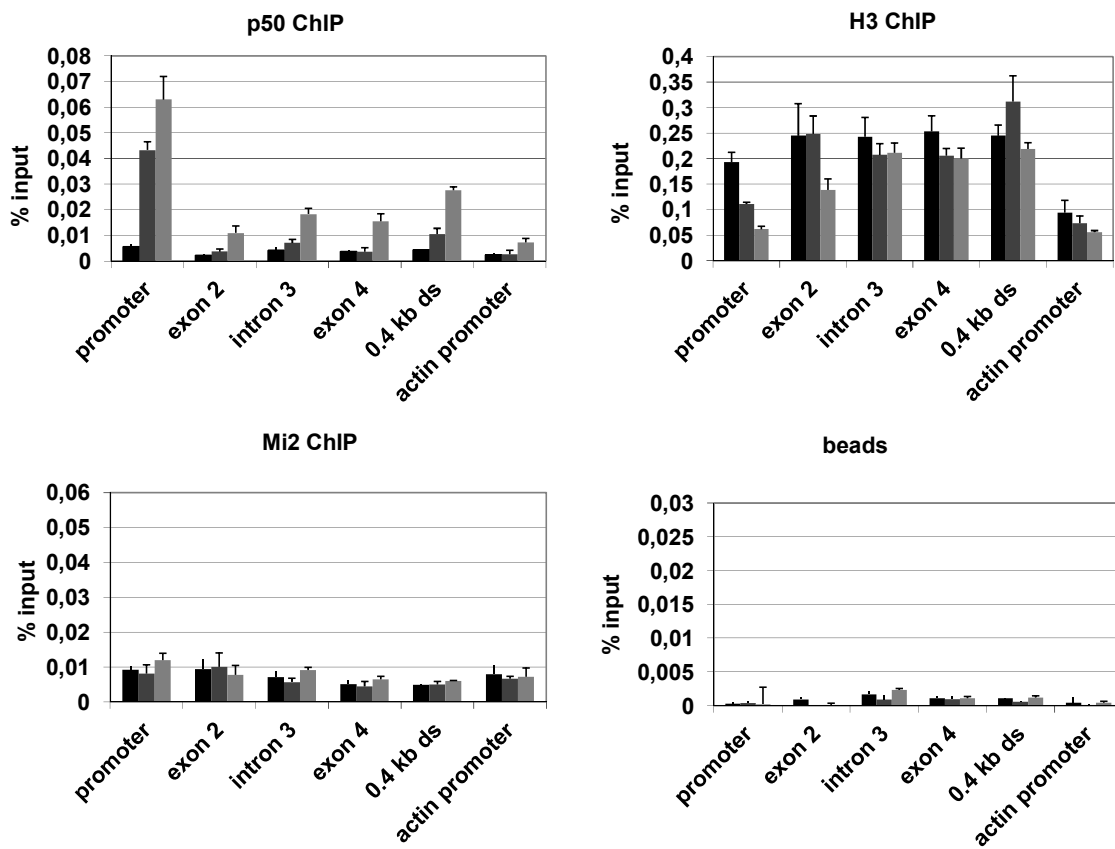
Fig. 4.37: p50, H3 and CHD4 ChIP experiments of the CXCL2 gene
ChIP experiments were performed in HEK293 cells after TNFα stimulation for 40 and 60 minutes using antibodies against the NF-kB subunit p50, Histon H3 and Mi2. As a negative control a sample with the exclusive addition of beads without antibody was used. Error bars represent the standard error.

■ untreated   ■ 40 min   ■ 60 min

As a second approach to evaluate whether CHD4 and BRG1 might be recruited by NF-κB to target genes, a co-immunoprecipitation using an antibody against the NF-κB subunit p65 was performed. HEK293 cells were stimulated with TNFα for one, two and four hours, nuclear extracts were prepared, subjected to p65-IP and analyzed in western blots for CHD4 and BRG1 co-immunoprecipitation. The fraction of p65 in nuclear extracts increased after TNFα stimulation, again indicating the nuclear re-localization of NF-κB (see Fig. 4.38, lanes 1-4). The p65 co-immunoprecipitation worked well (see Fig. 4.38, lanes 5, 7, 9 and 11) without unspecific binding to the beads (see Fig. 4.38, lanes 6, 8 and 10). Neither CHD4 nor BRG1 co-immunoprecipitated with p65 before or after TNFα stimulation. This suggests that no abundant and/or robust NF-κB-CHD4 and NF- κB-BRG1 complexes form after NF-κB activation by TNFα at the tested points in time but still cannot exclude such an interaction.
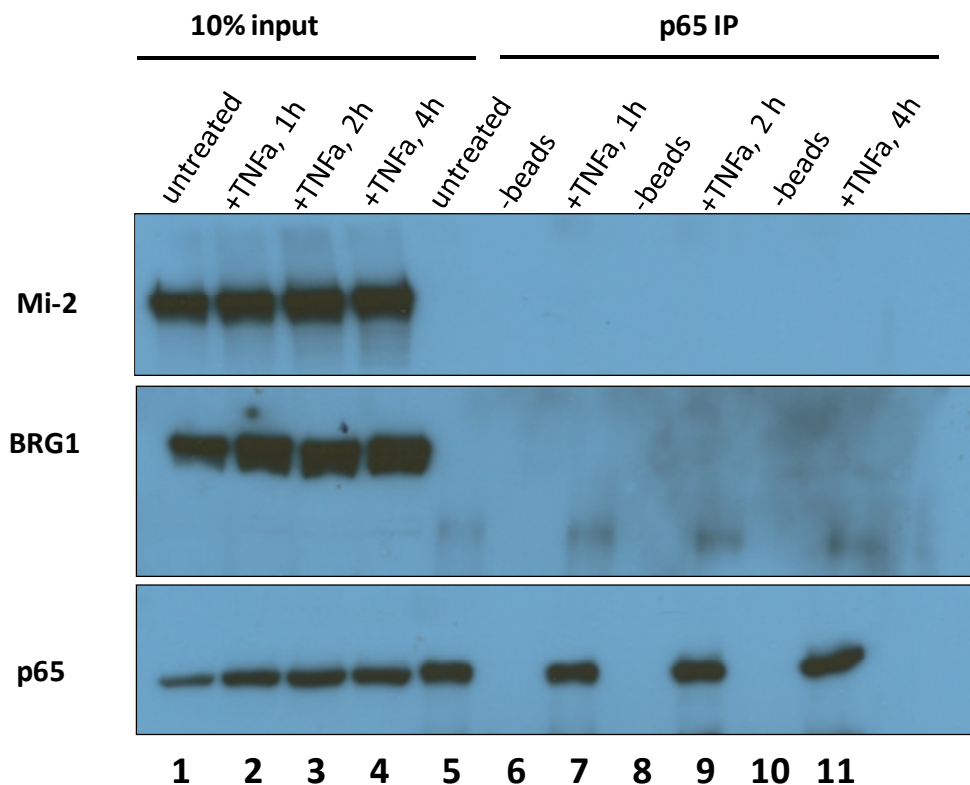
Fig. 4.38: Co-immunoprecipitation of RelA with Mi-2 and BRG1 upon TNFα stimulation
400 µg of nuclear extract were subjected to p65-IP. The following western blot was stained with antibodies against Mi2, BRG1 and p65. No interaction of RelA with Mi-2 and BRG1 upon TNFα stimulation (20 ng/ml) in HEK293 cells was observed.

5. Discussion

5.1. Discussion objective 1

5.1.1 Establishment of polyclonal antisera against CHD5

One major goal of this work was the characterization of polyclonal CHD5 antibodies and the evaluation of their use as a tool to investigate CHD5. Three polyclonal peptide antisera were successfully generated by Ina Wanandi. All of them specifically reacted with recombinant hCHD5 protein in Western Blot. In addition, antiserum R was able to detect endogenous CHD5 from mouse brain lysates. None of them could be successfully used in immunoprecipitation and immunofluorescence. For further studies all of them can be used in Western Blot in order to detect CHD5 from human origin, antiserum R additionally for murine CHD5. They are not suitable for immunoprecipitations or for immunofluorescence stainings. For these applications and for detection of rodent CHD5 in Western Blot the CHD5 antibody M-182 was shown to be better applicable in this study. Thus, for further experiments I mostly used this M-182 antibody.

Concurrently with this study, several CHD5 antibodies became available. Three of them detected a band at 250 kDa (α-CHD5 RabMAb from Epitomics[238]; rabbit polyclonal α-CHD5 from Abcam[239]; Y-1 as published by Yoshimura S et al[240]). This was somewhat surprising because we detected the recombinant CHD5 protein at 220 kDa, consistent with the estimated molecular mass of CHD5, with our antisera and additionally with the CHD5 antibody M-182. Furthermore, in this study, endogenous CHD5 from mouse brain lysates was shown to have the same electrophoretic mobility as the recombinant CHD5 protein. But most likely, this discrepancy is due to the use of different molecular markers as these can differ dramatically and allows no further conclusion.

The rabbit α-CHD5 monoclonal antibody from Epitomics was predicted to cross-react with hCHD3 by the manufacturer. The manufacturer suggested a detection of CHD5 with their antibodies in protein extracts from adrenal gland and the neuroblastoma

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[238] www.epitomics.com/products/product_info/10413/CHD5-antibody-5212-1.html.

[239] www.abcam.com/Chromodomain-helicase-DNA-binding-protein-5-antibody-ab66516.html.

[240] Yoshimura S et al, A rat monoclonal antibody against the chromatin remodeling factor CHD5. Hybridoma (Larchmt). (2010).

cell line SH-SY-5Y. According to this study and Potts et al[241] both were shown not to express CHD5 protein. Thus, these results argue that this antibody probably detect CHD3 instead of CHD5.

The rabbit α-CHD5 polyclonal antibody from Abcam (ab66516) was evaluated by the manufacturer on liver tissue lysates and protein extracts from HeLa cells. Also Yoshimura et al[242] established their monoclonal CHD5 antibody Y-1 on protein extracts from HeLa cells labelling the detected band as CHD5. I detected CHD5 in RNA and protein extracts neither from rat liver nor from HeLa cell protein extracts. The absent expression in HeLa cells is consistent with data from Potts et al.[243] Altogether, I doubt whether these antibodies indeed detect CHD5.

Two other commercial antibodies were described to detect a protein at 90 kDa (goat α-CHD5 antibody from Everest Biotech[244]; goat α-CHD5 from ProSci[245]). This distinct difference in molecular weight could be in principle explained by the existence of another isoform of CHD5 or degradation of CHD5 protein in the extracts tested. So far, besides the second isoform of CHD5 in mice, which differs only insignificantly from the predicted molecular weight of the first one, no other splicing variant is predicted to my knowledge that could explain this low electrophoretic mobility.

Although the use of different molecular markers decreases the comparability between different laboratories, as mentioned above, I would rigorously assess whether the proteins at 90 kDa and 250 kDa detected by the commercial antibodies indeed specifically react with CHD5. Possibly, the bands at 90 kDa and 250 kDa could be caused by cross-reactivity to CHD3 or CHD4. For further studies, I preferred to use the CHD5 antibody M-182.

5.1.2 CHD5 overexpression does not induce senescence in SAOS-2 cells

CHD5 was found to interfere with the p16-pRb-pathway for promotion of cellular senescence in MEFs[246] but the molecular mechanism remained unclear. In order to

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[241] Potts RC et al, CHD5, a Brain-Specific Paralog of Mi2 Chromatin Remodeling Enzymes, Regulates Expression of Neuronal Genes. PLoS One (2011).

[242] Yoshimura S et al, A rat monoclonal antibody against the chromatin remodeling factor CHD5. Hybridoma (Larchmt) (2010).

[243] Potts RC et al, CHD5, a Brain-Specific Paralog of Mi2 Chromatin Remodeling Enzymes, Regulates Expression of Neuronal Genes. PLoS One (2011).

[244] http://everestbiotech.com/product/goat-anti-chd5-antibody.

[245] www.prosci-inc.com/CHD5-Antibody-c-46-642.

[246] Bagchi A et al, CHD5 is a Tumorsuppressor at Human 1p36. Cell (2007).

gain mechanistic insight into the effects of CHD5 on Rb-induced senescence I decided to use the so-called 'flat cell assay'[247] in SAOS-2 cells. Rb-induced senescence was successfully induced by transfection of its large pocket domain. CHD5 overexpression itself did not induce senescence in that cell system. It appeared conceivable that CHD5 modulates senescence exclusively in the presence of pRb. Thus, we studied the formation of flat cells upon co-expression of pRb and CHD5. This co-expression did not further augment or diminish the pRb-induced senescence. I did not control whether co-expression was successful because the selection process during transfection complicated protein extraction. A successful transfection was assumed by the reproducible induction of senescence in Rb-transfected cells and expression of both proteins was driven by the same promoter (CMV). Thus, an inefficient co-transfection was unlikely, but could explain nevertheless any absent effect. A better method than Western Blot to control the successful co-transfection would be an immunofluorescence staining of CHD5 and pRb.

Furthermore, it is possible that CHD5 and pRb directly interact during the induction of senescence via a Rb-domain outside its large pocket domain. In this study, only the large pocket domain of Rb was transfected, thus, a possible interaction could be disturbed. Here, it could be worthwhile to repeat these experiments with transfection of the entire Rb.

However, CHD5 does not seem to influence senescence in this experimental system. Another system to study the influence of CHD5 on senescence could be a cell line with inducible CHD5 expression.

5.1.3 Overexpressed CHD5 does not directly interact with endogenous p53

It has been proposed that CHD5 regulates the p53 level via the p19[ARF] pathway.[248] Alternatively, CHD5 or a CHD5-containing complex could directly interact with p53. A similar model was suggested for a MTA2-containing NuRD complex regulating the p53-mediated transactivation by modulating the p53 acetylation status.[249] Since CHD5 was shown to be part of a NuRD-like complex[250], it may be found to function similarly.

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[247] Hinds PW et al, Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. Cell (1992).
[248] Bagchi A et al, CHD5 is a Tumorsuppressor at Human 1p36. Cell (2007).
[249] Luo J et al, Deacetylation of p53 modulates its effects on cell growth and apoptosis. Nature (2000).
[250] Potts RC et al, CHD5, a Brain-Specific Paralog of Mi2 Chromatin Remodeling Enzymes, Regulates Expression of Neuronal Genes. PLoS One (2011).

Thus, we tested the possible interaction of endogenous (functionally inactive) p53 and overexpressed Flag-tagged CHD5 in HEK293 cells. This approach was chosen because the classical method to show a co-immunoprecipitation of two overexpressed proteins failed since a simultaneous overexpression of both candidate interaction partners did not succeed. No co-immunoprecipitation of Flag-tagged CHD5 with endogenous p53 could be achieved, suggesting there is no direct interaction between overexpressed CHD5 and endogenous p53. Another possibility would be that the interaction is not strong enough for a robust co-immunoprecipitation. Furthermore, a possible interaction might be restricted to a cellular stress response. To test this hypothesis, the experiment could be repeated during cellular stress as induced via TNFα, heat-shock or cytotoxic treatment.

5.1.4 CHD5 expression profile in tissues and cell lines

So far, CHD5 expression was thought to be restricted to cells of neural origin as in the central nervous system and adrenal gland.[251] To gain further insight, I performed a detailed expression analysis of CHD5 in primary tissues and several cell lines.

In rat muscle, liver, heart and lung CHD5 was only detectable at levels of background transcription. This is in agreement with previous reports.[252] CHD5 protein was also absent. It is still possible that its expression is below our detection threshold and that it may have some important functions even at these low levels. Moreover, there may be specific cell types within these tissues that express CHD5. For example, an expression in peripheral muscle nerve cells appears plausible because of their neural origin. This would have been missed when analyzing tissue lysates in Western Blot and RT-qPCR. Thus, it might be indicated to also perform immunofluorescence stainings on primary tissue sections. The CHD5 antibody M-182 is suitable for that application as tested on tissue sections of mouse brain (data not shown).

As a second explanation, CHD5 expression could be restricted to specific developmental stages what would have also been missed. This assumption led to our

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[251] Thompson PM et al, CHD5, a new member of the chromodomain gene family, is preferentially expressed in the nervous system; Oncogene (2003).
[252] Potts RC et al, CHD5, a Brain-Specific Paralog of Mi2 Chromatin Remodeling Enzymes, Regulates Expression of Neuronal Genes. PLoS One (2011).
Thompson PM et al, CHD5, a new member of the chromodomain gene family, is preferentially expressed in the nervous system; Oncogene (2003).

experiments with Pc12 cells undergoing differentiation, but there are many other cell types and developmental processes which could also be assessed.

I tested four neuroblastoma cell lines for CHD5 expression. None of the four tested neuroblastoma cell lines expressed CHD5. Many neuroblastoma cell lines have been tested for CHD5 expression on transcript level so far and none was shown to harbor significant levels.[253] To my knowledge, this study was the first to analyze the cell lines SH-EP and SMS-KCN; the absence of CHD5 in IMR-32 and SH-SY-5Y are consistent with previous studies.[254] Furthermore, the absence of CHD5 in neuroblastoma cell lines is in agreement with the suggested role for CHD5 as tumor suppressor in neuroblastomas.[255]

As previously mentioned, several possibilities exist for the lack of CHD5 expression. The first possibility is a homozygous deletion of the short arm of chromosome 1 (1p) including the CHD5 locus. This possibility is excluded at least for SH-EP cells because they were shown to have an intact short arm of chromosome 1.[256] Another possible explanation could be a loss of heterozygosity (LOH). A LOH is defined as inactivation of one allele by deletion and silencing of the second allele by promoter hypermethylation or an inactivating mutation. A deletion of one 1p allele is known for IMR-32.[257] Although the CHD5 promoter was shown to be unmethylated in this cell line[258] I tested this hypothesis further by treating IMR-32 cells with the demethylating agent 5-azacytidine because the methylation status can differ even between different passages of a cell line (Frank Lyko, personal communication). However, 5-azacytidine

[253] Garcia I et al, Expression of the neuron-specific protein CHD5 is an independent marker of outcome in neuroblastoma; Mol Cancer (2010).
Fujita T et al, CHD5, a Tumor Suppressor Gene Deleted From 1p36.31 in Neuroblastomas, J Natl Cancer Inst (2008).
Thompson PM et al, CHD5, a new member of the chromodomain gene family, is preferentially expressed in the nervous system; Oncogene (2003).
[254] Mulero-Navarro S and Esteller M, Chromatin remodeling factor CHD5 is silenced by promoter CpG island hypermethylation in human cancer. Epigenetics (2008).
Potts RC et al, CHD5, a Brain-Specific Paralog of Mi2 Chromatin Remodeling Enzymes, Regulates Expression of Neuronal Genes. PLoS One (2011).
[255] Garcia I et al, Expression of the neuron-specific protein CHD5 is an independent marker of outcome in neuroblastoma; Mol Cancer (2010).
[256] Carr J et al, High-resolution analysis of allelic imbalance in neuroblastoma cell lines by single nucleotide polymorphism arrays. Cancer Genet Cytogenet (2007).
[257] Schleiermacher G et al, Combined 24-color karyotyping and comparative genomic hybridization analysis indicates predominant rearrangements of early replicating chromosome regions in neuroblastoma. Cancer Genet Cytogenet (2003).
[258] Mulero-Navarro S and Esteller M, Chromatin remodeling factor CHD5 is silenced by promoter CpG island hypermethylation in human cancer. Epigenetics (2008).

could not restore CHD5 expression at the protein level, thus it might not explain the absence of CHD5 in IMR-32 cells or the restored expression was still below our detection limit.

Nevertheless, a recent analysis of tissue samples from 188 neuroblastoma patients demonstrated no examples of somatically acquired CHD5 mutations, indicating that homozygous genomic inactivation is rare. Furthermore, a methylation of the CHD5 promoter was commonly found in the high-risk tumors, generally associated with both 1p deletion and MYCN amplification.[259] Because re-expression of CHD5 during induction therapy was found to be associated with a better clinical outcome[260], a possible CHD5 restoration induced by demethylating agents provides a promising new therapeutic strategy for the treatment of high-risk neuroblastomas.

Regarding CHD5 expression in brain I was interested which cell types in brain express CHD5. Assuming that it has to be an abundant cell type, I established successfully a primary astrocyte culture. Lysates from these cultures were negative for CHD5 in RT-qPCR and Western Blot, which is in agreement with recently published data.[261] Moreover, the staining pattern of a CHD5 in-situ-hybridization on mouse brain tissue sections from *The Allen Institute Mouse Brain Atlas* appeared more like a neuronal expression pattern.[262] Furthermore, a prominent staining of the hippocampus region, a part of the brain with an important function for memory, was remarkable. This region, or, more precisely, the subgranular zone of the hippocampal dentate gyrus, was shown to function as a stem cell niche in adult mammals.[263] Therefore, I tested protein lysates from Ht22 cells, a cell line derived from hippocampal neurons, and from murine neural stem cells for CHD5 expression. The Ht22 cells turned out to be negative although I expected that CHD5 should be expressed in these cells. Considering the possibility that CHD5 is involved in negative regulation of cellular proliferation, its expression might be lost in cell culture, thus enabling cells to proliferate with less restriction in culture.

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[259] Koyama H et al, Mechanisms of CHD5 Inactivation in Neuroblastomas. Clin Cancer Res. (2012).

[260] Garcia I et al, Expression of the neuron-specific protein CHD5 is an independent marker of outcome in neuroblastoma; Mol Cancer (2010).

[261] Garcia I et al, Expression of the neuron-specific protein CHD5 is an independent marker of outcome in neuroblastoma; Mol Cancer (2010).

[262] Internet source: www.brain-map.org.

[263] Gage FH, Mammalian neural stem cells. Science (2000).
Ma DK et al, Glial influences on neural stem cell development: cellular niches for adult neurogenesis. Curr Opin Neurobiol. (2005).

Immunofluorescence stainings of tissue sections from the hippocampus would be required to further support the data from *The Allen Institute Mouse Brain Atlas* showing abundant CHD5 expression in this region of the brain.

Surprisingly, the murine neural stem cells turned out to express CHD5. Given that neural stem cells in the adult brain account for only a small number of cells and 100 µg of nuclear extract were required to produce a signal in Western Blot that was less intense than that from rodent brain lysates, neural stem cells cannot be the main CHD5-expressing cell type in brain. It may be inferred that the bulk of CHD5 is expressed in neurons as it was recently shown by Garcia et al.[264] In addition, it needs to be determined whether CHD5 expression is maintained in long-term neural stem cell cultures. Considering its anti-proliferative properties,[265] a loss of expression appears plausible in principle.

However, this is the first evidence that neural stem cells express CHD5. More, they are the first known naturally CHD5-expressing cell type that can be kept in cell culture for further functional and biochemical experiments.

Nevertheless, for a comprehensive CHD5 expression profile of brain cells it may be indicated to perform a cell sort of brain cells, for example via antibody-coupled magnetic beads, followed by an analysis of protein lysates in Western Blot. In addition, immunofluorescence staining of mouse brain sections with adequate co-staining for characterization of the different cell types could be carried out.

Interestingly, CHD5 knockdown was found to reduce gene sets commonly found to be upregulated in Alzheimer's disease. Thus, suggesting that CHD5 might be required for the expression of these Alzheimer promoting genes. At the same time, CHD5 was demonstrated to constitute a NuRD-like complex interacting with HDAC2 in neurons.[266] An inhibition of class I HDACs by sodium valproate and other HDAC inhibitors was demonstrated to improve the memory deficits in a mouse model of Alzheimer's disease.[267] That could be at least partially related to the interaction of HDAC2 and

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[264] Garcia I et al, Expression of the neuron-specific protein CHD5 is an independent marker of outcome in neuroblastoma; Mol Cancer (2010).
[265] Bagchi A et al, CHD5 is a Tumorsuppressor at Human 1p36. Cell (2007).
[266] Potts RC et al, CHD5, a Brain-Specific Paralog of Mi2 Chromatin Remodeling Enzymes, Regulates Expression of Neuronal Genes. PLoS One (2011).
[267] Kilgore M et al, Inhibitors of class 1 histone deacetylases reverse contextual memory deficits in a mouse model of Alzheimer's disease. Neuropsychopharmacology (2010).

CHD5. Although I would assume CHD5 interacting with HDAC2 is mainly involved in repression of genes, a promotion of Alzheimer-promoting genes via an indirect mechanism appears possible. Thus, the inhibition of class I HDAC inhibitors could be an interesting therapeutic approach for the future treatment of Alzheimer's disease.

In this study, four human glioblastoma cell lines as well as the mouse glioblastoma cell line Gl261 were tested for CHD5 expression. None of them were found to express CHD5. Glioblastoma cell lines are brain-derived and, more precisely, they are of astrocytic origin. Thus, considering the absence of CHD5 in astrocytes, that may have been predicted and does not allow any conclusion on functional consequences. Interestingly, the CHD5 promoter was shown to be hypermethylated in three of our tested cell lines (T98G, A172 and U87),[268] suggesting a plausible mechanism of CHD5 silencing possibly during fate-commitment to the astrocytic lineage. For further insight into this topic a methylation analysis of the CHD5 promoter in astrocytes might be of interest. Moreover, neural stem cells could be differentiated into astrocytes in culture while monitoring the methylation status of the CHD5 promoter in parallel to its expression level.

Regarding the common deletion of 1p in gliomas, CHD5 was claimed to be a candidate tumor suppressor gene. More, Ichimura et al[269] found CHD5 mostly included in the deleted area of 1p36 in gliomas. Bagchi et al[270] found CHD5 RNA to be absent in a subset of glioma samples with deleted CHD5 compared to normal brain. Interestingly, in glioma samples without CHD5 deletion they found CHD5 expression present but to a lesser extent than in normal brain. I remain in doubt whether the detection of CHD5 in this subset really provides evidence for CHD5 expression. Gliomas usually infiltrate the brain by active migration through the extracellular spaces of neurons (see introduction). This complicates any microdissection of the tumor from the surrounding brain structures for proper analysis. Furthermore, in glioma samples without CHD5 deletion the indicated standard deviation was many times greater than in the other samples supporting my suspicion that these samples were contaminated to a varying

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[268] Mulero-Navarro S and Esteller M, Chromatin remodeling factor CHD5 is silenced by promoter CpG island hypermethylation in human cancer. Epigenetics (2008).
[269] Ichimura K et al, 1p36 is a preferential target of chromosome 1 deletions in astrocytic tumors and homozygously deleted in a subset of glioblastoma. Oncogene (2008).
[270] Bagchi A et al, CHD5 is a Tumorsuppressor at Human 1p36. Cell (2007).

degree. I would suggest exploring CHD5 expression again in these subsets of gliomas by immunofluorescence staining, thus avoiding contamination with surrounding neurons.

To postulate CHD5 as a possible tumor suppressor in gliomas based on its absent expression is a bold hypothesis. For that to be concluded, extensive functional studies are required. Even if the glioma origin would be assumed to be a cell type with stem-cell-like properties, CHD5 presence could be even favorable for tumorigenesis, as we demonstrated CHD5 expression in murine neural stem cells. Furthermore, it was shown that CHD5 promotes the expression of a subset of genes commonly upregulated in neural stem cells[271] suggesting a promotion of stem-cell maintenance, rather than the opposite. However, this remains at the level of speculation until detailed functional data are available. I would not exclude that CHD5 might play a role as tumor suppressor in gliomas and especially glioblastomas but the rationale behind this assumption decreases with the absence of CHD5 expression in astrocytes.

5.1.5 CHD5 expression in testes

Surprisingly, CHD5 protein was robustly expressed in mouse and rat testes. Although its testicular expression was predicted by SAGE, it was reported to be absent[272] or indeterminate[273] in previous studies. CHD5 expression on transcript level was strongly consistent with protein expression. Given the paucity of neural-related tissue within the testes it is very unlikely that the robust CHD5 expression is caused by that. CHD5 could be ubiquitously expressed in testes or restricted to any abundant cell type as cells from different stages of spermatogenesis, Sertoli or Leydig cells. Considering the massive expression of CHD5 in testes it may be assumed that it is expressed in cells of spermatogenesis. This is further supported by the finding that CHD5 is not yet expressed in testes of 3 week old mice, reflecting its possible involvement in spermatogenesis (J. Bergs, personal communication).

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[271] Potts RC et al, CHD5, a Brain-Specific Paralog of Mi2 Chromatin Remodeling Enzymes, Regulates Expression of Neuronal Genes. PLoS One (2011).
[272] Thompson PM et al, CHD5, a new member of the chromodomain gene family, is preferentially expressed in the nervous system; Oncogene (2003).
[273] Potts RC et al, CHD5, a Brain-Specific Paralog of Mi2 Chromatin Remodeling Enzymes, Regulates Expression of Neuronal Genes. PLoS One (2011).

I did not test CHD5 expression in tissue lysates of ovaries. This would be an important issue to assess in the future because the ovaries host the female germ cells like testes the male germ cells. Thus, an expression in ovaries is feasible.

In this context, the possible role of CHD5 as a tumor suppressor may again attract some attention. In order to explore this possibility of whether CHD5 may play a role as tumor suppressor in germ cell tumors (GCTs), I analyzed published cytogenetic data for chromosomal aberrations involving 1p, especially deletions of 1p36 including the CHD5 locus.

In most publications, cytogenetic analysis is focused on loci proximal to the CHD5 locus which is located subtelomerically. Thus, a detailed analysis from literature whether the CHD5 locus is included in common regions of deletion was complicated. In many publications, microsatellite markers for a region closed to the CHD5 locus were used and an absence of the markers made a simultaneous deletion of the CHD5 locus likely, but a final conclusion cannot be drawn. Furthermore, the majority of cytogenetic analyses in germ cell tumors were performed before 2003 when CHD5 was first described.[274] Thus, an explicit search for the CHD5 locus remains to be done. Nevertheless, the available data is summarized below:

Chromosomal aberrations including the short arm of chromosome 1 are frequently observed in germ cell tumors (GCTs) but they appear less specific than in other neoplasms.[275]

Zahn S et al[276] showed that deletion of 1p36 is a common event in pediatric GCTs but constitutes a rare aberration in adults. Several other authors detected cases of pediatric GCTs with deletions of 1p36, presumably including the CHD5 locus in most of them.[277]

[274] Thompson PM et al, CHD5, a new member of the chromodomain gene family, is preferentially expressed in the nervous system; Oncogene (2003).
[275] Saikevych IA et al, Cytogenetic study of a testicular tumor in a translocation (13;14) carrier. Cancer Genet Cytogenet. (1987).
[276] Zahn S et al, Imbalances of Chromosome Arm 1p in Pediatric and Adult Germ Cell Tumors Are Caused by True Allelic Loss: A Combined Comparative Genomic Hybridization and Microsatellite Analysis. Genes Chromosomes Cancer (2006).
[277] Stock C et al, Cytogenetic aspects of pediatric germ cell tumors. Klin Padiatr. (1995).
Stock C et al, Detection of numerical and structural chromosome abnormalities in pediatric germ cell tumors by means of interphase cytogenetics. Genes Chromosomes Cancer (1994).
Bussey KJ et al, Chromosome abnormalities of eighty-one pediatric germ cell tumors: sex-, age-, site-, and histopathology-related differences--a Children's Cancer Group study. Genes Chromosomes Cancer (1999). Schneider DT el at, Genetic analysis of mediastinal nonseminomatous germ cell tumors in children and adolescents. Genes Chromosomes Cancer (2002).
Schneider DT et al, Genetic analysis of childhood germ cell tumors with comparative genomic hybridization. Klin Padiatr. (2001).

Especially a large majority of pediatric yolk sac tumors harbor 1p36 deletions.[278] Since no larger studies on the significance of 1p36 deletions were completed so far, and all published data consists more or less of case collections, a precise estimation of its incidence cannot be carried out although it seems to be a common event. The same is true for a possible prognostic impact. Interestingly, yolk sac tumors are, like neuroblastomas, a disease of younger children.[279]

In adults, several cases of GCTs are described in which 1p/1p36 is involved in non-random chromosomal rearrangements and deletions.[280] Although it was my intention to analyze thoroughly the involvement of the CHD5 locus in the published cases, that was very difficult because markers specific for that locus were only infrequently used.

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[278] Jenderny J et al, Detection of chromosome aberrations in paraffin sections of seven gonadal yolk sac tumors of childhood. Hum Genet. (1995).
Jenderny J et al, Interphase cytogenetics on paraffin sections of paediatric extragonadal yolk sac tumours. Virchows Arch. (1996).
Perlman EJ et al, Genetic analysis of childhood endodermal sinus tumors by comparative genomic hybridization. J Pediatr Hematol Oncol. (2000).
Hu J et al, Deletion mapping of 6q21-26 and frequency of 1p36 deletion in childhood endodermal sinus tumors by microsatellite analysis. Oncogene (2001).
Perlman EJ et al, Cytogenetic analysis of childhood endodermal sinus tumors: a Pediatric Oncology Group study. Pediatr Pathol. (1994).
Perlman EJ et al, Deletion of 1p36 in childhood endodermal sinus tumors by two-color fluorescence in situ hybridization: a pediatric oncology group study. Genes Chromosomes Cancer (1996).
Stock C et al, Cytogenetic aspects of pediatric germ cell tumors. Klin Padiatr. (1995).
Stock C et al, Detection of numerical and structural chromosome abnormalities in pediatric germ cell tumors by means of interphase cytogenetics. Genes Chromosomes Cancer (1994).
Mostert et al, Comparative genomic and in situ hybridization of germ cell tumors of the infantile testis. Lab Invest. (2000).
Van Echten J et al, Infantile and adult testicular germ cell tumors. a different pathogenesis? Cancer Genet Cytogenet. (2002).
[279] Hu J et al, Deletion mapping of 6q21-26 and frequency of 1p36 deletion in childhood endodermal sinus tumors by microsatellite analysis. Oncogene (2001).
[280] Saikevych IA et al, Cytogenetic study of a testicular tumor in a translocation (13;14) carrier. Cancer Genet Cytogenet. (1987).
Castedo SM et al, Chromosomal changes in human primary testicular nonseminomatous germ cell tumors. Cancer Res. (1989).
Smolarek TA et al, Cytogenetic analyses of 85 testicular germ cell tumors: comparison of postchemotherapy and untreated tumors. Cancer Genet Cytogenet. (1999).
Summersgill B et al, Molecular cytogenetic analysis of adult testicular germ cell tumours and identification of regions of consensus copy number change. Br J Cancer (1998).
Van Echten J et al, No recurrent structural abnormalities apart from i(12p) in primary germ cell tumors of the adult testis. Genes Chromosomes Cancer (1995).
Atkin NB and Baker MC, Chromosome analysis of three seminomas. Cancer Genet Cytogenet. (1985).
Gibas Z et al, Chromosome Changes in Germ Cell Tumors of the Testis. Cancer Genet Cytogenet. (1986).
Samaniego F et al, Cytogenetic and molecular analysis of human male germ cell tumors: chromosome 12 abnormalities and gene amplification. Genes Chromosomes Cancer (1990).
Parrington JM et al, Chromosome analysis of parallel short-term cultures from four testicular germ-cell tumors. Cancer Genet Cytogenet. (1994).
Rodriguez E et al, Cytogenetic analysis of 124 prospectively ascertained male germ cell tumors. Cancer Res. (1992).
Kernek KM et al, Identical allelic losses in mature teratoma and other histologic components of malignant mixed germ cell tumors of the testis. Am J Pathol. (2003).

Nevertheless, several of the published cytogenetic data make an inclusion of CHD5 in the deleted region likely. In contrast, Mathew S et al[281] found the 1p22 region in particular involved in 1p deletions.

In synopsis of the described cases in adults, a preference of 1p36 deletions for specific histological subtypes of GCTs appears unlikely, although some authors found such predominance as Rodriguez et al[282] for teratomas. But one histological hallmark of GCTs, especially in non-seminomas, is the coexistence of various histological subtypes within one tumor.[283] In principle, a chromosomal aberration could be restricted to one of the specific histological components.

As for the pediatric tumors, as discussed above, the significance of the cytogenetic findings is limited by the lack of large systematic data collections. In addition, given the amazing histological variety of GCTs, a separated analysis of the different histological components within one tumor might be required.

Summarizing, it has to be mentioned that pediatric yolk sac tumors appears to be the sole tumor entity within GCTs displaying commonly 1p36 deletions including most likely the CHD5 locus.

Furthermore, I was interested whether CHD5 expression is altered in any genome-wide study of GCTs. No alteration of CHD5 expression in genome-wide studies was mentioned.[284] That does not exclude any alteration of CHD5 expression because the studies focused mainly on upregulated genes and, in addition, it is always possible that altered genes are missed in microarrays due to normalization or technical issues.

The hypothesis that CHD5 works as tumor suppressor in testicular cancer/GCTs might simplify the comprehensive view of a chromatin remodeler. Many of these enzymes, such as CHD4 and BRG1, display ambivalent roles during tumorigenesis (see introduction). Although CHD5 was shown to interact with HDAC2 in a NuRD-like

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[281] Mathew S et al, Loss of heterozygosity identifies multiple sites of allelic deletions on chromosome 1 in human male germ cell tumors. Cancer Res (1994).
[282] Rodriguez E et al, Cytogenetic analysis of 124 prospectively ascertained male germ cell tumors. Cancer Res. (1992).
[283] Wittekind C, Biologie und Pathologie von Keimzelltumoren des Hodens. Onkologe (2008).
[284] LeBron C et al, Genome-wide analysis of genetic alterations in testicular primary seminoma using high resolution single nucleotide polymorphism arrays. Genomics (2011).
Gashaw I et al, Novel germ cell markers characterize testicular seminoma and fetal testis. Mol Hum Reprod. (2007).

complex[285], thus it is possibly involved in repression of genes, such a complex has not been shown in testes so far. A function of CHD5 as monomer is equally likely as a NuRD-like or another multi-protein complex containing CHD5. In addition to the already demonstrated involvement in anti-proliferation and senescence,[286] CHD5 could be involved in stem cell maintenance or fine-tuning of differentiation as suggested by its expression in neural stem cells. Regarding GCTs, it should be considered that polyploidy is a frequently observed phenomenon.[287] As a sharp contrast to the tumor suppressor hypothesis, a polyploidy could lead to a functional important increase of CHD5 dosage as well.

5.1.6 Both isoforms of CHD5 are expressed in mouse brain and testes

According to the NCBI database, CHD5 is predicted to have two isoforms in *Mus musculus*. So far, the existence of more than one isoform is not suggested for any other organism besides the mouse. Thus, I was interested whether both isoforms are expressed in mouse brain and testes. Therefore, an isoform-specific RT-PCR was designed. This RT-PCR revealed the expression of both isoforms in brain and testes. No quantitative statement could be made because RT-PCR is a semi-quantitative approach. For that, a RT-qPCR approach could be designed. Furthermore, it would be interesting whether both isoforms are translated into protein or if their expression is regulated at a post-transcriptional level. To asses this question a generation of isoform-specific antibodies would be required.

CHD5 was shown to exist in a NuRD-like complex in mouse brain.[288] That raises the question whether both isoforms can constitute such a complex. Alternatively, the formation of a NuRD-like complex could be restricted to one isoform. Especially a restriction to isoform 2 appears plausible to me because it has a higher similarity to Mi-2. To test this hypothesis, the generation of an isoform-specific antibody would be also necessary.

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[285] Potts RC et al, CHD5, a Brain-Specific Paralog of Mi2 Chromatin Remodeling Enzymes, Regulates Expression of Neuronal Genes. PLoS One (2011).
[286] Bagchi A et al, CHD5 is a Tumorsuppressor at Human 1p36. Cell (2007).
[287] Wittekind C, Biologie und Pathologie von Keimzelltumoren des Hodens. Onkologe (2008).
[288] Potts RC et al, CHD5, a Brain-Specific Paralog of Mi2 Chromatin Remodeling Enzymes, Regulates Expression of Neuronal Genes. PLoS One (2011).

5.1.7 CHD5 expression is not upregulated upon differentiation of Pc12 cells

An explanation for the absence of CHD5 expression in tumor cell lines and its suggested function as a tumorsuppressor supported the hypothesis that CHD5 expression might be restricted to non-proliferating cells such as terminally differentiated cells. Thus, I used the rat pheochromocytoma cell line Pc12 and differentiated them in presence of NGF. The differentiation was successful as indicated by the active outgrowth of neurites and further changes in cell morphology. Western Blot analysis of protein samples taken at different points in time during differentiation revealed absent CHD5 expression. In conclusion, CHD5 was not induced upon differentiation in this cell system.

Although it was only a reasonable hypothesis, there are several explanations for CHD5 absence in Pc12 cells. Firstly, pheochromocytoma is a tumour originating in the chromaffin cells from the adrenal medulla.[289] CHD5 transcripts were shown to be present in the adrenal gland.[290] Given that the adrenal medulla is a part of the sympathetic nervous system, CHD5 expression could be predominantly expected there. This assumption was recently confirmed.[291] However, the opposite - a restriction of CHD5 expression to the adrenal cortex - would explain its absence in Pc12 cells but is now invalid.

Secondly, the restriction of CHD5 expression to the adrenal medulla and its function as tumor suppressor could explain the absence in the corresponding tumour e.g. by deletion. A deletion of 1p is described for Pc12 cells.[292] This could possibly provide an explanation for absence of CHD5.

Nevertheless, Pc12 cells undergoing differentiation may not be a suitable cell system to study CHD5 function.

5.1.8 Outlook

For continuation of the CHD5 project, I would initially attempt to distinguish which cells types express CHD5 in testes. I consider immunofluorescence staining as the most

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[289] Carlsen E et al, Pheochromocytomas, PASS, and immunohistochemistry. Horm Metab Res. (2009).
[290] Thompson PM et al, CHD5, a new member of the chromodomain gene family, is preferentially expressed in the nervous system; Oncogene (2003).
[291] Garcia I et al, Expression of the neuron-specific protein CHD5 is an independent marker of outcome in neuroblastoma; Mol Cancer (2010).
[292] Pfragner R et al, First continuous human pheochromocytoma cell line: KNA. Biological, cytogenetic and molecular characterization of KNA cells. J Neurocytol. (1998).

appropriate method for this and at least the CHD5 antibody M-182 is suitable for this application. Secondly, I would like to test (especially testicular) GCTs for CHD5 expression. If any of the tumor samples display CHD5 expression, I would consider studying a larger panel of testicular GCTs with regard to histological subtype and outcome.

After synopsis of the published cytogenetic data regarding 1p36 deletions in pediatric yolk sac tumors, I consider an independent study of CHD5 expression in this tumor entity as a very promising approach. The presumably common inclusion of the CHD5 locus in deletions observed in this tumor entity as well as the parallel with neuroblastomas makes a biological and hopefully diagnostic impact of CHD5 more likely.

5.2. Discussion objective 2

5.2.1 Induction of genes in response to TNFα requires BRG1 and CHD4

In this study, a RNAi strategy was used to explore the functional roles of the chromatin remodelers CHD4 and BRG1 during TNFα-induced NF-κB target gene induction. The results revealed that both, BRG1 and CHD4, were required for efficient NF-κB target gene activation at early as opposed to later points in time. These results were somewhat surprising because both remodelers have been repeatedly shown to work antagonistically[293] but not synergistically. Furthermore, although some studies suggest an activating role, CHD4/NuRD is thought to be one of the classical repressor complexes.[294] These findings raised many interesting questions. Are both remodelers involved in chromatin remodeling at the target genes themselves during early gene induction? Do they have unknown functions? Do they act on different target structures or on the same ones? Do they influence target gene induction via a direct or an indirect mechanism? Do they work in their most abundant and best characterized complexes (NuRD, BAF/PBAF), within novel complexes or as monomers?

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[293] Curtis CD and Griffin CT, The chromatin-remodeling enzymes BRG1 and CHD4 antagonistically regulate vascular Wnt signaling. Mol Cell Biol. (2012).
Ramirez-Carrozzi VR et al, Selective and antagonistic functions of SWI/SNF and Mi-2beta nucleosome remodeling complexes during an inflammatory response. Genes Dev. (2006).
[294] Denslow SA and Wade PA, The human Mi-2/NuRD complex and gene regulation. Oncogene. (2007).

Although several experiments were performed during this thesis in order to find the mechanism of action, it remained unknown. An impaired induction of inflammatory target genes upon TNFα stimulation can be caused at all stages of the pathway as a direct or indirect mechanism. In the following part, I will discuss the possible mechanisms of how CHD4 and BRG1 could act in the experimental scenario regarding the questions raised above. The direct mechanisms are discussed first followed by the indirect mechanisms.

5.2.2 The requirement of BRG1 and CHD4 during the TNFα-induced NF-κB response is exclusive to the induction of early response genes

Inflammatory response genes are often classified as primary, late primary and secondary response genes according to induction kinetics, transcription factors involved and dependency on *de novo* synthesis of these transcriptions factors. Primary response genes are induced during the first two hours of stimulation. Furthermore, they are regulated by transcription factors that are constitutively expressed and activated by signal-dependent post-translational modifications. The prime example of such a transcription factor is NF-κB. Secondary response genes rely on *de novo* synthesis of transcription factors. They are usually induced within a time frame of two to eight hours.[295]

With the exception of CXCL10, all target genes tested in this study exhibited their greatest induction one hour after TNFα stimulation. Although a dependence on *de novo* protein synthesis for their induction has not been explored, it was assumed that they are independent due to the short time period between stimulus and maximum expression. Thus, they are classified as primary response genes. This is in agreement to literature where they were clearly independent on *de novo* protein synthesis.[296] Regarding the induction kinetics, CXCL10 could be classified as primary as well as secondary response gene in this study because it was upregulated seventy fold after one hour of TNFα stimulation but rose further to 200-fold expression after four hours.

[295] Medzhitov R and Horng T, Transcriptional control of the inflammatory response. Nat Rev Immunol. (2009).

[296] Ramirez-Carrozzi VR et al, A unifying model for the selective regulation of inducible transcription by CpG islands and nucleosome remodeling. Cell (2009).

In literature, CXCL10 is classified as primary response gene[297] but there are no data available comparing its activation after 2 and 4 hours. In principle it appears plausible that it is activated with 2 peaks, the first without requirement of *de novo* protein synthesis and the second induced by newly synthesized transcription factors.

Recently, Ramirez-Carrozzi et al[298] explored the role of CHD4 and BRG1 in relation to the induction of inflammatory genes after LPS stimulation in macrophages. They found BRG1 was required for the induction of late primary and secondary response genes but not for early primary response genes. On the other hand, the limitation of this activation was dependent on CHD4. Thus, disruption of CHD4 resulted in a prolonged inflammatory response.

In contrast, I found that CHD4 and BRG1 are both required for induction of early primary response genes after one hour of TNFα stimulation. These genes were still upregulated after four hours and at this time a disruption of BRG1 and CHD4, respectively, did not temper their expression. This suggests that chromatin remodelers are mainly involved in the very early induction period of inflammatory genes in this specific context. The key mediator of this early response is NF-κB.[299] A robust interaction of RelA (as one of the most abundant NF-κB subunits) with both remodelers could not be observed in this study by co-immunoprecipitation experiments (see Fig. 4.38). But that does not exclude either a direct interaction with other subunits or the fact that chromatin remodeling by BRG1 and CHD4, respectively, is a prerequisite for NF-κB binding to its target genes. A key experiment to test whether NF-κB binding to target genes is dependent on activity of both remodelers would be a p50/p65 ChIP experiment upon knockdown of BRG1 and CHD4, respectively.

Different transcription factors and pathways are activated upon TNFα stimulation (see Fig. 2.5). After four hours another component of the TNFα pathway in addition to NF-κB might be actively involved in the induction of our target genes and would possibly not require CHD4 and BRG1 for chromatin remodeling. Alternatively, the

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[297] Ramirez-Carrozzi VR et al, A unifying model for the selective regulation of inducible transcription by CpG islands and nucleosome remodeling. Cell (2009).

[298] Ramirez-Carrozzi VR et al, Selective and antagonistic functions of SWI/SNF and Mi-2beta nucleosome remodeling complexes during an inflammatory response. Genes Dev. (2006).

[299] Covic M et al, Arginine methyltransferase CARM1 is a promoter-specific regulator of NF-kappaB-dependent gene expression. EMBO J. (2005).

target genes already have an open chromatin after 4 hours and there is no further need for chromatin remodeling. Thus, after four hours the expression of the tested target genes may not be diminished by knockdown of BRG1 and CHD4. A kinetic for RelA and p50 binding to the promoters of the studied genes could be helpful in determining whether the NF-κB recruitment increases or decreases after one hour. In the next step, a knockdown of both major NF-κB subunits could determine whether the transcription after four hours is dependent on them.

The different classes of response genes were further characterized in a genome-wide approach by Ramirez-Carrozzi et al[300]. They found that primary response genes harboured promoter CpG islands which facilitated induction from constitutively active chromatin without requiring SWI/SNF remodeling complexes. TNFα was found to preferentially induce this class of genes in macrophages. Thus, it was suggested that TNFα-induced genes are activated independently from SWI/SNF remodelers.

Interestingly, four of the seven tested genes in our study (CXCL2, TNFα, IκB and IRF-1) belong to the class of genes that have a CpG island in their promoter and are predicted to be SWI/SNF independent according to Ramirez-Carrozzi et al[301]. Two of the remaining studied genes, CCL2 and CXCL10, respectively, were described to be SWI/SNF-dependent without a CpG island in their promoter region.[302] The gene which was tested last, IL-8, was not included in their study and its promoter status is a contentious issue. However, there is no CpG island predicted by UCSC genome browser and the promoter was described as not containing a typical CpG island. [303]

Although the concept postulating that CpG islands containing promoters are SWI/SNF-independent in macrophages appears entirely convincing to me, we found a totally different situation. In HEK293 cells, all tested TNFα-induced genes, irrespective of their CpG island status, were SWI/SNF dependent at early points in time. Furthermore, they were additionally CHD4-dependent. Thus, a requirement for chromatin remodelers in the inflammatory response to diverse stimuli might not obey an overarching principle

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[300] Ramirez-Carrozzi VR et al, A unifying model for the selective regulation of inducible transcription by CpG islands and nucleosome remodeling. Cell (2009).

[301] Ramirez-Carrozzi VR et al, A unifying model for the selective regulation of inducible transcription by CpG islands and nucleosome remodeling. Cell (2009).

[302] Ramirez-Carrozzi VR et al, A unifying model for the selective regulation of inducible transcription by CpG islands and nucleosome remodeling. Cell (2009).

[303] Oliveira NF et al, DNA methylation status of the IL8 gene promoter in oral cells of smokers and non-smokers with chronic periodontitis. J Clin Periodontol. (2009).

according to the suggested theory regarding gene structure. This view might be limited by the use of different cell types because cell-type-specific differences in the classification of response genes are already known. For instance, IL-6 was shown to be a SWI/SNF-dependent secondary response gene in LPS-stimulated macrophages but a SWI/SNF-independent primary response gene in MEFs, most likely due to a different biological need in various cell types.[304] I used exclusively HEK293 cells for which there are no data available regarding the different classes of response genes. This might limit any conclusion from the published data in comparison with this work. In addition, we tested a limited amount of target genes; a genome-wide approach would allow a broader analysis of gene characteristics.

5.2.3 The role of CHD4 in transcriptional activation upon TNFα stimulation

In mammals, the bulk of CHD4 is thought to reside in the NuRD complex and no other stable Mi-2-containing complex has been discovered so far. Additionally, only vague hints exist that there could be a functional role for CHD4 outside the NuRD complex.[305] Recently, it was shown that dMi-2 is also part of the newly identified complex dMec. This complex constitutes the major Mi-2 containing complex in *Drosophila*. Furthermore, it was shown not to rely on histone deacetylation to effect transcriptional repression. [306]

In this study, due to time limitation I could not test whether CHD4 or the entire NuRD complex is required for gene activation. To evaluate the hypothesis that the entire NuRD complex is needed, it would be reasonable to perform sequential knockdown experiments for all NuRD subunits.

In both known Mi-2-containing complexes, NuRD and dMec, CHD4 was strongly linked to transcriptional repression.[307] Therefore, a requirement for gene activation, as shown here, is quite surprising and suggests a role in active transcription. Only few studies have hitherto implicated CHD4 in transcriptional activation. For example, during T-cell development, Mi-2 associates with the CD4 enhancer resulting in

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[304] Ramirez-Carrozzi VR et al, A unifying model for the selective regulation of inducible transcription by CpG islands and nucleosome remodeling. Cell (2009).
[305] As example, see Williams CJ et al, The chromatin remodeler Mi-2beta is required for CD4 expression and T cell development. Immunity. (2004).
[306] Kunert N et al, dMec: a novel Mi-2 chromatin remodelling complex involved in transcriptional repression. EMBO J. (2009).
[307] Murawska M and Brehm A, CHD chromatin remodelers and the transcription cycle. Transcription (2011).

recruitment of the E-box binding protein HEB and the histone acetyltransferase p300 to the CD4 enhancer. This causes histone H3-hyperacetylation of the regulatory region and enables CD4 transcription. With both factors, CHD4 was shown to interact in a HDAC independent manner suggesting this activity to be independent from NuRD.[308]

Furthermore, CHD4 was implicated to be involved in rRNA activation in the nucleolus.[309] Recently, an activating role of dMi-2 in *Drosophila* heat shock gene induction was demonstrated. dMi-2 was recruited to Drosophila heat shock genes in a PAR–dependent manner. There, it associated with nascent heat shock gene transcripts and its catalytic activity was required for efficient transcription and co-transcriptional RNA processing.[310] Whether a further role of Mi-2 exists in splicing is far from clear but it might recruit splicing machinery to RNA via binding to nascent transcripts.[311]

In summary, at least in some circumstances CHD4 appeared to be involved in transcriptional activation.

Several mechanisms are conceivable as to how CHD4 could act in transcriptional activation of TNFα-induced genes.

One possible mechanism could be a recruitment of CHD4 to the target genes followed by binding to nascent RNA and involvement in co-transcriptional RNA processing similar to the observed scenario upon heat shock gene activation in Drosophila. Interestingly, PARP-1 was demonstrated as interacting with NF-κB upon LPS stimulation in mammalian cells[312] and TNFα-induced transcriptional activation of a subset of NF-κB target genes was shown to be defective in PARP-1-deficient mice.[313] Thus, PARP might also be a candidate to modulate CHD4 action in the context of TNFα pathway.

Moreover, as described for the CD4 locus, CHD4 could associate with a histone acetyltransferase, thereby establishing activating histone marks. One might argue that

[308] Williams CJ et al, The chromatin remodeler Mi-2beta is required for CD4 expression and T cell development. Immunity. (2004).

[309] Shimono K et al, Microspherule protein 1, Mi-2beta, and RET finger protein associate in the nucleolus and up-regulate ribosomal gene transcription. J Biol Chem. (2005).

[310] Murawska M et al, Stress-induced PARP activation mediates recruitment of Drosophila Mi-2 to promote heat shock gene expression. PLoS Genet. (2011).

[311] Murawska M and Brehm A, CHD chromatin remodelers and the transcription cycle. Transcription (2011).

[312] Ullrich O et al, Regulation of microglial expression of integrins by poly(ADP-ribose) polymerase-1. Nat Cell Biol. (2001).

[313] Oliver FJ et al, Resistance to endotoxic shock as a consequence of defective NF-kappaB activation in poly (ADP-ribose) polymerase-1 deficient mice. EMBO J. (1999).

the observed high basal levels of active histone modifications like acetylation at primary response genes dispense with the requirement for additional histone modifications because the chromatin is already in an open conformation.[314] However, the histone acetyltransferase p300 was shown to be required for early target gene induction upon TNFα stimulation,[315] thus an additional requirement of activating histone modifications might be feasible. An approach to validate or exclude this hypothesis would be a co-immunoprecipitation of CHD4 and the most abundant acetyltransferases like p300.

In order to explore any direct mechanism in this study, a recruitment of CHD4 to the CXCL2 gene upon TNFα stimulation was tested by ChIP experiments. Such recruitment could not be demonstrated in these experiments. That does not exclude any recruitment because I studied only one model gene. For a reliable result more target genes would have to be studied. Furthermore, the used antibody was not established in ChIP experiments in our laboratory beforehand. In addition, experiments were performed after 20 (data not shown), 40 and 60 minutes. If CHD4 had been recruited in the very initial phase of TNFα-pathway activation and released soon thereafter, detection could have been missed. Furthermore, if our ChIP results indeed represent a very low but steady binding of CHD4 to CXCL2, it might undergo activation by a post-translational modification upon stimulation.

As a second approach, an interaction of CHD4/BRG1 and the NF-κB subunit RelA upon TNFα stimulation was evaluated revealing no robust interaction between the chromatin remodelers and RelA. These results do not support the view that the remodelers are recruited to the target genes via interaction with DNA-bound transcription factors and are acting directly at the affected genes. But so far, a technical cause cannot be excluded as a reason for my results. Because the key to all possible direct mechanisms is almost exclusively the demonstration of a positive recruitment to the tested target genes, for further studies this question should be re-addressed with considerable effort.

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[314] Ramirez-Carrozzi VR et al, A unifying model for the selective regulation of inducible transcription by CpG islands and nucleosome remodeling. Cell (2009).
[315] Covic M et al, Arginine methyltransferase CARM1 is a promoter-specific regulator of NF-kappaB-dependent gene expression. EMBO J. (2005).

In principle, any siRNA-mediated off-target effect can cause irregularities in gene expression. Considering the unaffected four-hour-response and housekeeping gene expression, these explanations become less likely. In addition, identical off-target effects for two siRNAs targeting different regions of the same transcript, as used in this study, seem implausible. Were there to be any remaining doubt, rescue experiments could be performed to ultimately exclude this kind of confounder. [316]

5.2.4 The role of BRG1 in transcriptional activation upon TNFα stimulation

BRG1 was repeatedly shown to be required for target gene induction in different inflammatory responses as to renal ischemia[317], LPS[318], IFNα[319], TGFß[320] and IL-6.[321] No doubt that BRG1 is of great significance to the fine-tuning of inflammatory responses to diverse stimuli. But depending on the specific context, the requirement for BRG1 differs considerably regarding the kinetics and modes of action. Further studies might improve insight into its detailed function in the context of inflammatory responses.

Since only one siRNA targeting BRG1/Brm was used in this study, off-target effects upon BRG1 knockdown leading to the observed reduction in early TNFα induced gene activation cannot be fully excluded. But considering the specificity for early gene induction and the unaffected expression of the housekeeping gene, off-target effects as confounders seem to be very unlikely. A possible control experiment besides rescue experiments could be a TNFα-induction of inflammatory genes in SW13 cells. These cells lack BRG1 and Brm expression.[322] In case of dependence on BRG1, early target genes should be induced less strongly. Furthermore, a re-expression of BRG1 should enable or multiply the inflammatory response.

[316] Li CC et al, Pursuing gene regulation 'logic' via RNA interference and chromatin immunoprecipitation. Nat Immunol. (2006).

[317] Naito M et al, BRG1 increases transcription of proinflammatory genes in renal ischemia. J Am Soc Nephrol. (2009).

[318] Ramirez-Carrozzi VR et al, Selective and antagonistic functions of SWI/SNF and Mi-2beta nucleosome remodeling complexes during an inflammatory response. Genes Dev. (2006).

[319] Liu H et al, Maximal induction of a subset of interferon target genes requires the chromatin-remodeling activity of the BAF complex. Mol Cell Biol. (2002).
Huang M et al, Chromatin-remodeling factor BRG1 selectively activates a subset of interferon-alpha-inducible genes. Nat Cell Biol. (2002).

[320] Xi Q et al, Genome-wide impact of the BRG1 SWI/SNF chromatin remodeler on the transforming growth factor beta transcriptional program. J Biol Chem. (2008).

[321] Ni Z et al, BRG1-dependent STAT3 recruitment at IL-6-inducible genes. J Immunol. (2007).

[322] Ni Z et al, BRG1-dependent STAT3 recruitment at IL-6-inducible genes. J Immunol. (2007).

A direct recruitment of BRG1 to the CXCL2 gene upon stimulation could not be shown (data not shown) possibly due to the lack of a highly specific BRG1 antibody established for ChIP experiments in our laboratory.

Although many indirect effects are in principal conceivable for the impaired TNFα response, the direct recruitment of BRG1 to target genes, or a requirement for constitutively bound BRG1 followed by subsequent involvement in opening chromatin remodeling, has been shown in several cases and therefore seems possible.[323] Moreover, BRG1 binding to a subset of target promoters in response to TGFß was shown to be required for recruitment of RNA polymerase II,[324] indicating its direct involvement in transcriptional activation.

In this study, the associations of histone H3 with the promoter of our model gene, CXCL2, decreased upon activation. This finding led to the conclusion that some chromatin remodeling definitively took place (see Fig. 4.37). An H3 ChIP experiment under BRG1 knockdown conditions could further specify whether the removal of H3 is BRG1-dependent.

BRG1 exists in different complexes. Due to time limitation I did not test whether BRG1 is required for NF-κB target gene induction with an entire complex or as a monomer. To explore the involvement of the most abundant BRG1-containing complexes, a knockdown of BAF250 (representative for BAF complex) and BAF180 (representative for PBAF) might enable us to answer this question.

Interestingly, the arginine methyltransferase CARM1 was shown to interact with the histone acetyltransferase p300 and RelA in HEK293 cells after 30 minutes of TNFα stimulation. Induction of CXCL2, CCL2 and CXCL10 was dependent on CARM1 and p300.[325] Remarkably, in a subsequent study CCL2 and CXCL10 were found to be

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[323] Ramirez-Carrozzi VR et al, Selective and antagonistic functions of SWI/SNF and Mi-2beta nucleosome remodeling complexes during an inflammatory response. Genes Dev. (2006).
Ni Z et al, BRG1-dependent STAT3 recruitment at IL-6-inducible genes. J Immunol. (2007).
Liu H et al, Maximal induction of a subset of interferon target genes requires the chromatin-remodeling activity of the BAF complex. Mol Cell Biol. (2002).
[324] Xi Q et al, Genome-wide impact of the BRG1 SWI/SNF chromatin remodeler on the transforming growth factor beta transcriptional program. J Biol Chem. (2008).
[325] Covic M et al, Arginine methyltransferase CARM1 is a promoter-specific regulator of NF-kappaB-dependent gene expression. EMBO J. (2005).

CARM1-independent after three hours of stimulation.[326] These kinetic properties and the target genes are reminiscent of this study.

Moreover, BRG1 was shown to interact with CARM1 within the NUMAC (<u>nu</u>cleosomal <u>m</u>ethylation <u>a</u>ctivator <u>c</u>omplex). In this context, CARM1 stimulates the ATPase activity of BRG1 and enhances ER-dependent transcription.[327] Together, these observations lead me to speculate that a NUMAC-like chromatin remodeler 'super-complex' interacts with RelA upon TNFα stimulation. A co-immunoprecipitation of BRG1 and CARM1 would provide further information but this will not be the question I address first when continuing this study.

5.2.5 Are CHD4 and BRG1 recruited to the same target structures?

Since BRG1 and CHD4 were both shown to positively regulate the induction of early response genes upon TNFα stimulation, the question arises whether they are involved in the same steps of transcriptional activation. Furthermore, there is already some evidence to show that both proteins interact or work in a type of 'super complex' of chromatin remodelers.[328] However, I did not observe any direct interaction between both upon TNFα stimulation (data not shown). Nevertheless, an interaction between CHD4 and BRG1 via linker proteins is still possible. So far, I am not able to answer this question.

5.2.6 Summary: Possible direct mechanisms of BRG1/CHD4 involvement

Although any suggestion of a mechanistic model for the involvement of BRG1 and CHD4 in the TNFα response is speculative at this stage, I will generate two models combining a possible function of both chromatin remodelers:

1) CHD4 as a monomer could be targeted to the responding genes upon TNFα stimulation by an unknown mechanism (perhaps by PARP). There, it assembles with a histone acetyltransferase, initiating a hyperacetylation of histones. The bromodomains of BRG1 recognize the acetylated histone tails, thereby mediating a recruitment of the

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[326] Jayne S et al, CARM1 but not its enzymatic activity is required for transcriptional coactivation of NF-kappaB-dependent gene expression. J Mol Biol. (2009).
[327] Xu W et al, A methylation-mediator complex in hormone signaling. Genes Dev. (2004).
[328] Shimono Y et al, Mi-2 beta associates with BRG1 and RET finger protein at the distinct regions with transcriptional activating and repressing abilities. J Biol Chem. (2003).
O'Neill DW et al, An ikaros-containing chromatin-remodeling complex in adult-type erythroid cells. Mol Cell Biol. (2000).

BAF/PBAF complex to the target genes. Finally, the BAF/PBAF complex decreases the nucleosome occupancy of the target gene's promoter, thus improving the access of NF-κB and the transcription machinery to the target gene.

2) BRG1 could be recruited to the promoter regions of the target genes upon TNFα stimulation. There, it could provide better access for transcription factors and the transcriptional machinery by a decrease of nucleosome occupancy at the promoter region. CHD4 could be recruited to the open-reading frame, bind to the nascent RNA and participate in co-transcriptional RNA processing.

5.2.7 Mechanisms which impair key events of the TNFα pathway

As mentioned above, any dysfunction of the TNFα signaling by disruption of the chromatin remodeler expression could be caused by alterations at any single stage of the pathway. Now, to exemplify this, I will highlight some key events that could possibly be affected by more indirect mechanisms.

<u>Release of cytoplasmic NF-κB from its inhibitor IκB and nuclear translocation</u>

This part of NF-κB activation could be impaired by indirect mechanisms targeting the proteasomal degradation of IκB.

IκB was not upregulated in unstimulated HEK293 cells when CHD4 and BRG1 expression was disrupted by RNAi (data not shown). Thus, an excess of IκB inhibiting the nuclear translocation of NF-κB appears unlikely. But there are still many ways in which this part of the signaling could be hampered. The key experiment to investigate whether this part of NF-κB signaling is intact would be to test if the p50/RelA heterodimer enters the nucleus under knockdown of BRG1 and CHD4, respectively. For instance, this could be performed by immunofluorescence staining. Any indirect effect that impairs the nuclear re-localization of NF-κB would lead to a sustained cytoplasmic staining.

<u>Regulation of NF-κB in the nucleus</u>

In contrast to the well-studied regulation of NF-κB in the cytoplasm, its nuclear regulation is less well understood. NF-κB activity could possibly be fine-tuned via post-

translational modifications as is shown in some cases.[329] Although CHD4 and BRG1 are mainly involved in chromatin remodeling, in principle their complexes could also modify non-DNA-associated proteins as shown for HDAC1/NuRD and p53,[330] thereby adjusting NF-κB activity. Exploring this possibility is the most challenging.

Binding of NF-κB to DNA

Although it might be feasible that κB-sites are less accessible when their DNA is tightly wrapped around histones, it was shown that the incorporation of κB-sites in positioned nucleosomes only slightly decreases the ability of NF-κB to bind to these sites.[331] But given the paucity of existing experimental data on this topic, it cannot be excluded that an open chromatin structure or nucleosome remodeling are obligatory for NF-κB recruitment to DNA.[332] I have observed that histone H3 is removed from the promoter region of CXCL2 upon TNFα stimulation. It remains unclear whether this is a prerequisite for NF-κB binding to its κB-site at the CXCL2 promoter. It might be worth performing a p50/p65 ChIP experiment upon knockdown of CHD4 and BRG1 respectively, although any other chromatin remodeling enzyme could in principle be involved in this specific step as well.

5.2.8 Perspectives

The surprising finding that CHD4 and BRG1 are both required for the efficient early target gene induction after TNFα stimulation has raised the many extremely interesting questions which have been discussed above. Of further interest might be to gain more insight into the precise regulation of transcription in inflammatory responses.

Since BRG1 is a known tumor suppressor often deleted in cancer and TNFα is a common mediator in tumor-related inflammation (see Introduction), this raises the additional question of whether there is any functional consequence for the impaired

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[329] Zhong H et al, The phosphorylation status of nuclear NF-kappa B determines its association with CBP/p300 or HDAC-1. Mol Cell. (2002).
Nowak DE et al, RelA Ser276 phosphorylation is required for activation of a subset of NF-kappaB-dependent genes by recruiting cyclin-dependent kinase 9/cyclin T1 complexes. Mol Cell Biol. (2008).
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early gene induction in response to TNFα. Might this uncouple a cancer cell from a subset of inhibiting signals generated in the microenvironment?

Another interesting question might be in the context of tumors depending on constitutive active NF-κB signaling like Hodgkin lymphoma or some subtypes of diffuse large B cell lymphoma.[333] Suggesting that not all NF-κB target genes have permanently open chromatin, it might be of interest whether there is a dependence on chromatin remodelers also in this context. Thus, an inhibition of chromatin remodelers or other subunits of their complexes might provide a new therapeutic approach in these tumor entities.

In conclusion, the findings of this study provide an interesting view on the involvement of ATP-dependent chromatin remodelers in inflammatory gene activation which could in future contribute to a better understanding of transcriptional control in inflammation.

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6. Appendix

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6.2. List of abbreviations and acronyms

aa	amino acid
ac	acetyl
ACF	ATP-utilizing chromatin assembly and remodeling factor
ADP	adenosindiphosphate
ATP	adenosintriphosphate
BAF	BRG1-associated factors
BAP	Brahma-associated proteins
BLAST	basic local alignment search tool
bp	base pair
BRG1	Brahma-associated gene 1
BRK	Brahma and Kismet domain
BRM	brahma
BSA	bovine serum albumine
C-	carboxy-
C/EBP	CCAAT/Enhancer-Binding Protein-β
CCL2	C-C motif ligand 2
cDNA	complementary DNA
CHD	chromodomain-helicase-DNA binding
CHD5	chromodomain, helicase, DNA binding protein 5
ChIP	chromatin immunoprecipitation
CHRAC	chromatin assembly complex
CK2	casein kinase 2
CpG	cytosine-phospatidyl-guanosine

Ct	cycle threshold
CT	computed tomography
CXCL2	C-X-C motif ligand 2
Da	dalton
DAPI	4',6-diamidino-2-phenylindole
dATP	desoxyadenosintriphosphate
dCTP	desoxycytosinosintriphosphate
dGTP	desoxyguanidintriphosphate
DIV	days in vitro
dMec	drosophila MEP-1-containing complex
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl suloxide
DNA	desoxyribonucleic acid
DNMT	DNA methyltransferase
dNTP	desoxyribonucleotidetriphosphate
DSB	double strand break
dsRNA	double stranded RNA
DTT	dithiotreithol
dTTP	desoxythyminidintriphosphate
EDTA	ethylenediaminetetraacetic acid
ER	estrogen receptor
FBS	fetal Bovine Serum
FCS	fetal calf serum
for	forward
g	gram
G1	gap phase 1
GCT	Germ cell tumor
GR	glucocorticoid receptor
H	histone
HAT	histone acetyltransferase
HBSS	Hank's Buffered Salt Solution
HDAC	histone deacetylase

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid N-(2-
HMG	high mobility group
HP1	heterochromatin protein 1
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
IF	immunofluorescence
IFNα	Interferon alpha
Ig	immunoglobuline
IκB	inhibitor of NF-κB
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's Medium
INO80	inositol requiring 80
INSS	International Neuroblastoma Staging System
IP	immunoprecipitation
IRF-1	Interferon regulatory factor 1
ISWI	imitation switch
KAP-1	krüppel-associated box domain–associated protein -1
kDA	kilodalton
LB	Luria-Bertani
LKB1	liver kinase B1
LPS	lipopolysaccharide
LSD1	lysine-specific demethylase 1
M	molar
mA	miiliampere
MBP	methyl-CpG-binding protein
MDa	mega dalton
me	methyl
MEF	mouse embryonic fibroblast
MEF	mouse embryonic fibroblast
min	minute
mNGF	murine Nerve growth factor
MRI	magnetic resonance imaging

mRNA	messenger RNA
MTA	metastasis associated protein
N-	amino-
NAD	nicotinamide adenine dinucleotide
NE	nuclear extract
NF-κB	nuclear factor kappa B
nm	nanometer
NoRC	nucleolar remodeling complex
NuRD	nucleosome remodeling and histone deacetylation
NURF	nucleosome remodeling factor
PAA	polyacrylamide
PAGE	polyacrylamide gel electrophoresis
PAR	poly(ADP-ribose)
PARP	poly(ADP-ribose) polymerase
PBAF	polybromo-associated BAF
PBAP	polybromo-associated BAP
PBS	phosphate buffered saline
Pc	polycomb
PCR	polymerase chain reaction
PEI	polyethyleneimine
PHD	plant homeo domain
PMSF	phenylmethane sulfonyl fluoride
PPAR	peroxisome proliferator-activated receptor
PS	penicilline/ streptomycine
P-TEFb	positive transcription elongation factor b
PVDF	polyvinylidine difluoride
QPCR	quantitative PCR
rDNA	ribosomal DNA
rev	reverse
RNA	ribonucleic acid
RNAi	RNA interference
RpAp	retinoblastoma associated protein

RPD3	reduced potassium dependency 3
rpm	revoltations per minute
RPMI	Roswell Park Memorial Institute
RSC	remodels the structure of chromatin
RT	room temperature
SAGE	serial analysis of gene expression
SANT	ySWI3, yADA2, hNCoR, hTFIIIB
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec	second
shRNA	short hairpin RNA
SLIDE	SANT-like ISWI domain
Snf2	sucrose non-fermenting protein 2 homolog
ssRNA	single stranded RNA
suc	sucrose
SUMO	small ubiquitin-like modifier
SWI/SNF	switch/sucrose non-fermenting
Swr1	Swi2/Snf2-related 1
Temed	N,N,N',N'-Tetramethylethylenediamine
TGFß	Transforming growth factor beta
TLR-9	Toll-like receptor 9
TNFα	Tumor necrosis factor alpha
Tris	tris(hydroxymethyl)aminomethane
v/v	volume per volume
w/v	weight per volume
WCE	whole cell extract
wt	wild-type

6.3 Curriculum vitae

Dieser Abschnitt enthält persönliche Daten und ist deshalb nicht Bestandteil der Online-Veröffentlichung.

6.4 Verzeichnis der akademischen Lehrer

Meine akademischen Lehrer waren die folgenden Damen und Herren Professoren, Doktoren und Dozenten

In Marburg:

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6.6 Ehrenwörtliche Erklärung

Dieser Abschnitt enthält persönliche Daten und ist deshalb nicht Bestandteil der Online-

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