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Function of the CHD4/Mi-2ß chromatin remodelling ATPase during neural development of *Xenopus laevis*

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1 Summary - Zusammenfassung

Multicellular organisms achieve cellular differentiation through precisely regulated gene expression. The informational content that is necessary for a fertilized egg to develop into approximately 260 different cell types is not restricted to its primary DNA sequence. An additional level of information includes DNA methylation, histone modifications, and ATP dependent chromatin remodelling of specific local chromatin structures. These additional sources of information are termed epigenetic regulatory mechanisms. The genetic information generally remains constant during development, whereas epigenetic information can be dynamically reprogrammed in response to a multitude of factors including cellular interactions and extracellular signalling events. Epigenetic regulation results in activation and repression of specific genes, which direct the establishment from stem cells to individual progenitor cells that develop into whole tissues and organs. Therefore, epigenetic mechanisms of gene expression have to be tightly regulated during stem cell differentiation and embryonic development. In this work, I investigate the role of CHD4/Mi-2ß, the catalytic ATPase subunit of the NuRD chromatin-remodelling complex during *Xenopus* neurogenesis. The analysis is specifically focused on how differentiation of prospective neural tissue is affected by CHD4/Mi-2ß misexpression at different stages during development and thus within different genetic and epigenetic chromatin contexts.

Based on our latest results, describing the direct interaction of CHD4/Mi-2ß and Sip1/ZFH1b as a crucial factor in the neuroectoderm/mesoderm germ layer formation of *Xenopus* embryos (Linder et al, 2007), the analysis in this work is focused on the influence, which the CHD4/Sip1 regulatory module exerts on neural development.

For this analysis, we applied two different strategies for CHD4 gain- and loss-of function studies. To interference during early developmental stages at neural induction and neural cell differentiation, wild type (wt) and dominant negative (dn) CHD4 and Sip1 is overexpressed by mRNA microinjection into two-cell stage *Xenopus* embryos. For further spatial and temporal specification of CHD4 misexpression during development, I established a procedure, which enables the expression of wt-/dnCHD4 at later stages of neural development by the means of an inducible heat-shock promoter plasmid construct. This inducible activation of CHD4 gene expression allows distinguishing between CHD4 and Sip1 functions before and after the formation of the neural plate, i.e. functions on neuroblast determination versus neural cell differentiation within a dynamic chromatin context. The resulting

phenotypes were analysed by whole mount in situ hybridization of marker genes, which spatially and temporally characterise the developing prospective neural tissue according to its state of differentiation. The results demonstrate two major different functions of the CHD4 ATPase subunit of the NuRD chromatin-remodelling complex during neural induction and neural cell differentiation. First, CHD4 chromatin remodelling induces prospective neural tissue at the expense of ectodermal derivates. The induced neuroblasts are kept in an undifferentiated proliferating state. Second, epigenetic chromatin remodelling by CHD4 promotes neural cell differentiation, when it is activated after neural induction from NF stage 13 onwards, i. e. after formation of the neural plate.
This work reveals an unknown role of chromatin remodelling by CHD4/Mi-2ß within different chromatin contexts, which regulate developmental processes during neural induction and neural stem cell differentiation.

Deutsche Übersetzung:

Zelluläre Differenzierungsprozesse während der Entwicklung von multizellulären Organismen benötigt eine genaue Regulation der Genexpression. Die nötigen Informationen für die Entwicklung von einer befruchteten Eizelle zu circa 260 unterschiedlichen Zelltypen ist nicht alleine beschränkt auf die primäre DNA Sequenz. Eine weitere Informationsebene stellen DNA Methylierung, Histon Modifikationen und ATP-abhängige Remodellierung des Chromatins an spezifischen, lokalen Chromatinstrukturen dar. Diese zusätzlichen Informationsquellen werden epigenetische Regulationsmechanismen bezeichnet. Während der Entwicklung bleiben genetische Informationen generell konstant, epigenetische Informationen hingegen können als Antwort auf extrazelluläre Einflüsse, wie Zell-Zell Kontakte und Signaltransduktionswege dynamisch verändert werden. Die epigenetische Regulation der Genexpression führt zur Aktivierung und Repression spezifischer Gene, die die Entwicklung von einer Stammzelle zu Vorläuferzellen hin zu differenzierten Geweben und Organen vorgeben. Für diese Differenzierungs-prozesse unterliegen die epigenetischen Mechanismen der Genexpression während der Stammzelldifferenzierung und Embryonalentwicklung einer genauen Regulation.
In dieser Arbeit untersuche ich die Funktion von CHD4/Mi-2ß, der katalytischen ATPase Untereinheit des NuRD Chromatin-Remodellierungskomplexes während der Neurogenese von *Xenopus laevis.*

Es soll analysiert werden, wie die Differenzierung des prospektiven neuralen Gewebes durch die Missexpression von CHD4/Mi-2ß beeinflusst wird. Diese Untersuchung wird zu verschiedenen Stadien der Embryonalentwicklung und somit in verschiedenen genetischen und epigenetischen Chromatin Kontexten durchgeführt. Aufbauend auf unseren letzten Ergebnissen, die eine direkte Interaktion von CHD4/Mi-2ß und Sip1/ZFH1b als einen wichtigen Faktor in der Keimblatt-Entwicklung von Neuroektoderm und Mesoderm beschreiben (Linder et al, 2007), sind die Untersuchungen dieser Arbeit auf den Einfluss des CHD4/SIP1 Regulations-Moduls auf die neurale Entwicklung fokussiert. Für diese Analyse verwenden wir zwei unterschiedliche Strategien um eine Zunahme und eine Abnahme der CHD4 Funktion zu studieren. Um die Genexpression während frühen Entwicklungsstadien der neuralen Induktion und neuralen Zelldifferenzierung zu beeinflussen, überexprimieren wir wildtyp (wt) und eine dominant negative (dn) Variante von CHD4 und Sip1 durch mRNA Mikroinjektionen im 2-zell Stadium von *Xenopus* Embryonen. Für eine räumliche und zeitliche Spezifikation der CHD4 Missexpression während der Entwicklung, etablierten wir eine Methode mit der es möglich ist die Expression von wt-/dnCHD4 in späteren Stadien der neuralen Entwicklung durch einen induzierbaren Hitzeschock-Promotor zu aktivieren. Dies ermöglicht die Differenzierung zwischen Funktionen von CHD4 und Sip1 vor, und nach der Formation der Neuralplatte, bzw. zwischen Funktionen während der Neuroblastendetermination und der neuralen Zelldifferenzierung zu unterscheiden.

Die resultierenden Phänotypen wurden durch in situ Hybridisierung von Markergenen analysiert, die räumlich und zeitlich das sich entwickelnde, prospektive neurale Gewebe hinsichtlich seines Differenzierungsgrades charakterisieren.

Die Ergebnisse zeigen zwei grundlegende, unterschiedliche Funktionen der CHD4 ATPase Untereinheit des NuRD Chromatin-Remodellierungs Komplexes während der neuralen Induktion und der neuralen Stammzelldifferenzierung.

Erstens, CHD4/Mi-2ß Chromatin-Remodellierung induziert prospektives neurales Gewebe auf Kosten von ektodermalen Zellen. Die induzierten Neuroblasten werden in einem undifferenzierten und proliferativen Stadium gehalten. Zweitens, CHD4/Mi-2ß Chromatin-Remodellierung unterstützt die neurale Differenzierung, wenn es nach der Gastrulation, d. h. nach Formation der Neuralplatte aktiviert wird. Diese Arbeit beschreibt eine bisher nicht bekannte Funktion von CHD4/Mi-2ß, die im Rahmen unterschiedlicher Chromatin Kontexte die Entwicklungsprozesse während der neuralen Induktion und der neuralen Stammzelldifferenzierung reguliert.

2 Introduction

2.1 Bridging the gap from basic research to human therapy: Translating neural development in *Xenopus* to human pluripotent cells.

During the last decades, basic embryological research has provided a major progress to understand the regulatory mechanism of cellular differentiation and specifically of neural induction and neural cell differentiation. From a medical perspective, the translational process from basic science to clinical medicine is known as a profound means to enhance the progression of human therapeutic development. The translational medicine, as a linkage between basic and medical research, is emphasized by the recent developments of human induced pluripotent stem cells. Pluripotent stem cells are powerful tools for bridging the gap from an accumulated knowledge of embryology to regenerative medicine. They serve for a wide spectrum of medical and pharmaceutical research. In addition, research on the differentiation mechanisms of pluripotent cells provides the basis for a profound understanding of cancer biology and metastasis formation.

Pluripotency can be defined as the cellular ability to differentiate into three germ-layer derivatives. The process of *in vitro* differentiation of embryonic stem (ES) cells has been shown to mimic many aspects of embryonic development *in vivo* (Wichterle et al, 2002; Wobus & Boheler, 2005). Consequently, ES cells can be used as a substitute for inner cell mass or epiblast tissues to study the differentiation of mammalian pluripotent tissues *in vitro*. To understand these evolutionary conserved mechanisms, major lessons were learned from basic science in the field of amphibian neural induction.

Amphibian neural induction has been extensively studied by the approach of classic experimental biology. In contrast, mammalian neural induction cannot experimentally be approached due to technical limitations. Therefore, various works demonstrated that culture of ES cells under differentiation condition provides a means to study the molecular and cellular mechanisms in early mammalian embryogenesis. ES cells mimic an embryonic pluripotent epithelium that is able to differentiate into numerous types of cells, depending on signalling factors added exogenously to culture. ES programming occurs physiologically during germ layer induction and pattern formation, e.g. during the boundary formation of neuroectoderm and mesoderm or

neuroectoderm and ectoderm. These investigations are strongholds of the *Xenopus* experimental system. The characterization of the molecular factors that influence germ layer boundary formation mostly derived from the knowledge obtained from basic embryological studies, as those of *Xenopus*. The insights, gained from years of classical embryology and molecular biology in model systems, such as *Xenopus* leads to direct applications in the identification of the molecular nature of neural induction in human cells. For example, our laboratory demonstrated that the chromatin remodelling factor CHD4 plays a crucial role for the positioning of the boundary formation between the germ layers neuroectoderm and mesoderm. This is specifically controlled by the Nodal signalling input via Sip1 for the Xbra transcription (Linder et al, 2007). Most recently, data provides evidence that Nodal signalling acts through Sip1 to regulate the cell-fate decision between neuroectoderm and mesendoderm in human pluripotent stem cells (Chng et al, 2010).
Thus, studies on the basic mechanism of cell fate induction helps to improve differentiation protocols for human stem cells *in vitro* and *in vivo*, as a necessary step in the development of stem cell based therapeutics for various neuronal disorders (Munoz-Sanjuan & Brivanlou, 2002).

2.2 Induction events in *Xenopus laevis*

During the early developmental stages of gastrula, the major part of cells that form the developing embryo are not determined, except one region. This region is the anterior part of the dorsal blastopore lip. When this tissue is transplanted to another region of the embryo, it develops autonomously as it would, and is able to induce the formation of secondary dorsal or ventral body axis. This region was discovered by the German embryologist Hans Spemann. He described the effect now known as embryonic induction. Induction is executed by specific parts of the developing embryo that direct the fate of cell populations to differentiate into particular tissue types and organs.
High ß-catenin levels on the dorsal side of the embryo, together with the vegetally located transcription factor VegT and the maternal TGF-ß family growth factor Vg1, generate a gradient of so called Nodal-related molecules, which are expressed in the endoderm. In turn, this gradient induces the formation of overlying mesoderm by Nodal-related molecules (Xnrs) leading to the formation of ventral mesoderm. High doses of Nodal-related molecules in the dorsal vegetal endoderm lead to the

establishment of the Nieuwkoop's centre that induces the Spemann organizer tissue. Figure 1 illustrates the different stages of *Xenopus* development and their contribution to germlayer formation.

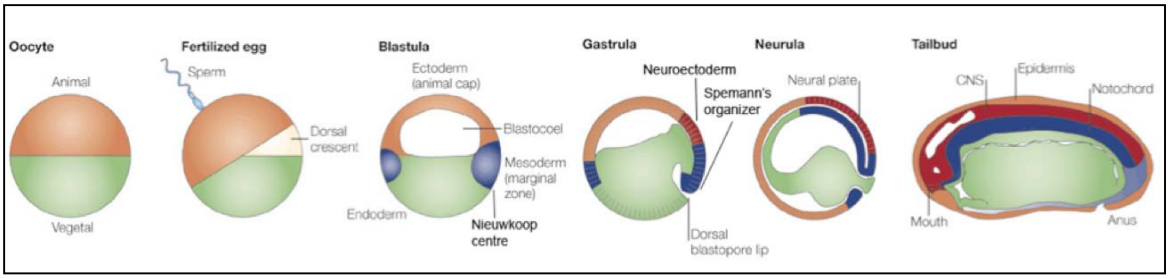

Figure 1: Developmental stages of *Xenopus laevis*

The ovarian oocyte is radially symmetrical and is divided into two domains: an animal and a vegetal one. As the embryo rapidly divides into smaller cells, a cavity called the blastocoel is formed, which defines the blastula stage. By the late blastula stage (9 h post fertilisation), the three germ layers become defined. The ectoderm, or animal cap, forms the roof of the blastocoel. The mesoderm is formed in a ring of cells in the marginal zone, located between the ectoderm and endoderm. At the gastrula stage (10 h post fertilisation), involution of the mesoderm towards the inside of the embryo starts at the dorsal blastopore lip. The organizer secretes signalling factors (Chordin, Noggin, Follistatin and Xnr-3) that refine the initial patterning. At the neurula stage (14 h post fertilisation), the neuroectoderm gives rise to the neural plate, the prospective central nervous system (CNS). By the tailbud stage (24-42 h post fertilisation), a larva with a neural tube, located between the epidermis and the notochord, has formed. Adapted from (De Robertis et al, 2000).

The different parts of the developing blastula stage embryos have different potentials to give rise to specific tissue types. The vegetal cap gives rise to endodermal cell types, whereas the animal cap develops to epidermal cell types. The marginal zone can give rise to most structures in the embryo, including mesoderm. The organizer induces ventral mesoderm to form lateral mesoderm, initiates the formation of dorsal structures in the endoderm and induces the ectodermal cells to form neural tissue. The basic transcriptional regulation of these induction events is mediated via the inhibition of the BMP4 (*b*one *m*orphogenic *p*rotein 4) signalling pathway. Four molecules are secreted from the organizer that directly interact with BMP4 and block its ability to bind to its receptor. These secreted molecules are Chordin, Noggin, Follistatin and Xnr-3 (*Xenopus nodal-related-3*), which create a gradient of BMP4 along the dorsal/ventral axis of the mesoderm.

Neural induction is the process that initiates the development of the nervous system in vertebrates. Molecular studies in *Xenopus* during the 90s identified neural inducer molecules such as Noggin, Chordin, and Follistatin (Hemmati-Brivanlou et al, 1994; Lamb et al, 1993; Sasai et al, 1995), and their function in *Xenopus* animal caps (Hemmati-Brivanlou & Melton, 1997; Sasai & De Robertis, 1997). The basic mechanism of neural inducer molecules mainly acts by inactivating BMP4, an inhibitor for neural differentiation (Fainsod et al, 1997; Piccolo et al, 1996; Zimmerman et al, 1996). Consequently, neural induction is understood as a process that is negatively controlled by the active BMP-signalling gradient. The dissociation of cells from *Xenopus* animal cap explants revealed the spontaneous induction of neural fate, due to de-repression of BMP antagonists. These observations led to the notion of the neural fate-default model (Hemmati-Brivanlou & Melton, 1997; Sasai & De Robertis, 1997). The neural default model postulates that signalling interactions, probably mediated by BMP signals, prevent the formation of neural tissue, which will form in the absence of extracellular cell-cell signalling (Munoz-Sanjuan & Brivanlou, 2002).

Beside BMP inhibition, FGF and Wnt signalling is necessary for the neural differentiation process of gastrula ectoderm to undergo neural differentiation (Delaune et al, 2005; Marchal et al, 2009). The molecular pattern that determines the development of neuroectodermal tissue proposes a common origin of primordial CNS throughout different species. This is indicated by the fact, that the nervous systems of vertebrates (frog) and invertebrates (fly) develop under the control of similar regulatory information (De Robertis & Sasai, 1996). This idea led to the hypothesis of an inversion of the dorso-ventral (DV) axis in vertebrates and invertebrates that was first proposed by E. Geoffroy Saint-Hilaire (Arendt & Nubler-Jung, 1994). A pivotal role in the upstream regulation of neuroectoderm determination plays the antagonism between BMP signalling and Chordin.

Beside a Chordin/BMP antagonism for embryonic patterning, the De Robertis group further demonstrated that the evolutionary conservation extends beyond these two factors and involves a complex, conserved regulatory network with several additional factors. For example, one of the Sox subclass-B genes have conserved roles in neural determination in both *Xenopus* (Sox2) and Drosophila (Sox-N), reviewed by (Kishi et al, 2000; Sasai, 1998).

2.2.1 Transcriptional regulation of neural induction and neuronal differentiation

Neural induction traditionally concentrates on signalling factors, secreted by the organiser, which are able to induce neural fates in other cells. A profound understanding of this process will only be achieved when we uncover the mechanisms responsible for directly activating neural-specific genes and repressing those required for specifying other cell identities (Stern, 2006).

Sox2 is the first general neural marker gene whose spatial and temporal expression pattern correlates with the commitment of cells to the neural plate. Hisato Kondoh's group described regulatory regions upstream and downstream of the Sox2 reading frame and revealed about 23 separate enhancers. Two of these enhancers are most relevant for neural induction, referred to as N1 and N2. N1 directs Sox2 expression to the caudal hindbrain and spinal cord, whereas N2 is necessary for Sox2 expression in the fore- and midbrain (Uchikawa et al, 2003).

In the chicken embryo, the same group analysed the role of the N1 enhancer and showed that it drives the expression of Sox2 in the hindbrain and spinal cord regions. They demonstrated a convergence of FGF and Wnt signals to regulate this expression independently of BMP signals (Takemoto et al, 2006).

A further crucial factor for neural induction is Churchill (ChCh). ChCh is a zinc finger transcription factor that regulates the choice between neural and mesodermal cell fates near the embryo's midline. ChCh activates its target gene, the *S*mad-*i*nteracting *p*rotein1, referred to as Sip1 (Sheng et al, 2003). Makoto Asashima's laboratory has shown that Sip1 is essential for neural plate specification in *Xenopus laevis* (Nitta et al, 2004), while Dale Frank's group described that the POU domain gene, Oct3/4 homologue POU91, is an essential regulator of ChCh expression. The loss of POU91 function, leads to the induction of high expression levels of the mesodermal marker gene *Xenopus bra*chyury (Xbra). The loss can be rescued by the expression of ChCh or Sip1 (Snir et al, 2006). This reveals Oct3/4/POU91 as a regulator of ChCh, which induces Sip1 expression. Thus, the reduced expression of one gene can be substituted by the down stream target genes during neural induction. Beside transcription factors and their roles in regulating specific elements to direct expression of critical target genes during neural development, a new area of investigation is starting to emerge: larger-scale changes in chromatin structure and the factors responsible for these modifications. As an example, Amanda Fisher's group showed the regulation of Mash1, a key locus involved in the acquisition of

neural fates by ES cells *in vitro*. They discovered that the replication timing of this locus during the cell cycle moves from late to early during S-phase after neural induction. Large-scale chromatin changes like histone acetylation and methylation play a key role during this regulation, suggesting that neural gene expression is regulated by changes in chromatin structure (Williams et al, 2006).

According to Claudio D. Stern, this has only been applied to study the acquisition of neural fate by various cells in culture, rather than in the context of the normal developing embryo. However, it is proposed only a matter of time before changes in chromatin structure are recognised more widely as a key regulatory mechanism of neural induction and other key developmental events (Stern, 2006). So far, the precise factors, which are responsible for the regulation of chromatin remodelling in the context of neural induction are unclear. This underlines the idea that global changes in chromatin are required to regulate neural gene transcription. Therefore, I will analyse the role of the chromatin-remodelling factor CHD4/Mi-2ß during neural stem cell differentiation in the context of embryonic development.

2.2.2 Chromatin in embryonic neural stem cell differentiation and neural development

Chromatin represents the basic regulatory entity of eukaryotic genetic material, and serves as a template for genetic information. To match the physiological demands of storage, processing and alteration of information during embryonic development, cellular differentiation and cell fate decision in regular or pathological development, this template is subjected to a variety of structural changes. The structural changes correlate with significant changes in the replicative and transcriptional activity and are orchestrated and controlled by epigenetic mechanisms.

Epigenetic mechanisms of gene regulation include the exchange and incorporation of different histone variants, which alter chromatin composition and the DNA methylation status. In addition, the precise regulation of eukaryotic gene expression is achieved by two further enzyme catalyzed dynamic ways to change chromatin structure and the DNA-histone interactions: covalent posttranslational histone modifications, which act in concert with ATP-dependent chromatin remodelling.

The changes in chromatin structure that accompany embryonic stem cells and particularly neuronal differentiation illustrate how chromatin serves as a platform to regulate cellular fate. These changes in chromatin architecture are particularly interesting as they affect the process of cell differentiation but also the self-renewing

potency of embryonic stem cells and cellular plasticity (Meshorer, 2007). Neuronal differentiation includes alteration in gene expression pattern, nuclear architecture, but also cellular-morphological changes such as neurite extension and synapse formation. Consequently, plasticity is crucial for ES cells to accommodate to rapid and substantial changes. Their unique chromatin is one potential key to their ability to adapt and react adequately. It is becoming increasingly clear that epigenetic regulation is one key to pluripotency, stem cell identity, regulation of differentiation and cellular fate (Meshorer, 2007). The complex regulation of gene expression and nuclear processes during development requires the modification of chromatin to provide the correct segment of DNA accessible to the transcription machinery at the correct time. A way to control the access to DNA is the covalent modification of histones and the subsequent alteration of interactions between DNA and histones, which results in new binding surfaces for further factors (Imhof, 2006).

The tight packing of genomic DNA into chromatin renders genes mostly inaccessible to the transcriptional machinery. Thus, the second major mechanism is provided by ATP-dependent chromatin remodelling complexes. These protein complexes use the energy of ATP hydrolysis to alter DNA-histone contacts, to translationally reposition nucleosomes or displace histone dimers/octamers along the DNA fibre. This remodelling is catalyzed by ATPases, which act in large chromatin remodelling protein complexes, whose composition determines their developmental role they play in specific tissues. In addition, their function depends on the stage of development in response to specific signalling pathways and transcription factors. A review of the diverse roles of the three major subclasses of ATP-dependent chromatin-remodelling complexes, SWI/SNF, ISWI and CHD during the development of neural tissue was given by (Brown et al, 2007). For review of chromatin remodelling during development see (Ho & Crabtree, 2010). A detailed description of the major chromatin-remodelling enzymes will be provided in 2.4.3.

In the following, the epigenetic regulation of cellular differentiation and the particular role of a changing chromatin context along with neural cell differentiation will be portrayed.

2.2.3 Regulation of cellular differentiation and development by the SNF2-like chromatin remodelling ATPase CHD4 and the transcription factor Sip1

In 2004, our laboratory published a database screening of 29 members of the SNF2 domain containing protein family for *Xenopus laevis* (see Table 1) (Linder et al, 2004). The analysis of the expression domains revealed that the family members are broadly expressed, but have stage and tissue specific mRNA expression profiles.
The ATPase subunit CHD4 of the NuRD chromatin remodelling complex was further analyzed by gain- and loss-of-function studies in regard to their influence on specific marker genes (Linder et al, 2007). We demonstrated that gain- and loss-of-function of CHD4 affects the expression domain of the mesodermal marker gene Xbra *(Xenopus brachyury)* and the neuroectoderm marker gene Sip1 (Smad-Interacting Protein*1)*. Both genes demarcate the mesodermal and the neuroectodermal germ layer during gastrulation. Xbra is expressed in response to Nodal/Smad2 and FGF/MAPK signalling and is induced shortly before gastrulation in the future mesoderm. For review see (Wardle & Smith, 2006). Xbra expression is repressed by Sip1 by a single bipartite binding site, which is located in the Xbra promoter region (Eisaki et al, 2000; Lerchner et al, 2000; Papin et al, 2002; Verschueren et al, 1999).
In addition to mesoderm repression, Sip1 has neural-inducing activity in *Xenopus laevis* animal cap explants (Eisaki et al, 2000; Nitta et al, 2004). In chicken embryos, Sip1 is part of a pathway, which promotes the formation of neurogenesis and suppresses mesoderm (Sheng et al, 2003). In *Xenopus*, Xbra and Sip1 genes are initially co-expressed at the beginning of gastrulation, but their domains are quickly separated into neighbouring domains, which mark the future mesoderm and the neuroectoderm, respectively (Papin et al, 2002). Our laboratory demonstrated by mRNA in situ hybridization experiments and animal cap explantation assays that CHD4 plays a crucial role for the positioning of the boundary formation between the germ layers neuroectoderm and mesoderm. This is specifically controlled by the Nodal signalling input via Sip1 for the Xbra transcription (Linder et al, 2007). Consequently, this study revealed the crucial role of epigenetic regulation by chromatin remodelling enzymes during fate decision of ES cells, germ layer formation and cellular differentiation during development. Further information about Sip1 will be given in 2.4.1. To gain an insight in epigenetic gene regulation, the following chapters will provide information about the underlyling mechanisms in regard to this work.

2.3 Epigenetic regulation of cellular differentiation

Epigenetics was first referred to by Conrad Waddington as, beside genetics, “the branch of biology, which studies the causal interactions between genes and their products, which bring the phenotype into being” (Waddington, 1942). Arthur Riggs and colleagues defined epigenetics as the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence. For review see (Bird, 2007).

Today, epigenetics is used to describe heritable alterations of phenotypes that are not based on changes in DNA sequence to explain many aspects of the transmission of genetic information, ranging from the mitotically stable silencing of whole chromosomes over the tissue specific maintenance of gene activity to the heritable affect of nutrition on gene expression patterns (Bouazoune et al, 2002).

Cellular differentiation may be considered an epigenetic phenomenon, largely governed by changes in what Waddington described as the epigenetic landscape, rather than alterations in genetic inheritance. Thus, epigenetics can be defined as the study of any potentially stable and, ideally, heritable change in gene expression or cellular phenotype that occurs without changes in Watson-Crick base-pairing of DNA (Goldberg et al, 2007). These epigenetic changes are mainly associated with changes in chromatin structure. The conformational changes of chromatin structure are executed by covalent posttranslational modifications of histones, DNA methylation and nucleosome remodelling. For the understanding of gene expression regulation due to epigenetic alteration of the chromatin architecture, the basics of chromatin structure is provided in the following chapter.

2.4 Chromatin

2.4.1 Chromatin structure

Historically, the term “chromatin” is derived from the Greek word “χρωμα” for colour and was first introduced in 1882 in “Zell-substanz, Kern und Zelltheilung” by the anatomist Walther Flemming: *“Therefore, we will designate as chromatin that substance, in the nucleus, which upon treatment with dyes known as nuclear stains does absorb the dye.”* (Flemming, 1882).

Today, the term “chromatin” is used to describe the functional state of eukaryotic genomes as a complex of DNA and proteins found inside the nuclei of eukaryotic cells. Based on histological observations, the botanist Emil Heitz suggested the distinction between “euchromatin” and “heterochromatin” (Heitz, 1928). He described the heterochromatin that remains basically condensed after cell division and the proper “eu”chromatin that undergoes de-condensation after mitosis. Beside differences in structural features, he proposed that “euchromatic pieces are rich, whereas heterochromatic ones are at least poor in genes” (Heitz, 1934). The theory that “euchromatin is genicly active, heterochromatin genicly passive” as hypothesized by Heitz is challenged by the theory of facultative heterochromatin (Bernstein & Hake, 2006).

Beside the histological observations, a biochemical approach to verify the character of chromatin structure was provided by Friedrich Miescher. He digested isolated leukocyte nuclei with pepsin and termed the remaining material “nuclein” (Miescher, 1871). In 1884, Albert Kossel separated basic proteins from nuclei by acid extraction and called them “histon” (Kossel, 1911). The revelation of a recurring structural feature of chromatin was supported by a further digestion experiments upon chromatin with micrococcal nuclease by Clark and Felsenfeld. They observed that distinct parts of the DNA were protected from digestion, resulting in fragments of about 100-200 base pairs (Clark & Felsenfeld, 1971).

Two years later, Hewish and Burgoyne suggested that “specific histones seems to be important to limiting the sites of action of deoxyribonucleases”, consequently, “any periodicity of regularity of positioning of the histones on the nuclear DNA will be reflected in the spectrum of molecular weights produced in the DNA after deoxyribonuclease digestion” (Hewish & Burgoyne, 1973).

Ada L. and Donald E. Olins first described the appearance of chromatin fibres as "particles on a string"-like structure by electron microscopy (Olins & Olins, 1974).

Roger D. Kornberg hypothesized the arrangement of histones and DNA as a flexibly jointed chain of repeating units, which differ in their spacing. These units were termed nucleosomes by P. Oudet. In one fraction, almost all of the DNA is condensed in nucleosomes, while the other fraction contains long stretches of free DNA (Doenecke et al, 1997; Gross-Bellard et al, 1973).

In 1997, Luger et. al presented the x-ray crystal structure of the nucleosome core particle of chromatin in atomic detail, showing how the histone complex is assembled with 147bp +/-2 to 3 of DNA organized into a superhelix around a protein octamer (Luger et al, 1997) (Figure 2). Nucleosomes, containing more than 150 bp are defined as mononucleosomes.

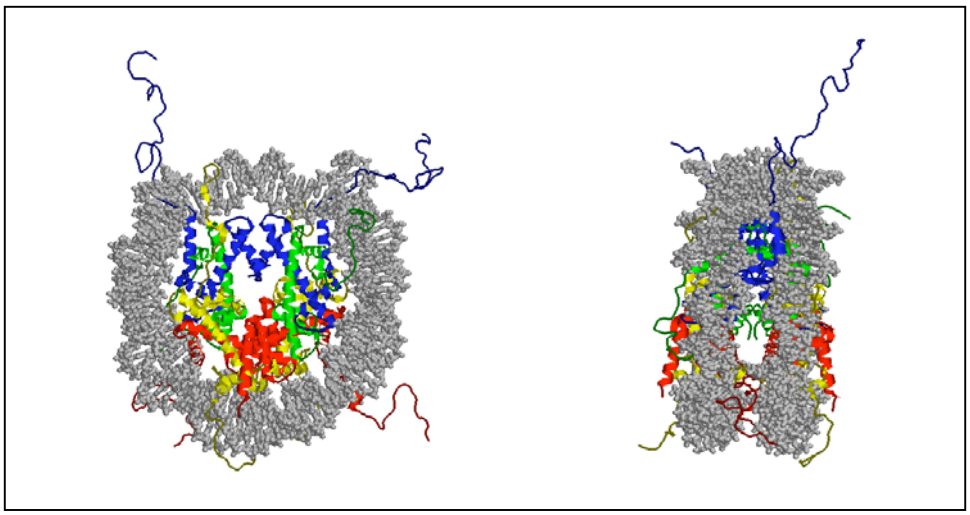

Figure 2: Nucleosome structure at 1,9-Å resolution

Colour code: H2As are represented in yellow, H2Bs in red, H3s in blue, and H4s in green. The DNA is represented in grey. Histone H1 is not illustrated. Adapted from (Davey et al, 2002).

The extended array of adjacent nucleosomes are linked via the linker DNA, which varies in length in a cell- and species-specific manner. Long chains of nucleosomes form an 11nm fibre and can be visualized by electron microscopy as a “beads-on-a-string” structure. However, they are very unlikely to exist as such in a living cell. Instead, these nucleosome arrays are very tightly compacted in a larger structure, called the 30nm fibre. For review see (Alberts et al, 2004). Additional histone proteins, the linker histones, are important for the formation of this structure. The linker histones consist of a central, globular domain and a highly positive charged C-terminal domain. With their globular domain, they anchor themselves to the nucleosome. The C-terminal domain binds to the linker DNA. For review see (Jerzmanowski, 2004).

Chromatin structure is dynamic and active changes directly influence the transcriptional activity. Chromatin binding proteins associate with chromatin dynamically (Phair & Misteli, 2000). This is particularly evident in undifferentiated embryonic stem cells and multipotent cells, where a loosely bound fraction of chromatin binding proteins is present in the nucleosplasm. Core histones, which are normally tightly associated with the DNA, revealed to have an unbound or loosely bound fraction in pluripotent cells. However, the free pool of histones becomes tightly associated with chromatin during ES cell neuronal differentiation (Meshorer et al, 2006). Consistent with the notion of a tighter binding of histones to chromatin in

differentiated cells, terminally differentiated neurons show a lower core histone turnover, demonstrated by a decrease in their rate of synthesis (Cestelli et al, 1992). This reveals a central role of histones and their variants in the formation of the chromatin structure. Consequently, further information about histone proteins is provided below.

2.4.1.1 Histones and their variants

The structure of eukaryotic chromatin consists of DNA and its linked proteins, which can be divided into two main groups: histones and non-histone proteins. The highly conserved basic histone proteins are further subdivided in two classes of histone proteins: The core histones, referred to as H2A, H2B, H3 and H4, and the protein family of closely related H1 linker histones.

Interaction of H2A and H2B leads to their dimerisation. H3 and H4 dimer complexes further interact in a H3-H3 homomeric manner, giving rise to a tetrasome structure.

The association of a (H3-H4)2 tetrameric complex by two H2A-H2B dimers result in the histone octamer. Beside the composition of different histones in one nucleosome, different eukaryotic histone variants are known, which function in different cellular and developmental contexts from transcription activation to gene silencing, detecting DNA damage and centromere formation. Conventional histones are encoded by multiple genes. In contrast, histone variants are usually encoded by single genes, which differ in their temporal and special expression profile in a replication dependent, replication independent or in a tissue specific manner (Doenecke et al, 1997; Wolffe & Pruss, 1996). The combination of different histone variants depends on the cellular context and influence cellular response on transcriptional influences. Chromatin remodelling alters the nucleosome architecture, which results in different functional outcomes. Thus, the following chapter will describe the different histone variants and their functions during development due to their nucleosomal compositions.

Variations of the histone variant H2A are diverse. Until today, up to five variants have been described in mammals, which strongly differ in their amino acid sequences.

The H2A variant H2A.X is highly conserved through out all analyzed species. H2A.X is the main form of H2A in *Saccharomyces cerevisiae* however, in mammals, the variant H2A.X represents up to 25% of H2A histones, and is equally disseminated throughout the genome. Its specific function can be attributed to its extended COOH-terminal domain. It is characterized by a distinctive serine-glutamine-glutamate

(SQE) motif that harbours serine at position 139, which can be post-translationally phosphorylated by different kinases (Burma et al, 2001; Stiff et al, 2004). The phosphorylation of H2.AX serine 139 was first observed as a consequence of DNA damage due to gamma radiation, thus this variant is referred to as gamma-H2A.X (Redon et al, 2002). Beside its role in the recruitment and accumulation of the DNA damage response proteins and its cellular protection against alkylation-induced DNA damage (Meador et al, 2008), gamma-H2A.X seems to have a repressive effect on tumour induction and genomic stability (Bassing et al, 2003; Celeste et al, 2003; Stucki et al, 2005). Gamma-H2A.X is removed from chromatin either by exchanging with the unphosphorylated histone version or by in situ dephosphorylation.

A further variant, called H2A.Z belongs to the best studied, but least understood variants of H2A. The first observations indicated that H2A.Z might be associated with actively transcribed chromatin. In *Tetrahymena thermophila*, H2A.Z is mainly located in the actively transcribed macronucleus (Allis et al, 1980). In contrast, in *Saccharomyces cerevisiae* the H2A.Z homologue Htz.1 is associated with the maintenance of the boundary between euchromatin and heterochromatin. In mammals, H2A.Z is mainly attributed to gene silencing due to its localisation to pericentric chromatin and its co-localisation with the heterochromatin protein HP1-α (Rangasamy et al, 2003; Rangasamy et al, 2004). The posttranslational modifications (PTMs) of H2A.Z, detected by mass spectrometry until today are lysine 4 (K4) and lysine 7 (K7) acetylation (Bonenfant et al, 2006). The biological indications of these findings are not determined yet.

Interestingly, induced neural differentiation could be achieved from human embryonic carcinoma stem cells, which was accompanied by significant changes in the acetylation and methylation patterns of histone H3, and the expression level of histone H2A.Z (Shahhoseini et al, 2010).

The vertebrate specific variant macroH2A1 has a 65% identical N-terminal tail region with H2A and a globular non-histone macro domain at its C- terminal end that is not present in other histone proteins. In mammalian cells, two separate genes exist, coding for macroH2A1 and macroH2A2, which are enriched on the inactive X chromosome (Xi) in female mammalian cells, where they function to maintain gene silencing by *XIST* RNA (Chow et al, 2005; Csankovszki et al, 1999). However, resent results indicate that the function of macroH2A1 histones is not only restricted to gene silencing. It is also involved in fine tuning the expression of specific genes that have increased expression levels in macroH2A1 knockouts (Changolkar et al, 2007). The macroH2A1 gene has two alternatively spliced variants in mammals, which differ only in a small region of the macro domain: macroH2A1.1 and macroH2A1.2.

The repressive function of macroH2A in a chromatin context has been attributed to inhibiting the chromatin remodelling effect of the chromatin remodelling factor SWI/SNF on the one hand, and to its repression on p300 on the other (see 2.4.2.1) (Angelov et al, 2003; Doyen et al, 2006). The PTMs of this H2A variant are diverse and not well understood. For detailed information, please see (Bernstein et al, 2008; Chu et al, 2006; Ogawa et al, 2005).
The least related variant of H2A, with only 48% sequence identity to the conventional H2A was identified as an H2A variant that is Barr-body deficient and is referred to as H2A.Bbd. Beside these five H2A variants, three H2B variants have been described, with a localisation throughout the genome.

The first H2B variant is the human-sperm-specific spH2B. It has a high affinity to the unique sequence TTAGGG of double stranded DNA, which is mainly localized at telomere regions of the genome (Gineitis et al, 2000).
The second H2B variant, which is tissue specific for testis or sperm, was named hTSH2B (Zalensky et al, 2002), and differs from the somatic H2B in various aspects, e.g. its amino acid sequence in the N-terminal tail region (Cheung et al, 2003; Kimmins & Sassone-Corsi, 2005).
The third H2B variant H2BFWT is exclusively restricted to testis with the least homology of only 45% with H2B and sequence differences mainly restricted to the N-terminal tail. Due to the conserved histone fold domain H2B and H2BFWT can be mobilized and remodelled by the chromatin remodelling complex SWI/SNF (Boulard et al, 2006). In contrast to the H2A and H2B variants, the well-studied variants of histone H3 strongly differ in their function.

The H3 variants described so far include H3.1, H3.2, H3.3, H3.1t and CENP-A, which are specialized due to their unique sequence, their expression and their large variety of PTMs. The first H3 variant called CENP-A (*cen*tromeric *p*rotein *A*) is conserved in all species analysed until today and compared to other H3 variants, revealed the strongest sequence variations in its N-terminal region (Kitagawa & Hieter, 2001). Its expression is replication and cell cycle independent (Ahmad & Henikoff, 2002), peaks in late S/G2 phase (Sullivan & Karpen, 2001) and functions in chromosome segregation. In early embryonic development, CENP-A plays a crucial role. CENP-A deficient mice failed to survive beyond 6.5 days post conception, due to severe mitotic problems, chromatin fragmentation and chromatin hyper-condensation (Howman et al, 2000). CENP-A is phosphorylated by Aurora-B kinase at serine 7 in a mitosis dependent manner (Zeitlin et al, 2001).

In contrast to CENP-A, the H3 variants H3.1 and H3.2 are expressed in a replication and cell cycle dependent manner and differ at position 96 in cysteine and serine, respectively. Despite their sequence similarity, H3.1 and H3.2 can be characterized by their functional differences. Beside their different expression profiles, they differ in their biological functions due to their PTMs. H3.1 shows an enrichment of lysine 9 (K9) di-methylation and K14 acetylation, whereas H3.2 is more associated with K27 di- and tri-methylation. In contrast to acetylation, di- and tri-methylation modifications are marks for transcriptional silencing of facultative heterochromatin.

The H3 variant H3.3 is highly conserved and only differs in up to five amino acids in its core region. It is expressed and integrated by the chaperon complex HIRA (*hi*stone *re*gulation) in a replication independent manner (Ray-Gallet et al, 2002). H3.3 is associated with transcriptional activation and is actively located at places in the genome with PTMs, which are associated with transcriptional activation (Hake et al, 2006; Janicki et al, 2004; Johnson et al, 2004b). Further specific functions are implicated by the unique phosphorylation of the H3.3 serine 31 (H3S31Ph) residue during metaphase. Interestingly, this modification is combined with the unphosphorylated status of H3 serine 28, a well-known maker for mitosis in its phosphorylated status. For this work, I tested H3 serine 28 phosphorylation as a marker to detect proliferating cells, as provided in the result part 4.2.2.1.

Summing up, the described differences in H3 variants have specific functions in gene regulation. This notion has recently been referred to as the “H3 barcode hypothesis”, which states that H3.1 might be associated with constitutive heterochromatin, H3.2 with facultative heterochromatin and H3.3 might be associated with euchromatin. Consequently, H3 variants are understood to play an important role in epigenetic inheritance and cell fate restriction (Hake & Allis, 2006). As an example, Gurdon’s group proposed that H3.3 is initially recruited to particular gene loci by differentiation signals or specific transcription factors with the subsequent incorporation of H3.3. This could establish a marking system for cellular memory that was proposed for histone H3.3 at the *MyoD* promoter in *Xenopus* embryos. This association suggests that epigenetic memory helps to stabilize gene expression in normal development (Ng & Gurdon, 2008). The example illustrates that the influence of different histone variants on gene expression and nucleosome assembly have to be considered, if differences in gene expression patterns and cellular differentiation are investigated, due to changes in chromatin structure during development. These histone variants assemble in different combinations to form different nucleosome versions. This provides an additional level of information, encoded in the chromatin structure.

2.4.1.2 Nucleosome assembly

The incorporation of the presented histone variants into nucleosomes has diverse effects on nucleosome structure, its stability, and the ability of nucleosomal arrays to condense into chromatin higher order structures. Two different theories are known, how nucleosome assembly is accomplished. The first is a two-step process, which is initiated by the deposition of two tetramers or dimers (H3-H4) on the DNA (Jackson, 1990; Smith & Stillman, 1991; Worcel et al, 1978). This tetrasome is completed by two (H2A-H2B) dimers, added to form the octamere nucleosome. In nucleosome assembly, two pathways have been described, which are referred to as a replication-dependent and a replication-independent mechanism.

The first depends on the CAF-1 (*c*hromatin *a*ssembly *f*actor-1) (Smith & Stillman, 1989), which acts together with the ASF1 (*a*nti-*s*ilencing *f*unction 1) (Mello et al, 2002; Tyler et al, 2001). The second, replication-independent pathway, depends on histone chaperone HIRA, which is associated with the histone variant H3.3. Further histone chaperones like nucleoplasmin and NAP-1 (*n*ucleosome *a*ssembly *p*rotein-1) are mainly associated with H2A-H2B dimers to promote their assembly into nucleosomes (Ishimi & Kikuchi, 1991). The biological functions of the different histone variants are under discussion. On the one hand, specific variants can serve as sequence modules that can be used in a context dependent manner due to their unique PTMs and are recognized by specific effector proteins, resulting in specific downstream effects. Consequently, differences in the composition of different histone variants result in distinct, variant-specific biological functions. On the other hand, the composition of different histone variants alters the architecture of the nucleosome octamere. This results in changes in chromatin structure at the regions, which harbour a special composition of histone variants. The composition of the histone variants is not randomly, but is limited to unique combinations and lead to the idea of the histone code hypothesis, which was primarily invented for PTMs (Strahl & Allis, 2000; Turner, 1993). A better insight how the nucleosome is constructed and chromatin is structured, was achieved by crystal structure analysis of the NCP (*n*ucleosome *c*ore *p*article) (Luger et al, 1997). Bernstein et. al proposed an uniform nomenclature to identify the NCP composition, regarding the incorporation of the different histone variants (Bernstein & Hake, 2006). According to this nomenclature, a nucleosome, which contains a combination of two variants from one histone family is a heterotypic single-variant nucleosome (HeSVN), a nucleosome, which contains only the same variant from one type is called a homotypic single-variant nucleosome (HoSVN), respectively. In contrast, no HeSVNs have been identified, yet. A

heterotypic multiple-variant nucleosome (HeMVN) is referred to as a mixture of different types of variants, whereas a homotypic multiple-variant nucleosome (HoMVN) would be composed of identical variants from different types. (Figure 3 A-G) Considering, that a certain combination of histone variants might be due to the function of chaperones, which recognise them, it is proposed that homotypic nucleosomes might actively participate in epigenetic inheritance mechanisms by marking specific chromatin domains. This is referred to as the nucleosome code.

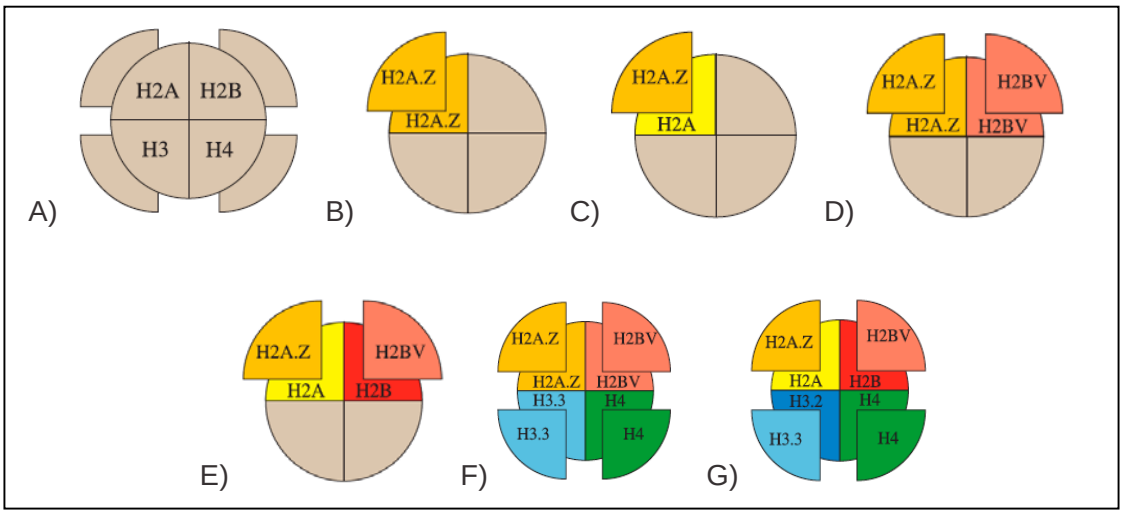

Figure 3: Combinatorial possibilities of histone variants within a nucleosome

A) Conventional nucleosomes, B) Homotypic single variant nucleosomes (HoSVN), C) Heterotypic single variant nucleosomes (HeSVN), D) Homotypic multiple variant nucleosomes (HoMVN), E) Heterotypic multiple variant nucleosomes (HeMVN), F) uniform homotypic variant nucleosomes (UHoVN), G) uniform heterotypic variant nucleosomes (UHeVN) nucleosomes. Adapted from (Bernstein & Hake, 2006).

Beside the illustrated core histones H2A, H2B, H3, H4 and their variants, the linker histones are less conserved. Schulze et al stated that in evolutionary history the vertebrate linker histones H1 zero, H5, and H1M diverged from the main group of histone H1 proteins before the vertebrate type of histone H1 was established in evolution (Schulze & Schulze, 1995). The variants of H1 can be distinguished into maternal and somatic linker histones. The maternal histone H1 subtype H1M, is only detectable in pre-neurula stages. For a review of gene regulation by H1, and its role during development, ES cell differentiation and links to PTMs (methylation) see Rupp and Becker and references therein (Rupp & Becker, 2005).

In consideration to my investigations, the combination of different histone variants establishes a chromatin structure during neuronal and ES cell differentiation and thus promotes a stage specific gene expression profile that differs according to their developmental state (Hake et al, 2006). These changes work in concert with covalent N-terminal histone modifications.

2.4.2 Covalent, post-translational histone modifications

Since Vincent Allfrey's discovery of histone methylation and acetylation more than 40 years ago (Allfrey et al, 1964), histone modifications, including phosphorylation, ubiquitinylation, sumoylation, biotinylation, ADP-ribosylation, prolyl isomerisation and tail clipping, have been strongly implicated in all genomic processes. Within the chromatin context, the combinatorial pattern of the N-terminal modifications results in a heterogeneous identity for each nucleosome form. The modifications are put in place and removed by specific and expanding enzyme families, which are responsible for setting and maintaining patterns of modifications that contribute to define cell fate and cellular identity.

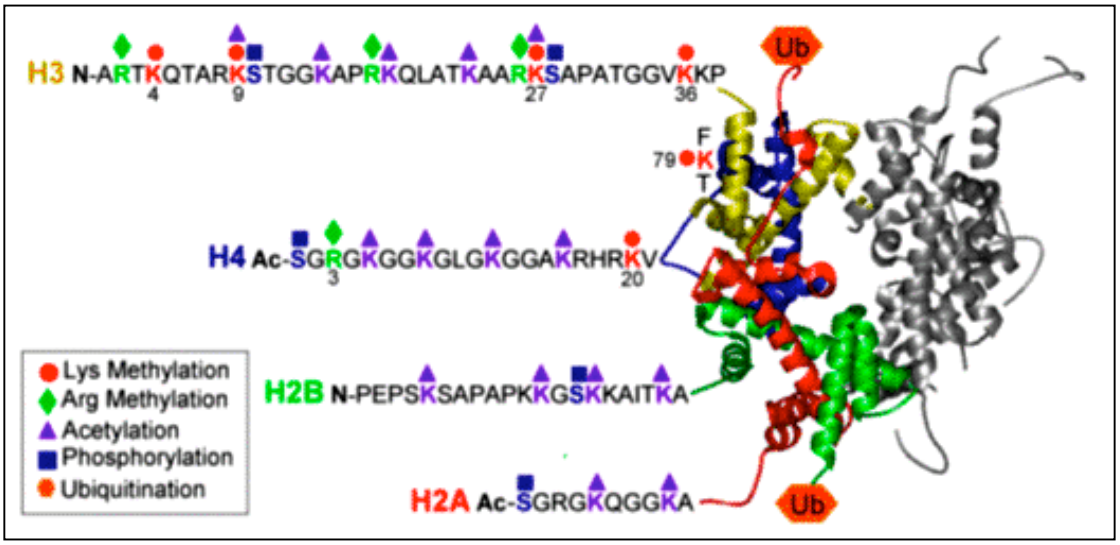

Figure 4: Nucleosome structure with its N-terminal covalent histone modifications
Illustration of posttranslational modifications of histones with specific amino acid sites of posttranslational modifications (acetylation, phosphorylation, ubiquitination and methylation) that are known to occur on histones are indicated by coloured symbols. Half of the histone octamer structure is coloured. H3 is represented in yellow, H4 in blue, H2A in red and H2B in green. The other half is represented in grey. After Scott D. Briggs' webpage: http://www.biochem.purdue.edu/faculty/briggs.htm

Enzymes, which catalyze covalent posttranslational histones modifications, such as acetylation, methylation, phosphorylation, ubiquitination or biotinylation and ATP-ribosylation, are mainly targeted to the N-terminal histone tails, which extend out of the nucleosomal core structure. See Figure 4.

The setting of these modifications is orchestrated in a spatial and temporal dependent manner by specific enzymes, as illustrated in Figure 5. These enzymes can read and alter specific combinations of histone modifications, which result in synergistic or antagonistic interaction affinities for chromatin-associated proteins. A distinct combination of modifications in this epigenetic marking system serves as a binding platform for specific chromatin-associated effector proteins, which translate this encoded epigenetic information into chromatin structural changes.

Thus, these modifications regulate the dynamic transitions between a transcriptionally active and a transcriptionally silent chromatin state. This notion implies a fundamental regulatory mechanism to regulate gene expression profiles, which direct development and cellular differentiation. This epigenetic code of histone modifications regulate the accessibility of the underlying DNA within a certain cellular context (Jenuwein & Allis, 2001). This results in a gene expression profile that depends on histone modifying and chromatin remodelling factors. The modifications are identified and set in place by specific enzymes (Kouzarides, 2007; Nightingale et al, 2006).

Mellor illustrates the histone modifications on histone H3 with its corresponding enzymes that specifically recognize and alter them. In Figure 5 B the red amino acid residues and enzymes represent repressive function, whereas the blue represent activating function. During this process, factors regulate each other, and lead to an integration of information encoded in the epigenetic regulatory pathways to form a complex network of interactions.

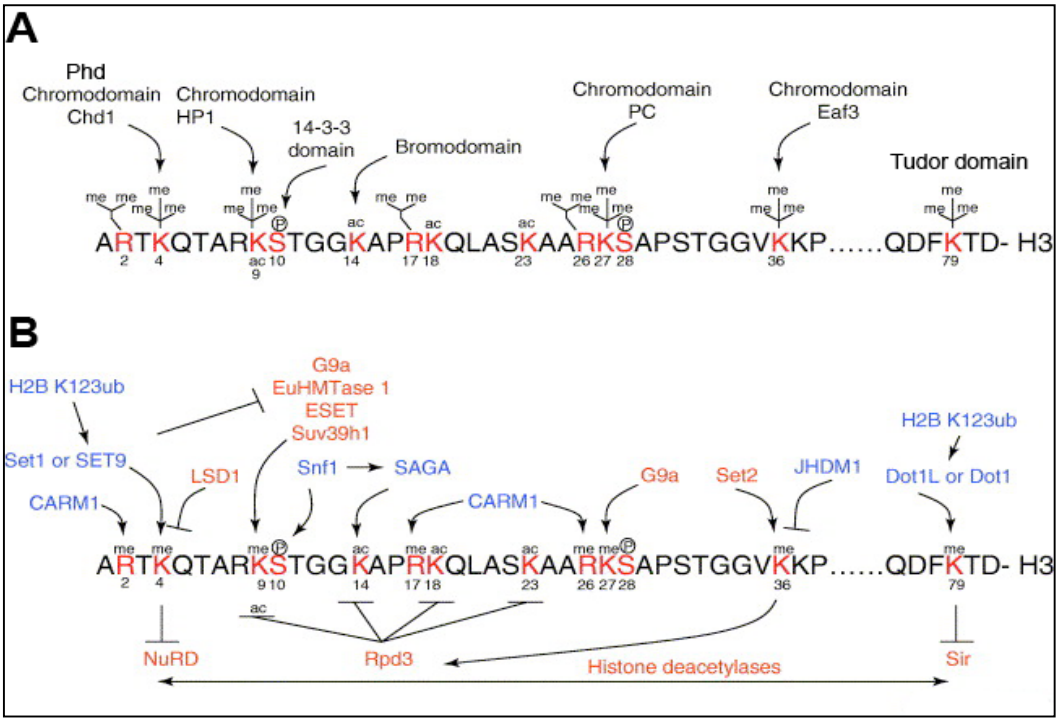

Figure 5: N-terminal histone H3 modifications and modifying binding factors

The figure shows the IUPAC code of the amino acid sequence of the N-terminus of histone H3. The modified amino acids are marked in red. The numbers underneath mark the position of the amino acid. me: methylation, ac: acetylation. Enzymes associated with transcription activation are marked in blue; those associated with repressive events are marked in red.

A) Factors and their domains, which bind to histone modifications.

B) Enzymes, which modify the histone residues. Adapted from (Mellor, 2006).

To clearly and unambiguously specify and facilitate discussion of the functional roles of histone modifications, Turner presented the Brno nomenclature, which was defined at the first meeting of the Epigenome Network of Excellence (NoE) at the Mendel Abbey in Brno, Czech Republic (Turner, 2005).

In 2007, Turner stated that histone modifications are proven to operate as a part of a predictive and heritable epigenetic code that specifies patterns of gene expression through differentiation and development (Turner, 2007). Nevertheless, the discussion about the functional implications of each modification and the complex integration of information they provide as an epigenetic code just started.

During development, toti- and pluripotent stem cells differentiate into all the specific cell types that constitute an organism. During this process, the genome encounters changes in histone modifications during differentiation that reflect changes in the

developmental potential and gene expression, e.g. H3K56ac redistribution (Xie et al, 2009), differential H3K4 di- and tri-methylation at tissue-specific genes during haematopoiesis (Orford et al, 2008), establishment of broad, differentiation-specific H3K9me2 regions, associated with gene silencing (Wen et al, 2009) and modulation of modifications in bivalent domains (Bernstein et al, 2006a; Mikkelsen et al, 2007). Thus, undifferentiated ES cells possess a unique epigenetic landscape, which is altered during the differentiation process. In mouse ES cells, differentiation is accompanied by an increase in tri-methylation of lysine residue 9 on histone H3 (H3K9me3) (Meshorer et al, 2006), and a decrease in H3K4me3 (Azuara, 2006; Bernstein & Hake, 2006). Together with a decrease in pan-acetylation of histones H3 and H4 (Lee et al, 2004b; Meshorer et al, 2006), stem cell neuronal differentiation clearly requires active modification by chromatin modifying enzymes, which include *h*istone *a*cetyl *t*ransferases (HATs), *h*istone *d*ea*c*etylases (HDACs), histone methyl-transferases (HMTs) and the histone demethylases. The following chapters will provide an overview of posttranslational modifications with a focus on epigenetic gene regulation that affects developmental processes of neural induction and neural cell differentiation.

2.4.2.1 Histone acetylation and deacetylation

The first description of histone acetylation by Philips dates back to 1963 (Phillips, 1963). Acetylation is placed on lysines (K) of the N-termini of H3 and H4 (e.g. H3K4ac). In 1964, Allfrey already hypothesized that acetylated histones reduce the capacity to inhibit in vitro ribonucleic acid synthesis (Allfrey et al, 1964). Until today, histone acetylation is positively correlated with active mRNA transcription (Nightingale et al, 2006) and is present in at least 80 non-histone proteins.

The covalent acetylation of the histone amino group at lysine residues affects chromatin at two levels: First, acetylation alters the chromatin structure by neutralising the positive charge of the amino group of lysines, thereby sterically reducing nucleosome/DNA interactions. This reduced nucleosome/DNA interactions facilitate chromatin de-condensation and increases the accessibility to the nucleosomal DNA.

Second, acetylation is recognized and targeted by specific factors with transcription regulation and/or ATP-dependent remodelling activities. Factors with a specific binding domain, e.g. a bromodomain, which has a high affinity to acetylated lysines, like the bromodomain proteins Brahma and Brg1 (see Figure 5) promote gene

transcription (Fischle et al, 2003). This results in a specific gene expression output through the recruitment of further factors and direct mechanical effects like nucleosome sliding, chromatin remodelling or the removal of nucleosomes (Jenuwein & Allis, 2001; Turner, 2000; Vaquero et al, 2007a). The acetylation of histones is performed by HATs (*h*istone *a*cetyl*t*ransferases). HATs act as a part of multisubunit protein complexes. The first nuclear HAT was identified in 1995 and cloned one year later from *Tetrahymena* by Brownell and Allis (Brownell & Allis, 1995) (Brownell et al, 1996). The first classification of HATs was divided into two types according to their intracellular localisation: Typ A is characterized by their cytoplasmatic localisation and type B by their nuclear localisation. A further classification was based on their conserved protein sequence and was grouped into three main families:

- The GNATs (*G*CN5-related *N*-*a*cetyl*t*ransferases) protein family, including Gcn5, PCAF, Hat1, Elp3, Hpa2 and ATF-2.
- The MYST family, containing the factors MOZ, YBF2/Sas3, Sas2, TIP60. Further members are MOF, Esa1, MORF and HBO1.
- The third family is the hormone receptor co-activator p160 (SRC), including p/CIP, ACTR, SRC-1/NCoA-1 and TIF2/GRIP-1/NcoA-2 (Gregory et al, 2001) (Vaquero et al, 2007b).

Factors, which are not grouped into this families are CBP/p300, TAFII250, TAFIIIC and Nut1, nevertheless they are functionally associated with transcriptional activation. In general, these enzymes modify more than one lysine. The acetylation sites that are characterized to date are located within the N-terminal tail of the histones, which are more accessible for modification, except lysine 56 (K56), which is located within the core domain of H3 (H3K56). The K56 residue is facing toward the major groove of the DNA within the nucleosome, so it is in a particularly good position to affect histone/DNA interactions when it is acetylated (Kouzarides, 2007). However, acetylation and gene activation are not mono causally interconnected due to a reduction of negative charges with a resulting weakened DNA-histone interaction. Some HATs are also connected to transcriptional repressors like Sas (*S*omething *a*bout *s*ilencing). According to the histone code hypothesis, acetylated lysines serve as a binding platform for bromodomain containing proteins (Jenuwein & Allis, 2001). The binding and recruiting of proteins or protein complexes affect the local chromatin structure, which leads to a specific biological outcome to dynamically adapt to its context. Therefore, the acetylation marks are reversible with a rapid turnover rate within minutes (Waterborg, 2001).

Apart from acetylation, the process of deacetylation is performed by HDACs (*h*istone *de**ac*etylases). The first HDAC was cloned in 1996 by Taunton and colleagues. In 1991, Vidal et al. described sequence homologies between HDAC1 and the yeast protein Rpd3 (*r*educed *p*otassium *d*ependency *3*) (Vidal & Gaber, 1991). Due to the fact, that Rpd3 is associated to transcription regulation, Schreiber hypothesized HDACs to play a crucial role in regulating eukaryotic transcription (Schreiber & Bernstein, 2002). HDACs seem to have evolved from enzymes, which are involved in bacterial metabolism and are known to be a part of multimeric protein complexes. Although, HDACs have opposing enzymatic activities, HATs can be integrated into HDAC complexes and establish a dynamic, balanced equilibrium of these opposing enzymatic activities in the cell (Yamagoe et al, 2003). HDACs can be classified according to their different characteristics, which are under constant revision. Based on phylogenetic sequence similarity to their yeast counterparts, co-factor dependency, localization and function, HDACs are grouped into four classes and two protein families.

The first is referred to as the classical family, the second as the silent information regulator 2 (Sir2)-related protein (sirtuin) family. The members of the classical family include: The class I Rpd3 orthologs HDAC1, -2, -3 and -8. The class II HDACs are defined based on their sequence homology in their catalytic domain with Hda1 in *Saccharomyces*. Additional domains allow a further subdivision into class IIa (HDAC4, -5, -7 and -9) and class IIb (HDAC6 and -10) enzymes. On the basis of pair wise sequence comparisons, Gregoretti et al. proposed that HDAC11-related enzymes should be given their own class designation, and are referred to as class IV HDACs (Gregoretti et al, 2004). Both classes require $Zn2^{+}$ ion for hydrolysis of the acetyl group and release the acetyl group in form of acetate as reviewed by (Blander & Guarente, 2004; Cress & Seto, 2000; Grozinger & Schreiber, 2002).

The second, sirtuin family of class III enzymes are related to the silent information regulator (Sir2) protein in yeast and its homologues in higher eukaryotes called SirT1–7. In the following, an inside to the functional characteristics of each HDAC class is provided.

Class I HDACs:

Class I HDACs contain HDAC1, HDAC2, HDAC3 and HDAC8 in vertebrates and the RPD3 protein in Drosophila. The activity of these nuclear localized proteins is independent from the co-factor nicotinamide adenine dinucleotide (NAD+). They are ubiquitously expressed and function in transcription repression and cellular

differentiation. HDAC1 and HDAC2 exist in similar chromatin remodelling complexes including CHD4/NuRD, Sin3 and CoREST (Grozinger & Schreiber, 2002). Although, HDAC1 and HDAC2 are two highly related proteins, HDAC2 cannot fulfil all HDAC1 functions, as homozygous disruption of mouse HDAC1 causes early embryonic lethality, and cannot be compensated by HDAC2 function. This demonstrates that significant differences in function or expression of these enzymes do exist (Lagger et al, 2002).

The HDAC3 subclass exists as a single protein from humans to Drosophila (Lagger et al, 2002). Human HDAC3 interacts with most class II proteins, including HDAC4, -5, and -7, as well as HDAC10 and has both nuclear and cytoplasmic localization (Fischle et al, 2002; Takami & Nakayama, 2000).

HDAC8 is restricted to vertebrates and has undergone significant functional specialization, relative to other class I HDACs. Phylogenetic tree analysis suggests that HDAC8 diverges from other class I human HDACs early in evolution that may represent a key point that distinguishes class I and class II HDACs in human (Hu et al, 2000).

Class II HDACs:

Class II (Hda1-like) HDACs are divided into two subclasses: Class IIa HDAC4, -5, -7, -9 and class IIb HDAC6, -10. Functionally, class II enzymes are expressed in a tissue specific manner and shuttle between the nucleus and the cytoplasm (Dequiedt et al, 2006) (Grozinger & Schreiber, 2000; Kao et al, 2001; Wang et al, 2000). Class IIa HDACs share the highest homology at their C-terminus that has full-HDAC activity *in vivo* when it is expressed as an isolated subdomain (Wang et al, 1999). The conserved amino acid motifs are specialized to bind an array of DNA-binding transcription factors, for which class IIa HDACs serve as transcriptional co-repressors. This is important, because class IIa HDACs are not able to bind DNA directly, so that deacetylase activity on promotor regions depends on the recruitment by sequence-specific DNA-binding proteins. An example is the association of class II HDACs with the MEF2 transcription factors via a 17 amino acid motif, which is conserved in all class IIa HDACs. This results in repression of MEF2-targeted promoters by the recruitment of class IIa-associated HDAC activity. The MEF2 family is involved in signal-dependent transcriptional regulation in myogenesis (Black & Olson, 1998) cardiac differentiation and metabolism (McKinsey et al, 2002), negative selection of thymocytes (Woronicz et al, 1994), vascular development (Lin et al, 1998) and most interestingly for my investigations, neuronal differentiation and

survival (Heidenreich & Linseman, 2004). A second example is the interaction of the N-terminal region of class IIa HDACs with CtBP (*C*-*t*erminal-*b*inding *p*rotein) and HP1 (*h*eterochromatin *p*rotein 1). This mediates the recruitment of deacetylase activity into higher order complexes, containing chromatin-modifying enzymes such as class I HDACs and histone methyltransferases (HMTs) (Shi & Massague, 2003). The N-terminal adapter domain is further subjected to various posttranslational modifications, e.g. proteolytic cleavage (Bakin & Jung, 2004; Liu et al, 2004; Paroni et al, 2004), ubiquitination, sumoylation (Kirsh et al, 2002) and phosphorylation. Phosphorylation of class IIa HDACs creates docking sites for 14-3-3 proteins, which induce the nuclear export (Grozinger & Schreiber, 2000). Therefore, this leads to de-repression of class II HDAC target promoters (Grozinger & Schreiber, 2000; Kao et al, 2001; Wang et al, 2000).

In the case of HDAC7, a targeting motif in the N-terminal domain leads HDAC7 to the mitochondria of apoptotic cells. This implicates a possible role in the initiation of programmed cell death (Bakin & Jung, 2004).

Taking into account that formation of skeletal muscle, cardiac hypertrophy, bone development, T-cell differentiation and neuronal survival are controlled by class IIa HDACs suggests therapeutical opportunities for many human pathologies as arteriosclerosis (Verma, 2004), stroke and aneurysms (Kadoglou & Liapis, 2004). As well as for tumour angiogenesis and metastasis (Ranieri & Gasparini, 2001), skeletal abnormalities (Mundlos & Olsen, 1997), autoimmune and lymphoproliferative syndromes (Sarvetnick & Ohashi, 2003; Siggs et al, 2006) and acute lymphoblastic leukaemia (Prima et al, 2005; Yuki et al, 2004). Alterations of MEF2 transcriptional activity have been implicated in neurodegenerative disorders (Camins et al, 2006) and cardiac hypertrophy (Czubryt & Olson, 2004).

The Class IIb HDAC subclasse consists of HDAC6 and -10. According to phylogenetic analysis of full-length human HDAC6 and HDAC10 protein alignments against the Drosophila HDAC6, -10 members indicate that HDAC10 derived from an HDAC6-like enzyme after the divergence from invertebrates to vertebrates. Nevertheless, they differ in various aspects. First, HDAC6 proteins contain two tandem HDAC domains, whereas HDAC 10 contains one full and one vestigial HDAC domain. Second, in contrast to HDAC10, which is predominantly located in the nucleus, HDAC6 is primarily found in the cytoplasm (Zhang et al, 2003) (Matsuyama et al, 2002). This suggests that HDAC6 and HDAC10 may have divided the functions of a single ancestral protein between them and specialized to substrates in their different compartments.

Class III HDACs:

The second sirtuin family of Class III enzymes introduced above, is related to the Sir2 (silent information regulator) protein in yeast, which is involved in the epigenetic silencing of three main yeast gene loci, called mating type loci, telomeres and nucleolar DNA tandem repeats (Guarente, 1999). Histone-related proteins appear to be absent from eubacteria. The characterized “histone-like” proteins in eubacteria are similar to histone proteins, but differ at the sequence and structural level. Nevertheless, histone deacetylases exist in eubacteria, demonstrating that these proteins have also functions in the absence of histone proteins. This suggests that all three classes of “histone deacetylase” enzymes evolved in the absence of histone proteins. Therefore, it is unlikely that HDAC classes became uniquely directed at histones in eukaryotes and lost their activity on their ancestral substrates. Therefore, HDACs have other physiologically important substrates, e.g. human HDAC6 deacetylases tubulin and HDAC1 acts on transcription factors, including p53. The class III members of the sirtuin family exist in species that range from prokaryotes to higher eukaryotes in a quite variable number. One to two members are described in prokaryotes, five in S. cerevisiae (Guarente, 1999), three in Schizosaccharomyces pombe, four in Caenorhabditis elegans (Tissenbaum & Guarente, 2001), five in Drosophila (Tissenbaum & Guarente, 2001) and seven in mammals, namely SirT1–7 or Sirtuins (Frye, 1999; Frye, 2000). In contrast to class I HDACs, the class III HDACs depend on the co-factor NAD+ (Haigis & Guarente, 2006).

SirT1, SirT2 and SirT3 are the only Sirtuin members with HDAC activity with similar specificity for H4K16Ac and H3K9Ac and deacetylate all four histones completely *in vitro*. Loss of SirT1 function results in an increase of H4K16 and H3K9 hyperacetylation and decreased levels of H3K9me3 and H4K20me1, two markers which are characteristic for heterochromatin (Vaquero et al, 2004). SirT1 recruitment to a reporter gene in euchromatic genomic regions promotes the formation of facultative heterochromatin. This is mediated by three different mechanisms. First, SirT1 deacetylates H4K16Ac and recruits histone H1. Second, SirT1 promotes the establishment of H4K20me1 and H3K9me3 by directly elevating and recruiting Suv39h (Vaquero et al., submitted). Third, SirT1 recruitment is concomitant with the loss of H3K79me2.

SirT2 is located in the cytoplasm, binds HDAC6 and deacetylates α-tubulin (North et al, 2003). During G2/M transition of prophase, SirT2 localizes to chromatin at the same time when H4K16Ac levels drop (Vaquero et al, 2006). Thus, SirT2 seems to regulate the global levels of H4K16 acetylation in response to cell-cycle and mitotic

entry. Considering SirT3, it is the closest related Sirtuin to SirT2 and is shuttled to the mitochondria under conditions of stress, where it targets AceCS2 (*Ace*tyl-*C*oA *s*ynthetase 2) (Schwer et al, 2006).
In the context of neural development, HDAC activity plays crucial different roles. In ES cells and neural progenitor cells HDAC influence neural differentiation. While the ES cells chromatin is globally acetylated, deacetylation is required for differentiation, in neural progenitor cells. The deacetylase activity (e.g. HDAC1) is required to suppress neuronal genes and inhibition of HDAC is therefore likely to drive the progenitors towards neuronal commitment.

2.4.2.2 Histone methylation and demethylation

Methylation of histone proteins was first shown to occur on the ε-amino group of lysine (K) by Murray in 1964 and on the guanidino group of arginine (R) by Paik and Kim, 1967 and 1969. Nevertheless, it took thirty years until Jenuwein and colleagues uncovered SUV39H1 as the first specific histone H3 lysine 9 methyltransferase (Margueron et al, 2005; Rea et al, 2000). The process of methylation is catalyzed by enzymes, which use S-adenosyl-L-methionine (SAM) as the methyl group donor (Kim & Paik, 1965; Paik & Kim, 1971).
Today, methylation is observed at various histone lysine (K) and arginine (R) residues. The methylation of these residues work both, as a marker for transcriptional activation and repression, as well as DNA damage response (Margueron et al, 2005). Lysine residues on histone H3, include H3K4, -9, -27, -36, and -79, as well as histone H4K20 and can be mono-, di-, or trimethylated by SET-domain containing HMTs (*h*istone *m*ethyl*t*ransferases) (Bannister & Kouzarides, 2004). Of all the enzymes that modify histones, the methyltransferases and kinases are the most specific. The methylation of H3K4 is catalyzed by the Trithorax-related SET1 domain containing protein family MLL (*m*ixed *l*ineage *l*eukemia) including, MLL1- 4, SET1A and SET1B. For review see (Schotta et al. 2009, submitted).
The H3K9 methylation mark is set by the Suv39 protein family members, called Suv39h, G9a, EST/SETB1 and EuHMTase I (Santos-Rosa & Caldas, 2005).
Similar to high levels of acetylation, the methylation of H3K4, H3K36 and H3K79 correlates with active transcription, whereas a silent heterochromatic state is associated with low levels of acetylation and high levels of methylation at H3K9, H3K27 and H4K20. The degree of methylation and the locations relative to the genome are context dependent and results in different transcriptional outcomes. For

example, H3K4 tri-methylation (H3K4me3) at the promoter, in contrast to H3K4 di-methylation (H3K4me2), is directly involved in active transcription (Santos-Rosa et al, 2002; Bernstein et al, 2002). In addition, H3K9me3 is linked to pericentric heterochromatin, whereas H3K9me3 within a gene region is linked to active gene expression (Vakoc et al, 2005). Mono- and di-methylated H3K9 is associated with transcriptionally silent domains within the euchromatic regions, mediated by co-repressors such as RB and KAP1 (Kouzarides, 2007; Lehnertz et al, 2003; Peters et al, 2003). Consequently, H3K9 methylation within the coding regions is activating, whereas H3K9me in the promoter regions has a repressive character (Vakoc et al, 2005).

The methylation at H3K27 is exclusively performed by EZH2, the mammalian homolog of the *Drosophila* protein Ez (*e*nhancer of *z*este), which is a member of the Polycomb (PcG) protein family. PcG is crucial for the maintenance of transcriptional repression of the developmentally important homeotic (Hox) genes. EZH1 is a homolog of EZH2 that is physically present in the PRC2 (*P*olycomb *R*epressive *C*omplex 2) complex and was identified as an H3K27 methyltransferase *in vivo* and *in vitro*. In the context of development and cellular differentiation, EZH1 co-localizes with the H3K27me3 mark on chromatin to partially complement EZH2 in executing pluripotency during ES cell differentiation (Shen et al, 2008).

H4K20 mono-methylation is exclusively catalyzed by the methyltransferase PR-SET7. In a developmental context H4K20me1 and H3K27me1 are not erased by histone lysine demethylases but remain stably methylated over several cell generations, at least at certain developmental stages (Trojer & Reinberg, 2006). Methylation marks are recognized and bound by proteins or chromatin remodelling complexes via specific domains called chromo-like domains of the Royal protein family chromo, tudor, MBT and PHD domains. But methyl histone modifications can also prevent binding of remodelling factors to chromatin, e.g. H3K4me disturbs the binding of the NuRD complex (Margueron et al, 2005). For an overview of binding domains for each modification and their interacting binding proteins, please see Figure 5 A. A review about specific H3 methylation functions is provided by (Sims & Reinberg, 2009).

In contrast to acetylation and phosphorylation, histone methylation was considered static and enzymatically irreversible (Jenuwein & Allis, 2001), although an enzyme-based reversibility provides the cell with a tool to respond quickly to changes by flexible alteration in its gene expression programs. The notion of static histone methylation was based on comparable turnover rates of bulk histones and the methyl

groups on histone lysine and arginine residues in mammalian cells with a low level of histone methyl group turnover of 2%/hour (Borun et al, 1972; Byvoet et al, 1972; Thomas et al, 1972). A further reason to see methylation of histones as permanent, was the discovery that methylation at H3K9 is responsible for the formation and maintenance of heterochromatin. The most prominent argument was the mere fact that a demethylating enzyme had not been discovered.

However, an enzyme with demethylase activity had already been reported by Paik and Kim in 1973 (Paik & Kim, 1973). Only few years ago, the first histone lysine demethylase LSD1 (*l*ysine-*s*pecific *d*emethylase *1*) was identified to be able to demethylate mono- or di-methylated H3K4 by an amine oxidase reaction (Shi et al, 2004). This led to the identification of numerous histone demethylases.

The regulation of LSD1 activity and its substrate specificity to demethylate H3K4 depends on interaction with the co repressor complex Co-REST, which allows accessibility to nucleosomal substrates. Interestingly, a recent study revealed LSD1 as a subunit of the NuRD complex that targets the metastasis programs in breast cancer (Wang et al, 2009). In addition, the surrounding histone modification context, e.g. H3 serine 10 phosphorylation also affects LSD1 activity (Forneris et al, 2005). For a recent review with additional roles of LSD1 see (Forneris et al, 2009). LSD1 only mediates mono- and di-demethylation, but lysines can also be tri-methylated. This discrepancy raised the hypothesis that further nuclear proteins may exist. The first group of proteins, which were suggested to function as histone demethylases were represented by proteins that contained the Jumonji C (JmjC) domain (Trewick et al, 2005). In Japanese, Jumonji means “cruciform” and was named after the transcription factor, whose loss of function in mice resulted in a cruciform neural plate deformation (Takeuchi et al, 1995; Tsukada et al, 2006).

The first histone demethylase, containing a JmjC domain was JHDM1 (*J*mjC domain-containing *h*istone *d*emethylase *1*), which reverses mono-, and dimethylation of histone H3K36. The first hypothesis that demethylases may also use oxidation of N-methylated lysines was finally strengthened by the identification of JHDM2, which mediates demethylation of H3K9me2 by the use of oxidative mechanisms to remove methyl groups in the form of formaldehyde (Yamane et al, 2006).

Although JHDM1 and JHDM2 are chemically compatible for the reversal of tri-methylated lysine, they only demethylate mono- and di-methylated lysine residues.

The demethylation of tri-methylated lysines has been solved by the identification of a new histone demethylase subfamily, consisting of four members JMJD2 A-D (Katoh, 2004), which are able to specifically reverse tri-methylated H3K9 and H3K36. The family member JMJD2A has been identified before as transcriptional repressor that

interacts with Rb-, HDAC1-, and N-CoR and is associated with the repression of the human neural specific ASH2 gene (Gray et al, 2005; Zhang et al, 2005).

Methylation of histone residues is associated with many biological processes such as stem cell maintenance and differentiation (Bernstein et al, 2006b; Boyer et al, 2006; Lee et al, 2006), X inactivation (Plath et al, 2003) and DNA damage response (Plath et al, 2003). Thus, the different histone methylation patterns account for a potential fine-tuning mechanism to regulate transcription.

In a medical context, increased LSD1 expression in prostate tumours correlates significantly with relapse during therapy (Metzger et al, 2006). Further, an alteration in heterochromatin formation by a significant reduction of H3K9me3 and H4K20me3 levels contributes to cancer development (Fraga et al, 2005; Pogribny et al, 2006). JMJD2C/GASC1 (*g*ene *a*mplified in *s*quamous *c*ell carcinoma 1) is overexpressed in oesophageal squamous carcinoma. In a developmental context, DNA methylation plays distinct roles in different chromatin contexts and seems to be important during neuronal differentiation and neuronal commitment during neurogenesis in particular. For example, knockout of DNMT1 (*DN*A-*m*ethyl*t*ransferase 1) has no effect on postmitotic neurons, however, a severe impairment of neurogenesis was observed (Fan et al, 2001). In contrast, the *de novo* methyltransferase DNMT3b has a central role during terminal neuronal differentiation. The knock-down of DNMT3b in rat pheochromocytoma PC12 cells, stimulated by neural growth factor (NGF), fail to produce post-mitotic neurons (Bai et al, 2005). The loss of the transcriptional repressor MBD1 (*m*ethyl-CpG *bind*ing protein 1) results in neural stem cell differentiation however, leads to adult neurogenesis defects (Zhao et al, 2003) and MeCP2 (*me*thyl-*C*pG binding *p*rotein) is known as a key player in neuronal differentiation (Jung et al, 2003; Matarazzo et al, 2004) and maturation (Kishi & Macklis, 2004). MeCP2-null mutation causes the neurological defect called Rett-syndrome with the classical signs of autism, ataxia and cogitative defects (Guy et al, 2001). Interestingly, MeCP2 binds the catalytic subunit of the SWI/SNF chromatin remodelling complex Brahma (Brm) (Harikrishnan et al, 2005), which will be described in 2.4.3. Furthermore, MeCP2 links neuronal activity and chromatin structure, because MeCP2 binds the methyl-CpG islands of the BDNF (*b*rain-*d*erived *n*eurotrophic *f*actor) promoter in resting neurons. Neuronal activation leads to MeCP2 phosphorylation and re-localization to heterochromatin loci with a release of MeCP2 from the BDNF promoter. This results in an activation of BDNF transcription (Chen et al, 2003; Martinowich et al, 2003). These examples illustrate the links between DNA methylation and chromatin remodelling that regulates gene expression during neurogenesis (Zlatanova, 2005) and demonstrate a dynamic interaction between

DNA methylation and methyl-CpG binding proteins in neurons. This reveals a close relationship between neuronal activity and chromatin structure. Chapter 2.4.3 will introduce ATP-dependent alteration of the chromatin structure due to chromatin remodelling factors, such as CHD4 and their role during neural cell differentiation.

2.4.2.3 Histone phosphorylation and dephosphorylation

In 1966, two independent groups detected histone phosphorylation (Kleinsmith et al, 1966; Ord & Stocken, 1966). Usually, phosphorylation occurs on serine (S) and threonine (Th), but also lysine (K) and arginine (Arg) have been described as well (Smith et al, 1978). Phosphorylation of H3S10 was first described on metaphase chromosomes and was consequently thought to be associated with chromosome condensation and segregation during mitosis and meiosis (Gurley et al, 1978). During mitosis, histone phosphorylation originates in the pericentric heterochromatin and spreads throughout the genome during the G2–M phase transition (Hendzel et al, 1997). The mitosis-specific phosphorylation of histone H3 also occurs at serine 28 (Goto et al, 1999) and at threonine 11 (Thr 11) (Preuss et al, 2003).

In 2001, it was confirmed that interestingly not H3, but H2B is essential for chromosome condensation and led to a revision of H3S10 function, which was then linked to anaphase procession. In correlation with this hypothesis, histone H3 phosphorylation is referred to as a ‘ready production label’, which is attached to histone H3, when chromosomes had passed through the various checkpoints and reached metaphase (de la Barre et al, 2001; Hans & Dimitrov, 2001).

Two different phosphorylation events in mammalian cells play an important role in chromatin condensation and decondensation during mitosis: First, the phosphorylation of H3S10 executed by members of the aurora AIR2–Ipl1 kinase family, which are counter-balanced by the activity of type1 phosphatases (PP1). Second, the phosphorylation at H3T3 is necessary for the normal chromosome alignment during metaphase (Dai et al, 2005). In addition, H3.3S31 phosphorylation (Ph) was shown to be a mitosis-specific modification that differs from other mitotic marks, because H3.3S31Ph is only present in late prometaphase and metaphase and is enriched in distinct chromosomal areas immediately adjacent to centromeres. In contrast, H3S10Ph and H3S28Ph first appear in prophase, persist until anaphase and localize to outermost peripheral regions of the condensed DNA (Hake et al, 2005).

In 1991, Mahadevan et al. postulated a link between phosphorylation and

transcription as they observed a rapid phosphorylation of histone H3 molecules concomitant with activation of the c-fos and c-jun immediate-early response genes (Clayton et al, 2000). Later studies demonstrated that this H3 phosphorylation differed from that in dividing cells. Furthermore, it is targeted only to a small fraction of nucleosomes and is tightly linked to acetylation of H3K9 and H3K14 phospho-acetylation (Barratt et al, 1994; Cheung et al, 2000; Clayton & Mahadevan, 2003; Mahadevan et al, 1991). The interplay between phosphorylation and acetylation of H3K9 or K14 works synergistically with H3S10Ph at the initiation of transcriptional activation (Lo et al, 2000). In addition, H3S10Ph prevented methylation of K9 by the SUV39H1 methyltransferase and the yeast GCN5 histone acetyltransferase displayed a binding preference to H3 if it was pre-phosphorylated at serine 10 (Cheung et al, 2000; Clayton et al, 2000). In medical approaches, H3S10Ph was described in patients with Coffin–Lowry syndrome (CLS). This syndrome is characterized by an impaired transcriptional activation of c-fos and a loss of EGF-induced H3 phosphorylation with a deficit in the RSK2 (*r*ibosomal S6 *s*erine-threonine *k*inase 2) (Sassone-Corsi et al, 1999).

In this work, I will investigate how CHD4 chromatin remodelling influences H3S10 phosphorylation in the developing neural tissue (please see results 4.2.2-4.2.3.1). Further histone modifications include ubiquitination, ADP-ribosylation, deimination and proline isomerisation.

2.4.2.4 Histone code hypothesis

The concept of a combinatorial pattern of PTMs as described above, which results in a specific, context dependent gene expression profile is referred to as the ‘histone code hypothesis’ (Strahl & Allis, 2000). A code is usually defined by simplicity, universality and predictability. Epigenetic information however, as described above, encoded in histone modifications, is characterized by complexity, diversity and more seen as a respond to genomic changes than to predict them. Thus, according to Nightingale et al., the term histone code should be used to refer to the combinations of modification that are known to be involved in ongoing cellular processes and the term epigenetic code to refer to the accepted heritable code that might support cellular memory (Nightingale et al, 2006).

In either case, the modifications are crucial for the understanding how the genome responds to environmental and metabolic change and for manipulating its activities for experimental and therapeutic benefits.

2.4.3 ATP-dependent chromatin remodelling enzymes

The extended array of adjacent nucleosomes is referred to as “beads on a string”.
The regulatory potential on the nucleosomal level, provided by histone variants and covalent modifications, as described, is supplemented by an additional means of gene regulation to partially unravel the tight and hence frequently repressive chromatin organization. Eukaryotes developed an extremely elaborate and sophisticated regulatory network to fine-tune the level of gene expression, which ranges from complete active to mainly repressed states, depending on the cellular context. To enable a flexible and responsive chromatin, histone modifying enzymes act and function in strong cooperation with ATP-dependent nucleosomal DNA remodelling that results in altered histone-DNA interactions such that nucleosomal DNA becomes accessible to interacting proteins. The nucleosome remodelling complexes are comprised of two to twelve different subunits that can be further subdivided into several families according to sequence features outside their ATPase domains. Evidence is emerging that these ATP dependent chromatin-remodelling enzymes have instructive and programmatic roles during development. For review see (Ho & Crabtree, 2010).
The ATP-dependent chromatin remodelling enzymes belong to the SNF2, helicase superfamily 2. They are characterized by seven motifs within their ATPase domain, which are typically found in helicases (Eisen et al, 1995). Based on the work of Gorbalenya and colleagues (Gorbalenya et al, 1989) and Bork and Koonin (Bork & Koonin, 1993), a phylogenetic approach in yeast identified the swi (*swi*tch) or snf (*s*ucrose *n*on *f*ermenting) mutations and led to the discovery of the helicase-containing SWI/SNF family of chromatin remodelling complexes. By sequences comparison of their ATPase domain, these proteins could further be divided into different subfamilies. In addition, the SWI2/SNF2 (*swi*tch 2/*s*ucrose-*n*on-*f*ermenting 2)-related proteins share a C-terminal bromodomain that recognizes acetylated lysines on the histone tails (see Figure 5). For review see (Zeng & Zhou, 2002). In contrast, CHD (*c*hromodomain-*h*elicase-*D*NA binding) family members possess chromodomains, Rad16 family members have a ring-finger and ISWI (*i*mitation *s*witch)-related proteins contain a SANT domain. Nucleosome remodelling activity was shown for the SWI2/SNF2-related enzymes, the ISWI/SNF2L-type ATPases, the CHD family member Mi-2, the Cockayne syndrome B factor and the INO80 complex.
An EST-based screen in our laboratory clustered *Xenopus laevis* and human homologues of these remodelers in a similarity tree and identified *Xenopus*

homologues, which were not described before (Linder et al, 2007).

These chromatin-remodelling enzymes play important roles during cellular differentiation processes to promote or prevent gene expression. Especially, the combined action of chromatin-remodelling factors helps to maintain the decondensed chromatin structure in undifferentiated ES cells. For review see (Bouazoune & Brehm, 2006). Furthermore, chromatin-remodelling enzymes are important for the chromatin assembly and the maintenance of the chromosome structure. For review see (Langst & Becker, 2001b). These factors include Brg1 (Bultman et al, 2000), Snf5 (Klochendler-Yeivin et al, 2000) SSRP1 (Cao et al, 2003) and Snf2h (Stopka & Skoultchi, 2003). Interruption of these factors leads to premature embryonic death at the blastocyst stage before implantation. This suggests a crucial role for chromatin remodelling during early differentiation and development. ATP-dependent chromatin-remodelling factors are abundant in undifferentiated ES cells (Kurisaki et al, 2005) and are important for early ES cell differentiation (Puente et al, 2006). Especially, the chromatin-remodelling NuRD complex is significant important for ES cell differentiation and stem cell self-renewal (Kaji et al, 2006; Yoshida et al, 2008).

The factor Brg1 (*b*rahma *r*elated *g*ene 1), which works as the catalytic subunit of the SWI/SNF chromatin-remodelling complex is critical for neuronal differentiation in *Xenopus* (Seo et al, 2005b). However, in mice Brg1 has an opposite effect, as Brg1 loss of function results in precocious terminal neuronal differentiation of the neural stem cells in the subventricular zone (Matsumoto et al, 2006). This example illustrates the different functions chromatin-remodelling complexes can play in a context dependent manner.

In conclusion, chromatin remodelling is a crucial factor during differentiation of both, ES cells and neuronal progenitor cells. Consequently, the following chapters will provide an overview of three main groups of chromatin remodelers, SWI/SNF, ISWI and CHD class of remodelers and will focus on their role in development and neural differentiation.

2.4.3.1 SWI/SNF family: BRM, BRG1

The identification of the SWI/SNF proteins was initiated by a screen for mutants in *Saccharomyces cerevisiae* that were deficient in activating the SUC2 gene. The SUC2 gene encodes an enzyme required for the utilization of sucrose/raffinose as a source of carbon. High concentrations of glucose result in SUC2 gene repression. In SUC2 deficient mutants, the snf (*s*ucrose *n*on-*f*ermenting) genes could be identified

(Neigeborn & Carlson, 1984). In a further screen for yeast mutants that failed to express the endonuclease, which is necessary for mating-type switching, identified the swi (*swi*tching) defective genes (Breeden & Nasmyth, 1987; Stern et al, 1984). The snf2 and swi2 genes revealed to be the same and were suggested to be components of the same multisubunit complex (Peterson & Herskowitz, 1992) and references therein). This hypothesis was confirmed by the purification of the SWI/SNF protein complex (Cote et al, 1994; Smith et al, 2003) and references therein). The association to chromatin was provided in a genetic screen and vitro studies, which confirmed that the SWI/SNF complex is able to dislocate nucleosomes from DNA, which results in an increase of transcription factor binding to chromatin (Cote et al, 1994).

The Drosophila brahma (brm) gene, named after the Hindu god of fate, is highly related to the yeast SWI2/SNF2 chromatin remodelling ATPase (Tamkun et al, 1992). In 1988, Kennison and Tamkun identified brahma (brm) to have typical properties of trithorax group (trxG) genes that counteract Polycomb group (PcG) repressors to determine cell fate. After the similarity to SWI2/SNF2 was shown, brm was purified as a component of a multisubunit protein complex (Papoulas et al, 1998). Homologues of *Drosophila melanogaster* brm have also been found in vertebrates like *Xenopus* (Gelius et al, 1999), humans (Chiba et al, 1994; Khavari et al, 1993; Muchardt & Yaniv, 1993) and mice (Randazzo et al, 1994).

In *Drosophila melanogaster,* brm is a regulator of homeotic genes and resides in the multisubunit brahma complex. Embryos, lacking brm function, die in late embryogenesis or reveal developmental defects. Beside its function in homeotic gene regulation, brm loss-of-function mutants and mutant flies, which express a *d*ominant *n*egative form of BRM, called dnBRM, revealed a general decrease in cell viability and defects in the development of the nervous system (Elfring et al, 1998).

In vertebrates the products of the paralogous genes, BRM and BRG1 work as alternative ATPase subunits of the SWI/SNF chromatin-remodelling complex. In zebrafish, BRG1 plays an interesting role in the development of retina, brain and neural crest cells. Brg1 null mutant embryos specify retinal cells as an early step in retinal development however; these cells fail to develop terminally differentiated retinal cells. This indicates, that BRG1 is necessary for the terminal differentiation but not for the earlier steps of retinal cell specification. The association with active RNA polymerase II and hyperacetylated chromatin suggests that BRM is functionally linked to gene activation (Armstrong et al, 2002; Mohrmann et al, 2004), and SWI2/SNF2 BRG1 can assist the Rb (*R*etino*b*lastoma protein) to regulate transcription (Dunaief et al, 1994; Trouche et al, 1997). BRG1 also interacts with

cyclin E and BRCA1, which are mutated in breast and ovarian cancer, suggesting a crucial role of BRG1 in tumour genesis. Further studies also implicate a role in transcriptional repression. For review see (Becker & Horz, 2002; Bouazoune & Brehm, 2006).

In summary, BRG1 and BRM-containing complexes have overlapping non the less non-redundant, selective functions in vertebrate development and transcription. BRG1 is associated with survival of dividing cells, maintenance of pluripotency and differentiation in early stages. BRM function is more restricted to terminal differentiation and transcriptional regulation of postmitotic cells.

2.4.3.2 The ISWI family: ACF, CHRAC, NURF

The ISWI (*i*mitation *swi*tch) chromatin remodelling ATPase family is a prominent subgroup of the SNF2 ATPase superfamily and can be found throughout all eukaryotes. It contains several domains and sequence motifs that are conserved among the ISWI subfamily (Eberharter et al, 2004). Because of its sequence homology to the yeast SWI2/SNF2 enzyme, it was first identified in *Drosophila* (Elfring et al, 1998). ISWI is a component of three known chromatin remodelling complexes: NURF (*nu*cleosome *r*emodelling *f*actor), ACF (*ATP*-utilizing *c*hromatin assembly and remodelling *f*actor) and CHRAC (*chr*omatin *a*ccessibility *c*omplex) (Ito et al, 1997; Tsukiyama et al, 1995; Tsukiyama & Wu, 1995; Varga-Weisz et al, 1997). Functionally, two protein-folding units are required for its nucleosome-stimulated ATPase activity (Grune et al, 2003). The structural features of the C-terminal folding are characterized by 12 helices, which fold into three structural domains. These are referred to as the HAND, the SANT and the SLIDE domains. These three domains play an important role for the binding of histone tails via the SANT domain and in ISWI-driven nucleosome remodelling (de la Cruz et al, 2005).

The analysis of Drosophila ISWI and ISWI-containing protein complexes revealed fundamental principles of the mechanisms, which are used by chromatin remodelers to move nucleosomes along the DNA. Längst and Becker and Heayes' laboratory showed that sliding, induced by ISWI, Mi-2 and SWI/SNF remodelers is not due to a twist-diffusion mechanism (Aoyagi & Hayes, 2002; Aoyagi et al, 2003; Guschin et al, 2000a; Langst & Becker, 2001a; Langst & Becker, 2001b; Strohner et al, 2005). Collectively these data hypothesized the "loop recapture" model as the most likely explanation for ATP-dependent chromatin remodelling observed *in vitro* (see Figure 6). For review see (Langst & Becker, 2004).

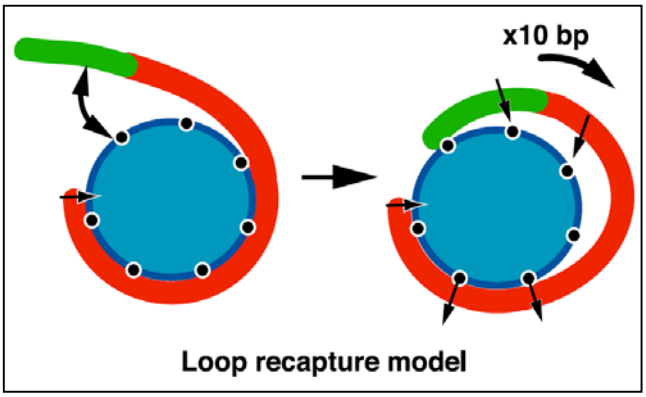

Figure 6: Model for nucleosome mobilization

The blue circle represents a histone octamer around which the DNA (red and green line) is wrapped. Nucleosome mobilization steps: First, 30-35 bp of DNA are dissociated from either edge of the nucleosome. The segment of detached DNA may then either rebind to reform the original nucleosome or interact with a different position on the histone octamer creating a DNA loop or bulge on the nucleosomal surface. Eventually, directional propagation of the DNA loop around the histone octamer will change the translational position of the nucleosome according to the loop size (Langst & Becker, 2004)

According to this model, an ACF heterotetramer binds the nucleosome near the DNA entry sites, then nucleosomal DNA is detached and pushed over the surface of the histone octamer. The DNA bulge traverses over the entire octamer surface. As a consequence, the nucleosome moves along the DNA with a distance, which is defined by the length of the DNA bulge that was generated by ACF (Bouazoune & Brehm, 2006). ACF is a dimer consisting of ISWI and ACF1. The specific function of the complex subunits influence the enzymatic properties of ISWI, which has been proposed by (Eberharter et al, 2001). ACF1 binding stimulates the ATP-dependent nucleosome mobilization in vitro and can change the direction of nucleosome movement along a linear fragment of DNA.

In contrast, NURF is suggested to bind the nucleosome near the DNA entry sites, followed by nucleosome sliding in increments of 10 base pairs (Schwanbeck et al, 2004). NURF is composed of the four subunits ISWI, NURF38, NURF55 and NURF301, whose DNA binding HMG box is crucial for nucleosome sliding by ISWI (Xiao et al, 2001). However, the influence of ACF1 on ISWI activity is not only due to the interaction with the enzyme (Eberharter et al, 2001; Fyodorov & Kadonaga, 2002). Moreover, ACF1 also seems to interact with the globular domains of core histones via its two PHD fingers in the C-terminus, which presents the nucleosome

substrate to the ISWI enzyme (Eberharter et al, 2004). The *in vivo* relevance of ACF1 chromatin remodelling was provided by acf1 mutant flies that demonstrated three chromatin-associated phenotypes: First, chromatin displays a lack of periodicity with a reduction of the average distance between adjacent nucleosomes. Second, heterochromatic genes, and genes regulated by the PcG are de-repressed. Third, progression through S-phase is accelerated. Recent data provide evidence that ACF1 expression is under strict developmental control. ACF1 expression is strongly diminished during embryonic development, but persists at high levels in undifferentiated cells, including the germ cell precursors and larval neuroblasts (Chioda et al, 2010).

The CHRAC (*chr*omatin *a*ccessibility *c*omplex) has been purified by the Becker laboratory from *Drosophila* embryo extracts, and is thought to "crack" open chromatin to increase DNA accessibility (Varga-Weisz et al, 1997). CHRAC shares two subunits with ACF. Further, it contains two CHRAC-specific, small histone-fold proteins called CHRAC14 and CHRAC16. Due to their ability as heterodimers to adopt a characteristic histone fold geometry, CHRAC14/16 have a weak DNA binding activity to stimulate ISWI-mediated nucleosome mobilization (Corona et al, 2000; Hartlepp et al, 2005; Kukimoto et al, 2004). *In vivo* studies demonstrated that ISWI function is necessary for the maintenance of a functional chromosome structure. In flies, the male X chromosome is dosage compensated and marked by chromosome-wide histone H4K16 acetylation (Straub et al, 2005).

2.4.3.3 The CHD family

In 1993, Robert Perry's laboratory isolated a DNA-binding protein that contained both, a chromodomain and an SNF2/SWI2-like helicase domain (Delmas et al, 1993). According to its structure, it was named CHD1 (*chr*omodomain *h*elicase *D*NA-binding protein). The common feature of the CHD protein family is the chromo (*chr*omatin *o*rganization *mo*difier) domain. Related proteins that share this domain in addition to the SNF2-related ATPase domain have been identified in many eukaryotes. Based on protein sequence and phylogenic analysis, Woodage and colleagues defined and divided the CHD family into three subfamilies:

Subfamily I	Subfamily II	Subfamily III
CHD1 and CHD2	CHD3 and CHD4	CHD5, CHD6, CHD7, CHD8, CHD9, Kidmet-L, T04D-14 and KIAA1416.

(Woodage et al, 1997)

CHD proteins are large and range from 233 kDa in the case of CHD5 to 252,5 kDa in the case of CHD7. Beside their two N-terminal chromodomains, and their centrally located SNF2-like helicase domain, they share a C-terminal DNA binding domain.
In addition, the CHD subfamily II is further characterized by two PHD (*p*lant *h*omeo*d*omain) zink finger domains and the lack of a defined DNA-binding domain (Schuster & Stoger, 2002). Most of the subclass III members possess a TCH (*t*erminal *c*onserved *h*airpin) motif and a DNA binding motif (SANT domain). Subclass II and III have little sequence homology with subfamily I. Thus, DNA binding may be mediated through their SANT domain or, in the case of CHD3/4, via recruitment by proteins within the chromatin-remodelling complexes, e.g. CHD4/NuRD (Aasland et al, 1996; Bowen et al, 2004). The following will give a further insight by considering important domains.
The chromodomain is well conserved between species from plants, amphibians to mammals (Brehm et al, 2004). It was originally identified as a 37-amino acid residue region of homology, shared by epigenetic repressors as HP1 (*h*eterochromatin *p*rotein *1*) and Pc (*P*oly*c*omb) in *Drosophila melanogaster* (Paro & Hogness, 1991). Today, the chromodomain is known as a 50-amino acid region, forming a folded three-stranded anti-parallel ß-sheet and α-helices. It has been found in ATP-dependent chromatin remodelling factors, histone acetyltransferases, and histone methyltransferases (Eissenberg, 2001). Deletion of the chromodomains in the *Drosophila melanogaster* chd3/chd4 gene homologue impaired nucleosome binding, mobilization, and ATPase functions (Bouazoune et al, 2002). In general, chromodomain harbouring proteins are seen as regulators of chromatin in different contexts. In contrast to HP1 and Pc, a structural difference that is specific for CHD proteins provides a methyl binding side for H3K4me (Pray-Grant et al, 2005). As an exception, analyses of the related human CHD3 and CHD4 (hCHD3 and hCHD4) protein structures do not predict binding to methylated lysine residues (Sims et al, 2005). This is consistent with the notion, that H3K4me is restricted to active chromatin, but CHD3 and CHD4 function is associated with transcriptional repression. Bouazoune and colleagues have described a new activity for the *Drosophila* chromodomains CHD3 and CHD4 as DNA-binding modules, which are

associated with ATP-dependent nucleosome mobilization, together with dCK2 phosphorylation (Bouazoune & Brehm, 2005; Bouazoune et al, 2002).

A further domain is the Snf2-like helicase-ATPase domain, which resembles the previously described SWI2/SNF2 catalytic subunit of the SWI/SNF complex. Functionally, the ATPase provides the energy for chromatin remodelling, either by histone displacement (Pazin et al, 1997) or nucleosome sliding (Becker & Horz, 2002). The structure of the SWI2/SNF2 ATPase consists of about 400 amino acids, divided into seven conserved helicase motifs. These motifs are divided into a N-terminal subdomain I and a C-terminal subdomain II. The first subdomain comprises the motifs I, Ia, II and III, which are required for ATP-binding and hydrolysis.

The second subdomain consists of the motifs IV-VI and is crucial for energy transduction (Eisen et al, 1995). According to Richmond and Peters, all seven motifs are necessary for the ATPase function (Richmond & Peterson, 1996). The DNA-binding domain is the least conserved and comprises the C-terminal 229 amino acids.

Subfamily I members of CHD proteins contain two motifs within their DNA-binding domain that recognize A and T rich sequences via DNA minor groove interaction. The first description of the binding specificity by (Delmas et al, 1993) was further characterized by (Stokes & Perry, 1995) two years later. Due to their limited homology between the family members, the subfamilies II and III do not show a canonical DNA-binding domain. A Myb-related binding motif present in subfamilies I and II is referred to as telobox, and probably provides a weak binding affinity to DNA for CHD3 and CHD4 (Woodage et al, 1997). CHD3 and CHD4 are part of the NuRD complex and may gain further possibilities for DNA binding through interaction with other subunits within the complex. Further, interaction with nucleosomes could be mediated through the PHD domain (Schuster & Stoger, 2002). The lack of a defined DNA binding domain is the most obvious difference between the subfamilies. CHD3 and CHD4 harbour a N-terminal PHD zincfinger DNA binding domain, resembling the RING structure, which is associated with chromatin. Although, CHD3 and CHD4 protein structures do not predict binding to methylated lysine residues, interactions of the zincfinger with methylated lysine have been described (Shi et al, 2006). CHD4 recognizes the N-terminus of histone H3 and this interaction is facilitated by acetylation or methylation of lysine 9 (H3K9ac and H3K9me, respectively) but is inhibited by methylation of lysine 4 (H3K4me) or acetylation of alanine 1 (H3A1ac). This suggests that the PHD2 finger plays a role in targeting of the CHD4/NuRD complex to chromatin (Musselman et al, 2009).

Further interactions of PHD zincfingers are described with HDAC1, the histone

deacetylase component of the NuRD complex. The SANT domain is associated with DNA and nucleosome binding and is restricted to the subfamily members CHD5, Kismet and potentially KIAA 1416 and To4D.14 (Boyer et al, 2004; Daubresse et al, 1999; Schuster & Stoger, 2002). The next chapter, provides an overview of the functional characteristics of the different CHD subfamilies.

CHD subfamily I:

In Drosophila, CHD1 was first found to localize to interbands and puffs of polytene chromosomes, which supported an active role of this protein in gene regulation (Stokes et al, 1996). Although, no direct interaction with active RNA polymerase II is known, CHD1 co-localizes extensively with its active forms (Srinivasan et al, 2005). Moreover, CHD1 can interact with SSRP1 (structure specific recognition protein 1), a subunit of the transcription elongation factor FACT (facilitates chromatin transcription) (Kelley et al, 1999; Orphanides et al, 1998). Further interaction with the transcriptional co-repressor NCoR was shown by yeast 2-hybrid assays. An interaction with HDAC1 was reported by the same group, which suggests that histone modifications are important for CHD1/2 recruitment to the DNA (Tai et al, 2003). This notion was underline, when it was shown that CHD1 interacts with the SAGA and SLIK histone acetyltranferase (Pray-Grant et al, 2005). An additional link to histone modification is the specific recognition of H3K4me by the chromodomains of CHD1 (Flanagan et al, 2005), which build the link to transcriptional elongation (Eissenberg et al, 2007). This reveals an interesting function of CHD1 in transcription regulation, because it interacts with the Pol II associated factors for transcriptional elongation (Simic et al, 2003). Controversially, this could not be shown for yeast CHD1 (Sims et al, 2005).

CHD subfamily II:

This study is focused on the developmental role of the class II ATPase CHD4 during neurogenesis. CHD4 and CHD3 are the central components of the NuRD (nucleosome remodelling and histone deacetylase) complex (see 2.4.3.5). Interestingly, CHD4 was initially detected in a medical context. Thus, I will portray how CHD4 made its way from a potential marker for dermatomyositis to play a crucial role in epigenetic gene regulation.
Dermatomyositis is defined as an idiopathic inflammatory myopathy with characteristic cutaneous findings. In 1975, Bohan and Peter first suggested a set of

criteria to aid in the diagnosis and classification of dermatomyositis (Bohan & Peter, 1975a; Bohan & Peter, 1975b). Dermatomyositis is a systemic disorder that frequently affects the oesophagus and lungs and, less commonly, the heart. The association between dermatomyositis (and possibly polymyositis) and malignancy has been recognized for a long time with 20-25% of patients eventually developing malignancy. The greatest risk areas for these patients are cancers of the ovary, lung, pancreas, stomach, colon/rectum and lymphomas (Hill et al, 2001). In the late 70s, Reichlin and colleagues and later Ira Targoff were eager to identify and characterize myositis-specific autoimmune-antibodies (Reichlin & Mattioli, 1976; Targoff & Reichlin, 1985). A positive reference serum was provided by the dermatomyositis patient called Mitchell (Mi-). An immunodiffusion assay resulted in the identification of the two antibodies anti-Mi-1 and anti-Mi-2. However, anti-Mi-1 was not active in complement fixation, suggesting that this antibody was not involved in the inflammatory process of dermatomyositis. Consequently, the focus was targeted towards the identification of anti-Mi-2 and revealed that the Mi-2 antigen was contained in a protein complex with a central polypeptide of about 240kD (Nilasena et al, 1995). The following investigation led to its cloning by Seelig and colleagues and identified Mi-2 as CHD4, a member of the SNF2 ATPase family (Seelig et al, 1995; Seelig et al, 1996).

CHD4 and CHD3 interact with components of the NuRD complex as HDAC1/2 (see chapter on histone deacetylation). The functional descriptions of CHD4 together with other subunits within the NuRD complex will be described in chapter 2.4.3.5 to introduce the objectives of my analysis of CHD4 function during neural development.

CHD subfamily III:

CHD5 plays a role in neural development and is manly expressed in the fetal brain, the adult brain and the adrenal glands (Thompson et al, 2003). CHD5 is also seen as a tumour suppressor that acts via the p53 pathway (Bagchi et al, 2007). Mutations of CHD5 affects recruitment of Pol II to promoters and affects the elongation process (Srinivasan et al, 2005).

CHD6 has also been linked to sites of Pol II, however independent of its phosphorylation state (Lutz et al, 2006). The expression pattern of CHD7 in mice is similar to those in human foetuses, including neural crest cells, cranial nerves, auditory and nasal tissues and neural retina (Sanlaville et al, 2006). The knowledge about CHD7 comes from mutation analysis. CHD7 mutation can lead to the CHARGE syndrome, which comprises coloboma of the eye, heart defect, choanae

atresia, retardation of growth, ear and genital abnormalities as well as palate and lip clefts. CHD8 regulates transcription by maintaining the boundaries between eu-and heterochromatin via the insulator binding protein CTCF. During early stages of development, CHD8 interacts with Duplin, a protein involved in ß-catenin and Wnt signalling maintenance (Nishiyama et al, 2004). CHD9 is functionally associated with skeletal muscle specific promoters like CBAF1 (Shur et al, 2006).
All members throughout the subfamilies provide an obvious common structure and are connected to chromatin. Most of them seem to act as co-activator or repressor, depending on their binding partner. A second crucial feature is their tissue specificity during development. Importantly, the repression or activation equilibrium works in an interesting way. CHD1 overexpression and knockout was shown to result in nearly identical phenotypes, which indicates a regulating feedback loop to keep CHD1 levels balanced. The CHD4 dependent chromatin condensation in *Drosophila* seems to work in a similar way. The next chapters will provide information about CHD4 within the chromatin-remodelling complex NuRD and its subunits.

2.4.3.4 CHD4/Mi-2ß chromatin remodelling complexes

Two years after the identification of CHD4/Mi-2ß as member of the SNF2 ATPase family by Seelig and colleagues, the purification of the NuRD complex was shown by several different groups (Tong et al, 1998; Wade et al, 1998; Xue et al, 1998; Zhang et al, 1998). Despite differences in the precise subunit composition of these complexes, all contain a Mi-2 ATPase of the CHD4-type, a histone deacetylase core, which is made of an Rpd3-like protein (Rpd3 in *Xenopus*, HDAC 1 and HDAC2 in humans) and the Rb-associated proteins p46/48. In addition, MTA and MBD protein family members were later identified as genuine components of these complexes (Fujita et al, 2003; Wade et al, 1999a; Zhang et al, 1999).
The slight differences in the precise subunit composition of CHD4/Mi-2 containing chromatin remodelling complexes ad unique biochemical properties. One complex is composed of the NuRD complex subunits in association with MBD2 (*m*ethyl-CpG-*b*inding *d*omain protein *2*) and the p66/68 proteins (Feng et al, 2002; Feng & Zhang, 2001). The composition of this complex was first identified in Adrian Bird's laboratory in the search for methyl-DNA binding activities. The first *me*thyl-*C*pG binding *p*rotein that was found was termed MeCP1 (Meehan et al, 1989). In addition to the properties of the NuRD complex, this renders the properties of MeCP1 to preferentially bind, remodel, and deacetylate nucleosomes, which contain methylated

DNA.

Most recently, a novel dMi-2 complex called dMec (dMEP-1 complex) has been purified from *Drosophila* that is distinct from NuRD. dMec is composed of dMi-2 and dMEP-1. It is far more abundant than dNuRD and constitutes the major dMi-2-containing complex. It is expressed in embryos, larval tissues and adult flies. In the context of neural development, dNuRD and dMec associate with and regulate proneural genes of the achaete-scute complex (ASH, see results). Although it is lacking a histone deacetylase subunit, only dMec contributes to the repression of proneural genes, which revealed an unexpected complexity in the composition and function of CHD4/Mi-2 complexes (Kunert & Brehm, 2009; Kunert et al, 2009).

2.4.3.5 Subunit composition and function of the NuRD complex

Despite differences in the subunit composition of CHD4/Mi-2ß containing complexes, the NuRD complexes of different species all contain a CHD4/Mi-2ß ATPase with a histone deacetylase core, which is made of HDAC1, HDAC2 and the histone binding proteins RbAp46 and RbAp48 (*R*etino*bl*astoma-*A*ssociated p46 and p48). Beside the deacetylase core, MTA, p66/68 and MBD protein family members were later characterized as unique components of CHD4/Mi-2ß-containing complexes (Brackertz et al, 2002; Brackertz et al, 2006; Feng et al, 2002; Feng & Zhang, 2001; Fujita et al, 2004; Fujita et al, 2003; Wade et al, 1999b).

The components RbAp46 and RbA p48, now termed Rbbp7 (RbAp46) and Rbbp4 (RbAp48) have been identified based on their interaction with the tumour suppressor Rb (*R*etino*b*lastoma), which belong to the highly conserved superfamily of tryptophan-aspartate repeat (WD-repeat) proteins (Qian et al, 1993). Both bind directly to the first helix of histone H4 and form a heteromer with associated HDACs. The HDAC-RbAps heteromer is incorporated in several histone deacetylase complexes. Since this association is conserved, it is often referred to as the „HDAC core" complex.

A further member of the NuRD complex is the subunit MTA1 (*m*e*t*astasis-*a*ssociated gene *1*). MTA1 was first identified in a screen for genes that were abundantly overexpressed in highly metastatic rat mammary adenocarcinoma cell lines (Toh et al, 1994). The MTA proteins represent a protein family, which is encoded by three distinct genes (MTA1-3) and six splicing-isoforms (MTA1, MTA1s, MTA1-ZG29p, MTA2, MTA3, and MTA3L). The family of MTA proteins share several common domain structures as the BAH (*b*romo-*a*djacent *h*omology) domain, the SANT, the

ELM (*eg*l-27 and *M*TA1 homology) domain as well as the highly conserved GATA-type zincfinger motif. MTA1 is characterized by two src-homology (SH)-binding motifs at its C-terminal region, which are also found in MTA2 and MTA3. These common domain structures indicate that the MTA family is involved in protein-protein and DNA binding interactions, which point to functions in signal transduction and transcriptional regulation (Fujita et al, 2003; Simpson et al, 2001). Xue et al. first associated the MTA1 proteins with the NuRD complex and described their strong transcription repressing activity (Xue et al, 1998). Subsequently, Zhang et al. reported that a protein similar to MTA1, named MTA2, was also a component of the NuRD complex and that MTA2 is highly expressed in rapidly dividing cells (Zhang et al, 1999). Later, MTA3 was identified as an oestrogen-inducible gene product that forms a distinct NuRD complex and is associated with an invasive growth pathway in breast cancer (Fujita et al, 2003). In summary, the fundamental functions of the MTA family members seem to be exerted through chromatin remodelling and histone deacetylation by the CHD4/NuRD complex.

In a medical context, MTA proteins are functionally associated with regulation of cell growth and metastasis. Using surgically resected human tissues, high levels of MTA1 mRNA expression were clinico-pathologically correlated to the invasiveness and growth properties of gastrointestinal cancers, including oesophageal, gastric and colorectal cancers (Toh et al, 1999; Toh et al, 1997). MTA1 overexpression in carcinogenesis and cancer progression was also shown for gastrointestinal tumours, such as pancreatic cancers and hepatocellular carcinomas (Hamatsu et al, 2003; Iguchi et al, 2000). High expression of MTA1 mRNA was further correlated clinico-pathologically with lymph node metastasis in human non-small cell lung cancers, ovarian cancers, and to the advanced stage and invasiveness of thymomas. (Sasaki et al, 2002; Sasaki et al, 2001; Yi et al, 2003) In addition, MTA1 was positively correlated to lymph node metastases of head and neck squamous cell carcinomas and oral squamous cell carcinoma (Kawasaki et al, 2008; Roepman et al, 2006).

Interestingly, MTA1 converts breast cancer cells to a more aggressive phenotype by repression of the oestrogen receptor (ER) through deacetylation of the chromatin in the ER-responsive element of ER-responsive genes such as pS2 and c-myc (Mazumdar et al, 2001). MTA2 physically interacts with ER and represses its transactivating function (Cui et al, 2006). MTA3 is the latest addition to the MTA family. It was identified as an oestrogen-dependent component of the CHD4/NuRD transcriptional co-repressor in breast epithelial cells (Fujita et al, 2003). The oestrogen dependent induction of MTA leads to the repression of the transcriptional repressor Snail, which is a regulating factor for EMT (*e*pithelial to *m*esenchymal

*t*ransitions) during germ layer formation. The resulting expression of the cell adhesion molecule E-cadherin is crucial in the maintenance of a differentiated, normal epithelial phenotype. Thus, MTAs execute important roles of the CHD4/NuRD complex during development, but also in cancer formation. This is underlined by the finding that protein targets for deacetylation by MTA-containing NuRD complexes also include non-histone proteins like the well-known tumour suppressor p53, which mediates inhibition of growth arrest and apoptosis (Luo et al, 2000; Moon et al, 2007). A further non-histone protein that is positively regulated by MTA proteins is HIF-1α (*h*ypoxia *i*nducible *f*actor-*1*α), which results in the expression of VEGF (*v*ascular *e*ndothelial *g*rowth *f*actor) (Yoo et al, 2006). Most recently, the NuRD complex was shown to be associated with LSD1 (Wang et al, 2009). (See chapter 2.4.2.2 about methylation and demethylation.)

DNA methylation is an essential modification for proper development and survival (Okano et al, 1999). Most of these modifications occur at CpG-dinucleotide sites. During the last decade, data suggest that the functional properties of methylated DNA are primarily due to the action of a conserved family of proteins that selectively bind methylated CpG-dinucleotides (Bird & Wolffe, 1999). The first methyl CpG binding protein that was described is MeCP2, a polypeptide capable of binding selectively to a single symmetrically methylated CpG (Lewis et al, 1992). MeCP2 consists of two functional domains. The MBD (*m*ethyl CpG *b*inding *d*omain) is both necessary and sufficient for methylated DNA binding (Nan et al, 1993). The second functional domain is referred to as TRD (*t*ranscriptional *r*epression *d*omain), which is necessary for transcriptional repression *in vitro* and *in vivo* (Jones et al, 1998; Kaludov & Wolffe, 2000; Nan et al, 1997). Hendrich and Bird characterized the family of mammalian proteins containing the MBD domain to its current size (Hendrich & Bird, 1998). Identification and characterization of MBD1 and MBD4 were discovered as EST clones with sequence similarity to the MBD motif of MeCP2. During the late 1990s, the notion of a biochemical link between the MBD family and enzymes that modify chromatin components had been established. First, MeCP2 was linked by biochemical and molecular analysis to histone deacetylase enzymes (Jones et al, 1998; Nan et al, 1998), then MBD2 and MBD3 have functionally been associated with the CHD4/NuRD complex (Wade et al, 1999a; Zhang et al, 1999). Later, MBD1 was shown to actively repress methylated genes (Ng et al, 2000).

Today, the function of MBD family members are understood to specifically bind to methylated DNA via the MBD motif, then recruit enzymatic activities such as HDACs and establish and/or maintain a locally repressive chromatin environment (Bird &

Wolffe, 1999). Although, the transcriptional repression of MBD1 is sensitive to HDAC inhibitors, it is not understood as a member of the previously defined NuRD complex. In contrast, MBD2 and MBD3 have been identified as core subunits of the CHD4/NuRD complex (Feng & Zhang, 2001; Ng et al, 1999). MBD2 is highly similar to MBD3 in a large region corresponding roughly to amino acids 140-400 (Hendrich & Bird, 1998). Compared to MBD3, which mRNA is abundant in most somatic tissues and in ES cells, MBD2 has a more restricted expression pattern (Hendrich & Bird, 1998).

MBD3 is the smallest member of the MBD family, coding for a protein of about 30 kDa. It presents a relatively rich variety of splice variants (MBD3 Long Form, MBD3D). The methyl binding properties of MBD3 appear to vary with species. MBD2 can selectively recognize methylated DNA (Hendrich & Bird, 1998). MBD3 has apparently lost this function during vertebrate evolution (Hendrich & Tweedie, 2003) due to crucial change in the amino acid sequence in the MBD fold (Saito & Ishikawa, 2002). Like all other known subunits of the complex, MBD is an interchangeable component, varying between the family members MBD2 and MBD3, so that MBD2/NuRD and MBD3/NuRD are seen as two distinct complexes with different biochemical and functional properties (Le Guezennec et al, 2006). In contrast to other species, the *Xenopus* MBD3 is an exception and binds methylated DNA with a quite similar affinity to the isolated MBD domain from MeCP2 (Wade et al, 1999a). This suggests that recruitment of the CHD4/NuRD complex to methylated loci defines a crucial regulatory pathway during the development of *Xenopus*.

A further interaction between MBD2 and another component of the CHD4/NuRD complex, known as p66, has been documented (Brackertz et al, 2002; Brackertz et al, 2006). The subunits, p66a or p66b, also known as Gatad2a (p66) and Gatad2b (p68), are incorporated into the NuRD complex and form additional NuRD versions (Brackertz et al, 2002; Feng et al, 2002; Wade et al, 1999a).

The CHD4/Mi-2ß subunit is the largest and the functional key component of the NuRD complex. Its first description was introduced in 2.4.3.3. It is physically associates with histone deacetylases. Two different genes have been annotated in the genome, encoding two CHD/Mi-2 proteins: Mi-2α, encoded by the *chd3* gene and Mi-2ß, encoded by the *chd4* gene. The latter is predominately associated with the NuRD complex (Seelig et al, 1996). CHD4/Mi-2ß protein belongs to the CHD (*c*hromo-*h*elicase-ATP-*D*NA binding) protein family, which are structurally characterized by two PHD (*p*lant *h*omeo *d*omain)-zinc finger domains and two chromodomains, which have DNA-binding ability (Bouazoune et al, 2002). The

SWI2/SNF2-type ATPase/helicase domain is responsible for the nucleosome remodelling activity (Bouazoune et al, 2002).

The biochemical properties of the CHD4 ATPase reveal that it facilitates nucleosome mobility through a nucleosome sliding mechanism (Brehm et al, 2000; Guschin et al, 2000b). This activity can strongly be activated by nucleosomes, however DNA alone only weakly stimulates ATP-hydrolysis and core histones alone fail to induce an enzymatic ATPase activity. A further question is if ATP-dependent remodelling and nucleosome deacetylation reactions are coupled and if ATP enhances nucleosome deacetylation. Tong et al. described a stimulation of histone deacetylation in the presence of ATP (Tong et al, 1998). Guschin and colleagues could only detect an ATP-dependent increase of deacetylation using mononucleosomes (Guschin et al, 2000b). *In vitro*, all histone lysines can be deacetylated by NuRD with the exception of H4K16, which becomes resistant to deacetylation if histones are structured into nucleosomes. However, like most SNF2 ATPases, CHD4/Mi-2ß can disrupt histone-DNA contacts and catalyze nucleosome mobilization in an ATP-dependent manner (Brehm et al, 2000; Guschin et al, 2000b) Längst and Becker proposed the “loop recapture” model as the most likely explanation for ATP-dependent chromatin remodelling observed *in vitro* as demonstrated in Figure 6.

The CHD4/NuRD complex is able to catalyze the conversion of an active, hyper-acetylated promoter region to that of an inactive gene. The deacetylation of histones is catalysed by the activity of HDAC1/2. Consequently, the NuRD complex physically combines the two basic mechanisms for chromatin remodelling: covalent histone modification (deacetylation) and ATP-dependent chromatin remodelling.

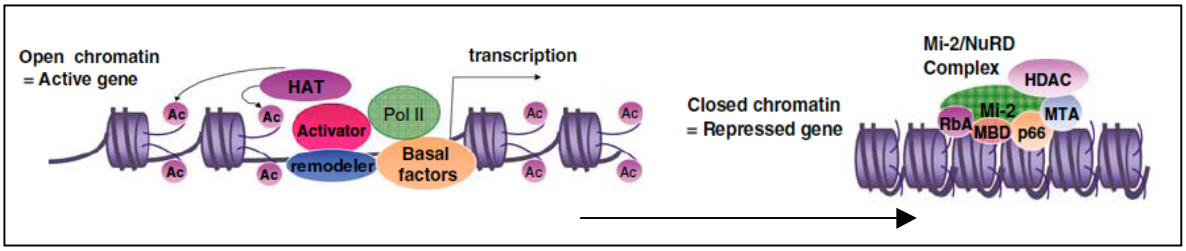

Figure 7: The CHD4/NuRD chromatin remodelling complex

The combined activity of two enzymatic moieties, i.e. covalent histone modification (deacetylation) and ATP-dependent chromatin remodelling, results in densely packed, hypo-acetylated nucleosomes. This leads to a transcriptionally repressed chromatin state. Adapted from (Denslow & Wade, 2007)

The result of the combined activity of these two enzymatic moieties leads to densely packed, hypo-acetylated nucleosomes. For illustration, see Figure 7.

Functionally, CHD/Mi-2 proteins mainly lead to transcriptional repression (Denslow & Wade, 2007). The repressive function of NuRD has been deduced from physical interaction studies in murine lymphocytes. In this context, CHD4/Mi-2ß interacts with Ikaros and Aiolos, two zincfinger DNA-binding factors and strong repressors, which are required for lymphoid cell development (Kim et al, 1999).

NuRD interacts with many different transcriptional repressors, suggesting that those different repressors are used in different cell types. In association with the NuRD complex, CHD4 can bind TRIM28 and BRG1 through its N- and C-terminal regions (Shimono et al, 2003). Further interaction partners are the ATR (*a*taxia *t*eleangiectasia and *R*ad3-related protein) and TRIM27 (Bowen et al, 2004). In the context of cell growth and gene regulation during development the Drosophila CHD4 (dMi-2) protein interacts with the transcription factors hunchback, the Polycomb group (PcG) (Kehle et al, 1998), Tram-track69 (Murawsky et al, 2001) and the DRE-binding factor (dDREF) (Hirose et al, 2002). Figure 8 summarizes different interaction partners, which work in concert with NuRD to establish a repressive function.

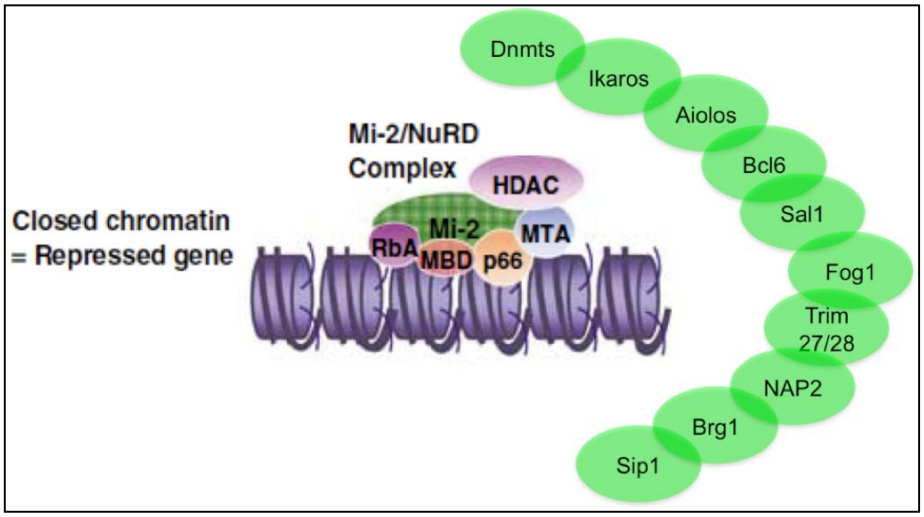

Figure 8: The NuRD complex and interacting transcriptional repressors

Schematic illustration of the NuRD multiprotein complex with its individual components and associated transcriptional repressors, indicated in green. The specificity and functionality of the complex are further increased by incorporating adaptor proteins that can recruit additional repressors and chromatin-modifying complexes. For further reading please see (McDonel et al, 2009).

CHD4 also interacts with the co-repressor NAP2, which to regulate the expression of EGF-family (*e*pidermal *g*rowth *f*actor) members (Srinivasan et al, 2006) and binds to and represses RORgamma-mediated transcriptional activation, which is necessary for thymocyte development and lymph node organogenesis (Johnson et al, 2004a).
Although, these interactions suggest a primary role in transcription repression, the N-terminus of CHD4/Mi-2ß also showed transcriptional activating ability by interacting with BRG1 (Shimono et al, 2003; Williams et al, 2004). In addition, CHD4/Mi-2ß has been associated with the active form of Pol II in euchromatic regions (Metivier et al, 2006; Srinivasan et al, 2005). On the one hand this may be a hint of NuRD-independent functions of CHD4, on the other, this may implicate that deacetylase function could result in transcriptional activation. This subject is under current discussion (Smith et al, 2008).
The transcriptional repression by the CHD4/NuRD complex has been connected to developmental functions in numerous model systems. In *Drosophila melanogaster*, CHD4/Mi-2ß is essential for embryogenesis as mutants arrest as first or second instar larvae. Furthermore, CHD4/Mi-2ß is connected with Polycomb group proteins, in hunchback associated HOX gene repression during embryo patterning and is required for germ cell development (Kehle et al, 1998). The protein *T*ram-*t*rack*69*, which represses neuronal specific genes, has been shown to interact genetically and physically with CHD4/Mi-2ß.
In *Caenorhabditis elegans*, the Mi-2 homologue *let-418* revealed to be required for proper vulval development (Solari & Ahringer, 2000) and is needed to maintain somatic differentiation, because let-418 deficient animals show inappropriate expression of germline specific genes in somatic cells (Unhavaithaya et al, 2002). In addition, let-418 antagonizes the cancer-related Ras signalling pathway in *C. elegans*. Recent studies provided evidence for a crucial role of CHD4/Mi-2ß in somatic stem cells. In mouse, embryonic stem (ES) cells, deficient in MBD3, exhibit self-renewal and showed a restricted potential to differentiate (Kaji et al, 2006). As such, CHD4/NuRD may play a role in ES cell pluripotency and represent a potential link between maintaining the undifferentiated state and the capacity to differentiate. The deletion of a *chd4* allele in the haematopoietic system unravelled an important role of CHD4/Mi-2ß in homeostasis and lineage choice of haematopoietic stem cells *in vivo* (Yoshida et al, 2008) as well as for CD4 expression and T cell development (Williams et al, 2004). The Georgopoulos lab discovered, that CHD4/Mi-2ß function was required for establishment of the basal epidermis and normal differentiation of its progeny by converting ectodermal progenitor cells to matrix stem cells (Kashiwagi et al, 2007). This requirement for a NuRD component during cellular transition stages

during development observed in the epidermis parallels the role of MBD3 in developmental transitions during the development of pluripotent cells *in vivo*.

Consequently, NuRD is particularly important for developmental transitions. Work with mice and cell lines mutant for NuRD components has indicated that this co-repressor complex plays an important role in maintaining stem cell homeostasis and lineage choice in both the haematopoietic system, in skin, and during maturation and lineage commitment of pluripotent cells in early embryos (McDonel et al, 2009).

These studies highlight the importance of CHD4/Mi-2 and NuRD in development and cell growth of various model organisms. Defining the target and genes, which are affected by CHD4/NuRD mediated chromatin remodelling, protein deacetylation and the proteins that target these actions, will allow us to further elucidate the molecular pathways, which control cellular lineage commitment and proliferation during development, stem cell programming and will inevitably lead to progress in cancer research.

Considering neural development, work in *Drosophila* identified a specific role for dCHD4/dMi-2 in the development of sensory bristles, which implicates a regulatory role in pro-neural gene expression (Yamasaki & Nishida, 2006). Additional data described a role of CHD4/Mi-2ß for terminal differentiation and nerve myelination. The repression of the endogenous Rad gene, which is normally repressed in Schwann cells during peripheral nerve myelination by NAB2, involves interaction with CHD4/Mi-2ß and demonstrate co-localization of NAB2 and CHD4/Mi-2ß on the Rad promoter in myelinating Schwann cells (Srinivasan et al, 2006).

Table 1 summarizes the developmental role of the CHD family of ATP-dependent chromatin remodelers in neural tissue across species.

Table 1: Members of the CHD family and their function in neural development

Proteins	*Expression pattern*	*Function*	*Reference*
cLET-418	Not done	Development of the first instar larva	(von Zelewsky et al, 2000)
CHD-3/dMi-2	Not done	Development of the first or second instar larva	(Kehle et al, 1998), (Yamasaki & Nishida, 2006)
dp66	Not done	For normal metamorphosis	(Kon et al, 2005)
xCHd2	Eyes, neural tube, branchial arches	Not done	
mCHD2	Not done	Survival for perinatal stage	(Marfella et al, 2006)
xCHD4	Eyes, neural tube, branchial arches, otic vesicle, somites	Establishing the boundary formation between mesoderm/neuroectoderm	(Linder et al, 2004)
mCHD4/Mi-2ß	Not done	Required for early stage thymocyte differentiation	(Williams et al, 2004), (Srinivasan et al, 2006)
xCHD5	Fetal and adult brain, otic vesicle	Possible role in development of neural tissue	(Linder et al, 2004)
mCHD5	Fetal and adult brain, adrenal glands	Possible role in development of neural tissue	(Thompson et al, 2003)
mCHD7	Precursors of eye, ear, kidney, vascular system, olfactory epithelium	Normal closure of inner ear, optic fissure, genitourinary tract and inner ear morphogenesis	(Sanlaville et al, 2006), (Aramaki et al, 2007)

Our laboratory demonstrated the dynamic mRNA expression patterns of the *Xenopus* orthologs of the mammalian SNF2-like ATPases during early embryogenesis. The stage and context specific expression levels suggest that quantitative and/or qualitative differences in nucleosome remodelling activities between cells could be of regulatory importance for embryonic gene regulation (Linder et al, 2004).

Based on this notion, we provided evidence that the neuroectoderm/mesoderm boundary is controlled by the chromatin remodelling ATPase CHD4/Mi-2β. Gain and loss of CHD4 function experiments shifted this boundary along the animal-vegetal axis at gastrulation. We could demonstrate that CHD4 together with the transcription factor Sip1, determines the “ON” threshold for Nodal-dependent, but not for FGF-dependent induction of *xbra* transcription (Linder et al, 2007). The CHD4/Sip1 epistasis thus constitutes a regulatory module, which balances the boundary between the mesoderm and neuroectoderm germ layer formation.

2.5 Objectives

Epigenetic regulation by histone modifications and specific local chromatin structures is known as a tightly regulated mechanism of gene expression during stem cell differentiation and embryonic development. The process of neural induction is complex and influenced by specific signalling events (BMP, FGF, Wnt). The factors responsible for the translation of this signalling information onto the chromatin level are not determined yet. The objective of this work is to investigate the role of the catalytic subunit CHD4/Mi-2ß of the NuRD complex during *Xenopus* neurogenesis, i.e. specifically, how neural induction and neural cell differentiation of prospective neural tissue is affected by CHD4/Mi-2ß misexpression. This will be investigated at different stages during neural development, which represent different chromatin contexts. Based on our latest results, describing the direct interaction of CHD4/Mi-2ß and Sip1 as a crucial factor in the neuroectoderm/mesoderm boundary formation, our further interest is focused on the influence of the CHD4/Sip1 regulatory module on neural development.

To approach this issue, I applied two different strategies for CHD4 gain- and loss-of function studies. First, for interference with early neural development, at a permissive chromatin stage, wild type (wt) and dominant negative (dn) CHD4 and Sip1 mRNAs were injected into two-cell stage *Xenopus* embryos. Second, for further spatial and temporal specification of CHD4 misexpression within the chromatin context of neural

differentiation, I established a procedure, which enables the expression of wtCHD4 and dnCHD4 at later stages of neural development by the means of an inducible heat-shock promoter driven plasmid construct. This method allows activating CHD4 misexpression after gastrulation to distinguish between CHD4 and Sip1 functions before and after the formation of the neural plate, i.e. functions on neuroblast determination versus neural differentiation in different chromatin contexts. The resulting phenotypes were analysed by whole mount in situ hybridization with neural marker genes to spatially characterise prospective neural tissue according to its state of differentiation. The influence on apoptosis and on the proliferation rate of differentiating neural cells was analysed by immunofluorescence against activated Caspase3 and phosphorylated serine 10 of histone 3, respectively. The fluorescence intensity was quantified by confocal microscopy.

3 Methods and materials

3.1 Reagents

3.1.1 Chemicals

The following chemicals have been used from the following companies: Fluka, Merck, Sigma, USB. Companies listed on top of the table.

Table 2:

Difco	Gibco/BRL	Sigma
Agar, Ampicillin, Yeast extract	Agarose, lamb serum	Human choriongonadotrophin

3.1.2 Enzymes and proteins

The following enzymes were ordered at the companies on the top row of the table:

Table 3:

Roche	Stratagene	New England Bio Labs	Fermentas	Sigma	Promega	Biorad
Alkaline phosphatase, Restriction endonuclease	DNase I	Restriction endonuclease	Restriction endonuclease	RnaseA Proteinase K, protein molecular weight standard	T3, T7 and SP6 RNA polymerase, Rnase free Dnase I	Precision Plus Protein Prestained Standard

3.2 Laboratory equipment

The following laboratory equipments were used. The companies are put in brackets.

Centrifuges: Eppendorf centrifuge 5417C (Eppendorf); Omnifuge 2.0 RS (Haereus); Sorvall RC-5B (Du Pont), Micro 22R (Hettich Zentrifugen), Optima LE-80K Ultracentrifuge (Beckman Coultier), PicoFuge (Stratagene)

Developer: Curix-60 (Agfa)

Microinjection experiments:

Glass injection needles: Glass 1BBL W/FIL 1.0 mm (World Precision Instrument).

Injector: Pli-100 (Digitimer Ltd.).

Incubator: Driblock DB1 and DB20 (Teche).

Microneedle Puller P-87 (Sutter Instrument).

Micromanipulator: Mm-33 (Science Products).

Microscopes: Stereomicroscopes Stemi SV6, Stemi SV11 (Zeiss), MZFCIII (Leica), Axiophot (Zeiss), Axiovert 200M (Zeiss)

Microsurgery: Gastromaster (Xenotek Engineering).

Nylon membrane: Hybond™ N (Amersham).

Data illustration:

CCD camera: ProGres C14 (Zeiss)

Software: Photoshop CS2 (Adobe); Illustrator CS2 (Adobe); MacVector 7.1 (Oxford Molecular Group); Office 2008 for Mac (Microsoft), Endnote X.1 (Thomson); ImageJ

Statistical analysis:

R version 2.8.1 (2008-12-22) Copyright I 2008 The R Foundation for Statistical Computing, Exel Microsoft

Nucleic acid quantification:

Spectrophotometer: GeneQuant II (Pharmacia Biotech), Nanodrop ND-1000 (PeqLab)

3.3 Nucleic acids

3.3.1 Standards

1kb ladder: GeneRuler™ 1kb DNA ladder (Fermentas), with the following 14 discrete fragments: 10000, 8000, 6000, 5000, 4000, 3500, 3000, 2500, 2000, 1500, 1000, 750, 500, 250 base pairs.

100bp ladder: GeneRuler™ 100bp DNA ladder plus (Fermentas). The DNA ladder yields the following 14 discrete fragments: 3000, 2000, 1500, 1200, 1031, 900, 800, 700, 600, 500, 400, 300, 200, 100 base pairs.

3.3.2 Plasmids

3.3.2.1 Cloning Vectors

pBS2 (Stratagene)
pcS2MT (Rupp et al, 1994)
pCS2+MT(Rupp et al, 1994)
pCS2+

3.3.2.2 Plasmids and *in vitro* transcription

The ORF of the *Xenopus* CHD4 was generated by PCR from an EST (BF047668; RZPD „Deutsches Ressourcenzentrum für Genomforschung GmbH“) and cloned into pCS2+ via BamHI/XhoI sites. *In vitro* transcription was performed by linearization with NotI and transcribed with Sp6 RNA polymerase.

Detailed information:

dbEST Id:	8588902	Clone information:	
EST name:	daf67d05.y1	Clone Id:	IMAGE:4743585 (5')
GenBank Acc:	BG814448	Source:	IMAGE
GenBank gi:	14185428	DNA type:	cDNA

Comments:

cDNA Library Preparation: Life Technologies, Inc.
cDNA Library Arrayed by: The I.M.A.G.E. Consortium (LLNL)
DNA Sequencing by: Washington University Genome Sequencing Center
Clone distribution: Xenopus clones from this library are available through the I.M.A.G.E. Consortium/LLNL at: info@image.llnl.gov

Library:

Lib Name:	LIBEST_008911 NICHD_XGC_Eye1
Organism:	Xenopus laevis
Organ:	eye
Develop. stage:	adult
Lab host:	DH10B (phage-resistant)
Vector:	pCMV-SPORT6, restriction site 1: NotI, restriction site 2: SalI

Description:

Cloned unidirectionally. Primer: Oligo dT. Average insert size 2.3 kb. Constructed by Life Technologies. Note: This is a Xenopus Gene Collection (XGC) library.

Submitter:

Name: Sandy Clifton, Ph.D.
Laboratory: WashU Xenopus EST project, 1999
Institution: Washington University School of Medicine
Address: 4444 Forest Park Parkway, Box 8501, St. Louis, MO 63108, USA

dnxCHD4 cloned into pCS2+ with via BamHI/XhoI sites,
In vitro transcription was performed by linearization with NotI and transcribed with Sp6 RNA polymerase. The dominant-negative variant of CHD4 was generated by point-mutating the lysine residue at postion 748 to arginin with a site mutagenesis kit (Stratagene) according to the manufacturer's instructions Analogous mutation in the SNF2 ATPase was shown not to disturb the integrity of the yeast SWI/SNF chromatin remodelling complex, but inhibits its enzymatic ATP-dependent remodelling function (Richmond & Peterson, 1996).

For the production of rat monoclonal antibodies against the c-terminal domain of xCHD4 (amino acid 1513-1891), the corresponding fragment was cloned into the pGEX-4T3 bacterial expression vector (Amersham), expressed in Escherica coli and purified.

xSip1 cloned into pCS2+ (Eisaki et al 2000)
In vitro transcription was performed by linearization with NotI and transcribed with Sp6 RNA polymerase

GFP cloned into pCS2+ with via BamHI/XhoI sites,
In vitro transcription was performed by linearization with NotI and transcribed with Sp6 RNA polymerase (Steinbach et al, 1997)

3.3.2.3 Plasmids for digoxigenin-labeled RNA in situ hybridization probes

- Plasmid # 315: xNSCL cloned into pBS II. Skt, Plasmid, a kind gift from Dr. Jaqueline E. Lee, Pediatric Oncology, Universty of Colorado, Denver
- anti-sense linearized with Not I,
- transcribed with T7 polymerase in Buffer Orange

Plasmid # 112: xAsh3a cloned into CS2-MT, (Turner & Weintraub, 1994)
- anti-sense linearised with HinD3,
- transcribed with T7 polymerase

Plasmid # 111: xAsh3b cloned into CS2-MT, (Turner & Weintraub, 1994)
- anti-sense linearised with HinD3,
- transcribed with T7 polymerase

Plasmid # 324: xNeuroD cloned into pCS2+MTx12A, provided by Jacquline E. Lee, Ph.D.
- anti-sense linearised with Xho I,
- transcribed with T3 polymerase
- GenBank accession number neuroD U28067

Plasmid # 697: Sox2 cloned into pCS2, a kind gift from Dr. Yoshiki Sasai (Mizuseki et al, 1998)
- anti-sense linearised with HinD3,
- transcribed with T7 polymerase in Buffer Red

Plasmid # 121: NCAM cloned into pCS2, (Tonissen & Krieg, 1993)
- anti-sense linearised with Asp 718,
- transcribed with T7 polymerase in Buffer B
- GenBank accession number M76710, ordered from MWG-Biotech

Plasmid # 121: Pax6 cloned into pGEM, a kind gift from Dr. Thomas Hollemann, Institut für Biochemie und Molekulare Zellbiologie, Universität Göttingen
- anti-sense linearised with Not I,
- transcribed with T7 polymerase in Buffer B

3.3.2.4 CHD4 and Sip1 Morpholinos

Sip1 mRNA translation was inhibited by Morpholinos described by (Nitta et al, 2007).
Sip1-Mo: CTTGCTTCATTGATAAGAGTGGGAT, purchased from Gene Tools, LLC, One Summerton Way, Philomath, OR 97370 USA.

CHD4 mRNA translation was inhibited by 25-mer anti-sense Morpholino oligo-nucleotide complementary to the *Xenopus* CHD4 translation start side:
xCHD4-Mo: 5'-CCATGCCCAGGAAGGAGCAAAATGG-3', purchased from Gene Tools, LLC, One Summerton Way, Philomath, OR 97370 USA.

3.3.3 Transformation with E. coli strains

The handling, transformation and preparations of competent cells have been performed as standard methods. For each transformation 1 vial of competent cells (e.g.xL1blue), stored at -80°C, has been thawed one ice. Then, 1 µl plasmid-DNA (1:100 diluted) or 10 µl ligation product was given in 1,5 ml vial, put on ice and mixed with the cell suspension, followed by a 30 minute incubation step on ice. Then, plasmids and cell suspension was heat-shocked in a thermoblock at 42°C for 45 sec at 42°C, and put on ice afterwards immediately, followed by a 3 minute incubation time on ice with 1 ml LB-medium without Ampicillin. After that, the cell and plasmid suspension was incubated for 1 hour at 37°C with shacking. After incubation for 1 hour, the suspension was centrifuged for 2 minutes at 3000 rpm. The supernatant was discarded and the cells were resuspended in 50-100 µl and streaked on a LB-Ampicillin plate, followed by incubation over night at 37°C.
After incubation of the transformed competent cells on a LB-Ampicillin plate over night, a single colony of cells was picked and incubated in 5 ml psi medium at 37°C over night. Then 400 ml of psi media was inoculated with 4 ml of the over night culture and incubated at 37°C until OD 600=0,5-0,6. After incubation, cells were chilled on ice for 5 minutes and poured into 50 ml polypropylene tubes and centrifuged at 3000 rpm for 10 minutes at 4°C. The supernatant was discarded and the pellet resuspended in 2 ml of Tfb2 media and kept on ice for 15 minutes. For storage, 200 µl aliquots were frozen in liquid nitrogen. According to (Hanahan et al, 1991).

Table 4: Overview of E. coli strains used for transformation procedures

Strain	Genotype	Company
BL21(DE3)	B F- dcm ompT hsdS(rB- mB-) gal (DE3)	Novagene
XL1Blue	F'::TN10 proA$^+$B$^+$lacI^q Δ(lacZ)M15/recA1 end A1 gyrA96(Nal^R) thi hadR17 ($r_K^-m_K^-$) glnV44 relA1 lac	Stratagene

3.4 Molecular biological methods

3.4.1 Buffers and solutions (in alphabetical order)

- AB buffer: 80% TBSX, 15% heat-inactivated lamb serum, 5% *Xenopus* egg extract.
- AP-Buffer: 100mM trichlorethane Tris/HCl 9.5; 100mM NaCl; 50mM $MgCl_2$
- Bleaching solution: 1% H_2O_2; 5% Formamid; 0.5x SSC
- DEPC-H_2O: ddH_2O with 0.1% Diethylpyrocarbonat (DEPC) agitated at 23°C over night and autoclaved afterwards.
- 10mM DIG NTP mixture: 10mM CTP, GTP, ATP, 6.5mM UTP and 3.5mM Dig-11-UTP.
- Hybridization solution: 5x SSC, 50% formamide, 1% Boehringer block, 0.1% Torula RNA, 0.01% Heparin, 0.1% Tween-20, 0.1% CHAPS, 5mM EDTA.
- Lamb Serum: Heat-inactivated lamb serum (30 min at 56°C), stored at -20°C.
- MEMFA: 0.1M 3-(N-Morpholino)-propanesulfonic acid (MOPS), 2mM EGTA, 1mM $MgSO_4$, 3.7% formaldehyde pH 7.4
- Paraformaldehyde: 4% paraformaldehyde in PBSw
- PBS: 137mM NaCl, 2.7mM KCl, 8mM Na_2HPO_4, 1.7mM KH_2PO_4 pH 7.2
- PBSw: 1xPBS, 0.1% Tween-20
- Proteinase K: 10µg/ml Proteinase K in PBSw
- 20xSSC: 3M NaCl, 0.3M sodium citrate (pH 7.0 at 23°C).
- TBE: 100mM Tris/HCl, 83mM borate, 0.1mM EDTA (pH 8.6 at 23°C).
- TBS: 50mM trichloroethylene (Tris)/HCl, 150mM of NaCl (pH 7.5 at 23°C).
- TBSX: 1xTBS, 0.1% Triton X-100 (pH 7.5 at 23°C).
- TE: 1mM EDTA, 10mM of Tris/HCl (pH 8.0 at 23°C).

Xenopus egg extract for in situ hybridization:
Dejellied in 2% cysteine, unfertilized eggs washed 3 times, add 1 volume of PBS, then lysed by 10 strokes of a Dounce homogenisators, and centrifuged (7500xg, Sorvall Rc-5b, rotors SS-34, 10000rpm, 4°C, 10min).
The supernatant was transferred into a new centrifuge tube and further centrifuged twice with the same conditions. The supernatant was aliquoted and stored at -20°C.

3.4.2 Nucleic acid isolation

3.4.2.1 Plasmids isolation from competent cells

Plasmid DNA mini-preparations were carried out with Qiagen mini-preparation kits according to the manufacturer's instructions: After incubating the transformed competent cells on a LB-Ampicillin plate over night, a single colony of cells was picked and cultured in 2-5 ml LB medium containing Ampicillin and incubated for 8h at 37°C with shaking. The started culture was diluted 1/500 into selective LB medium, inoculated in 500 ml medium and grown at 37°C for 12h with shacking. The bacterial cells were harvested by centrifugation at 6.000 x g for 15 minutes at 4°C. The pellet was resuspended in 10 ml Buffer P1 including RNase. Then 10 ml Buffer P2 was added and mixed gently, followed by an incubation step at room temperature for 5 minutes. After that, 10 ml of chilled Buffer P3 was added, mixed and incubated on ice for 15 minutes. Then, the mix was centrifuged at 20.000 x g for 30 minutes at 4°C and the supernatant, containing the plasmid DNA, was removed. After equilibrating the Qiagen-tip with Buffer QBT, the supernatant was applied to the tip and was allowed to enter the resin by gravity. After washing two times with Buffer QC, the DNA was eluted with Buffer QF in 15 ml volume. The DNA was precipitated with 0,7 volume of isopropanol and centrifuged at 15.000 x g for 30 minutes at 4°C. The supernatant was decanted. The DNA pellet was washed with 70% ethanol and centrifuged at 15.000 x g for 10 minutes. The supernatant was decanted. The pellet was air dried and redissolved in the volume of choice. The yield was quantified by NanoDrop ND-1000 spectrophotometer (Peqlab).

3.4.2.2 RNA isolation from *Xenopus* embryos

Five whole embryos were collected at the proper developmental stage in 1,5 ml Eppendorf tubes with a minimal amount of buffer as possible. In case, they were not

used immediately, they were shock frozen in liquid nitrogen and stored at -80°C. The cells were mixed with RLT buffer with beta-mecapoethanol. After homogenization by pipetting, it was centrifuged 5 min at 4°C with maximal speed. The upper phase was transferred into a new tube. RNA isolation was performed according to the manufactures instruction of Quiagen RNeasy Kit. The RNA was eluted in DEPC-treated H_2O and stored at -80°C immediately

3.4.3 Nucleic acid manipulation

3.4.3.1 Cloning method

The cloning of DNA has been performed according to standard methods. Cloning of wtCHD4 and dnCHD4 fragment into the hsp70pCS2+ plasmid: First, wtCHD4pCS2+ and dnCHD4pCS2+ plasmids were digested with BamHI/XhoI. Digestion was performed with 1 µg/µl wtCHD4pCS2+ or dnCHD4pCS2+ plasmids in 20 µl volume, containing 2 µl restriction enzymes (BamHI/XhoI), 2 µl Puffer, the volume of plasmid corresponding to 1 µg/µl, added by the volume of H_2O, which adds up to 20 µl volume. The plasmids were digested at 37°C over night. Fragments were separated by gel electrophoresis, cut out and extracted from the gel by heating at 50°C for 10 minutes in a heat-block, mixed with isopropanol and eluded. The cloning was done in 10 µl volume with 2 µl of hsp70pCS2+ vector, 2 µl of insert (wtCHD4 or dnCHD4), 1 µl of T4 Ligase in 1 µl of Ligase Puffer (10x) and 4 µl of H_2O. As a negative control, the same ligation mix was used with 2 µl H_2O instead of 2 µl of Insert (wtCHD4 or dnCHD4). The ligation was performed by 4°C overnight. The cloning products (i.e. hsp70pCS2+ wtCHD4 and hsp70pCS2+ dnCHD4) have been sequenced for correct insertion.

3.4.4 Nucleic acid analysis

3.4.4.1 Gel electrophoresis of nucleic acids

DNA or *in vitro* synthesized RNA was isolated in horizontal agarose gel. Depending upon fragment size, one to two percent TBE agarose gels were used. After electrophoresis, the gels were photographed. 1kb or 100bp DNA ladder was used as size standard.

3.4.4.2 Isolation of DNA fragments from agarose gel

In order to isolate DNA fragments after electrophoresis from agarose gel, the appropriate bands were cut out under long-wave UV light. The DNA was extracted from the gel with Qiagen gel-extraction kit.

3.4.5 *In vitro* transcription of nucleic acid

3.4.5.1 *In vitro* transcription for microinjection experiments

Capped mRNAs for microinjection were *in vitro* transcribed with RNA polymerase.
A total volume of 50µl reactions mix was set up as following:

Table 5:

Volume	***Substrate***	***Total amount***
8 µl	0,25 µg/µl linearized plasmid DNA	2µg
10 µl	of the supplied transcription buffer	1 x
5 µl	25 mM RNA cap structure analogue	2.5mM
5 µl	100 mM DTT	10mM
10 µl	10 mM dNTP-Mix	0,5mM
0,5 µl	40 U/µl RNasin	20U
2 µl	20 U/µl Sp6	40U
DEPC H2O to 50 µl		

The reaction was incubated for 4 hours at 37°C. Subsequently, the template DNA was digested with 10U RNase free DNaseI for 30 min at 37°C. The RNA was purified with the RNeasy Spin Kit (Qiagen). The concentration of the RNA was measured with a NanoDrop ND-1000 spectrophotometer (Peqlab). The quality of RNA products for injection was evaluated by gel electrophoresis.

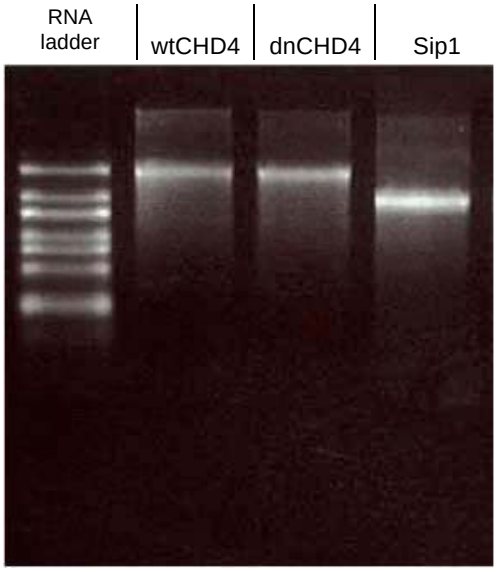

Figure 8: RNA gel electrophoresis

The gel demonstrates the quality control of synthesized mRNA for microinjection purpose. Only good quality RNA without signs of RNA degradation was used for each experiment.

3.4.5.2 *In vitro* transcription of digoxygenin labelled RNA probes

Plasmids were linearized and antisense RNA was generated by *in vitro* transcription to synthesise digoxygenin labelled RNA probes for whole mount in situ hybridization analysis The reactions were set up in a total volume of 50µl as following:

Table 6:

Volume	***Substrate***	***Total amount***
8 µl	0,25 µg/µl linearized template DNA	2 µg
10 µl	of 5 x supplied transcription buffer	
5 µl	1 mM Dig-NTPs labeling Mix	0,1 mM
5 µl	100 mM DTT	10 mM
2,5 µl	40 U/µl RNasin	100 U
2,5 µl	20 U/µl Sp6, T3, T7	50 U
DEPC H2O to 50 µl		

The reactions were incubated at 37°C for 4h and purified with the RNeasy Kit (Qiagen). The concentration of the RNA was determined by NanoDrop ND-1000 spectrophotometer (Peqlab).

3.4.6 Whole mount in situ hybridization

The embryos were fixed in the corresponding developmental stage in fresh MEMFA for 1,5-2 hours. After washing in PBS three times for 5 minutes the embryos were dehydrated over a period of one hour by replacing the PBS subsequently with 100% ethanol. The lipid membranes were dissolved overnight at 20°C in 100% ethanol. The rehydration of the embryos was performed by a 75, 50, 25% ethanol series in PBSw. Each ethanol step was incubated for 5 min at room temperature. Afterwards, three washes for 5 minutes with PBSw were performed. After that, Proteinase K in PBSw solution incubated for 20 min at 17°C, followed by a short rinse with PBSw. Again two washes for 5min each were performed with PBSw. After the digestion step with Proteinase K, the embryos were refixed with paraformaldehyde for 20 min. A short rinse with PBSw was performed followed by subsequent washing in PBSw for 5x5min. The PBSw was subsequently replaced with hybridization solution by 50% PBSw with 50% hybridization solution, followed by 100% hybridization for 3 min each step. 0.5 ml of fresh hybridization solution was added to each vial. To inactivate the endogenous phosphatases, the vials were incubated at 65°C for 1h. The embryos were then prehybridized at 60°C for 2-6 h. To 100 µl of hybridization solution 30-50 ng of digoxigenin-labelled RNA probe was added and incubated at 95°C for 2-5 min cooled immediately afterward on ice and added to the embryos in prehybridization solution. The RNA probe was hybridized to the mRNA over night at 60°C. To remove excessive RNA probe, the embryos were washed as follows: 2xSSC; 0.1% CHAPS short rinse; 2xSSC;0.1% CHAPS for 20 min; short rinse with 0.2xSSC;0.1% CHAPS; 2x for 30 min at 60°C in 0.2xSSC;0.1% CHAPS. Prior to the antibody binding, the embryos were transferred into TBSX (short Rinse in 50% TBS: 50% 0.2xSSC; 0.1% CHAPS), washed in TBS for 5min and rinsed in TBSx. To block unspecific antibody binding sites, the embryos were incubated in antibody buffer (0.5ml per vial) for 2h at 4°C. In parallel, AP-conjugated anti-DIG antibody (1/5000 diluted) was preabsorbed against *Xenopus* proteins present in antibody solution. 0.5 ml of preabsorbed antibody solution was added to the embryos and incubated overnight at 4°C. After antibody binding, the embryos were briefly rinsed with TBSx and washed 6 times for 1h in TBSx. Embryos were shortly rinsed in AP buffer and equilibrated for 15 min. AP-buffer was replaced with 0.5 ml staining solution and incubated overnight or up to 3 days at 17°C in the dark depending on the staining reaction. At the optimal signal to background ratio, the reaction was stopped by washing twice in PBS for 10 min. If the embryos were over-stained, some colour was removed by washing the embryos in 75% ethanol in PBS for 20 min. The stain was fixed in MEMFA for 90 min.

Bleaching of the embryos was performed to destroy endogenous colour pigments in bleaching solution on a light box for 2 h. The bleach solution was washed off with PBS 3 x 5 min. For long-term storage, the embryos were transferred to PBSw containing 0.2% Natriumazid (Fa VWR #1066880100) and kept at 4°C.

3.5 Embryological methods

3.5.1 Solutions

- Cystein**:** 2% L-Cystein in 0.1xMBS (pH7.8 at 23°C, adjusted with 5M NaOH).
- Human Chorionic Gonadotropin (HCG): 1000 I.U./ml HCG in ddH_2O.
- MEMFA: 0.1M 3-(N-Morpholino)-propanesulfonic acid (MOPS), 2mM EGTA, 1mM $MgSO_4$; 3.7% formaldehyde (pH 7.4 at 23°C).
- 1xModified Barth's Saline (MBS): 5mM HEPES, 88mM NaCl, 1mM KCl, 0.7mM $CaCl_2$, 1mM $MgSO_4$, 2.5mM $NaHCO_3$ (pH 7.6 at 23°C). Add the $CaCl_2$ before use.
- MBS/high salt: 1xMBS with 50mM NaCl
- 0.1xMBS/Gentamycin: 0.1xMBS, 10µg/ml Gentamycin
- 0.5xMBS/BSA: 0.5xMBS, 1mg/ml BSA, 10µg/ml Gentamycin
- MBS/CS: 0.8xMBS high salt with 20% chicken serum, 200U Penicillin/ml, 200µg/ml streptomycin stored at -20°C

3.5.2 Experimental model organism

Adult wild-type *Xenopus laevis* frogs (*Xenopus* Express) were used. The frogs were kept in charcoal filtered water with a temperature of 17-19°C with a population density of 5l water per frog. The animals were fed three times per week with Pondsticks Premium brittle (Interquell GmbH, Wehringen).

3.5.3 Ovulation stimulation

Female *Xenopus laevis* were injected 800 units of human chorionic gonadotropin (Sigma) into the dorsal lymph sac. After incubation at 18-20°C over night, females started to lay eggs 12-18 h later.

3.5.4 Isolation of testis

Male frogs were anaesthetized in 0.1% 3-Aminobenzoeacid-ethyl-ester in ddH_2O for 30 min and cooled down in iced water. The male frogs were killed by decapitation. The testes were removed from the abdominal cavity and separated from the frog body, to which they are connected to and isolated from the abdominal cavity. Testes were stored in MBS/CS at 4°C for maximal 7 days.

3.5.5 *In vitro* fertilization

In-vitro fertilization was performed by homogenizing testis tissue in 1 x MBS and mingle it with freshly laid eggs. The fertilized eggs were cultured in Petri dishes with 0.1xMBS at 16-23°C until the stage was reached for injection procedure. Embryos, which served as uninjected control, were cultured in parallel.

3.5.6 Dejellying of fertilized eggs

The fertilized eggs are covered by a jelly coat, which was removed about 60 minutes after fertilization by a 2% cysteine solution pH 7.8 for with gentle agitation in an Erlmaier glass flask until the eggs were in direct contact with each other as a sign for total removal of the jelly coat. Embryos were washed three times with 0.1 x MBS and cultured in Petri dishes with 0.1xMBS at 16-23°C.

3.5.7 Preparation of microinjection and needle calibration

Injection needles were pulled from capillaries with the Microneedle Puller (setting: heat: 800; pull: 35; vel: 140; time: 139; Sutter Instrument, model P-87). The needle

was adjusted to the holder of the injection equipment (Medical System, model Pi-100), and the tip of the injection needle was calibrated by cutting the tip at the point were the flexible part becomes more rigid with Dumont tweezers. Shortly before the injection, the needle was filled with 1-2 µl nucleotide acid containing RNase free DEPC water.

3.5.8 Microinjection of *Xenopus* embryos

The injection volume was adjusted by choosing the injection pressure of 25-30 psi with an injection time of 30 ms-1 s. With each shot, 5nl nucleotide solution containing was injected into a blastomere of the two to eight cell stage embryos into the animal hemisphere. After injection, the embryos were transferred into new dishes covered with 1% agarose in 0.1 x MBS with Gentamycin and incubated in 0.1 x MBS at 16-23°C until the desired developmental stages was reached. The saline was changed every day.

3.5.9 CHD4/Mi-2ß and Sip1 gain- and loss-of-function analysis

The gain of function of wtCHD4 or Sip1 was performed by microinjection of 1ng/5µl CHD4 or Sip1 mRNA in one blastomere of a two-cell stage *Xenopus* embryo respectively, as described in 3.5.8 to increase the CHD4 activity.
Microinjection of dnCHD4 mRNA on the other hand lead to the over expression of CHD4 protein with a functionally inactive ATPase domain, which diminishes the endogenous CHD4 protein ATPase function by competitive inhibition without disturbing the association with complex subunits or co-factors of the NuRD complex.
For loss of function analysis we designed Morpholino oligonucleotides, complementary to the translation start side of endogenous CHD4 mRNA.
The injection of these antisense nucleotides at a concentration of 40ng was shown to inhibit the translation of endogenous CHD4 protein. To test the efficiency of CHD4 Morpholinos to reduce translation of CHD4 protein, we subcloned the first 363 amino acids of the CHD4 in-frame upstream of the 6x Myc-tag cassette of the pCS2+MT6 vector, with and without the 5' untranslated region, which is complementary to the CHD4 Morpholino. The in-vitro translation of these test constructs was analysed with increasing amounts of CHD4 Morpholinos, followed by Western blot detection to evaluate the relative levels of the CHD4-Myc protein (Linder et al, 2007) (Figure 9).

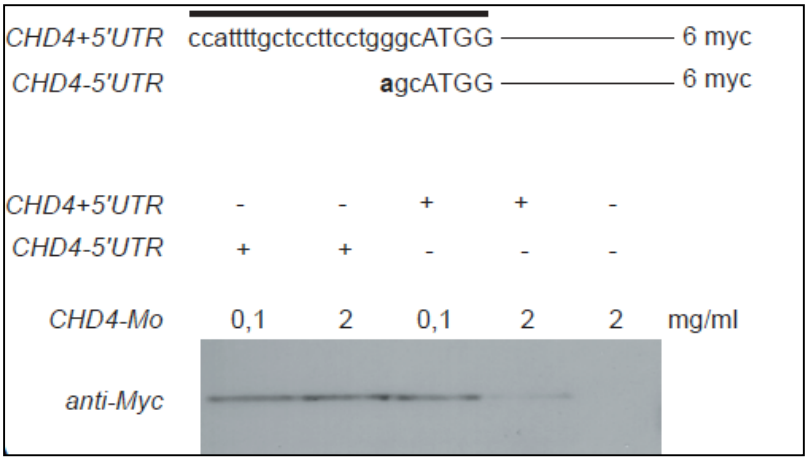

Figure 9: Western blot detection of CHD4-Myc protein

Western blot detection of CHD4-Myc protein to evaluate the efficiency of CHD4 Morpholinos to reduce CHD4 protein translation *in vitro*. Increasing amounts of CHD4 Morpholinos reduced the *in vitro* translation of CHD4 protein.

The described concentrations have been determined to be the optimal amount for functional interference without generating toxic side effects. To distinguish between left and right, embryos were co-injected with GFP mRNA or Alexa Dextrane and pre-sorted before fixation. The modulation of neural gene expression by CHD4 mis-expression was analyzed by whole mount in situ-hybridization as described in 3.4.6.
As controls, we compared the gene expression patterns and morphology of the injected side with the uninjected control side of the embryos, as well as with completely untouched wild-type embryos. Neither injection of gfp mRNA, nor the injection of 5% Alexa Dextrane in RNase free DEPC water led to phenotypic alterations of gene expression or morphology.

3.6 Histological methods

3.6.1 Solutions (in alphabetical order)

- AP buffer: 100mM Tris/HCl (pH 9.5), 50mM $MgCl_2$, 100mM NaCl, 0.1% Tween 20, 5mM Levamisole.
- AP staining solution: 4.5µl NBT, 3.5µl BCIP in 1ml AP buffer.
- A-PBS: 103mM NaCl, 2.7mM KCL, 0.15mM KH_2PO_4, 0.7mM NaH_2PO_4 pH7.5

- A-PBS-T: APBS with 0.1% Tween20
- Blocking buffer: PBT plus 10% heat inactivated serum
- Citrate buffer: Stock A: 0.1M citrate monohydrate (10.5g for 500ml solution)
- Stock B: 0.1M Trisodiumcitrate-dihydrate (14.7g for 500ml solution)
- Working Sol.: 9ml A with 41ml B to 450 ml ddH_2O. pH should be 6.
- DAPI: Hoechst dye (1mg/ml) 1:1000 in APBS-T
- Dent's Fixative: 80% methanol, 20% dimethyl sulfoxide (DMSO)
- Elvanol: 2.4g Moviol 4 (Hoechst, Frankfurt) were mixed with 6g glycerol and 6ml ddH_2O and stirred at least for 2h at room temperature. Then 2ml 0.2M Tris/HCl pH8.5 were added and incubated for 10min at 60°C. Afterwards 50mg/ml DABCO (1.4-Diazabicyclo(2.2.2) Octane) were added and centrifuged for 30 min at 17000 x g. Aliquots of the supernatant were stored at -20°C.
- MEMFA: 0.1M MOPS, 2mM EGTA, 1mM $MgSO_4$, 3.7% formaldehyde (pH7.4 at 23°C), prepare freshly.
- PBS: 137mM NaCl, 2.7mM KCl, 8mM Na_2HPO_4, 1.7mM KH_2PO_4 (pH7.2 at 23°C).
- PBT: PBS, 2mg/ml BSA, 0.1% Triton-X-100.

3.6.2 Immunocytochemistry

For immunocytochemistry, the first step was to remove the vitelline membrane from the embryos. Subsequently, the embryos were fixed in MEMFA for 1-2h at room temperature with rotation. After rinsing with PBS, the embryos were gradually dehydrated with methanol and incubated in methanol at -20°C over night. Rehydration was performed by 80%, 50%, 0% methanol in PBS for 5 min each, followed by a 5min rinse with PBS and one rinse in PBT for 15min. Unspecific antibody binding sites were blocked by incubating the embryos in PBT plus 10% heat inactivated goat serum at room temperature for 1h. The primary antibody was diluted in the blocking buffer and incubated over night at 4°C. Afterwards the embryos were washed 6 times with PBT for one hour. The secondary antibody solution, consisting of the secondary antibody, coupled with alkaline phosphatase, diluted 1:1000 with blocking buffer was added to the embryos and was incubated over night at 4°C. Subsequently, the embryos were washed 6 times with PBT for on hour. Prior to staining, the endogenous alkaline phosphatases were blocked by the addition of Levamisol to the AP buffer. The embryos were incubated twice in AP buffer for

30min. Staining was performed in 1 ml staining solution in the dark for 30 to 120min at room temperature. The staining reaction was stopped by washing the embryos in PBS to titrate out the staining solution. The stain was fixed in MEMFA over night. Bleaching of the embryos was reached by washing in bleaching solution for 2h on a light box.

3.6.3 Antibodies

3.6.3.1 Primary antibodies

Against:

xCHD4 as GST-fusion protein of the N-terminus (aa1-aa357) (Linder et al, 2007; Singhal, 2005):

CH4-N 3A11: subtype IgG2a (WB negative, positive in IP + ChIP, ICC negative)

CH4-N 5H4: subtype IgG1 (WB negative, positive in IP + ChIP, ICC negative)

CH4-N 5A2: subtype IgG2a (WB negative, positive in IP + ChIP, ICC negative)

Antigen xCHD4 as GST-fusion protein of the C-terminus (aa 1513- aa 1891) (Linder et al, 2007)

CH4-C 7C9: subtype IgM (WB negative, positive in ChIP, ICC positive)

CH4-C 7E8: subtype IgM (WB negative, positive in ChIP, ICC positive).

For the production of rat monoclonal antibodies, the C-terminal domain of xCHD4 (amino acids 1513-1891) was cloned into the pGEX-4T3 bacterial expression vector (Amersham), expressed in *Escherichia coli*, and purified. The antibodies were generated in collaboration with Dr. Elisabeth Kremmer, GSF Munich. The GST-fusion proteins were cloned and purified in our laboratory by Dr. rer. nat. Kathrin Mansperger. Positive primary hybridoma cell supernatants were prescreened by the Kremmer laboratory concerning their specificity to bind the antigen, but not to the GST-fusion part. Using Western blot and immunoprecipitation (IP) analyses, positive clones were further analyzed for their specific detection of *in vitro* translated antigens in our lab. Subsequently, the Kremmer laboratory stabilized the positive tested hybridoma clones. Clones were then tested in IPs and ChIP-IPs for their specificity and affinity to their antigen.

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Caspase3: (Upstate Biotechnology, Lake Placid, NY)
H3S10Ph: (Upstate Biotechnology, Lake Placid, NY)
H3S28P: (Upstate Biotechnology, Lake Placid, NY)

3.6.3.2 Secondary antibodies

Cy3 Indocarbocyanin, donkey anti-rat, Dianova, 1:200 diluted
Fluorescein- Isothiocyanat (FITC), donkey anti-mouse, Dianova, 1:200 diluted
AlkPhos, Fab Ig, sheep anti mouse, Chemicon, 1:1000 diluted

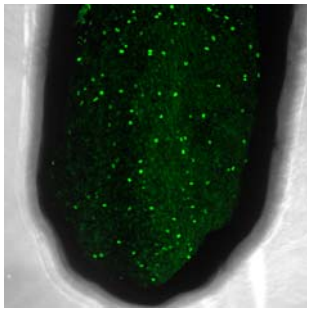
3.6.4 Immunofluorescence

For immunofluorescence analysis of whole embryos, the same protocol was used with exception that no blocking of endogenous phosphatase, fixation of staining and bleaching was performed or necessary.

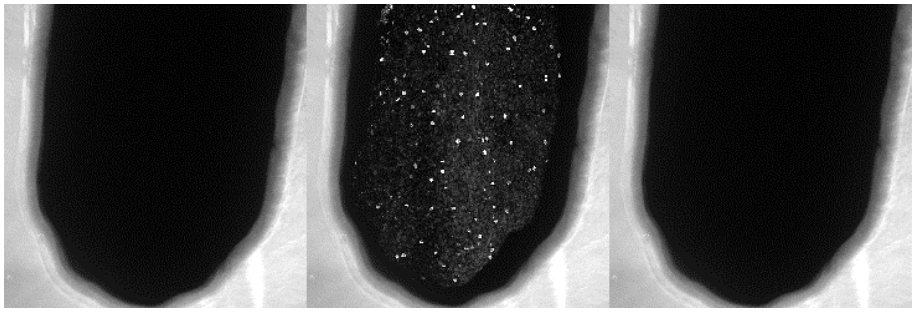
3.7 Confocal microscopy

Series of confocal sections through whole-mount embryos, focused on the central nervous system were collected with a Leica SP5 microscope equipped with a Plan/Apo 63X 1.4 NA oil immersion objective. For each optical section, images (512*512 pixels) were collected sequentially for three fluorochromes. The stacks of each sectios at 4 micrometers were recorded as separated eight-bit grayscale Z-planes with voxel size 120x120x200 nm (XxYxZ). The “Abi prism” software was used to convert .lsm (laser-scanning-microscopy) files, obtained by the confocal microscopy, to .tif files to be processes by ImageJ software. RGB stacks were reconstructed with the three channels function of ImageJ. The mean integrated fluorescence intensity was quantified with ImageJ according to the following steps:

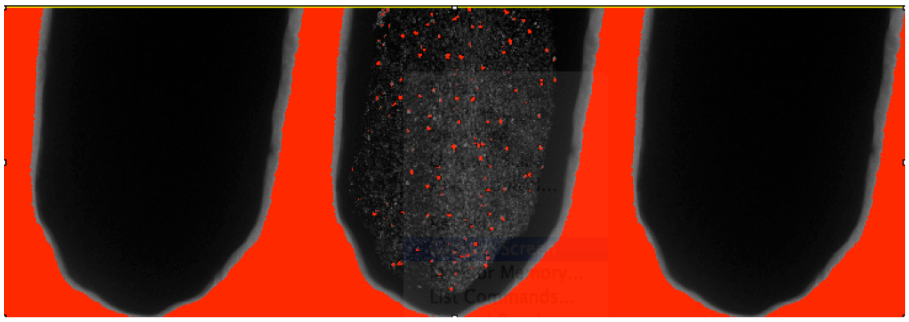
1) Open File of two-dimensionally reconstructed scan:



2) Image – Type – RGB Stack – Make Montage:

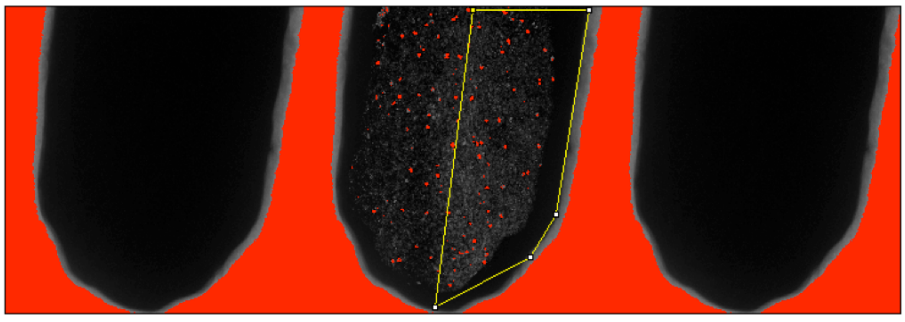


3) Image - Adjust - Thresholds:



4) Analyze - Set Measurements,

5) Creating a Mask



6) Analyze - Measurement of the integrated fluorescence intensities – Result

4 Results

4.1 CHD4/Mi-2ß function during neural induction and neuronal differentiation within an early chromatin stage

During gastrulation, the establishment of ectoderm, mesoderm and endoderm is achieved through the spatial and temporal coordination of multiple signalling events called induction. During these events, a cell's ability to respond to signalling as a function of time is referred to as cellular competence. In *Xenopus laevis,* the competence to respond to FGF (*f*ibroblast *g*rowth *f*actor) signals changes during development. At blastula stages, FGF induces mesoderm, whereas at gastrula stages FGF regulates the neuroectoderm formation (Hardcastle et al, 2000).

The processes of embryonic development, cellular differentiation and neural induction involve large alterations in chromatin architecture, resulting in different gene expression patterns. The process, how cell signalling is translated onto the chromatin level influences chromatin architecture, changes the chromatin context and the outcome of subsequent signalling events. The factors that translate and integrate these signalling information onto the chromatin level to establish regulated gene expression patterns from stem cell state to differentiated tissue are not determined yet. ATP-dependent chromatin remodelling plays a crucial role during cellular differentiation and works in concert with binding co-factors and transcription factors to regulate gene expression.

Figure 10 shows the spatial and temporal mRNA expression pattern of n-ß tubulin as a marker gene for neural tissue. The temporal co-expression of the ATPase subunit CHD4 and the transcription factor Sip1 is illustrated on the left and on the right, respectively. Their expression domains overlap with the spatial expression of n-ß tubulin in the developing neural tissue. This implicates a developmental role of the CHD4/Mi-2ß chromatin remodelling ATPase subunit and the transcription factor Sip1 during neural induction and neuronal differentiation.

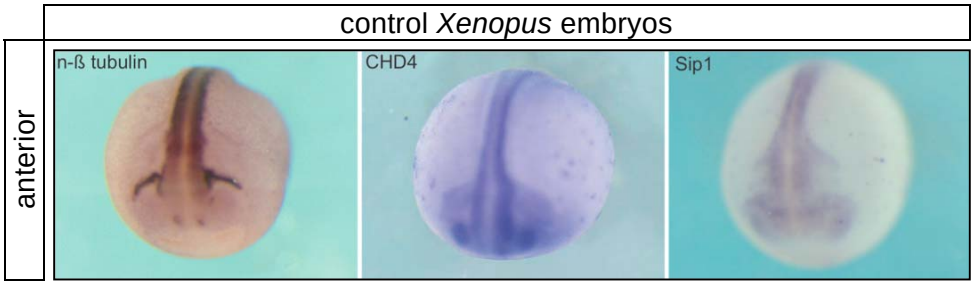


Figure 10: mRNA in situ hybridization of neural n-ß tubulin, CHD4 and Sip1
Xenopus embryos at NF stage 19 show n-ß tubulin expression, limited to the presumptive spinal cord and the brain area (CNS) (Oschwald et al, 1991). CHD4 and Sip1 reveal a spatial overlapping expression pattern with n-ß tubulin in the central nervous system (CNS). Spatial and temporal co-expression of CHD4 and Sip1 in the CNS implicates combined functions of CHD4 and Sip1 during neural development.

4.1.1 Analysis of CHD4/Mi-2ß gain-of-function and loss-of-function during neural development

To investigate the function of CHD4 during neural induction and neural cell differentiation, I analysed the influence of CHD4 gain- and loss-of-function experiments on neural marker gene expression. To alter CHD4 expression levels with a gain in chromatin remodelling function, I microinjected *Xenopus* embryos in one blastomere in the animal pole at 2-cell stage with CHD4 mRNA, coding for CHD4 protein. (See Figure 12) To reduce endogenous CHD4 protein levels for loss-of-function analysis, I injected Morpholinos against the ATG translation initiation side of endogenous CHD4 mRNA to block protein translation. The injection of dominant negative (dn) CHD4 mRNA leads to the translation of a CHD4 variant that lacks a functioning ATPase domain for ATP-dependent chromatin remodelling. (See Figure 11)

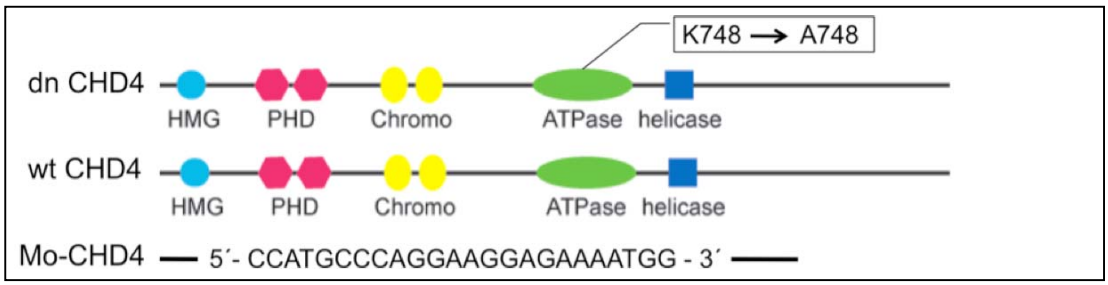


Figure 11: Microinjection of nucleic acids for CHD4 gain- and loss-of-function

The dominant negative variant of the CHD4 ATPase (dnCHD4) was created by point-mutating the lysine (K) residue at position 748 to arginine (A). The analogous mutation in the SNF2 ATPase maintains the integrity of the yeast SWI/SNF chromatin-remodelling complex, but abolishes transcriptional activation of target genes (Richmond & Peterson, 1996). Wild-type CHD4 mRNA (wtCHD4) increases CHD4/NuRD activity. For loss-of-function analysis, we designed a 25-mer antisense Morpholino oligonucleotide (Mo-CHD4) complementary to the *Xenopus* translational start site of endogenous CHD4 mRNA to block endogenous protein translation (Heasman et al, 2000).

The microinjection at 2-cell stage alters the CHD4 protein levels, depending on the injected condition. The embryos developed until NF stage 19 (neurula) with an altered CHD4 function on the injected side, as marked in red. The uninjected side served as a control.

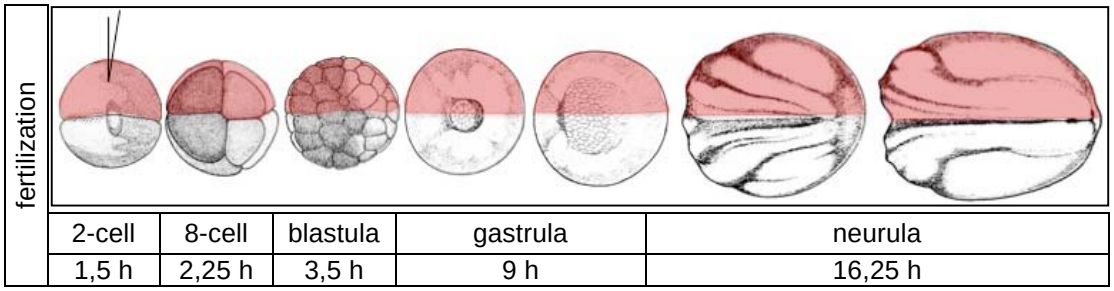


Figure 12: Illustration of gene expression interference by nucleic acid microinjection

mRNA or Morpholino microinjections after 1,5 h post fertilization of *Xenopus* eggs leads to translation of the injected mRNAs or reduction of endogenous CHD4 mRNA translation and subsequent alteration of the endogenous CHD4 protein levels during development. The gene expression interference is restricted to the injected side of the embryo, as indicated in red. The uninjected side serves as a negative control.

The analysis of the altered expression of the neural specific gene of interest was analyzed in whole embryos. The embryos are presented as demonstrated below.

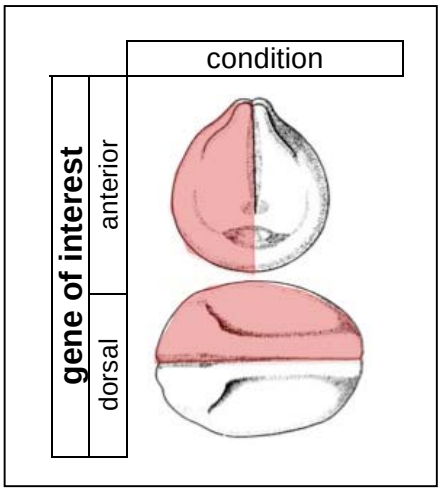


Figure 13: Schematic illustration of the analysis
The embryos are orientated in the illustrated manner for the following presentation of the analysis. The gene of interest is analysed by in situ hybridization. The different injection conditions are noted at the top. The injected side is on the right side, as indicated in red.

The following results demonstrate the altered expression pattern of neural marker genes, due to CHD4 misexpression. The analysed genes characterise the developing neural tissue according to its state of differentiation during neural induction and neural cell differentiation.

4.1.1.1 CHD4 upregulates the expression level of Churchill

The search of factors, that are responsible for how cells respond on signalling events, led to the identification of a zincfinger protein, which acts as a transcriptional activator, called Churchill (ChCh). Churchill contains two putative C4-type zincfingers named in analogy to Sir Winston's famous “Victory” gesture (Sheng et al, 2003). ChCh has been described to act as a gate, separating the two different functions of FGF signalling in mesendoderm formation on the one hand and in neural induction on the other hand.

The first step of my analysis to investigate the early regulating factor of neural inducing processes showed that alteration of CHD4 expression levels influenced the FGF dependent ChCh gene expression. Figure 14 A demonstrates the quantitative analysis of ChCh expression due to CHD4 misexpression. The injection of 1ng of wtCHD4 mRNA in the animal pole of a two-cell stage embryo led to a unilateral induction of ChCh expression on the injected side at NF stage 19 (Figure 14, black arrow). The injection of the dominant negative variant of CHD4 (dnCHD4) results in

the formation of CHD4/NuRD complex lacking a functional ATPase domain. The dnCHD4 overexpression resulted only in a slight induction of ChCh expression (blue arrow). Control embryos, which have not been injected, showed no phenotypic alteration. Neither did reduction of endogenous CHD4 function by CHD4 Morpholino (Mo-CHD4) injection result in the alteration of ChCh expression (white arrow), compared to the uninjected side of the same embryo, and compared to uninjected control embryos. Thus, no p-value was calculated for this condition.

A

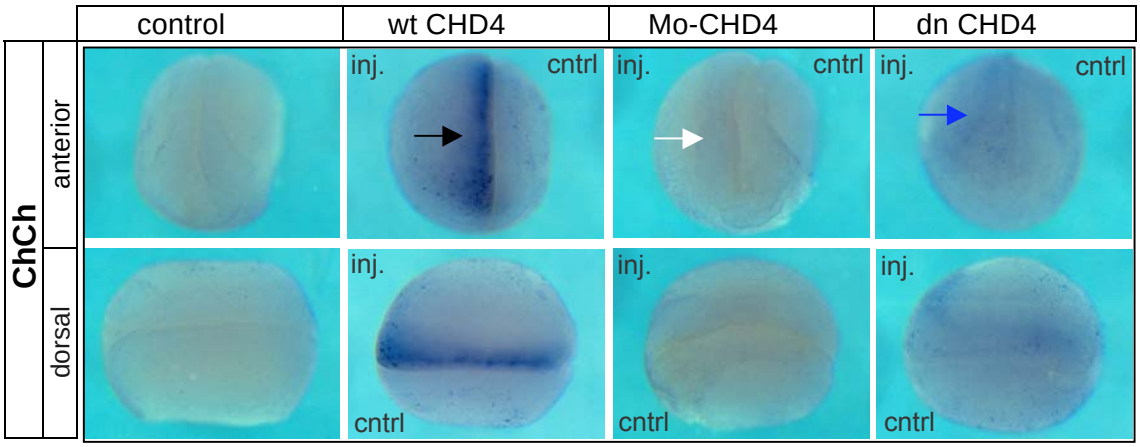


Figure 14 A: CHD4 overexpression induces the expression of ChCh

A) ChCh mRNA in situ hybridization (ISH) illustrates the qualitative difference of ChCh gene expression due to CHD4 misexpression (inj.), compared to the uninjected control side (cntrl). The injected conditions are wild type CHD4 (wt CHD4), dominant negative CHD4 (dn CHD4) and Morpholinos against CHD4 mRNA (Mo-CHD4). The control condition is uninjected (control). Embryos, which have been injected with gfp mRNA or Alexa Dextrane alone did not show any alteration of ChCh expression or morphology and resembled the uninjected control condition. The injected side is the right side of the embryos. The upper row shows the anterior view of NF stage 19 *Xenopus* embryos, the lower row displays the dorsal view of the same embryo for each condition.

B

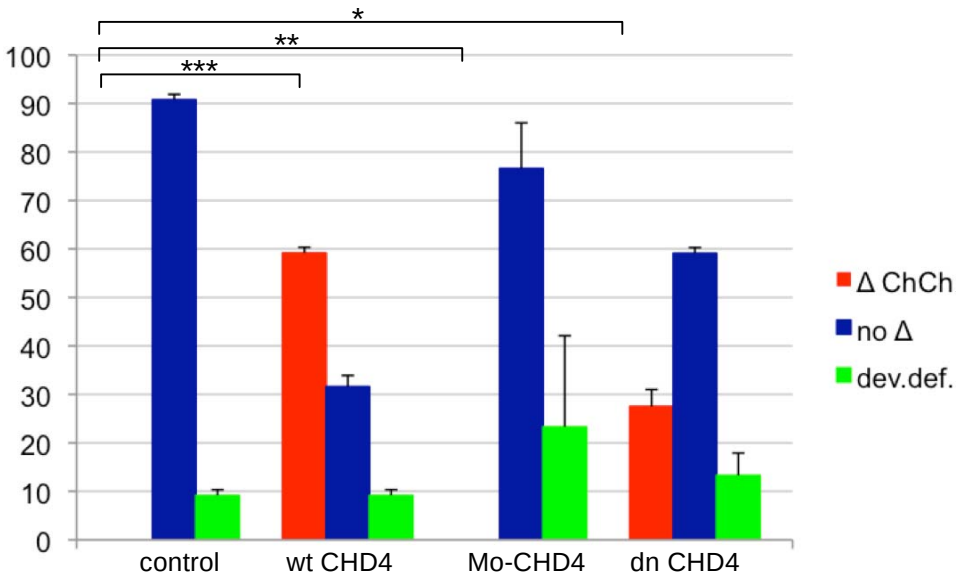


Figure 14 B: Quantitative analysis of induced ChCh expression

B) The chart illustrates the mean penetrance of altered ChCh expression in percent on the y-axis. Δ ChCh = different ChCh expression compared to the uninjected control embryos. no Δ = no difference compared to control embryos. dev.def. = embryos died from developmental defects. The standard deviation reflects two independent experiments. The significance level was set to $p \leq 0.05$.

	control	**wt CHD4**	**Mo-CHD4**	**dn CHD4**
n = ∑88	n = 22	n = 22	n = 22	n = 22
p-value, Δ ChCh	-	***p = 0.0007	**p = -	*p = 0.02

The results demonstrated in Figure 14 illustrate that CHD4 dependent chromatin remodelling promotes the induction of ChCh expression as an early regulating factor of neural induction.

Consequently, CHD4 is not only important for the boundary formation between mesoderm and neuroectoderm, as demonstrated in our laboratory (Linder et al, 2007), but also influences fade decision between neuroectoderm and ectoderm for the benefit of neuroectoderm, indicated by the induction of the early neural gene ChCh. To test, if CHD4 works as a gate opener for neural induction, I analysed, how CHD4 misexpression affects the expression of the neural gene that is expressed at the onset of neural development, i.e. the early neural marker gene Sox2.

4.1.1.2 CHD4 induces ectopic expression of Sox2

Sox2 belongs to the family of Sox genes, encoding a group of 20 Sox factors, which are classified into seven groups that are structurally related to the sex-determination factor Sry (Pevny & Lovell-Badge, 1997; Wegner, 1999). Sox2 carries a single 79-amino acid HMG (*h*igh *m*obility *g*roup) domain that binds to DNA by recognizing a 6 to 7 base pair sequence (ATTGTT or CTTTGTT), which is located in the minor groove of the target DNA (Kamachi et al, 2000). Sox2 function depends on distinct partners in a cell type-specific manner. In embryonic stem cells, Sox2 interacts with Oct3 protein on binding to the enhancers of the Fgf4 gene (Yuan et al, 1995). In the forming neural tissues, at least three distinct classes of Sox genes are expressed. Class one consists of Sox1, -2 and -3 (Group B, subgroup B1) (Collignon et al, 1996; Kamachi et al, 1995; Uwanogho et al, 1995). The expression pattern of subgroup B1 genes is restricted to *Xenopus* neuroectoderm from the late blastula stage onwards. Later, these genes are expressed in the forming lens, the neural crest cells and lateral line cells (Mizuseki et al, 1998; Penzel et al, 1997). The function of Sox2 during neural induction depends on the influence of FGF signals. While Sox2 is not sufficient to induce neural differentiation of *Xenopus* animal cap ectoderm it modifies the competence of the gastrula animal cap, thus the ectoderm can respond to the FGF neuralizing signal (Mizuseki et al, 1998). The *in vivo* role of Sox2 has been investigated in dominant negative studies by Kishi et al. (Kishi et al, 2000). The overexpression of dnSox2 in animal cap explants does not lead to differentiation into neural tissues, rendering Sox2 important for neural induction.

In my analysis, Figure 15 A shows that overexpression of wtCHD4 induces a strong and ectopic expression of Sox2 with upregulation in the spinal cord and strong ectopic expression in the presumptive ectodermal tissue on the injected side (inj.) of the embryo. The endogenous expression pattern of Sox2 is enhanced (Figure 15 A, black arrow), compared to the uninjected control side (cntrl) and compared to the uninjected control embryos. The reduction of endogenous CHD4 protein levels by CHD4 Morpholinos (Mo-CHD4) significantly reduced the expression of Sox2 on the injected side of the embryo, compared to the uninjected control side (Figure 15 A, white arrow). Interestingly, the presence of a functional ATPase domain is not a crucial factor for the induction of Sox2 gene expression, because overexpression of dnCHD4 also led to a phenotypic alteration of Sox2 expression that resembles the wtCHD4 condition (Figure 15 A, blue arrow).

A

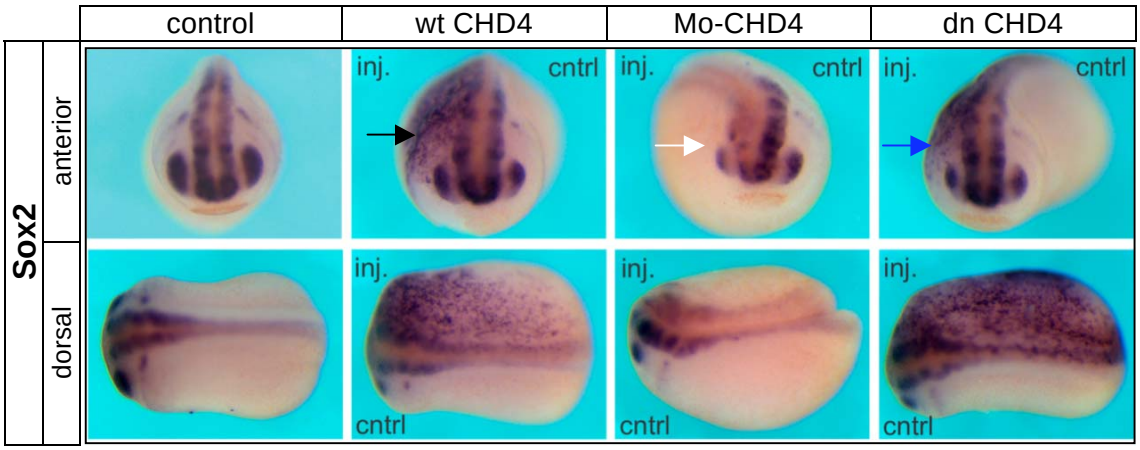


Figure 15 A: CHD4 induces ectopic expression of Sox2

A) The in situ hybridization illustrates the Sox2 gene expression on the mRNA level. The control embryo on the left illustrated the physiological endogenous Sox2 expression in the developing neural tissue. The control embryo shows a symmetric expression of endogenous Sox2 on the right and left half. The panel illustrates the qualitative differences of Sox2 gene expression due to CHD4 misexpression (inj.) compared to the uninjected control side (cntrl). Overexpression of wt- and dnCHD4 induces Sox2 gene expression within the endogenous expression domain and ectopically in the ectoderm (black and blue arrows). Reduced endogenous CHD4 levels (Mo-CHD4) lead to a reduced Sox2 expression (white arrows). The upper row shows the anterior view of stage 19 *Xenopus* embryos, the lower row displays the dorsal view.

B

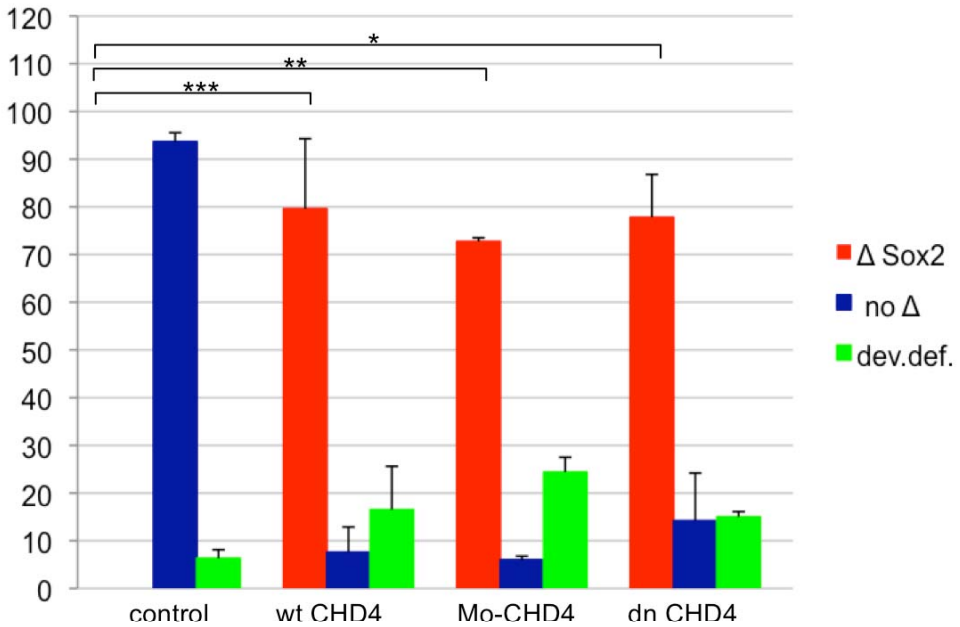


Figure 15 B: Quantitative analysis of Sox2 expression

B) The chart illustrates the mean phenotypic penetrance of altered Sox2 expression due to CHD4 misexpression in percent on the y-axis. Δ Sox2 = different Sox2 expression compared to the uninjected control. no Δ = no difference, compared to control. dev.def. = embryos died from developmental defects. Standard deviation reflects two independent experiments. The significance level was set to $p \leq 0.05$.

	control	wt CHD4	Mo-CHD4	dn CHD4
n = ∑123	n = 33	n = 23	n = 33	n = 31
p-value, Δ Sox2	-	***p = 0.002	**p = 0.0015	*p = 0.0013

4.1.1.3 CHD4 induces neural gene expression at the expense of ectodermal xK8 expression

The induction of neural progenitor formation was shown to be at the expense of non-neural ectodermal derivatives (Rogers et al, 2009a).

Consequently, CHD4 misexpression should affect the development of ectodermal tissue. The following analyses the influence of CHD4 misexpression on the expression domain of the ectodermal specific gene *Xenopus* Keratin cytoskeletal 8 (xK8), which is associated with epidermal induction and inhibition of neural fate (Jonas et al, 1985). Figure 16 A illustrates the qualitative alteration of xK8 gene expression due to CHD4 misexpression. Overexpression of wtCHD4 reduces the expression domain of xK8 mRNA and enhances the non-Keratine stained domain, indicated by a broadening of the dorsal neural tissue (marked by red dashed bar on the injected side of the embryo in the wtCHD4 condition).

In contrast, the reduction of endogenous CHD4 protein by injecting Morpholinos results in an intense xK8 staining of ectodermal tissue on the injected side with a reduction of the dorsal neural tissue, compared to the uninjected control side, and compared to the uninjected control embryos (marked by red dashed bar on the injected side of the embryos in the Mo-CHD4 condition). In agreement with the previous observations, the dnCHD4 phenotype resembles the wtCHD4 condition. Figure 16 B shows the quantitative analysis of xK8 mRNA expression alteration due to CHD4 misexpression.

A

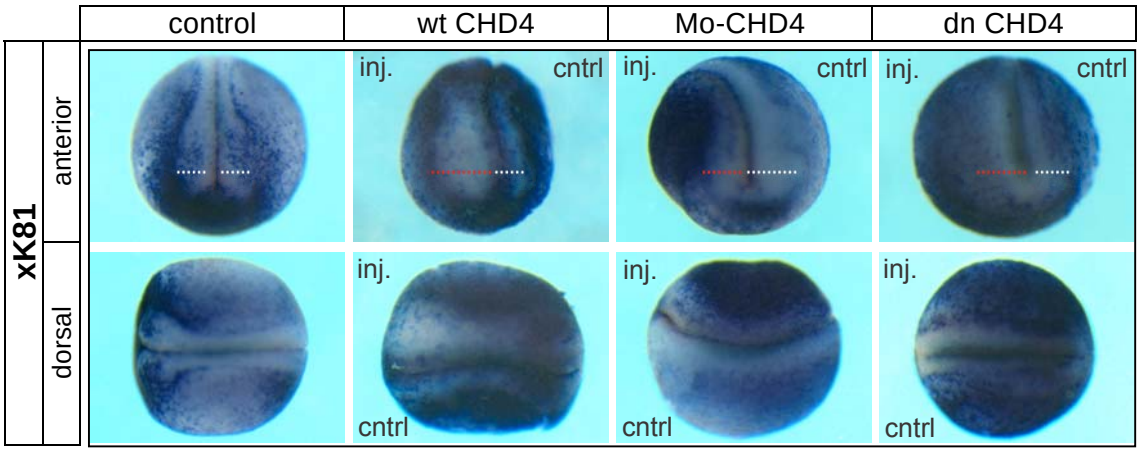


Figure 16 A: CHD4 reduces the xK81 expression domain

A) Representative illustration of mRNA in situ hybridization of Keratin (xK81) gene expression. The panel illustrates the qualitative alteration of xK81 gene expression due to CHD4 misexpression (inj.), compared to the uninjected control side (cntrl). The white lines indicate the unaffected control side compared to the altered expression domain, indicated by the red dashed lines. The injected side is the right side of the embryo and reveals a reduced xK81 staining due to wt/dn CHD4 expression. Mo-CHD4 results in an intense xK8 staining of ectodermal tissue on the injected side with a reduction of the dorsal neural tissue. The upper row shows the anterior view of NF stage 19 *Xenopus* embryos, the lower row displays the dorsal view. The control embryo on the left illustrates the physiological endogenous xK81 expression in the ectodermal domain.

B

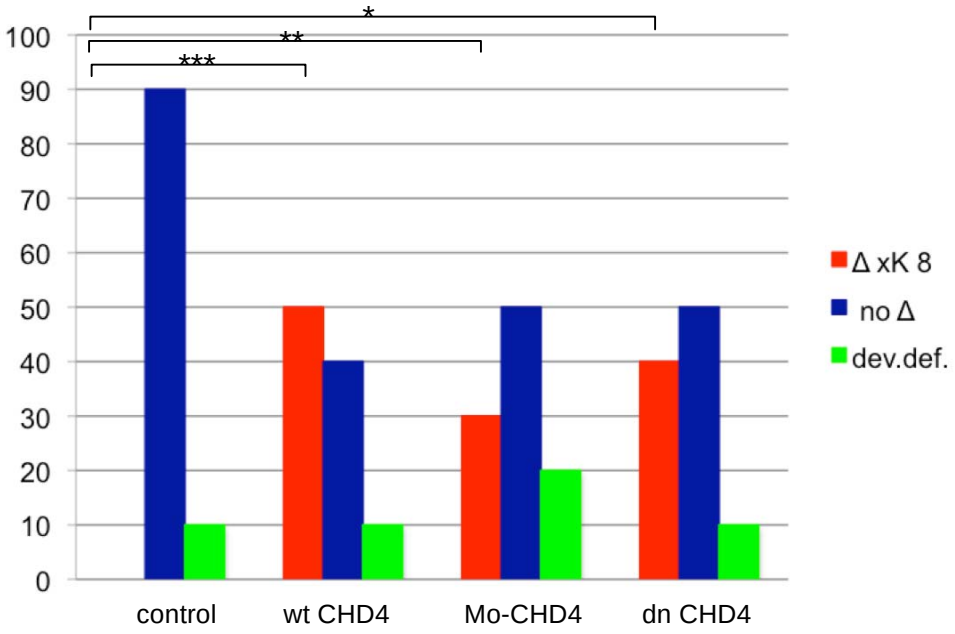


Figure 16 B: Quantitative analysis of reduced xK81 expression domains

B) The chart illustrates the mean phenotypic penetrance of altered xK81 gene expression in percent on the y-axis. Δ xK81 = different xK81 expression compared to the uninjected control. no Δ = no difference compared to control. dev.def. = embryos died from developmental defects. The significance level was set to $p \leq 0.05$.

	control	wt CHD4	Mo-CHD4	dn CHD4
n = ∑40	n = 10	n = 10	n = 10	n = 10
p-value, Δ xK81	-	***p = 0.013	**p = 0.058	*p = 0.026

In conclusion, CHD4 chromatin remodelling results in the formation of neural progenitor cells, with induced neural gene expression, e.g. ChCh and Sox2, at the expense of non-neural ectodermal derivates.

Based on this observation, CHD4 chromatin remodelling would modulate the expression of further neural specific genes, characterising the developing neural tissue. This will be analyzed in the following.

4.1.1.4 CHD4 regulates the expression of Ash3a/b

In Drosophila, neural development is promoted by the family of basic helix-loop-helix (bHLH) transcription factors encoded within the *achaete-scute-complex* (AS-C). In this context, a subset of pro-neural genes, which cause an enlargement of the CNS at the expense of ectodermal tissue comprise four genes in the *achaete-acute*-complex *(AS-C).* XAsh3, is a *Xenopus* homolog of the Drosophila AS-C genes. It is expressed during neural induction within a portion of the dorsal ectoderm that gives rise to the neural plate and the neural tube (Ferreiro et al, 1994). Turner and Weintraub described two achaete-scute homologs from *Xenopus*, named Ash3a and Ash3b *(achaete-scute homologs 3a* and *3b),* which are expressed during gastrulation in the prospective CNS (Turner & Weintraub, 1994). Ash3a/b are one of the earliest genes expressed in the prospective CNS after neural induction and regulate neuroblast formation. Ectopic expression of Ash3a/b in *Xenopus* embryos leads to a striking enlargement of the CNS, at the expense of ectodermal cell types (Turner & Weintraub, 1994). The expression of Ash3a/b mRNA in developing embryos was investigated by whole-mount in situ hybridization (Harland, 1991) and quantitative RT-PCR (Rupp & Weintraub, 1991). These analyses demonstrated that Ash3a/b is first expressed at NF stage 11.5 during midgastrulation in the presumptive neural

plate, beginning in the posterior of the prospective CNS. With the formation of the neural tube, the expression expands to include the future forebrain and retina. The expression of Ash3a/b is transient and restricted to zones within the CNS, which contains proliferating undifferentiated neural precursor cells. Ash3a/b expression prevents the differentiation of early forming primary neurons and maintains these cells as undifferentiated neuroblasts. Figure 17 A illustrates the qualitative alterations of Ash3a/b expression due to wtCHD4 overexpression. The wtCHD4 overexpression induces and enlarges the expression domain of Ash3a/b on the injected side of the embryos (black arrows) compared to the uninjected control side. The reduction of endogenous CHD4 protein by CHD4 Morpholinos results in a reduced Ash3a/b expression on the injected side (white arrows), compared to the uninjected control side of the embryo. Overexpression of dnCHD4 led to a phenotypic alteration of Ash3a/b expression, which resembles the wtCHD4 condition (blue arows). In accordance with the observations described above, the presence of a functional ATPase domain is not a crucial factor for the induction of Ash3a/b gene expression.

A

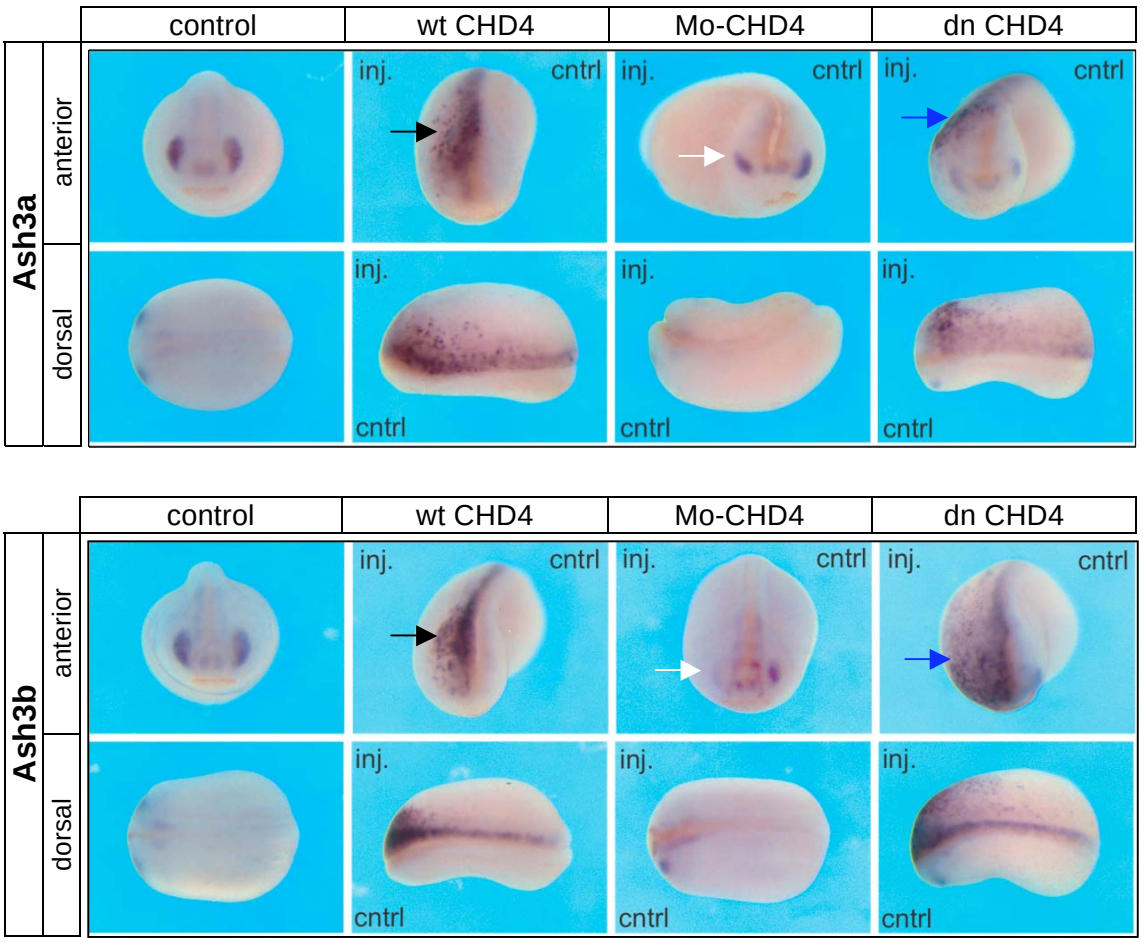


Figure 17 A: CHD4 regulates the expression of Ash3a/b

A) Representative illustrations of Ash3a/b mRNA in situ hybridizations. The control embryos on the left show the physiological endogenous Ash3a/b mRNA expression in the developing neural tissue. The panel illustrates the qualitative difference of Ash3a/b gene expression due to CHD4 misexpression (inj.) compared to the uninjected control side (cntrl). Overexpression of wt- and dnCHD4 induces Ash3a/b gene expression within the presumptive neural domain (black and blue arrows), even when the endogenous Ash3a/b expression is hardly detectable on the control side, compared to the control embryo. Reduction of endogenous CHD4 levels, due to Mo-CHD4 injection reduces Ash3a/b expression almost completely (white arrows).

Figure 17 B demonstrates the phenotypic penetrance of altered Ash3a and Ash3b gene expression due to CHD4 misexpression.

B

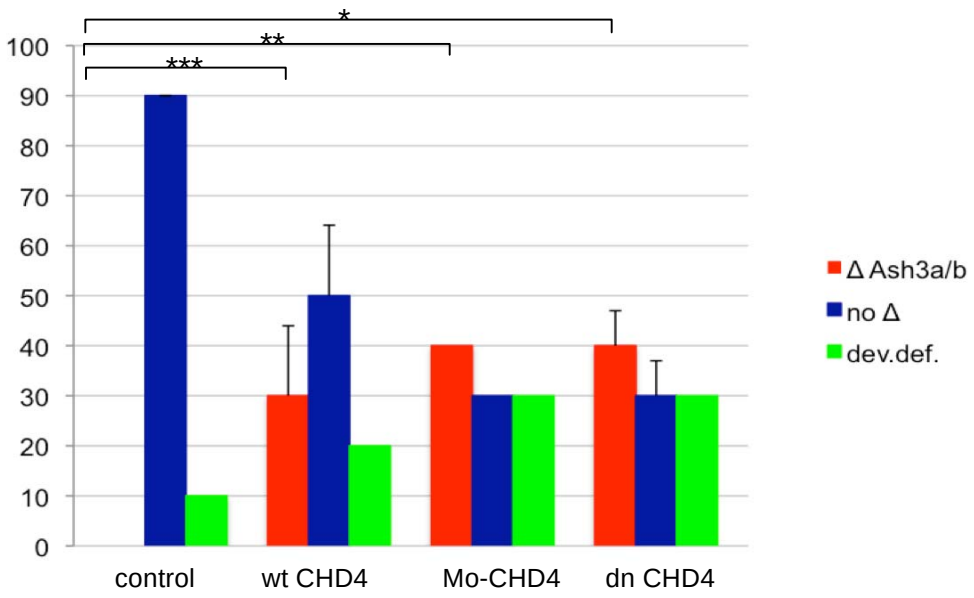


Figure 17 B: Quantitative analysis of altered Ash3a/b expression

B) The chart illustrates the mean phenotypic penetrance in percent on the y-axis. Δ Ash3a/b = different Ash3a/b expression compared to the uninjected control. no Δ = no difference compared to control. dev.def. = embryos died from developmental defects. The data was collected from two independent experiments. The significance level was set to $p \leq 0.05$.

	control	**wt CHD4**	**Mo-CHD4**	**dn CHD4**
n = ∑80	n = 20	n = 20	n = 20	n = 20
p-value, Δ Ash3a/b	-	***p = 0.028	**p = 0.014	*p = 0.015

In conclusion, these results demonstrate that epigenetic gene regulation by CHD4 chromatin remodelling regulates neural induction, resulting in the enlargement of the CNS at the expense of ectodermal cell types. In the following analyses I investigated, if CHD4 mediated neural induction also influences the expression of neural genes downstream of Ash3a/b. These genes are specific for neural determination and the initiation of neural cell differentiation.

4.1.1.5 CHD4 regulates the expression of NeuroD

Shortly after the identification of Ash3a/b and its function during neural development, a further gene, coding for a basic helix-loop-helix (bHLH) transcription factor has been identified, which is referred to as NeuroD. NeuroD is transiently expressed in differentiating neurons and displays several functional characteristics, which are consistent with its role as a differentiation factor for neurogenesis downstream of Ash3a/b. Further on, evidence was provided that the expression of Ash3 and NeuroD is coupled (Kanekar et al, 1997). This renders NeuroD to be functionally located in the centre of the neural transcriptional network. In *Xenopus* embryos, gain-of-function analyses demonstrated that overexpression of NeuroD can convert epidermal fate into neuronal fate (Lee et al, 1995). In *Xenopus*, expression of NeuroD is first detected at NF stage 14 in the trigeminal placodes and in rows of primary neurons adjacent to the midline of the spinal cord and the lateral edges of the neural plate. These locations correspond to the primary motor neurons and primary mechanosensory neurons; the latter are also referred to as Rohon-Beard cells. The primary neurons are the first neurons to differentiate and are responsible for establishing the earliest behavioural responses during early stages of *Xenopus* development. In vertebrates, neural crest cells give rise to skeletal components of the head, the ganglia of the peripheral nervous system and pigment cells. Among these derivatives, NeuroD expression is restricted to the cranial sensory ganglia.
NeuroD is only expressed after the time of neuronal commitment *in vivo*, while it is able to activate the entire neurogenesis program upon overexpression. The following analysis investigates, if CHD4 dependent chromatin remodelling regulates the expression of NeuroD as a key factor during neural development. In Figure 18 A, the overexpression of wtCHD4 results in an ectopic expression domain of NeuroD on the injected side of the embryos (black arrow), compared to the uninjected control side. The reduction of endogenous CHD4 protein by injecting Morpholinos against CHD4 mRNA results in a reduced NeuroD expression on the injected side (white arrow),

compared to the uninjected control side. Figure 18 B illustrates the penetrance of the different NeuroD expression phenotypes due to CHD4 misexpression. In agreement with the observations described previously, the presence of a functional ATPase domain is not necessary for the induction of NeuroD gene expression, as overexpression of dnCHD4 led to a phenotypic alteration of NeuroD expression comparable to the wtCHD4 condition (blue arrow).

A

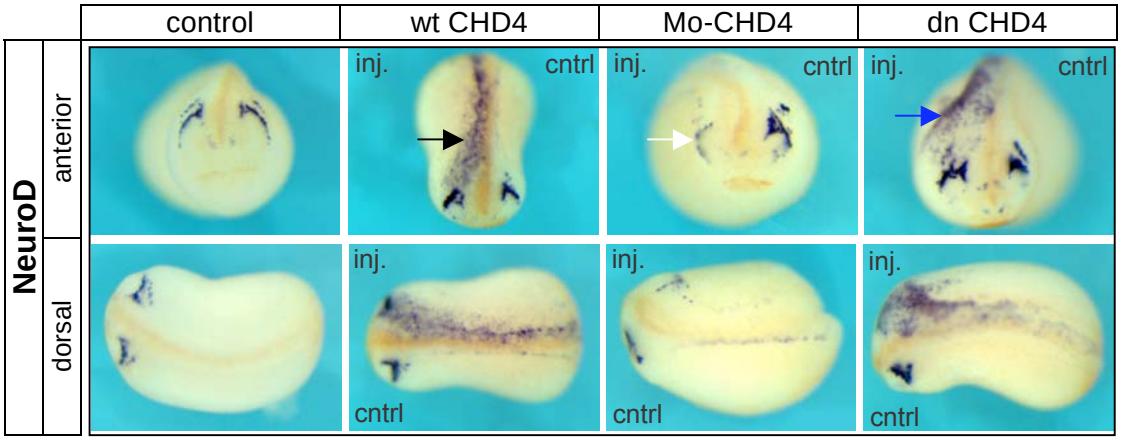


Figure 18 A: CHD4 regulates NeuroD expression

A) Representative illustration of NeuroD gene expression analysis on the mRNA level. The control embryos on the left show the unaltered endogenous expression of NeuroD. The panel illustrates the qualitative difference of NeuroD gene expression due to CHD4 misexpression (inj.) compared to the uninjected control side (cntrl) by in situ hybridization. Overexpression of wt- and dnCHD4 induces NeuroD gene expression within the domain of the developing neural tissue (black and blue arrows), compared to the control embryo. Reduction of endogenous CHD4 levels (Mo-CHD4) reduces the endogenous expression domain of NeuroD (white arrow) compared to the uninjected control side of the embryo.

B

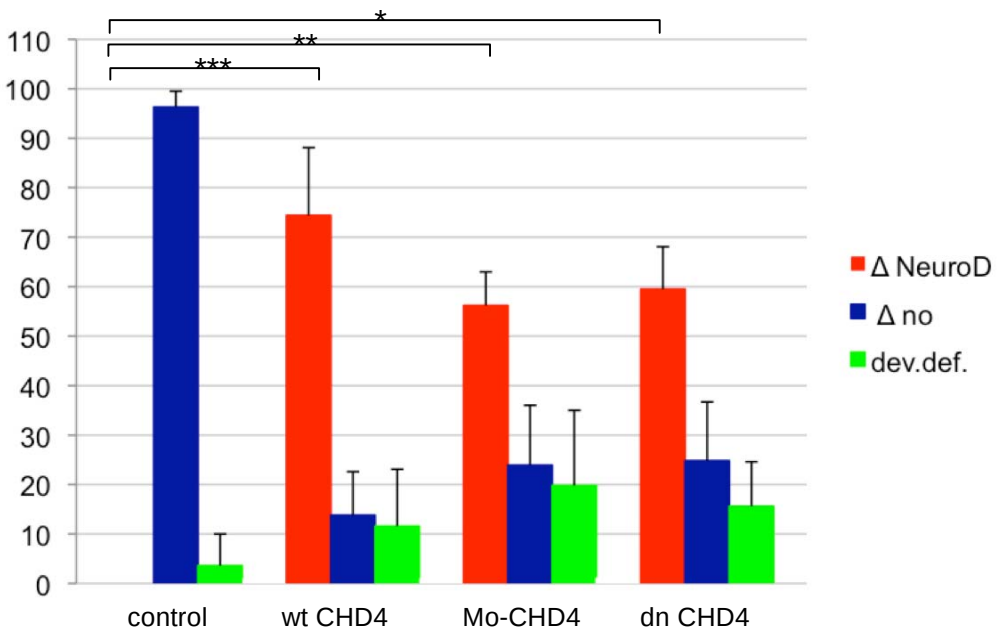


Figure 18 B: Quantitative analysis of altered NeuroD expression

B) The chart illustrates the mean phenotypic penetrance in percent on the y-axis. Δ NeuroD = different NeuroD expression compared to the uninjected control, no Δ = no difference compared to control, dev.def. = embryos died from developmental defects. Standard deviation reflects three independent experiments. The significance level was set to $p \leq 0.05$.

	control	**wt CHD4**	**Mo-CHD4**	**dn CHD4**
n = ∑198	n = 49	n = 48	n = 52	n = 49
p-value, Δ NeuroD	-	***p = 0.001	**p = 0.003	*p = 0.002

In conclusion, these results demonstrate that epigenetic chromatin remodelling by CHD4 promotes neural induction with the expression of early neural marker genes and is able to initiate neural fate determination. To further specify this data, and characterize the developing neural tissue, the influence of CHD4 chromatin remodelling on additional specific neural genes was investigated.

4.1.1.6 CHD4 affects the expression of N-CAM

N-CAM belongs to a heterogeneous group of cell surface glycoproteins, apparently encoded by one gene. These cell surface molecules are primarily expressed in the developing nervous system (Murray et al, 1986).

N-CAM expression first appears as an early response to neural induction at the neural plate stage and roughly corresponds with the developmental stage of neural induction (Kintner & Melton, 1987). N-CAM expression at early stages of neural plate formation does not occur homogeneously in all regions of the neural plate. At early stages, N-CAM expression is concentrated in the deep neuroectodermal layer with low expression in the superficial layer. Later, expression is more concentrated in medial regions of the deep neuroectoderm with very low expression in the neural folds. N-CAM is not expressed in the surrounding ectoderm or underlying mesoderm.

Figure 19 A illustrates that overexpression of CHD4 results in a heterogeneous phenotypic alteration of N-CAM expression. The overexpression of wtCHD4 shows slight effects on the N-CAM expression domain (black arrow). The embryos injected with dnCHD4 however show an enlarged expression domain with a broadening of the neural plate on the injected side of the embryo (blue arrow), compared to the uninjected control side. The reduction of endogenous CHD4 protein by injecting Morpholinos against CHD4 mRNA results in a reduced N-CAM expression on the

injected side (white arrow), compared to the uninjected control side. Figure 19 B illustrates the quantitative penetrance of the different N-CAM expression phenotypes.

A

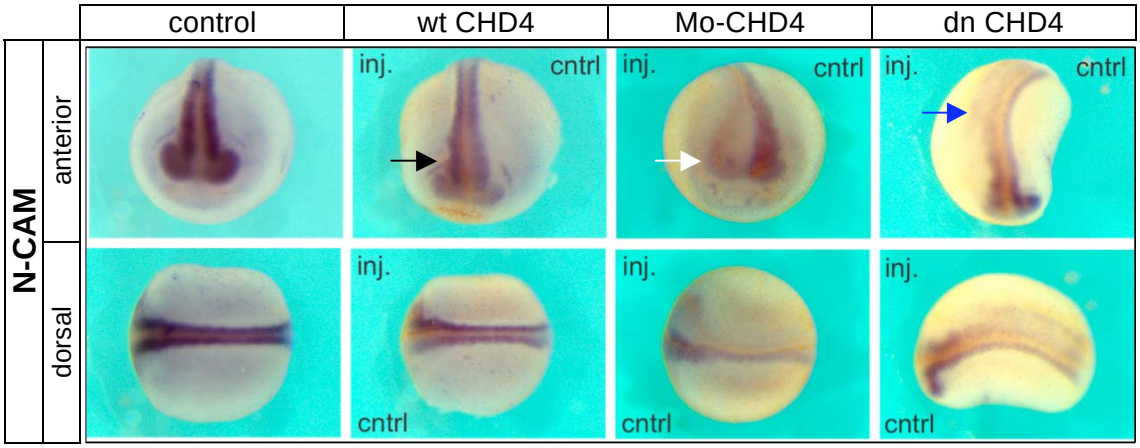


Figure 19 A: CHD4 affects N-CAM expression

A) The mRNA in situ hybridization of N-CAM mRNA illustrates the qualitative difference of N-CAM gene expression due to CHD4 misexpression (inj.) compared to the uninjected control side (cntrl). The injected side is the right side of the embryo. The upper row shows the anterior view of a stage 19 *Xenopus* embryos, the lower row displays a dorsal view. Overexpression of wtCHD4 shows slight alteration of the N-CAM expression domain. The embryos injected with dnCHD4 show an enlarged expression domain with a broadening of the neural plate. Morpholinos against endogenous CHD4 mRNA results in a reduced N-CAM expression.

B

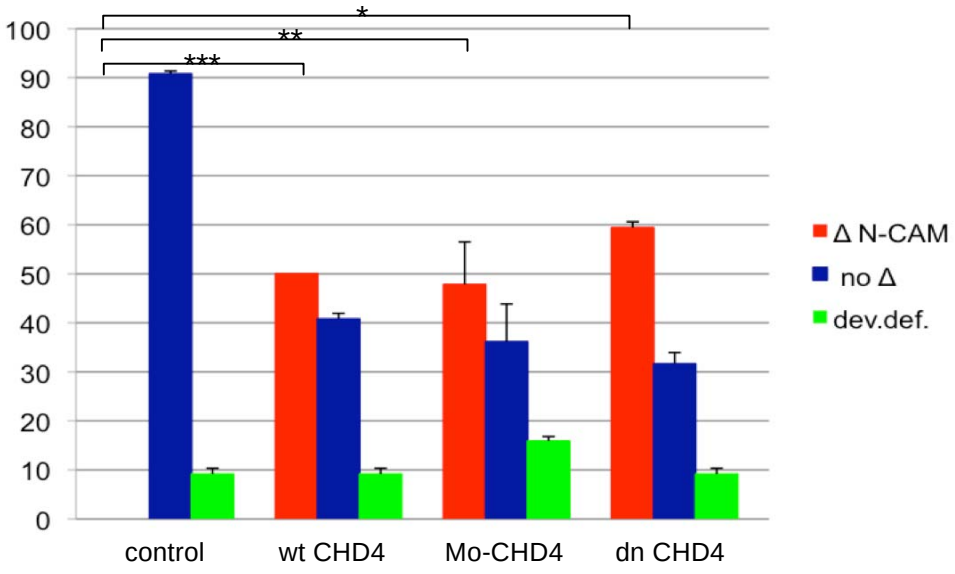


Figure 19 B: Quantitative analysis of affected N-CAM expression

B) The chart illustrates the mean phenotypic penetrance in percent on the y-axis. Δ N-CAM = different N-CAM expression compared to the uninjected control. no Δ = no difference compared to control. dev.def. = embryos died from developmental defects. Standard deviation reflects three independent experiments. The significance level was set to $p \leq 0.05$.

	control	**wt CHD4**	**Mo-CHD4**	**dn CHD4**
n = ∑91	n = 22	n = 22	n = 25	n = 22
p-value, Δ N-CAM	-	***p = 0.001	**p = 0.002	*p = 0.0007

These results indicate that CHD4 chromatin remodelling changes the expression of the neural marker gene N-CAM. In contrast to the CHD4 dependent alteration of neural marker gene expression analysed before, the alteration of N-CAM expression is more restricted to the endogenous expression domain and less ectopically induced in adjacent ectodermal tissue. This observed difference in neural gene expression alteration let to the question, if CHD4 chromatin remodelling does not only affect the neural induction process, but also neural cell differentiation. The following investigates, if CHD4 chromatin remodelling influences the process of neural cell differentiation.

4.1.1.7 CHD4 interferes with neural cell differentiation

The results demonstrate the induced, ectopic expression of Sox2 and, in contrast, the altered expression of N-CAM within its physiological expression domain by CHD4 overexpression. Sox2-class genes are essential for early neuroectoderm cells to consolidate their neural identity during secondary steps of neural differentiation (Kishi et al, 2000). Consistent with its function in maintaining a population of proliferating neural progenitors, Sox2 induces neural progenitors but delays neural cell differentiation at the same time at the expense of non-neural ectodermal derivatives (Rogers et al, 2009a). Consequently, I asked if CHD4 dependent induction of Sox2 expression affects the process of neural cell differentiation. As a marker for finally differentiated neurons, the expression of the neural gene n-ß tubulin was analysed. Figure 20 A shows that overexpression of wtCHD4 results in an induction of neural tissue as observed in the analysis of early neural marker genes, i.e. Sox2, which induces neural progenitor cells, but delays neuronal differentiation at the expense of non-neural ectodermal derivatives. Within the induced Sox2 expressing neural tissue, n-ß tubulin is not homogenously expressed (black arrow). In contrast,

dnCHD4 leads to a broadening of the neural tissue on the injected side of the embryo (blue arrow), compared to the uninjected control side. The reduction of endogenous CHD4 protein by injecting Morpholinos against CHD4 mRNA results in a reduced n-ß tubulin expression on the injected side (white arrow), compared to the uninjected control side and compared to the uninjected control embryos. Figure 20 B shows the quantitative analysis of alterated n-ß tubulin expression due to CHD4 misexpression.

A

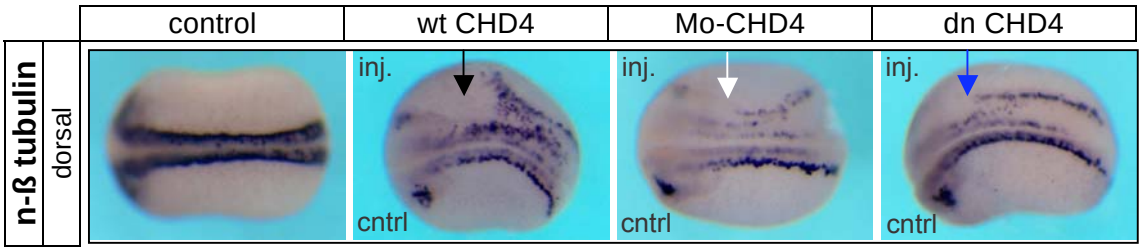


Figure 20 A: CHD4 reduces n-ß tubulin expression

A) Representative illustration of the n-ß tubulin gene expression analysis on the mRNA level. The control embryo on the left shows the unaltered endogenous expression of n-ß tubulin. The panel illustrates the qualitative difference of n-ß tubulin gene expression, due to CHD4 misexpression (inj.) compared to the uninjected control side (cntrl) by in situ hybridization. Overexpression of wt- and dnCHD4 results in reduced n-ß tubulin gene expression within injected domain of the developing neural tissue (black and blue arrows), compared to the control embryo. Reduction of endogenous CHD4 levels (Mo-CHD4) reduces the endogenous expression domain of n-ß tubulin (white arrow), compared to the uninjected control side of the embryo. The panel demonstrate the dorsal view of NF stage 19 embryos. The anterior view did not add any information and was omitted.

B

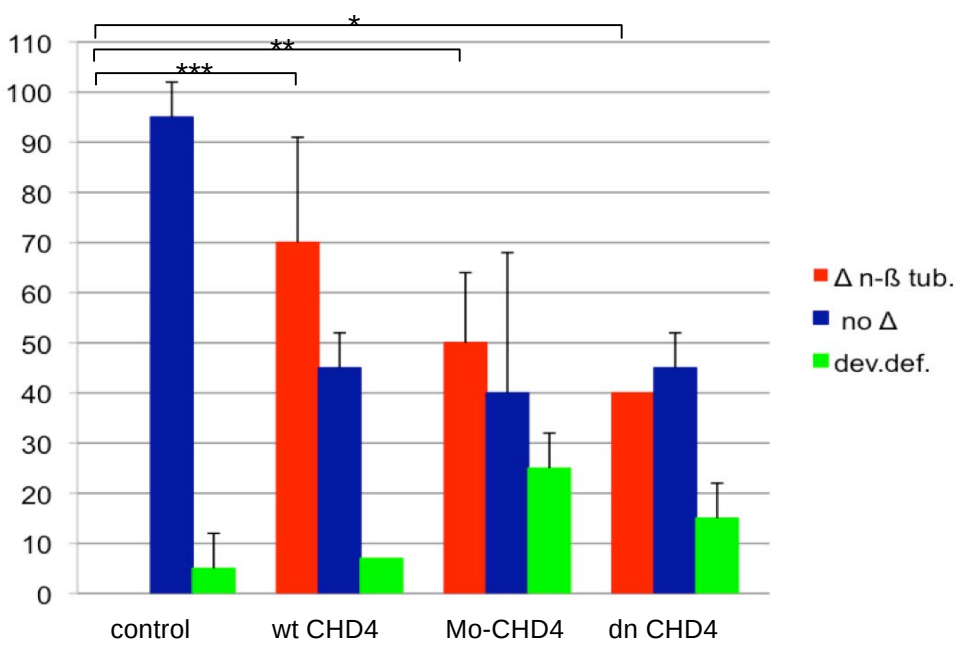


Figure 20 B: Quantitative analysis of n-ß tubulin expression

B) The chart illustrates the mean phenotypic penetrance in percent on the y-axis.

Δ n-ß tubulin = different n-ß tubulin expression compared to the uninjected control, no Δ = no difference compared to control, dev.def. = embryos died from developmental defects. Standard deviation reflects two independent experiments. The significance level was set to $p \leq 0.05$.

	control	wt CHD4	Mo-CHD4	dn CHD4
n = ∑92	n = 23	n = 23	n = 23	n = 23
p-value, Δ n-ß tubulin	-	***p = 0.0001	**p = 0.015	*p = 0.003

In summary, this data renders a specific function of CHD4 chromatin remodelling during the early neural induction process. In addition, an influence on neural cell differentiation could be observed: The induction of prospective neural tissue is accompanied by a delayed or blocked differentiation process of neural tissue, due to wtCHD4 overexpression, as fewer cells show positive staining for n-ß tubulin.

In conclusion, the analysis of n-ß tubulin revealed that CHD4 promotes the induction of a proliferating neural progenitor cell population, which is kept in a proliferating and undifferentiated state.

4.1.1.8 CHD4 affects Pax6 expression

Pax6 was first identified as a paired box (Pax) family member and cloned based on its homology to the *Drosophila* gene *paired.* It is highly conserved among vertebrate and invertebrate species. Pax6 is essential for the development of the central nervous system, including patterning of the neural tube, formation of neural circuits, including the eye, spinal cord and cerebral cortex, as well as pancreatic islet cells (Glaser et al, 1992; St-Onge et al, 1997). In *Xenopus*, Pax6 is expressed in the telencephalon at early developmental stages and, in particular, it was analyzed in relation to putative migratory cells in the primordial septum and olfactory bulb (Bachy et al, 2002; Moreno et al, 2008). Pax6 is mostly known to be critical for eye development (Callaerts et al, 1997). This was demonstrated in analyses showing that mutations in the Pax6 gene cause the *ANIRIDA* syndrome in humans (Glaser et al, 1992; Jordan et al, 1992), the *Small eye* phenotype in mouse (Hill et al, 2010) and the *eyeless* phenotype in flies (Quiring et al, 1994). Misexpression of Pax6 in *Xenopus laevis* leads to the formation of differentiated ectopic eyes (Chow et al,

1999). These observations suggest that the functions of Pax6 in eye development are conserved from invertebrates to vertebrates (Gehring, 1996; Quiring et al, 1994).

Figure 21 A illustrates the qualitative alteration of Pax6 gene expression, due to CHD4 misexpression. In wtCHD4 injected embryos a slight reduction of Pax6 expression could be detected (black arrow). This observation gains significance, when it is compared to the results presented below in 4.2.1.4, when CHD4 chromatin remodelling is activated at later developmental stages.

During the early developmental stages of gastrulation, Pax6 is restricted to its physiological expression domain. CHD4 misexpression affects Pax6 expression as it reduces the intensity of Pax6 expression on the injected side. Consequently, CHD4 affects Pax6 expression at this state.

A

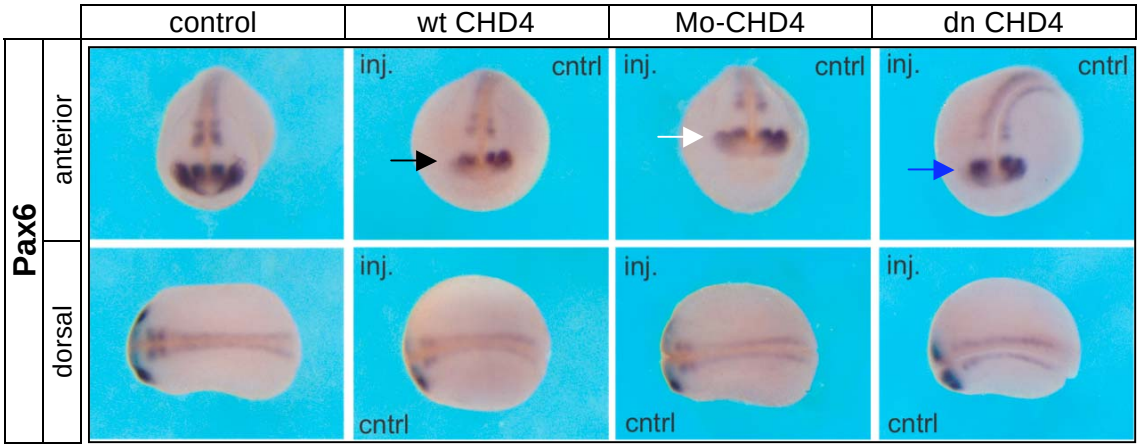


Figure 21 A: CHD4 influences Pax6 expression

A) The panel demonstrates a representative illustration of the Pax6 gene expression analysis on the mRNA level. The control embryo on the left shows the unaltered endogenous expression of Pax6 of a NF stage 19 embryo. The panel illustrates the qualitative difference of Pax6 gene expression due to CHD4 misexpression (inj.) compared to the uninjected control side (cntrl) by mRNA in situ hybridization. Overexpression of wt- and dnCHD4 reduces Pax6 gene expression within its domain (black and blue arrows), compared to the control embryos. Reduction of endogenous CHD4 levels (Mo-CHD4) also reduces the endogenous expression domain of Pax6 (white arrow), compared to the uninjected control side of the embryo.

B

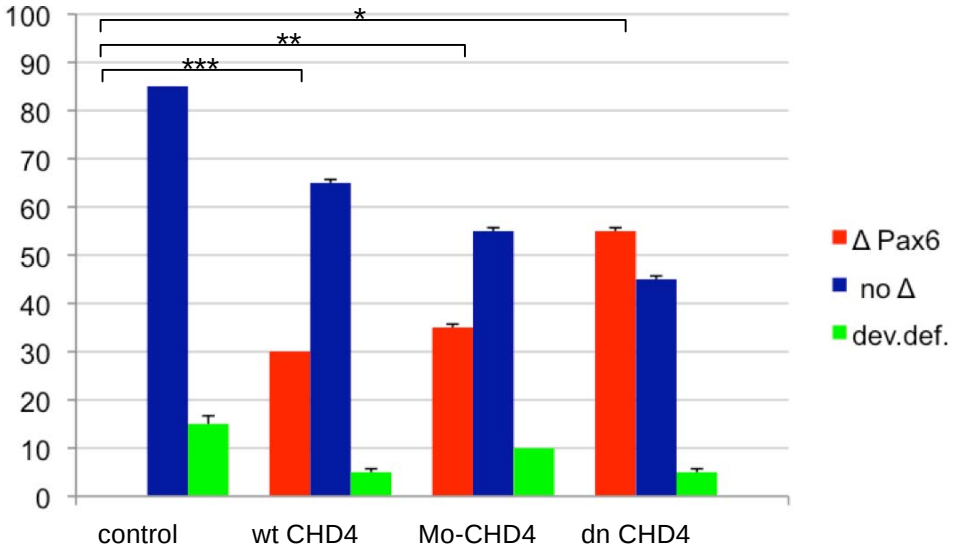


Figure 21 B: Quantiative analysis of Pax6 expression

B) The chart illustrates the mean phenotypic penetrance in percent on the y-axis of altered Pax6 expression. Δ Pax6 = different Pax6 expression compared to the uninjected control, no Δ = no difference compared to control, dev.def. = embryos died from develomental defects. Standard deviation reflects two independent experiments. The significance level was set to $p \leq 0.05$.

	control	**wt CHD4**	**Mo-CHD4**	**dn CHD4**
n = ∑81	n = 20	n = 20	n = 20	n = 21
p-value, Δ Pax6	-	***p = 0.028	**p = 0.015	*p = 0.0037

In conclusion, CHD4 chromatin remodelling influences neural induction and keeps neural progenitor cells in an undifferentiated state, as it delays or prevents neuronal differentiation during early developmental stages. The next intention was to find a potential transcriptional binding partner, which interact with CHD4 and works in concert with its function during neural development. As demonstrated in Figure 8, CHD4 and Sip1 have spatially overlapping expression patterns within the developing neural tissue. In the following section, I investigated, if Sip1 might act together with CHD4 during neural development to promote neural induction and affect neural cell differentiation.

4.1.2 Sip1 functions in concert with CHD4 chromatin remodelling

Sip1 belongs to the family of δEF1 proteins, δEF1/ZEB1/Zfhx1a and Smad interacting protein-1 SIP1/δEF2/ZEB2/Zfhx1b, which were originally identified as transcriptional repressors but can also work as transcriptional activators (van Grunsven et al, 2001) (Postigo et al, 2003). The proteins of this family have multiple conserved domains. Beside a *h*omeo*d*omain (HD) and a *C*-terminal *b*inding *p*rotein (CtBP) *b*inding *s*ite (CBS), Sip1 has two two-handed *z*incfinger *d*omains, one at the *N*-terminus, (NZf) and one at the *C*-terminus (CZf). Sip1 also harbours a *S*mad *b*inding *d*omain (SBD). The SBD interacts with regulatory Smads, which are the mediators of the TGF-β superfamily BMP signalling pathway (Funahashi et al, 1993; Verschueren et al, 1999; Yoshimoto et al, 2005). (See Figure 22)

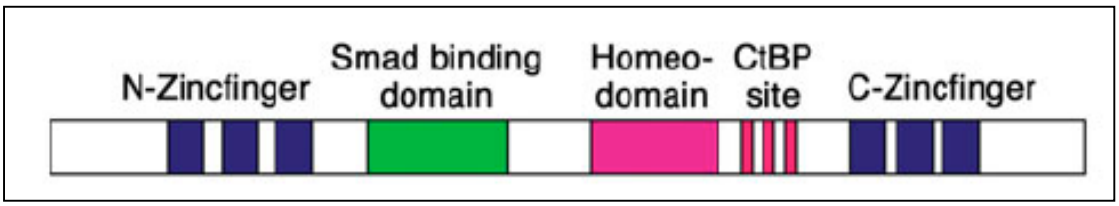


Figure 22: Conserved functional Sip1 domains. Adapted from (Nitta et al, 2007).

Inhibition of BMP signalling is a crucial step in neural induction, reviewed by (De Robertis & Kuroda, 2004; Harland, 2000; Rogers et al, 2009b; Weinstein & Hemmati-Brivanlou, 1999). *Xenopus* Sip1 (XSip1) was described to inhibit BMP signalling and downstream target genes to push the prospective epidermal tissue towards a neural fate (Eisaki et al, 2000; Nitta et al, 2004; Postigo, 2003; van Grunsven et al, 2006). The neuralizing activity involves the co-repressor CtBP and occurs through both BMP dependent and independent mechanisms (van Grunsven et al, 2007). Experiments with domain-deletion mutants of XSip1 suggest that the N-terminal zincfinger domain of XSip1 plays an important role in neural induction, however the repression on Xbra transcription by XSip1 involves a mechanism distinct from its neural inducing activity (Nitta et al, 2007).

As mentioned before, FGF signalling results in the induction of ChCh expression, which activates Sip1. In *Xenopus*, Sip1 is expressed in the neural region by nodal signals, where it antagonizes the induction of the mesodermal marker gene brachyury (xbra) and promotes neural induction. Consequently, Sip1 is a crucial factor to establish the neuroectdermal-mesodermal boundary formation. Our

laboratory could identify *Xenopus* Sip1 to directly interact with CHD4 and we could demonstrate that the CHD4/Sip1 module regulates the boundary formation between the mesodermal and the neuroectodermal germ layer (Linder et al, 2007; Verstappen et al, 2008). Moreover, CHD4 directly binds to the promoter region of the Sip1 gene.
Based on this data and my observation that CHD4 regulates neural gene expression, as presented above, I asked, if the CHD4/Sip1 regulatory module influences the boundary formation between the neural and neuroectodermal germ layer formation. First, I investigated the influence of Sip1 misexpression on neural marker genes, which characterise proliferating neural precursor cells and differentiated neurons. Furthermore, the influence of CHD4 misexpression in combination with altered Sip1 expression levels on neural development was evaluated.

4.1.2.1 CHD4/Sip1 increases the pool of neural progenitor cells (Sox2), while inhibiting neuronal differentiation (n-ß tubulin)

The analysis of CHD4 and Sip1 on neural development was performed by injecting 1ng Sip1 mRNA, or 1ng Sip1 in combination with 1ng CHD4 mRNA in one dorsal blastomere of a 4-cell stage embryo.
Figure 23 A illustrates the result of Sip1 overexpression, which resulted in the extension of Sox2 expressing prospective neural tissue on the injected side of the embryo (red dashed line), indicated by a broader and a thicker Sox2 positive neural tissue (see Figure 23 A and B). Sip1 overexpression results in more prospective neural tissue, however the induced tissue lacks the expression of n-ß tubulin (white dashed line). Consequently, consistent with the previous observations presented above, cells expressing Sox2 are delayed or hindered in their differentiation process. In Figure 23 B, the induction of neural tissue is demonstrated in a second dimension. The arrow indicates the increase of Sox2 expressing neural progenitor cells on the injected side. Figure 23 C illustrates the quantitative analysis of the altered Sox2 and n-ß tubulin expression due to Sip1 overexpression.

A

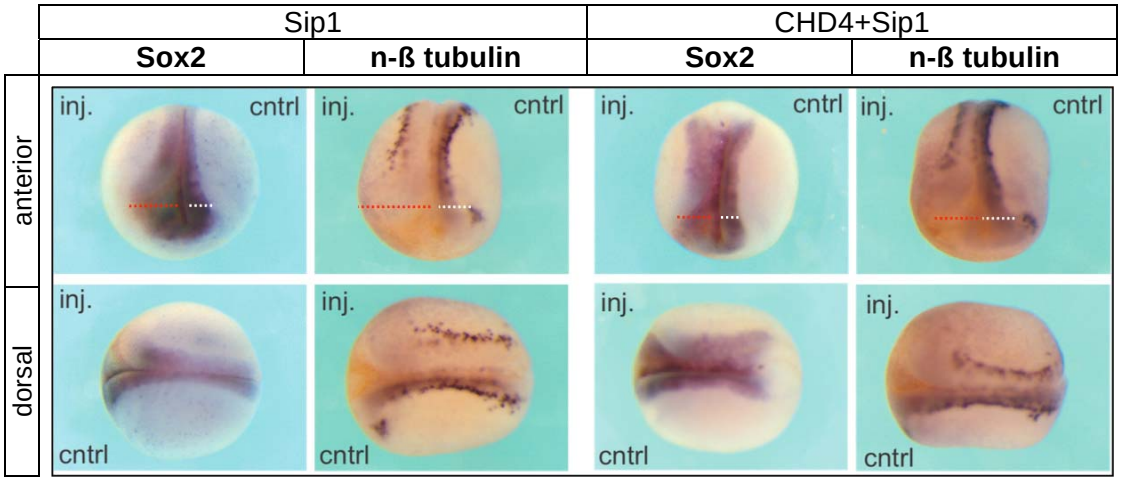


Figure 23: Sip1/CHD4 increases the pool of proliferating neural progenitor cells

A) The in situ hybridization of Sox2 and n-ß tubulin mRNA illustrates the qualitative difference of Sox2 and n-ß tubulin gene expression due to Sip1 overexpression and Sip1 in combination with CHD4 (inj.), compared to the uninjected control side (cntrl). The injected side is the right side of the embryo. The upper row shows the anterior view of a stage 19 *Xenopus* embryo, the lower row displays a dorsal view.

B

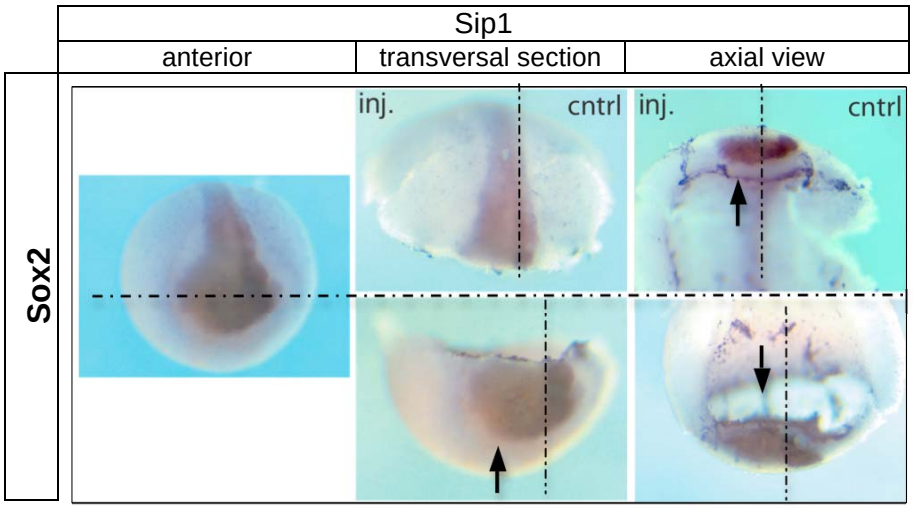


Figure 23 B: Sip1 induces the pool of neural progenitor cells

B) This illustration demonstrates the amount of induced neural progenitor cells indicated by Sox2. The embryo was sectioned along the horizontal dashed line. The upper half was flipped upwards 90° and the lower half was flipped downwards 90° to provide an axial view. The increased pool of proliferating neural progenitor cells are indicated by arrows.

C

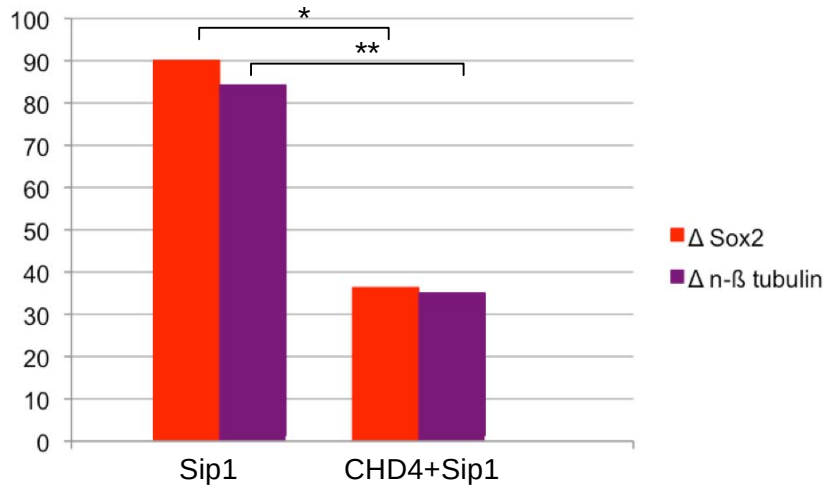


Figure 23 C: CHD4 reduces the penetrance of the Sip1 phenotype

C) The chart illustrates the difference of penetrance in percent on the y-axis. Δ n-ß tubulin = difference of n-ß tubulin expression compared to the uninjected control. Δ Sox2 = difference of Sox2 expression compared to the uninjected control. The significance level was set to $p \leq 0.05$.

	control	**Sip1**	**wtCHD4 + Sip1**
n = ∑42	n = 10	n = 10	n = 22
p-value, Δ Sox2	-	*p = 0.023	

	control	**Sip1**	**wtCHD4 + Sip1**
n = ∑49	n = 10	n = 19	n = 20
p-value, Δ n-ß tubulin	-	**p = 0.0027	

Interestingly, when Sip1 mRNA is co-injected with CHD4 mRNA in blastomers, which will contribute to neuroectodermal tissue, the penetrance of the Sip1 induced phenotype was reduced (see Figure 23 C). However, the morphology of the phenotype was not altered by Sip1/CHD4 co-injections. CHD4 overexpression reduces the endogenous Sip1 protein level. This feed back mechanism establishes a functional equilibrium of the CHD4/Sip1 regulatory module with an inhibitory effect on BMP signalling during the early developmental stages, which is necessary to induce neural tissue. Consequently, I asked, whether CHD4 or Sip1 misexpression could result in a typical phenotype that is associated with BMP inhibition: that is the formation of a secondary body axis.

4.1.2.2 Sip1 induces partial secondary body axes

Since the original experiment of Spemann and Mangold (1924) the isolation of molecules, which are responsible for the inductive activities of the organizer, has been referred to as Holy Grail of vertebrate embryologists. Screens of a cDNA library prepared from Spemann-Mangold organizer tissue identified several secreted factors, which function as antagonists of growth factors by binding them in the extracellular space. Among these factors, the BMP inhibitors Chordin and Noggin are thought to play a central role in organizer function (Lamb et al, 1993; Piccolo et al, 1996; Sasai et al, 1995; Sasai et al, 1994; Smith & Harland, 1992; Zimmerman et al, 1996). Overexpression of the BMP antagonists Chordin and Noggin can induce neural tissue in ectodermal explants and their expression is required for neural tissue formation in the absence of mesoderm (Kuroda et al, 2004). Furthermore, over expression of Chordin and Noggin in the *b*lastula *C*hordin and *N*oggin *e*xpressing centre (BCNE) was shown to result in the induction of secondary axis formation. This demonstrates that the organizer phenomenon can be reproduced by the injection of single molecules (De Robertis et al, 2001).

Taking my results into consideration, the BMP antagonists Chordin and Noggin has been describe to induce *Xenopus* Sip1 expression in animal cap explants, and the use of multiple dominant-negative BMP receptors (BMPRs), which block BMP signalling, greatly enhances secondary axis formation in the whole embryo (Yamamoto & Oelgeschlager, 2004). An exclusive inhibition of the BMP pathway in wild-type embryos results in the development of partial secondary axis with trunk and tail structures that lack head and forebrain tissues.

To investigate, if Sip1 inhibits BMP signalling, as a mechanism that would lead to neural induction as demonstrated above, I asked if Sip1 could induce secondary axis formation as a sign for BMP inhibition. Sip1 was overexpressed by injecting 1ng Sip1 mRNA in one dorsal blastomere of a 4-cell stage embryo. In accordance with the mechanisms of secondary axis formation described above, Sip1 overexpression had the potential to induce secondary axis formation (see Figure 24). Consequently, Sip1 has a neural inducing effect on the one hand and can mimic the function of the BCNE molecules Chordin and Noggin upstream of Sip1 on the other hand via inhibition of BMP signalling. The panel illustrates the secondary axis formation due to Sip1 overexpression by mRNA injection in one dorsal blastomere of a 4-cell stage embryo. The lower panel demonstrates the affected tissue, which forms secondary dorsal body axes, with green fluorescent Alexa Dextrane.

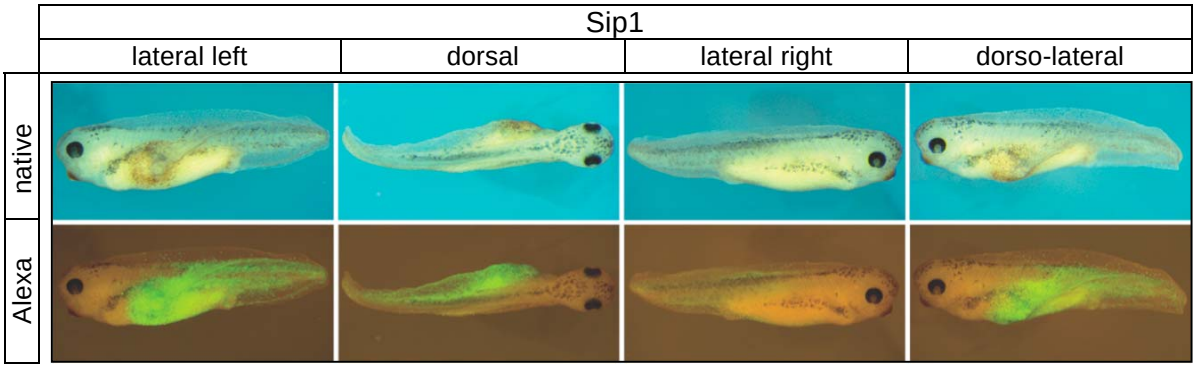


Figure 24: Sip1 induces the formation of secondary dorsal body axes
Secondary partial axes can be induced by Sip1 overexpression. The green fluorescent area indicates the induced axes on the injected dorsal side of the NF stage 40 embryos. Due to co-injection with Alexa Dextrane, cells that overexpress Sip1 can be traced by green fluorescence. The lower panel illustrates the affected tissue by Alexa Dextrane fluorescence that gives rise to the second partial body axes.

4.1.2.3 Sip1 induces ventral secondary body axes

The same experimental setup was used as described in 4.1.2.2, with an additional condition of embryos, which were injected on the ventral side instead of the dorsal side. Figure 25 demonstrates that overexpression of Sip1 in one ventral blastomere of a 4-8 cell stage *Xenopus* embryos can establish a secondary axis on the ventral side. Injected cells, which give rise to the secondary body axis, are traced with Alexa Dextrane. I emphasise that Sip1 does not give rise to secondary head formation.

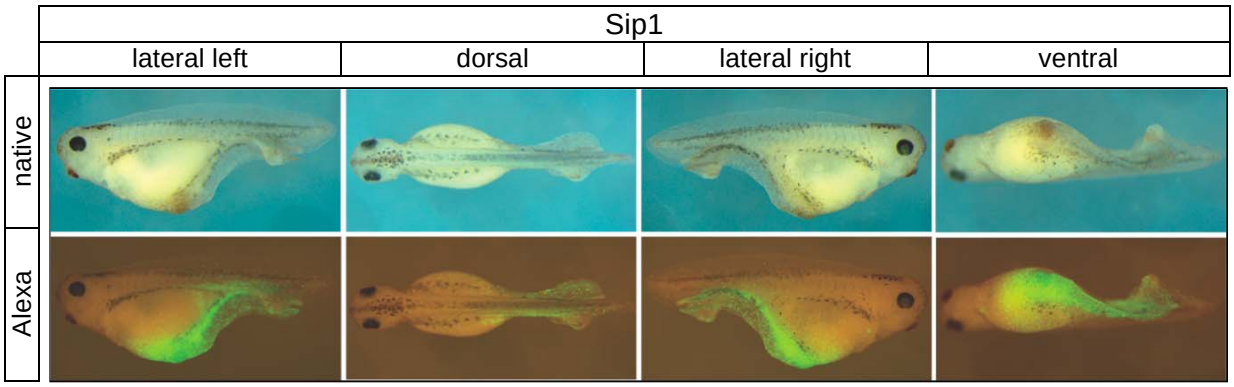


Figure 25: Sip1 induces secondary body axis formation on the ventral side
Secondary partial ventral axes are induced by Sip1 overexpression. The green fluorescent area indicates the induced axis on the injected ventral side of the NF stage 40 embryo. Due to co-injection with Alexa Dextrane, cells that overexpress Sip1 can be traced by green fluorescence. The lower panel illustrates the affected tissue by Alexa Dextrane green fluorescence that gives rise to the second partial body axis on the ventral side of the embryo.

In conclusion, these results indicate that inhibition of BMP signalling is one mechanism, which is executed by the CHD4/Sip1 regulatory module to influence neural development. The next step was to analyze, if altered CHD4 expression levels influence Sip1 dependent secondary axis formation.

4.1.2.4 The Sip1/CHD4 module regulates secondary axis formation

To determine a potential influence of CHD4 on the potency of Sip1 to induce secondary axis formation, I first overexpressed Sip1 and then Sip1 in combination with wt- or dnCHD4. Figure 26 A illustrates the results of secondary axis phenotypes, induced by Sip1 overexpression alone and Sip1 in combination with wtCHD4 and dnCHD4 overexpression. The injected side was traced with green fluorescent Alexa Dextrane, as demonstrated in the lower row.
The graph in Figure 26 B shows the potency of Sip1 to induce secondary axis formation. Co-injection of wtCHD4 together with Sip1 did not alter the penetrance of the secondary axis phenotype, but reduced the phenotype in its morphology. Sip1 overexpression in combination with dnCHD4 leads to the same penetrance but emphasises the characteristic of the phenotype. This is consistent with the repressive effect of CHD4 on the Sip1 gene locus (Linder et al, 2007).
Interestingly, Sip1 alone revealed a high rate of developmental defects, which are reduced by Sip1 and CHD4 co-expression.

A

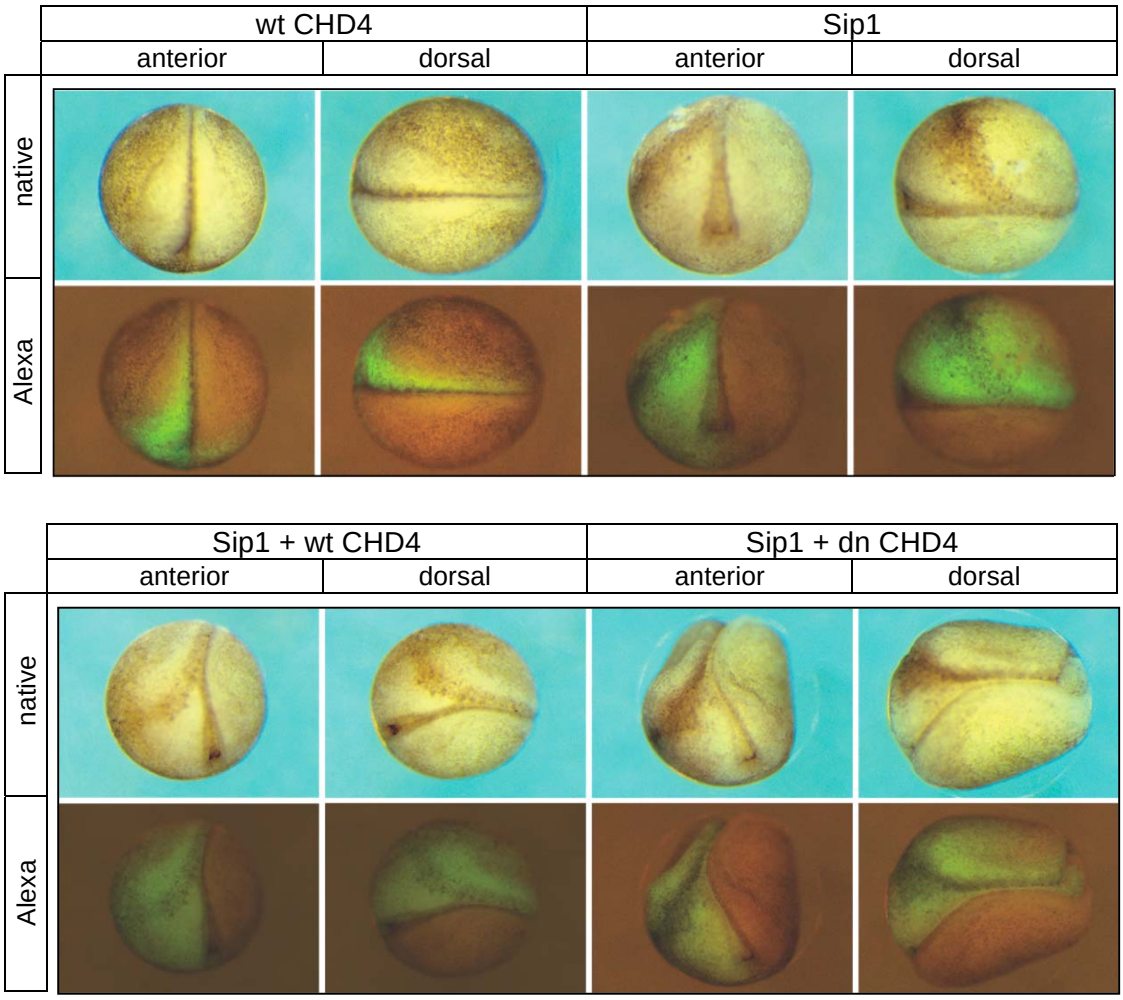


Figure 26 A: The CHD4/Sip1 module regulates secondary axis formation

A) The upper panels (native) illustrate the qualitative analysis of secondary axis formation due to Sip1 overexpression in combination with wt- or dnCHD4. In the lower panel, the injected side and the affected area is marked by green fluorescent Alexa Dextrane. Sip1 in combination with wtCHD4 reduces the characteristic of secondary axis formation. Sip1 overexpression in combination with dnCHD4 leads to the same penetrance but emphasises the characteristic of the phenotype.

B

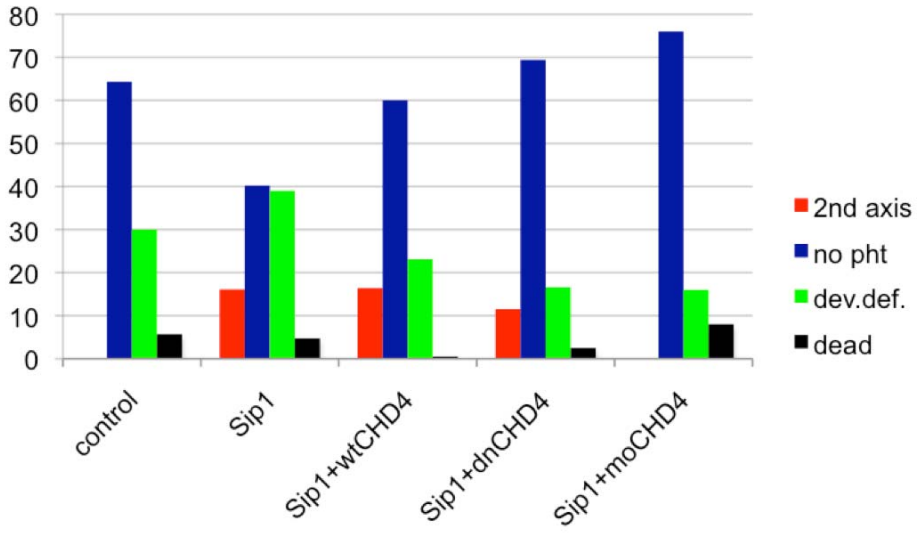


Figure 26 B: Quantitative analysis of secondary axis formation

B) The chart illustrates the quantitative penetrance of Sip1 dependant secondary axis formation of n=392 embryos analysed. Secondary axis formation depends on the endogenous levels of CHD4 protein, as reduction of endogenous CHD4 levels abolished Sip1 dependent secondary axis formation (see 4.1.2.5). Secondary axis formation is less dependent on the functioning CHD4 ATPase domain.

	control	**Sip1**	**Sip1 + wt CHD4**	**Sip1 + dn CHD4**
n = ∑392	n = 105	n = 105	n = 104	n = 78
2nd axis	-	+	+	+

The observation that Sip1 and CHD4 co-expression led to the same penetrance of secondary axis formation, let to the question, whether CHD4 is a necessary factor of the CHD4/Sip1 regulatory module to inhibit BMP, leading to secondary axis formation.

4.1.2.5 Sip1 function depends on endogenous CHD4 levels

To determine the influence of endogenous CHD4 and Sip1 levels, Sip1 and CHD4 were overexpressed in combination with the corresponding CHD4 and Sip1 Morpholino constructs.

First, I evaluated the optimal amount of Morpholinos by injecting a combination of CHD4 and Sip1 Morpholinos that does not cause a toxic effect or sever gastrulation

defects. The concentration of Morpholinos that did not interfere with the gastrulation process was used. The concentration was titrated from about 60ng, 50ng to the optimal amount of 30ng per blastomere. The embryos were injected in one dorsal blastomere at 4-8-cell stage.

Figure 27 demonstrates that overexpression of Sip1 in combination with reduced endogenous CHD4 levels by CHD4 Morpholino co-injection does not induce secondary axis formation. Instead, an additional, tumour-like, unorganized tissue was induced on the injected side of the embryos (see black arrow). The injected side is indicated by fluorescent Alexa Dextrane in the lower panel (see white arrow).

Injections with wtCHD4, MoSip1/MoCHD4 and MoSip1/wtCHD4 did not show any morphological phenotypic alterations on the injected side, compared to the uninjected control side of the embryo. Consequently, an equilibrium of Sip1 and endogenous CHD4 level is necessary for secondary axis formation.

A

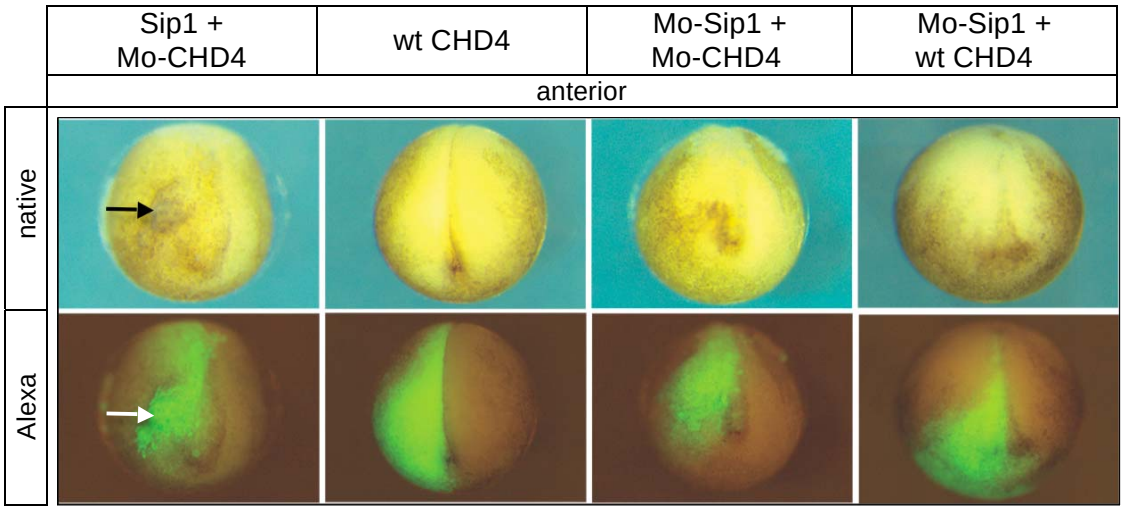


Figure 27: Endogenous CHD4 is necessary for Sip1 function

The upper panel illustrates the morphological alteration due to Sip1 and CHD4 misexpression. The lower panel demonstrates the tissue, which is affected by co-injection with Alexa Dextrane. The arrow indicates the additional, tumour-like tissue that was induced on the injected side of the embryos. The data represent two independent experiments to evaluate abnormal development (ab.dev.) due to loss of function of either, Sip1 or CHD4 or both. This demonstrates the necessity of Sip1 together with CHD4 protein for secondary axis induction.

	control	**Sip1+MoCHD4**	**wtCHD4**	**MoSip1+MoCHD4**	**MoSip1+wtCHD4**
n = ∑92	n = 290	n = 333	n = 105	n = 260	n = 263
ab.dev.	-	++	-	+/-	-

4.1.2.6 Second axis formation includes neural progenitor cells

In respect to the results demonstrated above, the tissue that forms the induced secondary axis was characterized. In analogy to Figure 23 A, the induced secondary axis was analysed by two marker genes for proliferating neural progenitor cells and finally differentiated neurons, i.e. Sox2 and n-ß tubulin, respectively.

Figure 28 demonstrates that the endogenous Sox2 domain along the proper axis overlaps with the endogenous expression domain of n-ß tubulin (see white arrows). This reveals a co-existence of undifferentiated and differentiated neural cells.

In contrast, the induced tissue, which forms the secondary axis, shows a different characteristic: Along the induced secondary axis, Sox2 is expressed within the induced tissue (see blue arrows). In contrast, along the induced secondary axis with a proliferating neural progenitor cell pool n-ß tubulin is not expressed within the Sox2 positive domain (see black arrows). This indicates that in agreement with the previous observations, illustrated in Figure 23 A, the induced Sox2 positive cells are delayed or blocked in their differentiation process.

Consequently, Sip1 and CHD4+Sip1 overexpression induces secondary axis formation of proliferating, prospective neural tissue, which lacks signs for neural differentiation.

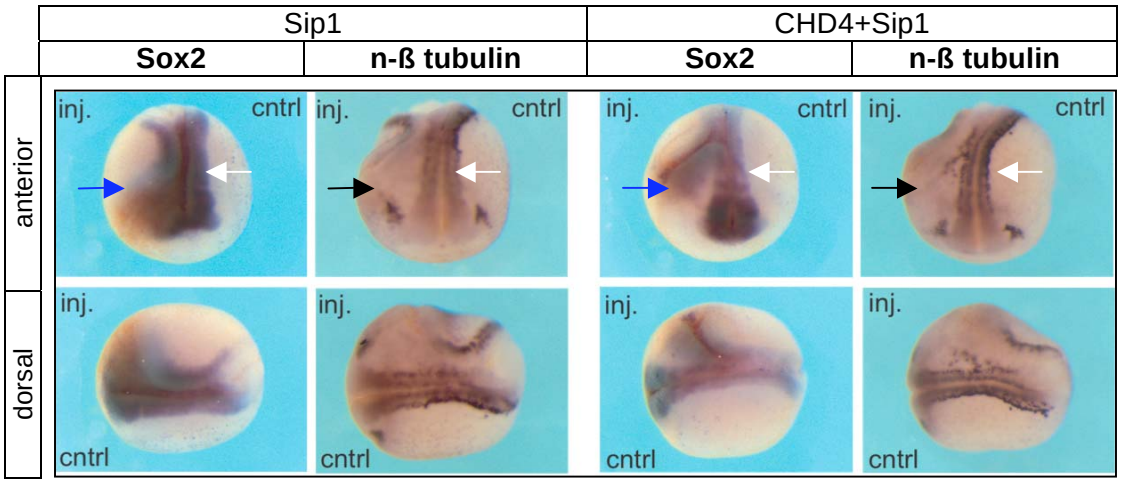


Figure 28: Second axis formation includes proliferating neural precursor cells

The mRNA in situ hybridization of Sox2 and n-ß tubulin illustrates the qualitative difference of Sox2 and n-ß tubulin gene expression due to Sip1 misexpression and Sip1 in combination with CHD4 (inj.) compared to the uninjected control side (cntrl). The injected side is the right side of the embryo. The upper row shows the anterior view of NF stage 19 *Xenopus* embryos, the lower row displays the dorsal view. White arrows indicate overlapping expression domains of Sox2 and n-ß tubulin along the endogenous axis. Along the induced axis, Sox2 positive cells (blue arrows) do not express n-ß tubulin (black arrows).

4.1.2.7 Second axis formation involves mesoderm formation

A complete secondary body axis formation would include not only ectodermal and neuroectodermal tissue but also mesodermal tissue. To ask whether the secondary axes formation could be defined as complete body axis, I analysed, if the observed secondary axes formation includes mesodermal derivatives. The same experimental setup was used as described in 4.1.2.4.

Figure 29 shows the overexpression of wt- and dnCHD4 in combination with Sip1. The endogenous axes of control embryos were stained as mesodermal tissue with the mesoderm marker gene cardiac actin by mRNA in situ hybridization. Interestingly, Sip1 dependent secondary axis formation includes the formation of mesoderm, as indicated by cardiac actin gene expression along the endogenous and the induced secondary axes (see black arrows).

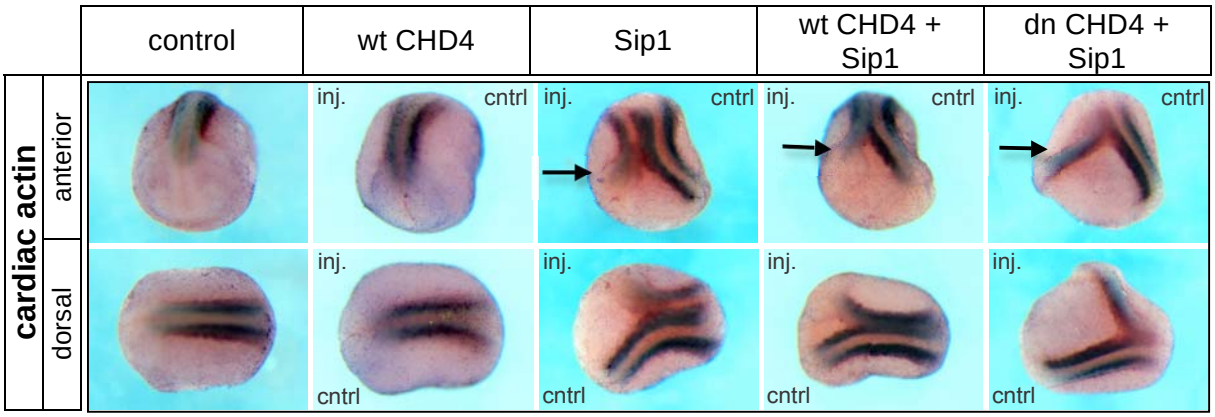


Figure 29: Sip1 and CHD4/Sip1 induced secondary axes include mesodermal tissue

The panel illustrates the secondary axis formation due to Sip1 over expression in combination with wt- or dnCHD4. Induced axes express the mesoderm marker cardiac actin along the secondary body axis (see black arrows). The statistical analysis of secondary axis formation corresponds to the data presented in Figure 26 B.

4.2 CHD4/Mi-2ß function in the chromatin context of neuronal differentiation

The processes of neural induction and neuronal differentiation depend on different influences at different time point during embryonic development. Cellular signals stimulate chromatin changes and influence chromatin organization (Gelato & Fischle, 2008), however, chromatin structure itself influences the gene expression pattern and modulates differentiation processes during development.

Therefore, I asked, whether CHD4 dependent chromatin remodelling may exert different functions within different chromatin contexts.

During early stages of development, my work demonstrates that CHD4 controls the decision between ectoderm and neuroectoderm and directs cells to a neural fate. Before NF stage 13, the developmental processes are directed by translation of maternally inherited mRNAs. At NF stage 8 *Xenopus* embryos undergo MBT (*m*id*b*lastula *t*ransition), a developmental stage where zygotic DNA transcription is activated. This developmental stage is accompanied by an alteration of the chromatin context. To be able to differentiate between observations, which were achieved by the early influences of CHD4 ATPase dependent chromatin remodelling from CHD4 functions during later developmental stages within a different chromatin context, I established a heat-shock promoter (hsp) driven expression construct, based on the *Xenopus* heat-shock protein 70 (Xhsp70) promoter. By the means of a heat-shock dependent inducible gene expression procedure, the expression of desired gene products, i.e CHD4 and GFP, could be activated upon mild heat treatment from neurula stage NF 13 onwards. This approach offers the possibility to distinguish temporal and spatial restricted effects of CHD4 dependent chromatin remodelling without affecting the early cell decision process during ectoderm and neuroectoderm formation, which I analysed before. To reduce cumulative effects due to alterations of the chromatin structure and histone modifications before NF stage 13, the injections were targeted to the dorsal blastomeres, which mainly give rise to neuroectodermal tissue, as indicated by the yellow area in Figure 30. Thus, it was possible to differentiate between early functions of CHD4 chromatin remodelling, as it controls the decision between ectodermal and neuroectodermal tissue, and late CHD4 functions during neurogenesis and neural cell differentiation.

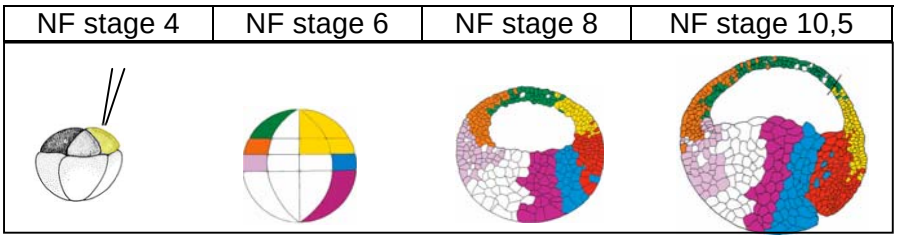


Figure 30: Fate map of cells that give rise to neuroectodermal tissue

Adapted from (Moody, 1987) and (Bauer et al, 1994). Yellow marked blastomeres give rise to neuroectodermal tissue. Targeted injection into the dorsal blastomeres will consequently affect gene expression restricted to neuroectodermal tissue.

4.2.1 Heat-shock inducible gene expression

To establish a heat-shock promoter driven gene expression system, I tested two different protocols to evaluate the efficacy of heat-shock induced transcription activation of the plasmid, potential toxicity of plasmid DNA for the injected embryos and to assess a potential promoter leakiness of the plasmid construct. First, embryos were injected with a plasmid construct, which harbours a heat-shock promoter and a gfp (*g*reen *f*luorescence *p*rotein) sequence (hspCS2+gfp), at 8-16 cell stage in one dorsal blastomere. Then the embryos were cultivated at 16°C for 28h, until NF stage 13. When the injected embryos are cultivated at 16°C the heat-shock promoter should not be activated. In previous experiments, any background activity of the heat-shock promoter was detected in transgene animals (Wheeler et al, 2000).

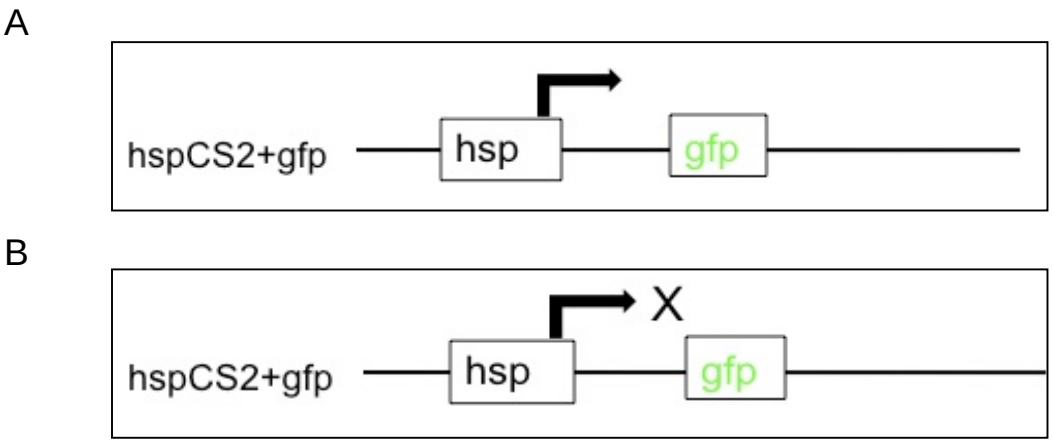


Figure 31: Heat-shock activated GFP expression

A) Mild heat treatment will activate the heat-shock 70 promoter (hsp) to express GFP protein of hspCS2+gfp plasmid constructs, which were injected in one dorsal blastomere

B) Without heat treatment, no activation of the heat-shock promoter occurs (indicated by X).

To test the efficacy of heat-shock dependent gene activation, the embryos were heat-shocked at 34°C three times for 15 minutes with a recovery time at 16°C for 15 minutes between the heat-shock procedures. For a second protocol, I used a recovery time of 45 minutes between the 15 minutes heat-shock procedures, respectively. Successful heat-shock dependent gfp gene activation was evaluated by GFP protein fluorescence. GFP fluorescence was monitored at NF stages 17, 25 and 36. Figure 32 A demonstrates that targeted, heat-shock dependent gfp gene activation in the neuroectoderm results in constant GFP protein fluorescence in the developing CNS.

A

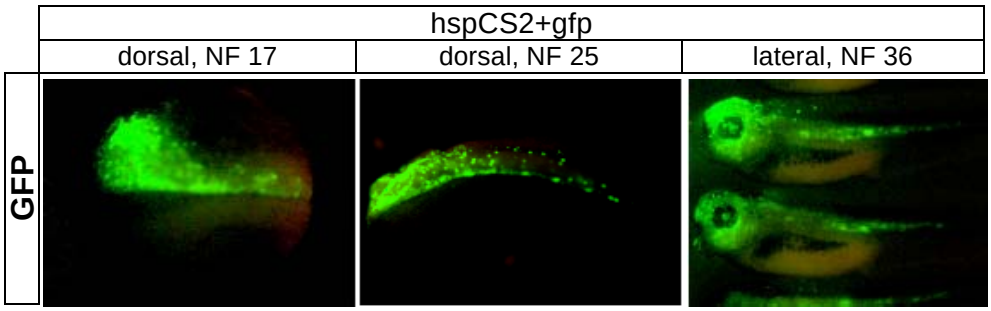


Figure 32 A: Stable hsp-driven GFP expression due to heat-shock promoter activation
A) Illustration of heat-shock dependent GFP expression, restricted to neuroectodermal tissue on the injected side of the embryo. Stable GFP expression was observed from the NF stage 17 onwards to tadpole stages at NF stage 36.

Beside successful gene activation, the positive fluorescence signal of GFP depends on the structure and the correct and complete folding of GFP. Incomplete or incorrect GFP folding would result in false negative results.
On the other hand, a false positive result of GFP detection could be observed due to gene activation without performing heat-shock. This could potentially occour, because of leakiness of the heat-shock promoter of the plasmid construct. To eliminate potential false negative and false positive fluorescence detection of GFP protein, I investigated the hsp driven GFP expression on the transcriptional level by whole mount in situ hybridization against gfp mRNA. The injected intervention group was heat-shocked according to the protocol described in 4.2.1, the control group was continuously cultured at 16°C. Both groups were grown until NF stage 19, followed by whole mount in situ hybridization against gfp mRNA. Figure 32 B shows the expression domain of gfp mRNA in the spatial restricted area of the developing

neural tissue on the injected side of the embryo due to heat-shock driven gfp gene expression. The uninjected control side did not show any in situ hybridization staining. In the control group no in situ hybridization staining was detectable in 95% of the cases, when embryos were grown at 16°C to keep the heat-shock promoter in a silent state. The resulting 5% of temperature independent gfp transcription correlates with the death rate of injected embryos and is due to perturbations in the developmental process, but not due to a temperature dependent leakiness of the promoter.

B

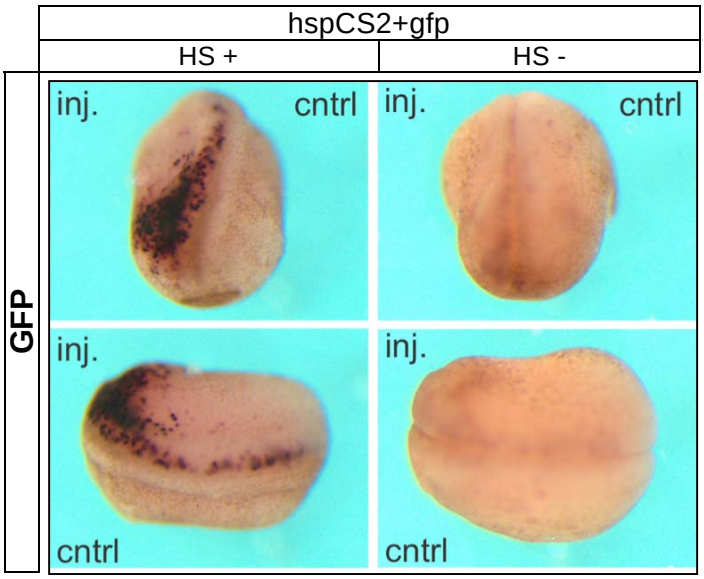
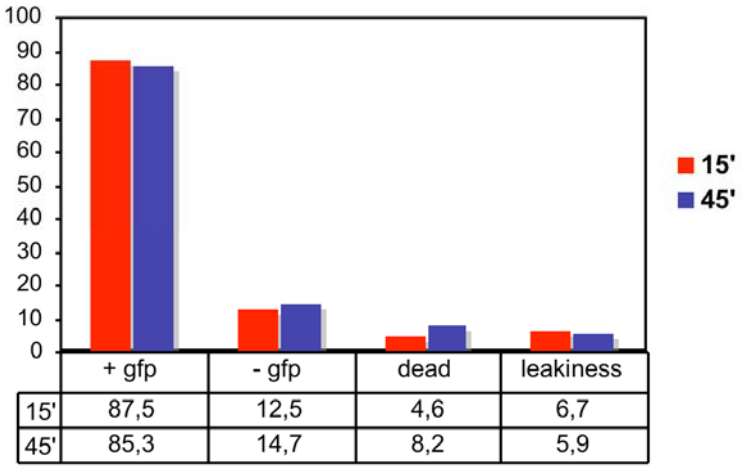


Figure 32 B: Qualitative analysis of heat-shock promoter induced gfp mRNA expression

B) Whole mount in situ hybridization against heat-shock induced expression of gfp mRNA. Embryos were injected as demonstrated in Figure 30.

Embryos, which were heat-shocked (HS +) at 34°C show in situ hybridization stain against gfp mRNA on the injected side. The activated transcription of gfp mRNA is restricted to the developing central nervous system as demonstrated and correlates with the GFP expression as shown in Figure 32 A. The control group has not been heat-shocked (HS -), and was continuously cultured at 16°C. At 16°C the heat-shock promoter was not activated, thus any sign of gfp mRNA staining was detected.

C



	+ gfp	- gfp	dead	leakiness
15'	87,5	12,5	4,6	6,7
45'	85,3	14,7	8,2	5,9

Figure 32 C: Quantitative analysis of heat-shock promoter driven GFP expression

C) The graph illustrates the potency of heat-shock dependent GFP induction according to two different protocols. The first protocol tested a recovery time of 15' (minutes) between the heat-shock procedures. For the second, I tested a recovery time of 45' (minutes), respectively. Embryos were evaluated due to GFP expression, death rate and false positive GFP expression (leakiness). Numbers represent percent of n = 115 embryos for each protocol.

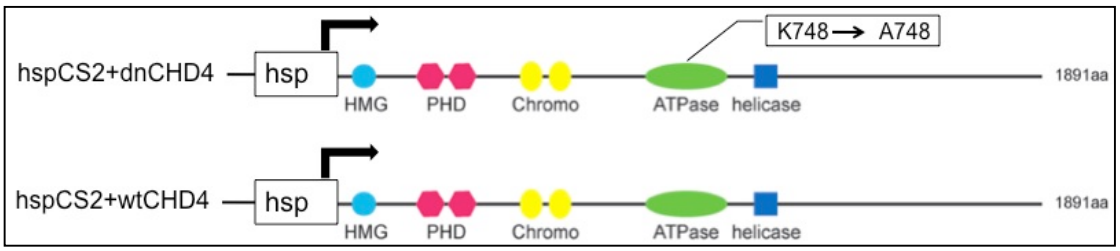
The results of the two protocols with n=115 embryos have been compared and evaluated in regard to the efficiency to induce GFP expression, in regard to toxicity and to leakiness of the hspCS2+gfp plasmid construct (Figure 32 C). The higher frequency of heat-shocks, with the lower recovery time of 15 minutes showed a higher efficiency of gene activation and presented a lower toxicity. Consequently, this protocol was chosen for the following experiments.

4.2.1.1 Heat-shock promoter driven CHD4 gene expression

After establishing this method for spatially and temporally restricted and targeted gene activation, I sub-cloned the wtCHD4 and dnCHD4 DNA sequences into the heat-shock promoter plasmid construct. As a result, CHD4 expression could be activated by heat-shock as established with GFP. This enables to analyse the influence of CHD4 dependent chromatin remodelling spatially restricted to the developing neural tissue. The activation of CHD4 misexpression from NF stage 13 onwards further enables to analyse CHD4 function independent from the early neural

induction process. Consequently, CHD4 function could be investigate from NF stage 13 onwards within the chromatin context of neuronal differentiation.

A



B

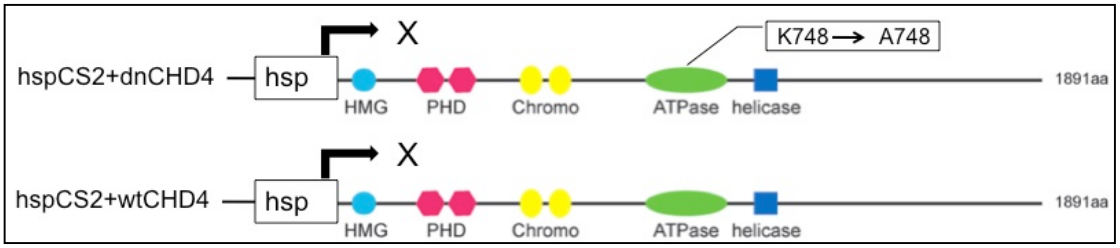


Figure 33: Heat-shock activated CHD4 gene expression

A) Injection of the heat-shock promoter plasmid construct in one dorsal blastomere that gives rise to neural tissue will activate the heat-shock promoter (hsp) upon heat treatment and express wtCHD4 and dnCHD4 protein in a heat-shock dependent manner.

B) Without the heat-shock procedure, the heat-shock promoter does not activate wtCHD4 or dnCHD4 expression as indicated by X.

The successful activation of CHD4 protein expression, due to heat-shock induction, was tested by immunohistochemistry against CHD4 protein.

Figure 33 C shows a coronary section of a NF stage 19 embryo. The embryo shows an increase in the staining signal after heat-shock, representing overexpressed CHD4 protein on the injected side, compared to the uninjected side (cntrl), which reflects the endogenous CHD4 protein levels.

C

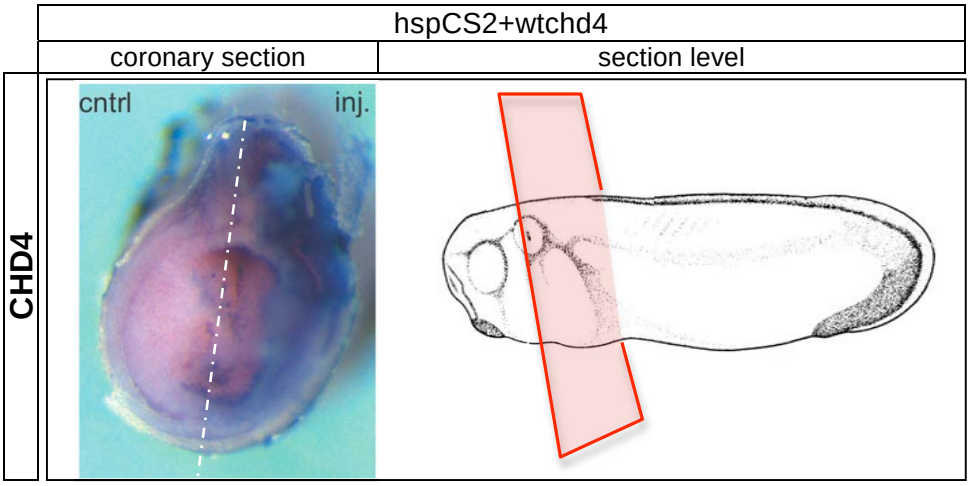


Figure 33 C: Heat-shock activated overexpression of CHD4 protein

C) Heat-shock induced overexpression of CHD4 protein on the injected side of the embryo. The embryo was dissected as demonstrated for better illustration and evaluation for induced CHD4 protein overexpression. The injected side (inj.) reveals induced CHD4 protein expression compared to the uninjected control side (cntrl)., which reveals the endogenous CHD4 protein expression level.

The influence of spatially and temporally restricted, heat-shock induced activation of wt- and dnCHD4 expression in the developing neural tissue was analysed by whole mount mRNA in situ hybridization against neural marker genes, representing uncommitted progenitors and differentiated neurons, marked by Sox2 and n-ß tubulin, respectively. For an additional analysis of neural specific genes, I analysed the influence of wt- and dnCHD4 expression on Pax6 gene expression.

4.2.1.2 Heat-shock induced CHD4 expression affects Sox2 expression

The previous results revealed an induced and ectopic Sox2 expression by mRNA mediated CHD4 overexpression during the early developmental gastrula stages (4.1.1.2). In contrast to this CHD4 function in transcriptional regulation, the heat-shock induced wtCHD4 expression from NF stage 13 (neurula) onwards was targeted to the prospective neural tissue and revealed a different influence on Sox2 expression, after the early neural inducing signals. Figure 34 demonstrates that CHD4 activation reduces expression of Sox2 within its expression domain on the

injected side of the embryo (black arrow), compared to the uninjected control side. The activated overexpression of dnCHD4, resulted in an enhancement of Sox2 expression (blue arrow). No alteration was observed in uninjected control embryos or by injection of a control heat-shock plasmid construct with a gfp sequence (white arrow).

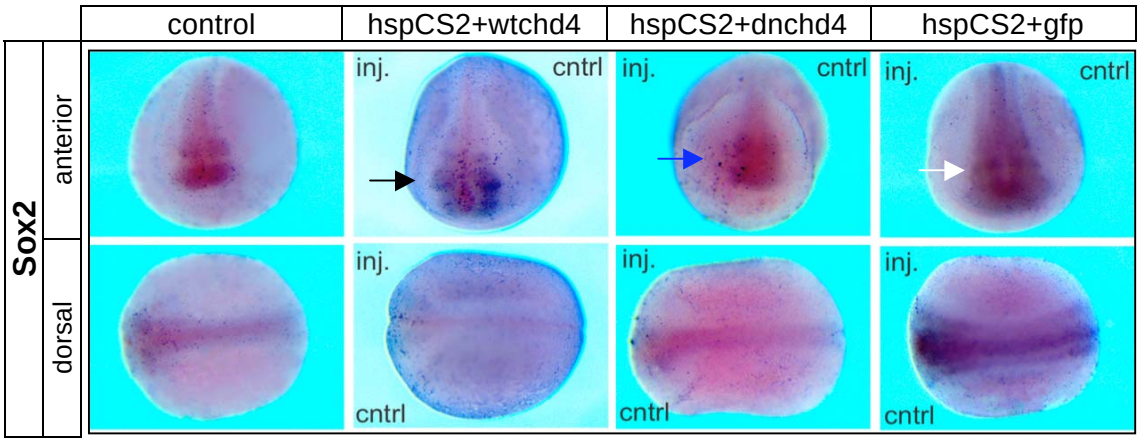


Figure 34: Heat-shock promoter driven CHD4 expression from neurula stage onwards affects Sox2 expression

The panel illustrates the alteration of Sox2 expression due to heat-shock dependent activation of the inducible heat-shock promoter plasmid construct for wt- and dnCHD4 expression (black and blue arrows). The plasmids were injected as demonstrated in Figure 30. The embryos have been grown at 16°C to keep the heat-shock promoter silent until NF stage 13 (neurula), until the heat-shock protocol was performed as described in 4.2.1. Control embryos, which were grown at 16°C did not show any alteration of Sox2 staining. Successful activation of the heat-shock promoter was controlled by GFP fluorescence of hspCS2+gfp injected embryos. The result represents at least two independent experiments with n = 10 embryos each. The penetrance of altered Sox2 expression was 20 %.

In summary, heat-shock dependent activation of CHD4 overexpression at neurula stage reduced the amount of neural progenitor cells, as indicated by the subtle alteration of Sox2. The next question was, whether the observed reduction of Sox2 expressing neural progenitor cells alters the ratio of undifferentiated to differentiated neural cells. Hence, I analysed if activation of CHD4 chromatin remodelling within this developmental context influences the process of neural differentiation.

4.2.1.3 Heat-shock induced CHD4 expression enhances n-ß tubulin expression

The previous results of 4.1.1.7 demonstrated that CHD4 overexpression reduced the expression of n-ß tubulin during the early developmental gastrula stages. In contrast, heat-shock induced wtCHD4 expression from NF stage 13 onwards in the developing neural tissue reveals a different influence on n-ß tubulin expression. Interestingly, an increase of n-ß tubulin staining could be observed by heat-shock dependent induction of wtCHD4 on the injected side (black arrow). In contrast, dnCHD4 reduced n-ß tubulin staining on the injected side (blue arrow), compared to the uninjected control side of the embryo. The control plasmid condition (white arrow), as well as the uninjected control embryos did not show any signs of altered n-ß tubulin expression.

Consistent with my observation that CHD4 misexpression leads to a reduction of proliferating neural cells during later developmental neurula stages, n-ß tubulin, as a marker for differentiated neurons, was enhanced on the injected side.

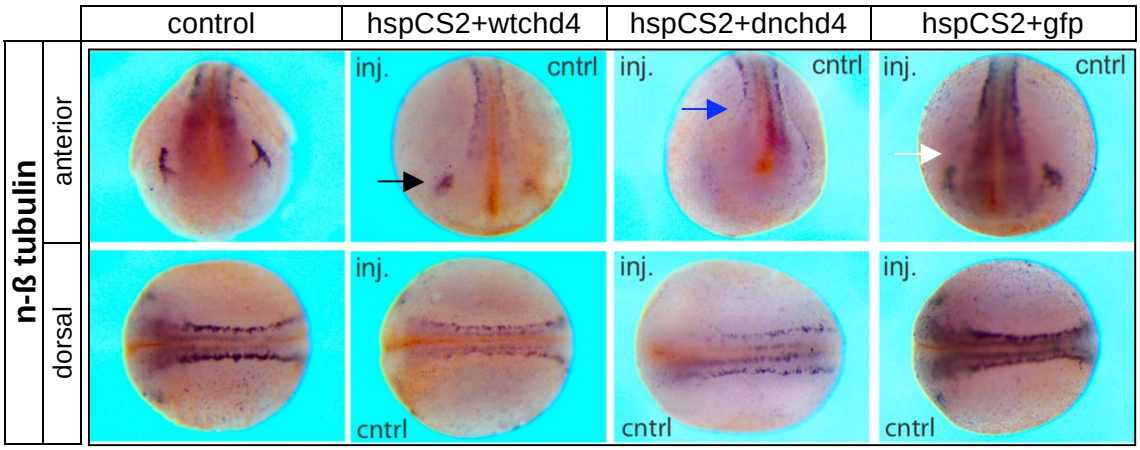


Figure 35: Heat-shock promoter driven CHD4 expression from neurula stage onwards enhances n-ß tubulin expression

The panel illustrates the alteration of n-ß tubulin expression due to heat-shock dependent activation of the inducible heat-shock promoter plasmid construct with wt and dnCHD4 sequences. Control embryos, which were grown at 16°C did not show any alteration of n-ß tubulin staining. Successful activation of the heat-shock promoter was controlled by GFP fluorescence of hspCS2+gfp injected embryos. The result represents at least two independent experiments with n = 12 embryos each. The penetrance of altered n-ß tubulin expression was 33 %.

In conclusion, CHD4 chromatin remodelling promotes the neural differentiation process within the chromatin context from neurula stage onwards. This function of CHD4 depends on a functioning ATPase domain, as dnCHD4 caused the opposite phenotype compared to wtCHD4 overexpression. The crucial point is that this reveals chromatin context dependent functions of CHD4 chromatin remodelling: First, during the early neural induction process CHD4 promotes neural induction but inhibits neural differentiation.

Second, if CHD4 expression is induced from NF stage 13 onwards, it promotes the neural differentiation process. Based on this, further characterisation of CHD4 chromatin remodelling during neural differentiation is provided by investigating the expression domain of the transcription factor Pax6.

4.2.1.4 Heat-shock induced CHD4 expression activates ectopic Pax6 expression

The analysis of Pax6 expression revealed interesting results. The embryos were injected with the heat-shock inducible plasmid as described before. The heat-shock dependent activation of CHD4 expression was performed as described above. The analysis of Pax6 expression revealed a spatial restricted area of cells, which stained positive for Pax6 in a mosaic manner in the restricted area of the developing CNS, where wtCHD4 protein expression was induced (black arrow). The induced Pax6 expression is located at the border of neural to ectodermal tissue and overlaps with the location of neural crest cells. None of the control conditions, or dnCHD4 did reveal ectopic alterations of Pax6 gene expression (blue and white arrows). The ectopic expression of Pax6 is located at the border of head ectoderm to neuroectoderm.

The suppressive effect of BMP signalling on the expression of neural genes, coupled with localized suppressors of BMP signalling results in the fine-tuning of neural gene expression in the anterior neural plate (Hartley et al, 2001). In consistence with the previous observation of the CHD4/Sip1 regulatory module, promoting a BMP inhibiting effect, this fine-tuning involves ATP-dependent chromatin remodelling, to induce the ectopic expression of Pax6 (black arrow).

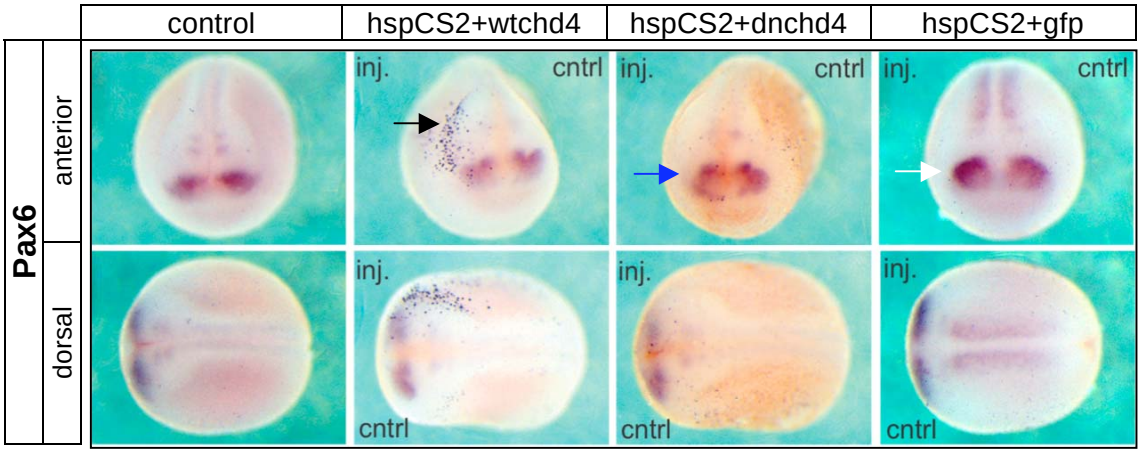


Figure 36: Heat-shock promoter driven CHD4 expression from neurula stage onwards activates ectopic Pax6 expression

The panel illustrates the induction of ectopic Pax6 expression due to heat-shock dependent CHD4 activation, according to the experimental setup as described above. None of the control embryos did show any alteration of Pax6 gene expression. The endogenous expression domain is unaltered. Successful activation of the heat-shock promoter was controlled by GFP fluorescence of hspCS2+gfp injected embryos. The result represents at least three independent experiments with n = 10 embryos each. The penetrance of altered Sox2 expression was 33 %.

This analysis describes differences between CHD4 function during the early developmental stages before neural induction, and functions of CHD4 during a later, more stable chromatin state. During later developmental stages, CHD4 overexpression promotes neural differentiation as indicated by a more prominent n-ß tubulin staining. This apparently involves ATPase activity, since dnCHD4 reduces or delays n-ß tubulin expression. In contrast to the previous observations during the initial steps of neural induction, a functional ATPase domain is crucial for the alteration of neural gene expression within a stable chromatin context.

These differences in gene expression, which indicate an influence on cellular differentiation, due to heat-shock promoter induced expression of wtCHD4 and dnCHD4, should result in an altered rate of cellular proliferation.

However, differences in gene expression could be biased due to an altered rate of apoptosis. Consequently, I analysed if heat-shock promoter driven CHD4 misexpression influences cellular proliferation or may provoke apoptosis.

4.2.2 Characterisation of cell proliferation and apoptosis by H310Ph and expression of activated Caspase3

First, to evaluate the Caspase3 expression as a marker for cells undergoing apoptosis, the ligand “Fas associated death domain” (FADD) was overexpressed by microinjection of 1 ng FADD mRNA in one blastomere of a two-cell stage *Xenopus* embryo. At NF stage 19, embryos were analysed by immunohistochemistry with antibodies against activated Caspase3.

The optimal concentration was evaluated by titrating the primary antibody concentration from 1:100 to 1:500. Cross-reactions of the second antibody with unspecific targets were tested by a second antibody control group (2.AB).

Figure 36 shows the results of Caspase3 detection on the injected side and the endogenous Caspase3 staining on the uninjected control side of embryos. A dilution of 1:500 was evaluated as the optimal amount with the best signal to background ratio. Second antibody alone did not show any staining (2.AB).

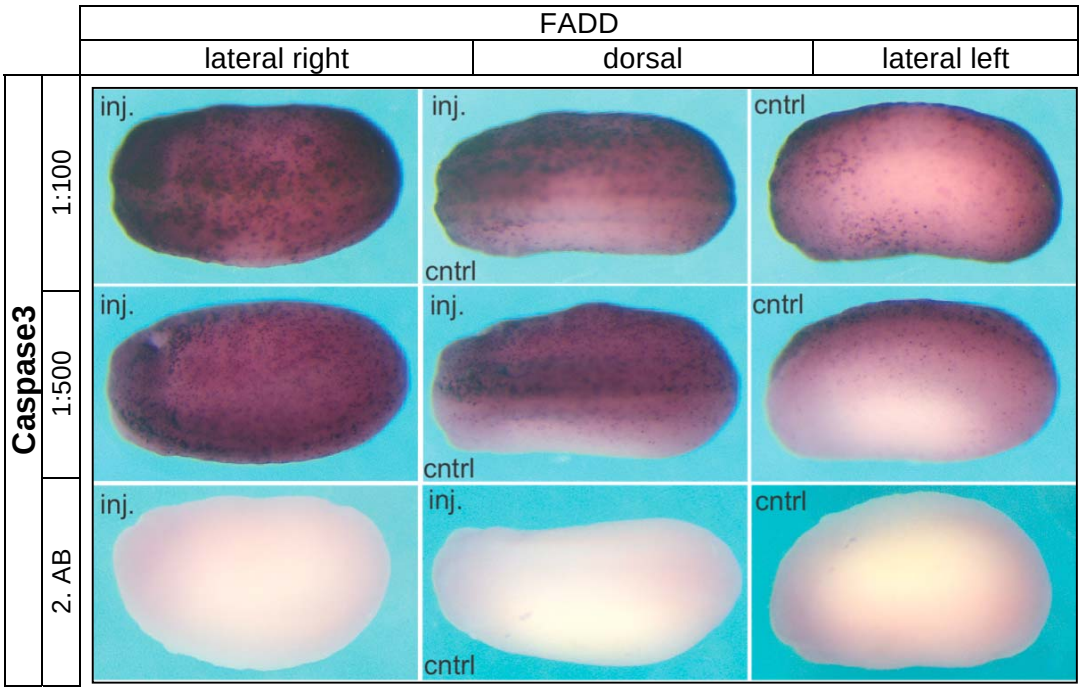


Figure 37: Detection of FADD activated Caspase3

Evaluation of the optimal concentration of Caspase3 antibodies. The second antibody concentrations are listed on the left. The side with FADD overexpression induced apoptosis, which could be detected by anti-Caspase3 antibodies. No background was detected in the second antibody control group, where the first antibody was omitted (2.AB)

After I demonstrated that activated Caspase3 could be applied as a marker for apoptotic cells, the next step was to characterize a marker for proliferating cells. For this intention, epigenetic histone modifications have been taken onto consideration, as described in the next section.

4.2.2.1 Detection of cell proliferation with H3S10 and H3S28 phospho marks

Epigenetic histone marks can be associated with specific cellular functions.
The phosphorylation of histone H3 at serine 10 (H3S10Ph) is described as a marker for proliferation since this modification was discovered to be associated with chromosome condensation and segregation during mitosis and meiosis (Gurley et al, 1978; Schmitt et al, 2002). For additional information, please see chapter 2.4.2.3 about phosphorylation and dephosphorylation in the introduction part.
Since this time, it has been found that mitosis-specific phosphorylation of histone H3 also occurs at serine 28 and at threonine 11 (Goto et al, 1999; Preuss et al, 2003). H3 phosphorylation at serine 10 was described to start during prophase, with peak levels during metaphase. It starts in the pericentric heterochromatin region and proceeds throughout the genome during transition of G2 to M phase. An analysis of histone variants and modifications during the development of *Xenopus laevis* described serine 10 phosphorylation to be most abundant in the early embryo.
Consistent with the rapid chromatin transitions that occur during early developmental stage and the known roles of these histone modifications during the cell cycle, I assessed the abundance of serine 10 and serine 28 phosphorylation by immunohistochemistry in whole *Xenopus* embryos. The aim of this approach was to answer the question, if alteration of neural-specific gene expression by heat-shock induced CHD4 expression is associated with an altered rate of cell proliferation and cell cycle progression or if the observations could be confounded by apoptosis. As a first step, the optimal amount of antibodies against H3 serine 10 and serine 28 phosphorylation was evaluated. Figure 38 shows the results of immuno-histochemistry of whole embryos. The optimal antibody concentration was determined for 1:500 with the best signal to background ratio. As the best results were achieved with anti-H3S10Ph antibodies the following experiments to evaluate the proliferation status were performed by H3S10Ph detection. Second antibodies alone did not show any background signal (2. AB).

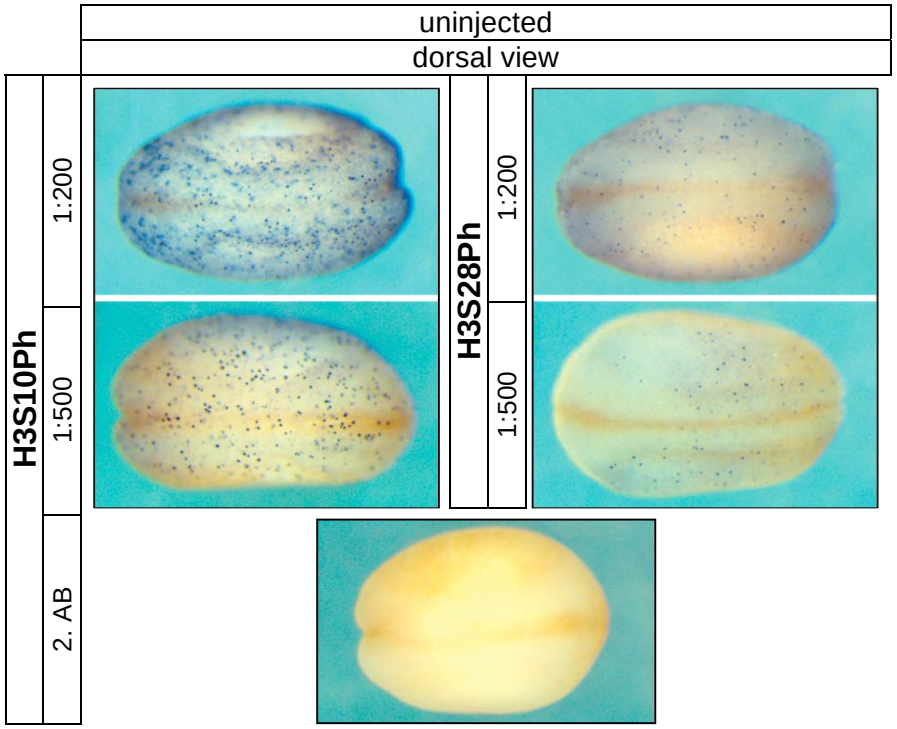


Figure 38: Immunohistochemistry of H3S10 and H3S28 phosphorylation

Detection of H3S10 and H3S28 phosphorylation pattern in whole *Xenopus* embryos. The best results were achieved with anti-H3S10Ph antibodies with a dilution of 1:500. No background was detected in the second antibody control, where the first antibody was omitted.

4.2.2.2 Heat-shock promoter driven CHD4 expression alters H3S10Ph pattern

The same experimental heat-shock procedure was performed as described in 4.2.1.2 - 4.2.1.4 to evaluate, if CHD4 misexpression alters the pattern of H3S10 phosphorylation. Figure 39 shows the result of a reduced H3S10Ph signal on the hspCS2+wtchd4 injected side after heat-shock, compared to the uninjected control side of the embryo. Interestingly, the reduction of proliferation goes along with a higher pigmentation of the eye field. In contrast, heat-shock dependent over expression of dnCHD4 results in more H3S10 phosphorylation positive cells on the injected side compared to uninjected control side. GFP overexpression did not alter the amount of H3S10Ph positive cells, neither did the heat-shock procedure affect the pattern of H3S10Ph. Control embryos, which were cultured at 16°C did show any alteration of the H3S10Ph histone modification pattern (not shown).

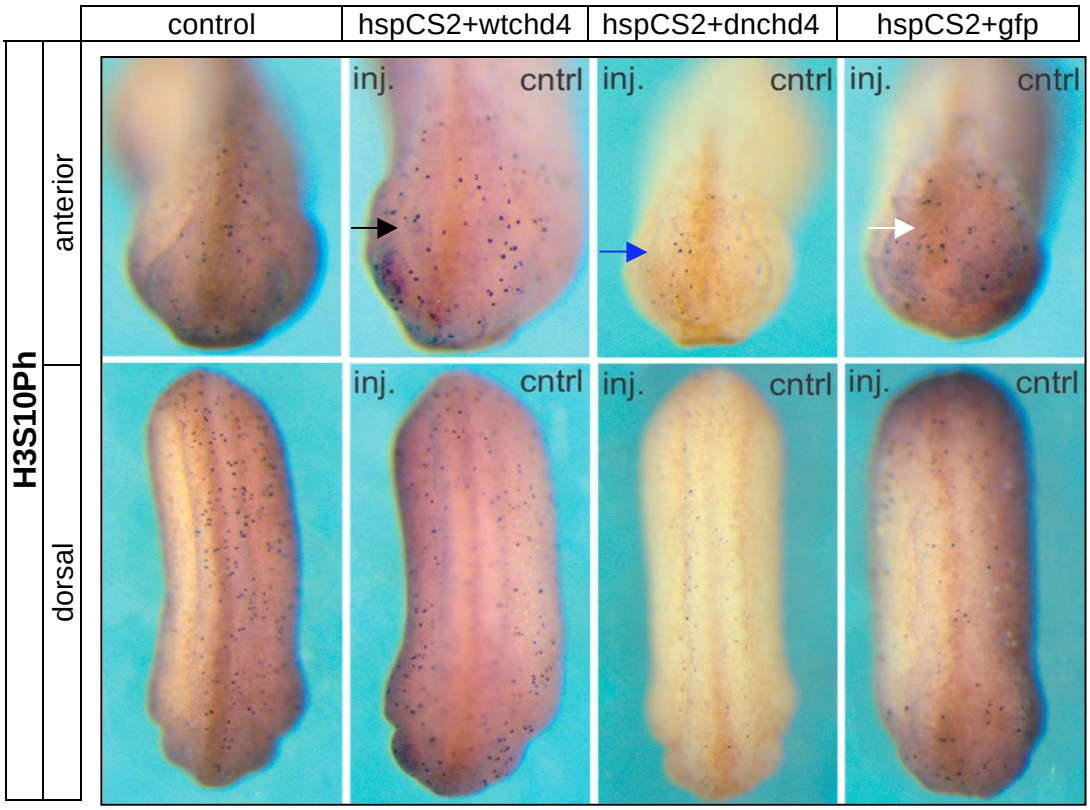


Figure 39: CHD4 expression alters the H3S10 phosphorylation pattern

The illustration gives the impression of the altered H3S10Ph due to heat shock activated CHD4 misexpression. A reduced amount of H3S10Ph positive cells can be counted on the injected side due to wtCHD4 overexpression within the CNS (black arrow). In contrast, dnCHD4 increases the amount of H3S10Ph positive cells on the injected side (blue arrow). Control plasmid injection did not alter the H3S10Ph pattern (white arrow).

In conclusion, the induced or accelerated neural cell differentiation by heat-shock activated wtCHD4 overexpression, as described in 4.2.1.3, is accompanied by a reduction of proliferating cells within the developing CNS, as indicated by a reduction of the active chromatin marker H3S10 phosphorylation (black arrow). In contrast, dnCHD4 overexpression enhanced H3S10Ph histone modification.

Interestingly, in contrast to CHD4 function within the chromatin context during gastrula, CHD4 function during neurula works in an ATPase dependent manner. The promotion of neural cell differentiation was demonstrated by enhanced n-ß tubulin stain in Figure 35. The reduction of proliferating neural progenitor cells was shown by Sox2 in Figure 34. Taken together, the promoted neural cell differentiation goes along with a reduced cellular proliferation rate, indicated by the reduction of

proliferating cells marked by H3S10Ph. Consequently, CHD4 chromatin remodelling promotes neural differentiation within the chromatin context after MBT. However, it is difficult to assess the precise difference of H3S10Ph staining between the investigated conditions. Thus, the next aim was to quantify the qualitative observation of the altered H3S10Ph modification pattern. In addition, I asked, if the observed alteration of neural gene expression and cell proliferation could be confounded due to induced apoptosis. To answer these questions, a further experimental approach was used by confocal microscopy to quantify proliferating cells and detect potential apoptosis.

4.2.3 Confocal microscopy analysis of H3S10Ph and Caspase3

For this experimental setup, the same heat-shock protocol was used as described above. Whole *Xenopus* embryos were subjected to immunohistochemistry with anti-H3S10Ph antibodies as performed in 4.2.2.2.

First, I tested if the antibodies could penetrate into the tissue to provide a throughout analysis of the neuroectodermal tissue. Therefore, the embryos were stained as whole mounts and sectioned as illustrated (see Figure 40 A-C). The red frame illustrates the neural tube and the notochord in an enlarged window. The phospho mark could be detected in both superficial and deep cell layers of the neural tube (see Figure 40 B). This ensured that the detection of H3S10Ph is not restricted to the outer surface of the embryo but is also detected in deeper layers of neuroectodermal tissue.

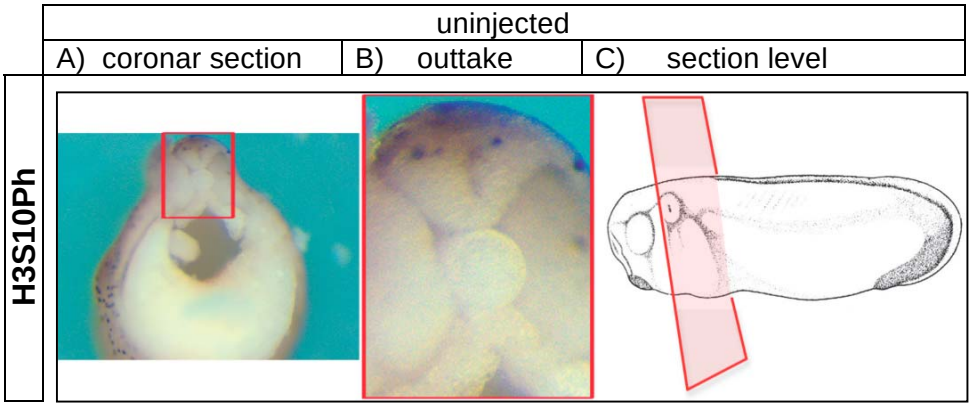


Figure 40: Immunohistochemistry to evaluate diffusion of H3S10Ph antibodies

A) Immunohistochemistry against H3S10Ph to evaluate antibody penetrance into the tissue.

B) Enlarged window of the red outtake of A. The stain is clearly located in superficial and deep cell layers of the neural tube

C) Illustration of the section level

To be able to detect H3S10Ph in deeper tissue layers, fluorescent second antibodies were used. The fluorescence was recorded by confocal microscopy. The data was collected from a z-stack of 70 optical sections through the CNS of NF stage 19 embryos (see Figure 41 A). After detection and data collection, the stacks of 70 slices were 2-dimensionally reconstructed to create an overlay of H3S10Ph positive cells in the restricted volume of the CNS (see Figure 41 B). The mean integrated intensity was measured by ImageJ software over a fixed mask, which enabled the investigation of a defined volume of tissue of the CNS (see Figure 41 C). A detailed description of the quantification process is described in the method section.

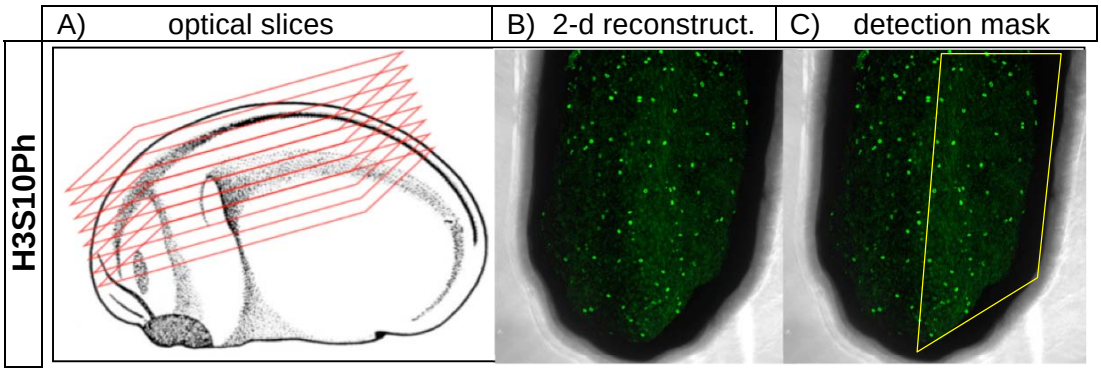


Figure 41: Confocal two-dimensional reconstruction of H3S10Ph marks

A) Embryos were embedded in agarose and scanned in 70 consecutive optical slices of 4 µm thickness.

B) The z-stacks of 70 images were two-dimensional reconstructed to create an overlay of each optical section.

C) Intensity of the fluorescence signal resulting from 70 consecutive optical slices was measured to evaluate the alteration of H3S10 phosphorylation pattern in a defined volume of tissue, which corresponds to the restricted CNS area, where targeted CHD4 misexpression was activated by the hsp70 promoter in a heat-shock dependent manner.

4.2.3.1 Quantification of histone H3S10Ph modification pattern

The same experimental setup was performed as described in 4.2.1.1. The embryos were scanned by confocal microscopy as describe in 4.2.3. To quantify H3S10Ph pattern, the mean integrated intensity of the fluorescent secondary antibodies was quantified. The method section gives a detailed explanation of the quantification procedure to measure the mean integrated intensity by ImageJ software.

Figure 42 A shows the 2-dimensional reconstruction of the qualitative mean integrated fluorescence intensities of secondary FITC and Cy5 coupled antibodies against the primary antibodies against CHD4 protein and the H3S10Ph modification. In Figure 42 B, the differences between mean integrated fluorescence intensities of the injected side relative to the uninjected control side are illustrated as percentage. The positive values refer to the injected side of the embryo. Control embryos with secondary antibodies alone and embyos, which were cultivated at 16°C without heat-shock (HS-) did not show any alteration in the H3S10 phosphorylation status. The quantitative analysis shows that heat-shock dependent overexpression of wtCHD4 led to a 43% increase of the integrated fluorescence intensity of red fluorescence, indicating CHD4 protein, on the injected side of the embryo, compared to the uninjected side. The integrated fluorescence intensity in green, detecting H3S10 phosphorylation, decreased 44% compared to the uninjected side of the embryo. Consequently, a negative correlation of wtCHD4 overexpression and the histone modification H3S10Ph was found.

In contrast, heat-shock dependent overexpression of dnCHD4 positively correlates with H3S10 hyper-phosphorylation, compared to the uninjected side. Uninjected control embryos and the injection of the control gfp heat-shock plasmid constructs revealed only slight differences of integrated intensities. (See Figure 42 B). Considering the biological function of H3S10Ph as described in 4.2.2.2, the influence of CHD4 chromatin remodelling on the histone phosphorylation status indicates an influence on cell cycle progression and on the activity status of the chromatin. According to these observations, the heat-shock promoter induced overexpression of wtCHD4 results in a less active chromatin with a reduced proliferation status of neural cells, which are prone to differentiation. This underlines the previously described observation that induced CHD4 activity after NF stage 13 promotes neural differentiation, as described in 4.2.1.3.

In summary, CHD4 overexpression within the chromatin context after MBT promotes neural cell differentiation in the CNS, accompanied by the reduction of neural cell

proliferation, as indicated by the reduction of epigenetic histone phospho marks, as a sign for an active chromatin status.

A

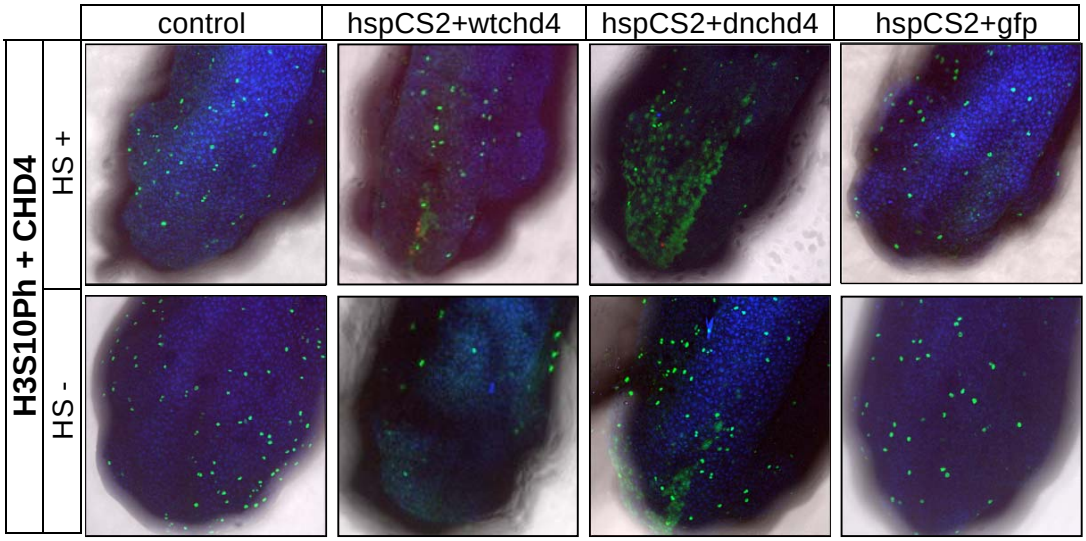


Figure 42 A: H3S10 phosphorylation and CHD4 immunofluorescence

A) The panel illustrates the 2-dimensional reconstruction of the mean integrated fluorescence intensities of secondary FITC and Cy5 coupled antibodies against the primary anti-CHD4 and H3S10Ph antibodies. The HS + panel shows the altered H3S10Ph pattern due to heat-shock dependent activation of the hsp70 plasmid constructs as indicated. The HS – panel serves as a negative control. Control embryos with secondary antibodies alone and embyos, which were cultivated at 16°C without heat-shock (HS -) did not show any alteration of the H3S10 phosphorylation status. Embryos were co-injected with Alexa Dextrane to determine the injected and the uninjected control side. The injection and activation of hspcs2+gfp did not significantly alter the H3S10Ph pattern, neither in the HS +, nor in the HS - condition. H3S10Ph was detected with green fluorescence; CHD4 was detected with red fluorescence. Chromatin was stained with DAPI as indicated by blue fluorescence.

B

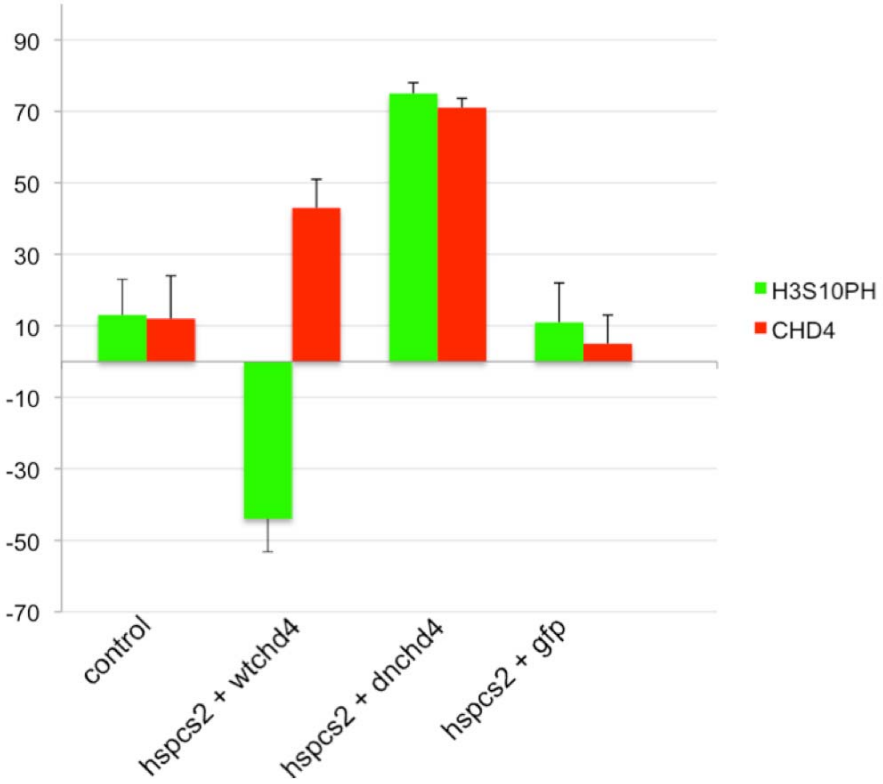


Figure 42 B: Quantification of H3S10 phosphorylation and CHD4 protein expression by immunofluorescence

Quantification of the mean integrated fluorescence intensities was performed with immunofluorescence histochemistry against H3S10Ph and CHD4. The differences between the integrated intensities of the injected sides are illustrated relative to the uninjected control sides. The y-axis represents percentage. 3 - 5 embryos were optically sectioned by confocal microscopy and the mean integrated fluorescence intensities were quantified with ImageJ software. The positive values refer to the injected side of the embryo. Heat-shock dependent overexpression of wtCHD4 led to a 43% increase of the integrated fluorescence intensity of red fluorescence, indicating CHD4 protein on the injected side of the embryo, compared to the uninjected side. The integrated fluorescence intensity in green, detecting H3S10 phosphorylation, decreased 44% compared to the injected side of the embryo. This shows a negative correlation of wtCHD4 overexpression and the histone modification H3S10Ph. Heat-shock dependent overexpression of dnCHD4 positively correlates with H3S10 hyper-phosphorylation, compared to the uninjected side. Uninjected control embryos and the injection of the control gfp heat-shock plasmid constructs revealed only slight differences of integrated intensities.

4.2.3.2 The results are not influenced by Caspase3 activity

These observed influences on cellular differentiation and the associated alteration of histone modifications due to heat-shock dependent CHD4 misexpression could be confounded by apoptosis. Therefore, the same experimental setup was used as described before and the expression level of activated Caspase3 was evaluated. As demonstrated in Figure 37, activated Caspase3 could successfully be detected by anti-Caspase3 antibodies, when apoptosis was triggered by FADD overexpression. To evaluate the potential influence of CHD4 overexpression on apoptosis, the experimental setup as described in 4.2.3.1 was used to detect activated Caspase3 expression. Figure 43 illustrates that no activation of Caspase3 could be detected by heat-shock alone or by heat-shock activated expression of wt- or dnCHD4 or the control gfp plasmid.

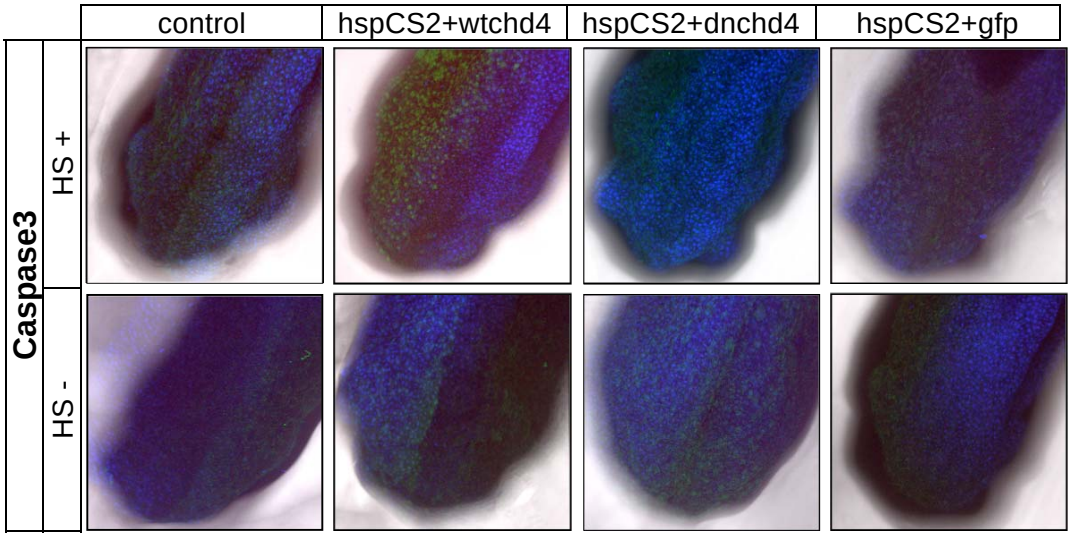


Figure 43: Immunofluorescence of putative Caspase3 expression

The panel illustrates that CHD4 overexpression did not induce Caspase3 expression. Embryos were co-injected with Alexa Dextrane to determine the injected and the uninjected control side. No activated Caspase3 was detected with specific FITC green fluorescence. Chromatin was stained with DAPI as indicated by blue fluorescence.

In summary, I demonstrate two different functions of the CHD4 chromatin remodelling ATPase. First, before neurulation, CHD4 induces the expression of proneural genes accompanied by the reduction of ectodermal gene expression. Together with Sip1, both factors work as a CHD4/Sip1 regulatory module. The results indicate that this module shifts the neuroectodermal boundary for the benefit of neural tissue and at the expense of ectodermal derivates. The induced neural tissue is comprised of proliferating, undifferentiated neural precursor cells. This function is mediated via BMP inhibition. Within the permissive chromatin context of early neural induction during gastrula, this function does not require a functioning ATPase domain of CHD4

The second function of CHD4 chromatin remodelling was demonstrated by its activation at neurula from NF stage 13 onwards. Within this chromatin context, CHD4 promotes neural cell differentiation, which is accompanied by a reduction of cellular proliferation. In contrast to its function during the early steps of neural induction, the effect of CHD4 chromatin remodelling on neural gene expression at neurula do depend on its ATPase function.

The presented results demonstrate a crucial role of chromatin remodelling, mediated by CHD4 in a chromatin and developmental context dependent manner. The data describes a regulatory mechanism, how information of signalling events during stem cell fate decision and tissue differentiation can be integrated onto the chromatin level to dynamically regulate gene expression within the complexity of embryonic development.

5 Discussion

The development from a single cell to a complex organism portrays the events that are investigated by developmental biology, physiology, and medicine. Developmental processes can be studied with genetic and protein interference analyses. These approaches revealed developmentally important genes. To establish hierarchies of these genes it was necessary to determine, whether the influences were direct or indirect. Therefore, one of the major challenges within this quest is to understand, how genetic information is interpreted and integrated to guide the unidirectional process of development, maintenance and adaption of physiological processes, but also disease development, cancer initiation and metastasis formation. Alterations of histone modifications, changes in the composition of DNA binding proteins and nucleosome remodelling are crucial steps in this process of information integration. In this work, I investigated the role of the CHD4 ATPase subunit of the NuRD chromatin-remodelling complex as an important factor during the establishment of neural tissue and the differentiation of neural cells.

5.1 Technical aspects of CHD4/Mi-2ß and Sip1 gain- and loss-of-function interference

5.1.1 Gene expression interference by nucleic acid microinjection

The interference with the endogenous expression levels of CHD4/Mi-2ß was provided by microinjection of 5nl nucleotide solution into the animal pole of one blastomere of a 2-cell stage embryo. CHD4 and Sip1 mRNAs were used at a concentration of 1ng/embryo. The injection of nucleotide acid solutions can cause toxic effects, thus I determined the optimal amount for functional interference without causing toxic effects.

As “controls” for the injected embryos, I injected gfp mRNA or Alexa Dextrane and compared the gene expression pattern and morphology of the injected side with the uninjected control side of the embryos, as well as with uninjected wild-type embryos. Nether injection of gfp mRNA, nor 5% Alexa Dextrane in RNase free DEPC water

resulted in phenotypic alterations of gene expression or did influence morphology. Consequently, I can exclude the following confounding effects:
- The alterations in gene expression, observed by in situ hybridization and/or morphology, could be caused by manipulation, due to the injection procedure.
- The alterations in gene expression, observed by in situ hybridization and/or morphology, could be caused by injection of foreign nucleotides.
- The alterations in gene expression, observed by in situ hybridization and/or morphology, could be caused by injection of Alexa Dextrane.

For the reduction of endogenous protein levels I used 25-mer antisense Morpholino oligonucleotides, complementary to the *Xenopus* CHD4 translation start side, at a concentration of 40ng/cell. The degree of translation reduction due to antisense Morpholino oligonucleotides was not known. To test the efficiency of CHD4 Morpholinos, we subcloned the first 363 amino acids of the CHD4 in-frame upstream of the 6xMyc-tag cassette of the pCS2+MT6 vector, with and without the 5' untranslated region, which is complementary to the CHD4 Morpholino. The in-vitro translation of these test constructs was analysed with increasing amounts of CHD4 Morpholinos, followed by Western blot detection to evaluate the relative levels of the CHD4-Myc protein, as illustrated in the methods section.
Sip1 mRNA translation was inhibited by the published Morpholinos described by (Nitta et al, 2004). Nevertheless, the *in vitro* analysis reveals only a limited impression of the *in vivo* effects of interference with CHD4 protein expression. Consequently, I first evaluated the influence of CHD4 protein overexpression and ablation in whole embryos by whole mount immunohistochemistry against CHD4 protein. This enabled to evaluate the *in vivo* expression of CHD4 protein, due to either mRNA injection or heat-shock dependent over expression. In conclusion, nucleic acid microinjection is a reliable means to induce and reduction *in vivo* CHD4 and Sip1 protein levels. An evaluation of the heat-shock promoter driven gene expression procedure will be provided in 5.3.1.

5.1.2 Quality control of nucleic acids for microinjection and in situ hybridization

The injected wtCHD4, dnCHD4 and Sip1 mRNAs have been individually synthesized for each experiment. The labelled in situ hybridization probes for each gene that was analysed was synthesised and used in control in situ hybridizations with uninjected

embryos to determine the correct identification of the physiological gene expression pattern. For quality control, each mRNA and in situ hybridization probe was checked by gel electrophoresis. Only non-degraded nucleic acids with the correct size were accepted and used for the experiments. Thus, this quality control secured that the investigations are based on correct nucleic acid sequences and not confounded by degraded nucleic acids.

5.2 CHD4/Mi-2ß function within the chromatin context of early neural induction

5.2.1 The role of CHD4/Mi-2ß and Sip1 in neural induction and germ layer formation

During gastrulation three germ layers are formed. In *Xenopus*, the boundary formation between neuroectoderm and mesoderm is established by the transcription factors Sip1 and Xbra, respectively (Papin et al, 2002; Wardle & Smith, 2006). One mechanisms for this boundary formation is the Sip1 mediated repression of the mesodermal gene Xbra (Lerchner et al, 2000; Papin et al, 2002). Furthermore, our laboratory provided evidence for a direct regulation of the Sip1 gene by CHD4, in chromatin immunoprecipitation (ChIP) analyses (Linder et al, 2007). Our group demonstrated a direct binding of CHD4 protein to the Sip1 gene locus and found that CHD4 binding was more than threefold enriched at the active exon xE1, which is located within the 5' part of the transcribed Sip1 gene body. Overexpression of CHD4 protein reduced Sip1 mRNA levels in *Xenopus* animal caps explants, which demonstrates CHD4 binding with a repressive effect at the active Sip1 locus, rather than the silent one. The result identified the Sip1 gene as a direct target of CHD4 in gastrula stage embryos (NF 11) when the neuroectoderm and mesoderm boundary is formed (Linder et al, 2007). The prevailing opinion is that the CHD4 containing NuRD complex is correlated with repressed loci (Bowen et al, 2004). However, Drosophila CHD4/Mi-2 is co-localized with activated RNA polymerase II in salivary gland polytene chromosome (Srinivasan et al, 2005) and the human CHD4/Mi-2 protein was shown be important for the re-initiation of the transcription elongation (Mellor, 2006).

An additional role of CHD4 in concert with Sip1 is implicated by the direct interaction of CHD4 and Sip1 protein (Linder et al, 2007). This has especially to be considered,

because we demonstrated the spatial and temporal co-expression of CHD4 and Sip1 in the developing neural tissue. This co-expression and the interaction of both proteins implicate a combined functional role during neural development. The overexpression of *Xenopus* Sip1 causes ectopic neural induction via inhibition of bone morphogenetic protein (BMP) signalling and inhibition of Xbra expression (van Grunsven et al, 2007).

Functional analysis of four domain-deletion mutants of *Xenopus* Sip1 by Nitta et al. demonstrated that deletion of the *N*-terminus *z*inc *f*inger domain (NZf) suppressed neural induction and BMP inhibition. However, these functions were not affected by deletion of the *S*mad *b*inding *d*omain (SBD), the DNA-binding *h*omeo*d*omain (HD) together with the *C*tBP *b*inding *s*ite (CBS) and the *C*-terminus *z*inc *f*inger (CZf). Consequently, Sip1 does not inhibit BMP signalling by binding to Smad proteins. Deletion of NZf caused the loss of Sox2 induction, while the activity was retained by the other deletion constructs. This indicates that NZf is required for both neural induction and suppression of BMP signalling. In contrast, all of the deletion constructs inhibited Xbra expression. These results suggest that the NZf domain of *Xenopus* Sip1 has an important role in neural induction and that Xbra suppression occurs via a mechanism separate from the neural inducing activity (Nitta et al, 2007; Nitta et al, 2004).

Here, I provide evidence of a regulatory role of the CHD4 ATPase subunit of the NuRD chromatin-remodelling complex in concert with Sip1 function during neural induction. This notion of CHD4 function rests on several independent lines of evidence. First, I demonstrate that an increase in wild-type CHD4 protein levels caused phenotypes that were opposite to those, observed by reducing endogenous CHD4 protein levels through a CHD4-specific antisense Morpholino strategy. Second, the normal development of the embryos and the gastrulation process was not affected, which argues against an unspecific perturbation of cellular transcription or of bulk chromatin architecture. Third, specific effects on neural gene expression could be analyzed. These neural genes characterize the developing neural tissue according to its state of differentiation. Consequently, it was possible to distinguish between effects on undifferentiated proliferating prospective neural tissue, neural precursor cells, which are determined to become neurons, and finally differentiated neurons. This gene inference analysis was done within two different chromatin contexts of the developing embryo.

5.2.2 CHD4 regulates the expression of neural specific genes by integrating information of early signalling pathways

5.2.2.1 Signalling events during neural induction

The BMP signalling pathway as key for neural induction was demonstrated by several studies, but as a negative regulator, not a positive inducer. Thus, neural ectoderm of vertebrates forms when the BMP signalling pathway is suppressed. Genes that induce a neural fate, are known as secreted antagonists of BMPs, i.e. Noggin (Zimmerman et al, 1996), Chordin (Fainsod et al, 1997; Piccolo et al, 1996), Cerberus (Bouwmeester et al, 1996), and XNr3 (Smith et al, 1995). These proteins are secreted from the organizer and bind to BMP in the extracellular space to inhibit the activation of the BMP receptor. When these antagonists bind BMP in the naïve embryonic ectoderm, neural tissue forms instead of epidermis. When the expression of one of these secreted proteins in the organizer is blocked, BMP signalling is maintained on the dorsal side of the embryo and the neural plate does not form (Wessely et al, 2004). In contrast, neural genes are directly induced when dominant-negative BMP4 protein, dominant-negative BMP receptors, or BMP antagonists are expressed in the ectodermal region. In addition, if BMP is inhibited on the ventral side of embryos, a secondary axis forms, which contains neural tissue (Hawley et al, 1995). There are more than thirty documented BMP proteins, but BMP4 is mainly involved in epidermal induction and neural inhibition (Sasai et al, 1995; Wilson & Hemmati-Brivanlou, 1995). Although inhibition of BMP signalling is sufficient to induce neural tissue in the dorsal ectoderm, it is not sufficient to induce it in the ventral, non-neural ectoderm of the embryo (Hawley et al, 1995; Wilson & Hemmati-Brivanlou, 1995). Consequently, additional signals are required. Several experiments in frog and chick demonstrate that neural tissue is induced in non-neural ectoderm if BMP signalling is inhibited in the presence of fibroblast growth factor (FGF) (Delaune et al, 2005; Wilson & Edlund, 2001). Additional studies describe that FGF act in neural induction however, it is not clear if FGF functions independently of its ability to inhibit BMP transcription (Delaune et al, 2005; Linker & Stern, 2004; Sheng et al, 2003; Wilson & Edlund, 2001). Different ways have been described, how FGF negatively regulates BMP signalling. In chick and zebrafish, FGF signalling directly inhibits BMP gene expression (Furthauer et al, 1997; Wilson et al, 2000). In frog and zebrafish, FGF signalling inhibits BMP signalling through the phosphorylation and inactivation of its effector protein, Smad1 (Pera et al, 2003; Schier, 2001). In summary, FGF inhibits the BMP pathway, but it is not clear if FGF is needed for

neural induction independent of BMP inhibition (Lamb & Harland, 1995; Rogers et al, 2008; Wilson et al, 2000). It is unclear if there is a specific role for Wnt signalling in neural induction. Maternal Wnt signalling and the inhibition of zygotic Wnt are important for the formation of neural tissue. Additional data demonstrated that inhibition of Wnt signalling is required for neural induction. In *Xenopus*, over-expression of a dominant-active form of ß-Catenin inhibited the expression of Sox2 and Sox3 (Heeg-Truesdell & LaBonne, 2006). However, Wnt signalling, like FGF, does play an important role in the anterior-posterior patterning of the neural plate (Gamse & Sive, 2000; Gould & Grainger, 1997).

5.2.2.2 Integration of two major biological information-processing mechanisms: cell signalling and epigenetic gene regulation.

The induction events presented above rise the question, how information of signalling events could be integrated onto the chromatin level by epigenetic mechanisms of gene regulation. In the following, I will discuss how the complex processes of information transport and integration during neural development and cellular differentiation could be executed by two major mechanism to process the biological information: Through cell signalling on the one hand, and the integration of the signalling information onto the chromatin level by the epigenetic regulatory network, on the other hand.

5.2.2.3 CHD4 chromatin remodelling upregulates ChCh expression

The competence of cells to respond to FGF signalling changes during development. At blastula stages, FGF induces mesoderm, whereas later on, at gastrula stages, FGF regulates neuroectoderm formation. Within this context, ChCh has been described to act as a gate, which separates these two different functions of FGF signalling for meso- and endoderm formation on the one hand, and for neural induction on the other (Sheng et al, 2003).

ChCh expression is initiated by FGF signalling and inhibits the induction of the mesoderm-inducing factor Brachyury (xBra). In this context, our laboratory demonstrated a crucial role of the CHD4/Mi-2ß ATPase to act in concert with Sip1 during germ layer formation as it balances the relative proportions of mesodermal and neuroectodermal territories. CHD4/Sip1 epistasis thus constitutes a regulatory module, which balances mesoderm and neuroectoderm formation (Linder et al,

2007). The results demonstrated in Figure 14 illustrate that CHD4 dependent chromatin remodelling induces ChCh expression. In addition, ATPase dependent remodelling of the chromatin structure by altering CHD4 expression levels with wild type and dominant negative ATPase subunits influence ChCh mRNA expression differently. ChCh expression is induced by FGF signalling as an early step in neural induction and formation of neuroectoderm. Consequently, I suggest that CHD4 chromatin remodelling is not only important for the boundary formation between mesoderm and neuroectoderm, as we have demonstrated before, but could also be crucial for the boundary formation between the neuroectoderm and the ectoderm.

ChCh is a late FGF response gene and a key factor to regulate FGF competence. I demonstrate that ChCh expression is strongly enhanced by CHD4 overexpression (Figure 14). At this stage, the endogenous expression of ChCh mRNA could hardly be detected. Interestingly, overexpression of the CHD4 variant, which lacks a functioning ATPase domain, reveals a weaker phenotype, but does not abolish ChCh induction completely. Consequently, my results indicate that neuroectodermal tissue could be sensitized for neural inducing FGF signals by CHD4 chromatin remodelling within the chromatin context of early neural induction.

Considering the multiple interaction partners, which bind and recruit CHD4, my observation indicates an additional function of CHD4, which is not exclusively exerted by its ATPase function. I suggest that not merely chromatin remodelling but also histone modifications, such as a changing acetylation status, mediated by co-factors such as *h*istone *de**ac*etylases (HDACs), which are incorporated in the CHD4/NuRD chromatin remodelling complex, could influence neural induction and early neural marker gene expression (compare 2.4.3.5). Interestingly, the histone deacetylase inhibitor valproic acid (VPA) was shown to induce the differentiation of neural progenitor cells (Hsieh et al, 2004). Consequently, HDACs could be involved to promote early neural gene expression and maintain an undifferentiated state of neural progenitor cells. This function is in agreement with the data presented in this work. In addition, recruiting factors, e.g. transcription factors such as Sip1, could further support CHD4 function. Thus, my observation gives an explanation, how information of signalling events could be integrated onto the chromatin level by epigenetic mechanisms of gene regulation to influence the fate of stem cell development and subsequent cellular differentiation processes. Interestingly, ChCh can induce the expression of the neural promoting factor Sip1 in the neural region. ChCh expression in *Xenopus* embryos leads to a very similar phenotype as overexpression with Sip1 (*S*mad-*i*nteracting-*p*rotein-1; also known as ZEB-2 and ZFHX1B), which is a direct transcriptional repressor of the mesodermal gene *Xbra*

(Lerchner et al, 2000; Papin et al, 2002; Verschueren et al, 1999). In addition to inhibiting mesoderm formation, Sip1 induces neural marker genes (Papin et al, 2002; Postigo et al, 2003). One neural gene that is expressed at the onset of neural development is Sox2. Sip1 binds to several sites in regulatory regions of the neural marker gene Sox2 (Uchikawa et al, 2003). Consistent with the hypothesis that the CHD4/Sip1 regulatory module may work as a gate opener for neural induction, the next section will discuss how CHD4 misexpression affects the expression of the early neural marker gene Sox2.

5.2.2.4 CHD4 chromatin remodelling induces ectopic Sox2 expression

Our hypothesis, that the biological information process during neural stem cell differentiation is integrated by epigenetic mechanisms such as chromatin remodelling is underlined by my analysis of Sox2 expression. Sox genes code for transcription factors that bind to the minor groove of DNA to regulating gene transcription (Guth & Wegner, 2008). Sox proteins are divided onto ten groups however, only SoxD (a *Xenopus* member of the SoxG group), SoxB (Sox1, 2, 3, 14, 21), SoxC (Sox4 and 11), and SoxE (Sox8, 9, 10) groups are involved in neural induction and neural development (Guth & Wegner, 2008).

Sox2 and Sox3 belong to the SoxB1 (Sox1, Sox2, Sox3) subfamily of transcriptional activators and are well documented as early neural marker genes (Kishi et al, 2000; Mizuseki et al, 1998; Penzel et al, 1997; Uwanogho et al, 1995) (Linker & Stern, 2004; Takemoto et al, 2006; Uchikawa et al, 2003).

Sox2 and Sox3 are expressed in neural progenitor cells throughout CNS development and are required for neural progenitor maintenance (Ellis et al, 2004; Graham et al, 2003; Pevny & Placzek, 2005; Rogers et al, 2009a). Sox2 expression begins at the onset of neural induction only in the neural ectoderm (Nitta et al, 2006) and promotes neural induction in combination with FGF signals. Neither exposure to FGF nor overexpression of Sox2 alone can initiate neural induction, suggesting that Sox2 can change the responsiveness of the ectoderm to FGF signalling to induce neural tissue (Mizuseki et al, 1998). In addition, in embryonic stem cells Sox2 interacts with Oct3/4 protein on binding the enhancers of the FGF gene (Yuan et al, 1995). Considering my data, I hypothesise that CHD4 facilitates the translation of FGF signal information onto the chromatin level. This could be executed in different ways. First CHD4 could have an inhibiting effect on BMP and consequently facilitate FGF signalling. Second, CHD4 could enhance FGF directly, which activates ChCh as

described above. ChCh in turn regulates FGF competence. Bearing in mind that BMP promotes ectoderm formation and FGF neuroectoderm induction, these two signalling events demarcate the boundary between the ectoderm and the neuroectoderm. The results of my work imply that the integration of these opposing signalling informations are modulated by CHD4 chromatin remodelling for the benefit of FGF signalling. This would results in a shift of the boundary between ectoderm and neuroectoderm for the benefit of neural progenitor cells, as indicated by the induced ectopic Sox2 expression at the expense of ectodermal derivates. Consequently, my results demonstrate that CHD4 overexpression not only enhances the endogenous expression of Sox2, but also ectopically enlarges the Sox2 expression domain into the prospective epidermis. The endogenous expression of Sox2 in the neural plate depends on endogenous CHD4 protein, because reduction of CHD4 strongly reduces the endogenous Sox2 expression domain and its staining intensity within the domain.

In conclusion, CHD4 dependent chromatin remodelling promotes Sox2 expression that is required for both CNS and neural crest formations during neurula stages. An analysis of Sox2 function showed that Sox2-mediated signalling is essential for all the neural markers studied at neurula stages, because dominant negative Sox2 (dnSox2) prevented the expression of the pan-neural marker N-CAM, the neuronal markers n-ß tubulin and neurogenin, the neural crest markers Slug and fkh6 (Kishi et al, 2000). The same study further provided evidence that dnSox2 inhibits differentiation of neuroectoderm cells without re-directing the cells into epidermal fate. The timeframe of Sox2 function during neural induction and neural differentiation is crucial during late gastrula, when neuroectoderm is not able to further differentiate into neural tissues in the absence of Sox2 activity. This suggests that Sox2 is essential for the presumptive neural ectoderm to further differentiate beyond gastrula stages and renders CHD4 induced Sox2 signalling to be necessary during secondary stages of neural differentiation, which is initiated at late gastrula stages.

Interestingly, I observed that the ATPase function of CHD4 seems not to be the crucial factor for the alteration of Sox2 expression as overexpression of dnCHD4 expands the Sox2 expression domain at the expense of ectodermal tissue comparable to the wtCHD4 condition. This could be explained by the fact, that the presence of dnCHD4 protein does not inhibit the binding of CHD4 interaction partners and co-factors. Thus, the putative CHD4/NuRD complex with recruited co-factors and its HDAC activity could be sufficient to alter Sox2 expression within the chromatin context during gastrulation, although a reduced ATPase function. The idea

of recruiting co-factors with an effect on transcription regulation will be discussed further in chapter 5.2.2.7.

5.2.2.5 CHD4 chromatin remodelling induces neural fate at the expense of ectodermal gene expression - shifting the boundary between ectoderm and neuroectoderm?

An additional fact, that underlines the hypothesis that CHD4 works as a pivotal factor for the boundary formation between neuroectoderm and ectoderm has recently been published. It has been demonstrated that the FGF dependent expression of Sox3 activates Sox2 and the neural promoting gene geminin. This results in additional neural progenitor formation at the expense of non-neural ectodermal derivates (Rogers et al, 2009a). Additional studies demonstrated that both, Sox2 and Sox3 in *Xenopus* and zebrafish leads to expansion of neural progenitors in the neural tube at the expense of epidermal development and neuronal differentiation (Graham et al, 2003; Kishi et al, 2000).

In relation to my work, I demonstrate that CHD4 induces additional Sox2 expressing neural progenitor cells at the expense of non-neural ectodermal derivates, indicated by a reduction of *Xenopus* Keratin, Type II cytoskeletal 8 (xK8), which is associated with epidermal induction and inhibition of neural fate (Figure 16). The reduction of endogenous CHD4 protein leads to the opposite phenotype.

Consistent with these findings, I conclude that CHD4, in concert with the BMP inhibitory effect of Sip1, could promote a BMP free area that is more prone to the neural inducing effect of FGF signalling. Hence, my results demonstrate a shift of the boundary between ectoderm and neuroectoderm for the benefit of neural tissue and at the expense of ectodermal tissue. This observation provides the explanation how information of signalling pathways, such as BMP and FGF signalling, could be integrated onto the chromatin level by CHD4 chromatin remodelling.

The data of my work renders CHD4 as a crucial factor in the boundary formation of germ layers. First, we demonstrated in our laboratory the function of CHD4 in the boundary formation between mesoderm and neuroectoderm (Linder et al, 2007). Second, my study provides evidence that CHD4 affects the boundary between the neuro- and the ectodermal germ layer formation. Thus, CHD4 regulates fate decision of stem cells that give rise to specific tissues during development.

5.2.2.6 CHD4 maintains neural precursor cells in a proliferating state

To further specify my observation of the induced neural progenitor cells, I analysed the expression of one of the earliest neural genes expressed in the prospective CNS after neural induction.

The expression of Ash3a/b (achaete-scute homolog 3) in *Xenopus* embryos converts ectodermal cells to a neural fate. Overexpression of Ash3a/b leads to an enlargement of the CNS at the expense of other ectodermal derivatives (Turner & Weintraub, 1994). Ash3a/b is first expressed at NF stage 11.5 during midgastrulation in the presumptive neural plate and is restricted to the zones within the CNS, which contain proliferating undifferentiated neural precursor cells (Ferreiro et al, 1994). My results reveal that CHD4 mediates an increase of ASH3a/b expressing cells within the induced, presumptive neural tissue that expands into the epidermal territory. This suggests not only an increase in the number of neural precursor cells, but also an increase in their proliferation rate. With an increase in their proliferation rate, the differentiation of the induced, presumptive neural cells is inhibited or delayed. Consequently, one would expect these cells to be delayed or hindered in expressing marker genes for finally differentiated neurons. This idea will be referred to below.

Downstream of ASH3a/b another bHLH transcription factor, named NeuroD is transiently expressed in neural cells. A recent study on NeuroD proposed that primary transcription factor targets of NeuroD such as Ebf2, Ebf3, HEN1 (Nhlh1), Hes6, MTGR1, MyT1, NeuroD, NeuroD4 (Ath3), and potentially Znf238 represent a core transcriptional network that mediates Ngn and NeuroD-regulated neurogenesis (Seo et al, 2007). This renders NeuroD to be functionally located in the centre of the neural transcriptional network. However, the timeframe of NeuroD expression is restricted (Dufton et al, 2005). Within this timeframe, NeuroD is a key player in the centre of a transcriptional network that mediates neurogenesis and cell migration. NeuroD is only expressed after the time of neuronal commitment *in vivo*. Although NeuroD is able to activate the entire neurogenesis program upon overexpression and its function is necessary during the neuronal differentiation process, it is not needed for the maintenance of differentiated cell types.

It was shown that Ash3 expression is followed by NeuroD expression in differentiating neural cells. Further on, evidence was provided that the expression of Ash3 and NeuroD is coupled (Kanekar et al, 1997).

My results provide evidence that overexpression of CHD4 induces the expression of the transcription factor NeuroD. Compared to the ectopic expression of the neural precursor specific genes, such as Sox2 and Ash3a/b, the expression of NeuroD is

more restricted to the developing CNS, e.g. the brain, spinal cord, the eye vesicle, and the trigeminal placode.

Interestingly, Brg1, as the catalytic subunit of the SWI/SNF chromatin-remodelling complex, is also required for vertebrate neurogenesis and mediates transactivation of Ngn (Neurogenin) and NeuroD (Seo et al, 2005b). It was shown that a reduction of Brg1 activity results in the expansion of the Sox2-positive domain. In contrast, expression of n ß-tubulin, which marks terminally differentiated neurons, was severely reduced or abolished in embryos injected with dominant negative Brg1 or Brg1 Morpholino. Brg1 was further identified as an interacting partner of Geminin that antagonizes Brg1 activity during neurogenesis to maintain an undifferentiated cell state (Seo et al, 2005a). Considering that Brg1 has opposing functions, compared to my observations of CHD4 function, it is possible that CHD4 could co-act with Geminin to promote FGF mediated neural induction with the subsequent expression of Sox3 and Sox2, as described above. Several facts point to a possible interaction of CHD4 and Geminin. First, Geminin expression can be induced by inhibiting BMP signalling, and is maintained in the proliferative regions of the developing nervous system (Kroll, 2007). Second, Geminin maintains a neural progenitor population by inhibiting re-initiation of DNA replication, thereby maintaining chromosomal integrity and preventing cell cycle exit (Seo & Kroll, 2006), which is controlled by an antagonistic interaction with the catalytic subunits of the SWI/SNF complex, Brg1 and Brahma (Seo et al, 2005a). Third, overexpression of Geminin inhibits epidermal development and neuronal differentiation, and expands the neural progenitor population by activating the N2 enhance of the Sox2 gene (Kroll et al, 1998; Papanayotou et al, 2008). Consequently, Geminin maintains cell cycling during neural plate stages and represses neural cell differentiation, allowing for the expansion of neural ectoderm (Luo & Kessel, 2004; Pitulescu et al, 2005; Seo & Kroll, 2006). Most recently, CHD4 was shown to act as an important regulator of the G1/S cell-cycle transition for cell-cycle progression (Polo et al, 2010).

This function of CHD4 to keep cells in an undifferentiated, proliferating state was also shown in muscle regeneration, where premature silencing of CHD4 with RNA interference *in vitro* resulted in accelerated myoblast differentiation (Mammen et al, 2009). These functions are in agreement with my results that demonstrate that CHD4 overexpresssion induces and maintains proliferating neural precursor cells.

In conclusion, my observations render Brg1 and CHD4 chromatin remodelling to exert opposing functions during vertebrate neurogenesis. Interestingly, functional and mechanistically opposing effects of Brg1/SWI/SNF and CHD4/NuRD have been reported in other cellular contexts, which underline this idea. For example, they exert

opposing functions in the reprogramming of genes by EBF and Pax5 during B-cell development and during inflammatory response (Gao et al, 2009; Ramirez-Carrozzi et al, 2006).

In concert with my observation on Sox2, which induces neural progenitor cells, but delays neuronal differentiation at the expense of non-neural ectodermal derivates (Rogers et al, 2009a), I demonstrate that gain of CHD4 chromatin remodelling results in an induction of prospective neural tissue. The induced proliferating prospective neural tissue is delayed or hindered in its differentiation process, as indicated by the reduced expression of n-ß tubulin (see 4.1.1.7).

The process from neural induction to neural progenitor maintenance to the onset of neural cell differentiation was suggested to be regulated by a certain set of transcription factors to regulate neural fate stabilization. According to Rogers et al. these genes can be divided into two functional groups: those that promote an immature, undifferentiated neural state (Geminin, Sox2, Sox3, FoxD5, Zic2), and those that promote the onset of neural differentiation (SoxD, Sox11, Zic1, Zic3, Xiro1, Xiro2, Xiro3) (Rogers et al, 2009b). It was shown that BMP inhibition initiates neural induction via FGF signalling and Zic genes (Marchal et al, 2009). CHD4 could act as a mediator to direct Zic gene expression by integrating BMP and FGF signalling information. This could explain, why CHD4 can promote an immature, undifferentiated neural state, i.e. by regulating transcription of Geminin, Sox2, Sox3, Zic2 on the one hand, and promote neural differentiation in a later chromatin context on the other hand, i.e. by regulating Zic1, Zic3.

I conclude that the expansion of the Sox2 positive territory, combined with the reduction of n ß-tubulin expression suggest that the induced neural progenitor cells are kept in an undifferentiated state, due to CHD4 overexpression. This observation demonstrates a specific role of CHD4-mediated chromatin remodelling within the chromatin context of early neural induction and neural cell differentiation.

5.2.2.7 CHD4 regulates early neural gene expression in an ATP hydrolysis independent manner – a matter of co-factor recruitment?

The results above indicate that the influence on neural gene expression depends on CHD4 expression as demonstrated in gain-and-loss of function analysis. However, at the early, permissive chromatin stage during blastula and early gastrula, the function of CHD4 did not dependent on its functional ATPase domain, because wt and dnCHD4 gain-of-function resulted in alike phenotypes. Consequently, ATP hydrolysis

is not an exclusive factor for early neural gene regulation. One possibility could be an additional effect by recruited co-factors, which work in concert with ATP dependent chromatin remodelling. These co-factors can function as transcription factors, DNA binding proteins or execute histone-modifying activity, e.g. histone deacetylation.

The interaction of CHD4 with binding co-factors is not inhibited by our deletion mutation within the ATPase domain by point-mutating the lysine residue at position 748 to arginine, resulting in the dnCHD4 variant. The analogous mutation in the SNF2 ATPase maintains the integrity of the yeast SWI/SNF chromatin-remodelling complex, but abolishes transcriptional activation of target genes (Richmond & Peterson, 1996).

Consequently, dnCHD4 can also form a functioning NuRD complex with its binding co-factors HDAC1 and HDAC2, the histone binding proteins RbAp46 and RbAp48, the metastasis-associated proteins MTA1, -2, or -3 and MBD3 methyl (CpG)-binding domain 3 proteins. Different studies demonstrated that co-factors of CHD4/NuRD play a relevant role during neural differentiation and have the potential to influence developmental processes. For example, NuRD associated HDAC activity plays a crucial role in the context of neural development. In ES cells and neural progenitor cells HDACs influence neural differentiation. While in the ES cells chromatin is globally acetylated, deacetylation is required for differentiation of neural progenitor cells. In ES cells, HDAC inhibition blocked the subsequent steps of differentiation (Lee et al, 2004b). In the context of neural differentiation, Class II HDACs, for example were shown to be upregulated during neuronal differentiation of cultured hippocampal neural progenitor cells (Ajamian et al, 2003).

In contrast, it was also demonstrated, that the deacetylase activity, e.g. by HDAC1, is required to suppress neuronal genes. Therefore, the inhibition of HDAC is likely to drive the progenitors towards neuronal commitment, as shown by HDAC inhibition, which promoted neuronal differentiation of cultured neural progenitor cells (Hsieh et al, 2004). In conclusion, this suggests that different chromatin contexts with differences in their global acetylation level influence the differentiation potential.

An influence on cell differentiation due to the acetylation status of chromatin was also demonstrated in the case of muscle development. In *Xenopus*, Steinbach et al. used the specific HDAC-inhibitor trichostatin A (TSA) to induce precocious histone hyperacetylation by TSA-treatment, which selectively blocked the transcriptional induction of the *myoD* gene, and severely reduced subsequent muscle differentiation (Steinbach et al, 2000). This demonstrates that endogenous changes in chromatin acetylation are necessary for the temporally programmed induction of skeletal myogenesis. Consequently, HDAC activity is required early for the formation of the

Xenopus embryonic musculature, apparently for the temporally induction of the MyoD-dependent muscle cell lineage.
In relation to my work, this data underlines the notion, that HDAC activity, mediated by the CHD4/NuRD complex, can temporally influence the regulation of early neural gene expression within the context of an undifferentiated and hyperacetylated chromatin state. Bearing the complexity of epigenetic regulation in mind, it is easy to imagine that within different chromatin contexts, which are regulated by dynamic epigenetic alterations during development, cell fate decisions and cellular differentiation acts and responds differently to chromatin remodelling complexes like CHD4/NuRD.

5.2.2.8 Sip1 as a potential recruiting co-factor for CHD4

CHD4/NuRD mainly acts as an inhibitory complex with interacting co-factors, which exert an inhibitory function during neural induction. Thus, they are interesting candidates to further investigate the integration of cell signalling to the chromatin level. Inhibition of BMP signalling is a crucial step in neural induction, reviewed by (De Robertis & Kuroda, 2004; Harland, 2000; Weinstein & Hemmati-Brivanlou, 1999) (Rogers et al, 2009b). *Xenopus* Sip1 (XSip1) was identified to inhibit BMP signalling and downstream targets to push the prospective epidermis towards a neural fate (Eisaki et al, 2000; Nitta et al, 2004; Postigo, 2003; van Grunsven et al, 2006). The neuralizing activity involves the co-repressor CtBP (*C*-*t*erminal *b*inding *p*rotein) and occurs through both BMP dependent and independent mechanisms (van Grunsven et al, 2007). Using multiple domain-deletion mutants to dissect XSip1 function, it was suggested that the N-terminal zinc finger domain of XSip1 plays an important role in neural induction, however the repressing effect of XSip1 on Xbra expression works via a mechanism separate from the neural inducing activity (Nitta et al, 2007). XSip1 was identified to directly interact with CHD4 (Verstappen et al, 2008). Our laboratory could demonstrate that the CHD4/Sip1 module regulates the boundary formation between the mesoderm and the neuroectoderm. Moreover, we reported that CHD4 directly binds to the 5' part of the Sip1 gene body (Linder et al, 2007).
FGF signalling induces the expression of ChCh, which in turn activates Sip1 (Sheng et al, 2003). XSip1 is expressed in the neural region and suppresses the induction of the mesodermal marker gene xbra (brachyury). This suggests that XSip1 helps to establish the boundary formation between neuroectodermal and mesodermal germ layers. Therefore, I investigated the influence of XSip1 misexpression on neural

marker genes for proliferating neural precursors and differentiated neurons. In addition, I investigated the influence of the CHD4/Sip1 regulator module on neural development by CHD4 misexpression in combination with altered Sip1 expression levels. In agreement with the previously described function of CHD4, Sip1 gain-of-function analysis resulted in an enlarged domain of Sox2 expressing cells that reached into ectodermal territories. As described before, Sox2 overexpression delays neuronal differentiation as demonstrated by a reduced expression of n-ß tubulin. However, the most lateral cells seemed not to be hindered in their differentiation process, as they are able to express n-ß-tubulin (see 4.1.2.1). This could be explained by potential influencing signalling factors at the neuroectoderm border.

The combination of Sip1 overexpression with CHD4 gain-of-function revealed an additional interesting characteristic of the CHD4/Sip1 regulatory module. Although the expression of Sox2 and n-ß tubulin is comparable to the Sip1 gain-of-function condition, the penetrance of the phenotype was reduced by approximately 30 percent when CHD4 and Sip1 were both overexpressed. Bearing in mind that we could demonstrate an inhibitory role of CHD4 on the Sip1 gene locus (Linder et al, 2007), this phenotype can be explained by a repressive feed back loop, which is executed by CHD4 on endogenous Sip1. Thus, overexpression of both CHD4 and Sip1 does not lead to an additional effect, but CHD4 rather binds to the Sip1 gene locus to restrict endogenous Sip1 expression. This regulatory feed back loop would aim to re-establish a functional equilibrium of the CHD4/Sip1 regulatory module.

Concluding, I propose that in physiological concentrations, CHD4 and Sip1 co-act to induce neural tissue. Elevated levels of Sip1 could provoke CHD4 to bind the 5' region of the Sip1 gene body and repress endogenous Sip1 expression in a negative feed back loop. This mechanism would secure a physiological equilibrium of the CHD4/Sip1 regulatory module to fine tune the information, which is provided by cell signalling onto the chromatin level to establish a boundary between neural and ectodermal tissue. In case, this equilibrium is shifted due to the unphysiological high Sip1 concentration, the repressive function of CHD4 may reduce the penetrance of the observed phenotype (see 4.1.2.1, Figure 22 C).

5.2.2.9 The transcription factor Sip1 versus BMP signalling

Based on the ideas discussed above the next chapter will consider how Sip1 can act as a neural inducing factor by influencing signalling events like BMP. Molecular studies demonstrated that early neural-inducing signals are secreted proteins such

as Chordin (Sasai et al, 1995), Noggin (Lamb et al, 1993), Follistatin (Hemmati-Brivanlou et al, 1994) and Xnr3 (Haramoto et al, 2004). These molecules directly bind to BMP (Rogers et al, 2009b). The activated BMP receptor recruits Smad1/Smad5/Smad8 proteins to the membrane and activates them via phosphorylation. This step allows them to bind to Smad4 and the complex translocates to the nucleus to regulate transcription. Since a previous study showed that XSip1 binds to XSmad1 like mouse SIP1 (van Grunsven et al, 2000; Verschueren et al, 1999), it is likely that XSip1 inhibits BMP signalling to promote neural cell fate in the neuroectoderm.

In *Xenopus*, XSip1 was first isolated from animal caps treated with activin (Eisaki et al, 2000). It is initially expressed in the neuroectoderm at the gastrula stage and maintained in neural tissue throughout development. Overexpression of XSip1 induces neural markers in animal cap causing a hyperneuralized phenotype (Eisaki et al, 2000). Furthermore, the epidermal marker E-cadherin is directly repressed by XSip1 (Comijn et al, 2001), suggesting that XSip1 may not only inhibit the expression of BMP responsive genes, but also directly repress epidermal genes to maintain the neural cell fate. Sip1 (*S*mad *i*nteracting *p*rotein-1/δEF2/ZEB2/Zfhx1b) was originally identified as a transcriptional repressor (van Grunsven et al, 2001). The proteins of this family have multiple conserved domains: a *h*omeo*d*omain (HD), a *C-t*erminal *b*inding *p*rotein (CtBP) *b*inding *s*ite (CBS) and two two-handed *z*inc *f*inger domains, one at the *N*-terminus (NZf) and one at the *C*-terminus (CZf) (see Figure 22). The *S*mad *b*inding *d*omain (SBD) interacts with regulatory Smads, which are the mediators of TGF-β superfamily signalling (Funahashi et al, 1993; Verschueren et al, 1999; Yoshimoto et al, 2005).

The deletion of the NZf causes the loss of Sox2 induction, while activity was retained by other deletion constructs. This indicates that NZf is required for both neural induction and suppression of BMP signalling, whereas the other conserved domains of Sip1 (SBD, HD, CBS and CZf) are not essential for these activities. Thus, although Sip1 was originally identified to interact with Smad proteins, these findings indicate that binding to Smad1 is not important for neural induction and BMP inhibition via XSip1. However, all of the deletion constructs inhibited Xbra expression. This suggested that the NZf of XSip1 has an important role in neural induction and that Xbra suppression occurs via a mechanism separate from the neural inducing activity (Nitta et al, 2007).

It has been reported that the zinc-finger clusters of the N-and C-terminal regions of δEF1 directly bind to the E2-box sequence and that the NR domain in the N-terminal region acts as an active repressor to silence a subset of E boxes with higher binding

affinity for the CACCTG sequence (Postigo & Dean, 1999a). The NR domain in the N-terminal region is also conserved in XSip1, suggesting that XSip1 works as an active repressor of genes downstream of BMP signalling. Interestingly, XSip1 interacts with the co-repressor CtBP at the promoter and is necessary for the repressor activity (Postigo & Dean, 1999b).

The early β-Catenin signal induces the BMP antagonists Chordin and Noggin in the BCNE (*b*lastula *c*hordin and *n*oggin *e*xpressing) centre, which are required for neural tissue formation in the absence of mesoderm (Kuroda et al, 2004). Chordin and Noggin in turn have been described to induce XSip1 expression in animal caps explants. Furthermore, the use of dominant-negative BMP receptors strongly enhances secondary axis formation in the whole embryo (Yamamoto & Oelgeschlager, 2004). This exclusive inhibition of the BMP pathway in wild-type embryos results in the development of partial secondary axes with trunk and tail structures that lack head and forebrain tissues. In consistence with the described pathway of BMP inhibition, I observed that Sip1 overexpression could mimic the function of BCNE molecules with the potential to induce secondary axes.

The analysis of Sox2 and n ß-tubulin expression in embryos with partial secondary axes revealed the same characteristics as described above. This could lead to the conclusion that Sip1 executes different functions, which leads to this phenotype. First, the BMP inhibitory effect of Sip1 could account for the secondary axis phenotype. Second, Sip1 affects the expression of neural gene expression. The induced expression of Sox2 by Sip1 explains the reduced or delayed neural cell differentiation that is indicated by the lack of n ß-tubulin expression along the induced secondary axis.

The additional analysis with Sip1 overexpression in combination with the CHD4 Morpholinos, which mediated reduction of endogenous CHD4 levels, revealed that the induction of a secondary axis depends on endogenous CHD4 protein levels (see 4.1.2.3. - 4.1.2.5). In summary, my analysis distinguishes between functions of Sip1 as a mere BMP inhibitor and Sip1 in combination with CHD4 as a regulator of neural induction and neural cell differentiation. The results imply that Sip1 requires endogenous CHD4 function for the repression of BMP.

5.2.2.10 The CHD4/Sip1 regulatory module

Neural induction does not work by BMP inhibition alone, but also involves signalling factors such as FGF and Wnt signals (Rogers et al, 2009b). For the formation of

complete secondary body axes, these signalling factors also play important roles. Supporting evidence for the requirement for Wnt signalling in axis formation was shown, as overexpression of either Wnt1 or Wnt3a, which are both expressed in neural tissue, induced a secondary axis (Steinbeisser et al, 1993).

In this work, I investigated the influence of CHD4 misexpression on secondary axis formation to ask, if the CHD4/Sip1 regulatory module influences secondary axis formation. Sip1 overexpression induced secondary axis formation in about 16% of the cases; however, co-expression of CHD4 did not affect secondary axis phenotype (see 4.1.2.4). This can be explained, due to the fact that neural induction is not exclusively mediated by BMP inhibition, but also involves a fine balanced network of additional signals such as FGF and Wnt signalling (Rogers et al, 2009b). Consequently, neural induction by the CHD4/Sip1 regulatory module cannot be reduced to a mere effect on BMP inhibition, which leads to secondary axis formation. This hypothesis is strengthened by data, which challenges the widely accepted "default model" for neural induction (Weinstein & Hemmati-Brivanlou, 1999; Wilson & Hemmati-Brivanlou, 1995). These authors suggest that neural induction requires BMP inhibition only as a late step, and involves signals other than FGF and Wnt antagonists (Linker & Stern, 2004). My results consequently render functions of Sip1 and as a co-factor of the CHD4/Sip1 regulatory module to fine tune signalling events to regulate transcription onto the chromatin level.

The combined function of CHD4 and Sip1 could also be mediated by additional chromatin binding and modifying factors. Sip1 interacts with the co-repressor CtBP (*C*-terminal *b*inding *p*rotein) by its CBS (*C*-terminal *b*inding *p*rotein (CtBP) *b*inding *s*ite). CtBPs exist as a multi-protein complex that includes the histone-methyltransferases (HMTs) G9a and Eu-HMTase1, but also HDAC1 and HDAC2 (Shi et al, 2003) (see introduction for further information). The neuralizing activity of Sip1 involves the co-repressor CtBP and occurs through both BMP dependent and independent mechanisms (van Grunsven et al, 2007). This implicates that functions of the CHD4/Sip1 regulatory module could be assisted by HDAC or HMT function.

A further observation accounts for a balanced equilibrium between Sip1 and CHD4 activities. I provide evidence that secondary axis formation by Sip1 overexpression depends on endogenous protein levels of CHD4 (see 4.1.2.4). Sip1 overexpression in combination with reduction of endogenous CHD4 does not lead to the secondary axis phenotype, but leads to proliferating tumour-like tissue, as indicated by Alexa Dextrane co-injection (see 4.1.2.5 Figure 27). Two major conclusions can be drawn from these observations: First, Sip1 dependent secondary axis formation is not altered by CHD4 overexpression, however Sip1 function depends on endogenous

CHD4 levels for BMP inhibition. Second, reduction of endogenous CHD4 protein could results in a reduced repression on the Sip1 gene locus, resulting in de-repressed Sip1 expression. Interestingly, Sip1 also acts as a repressor of E-Cadherin (Comijn et al, 2001). Consequently, the reduction of endogenous CHD4 levels would result in de-repressed and thus elevated Sip1 levels, which in turn repress E-Cadherin. The repression or loss of E-Cadherin is an important step in tumour genesis and metastasis formation. In summary, I suggest that the observation of proliferating tumour-like tissue can be explained by the deregulated de-repression of Sip1 due to the reduction of endogenous CHD4 proteins levels.

5.2.2.11 Sip1 induces secondary axes, including mesodermal gene expression

Sip1 has an important role in neural induction but the suppression of Xbra occurs via a separate mechanism. This concept is strengthened by my results as described in the following observation. In 4.1.2.7, it is demonstrated that Sip1 dependent secondary axis formation does include the expression of the mesodermal marker gene cardiac actin along the induced axis. The induction of a secondary axis includes the expression of mesodermal marker genes. This leads to several conclusions. First, BMP inhibition via Sip1 can mimic BCNE centre molecules. However, the induction of secondary head formation could not be observed. Inhibitors of Wnt signalling like Frzb (Leyns et al, 1997; Wang et al, 1997) and Dkk1 (Glinka et al, 1998) can co-operate with BMP inhibitors to induce secondary heads (Kazanskaya et al, 2000). To further discriminate the pathways, disturbed by Sip1 overexpression, I analysed a potential affection of the Wnt pathway and tested the potency of Sip1 on secondary head induction. In 4.1.2.6, secondary axis formation on the dorsal side of the embryo was induced by Sip1 mRNA injection in one dorsal blastomere of a 4-8-cell stage embryo. Alexa Dextrane co-injection indicates the secondary axis.

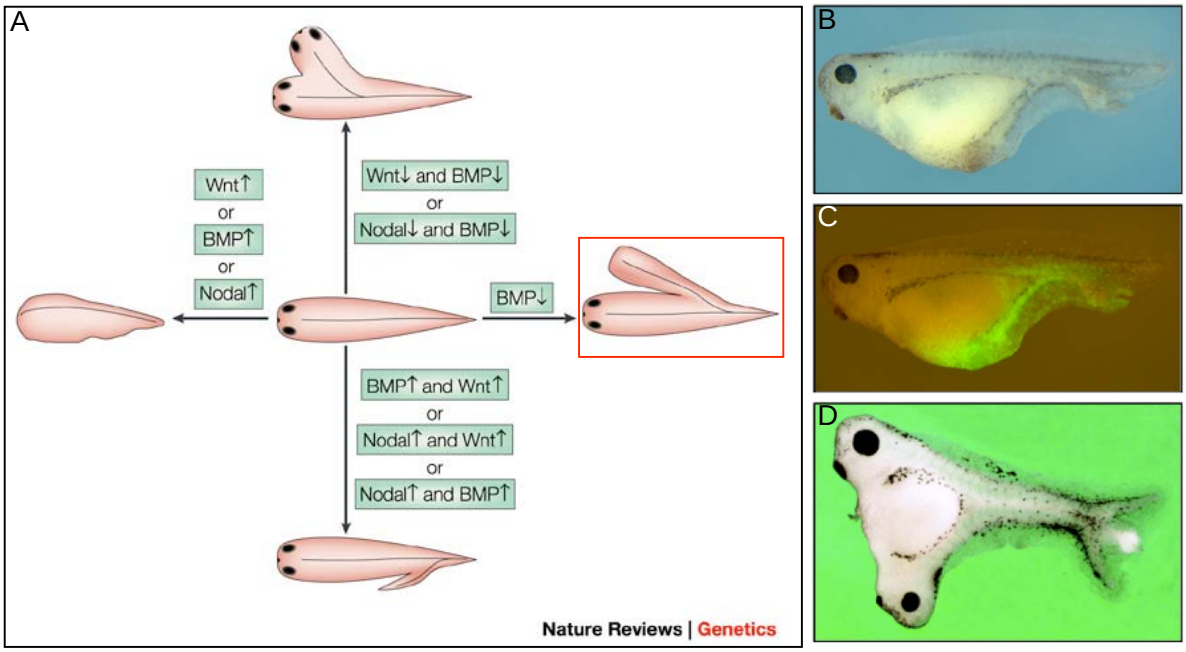


Figure 44: The illustration summarizes the effects due to overexpression or reduction of the three main signalling events alone or in combination as indicated.

A) Reduction of BMP signalling alone induces secondary trunks, whereas combined reduction of BMPs and either Wnt or Nodal induces extra heads. Sip1 consequently acts as a mere BMP inhibitor without interacting with Wnt signals, in a manner that would influence secondary axis formation. Adapted from (Niehrs, 2004).

B) Secondary axis formation due to Sip1 overexpression on the ventral side without head structures indicating a mere BMP inhibition (see chapter 4.1.2.2 and 4.1.2.3 Figure 25).

C) Cells that give rise to axis formation are traced with green fluorescence Alexa Dextrane

D) Illustration of the classical Spemann and Mangold organizer experiment repeated in Xenopus laevis. Organizer cells were transplanted on the ventral side. These cells carry the molecular information for signalling events (i.e. Wnt, BMP, Nodel inhibition) that induce complete axis formation, including head structures. Adapted from (De Robertis & Kuroda, 2004).

Beside this, Sip1 was injected in one ventral blastomere, which resulted in a ventral secondary axis along the belly of the embryo. This demonstrates a stable secondary axis formation in late developmental stages. In neither case, the induction of a secondary head structure was observed. It was demonstrated, that co-expression of a dominant-negative BMP receptor with inhibitors of the Wnt signalling pathway in *Xenopus* leads to the induction of complete secondary axes, including a head (Glinka et al, 1997). Hence, induction of head structures would not only include the exclusive

inhibition of BMP but also the inhibition of the Wnt pathway.
Consequently, the observed phenotypes, due to Sip1 misexpression did not include inhibition of the Wnt pathway in a way that would cause morphological alterations. These results indicate that inhibition of BMP signalling is one mechanism, which is executed by the CHD4/Sip1 regulatory module to influence neural development.
In summary, I conclude that the CHD4/Sip1 regulatory module induces neural fate at the expense of ectodermal cells. The induced cells express specific neural marker genes, which characterise early proliferating neural progenitor cells. In agreement to this observation, the ectodermal marker gene xK81 is reduced due to CHD4 overexpression. The induced neural cells are proliferating and do not express genes indicative of finally differentiated neurons. Hence, CHD4/Sip1 shifts the boundary between neuroectodermal and ectodermal germ layer formation for the benefit of neural and at the expense of ectodermal tissue. These observations are restricted to the function of the CHD4/Sip1 regulatory module within the chromatin context of early developmental stages. The next chapter will discuss the role of CHD4 chromatin remodelling within the chromatin context of neural cell differentiation.

5.3 CHD4/Mi-2ß chromatin remodelling during neural differentiation – different CHD4/Mi-2ß functions within a dynamic chromatin context?

The process of neural induction and neuronal differentiation are complex and depend on different influences at different time points during embryonic development. The chromatin architecture is under constant influence by signal input. In addition, chromatin structure itself influences the gene expression patterns and modulates differentiation processes during development. Hence, it is necessary to differentiate between influences of ATP-dependent chromatin remodelling during gastrula (NF stage 10), when neural induction occurs, from later stages after gastrulation (from NF stage 13 onwards), during neural differentiation. Many studies considered the effect of a dynamic chromatin context and demonstrated its influence during development (Srivastava et al, 2010). One influencing factor is the changing histone variant composition and the dynamics of linker histones integration. In *Xenopus*, levels of H1M, as well as somatic histones H1A, H1B, and H1C were determined during early embryogenesis. From MBT (*m*id*b*lastula *t*ransition) to the end of gastrulation,

maternal histone H1 is gradually exchanged by the somatic histones H1A, H1B, and H1C. Structural analysis showed that histone H1M does not significantly restrict the accessibility of linker DNA, but *in vitro* studies illustrated that incorporation of H1A results in a tighter compaction of chromatin, which is resistant to remodelling (Saeki et al, 2005). These transitions in chromatin composition correlate with significant changes in the replicative and transcriptional activity of embryonic nuclei and proposes that maternally expressed linker histones may function as key molecules to specify nuclear dynamics with respect to embryonic totipotency (Turner, 2005). In *Xenopus*, microinjection of targeted ribozymes into early embryos revealed that replacement of B4 with somatic H1 is required for selective gene repression and proper development and the accumulation of somatic H1 protein was shown to be rate limiting for the loss of mesoderm competence at midgastrula stages (Steinbach et al, 1997). A specific function for the linker histones in regulation of gene transcription was shown by the physical interaction of Msx1 and H1B, which bind to a key regulatory element of MyoD, where they induced repressed chromatin (Lee et al, 2004a).

In consideration to my investigations of chromatin remodelling during neural development it is interesting that a special role for histone variant combinations was discovered during neural and ES cell differentiation. The analysis of H3.1, H3.2 and H3.3 revealed that each of these histones are enriched for a special combination of post-translational modifications (Hake et al, 2006). In the context of neural differentiation, the transcription-associated variant H3.3 increases, whereas the levels of H3.1 and H3.2 exponentially decrease during neuronal differentiation (Bosch & Suau, 1995). Consistent with the observation during neural differentiation, the induced differentiation of mouse ES cells results in an increase of the relative fraction of H3.3, while H3.2 and H3.1 levels slightly decreases (Hake et al, 2006). These studies demonstrate that the effect of ATP-dependent chromatin remodelling on the neural gene expression profile could differ according to different chromatin contexts.

5.3.1 Evaluation of an inducible gene expression assay

Therefore, I aimed to distinguish between influences of ATP-dependent chromatin remodelling within different chromatin contexts: First, within the chromatin context of early gastrula stages, which represents the time of neural induction and second, during later neurula stages, the developmental stage of neural cell determination and

differentiation. I approached this question with an inducible heat-shock promoter driven gene expression procedure. By this means, CHD4 misexpression could be activated at neurulation from NF stage 13 onwards. To reduce cumulative effects due to alterations in the chromatin structure and histone modifications at early stages in adjacent tissue, I targeted the injection to the dorsal blastomeres, which mainly give rise to neuroectodermal tissue, as demonstrated in Figure 30 (Moody, 1987).

Other transgenesis procedures, such as restriction-enzyme-mediated integration (REMI) on decondensed sperm nuclei, followed by nuclear transplantation into unfertilized eggs or simplified versions (Sparrow et al, 2000), are not completely efficient and produces some embryos that have not integrated the transgene (Amaya & Kroll, 1999). In contrast, I established a reliable method with a high efficiency of heat-shock promoter driven gene expression from injected plasmids, which can be targeted to the desired tissue by microinjection.

As a first step towards a heat-shock promoter driven gene expression procedure by plasmid injection, we tested the toxicity, efficiency and leakiness of an hspCS2+gfp plasmid construct. Foreign DNA could have toxic effects on cells in a dose dependent manner. Thus, the toxicity of plasmid DNA was evaluated by injecting increasing concentrations of heat-shock promoter plasmid and evaluated gastrulation defects and loss of embryos as a sign for DNA derived abnormal development. The optimal plasmid concentration was determined by the best efficiency of gene induction, quantified by the number of successfully induced GFP protein expression with the least toxicity. The toxicity was evaluated by the death of plasmid injected embryos or unspecific developmental defects compared to control sibling embryos. The best concentration was determined at 100pg of plasmid DNA. The efficiency of successful heat-shock induced gene expression was evaluated with two different protocols as described in 4.2.1. To analyse CHD4 dependent chromatin remodelling, restricted to the neuroectodermal tissue without influencing neighbouring tissue, the stable heat-shock induced GFP expression was evaluated at NF stage 17, 25 and tadpole stages. Figure 32 A illustrates a stable expression of heat-shock induced GFP protein, restricted to the neuroectodremal tissue during all stages investigated.

The successful induction of protein expression could be demonstrated. However, possible false positive results could have been observed, resulting in positive GFP protein expression that was not exclusively due to the heat-shock procedure. One possibility of a false positive result could occur due to leakiness of the heat-shock promoter with heat-shock independent activation of the heat-shock promoter with subsequent GFP expression. In contrast, a false negative result of GFP protein

detection could occur due to incomplete, reduced or delayed folding of GFP protein, which would result in a lacking fluorescence signal.

To eliminate these possible confounders, I controlled the expression of heat-shock induced GFP expression on the transcriptional level by whole mount in situ hybridization against GFP mRNA (see Figure 32 B). This evaluation demonstrated a stable GFP mRNA expression due to heat-shock activation. The non heat-shocked control embryos did not show GFP mRNA expression.

As a next step, I subcloned wt- and dnCHD4 into the heat-shock expression plasmid and tested these constructs according to the established protocol described in 4.2.1. Heat-shock promoter driven activation of the hspCS2+wt/dnchd4 constructs resulted in a successful overexpression of CHD4 protein, as shown by immunohistochemistry (see Figure 33 C). In conclusion, I described an effective and reliable method for targeted and inducible gene expression assays. Consequently, this essay offers the possibility to be adapted for further applications, where a targeted activation of gene expression is needed.

5.3.2 Induced CHD4 expression influences neural cell differentiation

With this established assay, a temporal and spatial restricted ectopic CHD4 expression could be targeted to the developing neural tissue from NF stage 13 onwards. This developmental state reflects a transcriptionally mature and more stable chromatin context. The idea that the CHD4 chromatin remodelling function could depend on, and work differently in a dynamic chromatin context is underlined by a recent analysis. It describes that histones variants in *Xenopus laevis* revealed a distinct index of enriched histone variants and modifications in a developmental stage and cell type specific manner. The analysis of global histone modifications from embryonic cell types to adult somatic cell types was described to vary dramatically from cell type to cell type and from embryonic to somatic cells. (Shechter et al, 2009). PTMs (*p*ost *t*ranscriptional *m*odifications) are suggested to serve, in a localized fashion on discrete populations of nucleosomes within the cell, as a multivalent handle for effectors, referred to as “readers”. These “readers” work in concert with their associated remodelers and transcription machinery to regulate gene transcription (Shechter et al, 2009). Thus, the dynamics of chromatin structure has to be considered, when we investigate the influence of chromatin remodelling on cell differentiation. Rather than acting as static epigenetic landmarks in the genomic landscape, histone modifications may act as dynamic and transient operational

marks, exemplified by those modifications described at poised and actively transcribed genes. Precise quantitative analysis of the absolute levels and dynamics of these modifications is required to judge the likelihood that histone modifications can act epigenetically to program different gene loci in different cell types (Lee & Mahadevan, 2009).

A very interesting feature of changing histone modification during differentiation is the modulation of bivalent domains (Bernstein et al, 2006a; Mikkelsen et al, 2007). These bivalent domains were initially described in ESCs as broad regions of H3K27me3 with H3K4me3 peaks (Azuara et al, 2006; Bernstein et al, 2006a). During ESC differentiation, bivalent promoters may persist or decide for different chromatin states, i.e. H3K4me3 or H3K27me3, or neither H3K4me3 nor H3K27me3. Genes that decide for H3K4me3 alone become transcriptionally active. Thus, differential lineage-specification correlates with different outcomes of bivalent resolution and changes in gene expression (Bernstein et al, 2006a; Mikkelsen et al, 2007). Bivalent modifications were originally proposed to facilitate stem cell plasticity, i.e. to repress lineage-specific genes to maintain pluripotency, but keep them in a poised state for rapid activation in response to developmental signals. However, bivalent promoters have also been observed in differentiated cells (Barski et al, 2007; Cui et al, 2009). Interestingly, genes required for pluripotency, such as OCT4, Nanog and Sox2 become bivalent and silenced during differentiation (Pan et al, 2007). This possibility to switch between undifferentiated proliferation and differentiation has to be considered in relation to my results, because CHD4 can bind with its PHD and Chromodomain to H3K4me3 and H3K27me3, but CHD4/NuRD binding can be inhibited by H3K4me (see Figure 5). Hence, binding of CHD4/NuRD can depend on the methylation status of H3K4. Consequently, CHD4 chromatin remodelling depends on the chromatin context and could integrate signalling information via this mechanism, leading to either proliferation or differentiation in a chromatin context dependent manner.

Therefore, CHD4 dependent chromatin remodelling could execute different functions within a dynamic chromatin context from neural stem cell fate decision to neural cell differentiation. To approach this issue, I investigated the influence of CHD4 dependent chromatin remodelling on uncommitted neural progenitor cells and neurons, which are committed to final differentiation by analysing the marker genes Sox2 and n ß-tubulin, respectively. For an additional analysis how specific neural transcription factors are affected, the Pax6 gene expression was evaluated.

According to my observations described before, Sox2 expression is severely altered and ectopically expressed in early developmental stages. In contrast to this

observation, the pool of Sox2 positive neural progenitor cells reveals only a subtle alteration within the endogenous Sox2 expression domain when CHD4 expression was activated in early neural progenitors in a heat-shock dependent manner from NF stage 13 onwards. This leads to the following assumption: As soon as the pool of neural progenitor cells is established, the spatial expression domain of these cells is not altered due to CHD4 misexpression. Furthermore, epigenetic gene regulation by CHD4 dependent chromatin remodelling can modulate neural development in a stage specific manner.

In agreement with the conclusion that CHD4 could work in a context dependent manner to modulate BMP signalling during neural development, a study demonstrated that the conditional dexamethasone (DEX) induced BMP inhibition in *Xenopus* revealed stage-specific roles for BMPs in neural and neural crest induction (Wawersik et al, 2005). This indicates that BMP inhibition in early stages induces neural development. However, when DEX is added at NF stage 9 or later, ectopic neural tissue (Sox2) is decreased or absent compared to earlier BMP inhibition. As a result, BMP inhibition induces neural identity at early stages, however this response is lost between stage 8 and 9, and prior to the onset of gastrulation (Wawersik et al, 2005). This data correlates with the results of my work, as BMP inhibition by CHD4 chromatin remodelling in concert with Sip1, promotes neural progenitor cells formation before NF stage 13 however, this function is lost after NF stage 13.

Together, these observations point to different functions of CHD4 dependent chromatin remodelling during neural induction and neural cell differentiation. In early stages, CHD4 induced the progenitor pool of neural precursor cells, indicated by the ectopic expression of Sox2 and the analysed early neural marker genes. The analysis of differentiated neurons, marked by n ß-tubulin, however demonstrated a reduced expression. This observation was in agreement with the concept that an induction of a proliferating neural precursor cell population on the expense of ectodermal derivates results in a delayed or hindered differentiation of the developing neural tissue. Due to the fact, that CHD4 results in elevated expression levels of NeuroD, a regulator gene for neural differentiation, the proliferating neural progenitor cells are kept in a proliferating, undifferentiated state and thus are most likely not hindered, but delayed in their differentiation process. Interestingly, this function is independent from the CHD4 ATPase function, as dnCHD4 could alter gene expression comparable to the wild type CHD4 condition. In contrast to these observations during early developmental stages, the induced expression of CHD4 after gastrulation from NF stage 13 onwards and locally restricted to the neuroectodermal tissue, revealed different functions: First, the expression of n-ß

tubulin was enhanced by heat-shock activated CHD4 overexpression after NF stage 13. In contrast to the observations before, this demonstrates an induced maturation process of neural cells. This process clearly involves ATP hydrolysis, as dnCHD4 does show the opposite effect with a reduction of n-ß tubulin expression. The influence on Pax6 expression was also dependent on CHD4 ATPase function.

In conclusion, CHD4 mediated chromatin remodelling can induce the neural progenitor cell pool in an early chromatin context on the one hand, but promotes neural cell differentiation, on the other hand after gastrulation from NF stage 13 onwards within a different chromatin context. The later function can be observed on the cellular level as shown in the Pax6 analysis. Pax6 expression is induced by CHD4 overexpression in the restricted, mosaic like distribution, where the plasmids are expressed. The induced Pax6 expression is located at the border of the neural plate region, which is populated by neural crest cells. This ectopic expression depends on CHD4 ATPase function. Pax6 expression is regulated by subtle alterations of BMP signalling (Hartley et al, 2001). Consistent with my previous observations, CHD4 may function as a regulator that integrates and fine tunes signalling information to the chromatin level, resulting in the observed alteration of Pax6 expression.

This observation is consistent with the stage specific functions for BMP signalling as published by (Wawersik et al, 2005), demonstrating that late BMP signals do not inhibit neural identity, but ectopic neural crest cells. This suggests that BMP antagonists play temporally distinct roles in establishing neural and neural crest identity. Combining BMP inhibition with FGF signalling, the neural inductive response in whole embryos is greatly enhanced and no longer limited to pre-gastrula ectoderm. A further study revealed a reduced response to BMP4 signalling with a considerable variation to the degree of inhibition (Hartley et al, 2001). This study analysed whether genes expressed in the anterior neural plate change their responsiveness to BMP4 signalling during the neurula stages. Transgenic BMP expression from NF stage 13 onwards revealed widespread suppression of neural markers, which was unexpected due to several lines of evidence that suggest that neural induction takes place during gastrulation. Indeed, the expression of BMP antagonists on the dorsal side of the embryo is initiated at the late blastula to early gastrula stages (Hemmati-Brivanlou et al, 1994; Lamb et al, 1993; Sasai et al, 1995; Sasai et al, 1994). A possible explanation could be that BMP4 signalling is locally neutralized and therefore suppresses neural gene expression in spatially restricted regions of the neural plate. Such localized suppression would lead to the fine-tuning of neural gene expression. Thus, the available data so far support the idea that

sensitivity to BMP4 inhibition results in localized suppression of neural gene expression in areas of the neural plate, which do not express BMP inhibitors (Hartley et al, 2001). In the case of Pax6, its expression is initially uniform in the anterior neural plate but is suppressed in a central domain during the early neurula stage. Interestingly, this Pax6-negative domain reveals high level of underlying BMP4 expression. In accordance to my data, heat-shock induced CHD4 expression could result in the local suppression of BMP at the border of the neural plate with the result of local induction of usually suppressed Pax6 expression. Due to spatially and temporally co-expression of CHD4 and Sip1, this function could be executed by the CHD4/Sip1 regulatory module. Figure 45 summarizes the key points of my analysis.

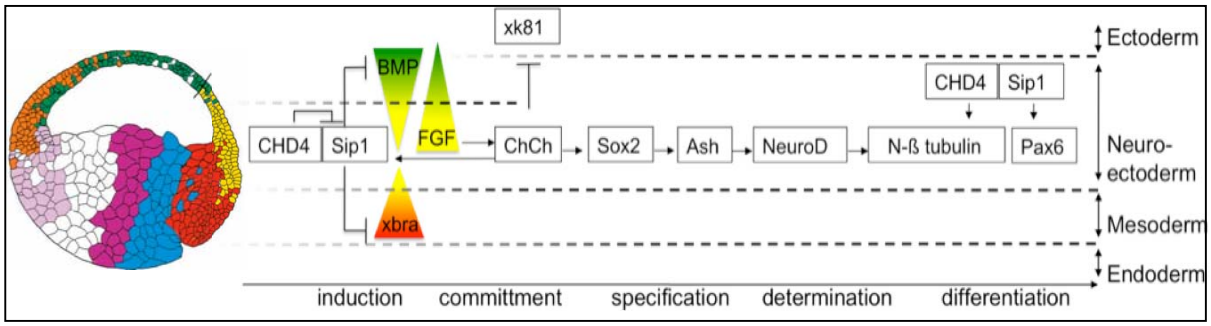


Figure 45: Model of CHD4 mediated integration of signalling information.
The model illustrates the CHD4/Sip1 regulatory module as an integrating factor for signalling information that influences germlayer formation and hence direct cell fate from induction to differentiation. The CHD4/Sip1 module is a pivotal factor the mesodermal (red) and neuroectodermal (yellow) boundary formation. This work ads further functions: First, for the boundary formation between ectoderm (green) and neuroectoderm (yellow). During gastrula stages, CHD4 could induce the pool of progenitor cells that are committed, specified and determined for neural differentiation. This CHD4 function represents regulation of gene expression due to chromatin remodelling within the chromatin context of early development. Within the chromatin context of neurula stages, CHD4 acts as a promoting factor for neural cell differentiation. This model hypothesizes the integration of signalling information onto the chromatin level.

In summary, BMP inhibition alone is not enough for neural induction. CHD4 probably modulates neural development not exclusive via BMP inhibition, but in concert with co-expressed Sip1, CHD4 integrates several signals that are necessary for neural development, e.g. FGF, to the chromatin level.

5.3.3 Evaluation of neural cell proliferation versus apoptosis

The described differences of gene expression, which are indicative of an enhanced differentiation, could be due to, or lead to an altered ratio of cell proliferation or could be confounded by apoptosis. Consequently, I analysed if heat-shock induced expression of CHD4 alters the ratio of activated (phosphorylated) Caspase3 as a marker for apoptosis and H3S10 phosphorylation as a sign for cell proliferation. The analysis of antibody staining as demonstrated in Figure 39 revealed a qualitative alteration of H3S10 phosphorylation in the area, where CHD4 misexpression was induced by the heat-shock procedure. To quantify H3S10 phosphorylation and Caspase3 expression, the mean integrated fluorescence intensity of optical sections, provided by confocal microscopy, were measured.

5.3.3.1 Confocal microscopy as a means to quantify H3S10 phosphorylation and activated Caspase3 expression

To establish a better means for quantification, I used confocal microscopy as an optical imaging technique to increase micrograph contrast and to reconstruct two-dimensional images by using a spatial pinhole. This technique eliminates light, which is out of focus in specimens that are thicker than the focal plane.
As described in 4.2.3 and 4.2.3.1, optical slices through the CNS area were two-dimensional reconstructed, where CHD4 misexpression was activated via heat-shock. The reconstruction rendered the overlay of fluorescence, which was detected in each slice. The area of detection was standardized for each embryo by a fixed mask. Thus, this assay allows the analysis of a fixed volume of tissue. The measurement of the mean integrated intensity, relative to the control side of the embryo delivered the intensity difference.
The results revealed that wtCHD4 overexpression causes a reduction of H3S10 phosphorylation. In contrast, dnCHD4 overexpression positively correlates with H3S10 hyper-phosphorylation. In agreement with my observation that wtCHD4 overexpression from NF stage 13 onwards promotes neural cell differentiation, the detected H3S10 hypo-phosphorylation indicates that cell differentiation is accompanied by a reduction of cellular proliferation. This result depends on the ATPase function of CHD4 chromatin remodelling, as dnCHD4 leads to H3S10 hyper-phosphorylation. In addition, this could work together with a potential connection to co-factor associated deacetylation by the NuRD complex. However, wtCHD4 and

dnCHD4 affected H3S10 phosphorylation differently, consequently it is unlikely that HDACs of NuRD can be attributed a central role for this function. Taking the described biological function of H3S10 phosphorylation status, as a mitotic marker for cell cycle progression into consideration, the observation could account for a promotion of cellular differentiation with reduced proliferation, characterised by a less active chromatin status. In conclusion, this works in an ATPase dependent manner as dnCHD4 overexpression resulted in a hyper-phosphorylated H3S10 status. As described in 4.2.3.2, this observation is not influenced by apoptosis, because no activation of Caspase3 could be detected.

These results demonstrate cell specific and developmental stage specific functions of the CHD4/Mi-2ß ATPase subunit of the NuRD chromatin remodelling complex during neural induction and neural cell differentiation. Chromatin remodelers consist of a large number of assembled complexes and co-factors. Interacting proteins such as Sip1 further modulate the integration of signalling information to the chromatin level. The observation of a co-factor and stage dependency leads to further implications of chromatin remodelling in a broader context, which will be discussed in the following chapter.

5.4 Implications of chromatin remodelling during development and cell (re-)programming

The diversity of ATP-dependent chromatin remodelling complexes has probably evolved to accommodate the major changes in chromatin regulation that occurred during the evolution from unicellular eukaryotes to vertebrates.

The SWI/SNF family is one of the most-studied of chromatin remodelling complexes. It has exchanged and rearranged subunits during evolution from yeast to vertebrates. The increase in combinatorial diversity of complexes, which regulate chromatin structure could be a strategy to handle the developing 30-fold increase of genetic regulatory information from files to vertebrates, excluding protein coding genes (Wu et al, 2009).

It has been demonstrated that many vertebrate chromatin-regulatory complexes are assembled in a combinatorial manner. Consequently, this offers an expanding potential for diverse gene expression patterns compared to unicellular eukaryotes. This notion is underlined, as during the evolution of multicellularity and complex body

plans, the demand for tissue-specific and developmental-stage specific expression of genes coincides with an increase of complexity in chromatin organization and chromatin regulation.

Considering that the greatest demand for diverse patterns of gene expression occurs in the development and function of the brain, it is probably no co-incidence that a diversity of neural phenotypes is emerging from genetic studies of the subunits of chromatin remodelers in the nervous system (Yoo & Crabtree, 2009). The dynamic context with different regulatory levels from the integration of signalling events to the complexity of epigenetic histone modifications and chromatin remodelling can in turn influence the output of gene regulatory information. Therefore, further contemplations are necessary to evaluate the data of this work.

5.4.1 A dynamic remodelling complex with different functions

Aside from CHD4/NuRD it has recently been described that ATP-dependent chromatin remodelling complexes, based on the alternative DNA dependent ATPases, Brg1 and Brm, play essential roles during neural development in both vertebrates and invertebrates. For example Brg1 misexpression affects the development of neural tissue development, as Brg1 Morpholinos injection into zebrafish embryos expands the expression domain of the fore brain marker gene six3, a reduction of the mid-brain boundary marker engrailed2 and the hindbrain marker gene krox20. Neural crest cells derive from the neuroectoderm and migrate laterally to become pigmented skin cells, peripheral neurons, and glia. In addition, they form cartilage and bones of the facial structures. The lack of Brg1 function severely reduces the prospective neural crest cells and neural crest derived structures fail to express the neural crest marker gene snail2.

In the context of *Xenopus* neurogenesis, Brg1 is expressed ubiquitously in early development and becomes restricted to the neural tissue a later stages. In situ studies demonstrated distinctive expression pattern for the brg1 and brm paralogs. In 2004, our laboratory could demonstrate the specific expression pattern of Brg1 and Brm. At tailbud, stage brm is expressed in the hindbrain, spinal cord, pronephros and somites. Brg1 however is restricted to the branchial arches (Linder et al, 2004). *Xenopus* Brg1 is important in the ß-catenin dependent determination of secondary body axis formation (Singhal, 2005). In addition, the transactivation of the bHLH transcription factors Neurogenin and NeuroD (see results) proves Brg1 to be required for neurogenesis (Seo et al, 2005a; Seo et al, 2005b). Loss of Brg1 function

in *Xenopus* results in a reduction in n-ß tubulin expression and a failure of Ngnr1 and NeuroD to promote neural differentiation of neurons from proneural cells (Seo et al, 2005a; Seo et al, 2005b). In mammals, Brg1 is expressed in neural stem cells, which give rise to neurons and glial cells (Matsumoto et al, 2006). Loss of Brg1 function reduces the expression of proteins associated to neural stem cell maintenance, e.g. Pax6 and Sox1. These remodelling complexes have dedicated functions at different stages of neural development that appear to arise by combinatorial assembly of its subunits (Yoo & Crabtree, 2009)

Considering the stage specific functions of CHD4 in its ATPase dependent and independent manner and the different potential interaction partners, forming a putative NuRD complex, my observations could also be influenced due to different interaction partners at different stages during development.

In mammals, NuRD includes the subunits CHD3/CHD4 and the histone deacetylases HDAC1 and HDAC2 to function as transcriptional repressors. Like BAF complexes, mammalian NuRD complexes achieve diversity in regulatory function through combinatorial assembly of its subunits. Beside the core ATPases CHD3 and CHD4, there are three main accessory subunits, which are encoded by different gene families. First, MTA (*m*e*t*astasis-*a*ssociated), MBD (*m*ethyl-CpG-*b*inding *d*omain) and the RbBP (*r*etino*b*lastoma-associated-*b*inding *p*rotein) are part of NuRD as described in the introduction. In addition, each complex contains one MTA protein, MTA1, MTA2 or MTA3, which are mutually exclusive and nucleate complexes with different, and sometimes opposite, functions. The composition of the NuRD complexes varies with cell type and in response to signals within a tissue (for review see (Denslow & Wade, 2007)), which gives rise to a diversity of complexes with distinct functions. Thus, CHD4 misexpression during development and cellular differentiation must be seen under this premise. As an example for different compositions of the NuRD complex, which lead to different CHD4 functions, the subunit MBD2 or MBD3, are functionally distinct and contribute to different forms of the complex (Feng & Zhang, 2001). This is also true for the subunits RbBP4 and/or RbBP7.

Further studies of the different subunits of the mammalian NuRD complex have shown additional functions during development. Inactivation of mouse Mbd3 results in death during mid-gestation, stemming from the failure of the inner cell mass to form an epiblast and the subsequent failure of embryonic and extra-embryonic tissues to organize properly after implantation (Kaji et al, 2007).

A further role of the NuRD complex is crucial during differentiation. Loss of Mbd3 results in the failure to assemble NuRD complexes and probably reflects a loss of function for these complexes. Mbd3-null ESCs (*e*mbryonic *s*tem *c*ells) have been

shown to be viable and can initiate differentiation in culture, but they fail to commit to developmental lineages, due to impaired silencing of pluripotency genes (Kaji et al, 2006). Conditional inactivation of CHD4 in the haematopoietic cells of mice leads to impaired haematopoietic stem-cell homeostasis and impaired differentiation into myeloid cells with a defective thymocyte development and defective activation of the *cd4* locus (Williams et al, 2004; Yoshida et al, 2008). Consequently, the NuRD complex is crucial for the correct silencing of genes during early development to allow proper patterning and cell lineage commitment.

In Drosophila, CHD4/Mi-2 also exists in a novel chromatin-remodelling complex, referred to as dMec that does not rely on histone deacetylation to affect transcriptional repression of proneural genes. Furthermore, the CHD4/Mi-2 related factor CHD3 acts as a monomer and does not associate with additional subunits *in vivo*. These results ad an additional complexity to the composition and function of CHD chromatin remodelling complexes (Kunert & Brehm, 2009). In relation to my work, these new insights in CHD4 function open the possibility that CHD4 gain-of-function could be mediated via additional functions, but HDAC activity.

In addition, our notion that CHD4/NuRD regulates signalling events to coordinate gene expression dynamically in a context dependent manner is underlined by very recent data that demonstrate that CHD4/NuRD orchestrates proper signalling in the context of DNA damage repair (Chou et al, 2010; Larsen et al, 2010; Polo et al, 2010; Smeenk et al, 2010).

5.4.2 Dynamic remodelling complexes to serve additional functions?

One essential question can be asked, why this diversity of different remodelers evolved and why the regulation of the genome requires functionally different ATP-dependent chromatin remodelers if they all function in an increase of nucleosome mobility?

One argument could be that beside their function as remodelers, additional roles and molecular functions have been discovered recently. For example, ISWI complexes have been shown to be required for maintaining the higher order structure of the *Drosophila melanogaster* male X chromosome 8, and INO80 complexes are involved in telomere regulation, chromosome segregation, checkpoint control, and DNA replication during cell division. For review see (Morrison & Shen, 2009).

Even within their traditional role of transcriptional regulation, ATP-dependent chromatin remodelers do not function in a consistent manner. For example, the

Brahma-associated factor (BAF) complexes, which belong to the SWI/SNF family, can function as both transcriptional activators, as well as repressors and can even switch between these two functions at the same gene (Chi et al, 2003). Moreover, tissue-specific BAF complexes have been reported to interact with a variety of transcription factors in different cell types. This enables the complexes to provide context dependent functions arising from their different interaction partners, as described above. This information-integrating network raises the question, how it can be entangled experimentally and which functions are crucial in different contexts? How can such a system be studied and which fundamental rules can be administered to a dynamic system, which evolved during development to flexibly provide a balanced system of information integration for the benefit of evolution?

I approached these questions to entangle this complexity by interfering at two different time points during development and could demonstrate changing roles of the CHD4 chromatin remodelling ATPase function during neural development. Bearing in mind that we approach truth, as we apply ontological, epistemological and methodological reductionism to the complexity of a developing organism, this is the most adequate manner to generate new knowledge. A profound understanding of these fundamental biological processes achieved by experimental approaches in model organism as *Xenopus* will enable to dissect the complexity of epigenetic regulatory network and translate it to human biology. This understanding of biological processes will provide opportunities to understand disease development and putative solutions for problems in the medical field.

5.5 Bridging the gap from basic epigenetic research to medical opportunities

The understanding of neural fate acquisition during embryogenesis is exciting in its own right. In addition to that however, from a medical point of view, the knowledge of the basic molecular developmental mechanisms is essential, because the same signalling factors and transcriptional regulators are also expressed in endogenous niches of neural stem cells in the adult brain.

Human embryonic stem cells (hESCs) can efficiently differentiate into functional neurons and glia with a mechanism akin to *in vivo* development (Hu et al, 2009; Li et al, 2005; Perrier et al, 2004; Roy et al, 2006; Yang et al, 2008). Therefore, the

developmental program that specifies neural fate in the embryo is important to programme neural stem cells to repopulate damaged tissue. However, due to ethical concerns, the opportunities surrounding hESCs is under discussion. In addition, a potential immune rejection due to their allograft character must not be ignored. According to studies in mice and with human somatic skin cells, which were reprogrammed by a set of core pluripotent transcription factors induced pluripotent stem cells (iPSCs), which are truly pluripotent (Boland et al, 2009; Okita et al, 2007; Park et al, 2008b; Takahashi et al, 2007; Yu et al, 2007; Zhao et al, 2009). Interestingly, neural differentiation of human iPSCs was shown to also follow developmental principles. In addition, human iPSCs have been shown to convert to neuro-epithelial cells following the same differentiation program as hESCs and could express neural marker genes. Their neural identity was determined by the expression of Pax6 and Sox1. The neural differentiation process was improved by regulating FGF and BMP signalling (Hu et al, 2010). This finding is very interesting in relation to this work, as I demonstrate the influence of CHD4 dependent chromatin remodelling during neural induction and neural cell differentiation that is regulated by FGF and BMP signalling processes. In addition, I could demonstrate that CHD4 chromatin remodelling regulates the expression of neural marker genes, including Pax6 and Sox2. This resembles the results achieved in hESCs. Consequently, epigenetic regulation plays a fundamental role during cellular (re-)programming processes and could thus provide opportunities to enhance cellular differentiation protocols for medical applications. Most recent evidence that epigenetic mechanisms, especially ATPase dependent chromatin remodelling, plays a role in the generation of iPSCs through somatic cell reprogramming is provided by Hans Schöler's group (Singhal et al, 2010). They demonstrated that the ATP-dependent BAF chromatin remodelling complex significantly increases the reprogramming efficiency when used together with the four transcription factors Oct4, Sox2, Klf4, and c-Myc. In addition to that, it was shown that gene suppression by pluripotency factors in ESCs is associated with the recruitment of repressive chromatin remodelling complexes, such as CHD4/NuRD (Kaji et al, 2006). A further example that underlines the role of chromatin remodelling during cell (re-)programming was provided by our laboratory, as we demonstrated that the chromatin remodelling factor CHD4 plays a crucial role for the boundary formation between neuroectoderm and mesoderm. We showed that this is specifically controlled by the Nodal input via Sip1 for the Xbra transcription (Linder et al, 2007). Most recent data provides evidence that Nodal signalling acts through Sip1 to regulate the cell-fate decision between neuroectoderm and mesendoderm in human pluripotent stem cells (hPSCs) (Chng et al, 2010).

Thus, it is possible that signalling information for the neuroectoderm/mesendoderm cell-fate decision in hPSCs by Sip1 is also regulated by CHD4 chromatin remodelling. As I demonstrate in this work that CHD4 and Sip1 could shift the ectoderm/neuroectoderm boundary for the benefit of neuroectoderm, my data can help to understand how stem cells could be programmed from neural commitment to neural differentiation, and reprogrammed back to a higher potent state by epigenetic chromatin remodelling.

A further step in cellular (re-)programming is provided by the recent results of Marius Wernig's group, which efficiently converted mouse embryonic and postnatal fibroblasts into functional neurons *in vitro*, referred to as induced neuronal cells (iN) (Vierbuchen et al, 2010). The induced cellular phenotypes are defined and reinforced by lineage-specific transcription factors, leading to cell-type-specific gene expression patterns. These patterns are further stabilized by epigenetic modifications that allow faithful transmission of cell-type-specific gene expression patterns over the lifetime of an organism (Bernstein et al, 2007; Jenuwein & Allis, 2001). Emphasising the crucial role of epigenetic gene regulation in their study, Wernig argues that changes in transcriptional activity result in a genome-wide adjustments of repressive and active epigenetic features such as DNA methylation, histone modifications and changes of chromatin remodelling complexes, which further stabilize the new transcriptional network (Jaenisch & Young, 2008; Zhou & Melton, 2008). A further possible notion that can be considered is that certain subpopulations of cells are 'primed' to respond to inducing factors, depending on their pre-existing transcriptional or epigenetic states (Yamanaka, 2009). Interestingly, the principle of reprogramming a differentiated cell into a different cell type by changing epigenetic patterns in combination with specific transcription factors has already been described very early in *Xenopus*. It was shown that fibroblasts could be converted to stable myoblasts by 5-azacitidine-treatment and transfection with the muscle specific transcription factor MoyD (Davis et al, 1987). Incorporation of 5-azacitidine into DNA inhibits methyltransferases, therefore leads to demethylation and de-repression of methylated gene loci. This data demonstrate that one type of differentiated cell can directly be converted into another, notably; this approach does not always require a stem-cell intermediate stage.

During the late 1950s, Briggs and King established the technique of somatic cell nuclear transfer (SCNT), or "cloning". This technique demonstrated the developmental potential of isolated nuclei derived from late stage embryos and tadpoles by transplanting them into enucleated oocytes (Briggs & King, 1952; King & Briggs, 1955). Together with work by Gurdon (Gurdon, 1962; Gurdon et al, 1975),

this illustrated that differentiated amphibian cells preserve the genetic information that is necessary to develop into cloned frogs. The conclusion that can be drawn from these early findings is that the genome undergoes reversible epigenetic, rather than irreversible genetic changes during cellular differentiation. This implies that these epigenetic changes have to be reversed, when the process of cell differentiation wants to be reprogrammed to generate iPSCs. Interestingly, this epigenetic contribution to cell reprogramming is emphasised by most recent data that describes an "epigenetic memory" in iPSCs by transcription factor-based reprogramming, compared to reprogramming by SCNT (Kim et al, 2010). They describe that iPSCs, derived by factor-based reprogramming of adult murine tissues harbour residual DNA methylation signatures characteristic of their somatic tissue of origin. This "epigenetic memory" of the donor tissue could be reset by differentiation and serial reprogramming, or by treatment of iPSCs with chromatin-modifying drugs. In contrast, the differentiation and methylation of nuclear-transfer-derived pluripotent stem cells were more similar to classical embryonic stem cells than iPSCs. Consequently, nuclear transfer was suggested to be more effective at establishing pluripotency than factor-based reprogramming, which can leave an "epigenetic memory" of the tissue of origin (Kim et al, 2010). That memory could influence applications in disease modelling or treatment. Nevertheless, in addition to serve for basic studies in development and epigenetic reprogramming, iPSCs have therapeutic potential for two fundamental concepts: First for custom-tailored or personalized cell therapy, and second for so-called "disease modelling". For cell therapy, the advantages are obvious, as therapy by organ transplantation is complicated, limited and require life long immunosuppression. Here, iPSCs from patients could provide the solution as they could be differentiated into the desired cell type that is already genetically matched with the patient. An additional approach could be the repairing of disease causing-mutations by homologous recombination. Promising data was provided by Jaenisch and colleagues in a mouse model for sickle cell anaemia (Hanna et al, 2007). They reprogrammed mouse skin cells into iPSCs, fixed the disease-causing mutation and differentiated the repaired cells into healthy blood-forming progenitors. The progenitor cells were transplanted into the anaemic mice, where they formed healthy red blood cells, and cured the disease. This method was also applied to correct haemophilia A in mice (Xu et al, 2009). The principle could be applied to any human disease with a known mutation that can be treated with cell transplantation.

The second approach is referred to as "disease modelling", which means that iPSCs, derived from patient skin cells could be differentiated *in vitro* into the diseased cell

type, thereby recapitulating the disease *in vitro*. This model of the disease could help to identify novel drugs to treat the disease. Several laboratories have isolated iPSCs from patients suffering from amyotrophic lateral sclerosis (ALS) (Dimos et al, 2008), Huntington's disease, Pakinson's disease (Soldner et al, 2009), juvenile diabetes, muscular dystrophy, Fanconi anaemia (Raya et al, 2009), Down syndrome, immunodeficiency (ADA-SCID), Shwachman-Bodian-Diamond syndrome, Gaucher disease type III, Duchenne and Becker muscular dystrophy and others (Park et al, 2008a). In addition, *in vitro* therapeutic approaches have been reported for spinal muscular atrophy (Ebert et al, 2009), familial dysautonomia (Lee et al, 2009) and the LEONARD syndrome (Carvajal-Vergara et al, 2010). For review about the history, the mechanisms and the applications of induced pluripotency please see (Stadtfeld & Hochedlinger, 2010).

The possibility to reverse the cellular differentiation process by a few transcription factors, combined with epigenetic mechanisms that enable and stabilize stage specific gene expression profiles also influenced our view back on normal and disease development. Comparable to reprogrammed iPSCs, the mechanisms that stabilize and regulate gene expression profiles are often reversed in cancer cells, which show characteristics of stem cells and de-differentiation (Stadtfeld & Hochedlinger, 2010). In contrast, induced neural differentiation could be achieved from human embryonic carcinoma stem cells. Interestingly, neural differentiation was accompanied by significant changes in the acetylation and methylation patterns of histone H3, and expression level of the histone variant H2A.Z. The epigenetic changes occurred on the regulatory regions of Oct4, Nanog, Nestin, and Pax6 (Shahhoseini et al, 2010).

Specific signalling pathways that are mutated cancer cells are also associated with iPSCs formation. This illustrates similarities of tumourgenesis and cellular re-programming and emphasises the role of epigenetic gene regulation in both healthy and diseased cells. The understanding of cancer development in recent years has identified epigenetic abnormalities as a common factor in tumourigenesis. One epigenetic factor is the dysregulation of histone deacetylases (HDACs) in both haematological and solid tumours. Research over the past decade consequently led to the development of HDAC inhibitors (HDACI) as anticancer agents. For a recent review about HDAC inhibitors and cancer therapy please see (Atadja, 2010).

In relation to my work, data suggests epigenetic regulation by ATPase dependent chromatin remodelling to be specifically crucial in cellular differentiation, especially for neural tissue. As an example, Chd1-deficient embryonic stem cells have been shown to be no longer pluripotent, because they are incapable to give rise to

primitive endoderm with a high propensity for neural differentiation. Furthermore, Chd1 is required for efficient reprogramming of fibroblasts to the pluripotent stem cell state. These results indicate that Chd1 is essential for open chromatin and pluripotency of embryonic stem cells and for somatic cell reprogramming to the pluripotent state (Gaspar-Maia et al, 2009).

A recent study revealed an evolutionarily conserved role for CHD7, which was identified to orchestrate neural crest gene expression programs, which provides insights into the synergistic control of distal elements by chromatin remodelers and illuminated the patho-embryology of the CHARGE syndrome. The CHARGE syndrome is a sporadic, autosomal dominant disorder characterized by malformations of the craniofacial structures, peripheral nervous system, ears, eyes, and heart. This observation is suggested to represent a broader function for CHD7 in the regulation of cell motility (Bajpai et al, 2010).

A further interesting syndrome that links epigenetic regulation in neural development with disease formation is the Mowat-Wilson syndrome (MWS). MWS is caused by Sip1 mutations. It was shown that aberrant Sip1 protein is unable to recruit NuRD/CHD4. This defective NuRD/CHD4 recruitment due to mutant human Sip1 can be a MWS-causing mechanism (Verstappen et al, 2008). MWS is characterized by a number of health defects including delayed growth and motor development, congenital heart disease, genitourinary anomalies and absence of the corpus callosum, mental retardation, and Hirschsprung's disease. Hirschsprung's disease arises when ganglion nerve cells in the gut fail to develop and mature correctly. Considering these human developmental defects, MWS could consequently be understood as a clinical representation of a disorder in the epigenetic regulatory mechanism by CHD4/Sip1, which I describe in this work.

During the last years, these examples demonstrated that epigenetic regulation of cell differentiation revealed promising opportunities in medical applications. The study of epigenetic mechanism for cell (re-)programming and cellular differentiation in developmental model organism are crucial steps to understand how cells can be modulated for the benefit of patients. The knowledge of the basic mechanism of epigenetic regulation during cellular differentiation and (re-)programming has been acquired by basic research in model organisms like *Xenopus*.

Concluding, in reference to the introduction, epigenetic cell modification has a high potential to serve as useful tools for medical applications. Epigenetically enhanced (re-)programming of iPSCs could overcome the problems of hESCs to study diseased cells for pathological studies, drug screening, and regenerative medical approaches.

6 Abbreviations

Å	Ångström = 10^{-10} metre
ACF	ATP-utilizing chromatin assembly and remodelling factor
ASF1	anti-silencing function 1
ATP	adenosine triphosphate
bHLH	basic helix-loop-helix
BMP4	bone morphogenic protein 4
bp	base pairs
CAF-1	chromatin assembly factor-1
cDNA	complementary DNA
CHD4	chromodomain-helicase-DNA binding4 protein
chd4	chromodomain-helicase-DNA binding4 gene
CHRAC	chromatin accessibility complex
chromo	chromatin organization modifier
CtBP	C-terminal-binding protein
CZf	C-terminus zincfinger
CK2	cyclin dependent kinase2
DEPC	diethlypryocarbonate
ddH_2O	double-distilled water
DNA	deoxyribonucleic acid
DNMT1	DNA-methyltransferase 1
e.g.	exempli gratia, for example
EMT	epithelial to mesenchymal transitions
ES	embryonic stem
EST	expressed sequence tag
et al.	et alii, and others
Ez	enhancer of zeste
FACT	facilitates chromatin transcription
FADD	Fas associated death domain”
FGF	fibroblast growth factor
GFP	green fluorescent protein
GST	glutathione S-transferase
h	hour
HAT	histone acetyltransferase
HDAC	histone deacetylase

HDACI	HDAC inhibitors
HD	homeodomain
hESCs	Human embryonic stem cells
HIF-1α	hypoxia inducible factor-1α
H3K4me2	example for abbreviation of histone modification, here: dimethylation of lysine 4 on Histone H3
HMG	high mobility group
HMT	histone methyl-transferase
HP1	heterochromatin protein 1
hESCs	Human embryonic stem cells
hpf	hours post fertilization
hsp	heat shock promoter
ICC	immunocytochemistry
i.e.	id est, it is
IF	immunofluorescence
ISH	in situ hybridization
ISWI	imitation switch
iPSCs	induced pluripotent stem cells
IUPAC	International Union of Pure and Applied Chemistry
l	litre
kDa	kilodaltons
LSD1	lysine-specific demethylase 1
M	molar
MBD	methyl-CpG-binding domain protein
MBT	mid-blastula transition
MeCP1	methyl-CpG binding protein
min	minutes
ml	millilitre
mM	millimolar
mRNA	messenger ribonucleic acid
MTA	metastasis-associated gene
NAP-1	nucleosome assembly protein-1
NCP	nucleosome core particle
NAD	nicotinamide adenine dinucleotide
NF	*Xenopus* developmental stages according to the normal table of staging of *Xenopus laevis* (Daudin) after (Niewkoop & Faber, 1994)
ng	nanogram

nm	nanometre
NoE	Epigenome Network of Excellence
NTPs	nucleotide triphosphate mixture containing adenosine, guanidine, uridine and cytosine
NZf	N-terminus zincfinger
NuRD	nucleosome remodelling and histone deacetylase
NURF	nucleosome remodelling factor
OD	optical density
PcG	Polycomb
PCR	polymerase chain reaction
PHD	plant homeodomain
PRC2	Polycomb Repressive Complex 2
pmol	picomol
PTMs	posttranslational modifications
Rb	Retinoblastoma
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gelelectrophoresis
sec	seconds
SIP1	Smad-interacting-protein-1
Sir2	silent information regulator
SSRP1	structure specific recognition protein 1
SWI2/SNF2	switch 2/sucrose-non-fermenting 2
TCH	terminal conserved hairpin
TRD	transcriptional repression domain
UV	ultraviolet
VEGF	vascular endothelial growth factor
WB	Western blot analysis
µg	microgram
µl	microlitre
µM	micromolar

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