The molecular function of AT-rich DNA binding proteins in the developing brain

PhD thesis

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I. Introduction

A central question in developmental neuroscience is how progenitor cells differentiate into diverse neuronal and glial phenotypes. Several developmental brain disorders are thought to be the consequence of abnormal regulation of this differentiation processes. Building an adult nervous system involves the production of a vast array of neuronal and glial cell types that must be produced at the correct number and appropriate positions. Diversity manifests in morphology, connectivity (afferent and efferent projections and synapse targeting), immunohistochemical character, molecular profile, electrophysiological nature, etc. Regulation of cell fate acquisition in the vertebrate central nervous system is complex and reliant on both cell-intrinsic factors and position-dependent extracellular cues.

In this study we investigated the transcriptional regulatory mechanisms that may affect neuronal fate decisions in the developing brain. We analyzed the regulation of the enkephalin (ENK) gene in the developing brain as a model system during the specification of enkephalinergic neurons.

Two AT-rich dsDNA binding proteins were identified, one with a molecular weight of approximately 45 kDa and another of 90 kDa. They were named as developmental-specific AT-rich DNA binding protein (DAT) 1 and 2, respectively. AUF1 was found to be identical to DAT1, and SATB2 to DAT2. Both of these structurally unrelated proteins are expressed in development- and cell-specific fashion in the rat brain and are likely to be involved in regulating gene expression in differentiating neurons (Agoston et al. 2006., in preparation).

1. Telencephalic development

The mechanisms involved in telencephalic development are not yet completely understood, however, some factors and pathways are already identified that induce transition from one progenitor stage to the next. Most factors control multiple steps, like self-renewal of neural stem cells as well as differentiation of neurons, and there is also a cross-talk between the different pathways, which makes the picture even more complex. (**Fig. 1**.)

1. 1. From neural stem cells to the neuronal progenitors

A number of signaling pathways and nuclear factors have been implicated in the self-renewal of neural stem cells, including in their proliferation and/or the maintenance of their undifferentiated state. For example the Notch signaling pathway and its main effectors, the Hes proteins, have a well-characterized role in keeping progenitors undifferentiated and insulating them from specification signals [1], so that progenitors that escape Notch inhibition at different times acquire distinct fates. [2, 3]. Numb is an inhibitor of the Notch pathway, and is required for the acquisition of distinct fates by daughter cells of asymmetrically dividing neural progenitors [4]. Lgl1-mediated localization of Numb to the apical membrane is required to maintain asymmetric divisions and neurogenesis. [5]

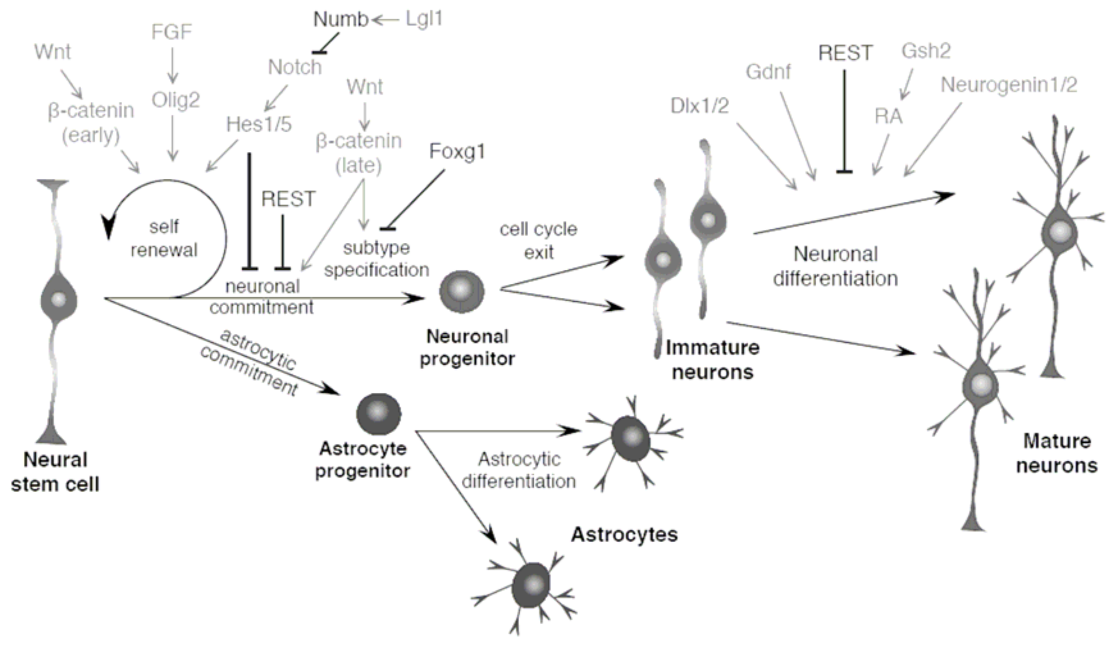

Figure 1.
Some of the pathways regulating the generation of telencephalic neurons or astrocytes. (Modified from [6])

1. 2. Nuclear factors involved in striatal development

In the striatum, Notch activity is only required during the period of neurogenesis, when progenitors divide asymmetrically, and different combinations of Notch receptors are required for the generation of early- and late-born striatal neurons [7]. The FGF signaling pathway, similarly to Notch, also promotes self-renewal, possibly through activation of Olig2 [8], and induces the radial glia phenotype, suggesting possible interactions between the two signaling pathways in maintaining this progenitor population [9]. Wnt signaling also has an important and complex role in regulating neural stem cell activity.

1. 3. Regulatory pathways of cortical development

In the cortex, a stabilized form of b-catenin promotes either the self renewal [10] or the neuronal differentiation of progenitors [11], depending on when it is

expressed during cortical development, suggesting that a timing mechanism modifies the response of cortical progenitors to the Wnt pathway.

The progressive onset of neuronal gene expression that marks the commitment of progenitors to the neuronal lineage and their subsequent differentiation involves not only transcriptional activators such as proneural bHLH proteins [12], but also transcriptional repressors. Indeed, REST/NRSF a factor known to repress neuronal genes in non-neuronal cells, turns out to be an important regulator of neuronal lineage progression [13]. The transition from pluripotent stem cells to neural stem cells and then to neurons involves a progressive reduction of REST binding to neuronal promoters. Moreover, the timing of neuronal gene expression may be regulated by differential affinity of REST binding sites, with early expression of the proneural gene Mash1 correlating with a lower binding of REST to the Mash1 gene than to neuronal genes expressed later on[1].

1. 4. Genes involved in neuronal subtype specification and differentiation

The commitment of multipotent stem cells to the neuronal lineage, and the specification of neuronal progenitors to a particular subtype identity, represents two conceptually distinct steps of neurogenesis. Yet they are mechanistically tightly linked, as genes known to promote neuronal commitment have now also been shown to control the specification of neuronal identities. The specification of neuronal identities involves not only factors acting instructively to promote a particular fate, but also factors that suppress alternative fates. Foxg1, a winged helix transcription factor expressed by most telencephalic progenitors, regulates proliferation and cell fates in both dorsal and ventral telencephalon [14]. In the cortex, Foxg1 suppresses the Cajal-Retzius cell fate[15].

Once a post-mitotic precursor has been specified to a particular neuronal fate, it still has to migrate to its final position, acquire its mature dendritic morphology and survive until its axon has reached an appropriate target. A few components of genetic programs that control the later steps of telencephalic neuron differentiation have recently been identified.

Differentiation of striatal projection neurons involves retinoic acid signaling induced by the patterning gene Gsh2 [16]. Glial-derived neurotrophic factor (Gdnf) is involved in axonal growth and the acquisition of neuronal morphology by cortical interneurons [17], while Dlx1 acts to promote the survival and morphological maturation of a subset of cortical interneurons [18]. (**Fig. 1**.)

Comparatively little is known about the factors that regulate the differentiation of cortical neurons, although Neurogenin1 and 2, have an early role in the process [19]. Obviously, much remains to be learned about the regulatory networks that underlie the differentiation of postmitotic precursors into various types of mature neurons.

2. AT-rich DNA in the mammalian genome

The above mentioned transcription factors (like Foxg1) usually bind to their cognate DNA sites. However, not all proteins that are involved in developmental gene expression regulation bind to DNA elements having a specific sequence: some binding sites attract their proteins by their special secondary structure rather than by a specific sequence. Such DNA regions are the AT-rich DNA elements.

2.1. The two major groups of AT-rich DNA regions

Two distinct groups of AT-rich DNA are present in the mammalian genome: long AT-islands and short AT-tracks. AT-islands are distinct large domains consisting of highly atypical AT-rich sequences[20]. The AT content of these domains ranges from ~80 to 100% AT over their entire length of 200-1000 bp or more. [21]

AT-islands represent repetitive DNA, but no single repeat consensus sequence is common to all these domains. The general consensus for AT-islands is (W)n, where "W" represents A or T and n=200-1000 or more bp with occasional S (G or C) not exceeding 10-15% of the total number of bp in an AT island. A common feature for AT-islands is not any specific sequence expressed using the four letter base designation, but just the presence of long tracts of "W"s. In a sense, AT-islands represent the counterpart of CpG islands, long stretches of highly GC-rich DNA [22].

2.2. S/MARs

Long stretches of AT are characterized by a narrower minor groove, which can be "read" by various interacting proteins through specialized peptide motifs called "AT hooks" [23]. For instance, *MLL* protein that contains a cluster of three AT hooks could bind a long AT-rich S/MAR DNA but not a DNA domain that lacked extended AT area [24]. Moreover, the long stretches of nearly pure AT DNA confer other highly unusual structural properties, such as long-range duplex destabilization [25], also known as base unpairing regions (BURs). Cellular roles of long AT-islands and short AT-enriched tracts are also likely to differ. Various short AT tracts are most often implicated in the regulation of transcription, serving as binding sites for some transcription factors and gene regulators [26]. In contrast, long AT-islands seem to be

involved in the long-range structural organization of the genome in association with the nuclear matrix [20].

Nuclear scaffold- or matrix attachment regions (S/MARs) have been implicated in the regulation of gene expression by altering the organization of eukaryotic chromosomes and augmenting the potential of enhancers to act over large distances [27].

S/MARs have been identified as AT-rich sequences that are associated with the nuclear matrix. The nuclear matrix, also referred to as the interchromatin space, contains multiple sub nuclear structures that can be visualized microscopically as speckles and that have been proposed to functionally compartmentalize the nucleus [28, 29]. The association of S/MARs with the nuclear matrix may serve to structurally define the borders of chromatin domains and participate in the regulation of transcription [30, 31].

3. Chromatin remodeling

Many transcription factors act by recruiting multiprotein complexes with chromatin modifying activities to specific sites on DNA. These findings have merged two fields that were previously disconnected, allowing the study of chromatin structure and sequence-specific transcriptional regulatory proteins, within the same conceptual framework. [32]

Chromatin remodeling is emerging as a critical mechanism of coordinating the expression of large numbers of genes [33-35], and recent publications highlighted the importance of correct epigenetic regulation during the nervous system development.[36] Mutations of MeCP2, a member of chromatin remodeling

complexes, cause Rett syndrome, an X-linked dominant neurodevelopmental disorder by the loss of epigenetic imprinting. [37].

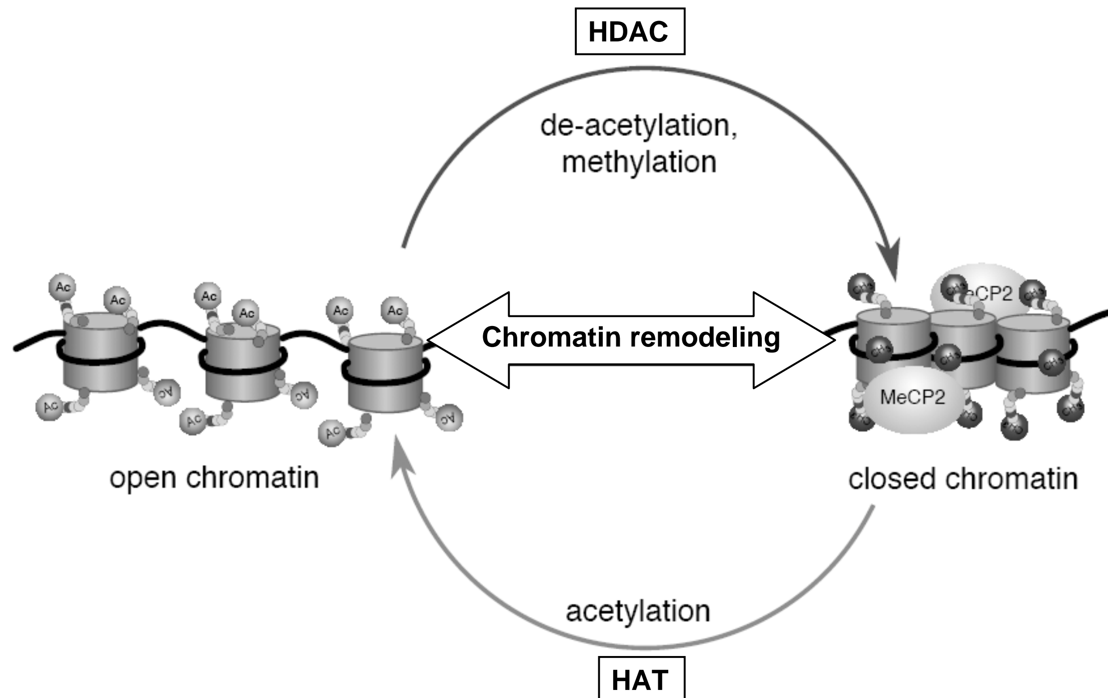

Figure 2.
Cartoon showing how the secondary modifications of histone tails (acetylation vs. methylation) modify the transcriptional availability of a gene. Acetylated histones mean an "open" chromatin structure enabling transcription factors to bind and initiate gene expression. Histone acetyl-transferase (HAT) acetylate histone tails promoting a more transcriptionally accessible structure, while histone de-acetylases (HDACs) remove these acetyl-groups, so that other proteins in the complex may remodel nucleosomes into a more "closed" structure.

3.1 Definition and function of epigenetic mechanisms

Epigenetics is the study of the heritable and potentially reversible covalent modifications of DNA and its binding proteins. These modifications determine whether the DNA strand is tightly wound in nucleosomal arrays (heterochromatin) and thus, poorly accessible to transcriptional proteins, or if the DNA strand resides in a more relaxed transcriptionally active state (euchromatin). Epigenetic modifications

are important in influencing the levels of gene expression and play a pivotal role in phenotypic variations. [38]

Faced with the problem of how to organize its large genome to fit the dimensions of a nucleus, but at the same time keep the DNA substrate sufficiently accessible to regulatory factors, the eukaryotic cell adapted by the use of the nucleosomes [39]. Nucleosomes consist of DNA segments of about 150 base pairs (bp) wrapped in almost two superhelical turns around molecular barrels, the histone octamers, which consist of two copies of four histone proteins, H2A, H2B, H3, and H4. Neighboring nucleosomes are separated by short segments of linker DNA that can be bound by histone H1. The N termini of the octamer-forming histones protrude from the compact particle to contact DNA, other histones, and non-histone proteins. These contacts contribute to the folding of the nucleosomal fiber into complex higher-order structures, collectively called chromatin [40].

3. 2. Chromatin Remodeling Complexes

Formation of closed chromatin prevents the transcription factors from interacting with the DNA strands and leads to transcriptional repression [41]. Dynamic changes in the chromatin structure, therefore, lead to either transcriptional activation or transcriptional repression. Alteration of the chromatin structure utilizing energy derived from ATP hydrolysis is called “chromatin remodeling” and is one of the known transcriptional regulatory mechanisms. The actual process of chromatin remodeling can be subdivided into a 3-step mechanism. The initial binding to the nucleosomal target may involve simple diffusion or scanning along a chromatin template or a more target-mediated model. After reaching the locus to be modified,

actual remodeling will take place. Finally, after histone displacement, the remodeling complex will need to be released to reach its next target. [42]

The interactions of the histone N-terminal tails can be modulated through acetylation, phosphorylation, methylation, ubiquitination, and ADP-ribosylation. The functional consequences of these modifications, which are likely to have profound effects on recognition of the nucleosomal fiber by regulatory proteins and its higher-order folding, are currently being explored with great intensity.[43]

Although covalent modifiers of the histone tails can contribute to the state of chromatin condensation in regulatory regions, such as at promoter sites, they are not sufficient to single-handedly promote histone displacement. Therefore, the action of chromatin-remodeling complexes is needed to alter the accessibility of long stretches of DNA by displacing individual or multiple core histones.

Remodelings can be divided into classes on the basis of different protein compositions and functions, and include the SWI/SNF (BAF), imitation switch (ISWI), and Mi-2/CHD groups. They are remarkably conserved throughout eukaryotes, and most are abundant complexes with important roles in many aspects of chromatin biology. For example, SWI/SNF-group remodelings have established roles in altering nucleosome positioning at promoters, which can regulate transcription either positively or negatively [44].

The SWI/SNF family of chromatin remodeling complexes plays a role in enhancing transcriptional activity in response to a variety of extracellular cues [45]. The SWI/SNF complex is approximately 2 MDa. SWI/SNF complexes, once recruited to specific regulatory regions, can disrupt nucleosome structure and increase accessibility to DNA in an ATP-dependent manner [45]. These complexes contain an

ATPase subunit at their catalytic cores that is related to the yeast SWI2/SNF2 family of nucleic acid stimulated ATPases (Eisen et al. 1995).

The ISWI group of enzymes was originally identified in *Drosophila*, and includes the ISWI-based remodeling complexes NURF (nucleosome-remodeling factor), ACF (ATP-dependent chromatin assembly and remodeling factor), and CHRAC (chromatin-accessibility complex) [45]. The common theme of these complexes is the presence of the ISWI ATPase subunit, which, like SWI/SNF, shares a homology with the SWI2/SNF2 subfamily of ATPases.

In vivo functions attributed to ISWI complexes include transcriptional activation and repression, chromatin assembly, nucleosome spacing or sliding, replication through heterochromatin, maintenance of higher-order chromatin structure, nuclear remodeling of somatic cells. [46]. In vitro studies have shown that CHRAC and ACF tend to cluster and assemble nucleosomes, and consequently promote transcription deactivation [44]. NURF however, though also ISWI-based, seems to act in vitro in a manner similar to SWI/SNF, in that it acts to perturb and destabilize the chromatin structure [45]

Gene-specific activation domains of transcription factors (transactivators) have been shown to function by directly interacting with coactivators (mediators and other components of the transcriptional machinery) to activate transcription [45].

Activation and repression of gene expression correlate with the acetylation state of histones. In general, acetylated histones are associated with more open chromatin and active gene expression, whereas deacetylated histones correspond with closed chromatin and repressed gene expression. Early work using histone deacetylase inhibitors showed that they disrupted normal development, indicating that the acetylation state of histones is developmentally important [45]. Recently, enzymes

that acetylate histones (histone acetyl-transferases, or HATs) and deacetylate them (histone deacetylases, or HDACs) have been identified [47]. These function in several different large multiprotein complexes that are associated with sequence-specific DNA-binding proteins, which are thought to target the complexes to specific genes, leading to local chromatin modification. Many *in vitro* or cell culture studies have been conducted, examining the biochemical activities of these complexes. However, recent *in vivo* work in several systems has provided some functional information during development.

4. The enkephalin (ENK) gene

We chose the ENK gene as our model to investigate neuronal differentiation, when trying to answer the intriguing question of how some of the multipotent neuronal progenitor cells become differentiated into enkephalinergic neurons.

The contrasting distribution of enkephalinergic cells (i.e. that express the ENK gene and use ENK peptides as neurotransmitters) is the result of the developmental process. The expression of the ENK gene in the adult brain is highly cell-specific and spatially restricted. Many of enkephalinergic neurons are localized in the striatum. Most of other brain structures, such as cerebral cortex, are devoid of enkephalinergic neurons [48].

The ENK gene encodes peptide neurotransmitters (primarily met- and leu-ENK) that bind to and activate various opioid receptors [49-51]. They act as signaling molecules in various systems, including the immune system [52]. In the peripheral nervous system (PNS) and in the CNS they act as peptide neurotransmitters [49].

4.1. Importance and function of ENK

The striatal enkephalinergic neurons mediate important neuropsychiatric functions involving emotion, social behavior, aggression and reward [53].

Abnormal enkephalinergic system has also been implicated in various developmental brain disorders including ADHD [54, 55], seizures [56, 57] autism [58, 59].

Experiments with rodents and primates have indicated that a normally developed striatal enkephalinergic system is critical for social interactions. Striatal enkephalinergic levels have been correlated with the social status and the behavior of rodents and also primates [60-62]. Mice lacking the ENK gene display abnormal behavior, including increased aggression [63]. There is a pronounced sex difference in ENK function, ENK knock-out females display different emotional responses, elevated fear / anxiety [64].

Rat and mouse strains with naturally low basal levels of striatal ENK peptides had an increased propensity for substance abuse (as defined by increased drug and alcohol self-administration) [65-67].

4.2. Enkephalinergic neurons are restricted to specific brain regions.

As mentioned before, in the adult mammalian brain, enkephalinergic neurons are restricted to anatomically well-defined brain regions. Enkephalinergic neurons are found in several of the hypothalamic nuclei, in the olfactory bulb and in the hippocampus. The majority of enkephalinergic neurons however are found in the striatum (caudate-putamen) where large numbers of the medium spiny projection neurons express the ENK gene and use ENK peptides as neurotransmitters [68].

4.3. Developmental expression of ENK

ENK expression starts around embryonic day 16 (E16) in the caudal–ventral part of the rat striatum; around E20, positive cells can be detected by immunocytochemistry in the entire striatum, and by postnatal day 14 (P14) they reach the adult levels [69]. In adulthood, enkephalinergic neurons alter their ENK expression levels in response to environmental signals. The DNA elements responsible for this are binding sites of AP-1 (activator protein 1), NF-1 (nuclear factor 1), (ENK) CRE1, (ENK) CRE2, AP-2 and NF-*j*B, and are located within the first approximate -200 bp relative to the transcriptional start site of the regulatory region of the gene for ENK. This region is also known as the Proximal Regulatory Cassette (PRC).

However, DNA elements that are required for the developmental “decision” enabling only a subset of neurons to become enkephalinergic were not yet identified.

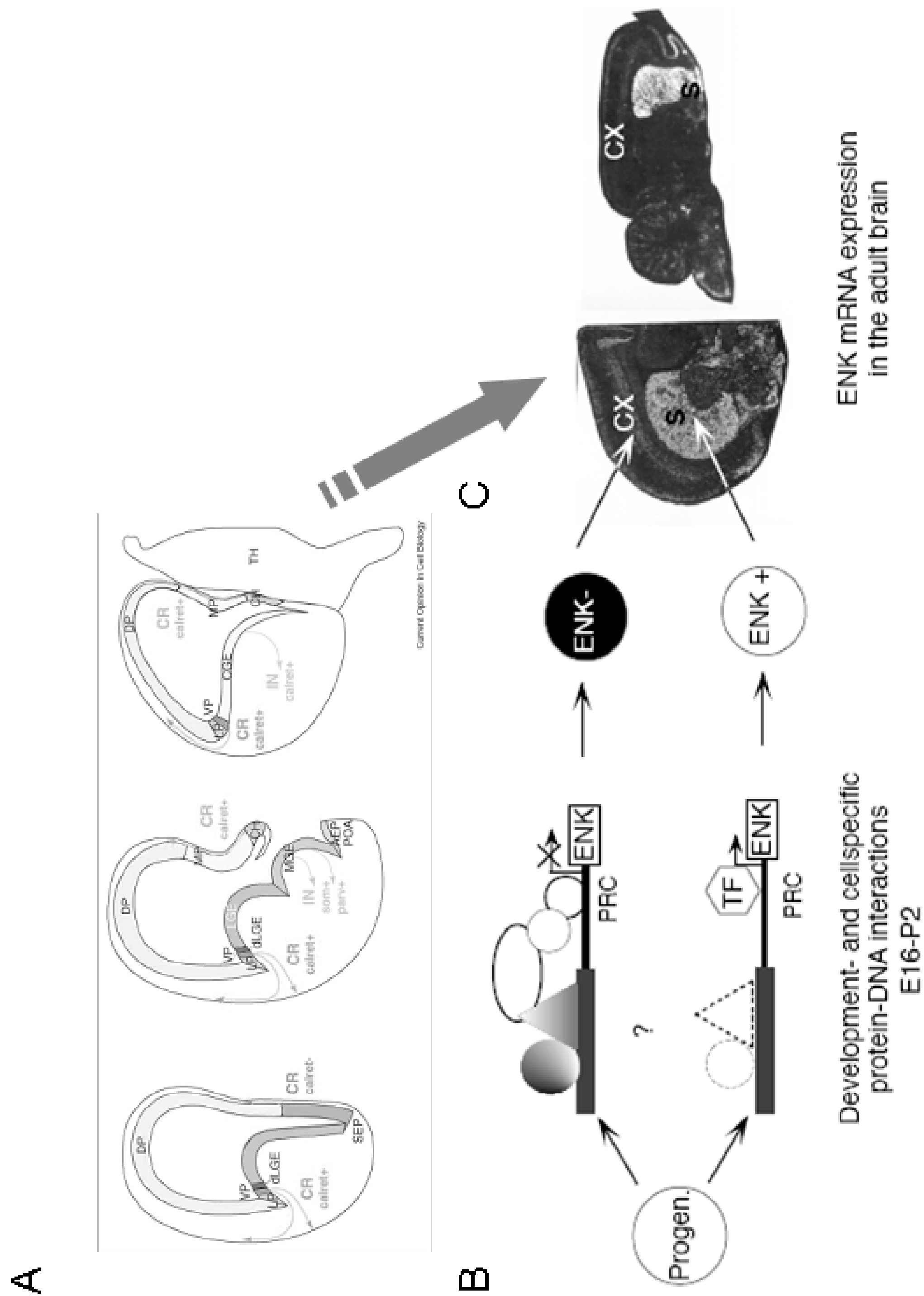

Figure 3.

A) Progenitors in the embryonic telencephalon are organized in distinct domains that generate defined neuronal subtypes. (Frontal sections). The dorsal pallidum (DP) will become the cortex, and the caudal ganglionic eminence (CGE) will form the striatum. (*Modified from* [6]).

B) Schematic representation of how a progenitor cell may become committed to the enkephalinergic phenotype; during the critical period in cortical

development (ages E16 to P2) specific proteins (filled circle and triangle) can bind to the 5' regulatory region of the ENK gene (shown as black rectangle). If this interaction occurs (upper panel), it can recruit other protein complexes that can inhibit the ENK gene expression by preventing the binding of transcription factors (TF) to the Proximal Regulatory Cassette (PRC). That results in an ENK negative (ENK-) phenotype in the cortex (CX) and other brain regions, as shown by the ENK mRNA in situ hybridization on the right side. If the cell- and developmental specific interaction does not take place, (lower panel), the ENK mRNA transcription will be made possible, resulting in an ENK+ phenotype, as shown in the striatum (S).

C) The resulting adult phenotypical distribution, where the majority of the enkephalinergic neurons are to be found in the striatum, and almost none in the cortex.

5. Isolation of the 5' key regions of ENK in development

Several developmental-specific protein-DNA interactions involving different parts of the 5' regulatory region of the rat ENK gene were previously identified using the "sample-and-probe" approach which is a novel combination of microdissection with electrophoretic mobility shift assay (EMSA) and DNaseI footprinting developed by the Agoston lab. [70] For this approach, distinct regions of the rat brain with a contrasting enkephalinergic neuronal population (e.g. caudate nucleus, cerebral cortex, thalamus) were microdissected at key developmental stages (E14, E16, E18, E20, E22, postnatal day 2 (P2), P8 and adult) and nuclear proteins ("samples") were isolated. Partly overlapping DNA fragments representing approx. 6 kb of the 5' regulatory region of the rat gene for ENK were generated and were labeled radioactively ("probes"). These probes were screened for development- and brain region-specific protein-DNA interactions with the various nuclear proteins by electrophoretic mobility-shift assay (EMSA). DNA-binding sites were identified by footprinting and competitive EMSA; the identified DNA motifs were used to isolate the various DNA-binding proteins by expression cloning, or DNA affinity purification.

Using this technique, our group elucidated the role of the septamer region, [71] and an Ikaros-like element that interacts with its specific binding proteins in a spatio-temporally restricted manner.[72]

Another upstream element, the d(TG/AC)*n* repeat was also identified earlier.[73] Ca^{2+} levels of 0.01-1 mM, identical to the physiological total intranuclear concentration, induced a conformational change in d(TG/AC)*n*. It was proposed that the observed conformational change *in vivo* results in a DNA region that provides the genome with a 'joint' or 'hinge' enabling far-distant DNA interactions.

Since DNA elements and their binding proteins that were identified with the `sample and probe' approach may regulate the developmental expression of the ENK gene through structural changes of the DNA, it was hypothesized that far-distant DNA-protein interaction between the proximal regulatory cassette and another upstream DNA element may be involved in the regulation.

This hypothesis was tested by developing a novel *in vitro* "Protein mediated DNA-DNA Interaction" assay (PMDDI) [74]. This assay has identified a far-distant DNA element that resembles nuclear scaffold or matrix associated regions (S/MARs).

5. 1. The AT-rich DNA region is a cis-regulatory element of the rat ENK gene

When differentiating striatal and cortical primary neuronal cultures were transiently transfected with reporter constructs containing either the (-2700; -703) region of the 5' rat enkephalin gene (rENK), or the PRC [75], the the observed reporter gene activities from the plasmid containing 2.7 kb upstream region were consistent with the levels of endogenous ENK mRNA (high in the striatum and very low in the cortex). In contrast, we observed no significant difference in reporter activities in striatal versus cortical cultures when the reporter plasmid containing only

the PRC was used. Based on these findings it was hypothesized that the difference in the observed reporter gene activities is the result of transcription factor(s) selectively expressed in cortical versus striatal neurons, and should directly interact with the PRC of the rENK gene. [76, 77].

5.2. Isolation of proteins that interact with the rAT^{ENK}

To identify these proteins and their DNA binding sites, a search was conducted for protein-DNA interactions that fulfilled the three criteria: first, the protein-DNA interaction should show either negative or positive correlation with the anatomical distribution of enkephalinergic neurons in the adult rat brain (i.e. striatum = high and cortex=low); secondly, the DNA-protein interactions should occur in the developing (~E16 to ~ P2) but not in the adult rat brain and #3, the proteins should mediate interaction between the *cis*-element and the TATA region.

To identify the protein(s) and their *cis*-regulatory DNA elements, a magnetic-bead based assay was designed enabling the detection of protein-mediated DNA-DNA interactions [70] (PMDDI, **Fig.4**).

Fragment "a" containing the PRC + TATA region of the rENK gene, was immobilized and used as bait, while other fragments (*b* through *k*) representing different portions of the 6 kb upstream regulatory region of the rENK gene were labeled radioactively and then individually tested for interaction using cortical nuclear extracts from different ages. Fragment "h" (-2,067 to -2,823) showed a developmental- and brain region-specific interaction in this assay, therefore smaller sub fragments were generated to precisely identify the exact region.

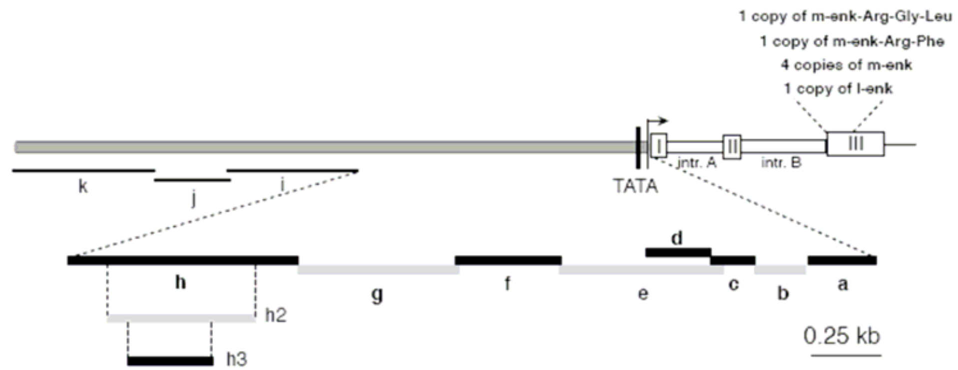

Figure 4.
PMDDI assay: various fragments from the 6000 bp of 5' region of the rENK gene were tested for protein mediated interaction with the TATA-region (named fragment "a").[78]

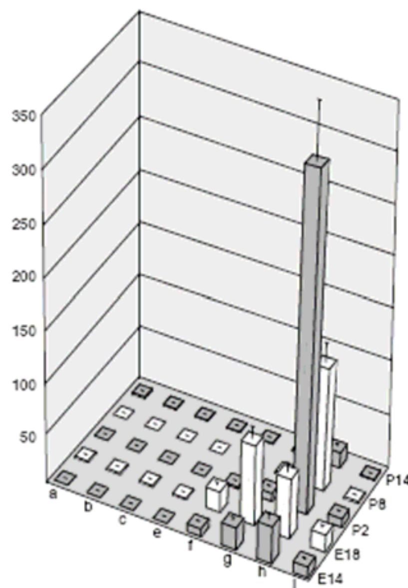

Figure 5.
Results of the PMDDI experiment: cortical nuclear extracts from E18 to P2 mediate specific interaction between the TATA-region (fragment "a") and fragment "h" containing the AT-rich region. Coordinates are as follows; Y: radioactivity measured (count x1000), X: fragments of the 5' region, Z: age of animals that were used to prepare the cortical nuclear extracts.[78]

Because of its relatively large size, a portion of fragment *h* was sub cloned and tested. The resulting fragment h_2 retained full activity as tested by PMDDI. For the identification of the DNA binding site, DNaseI I footprinting was performed on h_3, a sub fragment h_2 to further narrow the search for the regulatory region. Nuclear extracts of the neonatal cortex protected an AT-rich DNA repeat region [$(ATT)_{19}$] of the rENK gene. The ds[$(ATT)_{19}$] oligonucleotide (=rAT^{ENK}) fully retained the behavior of fragment h_2 in PMDDI.

The AT-rich DNA of the rENK gene resembles the core elements of matrix or scaffold attachment regions (MAR/SAR-s) [79, 80]. Since distamycin can compete

with proteins that bind the minor groove of AT-rich dsDNA [81-83], the effect of distamycin treatment was tested on reporter gene activity using cortical and striatal primary neuronal cultures.

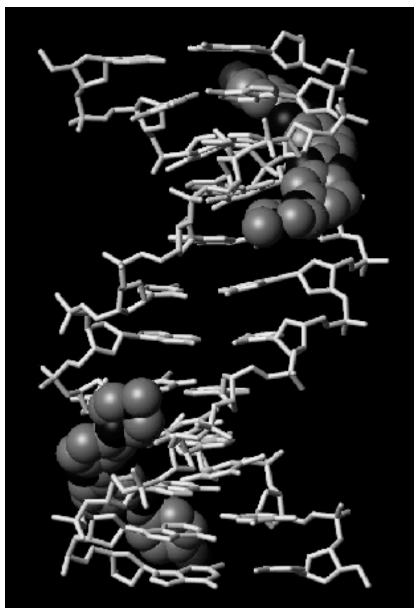

Figure 6.
A model showing how distamycin binds to the minor groove of AT-rich DNA.

Differentiating striatal and cortical neuronal cultures were transfected with the reporter plasmid where the firefly luciferase is under the control of the 5' (-2700; -703) region of the rENK gene. Distamycin treatment resulted in a substantial increase in reporter gene activity in cortical, but only moderate increase in striatal cultures. These result suggested that the protein-DNA interaction takes places in the the minor grove of the AT-rich dsDNA element; the increase of the reporter gene activity after dystamicin treatment suggests that this interaction mediates repression.

5.2.1. Identification of AUF1 as DAT1

The first step toward identification of the ds[$(ATT)_{19}$] binding protein(s) was a UV-crosslinking experiment, that was performed to assess the molecular mass of the protein(s). Because of the significant effect of distamycin treatment seen *in vivo*, half

of the nuclear extracts were pre-incubated with distamycin prior to UV crosslinking. (**Fig. 7**.)

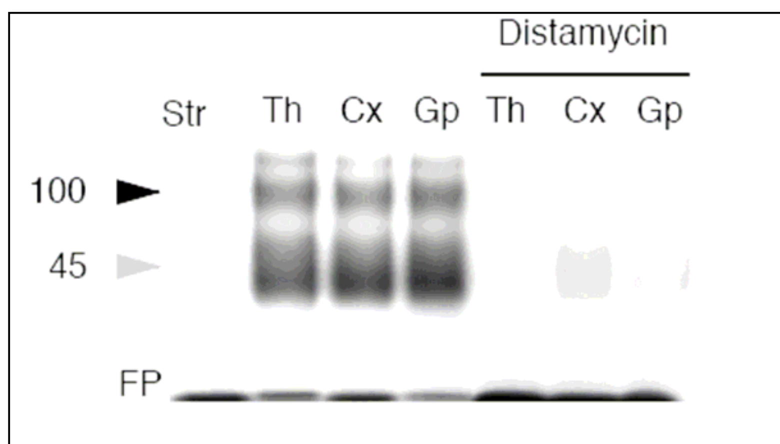

Figure 7.

Distamycin specifically binds to the minor groove of AT-rich DNA elements. Solution-mediated UV-crosslinking of nuclear proteins derived from neonatal Striatum (Str), thalamus (Th), cortex (Cx) and globus pallidus (GP) to a synthetic ds(ATT)19 DNA. AUF1 and SATB2 (identified later) appear as a ~40 kD and a ~90 kD proteins. The presence of distamycin prevents the formation of these complexes. FP: free probe [78]

UV-crosslinking showed a broad protein band with the average molecular mass at around 45 kDa, and a sharper second band with an apparent molecular weight ~ 100 kD (**Fig. 7.**).The broad appearance of the bands suggested that there may be multiple proteins of slightly different molecular weights present in the complex.

Two protein-DNA complexes were seen; the protein with an approximate size of 45 kDa was named development-specific AT-rich DNA binding protein 1 (DAT1) and one of 100 kDa was named DAT2. These proteins are expressed between E16 and P2 in the developing CNS, predominantly in neurons that do not express the gene for ENK (e.g. in cerebral cortex or thalamus).

Preincubation of the nuclear extracts with distamycin almost completely prevented the formation of both ~45 kD and 100 kD complexes with every nuclear extracts tested further implicating the AT-rich minor grove as binding site for both proteins.

To isolate the cDNA encoding the AT-rich dsDNA binding proteins an embryonic rat brain expression library was screened using radioactively labeled $ds(ATT)_{19}$ as probe. Sequence analysis of the positive clone showed that DAT1 is identical to p37, the smallest isoform of AUF1.

5.2.2. Identification of SATB2 as DAT2

To isolate additional proteins of the DAT2 complex, rAT^{ENK} dsDNA fragments were immobilized and DNA-affinity purification was performed using nuclear extracts form neonatal rat cerebral cortex. Bound proteins were separated by gel electrophoresis (**Fig. 8**.), individual bands were cut out and analyzed by mass spectrometry.

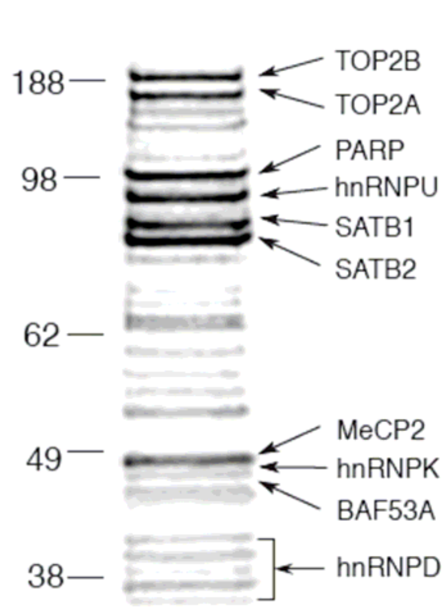

Figure 8.
Identification of AT-rich DNA binding protein following DNA affinity purification. Nuclear extract from developing rat cortex (from P2) was used for DNA affinity purification. The binding fraction was loaded on a 4-12% gradient polyacrylamide

gel, was subjected to electrophoresis and stained with colloid Coomassie Blue stain. After visualization the proteins were identified by mass spectrometry. These bands are marked with arrows. Abbreviations: TOP2B: DNA-Topoisomerase II beta; TOP2A: DNA-Topoisomerase II alpha; PARP: poly-ADP-ribose polymerase; SATB1: special AT-rich sequence binding protein 1; SATB2: special AT-rich sequence binding protein 2; hnRNP U and K: heterogeneous ribonucleoprotein U and K.[84]

As expected several known ubiquitous AT-binding proteins were found (**Table I**). These include DNA topoisomerase II A and B [85], poly(ADP-ribose) polymerase I [86, 87], hnRNP U (SAF-A)[88, 89] and hnRNP K [90]. The identity of DAT2 being SATB2 was confirmed with a supershift experiment.

Table I. AT-rich DNA binding proteins in the neonatal rat cerebral cortex.

Name	Description
DNA topoisomerase II, beta 2 isoform	MAR-binding
DNA topoisomerase II, alpha isoform	MAR-binding
poly(ADP-ribose) polymerase I	MAR-binding
hnRNP U (SAF-A)	matrix protein, MAR-binding
SATB1	A/T-binding
SATB2	A/T-binding
methyl CpG binding protein 2 (MeCP2)	part of NuRD, MAR-binding
hnRNP K	matrix-associated transcription factor
BAF53a	part of SWI-SNF
hnRNP D or C or C-like protein	AT-binding or transcription factor

These proteins are known to be either part of the nuclear matrix or frequently associated with it indicating that rAT^{ENK} binds to the nuclear matrix. In addition, the mass spectrometry analysis also showed that the hematopoietic regulator SATB1 is

also expressed in the developing cortex and binds to rAT^{ENK} dsDNA [91-93]. Finally, a homologue to SATB1 originally called KIAA1034 was identified [84], parallel with another group[27]. Because of its homology to SATB1, the protein was renamed as “special AT binding protein 2” or SATB2.

As during the time of the DNA affinity studies there were no information about the neuronal expression of SATB2 and because its homologue SATB1 represents a novel type of transcriptional regulation, we decided to characterize SATB2 and analyze its expression in the rat brain.

To verify the identity of SATB2 as the AT-rich DNA binding protein expressed in the developing rat cortex, its cDNA clone was isolated from a neonatal rat brain library using PCR cloning. [84]

5.3. Characterization of recombinant SATB2

To characterize the binding specificity of rSATB2 protein, the recombinant SATB2 was expressed and competitive EMSA was performed using rAT^{ENK} dsDNA as probe. Comparing the mobility of protein-DNA complexes formed with the recombinant rSATB2 protein and nuclear extracts isolated from P2 cortex showed similar mobility (**Fig. 9**.).

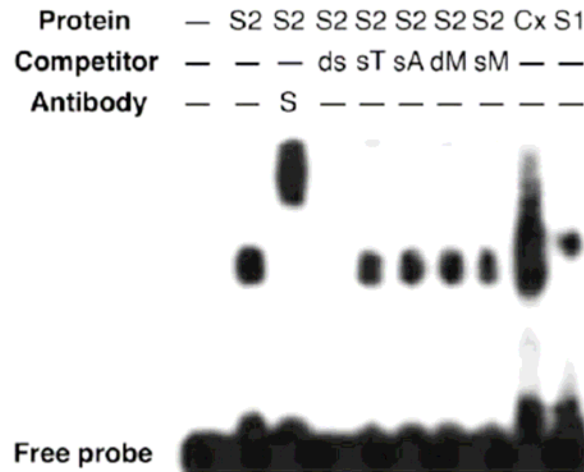

Figure 9.
Binding specificity of SATB2 to AT-rich DNA element. Competitive electro-mobility shift assay (EMSA) with different single- and double stranded AT-rich and mutant DNA. Abbreviations: S2: recombinant rSATB2; S1: recombinant rSATB1; Cx: nuclear extract from P2 rat cortex; ds: double stranded rAT(ENK); sT: single stranded rAT(ENK)-F; sA: single stranded rAT(ENK)-R; dM: double stranded rAT(ENK) mut; sM: single stranded rAT(ENK)mut-F; S: SATB2 antibody used for supershift.[84]

However, the complex formed with the nuclear extract appeared as a broader band indicating the presence of multiple proteins in the complex. This finding is consistent with our DNA affinity purification and mass spectrometry data that identified these additional AT-rich DNA binding proteins (see **Fig. 8.** and **Table I**). Competitive EMSA showed that the binding of SATB2 to the AT-rich dsDNA probe was specific. Neither single stranded specific DNA competitors (5'rAT^{ENK} or 3'rAT^{ENK}, see Table II. for details) nor mutant single-stranded competitors (5'rAT$^{ENK}_{mut}$ or 3'rAT$^{ENK}_{mut}$) or double-stranded mutant competitor (rAT$^{ENK}_{mut}$) interfered with the binding. We also compared the binding of rSATB2 to a previously identified AT-rich DNA binding protein, SATB1. Further experiments confirmed, that in the presence poly[d(I-C)], SATB2 bound to rAT^{ENK} dsDNA probe with an apparent higher affinity than that of the SATB1. In addition, consistent with the

different molecular weights and amino acid compositions, the two complexes also showed different mobility.

II. Aims

In the present study we explored intrinsic transcriptional regulatory mechanisms that affect neuronal fate decisions in the developing CNS. As a model system, we studied the regulation of the ENK gene in the developing brain, before and during the specification of enkephalinergic neurons.

Our first goal was to determine the expression pattern of AUF1 and SATB2 during development. Both proteins were known to be expressed in the brain, however, there was no information about their developmental expression pattern during the critical phase of cortical development, when the enkephalinergic "decision-making" takes place.

The second goal was to show that the interaction between the AT-binding proteins and the AT-rich DNA element of the ENK gene (AT^{ENK}) is present *in vitro* as well as *in vivo.* Even though both AUF1 and SATB2 were isolated based upon their binding to the AT^{ENK}, there was no direct proof if the binding is specific, or if it is present in the developing brain, *in vivo,* as well.

The third aim was to identify potential partner protein(s) which may interact with SATB2. Is SATB2 independently acting as a regulator of gene expression, or as a part of a complex? SATB2 is a close homologue to SATB1, the hematopoietic master regulator, which is known to interact with members of multiple chromatin remodeler complexes. As the two SATB proteins share a high homology at the aminoacid level, especially at DNA- and protein binding domains, it was reasonable to assume that SATB1 and SATB2 might have similar partner proteins.

Next, we wanted to determine the spatio-temporal distribution of these partner proteins during the critical phase of cortical development.

Since such interactions would likely influence the mechanism of gene regulation by SATB2, it was an important to find out the role of the interactory proteins.

The final goal was to determine how SATB2 regulates gene expression *in vivo*, and if it requires any co-factors for its regulation.

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III. Materials and Methods

1. Semi-quantitative Reverse Transcription PCR

Total RNA was purified from rat cortex dissected at different developmental ages and from P2 thymus using the TRIzol reagent (Invitrogen, Carlsbad, CA). Two µg of RNA was reverse transcribed with the Promega Reverse Transcription System (Madison, WI). The obtained cDNA was diluted 10-fold and served as a template in the following PCR. A SATB2-specific DNA fragment was amplified by using the primers 5'S2A and 3'S2A (for sequence information see **Table II.**). by YieldAce polymerase (Stratagene, La Jolla, CA). After an initial denaturizing step at 95 C for 5 min, the following temperature profile was followed during 35 cycles: 30 sec at 95 C, 30 sec at 55C and 1 min at 72C. The amplicons were subjected to gel electrophoresis on a 2% agarose gel and Bands were visualized under UV light after Ethydium Bromide staining, photographed using Kodak Gel Electrophoresis and Documentation System 120.

2. Immunoblot assay

Nuclear extracts were purified from microdissected cortexes from different ages: embryonic day 14 (E14), E18, postanatl day 2 (P2), and from an adult, using the NE-PER kit from Pierce (Rockford, IL). Protein concentrations of were determined by the BCA Protein Assay kit (Pierce, Rockford, IL). Twelve µg of nuclear proteins from developing cortexes were separated on NuPage 4-2% Bis-Tris gels, transferred onto PVDF membranes by electro blotting (Invitrogen, Carlsbad, CA) followed by incubating the membranes with various primary antibodies overnight at 4°C.

Immunoreactive protein bands were visualized using SuperSignal West Pico Chemiluminescent Kit (PIERCE, Rockford, IL). The membranes were stripped using the Re-Blot Plus Mild Antibody Stripping Solution (Chemicon, Temecula, CA) and re-probed with anti-GAPDH antibody (Sigma-Aldrich, St. Louis, MO) as described above.

3. DNA Affinity Preincubation Specifity Test of Recognition (DAPSTER) assay

The assay was performed as previously described with minor modification [94]. Nuclear extract were prepared as published previously [70]. 50 μg of nuclear extract was pre cleared with 30 μl streptavidin-agarose beads (PIERCE, Rockford, IL) washed in buffer Z (25 mM HEPES (pH 7.9), 20% glycerol, 0.1% Igepal, 0.1 M KCl, 12.5 mM $MgCl_2$, 1 mM DTT, 0.1 μM $ZnCl_2$) After separation by centrifuging, 75 pmol competitor ds- or ssDNA or equal volume of 1X kinase buffer (50 mM Tris-HCl (pH: 7.5), 10 mM $MgCl_2$) were added in the presence of 0.1 μg/μl poly[dI/dC)], 1 mM Na-Ortho vanadate and Complete Mini protease inhibitors (Roche, Indianapolis, IN), and incubated under gentle rotation for 15 min at 4 ^{o}C. 5 pmol of biotinylated ds- or ssDNA – rAT^{ENK} or rAT^{ENK}_{mut} - were added to the recovered nuclear extract, and mixed by gentle rotation for 2 hrs at 4 ^{o}C. (See sequences in **Table II**). Thirty μl of washed streptavidin-agarose bead slurry was added and the suspension was further incubated, gently rotating, for 2 h at 4 oC. The mixture was centrifuged briefly, the pelleted beads were washed 3 times in buffer Z and beads were boiled in the presence of LDS sample buffer (Invitrogen, Carlsbad, CA) under reducing conditions. The bound proteins were separated on NuPAGE® 4-12% Bis-Tris gels, transferred onto PVDF membranes by electro blotting (Invitrogen, Carlsbad, CA) followed by

incubating the membranes with various primary antibodies overnight at 4 ^{o}C. Immunoreactive protein bands were visualized using SuperSignal West Pico Chemiluminescent Kit (Pierce, Rockford, IL). The membranes were stripped using the Re-Blot™ Plus Mild Antibody Stripping Solution (Chemicon, Temecula, CA) and re-probed with anti-actin antibody (Sigma-Aldrich, St. Louis, MO) as described above.

4. Chromatin Immunoprecipitation (ChIP)

The assay was performed by optimizing existing protocols [95-98] for embryonic and neonatal brain tissues. Briefly, frozen, micro-dissected brain regions were placed into 0.5 ml ice-cold PBS containing 1% formaldehyde and incubated first on ice for 10 min then at 37 ^{o}C for 30 min.

Cross-linking reaction was stopped by the addition of 0.75 ml cold PBS, followed by 3 washes with 1 ml cold PBS. After the last wash, 0.9 ml RT lysis buffer (50 mM Tris/HCl pH 8.1, 10 mM EDTA, 1% SDS and protease inhibitors) was added, and the mixture was incubated on ice for 10 min and mixed with occasional, gentle pipetting.

The cells were disrupted by sonication on ice (four times 10 sec at level 4, separated by 30 sec breaks). Debris was removed by centrifuging, the supernatant was diluted tenfold with dilution buffer (16.7 mM Tris/HCl (pH 8.1), 167 mM NaCl, 0.01% SDS, 1.1% TritonX100, 1.2 mM EDTA and protease inhibitors), and filtered through 45 μm pore-size filter.

Aliquots (500 μl) were pre cleared with DNA- and BSA-blocked protein A/G-coupled sepharose beads (Amersham Pharmacia, Uppsala, Sweden) and with pre-immune serum and beads.

The supernatant was recovered and was incubated with 1 μg of the following antibodies at 4 ^{o}C overnight: anti-SATB2 antibody, anti-HDAC1 antibody (Abcam, Cambridge, MA), anti-MTA2 antibody (Abcam, Cambridge, MA), anti-CHRAC17 antibody (Santa Cruz, Biotechnology, Santa Cruz, CA) or EGFR antibody (Santa Cruz Biotechnology, Santa Cruz, CA) . Sixty μl DNA- and BSA-blocked, protein A/G-coupled sepharose beads were added to each sample and they were rotated for 1 h at 4°C. Subsequently, the samples were washed 5 times in ice-cold wash buffer 1 (20 mM Tris/HCl pH 8.1, 150 mM NaCl, 2 mM EDTA, 0.1 % SDS, 1% TritonX100), once in RT wash buffer 2 (Tris/HCl pH 8.0, 250 mM LiCl, 1 mM EDTA, 0.5% NP-40, 0.5% Na-deoxycholate) and twice in RT wash buffer 3 (Tris/HCl (pH 8.0), 1 mM EDTA).

After the final wash, beads were resuspended in 100 μl wash buffer in the presence of 0.5% SDS and 0.5 mg/ml Proteinase K, incubated at 37°C for 12 hrs then 65°C for 12 hrs to revert the crosslink. DNA was purified by phenol/chloroform/isoamyl alcohol (25:24:1) extraction and was ethanol precipitated.

After washing and drying, DNA was resuspended in 10 μl of 10 mM Tris/HCl (pH 7.5) and was size separated on a 1.5% TBE agarose gel. DNA in the 300-500 bp size range was isolated from each sample and was extracted from the gel by using the Agarose Gel DNA Extraction Kit (Roche, Indianapolis, IN). DNA was eluted in 50 μl of Tris/HCl (pH 8.3), and 3 μl was used as template in PCR using the qPCR™ Core Kit (Eurogentec, San Diego, CA) according to the manufacturer's instructions.

The first PCR was carried out with 25 cycles, using primers specific to the rAT^{ENK}, the proximal cassette and control upstream region, respectively. The 100 times diluted product of the first reaction was used as template in the (semi-) nested reaction, performed with 25 or 30 cycles. The following PCR primers were used: The

rAT^{ENK} region was amplified with the primer pair of 5'oATT and 3'oATT (-2654; -2395) in the first reaction, whereas the nested primer pair was 5'nATT and 3'nATT (-2612; -2471). The ENK TATA region was first amplified with the sense primer 5'oTATA and the antisense 3'oTATA (-103; +73). In semi-nested reaction the sense primer was used in pair with the antisense primer 3'snTATA (-103; +25). A distinct regulatory region containing the septamer site located between (-569; -247) of the rENK gene was used as control, and was amplified with primers 5'oSept and 3'oSept. The nested primers were 5'nSept and 3'nSept (–560; -287).

The PCR products were separated on 2.5% agarose gel by agarose gelelectrophoresis along with a 100 bp molecular weight ladder, and the bands were visualised with ethidium bromide.

Table II.

Sequences of oligonucleotides

Name	***Sequence 5'-3'***
5'S2F	GGT GAA TTC GCC GGT GGG AAC TTT GTC TC
3'S2F	ATT AGC GGC CGC GAA AGT CCT TGG ACC CAT GT
5'S2A	AAG AAG ATA AAA GTG GAA AGA GTG
3'S2A	GAT GGC TTG AGG ATG CTG GTG
5'rAT^{ENK}	GAA CCA AAA ATA ATA ATA ATA ATA ATA ATA ATA ATA ATA ATA ATA ATA ATA ATA ATT AAC CAT
3'rAT^{ENK}	ATG GTT AAT TAT TAT TAT TAT TAT TAT TAT TAT TAT TAT TAT TAT TAT TAT TAT TTT TGG TTC
5'rAT$^{ENK}_{Mut}$	GAA CTG TTC TTA CTA TCG CCA TCA CCG TTA TTG CTC AAT TCG TAC TAG TTC ATG AAG TCT TTG G
3'rAT$^{ENK}_{mut}$	CCA AAG ACT TCA TGA ACT AGT ACG AAT TGA GCA ATA ACG GTG ATG GCG ATA GTA AGA ACA GTT C
5'oATT	TTT AGC TCA GTG GTA GAG CGC
***3'*oATT**	GTT TCT GCC CTT TCC AAC TGC
5'nATT	CCC TGG GTT CGG TCC CC AGC
***3'*nATT**	GTT CCC AGA CCT GTC CAG TTC
5'oTATA	CTG GCG TAG GGC CTG CGT C
***3'*oTATA**	TGT GAG GTG GCC GCG TCG
***3'*snTATA**	CTG GGC TGC GGG GCT CTG T

5'oSept	TCC TGC TCC GGC AGT ATT GC
***3'*oSept**	ATG AAT CAC TTC GGT GGC AC
5'nSept	GGC AGT ATT GCT TTT GTG GTG
***3'*nSept**	AGA CGG ACT TGG ATT TCG TGA G

5. Immunohistochemistry

Rat embryos were obtained from timed pregnancies (Zivic Miller Laboratories, Pittsburgh, PA). Following anesthesia, brains were removed and snap frozen on powdered dry ice. Twenty µm frozen coronal sections containing the cerebral cortex, striatum, septum (or their primordia) were cut and processed for immunohistochemistry using anti-SATB2 antibody at 1:200 dilution and anti-HDAC1 polyclonal antibody in 1: 400 or anti-MTA2 polyclonal antibody in 1: 200 dilutions. After washing, sections were processed for immunofluorescent detection using FITC-conjugated secondary antibodies (1:1000, Molecular Probes, Eugene, Oregon). Sections were viewed in an Olympus 70 IX microscope equipped with a Spot digital camera or in a Leica DM RXA microscope, or the Zeiss Pascal Laser Scanning Confocal Microscope (LSCM) (Biomedical Instrumentation Center, USUHS). The appropriate fluorescent channels and images were merged using TIFFany3 (Caffeine Software, Inc.) to create the final pictures. The quantification of the SATB2 and MTA2 or HDAC1 double and single positive areas was done by analysing the scanned images of immunhistochemically stained hemispheres with a Mathematica 5.2 script (Wolfram Reaserch Inc., Urbana, IL).

6. Antibodies

The SATB2 specific antibody production was performed at ProteinTech Group Inc. (Chicago, IL). Based on the predicted amino acid sequence, the peptide: AEEESAEKNKVAPAETDQR was synthesized using FMOC chemistry on solid phase peptide synthesizer (Symphony, Protein Technologies, Inc. Arizona).

The following AUF1 antibodies were used: rabbit polyclonal pan-specific antibody kindly provided by Dr. Brewer [99], rabbit polyclonal pan-specific commercially available antibody (Upstate, Charlottesville, VA); rabbit polyclonal pan-specific AUF1 antibody, kindly provided by Dr. Tolnay [100]; a mouse monoclonal antibody kindly provided by Dr. Dreyfuss [101]; rabbit polyclonal antibody kindly provided by Dr. Schneider [102]. For immunoblotting, immunoprecipitation and immunoblot assays the following antibodies were used: anti-SATB2 antibody (produced as described above), anti-HDAC1 antibody (Abcam, Cambridge, MA), anti-MTA2 antibody (Abcam, Cambridge, MA), anti-CHRAC17 antibody (Santa Cruz, Biotechnology, Santa Cruz, CA), anti-GAPDH antibody (Sigma-Aldrich, St. Louis, MO), EGFR antibody (Santa Cruz Biotechnology, Santa Cruz, CA)anti-actin antibody (Sigma-Aldrich, St. Louis, MO).

7. Cell cultures, reporter assays, Trichostatin A (TSA) treatment

For DNA molecular decoy assays, primary neuronal cultures were co-transfected with a DNA mixture containing: 0.5 μg of the reporter plasmid 2700/703ENK-Luc, 2 μg of competitor dsDNA corresponding to the rAT^{ENK} region, or a control dsDNA ($rAT^{ENK}{}_{mut}$) as described for EMSA above; 0.1 μg of pCMV-Renila Luciferase.

Animals were handled according to protocols approved by the Committee on Animal Research at USUHS. Following pentobarbital anesthesia, rat embryos were obtained from timed pregnancies, brains removed and striatal and cortical primary embryonic neuronal cultures were prepared and maintained as published. C6 cells were obtained from ATCC and cultured as previously described.

Cells were transfected by using the poly ethyleneimine (PEI) gene delivery system [103]. To measure transactivation, cells were cotransfected with the 0.5 μg of reporter plasmid TK- rAT^{ENK} -Renila or TK- $rAT^{ENK}{}_{mut}$ -Renila, 1 μg of the mammalian expression plasmid pCI driving the expression of SATB2 (pCI-SATB2) and 0.1 μg of RSV-Luciferase used as control plasmid. Forty-eight hours after transfection, cultures were harvested and Firefly and Renila luciferase activities were measured using the Dual-Luciferase Assay System (Promega, Madison, WI). The control luciferase (Renila or Firefly) activities were used to normalize the activities of the reporter gene for transfection efficiency.

TSA treatment for the inhibition of HDAC1 activity was done by adding TSA or ethanol as a vehicle to the cell cultures 48h after plating in a final concentration of 1 mg/ml. Cells were harvested for measuring the reporter gene activity 12 hours after the treatment.

IV. Results

1. AUF1

1.1 Cell-specific expression of AUF1 in the developing rat brain

To characterize the distribution of AUF1 proteins in the developing brain, which could help us to elucidate its role in neurodevelopment, we have mapped the spatial and temporal distribution of AUF1+ cells by immunohistochemistry (**Fig. 10**.).

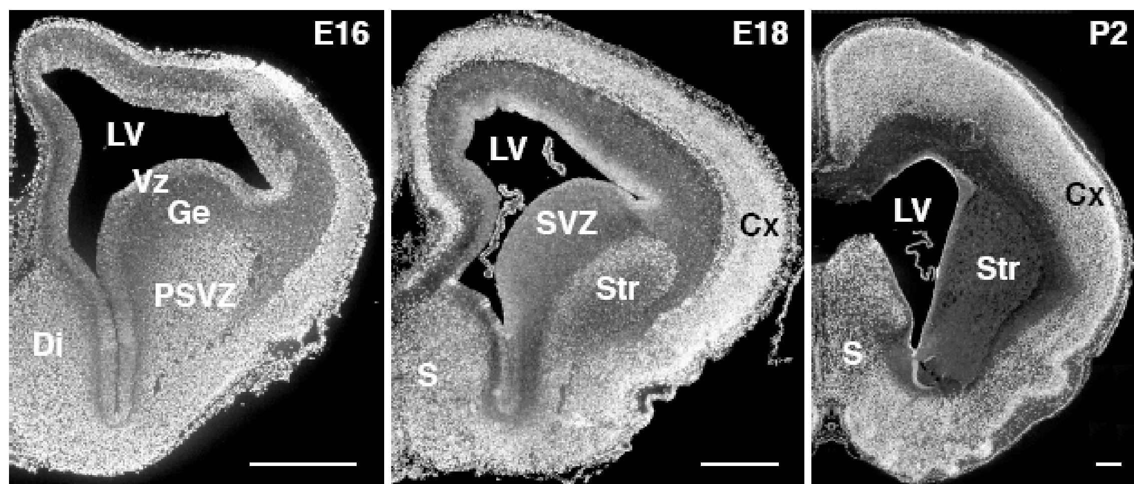

Figure 10.
AUF1 expressing cells are localized mainly in the specific layer of the cortex (Cx), the ventricular zone (VZ), and the subventricular zone (SVZ) of the lateral ventricle (LV). Abbreviations: Di: diencephalon; Ge: ganglionic eminence; Str: striatum; S: septum. Bar: 1mm.[78]

At embryonic day 16 (E16) practically all cells populating the ventricular zone (VZ) were immunoreactive for AUF1. In addition, many cells in the pallidal subventricular zone and in the telencephalic wall were positive for AUF1 suggesting its involvement in the proliferation and/or differentiation of neuronal progenitors. At E18, practically all cells in the developing cortex were AUF1 immunoreactive. Similarly, in the developing septum and rhinencephalon most cells were also AUF1+.

However, in the striatum only a subset of cells was immunoreactive. The proportion of AUF1+ cells in the striatum seemed proportional to the population of neurons that are expressing other neurotransmitter genes but not ENK in the adult striatum [104]. At postnatal day 2 (P2), the majority of AUF1 immunoreactive cells were found in regions that contain very few or no enkephalinergic neurons in the adult including the cerebral cortex, septum, thalamus, etc.

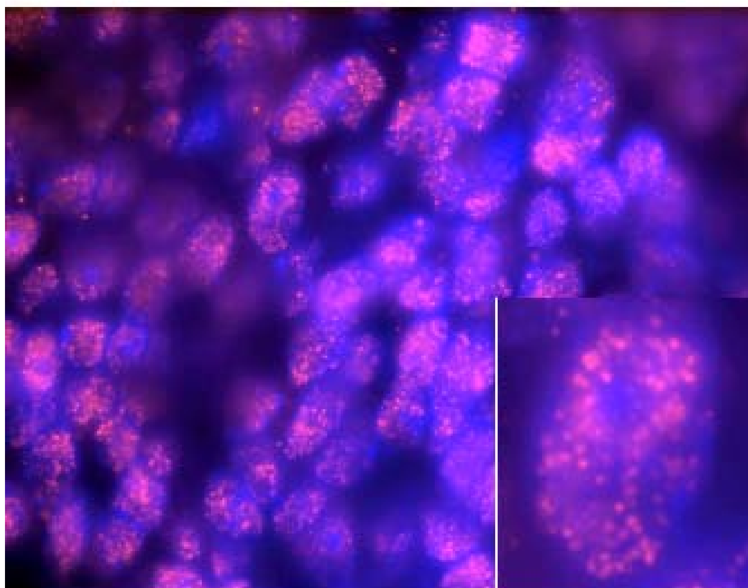

Figure 11.
The nuclei of differentiating cortical neurons are labeled positive for AUF1 proteins. AUF1 proteins are localized to sub nuclear speckle-like structures, while most of the nucleoplasm lacks immunoreactivity (insert). DNA is stained with DAPI (blue).[78]

Previous studies have shown that AUF1 proteins can be both cytoplasmic and also nuclear in their subcellular localization [105]. Using higher power imaging, we found that AUF1 immunoreactivity in the developing rat brain is exclusively nuclear (**Fig. 11**.) and we have found no immunohistochemical evidence for cytoplasmic localization. More over, within the nucleus, AUF1 immunoreactivity was not distributed evenly: it appeared by sub nuclear speckle-like “hot-spots” [106] while

most of the nucleoplasm lacked immunoreactivity suggesting that AUF1 might bind to multiple other genomic regions in addition to ENK (**Fig. 11**., insert).

1. 2. AUF1 binds to AT-rich dsDNA element *in vitro* and *in vivo*

Previous reports have shown distinct regulatory functions associated with the various isoforms of AUF1 [105, 107]. To test which isoforms are expressed in the developing rat brain and which one of them binds the AT-rich dsDNA *in vitro*, we performed DNA affinity preincubation specificity test of recognition (DAPSTER) assay followed by immunoblot assay using neonatal cortical nuclear extracts. (see Materials and Methods) (**Fig. 12**.).

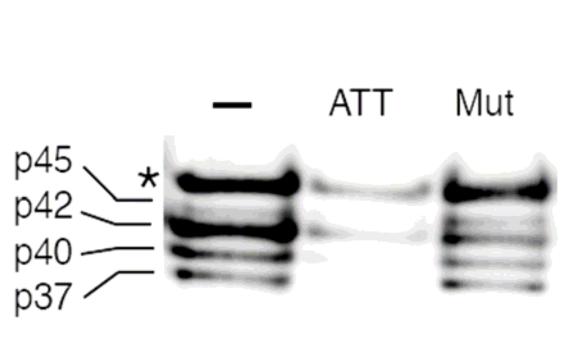

Figure 12.
DAPSTER : AUF1 specifically binds to AT-rich DNA. Nuclear proteins isolated from E18 rat cortex were incubated with biotinylated ds[(ATT)19] in the presence or absence or various competitor DNAs: dsATT, dsMutant. The bound proteins were separated on a 10% acrylamide gel and analyzed by immunoblotting using a pan-specific AUF1 antibody. The isoforms are shown by their respective position. Star shows the non-specific band seen with the commercial AUF1 antibody.[143]

We have found that all of the four isoforms, p37, p40/42 and p45 are expressed in the developing rat brain albeit their abundance is different. All isoforms bound specifically to rAT^{ENK}, but the p42, since it could not be completely competed out with AT-rich dsDNA.

To test if AUF1 proteins do bind the AT-rich dsDNA of the rENK gene *in vivo*, we performed chromatin immunoprecipitation (CHIP) assay using microdissected neonatal brain tissue. (**Fig. 12**.)

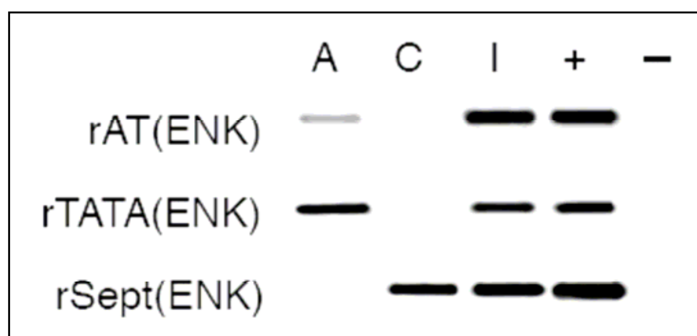

Figure 12.
ChIP assay: for chromatin immunoprecipitation, pan specific AUF1 antibody ("A") was used. As a control ("C") , we used an antibody against hnRNPA2/B1 and amplified its septamer cis-regulatory DNA element. I: Input. Positive controls ("+") were amplifications from plasmids coding the respective regulatory regions. EGFR antibody was used as a negative control (-).Horizontal lanes show the different regions of the rENK gene amplified after IP: rAT(ENK) = the AT-rich region of the rENK gene; TATA(ENK)= the TATA region of the rENK gene; rsept(ENK) = septamer, the binding site for hnRNPA2/B1. (courtesy of Dr. Marianna Szemes [78])

The cross linked protein-DNA complexes were immunoprecipitated by anti-AUF1 or by a control antibody against hnRNPA2/B1, which is known to bind to another regulatory region of the rENK gene, named septamer [71], thus could be used as a positive control. The DNA fragment containing the [$(ATT)_{19}$] region of the rENK gene was successfully amplified from the AUF1 antibody-precipitated chromatin with primers flanking the [$(ATT)_{19}$] region (**Fig. 12**., and **Table II**.) indicating *in vivo* interaction in the nucleus. Because our PMDDI assay showed a protein-dependent interaction between the AT-rich and the TATA region of the ENK gene, we also used primers flanking the TATA region of the rENK gene to test binding. Indeed, the DNA fragment containing the TATA region was also amplified following immunoprecipitation confirming the former *in vitro* data. The AUF1

antibody-precipitated samples were negative with the septamer-specific primer set even though the DNA region containing the other cis-regulatory element could be amplified both from the corresponding antibody precipitated chromatin and from the input sample. These experiments confirmed the results of *in vitro* studies and demonstrated that in the developing brain, AUF1 protein binds the AT-rich dsDNA element and also interacts with the TATA region of the rENK gene *in vivo*.

2. SATB2

The comparison of rat (rSATB2), mouse (mSATB2) and human (hSATB2) proteins has revealed high level of homology (**Fig. 13**). Amino acid substitutions of rSATB2 compared to the mouse of the human protein (marked with stars) were found on the C-terminus outside of known functional domains. Similar to a recent publication [108] we found significant homology between SATB1 and SATB2 at a region between amino acids 355 and 483 of SATB1 (Fig. 25.). This region, part of a CUT domain (**Fig. 25**.), has been shown to be responsible for the high affinity binding of SATB1 to AT-rich DNA [93, 109]

```
m SATB2      1   MERRSESPCL RDSPDRRSGS PDVKGPPPVK VARLEQNGSP MGARGRPNGA VAKAVGGLMI PVFCVVEQLD GSLEYDNREE HAEFVLVRKD 90
human SATB2  1   .......... .......... .......... .......... .......... .......... .......... .......... .......... 90
rat SATB2    1   .......... .......... .......... .......... .......... .......... .......... .......... .......... 90

m SATB2      91  VLFSQLVETA LLALGYSHSS AAQAQGIIKL GRWNPLPLSY VTDAPDATVA DMLQDVYHVV TLKIQLQSCS KLEDLPAEQW NHATVRNALK 180
human SATB2  91  .......... .......... .......... .......... .......... .......... .......... .......... .......... 180
rat SATB2    91  .......... .......... .......... .......... .......... .......... .......... .......... .......... 180

                                                                            s
m SATB2      181 ELLKEMNQST LAKECPLSQS MISSIVNSTY YANVSATKCQ EFGRWYKKYK KIKVERVERE NLSDYCVLGQ RPMHLPNMNQ LASLGKTNEQ 270
human SATB2  181 .......... .......... .......... .......... .......... .......... .......... .......... .......... 270
rat SATB2    181 .......... .......... .......... .......... .......... .......... .......... .......... .......... 270
                                                                                                   s
m SATB2      271 SPHSQIHHST PIRNQVPALQ PIMSPGLLSP QLSPQLVRQQ IAMAHLINQQ IAVSRLLAHQ HPQAINQQFL NHPPIPRAVK PEPTNSSVEV 360
human SATB2  271 .......... .......... .......... .......... .......... .......... .......... .......... .......... 360
rat SATB2    271 .......... .......... .......... .......... .......... .......... .......... .......... .......... 360

m SATB2      361 SPDIYQQVRD ELKRASVSQA VFARVAFNRT QGLLSEILRK EEDPRTASQS LLVNLRAMQN FLNLPEVERD RIYQDERERS MNPNVSMVSS 450
human SATB2  361 .......... .......... .......... .......... .......... .......... .......... .......... .......... 450
rat SATB2    361 .......... .......... .......... .......... .......... .......... .......... .......... .......... 450

m SATB2      451 ASSSPSSSRT PQAKTSTPTT DLPIKVDGAN VNITAAIYDE IQQEMKRAKV SQALFAKVAA NKSQGWLCEL LRWKENPSPE NRTLWENLCT 540
human SATB2  451 .......... .......... .......... I......... .......... .......... .......... .......... .......... 540
rat SATB2    451 .......... .......... .......... .......... .......... .......... .......... .......... .......... 540

m SATB2      541 IRRFLNLPQH ERDVIYEEES RHHHSERMQH VVQLPPEPVQ VLHRQQSQPT KESSPPREEA PPPPPPTEDS CAKKPRSRTK ISLEALGILQ 630
human SATB2  541 .......... .......... .......... .......... .........A .......... .......... .......... .......... 630
rat SATB2    541 .......... .......... .......... .......... .......... .......... .......... .......... .......... 630
                                                                                                 *          *
m SATB2      631 SFIHDVGLYP DQEAIHTLSA QLDLPKHTII KFFQNQRYHV KHHGKLKEHL GSAVDVAEYK DEELLTESEE NDSEEGSEEM YKVEAEEENA 720
human SATB2  631 .......... .......... .......... .......... .......... .......... .......... .......... .......... 720
rat SATB2    631 .......... .......... .......... .......... .......... .......... .......... .......D.. ........S. 720

                 * * *
m SATB2      721 DKSKAAPAET DQR 733
human SATB2  721 .........I ... 733
rat SATB2    721 E.N.V..... ... 733
```

Figure 13.
Comparison of amino acid sequences for mouse, human and rat SATB2. The number refers to the position of amino acids. The black and grey boxes indicate the conserved "cut" and homeodomains. Dots indicate identical amino acids. Amino acid substitutions in the rat are bold and marked with a star above. Possible SUMOilation sites are italicized and marked with an 's' above. The 19 amino acid peptide sequence (AEEESAEKNKVAPAETDQR) used to raise the rat SATB2 specific antibody is underlined on the C-terminal of the protein.[84]

Based on the predicted amino acid sequence, a peptide unique to rSATB2 was synthesized and antibody against the protein was raised.

2.1. Developmental expression of SATB2 mRNA and protein in the rat brain

We analyzed the developmental pattern of rSATB2 mRNA expression in the rat cerebral cortex by semi-quantitative RT-PCR using primers 5'S2A and 3'S2A (**Table II**). As shown on Fig. 16, we detected low levels of rSATB2 mRNA in the rat telencephalon at E14. (**Fig. 14**.)

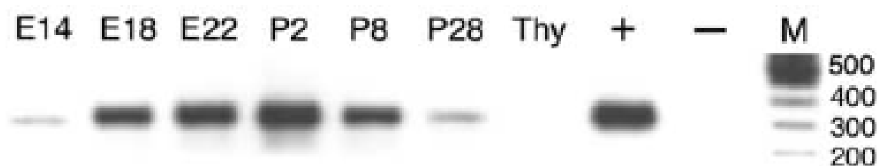

Figure 14.
SATB2 mRNA expression in the developing rat cortex. RT-PCR analysis of total RNA isolated from whole brains at different ages (E14-P28). Thy: total RNA derived from P2 thymus. (+): positive control where SATB2-coding plasmid was used as a template. (-): No template control. M: 100bp molecular weight marker. (courtesy of Dr. Marianna Szemes [84])

The abundance of rSATB2 mRNA gradually increases in the developing cortex and reaches the highest abundance at P2. SATB2 mRNA levels were gradually

decreasing after P2 and only very low level of rSATB2 mRNA was detected in the adult cortex. Interestingly, we detected no SATB2 mRNA in the neonatal thymus, a rich source of SATB1 mRNA at this developmental stage. [110].

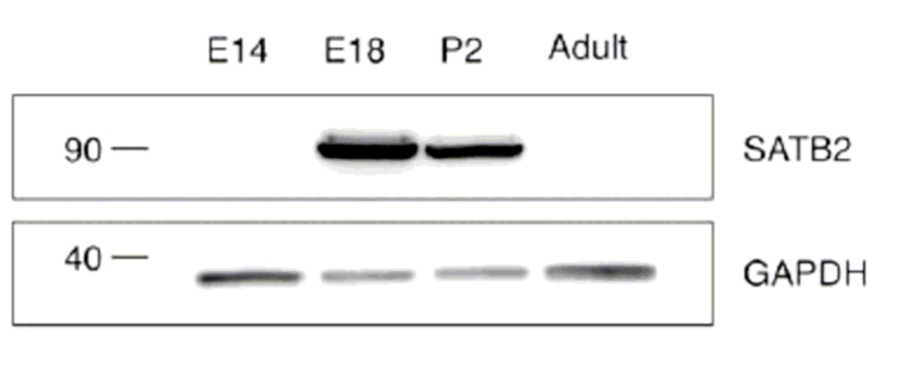

Figure 15.
SATB2 protein in the developing rat cortex. Immunoblot with anti-SATB2 antibody. All lanes contains equal amount of nuclear extracts isolated from whole brain from E14, or cortex from E18, P2 or adult rats. The antibody is specific to SATB2 and did not recognize any other protein. We used anti-GAPDH antibody as a loading control. [84]

We also analyzed the developmental distribution of SATB2 protein using immunoblot assay. As opposed to mRNA shown above, we did not detect SATB2 protein in the E14 cortical nuclear extract (**Fig. 15**.)

However, we detected high level of SATB2 protein in nuclear extracts isolated from E18 cortex and the abundance of SATB2 protein was maintained at P2. No SATB2 protein was seen in the adult rat cortex. Overall, the observed developmental pattern of SATB2 mRNA and protein expressions showed similar trend. The discrepancy between mRNA and protein levels is likely due to the lower sensitivity of the immunoblot assay.

2.2. Distribution and characterization of SATB2 expressing cell in the in the rat brain

To obtain information about the cell-specific expression of SATB2 in the rat brain, we performed immunohistochemical analysis using the same SATB2 antibody as in immunoblot analysis. We found that SATB2 immunoreactive cells were restricted to the developing cortex, and no immunoreactive cells were found in any of other brain regions. SATB2 immunoreactive cells were first detected at E16 in the superficial layers of the developing cortex (**Fig. 16**).

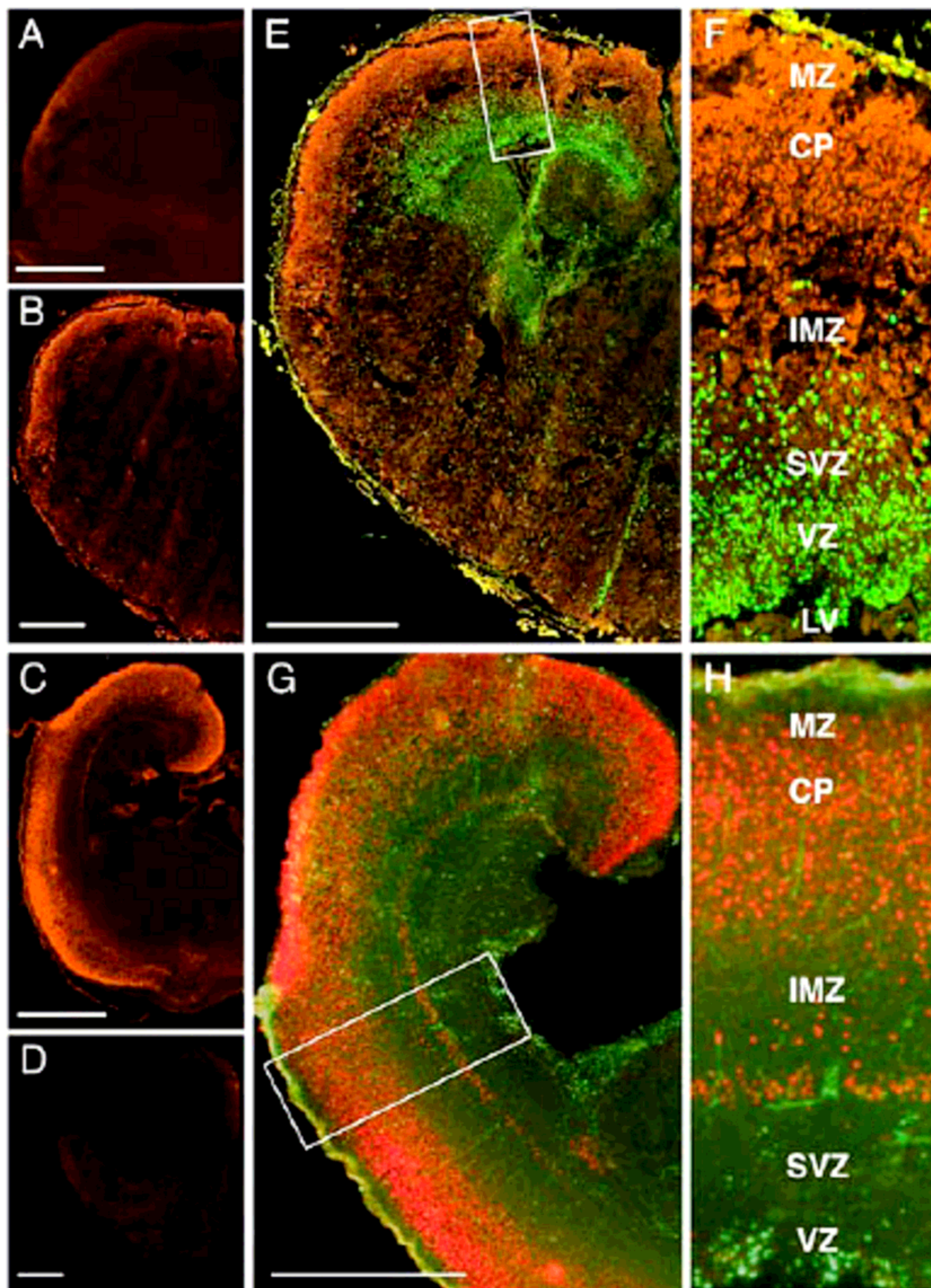

Figure 16.
Distribution of SATB2 expressing cells in the rat cortex. (a) – (d) Cross-sections derived from developing rat brains at ages E16, E18, P2 and P4 were immunostained with the SATB2 antibody (red). The low magnification images show immunoreactive cells that increase in numbers and staining intensity in the neocortex between E16 and P2 and decreases thereafter. (e) – (h) SATB2 expressing cells (red) and proliferating,

BrdU (green) are spatially separated at E18 (e-f). The insert on (e) indicate the location of the higher magnification image (f). SATB2+ (red) cells are located in the intermediate zone (IMZ), in the cortical plate (CP) and in the marginal zone (MZ). BrdU immunoreactive cells (green) cells are located in the ventricular (VZ) and subventricular zones (SVZ). Some BrdU+ cells are present in the IMZ but they do not express SATB2. At P2 (g-h) the separation between SATB2+ and BrdU+ cells is even more marked. Although there are both SATB2+ and BrdU+ cells in the IMZ, there were no double labeled cells found. Bars = 1mm. Abbreviation: LV= lateral ventricle. [84]

At E18, the number of SATB2 immunoreactive cells and the apparent staining intensity increased substantially. SATB2 expressing cells were present in the marginal zone, cortical plate and intermediate zone. Interestingly, SATB2 + cells were mostly restricted to the neocortex and cells at similar laminar locations in the piriform cortex were not expressing SATB2. This finding is consistent with a recent observation in the mouse brain using in situ hybridization histochemical analysis also showing that SATB2 mRNA expression is absent from the developing piriform cortex [108]. The number of immunoreactive cells and apparent staining intensity further increased in the rat neocortex at P2 and then sharply declined by P4.

The observed spatio-temporal distribution of SATB2 positive cells in the cortex suggested that they are postmitotic, differentiating neurons. To test hypothesis, we performed SATB2/BrdU double immunohistochemistry at ages E18 and P2. We found a clear boundary between BrdU and SATB2 immunoreactive cells at both ages investigated and we found no double-labelled cells. Consistent with previous observations BrdU+ proliferating cells populated the ventricular and the subventricular zones [111, 112] whereas SATB2 immunoreactive cells were located at the marginal zone and cortical plate. The few BrdU+ cells we detected in the intermediate zone at E18 were not expressing SATB2 and similarly, all SATB2 immunoreactive cells at this location were BrdU negative. At P2 when the number of

proliferating cells is substantially reduced and restricted to small number of cells at the ventricular zone, the separation of BrdU positive and SATB2 expressing cells was even more distinct. These findings demonstrated that SATB2 is expressed in a subpopulation of postmitotic, differentiating neurons in the rat cerebral cortex and that these cells were born before E17 in the rat. Our findings are consistent with a recent report showing that SATB2 is expressed in BrdU negative, postmitotic cells in the mouse cerebral cortex. [108]

2.3. SATB2 interacts with chromatin remodeling factors *in vitro*

The SATB2 homologue SATB1 has been shown to interact with proteins of the NuRD (nucleosome remodeling and histone deacetylase) complex, such as HDAC1 and MTA2. Our previous studies using DNA affinity followed by mass spectrometry indicated that SATB2 co-purifies with members of this chromatin remodeling complex as well as with other AT-binding proteins. [84] To determine if SATB2 can directly bind to HDAC1 or MTA2, we performed co-immunoprecipitation using SATB2 specific antibody. (**Fig. 17**.) SATB2 and the interacting proteins were precipitated from nuclear extracts isolated from the late embryonic (E18) cortex because major gene regulatory events take place at this age and SATB2 is highly expressed in differentiating cortical neurons. MTA2 and HDAC1 were both co-precipitated with SATB2.

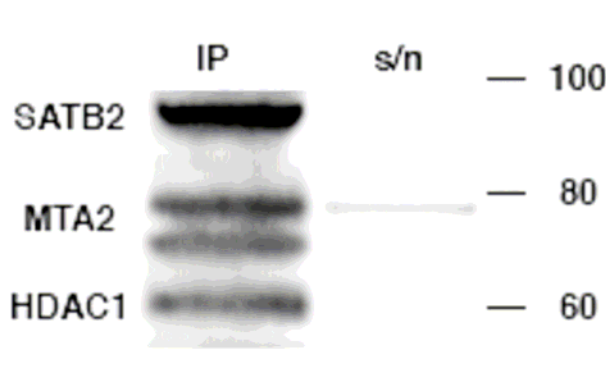

Figure 17.
Binding specificity of SATB2 to chromatin remodeling factors *in vitro*. E18 nuclear extracts were immunoprecipitated with SATB2-specific antibody. IP: bound fraction/precipitated proteins; s/n: supernatant. As shown, SATB2 formed an immuncomplex with both MTA2 and HDAC1. [143]

Our initial purpose in performing DNA binding experiments was to identify proteins that bind to the rAT^{ENK} DNA sequence, either in co-operation or in competition with SATB2 [84]. We postulated the existence of higher order complexes between SATB2, the rAT^{ENK} DNA motif and members of chromatin remodeling complexes, likely of NuRD. Because we thought that the complexes we were attempting to detect were higher order complexes, therefore perhaps more labile than smaller complexes, in which interactions of proteins and DNA are direct, we used the DNA-affinity preincubation specifity test of recognition (DAPSTER) assay to assess the specificity of binding to the rAT^{ENK} regulatory sequence, as this assay was successfully used previously to detect such interactions. [94]

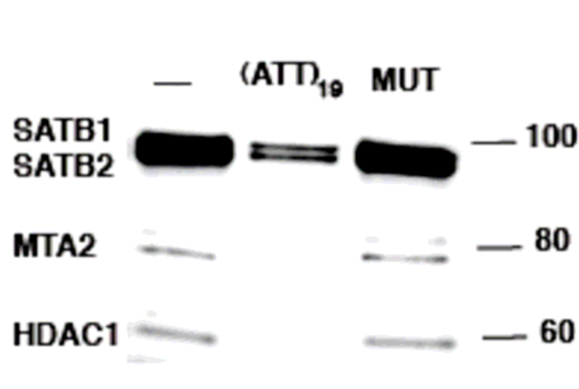

Figure 18.
SATB2 and two members of chromatin remodeling complexes (HDAC1 and MTA2) interact specifically with ds(ATT)19 DNA in DAPSTER assay. DNA affinity chromatography with 50 μg nuclear extracts were subjected to DNA affinity

chromatography with 50 µg of protein from NE was either performed directly (-) or after preincubation with a 3.5x excess of wild-type (ATT) or mutant (MUT) dsDNA. NE, nuclear extract (5 µg) directly loaded onto SDS–PAGE. Proteins were transferred and immunoblotted with a mixture of specific antibodies against SATB1/2, MTA2, and HDAC1. [143]

We detected both SATB2 and SATB1 along with MTA2 and HDAC1 in the bound fraction. (**Fig. 18**.) Binding of these four kinds of proteins was detected in the DAPSTER assay when nuclear extract was pre incubated with no oligo or with mutant but not wild-type rAT^{ENK} oligo prior to affinity chromatography. Preincubation with excess wild-type oligo virtually abolished visually detectable binding of the complexing proteins to the immobilized rAT^{ENK}. This confirmed our predictions for the specificity of the binding of SATB2 with its partner proteins to rAT^{ENK}.

2.4. SATB2 interacts with chromatin remodeling factors *in vivo*.

To ascertain whether SATB2 and its putative partners interact with the rAT^{ENK} locus in vivo in the developing brain and may direct chromatin remodeling complexes to this region, we turned to chromatin immunoprecipitation (ChIP) experiments using neonatal cortexes. (**Fig. 19**.) Along with the already shown interaction of HDAC1 and MTA2, members of the NuRD complex, this time we included CHRAC17, a histone fold protein, part of the CHRAC/ACF complex which is thought to play a role in the maintenance of heterochromatin. We used the EGFR antibody as a negative control. The DNA fragment containing the $[(ATT)_{19}]$ region of the rENK gene was successfully amplified from samples immunoprecipitated with SATB2, HDAC1 or MTA2 antibodies proving an already shown interaction, as well as from the CHRAC17, a histone fold protein-precipitated sample, indicating in vivo interaction of these proteins with the rAT^{ENK} region. The pre-immune serum never precipitated the rAT^{ENK} region and therefore could not be amplified by PCR under the same

circumstances. These experiments confirmed the results of in vitro studies and demonstrated that in the developing brain, SATB2 protein binds to the AT-rich dsDNA element of the rENK gene in vivo along with three proteins that are part of chromatin remodeling complexes.

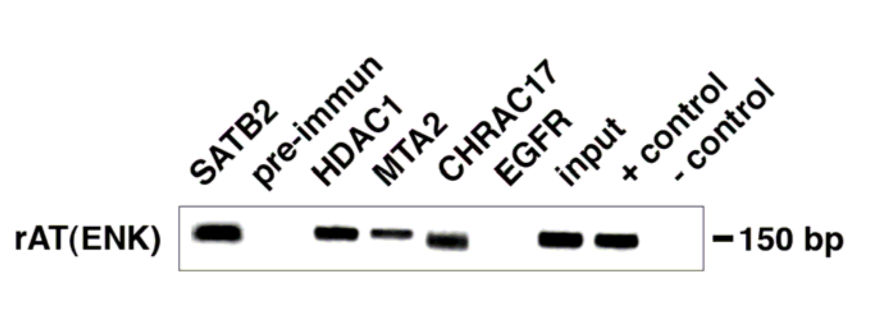

Figure 19.
Chromatin immunoprecipitation preformed on neonatal cortexes. The indicated various antibodies were used to precipitate the AT-rich region of the rENK gene. (EGFR was used as a negative control); (+) control: plasmid encoding the rAT^{ENK} region; (-) control: no antibody was used. (courtesy of Dr. Marianna Szemes [84])

2.5. Cellular co-localization of SATB2, MTA2 and HDAC1

Next, we tested if SATB2 and MTA2, or SATB2 and HDAC1 co-localize in the developing brain. We have mapped the spatial and temporal distribution of SATB2-MTA2 or SATB2-HDAC1 double positive cells by immunohistochemistry using pre- and postnatal brain sections. (**Fig. 20**.) At embryonic day 18 (E18) SATB2 (+) regions are present in the marginal zone, cortical plate and intermediate zone, mostly restricted to the neocortex as previously described [84]. MTA2 and HDAC1 expression is more widely spread. Surprisingly, even though chromatin remodeling complexes are thought to be abundant, we have found that HDAC1 as well as MTA2 expression are both limited to the neocortex at the age of P2. SATB2 is localized in the nucleus, but right after birth (P2), slight perinuclear localization appears as well. The nuclear localization of both proteins was confirmed by the nuclear Hoechst staining (not shown). When the SATB2 and HDAC1 or MTA2 stainings are merged,

there is a clear yellow colored overlap of the two labeling. SATB2 and HDAC1 or MTA2, respectively, were showing overlapping localization in the developing cortex in both ages. Confocal microscopy shows nuclear co-localization of HDAC1 + SATB2 and MTA2 + SATB2. These findings were consistent with our previous results obtained by using *in vivo* (ChIP) and *in vitro* (IP and DAPSTER) assays.

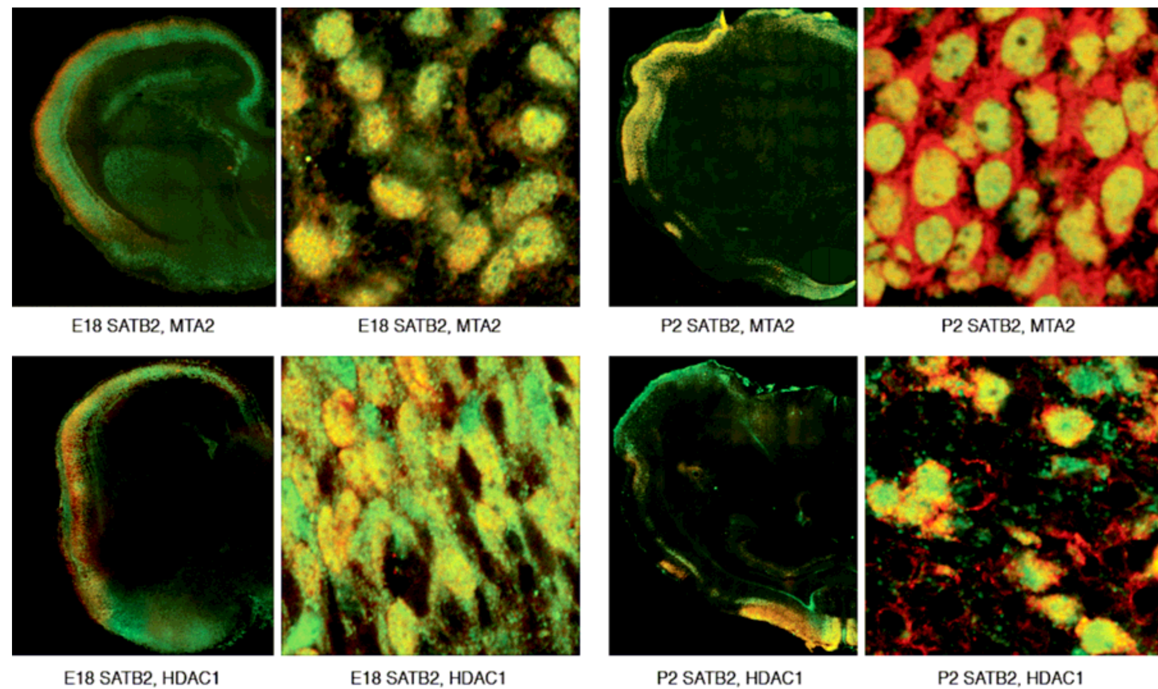

Figure 20.
Co-expression of SATB2 (red) and MTA2 (green), and SATB2 (red) and HDAC1 (green), respectively, in the developing rat cortex.
Cross-sections derived from developing rat brains at ages E18 and P2 were immunostained with the SATB2 antibody and MTA2, or HDAC1 antibody. (left sides) Confocal microscopy images (63x) of the hemispheres on their left side showing nuclear co-localization of and SATB2 (red) and MTA2 (green), or HDAC1 (green). [143]

The digital image analysis of the immunhistochemical images of the double stained E18 and P2 hemispheres (**Fig. 20**.) shows an interesting trend: the SATB2-MTA2 as well as the SATB2-HDAC1 double positive areas substantially increase from late embryonic (E18) to early postantal age (P2). (**Fig. 21.**) Paralelly, the MTA2 and HDAC1 single-positive areas decrease. The SATB2 single positive areas

are dimunitive at both ages. These results suggest that SATB2 may not regulate by itself, but rather in complex with members of the NuRD complex, while HDAC1 and MTA2 may have other co-factors and partners as well at earlier ages when SATB2 expression is not yet abundant.

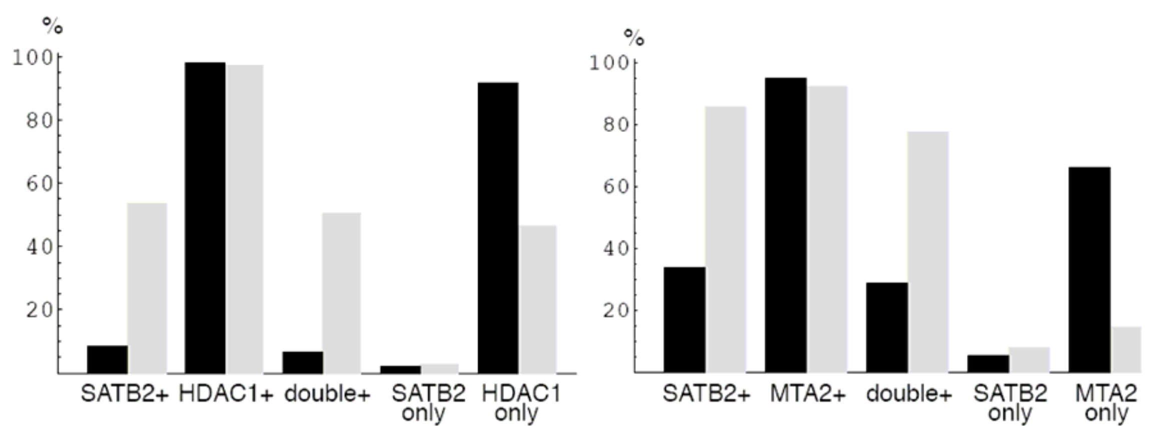

Figure 21.
The ratio of SATB2, MTA2 or SATB2, HDAC1 and double or single positive area on the images of hemispheres. The whole stained area was taken as 100% (SATB2 only + MTA2 or HDAC1 only + double positive areas). E18 = black bars, P2 = grey bars. The ratio of double positive area notably increases, while the MTA2 or HDAC1 single positive area decreases at P2 when compared to E18. The SATB2 single positive areas are notably small compared to the double positive ones. [143]

2.6. SATB2 represses reporter gene in AT-rich dsDNA-dependent manner, and requires HDAC1 for its repression

Our PMDDI assay (**Fig. 2.**) demonstrated that there is a nuclear protein-mediated interaction between the upstream rAT^{ENK} and the TATA regions of the rENK gene *in vitro*. Therefore we tested whether SATB2 can interact with the TATA region in an AT-rich DNA-dependent manner *in vivo*. C6 cells were co- transfected with the reporter plasmids pTK-[$(ATT)_{19}$]-REN or pTK-[(ATT^{mut})]-REN and pCI-SATB2 plasmids that express SATB2 proteins. The TK promoter driven expression of Renila luciferase of the reporter plasmid is modulated by proteins that bind to the inserted DNA elements, in our case either [$(ATT)_{19}$] or (ATT^{mut}).

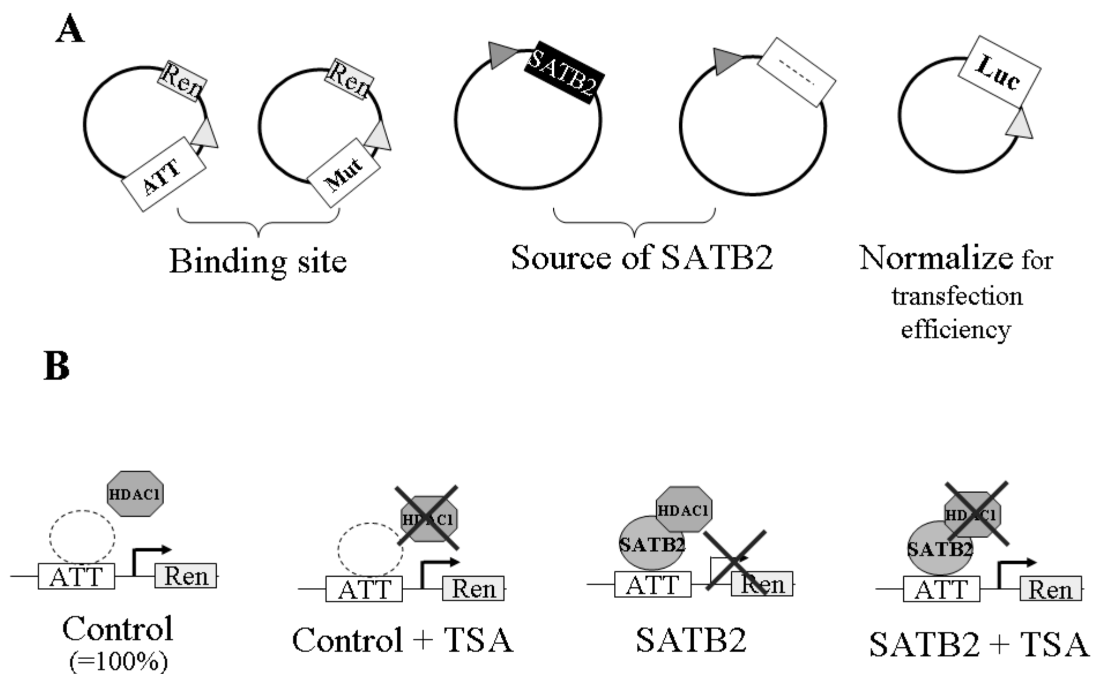

Figure 21.
A: schematic cartoon showing the plasmids used for the transient transfection experiment; reporter gene activity was modified if a regulatory protein (SATB2) was able to bind to the binding site: either wild type (ATT) or mutant (Mut); a separate plasmid was used for a source of recombinant SATB2, or in the case of control, an empty vector was co-transfected with the reporter plasmids. A different luciferase (Luc, not Ren) plasmid was used for enabling us to normalise to transfection efficiency differences.
B: Cartoon showing the expected outcome of different transfections and the effect of the HDAC1 inhibitor Trichostatin A (TSA).

We have found that SATB2 repressed the reporter gene decreasing its activity approximately 5-fold. (**Fig. 22.**).

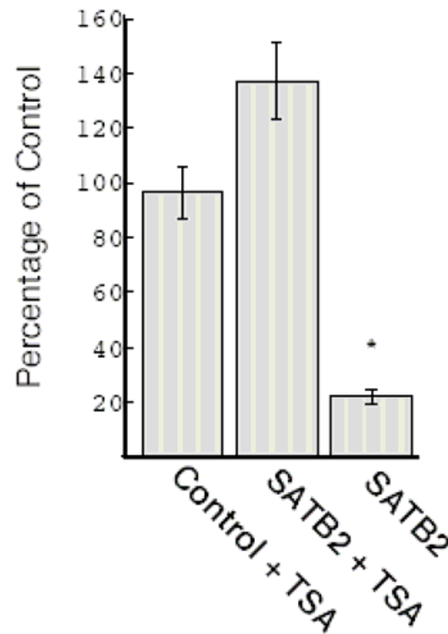

Figure 22.
HDAC1 modifies the effect of SATB2 regulation in an AT-dependent manner. HDAC inhibitor TSA modifies the effect of SATB2 in transient transfection experiments. The values resulting from the binding to the mutant site were subtracted from those from the wild type for normalization. Every value shown is compared to those of the empty vector (=control), which is 100% (not shown). [143]

The repression was however dependent on the presence of the AT-rich DNA element. When pTK-[$(ATT)_{19}$]-REN that carries the AT-rich DNA element was used as reporter plasmid there was significant change in Renila luciferase activity (Fig. 23.). When this reporter plasmid was replaced by the control that carried the mutated version of the AT-region -pTK-[(ATT^{mut})]-REN - there was no significant change in reporter gene activity. These results confirmed our *in vitro* data illustrated above and demonstrated that SATB2 can contribute to the regulation of the rENK gene expression through direct interactions between AT-rich dsDNA element and the TATA-region of the gene. To prove that HDAC1 mediates the effect of SATB2, we

used Trichostatin-A (TSA), a specific HDAC inhibitor. The effect of the TSA treatment was very robust: the repression was actually reversed to a slight (1.4-fold) transactivation by TSA, showing that SATB2 in itself is not enough to exert a repression on the reporter gene: it requires HDAC activity as well.

V. Discussion

Our goal was to identify nuclear proteins that are expressed in the developing brain and contribute to the developmental regulation of the ENK gene. Two proteins, that fit in this category, AUF1 and SATB2 have been identified. Interestingly, AUF1 and SATB2 are two structurally unrelated proteins, however, both bind specifically to the [$(ATT)_{19}$] cis- regulatory DNA element present on the upstream region of the rat ENK gene. In the developing brain the expression of both proteins is spatially and temporally restricted. SATB2 also regulates reporter gene expression in transient tranfection experiments in an AT-rich DNA dependent manner. Furthermore, we have found that SATB2 is interacting with other proteins that are members of chromatin remodeling complexes, suggesting a complex regulatory mechanism.

1. AT-rich DNA element of the upstream regulatory region of the ENK gene

AT-rich DNA elements form the core of MARs/SARs which are not unique in the genome [80]. Accordingly, AT-rich DNA binding proteins such as AUF1 and SATB2 have many potential binding sites present on many different genes [115-117].

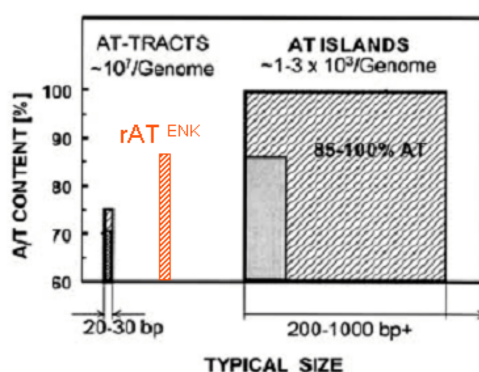

Figure 23.
AT-islands versus common AT-rich elements. The AT-rich region of the ENK gene is showed as a red bar: note that our sequences are in between the short abundant AT-

elements and the long AT-islands both in terms of length and in AT-content. (Modified from [19]).

Since the AT-rich regions identified in the 5' regulatory region of the ENK gene are longer than the before-mentioned short AT-tracks, but shorter than the AT-islands, it is likely that while regulating the expression of the ENK gene as short tracks often do, it could also have the secondary structure similar to the AT-islands, thus providing a binding site for proteins that recognize structure, rather than a specific sequence (**Fig. 23**.). As the mouse, rat and human AT^{ENK} sequences all can be recognized by the rat nuclear extracts derived from developing cortexes, this is a very tempting hypothesis.

The binding site for AT-rich DNA-binding proteins is a "pocket" formed on the minor groove of AT-rich DNA [21]. DNA sequences that are composed of higher than 70% of A or T nucleotides and span the length of 3 or more helical turns (>36 nucleotides) can form such "pockets". The precise dimensions of the "pocket" are determined by the nucleotide composition and by the frequency of A- and T nucleotides. Several proteins that specifically bind to AT-rich MAR/SAR DNA regions have been identified. The list includes nucleolin [118], histone H1 [119], HMGI/Y [120] and the thymus-specific developmental regulator SATB1 [91].

One of the cis-regulatory DNA elements that were identified was an AT-rich DNA site present on the upstream regulatory region of the mouse, rat and human ENK genes [113] (AT^{ENK}). Both AUF1 and SATB2 are acting upon binding to the AT-rich element of the ENK gene. (**Fig. 24.**)

The mouse and human genes lack the repetitive nature of the rat AT-rich DNA sequence however they have orthologous regions at similar upstream locations. This

further illustrates that in contrast to the typical short DNA binding sites, AT-rich DNA elements do not have a consensus sequence in a classical sense [20, 80, 114].

Rat: +2450
CATGG TTAATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTTTT GGTTCTTTTTT
Mouse: -3150
ATTTTTTAAATATTCTTATCAG AATATAGCTACAAATCTACCCCTTATATTTAA GAATATAATG ATAAAAA GATATTG
Human:-3980
GTTTCTATTATTTAAACTCATTCTATTATTTAATTTATATTATTTAAACTCTTTCTATTATTTAATCTG

Figure 24.
Sequences of the AT-rich regions of the rat, mouse and human ENK. Note that there is no “consensus” sequence or other similarity than the relative AT-richness among the three species, and only the rat AT-region has a repetitive nature.[78]

The size of the AT-rich region of the ENK gene is between the short (>20 bp) AT-enriched sequences and the long AT-islands (<200-1000 bp) with its 19x (ATT) repeats (in case of the rat sequence), however, it is over 90% AT-rich in mouse and human as well (**Fig. 23.**). This feature of the rAT sequence must play an important role in the formation of an identifiable secondary structure, which is likely to be “read” by proteins that can specifically recognize them.

2. AUF1

AUF1 have been identified as an AT-rich DNA binding protein that is binding to its site of recognition in a temporally and spatially defined manner in the developing CNS.

AUF1 has been known as a regulator of mRNA stability. Regulation of the half-life of mRNAs has been emerging as an important control mechanism during development [121, 122]. Frequently, such regulation is mediated via AU-rich sequences (AREs), located at the 3’ untranslated region of mRNAs [123].

Proteins that bind these sequences include the AU binding factor 1 (AUF1), also called hnRNP D [124, 125]. AUF1 has the characteristic domain structure that includes two RNA binding domains (RBDs), oligomerization domain and an RGG domain [126, 127]. This domain structure is shared by many other hnRNP proteins [125, 128, 129]. It has four isoforms, p37, (A), p40 (B), p42 (C) and p45 (D) that are the results of alternative splicing of exon2 and exon7 [127].

AUF1 has been described to participate in other regulatory mechanisms as well. As Tolnay et al. have shown, AUF1 also regulates the complement receptor gene expression in the immune system by binding to the promoter and interacting with the TATA-binding protein [130]. Thus it does not come as a surprise that it can also regulate gene expression in the central nervous system.

AUF1 also lacks the AT-hook and shows little or no structural homology to SATB1 or SATB2 and to other AT-binding proteins. In summary, how AUF1 proteins bind to AT-rich dsDNA elements is currently not known. One can only speculate that dimerization or multimerization may play a role in the binding. Previous studies demonstrated that AUF1 proteins can directly interact with nucleolin that binds MAR/SAR DNA elements [131] and with the MAR associated factor SAF-B [132] further implicating AUF1 in MAR/SAR-related regulation.

3. SATB2

We identified SATB2 as the other AT-rich DNA binding protein in the developing cortex. SATB2 has already been identified as a protein that plays a crucial role during development by a group that identified SATB2 as the cleft palate gene (CPO) [133]. It

has also been shown to bind to S/MAR regions, [108], although no specific gene has been identified so far that has been shown to be directly regulated by SATB2.

3.1. SATB2 and SATB1

Studies have shown that another AT-rich dsDNA binding protein, SATB1, that shares a high amino acid homology with SATB2 (**Fig. 24.**), exerts its regulatory role by regulating chromatin structure. Mi2/NuRD is believed to be recruited to specific DNA sequences by adaptor proteins such as Ikaros and Hunchback and to repress specific unmethylated promoters.

SATB1 is known to be a “global” coordinator of cellular differentiation in the hematopoietic lineage.

SATB1 recruits the histone deacetylase contained in the NURD chromatin remodeling complex to a SATB1-bound site in the IL-2Ra locus [138], and mediates the specific deacetylation of histones in a large domain within the locus. SATB1 also targets ACF1 and ISWI, subunits of CHRAC and ACF nucleosome mobilizing complexes, to this specific site and regulates nucleosome positioning over seven kilobases. SATB1 defines a class of transcriptional regulators that function as a ‘landing platform’ for several chromatin remodeling enzymes and hence regulate large chromatin domains.

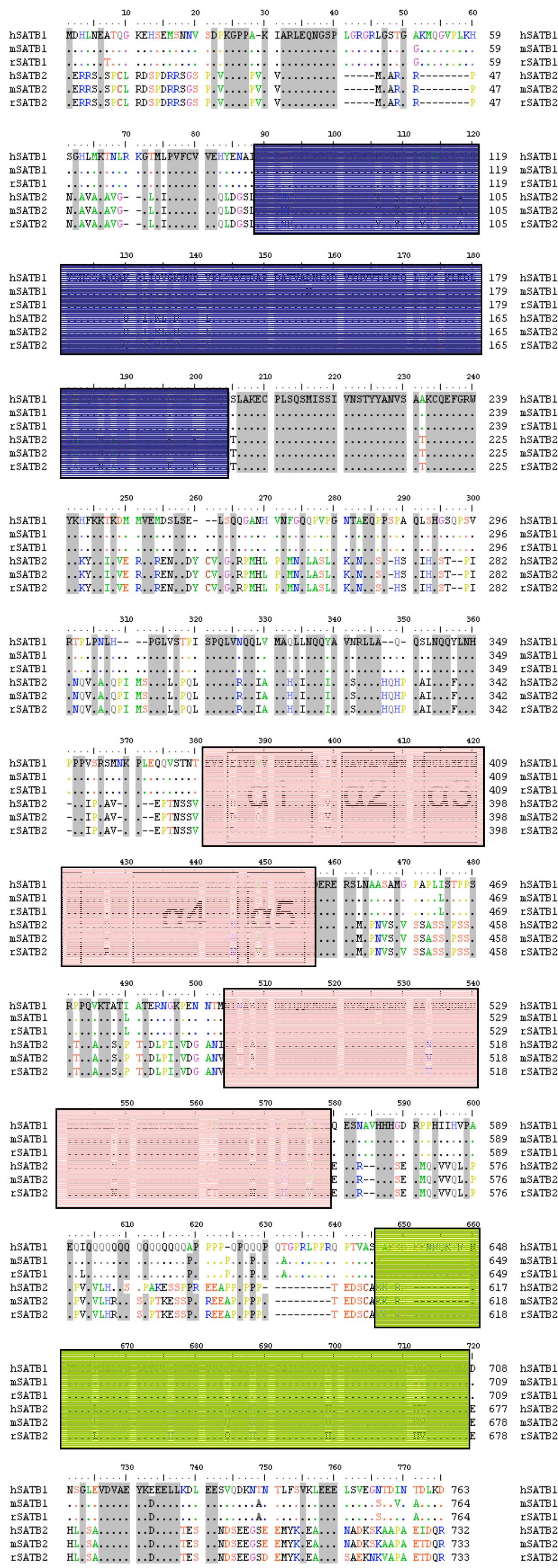
Dimerization/
PDZ-like domain:
protein-protein
interaction
CUT1
domain:
MAR
binding
CUT2
domain
Homeo-
domain

Figure 25.
Amino acid sequence alignment of the human, mouse and rat SATB2 and SATB1 with functional domains shown in boxes. As shown, SATB1 and SATB2 are share a high homology in all their important domains, including those 5 alpha-helixes that are required for MAR-binding, and also the PDZ-like dimerization domain on the N-terminal end that is essential for binding to partner proteins like HDAC1.

3.2. The mechanism of AT-binding of SATB2

Many AT-binding proteins have a specific domain, called the AT-hook, thought to be critical to bind to AT-rich dsDNA sequences [23]. However, several proteins, including AUF1 and SATB2, as well as SATB1 lack the AT-hook and have distinct domain for binding AT-rich DNA [120, 134] [93].

3.2.1. Major groove binding

A recent study by Yamagutchy et al. [135] determined the solution structure of the matrix attachment region-binding domain, possessing similarity to the CUT DNA-binding domain, of human SATB1 by NMR spectroscopy. The structure determined by this group consists of five alpha-helices, and according to them, the MBD domain recognizes B-DNA from the major groove side. **(Fig. 26.)**

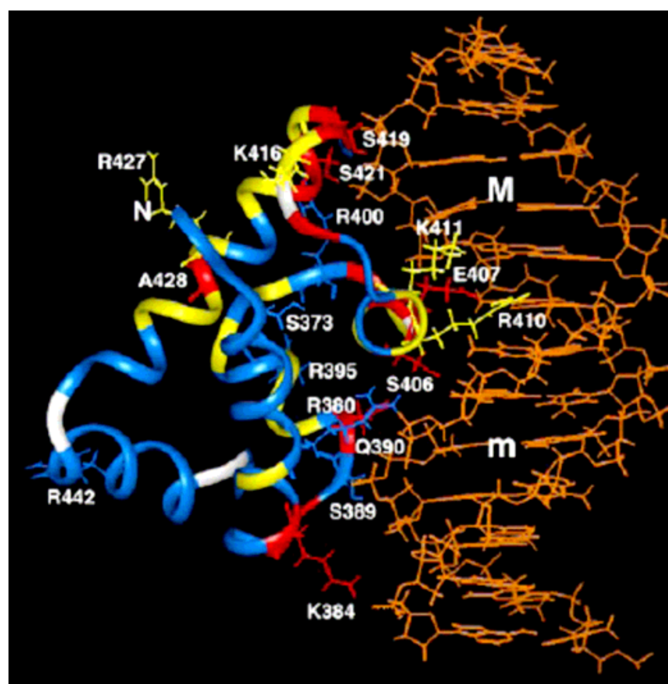

Figure 26.
DNA-recognition mode of the SATB1 MAR Binding Domain. [136] The presented region of the protein is Glu370–Arg450. A predicted model of the complex of SATB1-MBD (a ribbon of different colors) and a standard B-DNA molecule (orange wire) in stereo view. The major and minor grooves of DNA are indicated by *M* and *m*, respectively. Note that according to this prediction, SATB1 is binding to the major groove of the DNA.[135]

3.2.2. Minor groove binding

However, since we found that the formation of protein-DNA complexes between the nuclear extracts from developing cortexes and the AT-rich dsDNA could be completely prevented by the addition of a minor-groove binding drug, distamycin (see **Fig. 5. and 6**.), the exact means of how SATB2 and AUF1 are binding to AT-rich dsDNA elements is currently not known.

3.3. Functional significance of MAR-binding

As it has been described for SATB1, the protein-DNA complex may stabilize the double-stranded MAR-DNA in the B-form and protect it from the base-unpairing force. Therefore, it is likely that the binding of SATB1 to MAR competes with the binding to the nuclear matrix, if the base-unpaired DNA is assumed to be involved in the binding to the nuclear matrix. Nuclear matrix binding clearly has functional implications for transcription transcriptionally active DNA is tightly associated with the nuclear matrix, whereas inactive loci are not. Therefore, SATB1 might repress gene expression by preventing the gene to be attached to the nuclear scaffold [136].

Considering the high amino-acid homology between SATB1 and SATB2 in all the important domain involved in MAR-binding (MBD), as well as the dimerization/protein-protein interaction (PDZ-like) domain, it is reasonable to assume

that SATB2 might have a similar way of suppressing gene expression during cortical development.

4. Expression of AUF1 and SATB2 in the developing cortex.

We have found that in the rat brain, AUF1 proteins are expressed in development- and cell-specific manner. Their spatial and temporal pattern of expression as well as the cell culture experiments implicates that AUF1 proteins are developmental repressors of ENK gene expression.

Previous experiments using adult tissues indicated that AUF1 expression is quite ubiquitous and there is also evidence that distinct isoforms are expressed in tissue and cell specific manner [102]. We showed here that AUF1 proteins are present both in proliferating progenitors and in differentiating neurons suggesting that AUF1 proteins may be directly involved in regulating the expression of multiple, developmentally relevant genes during neuronal development.

Our immunohistochemical data and a recent study [108] showed that SATB2 is abundantly expressed in a subset of postmitotic differentiating neurons in the neocortex implicating that SATB2 regulates gene expression during cortical development. We believe that both SATB1 and SATB2 are major regulators of gene expression during cortical development [91, 108]. Our own data (not shown) and a recent publication [108] demonstrate that in the developing brain, the pattern of SATB1 and SATB2 expression is not overlapping suggesting that they regulate the differentiation of distinct populations of neurons. Accordingly, SATB1 and SATB2 can be used as markers to identify distinct subsets of differentiating cortical neurons.

5. Chromatin remodeling

Simultaneous regulation of multiple genes during development is necessary to produce the vast array of adult neuronal phenotypes. A good example is the regulation with the neuron-restrictive silencer factor (REST/NRSF), which was thought to be an ordinary repressor. The repressor protein REST/NRSF plays a crucial role in suppressing the neuronal phenotype outside of the developing nervous system. It has been shown that REST forms a complex with CoREST and mSin3a, utilizing epigenetics to repress multiple neuron-specific genes at the same time. [34]

We focused on the role of the major histone deacetylation complex, NuRD (*Nu*cleosome *R*emodeling and histone *D*eacetylation) and its associated proteins, as SATB1, a structurally related protein of SATB2 was known to interact with proteins of this complex.

NuRD complexes are of considerable interest since they physically combine aspects of the two essential strategies for chromatin remodeling: covalent modification (deacetylation of histones) and ATP-dependent nucleosome remodeling through the Mi-2 ATPase, which is an active enzyme for remodeling by itself - it is able to disrupt histone-DNA interactions and to induce nucleosome sliding on DNA fragments.

The Mi2/NuRD complex [40] is approximately 2 MDa in size and in mammalian cells comprises at least seven polypeptides. The histone deacetylases HDAC1 and HDAC2 and two histone-binding proteins (RbAp46 and RbAp48) are also found in the Sin3a complex. In addition to histone-deacetylase activity, the NuRD complex has ATP-dependent nucleosome remodeling activity because it contains Mi-2/CHD family proteins, which have a chromodomain, a DNA helicase/ATPase domain of the SWI/SNF family, and plant homeodomain (PHD)

fingers. Another distinguishing feature of NuRD is the inclusion of MTA1, originally identified as being overexpressed in metastatic carcinomas [42], or the similar protein MTA2. NuRD also contains MBD3 (similar to methyl CpG-binding domain proteins). Since hypoacetylated histone amino-termini are generally correlated with repression of transcription, NuRD complexes were thought to be involved in establishing a repressive chromatin environment. NuRD is thus targeted to bind, remodel, and deacetylate nucleosomes containing methylated DNA [38].

In our current work we investigated the role of two members of the NuRD complex, HDAC1 and MTA2. We have found that both of these proteins interact with SATB2 and the AT-rich element of the ENK gene *in vitro* and *in vivo*.

5.1. HDAC1

There have been at least 11 HDACs characterized to date in human. They fall into two classes: the class I HDACs (HDAC1, 2, 3 and 8) are thought to be widely expressed, and the class II HDACs (HDAC4, 5, 6, 7, 9 and 10) have cell-type-restricted patterns of expression (reviewed in [47]). A third class of HDACs is a group of proteins related to the yeast transcriptional repressor Sir2. From extensive studies of histone acetylation and deacetylation in cultured cells, the characterization of specific HDACs in vivo has revealed that they have essential functions in chondrocyte development, cardiac signaling and skeletal muscle physiology [refs]. [36]

Class I HDACs are supposedly expressed fairly ubiquitously, whereas Class II HDACs are enriched in heart, skeletal muscle and brain. Northern analysis of HDAC expression in embryonic rat hippocampal progenitors revealed that class II HDACS were specifically upregulated when cells were induced to differentiate, whereas class I

HDAC expression remained unchanged, suggesting a regulatory role for class II HDACs during neuronal differentiation [45]. [137]

Some target genes (i.e. otoferlin) are HDAC-dependent and can be reactivated with treatment of the HDAC inhibitor Trichostatin A (TSA), whereas other genes (i.e. SMARCe) are silenced by HDAC- and DNA-methylation-dependent mechanisms and are only reactivated upon stimulation with both TSA and the DNA demethylating reagent 5-aza-cytidine. Histone acetylation has been shown to be important for other aspects of neuronal development, including neuronal and oligodendrocyte lineage progression. Adult multipotent neuronal progenitor cells differentiated predominantly into neurons in the presence of the HDAC inhibitor valproic acid (VPA). [137]

Our finding that HDAC1 is not ubiquitously expressed in the developing rat brain points to the possibility that this protein and the complexes it is participating in are much more specific than it was originally thought.

5.2. MTA2

MTA1, MTA2, and MTA3 are homologous proteins. MTA1 has been linked to tumor metastasis, but experimental data suggest that MTA2 may not be associated with metastasis. The functions of MTA1 and MTA2 involve histone deacetylation and the formation of protein complexes. The divergent C-terminal regions of the MTA proteins suggest that MTA1 and MTA2 are regulated differently. Although both MTA1 and MTA2 repress transcription, are located in the nucleus, contain associated histone deacetylase activity, and form multiprotein complexes. However, MTA3 does not appear to repress transcription to a significant level despite sharing a high degree of homology in the N terminus. Whereas the MTA2 complex is similar to the HDAC1 complex, the MTA1 complex contains specific proteins for the MTA1 complex that

are absent in the MTA2 complex. In addition to forming stable complexes, MTA1 and MTA2 are able to make transient association with different transcription factors, reinforcing the idea that MTA1 and MTA2 have different biological functions.

MTA2 is involved in maintaining the normal transcriptional balance of the cell, whereas overexpression of MTA1 leads to perturbation of the cellular transcription program. Therefore, we focused on the possible role of the MTA2 protein in the developing cortex.

6. SATB2 and chromatin remodeling

We have found that the overwhelming majority of the subset of cortical neurons that expresses SATB2, also co-expresses two other members of the NuRD complex, HDAC1 and MTA2.

HDAC1 is member of the class I HDACs along with HDAC 2, 3, and 8, which are thought to be ubiquitously expressed. However, there are only a few detailed studies about its expression pattern, particularly during development. One study found that HDAC1 is first expressed in the preimplantation embryo, where the genome undergoes dynamic epigenetic changes [139]. Nonetheless, this would not be the first study which correlates a class I HDAC with differentiation: it was found that overexpression of HDAC1 and 2 in fetal intestine explants reverses the expression of certain maturation markers, [140] and another study identified HDAC1 as necessary to repress MyoD in undifferentiated myoblasts [141]. The MTA2-SATB2 coexpression in the neonatal rat cortex also emphasizes that SATB2 is not acting alone: HDAC1 and MTA2, members of the MI2/NuRD complex just are likely to be as important in repression of the regulated genes as SATB2 itself is.

7. Regulation of the telencephalic development

How AUF1 and SATB2 fit into the regulatory network of proteins that control gene expression during brain development is currently not understood.

For most species, the nervous system is the most complex part of the organism. In mammalian species, over one third of genes are expressed in the central nervous system (CNS). Furthermore, there is an abundance of gene expression and protein synthesis that underscore the structural and functional intrinsic complexity of the CNS. It is reasonable to assume that CNS-specific transcripts are necessary not only to encode the wide variety of cellular and molecular components of the mature CNS, but that a substantial part of them guides CNS morphogenesis and are primarily expressed during development. Key players in the developmental process are the transcription factors that are expressed and interact with one another in spatiotemporally defined ways. Their timely coordinated and cooperative interactions are critical to define mature neuronal phenotypes through selective activation or repression of developmentally important genes.

While more proteins are known to regulate the early events of neuronal differentiation, like Notch, the two factors we identified are acting at a later stage in differentiation, and might play a role in specifying the non-enkephalinergic phenotype in the cortex.

Summary

A central question in developmental neuroscience is how progenitor cells differentiate into diverse neuronal and glial phenotypes. We chose the enkephalin (ENK) gene as our model system to study neuronal differentiation in the developing brain.

Using a variety of techniques, several upstream DNS elements of the ENK gene have been identified that are involved the in the developmental expression regulation of the enkephalinergic phenotype. One of these regions was the AT-rich sequence, that resembles the secondary structures of MARs.

Two AT-rich DNA binding proteins were identified in the developing cortex: AUF1 and SATB2. We have determined using immunohistochemistry and immunblotting as well as RT-PCR, that both of the proteins have a spatially and temporally restricted expression pattern in the developing rat brain. This suggests a role in later events of neuronal differentiation, possibly in the restriction of enkephalinergic phenotype in the cortex.

The mechanism of AT-rich DNA binding of these structurally unrelated proteins is not currently known; the only common feature of SATB2 and AUF1 is that both lacks the AT-hook, however, both still specifically bind to rAT^{ENK} sequence *in vitro* and *in vivo* as well, as we determined by DNA Affinity Preincubation Specifity Test of Recognition (DAPSTER) and chromatin immunoprecipitaion (ChIP).

Because of the high sequence homology between SATB1 and SATB2, we set out to determine whether SATB2 acts upon binding MAR-like DNA sequences and targeting chromatin remodeling complexes to its cognate regulatory DNA, thereby repressing the ENK gene.

Using double immunohistochemistry, we found that SATB2 was co-expressed with HDAC1 and MTA2 in the developing brain both at embryonic daz 18 (E18), and at postnatal day 2 (P2). The area of double positive staining substantially increased by P2, as was shown with digital image processing.

Surprisingly, the expression of the two members of the NuRD chromatin remodeler complex, MTA2 and HDA1 also had a spatially and temporally resticted expression pattern, even though it was assumed that proteins with a similar function should be abundant in the developing brain.

Co-immunoprecipitation and DAPSTER assay results showed that SATB2 was bound to these co-factors *in vitro* in the presence as well as absence of the AT-rich DNA of the ENK gene.

We determined that SATB2 needed HDAC1 as its co-repressor when regulating reporter gene expression, as we found by inhibiting HDAC1 activity by TSA treatment in transient transfection experiments. This finding is in accordance with a recent publication about SATB1, where it was shown that SATB1 (just as SATB2) possesses a PDZ-like domain where it binds to HDAC1, and prevention of HDAC1 to interact with SATB1 hindered the repressor activity of SATB1 [142].

This observation, along with the in vivo evidence showing that HDAC1, MTA2 and CHRAC17 are bound to the rAT^{ENK} sequence during development, points us to the conclusion that SATB2, as well as SATB1 [138] suppresses gene expression by functioning as a "guide" for chromatin remodeling complexes. Our preliminary results also implied that AUF1 may utilize chromatin remodeling just as SATB2 (data not shown).

Future studies will be needed to elucidate the exact role that SATB2 and AUF1 are playing in the regulation of ENK during development, as well as possibly more other genes.

As the two identified rAT^{ENK} binders are not “sequence-specific” transcription factors in the classical sense, it is tempting to hypothesize that given the relative abundance of AT-rich sequences in the genome, they might regulate the phenotypical differentiation of cortical neurons through repressing multiple genes at the same time, which is a crucial pre-requisite of fate aquisition.

The regulation is likely to involve epigenetic mechanisms by de-acetylating the histones first, then remodeling the nucleosomes and thus achieving a closed chromatin structure that prevents the landing of the transcription machinery (**Fig. 27.**).

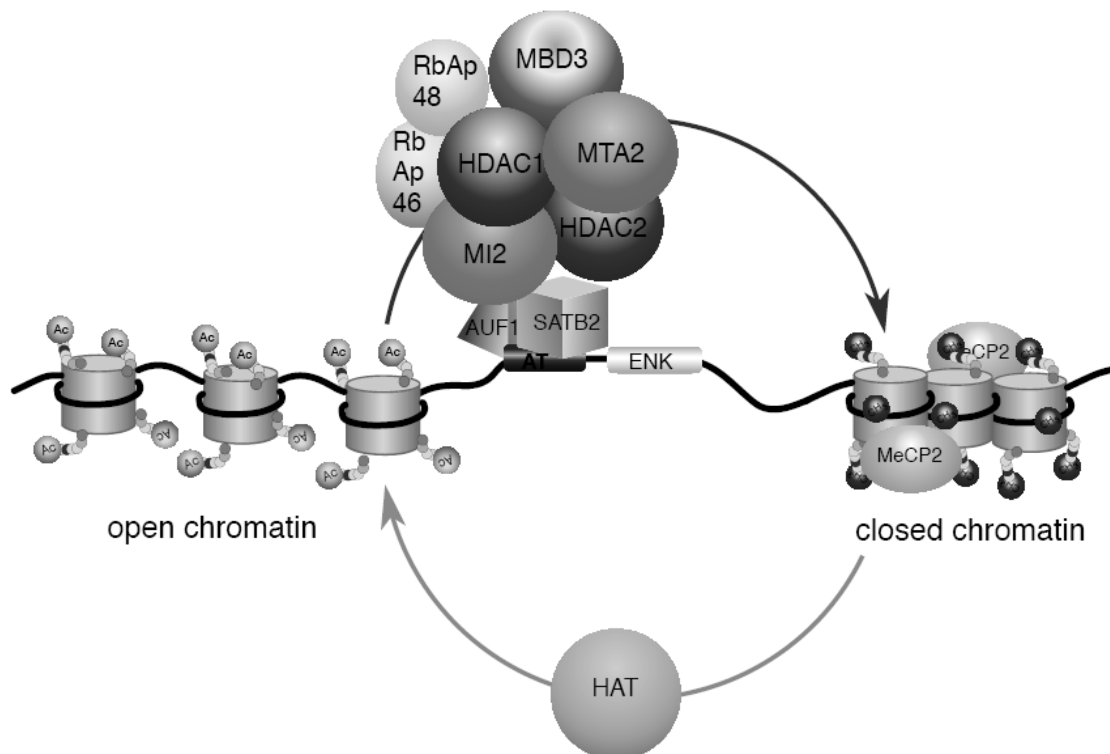

Figure 27.
Cartoon summarizing our working hypothesis about the mechanism of SATB2 and AUF1 regulation of gene expression. [143]

Összefoglaló

Az idegrendszer fejlődésének egyik legfontosabb része a sejtdifferenciáció szabályozása. Ebben a tanulmányban azokat a transzkripciós szabályozó mechanizmusokat vizsgáltuk, melyek a fejlődő agyban befolyásolhatják a sejtsorsról való döntést. Modellként az enkefalin (ENK) gént választottuk.

Különböző technikák alkalmazásával korábban azonosítottak több olyan szakaszt az ENK gén távolabbi 5' régiójában, amelyek szerepet játszhatnak az enkefalinerg fenotípus bizonyos neuronokra való korlátozásában. Ezek közül az egyik egy AT-gazdag DNS szakasz volt, mely megtalálható a patkány, az ember és az egér ENK génben egyaránt. Erre a szakaszra is jellemző a MAR szekvenciáknál ismert szűk kisárok, melyet az AT-kötő fehérjék felismernek.

Két fehérjét is azonosítottak, amelyek ehhez a régióhoz kötődnek: az egyik az mRNS-stabilitást szabályozó AUF1 volt, a másik egy ismert mátrix-kötő fehérjéhez, a SATB1-hoz hasonló SATB2.

Mindkét azonosított AT-gazdag DNS-kötő fehérje, a SATB2 és az AUF1 is kifejeződik a fejlődő agyban, és hozzájárulhat az ENK fejlődésbeni szabályozásához. A fejlődő agyban mindkét fehérje kifejeződése térben és időben meghatározott. Az AUF1 és a SATB2 kifejeződési mintázatát immunhisztokémiával, Western blot és a SATB2 esetében RT-PCR technikával térképeztük fel. Az immunhisztokémia nagy nagyítású elemzése azt mutatta, hogy mindkét fehérjét a sejtmagi kifejeződés jellemzi.

Érdekes módon ez a két fehérje szerkezetileg nem rokon egymással: nincsenek közös domainjeik, és aminosav szinten sem hasonlóak. Egyetlen közös tulajdonságuk, hogy egyik sem rendelkezik az AT-kötő fehérjékre jellemző AT-hurokkal. Noha a kötés pontos

mechanizmusa azonban jelenleg nem pontosan ismert, mindkét fehérje specifikusan kötődik az ENK gén AT-gazdag 5' régiójához mind *in vitro*, mind *in vivo*, amint ezt DAPSTER assay és kromatin immunoprecipitáció (ChIP assay) segítségével megállapítottuk.

A SATB2 aminosav szinten igen hasonlít a hozzá közel rokon SATB1-hoz, amiről ismert, hogy mind a NuRD, mind az CHRAC/ACF kromatin átrendezéséért felelős enzimkomplexek tagjaival kölcsönhat. Az is bizonyított, hogy akárcsak a SATB2, MAR szekvenciákhoz köt a genomban. Ebből kiindulva immunprecipitációval, DAPSTER assay segítségével és kromatin immunoprecipitációval bebizonyítottuk, hogy a SATB2 ugyancsak komplexet képez a NuRD két tagjával, az MTA2-val és az HDAC1-al az ENK AT-gazdag régiójához kapcsolódva.

Ezt alátámasztotta a kettős immunhisztokémia is, mely azt mutatta, hogy a SATB2 elsősorban olyan sejtekben fejeződik ki mind a 18. embrionális (E18), mind a második posztnatális napon (P2), melyek a vizsgált partnerfehérjéket is expresszálják. Ezt a nagy nagyítású konfokális mikroszkóppal készült képek is megerősítették. Idősebb állatokban (P2) a kettős pozitív terület aránya mindkét ko-faktor esetében jelentősen megnőtt.

Meglepő módon az általánosan elterjedtnek hitt két fehérje, az MTA2 és az HDAC1 is térben és időben meghatározott módon fejeződik ki a fejlődő agyban.

A SATB2 funkciójáról a tranziens transzfekció azt mutatta, hogy represssziót közvetít, de a SATB2 önmagában nem képes erre, hanem HDAC1 is aktivitást igényel funkciója betöltéséhez, amint azt a trichostatin A (TSA) kezelés mutatta.

További kísérletek fogják csak eldönteni, hogy mi a SATB2 és az AUF1 pontos szerepe az ENK és valószínűleg több más gén egyidejű szabályozásában az egyedfejlődés során.

A fenti eredmények ismeretében mi a következő hipotézist állítottuk fel az AUF1 és a SATB2 funkciójáról: valószínű, hogy mindkét fehérje több, hasonlóan AT-gazdag

szekvenciához kötődve a genomban, a kromatin átrendezéséért felelős enzim-komplexeket irányítja oda az AT-gazdag kötőhelyekhez, így több gén együttes szabályozását téve lehetővé, elősegítve a sejtdifferenciációhoz elengedhetetlen represssziót a kérgi neuronokban. Ez a szabályozás valószínűleg olyan epigenetikus mechanizmusokon keresztül történik, mint például a hisztonok de-acetilációja –amire az HDAC1 függő represszió is utal-, és a nukleoszómák átrendezése zárt kromatin struktúrává, ami megakadályozza, hogy a transzkripciós apparátus odakötődjön a represszált génekhez. (**Fig. 27.**)

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References

1. Kageyama, R., et al., *bHLH transcription factors and mammalian neuronal differentiation.* Int J Biochem Cell Biol, 1997. **29**(12): p. 1389-99.
2. Lewis, J., *Neurogenic genes and vertebrate neurogenesis.* Curr Opin Neurobiol, 1996. **6**(1): p. 3-10.
3. Okano, H., et al., *Evolutionarily conserved mechanisms regulating neural development: lessons from the development of Drosophila peripheral nervous systems.* Hum Cell, 1997. **10**(3): p. 139-50.
4. Verdi, J.M., et al., *Distinct human NUMB isoforms regulate differentiation vs. proliferation in the neuronal lineage.* Proc Natl Acad Sci U S A, 1999. **96**(18): p. 10472-6.
5. Klezovitch, O., et al., *Loss of cell polarity causes severe brain dysplasia in Lgl1 knockout mice.* Genes Dev, 2004. **18**(5): p. 559-71.
6. Guillemot, F., *Cellular and molecular control of neurogenesis in the mammalian telencephalon.* Curr Opin Cell Biol, 2005. **17**(6): p. 639-47.
7. Mason, H.A., et al., *Notch signaling coordinates the patterning of striatal compartments.* Development, 2005. **132**(19): p. 4247-58.
8. Marin, O. and J.L. Rubenstein, *A long, remarkable journey: tangential migration in the telencephalon.* Nat Rev Neurosci, 2001. **2**(11): p. 780-90.
9. Campbell, K., *Cortical neuron specification: it has its time and place.* Neuron, 2005. **46**(3): p. 373-6.
10. Malatesta, P., et al., *Neuronal or glial progeny: regional differences in radial glia fate.* Neuron, 2003. **37**(5): p. 751-64.
11. Haubensak, W., et al., *Neurons arise in the basal neuroepithelium of the early mammalian telencephalon: a major site of neurogenesis.* Proc Natl Acad Sci U S A, 2004. **101**(9): p. 3196-201.
12. Lee, J.E., *Basic helix-loop-helix genes in neural development.* Curr Opin Neurobiol, 1997. **7**(1): p. 13-20.
13. Lunyak, V.V. and M.G. Rosenfeld, *No rest for REST: REST/NRSF regulation of neurogenesis.* Cell, 2005. **121**(4): p. 499-501.
14. Martynoga, B., et al., *Foxg1 is required for specification of ventral telencephalon and region-specific regulation of dorsal telencephalic precursor proliferation and apoptosis.* Dev Biol, 2005. **283**(1): p. 113-27.
15. Hanashima, C., et al., *Foxg1 suppresses early cortical cell fate.* Science, 2004. **303**(5654): p. 56-9.
16. Corbin, J.G., et al., *The gsh2 homeodomain gene controls multiple aspects of telencephalic development [In Process Citation].* Development, 2000. **127**(23): p. 5007-20 [MEDLINE record in process].
17. Pozas, E. and C.F. Ibanez, *GDNF and GFRalpha1 promote differentiation and tangential migration of cortical GABAergic neurons.* Neuron, 2005. **45**(5): p. 701-13.
18. Cobos, I., et al., *Mice lacking Dlx1 show subtype-specific loss of interneurons, reduced inhibition and epilepsy.* Nat Neurosci, 2005. **8**(8): p. 1059-68.

19. Fode, C., et al., *A role for neural determination genes in specifying the dorsoventral identity of telencephalic neurons.* Genes Dev, 2000. **14**(1): p. 67-80.
20. Boulikas, T., *Chromatin domains and prediction of MAR sequences.* Int Rev Cytol, 1995.
21. Woynarowski, J.M., *AT islands - their nature and potential for anticancer strategies.* Curr Cancer Drug Targets, 2004. **4**(2): p. 219-34.
22. Catasti, P., et al., *DNA repeats in the human genome.* Genetica, 1999. **106**(1-2): p. 15-36.
23. Aravind, L. and D. Landsman, *AT-hook motifs identified in a wide variety of DNA-binding proteins.* Nucleic Acids Res, 1998. **26**(19): p. 4413-4421.
24. Broeker, P.L., et al., *The mixed lineage leukemia (MLL) protein involved in 11q23 translocations contains a domain that binds cruciform DNA and scaffold attachment region (SAR) DNA.* Curr Top Microbiol Immunol, 1996. **211**: p. 259-68.
25. Benham, C., T. Kohwi-Shigematsu, and J. Bode, *Stress-induced duplex DNA destabilization in scaffold/matrix attachment regions.* J Mol Biol, 1997. **274**(2): p. 181-96.
26. Yasuda, H., et al., *ATBF1, a multiple-homeodomain zinc finger protein, selectively down-regulates AT-rich elements of the human alpha-fetoprotein gene.* Mol Cell Biol, 1994. **14**(2): p. 1395-401.
27. Dobreva, G., J. Dambacher, and R. Grosschedl, *SUMO modification of a novel MAR-binding protein, SATB2, modulates immunoglobulin mu gene expression.* Genes Dev, 2003. **17**(24): p. 3048-61.
28. Berezney, R., et al., *The nuclear matrix: a structural milieu for genomic function.* Int Rev Cytol, 1995. **162A**: p. 1-65.
29. Hancock, R., *Internal organisation of the nucleus: assembly of compartments by macromolecular crowding and the nuclear matrix model.* Biol Cell, 2004. **96**(8): p. 595-601.
30. Berezney, R. and D.S. Coffey, *Identification of a nuclear protein matrix.* Biochem Biophys Res Commun, 1974. **60**(4): p. 1410-7.
31. Razin, S.V., *DNA interactions with the nuclear matrix and spatial organization of replication and transcription.* Bioessays, 1987. **6**(1): p. 19-23.
32. Sharma, R.P., *Schizophrenia, epigenetics and ligand-activated nuclear receptors: a framework for chromatin therapeutics.* Schizophr Res, 2005. **72**(2-3): p. 79-90.
33. de Laat, W. and F. Grosveld, *Spatial organization of gene expression: the active chromatin hub.* Chromosome Res, 2003. **11**(5): p. 447-59.
34. Brown, K.E., *Chromatin folding and gene expression: new tools to reveal the spatial organization of genes.* Chromosome Res, 2003. **11**(5): p. 423-33.
35. Recillas-Targa, F. and S.V. Razin, *Chromatin domains and regulation of gene expression: familiar and enigmatic clusters of chicken globin genes.* Crit Rev Eukaryot Gene Expr, 2001. **11**(1-3): p. 227-42.
36. Hsieh, J. and F.H. Gage, *Chromatin remodeling in neural development and plasticity.* Curr Opin Cell Biol, 2005. **17**(6): p. 664-71.

37. Bapat, S. and S. Galande, *Association by guilt: identification of DLX5 as a target for MeCP2 provides a molecular link between genomic imprinting and Rett syndrome.* Bioessays, 2005. **27**(7): p. 676-80.
38. Ahringer, J., *NuRD and SIN3 histone deacetylase complexes in development.* Trends Genet, 2000. **16**(8): p. 351-6.
39. Luger, K. and T.J. Richmond, *DNA binding within the nucleosome core.* Curr Opin Struct Biol, 1998. **8**(1): p. 33-40.
40. Becker, P.B. and W. Horz, *ATP-dependent nucleosome remodeling.* Annu Rev Biochem, 2002. **71**: p. 247-73.
41. Barton, M.C. and A.J. Crowe, *Chromatin alteration, transcription and replication: What's the opening line to the story?* Oncogene, 2001. **20**(24): p. 3094-9.
42. Yao, Y.L. and W.M. Yang, *The metastasis-associated proteins 1 and 2 form distinct protein complexes with histone deacetylase activity.* J Biol Chem, 2003. **278**(43): p. 42560-8.
43. Peterson, C.L. and M.A. Laniel, *Histones and histone modifications.* Curr Biol, 2004. **14**(14): p. R546-51.
44. Muchardt, C. and M. Yaniv, *ATP-dependent chromatin remodelling: SWI/SNF and Co. are on the job.* J Mol Biol, 1999. **293**(2): p. 187-98.
45. Drago, J., et al., *Targeted expression of a toxin gene to D1 dopamine receptor neurons by cre-mediated site-specific recombination.* J Neurosci, 1998. **18**(23): p. 9845-57.
46. Dirscherl, S.S. and J.E. Krebs, *Functional diversity of ISWI complexes.* Biochem Cell Biol, 2004. **82**(4): p. 482-9.
47. Zhang, Y., et al., *Analysis of the NuRD subunits reveals a histone deacetylase core complex and a connection with DNA methylation.* Genes Dev, 1999. **13**(15): p. 1924-35.
48. Loughlin, S.E., Leslie, F.M., Fallon, J.H., *Endogenous Opioid Systems*, in *The Rat Nervous System*, G. Paxinos, Editor. 1995, Academic Press: San Diego. p. 975-1001.
49. Akil, H., et al., *Endogenous opioids: overview and current issues.* Drug Alcohol Depend, 1998. **51**(1-2): p. 127-40.
50. Kieffer, B.L., *Opioids: first lessons from knockout mice.* Trends Pharmacol Sci, 1999. **20**(1): p. 19-26.
51. Silberstein, S.D. and D.C. McCrory, *Opioids.* Cephalalgia, 2000. **20**(10): p. 854-64; discussion 851-3.
52. McCarthy, L., et al., *Opioids, opioid receptors, and the immune response.* Drug Alcohol Depend, 2001. **62**(2): p. 111-23.
53. Angulo, J.A. and B.S. McEwen, *Molecular aspects of neuropeptide regulation and function in the corpus striatum and nucleus accumbens.* Brain Res Brain Res Rev, 1994. **19**(1): p. 1-28.
54. Hynd, G.W., et al., *Attention deficit-hyperactivity disorder and asymmetry of the caudate nucleus.* J Child Neurol, 1993. **8**(4): p. 339-47.
55. Modestin, J., B. Matutat, and O. Wurmle, *Antecedents of opioid dependence and personality disorder: attention-deficit/hyperactivity disorder and conduct disorder.* Eur Arch Psychiatry Clin Neurosci, 2001. **251**(1): p. 42-7.

56. Onishi, H., et al., *Developmental and regional alteration of methionine enkephalin-like immunoreactivity in seizure-susceptible E1 mouse brain.* Neurochem Res, 1990. **15**(1): p. 83-7.
57. Plotkin, S.R., et al., *Withdrawal from alcohol in withdrawal seizure-prone and -resistant mice: evidence for enkephalin resistance.* Pharmacol Biochem Behav, 2001. **68**(3): p. 379-87.
58. Lensing, P., et al., *Naltrexone open trial with a 5-year-old-boy. A social rebound reaction.* Acta Paedopsychiatr, 1992. **55**(3): p. 169-73.
59. Van Ree, J.M., et al., *Endogenous opioids and reward.* Eur J Pharmacol, 2000. **405**(1-3): p. 89-101.
60. Raab, A., B.R. Seizinger, and A. Herz, *Continuous social defeat induces an increase of endogenous opioids in discrete brain areas of the mongolian gerbil.* Peptides, 1985. **6**(3): p. 387-91.
61. Martin, L.J., et al., *Social deprivation of infant rhesus monkeys alters the chemoarchitecture of the brain: I. Subcortical regions.* J Neurosci, 1991. **11**(11): p. 3344-58.
62. Carden, S.E., et al., *Isolation alters striatal met-enkephalin immunoreactivity in rat pups.* Physiol Behav, 1996. **60**(1): p. 51-3.
63. Konig, M., et al., *Pain responses, anxiety and aggression in mice deficient in pre- proenkephalin.* Nature, 1996. **383**(6600): p. 535-8.
64. Ragnauth, A., et al., *Female preproenkephalin-knockout mice display altered emotional responses.* Proc Natl Acad Sci U S A, 2001. **98**(4): p. 1958-63.
65. Uhl, G.R., et al., *Synaptic regulation of the enkephalin gene and transcription factors in vivo: possible roles in drug abuse.* NIDA Res Monogr, 1991. **105**: p. 123-9.
66. Hurd, Y.L. and M. Herkenham, *Molecular alterations in the neostriatum of human cocaine addicts.* Synapse, 1993. **13**(4): p. 357-69.
67. Comings, D.E., et al., *The proenkephalin gene (PENK) and opioid dependence [In Process Citation].* Neuroreport, 1999. **10**(5): p. 1133-5 [MEDLINE record in process].
68. Loughlin, S.E., F.M. Leslie, and J.H. Fallon, *Endogenous Opioid Systems*, in *The Rat Nervous System*, G. Paxinos, Editor. 1995, Academic Press: San Diego. p. 975-1001.
69. Pert, C.B., M.J. Kuhar, and S.H. Snyder, *Autoradiograhic localization of the opiate receptor in rat brain.* Life Sci, 1975. **16**(12): p. 1849-53.
70. Dobi, A., et al., *Sample and probe: a novel approach for identifying development-specific cis-elements of the enkephalin gene.* Brain Res Mol Brain Res, 1997. **52**(1): p. 98-111.
71. Dobi, A., et al., *Septamer element-binding proteins in neuronal and glial differentiation.* J Neurosci, 2000. **20**(3): p. 1073-84.
72. Ring, M.A., Dobi, A., Georgopoulos, K., Agoston, D.v. *The role of the lymphoid transcription factor Ikaros in the developmental specification of the enkephalinergic neurons in the striatum.* in *Society for Neuroscience*. 1999. Miami Beach, FL.
73. Dobi, A. and D. Agoston, *Submillimolar levels of calcium regulates DNA structure at the dinucleotide repeat (TG/AC)n.* Proc Natl Acad Sci U S A, 1998. **95**(11): p. 5981-6.
74. Dobi, A.L., et al., *Protein-DNA interactions during phenotypic differentiation.* Mol Neurobiol, 1995. **10**(2-3): p. 185-203.

75. Agoston, D.V. and A. Dobi, *Complexity of transcriptional control in neuropeptide gene expression; enkephalin gene regulation during neurodevelopment.* Biochem Soc Trans, 2000. **28**(4): p. 446-51.
76. Hyman, S.E. and D. Borsook, *Mechanisms regulating proenkephalin gene expression: contributions of transgenic models.* NIDA Res Monogr, 1996. **161**: p. 59-71.
77. Comb, M., et al., *Mechanisms underlying synaptic regulation of proenkephalin transcription.* NIDA Res Monogr, 1991. **111**: p. 149-70.
78. Dobi, L., et al., *AUF1 is expressed in cell- and development-specific manner in the developing brain, binds to AT-rich dsDNA and regulates enkephalin gene expression*
Journal of Biological Chemistry, 2005. **submitted**.
79. Glazko, G.V., et al., *A significant fraction of conserved noncoding DNA in human and mouse consists of predicted matrix attachment regions.* Trends Genet, 2003. **19**(3): p. 119-24.
80. Boulikas, T., *Nature of DNA sequences at the attachment regions of genes to the nuclear matrix.* J Cell Biochem, 1993. **52**(1): p. 14-22.
81. Taylor, A., et al., *The anti-cancer agent distamycin A displaces essential transcription factors and selectively inhibits myogenic differentiation.* Mol Cell Biochem, 1997. **169**(1-2): p. 61-72.
82. Wemmer, D.E., *Designed sequence-specific minor groove ligands.* Annu Rev Biophys Biomol Struct, 2000. **29**: p. 439-61.
83. Reddy, B.S., S.K. Sharma, and J.W. Lown, *Recent developments in sequence selective minor groove DNA effectors.* Curr Med Chem, 2001. **8**(5): p. 475-508.
84. Agoston, M.S.A.G.C.P.A.D.D.V., *Isolation and characterization of SATB2, a novel AT-rich DNA binding*
protein expressed in development- and cell-specific manner in the rat brain. Neurochemical Research, 2006.
85. Kas, E., et al., *A model for chromatin opening: stimulation of topoisomerase II and restriction enzyme cleavage of chromatin by distamycin.* Embo J, 1993. **12**(1): p. 115-26.
86. Soldatenkov, V.A. and V.N. Potaman, *DNA-binding properties of poly(ADP-ribose) polymerase: a target for anticancer therapy.* Curr Drug Targets, 2004. **5**(4): p. 357-65.
87. Galande, S. and T. Kohwi-Shigematsu, *Caught in the act: binding of Ku and PARP to MARs reveals novel aspects of their functional interaction.* Crit Rev Eukaryot Gene Expr, 2000. **10**(1): p. 63-72.
88. Galande, S., *Chromatin (dis)organization and cancer: BUR-binding proteins as biomarkers for cancer.* Curr Cancer Drug Targets, 2002. **2**(2): p. 157-90.
89. Podgornaya, O.I., et al., *Structure-specific DNA-binding proteins as the foundation for three-dimensional chromatin organization.* Int Rev Cytol, 2003. **224**: p. 227-96.
90. Bomsztyk, K., O. Denisenko, and J. Ostrowski, *hnRNP K: one protein multiple processes.* Bioessays, 2004. **26**(6): p. 629-38.
91. Dickinson, L.A., et al., *A tissue-specific MAR/SAR DNA-binding protein with unusual binding site recognition.* Cell, 1992. **70**(4): p. 631-45.
92. Dickinson, L.A., C.D. Dickinson, and T. Kohwi-Shigematsu, *An atypical homeodomain in SATB1 promotes specific recognition of the key*

structural element in a matrix attachment region. J Biol Chem, 1997. **272**(17): p. 11463-70.
93. Nakagomi, K., et al., *A novel DNA-binding motif in the nuclear matrix attachment DNA-binding protein SATB1.* Mol Cell Biol, 1994. **14**(3): p. 1852-60.
94. Kumar, N.V. and L.R. Bernstein, *A new analytical scale DNA affinity binding assay for analyses of specific protein-DNA interactions.* Anal Biochem, 2001. **299**(2): p. 203-10.
95. Orlando, V., H. Strutt, and R. Paro, *Analysis of chromatin structure by in vivo formaldehyde cross-linking.* Methods, 1997. **11**(2): p. 205-14.
96. Weinmann, A.S., et al., *Use of chromatin immunoprecipitation to clone novel E2F target promoters.* Mol Cell Biol, 2001. **21**(20): p. 6820-32.
97. Bernstein, B.E., et al., *The use of chromatin immunoprecipitation assays in genome-wide analyses of histone modifications.* Methods Enzymol, 2004. **376**: p. 349-60.
98. Umlauf, D., Y. Goto, and R. Feil, *Site-specific analysis of histone methylation and acetylation.* Methods Mol Biol, 2004. **287**: p. 99-120.
99. Wilson, G.M. and G. Brewer, *Identification and characterization of proteins binding A + U-rich elements.* Methods, 1999. **17**(1): p. 74-83.
100. Tolnay, M., Y.T. Juang, and G.C. Tsokos, *Protein kinase A enhances, whereas glycogen synthase kinase-3 beta inhibits, the activity of the exon 2-encoded transactivator domain of heterogeneous nuclear ribonucleoprotein D in a hierarchical fashion.* Biochem J, 2002. **363**(Pt 1): p. 127-36.
101. Kamma, H., D.S. Portman, and G. Dreyfuss, *Cell type-specific expression of hnRNP proteins.* Exp Cell Res, 1995. **221**(1): p. 187-96.
102. Lu, J.Y. and R.J. Schneider, *Tissue distribution of AU-rich mRNA-binding proteins involved in regulation of mRNA decay.* J Biol Chem, 2004. **279**(13): p. 12974-9.
103. Boussif, O., M.A. Zanta, and J.P. Behr, *Optimized galenics improve in vitro gene transfer with cationic molecules up to 1000-fold.* Gene Ther, 1996. **3**(12): p. 1074-80.
104. Bayer, S.A. and J. Altman, *Neurogenesis and neuronal migration.* The rat nervous system, 2nd Edition, ed. G. Paxinos. 1995, San Diego: Academic Press. 1041-0198.
105. Sarkar, B., J.Y. Lu, and R.J. Schneider, *Nuclear import and export functions in the different isoforms of the AUF1/heterogeneous nuclear ribonucleoprotein protein family.* J Biol Chem, 2003. **278**(23): p. 20700-7.
106. Mattern, K.A., et al., *Spatial organization of four hnRNP proteins in relation to sites of transcription, to nuclear speckles, and to each other in interphase nuclei and nuclear matrices of HeLa cells.* Exp Cell Res, 1999. **246**(2): p. 461-70.
107. Xu, N., C.Y. Chen, and A.B. Shyu, *Versatile role for hnRNP D isoforms in the differential regulation of cytoplasmic mRNA turnover.* Mol Cell Biol, 2001. **21**(20): p. 6960-71.
108. Britanova, O., et al., *Novel transcription factor Satb2 interacts with matrix attachment region DNA elements in a tissue-specific manner and demonstrates cell-type-dependent expression in the developing mouse CNS.* Eur J Neurosci, 2005. **21**(3): p. 658-68.

109. Dickinson, L.A., C.D. Dickinson, and S.T. Kohwi, *An atypical homeodomain in SATB1 promotes specific recognition of the key structural element in a matrix attachment region.* J Biol Chem, 1997. **272**(17): p. 11463-70.
110. Kohwi-Shigematsu, T., K. Maass, and J. Bode, *A thymocyte factor SATB1 suppresses transcription of stably integrated matrix-attachment region-linked reporter genes.* Biochemistry, 1997. **36**(40): p. 12005-10.
111. Jossin, Y., *Neuronal migration and the role of reelin during early development of the cerebral cortex.* Mol Neurobiol, 2004. **30**(3): p. 225-51.
112. Uylings, H.B., *Development of the cerebral cortex in rodents and man.* Eur J Morphol, 2000. **38**(5): p. 309-12.
113. Agoston, D., et al., *Isolation and structural and genetic analysis of the mouse enkephalin gene and its d(AC/TG)n repeats.* DNA Seq, 1998. **9**(4): p. 217-26.
114. Boulikas, T., *Homeotic protein binding sites, origins of replication, and nuclear matrix anchorage sites share the ATTA and ATTTA motifs.* J Cell Biochem, 1992. **50**(2): p. 111-23.
115. Laemmli, U.K. and R. Tjian, *A nuclear traffic jam - unraveling multicomponent machines and compartments.* Curr Opin Cell Biol, 1996. **8**(3): p. 299-302.
116. Strick, R. and U.K. Laemmli, *SARs are cis DNA elements of chromosome dynamics: synthesis of a SAR repressor protein.* Cell, 1995. **83**(7): p. 1137-48.
117. Strick, T., et al., *Twisting and stretching single DNA molecules.* Prog Biophys Mol Biol, 2000. **74**(1-2): p. 115-40.
118. Dickinson, L.A. and S.T. Kohwi, *Nucleolin is a matrix attachment region DNA-binding protein that specifically recognizes a region with high base-unpairing potential.* Mol Cell Biol, 1995. **15**(1): p. 456-65.
119. Pauli, U., et al., *Specific interactions of histone H1 and a 45 kilodalton nuclear protein with a putative matrix attachment site in the distal promoter region of a cell cycle-regulated human histone gene.* J Cell Physiol, 1989. **139**(2): p. 320-8.
120. Reeves, R., *Structure and function of the HMGI(Y) family of architectural transcription factors.* Environ Health Perspect, 2000. **108**(Suppl 5): p. 803-9.
121. Wilson, G.M. and G. Brewer, *The search for trans-acting factors controlling messenger RNA decay.* Prog Nucleic Acid Res Mol Biol, 1999. **62**: p. 257-91.
122. Brewer, G., *Messenger RNA decay during aging and development.* Ageing Res Rev, 2002. **1**(4): p. 607-25.
123. Mitchell, P. and D. Tollervey, *mRNA stability in eukaryotes.* Curr Opin Genet Dev, 2000. **10**(2): p. 193-8.
124. Zhang, W., et al., *Purification, characterization, and cDNA cloning of an AU-rich element RNA-binding protein, AUF1.* Mol Cell Biol, 1993. **13**(12): p. 7652-65.
125. Kajita, Y., et al., *The UUAG-specific RNA binding protein, heterogeneous nuclear ribonucleoprotein D0. Common modular structure and binding properties of the 2xRBD-Gly family.* J Biol Chem, 1995. **270**(38): p. 22167-75.

126. Katahira, M., et al., *Structure of the C-terminal RNA-binding domain of hnRNP D0 (AUF1), its interactions with RNA and DNA, and change in backbone dynamics upon complex formation with DNA.* J Mol Biol, 2001. **311**(5): p. 973-88.
127. Wagner, B.J., et al., *Structure and genomic organization of the human AUF1 gene: alternative pre-mRNA splicing generates four protein isoforms.* Genomics, 1998. **48**(2): p. 195-202.
128. Del Gatto-Konczak, F., et al., *hnRNP A1 recruited to an exon in vivo can function as an exon splicing silencer.* Mol Cell Biol, 1999. **19**(1): p. 251-60.
129. Singh, O.P., *Functional diversity of hnRNP proteins.* Indian J Biochem Biophys, 2001. **38**(3): p. 129-34.
130. Tolnay, M., L.A. Vereshchagina, and G.C. Tsokos, *Heterogeneous nuclear ribonucleoprotein D0B is a sequence-specific DNA-binding protein.* Biochem J, 1999. **338 (Pt 2)**: p. 417-25.
131. Dempsey, L.A., L.A. Hanakahi, and N. Maizels, *A specific isoform of hnRNP D interacts with DNA in the LR1 heterodimer: canonical RNA binding motifs in a sequence-specific duplex DNA binding protein.* J Biol Chem, 1998. **273**(44): p. 29224-9.
132. Arao, Y., et al., *A nuclear matrix-associated factor, SAF-B, interacts with specific isoforms of AUF1/hnRNP D.* Arch Biochem Biophys, 2000. **380**(2): p. 228-36.
133. FitzPatrick, D.R., et al., *Identification of SATB2 as the cleft palate gene on 2q32-q33.* Hum Mol Genet, 2003. **12**(19): p. 2491-501.
134. Wilsker, D., et al., *ARID proteins: a diverse family of DNA binding proteins implicated in the control of cell growth, differentiation, and development.* Cell Growth Differ, 2002. **13**(3): p. 95-106.
135. Yamaguchi, H., M. Tateno, and K. Yamasaki, *Solution Structure and DNA-binding Mode of the Matrix Attachment Region-binding Domain of the Transcription Factor SATB1 That Regulates the T-cell Maturation.* J Biol Chem, 2006. **281**(8): p. 5319-27.
136. Seo, J., M.M. Lozano, and J.P. Dudley, *Nuclear matrix binding regulates SATB1-mediated transcriptional repression.* J Biol Chem, 2005. **280**(26): p. 24600-9.
137. Hsieh, J. and F.H. Gage, *Epigenetic control of neural stem cell fate.* Curr Opin Genet Dev, 2004. **14**(5): p. 461-9.
138. Yasui, D., et al., *SATB1 targets chromatin remodelling to regulate genes over long distances.* Nature, 2002. **419**(6907): p. 641-5.
139. Kantor, B., et al., *Expression and localization of components of the histone deacetylases multiprotein repressory complexes in the mouse preimplantation embryo.* Gene Expr Patterns, 2003. **3**(6): p. 697-702.
140. Tou, L., Q. Liu, and R.A. Shivdasani, *Regulation of mammalian epithelial differentiation and intestine development by class I histone deacetylases.* Mol Cell Biol, 2004. **24**(8): p. 3132-9.
141. Mal, A., et al., *A role for histone deacetylase HDAC1 in modulating the transcriptional activity of MyoD: inhibition of the myogenic program.* Embo J, 2001. **20**(7): p. 1739-53.
142. Kumar, P.P., et al., *Displacement of SATB1-bound histone deacetylase 1 corepressor by the human immunodeficiency virus type 1*

transactivator induces expression of interleukin-2 and its receptor in T cells. Mol Cell Biol, 2005. **25**(5): p. 1620-33.

143. Gyorgy, A. et al., *The special AT-rich DNA binding protein SATB2 is co-expressed with chromatin remodellers in a subset of developing neocortical neurons and regulates gene expression through altered histone acetylation* -In preparation
J. Neurochemistry, 2006.