# “FEDERICO II” UNIVERSITY OF NAPLES

*PhD PROGRAM IN NEUROSCIENCE*
*XXXI CYCLE*

**PhD Thesis:**

# “Identification of Epigenetic Mechanisms Regulating NCX3 in *in Vivo* Model of Brain Ischemic Preconditioning”


***Coordinator***

**Prof. Maurizio Taglialatela**

**Tutor**

**Dr. LUIGI FORMISANO**

**Candidate**

**Dr. LUIGI MASCOLO**


***Academic Year 2017 /2018***

# INDEX

**Summary**

**Introduction**

I.A. ISCHEMIC PRECONDITIONING IN BRAIN ........ 6
I.A.1. Mechanisms of Brain Ischemic Preconditioning ........ 7
*I.A.2.* Clinical Applications and Future Perspectives ........ 9
I.B. EPIGENETIC ........ 9
I.B.1. Nucleosome ........ 11
I.B.2. Epigenetic Modification ........ 13
I.B.3. Histone Lysine Methylation and Demethylation ........ 16
I.B.4. Histone Lysine Methyl Transferase (KMT) Families ........ 19
I.B.5. Histone Lysine Demethylases (KDM) Families ........ 22
I.B.6. Drugs Regulating Lysine Methylation ........ 24
I.B.7. The Role Of Kmts in Models of Neurological Disease ........ 24
*I.B.7.1.Huntington's disease and Friedreich's Ataxia* ........ 25
*I.B.7.2.Stroke inVivo and Vitro* ........ 26
*I.B.7.3 Weidemann Steiner Syndrome* ........ 26
*I.B.7.4.Kabuki Syndrome* ........ 27
*I.B.7.5.Schizophrenia* ........ 27
I.C. SODIUM CALCIUM EXCHANGER ........ 28
I.C.1. State of Art of $Na^+$\$Ca^{2+}$ Exchanger (NCX) ........ 29
I.C.2. Molecular Biology of NCX Isoforms ........ 32
I.C.3. Distribution of NCX Isoforms ........ 33
I.C.4. Regulation of NCX Isoforms ........ 35
I.D. NCX3 $Na^+/Ca^{2+}$ EXCHANGER 3 ........ 37
I.D.1. Role and Mechanism of NCX3 in Brain Ischemic Preconditioning ........ 39
I.D.2. Transcriptional Regulation of ncx3 Gene in the Brain ........ 41
*I.D.2.1. ncx3 brain promoter region and its putative transcription factor in binding sites* ........ 44
*I.D.2.2. ncx3 Is Transcriptionally Up-regulated by CREB* ........ 45
*I.D.2.3. ncx3 Is Transcriptionally Down-regulated by DREAM* ........ 47
I.E. GATA TRANSCRIPTION FACTORS ........ 47
I.E.1. The GATA Transcription Factor Family ........ 48
I.E.2. Structural Characteristics and Binding Site Specificity of the GATA Factors ........ 50

I.E.3. Physiological Function and Tissue Specificity of GATA Protein .............................. **55**
I.E.4. Specificity in the Regulation of Transcription by GATA Factor .............................. **55**
*I.E.4.1. Post-translational Modification* .............................................................. **57**

## Aim of the Thesis

II. AIM OF THE STUDY ........................................................................................ **59**

## Materials and Methods

III.1. Materials ........................................................................................................ **61**
III.2. Primary Cortical Neurons ............................................................................... **61**
III.3. Transfection with Expression Plasmids or Small Interfering RNA (siRNA) and Luciferase Reporter Assay in Cortical Neurons ................................................................ **62**
III.4. Quantitative Real-Time PCR (qRT-PCR) Analysis ......................................... **62**
III.5. Western Blotting ............................................................................................. **63**
III.6. Chromatin Immunoprecipitation (ChIP) .......................................................... **64**
III.7. *In Vivo* Studies ................................................................................................ **65**
III.7.1. Experimental groups ................................................................................... **65**
III.7.2. Transient Focal Ischemia and Ischemic Preconditioning ................................ **65**

III.8. Statistical Analysis .......................................................................................... **65**

## Results

IV.1. GATA3 binds and activates ncx3 by a specific GATA sequence ............................ **71**

IV.2.GATA3,KMT2A,NCX3 increase in the temporoparietal cortex after preconditioning+ischemia ........................................................................................ **73**

IV.3.GATA3,KMT2A and NCX3 increase in the temporoparietal cortex after preconditioning and preconditioning+ischemia but not after ischemia ..................................................... **76**

## Discussion

V. Discussion ......................................................................................................... **79**

## Conclusion

VI. Conclusion ........................................................................................................ **80**

## References

VII. References ...................................................................................................... **86**

## SUMMARY

Variations of the isoform 3 expression of the sodium / calcium exchanger play an important role in the response to neuronal damage after an ischemic insult. I found that the transcription factor (GATA binding protein) 3 GATA3 activates the transcription of ncx3 in rat cortical cultures. In fact, the overexpression of GATA3, obtained as a result of transient transfection of the plasmid containing the GATA3 cDNA in neurons, leads to a significant increase in the luciferase activity of the ncx3 promoter, in parallel with an increase in mRNA and protein expression of ncx3. In contrast, the transfection of a siRNA capable of reducing the protein expression of GATA3 by about 60% causes a reduction of the luciferase activity of the ncx3 promoter and a decrease in the ncx3 mRNA. The site-specific mutagenesis of the binding sequence of GATA3 on the ncx3 promoter demonstrates that the mechanism by which GATA3 activates NCX3 is site-specific. More important, *in vivo*, GATA3 recruitment to the ncx3 gene was increased in the temporoparietal cortex of rats subjected to Preconditioning (PC) followed by transient middle cerebral artery occlusion (tMCAO), with an increase of histone 3 lysine 3 trimethylation of the ncx3 promoter region. Interestingly, in the same experimental conditions histone acetylation on ncx3 promoter was unmodified. Furthermore, Re-ChIP experiments demonstrated that GATA3 forms a functional complex with the histone lysine methyl transferase KMT2A on the ncx3 gene during PC+tMCAO. Therefore, increasing KMT2A expression or activity might represent a new possible strategy in stroke intervention.

# I. INTRODUCTION

## I.A. ISCHEMIC PRECONDITIONING IN BRAIN

Brain ischemia is one of the most common causes of death and the leading cause of adult disability in the world. Brain ischemic preconditioning (BIP) refers to a transient, sublethal ischemia which results in tolerance to later, otherwise lethal, cerebral ischemia. neuroprotective mechanisms may involve a series of molecular regulatory pathways. Generally speaking, any stimulus capable of causing injury to a tissue or organ can, when applied close to (but below) the threshold of damage, activate endogenous protective mechanisms and thus potentially lessen the impact of a subsequent, more severe attack. a phenomenon known as ischemic preconditioning (IP) or ischemic tolerance (IT)(Liu et al. 2009). The terms 'tolerance' and 'preconditioning' were introduced for the first time by Janoff in 1964 (JANOFF 1964) ischemic preconditioning is an adaptive reaction to a potentially noxious stimulus, such as ischemia, hypoxia, hypoglycemia, or inflammation(Liu, Sheng et al. 2009) .The phenomenon of ischemic preconditioning has been observed in numerous organs such as brain (Kitagawa et al. 1991) and heart (Meldrum et al. 1997), as well as in a wide range of species like the gerbil (Kirino et al. 1991), the rat and the mouse. Various brain regions, hippocampus, cerebral cortex, basal ganglia and thalamus were often reported to acquire ischemic tolerance (Kitagawa, Matsumoto et al. 1991) The brain is one of the most sensitive organs to injury. A constant flow of blood to the brain is essential for delivering oxygen and glucose to neurons. If this flow is disrupted for even a short period of time, the result is cell damage or death. Neurons are rarely replaced once they have died, so the damage to affected regions may be permanent (Caplan 2000). there are two threshold values for cerebral blood flow. Reduction in blood flow below the first threshold results in electrical failure within neurons, and further reduction in blood flow below the second threshold leads to the failure of metabolism and ion pumps. Cells with lower perfusion than the second threshold are designated to die. Between the two thresholds, cells are electrically silent but maintain a low level of metabolic activity and can be stable for hours. If normal blood flow is restored within a reasonable amount of time, they may recover with no apparent damage. Longer periods of ischemia, however, will result in their death. So these cells have a variable fate, and constitute what is known as the ischemic penumbra.(Dirnagl et al. 1999) It is the cells within the penumbra that

receive the most benefit from ischemic preconditioning endogenous neuroprotective mechanisms and potentially, a window of opportunity to utilize these mechanisms in the clinic to treat patients with stroke and other CNS disorders.(Liu, Sheng et al. 2009)

### I.A.1. Mechanisms of Brain Ischemic Preconditioning

Two temporally distinct types of ischemic tolerance are afforded by sublethal pretreatment: early and delayed tolerance (Bhuiyan and Kim 2010),The first phase, named rapid or acute preconditioning ,starts 3–5min after the preconditioning Stimulus and ends 1h later and is due to rapid post-translational Protein modifications .The second phase, named delayed preconditioning ,starts2–3days after preconditioning And ends 1week later and mainly involves de novo protein synthesis (Cuomo et al. 2015).To induce tolerance by means of ischemic episodes, three factors should be considered: 1) the duration, 2) the time interval, and 3) the number of episodes. First, the preconditioning stimuli must be severe enough to initiate a response, but not so severe as to cause permanent damage. Second, some interval of time must exist between sublethal and lethal stress. Third, the number of short ischemic episodes should be considered for sufficient stimulation of the protective response against a lethal ischemic insult. In general, the process of tolerance induction can be divided into the following elements: sensors of the stress signal, transducers of the stimulus, and effectors of the tolerance. First, the preconditioning stimulus must be recognized by cellular sensors so that the cells can be prepared for upcoming stress. Neurotransmitter and cytokine receptors, ion channels and redox-sensitive enzymes generally work as molecular sensor of stress stimuli. These sensors activate enzymes, such as kinase protein Ras, Raf, mitogen-activated protein kinase (MAPK) kinase (MEK), extracellular regulated kinase (ERK), Akt, and protein kinase C, and signaling molecules, such as nitric oxide (NO), diacylglycerol, inositol triphosphate, $Ca^{2+}$, and ceramide, which transduce the signal and initiate an adaptive response. Finally, effectors of the preconditioning response confer tolerance to cells or tissues through anti-excitotoxicity, anti-apoptosis, anti-inflammation, protection of mitochondria and increased anti-oxidant mechanisms (Bhuiyan and Kim 2010). Ischemic tolerance thus reflects a fundamental change in the cellular response to injury that shifts the outcome from cell death to cell survival

(Dirnagl et al. 2003). Although transcription factors, such as HIF-1, CREB and NF-κB are already known to be driving neuroprotective gene expression upon an ischemic preconditioning stimulus, we are today more aware that apart from the transcription factors and DNA sequence, regulation of such transcriptional activity requires the cooperation of a third party, namely epigenetic alterations of the DNA and histones. Indeed, these modifications crucially regulate the accessibility of specific regulatory DNA elements for transcription machinery yet involvement of these mechanisms in brain ischemic preconditioning and neuroprotection is mostly unknown.

### I.A.2. Clinical Applications and Future Prospective

The preconditioning phenomenon has been successful as an experimental procedure for identifying the mechanisms responsible for brain protection and regeneration. Important examples of strategies to modulate these mechanisms include erythropoietin, activators of mitochondrial KATP channels, and volatile anesthetics. The phenomenon of ischemic tolerance has not only been found in cells, organs and animal experimental models; some clinical observational data indicate that this phenomenon may occur naturally in the human brain in the form of short episodes of ischemia without infarction, known as TIA. In a retrospective clinical study, evaluated 148 stroke patients with and without antecedent TIA and found that TIA before stroke is associated with significantly less severe stroke on admission and improved outcomes on follow-up. Another retrospective study, which included more than 2000 patients, confirmed these results 1 year later . In support of these findings, studies using magnetic resonance imaging and neuroradiological analysis showed that ischemic stroke patients with prodromal TIA have significantly smaller ischemic lesions after stroke than those patients without TIA . These observations suggest that endogenous preconditioning triggered by TIA is present in the human brain. Induction of ischemic tolerance in the brain has been suggested to be a promising clinical strategy for preparing the brain for situations of possible ischemia, such as cardiac or brain surgery and in patients with a high risk of stroke. However, because of ethical and safety concerns associated with ischemic preconditioning, researchers are trying to identify a safer preconditioning stimulus that would be both practical and effective or a biological agent that can mimic preconditioning pharmacologically. Many candidate pharmacological regulators of the stress response and inducers of

ischemic tolerance have been proposed, one of which is erythropoietin. Erythropoietin is approved for the treatment of anemia and seems safe and effective for critically ill patients who are anemic and have experienced trauma. The iron chelator desferrioxamine is clinically approved for various indications, including thalassemia and other iron-overload syndromes. Various inhalational anesthetics used in human beings (e.g., sevoflurane) induce tolerance against brain ischemia and act as brain protectants after ischemia in preclinical experiments. These compounds are safe and effective at eliciting early preconditioning in patients undergoing coronary artery bypass graft surgery in randomized controlled trials. These drugs also elicit delayed preconditioning in human beings. Another promising approach is remote preconditioning in which preconditioning of one organ or system leads to protection of a different (remote) organ. The prototypical approach for remote preconditioning is the initiation of short ischemic insult(s) to a limb to protect organs such as the heart and the brain. Remote preconditioning might indicate a crosstalk between the brain and the rest of the body in response to stress through the peripheral nervous system or paracrine signal. Randomized clinical trials have already shown the efficacy of this strategy for the heart. Remote preconditioning is a particularly attractive strategy for protecting organs that are highly susceptible to damage but that are difficult to target, such as the brain. Although many researchers are actively characterizing the signaling mechanisms of ischemic preconditioning in the nervous system, our knowledge of cerebral ischemic tolerance is still in its infancy and insufficient to be able to translate the laboratory results into application. Many issues need to be resolved to avoid disappointing results from the clinical application of ischemic preconditioning, e.g., whether the tolerant state can be maintained long term and provide chronic neuroprotection. Thus far, it appears that ischemic tolerance persists for approximately 1 week in the brain. The threshold for tolerance induction and for cell injury needs to be determined. Pharmacological substances used for tolerance induction need to be safe. In conclusion, the past two decades have provided interesting insights into the mechanisms and potential applications of ischemic tolerance in the brain. Current knowledge suggests that the preconditioning strategy and related interventions, such as remote preconditioning and pharmacological preconditioning, can protect neurons and improve neuronal survival after critical ischemia, and, thus, have promise for practical application in cases of vascular neurosurgery and endo-vascular therapy and possibly in the management

of brain trauma. As knowledge in this field advances, the unresolved issues concerning the preconditioning cascade will likely be resolved and will lead to pharmacological strategies for protecting the brain from ischemic injury, traumatic brain injury, and other neurodegenerative disorders.(Bhuiyan and Kim 2010).

## I.B. EPIGENETICS

Conrad Waddington introduced the term epigenetics in the early 1940s.He defined epigenetics as the branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being. In the original sense of this definition, epigenetics referred to all molecular pathways modulating the expression of a genotype into a particular phenotype. Epigenetics is generally accepted as ‘‘the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence. "In particular the epigenetic modifications described in current literature generally comprise histone variants, posttranslational modifications of amino acids on the amino-terminal tail of histones, and covalent modifications of DNA bases.(Dupont et al. 2009)

### I.B.1. Nucleosome

Chromatin is the complex of DNA wrapped around histonic proteins found in the eukaryotic nuclei. The functional unit of chromatin is the nucleosome. Nucleosomes elicit an initial ~7-fold linear compaction of genomic DNA. They provide a critical mechanism for stable repression of genes and other DNA-dependent activities by restricting binding of trans-acting factors to cognate DNA sequences. They are engineered to be nearly meta-stable and disassembled (and reassembled) in a facile manner to allow rapid access to the underlying DNA during processes such as transcription, replication and DNA repair. Nucleosomes protect the genome from DNA damaging agents and provide a lattice onto which a myriad of epigenetic signals are deposited. Moreover, vast strings of nucleosomes provide a framework for assembly of the chromatin fiber and higher-order chromatin structures. **(Figure 1-A).** Nucleosomes constitute the basic repeating subunit of chromatin. Each nucleosome can be considered as composed of a nucleosome ‘core’, linker DNA, and in most instances, a linker histone. The structure of the nucleosome core

includes a 147 bp segment of DNA and two copies each of four core histone proteins (H2A, H2B, H3 and H4).The core histones assemble into a spool-like structure onto which the core DNA is wrapped, in about 1¾ left-handed superhelical turns, forming a squat disc-like structure about 5.5 nm in height and 11 nm in diameter .The core DNA is in tight association with the core histones and is protected from nuclease digestion whereas the linker DNA is rapidly digested.(Cutter and Hayes 2015). Histones, enriched in basic amino acids, are proteins which structure is made of a globular domain and an N- terminal tail protruding from the nucleosome. Although the histones are classified among the most evolutionarily conserved proteins, they represent the most variable in terms of posttranslational modifications.The N-terminal tails of histones are usually targets for various covalent posttranslational modifications, including acetylation, phosphorylation,methylation, sumoylation and ubiquitination **(Figure 1).** The specific combinations in posttranslational modifications generate a sort of “histone code”. The role of these modifications is found in their particular combinatorial pattern since they decode for a selective chromatin affinity to the associated proteins, which determine whether the chromatin is active (relaxed state) or silent (condensed state) (Thiagalingam et al. 2003).Thus, histone code influences the structure and pattern of chromatin condensation and consequently it has been found involved in the gene regulation (Jenuwein and Allis 2001).

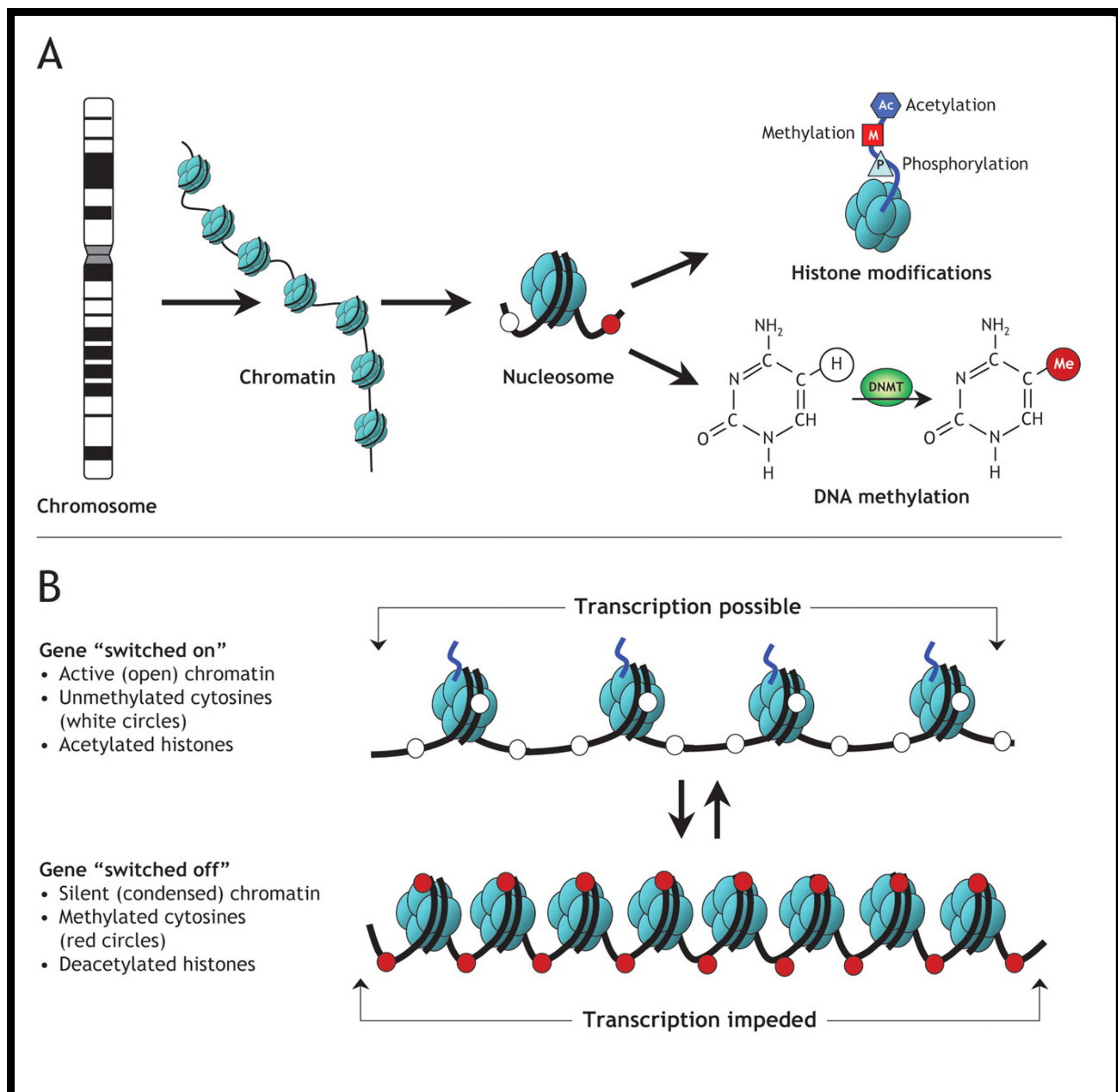


**Figure 1.** Chromatine organization into nucleosomes (A) and epigenetic modifications (B).

### I.B.2. Epigenetic Modifications

Two types of chromatin modification that regulate transcription of the protein-encoding genome are listed in **(Figure2).** These modifications have been broadly classified into repressing and activating — in other words, they correlate with, and perhaps directly regulate, gene repression and induction. DNA methylation occurs at cytosine residues usually within CG dinucleotides or CNG trinucleotides, and generally opposes transcription. DNA hypermethylation is a feature of certain human cancers, causing aberrant repression of tumour suppressor genes through methylation of the CpG islands in promoters. There are various histone PTMs,

including acetylation, phosphorylation, methylation, ubiquitylation and SUMOylation. These modifications decorate the canonical histones (H2A, H2B, H3 and H4), as well as variant histones (such as H3.1, H3.3 and HTZ.1). Most modifications localize to the amino- and carboxy-terminal histone tails, and a few localize to the histone globular domains. Lysine is a key substrate residue in histone biochemistry, because it undergoes many exclusive modifications, including acetylation, methylation, ubiquitylation and SUMOylation. Acetylation and methylation involve small chemical groups, whereas ubiquitylation and SUMOylation add large moieties, owing to their bulk, may lead to more profound changes in chromatin structure. Another degree of complexity is that methylation can occur several times (mono-, di- or trimethylation) on one lysine side chain, and each level of modification can have different biological outcomes. For example, there is abundant evidence that acetylation is activating, whereas SUMOylation seems to be repressing, and these two types of modification may mutually interfere. By contrast, methylation and ubiquitylation have variable effects, depending on the precise residues and contexts. For example, trimethylation of lysine 4 in histone H3 (H3K4me3) occurs at the 5´ ends of ORFs as genes become induced, whereas H3K9me3 occurs in compact pericentromeric heterochromatin, which is transcriptionally inert. In H3 and H4, arginine residues can also be mono- or dimethylated, and in the latter case the methyl groups can be placed symmetrically or asymmetrically on the side chain. Arginine methylation seems to be strictly activating to transcription Serine/threonine phosphorylation is also involved in transcription. All histone PTMs are removable. Histone deacetylases (HDACs) remove acetyl groups and Ser/Thr phosphatases remove phosphate groups. Ubiquitin proteases remove mono-ubiquitin from H2B. Arginine methylation is altered by deiminases, which convert the side chain to citrulline. Two classes of lysine demethylase have recently been identified: the LSD1/BHC110 class (which removes H3K4me1 and me2) and the jumonji class (which removes H3K4me2 and me3, H3K9me2 and me3, and H3K36me2 and me3). This finding put an end to heated debate about the reversibility of histone lysine methylation and whether it is the only ‘true’ epigenetic histone PTM. As mentioned above, the functional consequences of histone PTMs can be direct, causing structural changes to chromatin, or indirect, acting through the recruitment of effector proteins. In addition to the idea that histone PTMs directly alter nucleosome fibre structure is an expanding body of evidence that histone PTMs serve as binding surfaces for the

association of effector proteins. The initial finding was for acetyl lysine, which has been shown to associate with bromodomains. In this case, acetylated H3 stabilizes binding of the histone acetyltransferase GCN5 through its bromodomain14. Lysine methylation provides an important switch for binding of representatives of the 'royal family' of domains, including chromodomains and tudordomains. (Berger 2007)

**Table 1 | Chromatin modifications**

| Mark* | Transcriptionally relevant sites† | Transcriptional role‡ |
|---|---|---|
| **DNA methylation** | | |
| Methylated cytosine (meC) | CpG islands | Repression |
| **Histone PTMs** | | |
| Acetylated lysine (Kac) | H3 (9, 14, 18, 56), H4 (5, 8, 13, 16), H2A, H2B | Activation |
| Phosphorylated serine/threonine (S/Tph) | H3 (3, 10, 28), H2A, H2B | Activation |
| Methylated arginine (Rme) | H3 (17, 23), H4 (3) | Activation |
| Methylated lysine (Kme) | H3 (4, 36, 79)<br>H3 (9, 27), H4 (20) | Activation<br>Repression |
| Ubiquitylated lysine (Kub) | H2B (123§/120¶)<br>H2A (119¶) | Activation<br>Repression |
| Sumoylated lysine (Ksu) | H2B (6/7), H2A (126) | Repression |
| Isomerized proline (Pisom) | H3 (30–38) | Activation/repression |

*The modification on either DNA or a histone.
†Well-characterized sites with regard to genomic location for DNA methylation or residues within histones for PTMs.
‡Whether the epigenetic mark is associated with activation or repression.
§Yeast (*Saccharomyces cerevisiae*).
¶Mammals.

**Figure2** Chromatin modifications art the complex language of chromatin

### I.B.3. Histone Lisine Methilation and Histone Lisine Demethylation

The enzymes involved in lysine methylation were first found to target histone and thus were initially named histone methyltransferases and histone demethylases, following the naming model for histone acetyltransferase and histone deacetylase. With accumulating evidence that these modifications are not histone specific, a new nomenclature has been advocated for more generic names for these enzymes . The enzymes that add or remove the methylation mark on lysine residues are now named

lysine methyltransferases (KMTs) and lysine demethylases (KDMs).(Zhang et al. 2012). The SET domain is a 130 amino acid catalytic domain initially found to be conserved in Su(var)3-9, E(z) (enhancer of zeste) and trithorax. SET domain-containing enzymes are currently the larger of the two classes of KMTs. The second class of KMTs is represented solely by KMT4 (also known as Dot1p in yeast and Dot1L in human), which does not have a SET domain. Even though the catalytic domains of these two enzyme classes are distinct, both use S-adenosyl-Lmethionine (SAM) as the methyl group donor **(Figure 3B).** KMTs observe a high degree of enzymatic specificity for the lysine within the substrate and for the degree of methylation **(Figure 3C).** Therefore, KMTs can be highly specific, but their interacting partners can alter their target lysine or degree of activity. the first histone KDM, LSD1/KDM1A (Figure 3A and Table 1), as part of the C-terminal binding protein 1 (CtBP1) corepressor complex. KDM1A was found to be associated with other similar corepressor complexes, suggesting that this protein was a candidate repressor. KDM1A contains a flavin adenine dinucleotide (FAD)-dependent amine oxidase domain that demethylates H3K4me2 and H3K4me1 and modulates gene expression **(Figures 3B and 3C).** Subsequent to the discovery of KDM1A, an additional class of KDMs was discovered. This enzyme class utilizes the JmjC domain **(Figures 3A and 3C)** to catalyze demethylation through the oxidation of methyl groups. JmjC proteins rely on α- ketoglutarate, molecular oxygen, and Fe(II) as cofactors for demethylation **(Figure 3B).** In some cases, the JmjN domain is observed with the JmjC domain and is essential for enzymatic activity. The KDM2 and KDM3 families were unable to demethylate trimethylated lysines; however, this lack of activity was remedied by the discovery of the first tri-demethylase family, KDM4AKDM4D (also known as JMJD2A-JMJD2D) **(Figure3A).** KDM4A-KDM4D remove H3K9me3/H3K9me2, H3K36me3/H3K36me2, and H1.4K26me3/H1.4K26me2, but are unable to remove H3K9me1 or H3K36me1, emphasizing the specificity for both the site and degree of methylation. Subsequent to these discoveries, a multitude of groups proceeded to identify additional amine oxidase- and JmjC-containing KDMs **(Figure 3C).** However, no enzyme that is capable of demethylating H4K20me3 or H3K79me1/ H3K79me2/H3K79me3 has been discovered. Recently, LOXL2 has been demonstrated to remove methyl groups by deaminating lysine. Therefore, we hypothesize that LOXL2 or other LOX family members may catalyze demethylation of H4K20me3 or H3K79, which is an exciting area for future research (Black et al.

2012). Unlike acetylation, where positive charges on histones are removed, relaxing chromatin and activating genes, methylation or its removal does not affect charges on histones. Histone lysine residues can be monomethylated, dimethylated, or trimethylated (Bannister and Kouzarides 2004), while arginine residues can be monomethylated or dimethylated symmetrically or asymmetrically(Bedford and Richard 2005).The methylation of lysines 4, 36, or 79 of histone H3 is typically associated with active transcription, while the methylation of lysines 9 or 27 on histone H3 and lysine 20 on histone H4 contributes to repressed transcription.

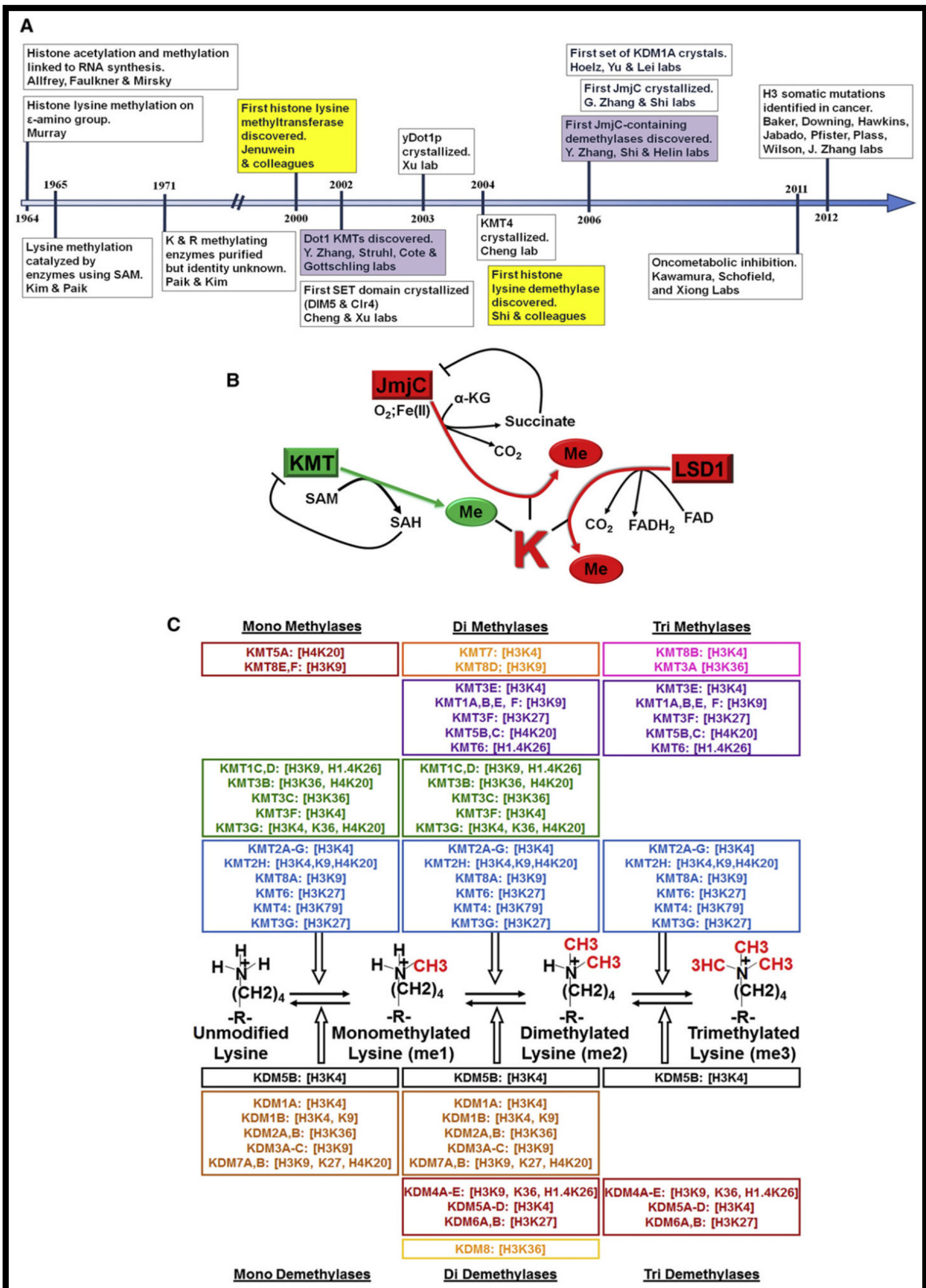


**Figure 3.** History, Mechanism, and Specificity of KMTs and KDMs (Black, Van Rechem et al. 2012)

### I.B.4. Histone Lisine Methilation Family (KMT)

KMTs catalyze mono-, di-, or tri-methylation by transferring one, two, or three methyl groups, respectively, from S-adenosyl-L-methionine to the 1-amino group of a lysine residue. Except for KMT4/DOT1L, all known KMTs contain a conserved SET (Su(var)3-9, Enhancer of Zeste, Trithorax) domain harboring the enzymatic activity. Besides the SET domain, most KMTs also contain some other defined protein domain or homologous sequence that is used to classify KMTs into distinct subfamilies. The enzymatic activities and substrate specificities of the eight KMT subfamilies are summarized here **(Figure 4).** Most **KMT1** group proteins are members of the SUV39 family that specifically methylate H3K9. This family includes KMT1A/SUV39H1, KMT1B/SUV39H2, KMT1C/G9a, KMT1D/EHMT1/GLP, KMT1E/SETDB1, and KMT1F/SETDB2. They are the dominant enzymes generating H3K9 trimethylation (H3K9me3) at pericentric heterochromatin, a highly compacted, transcriptionally silent chromatin domainThe KMT2 family includes Drosophila Trithorax homologs MLL family proteins (MLL1–MLL5, named KMT2A–KMT2E, respectively). All **KMT2** family proteins are H3K4 methyltransferases, and they share a distinct SET domain with an essential post-SET region at the C-terminus. KMT2A/MLL1 is a major regulator of hematopoiesis and embryonic development through regulation of HOX gene expression, and the MLL gene is frequently rearranged in human acute leukemias. The **KMT3** family includes yeast Set2 homolog SETD2/KMT3A, nuclear receptor binding SET domain protein 1, NSD1/KMT3B, and SET and MYND domain-containing proteins SMYD2/KMT3C, SMYD1/KMT3D, and SMYD3/KMT3E. This group of proteins methylates mainly histone H3K36, but their KMT activities are not restricted to H3K36. KMT3A is a Huntingtin interacting protein, and it is responsible for global transcription-dependent H3K36 trimethylation, a specific epigenetic mark for transcriptional activation enriched at the gene body. KMT3B is involved also shown to methylate H4K20. A recurrent translocation of KMT3B fused to nucleoporin-98 has been reported in childhood acute myeloid leukemia. **KMT4/DOT1L** is a H3K79-specific methyltransferase and represents the only class of KMT without a SET domain. KMT4-mediated H3K79 di- and tri-methylation are evolutionarily conserved from yeast to human and ubiquitously correlated with active transcription. It is the sole

enzyme responsible for H3K79 methylation.In mammals, KMT4 is an essential gene for embryogenesis, hematopoiesis, and cardiac function. Its H3K79 methylation activity is essential for MLL-AF4 and MLL-AF9 fusion-induced leukemias, suggesting that KMT4 could serve as a potential therapeutic target for MLL-rearranged leukemias. The **KMT5** family consists of enzymes that methylate H4K20. KMT5A/SET8 specifically mono-methylates H4K20, which has been shown to associate with both gene repression and activation, depending on its chromatin context. KMT5A and H4K20me1 are also essential for multiple other chromatin-associated processes, such as cell cycle progression, DNA replication, and DNA damage response. KMT5B/SUV420H1 and its homolog KMT5C/ SUV420H2 catalyze di- and tri-methylation of H4K20, which, along with H3K9me2/3, is essential for maintenance of repressive heterochromatin at pericentric and telomeric regions. **KMT6A/EZH2**, and **KMT6B/ EZH1** belong to the KMT6 family. The methylation activity of KMT6A and KMT6B requires other core components of their associated protein complex, polycomb repressive complex 2 (PRC2). As the catalytic subunit of PRC2, KMT6A participates in maintaining the transcriptional repressive state of chromatin and is upregulated in a broad range of human cancers. This family contains only one protein, SET7/9, which monomethylates histone at H3K4. **KMT7** was found to methylate, besides histone, a number of non-histone proteins, including p53, DNA methyltransferase 1 (DNMT1), estrogen receptor alpha (ERa), nuclear factor kappaB (NFkB), and components of the TATA binding protein (TBP) complex, TBP-associated factors TAF10 and TAF7. The consensus recognition sequence in substrates for SET7-mediated lysine methylation, K/R–S/T–K ,is also recognized by the H3K4 demethylase KDM1A/LSD1, which is capable of removing the methyl mark on most of these substrates. Therefore KMT7/KDM1A has emerged as a classic model for dynamic lysine methylation of both histone and non-histone proteins. Currently this family comprises only one member, PRDM2/RIZ1. **KMT8** was identified as retinoblastoma (RB) protein-interacting zinc-finger protein (RIZ1), and it belongs to the PRDM family of proteins which are characterized by the presence of a N-terminal positive regulatory (PR) domain (PRDI-BF1 and RIZ). The PR domain is a homolog of the SET domain and shares 20%–30% identity with the SET module. Some PR domains show intrinsic methyltransferase activity, whereas the methylation activity of most other PRDM proteins has not been identified. KMT8 possesses H3K9 methylation activity and functions as corepressor for gene regulation. Many other

PRDM proteins, although lacking methylation activity, also play a role in regulating chromatin dynamics during stem cell self-renewal, differentiation, and development.(Zhang, Wen et al. 2012).

**Table 1 Human KMTs and their histone and non-histone substrates**

| Family | Enzyme | Other name | Substrates | | Reference[a] |
|---|---|---|---|---|---|
| | | | Histone | Non-histone | |
| KMT1 | KMT1A | SUV39H1 | H3K9me3 | | |
| | KMT1B | SUV39H2 | H3K9me3 | | |
| | KMT1C | G9a/EHMT2 | H3K9me2 | p53, G9a, C/EBPβ, Reptin, RARα, DNMT1, | [4–9] |
| | | | H3K27me2 | CDYL1, WIZ, ACINUS | |
| | | | H1.4K26me2 | | |
| | | | H1.2K187me2 | | |
| | KMT1D | GLP/EHMT1 | H3K9me2 | p53 | [4] |
| | KMT1E | ESET/SETDB1 | H3K9me3 | Tat | [10] |
| | KMT1F | SETDB2 | H3K9me3 | | |
| KMT2 | KMT2A | MLL | H3K4me3 | | |
| | KMT2B | MLL2 | H3K4me3 | | |
| | KMT2C | MLL3 | H3K4me3 | | |
| | KMT2D | MLL4 | H3K4me3 | | |
| | KMT2E | MLL5 | H3K4me3 | | |
| | KMT2F | hSET1A | H3K4me3 | Dam1 (*Saccharomyces cerevisiae*) | [11] |
| | KMT2G | hSET1B | H3K4me3 | | |
| | KMT2H | ASH2 | H3K4me3 | | |
| KMT3 | KMT3A | SET2 | H3K36me3 | | |
| | KMT3B | NSD1 | H3K36me2 | NFκB | [12] |
| | | | H4K20me2 | | |
| | KMT3C | SMYD2 | H3K36me2 | p53, RB | [13,14] |
| | | | H3K4me | | |
| | KMT3D | SMYD1 | H3K4me | | |
| | KMT3E | SMYD3 | H3K4me3 | VEGFR | [15] |
| KMT4 | KMT4 | DOT1L | H3K79me2/3 | | |
| KMT5 | KMT5A | SET8 | H4K20me1 | p53 | [16] |
| | KMT5B | SUV420H1 | H4K20me3 | | |
| | KMT5C | SUV420H2 | H4K20me3 | | |
| KMT6 | KMT6A | EZH2 | H3K27me3 | | |
| | KMT6B | EZH1 | H3K27me3 | | |
| KMT7 | KMT7 | SET7/9 | H3K4me1 | p53, TAF7, TAF10, ERα, AR, DNMT1, NFκB, PCAF, RB, E2F1, STAT3, Tat | [17–31] |
| KMT8 | KMT8 | PRDM2/RIZ1 | H3K9me3 | | |

[a]Reference for non-histone substrates only.

**Figure4** Human KMTs and their histone and non-histone substrates (Zhang et al., 2012)

### I.B.5. Histone Lisine Demethylation Family (KDMs)

Histone lysine methylation was regarded as enzymatically irreversible for decades until the recent discovery of the first histone KDM, LSD1/KDM1A. Soon after, Jumonji (JmjC) domain was identified as another module that possesses enzymatic activity in removing methyl groups from lysine residues. As a large number of proteins in the human genome contain the JmjC domain, numerous JmjC domain-containing KDMs were discovered in the past few years, which in turn provide novel insights into the mechanisms of histone modification and epigenetic regulation. Like the KMTs, KDMs are classified according to the new nomenclature into several distinct groups based on their substrate specificities and protein domain organization **(Figure 5). KDM1A/LSD1** is a flavin adenine dinucleotide-dependent monoamine oxidase that can remove mono- and di-methyl, but not tri-methyl, groups from methylated lysines such as H3K4. Other core components of its protein complex, such as CoREST and BHC80, are essential for its demethylating activity on nucleosomes. KDM1 has been shown to alter its substrates' specificity toward H3K9 methylation when associated with nuclear receptors, there by acting as a transcriptional coactivator. Furthermore, KDM1 has a broad spectrum of non-histone substrates, such as p53 and DNMT1. As a homolog of KDM1A, KDM1B/LSD2 was recently identified and characterized as another H3K4 demethylase. In contrast to KDM1A, which functions at promoters, KDM1B removes intragenic H3K4 methylation for gene activation. Like KDM1A, KDM1B was reported to be able to remove mono- and di-methylation at histone H3K9. **KDM2A and KDM2B** demethylate mono- and di-methylation from H3K36, while KDM2B is also implicated in demethylation of H3K4. This family of proteins has been shown to function as transcriptional corepressors for regulation of several tumor-associated genes, including c-Jun and p15Ink4b. **KDM3** family of proteins was identified as the second family of JmjC histone demethylases (JHDM2). KDM3A/JHDM2A and KDM3B/JHDM2B have specific action toward mono- and di-methylation of H3K9, and they therefore function as transcriptional coactivators for gene expression. KDM3A and 3B have been shown to be involved in multiple biological processes such as androgen receptor (AR) signaling and spermatogenesis. Another JHDM2 family member, JHDM2C/ TRIP8, has not yet been shown to have enzymatic activity. The **KDM4** family encompasses four

homologous demethylases, KDM4A–4D (JMJD2A–2D, respectively). KDM4 proteins are the first demethylases that show demethylation activity on trimethylation. All KDM4 family members are able to remove di- and tri-methylation from H3K9 and/or H3K36. Besides the JmjC domain, they share a highly conserved JmjN domain, and three of them (all except KDM4D) contain tandem PHD fingers and Tudor domains that read distinct histone methylation. KDM4 family proteins function in hormone response. **KDM5** family members KDM5A–5D/JARID1A–1D specifically remove di- and tri-methylation from H3K4. They are multi domain-containing proteins characterized by a combination of JmjC and JmjN catalytic domains with an ARID DNA-binding domain, a C5HC2 zinc finger, and two to three PHD fingers. Distinct PHD fingers of KDM5A and KDM5C were shown to bind methylated H3K4 or H3K9, respectively.**KDM6** This family comprises two H3K27-specific histone demethylases, KDM6A/UTX and KDM6B/JMJD3, which are capable of removing di- and tri-methylation from H3K27. As H3K27 methylation is a repressive epigenetic mark elevated in multiple cancers, both KDM6A and KDM6B function as tumor suppressors, implicated in gene transcriptional activation, epigenetic reprogramming, and RB-dependent cell fate control. The **KDM7/ PHF2** family consists of three members: KDM7A/JHDM1D, KDM7B/PHF8, and KDM7C/PHF2, which are involved in regulation of the expression of ribosomal RNA and genes involved in X-linked mental retardation. This family of proteins possesses strong demethylation activity toward H3K9 and H3K27 mono and di-methylation. Furthermore, PHF8 is able to remove mono-methylation from H4K20. All three KDM7 family proteins contain a PHD finger that binds to histone H3K4me3, and this recognition is essential for their substrate specificity, genomic occupancy, and regulation of target gene expression**.**(Zhang, Wen et al. 2012)

| Table 2 Human KDMs and their substrates | | | | | |
|---|---|---|---|---|---|
| Family | Enzyme | Other name | Substrates | | Reference[a] |
| | | | Histone | Non-histone | |
| KDM1 | KDM1A | LSD1 | H3K4me1/2<br>H3K9me1/2 | p53, DNMT1, STAT3, MYPT1 | [17,24,103,104] |
| | KDM1B | LSD2 | H3K4me1/2<br>H3K9me1/2 | | |
| KDM2 | KDM2A | JHDM1A | H3K36me1/2 | NFκB | [12] |
| | KDM2B | JHDM1B | H3K36me1/2 | | |
| KDM3 | KDM3A | JHDM2A | H3K9me1/2 | | |
| | KDM3B | JHDM2B | H3K9me1/2 | | |
| KDM4 | KDM4A | JMJD2A | H3K9me2/3<br>H3K36me2/3 | | |
| | KDM4B | JMJD2B | H3K9me2/3<br>H3K36me2/3 | | |
| | KDM4C | JMJD2C | H3K9me2/3<br>H3K36me2/3 | | |
| | KDM4D | JMJD2D | H3K9me2/3 | | |
| KDM5 | KDM5A | JARID1A | H3K4me2/3 | | |
| | KDM5B | JARID1B | H3K4me2/3 | | |
| | KDM5C | JARID1C | H3K4me2/3 | | |
| | KDM5D | JARID1D | H3K4me2/3 | | |
| KDM6 | KDM6A | UTX | H3K27me2/3 | | |
| | KDM6B | JMJD3 | H3K27me2/3 | | |
| KDM7 | KDM7A | JHDM1D | H3K9me1/2<br>H3K27me1/2 | | |
| | KDM7B | PHF2 | H3K9me1/2 | | |
| | KDM7C | PHF8 | H3K9me1/2<br>H3K27me1/2<br>H4K20me1 | | |

[a]Reference for non-histone substrates only.

**Figure5** Human KDMs and their substrates( Zhang et al., 2012)

.

### I.B.6. Drugs Regulating Lysine Methylation

The emerging fundamental roles have implicated that development of inhibitors for KMTs are a new frontier for drug discovery. However, so far, only a few compounds targeting KMTs are available for preclinical and clinical development due to their toxicity. Some of the first-generation inhibitors for KMTs are derived from natural products KMT enzymes catalyze the transfer of one to three methyl groups from Sadenosylmethionine (SAM) to specific lysine residues on histones. Targeting the cofactor (SAM) binding site of protein methyltransferases appears to be the first approach for KMT inhibition. Sinefungin A, is the first SAMcompetitive and nonselective inhibitor of KMTs identified. Another natural KMT inhibitor is the Chaetocin a fungal metabolite, it is inhibitor of the Drosophila melanogaster Suv39 family including Suv39h1. A recent attempt at total synthesis of (+)-chaetocin enantiomers has showed that they also have inhibitory activity towards G9a. Chaetocin has also been reported to exhibit anti-myeloma activity, and can inhibit Suv39h1 in acute myeloid leukemia cells with hypermethylated tumor suppressor genes. These results support the potential of developing chaetocin H3K9 methyltransferase inhibitors as therapeutics to target reactivation of silenced genes. Gliotoxin analogs with a disulfide bond, show potent inhibitory activity of G9a and Suv39h1without affecting SET7/9. In another high throughput screen against a preselected chemical library, a highly selective small inhibitor of G9a, BIX-01294 (diazepinquinazolin-amine derivative) has been identified to lower bulk H3K9 me2 levels in mouse ES cells and fibroblasts, with levels restored upon removal of the inhibitor. BIX-01294 binds at the protein substrate channel of G9A and GLP1. Recently, second-generation inhibitors, such as E72, UNC321, UNC0638, and UNC0646 that are based on a 7-alkoxyamine tethered to the quinazoline core, have been developed with a marked improvement of potency and specificity against G9a/GLP. Among them, UNC0646 has also demonstrated improved potency of this quinazoline series in cell based assays. In addition, reported that BRD4770, a compound from a focused library of 2-substituted benzimidazoles as a potential SAM mimetic, reduced cellular levels of di- and trimethylated H3K9, induced senescence and inhibition of cell growth in the pancreatic cancer cell line PANC-1The DOT1L inhibitor EPZ004777 is also a SAM analogue and binds to the SAM binding site.EPZ004777 has been shown to kill mixed lineage leukemia cells with little effect

on non-MLL-translocated cells. In addition, EPZ004777 increases the survival of mice bearing tumors with MLL translocation. EZH2 is essential for cancer stem cell self-renewal. A potent SAM hydrolase inhibitor, 3- Deazaneplanocin A (DZNep), has been shown to selectively inhibit EZH2, leading to H3K27 demethylation and induction of apoptosis in breast cancer cells but not in normal breast epithelial cells. SMYD2 exhibits oncogenic properties by repressing the functional activities of p53 and retinoblastoma protein. Therefore, SMYD2 is an attractive drug target for the development of small-molecule inhibitors.(Tian et al. 2013)

### I.B.7. The Role of KMTs in Models of Neurological Disease

#### *I.B.7.1. Huntington's Disease (HD )and Friedreich's Ataxia*

From a genome-wide perspective, other types of histone modifications show a distribution that is highly complementary to most (histone) acetylation marks. For example, the di- and tri-methylated forms of histone H3-lysine 9 are typically enriched in heterochromatin and, when present at the sites of promoters, these marks are typically involved in transcriptional repression and silencing. These mechanisms likely contribute to the neurobiology of disease. For example, dysregulation of H3-methyl-lysine 9 was reported in postmortem brain studies of subjects diagnosed with Huntington's disease or Friedreich's Ataxia ,both of which are triplet repeat disorders. If the upregulation of histone acetylation generally seems to be beneficial for neuronal functions and behaviors, then what phenotype would be expected in genetically engineered animals with neuron-specific elevations of repressive chromatin marks such as H3-tri(di-)-methyl-lysine 9? This was examined using transgenic mice overexpressing the H3K9-specific histone methyltransferase, SET domain bifurcated 1 (Setdb1), also known as Erg-associated protein with SET domain. When expression in adult brain is upregulated (via transgenes expressed under control of neuron-specific promoters), levels of trimethylated H3 lysine 9 in heterochromatin surrounding pericentromeric repeat DNA became significantly elevated. Preliminary results from ongoing studies indicated that gross neurological function in these mice, as evaluated by rotarod and locomotor assays, body weight, and breeding behaviors were either normal or showed only subtle changes. However, preliminary findings suggest that Setdb1-overexpressing mice outperform their wild-type littermates in the Morris Water Maze and the Object Recognition tests,

which are thought to relate to hippocampus- and cortex-related memory functions . These findings were unexpected and suggest that therapeutic benefits in preclinical models of memory disorders are not limited to HDAC-mediated histone acetylation, but the mechanisms by which Setdb1- mediated H3-lysine 9 methylation alters neuronal function and behavior remain to be explored.(Jiang et al. 2008).

### *I.B.7.2. Stroke In Vivo and Vitro*

Stroke, caused due to the interruption of cerebral blood supply, is a major cause of death after heart disease, and cancer. It has worldwide prevalence and is a highly debilitating disorder where most of the survivors suffer from permanent neurological disorders. Unfortunately, the treatment options for alleviating the stroke-associated conditions are limited both in terms of available drugs and the narrow time window for the therapeutic intervention. In rodents, various reliable models for inducing cerebral ischemia have been developed to study pathophysiology, cellular, and molecular processes involved in the cerebral infarction and also in evaluating the efficacy of potentially therapeutic molecules. During last few decades, most of the cellular and molecular investigations employed the middle cerebral artery occlusion (MCAO) model in rodent that represents stroke conditions in human. However, many clinical studies have shown that 13– 25% stroke cases are due to the internal carotid artery occlusion -a cerebral ischemic condition that may be asymptomatic to symptomatic. So, they established an Internal Carotid Artery Occlusion (ICAO) model in CD1 mouse that resulted in mild to moderate level of neural damage mostly localized to the striatum. Recent molecular studies using various models of stroke in rodents have implicated epigenetic modifications that control transcription events such as DNA methylation and histone lysine acetylation and deacetylation, in ischemia-induced damage and recovery. Administration of compounds that attenuate the methylation of DNA and increase the lysine (K) acetylation of histones H3 and H4 by blocking histone deacetylases (HDACs), have been shown to slow down ischemia-induced neural damage. However, the role of other epigenetic mechanisms such as histone H3 and H4methylation and demethylation, in particular H3K9, H3K27 and H4K20 methylations, the transcriptionally repressive epigenetic modifications recently implicated in the etiopathology of most of the neurodegenerative and neuropsychiatric disorders, have little been investigated in ischemia or stroke models. Formisano et al. have recently shown that global ischemia induces

deacetylation of histones H3 and H4 and enhances H3K9me2 on MOR-1 (μ-opioid receptor) promoter in CA1 region of hippocampus causing a significant decrease in MOR-1 mRNA expression, leading to neurodegeneration. Another study by Schweizer et al., has also implicated H3K9 methylation and the role of histone lysine methyl transferases (KMTs) acting on this epigenetic modification, Suv39h1, and G9a, in hypoxia-induced oxidative stress and neuronal survival and death, using an in vitro OGD (oxygen glucose deprivation) model. The inhibition of transcriptionally repressive KMTs Suv39h1, and G9a using RNAi approach and specific inhibitor chaetocin, promoted neuronal survival partly mediated by enhancement of transcriptionally activating epigenetic mark H3K9ac (acetylation) on the promoter of brain-derived neurotrophic factor (BDNF) and its transcription in neurons.(Chakravarty et al. 2017)

#### *I.B.7.3. Wiedemann-Steiner Syndrome*

Mutations in KMT2A were reported to be associated withWiedemann-Steiner syndrome (WDSTS; OMIM 605130), an extremely rare neurodevelopmental condition accompanied by microcephaly, short stature, autism-like phenotype, and aggression. Interestingly, these abnormal brain functions were recapitulated in KMT2A heterozygous mutant mice, which displayed profound deficits in long-term contextual fear memory. In particular, neuronal ablation of KMT2A in the postnatal forebrain and adult prefrontal cortex exhibited increased anxiety and robust cognitive deficits in mice. In the same study, the analyzing H3K4me3 level and the gene expression profiles in KMT2A-deficient cortical neurons revealed that the homeodomain transcription factor, MEIS2, was repressed in these mice. Moreover, MEIS2 knockdown in prefrontal cortex phenocopied memory defects elicited by the deletion of KMT2A, thus proposing a critical role of MEIS2 in the pathogenesis of WDSTS.(Kim et al. 2017)

#### *I.B.7.4. Kabuki Syndrome 1*

The most well-studied neurodevelopmental disorder associated with dysregulated H3K4me is Kabuki syndrome 1 (KABUK1; OMIM 147920), which is a rare congenital syndrome characterized by a distinctive face (a reminiscent of the make-up of actors Kabuki, traditional Japanese music-drama) and mental retardation with additional features including autism, seizure, and microcephaly. Heterozygous mutations in

KMT2D were found in more than 50% of patients with KABUK1, with the majority of mutations resulting in the premature termination of the protein product. In addition, mutations in KDM6A, an H3K27me demethylase gene, were also reported to contribute to less than 10% of this syndrome, and this type is referred as Kabuki syndrome 2 (KABUK2; OMIM 300867). Recently, Bögershausen et al. identified two mutations in RAP1A/B, which encode the Ras family small GTPases, in patients with KABUK1 by whole exome sequencing. The authors also demonstrated that mutant RAP1 morphant phenocopied KDM6A and KMT2D mutants in zebrafish, and that the MEK/ERK pathway signaling was perturbed in RAP1- and KMT2D-defective cells. Interestingly, these phenotypes were rescued by treatment with an MEK inhibitor. On the other hands, the reduction in neurogenesis and hippocampal memory defects exhibited in a KABUK1 mouse model were ameliorated by the treatment with a histone deacetylase (HDAC) inhibitor, AR-42. Furthermore, a ketogenic diet rescued hippocampal memory defects through the elevation of beta-hydroxybutyrate, an endogenous HDAC inhibitor, in the same mice model. Taken together, these results potentially provide diverse therapeutic directions to treat, or at least mitigate, the symptoms of KABUK1.(Kim, Lee et al. 2017)

#### *I.B.7.5. Schizophrenia*

Extensive exome sequencing from over 200 patients with schizophrenia (SCZD; OMIM 181500) revealed two de novo mutations in SETD1A, which likely cause malfunction of SETD1A activity. Furthermore, a strong association between the loss-of-function mutation of SETD1A and SCZD was confirmed by analyzing the whole exome sequencing of over 4000 patients with SCZD. Interestingly, a recent bioinformatic analysis demonstrated that in addition to mutations in the protein coding region, mutations in the regulatory elements of SETD1A also contributed to the etiology of SCZD. De novo synonymous mutations within frontal cortex-derived DNase I-hypersensitive sites were enriched in SCZD, and SETD1A was identified as the highest statistical significant gene.(Kim, Lee et al. 2017)

# I.C. SODIUM CALCIUM EXCHANGER

## I.C.1. State of Art of $Na^+/Ca^{2+}$ Exchanger (NCX)

The $Na^+/Ca^{2+}$ exchanger (NCX) consists of 9 transmembrane segments that can mediate $Ca^{2+}$ and $Na^+$ fluxes across the plasma membrane(Blaustein and Lederer 1999) **(Figure 6).** In particular it is distributed throughout the brain and the heart. These laboratories individually discovered the presence of a countertransport mechanism that exchanged Na+ and $Ca^{2+}$ ions across the plasma membrane of different excitable but also non excitable cells. In 1988 and in 1990, Philipson and colleagues successfully performed the purification and cloning of the first isoform of NCX, the so-called NCX1, and some years later, the same investigation group cloned NCX2 (Li et al. 1994) and NCX3 (Nicoll et al. 1996). Among NCX isoforms, NCX2 and NCX3 are selectively expressed in the brain (Lee et al. 1994) and in the skeletal muscle (Nicoll, Quednau et al. 1996) .The regulation of intracellular ionic concentrations of the previously mentioned cations, plays important roles in several cellular homeostasis mechanisms in excitable cells. In fact, sodium regulates cellular osmolarity, plays a crucial role in the induction of action potential (Lipton 1999), and also acts in transducing signaling pathways (Yu and Colvin 1997). Importantly, also calcium is involved in several cytosolic intracellular signaling mechanisms as second messenger. The sodium calcium exchanger works in association with other selective ionic channels and ATP-dependent pumps involved in the physiological mechanism of regulation of cytosolic ions concentrations (Blaustein and Lederer 1999). Depending on the intracellular concentrations of $Ca^{2+}$ $[Ca^{2+}]_i$, and $Na^+$, $[Na^+]_i$, NCX can operate either in the *forward mode*, coupling the uphill extrusion of $Ca^{2+}$ to the influx of $Na^+$ ions, or in the *reverse mode*, mediating the extrusion of $Na^+$ and the influx of the $Ca^{2+}$ ions (Blaustein and Lederer 1999; Philipson and Nicoll 2000). The stechiometry of NCX is generally accepted to be three $Na^+$ ions/one $Ca^{2+}$ ion; however, at a later time, in addition to the major 3:1 transport mode, it has been demonstrate that ion flux ratio can vary from 1:1 to a maximum of 4:1, depending on $[Na^+]_i$ and $[Ca^{2+}]_i$ (Fujioka et al. 2000; Kang and Hilgemann 2004).

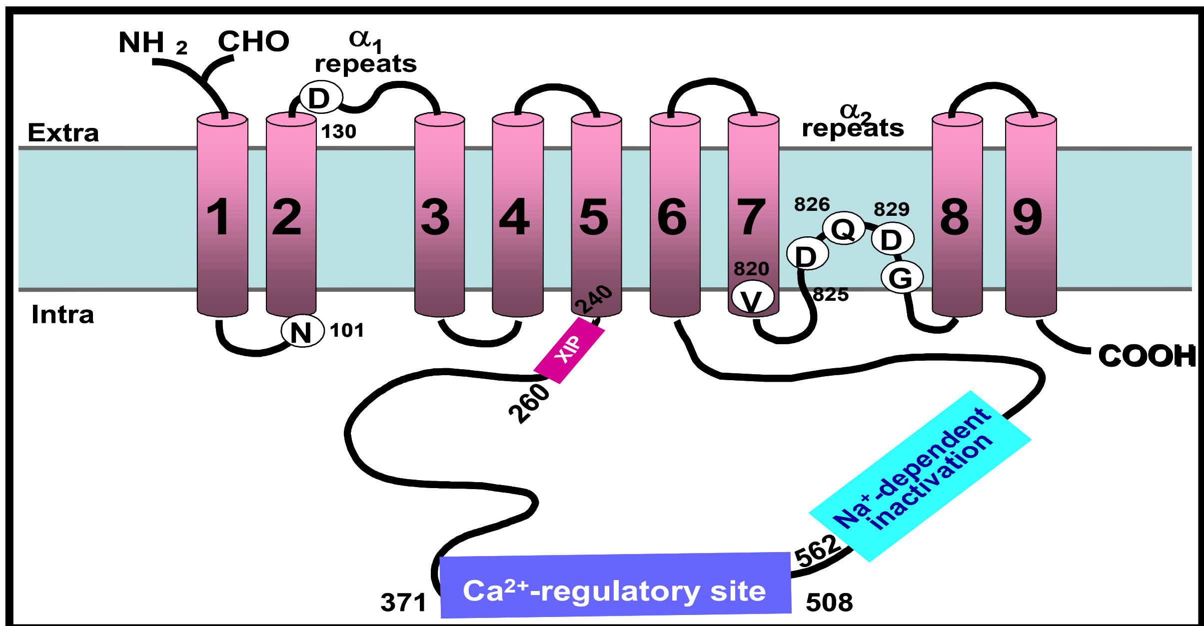


**Figure 6**. Molecular topology of NCX (Annunziato et al., 2004).

### I.C.2. Molecular Biology of NCX

The $Na^+/Ca^{2+}$ exchanger belongs to the superfamily of membrane proteins comprising the following members:

1. the NCX family, which exchanges three $Na^+$ ions for one $Ca^{2+}$ ion or four $Na^+$ ions for one $Ca^{2+}$ ion depending on $[Na^+]_i$ and $[Ca^{2+}]_i$ (Fujioka, Hiroe et al. 2000; Kang and Hilgemann 2004)
2. the $Na^+/Ca^{2+}$ exchanger $K^+$-dependent family, which exchanges four $Na^+$ ions for one $Ca^{2+}$ plus one $K^+$ ion (Lytton et al. 2002)
3. the bacterial family which probably promotes $Ca^{2+}/H^+$ exchange(Cunningham and Fink 1996)
4. the nonbacterial $Ca^{2+}/H^+$ exchange family, which is also the $Ca^{2+}$ exchanger of yeast vacuoles(Pozos et al. 1996)
5. the $Mg^{2+}/H^+$ exchanger, an electrogenic exchanger of protons with $Mg^{2+}$ and $Zn^{2+}$ ions (Shaul et al. 1999)

These membrane proteins are all peculiarly characterized by the presence of α-repeats, the regions involved in ion translocation. About the NCX family, three dominant genes coding for the three different NCX1, NCX2 and NCX3 proteins have been identified in mammals. These three genes appear to be dispersed, since NCX1, NCX2 and NCX3 have been mapped in mouse chromosomes 17, 7, and 12,

respectively (Nicoll, Quednau et al. 1996). At the post-transcriptional level, at least 17 NCX1 and 4 NCX3 proteins are generated through an alternative splicing of the primary nuclear transcripts (Quednau et al. 1997). These variants arise from a region of the large intracellular f loop, are encoded by six small exons defined A to F, and are used in different combinations in a tissue-specific manner (Lee, Yu et al. 1994). To maintain an open reading frame, all splice variants must include either exon A or B, which are mutually exclusive. Excitable tissues, such as those of the brain and heart, are usually characterized by the presence of exon A, whereas kidney, stomach, and skeletal muscle tissues comprise NCX with exon B (Quednau, Nicoll et al. 1997).

NCX1 is composed of 938 aminoacids, in the canine heart, having a theoretical molecular mass of 120 KDa and containing nine transmembrane segments (TMS). NCX1 amino-terminus is located in the extracellular space, whereas the carboxyl terminus is located intracellularly **(Figure 6**). The nine transmembrane segments can be divided into an N-terminal hydrophobic domain, composed of the first five TMS (1-5), and into a C-terminal hydrophobic domain, composed of the last four TMS (6-9). These two hydrophobic domains are important for the binding and the transport of ions. The first (1-5) TMS are separated from the last four (6-9) TMS through a large hydrophilic intracellular loop of 550 amino acids, named the f loop (Nicoll et al. 1999). Although the f loop is not implicated in $Na^+$ and $Ca^{2+}$ translocation, it is responsible for the regulation of NCX activity elicited by several cytoplasmic messengers and transductional mechanisms, such as $Ca^{2+}$ and $Na^+$ ions, NO, phosphatidylinositol 4,5 bisphosphate (PIP2), protein kinase C (PKC), protein kinase A (PKA), and ATP. In the center of the f loop, a region of approximately 130 amino acids in length has been reported to exert a $Ca^{2+}$ regulatory function. This region is characterized by two $Ca^{2+}$ binding domain (CBD1 and CBD2) that undergo conformational changes upon binding of $Ca^{2+}$ and look very similar in the $Ca^{2+}$ bound form, whereas in the absence of $Ca^{2+}$, the domains show dramatic structural differences. CBD1 binds $Ca^{2+}$ with $K_d$ values of 120 and 240 nM, whereas the respective values of CBD2 are 820 nM and 8,6 μM **(Figure 7)** (Hilge et al. 2006). At the N-terminal end of the f loop near the membrane lipid interface, an autoinhibitory domain, rich in both basic and hydrophobic residues, named exchange inhibitory peptide (XIP)(Matsuoka et al. 1997), has been identified. The f loops also characterized by alternative splicing sites named β1-repeat and β2-repeat. These β-repeats are characterized by similar

regions comprising 60 to 70 amino acids for which no functional role has been proposed (Hilgemann 1990).

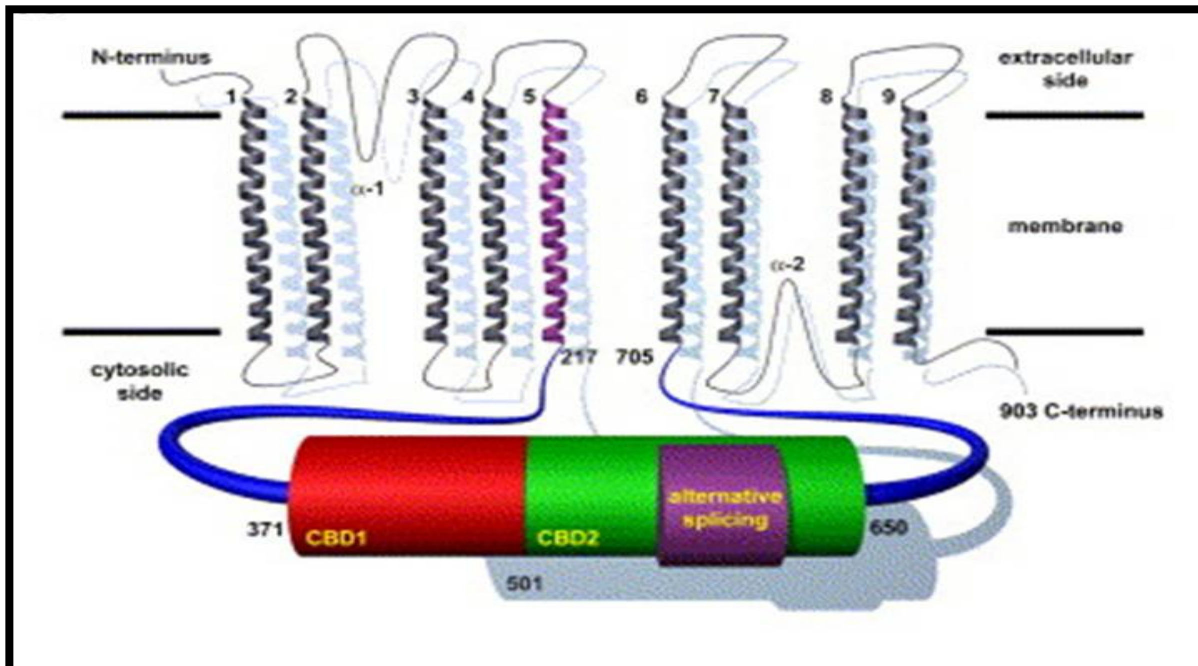


**Figure 7.** $Ca^{2+}$ binding in CBD1 and CBD2 domains. (Hilge et al., 2006).

The NCX protein aminoacid sequence found between TMS2 and TMS3 is called α-1 repeat, whereas the one found between TMS7 and TMS8 is named α-2 repeat. Both these regions are located on the opposite site of the membrane and include two segments composed of 12 and 9 highly conserved residues separated by a non conserved segment of 18 to 20 amino acids (Nicoll, Quednau et al. 1996; Nicoll et al. 2002). Since the putative α-helices of the α-repeats are amphipathic, the hydrophilic faces of these helices may form a portion of the ion translocation pathway (Nicoll, Quednau et al. 1996).

Interestingly, NCX2 and NCX3 have been found only in the brain and in the skeletal muscle. These two gene products consist of 921 and 927 amino acids and are characterized by molecular masses of 102 and 105 KDa, respectively. In addition, NCX2 displays a 65% sequence identity with NCX1, whereas NCX3 possesses a 73% sequence identity with NCX1 and 75% sequence identity with NCX2 (Nicoll, Quednau et al. 1996). All three NCX gene products share the same membrane topology.

In the central nervous system (CNS), the $Na^+/Ca^{2+}$ exchanger plays a fundamental role in controlling the changes in the intracellular concentrations of $Na^+$ and $Ca^{2+}$ ions. These cations are known to regulate neurotransmitter release, cell migration and differentiation, gene expression, and neurodegenerative processes. The NCX protein aminoacid sequence found between TMS2 and TMS3 is called α-1 repeat, whereas the one found between TMS7 and TMS8 is named α-2 repeat. Both these regions are located on the opposite site of the membrane and include two segments composed of 12 and 9 highly conserved residues separated by a non conserved segment of 18 to 20 amino acids (Nicoll, Ottolia et al. 2002). Since the putative α-helices of the α-repeats are amphipathic, the hydrophilic faces of these helices may form a portion of the ion translocation pathway (Nicoll, Quednau et al. 1996). Interestingly, NCX2 and NCX3 have been found only in the brain and in the skeletal muscle. These two gene products consist of 921 and 927 amino acids and are characterized by molecular masses of 102 and 105 KDa, respectively. In addition, NCX2 displays a 65% sequence identity with NCX1, whereas NCX3 possesses a 73% sequence identity with NCX1 and 75% sequence identity with NCX2 (Nicoll, Quednau et al. 1996). All three NCX gene products share the same membrane topology. In the central nervous system (CNS), the $Na^+/Ca^{2+}$ exchanger plays a fundamental role in controlling the changes in the intracellular concentrations of $Na^+$ and $Ca^{2+}$ ions. These cations are known to regulate neurotransmitter release, cell migration and differentiation, gene expression, and neurodegenerative processes.

### I.C.3.Distribution of NCX in Different Tissues

NCX1 is the most expressed protein of the SLC8 gene (solute carrier gene) family encoding for the $Na^+$- $Ca^{2+}$ exchangers. It was chiefly characterized and cloned as cardiac protein, then it has been disclosed in brain and kidney and minimally in other tissues (Quednau et al. 2004). NCX1 gene is alternatively spliced into two well-known sites giving tissue specificity (Lipton 1999). The first site is located at the 5' untranslated region (5′ UTR) and does not alter the structure of the encoded protein. Otherwise, the presence of three different promoters independently drives the tissue specific expression for NCX, supposedly in response to different physiological requirements. (Nicholas et al. 1998).The second site of splicing takes place into the coding region of NCX transcript, whereas two mutually exclusive and four cassette

exons encode for a huge number of isoforms that differs just for the cytosolic inner portion of the exchanger (Lee, Yu et al. 1994).
The two mutually exclusive exons includes the exon A in the transcript of excitable cells, primarily muscular and nervous cells, and the exon B in non-excitable cells. Nowadays, a combinatory pattern for cassette exons in tissues is not still available. Unlike NCX1, the other members of the SLC8 family show a more stringent tissue-specific expression pattern. In fact, NCX2 is present in neurons, but minimally in other sites and NCX3 was found mainly expressed in the brain and skeletal muscle(Papa et al. 2003). Recently, NCX3 has been detected also in the immune system and bones. Remarkably, NCX3, but not NCX2, is alternatively spliced and these spicing variants differ for the cytoplasmic region as it has already been demonstrated for NCX1. Since the three $Na^+$ - $Ca^{2+}$ exchangers, NCX1, NCX2 and NCX3 isoforms display a high homology of sequence, it is widely accepted that structure and the functional role of NCX1 can be generally extended also to NCX2 and NCX3 isoforms (Quednau, Nicoll et al. 2004).

### I.C.4. Regulation of $Na^+/Ca^{2+}$ Exchanger Isoforms

Several factors are involved in the regulation of $Na^+/Ca^{2+}$ exchanger activity: the intracellular pH; metabolic related compounds, ATP, PA, PIP2, PKA, and PKC; redox agents, hydroxyl radicals, $H_2O_2$, dithiothreitol (DTT), $O_2^-$ ,$Fe^{3+}$, $Fe^{2+}$, $Cu^{2+}$, OH, glutathione reduced (GSH), and glutathione oxidized (GSSG); and the gaseous mediator, NO. The site level at which $[Ca^{2+}]_i$ regulates NCX activity is different from the one required for $Ca^{2+}$ transport (Levitsky et al. 1994). The removal of intracellular $Ca^{2+}$ ions completely blocks NCX activity (Philipson and Nicoll 2000). The location of such regulatory site has been identified in the 134-amino acid region, situated in the center of the f loop. In addition to intracellular $Ca^{2+}$ regulatory site, an increase in $[Na^+]_i$ can also regulate the $Na^+/Ca^{2+}$ exchanger. In particular, when intracellular $Na^+$ increases, it binds to the transport site of the exchanger molecule, and after an initial fast outward the $Na^+/Ca^{2+}$ current, an inactivation process occurs (Hilgemann et al. 1992). This inactivation process, very similar to the phenomenon occurring in voltage-dependent ionic channels, is named $Na^+$-dependent inactivation. The region of the intracellular f loop, in which this regulatory site is located, has been identified in a 20-aminoacid portion of the N-terminal part of the loop named XIP (Matsuoka, Nicoll et al. 1997). Regarding on the mechanism by which XIP inhibits NCX activity, it

has been proposed that when the XIP-binding site is ligand-occupied, a conformational change is induced in C-terminal portion of the f loop, thus resulting in the inhibition of the ion transport (Li et al. 1991). XIP is provided with relevant pharmacological implications. In fact, those exogenous peptides, having the same amino acid sequence as XIP, act as potent inhibitors of NCX activity (Annunziato et al. 2004b); (Pignataro et al. 2004b). Interestingly, $Ca^{2+}$ ions, at low micromolar concentrations, binding its regulatory site, decrease the extent of this $Na^{+}$-dependent inactivation. In fact, mutations in the $Ca^{2+}$ regulatory binding site alter the activation and inactivation kinetics of exchange currents by modulating $Na^{+}$-dependent inactivation (Matsuoka et al. 1995). $H^{+}$ strongly inhibits NCX activity under steady-state conditions (Doering and Lederer 1993). The action exerted by $H^{+}$ ions is pathophysiologically relevant with regards to brain and heart ischemia. In fact, when intracellular $H^{+}$ and $Na^{+}$ ion homeostasis is deregulated, the anoxic conditions resulting in these cells may selectively interfere with the activity of the different NCX gene products. ATP may increase the activity of the exchanger in a number of ways. Firstly, ATP directly participates in the NCX molecule phosphorylation process by PKA and PKC (Caroni and Carafoli 1983). Secondly, it increases PIP2 production. This mechanism of activation is related to the relevant PIP2 influence on $Na^{+}$-dependent inactivation of NCX. In fact, PIP2 directly interacts with the XIP region of the exchanger, thus eliminating its inactivation and stimulating NCX function (Hilgemann and Ball 1996). Finally, by activating G-protein-coupled receptors, via endogenous and exogenous ligands, ATP can stimulate NCX activity through the pathway involving PKC or PKA activation (DiPolo and Beaugé 1998) .The mechanism underlying the phosphorylating effect on the exchanger seems to be related to an increase in its affinity for both internal $Ca^{2+}$ and external $Na^{+}$ and to a decrease in its inhibition by internal $Na^{+}$. In addition, ATP cellular depletion inhibits NCX1 and NCX2, but does not affect NCX3 activity (Secondo et al. 2007). Moreover, phosphoarginine (PA) can stimulate NCX activity. In particular, PA present in millimolar concentrations in the cytosol, activates $Na^{+}/Ca^{2+}$ exchanger function in the forward mode of operation by intracellular $Mg^{2+}$- and $Ca^{2+}$-dependent way (DiPolo et al. 2004). Several groups of investigators also have found that NCX is sensitive to different combinations of redox agents (Amoroso et al. 2000); (Santacruz-Toloza et al. 2000). In particular, the stimulation of the exchanger activity requires the combination of a reducing agent with an oxidizing agent. The effects of both agents

are mediated by metal ions. The antiporter's sensitivity to changes in the redox status can assume particular relevance during oxidative stress. In fact, in this condition, the modulation of reactive oxygen species (ROS) could affect the transport of $Na^{+}$ and $Ca^{2+}$ ions through the plasma membrane (Santacruz-Toloza, Ottolia et al. 2000). The ubiquitous gaseous mediator NO seems to be involved in the modulation of NCX activity. In fact, there are several evidences that NO, released by NO donors, is able to stimulate NCX in the *reverse mode* of operation in neuronal preparations and astrocytes. By contrast, in C6 glioma cells, the stimulatory action on NCX *reverse mode* of operation, elicited by the sodium nitroprusside (SNP), is not elicited by NO release but by the presence of iron in SNP molecule (Amoroso, Tortiglione et al. 2000). In addition, the relationship between the constitutive form of nitric oxide synthase (NOS) and NCX has recently been demonstrated. Indeed, heat stress by inducing NOS phosphorylation causes NOS complexation with NCX, thus decreasing its activity (Kiang et al. 2003).

## I.D. NCX3 $Na^{+}/Ca^{2+}$ EXCHANGER 3

The cloning of the $Na^{+}$- $Ca^{2+}$ exchanger isoform 3 (NCX3) was achieved from rat at the Philipson's laboratory in 1996 (Nicoll, Quednau et al. 1996).NCX3 displays approximately the 80% sequence homology with the other isoforms of the $Na^{+}$-$Ca^{2+}$ exchanger family NCX, including NCX1 and NCX2. The NCX structure expresses a different percentage of homology within the isoforms sequence. For istance, the 9 transmembrane domains share more than the 75% of homology within NCX1 and NCX2, but not the cytoplasmic f-loop with just the 60%, thus suggesting that the transport mechanism requires the conservation of the transmembrane regions, whereas the NCX3 capacity is specifically restricted to the cytoplasmic loop. The exact structure of calcium-binding domain (CBD1) of NCX3 remains unknown. Nonetheless, the conservation of some key acidic and basic residues leads to the same Ca2+ binding sites and a structure most likely similar to NCX1. Therefore, the regulation of NCX3 capacity of exchange by CBD1 is probably comparable to NCX1 (Hilge et al. 2009).The comparison of CBD2 has proven to be more difficult because of the alternative splicing of the NCX family causing various possibilities of sequence for CBD2. The NCX gene is spliced in different variants. It has been described more than 15 splicing variants for the isoform 1 and a exiguous number of variants for

NCX3. NCX2 is the unique gene that does not present alternative splicing forms. NCX1 gene displays a tissue specific distribution.(Kofuji et al. 1994; Quednau, Nicoll et al. 1997; Dyck et al. 1999).The NCX3 gene is composed of 9 exons (exons from 1 to 9) .(Quednau, Nicoll et al. 1997). Interestingly, exon 2 and 3– also named exon A and B, respectively - are mutually exclusive, whereas exon 4, named exon C, is optional. Thus, in the rat, three splice variants are detected. A variant containing exon A and C is found in skeletal muscle (NCX3-AC), while variants expressing the exon B are expressed in the brain (NCX3-B and NCX3-BC). Furthermore, in humans three truncated forms of NCX3 have been discovered of which two variants are expressed in the fetal brain (exons4- 9 and 6-9)(Lindgren et al. 2005)**.**The third truncated variant is expressed in skeletal muscle (exons 2 and 6- 9) (Gabellini et al. 2002).These human truncated forms are likely to be under the control of alternative promoters. Recently, the comparison of CBD2 deriving from NCX3-B and NCX1-AD exhibited a similar conformation in structures like the β-sandwich and the α-helix of the F-G loop (Breukels et al. 2012a). The splicing variants of NCX1 display a various orientation in the α-helix which has been found associated with the activation or inhibition during the rise in intracellular calcium [$Ca^{2+}$](Giladi et al. 2015).Conversely, the NCX3 helix found subsequently to the splicing region shows a unique orientation among the splicing variants, expressing an [$Ca^{2+}$]i-dependent activation. NCX1 exons A and B have been found not only individually involved in the signal transmission to the transmembrane domains, but also in the mechanism to relieve the Na+-mediated inactivation(Hilge, Aelen et al. 2009).The other NCX1 exons (C, D, E, and F) may regulate the $Ca^{2+}$-binding affinity of CBD1. Overall, NCX3 splicing exons A, B and C are evolutionary well conserved among species, even a minor sequence homology compared with the NCX1 and NCX2 exons, which has been estimated approximatively around 55 and 65 %.Thus, it is quite impossible to apply the effect observed during the alternative splicing of NCX1 to NCX3. In addition, among the splicing variants, NCX3 exhibited different $Ca^{2+}$ binding sites, which were identified three for NCX3-B and two for NCX3-AC. Interestingly, they expressed a different affinity for the $Ca^{2+}$(Breukels et al. 2012b). According to the NCX structure, we can infer that differences among the axon A and B may have effect both on the $Na^{+}$-dependent inactivation (Hilge, Aelen et al. 2009) and on the sensitivity to $Ca^{2+}$ through changes in conformation of CBD1-CBD2. Finally, we can assume that in the last years important works elegantly investigated the mechanism of translocation in

NCX and its regulation though CBD1 and CBD2 domains. Remains to reveal the mechanism that allows to the intracellular loop to influences the transmembrane segments and shed light on the role of the different alternatively splicing form of NCX3(Hilge, Aelen et al. 2009).

### I.D.1. Role and Mechanism of NCX3 in Brain Ischemic Preconditioning

Recent findings demonstrated that the two isoforms of the $Na^{+}/Ca^{2+}$ exchanger,NCX1 and NCX3, which are involved in several pathophysiological aspects of cerebral ischemia, can be included among the members of the growing family of the mediators involved in the ischemic brain tolerance due to their ability to regulate neuronal calcium homeostasis. In particular, we provide evidence that neuroprotection observed in preconditioned neurons exposed to OGD/reoxygenation is correlated to the increase in NCX1 and NCX3 protein expression. Indeed, the treatment with siRNA against NCX1and NCX3 prevents this effect. These data are in accordance with results recently observed *in vivo* in an animal model of IPC. Consistently with these results, the up regulation of NCX1 and NCX3 protein expression in neurons exposed to IPC was dependent on PI3K/Akt activation, since the treatment with LY294002 was able to abolish this increase. Interestingly, we demonstrated that NO plays a key role in the triggering PI3K/Akt pathway as the increase in the phosphorylated form of Akt observed within 30 min after IPC was completely abolished by the treatment with L-NAME. More importantly, the treatment with L NAME was able to inhibit NCX3 but did not affect NCX1 protein expression. It has recently demonstrated that in neurons NCX3, apart its localization on the plasma membrane, is also expressed on the outer mitochondrial membrane where it contributes to the extrusion of calcium from mitochondria. It is well known that mitochondria, in addition to the generation of cellular energy, play an important role in regulating cellular calcium homeostasis in concert with the sarco-endoplasmic reticulum $Ca^{2+-}$ATPase(SERCA),the plasma membrane $Ca^{2+}$-ATPase, and $Na^{+}/Ca^{2+}$ exchanger. On the other hand, the maintenance of mitochondrial calcium homeostasis is an important requirement ensuring mitochondrial function. Infact, $Ca^{2+}$-sensitive dehydrogenases can regulate oxidative phosphorylation and ATP synthes is during times of high cellular demand. Therefore, it is possible to hypothesize that the increase dexpression of NCX3,we observed within 48 h from the

sub-lethal insult, might exert neuroprotective effects regulating calcium handling and improving mitochondrial oxidative capacity. This finding is in line with the results previously obtained in an *in vitro* model of IPC and demonstrating that the increase in nNOS expression and NO production through the activation of Ras/ERK1/2 pathway stimulated mitochondrial MnSOD. These effects associate with a reduction in free radical production and cytocrome c release from mitochondria to cytosol, and in turn with an improvement of neuronal survival. The demonstration that NCX3 might represent a target of IPC-induced neuroprotection adds new insight into the molecular mechanisms involved in the ischemic brain preconditioning. Indeed, the over-expression of NCX3 might help mitochondria to preserve their energetic capacity making them less vulnerable to the subsequent lethal insult represented by OGD/reoxygenation. This hypothesis was strongly supported by the results obtained measuring the activity of NCX during IPC. In fact, we demonstrated that IPC induced an increase of NCX activity in the reverse mode of operation that was still observed in preconditioned neurons exposed to OGD/Reoxygenation. This is an early event since it occurred within 30 min after IPC stimulus was due to the contribution of NCX1and NCX3 isoforms and was promoted by NO because it was abolished by the treatment with L-NAME. Intriguingly, this effect was associated with an increase in ER calcium content. Consistently with data previously published in our Laboratory, we speculated that NCX1, working in the reverse mode of operation, plays a key role in the regulation of ER calcium refilling in the early phase of IPC. Indeed, the treatment with siNCX1,but not siNCX3, was able to prevent this phenomenon. The novel aspect of the study is the demonstration that NO promoted NCX1-induced ER refilling that was hampered by L-NAME pretreatment. This finding is in accordance with data recently published by Secondo et al. and demonstrating that NO was able to stimulate NCX1 to work in the reverse mode of operation, whereas NO did not affect NCX3 activity. The possibility that NCX1 activation in the early phase of IPC could affect mitochondrial calcium content promoting mitochondrial calcium up take could not be excluded. However, in the late phase of IPC mitochondrial calcium handling is mainly regulated by NCX3 that is able to promote mitochondrial calcium extrusion. These data are supported by the finding that NCX3 expression increased 48h after the IPC insult. We have previously demonstrated that NCX3 is distributed also at mitochondrial level; therefore, it is possible to speculate that during IPC, the increased expression of NCX3 on mitochondria might contribute to the efflux of

calcium from the organelle thus protecting neurons by the subsequent mitochondrial calcium overload induced by lethal OGD/reoxygenatione exposure. The finding that the treatment with CGP and siNCX3 counteracted the effect of IPC on mitochondrial calcium content, leading to the lack of IPC-neuroprotection, further supports this hypothesis. Collectively, we can conclude that a functional interplay between NCX1 and NCX3 occurs during IPC. This phenomenon is tightly dependent on NO and Akt activation, and by contributing to the modulation of intracellular ionic homeostasis, could represent one of the mechanisms responsible for neuroprotection induced by IPC(Sisalli et al. 2015).

### I.D.2. Transcriptional Regulation of ncx3 Gene in Brain

The human SLC8A3 minimal promoter was identified in the 250 bp region immediately upstream of the exon 1 . It shows features similar to the SLC8A1 brain promoter in that it includes SP1 and AP2 elements that confer constitutive transcription and tissue specific expression, respectively. The sequence of human SLC8A3 promoter and of the exon 1, as that of the rat brain SLC8A1 promoter, is very rich in GC bases. These form a typical cytosine and guanine dinucleotide (CpG) island as it occurs at the 5′ end of several housekeeping genes and oncogenes. While these GC rich sequences usually stay unmethylated, abnormal methylation occurs in some pathological conditions as during oncogenic transformation, e.g., the α2 (VI) collagene gene undergoes extensive methylation in SV-40 transformed fibroblasts that inhibit transcription from the AP2 sites. The sequence of the SLC8A3 promoter and that of SLC8A1 brain promoter include multiple AP2 sites (GCCNNNGGC) potentially important for regulation in neurons. Members of the AP2 transcription factor family have been shown to be important regulatory molecules for the vertebrate development of neuronal tube, neuronal crest derivatives, skin, and urogenital tissue. These members also show differences in spatial and temporal expression during mouse development . The tissue specific expression of AP2 is highest in ectodermal cell lineage, including neuroepithelium and neural crest. Several repeated consensus elements for transcription factors AP2 are found in neuron specific gene promoters (e.g., choline acetyltransferase, proenkephalin; human fragile mental retardation promoter [FMR1]) as well as in other cellular genes, (i.e.keratin, sodium-phosphate cotransporter [Npt2] of kidney proximal tubules and viral genomes as MMTV). In contrast to the SLC8A1 gene, whose transcription

initiates from multiple tissue-specific promoters, a single promoter region may determine the tissue-specific expression of the SLC8A3 gene. The SLC8A3 promoter indicates neuronal expression. Furthermore, it also includes elements for the muscle-specific transcription factors MyoD and GATA factors 2/3; in agreement is the fact that the orthologrodent gene is predominantly expressed in brain and skeletal muscles. The SLC8A3 promoter also includes elements potentially binding inducible transcription factors AP1, and Egr-1 (also known as NGF1-A, Krox24, Zif/268, or Tis 8). These are a subgroup of IEGs that are activated rapidly and transiently in neuronal cells in response to environmental stimuli, such as neurotransmitters and neurotrophin receptor stimulation. They regulate the expression of subsets of genes termed delayed-response genes. The AP1 complex is formed by the dimerization of the Jun proteins (c-Jun; JunB and Jun D) and Fos proteins (c-Fos, FosB, Fra-1, etc.) through their leucine zipper motifs. The AP1 complex binds to the consensus sequence TGACTCA. This element, also termed TPA response element (TRE), was first indicated as the element responsive to phorbol ester activation of protein kinase C (PKC). The Jun proteins can also form homodimers or heterodimers with Fos; dimerization with transcription factors of the ATF/CREB family is also possible. The composition of the AP1 complex can affect the affinity for consensus sequences and influence transactivation potential. The regulation of Jun and Fos proteins by phosphorylation has been deeply investigated and a considerable amount of literature is available. The transcription factors of the Egr or Krox family are rapidly induced in mammalian brain by a variety of stimuli; in contrast to Fos and Jun they lack the bZip domain and bind DNA by zinc finger motifs. The activation of the N-Methyl D-Aspartate (NMDA) receptor by glutamate transiently induces Egr-1 within minutes, similarly to the induction of c-fos . The induction of Egr-1-mRNA has been reported in a variety of primary cultures of cerebellar, corticostriatal, cortical, and hippocampal neurons. The *in vivo* expression of Egr-1 is strongest in the hippocampus and cerebellum of adult rats and considerably high in neocortex, striatum, and hippocampal CA1 neurons. Following stimulation by intraperitoneal administration of NMDA, the Egr-1 mRNA increases in the cortex, cerebellum, and hippocampus. The blockade of NMDA receptor by MK-801 selectively downregulates the expression of Egr-1 but does not alter c-fos and AP1 expression . It has been suggested that the activation of NMDA receptor by physiological synaptic activity specifically increases the expression of Egr-1, whereas kainateevoked seizures

induced all four members of the Egr family. Thus, although the role of Egr-1 element in the regulation of SLC8A3 gene expression has not yet been documented experimentally, it seems likely that NMDA receptor activation may cause the upregulation of SLC8A3 mRNA. The consequent increase in NCX3 protein activity may be specifically required to extrude $Ca^{2+}$ following its entry through the activated NMDA-receptor channel. A functional link between NMDA receptor and NCX3 is also suggested by their high expression in the hippocampus, especially in the CA1 region . The SLC8A3 promoter also indicates a CRE element–218 (TGACGTGC) originally identified in the promoter of the somatostatin gene as the 8-bp sequence TGACGTCA. The CRE element is included in several other cAMP-responsive gene promoters, such as cfos, Zif/268, or Egr-1 and proenkefalin. In addition, it was shown that the CRE element mediated the transactivation of the c-fos gene in response to $Ca^{2+}$ influx as well as cAMP thus also named Ca2+ response element (CaRE). The presence of the CRE element has attracted much attention since it could confer rapid inducibility to the SLC8A3 gene, similar to that of IEGs. The SLC8A3 gene also includes several DRE elements (with central core nucleotide sequence GTCA) in the region downstream of the TATA box and in the exon 1, potentially able to bind the cAMP and $Ca^{2+}$-regulated transcriptional repressor DREAM. The transcriptional regulation in response to BDNF, cAMP, and $Ca^{2+}$ signals via the CRE and DRE sites will be examined in the following subheading.(Gabellini 2004).

### I.D.2.1 *ncx3 Brain Promoter Region and Putative Transcription Factor in Binding Site*

The human genomic DNA region including the complete SLC8A3 gene was identified by similarity search (BLAST Human Genome) with the corresponding rat cDNA sequence and with a partial genomic sequence, which had been previously assigned to human isoform 2. The alignment of the predicted coding sequence with that of Rattus norvegicus Slc8a1 (AF109163), Slc8a2 (NM_078619) and Slc8a3 (U53420) clearly indicated high homology to rat isoform 3. The human SCL8A3 gene was identified within the genomic region 14q24.2 (sequence fragments AL160191.3 and AL135747.4, contig NT_010028.7, chr14:68875999–69023425). It includes nine exons, numbered 1–9. It should be noticed that exons 2–5 correspond to homologous exons 2–5 of the SLC8A1 gene; whereas the SLC8A3 gene exons 6–9

correspond to homologous NCX1 exons 9–12. Thus, SLC8A3 gene lacks the alternatively spliced exons 6–8. The sequence and the boundaries of exons 2–10 (now 2– 7) were already known and the mutual exclusiveness of exons 3 and 4 was previously demonstrated (also termed A and B, Kofuji et al., 1994). The sequence of the remainder exons (1, 8 and 9) was predicted by the alignment of cDNA sequences with the highly similar rat isoform 3 and by the consensus for splice sites. The predicted human NCX3 protein is encoded by eight exons (2–9). The coding sequence specifies 928 or 927 amino acids, depending on the inclusion of exons 3 or 4. The human SLC8A3 coding sequence exhibits 93% nucleotide conservation, and 97% amino acid identity with the orthologous rat sequence. Successful amplification of the exon 1 was performed with the cDNA from SH-SY5Y cells, a neuroblastoma cell line that expresses high levels of NCX3 protein and was used for the analysis of the SLC8A3 proximal promoter region. The segment (752 bp) was amplified starting from nucleotide 6 of the exon 1 and 66 nucleotides downstream of the 50 of the exon 2. Since exon 1, and the promoter region are located in a GC rich region, the yield of the amplification was low. Probably, the amplification of this segment from total human brain and human skeletal muscle cDNAs failed because the amount of SLC8A3 cDNA in tissues is lower than in the neuroblastoma cell lines. The 50 exon 1 sequence of the SLC8A3 gene is more extended than that reported previously from the rat cDNA. Only the 30 portion of exon 1 (228 bp) displays a significant similarity to the corresponding region of the rat sequence. The whole SLC8A3 coding sequence was amplified by RT-PCR from the RNA of the human neuroblastoma SHSY5Y cell line. The amplified segment (2766 bp) was cloned in the pCR vector and further analyzed by DNA sequencing. The coding sequence matched perfectly that predicted by the computational analysis, except the small exon 5 was absent. It consists of six exons: 2, 4, 6, 7, 8, 9 . As in the case of the SLC8A1, the ATG start codon is close (63 bp) to the 50 end of exon 2. This exon encodes the largest portion of the NCX3 protein, whereas the remainder coding exons (3–8) are much smaller. The coding sequence terminates with a stop codon located at base 375 of exon 9. The sequence continues with a long 30 UTR (1733 bp) as established by the alignment of genomic DNA and human EST sequences: N50099 (99.7% identity) AW022249 (98.7%); AW903279 (100%), and cDNAs AF086064 (99.7%) and AL359938 (99.8%). The polyadenylation signal (AATAAA) was found by NIX polyA program 23 nucleotides upstream of the putative polyA site. The alignment of the

deduced amino acids sequence of human NCX3 with that of human NCX1 and NCX2 showed 68% and 71% identities respectively, whereas that of NCX2 and NCX1 showed 65% identities. The amino acid sequence of the human NCX3 protein was analyzed by Simple Modular Architecture Research Tools (SMART) computer programs. The Signal program predicted an N-terminal signal peptide (residues 1–31), similar to that of NCX1. The TMHMM2 program predicted ten trans-membrane (TM) domains. The five N-terminal TM domains are clustered (amino acids 74–95, 133–155, 170–189, 202–224, 229– 251). The large hydrophilic loop is located from positions 252–744. Five potential TM domains are predicted at the C-terminus of the protein (745–767, 782–801, 821–843, 853–875 and 896–918). The alternative splicing in the SCL8A3 region encoding the C-terminal portion of the large intracellular loop was analyzed by RT-PCR on the cDNA from SH-SY5Y cells, human skeletal muscle and whole brain. The amplification of the cDNAs from human brain and neuroblastoma cells produced only one band of 267 bp (Fig. 3, lanes A and C). The DNA sequence of several clones of the fragment was identical: all of them included exon 4. Thus, the NCX3 isoform expressed in brain has the same composition of the NCX3 isoform expressed in the human neuroblastoma cell line (AF510501), corresponding to the murine isoform named NCX3.2. The alternative splicing pattern of the NCX3 cDNA from human skeletal muscle is more complex. Three DNA fragments were amplified from this tissue: the most abundant DNA band corresponded to the NCX3.2 isoform including exon 4 (267 bp), whereas the largest fragment (285 bp) corresponded to the NCX3.3 isoform including the exon 4 and the small exon 5 (AF510502). The sequence of the smallest DNA fragment (164 bp) revealed a NCX3 isoform lacking both exons 3 and 4. We propose to name this newly identified isoform NCX3.4 (AF510503). The skipping of both exons 3 and 4 caused a frame shift downstream of the exon 2. The NCX3.4 sequence encodes 620 amino acids, of which 595 are encoded by exons 2 and 25 by the different frame in the exon 6. The NCX3.4 isoform has a predicted molecular weight of about 68 kDa; its domain composition is similar to a truncated NCX1 isoform previously described. No NCX3 isoform containing exon 3 was found in the human cDNA analyzed. The sequence analysis performed by SMART similarity search with PFAM (protein families database) domains indicated that the sequence encoded by exons 3 and 4 (amino acids 595–630) overlaps the region encoding the second Calx-b domain (amino acids 519–619). Only 8 of the 25 amino acids specified by the different frame

of the exon 6 reconstruct a portion of the second Calx-b domain, the remaining C-terminal sequence of isoform 3.4 includes several positively charged residues.(Gabellini, Bortoluzzi et al. 2002)

### I.D.2.2 *ncx3 is Transcriptionally Up-Regulated by CREB*

In neurons, a wide range of extracellular stimuli activates signaling pathways like so CREB family members. Gene expression dependent by CREB pathway activation has been implicated in a complex and various series of processes ranging from development to plasticity, and eventually related also to diseases. CREB (cAMP response element binding protein) belongs to the bZIP superfamily of transcription factors. Within this superfamily, CREB and the closely related factors CREM (cAMP response element modulator) and ATF-1 (activating transcription factor 1) include a subcategory referred to as the CREB family. Belonging to the bZIP transcription factors, CREB family members contain a leucine zipper domain that facilitates CREB dimerization, and a C-terminal basic domain which role is binding with DNA. There is a high degree of homology among CREB, CREM, and ATF-1, allowing them to form both homo- and heterodimers, and each of these complexes takes place at the same side of cis-regulatory element as reviewed in (De Cesare et al. 1999; Shaywitz and Greenberg 1999). The remaining domains of CREB family members mediate the interaction with coactivators and components of the transcriptional machinery to the DNA, facilitating RNA synthesis of genes having an important role in the nervous system. The most common CREB isoforms, CREBα and CREBΔ, display a kinase inducible domain (KID) between two domains enriched of glutamine residues, the so-called Q1 and Q2/CAD (constitutive active domain). The unique difference between these two activators is the presence of an α domain. Following stimulation, the KID domain phosphorylated at the residue of Serine 133 (Ser-133), can bind the transcriptional coactivator, CREB binding protein (CBP) via its KIX domain (Chrivia et al. 1993; Kwok et al. 1994). As result of the interaction stimulus-dependent between these two domains, the transcriptional machinery starts the synthesis of the inducible genes. Q2/CAD domain interacts with components of the basal transcriptional machinery allowing the stimulus-independent CRE-driven gene expression (Ferreri et al. 1994). The SLC8A3 promoter also indicates a CRE element–218 (TGACGTGC) originally identified in the promoter of the somatostatin gene as the 8-bp sequence TGACGTCA. The CRE element is included in several other cAMP-responsive gene

promoters, such as cfos, Zif/268, or Egr-1 and proenkefalin. In addition, it was shown that the CRE element
mediated the transactivation of the c-fos gene in response to $Ca^{2+}$ influx as well as cAMP thus also named $Ca^{2+}$ response element (CaRE). The presence of the CRE element has attracted much attention since it could confer rapid inducibility to the SLC8A3 gene, similar to that of IEGs. (Gabellini 2004)

### I.D.2.3 *ncx3 is Transcriptionally Down-Regulated by DREAM*

DREAM was primarily identified as the transcriptional repressor of the human gene for the opioid peptide dynorphin. Dynorphins are members of the opioid peptide family which are involved in memory formation and chronic pain adaptation. Dynorphin peptide controls the release of neurotransmitters as well as it can block LTP in the hippocampus by inhibiting excitatory neurotransmission from synaptic terminals.(Wagner et al. 1993; Weisskopf et al. 1993) The DNA sequence required for the transactivation of prodynorphin transcription via cAMP and $Ca^{2+}$ has been identified in the first exon of the gene (at position +40), and termed dynorphin downstream regulatory element (DRE)(Carrion et al. 1998).The transcription factor binding to the DRE was subsequently identified and includes four $Ca^{2+}$ binding domains of the EF hand type. Nowadays, it represents the unique transcription factor known to be directly regulated by $Ca^{2+}$. When loaded with $Ca^{2+}$, DREAM undergoes conformational changes causing its detachment from DRE sites, thus relieving the transcriptional repression(Herdegen and Leah 1998; Osawa et al. 2001).The ability to bind DNA of some members of the basic helix-loop-helix (bHLH) transcription factor family is also regulated by $Ca^{2+}$, through the interaction with $Ca^{2+}$-loaded calmodulin (Corneliussen et al. 1994; Onions et al. 1997). The DREAM sequence presumably includes four EF-hands, displaying high homology to $Ca^{2+}$-binding proteins of the recoverin subfamily. Mutation of two residues within any of the functional EF-hands, the so-called EFmutDREAMs, did not affect the binding to the DRE but completely prevented the unbinding in the presence of increasing concentrations of $Ca^{2+}$. It seems like the derepression of DRE-dependent gene expression after PKA activation (Carrion, Mellstrom et al. 1998)is related to specific protein–protein interactions between DREAM and nuclear effectors of the transcriptional effects of cAMP. Analysis of the 5' regulatory regions of many $Ca^{2+}$-regulated genes has shown the

presence of one or several functional DRE sites downstream from their TATA boxes(Carrion, Mellstrom et al. 1998; Ledo et al. 2000).This suggests that DREAM mediated transcriptional derepression is a generally required step prior to transcriptional activation of many genes. A new search showed identity between DREAM and calsenilin, a protein able to interact with presenilin-2 found in a yeast two-hybrid screen using the carboxy terminal part of presenilin-2 as a bait(Buxbaum et al. 1998). Double immunostaining after overexpression of N-tagged calsenilin in Cos cells showed the colocalization with presenilin-2 in the cytoplasm and the endoplasmic reticulum and suggested that the colocalization at the ER, in particular, was increased after calcium stimulation (Buxbaum, Choi et al. 1998).More recently, also using a yeast two-hybrid screen to identify components of the protein complex responsible for the A-type potassium currents, a protein identical to DREAM called KchIP-3 (potassium channel interacting protein 3) was found to selectively interact with the amino terminal region of Kv4 potassium channels (An et al. 2000).Interestingly, the interaction with the potassium channel does not depend on the presence of calcium. However, the change in KchIP-3/DREAM conformation that follows binding to $Ca^{2+}$ profoundly affects channel properties (An, Bowlby et al. 2000). Interestingly, in this study two other proteins related to DREAM, KchIP-1 and -2, were identified. In terms of the interaction with potassium channels, KchIP-1 and -2 are similar to KchIP-3. However, it is not known whether they are also able to interact with presenilin-2 and especially, whether they are able to specifically bind to DNA. Thus, the possible existence of a DREAM family of nuclear repressors remains to be investigated. Taken together these results indicate that DREAM/KchIP-3/calsenilin might have pleiotropic functions through the interaction with specific DNA sequences and/or with proteins in different cell compartments (Agell et al. 1998).DREAM, calsenilin, and KChIP3 are the products of a unique gene(Buxbaum et al. 2000; Spreafico et al. 2001), although the proteins are localized in different cellular compartments: nucleus, endoplasmic reticulum, or lysosomes and plasma membrane, respectively. Regulation of mRNA translation at alternative start codons may generate proteins with variable N-terminal peptides, which could be important for their localization and function. Furthermore, isoforms lacking the EF-hand domains are generated by alternative splicing in mouse (Spreafico et al., 2001(Gabellini 2004). The SLC8A3 gene also includes several DRE elements (with central core nucleotide sequence GTCA) in the region downstream of the TATA box

and in the exon 1, potentially able to bind the cAMP and $Ca^{2+}$-regulated transcriptional repressor DREAM(Gabellini 2004).

## I.E. GATA TRANSCRIPTION FACTORS

### I.E.1. The GATA Transcription Factor Family

The GATA-binding proteins are a group of structurally related transcription factors that bind to the DNA consensus sequence GATA. Members of the GATA protein family (GATA1-6) function as lineage-specific transcription factors for a number of cell types in the hematopoietic system (Chen et al. 2012). In vertebrates, six GATA transcription factors have been identified. Based on phylogenetic analysis and tissue expression profiles, the GATA family can be divided into two subfamilies, GATA1/2/3 and GATA4/5/6 . Although in non-vertebrates GATA genes are linked together onto chromosomes, in humans they are segregated onto six distinct chromosomal regions, indicating segregation during evolution. Most GATA genes encode for several transcripts and protein isoforms. GATA proteins have two zinc finger DNA binding domains, Cys-X2-C-X17-Cys-X2-Cys (ZNI and ZNII), which recognise the sequences (A/T)GATA(A/G) **(Figure 8)**. In non-vertebrates GATA transcription factors have been identified that contain mostly one zinc finger, i.e. in Drosophila melanogaster and Caenorhabditis elegans. The C terminal zinc finger (ZNII) exists in both vertebrates and non-vertebrates indicating that ZNI was duplicated from ZNII (Lentjes et al. 2016)

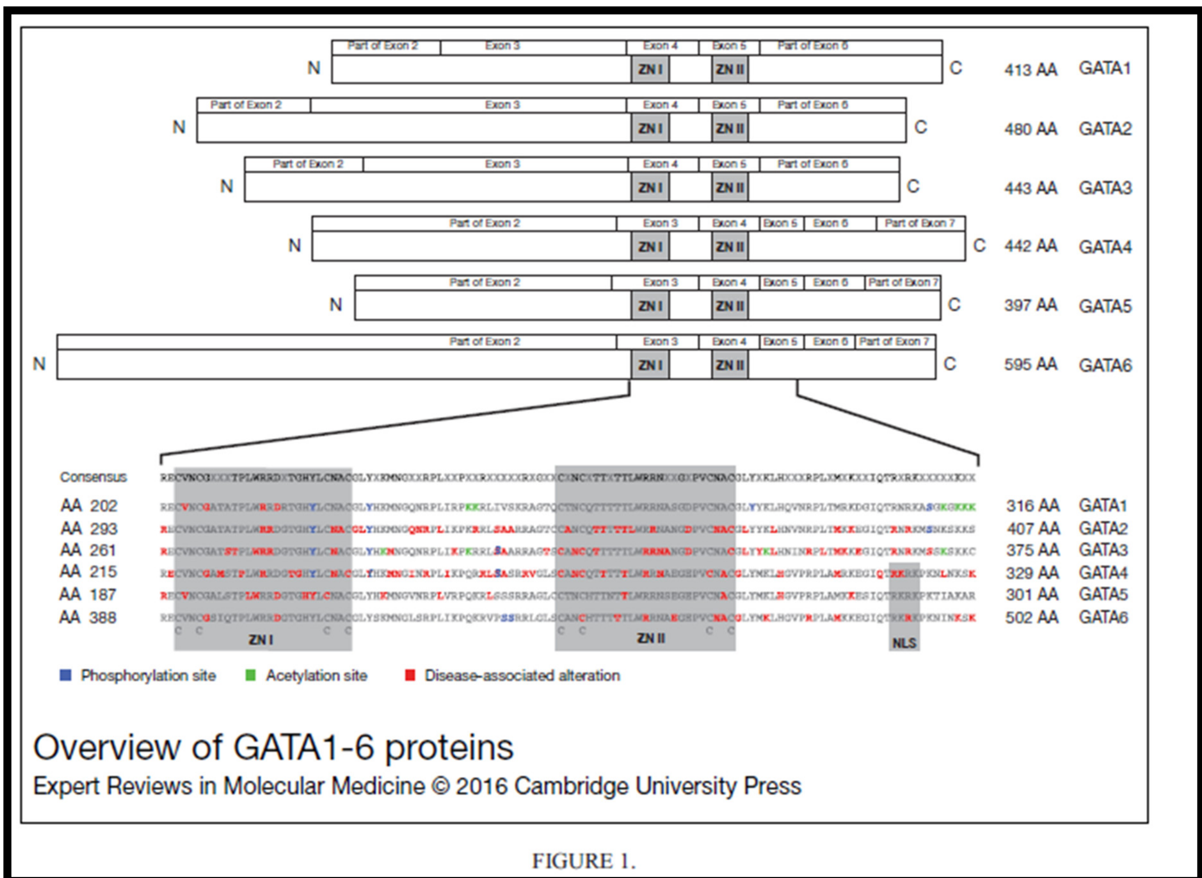


**Figure8** Overview of GATA1-6 proteins (Lentjes et al., 2016)

### I.E.2. Structural Characteristics and *Binding Site Specificity* of the GATA Factors

The function of GATA proteins depends critically on two highly conserved zinc fingers and nearby basic regions. The C-terminal ‘‘C-finger’’ and its adjacent basic region are necessary and sufficient for GATA to bind its cognate sequence, WGATAR (W = A/T, R = A/G). The N-terminal ‘‘N-finger’’ can also bind DNA independently but has a preference for GATC core motifs. Both fingers participate in binding the palindromic GATA motif ATCWGATA (W = A/T), resulting in markedly increased affinity. Recent chromatin immunoprecipitation sequencing (ChIP-seq) experiments on GATA1, GATA2, and GATA3 showed that GATA proteins mostly bind to a single GATA site ((A/T)GATAA) or a palindromic site (catctGATAAG;). It is known that GATA can bind palindromic sites with high affinity and that such binding may be functionally important. However, it was not known until recently how prevalent this DNA-binding mode of GATA is in vivo. Compared with classical Cys2His2 zinc finger proteins, such as Zif268 and TFIIIA, the GATA proteins have a longer linker region, which

allows more complex and versatile DNA-binding patterns. Zif268 and TFIIIA have shorter linkers and use multiple zinc finger domains to wrap around DNA and track along the major groove. Our structure is also different from most other Cys4 zinc finger proteins, such as nuclear receptor proteins. Although most nuclear receptor proteins also have two Cys4 zinc finger motifs, they use one for DNA binding and the other for dimerization. In addition, nuclear receptors form dimers to bind the same side of the DNA, the N-finger binds to one side of DNA in the major groove and then tracks along the minor groove without DNA binding. The C-finger binds to the major groove on the other side of the double helix and then tracks along the minor groove with extensive DNA binding. Finally, the end of the C-tail reaches the N-finger and completes the wrapping architecture. Several structural features may account for the enhanced binding affinity of GATA to the palindromic sites. First the N-finger and C-finger bind to adjacent sites on the DNA, making extensive protein-DNA contacts. Second, the binding of a zinc finger induces conformational changes of the DNA, and such changes may facilitate the binding of the second zinc finger to the adjacent site. Third, the N-finger and C-finger interact directly on palindromic sites, and these protein–protein interactions may lead to cooperative binding of the two fingers to DNA, similar to the cooperative binding of GATA C-fingers to adjacent sites. Finally, the N- and C-fingers, in conjunction with their C-terminal extensions, wrap around DNA in the palindromic complex, and such encirclement of DNA by the protein usually leads to enhanced kinetic stability of the protein/DNA complex. Given that it is well known that GATA proteins mediate long-range gene regulation, we believe that our model represents at least one of the mechanisms by which GATA proteins loop DNA in cells. In addition to direct DNA bridging, GATA proteins may loop DNA through indirect mechanisms, such as via their cofactors (e.g., FOG proteins), which have also been shown to play important roles in mediating DNA loop formation. Notably, these transcription factors are all implicated in lineage control during cellular differentiation. We thus propose that one mechanism whereby the epigenetic expression patterns of a given cell are controlled is specific folding of the three-dimensional structure of the genome by transcription factors that control a given lineage. There are two distinct DNA-binding modes of GATA transcription factors: (1) the wrapping mode, in which both zinc fingers synergistically enhance the binding affinity and kinetic stability; and (2) the bridging mode, in which a single GATA DBD bridges two pieces of DNA. Because the DBDs of GATA proteins are highly

conserved, the structural features described here will likely hold true for all six GATA family members. The fact that GATA can bind DNA in two distinct modes with different binding affinity/kinetic stabilities and conformations has important implications for transcriptional regulation by this family of proteins. At low expression levels, the GATA proteins may preferentially bind to the palindromic sites, and at high expression levels, the GATA proteins may be able to bind both palindromic sites and single sites, leading to more intra- and interchromosomal interactions, either by themselves or through their interaction with cofactors such as the FOG protein. The different DNA-bound conformations of GATA proteins may recruit different cofactors or change the local chromosomal conformation, leading to different biological functions(Chen, Bates et al. 2012).

### I.E.3. Physiological Function and Tissue Specificity of GATA-Proteins

GATA1/2/3 are required for differentiation of mesoderm and ectoderm-derived tissues, including the haematopoietic and central nervous system. GATA4/5/6 are implicated in development and differentiation of endoderm- and mesoderm-derived tissues such as induction of differentiation of embryonic stem cells, cardiovascular embryogenesis and guidance of epithelial cell differentiation in the adult.(Lentjes, Niessen et al. 2016)**(Figure 9).** The purification and cloning of GATA-1, ushered in studies that elucidated mechanistic principles governing GATA factor function. The zinc finger residing closest to the carboxy-terminus (C-finger) mediates sequence-specific DNA binding to WGATAR motifs, while the zinc finger proximal to the amino-terminus (N-finger) mediates an important protein–protein interaction with the nine zinc finger containing coregulator Friend of GATA-1. The N-finger may also stabilize DNA binding in certain contexts. Additional interactions involving the zinc fingers have been documented, including binding to the myeloid transcription factor PU.1, the erythroid transcription factor ELKF and the mediator complex component Med1. Much less is known about the structural basis and biological implications of these interactions. The broad GATA-1 N-terminus enhances endogenous target gene activation in a context-dependent manner. Missense mutations in the N-terminus trigger the usage of an alternative translational start site, yielding a mutant that is strongly associated with the development of transient myeloproliferative disease and acute megakaryoblastic leukemia. Despite approximately 7 million GATA motifs in the human genome, all capable of forming high-affinity complexes with GATA factors

and naked DNA in vitro, GATA-1 and GATA-2 occupy only 0.1–1% of these motifs in erythroblasts, based on chromatin immunoprecipitation coupled with massively parallel sequencing and real-time PCR validation. While the molecular determinants for this exquisite discrimination are not fully understood, FOG-1 facilitates GATA-1 occupancy at a subset of chromatin sites. Genome-wide analysis of cis-elements residing at endogenous GATA-1 and GATA-2 occupancy sites led to refinement of the GATA consensus from WGATAR to WGATAA. However, the percent of total WGATAA motifs occupied remains very low. Beyond GATA motif sequence composition, the most rudimentary determinant of chromatin occupancy, diagnostic patterns of histone posttranslational modifications demarcate occupied versus unoccupied sites, both containing conserved GATA motifs (**Figure 10, Principle 1**). In principle, the unique epigenetic signature of occupied sites may represent primed chromatin structures recognized by GATA-1 as a pivotal determinant of site selection. Alternatively, the signature may arise as a consequence of GATA-1 chromatin occupancy, followed by recruitment of GATA-1 coregulators that modify chromatin surrounding the occupancy site. GATA-1 chromatin occupancy leads to either activation or repression of target genes, both of which can be mediated by FOG-1(**Figure 10, Principle 2**). One mode of FOG-1 function involves interaction of its N-terminus with the NuRD chromatin remodeling complex, which can mediate both repression and activation. GATA-1 utilizes FOG-1 to induce higher order chromatin loops, based on chromosome conformation capture (3C) data. In principle, such loops can mediate activation or repression, dependent upon the physical relationship between the loop and functional features of a gene and the precise nature of the structure formed. GATA-1 also recruits the chromatin remodeler BRG1 to chromatin, which can mediate higher order looping. Additional GATA-1 mechanisms exist, including FOG-1-independent activation and repression, although these mechanisms remain poorly understood. GATA-1 commonly co-localizes on chromatin with the stem cell leukemia/T-cell acute lymphocytic leukemia-1 (Scl/TAL1) protein, and the co-localization commonly correlates with transcriptional activity (**Figure 10, Principle 3**). Scl/TAL1 is a master regulator of hematopoiesis that binds E-boxes and non-DNA-binding components including LMO2, LDB1, ETO2, and single-stranded DNA-binding proteins. In the context of naked DNA, optimal composite elements that support complex formation contain an E-box, a downstream GATA motif, and an 8-bp spacer. The 8-bp spacing is crucial for GATA-2-dependent enhancer activity in a

transient transfection assay using cells expressing endogenous GATA-2. However, GATA-1 and Scl/TAL1 also co-localize at certain chromatin sites lacking composite elements. Notably, the additional protein constituents of the complex modulate its transcriptional regulatory activity in a context-dependent manner and are linked to the development and/or progression of human hematologic malignancies. Sophisticated ChIP-seq analyses in the HPC-7 multipotent hematopoietic cell line demonstrated that additional components co-localized with GATA-2 and Scl/TAL1. This analysis revealed 1015 regions of 200 bp or less in which Scl/TAL1, LYL1, LMO2, GATA-2, ERG, FLI-1 and RUNX1 occupancy was detected. As each of these factors is likely to engage additional important partners, considerably more work is required to understand the structure/function of these higher order chromatin complexes containing multiple master regulators of hematopoiesis. GATA factor interplay appears to be a common mechanism for controlling developmental processes. During the development of erythrocytes, GATA-1 displaces GATA-2 from chromatin sites at target genes, and this GATA switch (defined as an exchange of different GATA factors at a chromatin site) is tightly coupled to an altered transcriptional output (**Figure 10, Principle 4**). GATA switches were first described at the Gata2 locus, at which GATA-1 binding instigates repression, thus explaining the differential GATA-1 and GATA-2 expression pattern during erythropoiesis. GATA-1 utilizes FOG-1 to displace GATA-2 from chromatin. The capacity of FOG-1 to bind the NuRD complex is required for the GATA switch, as GATA switches were impaired in a knock-in mouse strain expressing FOG-1 defective in NuRD complex binding. Ectopic FOG-1 expression in mast cell progenitors induces a GATA switch in which GATA-1 replaces GATA-2 from the –2.8 kb GATA switch site of the Gata2 locus, which was linked to Gata2 repression and generation of erythroid, megakaryocytic and granulocytic progeny. During the differentiation of trophoblast giant cells, GATA-2 displaces GATA-3 at Gata2, which is associated with transcriptional activation. Though GATA switches have not been studied in many systems, it is attractive to propose that they represent common devices to change transcriptional activity in diverse biological contents. Two aspects of the GATA switch paradigm merit careful consideration. First, erythroid GATA switches inform us that different GATA factors can exert qualitatively distinct functions through an identical chromatin site; one GATA factor mediates target gene activation, while the other confers repression or vice versa. Thus, while different GATA factors share certain biochemical attributes,

including their highly conserved zinc finger module, intrinsic differences underlie the qualitatively distinct activities. A notable difference is the relative high and low stabilities of GATA-1 and GATA-2, respectively. As proteasome inhibition stabilizes GATA-2 and blocks GATA switches, the low stability appears to be an important determinant of GATA switches. Another important implication of the GATA switch paradigm is that GATA switches and the requisite factors/signals that control the switches represent a novel tool to control developmental processes. Since certain non-hematopoietic cell types can express multiple GATA factors, it would not be surprising if the erythroid GATA switch mechanism were applicable to non-hematopoietic contexts. Despite major progress in elucidating GATA factor mechanistic principles, many questions remain unanswered regarding how cellular signaling pathways dynamically control GATA factor activities and GATA factor dependent biological processes. In summary, GATA factor mechanistic principles (**Figure 10**) include: (1) GATA factors target a small subset of chromatin sites containing a cis-element with the consensus sequence WGATAA; (2) GATA-1 activates or represses target genes in a FOG-1-dependent or –independent manner; (3) GATA-1 and GATA-2 commonly co-occupy chromatin sites with Scl/TAL1, and members of the Scl/TAL1 complex promote or suppress GATA factor-regulated transcription in a context-dependent manner; and (4) GATA switches can involve qualitatively distinct activities of different GATA factors through an identical chromatin site(Bresnick et al. 2012)

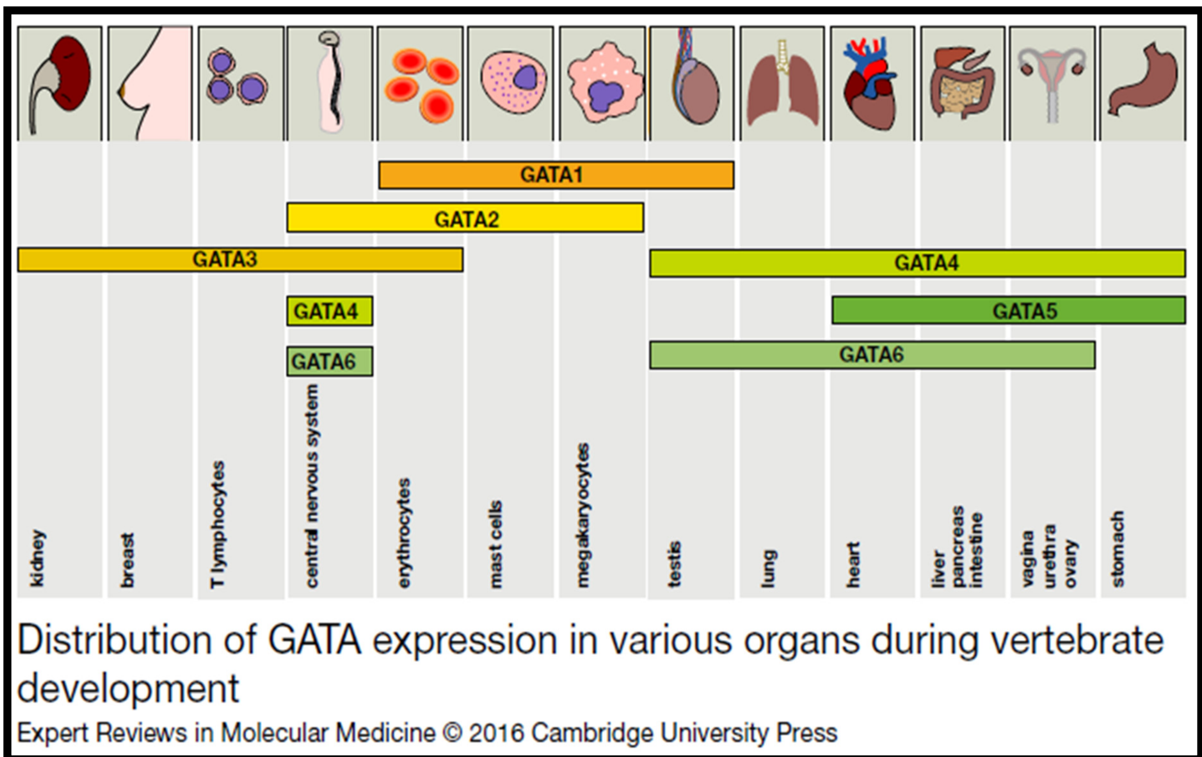


**Figure 9** Distribution of GATA expression in various organs during vertebrate development. The expression of all GATA factors is depicted in the corresponding tissues. The distribution of the expression patterns roughly reflects the two GATA subgroups (GATA1/2/3 versus GATA4/5/6). (Lentjes et al., 2016)

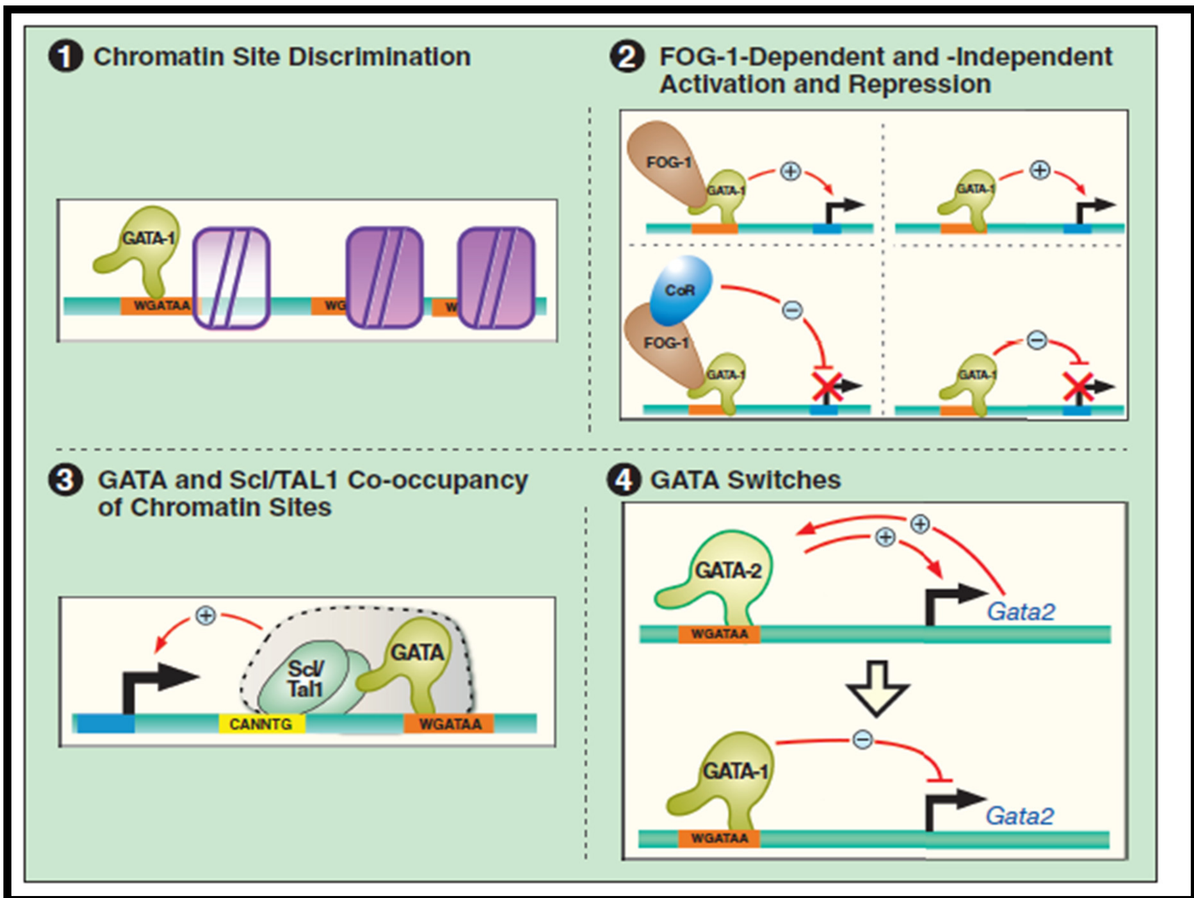


**Figure 10** GATA factor mechanistic principles. The models depict mechanistic principles derived from studies of GATA-1 and GATA-2. While the fundamental nature of these principles is likely to be shared by other GATA factors, additional GATA factor-specific mechanistic permutations are expected. (Bresnick, Katsumura et al. 2012)

### I.E.4. Specificity in the Regulation of Transcription by GATA Factors

The importance of GATA factors for development is illustrated by the embryonic lethality of most single GATA knockout mice. Moreover, GATA gene mutations have been described in relation to several human diseases, such as hypoparathyroidism, sensorineural deafness and renal insufficiency (HDR) syndrome, congenital heart diseases (CHDs) and cancer. GATA family members are emerging as potential biomarkers, for instance for the risk prediction of developing acute megalokaryblastic leakemia in Down syndrome and for the detection of colorectal- and breast cancer.(Lentjes, Niessen et al. 2016)

### I.E.4.1. *Post-translational Modifications*

Chromosomal alterations as well as regulation of GATA genes and proteins on transcriptional and post-transcriptional levels can also contribute to disease development. Recently it has been shown that combined tet methylcytosine dioxygenase 2 (TET2) and fms related tyrosine kinase 3 (FLT3) mutations regulate epigenetic silencing of GATA2 by promotor hypermethylation in human AML. In clear cell renal cell carcinomas downregulation of GATA3 expression by promoter hypermethylation results in decreased expression of TbetaRIII, a protein with tumour suppressor features, during disease progression . Presence of suppressive histone (H3K27) trimethylation of GATA3 together with absence of the GATA3 protein in anaplastic large cell lymphoma implicates epigenetical contribution in the pathogenesis of this disease. Clues about the transcriptional regulation of the GATA4 and GATA6 genes come from a SUMO-specific protease 2 (SENP2) knockout model. These mice have reduced expression of GATA4 and GATA6 and defects in the embryonic heart. In SENP2 deficient embryos sumoylation of CBX4, accumulates and occupies the promoters of GATA4 and GATA6, thereby leading to transcriptional repression. GATA4 is located at chromosome 8p, a chromosomal locus frequently deleted in multiple tumour types such as colorectal and oesophageal cancer. Alternatively GATA4 can be downregulated via epigenetic silencing, such as hypoacetylation of histones H3 and H4 and promoter CpG island hypermethylation, which has been observed in colorectal, gastric, oesophageal, lung, ovarian and HPV-driven oropharyngeal cancer, in GBM and in diffuse large B-cell lymphoma. In contrast, GATA4 amplification is recently described in certain gastric cancer which indicates a more oncogenic function. Further studies are needed to unravel the molecular mechanisms of GATA4 amplified in comparison with GATA4 methylated gastric cancers. GATA5 is located at chromosome 20q13, a locus which is often amplified and methylated in multiple cancer types. No coding sequence mutations in GATA4 and GATA5 have been described so far in colorectal- and breast cancer. However, promoter methylation of GATA5 might be established in order to downregulate increased gene expression imposed by amplification. Identified post-transcriptional modifications on GATA proteins include acetylation, phosphorylation and methylation. Protein stability of GATA2 and GATA3 is regulated by phosphorylation and ubiquitilation. Phosphorylation of GATA3 by respectively Cyclin-

dependent kinase 1 (CDK1) and CDK2 was required for F-box/ WD repeat-containing protein 7 (Fbw)-7 mediated ubiquitilation and degradation and contributed to precise differentiation of HSCs and T-cell lineages. How GATA acetylation influences transcriptional processes has been investigated for GATA1. It turns out that bromodomain protein Brd3 binds to acetylated GATA1 to regulate the chromatin occupancy at erythroid target genes. For GATA4 post-transciptional modifications have mainly been studied in the context of hypertrophy of the heart. Activation of GATA4 occurs in part through acetylation by the transcriptional coactivator p300. Takaya et al. identified 4 GATA4 lysine residues that, when mutated, lacked p300-induced acetylation, DNA binding and transcriptional activities. Phosphorylation of p300 by Cdk9 increases the ability of p300 to induce acetylation and DNA binding of GATA4. Alternatively, phosphorylation of GATA4 on serine 105 is critical for a productive cardiac hypertrophic response to stress stimulation in adult mice. Deacetylation of GATA4, and subsequent suppression of transcriptional activation, is mediated by histone deacetylase 2 (HDAC2) and the small homeodomain factor Hopx. Recently it was reported that the GATA4 protein is methylated by Polycomb-repressive complex 2 member Ezh2. This reduced the interaction with and acetylation by p300, thereby reducing GATA4’s transcriptional activity. Together, this emphasises how important post-transciptional modifications are for the regulation of GATA activity.(Lentjes, Niessen et al. 2016)

# Aim Of The Thesis

# II AIM OF THE STUDY

The Na+-$Ca^{2+}$ exchanger 3 (NCX3) is a plasmamembrane protein important for the cellular regulation of calcium and sodium homeostasis and is exclusively expresses in the brain and skeletal muscle (Annunziato et al. 2004a)). Several studies demonstrated that ncx3 silencing:(1) increased the infarct volume size after brain ischemia (Pignataro et al. 2004a; Molinaro et al. 2008)(Pignataro et al., 2004; Boscia et al., 2006; Molinaro et al., 2008), (2) partially reverted the preconditioning/postconditioning-induced neuroprotection (Pignataro et al., 2009; Pignataro et al., 2011b; Pignataro et al., 2011a) (3) caused an impairment in hippocampal long-term potentiation and in spatial learning and memory (Molinaro et al., 2011), (4) determined in in vitro model of Alzheimer Disease a hyperfunctional form delays caspase-12 activation and consequent neuronal death (Pannaccione et al., 2012). At transcriptional level it has been demonstrated that ncx3 mRNA expression is up-regulated by the transcriptional activator adenosine 3',5' cyclic monophosphate (cAMP) response element binding protein (CREB) (Gabellini et al., 2003) and is down-regulated by the transcriptional repressor downstream regulatory element antagonistic modulator DREAM (Gomez-Villafuertes et al., 2005) via a genetic mechanism . Interestingly, the ncx3 promoter (ncx3-pr) sequence contains several transcription factors (TF) motives, such as for the Early growth response protein 1 (EGR1), GATA transcription factors 1, 2 and 3 (GATA1, GATA2 and GATA3) and specificity protein 1, 3 and 4 (Sp1, Sp3 and Sp4) (Gabellini, 2004). Since until now only few papers have specifically studied the transcriptional regulation of ncx3 in the brain (Pignataro et al., 2009; Formisano et al., 2015), we investigated the role of above mentioned TF to modulate ncx3. Interestingly, among all the TF studied only GATA3 increased ncx3 expression. Then, we revealed the presence of sensitive GATA elements on the ncx3 brain promoter and the specificity of GATA3 to bind this sequence. In addition, because GATA3 regulates its target gene by modulating the methylation status of the histone 3 lysine 4 (H3K4me) (Wei et al., 2011), we investigated whether the GATA3 and the histone-lysine N-methyltransferase 2A (KMT2A) that is a H3K4-specific methyltransferase (Jakovcevski et al., 2015) form a complex able of activating ncx3 in *in vivo* model of brain ischemic preconditioning.

# Materials And Methods

## III. MATERIALS AND METHODS

### III.1. Materials

Luciferase reporter kits and luciferase vectors were from Promega (Milan, IT). Synthetic oligonucleotides were from Primm (Milan, IT). siRNAs for GATA3 (siGATA3) (sc-61845) was from Santa Cruz Biotechnology, INC. this compound was dissolved in dimethyl sulfoxide (DMSO) and diluted before application to a final DMSO concentration lower than 0.2%. All common reagents were of the highest quality and were purchased from Sigma (Milan, IT).

### III.2. Primary Cortical Neurons

Primary cortical neurons were prepared from 17-day-old Wistar rat embryos (Charles River, Calco, IT) and used after 7 days. Cytosine arabinoside (2.5 μM) was added the second day to reduce glial contamination. The experiments on primary cortical neurons were performed according the procedures described in experimental protocols approved by Ethical Committee of the "Federico II" University of Naples. Briefly, dissection and dissociation were performed in Ca2+/Mg2+-free phosphate-buffered saline (PBS) containing glucose (30 mM/l). Tissues were incubated with papain for 10 minutes at 37°C and dissociated by trituration in Earl's Balanced Salt Solution (EBSS) containing DNAse (0,16 U/ml), bovine serum albumin (10 mg/ml), and ovomucoid (10 mg/ml). Neurons were plated in plastic Petri dishes (Falcon™ Becton-Dickinson, Buccinasco, IT) pre-coated with poly-D-lysine (20 μg/ml), were grown in MEM/F12 containing glucose, 5% of deactivated fetal bovine serum (FBS) and 5% of horse serum (HS), glutamine (2 mM/l), penicillin (50 Units/ml), and streptomycin (50 μg/ml) (Invitrogen, Milan, IT). Cell density was 2x106 cells/well for 12-well plate for luciferase assay, 5x106 cells/well for 60 mm for qRT-PCR and 15x106 cells/well for 100 mm for Western Blot analysis, ChIP analysis and re-ChiP.

### III.3. Transfection with Expression Plasmids or Small Interfering RNA (siRNA) and Luciferase Reporter Assay, in Cortical Neurons

Cortical neurons were transfected with 50nM of negative control siRNA (siCTL) and 50nM of siRNA specific for GATA3 (siGATA3). To overexpress AP-2, EGR1, GATA1,

GATA2, GATA3 and Sp4 neurons were transfected with above mentioned constructs in the following amounts: 1.3 µg for 12-well plates, 7 µg for 60 mm plates and 15 µg for 100 mm plates. Each transfection was performed at 5 DIV in Optimem with Lipofectamine LTX (15338-100, Invitrogen, Milan, IT), as suggested by the producer. After 2 hours transfection mix was replaced with fresh medium. For luciferase assay experiments, cortical neurons were transfected in 12-well plates. Cells were co-transfected with 1 µg of total DNA vectors; the reporters (560 ng each) were the following: (1) the pGL3basic construct, (2) the pGL3-ncx3, that contains 500bp of the ncx3 brain promoter, (3) the pGL3-ncx3/GATA3mut, that contains a mutation in GATA binding site of ncx3 brain promoter. Mutagenesis of the GATA3 sites in the promoter was performed by using the QuickChange site-directed mutagenesis kit from Stratagene. Each transfection mix also contained 200 ng of the pRL-TK control vector expressing renilla luciferase gene. After 24hours of transfection lysates were analyzed with Dual-Luciferase Reporter Assay System kit (E1910) (Promega, Milan, IT), as already reported) (Formisano et al. 2013).

**III.4. Quantitative Real-Time PCR (qRT-PCR) Analysis**

The first-strand cDNA and quantitative real-time PCR was carried out as previously described (Formisano et al. 2007; Formisano, Guida et al. 2013). Using 1/10 of the cDNAs as a template, the quantitative real-time PCR was carried out in a 7500 fast real-time PCR system (Applied Biosystems, Monza, IT) by Fast SYBR Green Master Mix (cod. 4385610; Applied Biosystems, Monza, IT). Samples were amplified simultaneously in triplicate in one assay as follows: heating 2 min @ 50º C, denaturation 10 min @ 95º C, amplification and quantification 35 cycles of 15 sec @ 95º C, 1 min @ 60º C with a single fluorescence measurement. PCR data was collected using ABI Prism 7000 SDS software (Applied Biosystems). Normalization of the data was performed by HPRT as an internal control. Differences in mRNA content between groups were calculated as normalized values by using 2-ΔΔct formula and results were tested for significance using Relative Expression Software Tool (REST ©)(Formisano, Noh et al. 2007). The oligonucleotide sequences for NCX3 and Hypoxanthine phosphoribosyl-transferase (HPRT) were already published (Pignataro et al., 2011b; Formisano et al., 2012).

### III.5. Western Blotting

For Western blot analysis, cells or tissues were collected in ice-cold lysis buffer (Formisano, Guida et al. 2013) containing anti-protease cocktail (P8340 Sigma, Milan, IT). For NCX3, GATA3 and KMT2A expression, proteins (100 μg) were separated on 8% SDS polyacrylamide gels. Both were transferred onto Hybond ECL nitrocellulose membranes (Amersham, Milan, IT). Membranes were blocked with 5% non fat dry milk in 0.1% Tween 20 (Sigma, Milan, IT) (2 mM Tris-HCl and 50 mM NaCl pH 7.5) for 2 hours at room temperature, and then they were incubated overnight at 4°C in the blocking buffer with the 1:250 monoclonal antibodies against GATA3 (sc-268) and KMT2A (sc-374392) (Santa Cruz Biotechnology, INC), 1:1000 monoclonal antibodies against NCX-3 (Swant, Bellinzona, Switzerland) 1:1000 β-actin (A 4700) (Sigma, Milan, IT), 1:15000 α-tubulin (Sigma, Milan, IT). Finally, after the incubation with primary antibodies, membranes were washed with 0.1% Tween 20, followed by incubation with secondary antibodies for 1 h at room temperature. Immunoreactive bands were detected with the ECL reagent (Amersham). The optical density of the bands, normalized to β-actin or tubulin, was determined by Chemi-Doc Imaging System (Bio-Rad, Hercules, CA).

### III.6. Chromatin Immunoprecipitation (ChIP) and Re-Chip Assay

Brain tissue and cortical neurons were processed into chromatin by use of published protocols (Formisano et al., 2007; Formisano et al., 2012) with some modifications. Cells and tissues were cross-linked with 1% formaldehyde, and then reaction was stopped by adding glycine to a final concentration of 0.125 M. Brain tissue and cells were washed three times in cold PBS containing proteinase inhibitors and then collected in a buffer containing: 50mM Tris pH 8.1, 1% SDS, 10 mM EDTA, and anti-protease cocktail. For cell and tissue samples, chromatin was fragmented by sonication into 200–500 bp fragments (6 rounds for cells and 15 rounds for brain tissue of 15 1-s pulses at 50% of maximum potency) by a Bandelin Sonopuls HD 2070 ultrasonic homogenizer (Bandelin, Berlin, Germany). Equal amounts of chromatin lysates (50 μg for cells and 70 μg for tissues) were incubated overnight with 3μg of antibody for GATA3 (sc-268) and KMT2A (sc-374392) (Santa Cruz Biotechnology,INC), Tri-Methyl-Histone H3(Lys4) (17420, Thermo Fisher Scientific), acetyl-Histone H4 (06-866, MERCK) and RNA POL II (R1530, Sigma, Milan, IT). After immunoprecipitation, the DNA-histone complex was collected with 40 μl of

salmon sperm DNA/protein A- or G-agarose beads for 2 hours (16-157, 16-201) (Millipore, Milan, IT). After rotating for 2 hours at 4°C on a spinning wheel, the beads were washed once with each of the following buffers in the order shown: high-salt buffer (0.1% SDS, 1% Triton, 2mM EDTA, 20mM Tris HCl pH 8.1, 500 mM NaCl); low-salt buffer (0.1% SDS, 1% Triton, 2mM EDTA, 20mM Tris HCl pH 8.1, 150mM NaCl); LiCl buffer (0.25M LiCl, 1% NP40, 1% deoxycholate, 1mM EDTA, 10mM Tris HCl pH 8.1), and with TE buffer two times at room temperature (10mM Tris pH 8.1 and 1mM EDTA). The precipitated fragments were eluted with a buffer containing 1% SDS and 0.1M NaHCO3. DNA was analyzed by qRT-PCR using Fast SYBR Green Master Mix (cod 4385610; Applied Biosystems, Milan, IT). Heating 2 min @ 50ºC, denaturation 10 min @ 95ºC, amplification and quantification 35 cycles of 30 sec @ 95ºC; 1 min @ 60°C with a single fluorescence measurement. The binding activity was graphically represented as the percentage of total input of chromatin and the results were analyzed using a previously described formula (Renthal et al., 2007). The following oligonucleotides were used for the amplification of immunoprecipitated DNA: ncx3-Br Forward 5'-AGCACGGACCTAGCGTTCA-3' and Reverse 5'-GGGCTTGCATCCGCATT -3', ncx3-Br Forward 5'-GGGTGCAGAAGAGAGCGCTGGC-3' and Reverse 5'-GCACAAAGCGCGGCGGCCCG-3'. For each amplification, melting curves and gel electrophoresis of the PCR product were used to verify their identities. Samples were amplified simultaneously in triplicate in one assay run. Re-ChIP experiments were employed to detect the simultaneous presence of GATA3 or KMT2A, respectively. Beads from the first ChIP with anti-GATA3 and anti-KMT2A were incubated with an equal volume of 10 nM dithiothreitol at 37 °C for 30 min, centrifuged at 12000 rpm for 1 min to elute DNA-bound proteins. The elution was repeated twice. The final elute was diluted 1:10 in lysis buffer containing a protease inhibitor cocktail and re-immunoprecipitated with the anti-KMT2A, the anti-GATA3 antibodies. ChIP and input DNA were analyzed by PCR. Denaturation 10 min @ 95ºC, 35 cycles of 30 sec @ 95ºC; 1 min @ 60ºC; 1 min @ 72ºC. The PCR products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide. For ChIP and Re-ChIP experiments we used ncx3-Br primers, since the amplified sequence of ncx3-Br contains the binding sites forGATA3, whereas as negative control were used primers binding ncx1 promoter region already tested (Formisano et al. 2015).

### III.7. *In Vivo* Studies

#### III.7.1. Experimental Groups

Male Sprague-Dawley rats (Charles River) weighing 250 to 300 g were housed under diurnal lighting conditions (12 h darkness/light). Experiments were performed according to the international guidelines for animal research. All experiments were approved by the Institutional Animal Care and Use Committee of the “Federico II” University of Naples, IT.

#### III.7.2. Transient Focal Ischemia and Ischemic Preconditioning

Transient focal ischemia was induced as previously described (Valsecchi et al. 2011), by suture occlusion of the middle cerebral artery (MCA) in male rats anesthetized using 1.5% sevoflurane, 70% N2O, and 28.5% O2. Achievement of ischemia was confirmed by monitoring regional cerebral blood flow through laser Doppler (PF5001; Perimed). Animals not showing a cerebral blood flow reduction of at least 70% were excluded from the study. Animals that did not show a cerebral blood flow reduction of at least 70% were excluded from the experimental group, as well as animals that died after ischemia induction. Rats were divided into 4 experimental groups: (1) sham-operated rats (Sham); (2) preconditioned rats (PC); (3) ischemic rats, subjected to transient MCA occlusion (tMCAO); and (4) preconditioned ischemic rats (PC+tMCAO). The sham-operated animals underwent the same experimental conditions except that the filament was not introduced; in the ischemic group, the MCA was occluded for 100 minutes; in the preconditioned ischemic group, rats were subjected to 30 minutes of tMCAO 72 hours before of 100 minutes of tMCAO. All animals were euthanized 24 h after the 100 min tMCAO. Rectal temperature was maintained at 37±0.5°C with a thermostatically controlled heating pad and a catheter was inserted into the femoral artery to measure arterial blood gases before and after ischemia (Rapid Laboratory 860, Chiron Diagnostic). All surgical procedures were performed under an operating stereomicroscope.

### III.8. Statistical Analysis

The data were evaluated as means ± SEM. Statistically significant differences among means were determined by ANOVA followed by Student-Newman-Keuls test. The threshold for statistical significance data was set at $p<0.05$

# Results

# IV.RESULTS

## IV.1.GATA3 Binds and Activates ncx3 by a Specific GATA Sequence

NCX3 human gene promoter contains binding sites for the following transcription factors: activator protein-2 (AP-2), Early growth response protein 1 (EGR1), GATA transcription factors 1, 2 and 3 (GATA1, GATA2 and GATA3) and Specificity protein 1, 3 and 4 (Sp1, Sp3 and Sp4) (Gabellini 2004). To evaluate the role of these transcription factors on the ncx3 gene promoter activity, primary cortical neurons were co-transfected with vectors overexpressing AP-2, EGR1, GATA1, GATA2, GATA3 and Sp4, and with the pGL3basic construct enclosing the 650 bp genomic fragment containing the minimal ncx3 promoter region and exon 1-flanking region upstream of the ncx3 exon1 gene (pGL3-ncx3), previously used (Gomez-Villafuertes et al. 2005). Importantly, these constructs increased AP-2, EGR1, GATA1, GATA2, GATA3 and Sp4 mRNA expression by 177.1%, 122.46%, 119.35%, 113.03%, 103.97% and 93.38%, respectively (Figs.1 A–F).

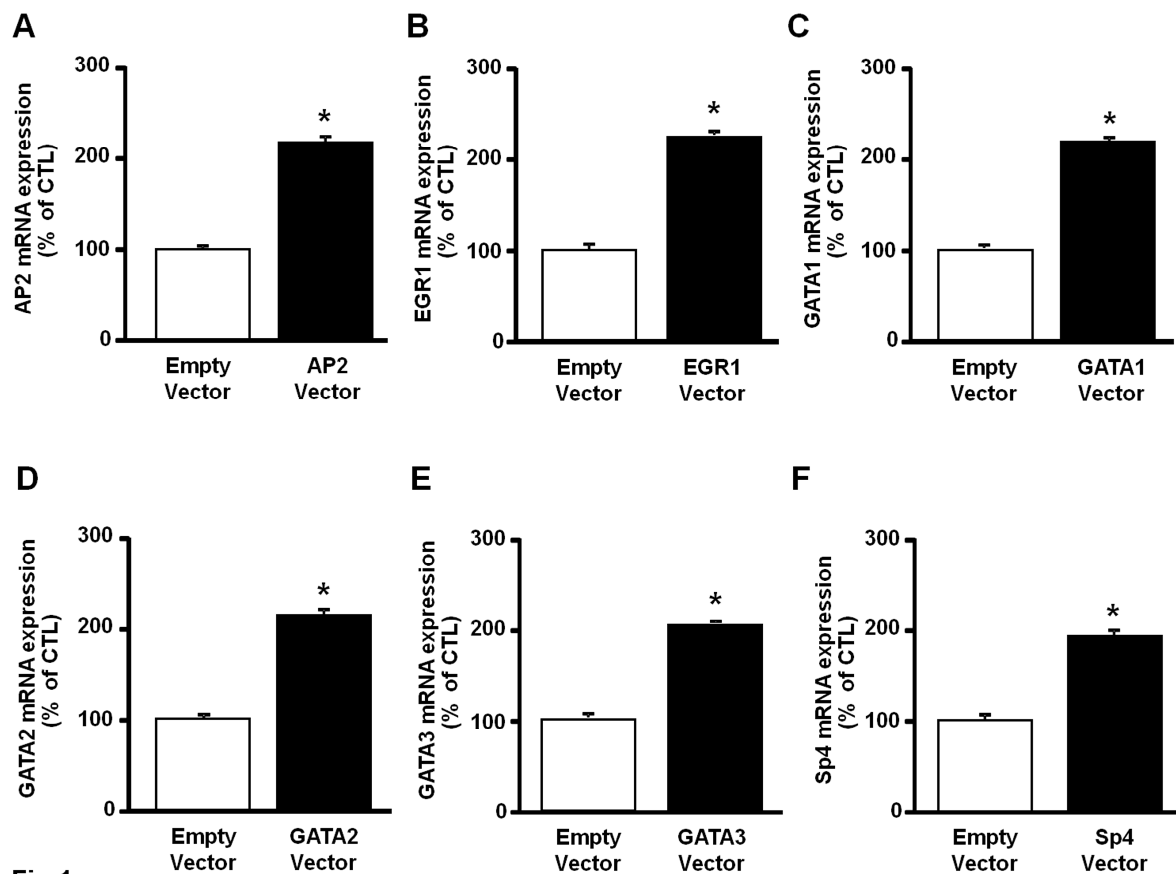


Fig.1

**Figure 1 Cortical neurons transfected with constructs for AP-2, EGR1, GATA1, GATA2, GATA3 and Sp4**.

**(A–F)**, qRT-PCR of AP-2, EGR1, GATA1, GATA2, GATA3 and Sp4 genes in transiently transfected neurons with Empty Vector and AP-2, EGR1, GATA1, GATA2, GATA3 and Sp4 constructs. Graphs show the quantification of the ratio of AP-2, EGR1, GATA1, GATA2, GATA3 and Sp4 to HPRT. Bars represent mean ± S.E.M. n = 3 per group. *p ≤ 0.05 versus EV.

As shown in the Luciferase Assay represented in Figure 2 A, only GATA3 overexpression significantly increased ncx3 gene promoter activity. Additionally, a new construct consisting in ncx3-pr containing a mutation in the GATA binding site induced by site direct mutagenesis was generated (pGL3-ncx3 GATA3mut). Cotransfection of pGL3-ncx3 GATA3mut into cortical neurons with an expression plasmid of GATA3 did not increase ncx3 promoter activity (Fig. 2B). Moreover, we performed a ChIP analysis in PC12 cells that demonstrated that the binding of GATA3 on its specific sequence on ncx3 brain promoter significantly increased in cell overexpressing GATA3 (Fig. 2C).These results indicate that GATA3 increased ncx3 gene promoter activity in a sequence specific manner.

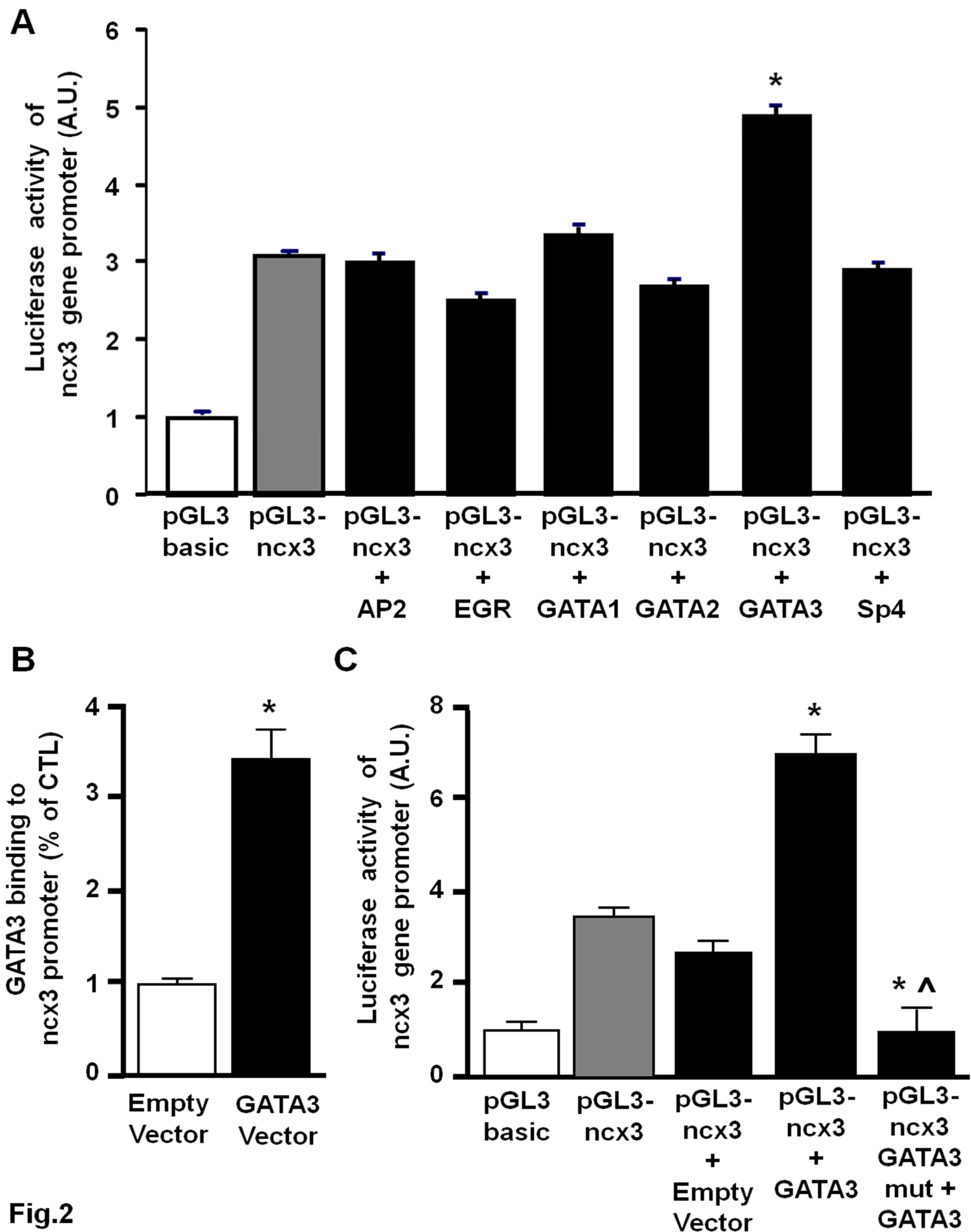


**Fig 2 GATA3 binds GATA sequence on ncx3 promoter.**

**(A)** Luciferase activity of cortical neurons cotransfected with pGL3 basic, pGL3-ncx3 alone or with AP2, EGR, GATA1, GATA2, GATA3 and Sp4 constructs. Bars represent mean ± S.E.M. n = 3 per group. $^{*}p \leq 0.05$ versus , pGL3-ncx3. **(B)** ChIP analysis of the ncx3 promoter containing the GATA/ncx3 region performed with anti-GATA3. The binding activity of GATA3 is graphically represented as the percentage of total input of chromatin DNA. Bars represent mean ± S.E.M. n = 3 per group. $^{*}p \leq 0.05$ versus EV. **(C)** Luciferase activity of cortical neurons cotransfected with the following constructs: **(1)** pGL3 basic, **(2)** pGL3-ncx3, **(3)** pGL3-ncx3 + Empty vector, **(4)** pGL3-ncx3 + GATA3 and **(5)** pGL3-ncx3 Mut + GATA3. Bars represent mean ± S.E.M. n = 3 per group. $^{*}p \leq 0.05$ versus , pGL3-ncx3 and ^ $p \leq 0.05$ versus , pGL3-ncx3 + GATA3.

Next, transfection of specific siRNAs for GATA3 (siGATA3) reduced its protein expression by 42.73% (Fig. 3A). Interestingly, siGATA3 significantly reduced ncx3 mRNA and protein expression (Figs. 3B, C), indicating that GATA3 is a ncx3 gene activator.

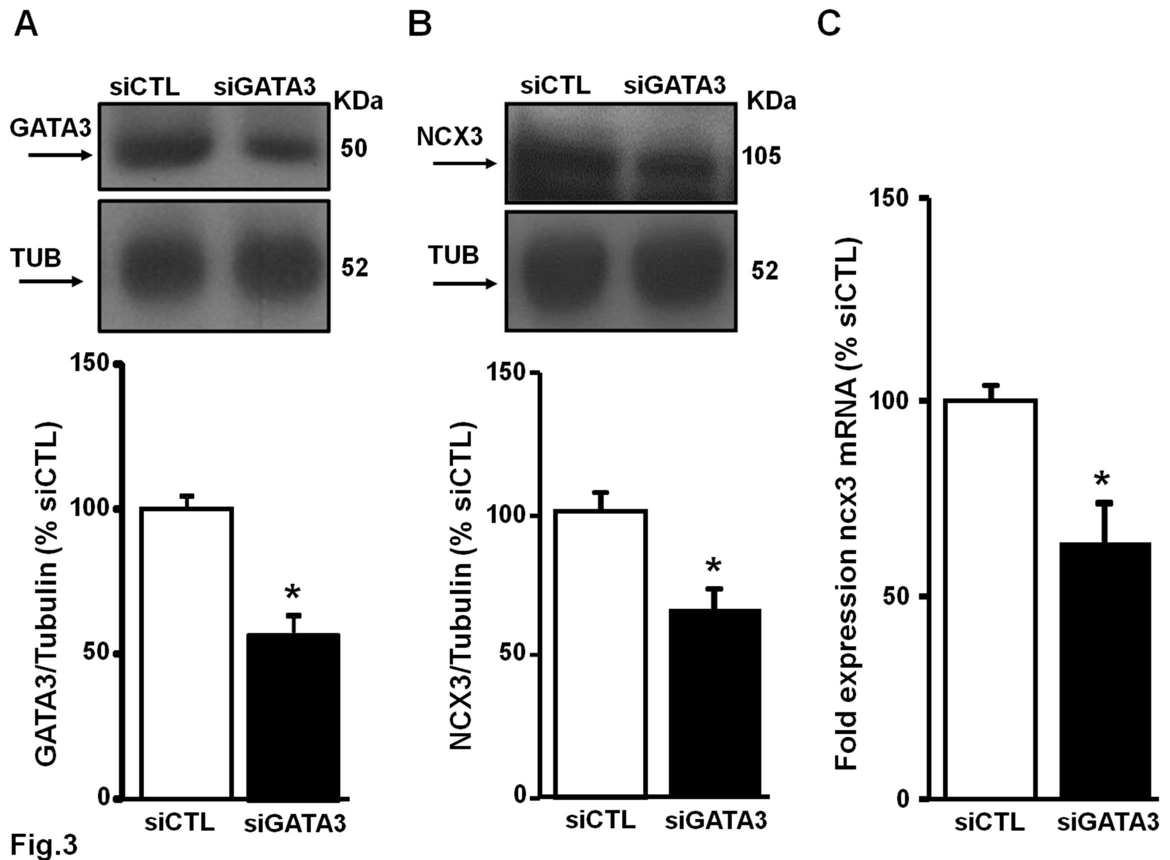


**Fig 3 GATA3 silencing reduces NCX3 mRNA and protein levels in primary cortical neurons**.

**(A,B)** Representative WB with quantification of GATA3 and NCX3 transfection with siCTL or siGATA3. Graphs show the quantification of the ratio of GATA3 and NCX3 to Tubulin. Bars represent mean ± S.E.M. n = 3-4 per group. *p ≤ 0.05 versus siCTL. **(C)** qRT-PCR of NCX3 gene in transiently transfected neurons with siCTL and siGATA3 constructs. Graphs show the quantification of the ratio of GATA3 to HPRT. Bars represent mean ± S.E.M. n = 3 per group. *p ≤ 0.05 versus siCTL

Coherently, neurons overexpressing GATA3 increased its protein expression by 42.73% (Fig. 4A), and significantly increased NCX3 mRNA and protein expression (Figs. 4B,C). These experiments confirm that GATA3 up-regulated ncx3 gene at transcriptional level.

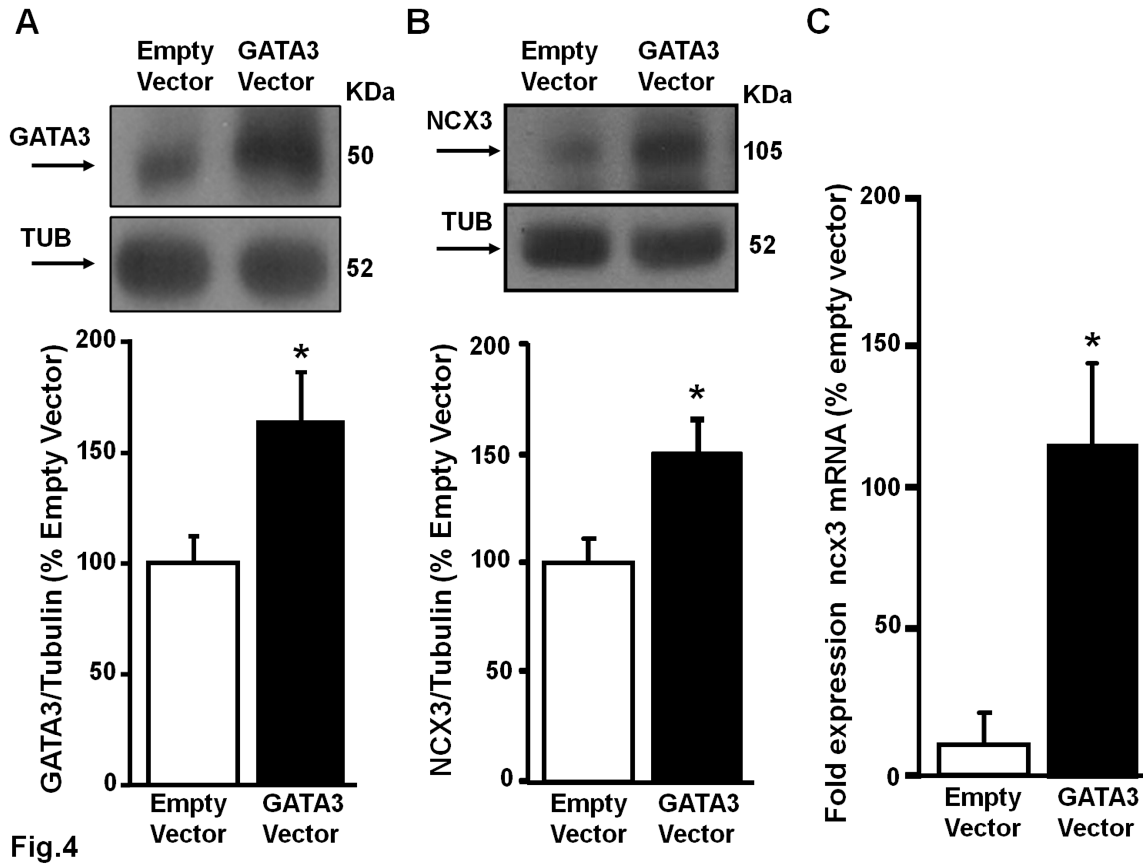


**Fig 4 GATA3 overexpression up-regulates NCX3 mRNA and protein levels in primary cortical neurons.** **(A,B)** Representative WB with quantification of GATA3 and NCX3 transfection with an empty vector or with a construct overexpressing GATA3. Graphs show the quantification of the ratio of GATA3 and NCX3 to Tubulin. Bars represent mean ± S.E.M. n = 3 per group. *p ≤ 0.05 versus Empty Vector. **(C)** qRT-PCR of NCX3 gene in transiently transfected neurons with empty vector and GATA3 constructs. Graphs show the quantification of the ratio of GATA3 to HPRT. Bars represent mean ± S.E.M. n = 3 per group. *p ≤ 0.05 versus Empty Vector.

### IV.2. GATA3, KMT2A and NCX3 Increase in the Temporoparietal Cortex After Preconditioning+Ischemia

Because ncx3 gene and protein are increased in an *in vivo* model of brain ischemic Preconditioning(Formisano, Guida et al. 2015) and GATA3 activates its target genes by increasing the levels of the lysine 4 tri-methylation of histone H3 (H3K4me3) (Wei et al. 2011), an epigenetic modification catalyzed by the histone methyl transferase 2A (KMT2A) enzyme, that is basically expressed in the brain (Jakovcevski et al. 2015), we studied in the temporoparietal cortex of rats subjected to Preconditioning+Ischemia (PC+tMCAO) GATA3, KMT2A and NCX3 protein expression. Western blot analysis revealed that PC+tMCAO after 12 and 24 hours increases the expression of GATA3 and KMT2A and returns to sham level at 48 and 72 hours (Figs.5 A, B). Interestingly, NCX3 protein expression levels increased 24, 48 and 72 hours after PC+tMCAO, compared with the sham (Fig. 5D). On the contrary, ncx3 gene increased in a time dependent manner at 12 and 24 hours, but it did not modify at 48 and 72 hours after PC+tMCAO, compared to sham (Fig. 5C).

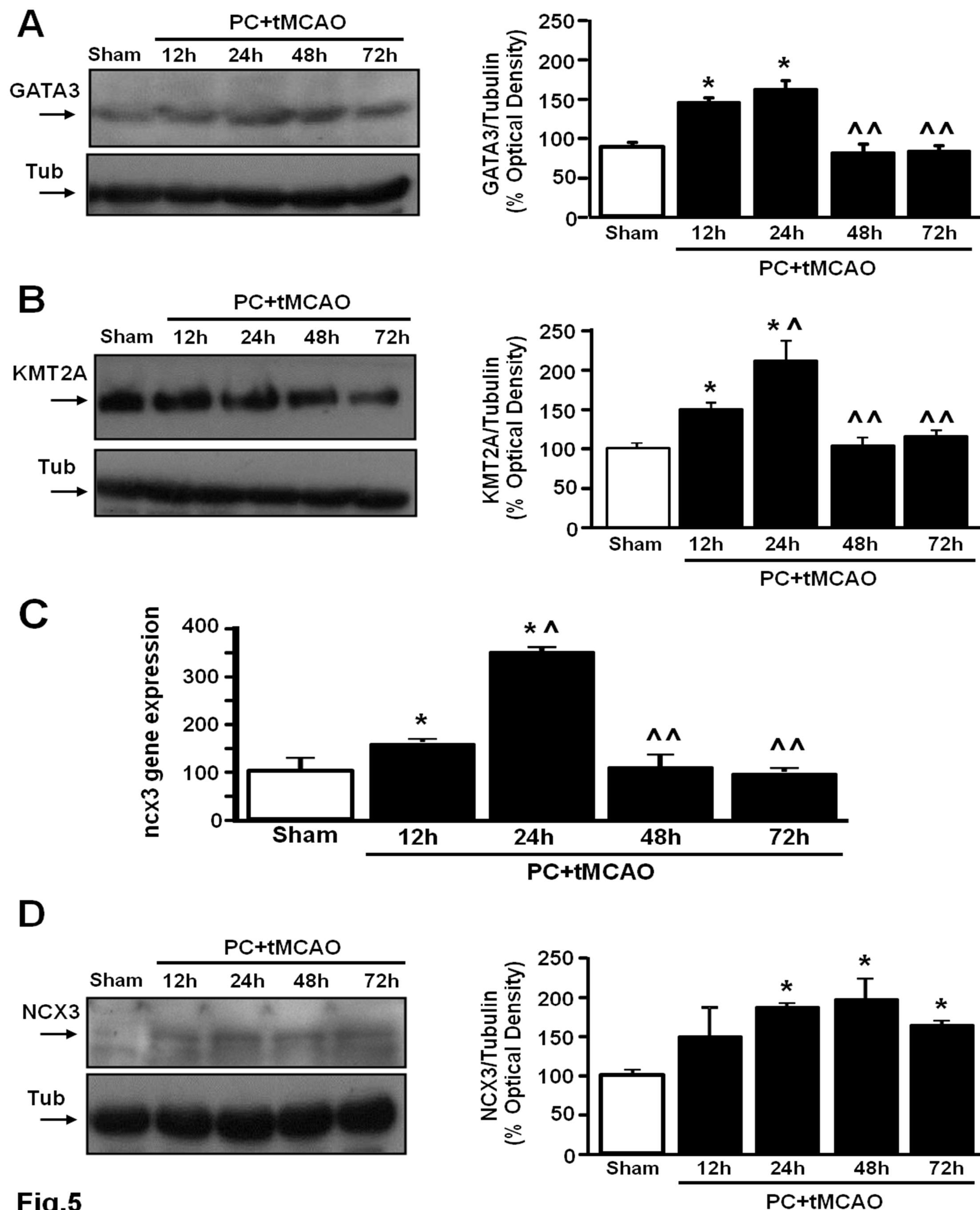


**Fig 5 Expression levels of GATA3, KMT2A and NCX3 protein expression and ncx3 gene after 12, 24, 48 and 72 hours of PC+tMCAO**

**(A,B,D)** Representative WB with quantification of GATA3, KMT2A and NCX3 in the rat ipsilateral–temporoparietal cortex in Sham and PC+tMCAO groups. Graphs show the quantification of the ratio of GATA3, KMT2A and NCX3 to Tubulin. Bars represent mean ± S.E.M. n = 4 per group. *p ≤ 0.05 versus Sham, ^ p ≤ 0.05 versus 12 hours of PC+tMCAO and ^^ p ≤ 0.05 versus 12 and 24 hours of PC+tMCAO. **(C)** qRT-PCR of NCX3 gene in the rat ipsilateral–temporoparietal cortex in Sham and PC+tMCAO groups. Graphs show the quantification of the ratio of NCX3 to HPRT. Bars represent mean ± S.E.M. n = 4 per group. *p ≤ 0.05 versus Sham , ^ p ≤ 0.05 versus 12 hours of PC+tMCAO and ^^ p ≤ 0.05 versus 12 and 24 hours of PC+tMCAO.

### IV.3. GATA3, KMT2A and NCX3 Increase in the Temporoparietal Cortex After, Preconditioning, and Preconditioning+Ischemia but not After Ischemia

In the ipsilesional-temporoparietal cortex, GATA3, KMT2A and NCX3 protein expression significantly increased after 24 hours of preconditioning alone (PC), or after PC+tMCAO. Interestingly, western blot after 24 hours of tMCAO revealed a reduction of NCX3, but not of GATA3 and KMT2A protein expression (Figs.6 A-C). To identify the possible interaction between GATA3 and KMT2A, their binding activity to the ncx3 promoter sequence was evaluated in rats subjected to tMCAO and PC+tMCAO by Re-ChIP experiments. As shown in figures 6 D and E chromatin from Sham, tMCAO, PC and PC+tMCAO groups was sequentially immunoprecipitated with GATA3 and KMT2A antibodies (Fig. 6 D), or conversely, with KMT2A antibody followed by GATA3 antibody (Fig. 6 E). Real-time PCR experiments identified the colocalization of GATA3 and KMT2A on the ncx3 promoter gene (Figs. 6 D, E). These results demonstrate that the binding between GATA3/KMT2A on ncx3 promoter increased after PC and PC+tMCAO

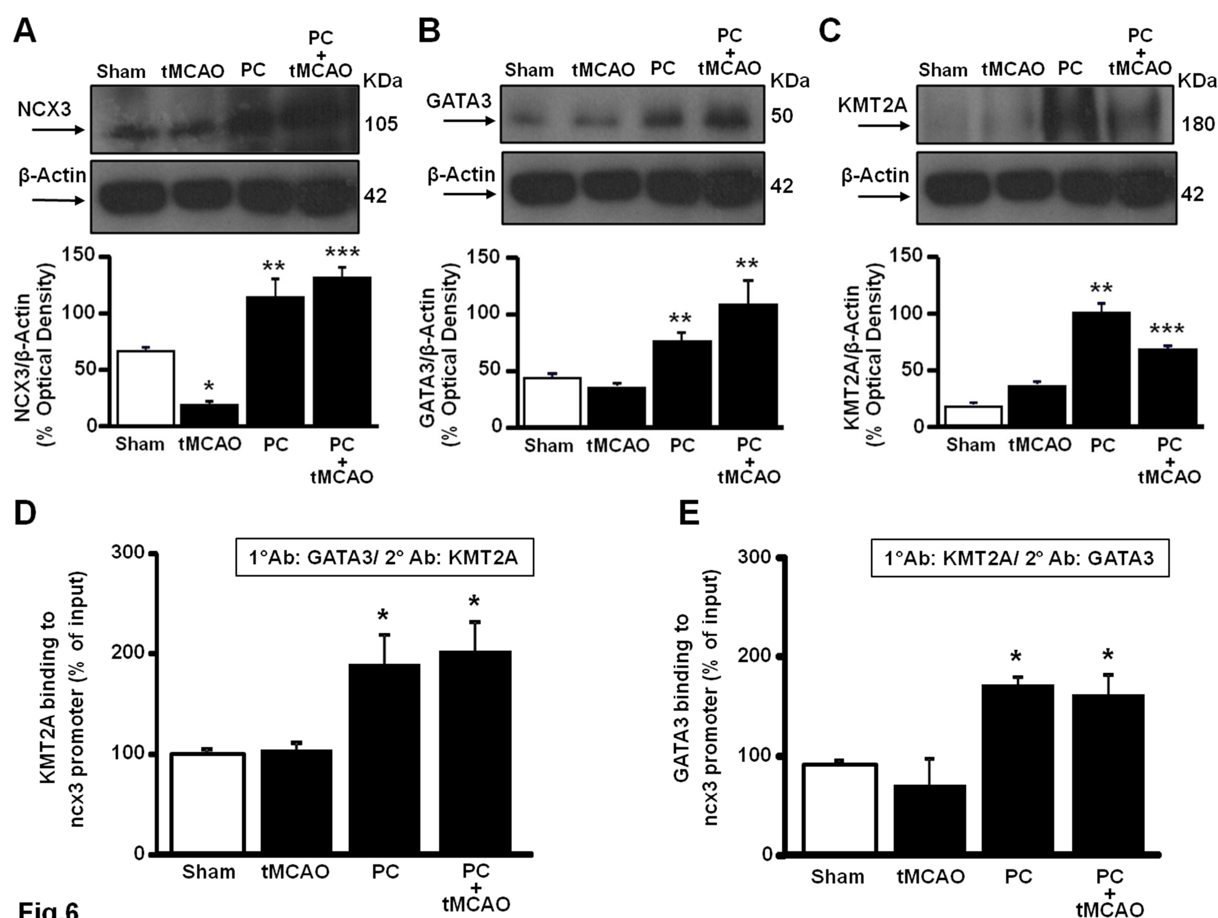


**Fig 6 Protein levels of GATA3, KMT2A and NCX3 and GATA3/KMT2A colocalization on ncx3-Br in the ipsilateral–temporoparietal cortex after 24 hours of tMCAO, PC and PC+tMCAO**

**(A-C)** Representative WB with quantification of GATA3, KMT2A and NCX3 in the rat ipsilateral–temporoparietal cortex in: (1) Sham, (2) tMCAO, (3) Preconditining alone (PC) and (4) PC+tMCAO groups. Graphs show the quantification of the ratio of GATA3, KMT2A and NCX3 to Tubulin. Bars represent mean ± S.E.M. n = 3 per group. Figure 6A *p ≤ 0.05 versus Sham, **p ≤ 0.05 versus Sham and tMCAO and ***p ≤ 0.05 versus all; Figure 6B *p ≤ 0.05 versus Sham, **p ≤ 0.05 versus Sham and tMCAO; Figure 6C **p ≤ 0.05 versus Sham and tMCAO and ***p ≤ 0.05 versus all. **(D-E)** Re-ChIP analysis of ncx3-pr in the ipsilateral–temporoparietal cortex in (1) Sham, (2) tMCAO, (3) Preconditining alone (PC) and (4) PC+tMCAO groups. Primary ChIP products for anti-GATA3 (1° Ab) were subjected to re-ChIP with anti-KMT2A (2° Ab). The binding activity of GATA3 and KMT2a is graphically represented as the percentage of total input of chromatin DNA. Bars represent mean ± S.E.M. n = 3 per group. *p ≤ 0.05 versus Sham and tMCAO.

Specifically, it is known that the transcription factor GATA3 regulates its target gene by histone lysine acetylation (Fields et al. 2002) and methylation(Wei, Abraham et al. 2011), that are both marker of transcriptional activation. Therefore, through experiments of ChIP assay, we studied the modification of the: (1) acetylated histone protein H4, (2) tri-methylated lysine 4 histone protein H3 and (3) the binding of RNA polymerase II (RNA-Pol II) on ncx3 promoter sequence in the ipsilesional–

temporoparietal cortex of rats subjected to tMCAO, PC and PC+tMCAO and compared it with Sham. We found that tMCAO reduced the amount of the ncx3 promoter associated with acetylated histone H4 and RNA-Pol II (Figs. 7A, C), but did not significantly alter the trimethylation status of residue K4 of histone H3 (Fig. 7B). Notably, ncx3 promoter association with acetylated H4 was not significantly modified by PC only and PC+tMCAO (Fig. 7A). On the other hand, PC+tMCAO increased H3K4me3 binding on ncx3 promoter sequence, in parallel with an increased amount of the ncx3 promoter associated with RNA-Pol II (Fig. 7B, C). Altogether, these results demonstrate that after PC and PC+tMCAO, GATA3 and H3K4 increase their binding on ncx3 determining an increase of ncx3 transcription

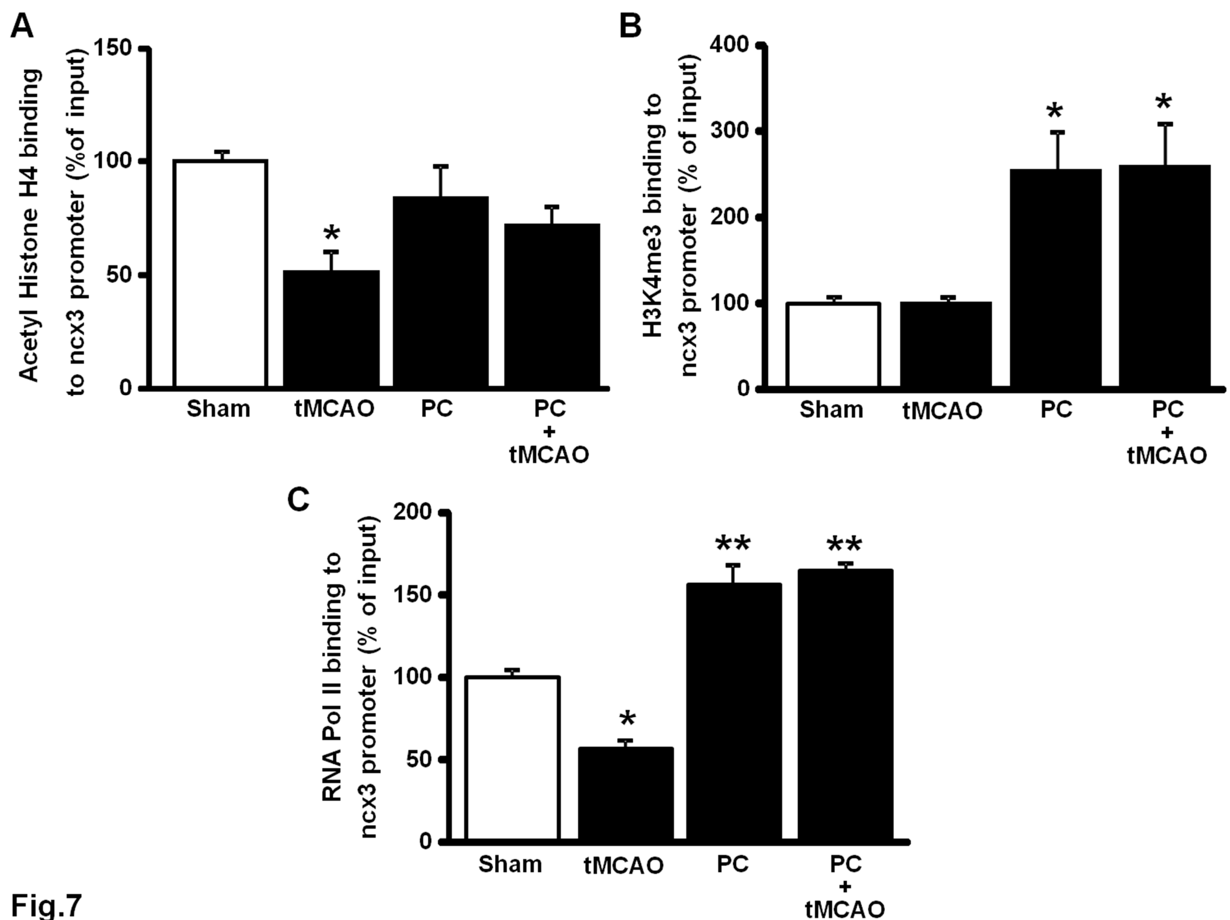


**Fig 7 Effect of tMCAO and PC+tMCAO rat models on H4 acetylation, , H3-K4me3 and RNA-Pol II to ncx3-Br (A-C)** ChIP analysis of ncx3-pr in the ipsilateral–temporoparietal cortex in: (1) Sham, (2) tMCAO, (3) Preconditining alone (PC) and (4) PC+tMCAO groups. The binding activity of H4 acetylation (H4 acetyl), H3-K4me3 and RNA-Pol II is graphically represented as the percentage of total input of chromatin DNA. Each column represents the mean SEM (n = 3 per group. Figure 7A *p ≤ 0.05 versus Sham; Figure 7B *p ≤ 0.05 versus Sham and tMCAO; Figure 7C *p ≤ 0.05 versus Sham and tMCAO and **p ≤ 0.05 versus all

# Discussion

## V. DISCUSSION

This work demonstrated that NCX3 is epigenetically increased in brain ischemic preconditioning by GATA3/KMT2A complex. Specifically, our findings indicate that these proteins form a transcriptional complex on the ncx3-pr sequence in brain ischemic preconditioning. Particularly, 24 hours after PC+tMCAO was the time point in which GATA3 and KMT2A protein and ncx3 mRNA arised the maximum increase. Furthermore, we found that ncx3 is a new GATA3 target gene. Indeed, GATA3 selectively activates neuronal NCX3 expression via GATA sequence. Interestingly , ncx3-GATA sequence on human genome is located on the Chr6:105.098.530-105.098.539, and has 100% interspecies homology between human, rat and mouse (Fig. 8). Interestingly, site-direct mutagenesis studies on ncx3-GATA3 sequence in cortical neurons totally blocked GATA3 effect on the activation of NCX3, due to a reduced GATA3 binding to the ncx3 promoter sequence. Moreover, GATA3, as occurred for NCX3, is neuroprotective, as evidenced by the fact that its expression is required for maintaining neuronal sympathetic survival (Tsarovina et al., 2010). By ChIP experiments we found that the transcriptional activator GATA3, by recruiting the epigenetic writer KMT2A, forms a complex upregulating ncx3 in an in vivo model of brain ischemic preconditioning. Interestingly, neuronal ablation of KMT2A, in mouse post-natal forebrain and adult prefrontal cortex (PFC) is associated with increased anxiety and robust cognitive deficits without locomotor dysfunction (Jakovcevski, Ruan et al. 2015). Regarding the mechanism by which GATA3 regulates its target gene it has been reported that in lymphoid cells it facilitates both H3K4me1 and H3K4me2, as well as H3K27me3 at enhancers to mediate gene activation and repression, respectively (Wei, Abraham et al. 2011). The results of the present study for the first time identified that the specific mechanism by which the transcription factor GATA3 increased its target gene ncx3 involves histone lysine methylation through KMT2A. Ischemic preconditioning promotes association of GATA3 and KMT2A on ncx3 brain promoter sequence in the temporoparietal cortex, in accordance with a casual relation between GATA3 and KMT2A induction and ncx3 up-regulation. We further show that PC+tMCAO promotes an increased trimethylation of H3-K4 (index of gene activation), in parallel with an increased binding of the RNA Pol II to the ncx3 promoter sequence, an epigenetic signature of gene activation. Furthermore, H4 acetylation was reduced in tMCAO group, but not in

PC+tMCAO group, indicating that lysine methylation and acetylation are not associated and that methylation occurred in the exclusively preconditioning phase and acetylation only after stroke.

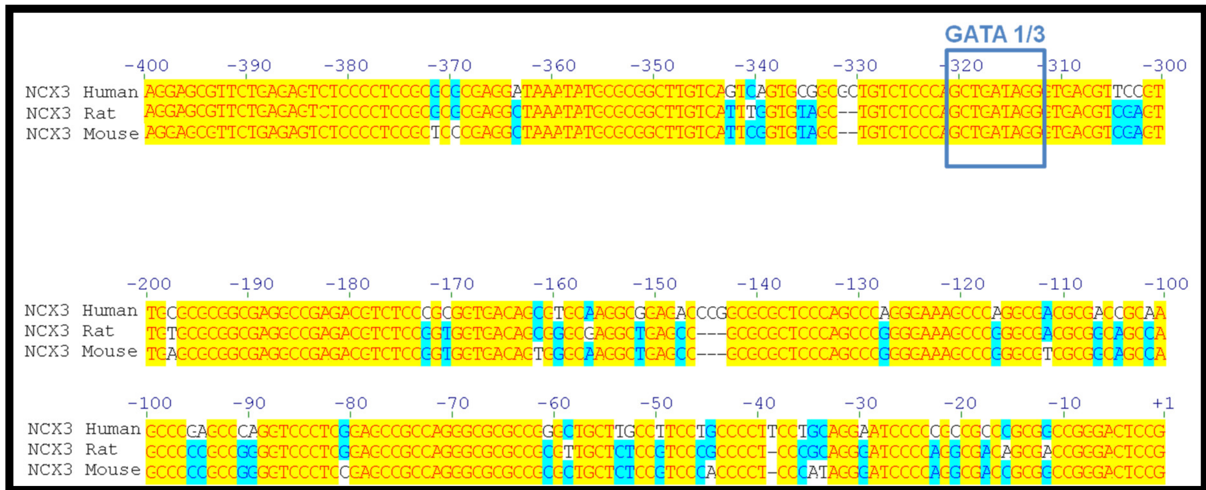


**Fig 8 Alignment GATA3 consensus sequence on NCX3 Promoter in Human/Rat/Mouse**

# VI. CONCLUSIONS

Collectively, these findings identify that the transcriptional activator GATA3, by recruiting KMT2A, up-regulates NCX3 in the brain through an increase of H3K4me3 on NCX3 gene (Fig.9). Moreover, the complex GATA3/KMT2A determines neuronal protection through the increase of NCX3 mRNA and protein expression, whose neurosurvival role has been demonstrated by several papers (Annunziato, Pignataro et al. 2004b; Secondo, Staiano et al. 2007; Formisano et al. 2008; Molinaro, Cuomo et al. 2008; Pignataro et al. 2011a; Pignataro et al. 2011b; Scorziello et al. 2013). Therefore, the development of drugs able to promote GATA3 and KMT2A up-regulation, and thus to increase NCX3 expression, could represent a newpharmacological strategy in stroke intervention.

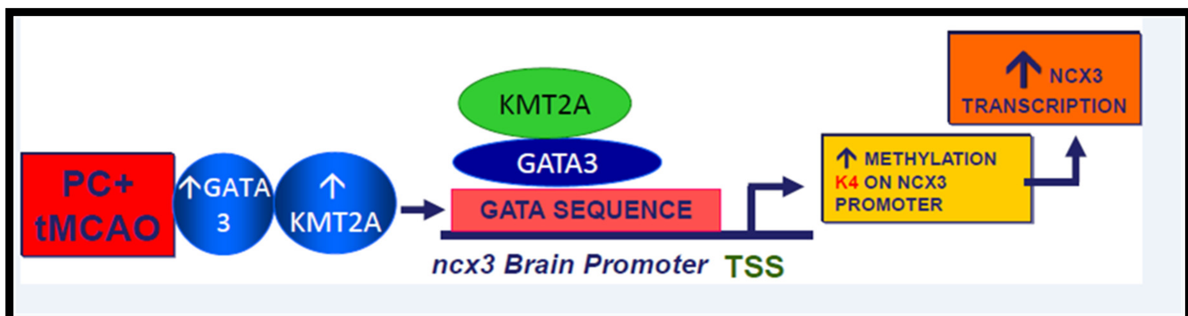


**Fig 9 Transcriptional Complex Regulating ncx3 expression in Brain Ischemic Preconditioning**

# References

# VII.REFERENCES

*Agell, N., R. Aligue, et al. (1998). "New nuclear functions for calmodulin." Cell Calcium **23**(2-3): 115-121.*

*Amoroso, S., A. Tortiglione, et al. (2000). "Sodium nitroprusside prevents chemical hypoxia-induced cell death through iron ions stimulating the activity of the Na+-Ca2+ exchanger in C6 glioma cells." J Neurochem **74**(4): 1505-1513.*

*An, W. F., M. R. Bowlby, et al. (2000). "Modulation of A-type potassium channels by a family of calcium sensors." Nature **403**(6769): 553-556.*

*Annunziato, L., G. Pignataro, et al. (2004a). "Pharmacology of brain Na+/Ca2+ exchanger: from molecular biology to therapeutic perspectives." Pharmacological reviews **56**(4): 633-654.*

*Annunziato, L., G. Pignataro, et al. (2004b). "Pharmacology of brain Na+/Ca2+ exchanger: from molecular biology to therapeutic perspectives." Pharmacol Rev **56**(4): 633-654.*

*Bannister, A. J. and T. Kouzarides (2004). "Histone methylation: recognizing the methyl mark." Methods Enzymol **376**: 269-288.*

*Bedford, M. T. and S. Richard (2005). "Arginine methylation an emerging regulator of protein function." Mol Cell **18**(3): 263-272.*

*Berger, S. L. (2007). "The complex language of chromatin regulation during transcription." Nature **447**(7143): 407-412.*

*Bhuiyan, M. I. and Y. J. Kim (2010). "Mechanisms and prospects of ischemic tolerance induced by cerebral preconditioning." Int Neurourol J **14**(4): 203-212.*

*Black, J. C., C. Van Rechem, et al. (2012). "Histone lysine methylation dynamics: establishment, regulation, and biological impact." Mol Cell **48**(4): 491-507.*

*Blaustein, M. P. and W. J. Lederer (1999). "Sodium/calcium exchange: its physiological implications." Physiol Rev **79**(3): 763-854.*

*Bresnick, E. H., K. R. Katsumura, et al. (2012). "Master regulatory GATA transcription factors: mechanistic principles and emerging links to hematologic malignancies." Nucleic Acids Res **40**(13): 5819-5831.*

*Breukels, V., W. G. Touw, et al. (2012a). "NMR structure note: solution structure of Ca(2)(+) binding domain 2B of the third isoform of the Na(+)/Ca(2)(+) exchanger." J Biomol NMR **54**(1): 115-121.*

*Breukels, V., W. G. Touw, et al. (2012b). "Structural and dynamic aspects of Ca2+ and Mg2+ binding of the regulatory domains of the Na+/Ca2+ exchanger." Biochem Soc Trans **40**(2): 409-414.*

*Buxbaum, J. D., E. K. Choi, et al. (1998). "Calsenilin: a calcium-binding protein that interacts with the presenilins and regulates the levels of a presenilin fragment." Nat Med **4**(10): 1177-1181.*

*Buxbaum, J. D., C. Lilliehook, et al. (2000). "Genomic structure, expression pattern, and chromosomal localization of the human calsenilin gene: no association between an exonic polymorphism and Alzheimer's disease." Neurosci Lett **294**(3): 135-138.*

*Caplan, L. R. (2000). "Acute stroke: seeing the full picture." Hosp Pract (1995) **35**(6): 65-71; discussion 71-62; quiz 113.*

*Caroni, P. and E. Carafoli (1983). "The regulation of the Na+ -Ca2+ exchanger of heart sarcolemma." Eur J Biochem **132**(3): 451-460.*

*Carrion, A. M., B. Mellstrom, et al. (1998). "Protein kinase A-dependent derepression of the human prodynorphin gene via differential binding to an intragenic silencer element." Mol Cell Biol **18**(12): 6921-6929.*

*Chakravarty, S., P. Jhelum, et al. (2017). "Insights into the epigenetic mechanisms involving histone lysine methylation and demethylation in ischemia induced damage and repair has therapeutic implication." Biochim Biophys Acta Mol Basis Dis **1863**(1): 152-164.*

*Chen, Y., D. L. Bates, et al. (2012). "DNA binding by GATA transcription factor suggests mechanisms of DNA looping and long-range gene regulation." Cell Rep **2**(5): 1197-1206.*

*Chrivia, J. C., R. P. Kwok, et al. (1993). "Phosphorylated CREB binds specifically to the nuclear protein CBP." Nature **365**(6449): 855-859.*

*Corneliussen, B., M. Holm, et al. (1994). "Calcium/calmodulin inhibition of basic-helix-loop-helix transcription factor domains." Nature **368**(6473): 760-764.*

*Cunningham, K. W. and G. R. Fink (1996). "Calcineurin inhibits VCX1-dependent H+/Ca2+ exchange and induces Ca2+ ATPases in Saccharomyces cerevisiae." Mol Cell Biol **16**(5): 2226-2237.*

*Cuomo, O., A. Vinciguerra, et al. (2015). "Ionic homeostasis in brain conditioning." Front Neurosci **9**: 277.*

*Cutter, A. R. and J. J. Hayes (2015). "A brief review of nucleosome structure." FEBS Lett **589**(20 Pt A): 2914-2922.*

*De Cesare, D., G. M. Fimia, et al. (1999). "Signaling routes to CREM and CREB: plasticity in transcriptional activation." Trends Biochem Sci **24**(7): 281-285.*

*DiPolo, R. and L. Beaugé (1998). "Differential up-regulation of Na+-Ca2+ exchange by phosphoarginine and ATP in dialysed squid axons." J Physiol **507 ( Pt 3)**: 737-747.*

*DiPolo, R., G. Berberián, et al. (2004). "Phosphoarginine regulation of the squid nerve Na+/Ca2+ exchanger: metabolic pathway and exchanger-ligand interactions different from those seen with ATP." J Physiol **554**(Pt 2): 387-401.*

*Dirnagl, U., C. Iadecola, et al. (1999). "Pathobiology of ischaemic stroke: an integrated view." Trends Neurosci **22**(9): 391-397.*

*Dirnagl, U., R. P. Simon, et al. (2003). "Ischemic tolerance and endogenous neuroprotection." Trends Neurosci **26**(5): 248-254.*

*Doering, A. E. and W. J. Lederer (1993). "The mechanism by which cytoplasmic protons inhibit the sodium-calcium exchanger in guinea-pig heart cells." J Physiol **466**: 481-499.*

*Dupont, C., D. R. Armant, et al. (2009). "Epigenetics: definition, mechanisms and clinical perspective." Semin Reprod Med **27**(5): 351-357.*

*Dyck, C., A. Omelchenko, et al. (1999). "Ionic regulatory properties of brain and kidney splice variants of the NCX1 Na(+)-Ca(2+) exchanger." J Gen Physiol **114**(5): 701-711.*

*Ferreri, K., G. Gill, et al. (1994). "The cAMP-regulated transcription factor CREB interacts with a component of the TFIID complex." Proc Natl Acad Sci U S A **91**(4): 1210-1213.*

*Fields, P. E., S. T. Kim, et al. (2002). "Cutting edge: changes in histone acetylation at the IL-4 and IFN-γ loci accompany Th1/Th2 differentiation." The Journal of Immunology **169**(2): 647-650.*

*Formisano, L., N. Guida, et al. (2015). "Sp3/REST/HDAC1/HDAC2 Complex Represses and Sp1/HIF-1/p300 Complex Activates ncx1 Gene Transcription, in Brain Ischemia and in Ischemic Brain Preconditioning, by Epigenetic Mechanism." J Neurosci **35**(19): 7332-7348.*

*Formisano, L., N. Guida, et al. (2013). "NCX1 is a new rest target gene: role in cerebral ischemia." Neurobiology of disease **50**: 76-85.*

*Formisano, L., K.-M. Noh, et al. (2007). "Ischemic insults promote epigenetic reprogramming of μ opioid receptor expression in hippocampal neurons." Proceedings of the National Academy of Sciences **104**(10): 4170-4175.*

*Formisano, L., M. Saggese, et al. (2008). "The two isoforms of the Na+/Ca2+ exchanger, NCX1 and NCX3, constitute novel additional targets for the prosurvival action of Akt/protein kinase B pathway." Molecular pharmacology **73**(3): 727-737.*

*Fujioka, Y., K. Hiroe, et al. (2000). "Regulation kinetics of Na+-Ca2+ exchange current in guinea-pig ventricular myocytes." J Physiol **529 Pt 3**: 611-623.*

*Gabellini, N. (2004). "Transcriptional regulation by cAMP and Ca2+ links the Na+/Ca2+ exchanger 3 to memory and sensory pathways." Mol Neurobiol **30**(1): 91-116.*

*Gabellini, N., S. Bortoluzzi, et al. (2002). "The human SLC8A3 gene and the tissue-specific Na+/Ca2+ exchanger 3 isoforms." Gene **298**(1): 1-7.*

*Giladi, M., S. Y. Lee, et al. (2015). "Structure-dynamic determinants governing a mode of regulatory response and propagation of allosteric signal in splice variants of Na+/Ca2+ exchange (NCX) proteins." Biochem J **465**(3): 489-501.*
*Gomez-Villafuertes, R., B. Torres, et al. (2005). "Downstream regulatory element antagonist modulator regulates Ca2+ homeostasis and viability in cerebellar neurons." Journal of Neuroscience **25**(47): 10822-10830.*
*Herdegen, T. and J. D. Leah (1998). "Inducible and constitutive transcription factors in the mammalian nervous system: control of gene expression by Jun, Fos and Krox, and CREB/ATF proteins." Brain Res Brain Res Rev **28**(3): 370-490.*
*Hilge, M., J. Aelen, et al. (2009). "Ca2+ regulation in the Na+/Ca2+ exchanger features a dual electrostatic switch mechanism." Proc Natl Acad Sci U S A **106**(34): 14333-14338.*
*Hilge, M., J. Aelen, et al. (2006). "Ca2+ regulation in the Na+/Ca2+ exchanger involves two markedly different Ca2+ sensors." Mol Cell **22**(1): 15-25.*
*Hilgemann, D. W. (1990). "Regulation and deregulation of cardiac Na(+)-Ca2+ exchange in giant excised sarcolemmal membrane patches." Nature **344**(6263): 242-245.*
*Hilgemann, D. W. and R. Ball (1996). "Regulation of cardiac Na+,Ca2+ exchange and KATP potassium channels by PIP2." Science **273**(5277): 956-959.*
*Hilgemann, D. W., S. Matsuoka, et al. (1992). "Steady-state and dynamic properties of cardiac sodium-calcium exchange. Sodium-dependent inactivation." J Gen Physiol **100**(6): 905-932.*
*Jakovcevski, M., H. Ruan, et al. (2015). "Neuronal Kmt2a/Mll1 histone methyltransferase is essential for prefrontal synaptic plasticity and working memory." Journal of Neuroscience **35**(13): 5097-5108.*
*JANOFF, A. (1964). "ALTERATIONS IN LYSOSOMES (INTRACELLULAR ENZYMES) DURING SHOCK; EFFECTS OF PRECONDITIONING (TOLERANCE) AND PROTECTIVE DRUGS." Int Anesthesiol Clin **2**: 251-269.*
*Jenuwein, T. and C. D. Allis (2001). "Translating the histone code." Science **293**(5532): 1074-1080.*
*Jiang, Y., B. Langley, et al. (2008). "Epigenetics in the nervous system." J Neurosci **28**(46): 11753-11759.*
*Kang, T. M. and D. W. Hilgemann (2004). "Multiple transport modes of the cardiac Na+/Ca2+ exchanger." Nature **427**(6974): 544-548.*
*Kiang, J. G., D. E. McClain, et al. (2003). "Constitutive NO synthase regulates the Na+/Ca2+ exchanger in human T cells: role of [Ca2+]i and tyrosine phosphorylation." J Cell Biochem **89**(5): 1030-1043.*
*Kim, J. H., J. H. Lee, et al. (2017). "Histone Lysine Methylation and Neurodevelopmental Disorders." Int J Mol Sci **18**(7).*
*Kirino, T., Y. Tsujita, et al. (1991). "Induced tolerance to ischemia in gerbil hippocampal neurons." J Cereb Blood Flow Metab **11**(2): 299-307.*
*Kitagawa, K., M. Matsumoto, et al. (1991). "'Ischemic tolerance' phenomenon detected in various brain regions." Brain Res **561**(2): 203-211.*
*Kofuji, P., W. J. Lederer, et al. (1994). "Mutually exclusive and cassette exons underlie alternatively spliced isoforms of the Na/Ca exchanger." J Biol Chem **269**(7): 5145-5149.*
*Kwok, R. P., J. R. Lundblad, et al. (1994). "Nuclear protein CBP is a coactivator for the transcription factor CREB." Nature **370**(6486): 223-226.*
*Ledo, F., W. A. Link, et al. (2000). "The DREAM-DRE interaction: key nucleotides and dominant negative mutants." Biochim Biophys Acta **1498**(2-3): 162-168.*
*Lee, S. L., A. S. Yu, et al. (1994). "Tissue-specific expression of Na(+)-Ca2+ exchanger isoforms." J Biol Chem **269**(21): 14849-14852.*
*Lentjes, M. H., H. E. Niessen, et al. (2016). "The emerging role of GATA transcription factors in development and disease." Expert Rev Mol Med **18**: e3.*
*Levitsky, D. O., D. A. Nicoll, et al. (1994). "Identification of the high affinity Ca(2+)-binding domain of the cardiac Na(+)-Ca2+ exchanger." J Biol Chem **269**(36): 22847-22852.*

*Li, Z., S. Matsuoka, et al. (1994). "Cloning of the NCX2 isoform of the plasma membrane Na(+)-Ca2+ exchanger." J Biol Chem **269**(26): 17434-17439.*
*Li, Z., D. A. Nicoll, et al. (1991). "Identification of a peptide inhibitor of the cardiac sarcolemmal Na(+)-Ca2+ exchanger." J Biol Chem **266**(2): 1014-1020.*
*Lindgren, R. M., J. Zhao, et al. (2005). "Molecular cloning and characterization of two novel truncated isoforms of human Na+/Ca2+ exchanger 3, expressed in fetal brain." Gene **348**: 143-155.*
*Lipton, P. (1999). "Ischemic cell death in brain neurons." Physiol Rev **79**(4): 1431-1568.*
*Liu, X. Q., R. Sheng, et al. (2009). "The neuroprotective mechanism of brain ischemic preconditioning." Acta Pharmacol Sin **30**(8): 1071-1080.*
*Lytton, J., X. F. Li, et al. (2002). "K+-dependent Na+/Ca2+ exchangers in the brain." Ann N Y Acad Sci **976**: 382-393.*
*Matsuoka, S., D. A. Nicoll, et al. (1997). "Regulation of cardiac Na(+)-Ca2+ exchanger by the endogenous XIP region." J Gen Physiol **109**(2): 273-286.*
*Matsuoka, S., D. A. Nicoll, et al. (1995). "Regulation of the cardiac Na(+)-Ca2+ exchanger by Ca2+. Mutational analysis of the Ca(2+)-binding domain." J Gen Physiol **105**(3): 403-420.*
*Meldrum, D. R., J. C. Cleveland, et al. (1997). "Early and delayed preconditioning: differential mechanisms and additive protection." Am J Physiol **273**(2 Pt 2): H725-733.*
*Molinaro, P., O. Cuomo, et al. (2008). "Targeted disruption of Na+/Ca2+ exchanger 3 (NCX3) gene leads to a worsening of ischemic brain damage." Journal of Neuroscience **28**(5): 1179-1184.*
*Nicholas, S. B., W. Yang, et al. (1998). "Alternative promoters and cardiac muscle cell-specific expression of the Na+/Ca2+ exchanger gene." Am J Physiol **274**(1 Pt 2): H217-232.*
*Nicoll, D. A., M. Ottolia, et al. (1999). "A new topological model of the cardiac sarcolemmal Na+-Ca2+ exchanger." J Biol Chem **274**(2): 910-917.*
*Nicoll, D. A., M. Ottolia, et al. (2002). "Toward a topological model of the NCX1 exchanger." Ann N Y Acad Sci **976**: 11-18.*
*Nicoll, D. A., B. D. Quednau, et al. (1996). "Cloning of a third mammalian Na+-Ca2+ exchanger, NCX3." J Biol Chem **271**(40): 24914-24921.*
*Onions, J., S. Hermann, et al. (1997). "Basic helix-loop-helix protein sequences determining differential inhibition by calmodulin and S-100 proteins." J Biol Chem **272**(38): 23930-23937.*
*Osawa, M., K. I. Tong, et al. (2001). "Calcium-regulated DNA binding and oligomerization of the neuronal calcium-sensing protein, calsenilin/DREAM/KChIP3." J Biol Chem **276**(44): 41005-41013.*
*Papa, M., A. Canitano, et al. (2003). "Differential expression of the Na+-Ca2+ exchanger transcripts and proteins in rat brain regions." J Comp Neurol **461**(1): 31-48.*
*Philipson, K. D. and D. A. Nicoll (2000). "Sodium-calcium exchange: a molecular perspective." Annu Rev Physiol **62**: 111-133.*
*Pignataro, G., O. Cuomo, et al. (2011a). "ASIC1a contributes to neuroprotection elicited by ischemic preconditioning and postconditioning." International journal of physiology, pathophysiology and pharmacology **3**(1): 1.*
*Pignataro, G., E. Esposito, et al. (2011b). "The NCX3 isoform of the Na+/Ca2+ exchanger contributes to neuroprotection elicited by ischemic postconditioning." Journal of Cerebral Blood Flow & Metabolism **31**(1): 362-370.*
*Pignataro, G., R. Gala, et al. (2004a). "Two sodium/calcium exchanger gene products, NCX1 and NCX3, play a major role in the development of permanent focal cerebral ischemia." Stroke **35**(11): 2566-2570.*
*Pignataro, G., R. Gala, et al. (2004b). "Two sodium/calcium exchanger gene products, NCX1 and NCX3, play a major role in the development of permanent focal cerebral ischemia." Stroke **35**(11): 2566-2570.*

*Pozos, T. C., I. Sekler, et al. (1996). "The product of HUM1, a novel yeast gene, is required for vacuolar Ca2+/H+ exchange and is related to mammalian Na+/Ca2+ exchangers." Mol Cell Biol **16**(7): 3730-3741.*

*Quednau, B. D., D. A. Nicoll, et al. (1997). "Tissue specificity and alternative splicing of the Na+/Ca2+ exchanger isoforms NCX1, NCX2, and NCX3 in rat." Am J Physiol **272**(4 Pt 1): C1250-1261.*

*Quednau, B. D., D. A. Nicoll, et al. (2004). "The sodium/calcium exchanger family-SLC8." Pflugers Arch **447**(5): 543-548.*

*Santacruz-Toloza, L., M. Ottolia, et al. (2000). "Functional analysis of a disulfide bond in the cardiac Na(+)-Ca(2+) exchanger." J Biol Chem **275**(1): 182-188.*

*Scorziello, A., C. Savoia, et al. (2013). "NCX3 regulates mitochondrial calcium handling through AKAP121-anchored signaling complex and prevents hypoxia-induced cell death." J Cell Sci: jcs. 129668.*

*Secondo, A., I. R. Staiano, et al. (2007). "The Na+/Ca2+ exchanger isoform 3 (NCX3) but not isoform 2 (NCX2) and 1 (NCX1) singly transfected in BHK cells plays a protective role in a model of in vitro hypoxia." Ann N Y Acad Sci **1099**: 481-485.*

*Shaul, O., D. W. Hilgemann, et al. (1999). "Cloning and characterization of a novel Mg(2+)/H(+) exchanger." EMBO J **18**(14): 3973-3980.*

*Shaywitz, A. J. and M. E. Greenberg (1999). "CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals." Annu Rev Biochem **68**: 821-861.*

*Sisalli, M. J., L. Annunziato, et al. (2015). "Novel Cellular Mechanisms for Neuroprotection in Ischemic Preconditioning: A View from Inside Organelles." Front Neurol **6**: 115.*

*Spreafico, F., J. J. Barski, et al. (2001). "Mouse DREAM/calsenilin/KChIP3: gene structure, coding potential, and expression." Mol Cell Neurosci **17**(1): 1-16.*

*Thiagalingam, S., K. H. Cheng, et al. (2003). "Histone deacetylases: unique players in shaping the epigenetic histone code." Ann N Y Acad Sci **983**: 84-100.*

*Tian, X., S. Zhang, et al. (2013). "Histone lysine-specific methyltransferases and demethylases in carcinogenesis: new targets for cancer therapy and prevention." Curr Cancer Drug Targets **13**(5): 558-579.*

*Valsecchi, V., G. Pignataro, et al. (2011). "NCX1 is a novel target gene for hypoxia-inducible factor-1 in ischemic brain preconditioning." Stroke: STROKEAHA. 110.597583.*

*Wagner, J. J., G. W. Terman, et al. (1993). "Endogenous dynorphins inhibit excitatory neurotransmission and block LTP induction in the hippocampus." Nature **363**(6428): 451-454.*

*Wei, G., B. J. Abraham, et al. (2011). "Genome-wide analyses of transcription factor GATA3-mediated gene regulation in distinct T cell types." Immunity **35**(2): 299-311.*

*Weisskopf, M. G., R. A. Zalutsky, et al. (1993). "The opioid peptide dynorphin mediates heterosynaptic depression of hippocampal mossy fibre synapses and modulates long-term potentiation." Nature **365**(6442): 188.*

*Yu, L. and R. A. Colvin (1997). "Regional differences in expression of transcripts for Na+/Ca2+ exchanger isoforms in rat brain." Brain Res Mol Brain Res **50**(1-2): 285-292.*

*Zhang, X., H. Wen, et al. (2012). "Lysine methylation: beyond histones." Acta Biochim Biophys Sin (Shanghai) **44**(1): 14-27.*