TALLINN UNIVERSITY OF TECHNOLOGY
DOCTORAL THESIS
14/2018

# Regulation of NFAT transcription factors by neuronal activity

HANNA VIHMA

TTÜ PRESS

TALLINN UNIVERSITY OF TECHNOLOGY
School of Science
Department of Chemistry and Biotechnology

**This dissertation was accepted for the defense of the degree of Doctor of Philosophy in Gene Technology on April 13, 2018.**

**Supervisors**: Professor Tõnis Timmusk, PhD,
Tallinn University of Technology, Department of Chemistry and Biotechnology, Estonia

Research scientist Priit Pruunsild, PhD
Tallinn University of Technology, Department of Gene Technology, Estonia
Present address: University of Heidelberg, Department of Neurobiology, Germany

**Opponents**: Professor Dan Lindholm, MD, PhD
University of Helsinki, Department of Biochemistry and Developmental Biology, Finland

Professor Allen Kaasik, PhD,
University of Tartu, Institute of Biomedicine and Translational Medicine, Estonia

**Defense of the thesis**: May 18, 2018

**Declaration**:
*Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for any academic degree.*

*/Hanna Vihma/*



ISSN 2585-6898 (publication)
ISBN 978-9949-83-234-7 (publication)
ISSN 2585-6901(PDF)
ISBN 978-9949-83-235-4 (PDF)

TALLINNA TEHNIKAÜLIKOOL
DOKTORITÖÖ
14/2018

# NFAT trankriptsioonitegurite närvitalitlusest sõltuv regulatsioon

HANNA VIHMA

TTÜ KIRJASTUS

**CONTENTS**

## INTRODUCTION

Nuclear factor of activated T-cells (NFAT) is a family of $Ca^{2+}$-regulated transcription factors that are activated via dephosphorylation by $Ca^{2+}$- and calmodulin (CaM)-dependent serine/threonine phosphatase calcineurin (CaN). The NFAT signaling pathway can be divided into multiple steps: NFAT protein dephosphorylation and its translocation to the nucleus, NFAT interaction with other nuclear proteins and regulation of its target genes, and NFAT rephosphorylation by different kinases that leads to its translocation back to the cytosol. Since NFAT proteins need partner proteins to activate transcription, they are important integrators of $Ca^{2+}$ signaling with many other signaling pathways. Thus, the NFAT pathway has significant impact on developmental and physiological processes that need coordinated responses to different signals.

30 years ago, NFAT was first discovered in the nucleus of activated T-cells, where it was thought to have a role in inducible gene transcription during immune response. Hence the name nuclear factor of activated T-cells. Since then, NFAT proteins have been shown to be expressed in many tissues and accordingly there are many developmental and physiological processes that involve NFAT signaling, which include development and regulation of the immune, cardiovascular, musculoskeletal, and nervous system.

In the following text, I will give an overview of the NFAT signaling pathway and the structural features of NFAT proteins. I will also cover main developmental and functional processes that are regulated by NFAT proteins, with an emphasis on their roles in the nervous system. Since NFAT proteins are $Ca^{2+}$-dependent transcription factors, I will first introduce the fundamental aspects of intracellular $Ca^{2+}$ signaling and $Ca^{2+}$-regulated signaling pathways controlling gene expression in neurons.

## ORIGINAL PUBLICATIONS

I. **Vihma H***, Pruunsild P*, Timmusk T
Alternative splicing and expression of human and mouse NFAT genes.
Genomics, 2008;92(5):279-91. doi: 10.1016/j.ygeno.2008.06.011.

II. **Vihma H**, Luhakooder M. Pruunsild P, Timmusk T
Regulation of different human NFAT isoforms by neuronal activity.
J Neurochem. 2016;137(3):394-408. doi: 10.1111/jnc.13568.

III. **Vihma H**, Timmusk T
Sumoylation regulates the transcriptional activity of different human NFAT isoforms in neurons.
Neurosci Lett. 2017;10;653:302-307. doi: 10.1016/j.neulet.2017.05.074

* Authors have equal contribution

## AUTHOR'S CONTRIBUTION TO THE PUBLICATIONS

I. I participated in experimental design, performed the majority of the experiments, and analyzed the data. I wrote most of the manuscript;
II. I designed and performed the majority of the experiments. I analyzed the data and wrote the manuscript;
III. I designed and performed all the experiments, analyzed the data and wrote the manuscript.

**ABBREVIATIONS**

AKAP5 – A-kinase anchoring protein 5
AKT1 – akt serine/threonine kinase 1
AMPA – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP-1 – activator protein 1
ATP – adenosine triphosphate
BDNF – brain-derived neurotrophic factor
BHK-21 – baby hamster kidney cells, litter number 21
bHLH – basic helix-loop-helix
CaM – calmoduline
CaMKII and IV – calcium/calmodulin-dependent kinase II and IV
cAMP – cyclic adenosine monophosphate
CaN – calcineurin
CaNA/B – calcineurin catalytic subunit A and B
CBP – CREB binding protein
CGN – cerebellar granule neurons
CK1 – casein kinase 1
CnBP-A/B – calcineurin binding peptide A and B
CRE – cyclic adenosine diphosphate ribose respose element
CREB – cyclic adenosine monophosphate response element-binding protein
CRM1 – chromosomal maintenance 1
CsA – cyclosporine A
DRG – dorsal root ganglion
DYRK1A/B – dual-specificity tyrosine phosphorylation regulated kinase 1A/B
EMT – epithelial-mesenchymal transformation
EP300 – E1A-associated protein p300
ER – endoplasmic reticulum
ERK – extracellular signal-regulated kinase
FASL – Fas ligand
FK506 – tacrolimus
GABA – gamma-aminobutyric acid
GABRA2/4 – gamma-aminobutyric acid receptor subunit alpha-2 and 4
GATA2, -4 – GATA binding protein 2 and 4
GSK3β – glycogen synthase kinase 3 beta
GTP – guanosine triphosphate
HDAC5 – histone deacetylase 5
HEK293-FT/T – human embryonic kidney 293-FT and T cells
HIV-1 – human immunodeficiency virus 1
HOMER – homer scaffolding protein
IL-2 – interleukin-2
$IP_3$ – inositol triphosphate
$IP_3R1$ – inositol trisphosphate receptor 1

JNK – c-Jun N-terminal kinase
LGIC – ligand-gated ionotropic channels
LTCC – L-type voltage-gated $Ca^{2+}$ channels
LTD – long-term depression
LTP – long-term potentiation
MAPK – mitogen-activates protein kinase
MEF2 – myocyte enhancer factor-2
miR-1, -9, -199b – micro RNA-1, -9, -199b
NCS – neuronal calcium sensor
NES – nuclear export signal
NFAT – nuclear factor of activated T-cells
NF-κB – nuclear factor kappa-light-chain-enhancer of activated B cells
NGF – nerve growth factor
NHR – NFAT homology region
NLS – nuclear localization signal
NMDA – N-methyl-D-aspartic acid
NMDAR – N-methyl-D-aspartic acid-type glutamate receptor
NPC – neural progenitor cells
NRON – non-coding repressor of NFAT
NSC – neural stem cells
ORAI1-3 – calcium release-activated calcium channel protein ORAI1-3
PCR – polymerase chain reaction
PKA – protein kinase A
PLC – phospholipase C
PTM – post-translational modification
RHD – Rel-homology domain
RSK – ribosome s6 kinase
RT – reverse transcription
RyR – ryanodine receptor
SERCA – sarco/endoplasmic reticulum calcium-adenylpyrophosphatase
SOC – store-operating $Ca^{2+}$ channels
SP – serine-proline
SR – sarcoplasmic reticulum
SRR – serine-rich region
SUMO1-5 – small ubiquitin-like modifier 1-5
TAD-N/C – amino/carboxy-terminal transactivation domain
TBI – traumatic brain injury
TRIM17 – tripartite motif containing 17
TRKA/B – tropomyosin receptor kinase A and B
TRPC – transient receptor potential channels
VEGF – vascular endothelial growth factor
VGCC – voltage-gated $Ca^{2+}$ channels

## REVIEW OF THE LITERATURE

A large number of cellular processes are controlled by $Ca^{2+}$ signaling. One of such processes is the activation of $Ca^{2+}$-dependent transcription, which extends the impact of $Ca^{2+}$ signaling into long-term cellular changes. In the first part of the review of the literature, I will give an overview of intracellular $Ca^{2+}$ signaling and components of its toolkit, since the main protein of interest of this thesis, nuclear factor of activated T-cells (NFAT), is a $Ca^{2+}$-regulated transcription factor. Furthermore, since the emphasis of this thesis is on the regulation of NFAT proteins in the nervous system, I will cover the main $Ca^{2+}$-regulated signaling pathways controlling gene expression in neurons. In the second part of the literature review, I will focus on the NFAT proteins themselves. I will describe the NFAT signaling pathway, the structural features of NFAT proteins, and NFAT functions in neuronal and non-neuronal tissues.

### 1. Intracellular $Ca^{2+}$ signaling – a brief overview

$Ca^{2+}$ is a remarkable versatile intracellular messenger that is capable of decoding extracellular stimuli to different intracellular actions. Signal $Ca^{2+}$ is derived either from extracellular space or from internal stores, such as the endoplasmic reticulum (ER) or the sarcoplasmic reticulum (SR) in muscle cells. There are three main types of channels that are capable of mediating $Ca^{2+}$ influx from extracellular space: (i) voltage-gated $Ca^{2+}$ channels (VGCCs); (ii) ligand-gated ionotropic channels (LGICs); and (iii) store-operated $Ca^{2+}$ channels (SOCs). Intracellular $Ca^{2+}$ release is mediated by the inositol trisphosphate receptor ($IP_3R$) and ryanodine receptor (RyR) channels on the ER/SR of most cell types and is controlled by $Ca^{2+}$ itself or by other messengers. Examples of such messengers include inositol trisphosphate ($IP_3$), cyclic adenosine diphosphate-ribose, nicotinic acid adenine dinucleotide phosphate, or sphingosine (reviewed in Nowycky and Thomas 2002; and Brini *et al.* 2014).

In all excitable cells, membrane depolarization activates $Ca^{2+}$ influx via VGCCs, which, based on their tissue distribution and pharmacological properties, belong to three main subfamilies: $Ca_v1.1$-1.4, i.e. L-type $Ca^{2+}$ channels; $Ca_v2.1$-2.3, i.e. P/Q/N/R-type $Ca^{2+}$ channels; and $Ca_v3.1$-3.3, i.e. T-type $Ca^{2+}$ channels. All these VGCC family members mediate a selected roles in signal transduction that include contraction of muscle cells, secretion of hormones in endocrine cells, regulation of gene expression, and initiation of synaptic transmission in neurons (reviewed in Catterall 2011).

Most numerous and varied voltage-independent $Ca^{2+}$-permeable channels are LGICs, that are a group of transmembrane ion channel proteins, which, when

activated by a ligand, allow $Na^+$, $K^+$, $Ca^{2+}$, and/or $Cl^-$ ions to pass through the membrane resulting in a change in intracellular ion concentration. Thus, LGICs are able to convert an extracellular chemical signal into an intracellular electrical signal. The $Ca^{2+}$ permeable LGICs include N-methyl-D-aspartic acid (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate, acetylcholine, serotonin, and adenosine triphosphate (ATP) receptors (reviewed in Nowycky and Thomas 2002).

In all metazoans, SOCs are the major $Ca^{2+}$ entry pathway in both electrically excitable and non-excitable cells, in which SOCs are activated by the depletion of $Ca^{2+}$ from the intracellular $Ca^{2+}$ stores. In general, binding of an extracellular agonist to its receptor, such as G-protein-coupled receptor or receptor tyrosine kinase, activates phospholipase C (PLC) through a G protein or tyrosine kinase, respectively. PLC then cleaves phosphatidylinositol 4,5-bisphosphate to diacylglycerol and $IP_3$, which releases $Ca^{2+}$ from ER through $IP_3R$. The reduction of $Ca^{2+}$ in ER activates $Ca^{2+}$ sensory proteins named stromal interaction molecule 1 or 2 located in the ER membrane, which then tether and activate highly $Ca^{2+}$-selective ion channels named $Ca^{2+}$ release-activated $Ca^{2+}$ channel protein ORAI (ORAI1-3) located in the plasma membrane. Experimentally, SOCs can be activated with agents that directly deplete $Ca^{2+}$ stores, such as thapsigargin, an inhibitor of sarco/endoplasmic reticulum $Ca^{2+}$-ATPase (SERCA) pumps that restore ER/SR $Ca^{2+}$ stores; ionomycin, that releases $Ca^{2+}$ from the ER; or $Ca^{2+}$ chelator acidegtazic acid (reviewed in Prakriya and Lewis 2015).

Another large superfamily of ion channels named the transient receptor potential channels (TRPCs) is a diverse group of non-selective, $Ca^{2+}$-permeable cation channels localized in the plasma membrane. TRPCs have polymodal activation properties, since some act as SOCs by mediating $Ca^{2+}$ entry in response to depletion of intracellular stores or activation of the PLC system, others are activated by changes in voltage, temperature, pressure, osmolarity, or some natural or chemical compounds. TRPCs can also form heteromultimers, which further alters their preferred mode of action (reviewed in Venkatachalam and Montell 2007; and Nilius and Owsianik 2011).

Since $Ca^{2+}$ plays a pivotal role in the survival, proliferation and growth of all cells, $Ca^{2+}$ homeostasis must be tightly regulated for cells to function properly. After the $Ca^{2+}$ transient induced by cell stimulation, the plasma membrane $Na^+/Ca^{2+}$ exchanger and plasma membrane $Ca^{2+}$ ATPase together with SERCA and the mitochondrial $Ca^{2+}$ uniporter help to restore resting cytosolic $Ca^{2+}$ and therefore play a key role in controlling the intracellular $Ca^{2+}$ homeostasis (reviewed in Brini and Carafoli 2011).

All these different sources of $Ca^{2+}$ influx can contribute to local cytoplasmic high $Ca^{2+}$ concentration microdomains in the immediate vicinity of the $Ca^{2+}$ entry sites. These microdomains enable the cell to regulate different processes within localized regions of the cell, which, for example, is needed for the regulation of contraction and relaxation of muscle cells or for the regulation of neurotransmitter release in the synaptic terminals of neurons.

### 1.1. $Ca^{2+}$-regulated signaling pathways controlling gene expression in neurons

From spontaneous activity-driven neuronal differentiation to experience-dependent maturation of complex neural networks, intracellular $Ca^{2+}$ transients are the driving force, which leads to changes in gene expression that are needed for the proper development and functioning of the nervous system. Therefore, neuronal activity-dependent gene expression can be also referred to as $Ca^{2+}$-dependent gene expression. In order to outline $Ca^{2+}$-regulated signaling pathways that activate transcription in neurons, I will give an overview of the molecular mechanisms by which $Ca^{2+}$-dependent transcription factors are regulated.

Dependent on the stimulus, changes in the intracellular $Ca^{2+}$ concentration could range from highly localized and transient $Ca^{2+}$ microdomains to longer lasting and global changes throughout the neuron, and thereby, they have different physiological outcomes. A good example is the regulation of long-term potentiation (LTP) and long-term depression (LTD), in which patterns of $Ca^{2+}$ concentration elevation have the opposite result: LTP is triggered by brief increases (few seconds) of $Ca^{2+}$ with high magnitude, whereas prolonged modest rises in $Ca^{2+}$ results in LTD (Yang *et al.* 1999). This is possible due to the functions of $Ca^{2+}$-binding proteins that act as $Ca^{2+}$ sensors, such as synaptotagmin I, annexins, calmodulin (CaM), and members of the $Ca^{2+}$-binding protein and neuronal $Ca^{2+}$ sensor (NCS) protein families, that differ in their spatiotemporal expression patterns, $Ca^{2+}$ affinity, and binding partners. The myriad of functions of these proteins ranges from direct regulation of exocytosis of neurotransmitter-containing synaptic vesicles to modulation of various ion channels and receptors (reviewed in Burgoyne and Haynes 2015).

However, as mentioned, $Ca^{2+}$ influx to the cell is also the driving force that leads to changes in gene expression. One possibility is that elevated $Ca^{2+}$ can directly bind to a transcriptional regulator, as has been shown for the NCS family member calsenilin/downstream regulatory element antagonist modulator, which when not bound to $Ca^{2+}$ acts as a transcriptional repressor by interacting with a specific DNA element (Carrión *et al.* 1999). More commonly, $Ca^{2+}$ regulates gene expression indirectly by acting via the ubiquitously expressed $Ca^{2+}$-binding effector protein CaM. $Ca^{2+}$ bound CaM contributes to a number of transcriptional pathways via activation of $Ca^{2+}$/CaM-dependent kinases (CaMKs) or the phosphatase calcineurin (CaN; also named protein phosphatase 2B, PP2B) that leads to either phosphorylation or dephosphorylation of their corresponding substrates, respectively. In neurons, for example, both CaMKII and CaMKIV can phosphorylate cyclic adenosine monophosphate (cAMP) response element (CRE) binding protein (CREB) (Matthews *et al.* 1994; Sun *et al.* 1994). In addition, CaMKII has been shown to phosphorylate histone deacetylase 5 (HDAC5) (Linseman *et al.* 2003), the basic helix-loop-helix (bHLH)

transcription factor NeuroD (Gaudillière *et al.* 2004) and methyl-CpG-binding protein 2, and CaMKIV has been shown to phosphorylate the bHLH transcription factor NeuroD2 (Ince-Dunn *et al.* 2006) and the histone acetyltransferase CREB binding protein (CBP) (Chawla *et al.* 1998). Target proteins of CaN are members of the MADS-box protein myocyte enhancer factor 2 (MEF2) (Flavell *et al.* 2006) and NFAT family proteins (Flanagan *et al.* 1991; Graef *et al.* 1999).

Intracellular signaling pathways often form complex networks. An important aspect that significantly influences $Ca^{2+}$-mediated crosstalk between different signaling pathways in neurons is the route by which $Ca^{2+}$ enters the cell. For example, the activation of CREB, a prototypical signal-regulated transcription factor in neurons, is different if it is initiated by $Ca^{2+}$ entry through NMDA-type glutamate receptors (NMDARs) or VGCCs (Bading *et al.* 1993). The transcriptional activity of CREB was first described to be activated by adenylyl cyclase/cAMP/protein kinase A (PKA) signaling via phosphorylation of its serine 133 (S133), which is required for the recruitment of the co-activator CBP to CRE-bound CREB (Montminy and Bilezikjian 1987; Gonzalez and Montminy 1989; Chrivia *et al.* 1993). However, in neurons, neuronal activity-dependent activation of CREB occurs predominantly via CaMK and Ras/Raf/mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK)/ribosomal s6 kinase (RSK) signaling cascades, which can be activated by $Ca^{2+}$ entry via both NMDAR and VGCCs (Xia *et al.* 1996; Dolmetsch *et al.* 2001). A study of the kinetics of the activity-dependent CREB phosphorylation has revealed that activation of the CaMK pathway results in fast, but transient phosphorylation of CREB, while phosphorylation of CREB via RSK is slower, but more sustained (Wu *et al.* 2001). CaMKIV has also been shown to phosphorylate CREB at S133, and thereby, it plays an important role in the S133-mediated CBP recruitment (Bito *et al.* 1996). Although $Ca^{2+}$ influx via both VGCC and NMDAR results in activation of the CaMK signaling pathway, only $Ca^{2+}$ entry through VGCCs, that results in increase in nuclear $Ca^{2+}$ concentration, can activate CaMKIV that is predominantly located in the nucleus (Hardingham *et al.* 1997; Chawla *et al.* 1998). $Ca^{2+}$ microdomains at the sites of $Ca^{2+}$ entry at the mouth of the activated VGCCs and NMDARs lead to increases in the levels of Ras-guanosine triphosphate (GTP) that eventually results in activation of CREB by RSK kinase (Hardingham *et al.* 2001). However, fast but weak CREB phosphorylation by nuclear CaM/CaMKIV primes CREB for a prolonged transcriptionally active state mediated by MAPK/ERK signaling (Wu *et al.* 2001), or for faster and less specifically VGCC-dependent CREB activation after subsequent stimulation (Mermelstein *et al.* 2001). In either case, CREB could be activated by a variety of extracellular signals (reviewed in Johannessen *et al.* 2004), which enables CREB-dependent transcription to regulate a number of biological functions from activity-dependent synaptic plasticity that underlies learning and memory formation to trophic factor-dependent neuronal survival (reviewed in Alberini

2009; and Walton and Dragunow 2000). However, neuronal activity-dependent gene regulation underlying proper development and functioning of the nervous system is not simply a bidirectional process of transcription factor activation and inactivation, but requires a coordinated activation of multiple transcription factors. One example, that I will discuss in more detail in the following text, would be the regulation of NFAT-dependent transcription where cooperative binding of nuclear partner proteins is needed to initiate transcription.

## 2. NFAT family of transcription factors

NFAT is a family of transcription factors evolutionary related to the Rel/NF-κB family (Chytil and Verdine 1996). It includes four classical members named NFATc1 (also named NFATc or NFAT2) (Northrop *et al.* 1994), NFATc2 (NFATp, NFAT1) (McCaffrey *et al.* 1993), NFATc3 (NFATx, NFAT4) (Masuda *et al.* 1995; Hoey *et al.* 1995), NFATc4 (NFAT3) (Hoey *et al.* 1995), and an atypical member NFAT5 (tonicity element binding protein, osmotic response element binding protein, or NFATz) (Lopez-Rodríguez *et al.* 1999; Miyakawa *et al.* 1999; Pan *et al.* 2000). Although NFAT5 shares DNA-binding specificity with NFATc1-c4, NFAT5 does not cooperate with well-known NFATc1-c4 transcriptional partner proteins. Moreover, the activity of NFATc1-c4 proteins is controlled by $Ca^{2+}$ and the $Ca^{2+}$-dependent protein phosphatase CaN, but NFAT5 is regulated by changes in osmotic tension (Lopez-Rodríguez *et al.* 1999). Therefore, as the study in hand is focusing on the classical $Ca^{2+}$/CaN regulated NFAT proteins, I will not discuss the atypical member NFAT5 in more detail here.

### 2.1. Overview of the NFAT signaling pathway

The NFAT signaling pathway can be divided into multiple steps: (i) NFAT dephosphorylation by the $Ca^{2+}$-dependent protein phosphatase CaN and its translocation to the nucleus; (ii) NFAT binding to DNA and interaction with other nuclear proteins to regulate its target genes; (iii) NFAT rephosphorylation by various kinases and its translocation back to the cytosol (Figure 1).

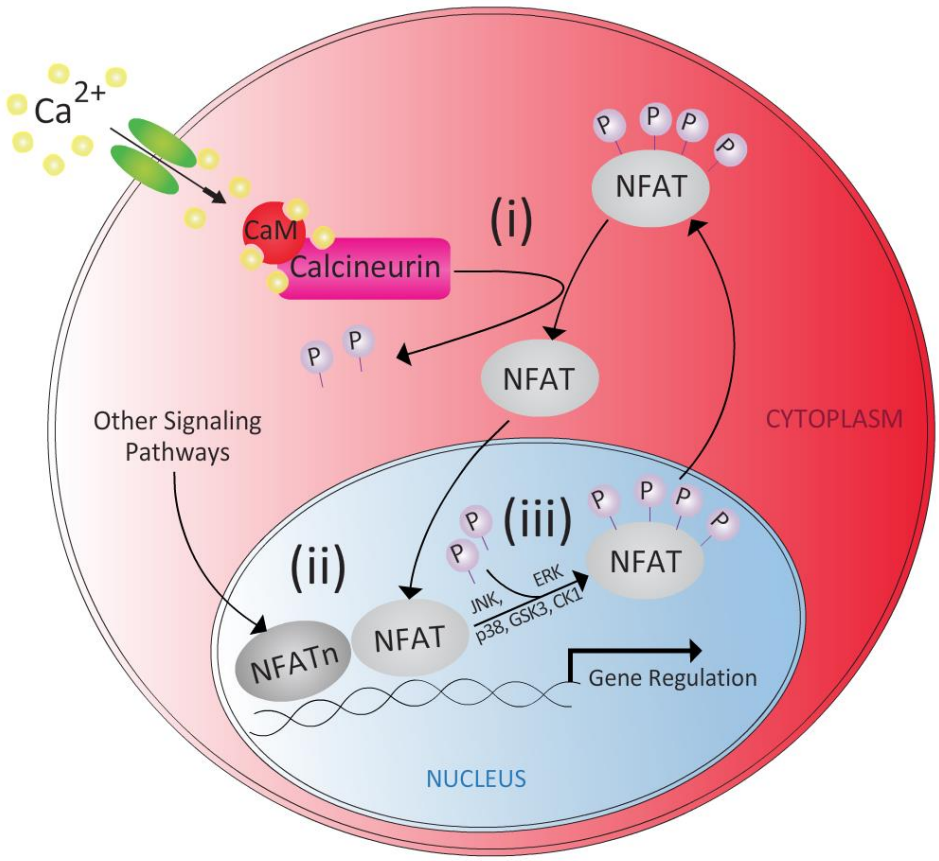



**Figure 1.** Schematic overview of the NFAT signaling pathway.
See text for details. CaM – calmodulin; CK1 – casein kinase 1; ERK – extracellular signal-regulated kinase; GSK3 – glycogen synthase kinase 3; JNK – c-Jun N-terminal kinase; NFAT – nuclear factor of activated T-cells; NFATn – nuclear partner protein of NFAT; p38 – p38 kinase.

NFAT proteins are highly modified by phosphorylation, which keeps them inactive in the cytoplasm. The activation of NFAT proteins is regulated by $Ca^{2+}$- and CaM-dependent serine/threonine phosphatase CaN, a protein that is widely distributed in mammalian tissues with the highest levels found in the brain (Klee *et al.* 1979; Jiang *et al.* 1997; Kincaid *et al.* 1987). CaN holoenzyme is a heterodimer that consists of a ~60 kDa catalytic subunit A (CaNA) and of a 19 kDa regulatory subunit B (CaNB) (reviewed in Nygren and Scott 2016). CaNA subunit contains four domains: catalytic, CaNB-binding, CaM-binding, and autoinhibitory domain. In a resting cell, where concentration of $Ca^{2+}$ is low, the autoinhibitory domain of CaNA is bound to the active site cleft of the catalytic domain of CaNA, keeping CaN in an inactive form (reviewed in Klee *et al.* 1998). Activation of CaN requires the participation of two distinct EF-hand $Ca^{2+}$-binding motifs-containing proteins, CaNB and CaM. Signaling pathways that lead to a

rise in intracellular $Ca^{2+}$ cause $Ca^{2+}$ binding to CaNB that results in a conformational change, which allows $Ca^{2+}$-dependent binding of CaM to CaNA, displacing of the autoinhibitory domain, and switching on CaN catalytic activity (Reviewed in Li *et al.* 2011a). Activated CaN mediates dephosphorylation of NFAT proteins, which results in rapid nuclear import of the NFATs (reviewed in Rao *et al.* 1997). Both CaN and activated NFAT are translocated to the nucleus where CaN helps to maintain the nuclear localization of NFAT, meaning that the activation of NFAT requires a sustained increase of intracellular $Ca^{2+}$ and CaN activity (Loh *et al.* 1996; Shibasaki *et al.* 1996; Al-Daraji *et al.* 2002).

NFAT proteins are transcription factors, thus they bind DNA and regulate their target gene expression in the nucleus. Unlike the evolutionarily related nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) protein that forms an obligate dimer in solution and on DNA, classical $Ca^{2+}$/CaN regulated NFAT proteins are monomeric in solution and can also bind DNA as monomers. However, this monomeric DNA binding is weak and in order to regulate transcription of their target genes, the NFAT family members need a partner (sometimes referred as NFATn). Therefore, the functional NFAT recognition sequence (GGAAA) is mostly positioned to close proximity of a DNA element capable of binding NFAT partner proteins (reviewed in Rao *et al.* 1997; Hogan *et al.* 2003; Natoli *et al.* 2005; and Macian 2005). The best-studied binding partners for NFATs are activator protein 1 (AP-1) proteins. AP-1 transcription factors are proteins of the basic leucine zipper transcription factor families Jun (cJun, JunB and JunD) and Fos (cFos, FosB, Fra1 and Fra2) that form homo- or heterodimers via their leucine zipper domain and bind to AP-1 regulatory elements via their conserved basic region (reviewed in Chinenov and Kerppola 2001). Antigen receptor–response element sites of the interleukin-2 (IL-2) promoter are probably the best-characterized examples where individual binding of NFAT and AP-1 proteins to their binding sites have relatively high dissociation rates, whereas cooperative binding of all three proteins to the composite site forms a strong and stable complex (Jain *et al.* 1993; Peterson *et al.* 1996; Chen *et al.* 1998). Furthermore, in order to form NFAT-AP-1 complexes, coordinate activation of different signaling pathways is necessary. NFAT activation requires activation of $Ca^{2+}$/CaN signaling, while different signaling pathways that involve protein kinase C, PKA, cyclin-dependent kinases, and MAPKs induce the activity of AP-1 proteins by posttranslational phosphorylation (reviewed in Macián *et al.* 2001 and; Pérez-Cadahía *et al.* 2011). Although NFAT proteins are present only in vertebrates, all the known NFATn proteins are also present in invertebrates (reviewed in Crabtree and Olson 2002), which diversifies NFAT function by integrating NFAT signaling pathway with many evolutionarily older signaling pathways.

The nuclear localization and activity of NFAT proteins is dependent upon the sustained activity of $Ca^{2+}$/CaN pathway, which is required to oppose several

serine/threonine-specific protein kinases that act either in the nucleus by rephosphorylating NFATs and promoting their rapid nuclear export, or in the cytoplasm to maintain the NFATs' phosphorylated state (reviewed in Zhu and McKeon 2000). Protein kinases that have been shown to phosphorylate NFAT proteins and to counteract their nuclear localization and activation include JNK (Chow *et al.* 1997), p38 kinase (Gómez del Arco *et al.* 2000), ERK (Porter *et al.* 2000), glycogen synthase kinase 3 (GSK3) (Beals *et al.* 1997b), and casein kinase 1 (CK1) (Zhu *et al.* 1998). In the absence of sustained CaN activity, NFAT proteins are rapidly rephosphorylated, which leads to relocation of NFAT to the cytoplasm (Okamura *et al.* 2000). Therefore, NFAT isoform-specific subcellular localization and activity is very much influenced by kinases that are available in certain cell types and/or active under specific conditions.

### 2.2. Modulators of the NFAT signaling pathway

As discussed, NFAT signaling pathway involves a coordinated action of CaN, nuclear partner proteins, and specific kinases. However, there are a number of modulatory mechanisms that are involved in the precise regulation of different NFAT family members in response to specific signaling inputs. Here I will cover the most important of these.

NFAT activity has been shown to be regulated by sumoylation, a lysine-targeted post-translational modification (PTM) by which the members of the small ubiquitin-like modifier (SUMO1-5) family are covalently bound to target proteins. Similarly to ubiquitination, SUMO conjugation to targets is achieved by three enzymatic steps catalyzed by activating enzyme E1 (SUMO-activating enzyme subunit 1 and 2 heterodimer in mammals), conjugating enzyme E2 (a single SUMO-conjugating enzyme UBC9), and various E3 SUMO ligases, which help to improve SUMO conjugation and substrate selection. Sumoylation is a highly dynamic process, where deconjugation of SUMO is performed by SUMO proteases (nine in mammals), which differ in their subcellular localization and specificity towards SUMO paralogs. The functional consequences of sumoylation are substrate specific, but often result in altered activity, stability, or subcellular localization. In case of transcription factors, sumoylation is generally associated with reduced or repressed transcription, which is achieved by sumoylation-dependent recruitment of HDACs (reviewed in Rosonina *et al.* 2017). It has been shown that sumoylation of NFATc1 represses its transcriptional activity by recruiting HDACs to IL-2 promoter (Nayak *et al.* 2009). Less frequently, sumoylation of transcription factors mediates increased transcriptional activity by promoting recruitment or association with coactivators, or by influencing transcription factor localization or abundance (reviewed in Rosonina *et al.* 2017). Terui and colleagues found that sumoylation of mouse NFATc2 positively regulates its transcriptional activity by increasing its nuclear

retention in BHK-21 (baby hamster kidney cells, litter number 21) fibroblasts (Terui *et al.* 2004). Sumoylation of NFATc3 mediates its interaction with tripartite motif containing 17 (Trim17) protein, an E3 ubiquitin ligase necessary for neuronal apoptosis, and this interaction prevents nuclear localization and transcriptional activity of NFATc3 in primary cerebellar granule neurons (CGNs) (Mojsa *et al.* 2015). Although sumoylation has been shown to influence other types of lysine-targeted PTMs on transcription factors and Trim17 is a known ubiquitin ligase, interaction with Trim17 dose-dependently increased the protein levels of NFATc3 rather than mediated NFATc3 ubiquitination/degradation, interaction with (reviewed in Rosonina *et al.* 2017; Lassot *et al.* 2010; Mojsa *et al.* 2015). Moreover, even though sumoylation and phosphorylation has been shown to interfere with each other at nearby residues on transcription factors (reviewed in Wilkinson and Henley 2010) and NFAT proteins are considered heavily phosphorylated, crosstalk between sumoylation and phosphorylation in NFATs has not been reported.

Additional lysine-targeted PTMs such as acetylation and ubiquitination have shown to add an additional layer to the regulation of NFAT activity. Acetylation of two sites in NFATc1 by E1A-associated protein p300 (EP300) has been shown to enhance transcriptional activity of NFATc1 in myoblasts (Meissner *et al.* 2011). It is to be noted that one of these acetylation sites is conserved between NFATc1-c4 family members across several species, which indicates that NFAT acetylation by EP300 might be a general mechanism for regulating NFAT activity. During osteoclast differentiation, NFATc1 acetylation contributes to the stability and transcriptional activity of NFATc1 in early-stage osteoclastogenesis, whereas in late-stage osteoclastogenesis, ubiquitination targets NFATc1 for degradation (Kim *et al.* 2010; Kim *et al.* 2011). Although, Kim and colleagues did not pinpoint the exact modified residues, it is possible that both of these modifications target the same residues and there is a regulatory crosstalk between acetylation and ubiquitination of NFATc1 during osteoclast differentiation. Crosstalk between different PTMs occurs also in the case of NFATc4, since GSK3-dependent phosphorylation of NFATc4 has been shown to trigger its ubiquitin-mediated proteolysis in cardiac myocytes (Fan *et al.* 2008).

The activity of NFAT proteins is also associated with large protein-protein or protein-RNA complexes that directly inhibit NFAT translocation and activity. For example, cytoplasmic NFAT has been found to form a complex with a long non-coding RNA named non-coding repressor of NFAT (NRON) and 11 additional proteins. Among them are CaM, CaM-binding scaffolding protein IQ motif containing GTPase activating protein 1, and kinases such as CK1, GSK3, and dual-specificity tyrosine phosphorylation regulated kinase (DYRK) (Willingham *et al.* 2005; Sharma *et al.* 2011). Liu and colleagues have found that the kinase leucine-rich repeat kinase 2, a major susceptibility gene for Crohn's disease and Parkinson's disease (reviewed in Lewis and Manzoni 2012),

negatively regulates NFAT-dependent innate immune responses in myeloid cells not by phosphorylating NFAT but by modulating the NRON complex in response to inducers such as lipopolysaccharide (Liu *et al.* 2011). However, the exact mechanism how this modulation occurs remains to be determined. Huang and colleagues showed that the homer scaffolding protein (HOMER) negatively regulates NFAT activation by competing with CaN for NFAT binding in resting T-cells (Huang *et al.* 2008). In addition, HOMER proteins are known to have a regulatory role in the maintenance of $Ca^{2+}$ homeostasis by regulating a number of $Ca^{2+}$-handling proteins (reviewed in Jardin *et al.* 2013). During skeletal muscle differentiation, HOMER proteins have shown to enhance NFAT-dependent signaling by binding to RyR and increasing RyR-dependent $Ca^{2+}$ release, whereas $IP_3R$-dependent $Ca^{2+}$ release, which promotes NFAT nuclear exit, is suppressed by HOMER (Stiber *et al.* 2005). A scaffolding protein named A-kinase anchoring protein 5 (AKAP5) has also been shown to couple $Ca^{2+}$ entry with NFAT activity by linking PKA and CaN to L-type voltage-gated $Ca^{2+}$ channels (LTCC) for localized activation and release of CaN from the complex to bind and activate NFAT upon depolarization in neurons (Li *et al.* 2012; Murphy *et al.* 2014; Dittmer *et al.* 2014). In addition, several proteins that inhibit CaN activity such as CaN-binding protein 1, CaNB homologous protein, and regulator of CaN have been shown to negatively regulate NFAT activity (reviewed in Lee and Park 2006).

A number of miRNAs have been shown to target components of the CaN/NFAT signaling pathway and thereby regulate NFAT activity. In heart development, miR-1 has been shown to regulate cardiomyocyte growth responses by negatively regulating NFAT signaling pathway via targeting CaM (Ikeda *et al.* 2009). miR-199b is involved in a NFATc2-dependent pathogenic feed-forward mechanism by targeting nuclear kinase DYRK1A and promoting NFAT signaling in mouse and human heart failure (da Costa Martins *et al.* 2010). In stimulated human T-cells, miR-9 enhances NFAT activity and promotes IL-2 production by targeting nuclear kinase DYRK1B and a nuclear transport factor karyopherin subunit beta 1, that have been shown to interact with NRON (Willingham *et al.* 2005; Zeng *et al.* 2015). A recent comprehensive pathway level analysis combining experimental data with computational predictions identified 191 miRNAs targeting 23 members of the CaN/NFAT signaling pathway in human immune cells and 32 miRNAs potentially induced by NFATs in activated T-cells (Kannambath 2016). Although these results need further experimental validation, this study illustrates the possible complexity that miRNAs can add to the modulation of the CaN/NFAT signaling pathway.

In addition to cellular modulators of the NFAT signaling pathway, some pharmacological drugs are known to inhibit CaN phosphatase activity and prevent activation of NFAT. These include immunosuppressants called cyclosporine A (CsA) and tacrolimus (FK506), which bind to their intracellular

receptors, immunophilins cyclophilin A and FK-binding protein 12, respectively. The drug-immunophilin complex binds to CaN at the interface of the two CaN subunits, the catalytic subunit A and the regulatory subunit B, and blocks the access of protein substrates to the active site of CaN, thereby inhibiting its phosphatase activity. However, the interaction of endogenous inhibitors with CaN is not affected by the drug-immunophilin complex, indicating that their binding regions are distinct (reviewed in Lee and Park 2006). CsA and FK506 are the inhibitors most frequently used experimentally for investigating the NFAT signaling pathway, and clinically, as immunosuppressants in treatments involving cell or organ transplantation (Scott *et al.* 2003; reviewed in Duncan and Craddock 2006). Furthermore, an inhibitory peptide VIVIT, more selective for NFAT inhibition than CsA or FK506, was developed to specifically interfere with the CaN-NFAT interaction without affecting CaN phosphatase activity (Aramburu *et al.* 1999). A cell penetrating peptide from the human transcription factor Sim-2 conjugated to VIVIT has been shown to have a clinical potential as an immunosuppressive agent in inflammatory diseases (Choi *et al.* 2012).

### 2.3. Structural features of NFAT proteins and their implications

All four $Ca^{2+}$/CaM-regulated NFAT proteins consist of three distinct domains – the NFAT homology region (NHR), the Rel-homology domain (RHD), and the C-terminal domain (Figure 2). The NHR contains an amino-terminal transactivation domain (TAD-N) and a regulatory domain, which is responsible for NFAT activation and translocation; the RHD is responsible for interaction with DNA and NFAT partner transcription factors, thereby regulating target gene expression; the C-terminal domain resides a carboxy-terminal transactivation domain (TAD-C) (reviewed in Macian 2005).

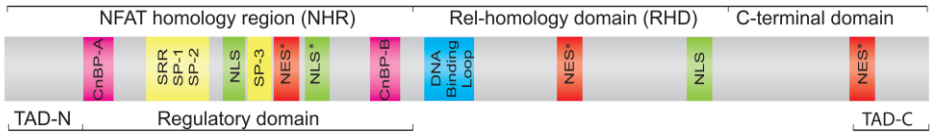



**Figure 2.** Schematic representation of the primary structure of NFAT proteins.
The location of each motif is well conserved among the NFAT proteins, except for the ones marked with asterisks. See text for further details. CnBP-A/B – calcineurin binding peptide A and B; SRR – serine-rich region; SP-1-3 – serine-proline motif 1-3; NLS – nuclear localization signal; NES – nuclear export signal; TAD-N/C – amino/carboxy-terminal transactivation domain.

NFAT proteins have two distinct CaN-binding sites, both located in the regulatory domain (Figure 2.). Aramburu and colleagues were the first to identify a conserved sequence motif of NFAT (PxIxIT), called CaN binding peptide A (CnBP-A), that is critical for effective recognition and dephosphorylation of

NFAT proteins by CaN (Aramburu *et al.* 1998). The non-conserved amino acids (x) in PxIxIT are thought to modulate the interaction between NFAT and CaN under different physiological conditions (Li *et al.* 2007). Later, another CaN binding site of NFAT (LxVP) was discovered, called CnBP-B, which synergizes with the previously identified docking site CnBP-A to increase the overall binding affinity for CaN (Park *et al.* 2000). CnBP-B has been shown to have different CaN binding potencies among the NFAT family members, which enables distinct regulation of different NFATs in the same cell type (Martínez-Martínez *et al.* 2006).

The regulatory domain of all NFAT proteins contains an extended serine-rich region (SRR) with the characteristic sequence $(SP)_{1-2}x(S)_{2-3}xSSxSxxS[D/E]$ and three serine-proline (SP) motifs (SP-1, SP-2, and SP-3) with the characteristic sequence $(SPxx)_{1-4}xxx[D/E]_{1-2}$. The serines in these motifs are phosphorylated in resting cells and dephosphorylated during NFAT activation by CaN. Between the second and the third SP motif lies a conserved monopartite nuclear localization signal (NLS) (PxxKR[K/R]xS) that is exposed by dephosphorylation of SRR and SP motifs (reviewed in Kiani *et al.* 2000). Okamura and colleagues have shown that dephosphorylation of at least thirteen conserved serines out of twenty one that are phosphorylated in resting cells is required for full exposure of the NLS in the regulatory domain and masking of the nuclear export signal (NES) of the NFATc2 protein (Okamura *et al.* 2000). Outside of the regulatory domain lies a second conserved monopartite NLS (NG[K/R]RK[K/R]SxxQ) that is located within the RHD of NFAT proteins (Masuda *et al.* 1995). The two conserved NLSs are partially redundant in their function, since both of these NLSs have to be mutated to block nuclear entry of NFAT in response to $Ca^{2+}$ elevation (Beals *et al.* 1997a).

NES motifs are not as clearly conserved among the different NFAT family members as are the NLS motifs. For example, Kiani and colleagues have pointed out that a NES located in the C-terminus of the NHR is conserved in NFATc1 and NFATc2 but not in NFATc3 and NFATc4, and a NES located in the N-terminus of the regulatory domain of NFATc3 is not conserved in other NFAT family proteins (reviewed in Kiani *et al.* 2000). Nuclear export of NFAT proteins is mediated by multiple nucleocytoplasmic shuttling factors, including Ran-GTPase and the nuclear export receptor chromosomal maintenance 1 (CRM1) (Kehlenbach *et al.* 1998). On NFATc3, however, binding of CaN and CRM1 is mutually exclusive because the NES located in the N-terminus of the regulatory domain overlaps with the CnBP-A motif. Thus, CaN binding abolishes NFATc3 nuclear export by CRM1 (Zhu and McKeon 1999). In any case, all in all the NFAT proteins can exist in at least two alternative conformations – one form in which the NLS is exposed and the NES is masked or *vice versa*. These different conformations are achieved via dephosphorylation or phosphorylation, respectively (Okamura *et al.* 2000).

Kinases that are important for NFAT cytoplasmic maintenance or rapid nuclear export can recognize and phosphorylate different motifs in NFAT proteins. For example, GSK3 phosphorylates SP-2 and SP-3 motifs (Beals *et al.* 1997b).

MAPKs p38 and JNK phosphorylate serines at the SRR (Chow *et al.* 2000; Yang *et al.* 2002). DYRK1A and DYRK2 phosphorylate the SP-3 motif of NFATc2 and therefore prime it for the subsequent phosphorylation by GSK3 and CK1 at SP-2 and SRR motifs, respectively (Gwack *et al.* 2006). Similarly, previous phosphorylation of serines in the SP-2 and SP-3 motifs and a serine just adjacent to the NLS located in the regulatory domain of NFATc1 by PKA is needed for its further phosphorylation by GSK3 (Sheridan *et al.* 2002).

The NFAT family of proteins is thought to have arisen approximately 500 million years ago from a common precursors with the Rel domain (Wu *et al.* 2007). Therefore each of the NFAT proteins contains a highly conserved DNA-binding domain in the N-terminal half of the RHD (reviewed in Rao *et al.* 1997). Despite the fact that the RHDs of NFAT proteins are only 18-20% identical to the Rel domain of NF-κB p50 (Graef *et al.* 2001c), their overall structural motifs governing DNA binding are very similar to other Rel family RHD domains (Jain *et al.* 1995). Within the Rel-family proteins, the RHD contains the conserved DNA recognition loop (RFRYxCEG) that in the NFAT family proteins is RAHYETEG, in which each of the underlined residues make a contact with DNA at the NFAT recognition site (Jain *et al.* 1995; Chen *et al.* 1998). Interestingly, mutating the histidine to arginine (as in NF-kB p50) in the DNA recognition sequence of NFAT proteins increases DNA binding affinity, which means that there has been a strong evolutionary selection for weak DNA binding of NFAT proteins (Wu *et al.* 2007). This means, in turn, that the NFAT partner proteins are required to increase its DNA binding energy (reviewed in Rao *et al.* 1997; Chen *et al.* 1998). The structures of monomeric NFAT-DNA complexes have revealed that a highly conserved nine amino acid linker located within the RHD provides conformational flexibility that allows many surfaces of the RHD domain to interact with different transcription factors (Stroud and Chen 2003). In addition to the previously mentioned AP-1 proteins, nuclear partners for NFAT that belong to different families of transcription factors include GATA binding proteins (GATA), MEF2 proteins, Sry-related HMG box containing SOX proteins, and many more (Kao *et al.* 2009; Singh *et al.* 2015; reviewed in McKinsey *et al.* 2002; Hogan *et al.* 2003; and Mognol *et al.* 2016). Therefore, NFAT-dependent gene regulation involves a coordinated action of many signaling pathways, although it is also possible that the partner for NFAT can be another NFAT protein. For example, NFAT homo- or heterodimers have been shown to bind NF-κB-like NFAT sites, such as in the tumor necrosis factor alpha, *IL8*, and *Grail* gene promoters, and in the HIV-1 long terminal repeat (McCaffrey *et al.* 1994; Giffin *et al.* 2003; Jin *et al.* 2003; Falvo *et al.* 2008; Bates *et al.* 2008; Soto-Nieves *et al.* 2009).

The highly conserved RHD of NFAT proteins allows them to bind to the same DNA sequence and interact with common partner proteins. Therefore, different NFATs have some redundancy in their functions as can be concluded from knock-out studies (reviewed in Hogan *et al.* 2003). However, NFATs have also non-redundant roles, which could be partially explained by the less conserved TADs located in the N- and C-terminal regions of NFAT proteins. As said, the

TADs are not conserved but are recognizable in different NFATs. For example the N-terminal TAD of NFATc2 has been localized to ~100 N-terminal amino acids, many of them being acidic and hydrophobic residues, which resembles the corresponding regions of NFATc3, NFATc4 and one of the isoforms of NFATc1 (reviewed in Rao *et al.* 1997). The transactivation potency of this region has been demonstrated in both NFATc1 and NFATc2 *in vitro* (Luo *et al.* 1996; Hock and Brown 2003). Similarly, the C-terminal TADs with limited sequence similarity can be identified in each of the NFAT family member. Using different reporter assays, C-terminally longer isoforms of NFATc1 and NFATc3 have been shown to have greater transactivation activity than shorter isoforms without a C-terminal TAD (Luo *et al.* 1996; Imamura *et al.* 1998). Later, several transcriptional co-regulators have been shown to interact with the TAD regions (reviewed in Mognol *et al.* 2016). For example, EP300 has been shown to interact with the TAD-N of NFATc2 and both the TAD-N and the TAD-C regions of NFATc1 and NFATc4, respectively (García-Rodríguez and Rao 1998; Avots *et al.* 1999; Yang *et al.* 2001).

### 2.4. Expression and function of NFAT genes

NFAT proteins are expressed in almost every tissue and, accordingly, there are many developmental and physiological functions that require NFAT signaling (reviewed in Rao *et al.* 1997). In the immune system, where NFATc2 was the first NFAT family member to be discovered in T-cells, all NFATs, except NFATc4, are expressed (Lyakh *et al.* 1997; reviewed in Rao *et al.* 1997). In the musculoskeletal, cardiovascular, and nervous system the expression of all four NFATs has been detected (reviewed in Schulz and Yutzey 2004 and; Lambrechts and Carmeliet 2004 and; Sitara and Aliprantis 2010 and; Kipanyula *et al.* 2016). However, the expression patterns and levels of each NFAT gene in different organ systems are dependent on the specific tissue and cell type, as well as the developmental stage and health condition. Moreover, NFAT family members often display redundant functions, as can be concluded from knock-out studies where compound NFAT mutants often display more severe phenotypes than single mutants (summarized in Wu *et al.* 2007). In the following subchapters I will provide a brief summary of the biological processes governed by CaN/NFAT signaling and of the related diseases associated with the disturbance of it, both which are tightly connected to NFAT expression in the respective tissue. Since the focus of this work is NFAT regulation in neurons, I will concentrate more on NFAT functions in the nervous system.

### 2.4.1. NFATs in non-neuronal tissues

NFAT signaling plays a critical role in the development and function of lymphocytes by regulating the expression of inducible genes encoding cytokines and cell-surface receptors (reviewed in Macian 2005). An efficient way to elucidate the contribution of different NFATs to lymphoid development and activation is to analyze the effects of mutations and deletions of NFATs. Since *Nfatc1* null mutant mice die *in utero* from congestive heart failure (Ranger *et al.* 1998a; de la Pompa *et al.* 1998), *Nfatc1*-deficient chimeras have been used in studies involving NFATc1-deficient lymphoid systems (Ranger *et al.* 1998b; Yoshida *et al.* 1998). NFAT knockout studies have revealed that NFATc1 and NFATc2 are indispensable in cytokine production, since T-cells from *Nfatc1*/*Nfatc2* double-deficient chimeras were unable to produce an immune response (Peng *et al.* 2001). However, studies using single mutant mice have revealed that NFATc1 and NFATc2 are antagonistic in their function in lymphocytes: *Nfatc1*-deficiency has been shown to impair proliferation of T-lymphocytes, whereas *Nfatc2*-deficiency has been shown to lead to hyperproliferation of both B- and T-lymphocytes (reviewed in Graef *et al.* 2001b). *Nfatc3*-deficient mice express normal cytokine levels but have defects in generation of normal number of cluster of differentiation $4^+$ and $8^+$ single positive thymocytes and mature T-cells during thymic development (Oukka *et al.* 1998). *Nfatc2/c3* double knockout mice develop a severe lymphoproliferative disorder named splenomegaly with dramatic increase of type 2 cytokines and IgG1 and IgE levels, resulting in inflammatory symptoms resembling a severe allergic response (reviewed in Graef *et al.* 2001b). Since fine balancing of CaN/NFAT signaling in lymphocytes is important in determining their fate, NFATc1, NFATc2, or NFATc3 misregulation is common in several types of lymphomas (reviewed in Medyouf and Ghysdael 2008 and; Mognol *et al.* 2016).

NFAT signaling plays a crucial role in heart development and function. During heart development, NFATc1 has been shown to be expressed in endocardial cells, where it is essential for the morphogenesis of vertebrate heart valves (Wu *et al.* 2011). *Nfatc1* null mutant mice die *in utero* from selective absence of the aortic and pulmonary valves at embryonic day 14.5 (Ranger *et al.* 1998a; de la Pompa *et al.* 1998). The activation of NFATc1 in cardiac valve growth and remodeling has been shown to be mediated by vascular endothelial growth factor (VEGF) signaling through VEGF receptor 2 (Johnson *et al.* 2003; Combs and Yutzey 2009; Park *et al.* 2010). NFATc2, NFATc3, and NFATc4, however, have been shown to be expressed in the myocardium during epithelial-mesenchymal transformation (EMT), where they prevent VEGF expression that, while being necessary for initiation of EMT, subsequently terminates this process (reviewed in Lambrechts and Carmeliet 2004). Mouse embryos bearing combined deletions of *Nfatc2*, *Nfatc3*, and *Nfatc4* die at around embryonic day 11 due to endocardial cushion defects with a hypocellular cardiac jelly (Chang *et al.* 2004). Furthermore, in addition to decrease in ventricular cardiomyocyte proliferation *Nfatc3*/*Nfatc4* double mutant mice have been shown to have reduced cardiac

metabolic function (Bushdid *et al.* 2003). In adult heart, NFATc3 and NFATc4 have been both shown to be able to induce cardiac hypertrophy *in vivo*, which could result in progression of heart failure and sudden death (reviewed in Molkentin 2000 and; Frey and Olson 2003; Wilkins *et al.* 2002). Cardiac hypertrophy is also associated with increased activity of CaN and transcription factors MEF2 and GATA4, known binding partners for NFATs, making all of them attractive therapeutic targets in the treatment of cardiac hypertrophy (reviewed in Kohli *et al.* 2011).

Since NFATs have been shown to modulate the expression of VEGF, a central regulator of the development and maintenance of heart, lung, and vascular tissues, it is not surprising that NFAT signaling plays a role in vasculogenesis and angiogenesis (reviewed in Graef *et al.* 2001b). *Nfatc3*/*Nfatc4* double mutant mice, but not single *Nfatc3* or *Nfatc4* mutants alone, die around embryonic day 11 having defects in vascular patterning and angiogenesis together with increased expression of VEGF (Graef *et al.* 2001a). Since coordinate maturation of the heart and blood vessels is needed for the development of the cardiovascular system, *Nfatc3*/*Nfatc4* double mutant mice, as mentioned beforehand, have also reported to die due to heart failure (Bushdid *et al.* 2003; Chang *et al.* 2004). NFAT signaling has been shown to control angiogenesis via negative regulation of VEGF by upregulating VEGF receptor 1, so called decoy receptor that, by sequestering VEGF, inhibits VEGF signaling. This means that NFAT downregulation leads to increased and aberrant expression of VEGF (reviewed in Qin *et al.* 2014). It has been proposed that during angiogenesis, high expression of NFATc4 in tissues surrounding blood vessels prevents VEGF-induced branching of blood vessels into these areas thereby promoting proper patterning of the vascular system (Graef *et al.* 2001a). Furthermore, in different experimental settings, NFATc1 and NFATc3 have been identified to promote VEGF-induced angiogenesis via upregulation of cyclooxygenase-2, an important mediator of angiogenesis (Urso *et al.* 2011; Mena *et al.* 2014; Suehiro *et al.* 2014).

The NFAT pathway has been shown to be important also in the development and maintenance of the musculoskeletal system (reviewed in Schulz and Yutzey 2004 and; Sitara and Aliprantis 2010). During skeletal muscle development, each NFAT protein seems to be specialized to regulate a specific stage of myogenesis, such as proliferation and differentiation of myoblasts, fusion of myoblasts into multinucleated myotubes, and myofiber maturation (reviewed in Horsley and Pavlath 2002). NFATc4 has been shown to be expressed in proliferating human primary myoblasts where it regulates self-renewal of quiescent reserve muscle cells (Perroud *et al.* 2017). Both single-mutant *Nfatc2* and *Nfatc3* mice have reduced muscle mass in the adult, however, for different reasons. *Nfatc3* mutant mice have defects in the formation of primary myofibers at early stages of embryogenesis, whereas, *Nfatc2* null mice have defects in recruitment and/or fusion of myoblasts into multinucleated muscle cells, which affects both embryonic and postnatal muscle growth (Kegley *et al.* 2001; Horsley *et al.* 2001). NFATc1 has been shown to be important for myotube growth and late myofiber

maturation (Ehlers *et al.* 2014; Perroud *et al.* 2017). Mice with conditional *Nfatc1* knockout in skeletal muscle have defects in muscle fiber typing and fast-to-slow fiber type switching in response to exercise *in vivo* (Ehlers *et al.* 2014). NFATc1, together with GATA-2, has also been associated with muscle regeneration after muscle damage, and with skeletal muscle hypertrophy in response to insulin-like growth factor 1 stimulation (Musarò *et al.* 1999; Sakuma *et al.* 2003).

In the musculoskeletal system, NFAT signaling plays a role also in bone and cartilage formation (reviewed in Sitara and Aliprantis 2010). Bone homeostasis depends on a balance between bone-forming osteoblasts and bone-resorbing osteoclasts, whereas their imbalance leads to various bone diseases. NFATc1 has been shown to be essential for regulating this balance (reviewed in Sitara and Aliprantis 2010). *Nfatc1*-deficient mouse embryonic stem cells are unable to differentiate into osteoclasts, whereas mice with constitutively nuclear NFATc1 have increased osteoclastogenesis and develop a rare skeletal disorder named osteopetrosis, literally "stone bone" (reviewed in Takayanagi 2007). Furthermore, all four $Ca^{2+}$-regulated NFAT proteins are expressed in cartilage (Ranger *et al.* 2000), where NFAT signaling has been shown to regulate the formation of joints and is thereby a focal point in the pathogenesis of osteoarthritis (reviewed in Beier 2014).

In addition to above-mentioned associations between NFAT signaling and development of different lymphomas, NFATs are implicated in pathophysiology of several types of solid cancers, such as lung, colon, skin, breast, prostate, and pancreatic tumors (reviewed in Daniel *et al.* 2014 and; Mognol *et al.* 2016), where NFATs play a role in both initiation and progression of cancer, including angiogenesis and lymphangiogenesis, as well as in drug resistance (reviewed in Shou *et al.* 2015). Since widely used inhibitors of NFAT signaling, such as CsA and FK506, lead to toxic side effects, such as hypertension, nephrotoxicity, and diabetic nephropathy (reviewed in Rühlmann and Nordheim 1997), clinical usage of these inhibitors is limited. Thus, there is a need for cancer therapies that target tumor- and isoform-specific NFATs.

### 2.4.2. NFATs in the nervous system

The first evidence that NFAT proteins may have roles in the nervous system emerged in 1994 when NFATc2 expression was detected in the brain (Northrop *et al.* 1994; Ho *et al.* 1994). The first proof of NFAT functionality in the brain came from the study by Graef and colleagues who showed that NFATc4 is expressed in hippocampal neurons where it regulates the expression of the neuron-specifically expressed *$IP_3R1$* gene (Graef *et al.* 1999). This study showed that the activation of NFAT-dependent transcription required $Ca^{2+}$ influx via LTCC (Graef *et al.* 1999). Later it was shown that in hippocampal dendritic spines $Ca^{2+}$ entry via LTCC is tightly linked to NFAT activation by the anchoring protein AKAP5, which couples PKA and CaN to CaM-bound $Ca^{2+}$ channels for localized activation upon depolarization (Li *et al.* 2012; Murphy *et al.* 2014;

Dittmer *et al.* 2014). Furthermore, activation of tropomyosin receptor kinase B (TrkB) receptors by brain-derived neurotrophic factor (BDNF) resulted in increased expression of $IP_3R1$ via NFATc4 signaling in hippocampal neurons (Groth and Mermelstein 2003). In line with a study showing that $IP_3R1$-deficient mice have impairments in hippocampal synaptic plasticity (Fujii *et al.* 2000), it was proposed that NFAT-dependent gene transcription is needed for the proper induction of hippocampal synaptic plasticity and memory formation. Since it has been shown in NFAT luciferase reporter transgenic mice that the brain is the organ with the highest NFAT transcriptional activity (Plyte *et al.* 2001), it is not surprising that since then many other functions of NFAT signaling in both the adult and developing nervous system have been discovered.

NFAT signaling has been found to be crucial already at the very early stages of nervous system development (Kao *et al.* 2009; Li *et al.* 2011b; Huang *et al.* 2011; Ding *et al.* 2013; Quadrato *et al.* 2014; Moreno *et al.* 2015; Serrano-Pérez *et al.* 2015; Artegiani *et al.* 2015). A transcriptome analysis of isolated proliferating neural stem cells (NSCs), differentiating neural progenitor cells (NPCs), and newborn neurons identified NFATc3 and NFATc4 as potential regulators of mouse corticogenesis (Aprea *et al.* 2013). NFATc4, for example, has been shown to promote proliferation and self-renewal, and to prevent differentiation of mouse embryonic NSCs (and NPCs) in hypoxia, which is a physiological condition during embryonic development (Moreno *et al.* 2015). Similarly, NFATc4 activity has been shown to inhibit dendritogenesis in mouse immature CGNs, whereas in mature CGNs, that have become hyperpolarized, decreased NFATc4 activity leads to upregulation of many synapse-related genes needed for dendritic and synaptic maturation (Ding *et al.* 2013). In adult-born hippocampal neurons, NFATc4 has been shown to promote neurogenesis via positive excitatory feedback loop between gamma-aminobutyric acid (GABA)-ergic signaling and NFATc4 by upregulating gamma-aminobutyric acid receptor subunit alpha-2 and -4 (*GABRA2* and *GABRA4*) upon GABA receptor activation (Quadrato *et al.* 2014). NFATc3 has been shown to reduce proliferation of NSCs and to promote neurogenesis of NSCs as well as migration and differentiation of NPCs, astrocytes, and newborn neurons during early postnatal corticogenesis (Serrano-Pérez *et al.* 2015; Artegiani *et al.* 2015). In the peripheral nervous system, both NFATc3 and NFATc4 have been shown to be activated in neuregulin 1-dependent differentiation of Schwann cells from Schwann cell precursor cells (Kao et al. 2009). Later, in promyelinating Schwann cells, NFATc4 and transcription factor Sox10 synergistically activate expression of early growth response protein 2, which in turn regulates genes necessary for myelination (Kao *et al.* 2009). Collectively, these examples illustrate that although both NFATc3 and NFATc4 regulate neural development from the early stages of embryogenesis to the adulthood and play a role in both neuro- and gliogenesis, they can evoke different responses in same type of cells.

NFAT signaling has also been shown to play a role in both neuronal survival and apoptosis, two reciprocal processes that determine cell fate during normal development of the nervous system. In the developing cerebellum, NFATc4 is

required for the survival of primary CGNs (Benedito *et al.* 2005), whereas NFATc3 mediates a proapoptotic effect in CGNs by interacting, in a SUMO-dependent manner, with Trim17, a crucial E3 ubiquitin ligase that is necessary and sufficient for neuronal apoptosis (Mojsa *et al.* 2015). NFATc4 has been shown to mediate the prosurvival effect of BDNF, which is one of the key survival factors for various populations of neurons in the developing nervous system (reviewed in Bibel and Barde 2000). In primary cortical neurons, NFATc4 has been shown to increase *BDNF* expression downstream of NMDA receptor signaling, thereby promoting neuronal survival (Vashishta *et al.* 2009). NFATc4 has been also shown to mediate the survival of hippocampal adult-born neurons downstream of BDNF/TrkB signaling (Quadrato *et al.* 2012). A few studies have shown that NFAT also mediates neural apoptosis by directly upregulating membrane-bound death receptor Fas ligand (FasL) expression (Jayanthi *et al.* 2005; Luoma and Zirpel 2008; Gómez-Sintes and Lucas 2010). For example, during the development and maturation of sensory neurons, deafferentation-induced neuronal loss in the cochlear nucleus during a critical period has been shown to be mediated by NFATc4 due to upregulation of FasL (Luoma and Zirpel 2008). NFAT/Fas-mediated neuronal apoptosis has also been shown to be induced after methamphetamine (Jayanthi *et al.* 2005) or lithium administration (Gómez-Sintes and Lucas 2010). Therefore, whether NFAT signaling promotes apoptosis or mediates neuronal survival is dependent on the activity of the specific member of the NFAT family in a particular tissue under certain conditions.

During brain development, neurons respond to neurotrophins and netrins with axonal outgrowth, which is the basis in the formation of neuronal connections. NFAT signaling has been shown to be involved in axonal growth (Graef *et al.* 2003; Nguyen *et al.* 2009; Yoshida and Mishina 2005). Mouse embryos bearing combined deletions of *Nfatc3* and *Nfatc4*, or a combination of deletions of *Nfatc2*, *Nfatc3*, and *Nfatc4* have been shown to have defects in neurotrophin- and netrin-dependent sensory neuron axon outgrowth (Graef *et al.* 2003). The fact that axonal outgrowth was not affected in single *NFAT* gene mutants indicates yet again that NFAT family members have some redundancy in their function (Graef *et al.* 2003). Furthermore, a study using zebrafish olfactory sensory neurons showed that NFAT activity positively regulates axon terminal differentiation during synaptogenesis (Yoshida and Mishina 2005). Opposite to the previous studies, Nguyen and colleagues showed that NFATc4 acts as a negative regulator of neuronal outgrowth and maturation by directly repressing the expression of axonal growth cone marker growth associated protein 43 in rat primary cortical neurons in response to neurotrophin signaling (Nguyen *et al.* 2009). In *Drosophila*, the single NFAT homolog has been shown to negatively regulate synapse growth and neurotransmitter release in *Drosophila* larval motor neurons (Freeman *et al.* 2011). Furthermore, CaN/NFAT activity has been shown to negatively regulate synaptic development and dendritic branch addition of individual optic tectal neurons in the living *Xenopus* tadpole (Schwartz *et al.* 2009). These seemingly contradictory results may probably be due to different

neuron types and/or developmental stages that were under examination. Future studies revealing possible binding partners and target genes would help to define the exact roles of NFATs in the processes that regulate neurite growth and synapse formation.

### 2.4.3. NFAT signaling in pathologies of the nervous system

Alterations in CaN/NFAT signaling have been associated with numerous pathologies of the nervous system. It has been shown that CaN activity and expression is increased in mouse models of Alzheimer's disease and Parkinson's disease (reviewed in Abdul *et al.* 2010; Luo *et al.* 2014). Increased nuclear levels of NFATc3 have been reported in midbrain dopaminergic neurons of Parkinson's disease mouse model (Luo *et al.* 2014), and nuclear staining of NFATc4 is increased in human α-synucleinopathy patients' brains compared to non-diseased controls (Caraveo *et al.* 2014). Moreover, increased NFATc4 levels were shown in hippocampal tissue of AD mouse model, where it was shown to directly regulate amyloidogenic processing (Mei *et al.* 2015). Furthermore, there is an increase in NFATc2 and NFATc4 nuclear levels in human hippocampal astrocytes of patients diagnosed with mild cognitive impairment and Alzheimer's disease, which correlated with cognitive decline severity. Therefore, it has been proposed that increased NFATc2 activity contributes to the neuroinflammatory responses in early stages of cognitive decline and increased activity of NFATc4 leads to the neurodegerative processes, such as increased cellular death and dementia (reviewed in Abdul *et al.* 2010). Similarly, increased CaN/NFATc4 activity is associated also with neuronal death of hippocampal CA1 neurons after ischemic insult through activation of FasL expression (Shioda *et al.* 2007).

Neuroinflammation is associated with short- and long-term consequences of traumatic brain injury (TBI) and in some cases TBI is also associated with neurodegenerative symptoms that could lead to early onset of dementia, Alzheimer's disease or Parkinson's disease (reviewed in Chiu *et al.* 2016). Several studies have shown increased nuclear NFATc3 levels in hippocampal astrocytes immediately after TBI, which is associated with regulation of neuroinflammatory processes (Serrano-Pérez *et al.* 2011; Neria *et al.* 2013; Yan *et al.* 2014; Furman *et al.* 2016). Furthermore, administration of CaN inhibitor FK506 to rats immediately after TBI has been shown to significantly decrease IL-2 and interferon gamma expression levels and NFATc1 nuclear staining in cortical tissue compared to control TBI animals (Wu *et al.* 2016). Increased levels of nuclear NFATc4 in hippocampal neurons were seen at subacute to chronic time points after cortical impact injury model of TBI (Yan *et al.* 2014), which could underlie neurodegenerative symptoms associated with TBI (reviewed in Chiu *et al.* 2016).

Since CaN/NFAT signaling plays a role in nociception by upregulating pro-nociceptive genes in dorsal root ganglia sensory neurons in response to nerve or tissue injury, upregulation of NFAT signaling is associated with hyperalgesia and

development of neuropathic pain (Groth *et al.* 2007; Jackson *et al.* 2007; Cai *et al.* 2013; Kim *et al.* 2014). In the processes of inflammatory pain, nerve growth factor (NGF) levels increase in peripheral tissue, which leads to tonic activation and sensitization of sensory afferent neurons via TrkA receptors. Furthermore, exogenous administration of NGF results in hyperalgesic responses similar to those observed in inflammatory pain models (reviewed in Siniscalco *et al.* 2011). It has been shown that NGF facilitates depolarization-induced activation of NFATc3 in dorsal root ganglion (DRG) sensory neurons via $PI_3K$/Akt1 pathway downstream of the TrkA receptors (Kim *et al.* 2014). NGF also triggers BDNF upregulation in DRG neurons where it is anterogradely transported to the central terminals of the primary afferents of the spinal dorsal horn. When BDNF is released in the dorsal horn, it binds to TrkB receptors on secondary sensory neurons thereby modulating synaptic transmission in nociception (reviewed in Obata and Noguchi 2006). NFATc4 has been shown to mediate the upregulation of BDNF by NGF in primary afferent neurons in the DRG. BDNF, in turn, triggers upregulation of pro-nociceptive genes involved in inflammatory pain via NFAT-dependent transcription in spinal cord neurons (Groth *et al.* 2007).

NFAT proteins have also been shown to constraint long-term plasticity by dampening neuronal excitability (Zhang and Shapiro 2012; Yao *et al.* 2016). Therefore, aberrant NFAT signaling could play a role in hyperexcitability disorders such as epilepsy, and produce chronic pain, and cognitive deficits (reviewed in Tsantoulas and McMahon 2014; Greene and Hoshi 2017). In rat superior cervical ganglion sympathetic neurons, NFATc1 and NFATc2 have been shown to regulate the dampening of excessive neuronal excitability by upregulating the expression of neuron-specific $K^+$ channels Kv7.2 and Kv7.3 upon LTCC activation (Zhang and Shapiro 2012). LTCC activation-induced augmentation of Kv7.2 and Kv7.3 transcription was shown to be orchestrated by AKAP5 that binds CaN and CaM to the microdomain of L-type $Ca_V1.3$ $Ca^{2+}$ channel and thereby links neuronal activity to NFAT-mediated transcriptional regulation (Zhang and Shapiro 2012). It is possible that the function of NFAT signaling for controlling over-excitability could be universal throughout the nervous system since neuron-specific Kv7 $K^+$ channels are expressed in various central and peripheral neurons, such as sympathetic neurons, hippocampal pyramidal cells, and striatal neurons (reviewed in Delmas and Brown 2005 and; Birnbaum *et al.* 2004).

In conclusion, since functions of the different members of the NFAT family are dependent of their specific target genes, which, when misregulated, could lead to different pathologies, understanding the roles and regulation of specific NFAT isoforms in these processes could have possible therapeutic potential.

## AIMS OF THE STUDY

Although NFAT proteins have been shown to $Ca^{2+}$-dependently regulate gene transcription in various developing and adult tissues, a systematic analysis of the structure and expression of the NFAT genes themselves has not been performed. Furthermore, increasing amount of evidence indicates the importance of different NFAT family members in the nervous system. Yet, how and to what extent the different NFAT proteins and, moreover, their isoforms contribute to the NFAT-dependent gene regulation in neurons has remained unclear. Thus, the aims of the current study were to:

- characterize the structure, alternative splicing and expression of the members of the human and mouse NFAT gene family, with the emphasis on their expression in the brain;

- study the subcellular localizations and the transactivation capacities of different over-expressed human NFAT isoforms at basal conditions and in response to membrane depolarization in rat cultured primary cortical and hippocampal neurons;

- determine whether sumoylation is involved in the regulation of the transcriptional activity of human NFAT proteins in neurons.

## MATERIALS AND METHODS

I used following experimental methods during the study that are described in the indicated publications:

- Bioinformatic analyses of gene and mRNA sequences (publication I)
- Cloning (publications I-III)
- Culturing of immortalized mammalian cells and rat primary cortical and hippocampal neurons (publications II and III)
- Immunocytochemistry (publication II)
- *In situ* hybridization (publication I)
- Luciferase reporter assay (publication II and III)
- Preparation of cell lysates and immunoblotting (publications II and III)
- RNA isolation and reverse transcription polymerase chain reaction (RT-PCR) (publications I and II)
- Site directed mutagenesis (publication III)
- Transfection of mammalian cells (publications II and III)

## RESULTS AND DISCUSSION

### 1. Structure and alternative splicing of human and mouse *NFAT* genes (publication I)

The existence of multiple *NFAT* transcripts has been demonstrated, but a systematic analysis of the structures and alternative splicing of human and mouse *NFAT* genes has not been performed. Such information would enable us to understand the possible specialized functions of alternative *NFAT* transcripts in different tissues. Thus, our first aim was to characterize the exon/intron structures of the human and mouse *NFATC1, NFATC2, NFATC3,* and *NFATC4* genes and to analyze the alternative splicing patterns of each NFAT gene in both human and mouse.

According to our data, both human and mouse *NFATC1* and *NFATC2,* and mouse *Nfatc3* and *Nfatc4,* have two alternative 5′ exons. In human, we detected six alternative 5′ exons for *NFATC3* and seven alternative 5′ exons for *NFATC4.* Our results also showed that in both human and mouse, *NFATC1* and *NFATC3* have two alternative 3′ exons and *NFATC4* has one 3′ exon. For *NFATC2* we detected one 3′ exon in human but three alternative 3′ exons in mouse. In addition, there are several alternative splice variants of internal exons. Exon IX of *NFATC1* has three splice variants in human and two in mouse, respectively. Exon II have three splice variant in both human and mouse *NFATC2*. There are two additional alternative exons downstream of exon IX in human and one in mouse *NFATC3,* respectively. For *NFATC4,* there are three splice variants of exon IX in human and two in mouse, respectively. Hence, for human NFAT genes, for example, alternative splicing in combination with the usage of alternative 5′ and 3′ exons could theoretically give rise to 44 different NFAT protein: 8 different protein isoforms of NFATc1, 6 different isoforms of both NFATc2 and NFATc3, and 24 different isoforms of NFATc4. We have named the *NFAT* alternative transcripts and protein isoforms according to the usage of an alternative 5′ exon (e.g. IA), alternative 3′ exon (e.g. VIII), and splice variants, such as skipping of an exon (e.g. ΔXa), long or short variant of an exon (e.g. IXL or IXS, respectively), or retention of an intron (e.g. IXi), in the respective mRNA.

Our results on the structures of the NFAT genes are in agreement with previous data from other groups (Imamura *et al.* 1998; Chuvpilo *et al.* 1999; Plyte *et al.* 2001) but add plenty of new data about the complex splicing and expression of this gene family. In general, *NFAT* genes encode proteins that are very similar in their central region, encoded by rarely alternatively spliced exons II–VIII that are translated into the regulatory domain and the RHD, which are needed for NFAT activation, translocation, and interaction with DNA and NFAT partner transcription factors. However, due to complex usage of alternative 5′ and 3′ exons and alternative splicing, NFAT isoforms differ greatly in their N- and C-terminal parts, which have been shown to interact with different transcriptional co-regulators (reviewed in Mognol *et al.* 2016) and could thereby give NFAT isoforms different transactivational potentials.

## 2. Expression of human and mouse *NFATc1, NFATc2, NFATc3,* and *NFATc4* mRNAs in the brain (publication I)

To determine the expression of different *NFATC1, NFATC2, NFATC3,* and *NFATC4* mRNA splice variants, we analyzed the expression patterns of different *NFAT* alternative transcripts by RT-PCR in different human and mouse tissues with the emphasis on the brain. Furthermore, we used *in situ* hybridization to analyze the expression of *NFAT* genes in adult mouse brain and in adult human hippocampus.

Our expression analyses are consistent with previous studies showing *Nfatc1* expression predominantly in the immune system (Hoey *et al.* 1995; Northrop *et al.* 1994; Cockerill *et al.* 1995; Masuda *et al.* 1995; Weiss *et al.* 1996). However, our study is the first to show that *Nfatc1* is also expressed in the brain. Our *in situ* hybridization analyses detected the highest expression levels of *Nfatc1* in the granular and glomerular cell layers of the mouse olfactory bulb and moderate expression was seen in the cerebellar granule cells. It is to be noted, that in the human brain, all the NFATs, among other regions, were expressed in the corpus callosum and optic nerve – regions of the brain containing mainly neuronal projections and glial cells. Furthermore, by *in situ* hybridization, we found that the majority of the signal in the mouse brain accumulated in certain neuronal populations, although for all NFATs, very low signal was detected all over the brain, including glial cells. Thus, it is possible that in addition to neurons, NFAT genes are also expressed in glial cells. In the human hippocampus, all NFATs were expressed in the CA3 pyramidal neurons and dentate gyrus granular cell layer and also in the hippocampal fissure, which further supports the possibility that NFAT genes are expressed in both neurons and glial cells. These findings are in agreement with studies showing that all NFATs, including NFATc1, are expressed in mouse primary astrocytes (Pérez-Ortiz *et al.* 2008; Canellada *et al.* 2008) and NFATc1 is expressed in both neurons and glial cells in mouse brain cortex (Wu *et al.* 2016), where it is associated with astrogliosis and neuroinflammation following TBI. Similarly, increased nuclear levels of NFATc2 and NFATc3, or NFATc4 have been shown in astrocytes in patients with mild cognitive impairment, Alzheimer's disease, or TBI, respectively (reviewed in Abdul *et al.* 2010; Serrano-Pérez *et al.* 2011; Neria *et al.* 2013; Yan *et al.* 2014; Furman *et al.* 2016). Altogether, it seems that all NFATs are expressed in glial cells where they could probably participate in the regulation of responses to pathological conditions.

Prior to our work, expression of *Nfatc2* had been shown only in some regions of the brain – in the hypothalamus and olfactory bulb (Northrop *et al.* 1994; Ho *et al.* 1994; Asai *et al.* 2004). In addition to these regions, we detected the highest *Nfatc2* mRNA levels in the pyramidal cell layer of the CA1–CA3 regions of the hippocampus, in the Purkinje and granule cell layers of the cerebellum, and in the thalamus. Furthermore, analysis of *Nfatc2* expression on adult mouse brain slices showed that *Nfatc2* is the most dominantly expressed NFAT with a broad expression pattern throughout the brain. Interestingly, to date, no studies have

analyzed the role of NFATc2 in the central nervous system. However, in parasympathetic nervous system, exogenous NFATc2 and endogenous NFATc1 have shown to be activated by either membrane depolarization or acetylcholine stimulation in rat superior cervical ganglion neurons, where they directly upregulate the expression of neuron-specific $K^+$ channels Kv7.2 and Kv7.3 and thus reduce excitability (Hernández-Ochoa *et al.* 2007; Zhang and Shapiro 2012). Therefore, it is possible that NFATc1 and/or NFATc2 are controlling over-excitability throughout the nervous system since neuronal Kv7 channels are widely expressed in various central and peripheral neurons (reviewed in Greene and Hoshi 2017).

Preceding our study, *Nfatc3* expression in the brain had been demonstrated only in the hypothalamus (Asai *et al.* 2004) and striatum (Jayanthi *et al.* 2005), whereas a functional role of Nfatc3 was demonstrated only in the latter. Namely, increased activity of Nfatc3 was associated with methamphetamine-induced death of dopaminergic neurons (Jayanthi *et al.* 2005). Similarly, increased nuclear levels of Nfatc3 have been reported in midbrain dopaminergic neurons of Parkinson's disease mouse model (Luo *et al.* 2014). Interestingly, our expression analysis showed relatively low levels of *Nfatc3* in different areas of basal ganglia, which, however, does not exclude its role in regulating neuronal survival in these brain areas. In addition, by *in situ* hybridization, we detected high levels of *Nfatc3* in the CGN and in Purkinje cells and, to a lesser extent, in the granule cells of the dentate gyrus and olfactory bulb. Later, *Nfatc3* expression in hippocampal neurons and CGN were also confirmed by others. In hippocampal neurons, silencing of *Nfatc3* has shown to significantly reduce NFAT-mediated luciferase expression in response to depolarization, showing that activity-dependent NFAT-mediated transcription in hippocampal neurons strongly depends on Nfatc3 (Ulrich *et al.* 2012). In CGN, Nfatc3 was shown to facilitates proapoptotic effect by upregulating the expression of Trim17, a E3 ubiquitin ligase involved in neuronal apoptosis (Mojsa *et al.* 2015). In addition to its involvement in regulating neuronal death, Nfatc3 has also shown to be necessary for regulation of neural development from the early stages of embryogenesis to the adulthood (Li *et al.* 2011b; Huang *et al.* 2011; Serrano-Pérez *et al.* 2015; Artegiani *et al.* 2015). These studies are in line with our finding that during mouse development the levels of *Nfatc3* in the brain remain unchanged from embryonic day 13 up to adult.

*Nfatc4* was long been thought to be the most abundant NFAT expressed in the nervous system, since only *Nfatc4* expression had been characterized in more detail in the adult nervous system and cultured primary neurons before our study (Ranger *et al.* 1998a; Graef *et al.* 1999; Jayanthi *et al.* 2005; Benedito *et al.* 2005; Bradley *et al.* 2005; Groth *et al.* 2007; Groth *et al.* 2008). However, our *in situ* hybridization results showed that *Nfatc4* is indeed expressed in the brain but at lower levels compared to the other NFATs. On the other hand, according to our PCR analysis of NFAT expression during mouse brain development, *Nfatc4* was more abundantly expressed in the earlier stages of development and the levels were decreased during later stages, observation that was later confirmed also by

others (Nguyen *et al.* 2009). Therefore, Nfatc4 function might be especially important in the developing brain. This has been shown to be a case during cerebellar development, where first, Nfatc4 activity is required to regulate temporal switch program leading to dendritogenesis in developing CGN (Ding *et al.* 2013), and second, Nfatc4 is required for the survival of primary CGNs (Benedito *et al.* 2005). In adult brain, however, the role of Nfatc4 may be less prominent, as our *in situ* hybridization analysis showed only moderate expression of *Nfatc4* in the cerebellar of adult mouse, as well as dentate gyrus granule cells, and in the mitral cells of the olfactory bulb. Altogether, our results argue that *Nfatc4*, which has previously described to be the major contributor to neuronal gene regulation by NFATs, is in fact less abundant in brain than *Nfatc2*. The idea that NFATc2 and NFATc3 might also have important functions in neurons is supported by the study showing that mice embryos bearing combined deletions of *Nfatc2*, *Nfatc3*, and *Nfatc4* have defects in neurotrophin- and netrin-dependent sensory neuron axon outgrowth (Graef *et al.* 2003), whereas NFAT single-knockout mice have milder neuronal defects (Graef *et al.* 2001a).

Collectively, our work is the most comprehensive investigation of the expression of $Ca^{2+}$-regulated *NFAT* family members to date and demonstrates that all *NFAT*s, although with different patterns, are expressed in the brain and could therefore participate in the regulation of neuronal activity-dependent gene regulation.

## 3. Regulation of the subcellular localization of over-expressed human NFAT isoforms by $Ca^{2+}$ signaling in rat primary cortical and hippocampal neurons (publication II)

NFAT isoforms differ mainly in their C- and N-terminal parts. For a better understanding of how these differences contribute to the regulation of human NFAT isoforms we analyzed the subcellular localization of NFAT family proteins in neurons. For that, we chose 18 human NFAT protein isoforms that, based on our previous mRNA expression analysis, are predominantly expressed in the brain. We subcloned these cDNAs to encode a C-terminal EGFP-tag. The NFAT-EGFP-fusion protein-encoding plasmids that we used included eight NFATc1 isoforms: NFATc1-IA-VIII, NFATc1-IA-ΔIX, NFATc1-IA-IXS, NFATc1-IA-IXL, NFATc1-IB-VIII, NFATc1-IB-ΔIX, NFATc1-IB-IXS, and NFATc1-IB-IXL; four NFATc2 isoforms: NFATc2-IA-IIL-ΔXa, NFATc2-IA-IIL-Xa, NFATc2-IB-IIL-ΔXa, and NFATc2-IB-IIL-Xa (hereafter referred to as NFATc2-IA-ΔXa, NFATc2-IA-Xa, NFATc2-IB-ΔXa, and NFATc2-IB-Xa, respectively); three NFATc3 isoforms: NFTAc3-IB-IX, NFATc3-IB-ΔXa, and NFATc3-IB-Xa; and three NFATc4 isoforms: NFATc4-ID-IXS, NFATc4-ID-IXL, and NFATc4-ID-IXi. We transfected these plasmids into rat primary cortical or hippocampal neurons, and on the following day, performed an

intracellular translocation assay by activating $Ca^{2+}$ signaling with high extracellular $K^+$ that induces $Ca^{2+}$ influx through LTCCs. In this study, we aimed to address the following questions. First, is the $Ca^{2+}$ signaling-mediated regulation of subcellular localization of all the chosen NFAT protein isoforms in neurons CaN dependent? Second, do the subcellular localizations of NFAT family isoforms differ in their response to membrane depolarization? And third, is there any variation in the localization of different NFAT isoforms in neurons at basal conditions?

By using a one-hour pre-treatment with CsA, an immunosuppressive drug that inhibits the $Ca^{2+}$-dependent serine-threonine phosphatase CaN, we confirmed that, in general, the membrane depolarization-dependent translocation of NFAT isoforms to the nucleus in neurons is reliant on the CaN. However, we detected both similarities and differences in the regulation of localization of NFAT isoforms in neurons. At basal conditions, all NFAT isoforms studied were located mostly in the cytosol in hippocampal neurons, whereas, in cortical neurons, the localizations of different NFAT isoforms varied greatly even within the same NFAT subfamily. For example, we found that in untreated cortical neurons NFATc1-IB and NFATc2-IB isoforms were localized predominantly in the cytoplasm, whereas NFATc1-IA and NFATc2-IA isoforms were localized in the nucleus. Yet, blocking spontaneous neuronal activity of transfected cortical neurons with an overnight pre-treatment with sodium channel blocker tetrodotoxin resulted in cytoplasmic localization of all NFAT isoforms analyzed. Thus, these results show that spontaneous neuronal activity in our cortical neuron cultures may be greater than in our hippocampal neuron cultures (an observation also made by others in a comparative study (Harrill *et al.* 2015)), and that the possible differences in the regulatory mechanisms (such as kinase or other regulatory protein activities) acting on N-terminally variable NFAT isoforms cause distinct subcellular localizations of the NFAT isoforms under spontaneous neuronal activity.

Membrane depolarization of hippocampal neurons results in nuclear localization of all NFAT isoforms tested, although the NFATc1, NFATc2 and NFATc3 isoforms translocated comparatively rapidly, whereas the NFATc4 isoforms translocated slowly to the nucleus. Similarly, in cortical neurons, neuronal activity resulted in nuclear localization of NFATc1, NFATc2, and NFATc3 isoforms, albeit NFATc1-IA and NFATc2-IA isoforms were already localized to the nucleus in untreated cells. As for NFATc4 isoforms, they were unresponsive to the membrane depolarization in cortical neurons. These findings suggest that CaN phosphatase activity acting on NFATc4 isoforms might in general be more readily neutralized compared to other NFAT family members in neurons. This has been shown to be a case in hippocampal neurons, where NFATc4, but not NFATc3, is persistently being phosphorylated by GSK3β that counteracts CaN dephosphorylating activity (Ulrich *et al.* 2012). Furthermore, it

has been described that synaptogenesis, maturation, and thus activity of neurons are correlated with an increase in inactive GSK3β levels (Rui *et al.* 2013). Therefore, it could be that decreased GSK3β activity is reflected in our results with NFATc4 isoforms in the spontaneously more active cortical neuron culture.

In conclusion, we have shown that although the neuronal activity-induced nuclear translocation of all NFAT isoforms requires CaN, the precise effects of $Ca^{2+}$ signaling on the subcellular localization of the NFAT proteins is isoform-specific and can vary between neuron types.

## 4. Transactivation capacities of over-expressed wild-type and SUMO mutant isoforms of human NFAT in rat primary cortical and hippocampal neurons (publications II and III)

NFAT proteins are highly conserved in their Rel-homology domain, which is responsible for interactions with DNA and NFAT partner transcription factors. However, different NFAT isoforms, even within the same NFAT subfamily, differ greatly in their C- and N-terminal parts that have been shown to contain less conserved TADs. Although several studies have demonstrated the transactivation potencies of these TADs in different immune and breast cancer cell lines (Luo *et al.* 1996; Imamura *et al.* 1998; García-Rodríguez and Rao 1998; Avots *et al.* 1999; Yang *et al.* 2001; Hock and Brown 2003; Zhang *et al.* 2005; Qin *et al.* 2008), transactivation capacities of different NFAT isoforms in neurons have not been studied before. Therefore, our goal was to analyze the transactivation capacities of human NFAT isoforms in rat primary cortical and hippocampal neurons in response to membrane depolarization (publication II). Additionally, since the transcriptional activity of NFAT proteins has also been shown to be regulated by sumoylation (Terui *et al.* 2004; Nayak *et al.* 2009; Mojsa *et al.* 2015), we wanted to study how sumoylation regulates the transcriptional activity of different human NFAT isoforms in neurons (publication III).

By using an *in silico* pattern recognition tool (Zhao *et al.* 2014), we identified three putative SUMO sites for both NFATc1 and NFATc3, two SUMO sites for NFATc2, and one SUMO site for NFATc4. The most N-terminal sumoylation sites of NFATc1 and NFATc3 are both located in the DNA-binding domain of NFAT, whereas all other sumoylation sites predicted are located near the C-terminal TAD of NFATs, which indicates that SUMO conjugation may alter the transactivation capacities of NFAT proteins. Next, we used site-directed mutagenesis for the generation of different SUMO site mutant human NFATc1-c4 isoforms. For analyzing the transactivational activities of different wild-type and SUMO mutant NFATc1-c4 isoforms in neurons, we carried out reporter assays in rat primary cortical or hippocampal neurons using firefly luciferase

construct carrying three tandem NFAT-response elements with and without membrane depolarization. We conducted similar luciferase reporter assays also in human embryonic kidney cells 293-FT (HEK293-FT) and BHK-21 cells in response to $Ca^{2+}$ signaling to test whether the potential effect of sumoylation on the transactivation capacities of the representative NFAT isoforms may be neuron-specific.

First, we analyzed the transactivation capacity of a representative NFAT isoform from each NFAT family in cortical and in hippocampal neurons. At basal conditions, NFAT proteins are less active in hippocampal neurons than in cortical neurons, which correlates with our previous results showing predominantly cytoplasmic localization of NFATs in untreated hippocampal neurons. In response to membrane depolarization, we detected an increase in NFAT-dependent luciferase expression only for the representative isoforms NFATc3-IB-IX and NFATc4-ID-IXL in both cell types, whereas neuronal activity did not induce the transcriptional activities of NFATc1-IA-IXL and NFATc2-IA-Xa. However, SUMO site mutations significantly increased the transactivation activity of representative NFATc1-IA-IXL and NFATc2-IA-Xa isoforms in both cortical and hippocampal neurons and NFATc3-IB-IX in hippocampal neurons. Contrary to our findings, Terui and colleagues have reported that sumoylation of mouse NFATc2 positively regulates its transcriptional activity in BHK-21 cells (Terui *et al.* 2004). Although, generally, sumoylation has been shown to have both negative and positive effects on transcription factor activity (reviewed in Chymkowitch *et al.* 2015), we could not replicate the results of Terui and colleagues neither in HEK293-FT cells nor BHK-21 cells where NFATc2 SUMO mutants had higher transcriptional activity than wild-type isoforms. Furthermore, inhibition of sumoylation with 2-D08, a cell-permeable compound that prevents the transfer of SUMO from the E2 conjugating enzyme to the substrate, enhanced endogenous NFAT-dependent transcriptional activity at basal conditions in both hippocampal and cortical neurons and after membrane depolarization in cortical neurons. Jointly, these results indicate that sumoylation represses NFATc1- and/or NFATc2-dependent transcriptional activity in cortical neurons, and NFATc1-, NFATc2- and/or NFATc3-dependent transcriptional activity in hippocampal neurons.

Next, we analyzed the transactivation capacities of all our cloned 18 NFAT protein isoforms in cortical neurons. We determined that in response to $Ca^{2+}$ signaling, all NFATc3 and NFATc4 isoforms tested were strong activators of transcription, and mutation of sumoylation sites had no effect on their transactivation activities in cortical neurons. Transactivation by NFATc1 and NFATc2, however, was dependent on the specific isoform. NFATc1 isoforms NFATc1-IA-VIII, NFATc1-IB-VIII, and NFATc1-IA-ΔIX, that differ C-terminally from the other NFATc1 isoforms, significantly increased NFAT-RE controlled luciferase expression upon high $K^+$ stimulation of cortical neurons.

Interestingly, these isoforms and isoform NFATc1-IB-ΔIX contain only the most N-terminal sumoylation site and displayed no differences in transactivation capacities between wild-type and SUMO mutant at basal level as well as after membrane depolarization. C-terminally longer NFATc1 isoforms NFATc1-IA/IB-IXS and NFATc1-IA/IB-IXL, that contain two or all three putative SUMO sites, respectively, could increase NFAT response element controlled luciferase expression at both basal level and after membrane depolarization only when their SUMO sites were mutated. These findings were in good agreement with previous study showing that two C-terminal SUMO sites of NFATc1 are the most important sites in HEK293-T cells and N-terminal SUMO site is irrelevant or dependent on other two sumoylation sites (Nayak *et al*. 2009). The transactivation capacities of all four NFATc2 isoforms analyzed are significantly inhibited by sumoylation in cortical neurons. However, wild-type NFATc2 isoforms displayed great variation in their transactivation capacities between biological replicates, which could be partly explained by the highly dynamic nature of sumoylation process where not all SUMO substrates could be sumoylated simultaneously, especially when being overexpressed. In addition, analysis of sumoylation of overexpressed representative NFAT isoforms by co-immunoprecipitation assay showed that all analyzed NFAT proteins were sumoylated in HEK293-FT cells. In cortical neurons, Western blot analysis showed that only the overexpressed representative NFATc1 and NFATc2 isoforms were sumoylated, with higher levels in depolarized neurons, whereas sumoylation of the representative NFATc3 and NFATc4 isoforms was not detected. This is in good agreement with our results from luciferase reporter assays showing that mutation of sumoylation sites promotes the transcriptional activity of all representative NFAT isoforms in HEK293-FT cells, while in cortical neurons only the activities of NFATc1 and NFATc2 are increased.

Collectively, our results show that the transcription activation capacity of NFAT proteins in neurons is isoform-specific. However, the presence of a known TAD in a certain NFAT isoform does not clearly correlate with its membrane depolarization-induced transactivation capacity. Sumoylation represses the transcriptional activity of NFATc1-c4 in general, but the effect of sumoylation on different NFAT isoforms varies between cell types. This could be due to differential expression patterns of substrate-specific SUMO ligases or proteases.

## CONCLUSIONS

The principal findings of the present thesis are:

- Alternative transcripts of the *NFAT* genes are generated predominantly by usage of alternative 5′ and 3′ exons.
- Alternatively spliced *NFAT* mRNAs display tissue-specific expression patterns.
- *NFAT* mRNAs are expressed in mouse brain with a specific pattern for each *NFAT* gene. *Nfatc2* is the most predominantly expressed *Nfat* gene in the mouse brain being broadly expressed throughout the brain.
- The subcellular distribution of the NFAT proteins is isoform-specific and varies between neuron types. In hippocampal neurons, all NFAT isoforms studied were located mostly in the cytosol, whereas in cortical neurons, the localizations of different NFAT isoforms varied greatly even within the same NFAT subfamily.
- Neuronal activity-induced nuclear translocation of all NFAT isoforms requires calcineurin activity.
- The extent and kinetics of neuronal activity-induced nuclear translocation of the NFAT proteins is isoform-specific and varies between neuron types. In hippocampal neurons, membrane depolarization resulted in nuclear localization of all NFAT isoforms tested. However, in cortical neurons, NFATc4 localization was unresponsive to depolarization.
- The transcription activation capacity of the NFAT proteins in neurons is isoform-specific: NFATc3 and NFATc4 isoforms lead to the highest induction of transcription, whereas NFATc1 and NFATc2 display isoform-specific transcription activation capacities.
- Sumoylation, in general, represses the transcriptional activity of all NFATs.
- The effect of sumoylation on different NFAT isoforms varies between cell types. In cortical neurons, sumoylation represses the transcriptional activity of NFATc1 and NFATc2 isoforms, whereas in hippocampal neurons, transcriptional activity of NFATc1, NFATc2, and NFATc3 isoforms is repressed by sumoylation.

## REFERENCES


Abdul H. M., Furman J. L., Sama M. A., Mathis D. M., Norris C. M. (2010) NFATs and Alzheimer's Disease. *Mol. Cell. Pharmacol.* **2**, 7–14.

Alberini C. M. (2009) Transcription Factors in Long-Term Memory and Synaptic Plasticity. *Physiol. Rev.* **89**:121-45

Al-Daraji W. I., Grant K. R., Ryan K., Saxton A., Reynolds N. J. (2002) Localization of Calcineurin/NFAT in Human Skin and Psoriasis and Inhibition of Calcineurin/NFAT Activation in Human Keratinocytes by Cyclosporin A. *J. Invest. Dermatol.* **118**, 779–788.

Aprea J., Prenninger S., Dori M., Ghosh T., Monasor L. S., Wessendorf E., Zocher S., et al. (2013) Transcriptome sequencing during mouse brain development identifies long non-coding RNAs functionally involved in neurogenic commitment. *EMBO J.* **32**, 3145–3160.

Aramburu J., Garcia-Cózar F., Raghavan A., Okamura H., Rao A., Hogan P. G. (1998) Selective inhibition of NFAT activation by a peptide spanning the calcineurin targeting site of NFAT. *Mol. Cell* **1**, 627–637.

Aramburu J., Yaffe M. B., López-Rodríguez C., Cantley L. C., Hogan P. G., Rao A. (1999) Affinity-driven peptide selection of an NFAT inhibitor more selective than cyclosporin A. *Science* **285**, 2129–2133.

Artegiani B., Jesus Domingues A. M. de, Bragado Alonso S., Brandl E., Massalini S., Dahl A., Calegari F. (2015) Tox: a multifunctional transcription factor and novel regulator of mammalian corticogenesis. *EMBO J.* **34**, 896–910.

Asai M., Iwasaki Y., Yoshida M., Mutsuga-Nakayama N., Arima H., Ito M., Takano K., Oiso Y. (2004) Nuclear factor of activated T cells (NFAT) is involved in the depolarization-induced activation of growth hormone-releasing hormone gene transcription in vitro. *Mol. Endocrinol. Baltim. Md* **18**, 3011–3019.

Avots A., Buttmann M., Chuvpilo S., Escher C., Smola U., Bannister A. J., Rapp U. R., Kouzarides T., Serfling E. (1999) CBP/p300 integrates Raf/Rac-signaling pathways in the transcriptional induction of NF-ATc during T cell activation. *Immunity* **10**, 515–524.

Bading H., Ginty D. D., Greenberg M. E. (1993) Regulation of gene expression in hippocampal neurons by distinct calcium signaling pathways. *Science* **260**, 181–186.

Bates D. L., Barthel K. K. B., Wu Y., Kalhor R., Stroud J. C., Giffin M. J., Chen L. (2008) Crystal structure of NFAT bound to the HIV-1 LTR tandem kappaB enhancer element. *Struct. Lond. Engl. 1993* **16**, 684–694.

Beals C. R., Clipstone N. A., Ho S. N., Crabtree G. R. (1997a) Nuclear localization of NF-ATc by a calcineurin-dependent, cyclosporin-sensitive intramolecular interaction. *Genes Dev.* **11**, 824–834.

Beals C. R., Sheridan C. M., Turck C. W., Gardner P., Crabtree G. R. (1997b) Nuclear Export of NF-ATc Enhanced by Glycogen Synthase Kinase-3. *Science* **275**, 1930–1933.

Beier F. (2014) NFATs are good for your cartilage! *Osteoarthritis Cartilage* **22**, 893–895.

Benedito A. B., Lehtinen M., Massol R., Lopes U. G., Kirchhausen T., Rao A., Bonni A. (2005) The transcription factor NFAT3 mediates neuronal survival. *J. Biol. Chem.* **280**, 2818–2825.

Bibel M., Barde Y.-A. (2000) Neurotrophins: key regulators of cell fate and cell shape in the vertebrate nervous system. *Genes Dev.* **14**, 2919–2937.

Birnbaum S. G., Varga A. W., Yuan L.-L., Anderson A. E., Sweatt J. D., Schrader L. A. (2004) Structure and Function of Kv4-Family Transient Potassium Channels. *Physiol. Rev.* **84**, 803–833.

Bito H., Deisseroth K., Tsien R. W. (1996) CREB Phosphorylation and Dephosphorylation: A Ca2+- and Stimulus Duration–Dependent Switch for Hippocampal Gene Expression. *Cell* **87**, 1203–1214.

Bradley K. C., Groth R. D., Mermelstein P. G. (2005) Immunolocalization of NFATc4 in the adult mouse brain. *J. Neurosci. Res.* **82**, 762–770.

Brini M., Calì T., Ottolini D., Carafoli E. (2014) Neuronal calcium signaling: function and dysfunction. *Cell. Mol. Life Sci. CMLS* **71**, 2787–2814.

Brini M., Carafoli E. (2011) The Plasma Membrane Ca2+ ATPase and the Plasma Membrane Sodium Calcium Exchanger Cooperate in the Regulation of Cell Calcium. *Cold Spring Harb. Perspect. Biol.* **3**. pii: a004168

Burgoyne R. D., Haynes L. P. (2015) Sense and specificity in neuronal calcium signalling. *Biochim. Biophys. Acta* **1853**, 1921–1932.

Bushdid P. B., Osinska H., Waclaw R. R., Molkentin J. D., Yutzey K. E. (2003) NFATc3 and NFATc4 Are Required for Cardiac Development and Mitochondrial Function. *Circ. Res.* **92**, 1305–1313.

Cai Y.-Q., Chen S.-R., Pan H.-L. (2013) Upregulation of Nuclear Factor of Activated T-Cells by Nerve Injury Contributes to Development of Neuropathic Pain. *J. Pharmacol. Exp. Ther.* **345**, 161–168.

Canellada A., Ramirez B. G., Minami T., Redondo J. M., Cano E. (2008) Calcium/calcineurin signaling in primary cortical astrocyte cultures: Rcan1-4 and cyclooxygenase-2 as NFAT target genes. *Glia* **56**, 709–722.

Caraveo G., Auluck P. K., Whitesell L., Chung C. Y., Baru V., Mosharov E. V., Yan X., et al. (2014) Calcineurin determines toxic versus beneficial responses to α-synuclein. *Proc. Natl. Acad. Sci. U. S. A.* **111**, E3544–E3552.

Carrión A. M., Link W. A., Ledo F., Mellström B., Naranjo J. R. (1999) DREAM is a Ca2+-regulated transcriptional repressor. *Nature* **398**, 80–84.

Catterall W. A. (2011) Voltage-Gated Calcium Channels. *Cold Spring Harb. Perspect. Biol.* **3**:a003947

Chang C.-P., Neilson J. R., Bayle J. H., Gestwicki J. E., Kuo A., Stankunas K., Graef I. A., Crabtree G. R. (2004) A field of myocardial-endocardial NFAT signaling underlies heart valve morphogenesis. *Cell* **118**, 649–663.

Chawla S., Hardingham G. E., Quinn D. R., Bading H. (1998) CBP: a signal-regulated transcriptional coactivator controlled by nuclear calcium and CaM kinase IV. *Science* **281**, 1505–1509.

Chen L., Glover J. N. M., Hogan P. G., Rao A., Harrison S. C. (1998) Structure of the DNA-binding domains from NFAT, Fos and Jun bound specifically to DNA. *Nature* **392**, 42–48.

Chinenov Y., Kerppola T. K. (2001) Close encounters of many kinds: Fos-Jun interactions that mediate transcription regulatory specificity. *Oncogene* **20**, 2438–2452.

Chiu C.-C., Liao Y.-E., Yang L.-Y., Wang J.-Y., Tweedie D., Karnati H. K., Greig N. H., Wang J.-Y. (2016) Neuroinflammation in animal models of traumatic brain injury. *J. Neurosci. Methods* **272**, 38–49.

Choi J.-M., Sohn J.-H., Park T.-Y., Park J.-W., Lee S.-K. (2012) Cell permeable NFAT inhibitory peptide Sim-2-VIVIT inhibits T-cell activation and alleviates allergic airway inflammation and hyper-responsiveness. *Immunol. Lett.* **143**, 170–176.

Chow C. W., Dong C., Flavell R. A., Davis R. J. (2000) c-Jun NH(2)-terminal kinase inhibits targeting of the protein phosphatase calcineurin to NFATc1. *Mol. Cell. Biol.* **20**, 5227–5234.

Chow C.-W., Rincón M., Cavanagh J., Dickens M., Davis R. J. (1997) Nuclear Accumulation of NFAT4 Opposed by the JNK Signal Transduction Pathway. *Science* **278**, 1638–1641.

Chrivia J. C., Kwok R. P., Lamb N., Hagiwara M., Montminy M. R., Goodman R. H. (1993) Phosphorylated CREB binds specifically to the nuclear protein CBP. *Nature* **365**, 855–859.

Chuvpilo S., Zimmer M., Kerstan A., Glöckner J., Avots A., Escher C., Fischer C., et al. (1999) Alternative polyadenylation events contribute to the induction of NF-ATc in effector T cells. *Immunity* **10**, 261–269.

Chymkowitch P., Nguéa P A., Enserink J. M. (2015) SUMO-regulated transcription: Challenging the dogma. *BioEssays* **37**, 1095–1105.

Chytil M., Verdine G. L. (1996) The Rel family of eukaryotic transcription factors. *Curr. Opin. Struct. Biol.* **6**, 91–100.

Cockerill G. W., Bert A. G., Ryan G. R., Gamble J. R., Vadas M. A., Cockerill P. N. (1995) Regulation of granulocyte-macrophage colony-stimulating factor and E-selectin expression in endothelial cells by cyclosporin A and the T-cell transcription factor NFAT. *Blood* **86**, 2689–2698.

Combs M. D., Yutzey K. E. (2009) VEGF and RANKL regulation of NFATc1 in heart valve development. *Circ. Res.* **105**, 565–574.

Costa Martins P. A. da, Salic K., Gladka M. M., Armand A.-S., Leptidis S., Azzouzi H. el, Hansen A., et al. (2010) MicroRNA-199b targets the nuclear kinase Dyrk1a in an auto-amplification loop promoting calcineurin/NFAT signalling. *Nat. Cell Biol.* **12**, 1220–1227.

Crabtree G. R., Olson E. N. (2002) NFAT signaling: choreographing the social lives of cells. *Cell* **109 Suppl**, S67-79.

Daniel C., Gerlach K., Väth M., Neurath M. F., Weigmann B. (2014) Nuclear factor of activated T cells - a transcription factor family as critical regulator in lung and colon cancer. *Int. J. Cancer* **134**, 1767–1775.

Delmas P., Brown D. A. (2005) Pathways modulating neural KCNQ/M (Kv7) potassium channels. *Nat. Rev. Neurosci.* **6**, 850–862.

Ding B., Wang W., Selvakumar T., Xi H. S., Zhu H., Chow C.-W., Horton J. D., Gronostajski R. M., Kilpatrick D. L. (2013) Temporal Regulation of Nuclear Factor One Occupancy by Calcineurin/NFAT Governs a Voltage-Sensitive Developmental Switch in Late Maturing Neurons. *J. Neurosci. Off. J. Soc. Neurosci.* **33**, 2860–2872.

Dittmer P. J., Dell'Acqua M. L., Sather W. A. (2014) Ca2+/Calcineurin-Dependent Inactivation of Neuronal L-Type Ca2+ Channels Requires Priming by AKAP-Anchored Protein Kinase A. *Cell Rep.* **7**, 1410–1416.

Dolmetsch R. E., Pajvani U., Fife K., Spotts J. M., Greenberg M. E. (2001) Signaling to the nucleus by an L-type calcium channel-calmodulin complex through the MAP kinase pathway. *Science* **294**, 333–339.

Duncan N., Craddock C. (2006) Optimizing the use of cyclosporin in allogeneic stem cell transplantation. *Bone Marrow Transplant.* **38**, 169–174.

Ehlers M. L., Celona B., Black B. L. (2014) NFATc1 controls skeletal muscle fiber type and is a negative regulator of MyoD activity. *Cell Rep.* **8**, 1639–1648.

Falvo J. V., Lin C. H., Tsytsykova A. V., Hwang P. K., Thanos D., Goldfeld A. E., Maniatis T. (2008) A dimer-specific function of the transcription factor NFATp. *Proc. Natl. Acad. Sci.* **105**, 19637–19642.

Fan Y., Xie P., Zhang T., Zhang H., Gu D., She M., Li H. (2008) Regulation of the stability and transcriptional activity of NFATc4 by ubiquitination. *FEBS Lett.* **582**, 4008–4014.

Flanagan W. M., Corthésy B., Bram R. J., Crabtree G. R. (1991) Nuclear association of a T-cell transcription factor blocked by FK-506 and cyclosporin A. *Nature* **352**, 803–807.

Flavell S. W., Cowan C. W., Kim T.-K., Greer P. L., Lin Y., Paradis S., Griffith E. C., Hu L. S., Chen C., Greenberg M. E. (2006) Activity-dependent regulation of MEF2 transcription factors suppresses excitatory synapse number. *Science* **311**, 1008–1012.

Freeman A., Franciscovich A., Bowers M., Sandstrom D. J., Sanyal S. (2011) NFAT regulates pre-synaptic development and activity-dependent plasticity in Drosophila. *Mol. Cell. Neurosci.* **46**, 535–547.

Frey N., Olson E. N. (2003) Cardiac hypertrophy: the good, the bad, and the ugly. *Annu. Rev. Physiol.* **65**, 45–79.

Fujii S., Matsumoto M., Igarashi K., Kato H., Mikoshiba K. (2000) Synaptic plasticity in hippocampal CA1 neurons of mice lacking type 1 inositol-1,4,5-trisphosphate receptors. *Learn. Mem. Cold Spring Harb. N* **7**, 312–320.

Furman J. L., Sompol P., Kraner S. D., Pleiss M. M., Putman E. J., Dunkerson J., Mohmmad Abdul H., Roberts K. N., Scheff S. W., Norris C. M. (2016)

Blockade of Astrocytic Calcineurin/NFAT Signaling Helps to Normalize Hippocampal Synaptic Function and Plasticity in a Rat Model of Traumatic Brain Injury. *J. Neurosci. Off. J. Soc. Neurosci.* **36**, 1502–1515.

García-Rodríguez C., Rao A. (1998) Nuclear Factor of Activated T Cells (NFAT)-dependent Transactivation Regulated by the Coactivators p300/CREB-binding Protein (CBP). *J. Exp. Med.* **187**, 2031–2036.

Gaudillière B., Konishi Y., Iglesia N. de la, Yao G., Bonni A. (2004) A CaMKII-NeuroD Signaling Pathway Specifies Dendritic Morphogenesis. *Neuron* **41**, 229–241.

Giffin M. J., Stroud J. C., Bates D. L., Koenig K. D. von, Hardin J., Chen L. (2003) Structure of NFAT1 bound as a dimer to the HIV-1 LTR kappa B element. *Nat. Struct. Biol.* **10**, 800–806.

Gómez del Arco P., Martínez-Martínez S., Maldonado J. L., Ortega-Pérez I., Redondo J. M. (2000) A role for the p38 MAP kinase pathway in the nuclear shuttling of NFATp. *J. Biol. Chem.* **275**, 13872–13878.

Gómez-Sintes R., Lucas J. J. (2010) NFAT/Fas signaling mediates the neuronal apoptosis and motor side effects of GSK-3 inhibition in a mouse model of lithium therapy. *J. Clin. Invest.* **120**, 2432–2445.

Gonzalez G. A., Montminy M. R. (1989) Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. *Cell* **59**, 675–680.

Graef I. A., Chen F., Chen L., Kuo A., Crabtree G. R. (2001a) Signals Transduced by Ca2+/Calcineurin and NFATc3/c4 Pattern the Developing Vasculature. *Cell* **105**, 863–875.

Graef I. A., Chen F., Crabtree G. R. (2001b) NFAT signaling in vertebrate development. *Curr. Opin. Genet. Dev.* **11**, 505–512.

Graef I. A., Gastier J. M., Francke U., Crabtree G. R. (2001c) Evolutionary relationships among Rel domains indicate functional diversification by recombination. *Proc. Natl. Acad. Sci. U. S. A.* **98**, 5740–5745.

Graef I. A., Mermelstein P. G., Stankunas K., Neilson J. R., Deisseroth K., Tsien R. W., Crabtree G. R. (1999) L-type calcium channels and GSK-3 regulate the activity of NF-ATc4 in hippocampal neurons. *Nature* **401**, 703–708.

Graef I. A., Wang F., Charron F., Chen L., Neilson J., Tessier-Lavigne M., Crabtree G. R. (2003) Neurotrophins and netrins require calcineurin/NFAT signaling to stimulate outgrowth of embryonic axons. *Cell* **113**, 657–670.

Greene D. L., Hoshi N. (2017) Modulation of Kv7 channels and excitability in the brain. *Cell. Mol. Life Sci. CMLS* **74**, 495–508.

Groth R. D., Coicou L. G., Mermelstein P. G., Seybold V. S. (2007) Neurotrophin activation of NFAT-dependent transcription contributes to the regulation of pro-nociceptive genes. *J. Neurochem.* **102**, 1162–1174.

Groth R. D., Mermelstein P. G. (2003) Brain-Derived Neurotrophic Factor Activation of NFAT (Nuclear Factor of Activated T-Cells)-Dependent

Transcription: A Role for the Transcription Factor NFATc4 in Neurotrophin-Mediated Gene Expression. *J. Neurosci.* **23**, 8125–8134.

Groth R. D., Weick J. P., Bradley K. C., Luoma J. I., Aravamudan B., Klug J. R., Thomas M. J., Mermelstein P. G. (2008) D1 dopamine receptor activation of NFAT-mediated striatal gene expression. *Eur. J. Neurosci.* **27**, 31–42.

Gwack Y., Sharma S., Nardone J., Tanasa B., Iuga A., Srikanth S., Okamura H., et al. (2006) A genome-wide Drosophila RNAi screen identifies DYRK-family kinases as regulators of NFAT. *Nature* **441**, 646–650.

Hardingham G. E., Arnold F. J., Bading H. (2001) A calcium microdomain near NMDA receptors: on switch for ERK-dependent synapse-to-nucleus communication. *Nat. Neurosci.* **4**, 565–566.

Hardingham G. E., Chawla S., Johnson C. M., Bading H. (1997) Distinct functions of nuclear and cytoplasmic calcium in the control of gene expression. *Nature* **385**, 260–265.

Harrill J. A., Chen H., Streifel K. M., Yang D., Mundy W. R., Lein P. J. (2015) Ontogeny of biochemical, morphological and functional parameters of synaptogenesis in primary cultures of rat hippocampal and cortical neurons. *Mol. Brain* **8**, 10.

Hernández-Ochoa E. O., Contreras M., Cseresnyés Z., Schneider M. F. (2007) Ca2+ signal summation and NFATc1 nuclear translocation in sympathetic ganglion neurons during repetitive action potentials. *Cell Calcium* **41**, 559–571.

Ho A. M., Jain J., Rao A., Hogan P. G. (1994) Expression of the transcription factor NFATp in a neuronal cell line and in the murine nervous system. *J. Biol. Chem.* **269**, 28181–28186.

Hock M. B., Brown M. A. (2003) Nuclear factor of activated T cells 2 transactivation in mast cells: a novel isoform-specific transactivation domain confers unique FcepsilonRI responsiveness. *J. Biol. Chem.* **278**, 26695–26703.

Hoey T., Sun Y. L., Williamson K., Xu X. (1995) Isolation of two new members of the NF-AT gene family and functional characterization of the NF-AT proteins. *Immunity* **2**, 461–472.

Hogan P. G., Chen L., Nardone J., Rao A. (2003) Transcriptional regulation by calcium, calcineurin, and NFAT. *Genes Dev.* **17**, 2205–2232.

Horsley V., Friday B. B., Matteson S., Kegley K. M., Gephart J., Pavlath G. K. (2001) Regulation of the Growth of Multinucleated Muscle Cells by an Nfatc2-Dependent Pathway. *J. Cell Biol.* **153**, 329–338.

Horsley V., Pavlath G. K. (2002) NFAT: ubiquitous regulator of cell differentiation and adaptation. *J. Cell Biol.* **156**, 771–774.

Huang G. N., Huso D. L., Bouyain S., Tu J., McCorkell K. A., May M. J., Zhu Y., et al. (2008) NFAT Binding and Regulation of T Cell Activation by the Cytoplasmic Scaffolding Homer Proteins. *Science* **319**, 476–481.

Huang T., Xie Z., Wang J., Li M., Jing N., Li L. (2011) Nuclear Factor of Activated T Cells (NFAT) Proteins Repress Canonical Wnt Signaling via

Its Interaction with Dishevelled (Dvl) Protein and Participate in Regulating Neural Progenitor Cell Proliferation and Differentiation. *J. Biol. Chem.* **286**, 37399–37405.

Ikeda S., He A., Kong S. W., Lu J., Bejar R., Bodyak N., Lee K.-H., et al. (2009) MicroRNA-1 Negatively Regulates Expression of the Hypertrophy-Associated Calmodulin and Mef2a Genes. *Mol. Cell. Biol.* **29**, 2193–2204.

Imamura R., Masuda E. S., Naito Y., Imai S., Fujino T., Takano T., Arai K., Arai N. (1998) Carboxyl-terminal 15-amino acid sequence of NFATx1 is possibly created by tissue-specific splicing and is essential for transactivation activity in T cells. *J. Immunol. Baltim. Md 1950* **161**, 3455–3463.

Ince-Dunn G., Hall B. J., Hu S.-C., Ripley B., Huganir R. L., Olson J. M., Tapscott S. J., Ghosh A. (2006) Regulation of thalamocortical patterning and synaptic maturation by NeuroD2. *Neuron* **49**, 683–695.

Jackson J. G., Usachev Y. M., Thayer S. A. (2007) Bradykinin-induced nuclear factor of activated T-cells-dependent transcription in rat dorsal root ganglion neurons. *Mol. Pharmacol.* **72**, 303–310.

Jain J., Burgeon E., Badalian T. M., Hogan P. G., Rao A. (1995) A similar DNA-binding motif in NFAT family proteins and the Rel homology region. *J. Biol. Chem.* **270**, 4138–4145.

Jain J., Miner Z., Rao A. (1993) Analysis of the preexisting and nuclear forms of nuclear factor of activated T cells. *J. Immunol. Baltim. Md 1950* **151**, 837–848.

Jardin I., López J. J., Berna-Erro A., Salido G. M., Rosado J. A. (2013) Homer Proteins in Ca2+ Entry. *IUBMB Life* **65**, 497–504.

Jayanthi S., Deng X., Ladenheim B., McCoy M. T., Cluster A., Cai N.-S., Cadet J. L. (2005) Calcineurin/NFAT-induced up-regulation of the Fas ligand/Fas death pathway is involved in methamphetamine-induced neuronal apoptosis. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 868–873.

Jiang H., Xiong F., Kong S., Ogawa T., Kobayashi M., Liu J. O. (1997) Distinct tissue and cellular distribution of two major isoforms of calcineurin. *Mol. Immunol.* **34**, 663–669.

Jin L., Sliz P., Chen L., Macián F., Rao A., Hogan P. G., Harrison S. C. (2003) An asymmetric NFAT1 dimer on a pseudo-palindromic kappa B-like DNA site. *Nat. Struct. Biol.* **10**, 807–811.

Johannessen M., Delghandi M. P., Moens U. (2004) What turns CREB on? *Cell. Signal.* **16**, 1211–1227.

Johnson E. N., Lee Y. M., Sander T. L., Rabkin E., Schoen F. J., Kaushal S., Bischoff J. (2003) NFATc1 mediates vascular endothelial growth factor-induced proliferation of human pulmonary valve endothelial cells. *J. Biol. Chem.* **278**, 1686–1692.

Kannambath S. (2016) Micro-RNA Feedback Loops Modulating the Calcineurin/NFAT Signaling Pathway. *Non-Coding RNA* **2**, 3.

Kao S.-C., Wu H., Xie J., Chang C.-P., Ranish J. A., Graef I. A., Crabtree G. R. (2009) Calcineurin/NFAT signaling is required for neuregulin-regulated Schwann cell differentiation. *Science* **323**, 651–654.

Kegley K. M., Gephart J., Warren G. L., Pavlath G. K. (2001) Altered primary myogenesis in NFATC3(-/-) mice leads to decreased muscle size in the adult. *Dev. Biol.* **232**, 115–126.

Kehlenbach R. H., Dickmanns A., Gerace L. (1998) Nucleocytoplasmic shuttling factors including Ran and CRM1 mediate nuclear export of NFAT In vitro. *J. Cell Biol.* **141**, 863–874.

Kiani A., Rao A., Aramburu J. (2000) Manipulating Immune Responses with Immunosuppressive Agents that Target NFAT. *Immunity* **12**, 359–372.

Kim J. H., Kim K., Jin H. M., Song I., Youn B. U., Lee S.-H., Choi Y., Kim N. (2010) Negative Feedback Control of Osteoclast Formation through Ubiquitin-mediated Down-regulation of NFATc1. *J. Biol. Chem.* **285**, 5224–5231.

Kim J. H., Kim K., Youn B. U., Jin H. M., Kim J.-Y., Moon J. B., Ko A., Seo S.-B., Lee K.-Y., Kim N. (2011) RANKL induces NFATc1 acetylation and stability via histone acetyltransferases during osteoclast differentiation. *Biochem. J.* **436**, 253–262.

Kim M.-S., Shutov L. P., Gnanasekaran A., Lin Z., Rysted J. E., Ulrich J. D., Usachev Y. M. (2014) Nerve Growth Factor (NGF) Regulates Activity of Nuclear Factor of Activated T-cells (NFAT) in Neurons via the Phosphatidylinositol 3-Kinase (PI3K)-Akt-Glycogen Synthase Kinase 3β (GSK3β) Pathway. *J. Biol. Chem.* **289**, 31349–31360.

Kincaid R. L., Balaban C. D., Billingsley M. L. (1987) Differential localization of calmodulin-dependent enzymes in rat brain: evidence for selective expression of cyclic nucleotide phosphodiesterase in specific neurons. *Proc. Natl. Acad. Sci. U. S. A.* **84**, 1118–1122.

Kipanyula M. J., Kimaro W. H., Etet P. F. S. (2016) The Emerging Roles of the Calcineurin-Nuclear Factor of Activated T-Lymphocytes Pathway in Nervous System Functions and Diseases. *J. Aging Res.* **2016**, e5081021.

Klee C. B., Crouch T. H., Krinks M. H. (1979) Calcineurin: a calcium- and calmodulin-binding protein of the nervous system. *Proc. Natl. Acad. Sci. U. S. A.* **76**, 6270–6273.

Klee C. B., Ren H., Wang X. (1998) Regulation of the calmodulin-stimulated protein phosphatase, calcineurin. *J. Biol. Chem.* **273**, 13367–13370.

Kohli S., Ahuja S., Rani V. (2011) Transcription Factors in Heart: Promising Therapeutic Targets in Cardiac Hypertrophy. *Curr. Cardiol. Rev.* **7**, 262–271.

Lambrechts D., Carmeliet P. (2004) Sculpting heart valves with NFATc and VEGF. *Cell* **118**, 532–534.

Lassot I., Robbins I., Kristiansen M., Rahmeh R., Jaudon F., Magiera M. M., Mora S., et al. (2010) Trim17, a novel E3 ubiquitin-ligase, initiates neuronal apoptosis. *Cell Death Differ.* **17**, 1928–1941.

Lee M., Park J. (2006) Regulation of NFAT activation: a potential therapeutic target for immunosuppression. *Mol. Cells* **22**, 1–7.

Lewis P. A., Manzoni C. (2012) LRRK2 and human disease: a complicated question or a question of complexes? *Sci. Signal.* **5**, pe2.

Li H., Pink M. D., Murphy J. G., Stein A., Dell'Acqua M. L., Hogan P. G. (2012) Balanced interactions of calcineurin with AKAP79 regulate Ca2+-calcineurin-NFAT signaling. *Nat. Struct. Mol. Biol.* **19**, 337–345.

Li H., Rao A., Hogan P. G. (2011a) Interaction of calcineurin with substrates and targeting proteins. *Trends Cell Biol.* **21**, 91–103.

Li H., Zhang L., Rao A., Harrison S. C., Hogan P. G. (2007) Structure of calcineurin in complex with PVIVIT peptide: portrait of a low-affinity signalling interaction. *J. Mol. Biol.* **369**, 1296–1306.

Li X., Zhu L., Yang A., Lin J., Tang F., Jin S., Wei Z., Li J., Jin Y. (2011b) Calcineurin-NFAT signaling critically regulates early lineage specification in mouse embryonic stem cells and embryos. *Cell Stem Cell* **8**, 46–58.

Linseman D. A., Bartley C. M., Le S. S., Laessig T. A., Bouchard R. J., Meintzer M. K., Li M., Heidenreich K. A. (2003) Inactivation of the myocyte enhancer factor-2 repressor histone deacetylase-5 by endogenous Ca(2+) //calmodulin-dependent kinase II promotes depolarization-mediated cerebellar granule neuron survival. *J. Biol. Chem.* **278**, 41472–41481.

Liu Z., Lee J., Krummey S., Lu W., Cai H., Lenardo M. J. (2011) The kinase LRRK2 is a regulator of the transcription factor NFAT that modulates the severity of inflammatory bowel disease. *Nat. Immunol.* **12**, 1063–1070.

Loh C., Shaw K. T.-Y., Carew J., Viola J. P. B., Luo C., Perrino B. A., Rao A. (1996) Calcineurin Binds the Transcription Factor NFAT1 and Reversibly Regulates Its Activity. *J. Biol. Chem.* **271**, 10884–10891.

Lopez-Rodríguez C., Aramburu J., Rakeman A. S., Rao A. (1999) NFAT5, a constitutively nuclear NFAT protein that does not cooperate with Fos and Jun. *Proc. Natl. Acad. Sci. U. S. A.* **96**, 7214–7219.

Luo C., Burgeon E., Carew J. A., McCaffrey P. G., Badalian T. M., Lane W. S., Hogan P. G., Rao A. (1996) Recombinant NFAT1 (NFATp) is regulated by calcineurin in T cells and mediates transcription of several cytokine genes. *Mol. Cell. Biol.* **16**, 3955–3966.

Luo J., Sun L., Lin X., Liu G., Yu J., Parisiadou L., Xie C., Ding J., Cai H. (2014) A calcineurin- and NFAT-dependent pathway is involved in α-synuclein-induced degeneration of midbrain dopaminergic neurons. *Hum. Mol. Genet.* **23**, 6567–6574.

Luoma J. I., Zirpel L. (2008) Deafferentation-Induced Activation of NFAT (Nuclear Factor of Activated T-Cells) in Cochlear Nucleus Neurons during a Developmental Critical Period: A Role for NFATc4-Dependent Apoptosis in the CNS. *J. Neurosci.* **28**, 3159–3169.

Lyakh L., Ghosh P., Rice N. R. (1997) Expression of NFAT-family proteins in normal human T cells. *Mol. Cell. Biol.* **17**, 2475–2484.

Macian F. (2005) NFAT proteins: key regulators of T-cell development and function. *Nat. Rev. Immunol.* **5**, 472–484.

Macián F., López-Rodríguez C., Rao A. (2001) Partners in transcription: NFAT and AP-1. *Oncogene* **20**:2476-89.

Martínez-Martínez S., Rodríguez A., López-Maderuelo M. D., Ortega-Pérez I., Vázquez J., Redondo J. M. (2006) Blockade of NFAT activation by the second calcineurin binding site. *J. Biol. Chem.* **281**, 6227–6235.

Masuda E. S., Naito Y., Tokumitsu H., Campbell D., Saito F., Hannum C., Arai K., Arai N. (1995) NFATx, a novel member of the nuclear factor of activated T cells family that is expressed predominantly in the thymus. *Mol. Cell. Biol.* **15**, 2697–2706.

Matthews R. P., Guthrie C. R., Wailes L. M., Zhao X., Means A. R., McKnight G. S. (1994) Calcium/calmodulin-dependent protein kinase types II and IV differentially regulate CREB-dependent gene expression. *Mol. Cell. Biol.* **14**, 6107–6116.

McCaffrey P. G., Goldfeld A. E., Rao A. (1994) The role of NFATp in cyclosporin A-sensitive tumor necrosis factor-alpha gene transcription. *J. Biol. Chem.* **269**, 30445–30450.

McCaffrey P. G., Luo C., Kerppola T. K., Jain J., Badalian T. M., Ho A. M., Burgeon E., et al. (1993) Isolation of the cyclosporin-sensitive T cell transcription factor NFATp. *Science* **262**, 750–754.

McKinsey T. A., Zhang C. L., Olson E. N. (2002) MEF2: a calcium-dependent regulator of cell division, differentiation and death. *Trends Biochem. Sci.* **27**, 40–47.

Medyouf H., Ghysdael J. (2008) The calcineurin/NFAT signaling pathway: a novel therapeutic target in leukemia and solid tumors. *Cell Cycle Georget. Tex* **7**, 297–303.

Mei Z., Yan P., Tan X., Zheng S., Situ B. (2015) Transcriptional regulation of BACE1 by NFAT3 leads to enhanced amyloidogenic processing. *Neurochem. Res.* **40**, 829–836.

Meissner J. D., Freund R., Krone D., Umeda P. K., Chang K.-C., Gros G., Scheibe R. J. (2011) Extracellular signal-regulated kinase 1/2-mediated phosphorylation of p300 enhances myosin heavy chain I/β gene expression via acetylation of nuclear factor of activated T cells c1. *Nucleic Acids Res.* **39**, 5907–5925.

Mena M.-P., Papiewska-Pajak I., Przygodzka P., Kozaczuk A., Boncela J., Cierniewski C. S. (2014) NFAT2 regulates COX-2 expression and modulates the integrin repertoire in endothelial cells at the crossroads of angiogenesis and inflammation. *Exp. Cell Res.* **324**, 124–136.

Mermelstein P. G., Deisseroth K., Dasgupta N., Isaksen A. L., Tsien R. W. (2001) Calmodulin priming: nuclear translocation of a calmodulin complex and the memory of prior neuronal activity. *Proc. Natl. Acad. Sci. U. S. A.* **98**, 15342–15347.

Miyakawa H., Rim J. S., Handler J. S., Kwon H. M. (1999) Identification of the second tonicity-responsive enhancer for the betaine transporter (BGT1) gene. *Biochim. Biophys. Acta* **1446**, 359–364.

Mognol G. P., Carneiro F. R. G., Robbs B. K., Faget D. V., Viola J. P. B. (2016) Cell cycle and apoptosis regulation by NFAT transcription factors: new roles for an old player. *Cell Death Dis.* **7**, e2199.

Mojsa B., Mora S., Bossowski J. P., Lassot I., Desagher S. (2015) Control of neuronal apoptosis by reciprocal regulation of NFATc3 and Trim17. *Cell Death Differ.* **22**, 274–286.

Molkentin J. D. (2000) Calcineurin and beyond: cardiac hypertrophic signaling. *Circ. Res.* **87**, 731–738.

Montminy M. R., Bilezikjian L. M. (1987) Binding of a nuclear protein to the cyclic-AMP response element of the somatostatin gene. *Nature* **328**, 175–178.

Moreno M., Fernández V., Monllau J. M., Borrell V., Lerin C., Iglesia N. de la (2015) Transcriptional Profiling of Hypoxic Neural Stem Cells Identifies Calcineurin-NFATc4 Signaling as a Major Regulator of Neural Stem Cell Biology. *Stem Cell Rep.* **5**, 157–165.

Murphy J. G., Sanderson J. L., Gorski J. A., Scott J. D., Catterall W. A., Sather W. A., Dell'Acqua M. L. (2014) AKAP-Anchored PKA Maintains Neuronal L-type Calcium Channel Activity and NFAT Transcriptional Signaling. *Cell Rep.* **7**, 1577–1588.

Musarò A., McCullagh K. J., Naya F. J., Olson E. N., Rosenthal N. (1999) IGF-1 induces skeletal myocyte hypertrophy through calcineurin in association with GATA-2 and NF-ATc1. *Nature* **400**, 581–585.

Natoli G., Saccani S., Bosisio D., Marazzi I. (2005) Interactions of NF-kappaB with chromatin: the art of being at the right place at the right time. *Nat. Immunol.* **6**, 439–445.

Nayak A., Glöckner-Pagel J., Vaeth M., Schumann J. E., Buttmann M., Bopp T., Schmitt E., Serfling E., Berberich-Siebelt F. (2009) Sumoylation of the Transcription Factor NFATc1 Leads to Its Subnuclear Relocalization and Interleukin-2 Repression by Histone Deacetylase. *J. Biol. Chem.* **284**, 10935–10946.

Neria F., Carmen Serrano-Perez M. del, Velasco P., Urso K., Tranque P., Cano E. (2013) NFATc3 promotes Ca(2+) -dependent MMP3 expression in astroglial cells. *Glia* **61**, 1052–1066.

Nguyen T., Lindner R., Tedeschi A., Forsberg K., Green A., Wuttke A., Gaub P., Di Giovanni S. (2009) NFAT-3 Is a Transcriptional Repressor of the Growth-associated Protein 43 during Neuronal Maturation. *J. Biol. Chem.* **284**, 18816–18823.

Nilius B., Owsianik G. (2011) The transient receptor potential family of ion channels. *Genome Biol.* **12**, 218.

Northrop J. P., Ho S. N., Chen L., Thomas D. J., Timmerman L. A., Nolan G. P., Admon A., Crabtree G. R. (1994) NF-AT components define a family of transcription factors targeted in T-cell activation. *Nature* **369**, 497–502.

Nowycky M. C., Thomas A. P. (2002) Intracellular calcium signaling. *J. Cell Sci.* **115**, 3715–3716.

Nygren P. J., Scott J. D. (2016) Regulation of the phosphatase PP2B by protein-protein interactions. *Biochem. Soc. Trans.* **44**, 1313–1319.

Obata K., Noguchi K. (2006) BDNF in sensory neurons and chronic pain. *Neurosci. Res.* **55**, 1–10.

Okamura H., Aramburu J., García-Rodríguez C., Viola J. P. B., Raghavan A., Tahiliani M., Zhang X., Qin J., Hogan P. G., Rao A. (2000) Concerted Dephosphorylation of the Transcription Factor NFAT1 Induces a Conformational Switch that Regulates Transcriptional Activity. *Mol. Cell* **6**, 539–550.

Oukka M., Ho I.-C., Brousse F. C. de la, Hoey T., Grusby M. J., Glimcher L. H. (1998) The Transcription Factor NFAT4 Is Involved in the Generation and Survival of T Cells. *Immunity* **9**, 295–304.

Pan S., Tsuruta R., Masuda E. S., Imamura R., Bazan F., Arai K., Arai N., Miyatake S. (2000) NFATz: a novel rel similarity domain containing protein. *Biochem. Biophys. Res. Commun.* **272**, 765–776.

Park I.-S., Jang G. H., Yang J. H., Bischoff J., Lee Y. M. (2010) Differential function of genes regulated by VEGF-NFATc1 signaling pathway in migration of pulmonary valve endothelial cells. *FEBS Lett.* **584**, 141–146.

Park S., Uesugi M., Verdine G. L. (2000) A second calcineurin binding site on the NFAT regulatory domain. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 7130–7135.

Peng S. L., Gerth A. J., Ranger A. M., Glimcher L. H. (2001) NFATc1 and NFATc2 together control both T and B cell activation and differentiation. *Immunity* **14**, 13–20.

Pérez-Cadahía B., Drobic B., Davie J. R. (2011) Activation and function of immediate-early genes in the nervous systemThis paper is one of a selection of papers in a Special Issue entitled 31st Annual International Asilomar Chromatin and Chromosomes Conference, and has undergone the Journal's usual peer review process. *Biochem. Cell Biol.* **89**, 61–73.

Pérez-Ortiz J. M., Serrano-Pérez M. C., Pastor M. D., Martín E. D., Calvo S., Rincón M., Tranque P. (2008) Mechanical lesion activates newly identified NFATc1 in primary astrocytes: implication of ATP and purinergic receptors. *Eur. J. Neurosci.* **27**, 2453–2465.

Perroud J., Bernheim L., Frieden M., Koenig S. (2017) Distinct roles of NFATc1 and NFATc4 in human primary myoblast differentiation and in the maintenance of reserve cells. *J Cell Sci* **130**, 3083–3093.

Peterson B. R., Sun L. J., Verdine G. L. (1996) A critical arginine residue mediates cooperativity in the contact interface between transcription factors NFAT and AP-1. *Proc. Natl. Acad. Sci. U. S. A.* **93**, 13671–13676.

Plyte S., Boncristiano M., Fattori E., Galvagni F., Paccani S. R., Majolini M. B., Oliviero S., Ciliberto G., Telford J. L., Baldari C. T. (2001) Identification

and characterization of a novel nuclear factor of activated T-cells-1 isoform expressed in mouse brain. *J. Biol. Chem.* **276**, 14350–14358.

Pompa J. L. de la, Timmerman L. A., Takimoto H., Yoshida H., Elia A. J., Samper E., Potter J., et al. (1998) Role of the NF-ATc transcription factor in morphogenesis of cardiac valves and septum. *Nature* **392**, 182–186.

Porter C. M., Havens M. A., Clipstone N. A. (2000) Identification of amino acid residues and protein kinases involved in the regulation of NFATc subcellular localization. *J. Biol. Chem.* **275**, 3543–3551.

Prakriya M., Lewis R. S. (2015) Store-Operated Calcium Channels. *Physiol. Rev.* **95**, 1383–1436.

Qin J.-J., Nag S., Wang W., Zhou J., Zhang W.-D., Wang H., Zhang R. (2014) NFAT as cancer target: mission possible? *Biochim. Biophys. Acta* **1846**, 297–311.

Qin X., Wang X.-H., Yang Z.-H., Ding L.-H., Xu X.-J., Cheng L., Niu C., Sun H.-W., Zhang H., Ye Q.-N. (2008) Repression of NFAT3 transcriptional activity by estrogen receptors. *Cell. Mol. Life Sci. CMLS* **65**, 2752–2762.

Quadrato G., Benevento M., Alber S., Jacob C., Floriddia E. M., Nguyen T., Elnaggar M. Y., Pedroarena C. M., Molkentin J. D., Giovanni S. D. (2012) Nuclear factor of activated T cells (NFATc4) is required for BDNF-dependent survival of adult-born neurons and spatial memory formation in the hippocampus. *Proc. Natl. Acad. Sci.* **109**, E1499–E1508.

Quadrato G., Elnaggar M. Y., Duman C., Sabino A., Forsberg K., Di Giovanni S. (2014) Modulation of GABAA receptor signaling increases neurogenesis and suppresses anxiety through NFATc4. *J. Neurosci. Off. J. Soc. Neurosci.* **34**, 8630–8645.

Ranger A. M., Gerstenfeld L. C., Wang J., Kon T., Bae H., Gravallese E. M., Glimcher M. J., Glimcher L. H. (2000) The Nuclear Factor of Activated T Cells (Nfat) Transcription Factor Nfatp (Nfatc2) Is a Repressor of Chondrogenesis. *J. Exp. Med.* **191**, 9–22.

Ranger A. M., Grusby M. J., Hodge M. R., Gravallese E. M., Brousse F. C. de la, Hoey T., Mickanin C., Baldwin H. S., Glimcher L. H. (1998a) The transcription factor NF-ATc is essential for cardiac valve formation. *Nature* **392**, 186–190.

Ranger A. M., Hodge M. R., Gravallese E. M., Oukka M., Davidson L., Alt F. W., Brousse F. C. de la, Hoey T., Grusby M., Glimcher L. H. (1998b) Delayed lymphoid repopulation with defects in IL-4-driven responses produced by inactivation of NF-ATc. *Immunity* **8**, 125–134.

Rao A., Luo C., Hogan P. G. (1997) Transcription factors of the NFAT family: regulation and function. *Annu. Rev. Immunol.* **15**, 707–747.

Rosonina E., Akhter A., Dou Y., Babu J., Sri Theivakadadcham V. S. (2017) Regulation of transcription factors by sumoylation. *Transcription* **8**, 220–231.

Rühlmann A., Nordheim A. (1997) Effects of the Immunosuppressive Drugs CsA and FK506 on Intracellular Signalling and Gene Regulation. *Immunobiology* **198**, 192–206.

Rui Y., Myers K. R., Yu K., Wise A., De Blas A. L., Hartzell H. C., Zheng J. Q. (2013) Activity-Dependent Regulation of Dendritic Growth and Maintenance by Glycogen Synthase Kinase 3β. *Nat. Commun.* **4**:2628

Sakuma K., Nishikawa J., Nakao R., Watanabe K., Totsuka T., Nakano H., Sano M., Yasuhara M. (2003) Calcineurin is a potent regulator for skeletal muscle regeneration by association with NFATc1 and GATA-2. *Acta Neuropathol. (Berl.)* **105**, 271–280.

Schulz R. A., Yutzey K. E. (2004) Calcineurin signaling and NFAT activation in cardiovascular and skeletal muscle development. *Dev. Biol.* **266**, 1–16.

Schwartz N., Schohl A., Ruthazer E. S. (2009) Neural Activity Regulates Synaptic Properties and Dendritic Structure In Vivo through Calcineurin/NFAT Signaling. *Neuron* **62**, 655–669.

Scott L. J., McKeage K., Keam S. J., Plosker G. L. (2003) Tacrolimus: a further update of its use in the management of organ transplantation. *Drugs* **63**, 1247–1297.

Serrano-Pérez M. C., Fernández M., Neria F., Berjón-Otero M., Doncel-Pérez E., Cano E., Tranque P. (2015) NFAT transcription factors regulate survival, proliferation, migration, and differentiation of neural precursor cells. *Glia*, **63**, 987-1004

Serrano-Pérez M. C., Martín E. D., Vaquero C. F., Azcoitia I., Calvo S., Cano E., Tranque P. (2011) Response of transcription factor NFATc3 to excitotoxic and traumatic brain insults: identification of a subpopulation of reactive astrocytes. *Glia* **59**, 94–107.

Sharma S., Findlay G. M., Bandukwala H. S., Oberdoerffer S., Baust B., Li Z., Schmidt V., Hogan P. G., Sacks D. B., Rao A. (2011) Dephosphorylation of the nuclear factor of activated T cells (NFAT) transcription factor is regulated by an RNA-protein scaffold complex. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 11381–11386.

Sheridan C. M., Heist E. K., Beals C. R., Crabtree G. R., Gardner P. (2002) Protein Kinase A Negatively Modulates the Nuclear Accumulation of NF-ATc1 by Priming for Subsequent Phosphorylation by Glycogen Synthase Kinase-3. *J. Biol. Chem.* **277**, 48664–48676.

Shibasaki F., Price E. R., Milan D., McKeon F. (1996) Role of kinases and the phosphatase calcineurin in the nuclear shuttling of transcription factor NF-AT4. *Nature* **382**, 370–373.

Shioda N., Han F., Moriguchi S., Fukunaga K. (2007) Constitutively active calcineurin mediates delayed neuronal death through Fas-ligand expression via activation of NFAT and FKHR transcriptional activities in mouse brain ischemia. *J. Neurochem.* **102**, 1506–1517.

Shou J., Jing J., Xie J., You L., Jing Z., Yao J., Han W., Pan H. (2015) Nuclear factor of activated T cells in cancer development and treatment. *Cancer Lett.* **361**, 174–184.

Singh S. K., Chen N.-M., Hessmann E., Siveke J., Lahmann M., Singh G., Voelker N., et al. (2015) Antithetical NFATc1–Sox2 and p53–miR200

signaling networks govern pancreatic cancer cell plasticity. *EMBO J.* **34**, 517–530.

Siniscalco D., Giordano C., Rossi F., Maione S., Novellis V. de (2011) Role of Neurotrophins in Neuropathic Pain. *Curr. Neuropharmacol.* **9**, 523–529.

Sitara D., Aliprantis A. O. (2010) Transcriptional regulation of bone and joint remodeling by NFAT. *Immunol. Rev.* **233**, 286–300.

Soto-Nieves N., Puga I., Abe B. T., Bandyopadhyay S., Baine I., Rao A., Macian F. (2009) Transcriptional complexes formed by NFAT dimers regulate the induction of T cell tolerance. *J. Exp. Med.* **206**, 867–876.

Stiber J. A., Tabatabaei N., Hawkins A. F., Hawke T., Worley P. F., Williams R. S., Rosenberg P. (2005) Homer modulates NFAT-dependent signaling during muscle differentiation. *Dev. Biol.* **287**, 213–224.

Stroud J. C., Chen L. (2003) Structure of NFAT bound to DNA as a monomer. *J. Mol. Biol.* **334**, 1009–1022.

Suehiro J., Kanki Y., Makihara C., Schadler K., Miura M., Manabe Y., Aburatani H., Kodama T., Minami T. (2014) Genome-wide Approaches Reveal Functional Vascular Endothelial Growth Factor (VEGF)-inducible Nuclear Factor of Activated T Cells (NFAT) c1 Binding to Angiogenesis-related Genes in the Endothelium. *J. Biol. Chem.* **289**, 29044–29059.

Sun P., Enslen H., Myung P. S., Maurer R. A. (1994) Differential activation of CREB by Ca2+/calmodulin-dependent protein kinases type II and type IV involves phosphorylation of a site that negatively regulates activity. *Genes Dev.* **8**, 2527–2539.

Takayanagi H. (2007) The role of NFAT in osteoclast formation. *Ann. N. Y. Acad. Sci.* **1116**, 227–237.

Terui Y., Saad N., Jia S., McKeon F., Yuan J. (2004) Dual role of sumoylation in the nuclear localization and transcriptional activation of NFAT1. *J. Biol. Chem.* **279**, 28257–28265.

Tsantoulas C., McMahon S. B. (2014) Opening paths to novel analgesics: the role of potassium channels in chronic pain. *Trends Neurosci.* **37**, 146–158.

Ulrich J. D., Kim M.-S., Houlihan P. R., Shutov L. P., Mohapatra D. P., Strack S., Usachev Y. M. (2012) Distinct activation properties of the nuclear factor of activated T-cells (NFAT) isoforms NFATc3 and NFATc4 in neurons. *J. Biol. Chem.* **287**, 37594–37609.

Urso K., Alfranca A., Martínez-Martínez S., Escolano A., Ortega I., Rodríguez A., Redondo J. M. (2011) NFATc3 regulates the transcription of genes involved in T-cell activation and angiogenesis. *Blood* **118**, 795–803.

Vashishta A., Habas A., Pruunsild P., Zheng J.-J., Timmusk T., Hetman M. (2009) Nuclear factor of activated T-cells isoform c4 (NFATc4/NFAT3) as a mediator of antiapoptotic transcription in NMDA receptor-stimulated cortical neurons. *J. Neurosci. Off. J. Soc. Neurosci.* **29**, 15331–15340.

Venkatachalam K., Montell C. (2007) TRP Channels. *Annu. Rev. Biochem.* **76**, 387–417.

Walton M. R., Dragunow M. (2000) Is CREB a key to neuronal survival? *Trends Neurosci.* **23**, 48–53.

Weiss D. L., Hural J., Tara D., Timmerman L. A., Henkel G., Brown M. A. (1996) Nuclear factor of activated T cells is associated with a mast cell interleukin 4 transcription complex. *Mol. Cell. Biol.* **16**, 228–235.

Wilkins B. J., De Windt L. J., Bueno O. F., Braz J. C., Glascock B. J., Kimball T. F., Molkentin J. D. (2002) Targeted disruption of NFATc3, but not NFATc4, reveals an intrinsic defect in calcineurin-mediated cardiac hypertrophic growth. *Mol. Cell. Biol.* **22**, 7603–7613.

Wilkinson K. A., Henley J. M. (2010) Mechanisms, regulation and consequences of protein SUMOylation. *Biochem. J.* **428**, 133–145.

Willingham A. T., Orth A. P., Batalov S., Peters E. C., Wen B. G., Aza-Blanc P., Hogenesch J. B., Schultz P. G. (2005) A strategy for probing the function of noncoding RNAs finds a repressor of NFAT. *Science* **309**, 1570–1573.

Wu B., Wang Y., Lui W., Langworthy M., Tompkins K. L., Hatzopoulos A. K., Baldwin H. S., Zhou B. (2011) Nfatc1 coordinates valve endocardial cell lineage development required for heart valve formation. *Circ. Res.* **109**, 183–192.

Wu G.-Y., Deisseroth K., Tsien R. W. (2001) Activity-dependent CREB phosphorylation: Convergence of a fast, sensitive calmodulin kinase pathway and a slow, less sensitive mitogen-activated protein kinase pathway. *Proc. Natl. Acad. Sci.* **98**, 2808–2813.

Wu H., Peisley A., Graef I. A., Crabtree G. R. (2007) NFAT signaling and the invention of vertebrates. *Trends Cell Biol.* **17**, 251–260.

Wu Q., Liu G., Xu L., Wen X., Cai Y., Fan W., Yao X., Huang H., Li Q. (2016) Repair of Neurological Function in Response to FK506 Through CaN/NFATc1 Pathway Following Traumatic Brain Injury in Rats. *Neurochem. Res.* **41**, 2810–2818.

Xia Z., Dudek H., Miranti C. K., Greenberg M. E. (1996) Calcium influx via the NMDA receptor induces immediate early gene transcription by a MAP kinase/ERK-dependent mechanism. *J. Neurosci. Off. J. Soc. Neurosci.* **16**, 5425–5436.

Yan H. Q., Shin S. S., Ma X., Li Y., Dixon C. E. (2014) Differential effect of traumatic brain injury on the nuclear factor of activated T Cells C3 and C4 isoforms in the rat hippocampus. *Brain Res.* **1548**, 63–72.

Yang S. N., Tang Y. G., Zucker R. S. (1999) Selective induction of LTP and LTD by postsynaptic [Ca2+]i elevation. *J. Neurophysiol.* **81**, 781–787.

Yang T., Davis R. J., Chow C. W. (2001) Requirement of two NFATc4 transactivation domains for CBP potentiation. *J. Biol. Chem.* **276**, 39569–39576.

Yang T. T. C., Xiong Q., Enslen H., Davis R. J., Chow C.-W. (2002) Phosphorylation of NFATc4 by p38 mitogen-activated protein kinases. *Mol. Cell. Biol.* **22**, 3892–3904.

Yao J.-J., Zhao Q.-R., Liu D.-D., Chow C.-W., Mei Y.-A. (2016) Neuritin Up-regulates Kv4.2 α-Subunit of Potassium Channel Expression and Affects

Neuronal Excitability by Regulating the Calcium-Calcineurin-NFATc4 Signaling Pathway. *J. Biol. Chem.* **291**, 17369–17381.

Yoshida H., Nishina H., Takimoto H., Marengère L. E. M., Wakeham A. C., Bouchard D., Kong Y.-Y., et al. (1998) The Transcription Factor NF-ATc1 Regulates Lymphocyte Proliferation and Th2 Cytokine Production. *Immunity* **8**, 115–124.

Yoshida T., Mishina M. (2005) Distinct Roles of Calcineurin-Nuclear Factor of Activated T-Cells and Protein Kinase A-cAMP Response Element-Binding Protein Signaling in Presynaptic Differentiation. *J. Neurosci.* **25**, 3067–3079.

Zeng Y., Wang Y., Wu Z., Kang K., Peng X., Peng W., Qu J., Liu L., Raj J. U., Gou D. (2015) miR-9 enhances the transactivation of nuclear factor of activated T cells by targeting KPNB1 and DYRK1B. *Am. J. Physiol. Cell Physiol.* **308**, C720-728.

Zhang H., Xie X., Zhu X., Zhu J., Hao C., Lu Q., Ding L., et al. (2005) Stimulatory cross-talk between NFAT3 and estrogen receptor in breast cancer cells. *J. Biol. Chem.* **280**, 43188–43197.

Zhang J., Shapiro M. S. (2012) Activity-Dependent Transcriptional Regulation of M-Type (Kv7) K+ Channels by AKAP79/150-Mediated NFAT Actions. *Neuron* **76**, 1133–1146.

Zhao Q., Xie Y., Zheng Y., Jiang S., Liu W., Mu W., Liu Z., Zhao Y., Xue Y., Ren J. (2014) GPS-SUMO: a tool for the prediction of sumoylation sites and SUMO-interaction motifs. *Nucleic Acids Res.* **42**, W325-330.

Zhu J., McKeon F. (1999) NF-AT activation requires suppression of Crm1-dependent export by calcineurin. *Nature* **398**, 256–260.

Zhu J., McKeon* F. (2000) Nucleocytoplasmic shuttling and the control of NF-AT signaling. *Cell. Mol. Life Sci. CMLS* **57**, 411–420.

Zhu J., Shibasaki F., Price R., Guillemot J.-C., Yano T., Dötsch V., Wagner G., Ferrara P., McKeon F. (1998) Intramolecular Masking of Nuclear Import Signal on NF-AT4 by Casein Kinase I and MEKK1. *Cell* **93**, 851–861.

**ACKNOWLEDGEMENTS**

First and foremost, I would like to thank my supervisor Tõnis Timmusk for accepting me into his lab. I am grateful for his mentorship, his constant support and advice over the course of so many years. In addition, I would like to thank my supervisor and mentor Priit Pruunsild for teaching me everything from experimental design to writing a manuscript. His fingerprints are on virtually every page of this thesis. I am thankful also to my former student and co-author Mirjam Luhakooder for being a quick learner and hard-worker – thank you for your contribution.

I thank Urmas Arumäe for critically reading the manuscript of the thesis. I am also grateful for my first supervisor Jaanus Remme and his group at the University of Tartu for leading my way to the world of molecular biology. I would like to thank Holger Jahr at the Erasmus University Rotterdam for inviting me as a collaborator to his lab. The time I spent in Rotterdam really broadened my horizon and extended my technical skills.

I would like to acknowledge all the wonderful people I have had the pleasure of working together at the Department of Chemistry and Biotechnology, former Department of Gene Technology. I am thankful to all the past and present members of Neuro lab, especially my predecessor PhD students Mari, Indrek, Kaur and Kristi, who have helped me a lot with my experimental work. Special thanks go to Jürgen for helping me with statistical analysis, Epp, Maila and Ants for technical assistance, and Heiti for help in confocal microscopy. Working with all of you has been a blast!

Finally, I would like to thank my friends and family for providing me with emotional support. I thank my brothers Peeter and Markus for always keeping me on my toes and taking such good care of me, and my "sisters" Eva and Mari for always being there for me. I thank my parents for their guidance, encouragement and good food (!).

My very special thank you goes to Kaspar for his support, kindness and love.

This work was supported by Welcome Trust International Senior Research Fellowship (Grant 067952), Estonian Ministry of Education and Research (Grants 0222602 and 0140143), Estonian Science Foundation (Grant 7257), Estonian Research Council (institutional research funding IUT19-18 and Grant 8844), Estonian Academy of Sciences, and European Union through the European Regional Development Fund (Project No. 2014–2020.4.01.15- 0012). I would also like to thank European Regional Development Fund's Dora Plus program, and Archimedes Foundation's national scholarship program Kristjan Jaak. This work has been partially supported by "TTÜ arenguprogramm aastateks 2016-2022" Graduate School in Clinical medicine receiving funding from the European Regional Development Fund under program ASTRA 2014-2020.4.01.16-0032 in Estonia.

## ABSTRACT

Four members of the nuclear factor of activated T cells (NFAT) family (NFATc1, NFATc2, NFATc3, and NFATc4) are $Ca^{2+}$-dependent transcription factors that regulate several cellular processes in vertebrates, including the development and function of the immune, cardiovascular, musculoskeletal, and nervous system. Upon activation by the $Ca^{2+}$-dependent phosphatase calcineurin (CaN), NFATs translocate from cytosol to the nucleus, where they regulate the expression of their target genes. Increasing amount of evidence indicate the importance of different NFAT family members in the nervous system, where NFAT-dependent gene regulation is associated with corticogenesis, synaptogenesis, synaptic plasticity and neurotransmission.

Given the critical processes that NFAT proteins regulate in the nervous system, relatively little is known about the expression and regulation of different NFAT protein isoforms derived from the four NFAT genes in neurons. Hence, the aims of the current study were (i) to characterize the structure, alternative splicing and expression of the human and mouse NFAT gene family members, with the emphasis on their expression in the brain, (ii) to study the subcellular localization and the transactivation capacities of different over-expressed human NFAT proteins at basal conditions and in response to membrane depolarization in rat cultured primary cortical and hippocampal neurons, and (iii) to determine whether sumoylation is involved in the regulation of the transcriptional activity of human NFAT proteins in neurons.

The results of the current study demonstrate that due to complex usage of alternative 5′ and 3′ exons and alternative splicing, the NFAT isoforms derived from each of the four NFAT genes differ greatly in their N- and C-terminal regions. Expression analysis of *NFATC1*, *NFATC2*, *NFATC3*, and *NFATC4* mRNAs showed that all NFATs, although expressed with different tissue-specific patterns, are expressed in the brain. Next, we studied how the structural differences between different human NFAT proteins contribute to the activity and localization of these proteins in neurons. Functional analysis of NFAT isoforms showed that nuclear translocation of all studied NFAT isoforms in rat cultured primary cortical and hippocampal neurons is dependent on CaN activity. However, the precise effects of $Ca^{2+}$ signaling on the subcellular localization of the NFAT proteins are isoform-specific and can vary between neuron types. Furthermore, the ability of different NFAT isoforms to regulate NFAT response element-controlled transcription in neurons is isoform-specific. We also found that, in general, sumoylation represses the transcriptional activity of NFAT proteins, but the effect of sumoylation on specific NFAT isoforms varies between cell types.

Collectively, the results of this study outline how and to what extent differential expression and regulation of NFAT isoforms could contribute to the diversity of neuronal activity-dependent functions of the NFAT proteins in neurons.

## KOKKUVÕTE

NFATc1, NFATc2, NFATc3 ja NFATc4 on perekond kaltsium-sõltuvaid transkriptsioonifaktoreid, mis reguleerivad selgroogsetes loomades mitmeid olulisi rakulisi protsesse. Muuhulgas on NFAT perekonna rolli kirjeldatud nii immuunsüsteemi, südame-veresoonkonna, lihaskonna, kui ka närvisüsteemi arengus ja talitluses. NFAT signaaliraja aktiveerib seriin/treoniin-sõltuv fosfataas kaltsineuriin, mis defosforüleerib NFAT valgud rakusisese kaltsiumi kontsentratsiooni tõusu korral. See indutseerib NFAT valkude liikumise raku tuuma, kus käivitatakse NFAT-sõltuv geeniekspressioon. Aina kasvav hulk tõendeid viitab NFAT valkude olulisusele närvisüsteemis, kus NFAT rolli seostatakse kortikogeneesiga, sünaptogeneesiga, sünaptilise plastilisusega ning neurotransmissiooniga.

Arvestades kuivõrd olulistes protsessides NFAT valgud närvisüsteemis osalevad, on üllatavalt vähe teada erinevate NFAT valkude isovormide ekspressioonist ja regulatsioonist närvirakkudes. Sellest tulenevalt oli käesoleva töö eesmärkideks: (i) iseloomustada inimese ja hiire NFAT geenide struktuuri ja ekspressiooni, rõhuasetusega nende ekspressioonianalüüsile ajus, (ii) analüüsida roti primaarsetes kortikaalsetes ja hipokampaalsetes närvirakkudes üleekspresseeritud inimese NFAT valguisovormide rakusisest lokalisatsiooni ja aktiivsust nii basaaltasemel, kui ka vastusena membraani depolarisatsioonile, ja (iii) teha kindlaks, kas NFAT valkude sumoüleerimine mõjutab nende transkriptsioonilist aktiivsust närvirakkudes.

Antud töö tulemused näitavad, et tulenevalt keerukast 5′ ja 3′ eksonite kasutusest ning alternatiivsest splaissimisest, erinevad NFAT isovormid suuresti oma amino- ja karboksüülterminuste poolest. *NFATC1, NFATC2, NFATC3* ja *NFATC4* mRNA'de ekspressioonianalüüsi kohaselt on kõik NFAT geenid, kuigi erineva koespetsiifilise avaldumismustriga, ajus ekspresseeritud. Sellest lähtuvalt tõstatasime küsimuse, mil määral mõjutavad NFAT isovormide struktuurilised erinevused nende regulatsiooni närvirakkudes. Meie tulemused näitavad, et roti primaarsetes kortikaalsetes ja hipokampaalsetes närvirakkudes on kõikide NFAT isovormide liikumine tuuma sõltuv kaltsineuriini aktiivsusest. Samas on kaltsiumi signaliseerimise mõju NFAT valkude rakusisesele lokalisatsioonile sõltuv konkreetsest NFAT isovormist ning varieerub rakutüübiti. Enamgi veel, erinevate NFAT isovormide võime aktiveerida transkriptsiooni närvirakkudes on samuti sõltuv konkreetsest isovormist. Kuigi me näitasime, et sumoüleerimine mõjutab negatiivselt kõikide NFAT valkude transkriptsioonilist aktiivsust, on see mõju erinevatele NFAT isovormidele rakutüübist sõltuv.

Kokkuvõtvalt kirjeldavad käesoleva töö tulemused kuidas ja mil määral võivad NFAT isovormide ekspressiooni ja regulatsiooni erinevused panustada NFAT närvitalitlusest sõltuvate funktsioonide mitmekesisusse.

**PUBLICATION I**

**Vihma H***, Pruunsild P*, Timmusk T
Alternative splicing and expression of human and mouse NFAT genes. Genomics, 2008;92(5):279-91. doi: 10.1016/j.ygeno.2008.06.011.

Genomics 92 (2008) 279–291

Contents lists available at ScienceDirect

Genomics

journal homepage: www.elsevier.com/locate/ygeno

# Alternative splicing and expression of human and mouse NFAT genes☆

Hanna Vihma [1], Priit Pruunsild [1], Tõnis Timmusk *

*Department of Gene Technology, Tallinn University of Technology, Akadeemia Tee 15, Tallinn 19086, Estonia*

ARTICLE INFO

*Article history:*
Received 13 May 2008
Accepted 24 June 2008
Available online 26 August 2008

*Keywords:*
NFAT
Alternative splicing
$Ca^{2+}$-regulated transcription factor
Calcineurin
Neurons
Brain
Nervous system

ABSTRACT

Four members of the nuclear factor of activated T cells (NFAT) family (NFATC1, NFATC2, NFATC3, and NFATC4) are $Ca^{2+}$-regulated transcription factors that regulate several processes in vertebrates, including the development and function of the immune, cardiovascular, musculoskeletal, and nervous systems. Here we describe the structures and alternative splicing of the human and mouse NFAT genes, including novel splice variants for *NFATC1, NFATC2, NFATC3,* and *NFATC4,* and show the expression of different NFAT mRNAs in various mouse and human tissues and brain regions by RT-PCR. Our results show that alternatively spliced NFAT mRNAs are expressed differentially and could contribute to the diversity of functions of the NFAT proteins. Since NFAT family members are $Ca^{2+}$-regulated and have critical roles in neuronal gene transcription in response to electrical activity, we describe the expression of *NFATC1, NFATC2, NFATC3,* and *NFATC4* mRNAs in the adult mouse brain and in the adult human hippocampus using in situ hybridization and show that all NFAT mRNAs are expressed in the neurons of the mouse brain with specific patterns for each NFAT.



Nuclear factor of activated T cells (NFAT) is a family of transcription factors evolutionarily related to Rel/NF-κB [1]. The family consists of the $Ca^{2+}$-regulated members NFATC1 (NFATc, NFAT2), NFATC2 (NFATp, NFAT1), NFATC3 (NFATx, NFAT4), and NFATC4 (NFAT3) and osmotic tension-regulated NFAT5. The approved human symbols for the NFAT family members are NFATC1, NFATC2, NFATC3, NFATC4 and NFATC5 and the approved mouse symbols are Nfatc1, Nfatc2, Nfatc3, Nfatc4 and Nfatc5. The $Ca^{2+}$-regulated NFAT proteins consist of two conserved domains—a regulatory domain in the N-terminus and a Rel homology domain (RHD) in the C-terminus [2]. The regulatory domain consists of two conserved binding sites for the protein phosphatase calcineurin (CaN) [3,4], an extended serine-rich region, and a nuclear localization signal (NLS) [5]. The Rel homology domain binds DNA and interacts with partner proteins (also referred as NFATn) to transactivate gene transcription. The partner transcription factors include AP-1 (FOS or JUN) [6], GATA4 [7], and MEF2 [8], for example. The N- and C-terminal ends of each NFAT family protein are unique and contain transcription activation domains (TADs) [9].

The NFAT proteins exist in at least two alternative conformations—one in which the NLS is exposed and the nuclear export signal (NES) is masked or vice versa. This is achieved by dephosphorylation or phosphorylation, respectively, of serines in the regulatory domain [10]. NFATs are dephosphorylated by CaN [11] and phosphorylated by various kinases [10] such as casein kinase 1 (CSNK1A1), glycogen synthase kinase 3 (GSK3A or GSK3B), p38 MAP kinase (MAPK14), and JUN N-terminal kinase (MAPK8), [12–16]. Exposure of the NLS leads to rapid import of the NFAT proteins into the nucleus, where they bind to DNA and regulate target gene expression [17], whereas phosphorylation causes rapid relocalization out of the nucleus, terminating NFAT-dependent transcription regulation [18].

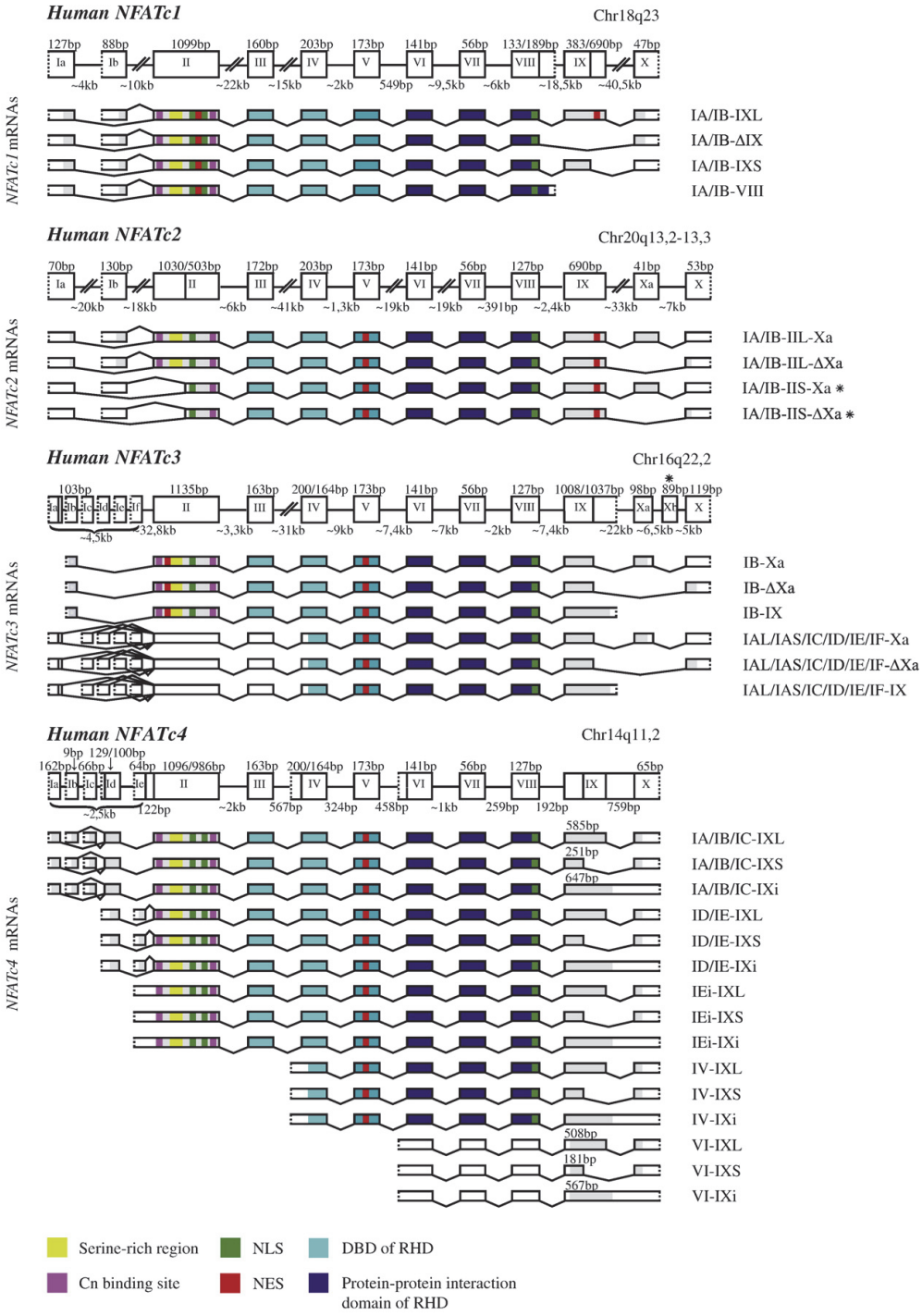
NFAT proteins regulate gene transcription in various developing and adult tissues. For example, their roles in the immune system [2], cardiovascular system [2,19], skeletal muscle [20], and nervous system [21,22] have been described. Accordingly, NFAT genes are expressed in almost all tissues. However, the expression levels and patterns for each NFAT are rather distinct [17,23]. All of the NFAT genes except *NFATC4* are strongly expressed in the immune system, in the thymus, spleen, and peripheral blood lymphocytes [23–28], but are also expressed at lower levels in other tissues. *NFATC1* has been detected in the cardiovascular and digestive systems, for example [26,29,30], and *NFATC2* expression has been detected in the testis, pancreas, placenta, and brain–in the hypothalamus, hippocampus, cerebellum, olfactory bulb, and frontal cortex [23,24,31–33]. In addition to the immune system, *NFATC3* is expressed in the skeletal and smooth muscle, kidney, and lung and in the brain, where it has been shown to be expressed in the hypothalamus and striatum [26,28,31,34,35]. *NFATC4* is more evenly expressed than the other NFAT genes and its expression

☆ Sequence data from this article have been deposited in the GenBank Data Libraries under Accession Nos. EU887559- EU887566 (Human NFATC1 transcript types); EU887573- EU887580 (Human NFATC2 transcript types); EU887605- EU887625 (Human NFATC3 transcript types); EU887632- EU887655 (Human NFATC4 transcript types); EU887567- EU887572 (Mouse Nfatc1 transcript types); EU887581- EU887604 (Mouse Nfatc2 transcript types); EU887626- EU887631 (Mouse Nfatc3 transcript types); EU887656- EU887661 (Mouse Nfatc4 transcript types).

* Corresponding author. Fax: +372 620 4401.
*E-mail address:* tonis.timmusk@ttu.ee (T. Timmusk).

[1] These authors contributed equally to this work.

0888-7543 © 2008 Elsevier Inc. Open access under CC BY license.
doi:10.1016/j.ygeno.2008.06.011


Human NFATc1
Chr18q23
127bp 88bp 1099bp 160bp 203bp 173bp 141bp 56bp 133/189bp 383/690bp 47bp
Ia Ib II III IV V VI VII VIII IX X
~4kb ~10kb ~22kb ~15kb ~2kb 549bp ~9,5kb ~6kb ~18,5kb ~40,5kb
NFATc1 mRNAs
IA/IB-IXL
IA/IB-ΔIX
IA/IB-IXS
IA/IB-VIII
Human NFATc2
Chr20q13,2-13,3
70bp 130bp 1030/503bp 172bp 203bp 173bp 141bp 56bp 127bp 690bp 41bp 53bp
Ia Ib II III IV V VI VII VIII IX Xa X
~20kb ~18kb ~6kb ~41kb ~1,3kb ~19kb ~19kb ~391bp ~2,4kb ~33kb ~7kb
NFATc2 mRNAs
IA/IB-IIL-Xa
IA/IB-IIL-ΔXa
IA/IB-IIS-Xa *
IA/IB-IIS-ΔXa *
Human NFATc3
Chr16q22,2
103bp 1135bp 163bp 200/164bp 173bp 141bp 56bp 127bp 1008/1037bp 98bp 89bp 119bp
Ia Ib Ic Id Ie If II III IV V VI VII VIII IX Xa Xb X
~4,5kb ~32,8kb ~3,3kb ~31kb ~9kb ~7,4kb ~7kb ~2kb ~7,4kb ~22kb ~6,5kb ~5kb
NFATc3 mRNAs
IB-Xa
IB-ΔXa
IB-IX
IAL/IAS/IC/ID/IE/IF-Xa
IAL/IAS/IC/ID/IE/IF-ΔXa
IAL/IAS/IC/ID/IE/IF-IX
Human NFATc4
Chr14q11,2
9bp 129/100bp
162bp 66bp 64bp 1096/986bp 163bp 200/164bp 173bp 141bp 56bp 127bp 65bp
Ia Ib Ic Id Ie II III IV V VI VII VIII IX X
~2,5kb 122bp ~2kb 567bp 324bp 458bp ~1kb 259bp 192bp 759bp
NFATc4 mRNAs
585bp
IA/IB/IC-IXL
251bp
IA/IB/IC-IXS
647bp
IA/IB/IC-IXi
ID/IE-IXL
ID/IE-IXS
ID/IE-IXi
IEi-IXL
IEi-IXS
IEi-IXi
IV-IXL
IV-IXS
IV-IXi
508bp
VI-IXL
181bp
VI-IXS
567bp
VI-IXi
Serine-rich region
NLS
DBD of RHD
Cn binding site
NES
Protein-protein interaction domain of RHD

has been detected in the placenta, lung, kidney, adipose tissue, cardiac muscle, testis, ovary, digestive system, and spinal cord and, at lower levels, in the brain—in the hippocampus, cerebellum, olfactory bulb, and various hypothalamic nuclei [19,21,23,34,36–40].

Despite this information, the expression of different NFAT isoforms generated by splicing or usage of alternative 5′ and 3′ exons has not been studied. Therefore, here we describe the structures of the human and mouse *NFATC1, NFATC2, NFATC3,* and *NFATC4* genes and analyze their alternative splicing and coding potentials. Furthermore, we have studied the expression of different *NFATC1, NFATC2, NFATC3,* and *NFATC4* mRNA splice variants in various mouse and human tissues and brain regions by RT-PCR and describe here the expression of the NFAT mRNAs in the adult mouse brain and in the adult human hippocampus using in situ hybridization.

## Results

### *The structure of the human and mouse NFAT genes*

The exon/intron structures of the human and mouse NFAT genes were characterized and the alternative splicing patterns of each NFAT gene in both human and mouse were analyzed using bioinformatics and RT-PCR. For each NFAT a search for mRNA sequences and expressed sequence tags (ESTs) was performed. RT-PCR analyses were used for the characterization of the expression patterns of the alternative transcripts in human and mouse.

The lengths of the four human NFAT genes vary from 10kb for *NFATC4* up to 170kb for *NFATC2* (Fig. 1). The NFAT genes are conserved in their central regions but are less similar in the 5′ and 3′ parts. The identity on the nucleotide level and the identity and strong similarity of the amino acids on the protein level among the most conserved part of the genes, encoded by exons V–VII, is ~ 80%. Although these exons are strongly similar, other exons are not so conserved. The identity among the full-length NFAT coding regions is ~ 50% on the nucleotide level and the amino acid identity or strong similarity in sum of the human NFAT proteins is ~ 56%.

There are several alternative transcripts for the NFAT genes, which are generated by usage of alternative 5′ and 3′ exons and alternative splicing. According to our data, human and mouse *NFATC1* and *NFATC2,* and mouse *Nfatc3* and *Nfatc4,* have two alternative 5′ exons. In human we detected six alternative 5′ exons for *NFATC3* and seven alternative 5′ exons for *NFATC4.* Our results also showed that in both human and mouse, *NFATC1* and *NFATC3* have two alternative 3′ exons and *NFATC4* has one 3′ exon. For *NFATC2* we detected one 3′ exon in human but three alternative 3′ exons in mouse. Due to these differences, the amino acid sequences within the C- and N-termini of different NFAT protein isoforms are distinct (Supplementary Figs. 1 and 2). In this study the alternative transcripts and protein isoforms have been given names according to the alternative exons used in the respective mRNAs (Fig. 1).

### *Alternative splicing and expression of NFATC1 in human and mouse*

For both human and mouse *NFATC1,* there are two alternative 5′ exons, exons IA and IB, and two alternative 3′ exons, exons VIII and X (Fig. 1; Table 1). Also, in both human and mouse exon IX has two alternative splice variants, designated here IXL and IXS, which are generated by the usage of alternative splice donor sites. In addition, we detected a novel splice variant for human *NFATC1* lacking exon IX, indicated here as ΔIX. In human, exon IA encodes 42 amino acids (aa).

**Table 1**
Usage of alternative 5′ and 3′ exons and alternative splicing of human and mouse NFAT genes

| Gene | | | Human | Mouse |
|---|---|---|---|---|
| *NFATC1* | 5′ exons | IA | [24] | [41] |
| | | IB | [29] | [42] |
| | 3′ exons | VIII | [24] | [42] |
| | | X | [29] | [41] |
| | Alternative splicing | IXL | [45] | + |
| | | IXS | [29] | [41] |
| | | ΔIX | + | ND |
| *NFATC2* | 5′ exons | IA | + | + |
| | | IB | [43] | [44] |
| | 3′ exons | III | ND | + |
| | | VIIA | ND | [33] |
| | | X | [43] | [43] |
| | Alternative splicing | IIL | [43] | [43] |
| | | IIS | + | + |
| | | ΔII | ND | + |
| | | XA | [43] | [43] |
| *NFATC3* | 5′ exons | IAL | + | [32] |
| | | IB | [23] | [46] |
| | | IC | + | ND |
| | | ID | + | ND |
| | | IE | + | ND |
| | | IF | + | ND |
| | 3′ exons | IV | ND | + |
| | | IX | [26] | ND |
| | | X | [26] | [32] |
| | Alternative splicing | IAS | + | ND |
| | | XA | [23] | [46] |
| | | XB | [26] | ND |
| *NFATC4* | 5′ exons | IA | + | ND |
| | | IB | + | ND |
| | | IC | + | ND |
| | | ID | [23] | [1] |
| | | IE | + | ND |
| | | IV | + | ND |
| | | VI | + | + |
| | 3′ exon | X | [23] | [1] |
| | Alternative splicing | IEi | + | ND |
| | | VIi | ND | + |
| | | IXL | [23] | [1] |
| | | IXS | + | ND |
| | | IXi | + | + |

+, novel transcript identified in this study; ND, not detected; references indicate studies describing the respective transcript variant.

Exon IB, located downstream from exon IA, encodes 29 aa. Transcripts that have the polyadenylation signal in exon VIII (VIII3′UTR) encode 63 aa from exon VIII, whereas transcripts containing exon X as the 3′ exon encode a C-terminal region that includes 44 aa identical to the exon VIII3′UTR isoforms in the region encoded by exon VIII, but contain an additional 245 aa or 127 aa, in the case of IXL or IXS usage, respectively. If exon IX is skipped the corresponding protein isoform lacks the 230 aa encoded by exon IXL but contains 15 C-terminal amino acids identical to the C-terminus of the exon IXL-comprising isoform. Altogether, according to our data, the possible transcript types of *NFATC1* in human are: (1) *NFATC1-IA/IB-IXL,* containing 5′ exon IA or IB, exon IXL, and 3′ exon X, having a protein coding region of 2829bp when exon IA is used or 2790bp when exon IB is used; (2) *NFATC1-IA/IB-ΔIX,* containing 5′ exon IA or IB, no exon IX, and 3′ exon X, having a protein coding region of 2139bp when exon IA is used or 2100bp when exon IB is used; (3) *NFATC1-IA/IB-IXS,* containing 5′ exon IA or IB, IXS, and 3′ exon X; exon IXS changes the open reading frame, introducing a stop codon in exon X that is 44bp upstream of the stop codon in type 1 and type 2 transcripts; the protein coding regions

**Fig. 1.** Structure and alternative transcripts of human NFAT genes. The structural organization of human *NFATC1, NFATC2, NFATC3,* and *NFATC4* was determined by analyzing genomic and mRNA sequence data using bioinformatics and RT-PCR. Exons are shown as boxes and introns are shown as lines. The numbers above the exons indicate the size of the protein coding part of the exon. Protein coding sequences of the mRNAs are shown as filled boxes and open boxes indicate UTRs of the mRNAs. Numbers below the introns indicate their size. Exon numbers are shown in roman characters. Asterisks mark rarely used exons and rarely transcribed mRNA variants. NES, nuclear export signal; NLS, nuclear localization signal; DBD, DNA binding domain; RHD, Rel homology domain; Cn, calcineurin A.

of *NFATC1* type 3 mRNAs are 2475bp when exon IA is used or 2436bp when exon IB is used; and (4) *NFATC1-IA/IB-VIII,* containing 5′ exon IA or IB and 3′ exon VIII; the coding region of exon VIII in type 4 transcripts is 56bp longer than in type 1, 2, and 3 transcripts; the protein coding regions are 2148bp when exon IA is used or 2109bp when exon IB is used (Fig. 1 and Supplementary Table 1). A human EST sequence corresponding to the *NFATC1* transcript lacking exon II is present in the databases; however, we did not detect it with RT-PCR and there are no references in the literature to confirm the generation of this transcript. According to our data *Nfatc1* transcripts for mouse are the same as in human, except for differences in the lengths of exons II, III, IXL, and IXS and in the length of the protein coding region of exon IB (data not shown).

In mouse, *Nfatc1* transcripts containing exon IA or exon IB were both predominantly expressed in the lung, thymus, and spleen (Fig. 2). Both alternative 3′ exon transcripts were detected in all tissues analyzed, with the levels being highest in the lung, thymus, and spleen. Exon IXS was more abundantly used than exon IXL in 3′ exon X-containing transcripts. In the adult mouse brain and during postnatal development of the brain the expression levels of transcripts containing exon IA or exon IB were similarly low (Fig. 2). In embryonic mouse brain, only *Nfatc1* transcripts comprising exon IA were expressed. In all the brain regions tested the expression levels of both of the 3′ exon transcripts were lower compared to the levels of respective mRNAs in the thymus, spleen, or lung (Fig. 2).

In human, *NFATC1* transcripts comprising exon IA were expressed more widely than transcripts comprising exon IB (Fig. 3). Both 5′ exon transcripts were highly expressed in the thymus and muscle. In addition, exon IA transcripts were expressed at high levels in the colon, small intestine, stomach, heart, uterus, testis, and thyroid and were detected also in other tissues. Exon IB mRNAs were highly expressed also in the fetal brain, cerebellum, and placenta and were undetectable in the stomach, uterus, liver, fetal liver, pancreas, salivary gland, trachea, and adrenal gland. Both 3′ exon transcripts were expressed in all the tissues analyzed, with highest levels observed in the testis, thymus, and muscle. Transcripts containing the 3′ exon X and exon IXS were expressed more predominantly than transcripts containing exon IXL or those in which exon IX was skipped. mRNAs

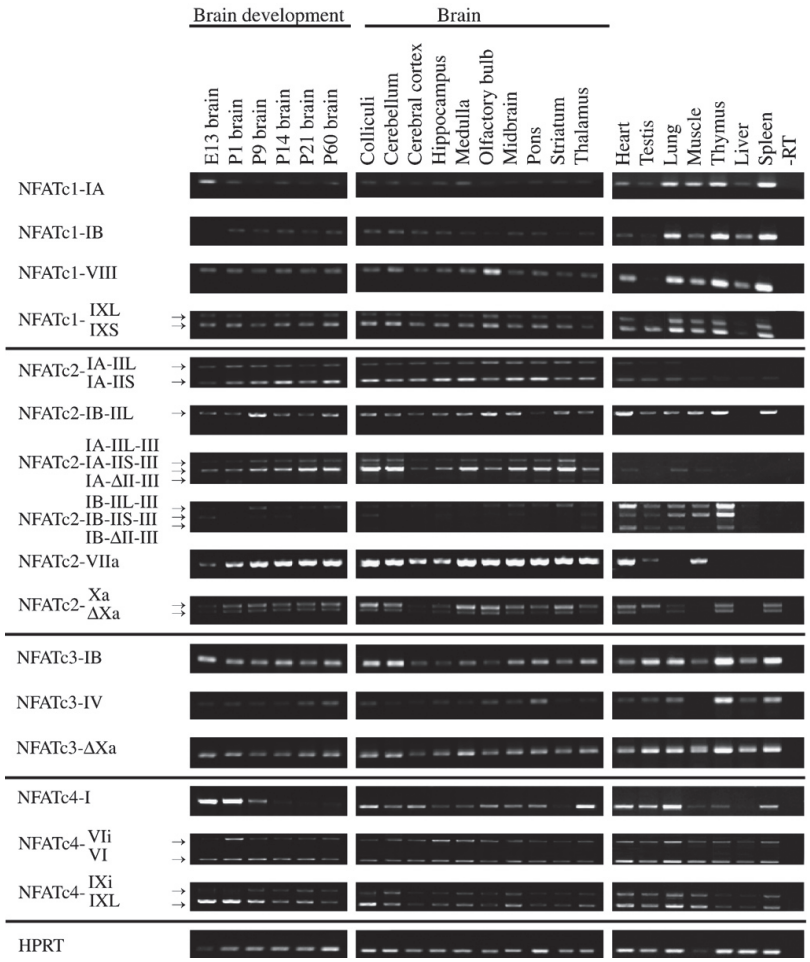



**Fig. 2.** Semiquantitative analysis of *Nfatc1, Nfatc2, Nfatc3, Nfatc4,* and control *Hprt* mRNA expression by RT-PCR in different mouse brain regions, in mouse brain at the indicated developmental time points, and in various mouse tissues.

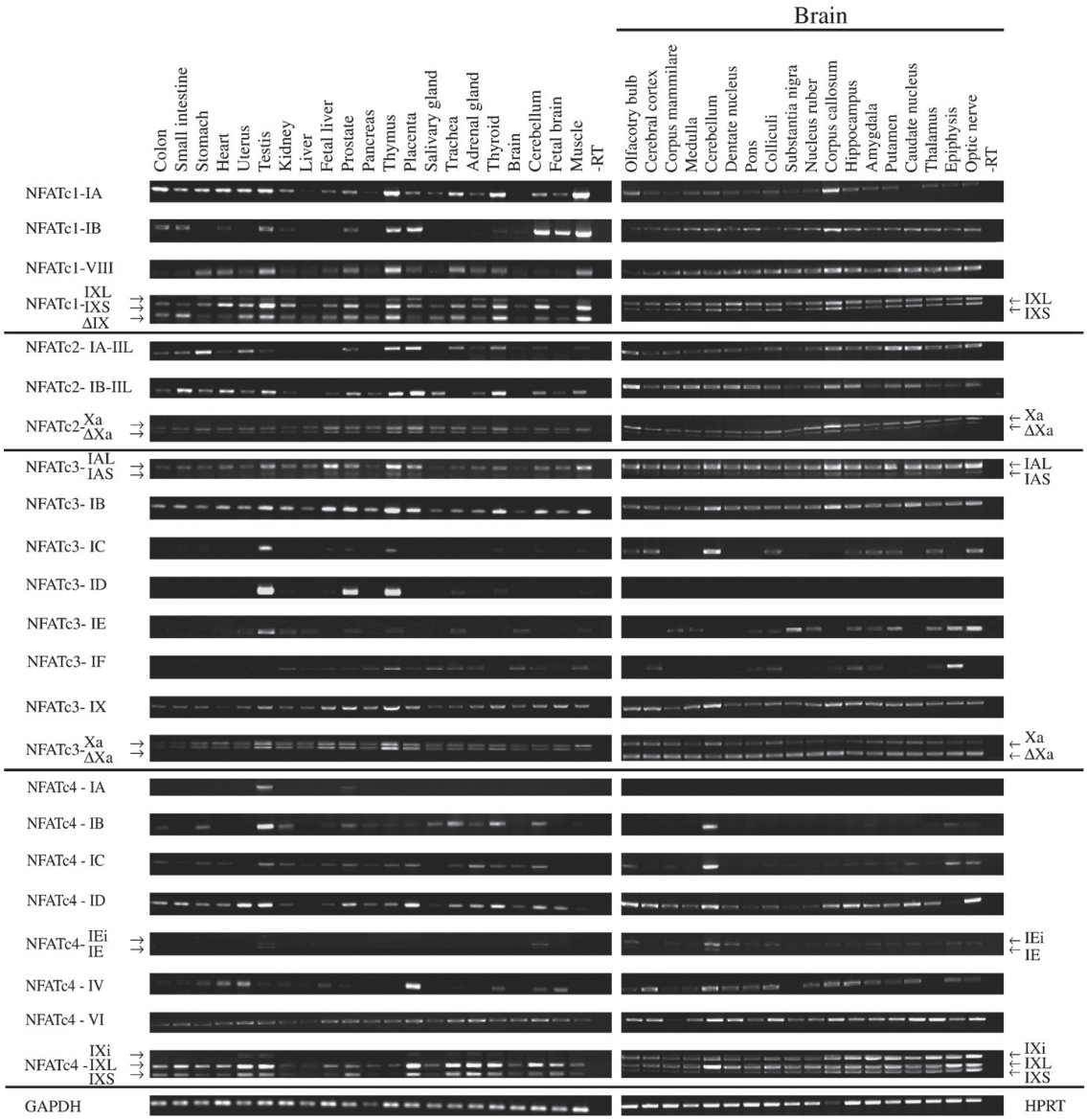



**Fig. 3.** Semiquantitative analysis of *NFATC1, NFATC2, NFATC3, NFATC4,* and controls *GAPDH* and *HPRT* mRNA expression by RT-PCR in various human tissues and brain regions.

with exon IXL were expressed at significantly lower levels, particularly in the small intestine, heart, liver, pancreas, salivary gland, brain, and fetal brain. Transcripts lacking exon IX or containing exon IXS were both highly and at comparable levels expressed in the thymus, thyroid, and muscle. The analysis of the expression of *NFATC1* transcripts in various human brain regions showed that transcripts comprising exon IA or exon IB were present in all regions tested, with highest levels in the corpus callosum (Fig. 3). Exon IA transcripts were expressed at relatively higher levels also in the olfactory bulb. Both of the alternative 3′ exons were also expressed in all of the regions analyzed, with the highest levels in the corpus callosum. The relative ratio of transcripts containing IXL or IXS was similar in most of the regions analyzed, except in the olfactory bulb, cerebral cortex, corpus mammilare, medulla, pons, and substantia nigra, where the expression levels of transcripts containing IXL were slightly higher. Transcripts lacking exon IX were not detected in the brain (Fig. 3).

*Alternative splicing and expression of NFATC2 in human and mouse*

Both human and mouse *NFATC2* contain two alternative 5′ exons, IA and IB, whereas only exon IB has been previously described (Fig. 1 and [41]). There is one 3′ exon in human, exon X, and three 3′ exons in

mouse, exons III, VIIa, and X (Table 1). Due to the usage of alternative splice acceptor sites exon II has two splice variants—IIL and IIS. Exon IIL is 1030 or 1036bp in length in human or mouse, respectively, and exon IIS is 516bp in both organisms. The shorter splice variant of exon II has not been described before. In mouse, exons IIS and IIL are both used and transcripts without exon II are also expressed, whereas in human the transcripts containing IIL are predominant and the usage of exon IIS or skipping of exon II is barely detectable. In both human and mouse exon IA encodes 23 aa and exon IB encodes 43 aa. Regardless of the 5′ exon, the translation start codon is positioned in exon II when exon IIS is used in transcripts. This leads to N-terminally truncated isoforms that are 199 and 201 aa (IA) or 219 and 221 aa (IB) shorter than the isoforms encoded by transcripts containing exon IIL in human and mouse, respectively. In both human and mouse *NFATC2* there is an additional exon compared to *NFATC1,* located upstream of exon X, named Xa here, that is either spliced in the mRNA or skipped. Usage of exon Xa leads to a translation stop codon in exon Xa. If exon Xa is not used then the translation stop codon is in exon X. This generates unique C-terminal sequences of 17 or 13 aa, for the respective NFATC2 protein isoforms. Taken together, our results showed that the possible transcript types of *NFATC2* in human are: (1) *NFATC2-IA/IB-IIL-Xa,* containing 5′ exon IA or IB, exon IIL, exon Xa, and 3′ exon X, having a protein coding region of 2703bp when exon IA is used or 2763bp when exon IB is used; (2) *NFATC2-IA/IB-IIS-Xa,* containing 5′ exon IA or IB, exon IIS, exon Xa, and 3′ exon X, having a protein coding region of 2106bp with either exon IA or exon IB; (3) *NFATC2-IA/IB-IIL-ΔXa,* containing 5′ exon IA or IB, exon IIL, and 3′ exon X and lacking exon Xa, having a protein coding region of 2715bp when exon IA is used or 2775bp when exon IB is used; (4) *NFATC2-IA/IB-IIS-ΔXa,* containing 5′ exon IA or IB, exon IIS, and 3′ exon X and lacking exon Xa, having a protein coding region of 2118bp with either exon IA or exon IB (Supplementary Table 1).

There are several differences in the mouse *Nfatc2* transcripts compared to the human ones in addition to the length of exon II. First, there are three alternative 3′ exons in mouse: exon III, exon VIIa, and exon X. The proteins encoded by transcripts using exon III or VIIa as 3′ exon lack the whole or a part of the Rel homology domain, respectively. Second, in mouse we detected *Nfatc2* transcripts lacking exon II (ΔII). Of note, according to the mouse EST and mRNA data in the NCBI databases there are transcripts in which exon V is used as the 3′ exon and transcripts with an alternative 5′ exon between exons III and IV. However, we did not detect these transcripts with PCR and there are no references to these transcripts in the literature.

Mouse *Nfatc2* transcripts containing exon IA, including transcripts with the mouse-specific 3′ exon III and transcripts with the 3′ exon VIIa, were all highly expressed in the brain, where the expression levels increased during postnatal development. In the adult mouse brain high levels were observed in the colliculi, cerebellum, medulla, olfactory bulb, and striatum (Fig. 2). Exon VIIa transcripts were highly expressed also in the heart and muscle. The levels of transcripts with exon X as the 3′ exon, either containing or lacking exon Xa, were relatively higher in the heart, testis, thymus, spleen, and brain, where they were more abundant in the colliculi, cerebellum, medulla, olfactory bulb, and striatum. Transcripts including exon IB predominantly contained exon IIL and were more broadly expressed, with relatively higher expression levels in the heart, thymus, and spleen. Transcripts containing exons IB and III3′UTR showed the highest expression levels in the heart and thymus and were very weakly expressed in the brain (Fig. 2).

In human, *NFATC2* transcripts including exon IA spliced to exon IIL were highly expressed in the stomach, uterus, thymus, placenta, trachea, and thyroid (Fig. 3). Transcripts containing exons IB and IIL were expressed highly in the small intestine, heart, testis, prostate, thymus, placenta, and thyroid. Transcripts containing IIS were hardly detectable (data not shown). Transcripts of *NFATC2* containing the 3′ exon X and either comprising or lacking exon Xa (ΔXa) were both present in all of the tissues analyzed, with exon Xa-containing transcripts being expressed at slightly higher levels. In the brain *NFATC2* was widely expressed. The expression levels were highest in the caudate putamen for exon IA and the olfactory bulb and corpus callosum for exon IB transcripts. Similar levels of exon Xa and ΔXa transcripts were expressed in almost all regions of the human brain except in the olfactory bulb, colliculi, nucleus ruber, corpus callosum, and caudate nucleus, where exon Xa transcripts were predominant (Fig. 3).

*Alternative splicing and expression of NFATC3 in human and mouse*

The human *NFATC3* contains six 5′ exons located within ~ 4500bp in the genome (Fig. 1), whereas in mouse there are only two alternative 5′ exons in *Nfatc3.* The human exon IB has been described before [23]. However, exons IA, IC, ID, IE, and IF are first described in this study (Table 1). Exon IB in human is homologous to exon IB in mouse and encodes 34 N-terminal amino acids of the respective proteins. Mouse 5′ exon IA is not homologous to human exon IA and encodes a unique N-terminus of 26 aa. In human, exon IB is the only 5′ exon that is a protein-coding exon. Exons IA, IC, ID, IE, and IF all lack an in-frame translation start codon and translation of *NFATC3* transcripts containing these exons could start from the ATG located in exon IV. Thus, proteins encoded by these transcripts would not contain the 485 N-terminal amino acids present in NFATC3 isoforms translated from transcripts including exon IB. Exon IA in human contains two alternative splice donor sites and the respective splice variants are named here IAL and IAS. There are two alternative 3′ exons in *NFATC3* genes: exons IX and X in human and exons IV and X in mouse. Transcripts in which exon IX is used as the 3′ exon encode proteins with 9 unique C-terminal amino acids that are not present in the proteins encoded by exon X-containing transcripts. Like in human *NFATC2* there is an alternative exon located between exon IX and exon X, designated here Xa, in both human and in mouse *NFATC3* genes. If exon Xa is used in the transcripts a stop codon is introduced. Therefore, different C-terminal sequences of 32 or 39 aa are encoded by exon X3′UTR transcripts depending on the usage or skipping, respectively, of exon Xa. Altogether, according to our data, the possible transcript types of *NFATC3* in human are: (1) *NFATC3-IB-Xa,* containing 5′ exon IB, exon Xa, and 3′ exon X, having a protein coding region of 3204bp; (2) *NFATC3-IB-ΔXa,* containing 5′ exon IB and 3′ exon X and lacking exon Xa, having a protein coding region of 3225bp; (3) *NFATC3-IB-IX,* containing 5′ exon IB and exon IX as the 3′ exon, having a protein coding region of 3135bp; (4) *NFATC3-IAL/IAS/IC/ID/IE/IF-Xa,* containing 5′ exon IAL or IAS or IC or ID or IE or IF, exon Xa, and 3′ exon X, having a protein coding region of 1767bp; (5) *NFATC3-IAL/IAS/IC/ID/IE/IF-ΔXa,* containing 5′ exon IAL or IAS or IC or ID or IE or IF and 3′ exon X and lacking exon Xa, having a protein coding region of 1788bp; and (6) *NFATC3-IAL/IAS/IC/ID/IE/IF-IX,* containing 5′ exon IAL or IAS or IC or ID or IE or IF and exon IX as the 3′ exon, having a protein coding region of 1698bp (Supplementary Table 1). Masuda et al. have described a human *NFATC3* transcript containing an additional exon upstream of exon X and downstream of exon Xa [26]. However, we were not able to detect this transcript in any of the tissues analyzed in this study.

In mouse, the expression of *Nfatc3* transcripts containing exon IB was detected in all tissues analyzed, with relatively higher levels in the testis, lung, thymus, and spleen (Fig. 2). Exon IA transcripts were barely detectable only in the testis and thymus (data not shown). *Nfatc3* transcripts containing the mouse-specific 3′ exon IV were expressed at moderate levels in the thymus and spleen. However, with the exception of muscle, low levels of this transcript were seen in all the tissues analyzed. Transcripts containing the 3′ exon X were also observed in all the tissues analyzed, with higher levels in the testis, lung, thymus, and spleen. In the mouse brain exon IB transcripts were expressed in all the regions analyzed (Fig. 2). Higher levels were seen

in the colliculi and cerebellum. During mouse brain development the levels of *Nfatc3* transcripts containing exon IB remained unchanged from embryonic day 13 (E13) up to adult, the developmental period studied here. Transcripts with 3′ exon X were expressed in all brain regions analyzed with, ΔXa transcripts being the predominant mRNAs (Fig. 2).

In human, *NFATC3* transcripts comprising exon IA or IB were more widely expressed than exon IC, ID, IE, or IF transcripts (Fig. 3). Both IA and IB transcripts were expressed at relatively higher levels in the testis, fetal liver, thymus, and muscle. In addition, exon IB was highly expressed in the prostate, placenta, thyroid, and cerebellum. IAL-containing transcripts were expressed at higher levels than IAS-containing transcripts. Transcripts containing exon IC were highly expressed in the testis. Transcripts containing exon ID had high expression in the testis, prostate, and thymus and lower expression in the kidney, liver, fetal liver, trachea, thyroid, and muscle. Exon IE was most strongly expressed in the testis and exon IF in the thymus. All the *NFATC3* 3′ exon transcript variants were expressed in all the tissues analyzed, with higher levels detected in the testis, fetal liver, prostate, thymus, and placenta. In the heart and muscle, transcripts containing exon Xa were expressed at slightly higher levels than transcripts lacking exon Xa. In the human brain, transcripts comprising exons IA and IB were expressed in all the regions analyzed, whereas exon IC transcripts were expressed only in some regions, with relatively higher levels in the cerebellum (Fig. 3). Exon ID transcripts were not detected in the human brain. Exon IE was relatively more expressed in the substantia nigra, optic nerve, and epiphysis and exon IF in the epiphysis. Expression levels of both of the alternative 3′ exons were similar in all the regions analyzed. Compared to the other brain regions exon IX transcripts were present at slightly higher levels in the olfactory bulb, cerebral cortex, cerebellum, and corpus callosum. Transcripts containing exon Xa were mostly expressed at higher levels than transcripts lacking exon Xa (Fig. 3).

*Alternative splicing and expression of NFATC4 in human and mouse*

Before our study, only one exon, named here exon ID, had been described as a 5′ exon in human *NFATC4* [23]. Our data show that human *NFATC4* has five 5′ exons within ~ 2.5kb of the most upstream part of the gene, named here IA, IB, IC, ID, and IE. In addition, 5′-extended exons IV and VI are also used as 5′ exons (Fig. 1 and Table 1). For mouse *Nfatc4* we identified two 5′ exons: the previously described 5′ exon, named exon I here, which is homologous to the human exon ID [26], and the 5′-extended exon VI, which has not been described before. With the usage of the 5′-extended exon VI in mouse, exclusion and retention of the intron between exons VI and VII were detected. Human exon ID and mouse exon I encode 33 aa. The human exon ID is in addition used as an internal exon: 129bp of its 3′ part are always inserted as the second exon in the transcripts starting upstream of exon ID. This is due to a cryptic splice acceptor site inside exon ID. Human *NFATC4* transcripts that use the 5′ exon IA, IB, or IC encode proteins with an additional 63, 13, or 32 aa, respectively, in their N-termini compared to the protein encoded by the transcripts containing exon ID as the 5′ exon. If exon IEi (retention of intron between exons IE and II), exon IV, or exon VI is used as the 5′ exon, the corresponding human *NFATC4* transcripts encode protein isoforms that are 70, 465, or 711 aa, respectively, shorter in their N-terminus compared to the protein encoded by the transcripts containing exon ID as the 5′ exon. Exon X is used as the 3′ exon in all mouse and human *NFATC4* mRNAs (Fig. 1). In human, there are two splice variants of exon IX, named here IXL and IXS, due to the usage of alternative splice donor sites. In addition, retention of the intron between exons IX and X leads to transcript variants indicated by IXi here. If exon IXS is used, the respective protein isoforms lack 108 aa in the C-terminal region compared to protein isoforms encoded by exon IXL-containing transcripts. IXi usage leads to protein isoforms with 20 unique amino acids in the C-terminus. In mouse, only exons IXL and IXi are used. Taken together, our results showed that the possible transcript types of *NFATC4* in human are: (1) *NFATC4-IA-IXL,* containing 5′ exon IA, exon IXL, and 3′ exon X, having a protein coding region of 2895bp; (2) *NFATC4-IA-IXS,* containing 5′ exon IA, exon IXS, and 3′ exon X, having a protein coding region of 2571bp; (3) *NFATC4-IA-IXi,* containing 5′ exon IA, exon IXi, and 3′ exon X, having a protein coding region of 2892bp; (4) *NFATC4-IB-IXL,* containing 5′ exon IB, exon IXL, and 3′ exon X, having a protein coding region of 2745bp; (5) *NFATC4-IB-IXS,* containing 5′ exon IB, exon IXS, and 3′ exon X, having a protein coding region of 2421bp; (6) *NFATC4-IB-IXi,* containing 5′ exon IB, exon IXi, and 3′ exon X, having a protein coding region of 2742bp; (7) *NFATC4-IC-IXL,* containing 5′ exon IC, exon IXL, and 3′ exon X, having a protein coding region of 2802bp; (8) *NFATC4-IC-IXS,* containing 5′ exon IC, exon IXS, and 3′ exon X, having a protein coding region of 2478bp; (9) *NFATC4-IC-IXi,* containing 5′ exon IC, exon IXi, and 3′ exon X, having a protein coding region of 2799bp; (10) *NFATC4-ID-IXL,* containing 5′ exon ID, exon IXL, and 3′ exon X, having a protein coding region of 2706bp; (11) *NFATC4-ID-IXS,* containing 5′ exon ID, exon IXS, and 3′ exon X, having a protein coding region of 2382bp; (12) *NFATC4-ID-IXi,* containing 5′ exon ID, exon IXi, and 3′ exon X, having a protein coding region of 2703bp; (13) *NFATC4-IE-IXL,* containing 5′ exon IE, exon IXL, and 3′ exon X, having a protein coding region of 2670bp; (14) *NFATC4-IE-IXS,* containing 5′ exon IE, exon IXS, and 3′ exon X, having a protein coding region of 2346bp; (15) *NFATC4-IE-IXi,* containing 5′ exon IE, exon IXi, and 3′ exon X, having a protein coding region of 2667bp; (16) *NFATC4-IEi-IXL,* containing 5′ exon IEi, exon IXL, and 3′ exon X, having a protein coding region of 2496bp; (17) *NFATC4-IEi-IXS,* containing 5′ exon IEi, exon IXS, and 3′ exon X, having a protein coding region of 2172bp; (18) *NFATC4-IEi-IXi,* containing 5′ exon IEi, exon IXi, and 3′ exon X, having a protein coding region of 2493bp; (19) *NFATC4-IV-IXL,* containing exon IV as the 5′ exon, exon IXL, and 3′ exon X, having a protein coding region of 1311bp; (20) *NFATC4-IV-IXS,* containing exon IV as the 5′ exon, exon IXS, and 3′ exon X, having a protein coding region of 987bp; (21) *NFATC4-IV-IXi,* containing exon IV as the 5′ exon, exon IXi, and 3′ exon X, having a protein coding region of 1308bp; (22) *NFATC4-VI-IXL,* containing exon VI as the 5′ exon, exon IXL, and 3′ exon X, having a protein coding region of 570bp; (23) *NFATC4-VI-IXS,* containing exon VI as the 5′ exon, exon IXS, and 3′ exon X, having a protein coding region of 246bp; and (24) *NFATC4-VI-IXi,* containing exon VI as the 5′ exon, exon IX, and 3′ exon X, having a protein coding region of 567bp (Supplementary Table 1).

Expression of mouse *Nfatc4* exon I mRNA was detected in all tissues analyzed, with higher levels in the lung, heart, testis, and spleen (Fig. 2). During brain development, the highest levels were observed at E13, the first developmental stage analyzed, and the expression decreased thereafter, reaching the lowest levels at postnatal day 14 and remaining unchanged thereafter. In the adult mouse brain the highest levels of *Nfatc4* exon I transcripts were detected in the thalamus and colliculi. Transcripts containing the extended 5′ exon VI or VIi were evenly expressed in all the peripheral tissues tested (Fig. 2). The highest expression levels of *Nfatc4* exon IXL and IXi transcripts were detected in the lung, heart, and muscle. In the brain IXL transcript levels decreased during development, while IXi transcripts were higher during postnatal development. In adult mouse brain these transcripts were most strongly expressed in the colliculi, midbrain, and cerebellum (Fig. 2).

In human, *NFATC4* transcripts comprising 5′ exons IB, IC, ID, and VI were expressed more widely than 5′ exon IA, IE, and IV transcripts (Fig. 3). Exon IA transcripts were detected only in the testis and prostate. Exon IB and IC transcripts were both expressed in several tissues, including the stomach, testis, kidney, trachea, adrenal gland, thyroid, and cerebellum. Transcripts containing exon ID were expressed in almost all tissues analyzed, with the highest levels in the small intestine, uterus, testis, prostate, placenta, thyroid, and cerebellum. Low levels of exon IE- and IEi-containing transcripts were seen only in the

testis. The 5′ exon IV transcript levels were highest in the placenta and relatively high also in the heart and uterus. Expression levels of transcripts containing exon VI as the 5′ exon were moderate in all tissues tested (Fig. 3). The 3′ exon X-containing transcripts of *NFATC4* were expressed according to the sum of the expression patterns of the 5′ exons. Highest levels were detected in the uterus, testis, placenta, trachea, adrenal gland, thyroid, and cerebellum. In most tissues, transcripts containing exon IXL were relatively more abundant than transcripts containing exon IXS. Transcripts containing the 3′ exon IX were expressed at low levels in the testis, kidney, and thymus. In the brain, all the transcript types of *NFATC4*, except exon IA transcripts, were expressed in the cerebellum (Fig. 3). In addition, high levels of 5′ exon ID, IV, and VI transcripts were detected in other brain regions: exon ID mRNAs in the olfactory bulb, hippocampus, caudate nucleus, and optic nerve; exon IV transcripts in the cerebral cortex, corpus callosum, and hippocampus; and exon VI transcripts in the olfactory bulb and cerebellum. Expression of exon X-containing transcripts, corresponding to the sum of all *NFATC4* mRNAs, was detected in all brain regions, with highest levels in the cerebellum, where exon IXL-containing transcripts were the predominant ones (Fig. 3).

*In situ hybridization analyses of NFATC1, NFATC2, NFATC3, and NFATC4 expression in adult mouse brain and human hippocampus*

Expression of NFAT mRNAs at the cellular level has not been studied thoroughly by in situ hybridization before. Therefore we analyzed the expression of NFAT mRNAs in adult mouse brain by in situ hybridization. The hybridization probes for each NFAT mRNA were constructed to recognize all of the major splice variants and therefore were targeted to the conserved RHD coding region of NFAT mRNAs. To distinguish different cell types, Nissl counterstaining of the tissue sections, which allows one to distinguish the large and weakly stained nuclei of neurons from the small and strongly stained nuclei of glial cells, was used. All of the NFAT mRNAs were expressed in the neurons of the brain, with specific patterns for each NFAT.

We observed similarities and differences in the expression patterns of *Nfatc1, Nfatc2, Nfatc3,* and *Nfatc4* (Figs. 4 and 5 and Supplementary Fig. 3). In the olfactory system *Nfatc1* was highly expressed in the granular layer and glomerular cell layer and *Nfatc2* in the mitral cell layer (Figs. 4 and 5). A moderate signal was detected in the glomerular and granular cell layer for *Nfatc2* (Figs. 4A, 4C, 5A, and 5D). *Nfatc3* and

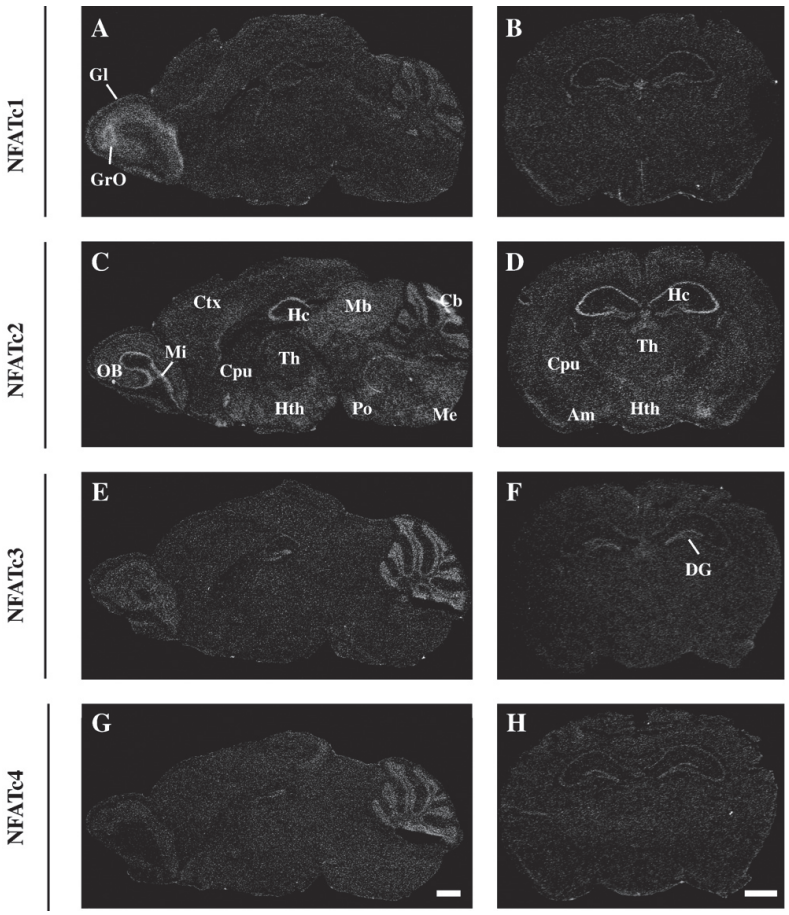



**Fig. 4.** In situ hybridization analysis of *Nfatc1, Nfatc2, Nfatc3,* and *Nfatc4* mRNA expression in adult mouse brain. Dark-field emulsion autoradiographs from sagittal sections (A, C, E, and G) and coronal sections at the level of thalamus (B, D, F, and H). The sections were hybridized with a probe for *Nfatc1* (A and B), *Nfatc2* (C and D), *Nfatc3* (E and F), or *Nfatc4* (G and H). Gl, glomerular layer of olfactory bulb; GrO, granular layer of olfactory bulb; OB, olfactory bulb; Mi, mitral layer of olfactory bulb; Ctx, cortex; Cpu, caudate putamen; Hc, hippocampus; Th, thalamus; Hth, hypothalamus; Mb, midbrain; Po, pons; Cb, cerebellum; Me, medulla; Am, amygdala; DG, dentate gyrus. Scale bars, 1 mm.

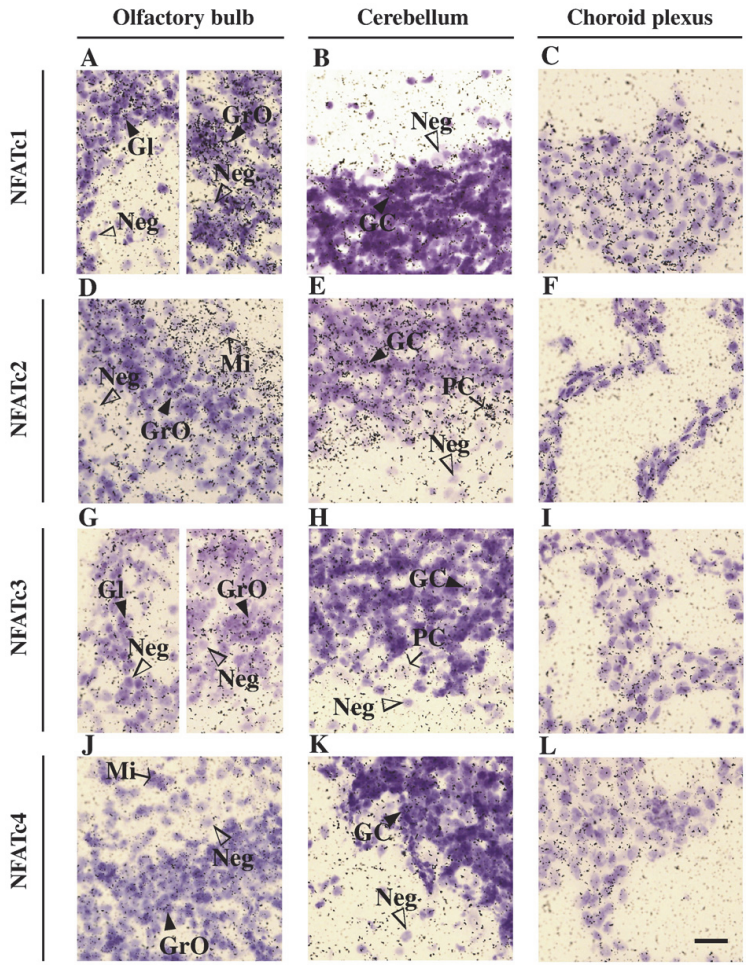



**Fig. 5.** In situ hybridization analysis of *Nfatc1*, *Nfatc2*, *Nfatc3*, and *Nfatc4* mRNA expression in adult mouse brain. Bright-field higher magnification pictures of the olfactory bulb (A, D, G, and J), cerebellum (B, E, H, and K), and choroid plexus (C, F, I, and L) are shown. Filled arrowheads denote some positive neurons, some negative neurons are marked with unfilled arrowheads. The sections were hybridized with a probe for *Nfatc1* (A, B, and C), *Nfatc2* (D, E, and F), *Nfatc3* (G, H, and I), or *Nfatc4* (J, K, and L). Gl, glomerular cell layer of the olfactory bulb; GrO, granular cells of the olfactory bulb; Mi, mitral cell layer, PC, Purkinje cells; GC, granular cells of the cerebellum; Neg, negative cell. Scale bar, 50 μm.

*Nfatc4* were expressed at low levels in the glomerular and granular layer (Figs. 4E, 4G, 5G, and 5J). In addition, *Nfatc4* was expressed in the mitral cell layer (Figs. 4G and 5J).

In the cerebral cortex *Nfatc2* mRNA expression was detected at relatively high levels in the neurons of layers II–VI (Fig. 4C and Supplementary Figs. 3A–D). The expression levels of other NFAT family members in this brain region were below the detection limit of our in situ hybridization method. Although all NFAT mRNAs were detected in the hippocampal formation, there were differences in the distribution and level of expression between different NFAT genes. *Nfatc2* showed a strong signal in the hippocampus, particularly in the CA1–CA3 pyramidal layers, and slightly lower levels in the granular layer of the dentate gyrus (Figs. 4C and D and Supplementary Figs. 3B–D). *Nfatc3* mRNA was moderately expressed only in the granular layer of the dentate gyrus and was detected at low levels in the CA1–CA3 pyramidal layers (Figs. 4E and F). *Nfatc1* and *Nfatc4* mRNAs were expressed at evenly low levels in both the CA1–CA3 pyramidal cells and the dentate gyrus granular cells (Figs. 4A and B).

In the basal ganglia, evenly distributed moderate signal was detected for *Nfatc2* in the caudate putamen, ventral pallidum, accumbens, and septum (Fig. 4C and Supplementary Fig. 3A), whereas *Nfatc1* mRNA was expressed at moderate levels only in the region of bed nucleus of stria terminalis (data not shown). *Nfatc3* and *Nfatc4* mRNAs were not expressed significantly in these brain structures (Figs. 4E and G). In the thalamus, hypothalamus, and midbrain *Nfatc2* mRNA was widely expressed at high or moderate levels in most of the nuclei (Figs. 4C and D and Supplementary Figs. 3B and C). In contrast, signal for *Nfatc1*, *Nfatc3*, and *Nfatc4* was barely detectable in these structures (Figs. 4A, B, and E–H).

In the cerebellum all NFAT transcripts were expressed (Figs. 4 and 5). *Nfatc1* and *Nfatc4* were moderately expressed only in the granular neurons (Figs. 4A, 4G, 5B, and 5K), whereas *Nfatc2* and *Nfatc3* were expressed in the granular cell layer and also in the Purkinje cell layer (Figs. 4C, 4E, 5E, and 5H). In the granular layer cells the signal for *Nfatc2* and *Nfatc3* was relatively stronger than that for *Nfatc1* and *Nfatc4*. In the pons and medulla only *Nfatc2* showed moderate expression all over the region (Fig. 4C and Supplementary Figs. 3E and F).

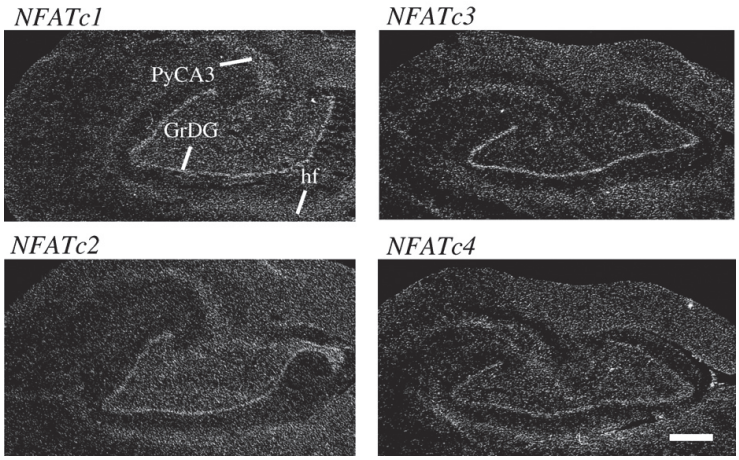



**Fig. 6.** In situ hybridization analysis of *NFATC1, NFATC2, NFATC3,* and *NFATC4* mRNA expression in adult human hippocampus. Dark-field emulsion autoradiographs from coronal sections at the level of hippocampus. GrDG, granular cell layer of the dentate gyrus; PyCA3, CA3 pyramidal cells; hf, hippocampal fissure. Scale bar, 1 mm.

In the choroid plexus and ependymal cells *Nfatc1* and *Nfatc2* were expressed at high levels and *Nfatc3* and *Nfatc4* at moderate levels (Figs. 5C, F, I, and L).

In the human hippocampus all four NFATs were expressed. For all NFATs stronger signal was detected in the granular layer of the dentate gyrus, in the pyramidal neurons of CA3 region, and in the hippocampal fissure (Fig. 6).

## Discussion

The aim of this study was to characterize the structures, alternative splicing, and expression of the NFAT genes in human and in mouse. Our results on the structures of the NFAT genes are in agreement with previous data from other groups [28,29,33,42,43] and also add important new data about the complex splicing and expression of this gene family. NFAT genes encode proteins that are very similar in their central region, which encodes the Rel homology domain, but are clearly variable in their N- and C-terminal parts due to the less conserved 5′ and 3′ regions of the paralogs of the genes, which differ significantly in their protein-coding potencies. Here we show that the NFAT genes are even more diverse in the 5′ and 3′ regions than previously described. All NFAT genes in human and mouse have multiple alternatively used 5′ exons: according to our data human and mouse *NFATC1* and *NFATC2* and mouse *Nfatc3* and *Nfatc4* have two alternative 5′ exons; human *NFATC3* has six and *NFATC4* has seven alternative 5′ exons. We show that usage of 3′-terminal exons is also complex: human and mouse *NFATC1* and *NFATC3* genes contain two and the mouse *Nfatc2* contains three alternative 3′ exons. Human *NFATC2* and both human and mouse *NFATC4* have one 3′ exon. In addition, alternative splicing is used for all NFAT genes and in combination with the usage of alternative 5′ and 3′ exons this could theoretically lead to 8 different protein isoforms of NFATC1, 6 different isoforms of both NFATC2 and NFATC3, and 24 different isoforms of NFATC4 in human.

The alternative 5′ exons of *NFATC1* have been described before [24,29,44,45] and the results of our bioinformatic and expression studies confirm that the *NFATC1* gene has two alternative 5′ exons in both mouse and human. Also, our expression analyses are consistent with studies showing *NFATC1* expression predominantly in the immune system [23–26,46]. For *NFATC2* only one 5′ exon had been previously described [41]. Here we have identified and characterized a novel alternative 5′ exon for both human and mouse *NFATC2*. We show that in human the previously known *NFATC2* 5′ exon, exon IB, and the novel alternative 5′ exon, named here exon IA, are expressed at similar levels in most of the tissues, except in the heart, where exon IB-containing mRNAs are the predominant transcripts. In mouse though, exon IA is used predominantly in the brain and exon IB both in the brain and in nonneural tissues. In addition to the novel *NFATC2* 5′ exon we describe a novel splice variant of exon II that is conserved in human and mouse and results in a short form of exon II, named IIS here. Usage of the short variant of exon II is the predominant splicing event in mouse for transcripts starting with exon IA. However, in human, exon IIS transcripts of *NFATC2* are barely detectable. *NFATC2* exon IIS-containing transcripts encode proteins without the N-terminal CaN binding site and serine-rich region. The functions of such isoforms are yet to be elucidated.

We have shown here that human *NFATC3* and *NFATC4* contain six and five 5′ exons, respectively. The human *NFATC3* exon IB and *NFATC4* exon ID are the only 5′ exons homologous to a 5′ exon in mouse *Nfatc3* and *Nfatc4,* respectively. These homologous exons are the only 5′ exons that have been previously described for *NFATC3* and *NFATC4* [23]. In human *NFATC3* and *NFATC4* the 5′ exons are located very close to each other in the 5′ ends of the genes. The close placement of the exons leaves open the possibility that they compile one exon with multiple transcription start sites. However, bioinformatic and PCR analyses showed that the 5′ sequences of both human *NFATC3* and *NFATC4* transcripts are not overlapping and use distinct splice donor sites, indicating that they are derived from different exons. In addition, there is an alternative transcription start site in human *NFATC4* located upstream of exon IV. Usage of this transcription start site generates protein isoforms that are similar to the *NFATC3* isoforms encoded by transcripts with the novel 5′ exons IA, IC, and ID of *NFATC3,* which yield NFAT isoforms without the whole regulatory domain. The function of these proteins is unclear. However, since we found generation of such isoforms for both *NFATC3* and *NFATC4* in human, these isoforms could be important in functions yet undefined. Another interesting observation about the putative N-termini of the human *NFATC3* and *NFATC4* is that although exon IB of human *NFATC3* and exons IA, IB, IC, and ID of human *NFATC4* encode N-termini that are different comparing the whole sequence, they clearly contain a conserved stretch of 12 amino acids—[E/D]EL[E/D]FKLVFGE[E/D] (Supplementary Fig. 1). The N-termini of the NFAT proteins have previously been shown to contain TADs [17]. We suggest that the TADs of the NFAT proteins contain important motifs that probably function

similarly but have also evolved to be distinct, potentially to convey different gene regulation activities.

The 3′ regions of NFAT genes are also diverse due to alternative splicing and usage of alternative 3′ exons. For example, in human, exon IX of *NFATC1* can be alternatively spliced in three ways, leading to transcripts lacking exon IX (ΔIX), with a long variant of exon IX (IXL) or with a short variant of exon IX (IXS). ΔXI transcripts have not been described before. Similar to the N-termini of NFAT proteins, there is a conserved motif (LDQ[T/L]YLDD(VN)E[I/L]I[R/D]) in the C-terminal sequences that locates to the C-terminal TAD of the NFATC1 and NFATC2 proteins [17] (Supplementary Fig. 2). This sequence is present only in the NFATC1 isoform NFATC1-IXL, whereas it is present in all human NFATC2 isoforms (Supplementary Fig. 2). In addition, by bioinformatics we found that this conserved motif contains a putative NES (Supplementary Fig. 2). Hence the isoforms lacking the sequence may act differently compared to the isoforms containing the TAD/NES. Our expression analysis of *NFATC1* showed that splice variants with exon IXS, encoding protein isoforms lacking the conserved motif, are the most abundantly expressed transcripts.

Alternative 3′ exons of NFAT genes are used in a species-specific manner. For example, for the mouse *Nfatc2,* exon VIIa has been shown to be an alternative 3′ exon and transcripts that include this exon are expressed specifically in neurons and encode a constitutively active isoform [33]. Our expression analysis confirmed that this *Nfatc2* transcript, named here *Nfatc2IB-IIS-VIIa,* is expressed in the mouse brain. However, in human, the homolog of this *Nfatc2* transcript is not expressed. Our analysis showed that similar species-specific differences are present also for human and mouse *NFATC3.* In human, and not in mouse, exon IX is used as an alternative 3′ exon. Transcripts including this exon encode the human-specific NFATC3 protein isoform containing a C-terminal TAD with a different transactivation potential compared to other NFATC3 C-terminal TADs [28]. In this study we have shown that this transcript is expressed in all human tissues analyzed. Furthermore, we show that *Nfatc3* transcripts using exon IV as the 3′ exon encoding a protein isoform lacking most of the Rel homology domain are expressed in mouse, in most of the tissues analyzed, and not in human. These data combined show that different NFAT C-termini could potentially have functionally unique characteristics, emphasizing that although NFAT proteins may have redundancy in their functions, as can be concluded from knockout studies [47,48], the specific functions of different NFAT isoforms could have an impact on $Ca^{2+}$-dependent gene expression in different tissues and species depending on the isoform expressed. Species-specific splicing and usage of alternative 5′ exons have also been shown to regulate other genes, for example, splicing of the *p53* tumor suppressor gene [49] and usage of 5′ exons of the transcription factor *NR5A1* [50].

In previous studies only *NFATC4* expression has been characterized in more detail in some regions of adult nervous system and cultured primary neurons [21,35,36,38,39,51,52]. However, there is evidence that NFATC2 and NFATC3 might also have important functions in the nervous system. Mice with the combination of *Nfatc2, Nfatc3,* and *Nfatc4* mutations have a complete defect of midline crossing of the commissural neurons in response to netrins and neurons from these mice are unable to respond to neurotrophins [47], whereas NFAT single-knockout mice have lesser neuronal defects [2,19]. In addition, there is evidence that *NFATC2* and *NFATC3* are expressed in some regions of the brain—in the hypothalamus and olfactory bulb [24,31,32]. To date there are no indications of *NFATC1* expression in the brain. To find out which of the NFAT genes are expressed in the adult brain and in which cells they are expressed we analyzed expression of NFAT mRNAs in adult mouse brain using in situ hybridization. Our results showed that *Nfatc2* is broadly expressed throughout the adult mouse brain and is the predominantly expressed NFAT in the mouse brain. The highest *Nfatc2* mRNA levels were detected in the pyramidal cell layer of the CA1–CA3 regions of the hippocampus, in the Purkinje and granule cell layers of the cerebellum, olfactory bulb, hypothalamus, and thalamus. Surprisingly, we found that *Nfatc1* is also expressed in mouse brain, having the highest expression levels in the granular and glomerular cell layers of the olfactory bulb. Moderate expression was seen also in the cerebellar granule cells. This suggests that NFATC1 may have important functions in the brain in addition to the functions in the tissues described earlier by others [24,26,52]. Before this study *NFATC3* expression in the brain had been demonstrated only in the hypothalamus and striatum and in certain cell lines of neuronal origin [31,35]. Here we describe *NFATC3* expression in the cerebellar granule cells and in Purkinje cells and, to a lesser extent, in the granule cells of the dentate gyrus and olfactory bulb. Therefore, our findings are consistent with the knockout studies showing that both *NFATC2* and *NFATC3* contribute to the development and function of the nervous system [47].

Several studies have shown that *NFATC4* is expressed in the brain, giving the notion that *NFATC4* might be the most abundant NFAT expressed in the nervous system. Expression has been shown in the adult rat hippocampus using RT-PCR and in situ hybridization and in the adult mouse hippocampus using RT-PCR [21], in cultured rat striatal cells using Western blot analysis [35] and in the adult mouse striatum using immunohistochemistry [51], in adult mouse dorsal root ganglion and spinal cord using in situ hybridization [36], in cultured rat cerebellar granule cells using immunocytochemistry [38], in adult mouse hippocampus using immunohistochemistry [39], and in cultured rat hippocampal neurons using immunocytochemistry [53]. However, we detected only low levels of *Nfatc4* expression in the brain. To ensure credibility we repeated the assay twice using two different hybridization probes. We show here that *Nfatc4* is indeed expressed in the brain but at lower levels compared to the other NFATs. Moderate expression was detected only in the cerebellar, olfactory bulb, and dentate gyrus granule cells and in the mitral cells of the olfactory bulb. On the other hand, according to our PCR analysis of NFAT expression during mouse brain development, *Nfatc4* was more abundantly expressed in the earlier stages of development and the levels were decreased during later stages. Therefore, Nfatc4 function might be especially important in the developing brain. Surprisingly, our PCR results showed that *NFATC4* is relatively highly expressed in the human brain, indicating again that NFAT signaling could have species-specific features. Moreover, in situ hybridization analysis showed that in the adult human brain all NFAT genes are expressed in the CA1–CA3 pyramidal neurons and dentate gyrus granule cells.

Notably, in the human brain all the NFATs, among other regions, were expressed in the corpus callosum and optic nerve—regions of the brain containing mainly neuronal projections and glial cells. By in situ hybridization, we found that the majority of the signal in the mouse brain accumulated in certain neuronal populations, although for all NFATs, very low signal was detected all over the brain, including glial cells. Thus, it is possible that in addition to neurons, NFAT genes are also expressed in glial cells. In the human hippocampus all NFATs were expressed in the CA3 pyramidal neurons and dentate gyrus granular cell layer and also in the hippocampal fissure, which further supports the possibility that NFAT genes are expressed in both neurons and glial cells.

In conclusion, the results of this study provide comprehensive data about the structure, splicing pattern, and expression of NFAT genes in human and mouse, providing useful information for future studies aiming to elucidate the functions of different NFAT isoforms.

## Materials and methods

### *Structure, expression analysis, and cloning of the NFAT genes*

Human NFAT gene structures and human and mouse NFAT mRNAs were identified by analyzing genomic, mRNA, and EST databases using bioinformatics tools (http://www.ncbi.nlm.nih.gov and http://genome.ucsc.edu). All homology searches were performed using various BLAST

tools (http://www.ncbi.nlm.nih.gov). NESs were predicted using the software at http://www.cbs.dtu.dk/services/NetNES/ site and NLSs were predicted using software at http://wolfpsort.org/ site. Rel homology and serine-rich sequence motifs were predicted using the software at the http://expasy.org/prosite/ site. Based on the sequence information acquired, primers were designed to analyze the expression of human and mouse NFAT mRNAs and to construct plasmids for mouse and human NFAT riboprobe generation (Supplementary Table 2). Total RNAs from various human and mouse brain regions and mouse tissues were purified with RNAwiz (Ambion, USA) as recommended by the manufacturer and treated with DNase using a DNA-free kit (Ambion). Human RNAs from different tissues were obtained from BD Biosciences (USA). First-strand cDNAs were synthesized from 5 μg of total RNA with Superscript III reverse transcriptase (Invitrogen, USA) as recommended by the manufacturer. Fire polymerase, Hot-Fire polymerase (Solis Biodyne, Estonia), or the GC-Rich PCR System (Roche, Switzerland) was used in PCR for expression analyses and riboprobe template plasmid construction, all according to the manufacturer's instructions. An annealing temperature of 57°C was used for all combinations of primers. Cycle numbers were optimized so that the PCR products were analyzed in the exponential phase of the PCR. Depending on the primers used, 30–40 cycles of PCR were performed. PCR fragments were resolved by agarose gel electrophoresis. PCR with primers specific for the ubiquitously expressed glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), 25 cycles, was performed as a control to determine the amount of human template cDNA in different PCRs. For normalizing the amount of mouse template cDNA and the amount of template cDNA in the panel of human brain regions, a 30-cycle PCR with primers specific for the ubiquitously expressed hypoxanthine guanine phosphoribosyl transferase (*HPRT*) was performed. All PCRs were performed in a volume of 10 μl containing 1/80 of the reverse transcription reaction product as a template. All PCR products were verified by sequencing.

*In situ hybridization*

For both human and mouse the in situ hybridization probes were generated as follows. The NFAT riboprobe fragments were selected to cover the parts of the NFAT mRNAs that are present in all mainly expressed splice variants of *NFATC1, NFATC2, NFATC3,* or *NFATC4.* DNA fragments amplified for NFAT riboprobe generation (Supplementary Table 2) were excised from the gel and cloned into pSC-A PCR cloning vector (Stratagene, USA). The mouse NFAT plasmids were linearized adjacent to the 5′ ends of the cloned fragments. The hybridization probes for mouse were 519, 524, 436, and 831 nt in length for *Nfatc1, Nfatc2, Nfatc3,* and *Nfatc4,* respectively. For *Nfatc4* the template for the second assay was generated by digestion at the BglII site inside the cloned *Nfatc4* sequence and the length of the probe was 456 bp. For human in situ hybridization the plasmids with *NFATC1, NFATC2, NFAT3,* and *NFATC4* were linearized with Eco45III, NcoI, NcoI, or HindIII restriction enzyme inside the cloned sequences, respectively. The lengths of the resulting riboprobes were 697 nt for *NFATC1,* 615 nt for *NFATC2,* 876 nt for *NFATC3,* and 816 nt for *NFATC4.* cRNA probes were synthesized with a MAXIScript in vitro transcription kit (Ambion), using [α-$^{35}$S]UTP (Amersham Biosciences) for labeling, according to the manufacturer's instructions. Serial sagittal and coronal sections (20 μm) from fresh-frozen adult male Black 6 mouse brains and coronal sections (16 μm) from fresh-frozen adult male human hippocampus were subjected to in situ hybridization using a protocol described elsewhere by Timmusk et al. [54]. Emulsion-dipped sections were developed after 7 weeks using D-19 developer (Eastman Kodak, USA), fixed (sodium fixer; Kodak), and counterstained with hematoxylin (Shandon, USA).

## Acknowledgments

We thank Enn Jõeste and Liina Kiho from North Estonian Regional Hospital, Tallinn, for help in collecting tissue samples; Epp Väli for technical assistance; and Richard Tamme and Liis Leitsalu for help in preparing cryostat sections. This work was supported by the following grants to T.T.: Wellcome Trust International Senior Research Fellowship (Grant 067952), Estonian Ministry of Education and Research (Grants 0222602 and 0140143), and Estonian Science Foundation (Grant 7257).

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ygeno.2008.06.011.

## References

[1] I.A. Graef, J.M. Gastier, U. Francke, G.R. Crabtree, Evolutionary relationships among Rel domains indicate functional diversification by recombination, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 5740–5745.
[2] G.R. Crabtree, E.N. Olson, NFAT signaling: choreographing the social lives of cells, Cell 109 (2002) S67–S79 (Suppl.).
[3] J. Aramburu, F. Garcia-Cozar, A. Raghavan, H. Okamura, A. Rao, P.G. Hogan, Selective inhibition of NFAT activation by a peptide spanning the calcineurin targeting site of NFAT, Mol. Cell 1 (1998) 627–637.
[4] S. Park, M. Uesugi, G.L. Verdine, A second calcineurin binding site on the NFAT regulatory domain, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 7130–7135.
[5] A. Kiani, A. Rao, J. Aramburu, Manipulating immune responses with immunosuppressive agents that target NFAT, Immunity 12 (2000) 359–372.
[6] J. Jain, P.G. McCaffrey, V.E. Valge-Archer, A. Rao, Nuclear factor of activated T cells contains Fos and Jun, Nature 356 (1992) 801–804.
[7] J.D. Molkentin, J.R. Lu, C.L. Antos, B. Markham, J. Richardson, J. Robbins, S.R. Grant, E.N. Olson, A calcineurin-dependent transcriptional pathway for cardiac hypertrophy, Cell 93 (1998) 215–228.
[8] F. Blaeser, N. Ho, R. Prywes, T.A. Chatila, Ca(2+)-dependent gene expression mediated by MEF2 transcription factors, J. Biol. Chem. 275 (2000) 197–209.
[9] C. Luo, E. Burgeon, A. Rao, Mechanisms of transactivation by nuclear factor of activated T cells-1, J. Exp. Med. 184 (1996) 141–147.
[10] H. Okamura, J. Aramburu, C. Garcia-Rodriguez, J.P. Viola, A. Raghavan, M. Tahiliani, X. Zhang, J. Qin, P.G. Hogan, A. Rao, Concerted dephosphorylation of the transcription factor NFAT1 induces a conformational switch that regulates transcriptional activity, Mol. Cell 6 (2000) 539–550.
[11] P.G. Hogan, L. Chen, J. Nardone, A. Rao, Transcriptional regulation by calcium, calcineurin, and NFAT, Genes Dev. 17 (2003) 2205–2232.
[12] C.R. Beals, C.M. Sheridan, C.W. Turck, P. Gardner, G.R. Crabtree, Nuclear export of NF-ATc enhanced by glycogen synthase kinase-3, Science 275 (1997) 1930–1934.
[13] C.W. Chow, M. Rincon, J. Cavanagh, M. Dickens, R.J. Davis, Nuclear accumulation of NFAT4 opposed by the JNK signal transduction pathway, Science 278 (1997) 1638–1641.
[14] P. Gomez del Arco, S. Martinez-Martinez, J.L. Maldonado, I. Ortega-Perez, J.M. Redondo, A role for the p38 MAP kinase pathway in the nuclear shuttling of NFATp, J. Biol. Chem. 275 (2000) 13872–13878.
[15] H. Okamura, C. Garcia-Rodriguez, H. Martinson, J. Qin, D.M. Virshup, A. Rao, A conserved docking motif for CK1 binding controls the nuclear localization of NFAT1, Mol. Cell. Biol. 24 (2004) 4184–4195.
[16] J. Zhu, F. Shibasaki, R. Price, J.C. Guillemot, T. Yano, V. Dotsch, G. Wagner, P. Ferrara, F. McKeon, Intramolecular masking of nuclear import signal on NF-AT4 by casein kinase I and MEKK1, Cell 93 (1998) 851–861.
[17] A. Rao, C. Luo, P.G. Hogan, Transcription factors of the NFAT family: regulation and function, Annu. Rev. Immunol. 15 (1997) 707–747.
[18] L.A. Timmerman, N.A. Clipstone, S.N. Ho, J.P. Northrop, G.R. Crabtree, Rapid shuttling of NF-AT in discrimination of $Ca^{2+}$ signals and immunosuppression, Nature 383 (1996) 837–840.
[19] I.A. Graef, F. Chen, L. Chen, A. Kuo, G.R. Crabtree, Signals transduced by Ca(2+)/calcineurin and NFATc3/c4 pattern the developing vasculature, Cell 105 (2001) 863–875.
[20] D.C. Hill-Eubanks, M.F. Gomez, A.S. Stevenson, M.T. Nelson, NFAT regulation in smooth muscle, Trends Cardiovasc. Med. 13 (2003) 56–62.
[21] I.A. Graef, P.G. Mermelstein, K. Stankunas, J.R. Neilson, K. Deisseroth, R.W. Tsien, G.R. Crabtree, L-type calcium channels and GSK-3 regulate the activity of NF-ATc4 in hippocampal neurons, Nature 401 (1999) 703–708.
[22] T. Nguyen, S. Di Giovanni, NFAT signaling in neural development and axon growth, Int. J. Dev. Neurosci. 26 (2008) 141–145.
[23] T. Hoey, Y.L. Sun, K. Williamson, X. Xu, Isolation of two new members of the NF-AT gene family and functional characterization of the NF-AT proteins, Immunity 2 (1995) 461–472.
[24] J.P. Northrop, S.N. Ho, L. Chen, D.J. Thomas, L.A. Timmerman, G.P. Nolan, A. Admon, G.R. Crabtree, NF-AT components define a family of transcription factors targeted in T-cell activation, Nature 369 (1994) 497–502.
[25] G.W. Cockerill, A.G. Bert, G.R. Ryan, J.R. Gamble, M.A. Vadas, P.N. Cockerill, Regulation of granulocyte–macrophage colony-stimulating factor and E-selectin expression in endothelial cells by cyclosporin A and the T-cell transcription factor NFAT, Blood 86 (1995) 2689–2698.
[26] E.S. Masuda, Y. Naito, H. Tokumitsu, D. Campbell, F. Saito, C. Hannum, K. Arai, N. Arai, NFATx, a novel member of the nuclear factor of activated T cells family that is expressed predominantly in the thymus, Mol. Cell. Biol. 15 (1995) 2697–2706.

[27] D.Z. Wang, P.G. McCaffrey, A. Rao, The cyclosporin-sensitive transcription factor NFATp is expressed in several classes of cells in the immune system, Ann. N. Y. Acad. Sci. 766 (1995) 182–194.
[28] R. Imamura, E.S. Masuda, Y. Naito, S. Imai, T. Fujino, T. Takano, K. Arai, N. Arai, Carboxyl-terminal 15-amino acid sequence of NFATx1 is possibly created by tissue-specific splicing and is essential for transactivation activity in T cells, J. Immunol. 161 (1998) 3455–3463.
[29] J. Park, A. Takeuchi, S. Sharma, Characterization of a new isoform of the NFAT (nuclear factor of activated T cells) gene family member NFATc, J. Biol. Chem. 271 (1996) 20914–20921.
[30] J.L. de la Pompa, L.A. Timmerman, H. Takimoto, H. Yoshida, A.J. Elia, E. Samper, J. Potter, A. Wakeham, L. Marengere, B.L. Langille, et al., Role of the NF-ATc transcription factor in morphogenesis of cardiac valves and septum, Nature 392 (1998) 182–186.
[31] M. Asai, Y. Iwasaki, M. Yoshida, N. Mutsuga-Nakayama, H. Arima, M. Ito, K. Takano, Y. Oiso, Nuclear factor of activated T cells (NFAT) is involved in the depolarization-induced activation of growth hormone-releasing hormone gene transcription in vitro, Mol. Endocrinol. 18 (2004) 3011–3019.
[32] A.M. Ho, J. Jain, A. Rao, P.G. Hogan, Expression of the transcription factor NFATp in a neuronal cell line and in the murine nervous system, J. Biol. Chem. 269 (1994) 28181–28186.
[33] S. Plyte, M. Boncristiano, E. Fattori, F. Galvagni, S.R. Paccani, M.B. Majolini, S. Oliviero, G. Ciliberto, J.L. Telford, C.T. Baldari, Identification and characterization of a novel nuclear factor of activated T-cells-1 isoform expressed in mouse brain, J. Biol. Chem. 276 (2001) 14350–14358.
[34] B.J. Wilkins, L.J. De Windt, O.F. Bueno, J.C. Braz, B.J. Glascock, T.F. Kimball, J.D. Molkentin, Targeted disruption of NFATc3, but not NFATc4, reveals an intrinsic defect in calcineurin-mediated cardiac hypertrophic growth, Mol. Cell. Biol. 22 (2002) 7603–7613.
[35] S. Jayanthi, X. Deng, B. Ladenheim, M.T. McCoy, A. Cluster, N.S. Cai, J.L. Cadet, Calcineurin/NFAT-induced up-regulation of the Fas ligand/Fas death pathway is involved in methamphetamine-induced neuronal apoptosis, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 868–873.
[36] R.D. Groth, L.G. Coicou, P.G. Mermelstein, V.S. Seybold, Neurotrophin activation of NFAT-dependent transcription contributes to the regulation of pro-nociceptive genes, J. Neurochem. 102 (2007) 1162–1174.
[37] I.C. Ho, J.H. Kim, J.W. Rooney, B.M. Spiegelman, L.H. Glimcher, A potential role for the nuclear factor of activated T cells family of transcriptional regulatory proteins in adipogenesis, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 15537–15541.
[38] A.B. Benedito, M. Lehtinen, R. Massol, U.G. Lopes, T. Kirchhausen, A. Rao, A. Bonni, The transcription factor NFAT3 mediates neuronal survival, J. Biol. Chem. 280 (2005) 2818–2825.
[39] K.C. Bradley, R.D. Groth, P.G. Mermelstein, Immunolocalization of NFATc4 in the adult mouse brain, J. Neurosci. Res. 82 (2005) 762–770.
[40] V.S. Seybold, L.G. Coicou, R.D. Groth, P.G. Mermelstein, Substance P initiates NFAT-dependent gene expression in spinal neurons, J. Neurochem. 97 (2006) 397–407.
[41] C. Luo, E. Burgeon, J.A. Carew, P.G. McCaffrey, T.M. Badalian, W.S. Lane, P.G. Hogan, A. Rao, Recombinant NFAT1 (NFATp) is regulated by calcineurin in T cells and mediates transcription of several cytokine genes, Mol. Cell. Biol. 16 (1996) 3955–3966.
[42] I.A. Graef, F. Chen, G.R. Crabtree, NFAT signaling in vertebrate development, Curr. Opin. Genet. Dev. 11 (2001) 505–512.
[43] S. Chuvpilo, M. Zimmer, A. Kerstan, J. Glockner, A. Avots, C. Escher, C. Fischer, I. Inashkina, E. Jankevics, F. Berberich-Siebelt, et al., Alternative polyadenylation events contribute to the induction of NF-ATc in effector T cells, Immunity 10 (1999) 261–269.
[44] X. Li, S.N. Ho, J. Luna, J. Giacalone, D.J. Thomas, L.A. Timmerman, G.R. Crabtree, U. Francke, Cloning and chromosomal localization of the human and murine genes for the T-cell transcription factors NFATc and NFATp, Cytogenet. Cell Genet. 68 (1995) 185–191.
[45] S. Pan, N. Koyano-Nakagawa, L. Tsuruta, Y. Amasaki, T. Yokota, S. Mori, N. Arai, K. Arai, Molecular cloning and functional characterization of murine cDNA encoding transcription factor NFATc, Biochem. Biophys. Res. Commun. 240 (1997) 314–323.
[46] D.L. Weiss, J. Hural, D. Tara, L.A. Timmerman, G. Henkel, M.A. Brown, Nuclear factor of activated T cells is associated with a mast cell interleukin 4 transcription complex, Mol. Cell. Biol. 16 (1996) 228–235.
[47] I.A. Graef, F. Wang, F. Charron, L. Chen, J. Neilson, M. Tessier-Lavigne, G.R. Crabtree, Neurotrophins and netrins require calcineurin/NFAT signaling to stimulate outgrowth of embryonic axons, Cell 113 (2003) 657–670.
[48] J. Aramburu, J. Heitman, G.R. Crabtree, Calcineurin: a central controller of signalling in eukaryotes, EMBO Rep. 5 (2004) 343–348.
[49] M. Laverdiere, J. Beaudoin, A. Lavigueur, Species-specific regulation of alternative splicing in the C-terminal region of the p53 tumor suppressor gene, Nucleic Acids Res. 28 (2000) 1489–1497.
[50] R. Kimura, H. Yoshii, M. Nomura, N. Kotomura, T. Mukai, S. Ishihara, K. Ohba, T. Yanase, O. Gotoh, H. Nawata, et al., Identification of novel first exons in Ad4BP/SF-1 (NR5A1) gene and their tissue- and species-specific usage, Biochem. Biophys. Res. Commun. 278 (2000) 63–71.
[51] R.D. Groth, J.P. Weick, K.C. Bradley, J.I. Luoma, B. Aravamudan, J.R. Klug, M.J. Thomas, P.G. Mermelstein, D1 dopamine receptor activation of NFAT-mediated striatal gene expression, Eur. J. Neurosci. 27 (2008) 31–42.
[52] A.M. Ranger, M.J. Grusby, M.R. Hodge, E.M. Gravallese, F.C. de la Brousse, T. Hoey, C. Mickanin, H.S. Baldwin, L.H. Glimcher, The transcription factor NF-ATc is essential for cardiac valve formation, Nature 392 (1998) 186–190.
[53] J.L. Belfield, C. Whittaker, M.Z. Cader, S. Chawla, Differential effects of $Ca^{2+}$ and cAMP on transcription mediated by MEF2D and cAMP-response element-binding protein in hippocampal neurons, J. Biol. Chem. 281 (2006) 27724–27732.
[54] T. Timmusk, K. Palm, M. Metsis, T. Reintam, V. Paalme, M. Saarma, H. Persson, Multiple promoters direct tissue-specific expression of the rat BDNF gene, Neuron 10 (1993) 475–489.

## Supplementary Table 1.

**Transcript types of human and mouse *NFAT genes*.**

| Transcript | Human | | Mouse | |
|---|---|---|---|---|
| | bp | aa; Mw (Da) | bp | aa; Mw (Da) |
| *NFATc1-IA-IXL* | 2829 | 943; 101243 | 2817 | 939; 101610 |
| *NFATc1-IB-IXL* | 2790 | 930; 100336 | 2775 | 925; 100468 |
| *NFATc1-IA-ΔIX* | 2139 | 713; 77376 | | ND |
| *NFATc1-IB-ΔIX* | 2100 | 700; 76469 | | ND |
| *NFATc1-IA-IXS* | 2475 | 825; 88763 | 2481 | 827; 89471 |
| *NFATc1-IB-IXS* | 2436 | 812; 87857 | 2439 | 813; 88328 |
| *NFATc1-IA-VIII* | 2148 | 716; 77785 | 2151 | 717; 77834 |
| *NFATc1-IB-VIII* | 2109 | 703; 76879 | 2109 | 703; 76692 |
| *NFATc2-IA-IIL-Xa* | 2703 | 901; 97693 | 2709 | 903; 97617 |
| *NFATc2-IB-IIL-Xa* | 2763 | 921; 99784 | 2769 | 923; 99642 |
| *NFATc2-IA/IB-IIS-Xa* | 2106 | 702; 76700 | 2106 | 702; 76589 |
| *NFATc2-IA/IB-ΔII-Xa* | | ND | 1419 | 473; 52311 |
| *NFATc2-IA-IIL-ΔXa* | 2715 | 905; 98054 | 2721 | 907; 97995 |
| *NFATc2-IB-IIL-ΔXa* | 2775 | 925; 100146 | 2781 | 927; 100020 |
| *NFATc2-IA/IB-IIS-ΔXa* | 2118 | 706; 77061 | 2118 | 706; 76966 |
| *NFATc2-IA/IB-ΔII-ΔXa* | | ND | 1431 | 477; 52689 |
| *NFATc2-IA-IIL-III* | | ND | 1377 | 459; 48661 |
| *NFATc2-IB-IIL-III* | | ND | 1437 | 479; 50686 |
| *NFATc2-IA/IB-IIS-III* | | ND | 774 | 258; 27633 |
| *NFATc2-IA/IB-ΔII-III* | | ND | 144 | 48; 5423 |
| *NFATc2-IA-IIL-VIIa* | | ND | 1959 | 653; 69937 |
| *NFATc2-IB-IIL-VIIa* | | ND | 2019 | 673; 71962 |
| *NFATc2-IA/IB-IIS-VIIa* | | ND | 1356 | 452; 48908 |
| *NFATc2-IA/IB-ΔII-VIIa* | | ND | 669 | 223; 24631 |
| *NFATc3-IAL-Xa* | 1767 | 589; 63608 | 3180 | 1060; 114491 |
| *NFATc3-IAL-ΔXa* | 1788 | 596; 63966 | 3204 | 1068; 114957 |
| *NFATc3-IAL-IV* | | ND | 1671 | 557; 60907 |
| *NFATc3-IB-Xa* | 3204 | 1068; 115237 | 3204 | 1068; 115071 |
| *NFATc3-IB-ΔXa* | 3225 | 1075; 115594 | 3228 | 1076; 115537 |
| *NFATc3-IB-IX* | 3135 | 1045; 112642 | | ND |
| *NFATc3-IB-IV* | | ND | 1695 | 565; 61486 |
| *NFATc3-IAS/IC/ID/IE/IF-Xa* | 1767 | 589;63608 | | ND |
| *NFATc3-IAS/IC/ID/IE/IF-ΔXa* | 1788 | 596; 63966 | | ND |
| *NFATc3-IAL/IAS/IC/ID/IE/IF-IX* | 1698 | 566; 61014 | | ND |
| *NFATc4-IA-IXL* | 2895 | 965; 101680 | | ND |
| *NFATc4-IA-IXS* | 2571 | 857; 90525 | | ND |
| *NFATc4-IA-IXi* | 2892 | 964; 101513 | | ND |
| *NFATc4-IB-IXL* | 2745 | 915; 96662 | | ND |
| *NFATc4-IB-IXS* | 2421 | 807; 85508 | | ND |
| *NFATc4-IB-IXi* | 2742 | 914; 96495 | | ND |
| *NFATc4-IC-IXL* | 2802 | 934; 98436 | | ND |
| *NFATc4-IC-IXS* | 2478 | 826; 87282 | | ND |
| *NFATc4-IC-IXi* | 2799 | 933; 98269 | | ND |
| *NFATc4-ID-IXL* | 2706 | 902; 95449 | 2703 | 901; 95782 |

| Transcript | Human | | Mouse | |
|---|---|---|---|---|
| | **bp** | **aa; Mw (Da)** | **bp** | **aa; Mw (Da)** |
| *NFATc4-ID-IXS* | 2382 | 794; 84294 | | ND |
| *NFATc4-ID-IXi* | 2703 | 901; 95282 | 2670 | 890; 94459 |
| *NFATc4-IE-IXL* | 2670 | 890; 94146 | | ND |
| *NFATc4-IE-IXS* | 2346 | 782; 82991 | | ND |
| *NFATc4-IE-IXi* | 2667 | 889; 93978 | | ND |
| *NFATc4-IEi-IXL* | 2496 | 832; 88270 | | ND |
| *NFATc4-IEi-IXS* | 2172 | 724; 77115 | | ND |
| *NFATc4-IEi-IXi* | 2493 | 831; 88103 | | ND |
| *NFATc4-IV-IXL* | 1311 | 437; 47373 | | ND |
| *NFATc4-IV-IXS* | 987 | 329; 36219 | | ND |
| *NFATc4-IV-IXi* | 1308 | 436; 47206 | | ND |
| *NFATc4-VI-IXL* | 570 | 190; 20104 | 630 | 210; 23170 |
| *NFATc4-VI-IXS* | 246 | 82; 8950 | | ND |
| *NFATc4-VI-IXi* | 567 | 189; 19937 | 744 | 248; 27055 |
| *NFATc4-VIi-IXL* | | ND | 630 | 210; 23170 |
| *NFATc4-VIi-IXi* | | ND | 744 | 248; 27055 |

## Supplementary Table 2.

1. **Primers used to analyse the expression of human and mouse *NFAT* mRNAs**:

| Transcript type/ Amplicon size | Primers |
|---|---|
| **Human *NFATc1*** | |
| ***NFATc1-IA*/** 1542 bp | hmrNFATc1exIAs, ACCAGCTTTCCAGTCCCTTCCAAG<br>hmrNFATc1exIVas, GGGATCTCCAGGACTTTGGTGTTG |
| ***NFATc1-IB*/** 1575 bp | hNFATc1exIBs, TCCTCTTCGAGTTTAACCAGCG<br>hrNFATc1exIVas, GGGATCTCCAGGACTTTGGTGTTG |
| ***NFATc1-VIII*/** 276 bp | hrNFATc1exVIIs, TGGCCACCATGTCTGGGAGATG<br>hNFATc1exVIIIUTRas, TGCTTTACGGCGACGTCGTTTC |
| ***NFATc1-ΔIX*/** 245 bp | hrNFATc1exVIIs, TGGCCACCATGTCTGGGAGATG<br>hNFATc1exXas, GTGGCAACTAGGAGTGGG |
| ***NFATc1-IXS*/** 628 bp | hrNFATc1exVIIs, TGGCCACCATGTCTGGGAGATG<br>hNFATc1exXas, GTGGCAACTAGGAGTGGG |
| ***NFATc1-IXL*/** 935 bp | hrNFATc1exVIIs, TGGCCACCATGTCTGGGAGATG<br>hNFATc1exXas, GTGGCAACTAGGAGTGGG |
| **Mouse *NFATc1*** | |
| ***NFATc1-IA/*** 462 bp | hmrNFATc1exIAs, ACCAGCTTTCCAGTCCCTTCCAAG<br>mNFATc2exIIas, TGCAGGGTTGCTGTAGACGGTG |
| ***NFATc1-IB/*** 406 bp | mNFATc1exIBs, GGAGTTCGACTTCGATTTCCTC<br>mNFATc2exIIas, TGCAGGGTTGCTGTAGACGGTG |
| ***NFATc1-VIII/*** 347 bp | mNFATc1exVIIs, CTGGGAGATGGAAGCAAAGAC<br>mrNFATc1exVIIIUTRas, GCGACTTGGTCTTGTGAATAGGG |
| ***NFATc1-IXS/*** 596 bp | mNFATc1exVIIs, CTGGGAGATGGAAGCAAAGAC<br>mrNFATc1exXas, GTGCTGGAGAGGTCGTTACG |
| ***NFATc1-IXL/*** 885 bp | mNFATc1exVIIs, CTGGGAGATGGAAGCAAAGAC<br>mrNFATc1exXas, GTGCTGGAGAGGTCGTTACG |
| **Human *NFATc2*** | |
| ***NFATc2-IA/*** 620 bp | hNFATc2exIAs, CTTTCCAAACACGCGCCAAG<br>hmNFATc2exII_LONGas, CTGGGGGAATAATGAGCAGGG |
| ***NFATc2-IB-IIS/*** 154 bp | hNFATc2exIBs, GACTATGAGTATTTGAATCCG<br>hmrNFATc2exII_BOTHas, GCGCACGAATGCCTCCGCTTG |
| ***NFATc2-IB-IIL/*** 532 bp | hNFATc2exIBs, GACTATGAGTATTTGAATCCG<br>hmrNFATc2exII_BOTHas, GCGCACGAATGCCTCCGCTTG |
| ***NFATc2-ΔXa/*** 857 bp | hmrNFATc2exVIIIs, CATGCTTTTTGTTGAGATCCC<br>hNFATc2exXas, CTGATTTCTGGCAGGAGGTC |
| ***NFATc2-Xa/*** 901 bp | hmrNFATc2exVIIIs, CATGCTTTTTGTTGAGATCCC<br>hNFATc2exXas, CTGATTTCTGGCAGGAGGTC |
| **Mouse *NFATc2*** | |
| ***NFATc2-IA-IIS/*** 245 bp | mNFATc2exIAs, GCCAGATCACAGCACACGGTC<br>hmNFATc2exII_BOTHas, GCGCACGAATGCCTCCGCTTG |
| ***NFATc2-IA-IIL/*** 765 bp | mNFATc2exIAs, GCCAGATCACAGCACACGGTC<br>hmNFATc2exII_BOTHas, GCGCACGAATGCCTCCGCTTG |
| ***NFATc2-IB-IIS/*** 165 bp | mrNFATc2exIBs, CAAGACGAGCTGGACTTTTC<br>hmrNFATc2exII_BOTHas, GCGCACGAATGCCTCCGCTTG |
| ***NFATc2-IB-IIL/*** 703 bp | mrNFATc2exIBs, CAAGACGAGCTGGACTTTTC<br>hmrNFATc2exII_BOTHas, GCGCACGAATGCCTCCGCTTG |

| Transcript type/ Amplicon size | Primers |
|---|---|
| ***NFATc2-VIIa/*** 635 bp | mrNFATc2exIVs, CACGGCTACATGGAGAACAAG<br>mrNFATc2exVIIUTRas, GGAAGGAGCACGGAGCATCTGAAG |
| ***NFATc2-ΔXa/*** 911 bp | hmrNFATc2exVIIIs, CATGCTTTTTGTTGAGATCCC<br>mNFATc2exXas, GGTCCTGAAAACTCCTTCCTGATG |
| ***NFATc2-Xa/*** 997 bp | hmrNFATc2exVIIIs, CATGCTTTTTGTTGAGATCCC<br>mNFATc2exXas, GGTCCTGAAAACTCCTTCCTGATG |
| **Human *NFATc3*** | |
| ***NFATc3-IAS/*** 443 bp | hNFATc3exIAs, CAGCCATTAAAGTTGAGGTGGG<br>hNFATc3exIIas, CTGCTGGCAGGACTAGGACTA |
| ***NFATc3-IAL/*** 698 bp | hNFATc3exIAs, CAGCCATTAAAGTTGAGGTGGG<br>hNFATc3exIIas, CTGCTGGCAGGACTAGGACTA |
| ***NFATc3-IB/*** 528 bp | hmrNFATc3Ibs, CACGCCGATGACTACTGCAAACTG<br>hNFATc3exIIas, CTGCTGGCAGGACTAGGACTA |
| ***NFATc3-IC/*** 470 bp | hNFATc3ICs, GATGACCGGAGACCGATAACCC<br>hNFATc3exIIas, CTGCTGGCAGGACTAGGACTA |
| ***NFATc3-ID/*** 494 bp | hNFATc3IDs, CCCTGTTACATATGCATACGAAC<br>hNFATc3exIIas, CTGCTGGCAGGACTAGGACTA |
| ***NFATc3-IE/*** 435 bp | hNFATc3exIEs, CCTGAACGTGAGGCATGAGGATTCT<br>hNFATc3exIIas, CTGCTGGCAGGACTAGGACTA |
| ***NFATc3-IF/*** 495 bp | hNFATc3exIFs, GGCTGCAGTGGTTTTACATCTCTG<br>hNFATc3exIIas, CTGCTGGCAGGACTAGGACTA |
| ***NFATc3-IX/*** 1161 bp | hNFATc3exVIIIs, CCTCCATATCATAACCCAGCAG<br>hNFATc3exIXUTRas, GTCCCTGAATCTTCACTACTTC |
| ***NFATc3-ΔXa/*** 91 bp | hNFATc3exIXs, CAGAAGATCGAGAGCCTAACTTTGC<br>hmNFATc3exXas, CAGAAATCTGGGACATGTCTCTC |
| ***NFATc3-Xa/*** 195 bp | hNFATc3exIXs, CAGAAGATCGAGAGCCTAACTTTGC<br>hmNFATc3exXas, CAGAAATCTGGGACATGTCTCTC |
| **Mouse *NFATc3*** | |
| ***NFATc3-IA/*** 190 bp | mNFATc3exIAs, TCTCTGTGTCTGCTCAACTTCCG<br>mrNFATc2exIIas, CCTTGGAGCTGAAATGATGGTGAC |
| ***NFATc3-IB/*** 246 bp | hmrNFATc3IBs, CACGCCGATGACTACTGCAAACTG<br>mrNFATc2exIIas, CCTTGGAGCTGAAATGATGGTGAC |
| ***NFATc3-IV/*** 392 bp | hmrNFATc3exIIIs, TGAAACTGAAGGTAGCCGAGGG<br>mNFATc3exIVUTRas, GGCTGGAGATAGAGGCAGGTGA |
| ***NFATc3-ΔXa/*** 555 bp | mNFATc3exIXs, CGACAGGACATCTCTTAGC<br>hmNFATc3exXas, CAGAAATCTGGGACATGTCTCTC |
| ***NFATx3-Xa/*** 659 bp | mNFATc3exIXs, CGACAGGACATCTCTTAGC<br>hmNFATc3exXas, CAGAAATCTGGGACATGTCTCTC |
| **Human *NFATc4*** | |
| ***NFATc4-IA/*** 1282 bp | hNFATc4exIA, CCGTTTAGTTGCTGGGATGGGGC<br>hNFATc4exIIas, GGATGCTCTCAGCTGGTGGGGCC |
| ***NFATc4-IB/*** 1100 bp | hNFATc4exIB, GAAGAGGAGGGGAACCCACAGG<br>hNFATc4exIIas, GGATGCTCTCAGCTGGTGGGGCC |
| ***NFATc4-IC/*** 1094 bp | hNFATc4exIC, CTTTGGGGGTCCTGGAGGAATGGC<br>hNFATc4exIIas, GGATGCTCTCAGCTGGTGGGGCC |

| Transcript type/ Amplicon size | Primers |
|---|---|
| ***NFATc4-ID/*** 689 bp | hNFATc4exIDs, ACCCGGGTGAAGATACAGCAG<br>hNFATc4exIIas, CTCATTTAGCTCAGACTCCACCTC |
| ***NFATc4-IE.*** 613 bp | hNFATc4exIeS, GTCCTAGGATCCAGGGGCCAGTG<br>hNFATc4exIIas, CTCATTTAGCTCAGACTCCACCTC |
| ***NFATc4-IEi/*** 738 bp | hNFATc4exIeS, GTCCTAGGATCCAGGGGCCAGTG<br>hNFATc4exIIas, CTCATTTAGCTCAGACTCCACCTC |
| ***NFATc4-IV/*** 536 bp | hNFTAc4exIVUTRs, GGATGAGACGGTGGGGATTTC<br>hmrNFATc4exVIas, GAGTCTGGCAGGAAGTTGGA |
| ***NFATc4-VI/*** 429 bp | hNFATc4exIV5'UTRs, GTTTAACCCTCTCTCTGCTCTG<br>hmNFATc4exVIIIas, CTTGTTGCTGTACTCGGGGA |
| ***NFAT-IXS/*** 492 bp | hNFATc4exVIIs, AGGAGGCCACAGTGAACCGA<br>hmrNFATc4exXas, TTCAGGCAGGAGGCTCTTCTC |
| ***NFATc4-IXL/*** 816 bp | hNFATc4exVIIs, AGGAGGCCACAGTGAACCGA<br>hmrNFATc4exXas, TTCAGGCAGGAGGCTCTTCTC |
| ***NFATc4-IXi/*** 1575 bp | hNFATc4exVIIs, AGGAGGCCACAGTGAACCGA<br>hmrNFATc4exXas, TTCAGGCAGGAGGCTCTTCTC |
| **Mouse *NFATc4*** | |
| ***NFATc4-VI/*** 277 bp | mrNFATc4exVI5'UTRs, CACTCAGCCCAGCCAGGCTTC<br>hmNFATc4exVIIIas, CTTGTTGCTGTACTCGGGGA |
| ***NFATc4-VIi/*** 1332 bp | mrNFATc4exVI5'UTRs, CACTCAGCCCAGCCAGGCTTC<br>hmNFATc4exVIIIas, CTTGTTGCTGTACTCGGGGA |
| ***NFATc4-IXL/*** 831 bp | mNFATc4exVIIs, GAAAACTGCAGTGGGAGGAAGAGG<br>hmrNFATc4exXas, TTCAGGCAGGAGGCTCTTCTC |
| ***NFATc4-IXi/*** 1583 bp | mNFATc4exVIIs, GAAAACTGCAGTGGGAGGAAGAGG<br>hmrNFATc4exXas, TTCAGGCAGGAGGCTCTTCTC |
| **Human *GAPDH*** | |
| ***GAPDH/*** 261 bp | GAPDHs, TCCCCACTGCCAACGTGTCAGTG<br>GAPDHas, ACCCTGTTGCTGTAGCCAAATTCG |
| **Mouse *HPRT*** | |
| ***HPRT/*** 331 bp | hmrHPRTs, GATGATGAACCAGGTTATGAC<br>hmrHPRTas, GTCCTTTTCACCAGCAAGCTTG |

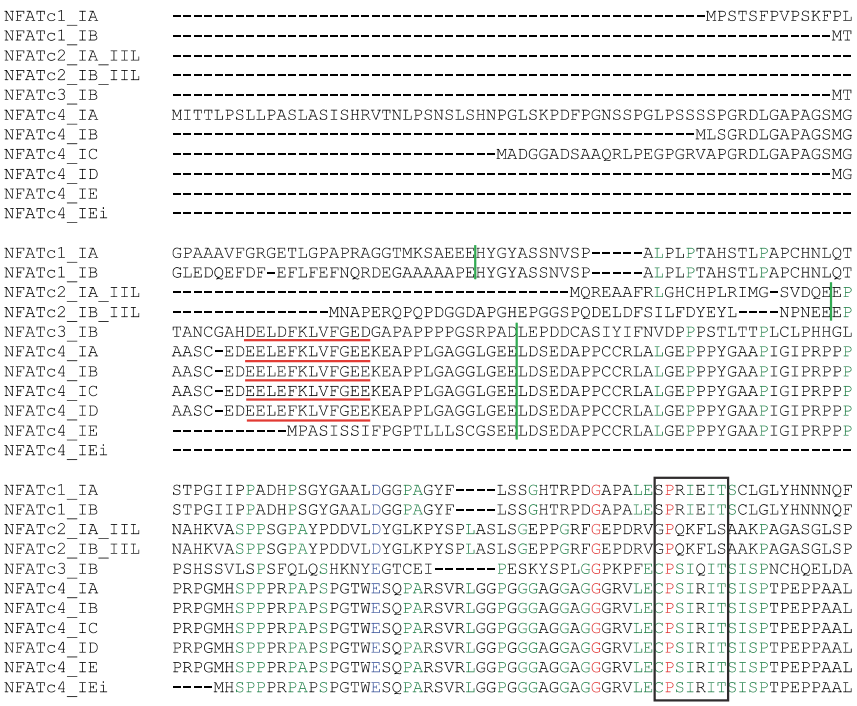
**2. Primers used for mouse *NFAT* riboprobe generation:**

| Mouse gene Amplicon size | Primers |
|---|---|
| ***NFATc1/*** 519 bp | insitu_mNFATc1exIVs, GCTACAGCTGTTCATTGGG<br>insitu_mNFATc1exVIIas, GTCTTTGCTTCCATCTCCCAG |
| ***NFATc2/*** 524 bp | insitu_mNFATc2exIIIs, CGCTGTCAAAGCCCCAACAGGA<br>insitu_mNFATc2exVIas, CCTTGGACTCCGCTGTGAAG |
| ***NFATc3/*** 436 bp | insitu_mNFATc3exIIs, CCCTGCACCGTTTCCATTTCAGTAC<br>insitu_mNFATc3exIIIas, CTTCACAACAGGATGGCCACCAGT |
| ***NFATc4/*** 831 bp | mNFATc4exVIIs, GAAAACTGCAGTGGGAGGAAGAGG<br>hmrNFATc4exXas, TTCAGGCAGGAGGCTCTTCTC |

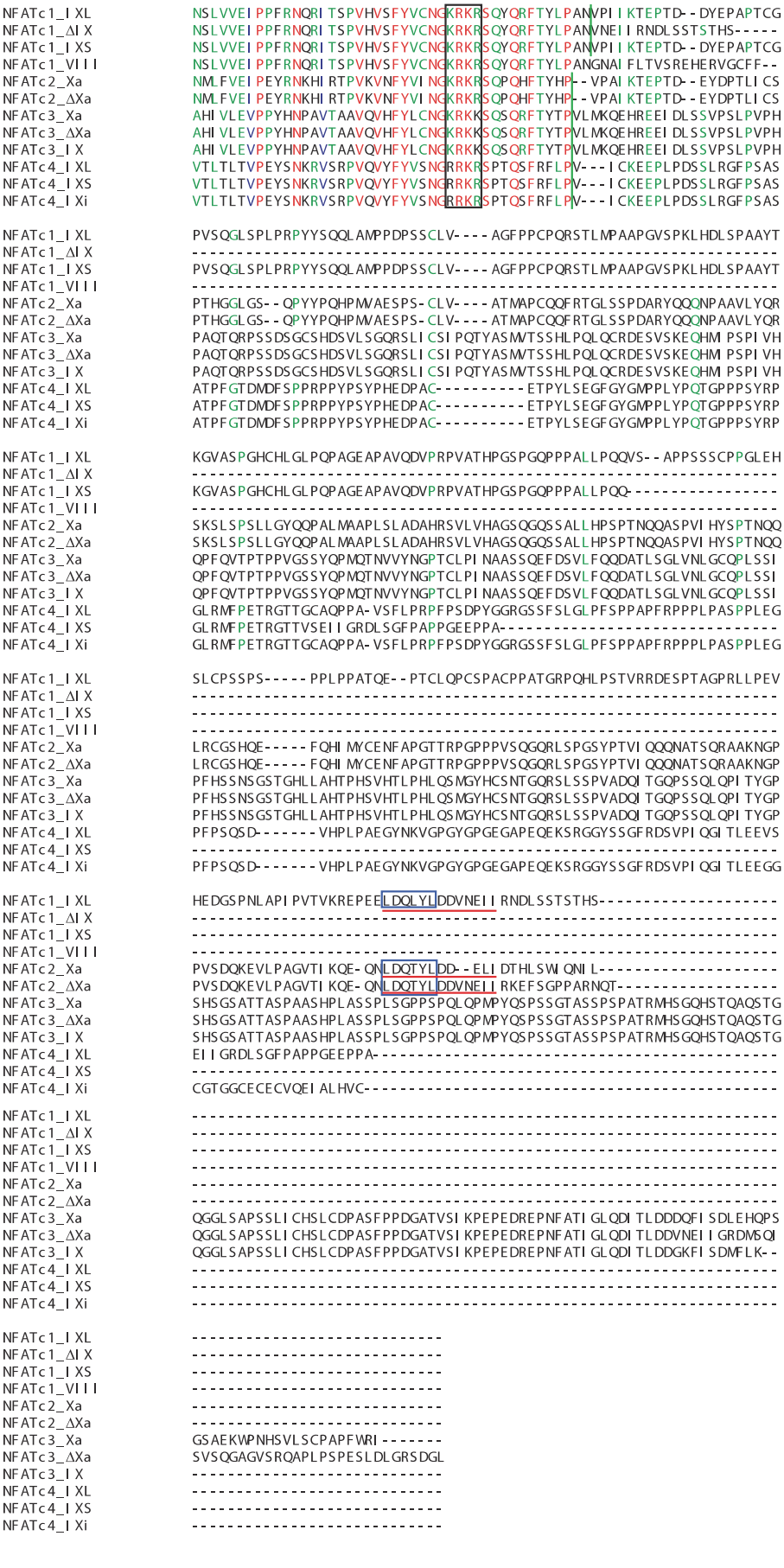
**3. Primers used for human *NFAT* riboprobe generation:**

| Human gene<br>Amplicon size | Primers |
|---|---|
| ***NFATc1/***<br>2479 bp | hNFATc1ex1A_ATGs, CACCATGCCAAGCACCAGCTTTCC<br>hNFATc1ex9S_woSTOPas, CTGCTGTGGCAGCAGGGCCGG |
| ***NFATc2/***<br>2715 bp | hNFATc2ex1A_ATGs, CACCATGCAGAGAGAGGCTGCGTTCAG<br>hNFATc2ex10_wSTOPas, GGCTTCTTTTACGTCTGATTTCTGGC |
| ***NFATc3/***<br>1698 bp | hNFATc3ex4_ATGs, CACCATGTTTATTGGGACAGCAGATGATCG<br>hNFATc3ex9_wSTOPas, GTCCCTGAATCTTCACTACTTC |
| ***NFATc4/***<br>1311 bp | hNFATc4ex4_ATGs, CACCATGTTCATCGGCACTGCAG<br>hNFATc4ex10_wSTOPas, CAGTTCACGTGGTTCAGGCAGGAGG |

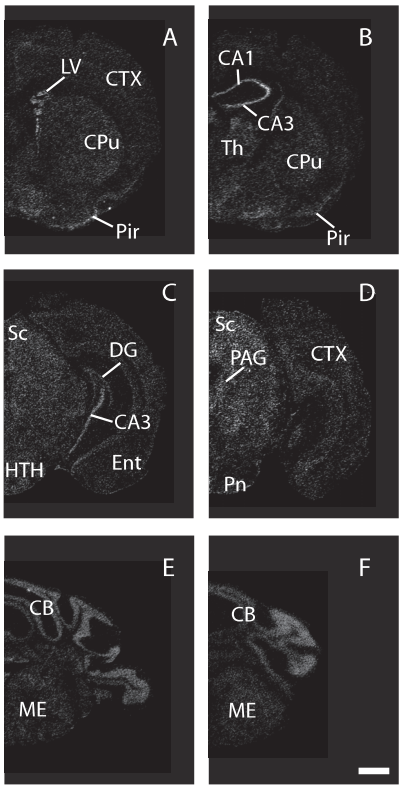
Supplementary Fig. 1



Supplementary Fig. 2



Supplementary Fig. 3



**Supplementary Fig.1.** Aligment of the human N-terminal regions of the NFAT protein isoforms translated from alternative splice variants compiled with MULTALIN (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_multalin.html). Red characters indicate identical amino acids and green characters indicate amino acids conserved 50% - 90 % among all the sequences aligned. Blue characters indicate similar amino acids between all the sequences aligned. The conserved N-terminal calcineurin binding site in the regulatory domain is highlighted with a black box. Stretch of 12 aa conservation is indicated with a red underline. Vertical green lines denote the start of exon II encoded sequences.

**Supplementary Fig.2**. Aligment of the human C-terminal regions of the NFAT protein isoforms translated from alternative splice variants compiled with MULTALIN (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_multalin.html). Red characters indicate identical amino acids and green characters indicate amino acids conserved 50% - 90 % among all the sequences aligned. Blue characters indicate similar amino acids between all the sequences aligned. The conserved C-terminal nuclear localisation signal (NLS) is highlighted with a black box. Conserved stretch of 13 amino acids containing a nuclear export signal (NES, blue box) is indicated with a red underline. Vertical green lines denote the end of exon VIII encoded sequences.

**Supplementary Fig.3.** *In situ* hybridization analysis of *NFATc2* mRNA expression in adult mouse brain. Dark-field emulsion autoradiographs from coronal sections at the level of striatum (A), thalamus (B), hippocampus (C and D) and cerebellum (E and F). LV, lateral ventricle; CPu, caudate putamen; CTX, cortex; Pir, piriform cortex; Th, thalamus; CA1, field CA1 of hippocampus; CA3, field CA3 of hippocampus; Sc, superior colliculi; Ent, entorhinal cortex; HTH, hypothalamus; PAG, periaqueductal gray; Pn, pontine nuclei; DG, dentate gyrus; CB, cerebellum; ME, medulla. Scale bar is 1 mm.

**PUBLICATION II**

**Vihma H**, Luhakooder M. Pruunsild P, Timmusk T
Regulation of different human NFAT isoforms by neuronal activity.
J Neurochem. 2016;137(3):394-408. doi: 10.1111/jnc.13568.

Journal of Neurochemistry

JOURNAL OF NEUROCHEMISTRY | 2016 | 137 | 394–408 doi: 10.1111/jnc.13568

ORIGINAL ARTICLE

# Regulation of different human NFAT isoforms by neuronal activity

Hanna Vihma, Mirjam Luhakooder, Priit Pruunsild[1] and Tõnis Timmusk

*Department of Gene Technology, Tallinn University of Technology, Tallinn, Estonia*

**Abstract**

Nuclear factor of activated T-cells (NFAT) is a family of transcription factors comprising four calcium-regulated members: NFATc1, NFATc2, NFATc3, and NFATc4. Upon activation by the calcium-dependent phosphatase calcineurin (CaN), NFATs translocate from cytosol to the nucleus and regulate their target genes, which in the nervous system are involved in axon growth, synaptic plasticity, and neuronal survival. We have shown previously that there are a number of different splice variants of *NFAT* genes expressed in the brain. Here, we studied the subcellular localizations and transactivation capacities of alternative human NFAT isoforms in rat primary cortical or hippocampal neurons in response to membrane depolarization and compared the induced transactivation levels in neurons to those obtained from HEK293 cells in response to calcium signaling. We confirm that in neurons the translocation to the nucleus of all NFAT isoforms is reliant on the activity of CaN. However, our results suggest that both the regulation of subcellular localization and transcriptional activity of NFAT proteins in neurons is isoform specific. We show that in primary hippocampal neurons NFATc2 isoforms have very fast translocation kinetics, whereas NFATc4 isoforms translocate relatively slowly to the nucleus. Moreover, we demonstrate that the strongest transcriptional activators in HEK293 cells are NFATc1 and NFATc3, but in neurons NFATc3 and NFATc4 lead to the highest induction, and NFATc2 and NFATc1 display isoform-specific transcription activation capacities. Altogether, our results indicate that the effects of calcium signaling on the action of NFAT proteins are isoform-specific and can differ between cell types.

**Keywords:** calcium-regulated transcription factor, neuronal activity, neurons, NFAT, subcellular localization, transactivation.

*J. Neurochem.* (2016) **137**, 394–408.

Activity-dependent transcription factors are important components of signaling pathways underlying neuronal adaptation. Neuronal activity regulates the action of these proteins by altering their expression, subcellular localization, and/or function (reviewed in West and Greenberg 2011). Four members of the nuclear factor of activated T-cells (NFAT) family, NFATc1, NFATc2, NFATc3, and NFATc4, are activity-dependent transcription factors that translocate from the cytosol to the nucleus following dephosphorylation by the calcium/calmodulin-dependent protein phosphatase calcineurin (CaN). Once in the nucleus, NFAT proteins regulate the expression of their target genes, which in turn affect a wide range of cellular processes (reviewed in Crabtree and Olson 2002 and Graef *et al.* 2001). In the nervous system, NFAT-dependent gene regulation has been shown to play a role in neuronal excitability (Oliveria *et al.* 2007; Zhang and Shapiro 2012; Dittmer *et al.* 2014; Murphy *et al.* 2014), axonal growth (Graef *et al.* 2003; Yoshida and Mishina 2005; Nguyen *et al.* 2009), neuronal survival (Benedito *et al.* 2005; Vashishta *et al.* 2009), as well as apoptosis (Jayanthi *et al.* 2005; Luoma and Zirpel 2008; Gómez-Sintes and Lucas 2010; Mojsa *et al.* 2015), synaptic plasticity and memory formation (Groth and Mermelstein 2003; de la Fuente *et al.* 2011; Quadrato *et al.* 2012), neuronal development (Arron *et al.* 2006; Schwartz *et al.* 2009;

Received October 19, 2015; revised manuscript received January 4, 2016; accepted January 29, 2016.

Address correspondence and reprint requests to Hanna Vihma and Tõnis Timmusk, Department of Gene Technology, Tallinn University of Technology, Akadeemia tee 15, 12618 Tallinn, Estonia. E-mails: hanna.vihma@ttu.ee, tonis.timmusk@ttu.ee

[1]Present address: Department of Neurobiology, Interdisciplinary Centre for Neurosciences, University of Heidelberg, INF 364, 69120 Heidelberg, Germany.

*Abbreviations used*: AP-1, activator protein 1; CaN, calcineurin; CMV, cytomegalovirus; CRE, cAMP/calcium-response element; CsA, cyclosporine A; DAPI, 4′,6-diamidino-2-phenylindole; EF1α, elongation factor-1 alpha; EGFP, enhanced green fluorescent protein; GSK3β, glycogen synthase kinase 3 beta; IL-2, interleukin 2; KCl, potassium chloride; NFAT, nuclear factor of activated T-cells; NHR, NFAT homology domain; NLS, nuclear localization signal; PdBu, phorbol dibutyrate; PKC, protein kinase C; PMA, phorbol myristate acetate; RHR, Rel homology domain; SP, serine–proline; SRR, serine-rich region; TAD, transactivation domain; TTX, tetrodotoxin.





Serrano-Pérez *et al.* 2015), and nociception (Cai *et al.* 2013; reviewed in Smith 2009).

Despite having different C- and N-terminal sequences, all NFAT proteins contain two conserved regions: a Rel homology domain in the C-terminus and a regulatory NFAT homology domain in the N-terminus. The Rel homology domain of NFAT proteins is responsible for DNA binding and for interactions with NFAT transcriptional partner proteins (reviewed in Macian 2005). The NFAT homology domain contains two CaN-binding sites (Aramburu *et al.* 1998; Park *et al.* 2000), an extended serine-rich region, three serine–proline-rich motifs, and a conserved nuclear localization signal (NLS) (reviewed in Kiani *et al.* 2000). In resting cells the NLS is masked by highly phosphorylated serine-rich region and serine–proline motifs. Upon dephosphorylation by CaN, the NLS is exposed resulting in NFAT translocation to the nucleus (reviewed in Rao *et al.* 1997). Dephosphorylation of NFAT proteins by CaN is opposed by several kinases that either act in the nucleus by rephosphorylating NFATs, leading to NFAT nuclear export and therefore termination of NFAT-dependent transcription, or act in the cytosol to maintain NFAT phosphorylation and cytosolic localization (reviewed in Hogan *et al.* 2003).

Regulation of NFATc3 and NFATc4 subcellular localization in neurons has been studied by several groups. Activation of L-type calcium channels by high extracellular $K^+$ has been shown to result in NFATc3 and NFATc4 nuclear translocation in hippocampal and dorsal root ganglion neurons (Graef *et al.* 1999; Oliveria *et al.* 2007; Ulrich *et al.* 2012; Murphy *et al.* 2014). Brain-derived neurotrophic factor application has been shown to trigger rapid nuclear translocation of NFATc4 in hippocampal neurons (Groth and Mermelstein 2003) and in cortical neurons (Graef *et al.* 2003). D1 dopamine receptor signaling has been demonstrated to trigger nuclear translocation of NFATc4 in striatal neurons (Groth *et al.* 2008) and *N*-methyl-D-aspartate treatment has been shown to induce nuclear translocation of both NFATc3 and NFATc4 in cortical neurons (Vashishta *et al.* 2009). Furthermore, it has been shown that in a transgenic mouse model of Parkinson's disease increased cytosolic calcium concentration and CaN activity leads to significantly increased nuclear distribution of NFATc3 in midbrain dopaminergic neurons (Luo *et al.* 2014), and that in human α-synucleinopathy patients' brains nuclear staining of NFATc4 is increased compared to non-diseased controls (Caraveo *et al.* 2014). However, all NFATs, not only NFATc3 and NFATc4, are expressed in the nervous system (Ho *et al.* 1994; Plyte *et al.* 2001; Asai *et al.* 2004; Jayanthi *et al.* 2005; Vihma *et al.* 2008; Abdul *et al.* 2009). And moreover, we have previously shown that for human *NFAT* genes alternative splicing in combination with the usage of alternative 5′ and 3′ exons could theoretically give rise to 44 different NFAT protein isoforms: 8 for NFATc1, 6 for both NFATc2 and NFATc3, and 24 for NFATc4 (Vihma *et al.* 2008). How the specific NFAT alternative isoforms are regulated by neuronal activity remains unclear. Here, based on mRNA expression analysis of different transcripts in various human tissues and brain regions (Vihma *et al.* 2008), we have chosen 18 NFAT protein isoforms predominantly expressed in the brain for further analysis (Fig. 1). We have investigated how the subcellular localizations and the transactivation capacities of NFAT family alternative isoforms are regulated by membrane depolarization in rat cultured primary cortical and hippocampal neurons. In addition, we have analyzed transactivation capacities of different NFAT isoforms in depolarized neurons and in HEK293 cells treated with the calcium ionophore ionomycin in combination with the protein kinase C (PKC)-activating phorbol dibutyrate (PdBu).

## Materials and methods

### Plasmid constructs

Primers used to generate full open-reading frame PCR amplicons were as follows. For NFATc1 isoforms NFATc1-IA-IXL, NFATc1-IA-IXS, NFATc1-IA-ΔIX, and NFATc1-IA-VIII, forward primer (5′-ACCATGCCAAGCACCAGCTTTCC-3′) and reverse primer (5′-CGTATTATTTCATTTACTGCTGTGG-3′), and for isoforms NFATc1-IB-IXL, NFATc1-IB-IXS, NFATc1-IB-ΔIX, and NFATc1-IB-VIII, forward primer (5′-CACCATGACGGGGCTG GAGGACCAGGAG-3′) and reverse primer (5′-CGTATTATTT CATTTACTGCTGTGG-3′). For NFATc2 isoforms NFTAc2-IA-Xa and NFATc2-IA-ΔXa, forward primer (5′-CACCATGCAGA GAGAGGCTGCGTTCAG-3′) and reverse primer (5′-GGCTT CTTTTACGTCTGATTTCTGGC-3′), and for isoforms NFATc2-IB-Xa and NFATc2-IB-ΔXa, forward primer (5′-CACCATGAA CGCCCCCGAGCGGC-3′) and reverse primer (5′-GGCTTC TTTTACGTCTGATTTCTGGC-3′). For NFATc3 isoforms NFATc3-IB-IX, NFATc3-IB-ΔXa, and NFATc3-IB-Xa, forward primer (5′-CACCATGACTACTGCAAACTGTGG-3′) and reverse primers (5′-GTCCCTGAATCTTCACTACTTC-3′), (5′-GTTAGA GCCCATCAGATCTTCC-3′), and (5′-CTCGTTCACCTCTAGAT TCTCCAG-3′), respectively. For NFATc4 isoforms NFATc4-ID-IXS, NFATc4-ID-IXL, and NFATc4-ID-IXi, forward primer (5′-CACCATGGGGGCGGCCAGCTGCGAG-3′) and reverse primer (5′-CAGTTCACGTGGTTCAGGCAGGAGG-3′). cDNAs used as template for NFATc1, NFATc2, NFATc3, and NFATc4 PCR were generated with oligo(dT) primer from human muscle, thymus, cerebellum, and heart RNA (Ambion, Austin, TX, USA) and selected based on the expression analyses conducted in our lab beforehand (Vihma *et al.* 2008). PCR reactions were performed using Expand High-Fidelity PCR Enzyme Mix (Roche Diagnostics, Risch-Rotkreuz, Switzerland) according to manufacturer's instructions. 3′ end A-overhangs were synthesized using FirePol Taq polymerase (Solis BioDyne, Tartu, Estonia) prior cloning amplified products into pTZ57R/T vector of InsTAclone PCR cloning kit (ThermoFisherScientific, Waltham, MA, USA). Cloned full-length coding regions of NFAT isoforms were excised from pTZ57R/T vector and subcloned into pEGFP-C1, -C2, or -C3 mammalian

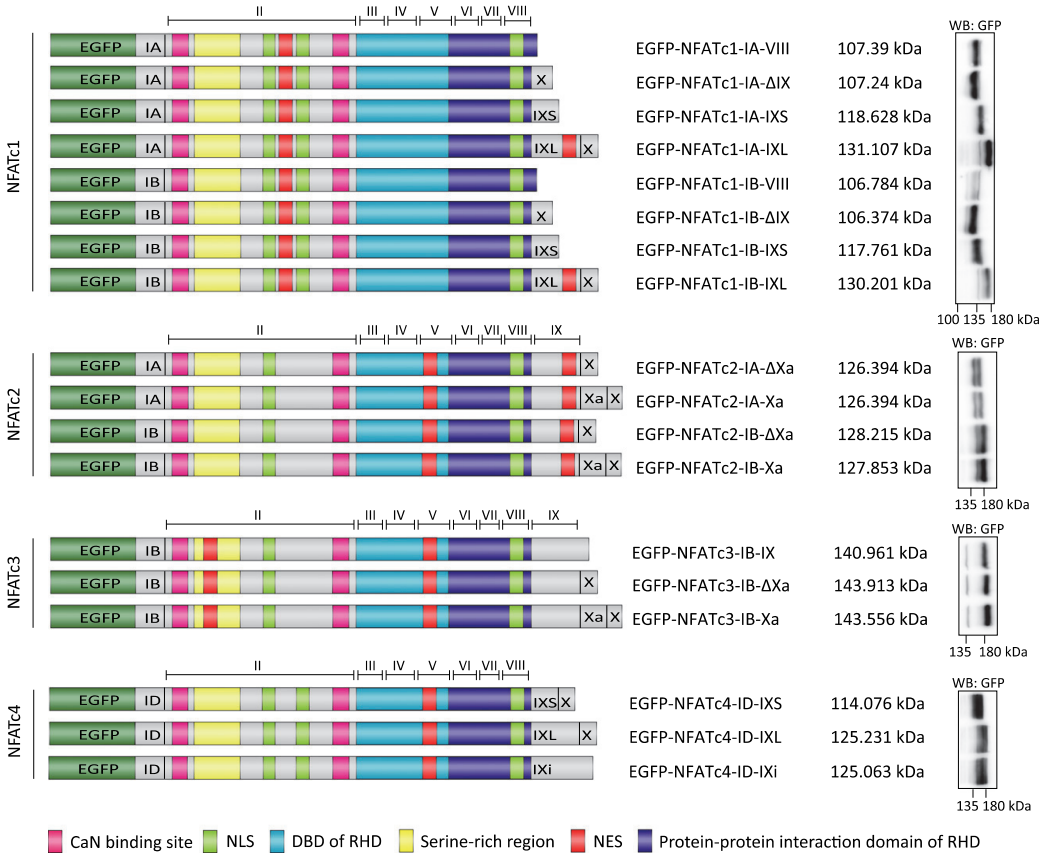



**Fig. 1** EGFP-tagged nuclear factor of activated T-cells (NFAT) isoforms used in this study. Shown are the schematic representations of each of the EGFP-tagged NFATc1, NFATc2, NFATc3, and NFATc4 isoform analyzed, calculated molecular weights in kilodaltons, and western blot analysis of the respective isoforms over-expressed in HEK293 cells. The names of the isoforms are according to Vihma *et al.* 2008. EGFP, enhanced green fluorescent protein; kDA, kilodalton; WB, western blot; CaN, calcineurin; NLS, nuclear localization signal; DBD, DNA-binding domain; RHD, Rel homology domain; NES, nuclear export signal.

expression vector (Clontech Laboratories, Mountain View, CA, USA). All constructs were confirmed by sequencing.

### Cell culture

Human embryonic kidney HEK293 cells (ATCC, Manassas, VA, USA) were grown in Eagle's minimum essential medium (PAA Laboratories, Dartmouth, MA, USA) supplemented with 10% fetal bovine serum (PAA Laboratories), 100 U/mL penicillin (PAA Laboratories), and 0.1 mg/mL streptomycin (PAA Laboratories) at 37°C in 5% CO2. Rat primary hippocampal and cortical neuronal cultures were prepared from E21 embryo brains (Sprague–Dawley, Scanbur BK AB, Sollentuna, Sweden). All animal procedures were performed in agreement with the local ethics committee. The dissected cortices or hippocampi were dissociated with 0.25% trypsin (Gibco, Rockville, MD, USA), treated with 0.05% DNase I (Roche Molecular Biochemicals, Indianapolis, IN, USA), and the cells were plated at density of 100 000–200 000 cells per well of a 48-well plate and grown on poly-L-lysine-coated dishes in Neurobasal-A medium (Gibco) with B27 supplement (Gibco), penicillin (100 U/mL; PAA), streptomycin (0.1 mg/mL; PAA), and L-glutamine (1 mM; PAA), respectively. Mitotic inhibitor 5-fluoro-2′-deoxyuridine (Sigma-Aldrich, St. Louis, MO, USA) was added to the medium (final concentration 10 μM) at 2 days *in vitro* (DIV).

### SDS–PAGE and western blotting

HEK293 cells were grown on a 6-well plate. Cell confluency at transfection was 60–70%. For transfection, 3 μg of DNA and 9 μL of LipoD293 reagent (SignaGen Laboratories, Gaithersburg, MD, USA) were used per well. Thirty-six hours after transfection cells were collected and lysed on ice using radioimmunoprecipitation

assay buffer [50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate, 0.2% SDS, and protease inhibitor cocktail Complete (Roche Applied Science, Penzberg, Upper Bavaria, Germany)]. The extracts were sonicated and centrifuged to remove insoluble cell debris. Protein concentrations were determined using bicinchoninic acid assay (Pierce, Rockford, IL, USA). Equal amounts of proteins were separated in 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to polyvinylidene fluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA) using Hoefer Semiphor (Amersham Biosciences, Amersham, UK) device. Membranes were blocked in 5% skim milk in 0.05% Tween 20 in phosphate-buffered saline (PBS). For western blotting, the antibodies were diluted in 2% skim milk in 0.05% Tween 20 in PBS. As the primary antibody, rabbit anti-EGFP (1 : 200 000, gift from professor Andres Merits) was used and, as the secondary antibody, horseradish peroxidase-conjugated secondary antibody (1 : 5000; Pierce) was used. Chemoluminescence reaction was performed using SuperSignal West Femto Chemiluminescent Substrate (Pierce). Chemoluminescence was detected using ImageGuant 400 imaging system (GE Healthcare, Little Chalfont, UK).

### Luciferase assay

For transfection of HEK293 cells, 0.375 μg of DNA and 0.75 μL of LipoD293 reagent (SignaGen Laboratories) were used per well of a 48-well plate. Cell confluency at transfection was 60–70%. Neuronal cultures were transfected at 6 DIV, using 0.5 μg of DNA and 1 μL of Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) per well of a 48-well plate. Cells were cotransfected with equal amounts of human *NFATc1*, *NFATc2*, *NFATc3* or *NFATc4* construct or empty vector, firefly luciferase construct carrying three tandem NFAT-response elements (NFAT-RE) in front of minimal promoter, pGL4.30[luc2P/NFAT-RE/Hygro] (Promega, Madison, WI, USA), and *Renilla* luciferase reporter vector pRL-TK (Promega) for normalization. Negative control of reporter assay was conducted using non-inducible firefly luciferase construct pGL4.29[luc2P/Hygro], generated by removal of cAMP/calcium-response element-binding site from reporter vector pGL4.29[luc2P/CRE/Hygro] (Promega). To rule out any influence of the EGFP tag, luciferase assay was also conducted with representative *NFAT* constructs without the EGFP tag. Two days post-transfection cultured rat primary cortical and hippocampal neurons were stimulated with 25 mM KCl and HEK293 cells were stimulated with 1 μM ionomycin (AppliChem, Darmstadt, Germany) and 1 μM PdBu (phorbol 12,13-dibutyrate; Sigma-Aldrich), respectively, for the time indicated. Cell lysates were prepared using Passive Lysis Buffer (Promega). Luciferase assays were performed using Dual-Glo Luciferase Assay System (Promega) and GENios pro (Tecan Group Ltd, Männedorf, Zürich, Switzerland) plate reader. Three independent experiments were performed in duplicates, all data were log transformed and autoscaled. Means and standard deviations were calculated. Using GraphPad Prism 6 Software (GraphPad Software Inc., San Diego, CA, USA), one-way ANOVA with Holm–Sidak pairwise comparisons were used for each time point to determine statistical significance between luciferase activities in cells transfected with the respective NFAT construct and the EGFP plasmid (Figs 4a–c, 5a–d, and Figure S2a and b). For the data representing transactivation at basal level, one-way ANOVA with Holm–Sidak *post hoc* test was used separately within data obtained from each cell type (Fig. 4d) or within data obtained with the constructs of each NFAT family (Fig. 5e). The data were back transformed to the original scale. Error bars represent upper and lower limits back transformed as mean ± standard deviation.

### Intracellular localization assay

Rat primary hippocampal or cortical neurons grown on glass cover slips were transfected at 6 DIV with plasmids encoding one of the different EGFP-tagged NFATc1, NFATc2, NFATc3, or NFATc4 isoforms, respectively, using 0.5 μg of DNA and 1 μL of Lipofectamine 2000 reagent (Invitrogen) per well of a 48-well plate. The next day, calcium-dependent cellular translocation of expressed isoforms was induced by depolarizing the membranes with 25 mM KCl. An additional cyclosporine A (5 μM; Tocris Cookson, Ballwin, MO, USA) one-hour pre-treatment followed by a three-time wash with Neurobasal-A (Gibco) was conducted before depolarizing the membranes with 25 mM KCl where indicated. For inhibiting spontaneous activity of transfected cortical neurons, overnight pre-treatment with tetrodotoxin (1 μM; Tocris) was applied. Cells were fixed with 4% paraformaldehyde, neutralized with 50 mM $NH_4Cl$ in PBS, and permeabelized in 0.5% Triton X-100 in PBS. Cells were mounted with 4′,6-diamidino-2-phenylindole containing mounting medium. EGFP localization in cells was determined with 63× magnification using confocal microscope LSM 510 (Carl Zeiss AG, Jena, Germany) (Figure S1). Up to 500 transfected cells per sample were counted and the subcellular distribution of each isoform was assessed in a single-cell basis and scored as cytoplasmic if EGFP signal localized predominantly in the cytoplasm or scored as nuclear if the EGFP signal localized predominantly in the nucleus. The percentage of cells with EGFP signal present in the nucleus was calculated. Three independent experiments were performed in duplicates. All data were arcsine transformed and means and standard deviations were calculated. Using GraphPad Prism 6 Software, analysis of statistical significance within data obtained with the indicated NFAT construct was determined by one-way ANOVA with Holm–Sidak pairwise comparisons between each treatment and untreated control, and between 6-hour depolarization with and without cyclosporine A (CsA) pre-treatment. The data were back transformed to the original scale.

## Results

### Regulation of the subcellular localization of over-expressed human NFAT isoforms by calcium signaling in rat primary cortical and hippocampal neurons

NFAT isoforms differ in their C- and N-terminal sequences (Vihma *et al.* 2008; Fig. 1). For a better understanding of how these differences contribute to the regulation of alternative human NFAT isoforms we first analyzed the localization of NFAT family proteins in neurons. For this, we transfected rat primary cortical and hippocampal neurons with plasmid constructs encoding different NFAT isoforms fused with the EGFP tag (Fig. 1). For activating calcium signaling, we used depolarization of neurons with high extracellular $K^+$ that induces calcium influx through voltage-gated L-type calcium channels. We analyzed the localization of the different C-terminally EGFP-tagged NFAT isoforms

by confocal fluorescence microscopy. Our transfections included plasmids encoding eight NFATc1 isoforms: NFATc1-IA-VIII, NFATc1-IA-ΔIX, NFATc1-IA-IXS, NFATc1-IA-IXL, NFATc1-IB-VIII, NFATc1-IB-ΔIX, NFATc1-IB-IXS, and NFATc1-IB-IXL; four NFATc2 isoforms: NFATc2-IA-IIL-ΔXa, NFATc2-IA-IIL-Xa, NFATc2-IB-IIL-ΔXa, and NFATc2-IB-IIL-Xa (hereafter referred to as NFATc2-IA-ΔXa, NFATc2-IA-Xa, NFATc2-IB-ΔXa, and NFATc2-IB-Xa, respectively); three NFATc3 isoforms: NFTAc3-IB-IX, NFATc3-IB-ΔXa, and NFATc3-IB-Xa; and three NFATc4 isoforms: NFATc4-ID-IXS, NFATc4-ID-IXL, and NFATc4-ID-IXi (names according to Vihma *et al.* 2008; Fig. 1).

In unstimulated cortical neurons the analyzed N-terminally different NFATc1 isoforms showed clear localization differences. The results showed that the majority of unstimulated cortical neurons displayed nuclear localization of all four NFATc1-IA isoforms analyzed with approximately 80% of transfected cells having nuclear EGFP signal (Fig. 2a). In contrast, all four NFATc1-IB isoforms were located in the cytoplasm where the percentage of transfected cells with nuclear EGFP ranged from 8% for NFATc1-IB-IXL to 17% for NFATc1-IB-ΔIX (Fig. 2c). From all NFATc1-IA isoforms, only NFATc1-IA-VIII had a trend toward being even more nuclear upon depolarization. After 6 h of membrane depolarization the EGFP signal was nuclear in 95% of NFATc1-IA-VIII-expressing cells ($p = 0.436$ vs. untreated) (Fig. 2a). For all four NFATc1-IB isoforms, membrane depolarization of cortical neurons resulted in nuclear localization. After 6 h of membrane depolarization, the percentage of transfected cells with nuclear EGFP ranged from 72% for NFATc1-IB-IXL ($p < 0.01$ vs. untreated) to 79% for NFATc1-IB-IXS ($p < 0.01$ vs. untreated) (Fig. 2c). In hippocampal neurons the results were different. All eight NFATc1 isoforms analyzed were predominantly localized in the cytoplasm in unstimulated cells. The percentage of transfected cells with nuclear EGFP ranged from 15% for NFATc1-IB-VIII to 38% for NFATc1-IA-ΔIX (Fig. 2b and d). Upon depolarization all eight NFATc1 isoforms showed a change toward nuclear localization (Fig. 2b and d). After 6 h of membrane depolarization, the most nuclear was NFATc1-IA-IXL and the least nuclear was NFATc1-IB-ΔIX with 94% ($p < 0.01$ vs. untreated) and 76% ($p < 0.01$ vs. untreated) of transfected cells having nuclear EGFP signal, respectively (Fig. 2b and d).

In unstimulated cortical neurons, similarly to NFATc1 isoforms, the analyzed N-terminally different NFATc2 isoforms showed clear localization differences. Over 90% of the NFATc2-IA-ΔXa- and NFATc2-IA-Xa-expressing cells had the EGFP signal predominantly in the nucleus of transfected cells. In contrast, isoforms containing the N-terminus encoded by exon IB were located in the cytoplasm – nuclear EGFP signal was detected in 22% and 25% of NFATc2-IB-ΔXa- and NFATc2-IB-Xa-transfected cells, respectively (Fig. 2e). Membrane depolarization of cortical neurons resulted in nuclear localization of all NFATc2 isoforms (Fig. 2e). In hippocampal neurons, by contrast, all four NFATc2 isoforms were located in the cytoplasm in unstimulated cells. The percentage of transfected cells with nuclear EGFP ranged from 11% for NFATc2-IB-Xa to 22% for NFATc2-IA-Xa (Fig. 2f). Fifteen minutes of membrane depolarization induced a rapid change in the nuclear localization of all four NFATc2 isoforms ($p < 0.01$ vs. untreated) (Fig. 2f). Six-hour depolarization resulted in over 98% of transfected cells with nuclear EGFP signal for all NFATc2 isoforms ($p < 0.01$ vs. untreated) (Fig. 2f).

For NFATc3, we observed variable distribution among different isoforms in unstimulated cortical neurons. 20%, 43%, and 53% of NFATc3-IB-Xa-, NFATc3-IB-ΔXa-, and NFTAc3-IB-IX-transfected cells, respectively, displayed nuclear EGFP localization (Fig. 2g). Depolarization resulted in a gradual increase in the percentage of cells with nuclear EGFP localization for all NFATc3 isoforms (Fig. 2g). In hippocampal neurons all three NFATc3 isoforms displayed predominantly cytoplasmic localization in unstimulated cells. The percentage of transfected cells with nuclear EGFP ranged from 20% to 27% for NFATc3-IB-ΔXa and for NFATc3-IB-IX, respectively (Fig. 2h). Similarly to cortical neurons, membrane depolarization of hippocampal neurons resulted in an increase in the percentage of cells with nuclear EGFP localization reaching 97% ($p < 0.01$ vs. untreated) for NFATc3-IB-ΔXa and 87% ($p = 0.018$ vs. untreated) for NFATc3-IB-Xa after one-hour depolarization (Fig. 2h).

Variability in subcellular localization of different NFATc4 isoforms was observed in unstimulated cortical neurons. For NFATc4-ID-IXi, NFATc4-ID-IXL, and NFATc4-ID-IXS, the percentage of transfected cells with nuclear localization was 82%, 72%, and 48%, respectively (Fig. 2i). Membrane depolarization did not increase the percentage of transfected cells with nuclear localization of any of the NFATc4 isoforms analyzed (Fig. 2g). Contrary to cortical neurons, in hippocampal neurons all three NFATc4 isoforms analyzed had similar predominantly cytoplasmic localization in unstimulated cells with nuclear EGFP signal in 17—27% of transfected cells (Fig. 2j). After 6 h of depolarization, the percentage of transfected cells with nuclear EGFP reached approximately 65% for all the three NFATc4 isoforms, with the increase being most significant for isoform NFATc4-ID-IXL ($p < 0.01$ vs. untreated) (Fig. 2j).

Next, we studied whether the regulation of NFAT protein localization in neurons by calcium signaling is CaN dependent. For that we used one-hour pre-treatment with CsA, an immunosuppressive drug that binds to the intracellular protein cyclophilin A and thereby inhibits the phosphatase CaN. Significant decrease in nuclear localization of EGFP after CsA pre-treatment was detected with all of the NFATc1-

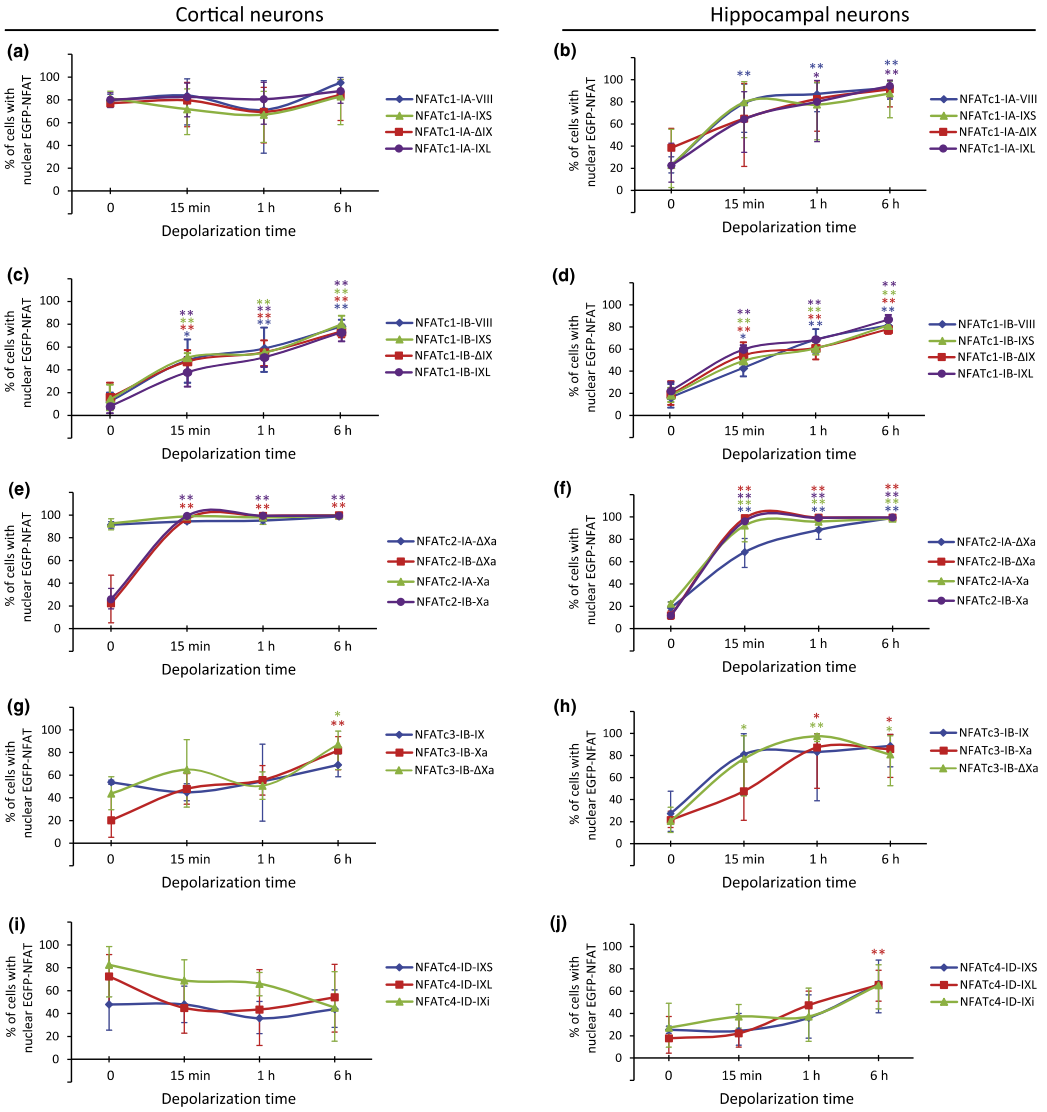



**Fig. 2** Temporal regulation of subcellular localization of EGFP-tagged human NFAT isoforms by depolarization in rat cultured primary neurons. Proportions of transfected cells showing nuclear localization of different EGFP-tagged NFATc1 (a, b, c, and d), NFATc2 (e and f), NFATc3 (g and h), and NFATc4 (i and j) isoforms in rat primary cortical (a, c, e, g, and i) and hippocampal (b, d, f, h and j) neurons. Neurons were either untreated or depolarized with the addition of 25 mM KCl for the time indicated. All data were arcsine transformed and are presented as mean values obtained from three independent experiments. Error bars represent standard deviations. Statistical significance shown with asterisks is relative to results from untreated cells (ANOVA with Holm–Sidak's *post hoc* test $*p < 0.05$; $**p < 0.01$). EGFP, enhanced green fluorescent protein.

IA isoforms analyzed in cortical neurons (Fig. 3a). For all NFATc1-IB isoforms CsA pre-treatment of cortical neurons resulted in cytoplasmic retention (Fig. 3c). CsA pre-treatment of hippocampal neurons resulted in cytoplasmic retention of all NFATc1 isoforms analyzed after 6 h of depolarization (Fig. 3b and d).

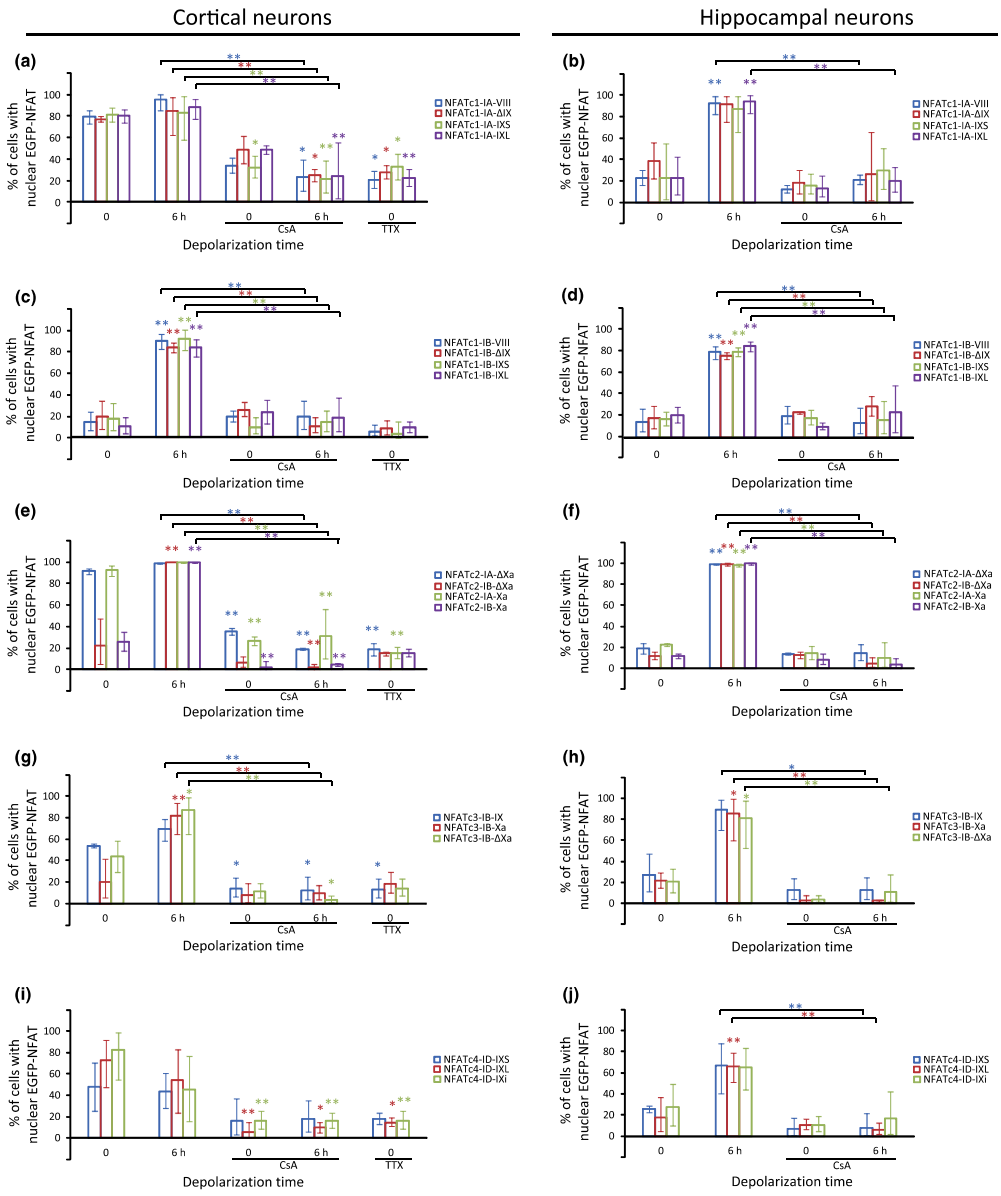



**Fig. 3** Effects of inhibition of calcineurin activity or synaptic activity on the subcellular localization of EGFP-tagged human NFAT isoforms in rat cultured primary neurons. Proportions of transfected cells showing nuclear localization of different EGFP-tagged NFATc1 (a, b, c, and d), NFATc2 (e and f), NFATc3 (g and h), and NFATc4 (i and j) isoforms in rat primary cortical (a, c, e, g, and i) and hippocampal (b, d, f, h, and j) neurons. Neurons were either untreated (0), depolarized with the addition of 25 mM KCl for 6 h (6 h), pre-treated with 5 μM CsA for 1 h before depolarization, or treated overnight with 1 μM TTX. All data were arcsine transformed and are presented as mean values obtained from three independent experiments. Error bars represent standard deviations. Statistical significance shown with asterisks is relative to results from untreated cells and between the bars connected with lines (ANOVA with Holm–Sidak's *post hoc* test $*p < 0.05$; $**p < 0.01$). CsA, cyclosporine A; EGFP, enhanced green fluorescent protein; TTX, tetrodotoxin.

CsA pre-treatment significantly decreased the percentage of cortical neurons with nuclear EGFP for NFATc2 isoforms with exon IA and isoform NFATc2-IB-Xa ($p < 0.01$ vs. untreated) in unstimulated neurons and resulted in cytoplasmic retention of all the NFATc2 isoforms analyzed after depolarization ($p < 0.01$ vs. untreated) (Fig. 3e). In hippocampal neurons, where all the four NFATc2 isoforms analyzed were located in the cytoplasm in unstimulated cells, CsA pre-treatment led to their cytoplasmic retention after depolarization (Fig. 3f).

In cortical neurons, pre-treatment with CsA decreased the proportion of cells with nuclear EGFP localization for isoform NFATc3-IB-IX ($p = 0.038$ vs. untreated) and resulted in cytoplasmic retention of all the NFATc3 isoforms analyzed after depolarization (Fig. 3g). In hippocampal neurons, CsA pre-treatment resulted in cytoplasmic retention of all three NFATc3 isoforms analyzed after depolarization (Fig. 3h).

CsA pre-treatment of cortical neurons decreased significantly the proportion of cells with nuclear EGFP localization for isoforms NFATc4-ID-IXL and NFATc4-ID-IXi ($p < 0.01$ vs. untreated) (Fig. 3i) and resulted in cytoplasmic retention of all NFATc4 isoforms analyzed after depolarization in hippocampal neurons (Fig. 3j).

Next, we asked whether variation in the localization of different NFAT isoforms at basal conditions in cortical neurons could result from different sensitivity of NFAT isoforms to spontaneous synaptic activity. To address this question, we pre-treated transfected cortical neurons overnight with the sodium channel blocker tetrodotoxin. The results showed that tetrodotoxin pre-treatment resulted in a decrease in the percentage of cortical neurons with nuclear EGFP for all isoforms analyzed and that decrease was statistically significant for isoforms NFATc1-IA-VIII, NFATc1-IA-ΔIX, NFATc1-IA-IXS, NFATc1-IA-IXL, NFATc2-IA-ΔXa, NFATc2-IA-Xa, NFATc3-IB-IX, NFATc4-ID-IXL, and NFATc4-ID-IXi ($p < 0.05$ vs. untreated) (Fig. 3a, c, e, g, and i).

Collectively, these results show that in primary cortical and hippocampal neurons the subcellular localizations of NFAT family alternative isoforms are regulated isoform specifically. In addition, in both cortical and hippocampal neurons, CsA pre-treatment of cells resulted in cytoplasmic retention of all NFAT isoforms analyzed showing that nuclear localization of different NFAT isoforms is dependent on the protein phosphatase CaN.

### Transactivation capacities of over-expressed human NFAT isoforms in rat primary cortical and hippocampal neurons and in HEK293 cells

Next, we studied the transactivation capacities of different human NFAT isoforms in primary neurons in response to neuronal activity. For that, we transfected a firefly luciferase construct carrying three tandem NFAT-response elements in front of a minimal promoter along with a construct encoding for one of the different human EGFP-tagged NFATc1, NFATc2, NFATc3, or NFATc4 isoforms into rat primary cortical or hippocampal neurons, and measured luciferase activities with and without depolarization. First, we analyzed the transactivation capacity of a representative EGFP-tagged NFAT isoform from each NFAT family in cortical neurons (Fig. 4a and d). For isoforms NFATc1-IA-IXL and NFATc2-IA-Xa, we did not detect an increase in NFAT-dependent luciferase expression in response to depolarization (Fig. 4a). For isoform NFATc4-ID-IXL a significant increase in luciferase levels was measured after 4 h of depolarization (Fig. 4a). For both NFATc3-IB-IX and NFATc4-ID-IXL isoforms we observed a significant increase in luciferase levels after 8 h of depolarization (Fig. 4a). Approximately three-fold and seven-fold increase in luciferase levels with NFATc3-IB-IX and NFATc4-ID-IXL, respectively, was detected compared to control EGFP-expressing cells. After 12 h of depolarization the transactivation capacity in response to depolarization was the highest for NFATc4-ID-IXL with an approximate 12-fold increase in luciferase activity compared to control cells (Fig. 4a). Similar results were obtained from a control assay where transactivation capacities of the representative NFAT isoforms without the EGFP tag were used (Figure S2a), demonstrating that EGFP fusion does not influence the activity of native NFAT isoforms. Another control experiment using a firefly luciferase construct without the NFAT-response elements demonstrated that none of the NFAT isoforms increased luciferase levels from the minimal promoter in cortical neurons after 12 h of stimulation (Figure S2b), indicating that the measured inductions were dependent on the NFAT-response elements.

In hippocampal neurons, similarly to cortical neurons, we did not observe an increase in NFAT-dependent luciferase expression in response to membrane depolarization when NFATc1-IA-IXL or NFATc2-IA-Xa was transfected (Fig. 4b). In both NFATc3-IB-IX- and NFATc4-ID-IXL-transfected cells, 4-h membrane depolarization resulted in significantly increased luciferase levels compared to untreated hippocampal neurons and the levels further increased several fold after 12 h of depolarization (Fig. 4b). In hippocampal neurons the CMV promoter, driving the expression of all cloned NFAT isoforms used in this study, was too weak to lead to detectable luciferase levels in basal conditions with the NFATc3-IB-IX construct. Therefore, the CMV promoter was replaced with the EF1α promoter in this construct for the luciferase assay.

We next conducted similar luciferase reporter assays also in HEK293 cells to see whether different human NFAT isoforms, especially NFATc1 and NFATc2 isoforms that did not increase NFAT-dependent luciferase expression in primary neurons upon membrane depolarization, act differently in non-neuronal cells. For activating the NFAT signaling

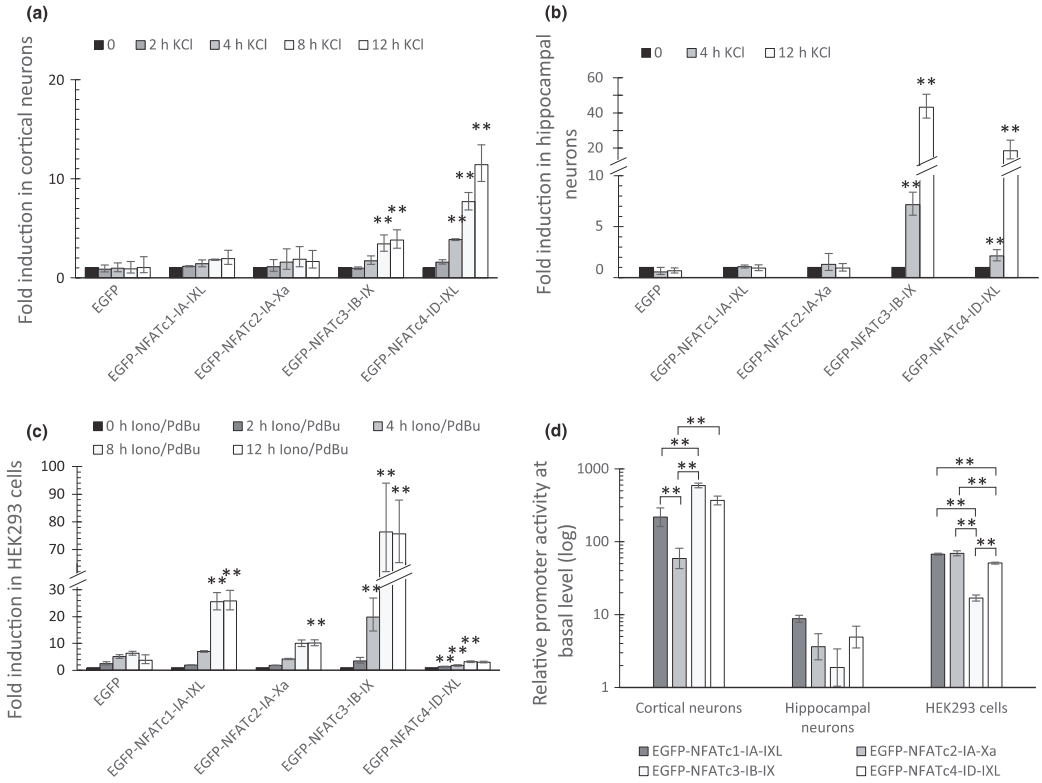



**Fig. 4** Transactivation capacities of representative EGFP-tagged isoforms from different human NFAT families. Results of luciferase reporter assays with rat cultured primary cortical neurons (a and d) and hippocampal neurons (b and d), or HEK293 cells (c and d) are shown. Cells were transfected with either EGFP- or the indicated NFAT isoform-encoding plasmid together with a firefly luciferase construct carrying three tandem NFAT response elements in front of a minimal promoter and *Renilla* luciferase reporter vector pRL-TK for normalization. Neurons (a and b) were left untreated (0) or treated with 25 mM KCl for the time indicated. HEK293 cells (c) were left untreated (0) or treated with 1 μM ionomycin and 1 μM PdBu for the time indicated. Data in (a–c) are presented as fold change in firefly luciferase activity normalized to *Renilla* luciferase activity measured from stimulated cells over that measured from untreated cells. In (d), fold difference in firefly luciferase activity normalized to *Renilla* luciferase activity measured from NFAT isoform-expressing untreated cells over that measured from EGFP-expressing untreated cells is shown. All data were autoscaled. Means and standard deviations were calculated using results from three independent experiments performed in duplicates. Statistical significance shown with asterisks is relative to the luciferase activity measured from the cells transfected with EGFP plasmid at the respective time point (a–c) and between the bars connected with lines (d) (ANOVA with Holm–Sidak's *post hoc* test $*p < 0.05$; $**p < 0.01$). EGFP, enhanced green fluorescent protein; Iono, ionomycin; PdBu, phorbol dibutyrate.

pathway in HEK293 cells we stimulated the cells with the calcium ionophore ionomycin in combination with a PKC-activating analog to phorbol myristate acetate (PMA), PdBu, a treatment previously used by Grigoriu and colleagues (Grigoriu *et al.* 2013). Representative isoforms NFATc1-IA-IXL, NAFTc2-IA-Xa, and NFATc3-IB-IX significantly induced luciferase expression upon stimulation, whereas isoform NFATc4-ID-IXL, which was the strongest transcription activator in primary neurons, did not increase luciferase levels after 12 h of stimulation. Rather, NFATc4-ID-IXL lead to weaker induction of luciferase levels after 2, 4, and 8 h of stimulation compared to the induction measured from control cells transfected with the EGFP-encoding plasmid (Fig. 4c).

In addition to analyzing the transactivation fold upon stimulation, we also compared the activation of NFAT-response element-dependent luciferase expression by the representative NFAT isoforms at basal conditions (Fig. 4d).

We observed that the over-expressed NFAT isoforms produced the greatest disparity of luciferase expression levels at basal conditions in cortical neurons as compared to hippocampal neurons and HEK293 cells. For example, there was a 10-fold difference in basal luciferase levels between isoforms NFATc2-IA-Xa and NFATc3-IB-IX in cortical neurons, but only a two-fold and four-fold difference in hippocampal neurons and HEK293 cells, respectively (Fig. 4d).

We went on to analyze the transactivation capacities of all cloned 18 NFAT protein isoforms in cortical neurons (Fig. 5). For NFATc1, eight different isoforms were used (Fig. 5a). Like before, the representative isoform NFATc1-IA-IXL did not increase the NFAT-dependent luciferase expression upon depolarization. Interestingly three of the other C-terminally different isoforms that were used, namely, NFATc1-IA-VIII, NFATc1-IA-ΔIX and NFATc1-IB-VIII, significantly increased transactivation after 12 h of depolarization resulting in an approximate seven-fold increase in luciferase levels for NFATc1-IA-VIII and three-fold increase for NFATc1-IA-ΔIX and NFATc1-IB-VIII (Fig. 5a). For NFATc2, four different isoforms were analyzed (Fig. 5b). In this assay all four NFATc2 isoforms, including NFATc2-IA-Xa, significantly increased NFAT-dependent luciferase expression after 12-hour depolarization reaching from approximately four-fold increase for NFATc2-IA-ΔXa to eight-fold for NFATc2-IB-Xa (Fig. 5b). The induction with the isoform NFATc2-IA-Xa displayed great variation between biological replicates (Fig. 5b), and also had lower basal levels of NFAT-dependent promoter activity than in the assay with the representative isoforms (Figs 4d and 5e), which combined could partly explain the difference from our previous result with this isoform. For both NFATc3 and NFATc4, we used three different isoforms (Fig. 5c and d). All three NFATc3 isoforms displayed almost identical induction patterns resulting in an approximate four-fold increase in luciferase levels after 12-hour depolarization (Fig. 5c). Similar to NFATc3, all the three NFATc4 isoforms used significantly increased NFAT-dependent gene transcription almost identically upon depolarization resulting in an approximate five-fold increase in luciferase levels after 12-hour depolarization (Fig. 5d).

At basal conditions in cortical neurons all of the eight NFATc1 isoforms analyzed had relatively similar NFAT-response element-dependent transactivation capacities (Fig. 5e). All four NFATc2 isoforms activated the NFAT-dependent reporter similarly and produced the lowest luciferase levels at basal conditions compared to other members of the NFAT family (Figs 4d and 5e). All three NFATc3 isoforms analyzed had similar transactivation capacities at basal conditions (Fig. 5e). NFATc4 isoform NFATc4-ID-IXL activated NFAT-dependent luciferase expression slightly more without depolarization compared to the other two NFATc4 isoforms analyzed ($p = 0.072$ vs. NFATc4-ID-IXS; $p = 0.051$ vs. NFATc4-ID-IXi) (Fig. 5e).

In summary, our results demonstrate that the transactivation capacities of different human NFAT proteins are dependent upon the interplay between the specific NFAT isoform and cell type.

## Discussion

In this study, we analyzed the subcellular localizations and transactivation capacities of different over-expressed human NFAT isoforms in rat primary cortical and hippocampal neurons. In general, our results confirmed that the translocation of NFAT isoforms to the nucleus in neurons is reliant on the calcium-dependent serine–threonine phosphatase CaN. However, we detected both similarities and differences in the regulation of localization of alternative NFAT isoforms in neurons and furthermore, we found that alternative human NFAT isoforms have variable transactivation capacities in primary cortical and hippocampal neurons and in HEK293 cells.

We observed that in hippocampal neurons all NFAT isoforms studied were located mostly in the cytosol at basal conditions. In cortical neurons, however, the localizations of different NFAT isoforms at basal conditions varied greatly even within the same NFAT subfamily. Yet, blocking spontaneous neuronal activity of transfected cortical neurons with an overnight tetrodotoxin pre-treatment resulted in cytoplasmic localization of all NFAT isoforms analyzed. It has been found that both firing and bursting rates are increased in rat primary cortical cultures compared to hippocampal cultures (Harrill *et al.* 2015). Although both culturing conditions and time play significant roles in the maturation of primary neurons in culture (Biffi *et al.* 2013), our results with tetrodotoxin indicate that a similar difference in spontaneous activity between our cortical and hippocampal cultures exists. This explains the tendency of NFAT isoforms to be more nuclear in cortical neurons at basal conditions.

Although alternative NFAT isoforms of the same NFAT family share common regulatory domains and should therefore share mechanisms underlying their subcellular localization regulation, our results show that some N-terminally different NFAT isoforms are more sensitive to spontaneous neuronal activity than others. For example, we found that in untreated cortical neurons NFATc1-IB and NFATc2-IB were localized predominantly to the cytoplasm, whereas NFATc1-IA and NFATc2-IA isoforms were localized to the nucleus. This result could be explained by a conserved docking site for casein kinase 1, motif F-X-X-X-F, that has been shown to be located in the region encoded by NFATc2 exon IB and found to be responsible for cytoplasmic maintenance of NFATc2-IB isoform in resting T-cells (Okamura *et al.* 2004). Interestingly, this motif is present not only in NFATc1-IB but also in the protein sequences encoded by *NFATc3* exon IB and *NFATc4* exon ID

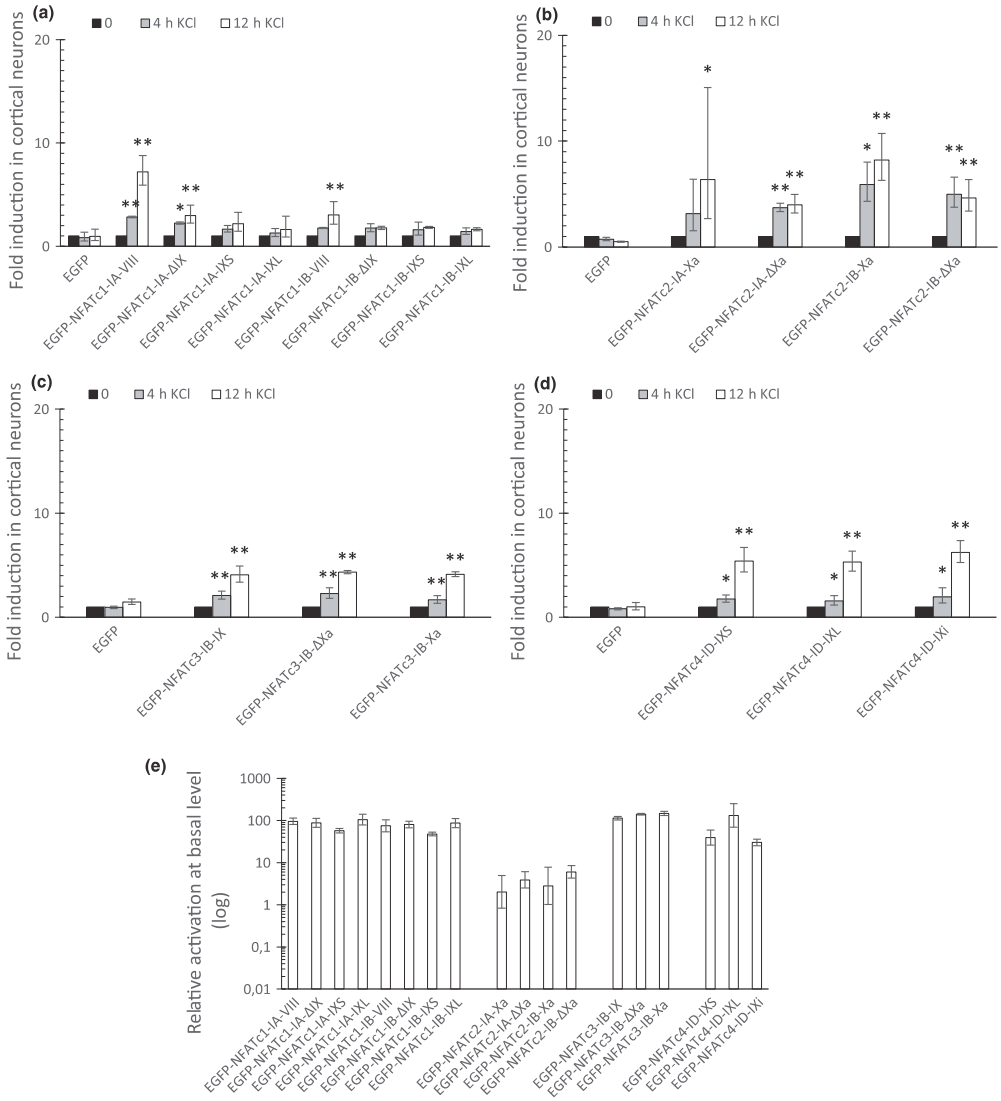



**Fig. 5** Transactivation capacities of alternative EGFP-tagged human NFAT isoforms in rat primary cortical neurons. Results of luciferase reporter assays with different EGFP-tagged human NFATc1 (a and e), NFATc2 (b and e), NFATc3 (c and e), and NFATc4 (d and e) isoforms are shown. Cells were transfected with either EGFP- or the indicated NFAT isoform-encoding plasmid together with a firefly luciferase construct carrying three tandem NFAT response elements in front of a minimal promoter and *Renilla* luciferase reporter vector pRL-TK for normalization. Cortical neurons (a–d) were left untreated (0) or treated with 25 mM KCl for the time indicated. Data in (a–d) are presented as fold change in firefly luciferase activity normalized to *Renilla* luciferase activity measured from stimulated cells over that measured from untreated cells. In (e), fold difference in firefly luciferase activity normalized to *Renilla* luciferase activity measured from NFAT isoform-expressing untreated cells over that measured from EGFP-expressing untreated cells is shown. All data were autoscaled. Means and standard deviations were calculated using results from three independent experiments performed in duplicates. Statistical significance shown with asterisks is relative to the luciferase activity measured from the cells transfected with EGFP plasmid at the respective time point (a–d). (ANOVA with Holm–Sidak's *post hoc* test $*p < 0.05$; $**p < 0.01$). EGFP, enhanced green fluorescent protein.

(Okamura *et al.* 2004; see sequence alignments in Vihma *et al.* 2008). However, our results show that for NFATc3 and NFATc4 alternative isoforms cytoplasmic maintenance of the respective N-termini-containing proteins in untreated cortical neurons is not obvious. If the conserved casein kinase 1 docking motif is involved in the regulation of subcellular localization of NFAT isoforms in neurons, this would indicate that the same regulatory unit can have diverse effects on different NFAT families. In addition, in resting T-cells the scaffolding protein Homer2 has been identified as a negative regulator of NFATc2 by interacting with amino acids 1–105 of the mouse NFATc2-IB isoform and competing with CaN for NFATc2 binding (Gwack *et al.* 2006; Huang *et al.* 2008). Although the regulation of human NFATc2 by Homer2 has not been documented, these results from others together with our data from this study suggest that the differences in the regulatory mechanisms acting on N-terminally variable NFAT isoforms cause distinct subcellular localizations of the NFAT isoforms under spontaneous neuronal activity.

Our results showed that membrane depolarization of hippocampal neurons, which increases CaN activity among other effects, results in nuclear localization of all NFAT isoforms tested. In addition to the very fast translocation kinetics of NFATc2-IB isoforms in hippocampal cells, and also in cortical cells, we observed comparatively rapid translocation of NFATc3-IB-IX and NFATc3-IB-ΔXa to the nucleus upon neuronal activity in hippocampal neurons. The latter is in accord with previous studies showing that membrane depolarization results in rapid translocation of NFATc3 to the nucleus in dorsal root ganglia neurons (Kim and Usachev 2009; Ulrich *et al.* 2012) and in hippocampal neurons (Ulrich *et al.* 2012; Murphy *et al.* 2014). Of note, NFATc2 and NFATc3 possess distinct nuclear localization dynamics in response to cell stimulation in mast cells, with NFATc3 showing a fast response and NFATc2 showing a slow, continuous response (Yissachar *et al.* 2013). Interestingly, the results with NFATc4 isoforms differed from the results with other NFATs. In hippocampal neurons we observed a relatively slower translocation to the nucleus of all NFATc4 isoforms analyzed compared to other NFAT family members and in cortical neurons NFATc4 localization was unresponsive to depolarization. These findings suggest that CaN phosphatase activity acting on NFATc4 isoforms might in general be more readily neutralized compared to other NFAT family members in hippocampal cells. In support of that concept a previous study has shown that in hippocampal neurons NFATc4, but not NFATc3, is persistently being phosphorylated by glycogen synthase kinase 3 beta (GSK3β) that counteracts CaN dephosphorylating activity (Ulrich *et al.* 2012). Alternatively, as it has been described that synaptogenesis, maturation, and thus activity of neurons are correlated with an increase in inactive GSK3β levels (Rui *et al.* 2013), it could be that decreased GSK3β activity is reflected in our results with NFATc4 isoforms in cortical cells. Considering that the maximum percentage of transfected hippocampal cells having nuclear NFATc4 was approximately 65, it could be that in the spontaneously active cortical neurons the shifted balance toward dephosphorylation, owing to continuous inactivation of GSK3β, leads to near-maximal levels of nuclear NFATc4 isoforms already at basal conditions. In addition to these possibilities, a reason for the localization variability and relative unresponsiveness to depolarization detected here for NFATc4 isoforms, and also for NFATc3 isoforms in cortical cells, could be that if the respective NFAT isoform has pulsatile translocation dynamics, as has been shown for NFATc3 in mast cells (Yissachar *et al.* 2013), determining the actual proportion of cells displaying nuclear translocation without live imaging is difficult. Anyhow, as CsA treatment of cells resulted in cytoplasmic retention of all NFAT isoforms analyzed, we have confirmed here that CaN activity is required for the nuclear translocation of all human NFAT proteins.

Our analysis of NFAT-dependent transcriptional activation revealed that at basal conditions NFAT proteins are the least active in hippocampal neurons compared to cortical neurons and HEK293 cells. This correlates with the predominantly cytoplasmic localization of NFATs in untreated hippocampal neurons. In cortical neurons, however, the variation in localization of different NFAT isoforms at basal conditions is also reflected in the transcriptional activation capacities, which vary significantly between different isoforms. We determined that in response to calcium signaling in neurons, all NFATc3 and NFATc4 isoforms tested were strong activators of transcription, whereas transactivation by NFATc1 and NFATc2 was dependent on the specific isoform. Importantly, we showed that in HEK293 cells, NFATc4 transcriptional activity is not increased upon the stimulation of calcium signaling. This demonstrates that different cellular contexts can lead to differences in calcium-dependent activation of NFAT proteins and using neuronal cells is essential for elucidating mechanisms of NFAT regulation in the nervous system.

It has been shown that NFAT isoforms NFATc1-IXL, NFATc2-ΔXa, NFATc3-ΔXa, and NFATc4-ID-IXL contain a transactivation domain (TAD) in the C-terminus (reviewed in Rao *et al.* 1997) and it contributes to the induction of transactivation by NFATc2-ΔXa in COS cells (Luo *et al.* 1996) and NFATc3-ΔXa in Jurkat cells (Imamura *et al.* 1998). We found here that in neurons the presence of this C-terminal TAD did not clearly influence membrane depolarization-induced transactivation capacities of different NFAT isoforms. However, we demonstrated that only the NFATc1 isoforms NFATc1-IA-VIII, NFATc1-IB-VIII, and NFATc1-IA-ΔIX (differing C-terminally from the other NFATc1 isoforms) significantly increased NFAT-dependent luciferase expression upon high $K^+$ stimulation of cortical neurons. In T-cells the NFATc1 isoform containing the

C-terminus encoded by exon IX, but not other NFATc1 isoforms, is changed from a transcriptional activator to a repressor via sumoylation of the protein at sumoylation sites in its C-terminus (Nayak *et al.* 2009). Our results indicating that NFATc1 isoforms containing the C-terminus encoded by exon IX do not activate transcription upon depolarization suggest that a similar sumoylation-dependent suppression of NFATc1 activity also takes place in neurons. In general, our luciferase assays show that although membrane depolarization resulted in induction of transcriptional activity by at least three of the isoforms examined from each of the NFAT subfamily, neuron type-specific differences in the localization of over-expressed N- and C-terminally diverse NFAT isoforms did not predict their capacity to induce transcription from the NFAT/activator protein 1 (AP-1) site-containing promoter in hippocampal or cortical neurons.

It is possible that additional cellular signals may be needed to fully activate NFAT isoform-specific coactivators in neurons and that the lack of these supplementary signals affect membrane depolarization-induced transactivation capacities of NFAT isoforms obtained in this study. In T-cells, for example, ionomycin treatment together with PKC activation by PMA has been used to demonstrate the effect of the C-terminal TADs on transactivation induction capacities of different NFATc2 and NFATc3 isoforms (Luo *et al.* 1996; Imamura *et al.* 1998). In both of these studies, and also in this study, a firefly luciferase construct carrying three tandem NFAT-response elements composed of the NFAT/AP-1 consensus sequence derived from the interleukin-2 promoter was used to measure NFAT-dependent transcription activity. It is likely that in non-neuronal cells the PMA cotreatment is needed for inducing cooperation of NFATs with PMA-stimulated AP1 family transcription factors, such as c-Fos and c-Jun (reviewed in Macián *et al.* 2001). The latter is supported also by our observation that the over-expressed NFATs increased NFAT-dependent luciferase expression in HEK293 cells in response to ionomycin and PKC-activating PdBu but not to ionomycin alone (data not shown). This suggests that activation of PKC is needed for the action of AP-1 proteins in addition to NFATs to induce transcription from the NFAT/AP-1 consensus sequence in HEK293 cells. In neurons, on the contrary, it is well established that calcium signaling induces expression of certain AP1 proteins, including c-Fos and c-Jun, for example (reviewed in Pérez-Cadahía *et al.* 2011). Thus, although transactivation induction by different NFAT isoforms in neurons may be facilitated by distinct interactions of NFATs with diverse coactivators, which in addition to membrane depolarization require other activation mechanisms, we consider the results of this study to reflect the neuronal activity-mediated component of NFAT regulation.

In conclusion, in this study we have shown that although the neuronal activity-induced nuclear translocation of all NFAT isoforms requires CaN, the precise effects of calcium signaling on the subcellular localization and transcription activation capacity of the NFAT proteins are isoform-specific and can vary between neuron types.

## Acknowledgements and conflict of interest disclosure

We thank Enn Jõeste from North Estonian Regional Hospital, Tallinn, for collaboration; Epp Väli and Maila Rähn for technical assistance; Heiti Paves for help in confocal microscopy; Jürgen Tuvikene for helping with statistical analysis; and Indrek Koppel for critical reading of the manuscript. This study was supported by Estonian Research Council (institutional research funding IUT19-18 and Grant 8844) and Estonian Academy of Sciences. The authors declare no conflict of interests.

All experiments were conducted in compliance with the ARRIVE guidelines.

## Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

**Figure S1.** Representative florescence microscopy images of the temporal regulation of subcellular localization of different EGFP-tagged human NFAT isoforms by depolarization in rat cultured primary neurons.

**Figure S2.** Transactivation capacities of alternative human NFAT isoforms in rat cultured cortical neurons.

## References

Abdul H. M., Sama M. A., Furman J. L. *et al.* (2009) Cognitive decline in Alzheimer's disease is associated with selective changes in calcineurin/NFAT signaling. *J. Neurosci.* **29**, 12957–12969.

Aramburu J., Garcia-Cózar F., Raghavan A., Okamura H., Rao A. and Hogan P. G. (1998) Selective inhibition of NFAT activation by a peptide spanning the calcineurin targeting site of NFAT. *Mol. Cell* **1**, 627–637.

Arron J. R., Winslow M. M., Polleri A. *et al.* (2006) NFAT dysregulation by increased dosage of DSCR1 and DYRK1A on chromosome 21. *Nature* **441**, 595–600.

Asai M., Iwasaki Y., Yoshida M., Mutsuga-Nakayama N., Arima H., Ito M., Takano K. and Oiso Y. (2004) Nuclear factor of activated T cells (NFAT) is involved in the depolarization-induced activation of growth hormone-releasing hormone gene transcription in vitro. *Mol. Endocrinol.* **18**, 3011–3019.

Benedito A. B., Lehtinen M., Massol R., Lopes U. G., Kirchhausen T., Rao A. and Bonni A. (2005) The transcription factor NFAT3 mediates neuronal survival. *J. Biol. Chem.* **280**, 2818–2825.

Biffi E., Regalia G., Menegon A., Ferrigno G. and Pedrocchi A. (2013) The influence of neuronal density and maturation on network activity of hippocampal cell cultures: a methodological study. *PLoS ONE* **8**, e83899.

Cai Y.-Q., Chen S.-R. and Pan H.-L. (2013) Upregulation of nuclear factor of activated T-cells by nerve injury contributes to development of neuropathic pain. *J. Pharmacol. Exp. Ther.* **345**, 161–168.

Caraveo G., Auluck P. K., Whitesell L. *et al.* (2014) Calcineurin determines toxic versus beneficial responses to α-synuclein. *Proc. Natl Acad. Sci. USA* **111**, E3544–E3552.

Crabtree G. R. and Olson E. N. (2002) NFAT signaling: choreographing the social lives of cells. *Cell* **109**(Suppl), S67–S79.

Dittmer P. J., Dell'Acqua M. L. and Sather W. A. (2014) $Ca^{2+}$/calcineurin-dependent inactivation of neuronal L-type $Ca^{2+}$ channels requires priming by AKAP-anchored protein kinase A. *Cell Rep.* **7**, 1410–1416.

de la Fuente V., Freudenthal R. and Romano A. (2011) Reconsolidation or extinction: transcription factor switch in the determination of memory course after retrieval. *J. Neurosci.* **31**, 5562–5573.

Gómez-Sintes R. and Lucas J. J. (2010) NFAT/Fas signaling mediates the neuronal apoptosis and motor side effects of GSK-3 inhibition in a mouse model of lithium therapy. *J. Clin. Invest.* **120**, 2432–2445.

Graef I. A., Mermelstein P. G., Stankunas K., Neilson J. R., Deisseroth K., Tsien R. W. and Crabtree G. R. (1999) L-type calcium channels and GSK-3 regulate the activity of NF-ATc4 in hippocampal neurons. *Nature* **401**, 703–708.

Graef I. A., Chen F. and Crabtree G. R. (2001) NFAT signaling in vertebrate development. *Curr. Opin. Genet. Dev.* **11**, 505–512.

Graef I. A., Wang F., Charron F., Chen L., Neilson J., Tessier-Lavigne M. and Crabtree G. R. (2003) Neurotrophins and netrins require calcineurin/NFAT signaling to stimulate outgrowth of embryonic axons. *Cell* **113**, 657–670.

Grigoriu S., Bond R., Cossio P., Chen J. A., Ly N., Hummer G., Page R., Cyert M. S. and Peti W. (2013) The molecular mechanism of substrate engagement and immunosuppressant inhibition of calcineurin. *PLoS Biol.* **11**, e1001492.

Groth R. D. and Mermelstein P. G. (2003) Brain-derived neurotrophic factor activation of NFAT (nuclear factor of activated T-cells)-dependent transcription: a role for the transcription factor NFATc4 in neurotrophin-mediated gene expression. *J. Neurosci.* **23**, 8125–8134.

Groth R. D., Weick J. P., Bradley K. C., Luoma J. I., Aravamudan B., Klug J. R., Thomas M. J. and Mermelstein P. G. (2008) D1 dopamine receptor activation of NFAT-mediated striatal gene expression. *Eur. J. Neurosci.* **27**, 31–42.

Gwack Y., Sharma S., Nardone J. *et al.* (2006) A genome-wide *Drosophila* RNAi screen identifies DYRK-family kinases as regulators of NFAT. *Nature* **441**, 646–650.

Harrill J. A., Chen H., Streifel K. M., Yang D., Mundy W. R. and Lein P. J. (2015) Ontogeny of biochemical, morphological and functional parameters of synaptogenesis in primary cultures of rat hippocampal and cortical neurons. *Mol. Brain* **8**, 10.

Ho A. M., Jain J., Rao A. and Hogan P. G. (1994) Expression of the transcription factor NFATp in a neuronal cell line and in the murine nervous system. *J. Biol. Chem.* **269**, 28181–28186.

Hogan P. G., Chen L., Nardone J. and Rao A. (2003) Transcriptional regulation by calcium, calcineurin, and NFAT. *Genes Dev.* **17**, 2205–2232.

Huang G. N., Huso D. L., Bouyain S. *et al.* (2008) NFAT binding and regulation of T cell activation by the cytoplasmic scaffolding homer proteins. *Science* **319**, 476–481.

Imamura R., Masuda E. S., Naito Y., Imai S., Fujino T., Takano T., Arai K. and Arai N. (1998) Carboxyl-terminal 15-amino acid sequence of NFATx1 is possibly created by tissue-specific splicing and is essential for transactivation activity in T cells. *J. Immunol.* **1950**, 3455–3463.

Jayanthi S., Deng X., Ladenheim B., McCoy M. T., Cluster A., Cai N.-S. and Cadet J. L. (2005) Calcineurin/NFAT-induced up-regulation of the Fas ligand/Fas death pathway is involved in methamphetamine-induced neuronal apoptosis. *Proc. Natl Acad. Sci. USA* **102**, 868–873.

Kiani A., Rao A. and Aramburu J. (2000) Manipulating immune responses with immunosuppressive agents that target NFAT. *Immunity* **12**, 359–372.

Kim M.-S. and Usachev Y. M. (2009) Mitochondrial $Ca^{2+}$ cycling facilitates activation of the transcription factor NFAT in sensory neurons. *J. Neurosci.* **29**, 12101–12114.

Luo C., Burgeon E., Carew J. A., McCaffrey P. G., Badalian T. M., Lane W. S., Hogan P. G. and Rao A. (1996) Recombinant NFAT1 (NFATp) is regulated by calcineurin in T cells and mediates transcription of several cytokine genes. *Mol. Cell. Biol.* **16**, 3955–3966.

Luo J., Sun L., Lin X., Liu G., Yu J., Parisiadou L., Xie C., Ding J. and Cai H. (2014) A calcineurin- and NFAT-dependent pathway is involved in α-synuclein-induced degeneration of midbrain dopaminergic neurons. *Hum. Mol. Genet.* **23**, 6567–6574.

Luoma J. I. and Zirpel L. (2008) Deafferentation-induced activation of NFAT (nuclear factor of activated T-Cells) in cochlear nucleus neurons during a developmental critical period: a role for NFATc4-dependent apoptosis in the CNS. *J. Neurosci.* **28**, 3159–3169.

Macian F. (2005) NFAT proteins: key regulators of T-cell development and function. *Nat. Rev. Immunol.* **5**, 472–484.

Macián F., López-Rodríguez C. and Rao A. (2001) Partners in transcription: NFAT and AP-1. *Oncogene* **20**, 2476–2489.

Mojsa B., Mora S., Bossowski J. P., Lassot I. and Desagher S. (2015) Control of neuronal apoptosis by reciprocal regulation of NFATc3 and Trim17. *Cell Death Differ.* **22**, 274–286.

Murphy J. G., Sanderson J. L., Gorski J. A., Scott J. D., Catterall W. A., Sather W. A. and Dell'Acqua M. L. (2014) AKAP-anchored PKA maintains neuronal L-type calcium channel activity and NFAT transcriptional signaling. *Cell Rep.* **7**, 1577–1588.

Nayak A., Glöckner-Pagel J., Vaeth M., Schumann J. E., Buttmann M., Bopp T., Schmitt E., Serfling E. and Berberich-Siebelt F. (2009) Sumoylation of the transcription factor NFATc1 leads to its subnuclear relocalization and interleukin-2 repression by histone deacetylase. *J. Biol. Chem.* **284**, 10935–10946.

Nguyen T., Lindner R., Tedeschi A., Forsberg K., Green A., Wuttke A., Gaub P. and Di Giovanni S. (2009) NFAT-3 Is a transcriptional repressor of the growth-associated protein 43 during neuronal maturation. *J. Biol. Chem.* **284**, 18816–18823.

Okamura H., Garcia-Rodriguez C., Martinson H., Qin J., Virshup D. M. and Rao A. (2004) A conserved docking motif for CK1 binding controls the nuclear localization of NFAT1. *Mol. Cell. Biol.* **24**, 4184–4195.

Oliveria S. F., Dell'Acqua M. L. and Sather W. A. (2007) AKAP79/150 anchoring of calcineurin controls neuronal L-type $Ca^{2+}$ channel activity and nuclear signaling. *Neuron* **55**, 261–275.

Park S., Uesugi M. and Verdine G. L. (2000) A second calcineurin binding site on the NFAT regulatory domain. *Proc. Natl Acad. Sci. USA* **97**, 7130–7135.

Pérez-Cadahía B., Drobic B. and Davie J. R. (2011) Activation and function of immediate-early genes in the nervous systemThis paper is one of a selection of papers in a Special Issue entitled 31st Annual International Asilomar Chromatin and Chromosomes Conference, and has undergone the Journal's usual peer review process. *Biochem. Cell Biol.* **89**, 61–73.

Plyte S., Boncristiano M., Fattori E., Galvagni F., Paccani S. R., Majolini M. B., Oliviero S., Ciliberto G., Telford J. L. and Baldari C. T. (2001) Identification and characterization of a novel nuclear factor of activated T-cells-1 isoform expressed in mouse brain. *J. Biol. Chem.* **276**, 14350–14358.

Quadrato G., Benevento M., Alber S., Jacob C., Floriddia E. M., Nguyen T., Elnaggar M. Y., Pedroarena C. M., Molkentin J. D. and Giovanni S. D. (2012) Nuclear factor of activated T cells (NFATc4) is required for BDNF-dependent survival of adult-born neurons and spatial memory formation in the hippocampus. *Proc. Natl Acad. Sci.* **109**, E1499–E1508.

Rao A., Luo C. and Hogan P. G. (1997) Transcription factors of the NFAT family: regulation and function. *Annu. Rev. Immunol.* **15**, 707–747.

Rui Y., Myers K. R., Yu K., Wise A., De Blas A. L., Hartzell H. C. and Zheng J. Q. (2013) Activity-dependent regulation of dendritic growth and maintenance by glycogen synthase kinase 3β. *Nat. Commun.* **4**, 2628.

Schwartz N., Schohl A. and Ruthazer E. S. (2009) Neural activity regulates synaptic properties and dendritic structure in vivo through calcineurin/NFAT signaling. *Neuron* **62**, 655–669.

Serrano-Pérez M. C., Fernández M., Neria F., Berjón-Otero M., Doncel-Pérez E., Cano E. and Tranque P. (2015) NFAT transcription factors regulate survival, proliferation, migration, and differentiation of neural precursor cells. *Glia* **63**, 987–1004.

Smith H. S. (2009) Calcineurin as a nociceptor modulator. *Pain Physician* **12**, E309–E318.

Ulrich J. D., Kim M.-S., Houlihan P. R., Shutov L. P., Mohapatra D. P., Strack S. and Usachev Y. M. (2012) Distinct activation properties of the nuclear factor of activated T-cells (NFAT) isoforms NFATc3 and NFATc4 in neurons. *J. Biol. Chem.* **287**, 37594–37609.

Vashishta A., Habas A., Pruunsild P., Zheng J.-J., Timmusk T. and Hetman M. (2009) Nuclear factor of activated T-cells isoform c4 (NFATc4/NFAT3) as a mediator of antiapoptotic transcription in NMDA receptor-stimulated cortical neurons. *J. Neurosci.* **29**, 15331–15340.

Vihma H., Pruunsild P. and Timmusk T. (2008) Alternative splicing and expression of human and mouse NFAT genes. *Genomics* **92**, 279–291.

West A. E. and Greenberg M. E. (2011) Neuronal activity-regulated gene transcription in synapse development and cognitive function. *Cold Spring Harb. Perspect. Biol.* **3**, a005744.

Yissachar N., Sharar Fischler T., Cohen A. A., Reich-Zeliger S., Russ D., Shifrut E., Porat Z. and Friedman N. (2013) Dynamic response diversity of NFAT isoforms in individual living cells. *Mol. Cell* **49**, 322–330.

Yoshida T. and Mishina M. (2005) Distinct roles of calcineurin-nuclear factor of activated T-cells and protein kinase A-cAMP response element-binding protein signaling in presynaptic differentiation. *J. Neurosci.* **25**, 3067–3079.

Zhang J. and Shapiro M. S. (2012) Activity-dependent transcriptional regulation of M-Type (Kv7) K+ channels by AKAP79/150-mediated NFAT actions. *Neuron* **76**, 1133–1146.

**Supporting information**

# Regulation of different human NFAT isoforms by neuronal activity

Hanna Vihma, Mirjam Luhakooder, Priit Pruunsild[1], Tõnis Timmusk

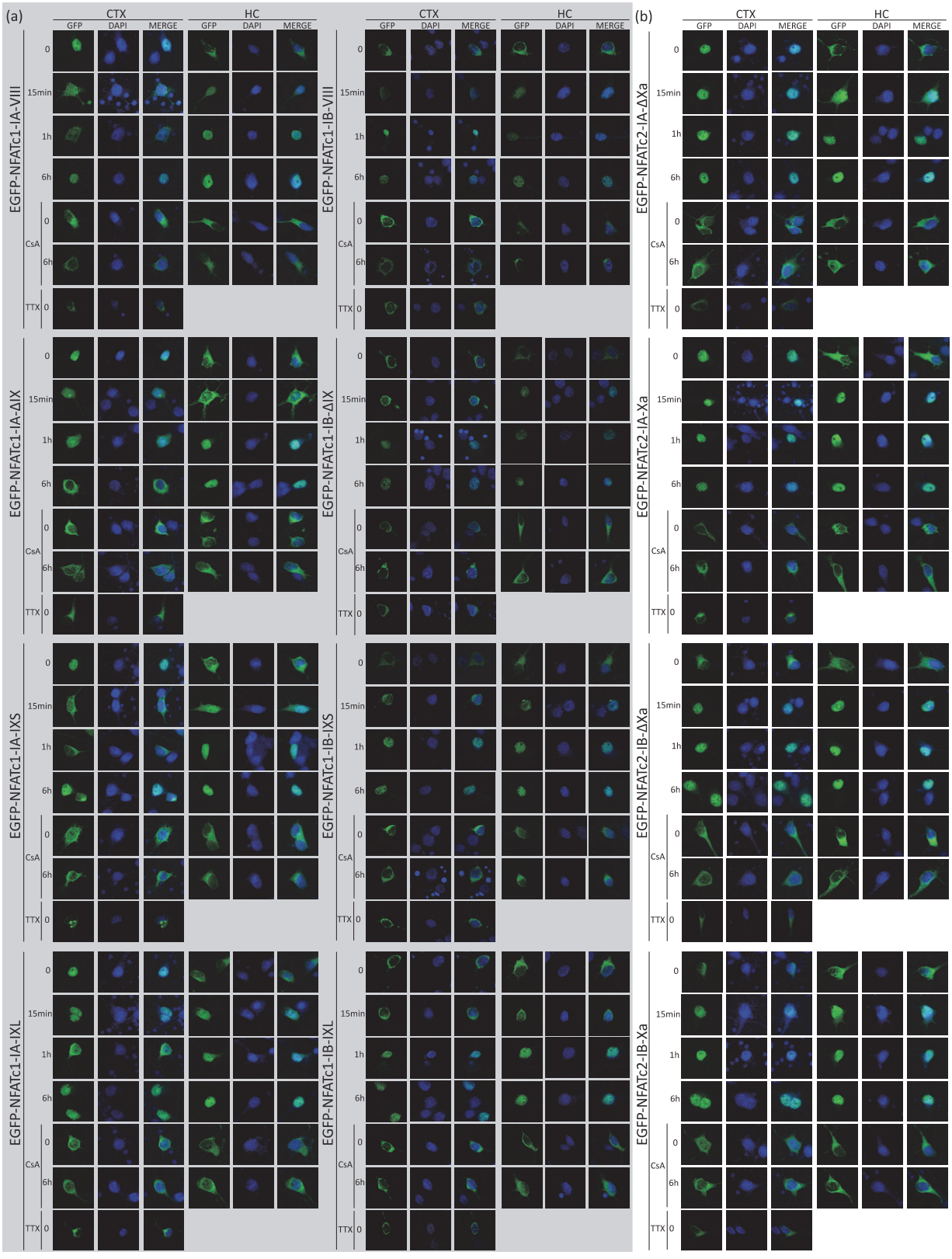
Department of Gene Technology, Tallinn University of Technology, Estonia

[1]Present address: Department of Neurobiology, Interdisciplinary Centre for Neurosciences, University of Heidelberg, INF 364, 69120 Heidelberg, Germany

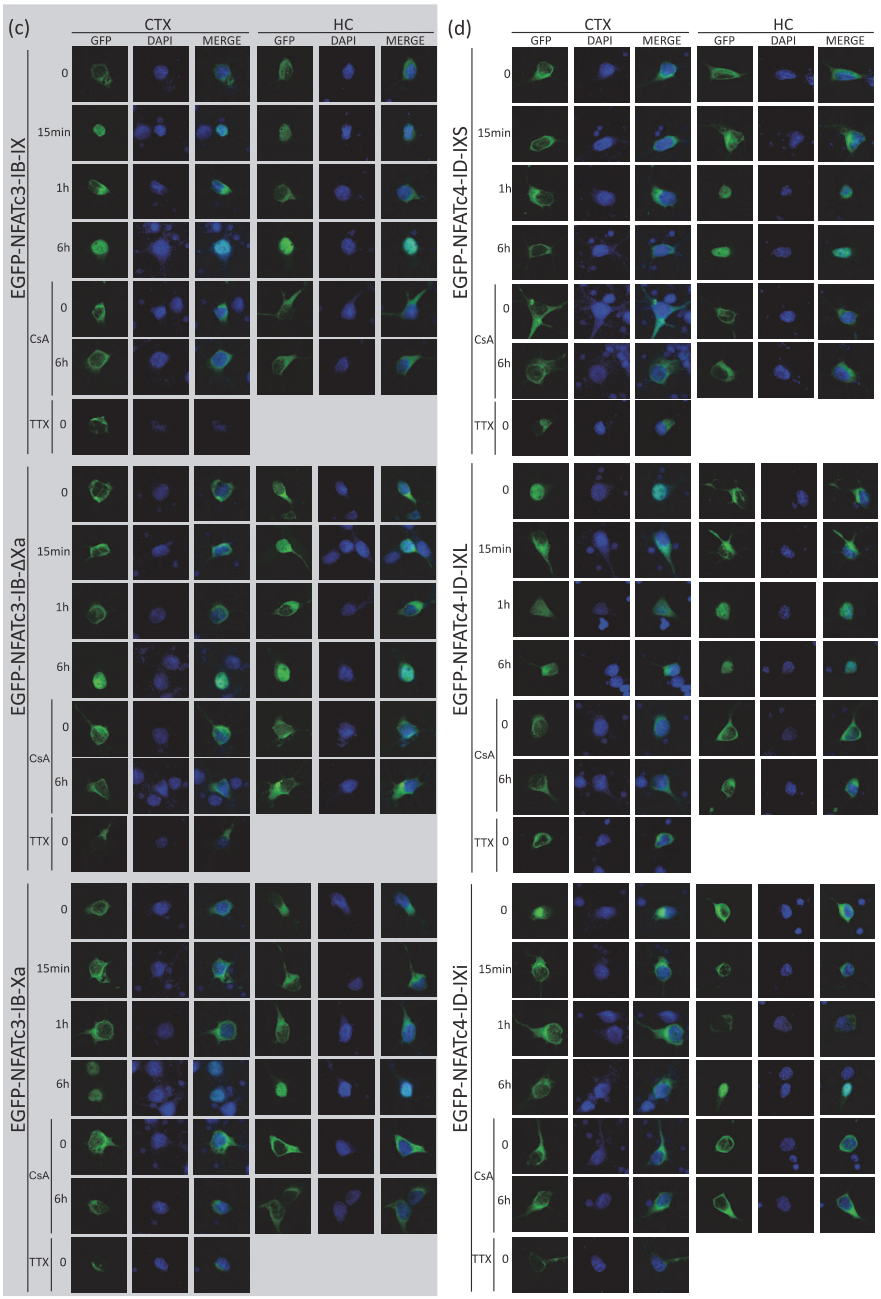
Address correspondence and reprint requests to Hanna Vihma and Tõnis Timmusk, Department of Gene Technology, Tallinn University of Technology, Akadeemia tee 15, 12618 Tallinn, Estonia. E-mails: hanna.vihma@ttu.ee, tonis.timmusk@ttu.ee

**Supplementary figure 1.** Representative florescence microscopy images of the temporal regulation of subcellular localization of different EGFP-tagged human NFAT isoforms by depolarization in rat cultured primary neurons. Localization of EGFP-tagged NFATc1 (a), NFATc2 (d), NFATc3 (c) and NFATc4 (d) isoforms in rat cultured primary cortical and hippocampal neurons. Neurons were either untreated (0), depolarized with the addition of 25 mM KCl for 6h (6h), pre-treated with 5 μM CsA for one hour before depolarization or treated overnight with 1 μM TTX. The EGFP-tagged NFAT isoforms are visible in green (EGFP) and the nuclei of the cells in blue (DAPI staining). Scale bar is 10 μm. EGFP, enhanced green fluorescent protein; DAPI, 4′-6-diamidino-2-phenylindole; CsA, cyclosporine A; TTX, tetrodotoxin.

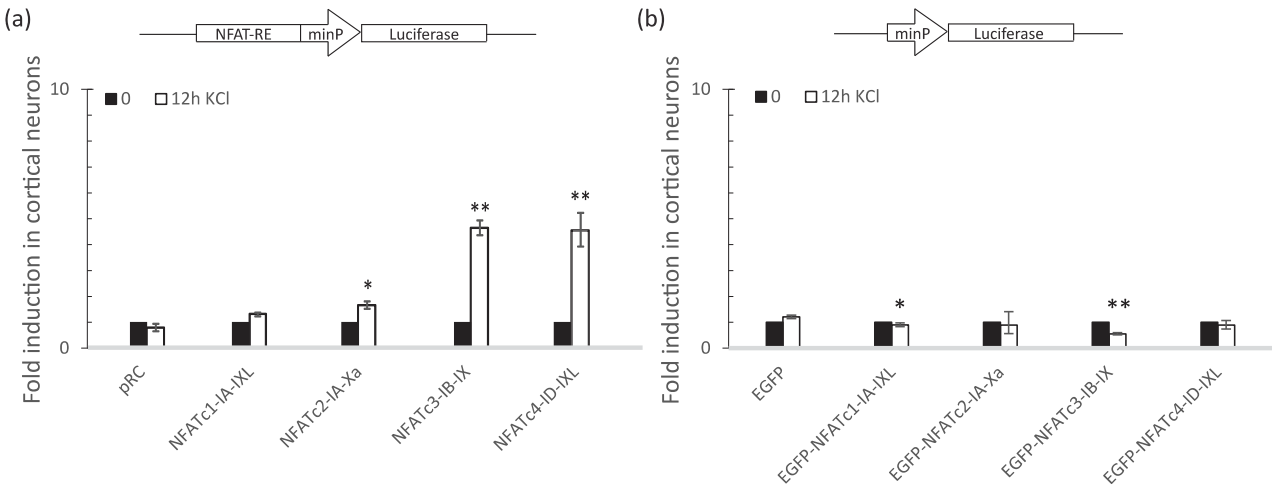
# Supplementary Figure 1



**Supplementary Figure 1** (continued)



## Supplementary Figure 2




**Supplementary figure 2.** Transactivation capacities of alternative human NFAT isoforms in rat cultured cortical neurons. Neurons in (a) were transfected with either empty vector pRC or the indicated not-EGFP-fused NFAT isoform-encoding plasmid together with a firefly luciferase construct carrying three tandem NFAT response elements in front of a minimal promoter and Renilla luciferase reporter vector pRL-TK for normalization. Neurons in (b) were transfected with either EGFP- or the indicated EGFP-tagged NFAT isoform-encoding plasmid together with a firefly luciferase construct carrying only a minimal promoter and Renilla luciferase reporter vector pRL-TK for normalization. Cortical neurons (a, b) were left untreated (0) or treated with 25 mM KCl for the time indicated. Data in (a, b) is presented as fold change in firefly luciferase activity normalized to Renilla luciferase activity measured from stimulated cells over that measured from untreated cells. All data were autoscaled. Means and standard deviations were calculated using results from three independent experiments performed in duplicates. Statistical significance shown with asterisks is relative to the luciferase activity measured from the cells transfected with EGFP plasmid (a) or empty vector pRC (b) at respective time point (Student's t-test *, $p<0.05$; **, $p<0.005$). minP, minimal promoter; NFAT-RE, NFAT response element.

**PUBLICATION III**

**Vihma H**, Timmusk T
Sumoylation regulates the transcriptional activity of different human NFAT isoforms in neurons.
Neurosci Lett. 2017;10;653:302-307. doi: 10.1016/j.neulet.2017.05.074

Neuroscience Letters 653 (2017) 302–307

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Research article

# Sumoylation regulates the transcriptional activity of different human NFAT isoforms in neurons

Hanna Vihma*, Tõnis Timmusk*

*Department of Chemistry and Biotechnology, Tallinn University of Technology, Akadeemia tee 15, 12618 Tallinn, Estonia*

## HIGHLIGHTS

- Sumoylation represses the transcriptional activity of $Ca^{2+}$-regulated NFAT proteins.
- Repression of the transcriptional activity of NFAT proteins is cell-type specific.
- In cortical neurons, sumoylation represses the transcriptional activity of NFATc1 and NFATc2 isoforms.
- In hippocampal neurons, sumoylation represses the transcriptional activity of NFATc1, NFATc2, and NFATc3 isoforms.
- Sumoylation represses the transcriptional activity of all NFATs in HEK293-FT cells.

## ARTICLE INFO

*Article history:*
Received 28 October 2016
Received in revised form 23 May 2017
Accepted 31 May 2017

*Keywords:*
NFAT $Ca^{2+}$-regulated transcription factor
Neuronal activity
Sumoylation
Transactivation
Neurons

## ABSTRACT

In the nervous system, four calcium/calcineurin-regulated members of the nuclear factor of activated T-cells (NFAT) family of transcription factors, NFATc1-c4, are involved in many developmental and functional processes, such as corticogenesis, synaptogenesis, synaptic plasticity and neurotransmission, that all need precise gene regulation. Therefore it is important to understand molecular events that contribute to the regulation of the transcriptional activity of specific NFAT isoforms. Previously, we have shown that there are a number of alternative splice variants of NFAT genes expressed in the brain and that neuronal activity leads to isoform-specific transactivation capacities of different human NFAT proteins. Here we looked at the effect of sumoylation as a possible regulator of the transcriptional activity of different human NFAT isoforms in rat primary cortical and hippocampal neurons in response to membrane depolarization and compared the results to those obtained from non-neuronal HEK293-FT and BHK-21 cells in response to calcium signaling. Our results show that in primary hippocampal neurons, sumoylation represses the transcriptional activity of NFATc1, NFATc2, and NFATc3 isoforms, whereas in cortical neurons, transactivation capacity of only NFATc1 and NFATc2 is repressed by sumoylation. In non-neuronal cells, however, transcriptional activity of all four NFAT isoforms is repressed by sumoylation in HEK293-FT cells, while only NFATc1 and NFATc2 isoforms are affected by sumoylation in BHK-21 cells. Altogether, our results show that sumoylation represses the transcription activation capacities of NFAT isoforms and that the effect is cell type-specific.



## 1. Introduction

Gene transcription is largely regulated by DNA sequence-specific transcription factors. Yet, various aspects of transcription factor function can be regulated by post-translational modifications (PTMs). In the case of nuclear factor of activated T-cells (NFAT), four members of this transcription factor family, NFATc1-c4, are highly modified by phosphorylation in their N-terminal regulatory domain, which keeps them inactive in the cytoplasm; dephosphorylation by the calcium/calmodulin-dependent protein phosphatase calcineurin triggers a conformational change exposing nuclear localization signals which allows transcriptionally active forms of NFATs to be translocated to the nucleus [1]. In the nucleus, NFAT proteins bind their DNA consensus sequence together with their nuclear partner proteins via C-terminal Rel homology domain (RHD), thereby regulating their target genes, which in the nervous system are involved in many developmental and functional processes such as corticogenesis, synaptogenesis, synaptic plasticity and neurotransmission [2]. The regulation of

* Corresponding authors.
*E-mail addresses:* hanna.vihma@ttu.ee (H. Vihma), tonis.timmusk@ttu.ee (T. Timmusk).

http://dx.doi.org/10.1016/j.neulet.2017.05.074
0304-3940/

NFATs' activity is achieved by several kinases that either act in the cytoplasm to maintain their phosphorylated state or in the nucleus by rephosphorylating NFATs and promoting their nuclear export [2]. Additionally, transcriptional activity of NFAT proteins has also been shown to be regulated by sumoylation [3–5].

Sumoylation is a lysine-targeted PTM in which the members of the small ubiquitin-like modifier (SUMO) family are covalently bound to target proteins. Similarly to ubiquitination, SUMO conjugation to targets is achieved by three enzymatic steps catalyzed by activating enzyme E1 (Sae1/Sae2 heterodimer in mammals), conjugating enzyme E2 (a single protein UBC9), and various E3 SUMO ligases, which help to improve SUMO conjugation and substrate selection. Sumoylation is a highly dynamic process, where deconjugation of SUMO is performed by SUMO proteases (nine in mammals), which differ in their subcellular localization and specificity towards SUMO paralogs [6]. Although sumoylation plays an important role in a wide range of cellular processes, it has emerged as an important regulator of neuronal and synaptic function [7]. The components of the sumoylation machinery are temporally and spatially regulated in the developing rat brain [8] and are redistributed upon membrane depolarization at hippocampal synapses [9]. These findings suggest that sumoylation is involved in neuronal differentiation in the developing brain and in synaptic plasticity in the adult – processes implicated also by NFAT proteins.

Previously, we have shown that in response to membrane depolarization, the transcriptional activity of different human NFAT proteins is isoform-specific in neurons [10]. For example, NFATc3 and NFATc4 are the strongest transcriptional activators in neurons, and NFATc1 and NFATc2 display isoform-specific transcription activation capacities [10]. Here, we studied whether sumoylation is involved in the regulation of the transcriptional activity of human NFAT proteins in neurons. For that, we mutated the predicted sumoylation sites of different human NFAT isoforms and studied the transactivation capacities of mutated and wild-type NFAT isoforms in rat primary cortical and hippocampal neurons in response to membrane depolarization. In addition, we analyzed the transactivation capacities of different wild-type and SUMO-mutant NFAT isoforms in non-neuronal HEK293-FT and BHK-21 cells treated with the calcium ionophore ionomycin in combination with the protein kinase C (PKC)-activating phorbol dibutyrate (PdBu).

## 2. Material and methods

### 2.1. Plasmid constructs

The generation of plasmid constructs encoding wild-type NFAT isoforms has been described elsewhere [10]. For mutating lysine residues to arginine of all predicted sumoylation sites on NFAT isoforms, we designed primers for site-directed mutagenesis (Supplementary Table 1). PCR reactions were performed using Expand High-Fidelity PCR Enzyme Mix (Roche Diagnostics, Risch-Rotkreuz, Switzerland) according to manufacturer's instructions. All constructs were confirmed by sequencing.

### 2.2. Cell culture

Human Embryonic Kidney 293-FT (HEK293-FT) cells (Invitrogen, Carlsbad, CA, USA) and Baby Hamster Kidney 21 (BHK-21) cells (ATCC, Manassas, VA, USA) were grown as described before [10,11]. Rat primary hippocampal and cortical neurons were cultured as before [10]. All cells were grown at 37 °C in a 5% $CO_2$ atmosphere.

### 2.3. Protein electrophoresis and Western blotting

HEK293-FT cells grown on a 6-well plate were transfected with 2 μg of DNA and 4 μg of polyethylenimine reagent (Sigma-Aldrich, St. Louis, MO, USA) per well at ~60–70% confluence. 36 h after transfection, sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blot were performed as described in [10].

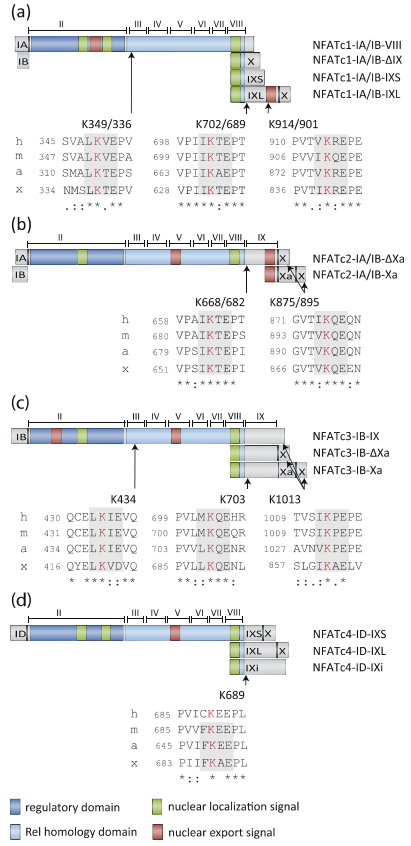



**Fig. 1.** Mapping sumoylation sites of human NFATc1-c4. Schematic representation of putative sumoylation sites of human NFATc1 (a), NFATc2 (b), NFATc3 (c), and NFATc4 (d) proteins together with multiple protein sequence alignments of sumoylation motifs of *Homo sapiens* (h), *Mus musculus* (m), *Anolis carolinensis* (a), and *Xenopus tropicalis* (x). Potential sumoylated lysines of NFATc1-c4 were predicted using GPS-SUMO algorithm [13] and are indicated with the arrow or highlighted in red in the sequence. Sumoylation motifs with high confidence scores are highlighted in grey. Sequence alignments were generated using Clustal Omega [15]. The names of the isoforms are according to [16]. Exons are numbered with Roman numbers.

### 2.4. Luciferase assay

For transfection of HEK293-FT and BHK-21 cells, 0.5 μg of DNA and 1 μg of polyethylenimine reagent (Sigma-Aldrich) were used per well of a 48-well plate at ~60–70% confluence. Neuronal cultures were transfected at 6 DIV using 0.5 μg of DNA and 1 μl of Lipofectamine 2000 reagent (Invitrogen) per well of a 48-well plate. Cells were cotransfected with equal amounts of human NFAT wild-type or SUMO mutant construct or empty EGFP vector, NFAT luciferase reporter plasmid pGL4.30[luc2P/NFAT-RE/Hygro] (Promega, Madison, WI, USA), and *Renilla* luciferase reporter vector pRL-TK (Promega) for normalization. Two days post-transfection, cultured neurons were stimulated with 25 mM KCl, and HEK293-FT and BHK-21 cells were stimulated with 1 μM ionomycin (AppliChem, Darmstadt, Germany) plus 1 μM PdBu

(Sigma-Aldrich) for 12 h. For studying the effect of sumoylation on endogenous NFAT-dependent transcription, 50 uM sumoylation inhibitor 2-D08 (Millipore, Billerica, MA, USA) or DMSO (0.1%) was added to the neurons one hour before cells were left untreated or treated with 25 mM KCl for 12 h. Luciferase assays were performed as described in [10]. At least three independent experiments were performed in duplicates. All data were log-transformed and means and standard deviations were calculated. Using GraphPad Prism 6 Software (GraphPad Software Inc., San Diego, CA, USA), analysis of statistical significance was determined by one-way ANOVA with Holm–Sidak pairwise comparisons between wild-type NFAT construct and its SUMO mutant (Fig. 2a–p and Supplementary Fig. 3a–d) or by Student's *t*-test (Fig. 2q–t) or by one-way ANOVA with Holm–Sidak post hoc test within data obtained with the wild-type and SUMO mutant constructs of each NFAT subfamily (Fig. 3 and Supplementary Fig. 3e and f). For graphical representation, the data were back-transformed to the original scale.

## 3. Results and discussion

### 3.1. Mapping sumoylation sites of human NFATc1-c4 proteins

Although most of the sumoylation sites follow a canonical consensus motif of ψ-K-x-E, where ψ is a hydrophobic amino acid and x any amino acid [12], Zhao and colleagues reported that about 40% of known sumoylation sites do not fit to the above consensus [13]. Using an updated *in silico* pattern recognition tool, we identified putative sumoylation sites in different human NFATc1-c4 isoforms (Fig. 1, [13]). Additionally, we analyzed the conservation of human NFAT sumoylation sites to the amino acid sequences of NFAT proteins from several vertebrates, including mammals (mouse; *Mus musculus*), reptiles (lizard; *Anolis carolinensis*), and amphibians (frog; *Xenopus tropicalis*) (Fig. 1). For human NFATc1, we identified three evolutionary conserved sumoylation sites with high confidence scores that correspond to K349, K702, and K914 in the longest NFATc1 isoform NFATc1-IA-IXL and to K336, K689, and K901 in NFATc1-IB-IXL isoform, respectively (Fig. 1a). Shorter isoforms NFATc1-IA-IXS and NFATc1-IB-IXS contain only two of these lysines, lacking the most C-terminal K914 or K901, respectively (Fig. 1a). Shortest NFATc1 isoforms NFATc1-IA-VIII, NFATc1-IA-ΔIX, NFATc1-IB-VIII, and NFATc1-IB-ΔIX contain only the most N-terminal lysine, K349 or K336, respectively (Fig. 1a). For human NFATc2, we identified two evolutionary conserved sumoylation sites with high confidence scores that are present in all NFATc2 isoforms analyzed (Fig. 1b). These sites correspond to K668 and K875 in isoforms NFATc2-IA-ΔXa and NFATc2-IA-Xa, and to K682 and K895 in isoforms NFATc2-IB-ΔXa and NFATc2-IB-Xa, respectively (Fig. 1b). For human NFATc3, we identified three evolutionary conserved sumoylation sites with high confidence scores that are present in all NFATc3 isoforms analyzed (Fig. 1c). These sites correspond to K434, K703, and K1013 in isoforms NFATc3-IB-IX, NFATc3-IB-ΔXa, and NFATc3-IB-Xa (Fig. 1c). For human NFATc4, we could identify one putative sumoylation site with moderate confidence score that corresponded to K689 in all three NFATc4 isoforms analyzed, NFATc4-ID-IXS, NFATc4-ID-IXL, and NFATc4-ID-IXi (Fig. 1d). Interestingly, in evolutionarily distant species, corresponding sequences fit this sumoylation consensus motif with high confidence scores and are very well conserved (Fig. 1d).

Our *in silico* prediction of NFAT sumoylation sites is in good agreement with previous experimental data. Using *in vitro* sumoylation assay, SUMO-1 has been shown to sumoylate K684 and K897 of mouse NFATc2 [3] and K435, K704, and K1013 of mouse NFATc3 [5]. SUMO-1 has been shown to sumoylate K349, K702, and K914 of human NFATc1 in HEK293-T cells [4] and K684 and K897 of mouse NFATc2 in BHK-21 cells [3]. Recent meta-analysis combined 22 published proteomics studies of human SUMO target proteins into a database [14]. Accordingly, NFATc1 (K349, K702, and K914) and NFATc2 (without specific sites) were identified as SUMO-2 targets, and NFATc4 (K689) as a SUMO-1 target [14]. Furthermore, multiple sequence alignment of human NFATc1-c4 proteins revealed homology between K702/689 of NFATc1-IA/IB, respectively, K668/682 of NFATc2-IA/IB, respectively, K703 of NFATc3, and K689 of NFATc4, and also between K914/901 of NFATc1-IA/IB, respectively, K875/895 of NFATc2-IA/IB, respectively, and K1013 of NFATc3 (Supplementary Fig. 1). These homologous sumoylation sites are all located near the C-terminal transactivation domain of NFATs [1]. The nonhomologous sumoylation sites of NFATc1 and NFATc3, K349/336 and K434, respectively, are both located in the DNA-binding domain of NFAT (Fig. 1 and Supplementary Fig. 1). NFATs are known to be highly phosphorylated in their N-terminal regulatory domain and bipartite phosphorylation-dependent sumoylation motifs (ψ-K-x-E-x-x-[pS]-P) and phosphorylated sumoylation motifs (ψ-K-x-[pS]-P) have been described. However, none of the predicted NFAT sumoylation sites are located in the regulatory domain or contain consensus for either of these motifs ([6], Supplementary Fig. 1). Therefore, the positions of sumoylation sites in NFATs indicate that SUMO conjugation may be involved in altering the transactivation capacities of NFAT proteins.

### 3.2. Analysis of the effect of sumoylation on the transcriptional activities of human NFATc1-c4 isoforms in primary rat neurons and in human non-neuronal cell lines

To analyze the effect of sumoylation on the transactivation capacities of NFAT isoforms we first mutated lysine residues to arginine of all putative sumoylation sites in different human NFAT isoforms and analyzed the expression levels of overexpressed wild-type and mutant NFATs in HEK293-FT cells by Western blotting (Supplementary Fig. 2). Next, we transfected a plasmid construct encoding firefly luciferase under the control of a minimal promoter with NFAT response elements (RE-s) together with one of the plasmid constructs encoding different EGFP-fused human NFATc1, NFATc2, NFATc3, NFATc4 wild-type or SUMO mutant isoforms into rat primary neurons (Figs. 2 and 3), HEK293-FT human embryonic kidney cells, and BHK-21 baby hamster kidney fibroblast cells (Supplementary Fig. 3). Our preliminary analysis, conducted in cortical and hippocampal neurons, included a representative human wild-type and its single and complete SUMO mutant NFAT isoforms from each NFAT subfamily: NFATc1-IA-IXL (Fig. 2a–d), NFATc2-IA-Xa (Fig. 2e–h), NFATc3-IB-IX (Fig. 2i–l), and NFATc4-ID-IXL (Fig. 2m–p) (Fig. 2a–p). We measured the luciferase activities at basal level (Fig. 2a, c, e, g, i, k, m, o) and also after 12-h of membrane depolarization with high levels of extracellular potassium (Fig. 2b, d, f, h, j, l, n, p), and analyzed whether the transactivation capacity of wild-type NFAT isoforms differ from their SUMO mutants. Additionally, we used sumoylation inhibitor 2-D08, a cell-permeable compound that prevents the transfer of SUMO from the E2 conjugating enzyme to the substrate, to study whether inhibition of sumoylation has an effect on the endogenous NFAT-dependent transcription in rat primary cortical and hippocampal neurons at basal level and after membrane depolarization (Fig. 2q–t). To confirm the sumoylation effects of representative NFAT isoforms found in hippocampal and cortical neurons, we conducted similar analysis in cortical neurons by comparing the transactivation capacities of all the 18 NFAT wild-type protein isoforms used in our previous study [10] to their complete SUMO mutants at basal level (Fig. 3a, c, e, g) and after membrane depolarization (Fig. 3b, d, f, h). Similar luciferase reporter assays were conducted also in non-neuronal HEK293-FT and BHK-21 cells to see whether the effects of sumoylation on the transactivation capacities of representative

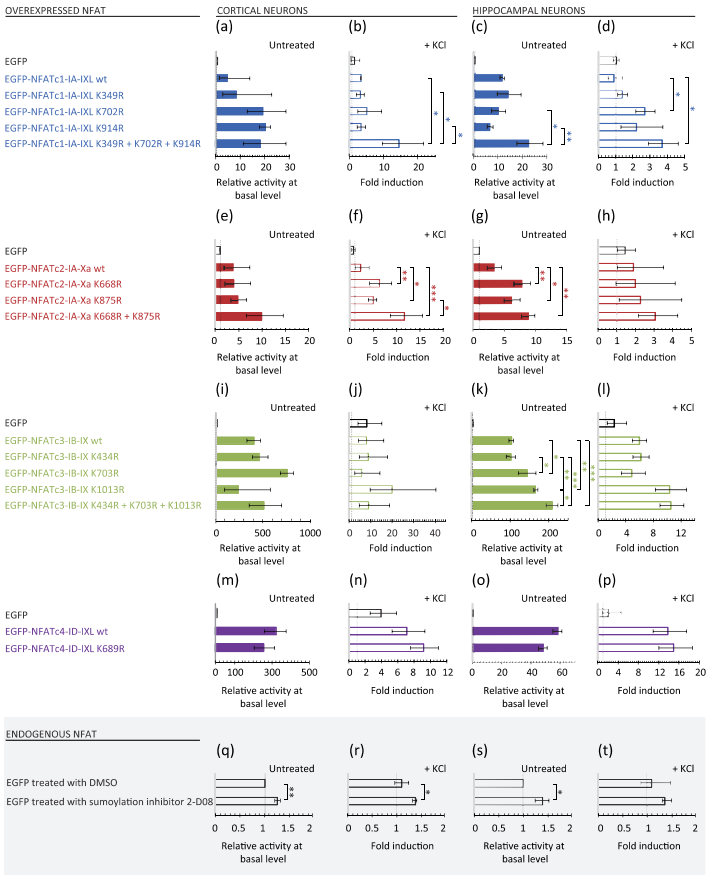



**Fig. 2.** Transcriptional activities of (a-p) representative EGFP-tagged human NFAT wild-type and SUMO mutant isoforms and (q–t) endogenous NFATs with or without sumoylation inhibitor 2-D08 in cultured rat primary cortical and hippocampal neurons. In (a–p), results of luciferase reporter assays with different EGFP-tagged wild-type and SUMO mutant human NFAT isoforms in cortical and hippocampal neurons are shown. In (q–t), results of luciferase reporter assays detecting endogenous NFAT activity with or without sumoylation inhibitor 2-D08 in cortical (q–r) and hippocampal neurons (s–t) are shown. Two days post transfection, cells were left untreated or treated with 25 mM KCl for 12 h, and luciferase activities were measured. In (q–t), 50 uM 2-D08 or DMSO (0.1%) was added to the neurons one hour before cells were left untreated (q, s) or treated with 25 mM KCl for 12 h (r, t). Data in untreated cells are presented as fold change relative to EGFP-expressing untreated cells. In KCl treated cells, fold change relative to untreated cells is shown. Error bars represent standard deviation. In (a–p), statistical significance shown with asterisks between bars was determined by one-way ANOVA with Holm-Sidak post-hoc test within data obtained with the wild-type and SUMO mutant constructs of each NFAT subfamily, and in (q–t) determined by Student's *t*-test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). EGFP, enhanced green fluorescent protein.

NFAT isoforms are neuron-specific (Supplementary Fig. 3). For activating the NFAT signaling pathways in non-neuronal cells, we used calcium ionophore ionomycin together with PKC-activating PdBu (Supplementary Fig. 3b, d, f) as we have used previously [10]. To confirm that NFAT proteins are sumoylated in our cellular models we also analyzed sumoylation of overexpressed representative NFAT isoforms in HEK293-FT cells and cortical neurons (Supplementary Fig. 4a and b).

Our results showed that in basal conditions there were no differences in NFAT RE controlled promoter activation for representative wild-type and SUMO mutant NFATc1-IA-IXL isoforms in cortical neurons (Fig. 2a). However, in response to membrane depolarization, there were significant differences between wild-type and triple mutant K349R and triple mutant, and K914R and triple mutant, NFATc1-IA-IXL isoforms (Fig. 2b). In hippocampal neurons, however, we observed significant differences of NFAT RE controlled promoter activation already at basal level between K702R and triple mutant, and between K914R and triple mutant NFATc1-IA-IXL isoforms (Fig. 2c). Membrane depolarization of hippocampal neurons resulted in significant differences in the activation of NFAT RE controlled promoter by wild-type and triple mutant, and wild-type and K702R NFATc1-IA-IXL isoforms, respectively (Fig. 2d). Interestingly, none of the single SUMO site mutations affected the transactivation capacity of NFATc1-IA-IXL isoform in cortical neurons, whereas in hippocampal neurons K702R mutation strongly up-regulated the reporter activity compared to wild-type NFATc1. This was also observed when similar assay with all the NFATc1 wild-type and SUMO mutant isoforms was conducted in cortical neurons (Fig. 3). Isoforms NFATc1-IA/IB-VIII and NFATc1-IA/IB-ΔIX that contain only the most N-terminal sumoylation site displayed no differences in transactivation capacities between wild-type and SUMO mutant at basal level nor after membrane depolarization (Fig. 3a and b). In agreement with our data, Nayak and colleagues have shown that lysines K702 and K914 are the most important sites for NFATc1 sumoylation in HEK293-T cells and K349 is irrelevant or dependent on other two sumoylation sites [4]. Overall, our

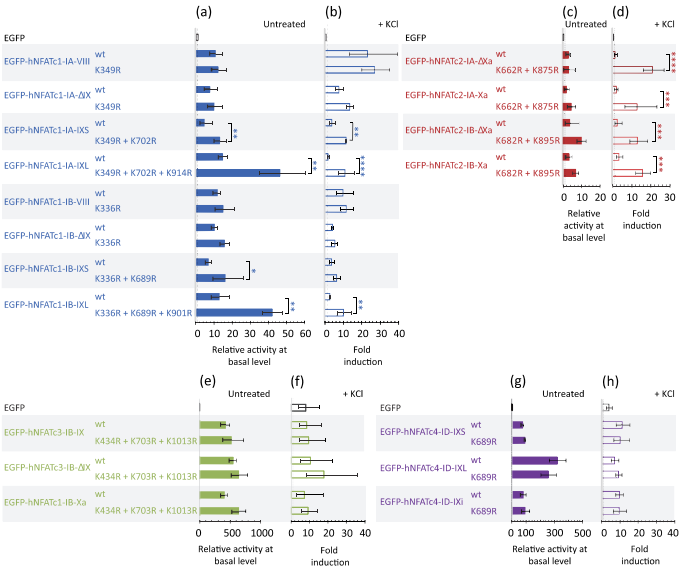



**Fig. 3.** Transcriptional activities of EGFP-tagged human NFAT wild-type and SUMO mutant isoforms in cultured rat primary cortical neurons. Results of luciferase reporter assays with different EGFP-tagged wild-type and SUMO mutant human NFAT isoforms are shown. Two days post transfection, cells were left untreated or treated with 25 mM KCl for 12 h, and luciferase activities were measured. Data in untreated cells are presented as fold change relative to EGFP-expressing untreated cells. In KCl treated cells, fold change relative to untreated cells is shown. Error bars represent standard deviation. Statistical significance shown with asterisks between bars was determined by one-way ANOVA with Holm–Sidak pairwise comparisons between wild-type NFAT construct and its SUMO mutant (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$). EGFP, enhanced green fluorescent protein.

results demonstrate that sumoylation represses the transcriptional activity of NFATc1 in both cortical and hippocampal neurons. This has also been shown by others in HEK293-T cells [4] and confirmed by us in this study in HEK293-FT (Supplementary Fig. 3a and b) and BHK-21 cells (Supplementary Fig. 3c and d).

For the representative NFATc2 isoform NFATc2-IA-Xa, we detected statistically significant differences in NFAT RE controlled luciferase expression between wild-type and all studied SUMO mutants in depolarized cortical neurons (Fig. 2f) and in untreated hippocampal neurons (Fig. 2g). Similar results were obtained for all four NFATc2 isoforms analyzed in cortical neurons where sumoylation significantly repressed NFATc2 transcriptional activity in depolarized neurons (Fig. 3d). Therefore, we can conclude that sumoylation of NFATc2 inhibits its transcriptional activity in both cortical and hippocampal neurons. Contrary to our findings, Terui and colleagues have reported that sumoylation of mouse NFATc2 positively regulates its transcriptional activity in BHK-21 cells [3]. Although, generally, sumoylation has been shown to have both negative and positive effects on transcription factor activity [6], we could not replicate the results of Terui and colleagues neither in HEK293-FT cells (Supplementary Fig. 3a and b) nor BHK-21 cells (Supplementary Fig. 3c–f) where NFATc2 SUMO mutants had higher transcriptional activity than wild-type isoforms.

NFATc3 wild-type and SUMO mutant isoforms displayed no differences in transcriptional activity in cortical neurons when representative NFATc3-IB-IX isoform (Fig. 2i and j) or all three NFATc3 isoforms were analyzed (Fig. 3e and f). In untreated hippocampal neurons, however, we detected significantly lower transcriptional activity of the wild-type representative NFATc3-IB-IX isoform compared to its SUMO mutants (Fig. 2k). Our results also showed no significant effect of the single SUMO mutation K434R on NFATc3-IB-IX transcriptional activity in hippocampal neurons (Fig. 2k and l). This is in agreement with published data showing that mutation of the most N-terminal sumoylation site of murine NFATc3 did not significantly affect its sumoylation analyzed by *in vitro* sumoylation assay [5]. Our results using non-neuronal cells showed that mutation of sumoylation sites in NFATc3 increased its transcriptional activity in HEK293-FT cells but has no effect in BHK-21 cells (Supplementary Fig. 3a–d). Interestingly, it has been shown that in BHK-21 cells SUMO mutant NFATc3 displayed differences in subcellular localization and transcriptional activity only when co-expressed with Trim17, a protein involved in neuronal apoptosis, that binds NFATc3 SUMO-dependently [5]. Collectively, our data indicate that sumoylation regulates transcriptional activity of NFATc3 isoforms in a cell-specific manner.

Similarly to NFATc3, NFATc4 also displayed cell type-specific effect of sumoylation on its transcriptional activity. In neurons, however, mutation of K689 had no effect on the transactivation capacities of the NFATc4 isoforms analyzed (Fig. 2m–p and Fig. 3g and h). We only observed a differences in transactivation capacities between wild-type NFATc4-ID-IXL and its SUMO mutant in non-neuronal HEK293-FT cells at basal level (Supplementary Fig. 3a). To date, only a meta-analysis of previously published mass spectrometry data have identified K689 of human NFATc4 as a SUMO-1 target in HeLa cells [14] which could mean that sumoylation of NFATc4 is a rare event.

Inhibition of sumoylation with 2-D08 enhanced endogenous NFAT-dependent transcriptional activity in basal conditions in both hippocampal and cortical neurons (Fig. 2q and s) and after membrane depolarization in cortical neurons (Fig. 2r). This is in good agreement with our findings showing that mutation of sumoylation sites activates the transcriptional activity of overexpressed NFAT isoforms in hippocampal and cortical neurons. In addition, analysis of sumoylation of overexpressed representative NFAT isoforms showed that all analyzed NFAT proteins were sumoylated in HEK293-FT cells (Supplementary Fig. 4a). In cortical neurons the representative NFATc1 and NFATc2 isoforms were sumoylated, with higher levels in KCl-treated neurons. However, sumoylation of

the representative NFATc3 and NFATc4 isoforms was not detected in cortical neurons (Supplementary Fig. 4b). This is in good agreement with our results showing that mutation of sumoylation sites activates the transcriptional activity of all representative NFAT isoforms in HEK293-FT cells while in cortical neurons only the activities of NFATc1 and NFATc2 are increased.

In conclusion, in this study we have shown that although in general sumoylation represses the transcriptional activity of NFATc1-c4, the effect of sumoylation on NFAT isoforms varies between cell types. It has been shown that SUMO activating enzymes SAE1/SAE2 and conjugating enzyme Ubc9 are sufficient for protein sumoylation [12], however, under physiological conditions SUMO substrate selection is improved by SUMO ligases [6]. We propose that the cell type-specific effect of sumoylation on the transactivation capacities of different NFATs results from differential expression pattern of substrate specific SUMO ligases or proteases.

## Acknowledgements

We thank Epp Väli for technical assistance and Jürgen Tuvikene for help in statistical analysis and critical comments on the manuscript. This project was supported by Estonian Research Council (IUT19-18) and European Union through the European Regional Development Fund (Project No. 2014–2020.4.01.15-0012).

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neulet.2017.05.074.

## References

[1] G.P. Mognol, F.R.G. Carneiro, B.K. Robbs, D.V. Faget, J.P.B. Viola, Cell cycle and apoptosis regulation by NFAT transcription factors: new roles for an old player, Cell. Death. Dis. 7 (2016) e2199, http://dx.doi.org/10.1038/cddis.2016.97.

[2] M.J. Kipanyula, W.H. Kimaro, P.F.S. Etet, The emerging roles of the calcineurin-nuclear factor of activated T-lymphocytes pathway in nervous system functions and diseases, J. Aging Res. 2016 (2016) e5081021, http://dx.doi.org/10.1155/2016/5081021.

[3] Y. Terui, N. Saad, S. Jia, F. McKeon, J. Yuan, Dual role of sumoylation in the nuclear localization and transcriptional activation of NFAT1, J. Biol. Chem. 279 (2004) 28257–28265, http://dx.doi.org/10.1074/jbc.M403153200.

[4] A. Nayak, J. Glöckner-Pagel, M. Vaeth, J.E. Schumann, M. Buttmann, T. Bopp, E. Schmitt, E. Serfling, F. Berberich-Siebelt, Sumoylation of the transcription factor NFATc1 leads to its subnuclear relocalization and interleukin-2 repression by histone deacetylase, J. Biol. Chem. 284 (2009) 10935–10946, http://dx.doi.org/10.1074/jbc.M900465200.

[5] B. Mojsa, S. Mora, J.P. Bossowski, I. Lassot, S. Desagher, Control of neuronal apoptosis by reciprocal regulation of NFATc3 and Trim17, Cell Death Differ. 22 (2015) 274–286, http://dx.doi.org/10.1038/cdd.2014.141.

[6] P. Chymkowitch, P.A. Nguéa, J.M. Enserink, SUMO-regulated transcription: challenging the dogma, Bioessays 37 (2015) 1095–1105, http://dx.doi.org/10.1002/bies.201500065.

[7] J.M. Henley, T.J. Craig, K.A. Wilkinson, Neuronal SUMOylation: mechanisms, physiology, and roles in neuronal dysfunction, Physiol. Rev. 94 (2014) 1249–1285, http://dx.doi.org/10.1152/physrev.00008.2014.

[8] C. Loriol, J. Parisot, G. Poupon, C. Gwizdek, S. Martin, Developmental regulation and spatiotemporal redistribution of the sumoylation machinery in the rat central nervous system, PLoS One 7 (2012), http://dx.doi.org/10.1371/journal.pone.0033757.

[9] C. Loriol, A. Khayachi, G. Poupon, C. Gwizdek, S. Martin, Activity-dependent regulation of the sumoylation machinery in rat hippocampal neurons, Biol. Cell. 105 (2013) 30–45, http://dx.doi.org/10.1111/boc.201200016.

[10] H. Vihma, M. Luhakooder, P. Pruunsild, T. Timmusk, Regulation of different human NFAT isoforms by neuronal activity, J. Neurochem. 137 (2016) 394–408, http://dx.doi.org/10.1111/jnc.13568.

[11] K. Ratnik, L. Viru, A. Merits, Control of the rescue and replication of semliki forest virus recombinants by the insertion of miRNA target sequences, PLoS One 8 (2013) e75802, http://dx.doi.org/10.1371/journal.pone.0075802.

[12] M.S. Rodriguez, C. Dargemont, R.T. Hay, SUMO-1 conjugation in vivo requires both a consensus modification motif and nuclear targeting, J. Biol. Chem. 276 (2001) 12654–12659, http://dx.doi.org/10.1074/jbc.M009476200.

[13] Q. Zhao, Y. Xie, Y. Zheng, S. Jiang, W. Liu, W. Mu, Z. Liu, Y. Zhao, Y. Xue, J. Ren, GPS-SUMO: a tool for the prediction of sumoylation sites and SUMO-interaction motifs, Nucleic Acids Res. 42 (2014) W325–330, http://dx.doi.org/10.1093/nar/gku383.

[14] I.A. Hendriks, A.C.O. Vertegaal, A comprehensive compilation of SUMO proteomics, Nat. Rev. Mol. Cell Biol. 17 (2016) 581–595, http://dx.doi.org/10.1038/nrm.2016.81.

[15] F. Sievers, A. Wilm, D. Dineen, T.J. Gibson, K. Karplus, W. Li, R. Lopez, H. McWilliam, M. Remmert, J. Söding, J.D. Thompson, D.G. Higgins, Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega, Mol. Syst. Biol. 7 (2011) 539, http://dx.doi.org/10.1038/msb.2011.75.

[16] H. Vihma, P. Pruunsild, T. Timmusk, Alternative splicing and expression of human and mouse NFAT genes, Genomics 92 (2008) 279–291, http://dx.doi.org/10.1016/j.ygeno.2008.06.011.

**SUPPLEMENTARY DATA**

**Supplementary Table 1**. Primers used for human NFATc1-c4 site-directed mutagenesis

| Mutant name | Forward primer sequence | Reverse primer sequence |
|---|---|---|
| NFATc1-IA/IB K349/336R | CTCAGTGGCGCTCAGGGTGGAG | TCCACCCTGAGCGCCACTGAG |
| NFATc1-IA/IB K702/689R | CCAATTATAAGAACAGAACCCAC | GTGGGTTCTGTTCTTATAATTGG |
| NFATc1-IA/IB K914/901R | CTATTCCTGTAACGGTCAGGCGAGAG | CTCTCGCCTGACCGTTACAGGAATAG |
| NFATc2-IA/IB K668/682R | GTCCCAGCCATCAGGACGGAGCC | GGCTCCGTCCTGATGGCTGGGAC |
| NFATc2-IA/IB K875/895R | GTGACCATTAGACAGGAGCAGAAC | GTTCTGCTCCTGTCTAATGGTCAC |
| NFATc3-IB K434R | AACTGCGAATAGAAGTGCAACCTAAAA | TTTTAGGTTGCACTTCTATTCGCAGTT |
| NFATc3-IB K703R | GATGCGGCAAGAACACAGAGAAGAG | CTCTTCTCTGTGTTCTTGCCGCATC |
| NFATc3-IB K1013R | CAACTGTGAGCATTCGACCTGAACC | GTTCAGGTCGAATGCTCACAGTTG |
| NFATc4-ID K689R | TGATCTGCCGAGAGGAGCCCCTA | TAGGGGCTCCTCTCGGCAGATCA |

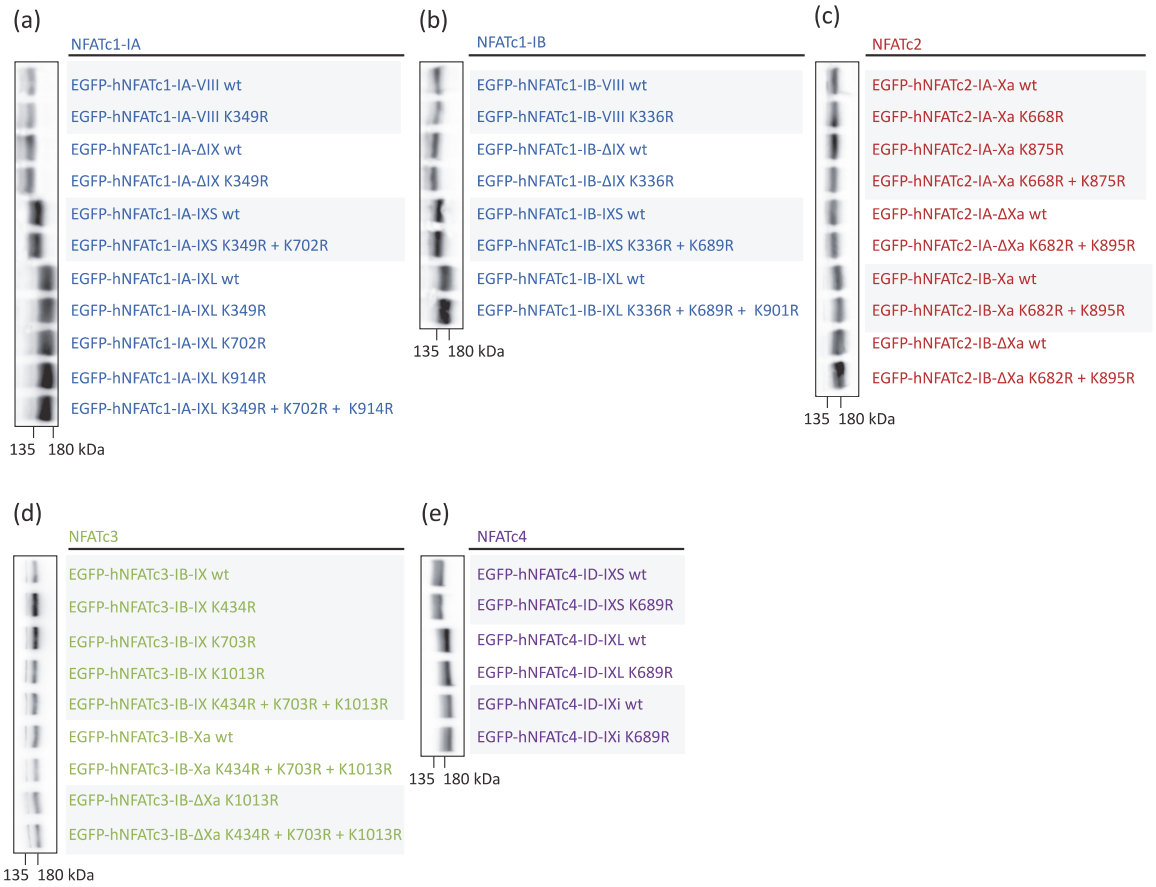
## Supplementary Figure 1

```
hNFATc1      MPSTSF--PVPSKFPLGPAAAVFGRGETLGPAPRAGGTMKSAE-EE-----HYGYASSNV
hNFATc2      -----M--QREAAFRLGHCHPLR-----------IMGSVDQEE-PN-----AHKVASPPS
hNFATc3      MTTANCGAHDELDFK-----LVFGEDGAPAPPP-PGSRPADLEPDDCASIYIFNVDPPPS
hNFATc4      MGAASC-EDEELEFK-----LVFGEEKEAPPLG-AGGLGEELDSEDAPPCCRLALGEPPP
                          *       :             .   . :  :

hNFATc1      SPALPLPTAHSTLPAPCHNLQTSTPGIIPPADHPSGYGA-----ALDGGPAGYFLSSGHT
hNFATc2      GPAYPDDVLDY-------GLKPYSPLASLSGEPPGRFGE-----PDRVGPQKFLSA---A
hNFATc3      TLTTPLCLPHHGLPSHSSVLSPSFQLQ-----SHKNYE------GTCEIPESKYS-----
hNFATc4      YGAAPIGIPRPPPPRPGMHSPPPRPAP-----SPGTWESQPARSVRLGGPGGGAG-----
               : *                                :           *

hNFATc1      RPDGAPALESPRIEITSCLGLYHNNNQFF-HD--VEVEDVLPSSK-RSPSTATLSLPSLE
hNFATc2      KPAGASG-LSPRIEITPSHELIQAVGPLRMRDAGLLVEQPPLAGV-AASPRFTLPVPGFE
hNFATc3      PLGGPKPFECPSIQITSISPNCHQE--LDAHEDDLQINDP-EREFLERPSRDHLYLPLEP
hNFATc4      GAGGGRVLECPSIRITSISPTPEPP--AALEDNPDAWGDGSPRDYP-PPEGFGGYREAGG
                *     .* *.**      .       .:      :

hNFATc1      AYRDPSCLSP---ASSLSSRSCNSEASSYESNYSYP-----------YASPQTSPWQSPC
hNFATc2      GYREPLCLSP---ASSGSSAS-------------------------FISDTFSPYTSPC
hNFATc3      SYRE-SSLSPSP-ASSISSRSWFSDASSCESLSHIYDDVDSELNEAAARFTLGSPLTSPG
hNFATc4      QGGG-AFFSPSPGSSSLSSWSFFSDASDEAALYAACDEVESELNEAASRFGLGSPLPSPR
                    :**   :** ** *                                **  **

hNFATc1      VSPKTTDPEEGFPRGLGACTLLGSPRHSPSTSPRASVTEESWLGARSSRPAS-------P
hNFATc2      VSPNNGGPDDLCPQF-QNIPAHYSPRTSPIMSPRTSLAEDSCLGRHSPVPRP-ASRSSSP
hNFATc3      GSPGGCPGEETWHQQYGL-GHSLSPRQSPCHSPRSSVTDENWLSPR--PASGPSSRPTSP
hNFATc4      ASPRPWTPEDPWSLY-GP-------------SPGGRGPEDSWLLLSAPGPTPASPRPASP
              **     ::                     **     ::. *                *

hNFATc1      CNKRKYSLN----GRQPPYSPHHSPTPSPHGSPRVSVTDDS-WLGNTTQYTS----SAIV
hNFATc2      GAKRRHSCAEALVALPPGASPQRSRSPSPQPSSHVAPQDHGSPAGYPPVAGS----AVIM
hNFATc3      CGKRRHSSAEVCY--AGSLSPHHSPVPSPGHSPRGSVTEDT-WLNA-----SVHGGSGLG
hNFATc4      CGKRRYSSSGTPS--SA----------SPALSRRGSLGEEG-SEPPPPPPLPLARDPG-S
               **::*                    **  * : :  :.

hNFATc1      AAINALTTDSSLDLGDGVPVKSRKTTLEQPPSVALKVEPVGEDLGSPPPPADFAPEDYSS
hNFATc2      DALNSLATDSPC----GIPPKMWKTSPDPSP------------VSAAPSKAGLPRHIYPA
hNFATc3      PAVFPF-QYC---VETDIPLKTRKTSEDQAAILPGKLELCSDDQGSLSPARETSIDDGLG
hNFATc4      --PGPF-DYVGAPPAESIPQKTRRTSSEQAVALPRSEEPASC-NGKLPLGAEE----SVA
                  :           :* *  :*: :                .            .

hNFATc1      FQ-HIRKGGFCDQYL-------AVPQHPYQWAKPKPLS---PTSYMSPTLPALDWQLPSH
hNFATc2      ----VEFLGPCEQGERRNSAPESILLVPPTWPKPLVPAIPICSIPVTASLPPLEWPLSSQ
hNFATc3      SQYPLKKDSCGDQFL--------SVPSPFTWSKPK-PGH--TPIFRTSSLPPLDWPLPAH
hNFATc4      PPGGSRKEVAGMDYL--------AVPSPLAWSKARIGGH--SPIFRTSALPPLDWPLPSQ
                  .      :             *  * *    .        : :** *:* * ::
```

**Supplementary Figure 1 (continued)**

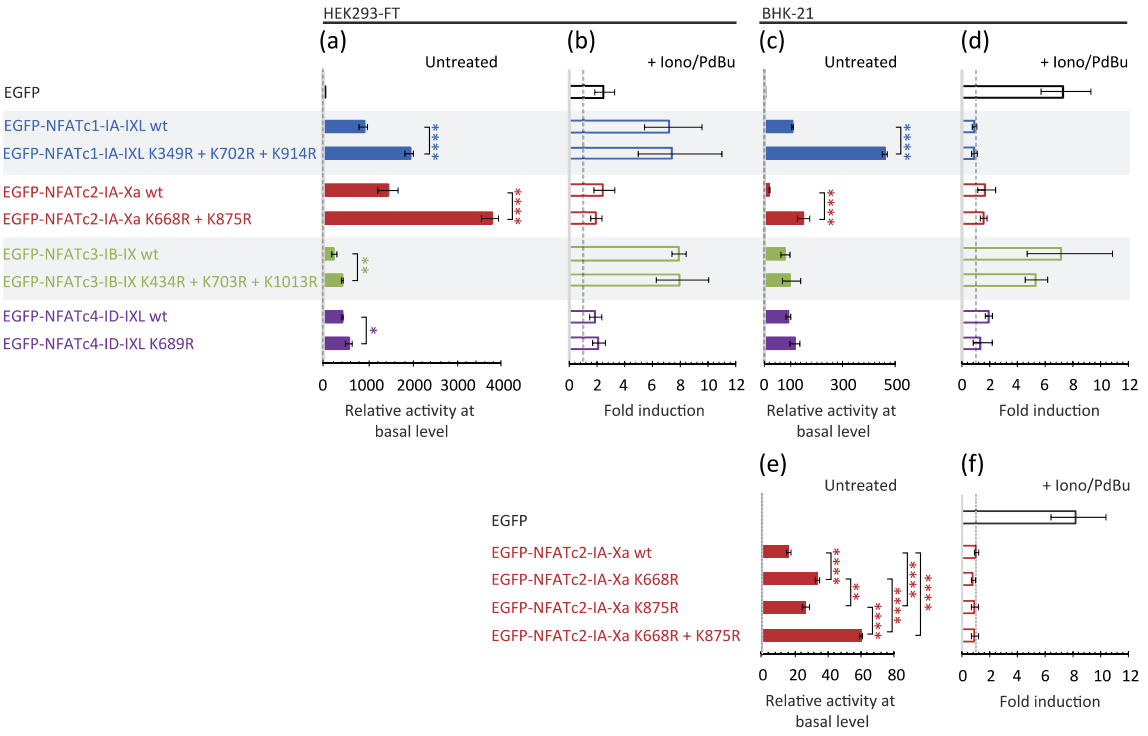

Supplementary Figure 1. Alignment of the human NFAT protein isoforms. Multiple protein sequence alignment of representative human isoforms NFATc1-IA-IXL, NFATc2-IA-Xa, NFATc3-IB-IX, and NFATc4-ID-IXL was carried out using Clustal Omega [1]. Putative sumoylated lysines of NFATc1-c4 were predicted using GPS-SUMO algorithm [2] and are highlighted in red. Sumoylation motifs with high confidence scores are highlighted in grey.

**Supplementary Figure 2**



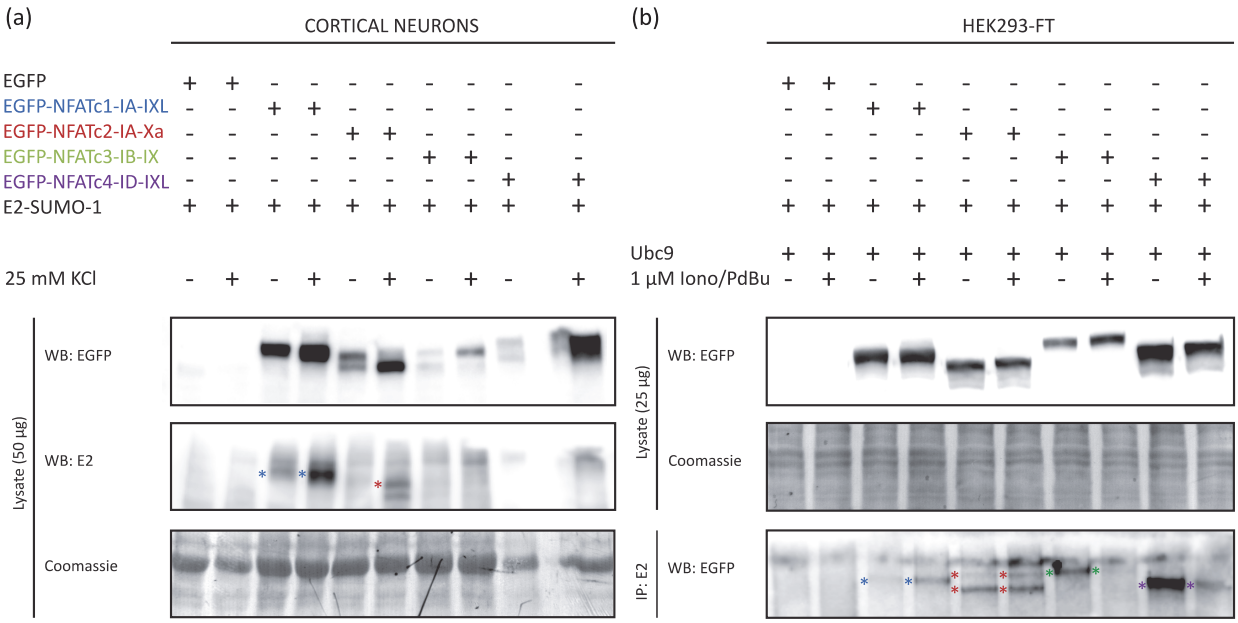

Supplementary Figure 2. Western blot analysis of EGFP-tagged nuclear factor of activated T-cells (NFAT) isoforms used in this study. Human NFATc1-IA (a), NFATc1-IB (b), NFATc2 (c), NFATc3 (d), and NFATc4 (e) wild-type and SUMO mutant isoforms were overexpressed in HEK293-FT cells. Equal amounts of protein were separated in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After transfer to polyvinylidene fluoride membranes, proteins were probed with rabbit anti-EGFP antibody (1:200 000, gift from Andres Merits) and subsequently visualized with horseradish peroxidase- conjugated antibody. EGFP, enhanced green fluorescent protein; kDA, kilodalton.

**Supplementary Figure 3**




Supplementary Figure 3. Transcriptional activities of representative EGFP-tagged human NFAT wild-type and SUMO mutant isoforms in HEK293-FT and BHK-21 cells. Results of luciferase reporter assays with different EGFP-tagged wild-type and SUMO mutant human NFATc1, NFATc2, NFATc3, and NFATc4 isoforms in HEK293-FT cells (a and b) and BHK-21 cells (c, d, e, f) are shown. HEK293-FT or BHK-21 cells were transfected with either EGFP-encoding or the indicated NFAT isoform-encoding wild-type or SUMO mutant plasmid together with a firefly luciferase construct carrying three tandem NFAT response elements in front of a minimal promoter, and *Renilla* luciferase reporter vector pRL-TK for normalization. 24 hours later, cells were left untreated (a, c, e) or treated with 1 µM ionomycin and 1 µM PdBu for 12 hours (b, d, f) and luciferase activities were measured. Data in (a, c, e) are presented as fold difference in firefly luciferase activity normalized to *Renilla* luciferase activity measured from NFAT wild-type or SUMO mutant isoform expressing untreated cells over that measured from EGFP-expressing untreated cells. In (b, d, f), fold change in firefly luciferase activity normalized to *Renilla* luciferase activity measured from treated cells over that measured from untreated cells is shown. Results of three independent experiments performed in duplicates are show. Error bars represent standard deviation. Statistical significance shown with asterisks between bars was determined by one-way ANOVA with Holm-Sidak post-hoc test within data obtained with the wild-type and SUMO mutant constructs of each NFAT subfamily (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$). EGFP, enhanced green fluorescent protein; Iono, ionomycin; PdBu, phorbol 12,13-dibutyrate.

**Supplementary Figure 4**




Supplementary Figure 4. Analysis of NFAT sumoylation in (a) rat primary cortical neurons and in (b) HEK293-FT cells. (a) Western blot analysis of representative EGFP-tagged NFAT isoforms overexpressed in rat primary cortical neurons together with E2-tagged SUMO-1 protein. Rat primary cortical neurons were grown on a 6-well plate and transfected at 6DIV using 1.5 µg of DNA and 3 µl of Lipofectamine 2000 reagent (Invitrogen) per well. Cells were cotransfected with equal amounts of human NFATc1-IA-IXL, NFATc2-IA-Xa, NFATc3-IB-IX or NFATc4-ID-IXL construct or empty EGFP vector, and E2-SUMO-1 construct. E2-SUMO-1 construct was generated by inserting SUMO-1 coding sequence from pSG5-His-SUMO-1 plasmid (Addgene plasmid #17271) into pQM-CMV-E2-N vector (Icosagen) behind the E2-tag. 24 hours after transfection cells were stimulated with 25 mM KCl. 12 hours later cells were collected and lysed on ice using RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate) supplemented with 0.2% SDS, protease inhibitor cocktail Complete (Roche Applied Science) and 20 mM N-Ethylmaleimide (Sigma-Aldrich). The extracts were sonicated and centrifuged to remove insoluble cell debris. 50 µg of proteins were separated in 4-15% Mini-PROTEAN TGX Precasat Gel (Bio-Rad Laboratories) and Western blot were performed as described in [3]. The blotted bands were immunodetected with rabbit anti-EGFP antibody (gift from professor Andres Merits) and subsequently visualized with horseradish peroxidase- conjugated anti rabbit antibody. Before reprobing the membrane with mouse monoclone HRP-conjugated anti E2 antibody (1:5000, Icosagen, 5E11), HRP activity was inactivated using 30% H2O2 (Sigma-Aldrich) as described in [4]. Coomassie staining was used as loading control. Asterisks indicate sumoylated NFAT proteins. (b) Co-immunoprecipitation assay using HEK293-FT cells overexpressing EGFP-tagged NFAT isoforms, E2-tagged SUMO-1 and sumoylation conjugating enzyme Ubc9. HEK293-FT cells grown on 10 cm culture dish were transfected with 20 µg of DNA and 40 µg of polyethylenimine reagent (Sigma-Aldrich) per well at ~60-70% confluence. Cells were cotransfected with equal amounts of human NFATc1-IA-IXL, NFATc2-IA-Xa, NFATc3-IB-IX or NFATc4-ID-IXL construct or empty EGFP vector, E2-SUMO-1 construct, and pcDNA3-Ubc9 construct (Addgene plasmid #20082). 24 hours after transfection cells were stimulated with 1 µM ionomycin plus PdBu. 12 hours later cells were collected and lysed on ice using RIPA buffer supplemented with 0.1% SDS, protease inhibitor cocktail Complete and 20 mM N-Ethylmaleimide. The extracts were sonicated and centrifuged to remove insoluble cell debris. 1.5 mg of protein lysate was brought to 1500 µl using RIPA buffer supplemented with protease inhibitor cocktail Complete and 20 mM N-Ethylmaleimide. Immunoprecipitation was carried out by incubating the extracts with 1.5 ug E2 antibody (Icosagen, 5E11) immobilized onto Protein A Sepharose beads (GE Healthcare) overnight at 4°C. Beads were washed four times with RIPA buffer and bead-bound complexes were eluted by boiling in SDS loading buffer for 5 minutes. The immunoprecipitates and 25 ug of input were separated in 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blot were performed as described in [3]. The blotted bands were immunodetected with rabbit anti-EGFP antibody and subsequently visualized with horseradish peroxidase-conjugated anti rabbit antibody. Coomassie staining was used as loading control. Asterisks indicate sumoylated NFAT proteins. EGFP, enhanced green fluorescent protein.

## References


[1] F. Sievers, A. Wilm, D. Dineen, T.J. Gibson, K. Karplus, W. Li, R. Lopez, H. McWilliam, M. Remmert, J. Söding, J.D. Thompson, D.G. Higgins, Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega, Mol. Syst. Biol. 7 (2011) 539. doi:10.1038/msb.2011.75.

[2] Q. Zhao, Y. Xie, Y. Zheng, S. Jiang, W. Liu, W. Mu, Z. Liu, Y. Zhao, Y. Xue, J. Ren, GPS-SUMO: a tool for the prediction of sumoylation sites and SUMO-interaction motifs, Nucleic Acids Res. 42 (2014) W325-330. doi:10.1093/nar/gku383.

[3] H. Vihma, M. Luhakooder, P. Pruunsild, T. Timmusk, Regulation of different human NFAT isoforms by neuronal activity, J. Neurochem. 137 (2016) 394–408. doi:10.1111/jnc.13568.

[4] A.D. Sennepin, S. Charpentier, T. Normand, C. Sarré, A. Legrand, L.M. Mollet, Multiple reprobing of Western blots after inactivation of peroxidase activity by its substrate, hydrogen peroxide, Anal. Biochem. 393 (2009) 129–131. doi:10.1016/j.ab.2009.06.004.

## *CURRICULUM VITAE*


| | |
|---|---|
| Name: | Hanna Vihma |
| Date and place of birth: | 15.01.1984, Tallinn |
| Citizenship: | Estonian |


### Contact information


Department of Chemistry and Biotechnology, Tallinn University of Technology, Akadeemia tee 15, 12618 Tallinn
Tel.: +372 5527668
Email: hanna.vihma@gmail.com, hanna.vihma@ttu.ee


### Education

| | |
|---|---|
| 2008-… | Tallinn University of Technology, Department of Gene Technology, doctoral studies |
| 2005-2007 | Tallinn University of Technology, Department of Gene Technology, Master of Science (MSc) |
| 2002-2005 | University of Tartu, Institute of Molecular and Cell Biology, Bachelor's degree (BSc) |
| 1999-2002 | Collegium Educationis Revaliae Gymnasium |
| 1990-1999 | Tallinn Music High School |

### Employment

| | |
|---|---|
| 2017-… | Tallinn University of Technology, School of Science, Department of Chemistry and Biotechnology, engineer |
| 2008-2016 | Tallinn University of Technology, Department of Gene Technology, engineer |

### Awards and stipends

| | |
|---|---|
| 2016 | DoRa Pluss Scholarship of Archimedes Academic Fund (activity T1.1) |
| 2012 | Kristjan Jaak's Scholarship of Archimedes Academic Fund |
| 2012 | Tiina Mõis stipend; Development Foundation of Tallinn University of Technology |
| 2010 | European Social Fund, DoRa Research Fellowship (activity 6) |
| 2009 | European Social Fund, DoRa Research Fellowship (activity 8) |

### Courses and conferences

| | |
|---|---|
| June 2017 | „*2nd Nordic Neuroscience*" meeting, poster presentation; Stockholm, Sweden |

November 2016 Society for Neuroscience, 47nd Annual Meeting, poster presentation; San Diego, USA

August 2016 "Behavioral Phenotyping of Rodent Disease Models – Potential and Pitfalls", course, University of Tartu, University of Helsinki, University of Eastern Finland; Tartu/Otepää, Estonia

December 2015 "From Gene to Function: Genome Editing tools", practical course, Centre for Genomic Regulation; Barcelona, Spain

October 2012 Society for Neuroscience, 42nd Annual Meeting, poster presentation; New Orleans, USA

August 2012 "Quantitative proteomics", practical course, University of Tartu Institute of Technology; Tartu, Estonia

2010-2011 Visiting PhD student in Erasmus University, Erasmus University Medical Center, Department of Orthopedics; Rotterdam, The Netherlands

October 2010 Society for Neuroscience, 40th Annual Meeting, poster presentation; San Diego, USA

June 2010 NGF meeting „Neurotrophic factors in health and disease"; Helsinki, Finland

September 2009 "European Bioinformatics Institute Roadshow: Genomes, Transcriptomics, Pathways, Proteins", practical course, University of Tartu Computer Science Institute; Tartu, Estonia

Spring 2006 "Laboratory Animal Science and Techniques", practical course, certificate, University of Helsinki; Tallinn, Estonia

**Supervised dissertations**

Mariliis Raud, BSc, 2017;
Supervised by Hanna Vihma and Tõnis Timmusk
Lentivirus-mediated overexpression of helix-loop-helix transcription factor TCF4 in rat primary neurons

Mirjam Luhakooder, MSc, 2012;
Supervised by Hanna Vihma and Tõnis Timmusk
Regulation of subcellular localization of human NFAT proteins by neuronal activity.

Mirjam Luhakooder, BSc, 2009;
Supervised by Hanna Vihma and Tõnis Timmusk
Analysis of the expression of human NFATc2 alternative transcripts and protein isoforms.

**Publications**

Sepp M, **Vihma H**, Nurm K, Urb M, Page SC, Roots K, Hark A, Maher BJ, Pruunsild P, Timmusk T. (2017). The intellectual disability and schizophrenia associated transcription factor TCF4 is regulated by neuronal activity and protein kinase A. J Neurosci.;37(43):10516-10527. doi: 10.1523/JNEUROSCI.1151-17.2017.

**Vihma H**, Timmusk T. (2017). Sumoylation regulates the transcriptional activity of different human NFAT isoforms in neurons. Neurosci Lett.;653:302-307. doi: 10.1016/j.neulet.2017.05.074.

**Vihma H**., Luhakooder M., Pruunsild P., Timmusk T. (2016). Regulation of different human NFAT protein by neuronal activity. J Neurochem.;137(3):394-408. doi:10.1111/jnc.13568

**Vihma H**, Pruunsild P, Timmusk T. (2008). Alternative splicing and expression of human and mouse NFAT genes. Genomics, 92(5):279-91. doi:10.1016/j.ygeno.2008.06.011

**ELULOOKIRJELDUS**


| | |
|---|---|
| Nimi: | Hanna Vihma |
| Sünniaeg ja -koht: | 15.01.1984, Tallinn |
| Kodakondsus: | Eesti |

**Kontaktandmed**

Keemia ja biotehnoloogia instituut, Tallinna Tehnikaülikool, Akadeemia tee 15, 12618 Tallinn
Tel.: +372 5527668
E-kiri: hanna.vihma@gmail.com, hanna.vihma@ttu.ee


**Hariduskäik**

| | |
|---|---|
| 2008-…. | Tallinna Tehnikaülikool, Geenitehnoloogia instituut, doktoriõpe |
| 2005-2007 | Tallinna Tehnikaülikool, Geenitehnoloogia instituut, loodusteaduste magistrikraad (MSc) |
| 2002-2005 | Tartu Ülikool, Molekulaar- ja rakubioloogia instituut, bakalaureusekraad (BSc) |
| 1999-2002 | Vanalinna Hariduskolleegium |
| 1990-1999 | Tallinna Muusikakeskkool |

**Kursused ja konverentsid**

| | |
|---|---|
| Juuni 2017 | „*2nd Nordic Neuroscience*“ konverents, posterettekanne; Stockholm, Rootsi |
| November 2016 | Neuroteaduste ühingu (*Society for Neuroscience*) 47. aastakonverents, posterettekanne; San Diego, USA |
| August 2016 | Kursus: “*Behavioral Phenotyping of Rodent Disease Models – Potential and Pitfalls*”, Tartu Ülikool, Helsinki Ülikool, Ida-Soome Ülikool; Tartu/Otepää, Eesti |
| Detsember 2015 | Praktiline kursus: “*From Gene to Function: Genome Editing Tools*”, Centre for Genomic Regulation; Barcelona, Hispaania |
| Oktoober 2012 | Neuroteaduste ühingu (*Society for Neuroscience*) 42. aastakonverents, posterettekanne; New Orleans, USA |
| August 2012 | Praktiline kursus: “Kvantitatiivne proteoomika”, Tartu Ülikooli Tehnoloogiainstituut; Tartu, Eesti |
| 2010-2011 | Külalisdoktorant, Erasmus University, Erasmus University Medical Center, Department of Orthopedics; Rotterdam, Holland |
| Oktoober 2010 | Neuroteaduste ühingu (*Society for Neuroscience*) 40. aastakonverents, posterettekanne; San Diego, USA |
| Juuni 2010 | NGF konverents „*Neurotrophic factors in health and disease*“; Helsinki, Soome |

September 2009 Praktiline kursus: "*European Bioinformatics Institute Roadshow: Genomes, Transcriptomics, Pathways, Proteins*", Tartu Ülikooli Arvutiteaduste Instituut; Tartu, Eesti

Kevad 2006 Praktiline kursus: *"Laboratory Animal Science and Techniques*", omandatud sertifikaat, Helsinki Ülikool; Tallinn, Eesti

**Teaduspreemiad ja –tunnustused**

2016 DoRa Pluss stipendium (tegevus T1.1); Sihtasutus Archimedes

2012 Kristjan Jaagu stipendium; Sihtasutus Archimedes

2012 Tiina Mõisa nimeline stipendium; Sihtasutus Tallinna Tehnikaülikooli Arengufond

2010 Euroopa Sotsiaalfondi DoRa 6 stipendium

2009 Euroopa Sotsiaalfondi DoRa 8 stipendium

**Erialane töökogemus**

2017-… Tallinna Tehnikaülikool, Keemia ja biotehnoloogia instituut, insener

2008-2016 Tallinna Tehnikaülikool, Geenitehnoloogia instituut, insener

**Juhendatud diplomitööd**

Mariliis Raud, bakalaureusetöö, 2017
Juhendajad Hanna Vihma ja Tõnis Timmusk
Aluselise heeliks-ling-heeliks transkriptsioonifaktori TCF4 üleekspressioon roti primaarsetes neuronites lentiviirusvektorite abil

Mirjam Luhakooder, magistritöö, 2012
Juhendajad Hanna Vihma ja Tõnis Timmusk
Neuronaalse aktiivsuse poolt vahendatud inimese NFAT valkude rakusisese lokalisatsiooni regulatsioon.

Mirjam Luhakooder, bakalaureusetöö, 2009
Juhendajad Hanna Vihma ja Tõnis Timmusk
Inimese NFATc2 alternatiivsete transkriptide ja valgu isovormide ekspressiooni analüüs.

**Publikatsioonid**

Sepp M, **Vihma H**, Nurm K, Urb M, Page SC, Roots K, Hark A, Maher BJ, Pruunsild P, Timmusk T. (2017). The intellectual disability and schizophrenia associated transcription factor TCF4 is regulated by neuronal activity and protein kinase A. J Neurosci.;37(43):10516-10527. doi: 10.1523/JNEUROSCI.1151-17.2017.

**Vihma H**, Timmusk T. (2017). Sumoylation regulates the transcriptional activity of different human NFAT isoforms in neurons. Neurosci Lett.;653:302-307. doi: 10.1016/j.neulet.2017.05.074.

**Vihma H**., Luhakooder M., Pruunsild P., Timmusk T. (2016). Regulation of different human NFAT protein by neuronal activity. J Neurochem.;137(3):394-408. doi:10.1111/jnc.13568

**Vihma H**, Pruunsild P, Timmusk T. (2008). Alternative splicing and expression of human and mouse NFAT genes. Genomics, 92(5):279-91. doi:10.1016/j.ygeno.2008.06.011