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DEPARTAMENT DE CIÈNCIES EXPERIMENTALS I DE LA SALUT

REGULATION OF NFAT5 BY SIGNALING PATHWAYS INVOLVED IN OSMOTIC STRESS RESPONSES AND CELL GROWTH

PhD Thesis

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Barcelona, May 2008

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To be presented for the obtention of the PhD degree from Universitat Pompeu Fabra.
This work was done in the Unitat d'Immunologia, Departament de Ciències Experimentals i de la Salut, Universitat Pompeu Fabra, with the direction of Dr. José Aramburu.

José Aramburu

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ABBREVIATIONS

AED	Auxiliary Export Domain
AMPK	AMP-activated Protein Kinase
AP-1	Activator Protein-1
AR	Aldose Reductase
ATM	Ataxia-Telangiectasia Mutated
ATR	Ataxia-Telangiectasia and Rad3-related
BGT-1	Betaine/GABA transporter-1
CaMKK	Calmodulin-dependent Protein Kinase Kinase
CK	Casein Kinase
CsA	Cyclosporine A
DBD	DNA binding domain
DD5	Dimerization Domain of NFAT5
DNA-PK	DNA-dependent Protein Kinase
DYRK	Dual-specificity Y (tyrosine) Regulated Kinase
ERK	Extracellular signal-Regulated Kinase
eEF2	eukaryotic Elongation Factor 2
eEF2K	eukaryotic Elongation Factor 2 Kinase
eIF3	eukaryotic Initiation Factor 3
eIF4E	eukaryotic translation Initiation Factor 4E
FKBP12	FK506-Binding Protein 12kDa
GAP	GTPase-activating protein
GSK-3	Glycogen-synthase kinase 3
hSMG-1	Human Suppressor of Morphogenetic effect on Genitalia-1
HIF-1	Hypoxia inducible Factor 1
HOG-1	High-Osmolarity Glycerol-1
HSP	Heat Shock Protein
hVps34	human Vacuolar protein sorting-34
JNK	Jun N-terminal kinase
LT-β	Lymphotoxin beta
MAPK	Mitogen-activated Protein Kinase
MEF	Mouse Embryo Fibroblasts
MIOX	Myoinositol Oxygenase
MKP-1	MAPK phosphatase-1
mTOR	mammalian Target Of Rapamycin
mTORC	mammalian Target Of Rapamycin Complex
NES	Nuclear Export Sequence

NFAT	Nuclear Factor of Activated T cells
NF-κB	Nuclear Factor kappa B
NLS	Nuclear Localization Signal
OREBP	Osmotic Stress Response Binding Protein
PARP-1	Poly(ADP-ribose) Polymerase-1
PDK1	3-Phosphoinositide-Dependent Protein Kinase 1
PERK	PKR-like ER kinase
PJS	Peutz-Jegher Syndrome
PKA	Protein Kinase A
PKC	Protein Kinase C
PI3K	Phosphatidylinositol 3-Kinase
PIKK	PI3-kinase-related Kinases
PIP2	Phosphatidylinositol-4,5-phosphate
PIP3	Phosphatidylinositol-3,4,5-phosphate
PRAS40	Proline-Rich Akt Substrate 40kDa
Protor-1	Protein observed with Rictor-1
Raptor	Regulatory Associated Protein of mTOR
Rictor	Rapamycin-Insensitive Companion of mTOR
RHA	RNA Helicase A
RHD	Rel Homology Domain
RSK	p90 kDa ribosomal S6-Kinase
ROS	Reactive Oxygen Species
RVI	Regulatory Volume Increase
SAPK	Stress-Activated Protein Kinase
S6K	p70 ribosomal protein S6 Kinase
SMIT	Sodium/Myoinositol Transporter
TauT	Taurine Transporter
TCA	Tricarboxylic Acid
TcR	T cell receptor
TNF-α	Tumor Necrosis Factor alpha
TonEBP	Tonicity-responsive Enhancer Binding Protein
TSC1/2	Tuberous Sclerosis Complex 1 and 2
UT-A	Urea Transporter
UTR	Untranslated region
4E-BP1	eIF4E Binding Protein 1

SUMMARY

NFAT5 (Nuclear Factor of Activated T cells 5) belongs to the Rel family of transcription factors, which also comprises the NF-κB and NFATc (NFAT1-4) proteins, and thus shares several characteristics with both of them. NFAT5 binds DNA sites that resemble the NFATc ones and its DNA binding domain shares more homology with this domain of NFATc proteins than with NF-κB, but it lacks the calcineurin-docking sites and the phosphoserine clusters that are distinctive of NFATc. On the other hand, it structurally resembles NF-κB proteins, since it is a dimer and encircles the DNA. Functionally, NFAT5 has been involved in several processes such as embryonic development, integrin-induced cellular migration, muscle differentiation, T cell receptor activation, proliferation, doxorubicin-induced toxicity in cardiomyocytes and HIV replication. However, NFAT5 is best known for its capacity to respond to osmotic stress. Hypertonicity induces NFAT5 synthesis, nuclear localization and transcriptional activity, switching on a specific gene expression program whose products allow the accumulation of organic osmolytes and chaperones that protect the cell from the harmful increase in the intracellular concentration of inorganic ions. In accordance, NFAT5-deficient mice present severe renal abnormalities, as the kidney is the organ subjected to maximal tonicity levels.

In order to examine the transcriptional activity of NFAT5 in primary cells, it was necessary a new system to detect the activity of the endogenous protein. We considered that, since certain DNA sites can be recognized by both NFATc and NFAT5, some of the already existing models of transgenic mice harbouring NFATc-regulated luciferase reporters might also be useful to monitor NFAT5. We have studied the regulation of NFAT5 transcriptional activity in several types of primary cells obtained from transgenic mice carrying the 9xNFAT-Luc reporter developed by the Molkentin laboratory. Our results show that this reporter was induced by hypertonicity in lymphocytes, macrophages and mouse embryo fibroblasts (MEF) in an NFAT5-dependent manner, whereas its response to the phorbol ester PMA plus ionomycin was independent of NFAT5 and mediated by the NFATc proteins. The transcriptional activation of NFAT5 was detected at variable tonicity ranges depending on the cellular type: around 360-380 mOsm/kg in splenocytes, 430 mOsm/kg in bone marrow-derived macrophages and 480 mOsm/kg in MEF. Tonicity levels in the range between 360 to 430 mOsm/kg have been recorded in plasma in patients and experimental mouse models of osmoregulatory disorders, thus indicating that NFAT5 can be activated by pathophysiological stress conditions in diverse cell types outside of the renal medulla.

Several signaling pathways have been reported to regulate NFAT5 activity, but their function has often been studied in transformed cell lines using very high hypertonicity levels, which are only found *in vivo* in the renal inner medulla, and might not be encountered by cells that do not belong in this particular anatomycal niche. Our results using primary, non-transformed lymphocytes exposed to lower, pathophysiologically relevant tonicity levels, indicate that NFAT5 transcriptional activity was partially inhibited by the PI3-kinase inhibitor wortmannin, the PKA inhibitor H89, substantially downregulated by p38 inhibitors (SB203580 and SB202190) and by inhibition of PI3-kinase-related kinases (PIKK) with LY294002, and insensitive to the ERK inhibitor PD98059. In addition, hypertonicity-induced NFAT5 expression was downregulated by LY294002 at concentrations that inhibit PIKK. Thus, several signaling pathways activate NFAT5 and the contribution of diverse kinases might be necessary to modulate its activity.

We also observed that the response of the 9xNFAT-Luc reporter to hypertonicity was more effective in mitogen-activated splenocytes than in quiescent cells, and that expression of NFAT5 was higher in cells undergoing proliferation. Previous reports and our own findings here indicated that NFAT5 was regulated by PI3K, which is involved in cell growth-promoting pathways. For this reason, and also considering that hypertonicity can induce cellular responses similar to those caused by energy and nutrient deprivation, we investigated whether NFAT5 could be regulated by the energy-sensing kinase AMPK and by mTOR, a major regulator of cell growth and proliferation. We report that the mTOR inhibitor rapamycin downregulated the hypertonicity-induced expression and transcriptional activation of NFAT5, without affecting its nuclear localization nor its DNA binding capacity. Moreover, rapamycin inhibited the induction of Hsp70.1 in response to hypertonicity in splenocytes in an NFAT5-dependent manner. We also found that the AMPK activator AICAR inhibited the expression and transcriptional activity of NFAT5, and downregulated the mTOR pathway in diverse cell types exposed to hypertonic stress. These effects were also observed in AMPK-null cells, indicating that they were independent of AMPK. Notably, we found that despite its inhibitory effect on NFAT5 and the mTOR pathway, AICAR was able to enhance the hypertonicity-induced expression of Hsp70.1 in primary T cells by NFAT5-independent mechanisms.

In conclusion, our results indicate that NFAT5 is able to respond to pathological tonicity levels in primary cells and it requires a combination of signaling mediators, some of

which are more relevant in specific cell types. Besides, the regulation of NFAT5 by osmotic stress also involves energy and nutrient sensing pathways, that might be necessary to integrate the effectiveness of osmoprotective responses to metabolic and proliferative demands of the cell. Finally, our results reveal the existence of NFAT5-dependent and independent mechanisms of regulation of osmotic stress responses in mammalian cells by pharmacological modulators of nutrient and energy-sensing pathways. Since these pathways are attractive targets for drugs already being used in the clinic or under development for the treatment of metabolic disorders and some types of cancer, our findings might be relevant to better understand their action and potential side effects on cellular functions under stress.

INTRODUCTION

NFAT5 (Nuclear Factor of Activated T cells 5) belongs to the Rel family of transcription factors, which also comprises the NFATc and NF-κB proteins. Although the homology of its DNA binding domain classifies NFAT5 as an NFAT protein, it shares structural and functional features with both NFAT and NF-κB members, and also presents unique characteristics among the family. The best-known function of NFAT5 is its activation by osmotic stress and its central role inducing an osmoprotective gene expression program in response to hypertonicity, but it is also activated by other stimuli suggesting that NFAT5 may be involved in the regulation of distinct cellular functions.

1. NFAT FAMILY OF TRANSCRIPTION FACTORS

The NFAT (Nuclear Factor of Activated T cells) family consists of four NFATc proteins (NFAT1/c2/p, NFAT2/c1/c, NFAT3/c4, NFAT4/c3/x) and NFAT5/TonEBP. NFATc transcription factors are characterised by a highly conserved DNA binding domain, that recognize the consensus nucleotide sequence (T/A/C)GGA(A/C). NFAT1 to NFAT4 are regulated by the calmodulin-dependent serine/threonine phosphatase calcineurin (PPase-2B), which is activated by increases of the intracellular concentration of free calcium. In resting cells NFATc proteins are phosphorylated in the cytoplasm; upon cell activation and stimuli that trigger calcium mobilization, calcineurin binds to NFATc by their PxIxIT (Aramburu et al. 1998) and LxVP (Martinez-Martinez et al. 2006) motifs and dephosphorylates sequentially certain phosphoserine clusters (SP motifs), exposing nuclear localization sequences to allow their nuclear translocation, and enhancing their transcriptional activation. The inhibition of calcineurin by the immunosuppressants cyclosporin A (CsA) or FK506 results in the relocalization of NFATc to the cytosol (Hogan et al. 2003, Macian 2005).

In addition, NFATc activity is modulated by inputs coming from another signaling pathways, such as diverse kinases and NFAT partners. Several kinases phosphorylate NFATc proteins at their serine-rich motifs controlling their nuclear shuttling, including glycogen-synthase kinase 3 (GSK3), casein kinase 1 (CK1), DYRK, p38 and Jun N-terminal kinase (JNK). It is necessary to distinguish between maintenance kinases, which act in the cytosol to keep NFATc proteins in a fully phosphorylated state and prevent their translocation into the nucleus in resting cells, and export kinases, which rephosphorylate NFATc factors in the nucleus and promote its nuclear export. Thus, GSK3 phosphorylates NFAT1 and NFAT2 promoting their nuclear export, but cyclic-AMP-dependent protein kinase A (PKA) has to previously phosphorylate NFAT2 to

allow GSK3 phosphorylation (Beals et al. 1997). CK1 functions as both a maintenance and an export kinase for NFATc proteins, docking at their amino-terminal domain (Okamura et al. 2004, Zhu et al. 1998). DYRK-family kinases phosphorylate NFAT1, thereby priming further phosphorylation by GSK3 and CK1; cytoplasmic DYRK2 functions as a maintenance kinase whereas DYRK1A, which localises in the nucleus, is the export kinase (Gwack et al. 2006). Mitogen-activated kinases (MAPKs) differentially phosphorylate NFATc proteins: p38 phosphorylates NFAT1 (Gomez del Arco et al. 2000), whereas JNK phosphorylates NFAT2 (Chow et al. 1997). Once in the nucleus NFATc proteins can bind to their DNA targets alone or in cooperation with other factors, whose regulation adds an additional layer of modulation to the function of NFATc proteins. Prominent partners of NFATc factors that enhance their DNA binding affinity and stabilise the NFATc-DNA interaction, resulting in an increased transactivation activity, are AP-1, GATA3, MEF2 or FOXP3 (Avni et al. 2002, Rao, Luo & Hogan 1997, Wu et al. 2006, Youn, Chatila & Liu 2000).

Binding sites for NFATc proteins are found in genes such as the cytokines IL-2, IL-4, IL-5, IL-13, IFN-γ and TNF-β; the cell surface proteins CD40L, CTLA-4 and FasL; the enzyme cyclooxygenase-2 and the cell cycle regulator CDK4 (Gibson et al. 2007, Lindgren, Axcrona & Leanderson 2001, Savignac, Mellstrom & Naranjo 2007, Telliez et al. 2006). Thus, a large number of inducible genes that regulate cell proliferation, differentiation, survival and apoptosis are under the control of NFATc proteins. Therefore, even if NFAT was first described in activated T cells as a nuclear factor able to bind to the promoter of the human IL-2 (Shaw et al. 1988), over the past years several studies have demonstrated the capability of these transcription factors in regulating the expression of different genes, including signaling proteins, cytokines, cell surface receptors and cell cycle and apoptosis related proteins. Moreover, despite their first depiction as proteins expressed only in T cells, nowadays they are known to be expressed in other immune tissues: NFAT1, NFAT2 and NFAT4 are expressed at the protein level in peripheral T and B cells; NFAT1 is also expressed in mast cells, NK cells, and in certain monocytes and macrophages (Crabtree, Olson 2002, Macian, Lopez-Rodriguez & Rao 2001, Rao, Luo & Hogan 1997). NFAT1 and NFAT2 mRNAs are expressed in peripheral lymphoid tissues, and NFAT2 mRNA is upregulated in activated T cells and NK cells (Aramburu et al. 1995, Northrop et al. 1994). NFAT4 mRNA is expressed at high levels in the thymus and at low levels in peripheral lymphoid tissues (Ho et al. 1995, Masuda et al. 2002). Besides the immune system, NFATc proteins are expressed in non-immune cells such as neurons, glia, endothelial

cells, skeletal and heart muscle, chondrocytes, keratinocytes and adipocytes, among others (Crabtree, Olson 2002, Hogan et al. 2003). Initially, NFATc proteins were considered to have redundant functions, but now they are known to play distinct roles in cellular physiology: targeted disruption of genes involved in NFATc signaling and NFATc proteins themselves, alone or combinated, have demonstrated their individual properties (Table 1). Thus, NFATc transcription factors take part in early stages in the development and function of the cardiovascular, musculoskeletal and nervous systems, besides immune system.

PROTEIN	PHENOTYPE OF KNOCK-OUT MICE	REF
NFAT1	Immune hyperactivation and allergic responses. Suppression of chondrogenesis.	Xanthoudakis 1996 Ranger 2000
NFAT2	Lethal failure of cardiac morphogenesis. Defects in thymic development and T cell activation in Rag1$^{-/-}$ chimeras.	de la Pompa 1998 Yoshida 1998 Ranger 1998
NFAT3	Normal development, no apparent defects.	Graef 2001
NFAT4	Defects in thymic development and hyperproliferation of lymphocytes. Altered primary myogenesis and decreased muscle size in adults.	Oukka 1998 Kegley 2001
NFAT5	Renal atrophy, lack of tonicity-responsive gene expression. Impaired T cell function under hyperosmotic conditions. Decreased cellularity of thymus and spleen.	Lopez-Rodriguez 2004 Go 2004
NFAT1+2	In fetal liver chimeras: failure of T cell activation and immune response gene activation but hyperreactive B cells.	Peng 2001
NFAT1+4	Lymphoproliferative disorder and excessive allergic responses.	Ranger 1998
NFAT3+4	Lethal vascular patterning defects.	Graef 2001
NFAT1+3+4	Defects in sensory axon projection and commissural axon growth.	Graef 2003

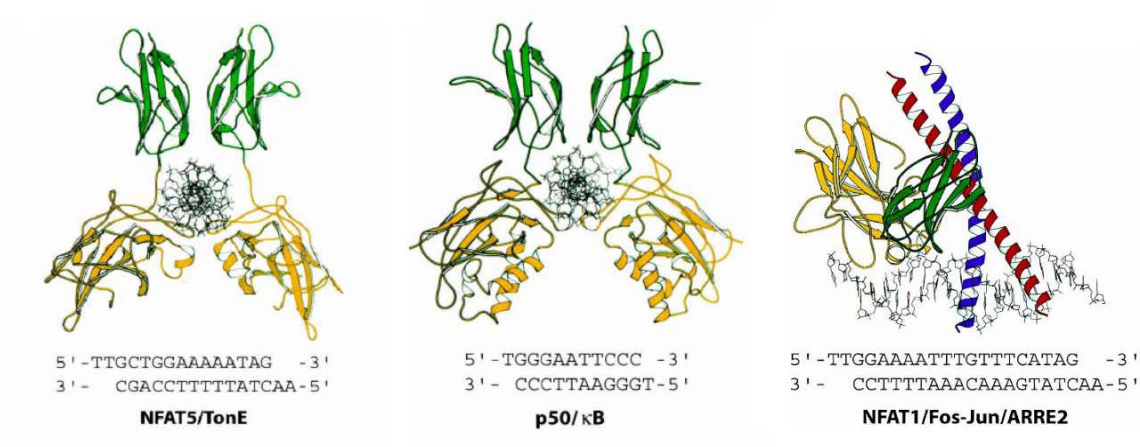
Adapted from Crabtree and Olson (2002)

Table 1. Phenotype of mice lacking NFAT proteins individually or in combination.

In addition to mouse models genetically modified to lack transcription factors, transgenic mice carrying reporter elements have brought an opportunity to assess directly their biological activity *in vivo*. For instance, the generation of bioluminescent mice by inserting reporters coupled to a luciferase gene have allowed to study the physiological roles and the spatio-temporal activity of several transcription factors such as AP-1 (Rincon, Flavell 1994), MEF2 (Naya et al. 1999) or NF-κB (Schmidt-Ullrich et al. 1996). More recently, Wilkins et al. developed transgenic mice containing an NFAT binding site-dependent luciferase reporter, in order to study NFATc activity in cardiac hypertrophic processes, by inserting nine copies of an NFAT binding site from the IL-4 promoter positioned 5' to a minimal promoter from the α-myosin heavy chain (Wilkins et al. 2004).

2. NFAT5 BELONGS TO THE REL FAMILY

NFAT5 (Nuclear Factor of Activated T cells 5) was identified by Lopez-Rodriguez et al. by its sequence homology to the DNA binding domain of NFATc proteins, called the Rel homology domain (RHD), which is also found in the NF-κB family of transcription factors. NFAT5 shares 43% amino acid identity with the RHD of NFATc proteins, but it is also the NFAT member with the greatest homology to the NF-κB family (17% identity to p50). Moreover, NFAT5 is a constitutively dimeric protein (Lopez-Rodriguez et al. 2001, Stroud et al. 2002), whereas NFATc proteins are predominantly monomers and NF-κB proteins can both homo- and heterodimerize among them (Hayden, Ghosh 2004). The dimerization surface of NFAT5 is found in the carboxy-terminal half of the RHD and it is similar to that of the NF-κBs. This surface, together with an additional dimer interface (E'F loop) in the amino-terminal half, allows NFAT5 to encircle DNA (Figure 1). As with NF-κB proteins, dimerization is also essential for NFAT5 DNA binding and transcriptional activity (Lopez-Rodriguez et al. 2001, Stroud et al. 2002). In addition to a higher overall homology of NFAT5 DNA binding domain to the DNA binding domain of NFATc proteins, NFAT5 also binds to DNA sites that closely resemble those recognised by the NFATc factors. Thus, NFAT5 recognises a consensus sequence (TGGAAANNYNY, where N represents any nucleotide and Y pyrimidine) in which is found the NFATc core GGAA (Lopez-Rodriguez et al. 1999). Furthermore, while the DNA binding site of NF-κB has to be symmetrical to allow both halves of the dimer to contact it, one monomer of NFAT5 contacts its cognate DNA sequence while the other can bind a non-consensus sequence (Stroud et al. 2002). However, outside the RHD there is no similarity between NFAT5 and NFATc or NF-κB proteins (Figure 2).

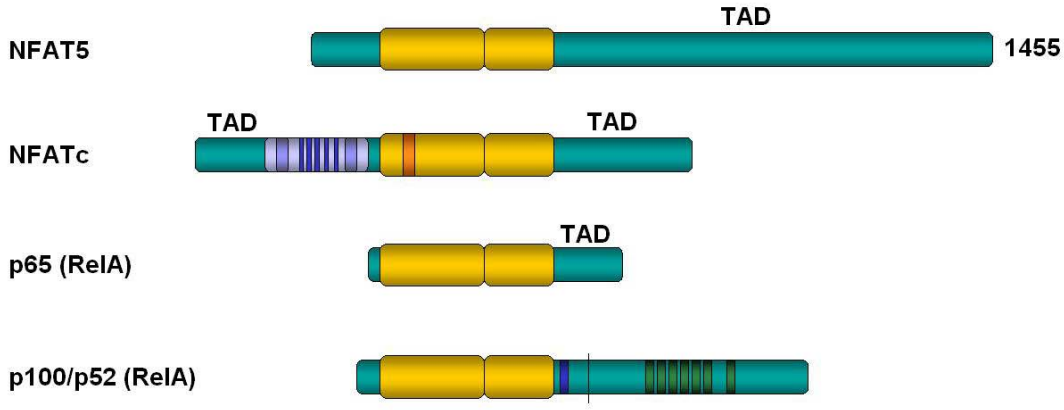


Adapted from Hogan et al. (2003)

Figure 1. Crystal structure of NFAT5/DNA, p50/DNA and NFAT1/Fos-Jun/DNA complexes (DNA binding domain of each protein).

The proteins are shown with the amino-terminal part of the Rel Homology Domain in green and the carboxy-terminal part in yellow. NFAT5 is bound to a TonE site, p50 to a κB site and NFAT1 is to the ARRE2 site of the IL-2 promoter, in cooperation with AP-1 (Fos-Jun). The DNA sequence is shown below the structure.

Hence, NFAT5 DNA binding domain presents a hybrid structure between NFATc and NF-κB proteins and these characteristics reflect evolutionary relationships. NF-κB proteins, and an NFAT5-like factor, are present in *Drosophila melanogaster* but not in *Caenorhabditis elegans* and NFATc members are only present in vertebrates (Graef, Chen & Crabtree 2001). In *Drosophila*, an ancient NFAT, called dNFAT, is found and its Rel domain resembles mammalian NFAT5 more than NFATc, as it presents 51% amino acid identity and conserves the characteristic dimerization residues. As NFAT5, dNFAT is a large protein that lacks the classical calcineurin docking sites and phosphoserine clusters that are found in NFATc proteins and presents glutamine repeats. Besides, functionally is also involved in salt stress tolerance, as the mammalian NFAT5 (Keyser, Borge-Renberg & Hultmark 2007), a unique feature not present in the other NFATc proteins. Referring to the osmotic stress regulation feature, NFAT5 was cloned at the same time by the Rao laboratory and by Moo Kwon and colleagues, who used a one-hybrid system approach using a hypertonicity response element. This sequence, known as TonE, was the reason to call it TonEBP (Tonicity-responsive enhancer binding protein) (Miyakawa et al. 1999).



Adapted from Aramburu et al. (2006)

Figure 2. Schematic diagram of mammalian Rel proteins.

Rel homology region is shown in yellow. In the amino-terminal domain of NFATc proteins their calcineurin-binding sites and SP motifs are in blue and the AP-1 binding site is located in the DNA binding domain. NF-κB proteins present diverse features: RelA comprises several ankyrin repeats (green boxes) and serine-rich regions (blue box). TAD (transactivation domain).

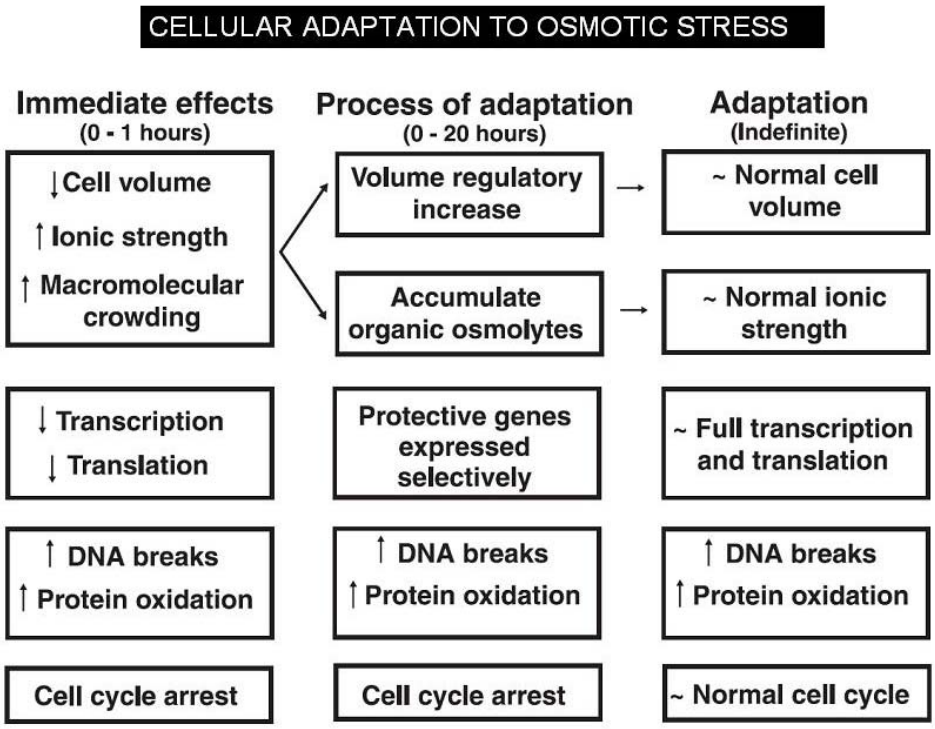
3. OSMOTIC STRESS RESPONSE

The bulk movement of water across a semi-permeable membrane is termed osmosis and the osmotic pressure is dependent on the total concentration of dissolved solute particles. The terms osmolality and osmolarity indicate the total number of particles present in a kilogram of solvent and a liter of solution, respectively, and are used interchangeably when referring to the relatively dilute solutions of the body. Cells can be exposed to anisosmotic, produced by alterations in extracellular osmolarity, or isosmotic, brought about by alterations in intracellular solute content. Under normal physiological conditions most cells are protected from anisosmotic volume changes by the kidney's regulation of plasma osmolarity, with the exception of the renal medulla and gastrointestinal cells. However, all cells can be exposed to isosmotic swelling or shrinkage when the balance between solute influx and efflux across the membrane, that is regulated by metabolic production and osmotically active substances, is perturbed (Strange 2004). In culture, cells are exposed to anisosmotic changes, hypertonic or hypotonic, depending on the extracellular final solute concentration.

Acute hypertonicity causes osmotic efflux of water, cell shrinkage and a nonspecific rise of all intracellular solutes. Cells respond rapidly to this increase in extracellular tonicity by activating transmembrane transporters, mediating uptake of NaCl, that

results in osmotically obligated water entry through the cell membrane, that is highly permeable to it, and recovery of cell volume (regulatory volume increase). NaCl uptake is achieved through the ADH/cAMP-dependent Na^+/H^+ and $Cl^-/HCO3^-$ exchangers. This Na^+ influx is later replaced by K^+ ions that are captured by Na^+/K^+-ATPase, whose activity is enhanced by the presence of Na^+. This restores cellular volume, but intracellular strength still remains elevated and it is necessary to lower it by accumulation of compatible organic osmolytes as a delayed, adaptative response that involves transcriptional activity. Compatible organic osmolytes, unlike most inorganic ions, raise cellular osmolarity without perturbing macromolecules (Garcia-Perez, Burg 1991). In mammals the predominant compatible osmolytes are polyhydric alcohols (myoinositol and sorbitol), methylamines (betaine and glycerophosphorylcholine) and amino acids (taurine, alanine, proline and, to a lesser extent, other amino acids). These non-perturbing solutes are accumulated as the result of changes in the activity of rate-limiting, sodium coupled transporters that carry the organic solutes into cells against steep concentration gradients (myoinositol, betaine, amino acids) and changes in activity of rate-limiting enzymes that catalyze solute synthesis (sorbitol) or degradation (glycerophosphorylcholine). The compatible osmolytes are relatively impermeable to the plasma membrane and, thus, their efflux from the cell is slow as long as cell swelling does not happen. Intracellular accumulation of betaine is mediated by its uptake from the extracellular media via BGT-1 (betaine/GABA transporter-1) (Rasola et al. 1995). BGT-1 couples three sodium and two chloride ions for each betaine, whereas SMIT (sodium/myoinositol transporter) couples the uptake of one myo-inositol to the entry of two Na^+. Hypertonicity enhances transcription of both BGT-1 and SMIT genes in the renal medulla. Sorbitol is produced from glucose by aldose reductase (AR) and it is subjected to pronounced osmotic regulation (Bagnasco et al. 1987). Free amino acids contribute less to the pool of organic osmolytes than polyols or trimethylamines (Sone et al. 1995), even if uptake of free amino acids by renal epithelial cells by system A and the Na^+ and Cl^--dependent taurine transporter (TauT) is activated by osmotic stress. The cellular accumulation of compatible osmolytes reduces the activation of caspases and apoptosis in hypertonic conditions (Horio et al. 2001). On the other hand, when the accumulation of inositol is prevented, cell death is promoted in hypertonic culture conditions (Kitamura et al. 1997) and also in the hypertonic kidney medulla, leading to acute renal failure (Kitamura et al. 1998). Failure to accumulate sorbitol due to targeted deletion of the aldose reductase gene is associated with a deffect in the urinary concentrating ability (Ho et al. 2000). Thus, accumulation of compatible organic osmolytes is necessary for cellular function and survival in presence of osmotic stress.

Altogether, a chronic increase in intracellular ionic strength may have numerous adverse effects on cell function such as DNA damage, growth arrest, inhibition of protein synthesis and disturbance of physiological transmembrane electrolyte gradients (Brigotti et al. 2003, Kultz, Chakravarty 2001). A schematic diagram of the stepwise cellular response to hyperosmotic stress is depicted in figure 3



Adapted from Burg, Ferraris & Dmitrieva (2007)

Figure 3. Cellular adaptation to osmotic stress.

3.1. The kidney, main regulator of tonicity

The kidney is structurally divided in the outer renal cortex and the inner renal medulla, which is divided in several cone-shaped renal pyramids that end as renal papillas into a calyx. The calyces are short tubes that receive urine from the renal papilla and empty into the large renal pelvis. Within the cortex and the medullary pyramids are found millions of structures called nephrons, the basic functional unit of the kidney. Each nephron is composed of the glomerulus, a capillary network that emerges from an afferent arteriole, which is surrounded by the Bowman's capsule or Bowman's space, continuous with the first part of the nephron. The nephron is divided in the proximal convoluted tubule, the proximal straight tubule (that extends from the cortex down into the medulla and back), the loop of Henle (which consists of a thin descending limb, a thin ascending limb, and a thick ascending limb), the distal convoluted tubule (again in

the cortex) and the collecting ducts (Figure 4). Filtrate passing from Bowman's capsule to the proximal convoluted tubule has an osmolarity of about 300 mOsm/L, the same as blood. As the filtrate flows through the proximal convoluted tubule, located in the kidney cortex, a large amount of water and salt is reabsorbed. Thus, the volume of filtrate decreases substantially at this stage, but the osmolarity remains about the same. Now the filtrate enters into the medulla through the descending limb of the loop of Henle, goes back up to the cortex by the ascending limb, and then down to the medulla one more time within the collecting duct. As the filtrate flows from cortex to medulla in the descending limb, water exits from the renal tubule by osmosis and the osmolarity of the filtrate increases. Increasing gradually from cortex to medulla, the salt concentration of the filtrate peaks at the elbow of the loop of Henle. This maximizes the diffusion of salt out of the tubule as the filtrate rounds the curve and enters the ascending limb, which actively pumps NaCl out of the tubule, making the filtrate more and more dilute. The loop of Henle can concentrate salt in the inner medulla because traffic in the descending limb opposes the osmolarity gradient produced by the ascending limb in the interstitial fluid. By the time the filtrate completes the loop of Henle, it is not hyperosmotic to body fluids, but is actually slightly hypoosmotic. Then, it flows from cortex to medulla through the collecting duct, losing water by osmosis as it encounters interstitial fluid of increasing osmolarity. This concentrates urea in the filtrate, and some of this urea leaks out of the lower portion of the collecting duct, making a major contribution to the high interstitial osmolarity of the inner medulla. Osmosis causes the filtrate in the collecting duct to equal the osmolarity of the interstitial fluid, which can be as high as 1200 mOsm/L in the inner medulla. Urine, even the most concentrated, is actually isosmotic to the interstitial fluid of the inner medulla, but hyperosmotic to blood and interstitial fluid elsewhere the body.

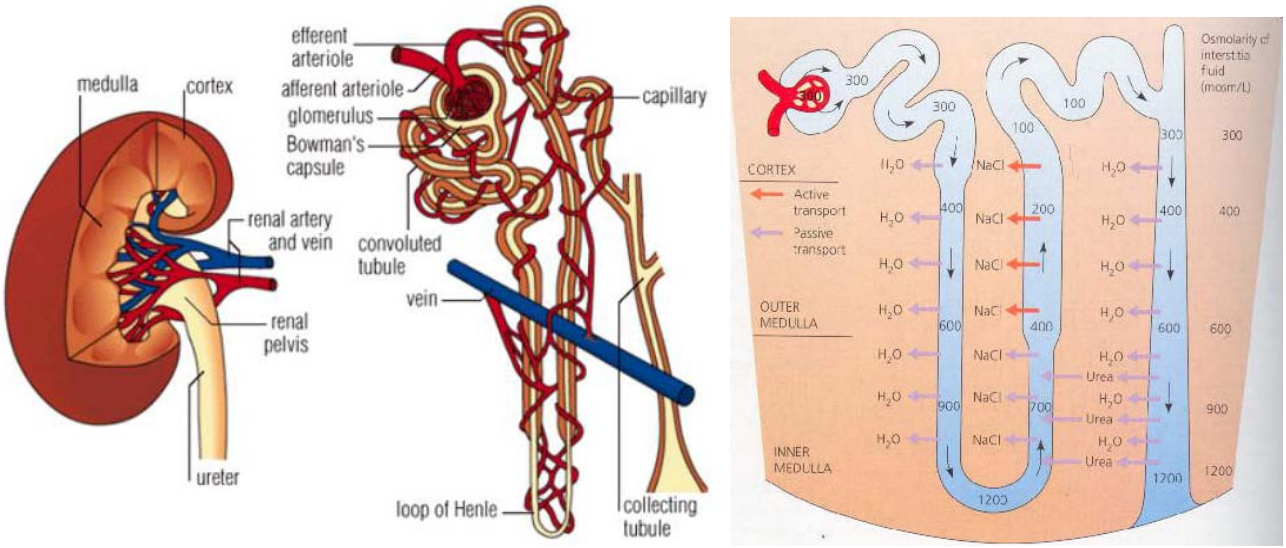


Figure 4. Cross-section of a kidney and nephron and osmolarity gradient in the kidney.

The kidney is the organ subjected to higher variations in tonicity and, depending on the hydration status, produces urine of widely varying osmolarity. During water deprivation, when concentrated urine is excreted, NaCl and urea are accumulated in the medullary interstitium and provide the driving force for water reabsorption across the collecting duct epithelium, a key function of the kidney for proper maintenance of body fluid volume. Conversely, during excessive water intake extracellular solute concentrations in the renal medulla decline, and dilute urine is excreted. Hypoosmolarity of the urine results form absorption of electrolytes along tubule segments. Thus, in the mammalian renal medulla interstitial solute concentrations widely fluctuate: the osmolarity of the interstitial fluid increases gradually along the outer and inner medulla reaching the highest level at the papilla, whereas the cortex is isotonic. As the cellular basolateral membrane is highly permeable to water and faces the interstitial fluid, the cells are in osmotic equilibrium with the interstitium. The ability to excrete highly concentrated urine during stages of water shortage is linked intimately to the specific transport properties and to the unique architecture of the tubular and vascular structures in the renal medulla. Although these unique features allow the establishment and maintenance of high medullary interstitial solute concentrations (predominantly NaCl and urea), in antidiuresis cells of the renal medulla are subjected to high salt and urea concentrations. In addition, during the transitions from antidiuresis to diuresis and viceversa, these cells are challenged by massive changes in extracellular solute concentrations. The interstitial fluid of the renal papilla of euhydrated rats typically

contains 700 mOsm/l of electrolytes, mostly Na^+ and Cl^-, and 1200 mOsm/l of urea (Beck, Burger-Kentischer & Muller 1998, Beck, Guder & Schmolke 1998). In antidiuresis, NaCl tops 1000 mOsm/l and urea 2000 mOsm/l. Consequently, these cells have developed several additional strategies to survive and function. As an example, inner medullary cells are able to maintain an intracellular Na^+ concentration not substantially higher than cortical cells, despite elevated extracellular NaCl concentrations, and due to enhanced expression and activity of the Na^+/K^+-ATPase (Capasso et al. 2003). This enables them to maintain transmembrane transport pathway indispensable for cell homeostasis and transepithelial electrolyte transport.

Moreover, it has also been described that chronic exposure of cultured cells to elevated concentrations of NaCl, but less to membrane-permeant solutes (vg. urea or glycerol), may cause chromosome aberrations, sister-chromatid exchanges and DNA single and double-strand breaks (Galloway et al. 1987, Kultz, Chakravarty 2001). These phenomena are accompanied by growth arrest and increased incidence of apoptosis (Kultz, Madhany & Burg 1998, Michea et al. 2000). Although it is not clear, hypertonic-induced DNA damage may be induced by direct break of the DNA backbone and inhibition of DNA damage repair mechanisms. However, as DNA damage occurs during normal cell replication, cells with high rates of proliferation display increased incidence of hypertonic-induced DNA damage and apoptosis. Conversely, the low proliferation rate of inner medullary cells in the kidney *in vivo* may contribute to the high resistance of these cells to apoptosis (Zhang et al. 2002).

Kidney also presents the highest urea concentration in the body and, due to the relatively permeability of cell membranes to this solute, the intracellular urea concentration is also high. At these concentrations urea compromises protein structure and function, reducing the activity of enzymes and, even more, inducing apoptosis (Colmont et al. 2001, Neuhofer, Fraek & X Beck 2002). The vasopressin-regulated urea transporters (UT-A) are expressed in the renal medulla and play a key role for urea accumulation (Bagnasco 2005). Specific Heat Shock Proteins (HSPs) are key factors in the inner medulla to resist this urea concentration and some are induced in hypertonic conditions. HSP70 abundance, for example, correlates with the diuretic state (Medina et al. 1996) and protects cells from high urea concentrations by counteracting urea-mediated inhibition of enzymes and protecting from apoptosis (Neuhofer et al. 1999). In addition, targeted disruption of the tonicity-inducible Hsp70 gene in mice results in an increased apoptosis in the renal medulla following osmotic

stress (Shim et al. 2002). The mechanism underlying these effects may be the chaperone properties of HSPs, promoting reestablishment of the correct conformation unfolded by urea (Fink, Oberg & Seshadri 1998), and the prevention of apoptosis by inhibiting Apaf-1 and cytochrome-c release from mitochondria (Mosser et al. 2000, Ravagnan et al. 2001).

The hypertonic conditions that can be found in the kidney are not present anywhere else in the organism, but some organs such as liver and lymphoid tissues are slightly hyperosmolar (330 mOsm/kg) compared with serum, brain or lung (Go et al. 2004). This suggests that the mechanisms and factors involved in the response to osmotic stress found in kidney cells may be present in other organs and tissues.

4. ROLE OF NFAT5 IN PROTECTION AGAINST HYPEROSMOLARITY

NFAT5 is also called TonEBP (Tonicity-responsive enhancer binding protein) and OREBP (Osmotic stress response binding protein) owing to its importance in the cellular response against osmotic stress. It is stimulated by hypertonicity in the renal medulla and plays a central role in the cellular accumulation of organic osmolytes. NFAT5 stimulates the transcription of genes encoding BGT-1, SMIT and AR (Burg, Ferraris & Dmitrieva 2007) and it also enhances the expression of myoinositol oxygenase (MIOX) that catalyzes the oxidative cleavage of myoinositol (Prabhu et al. 2005). NFAT5 is involved in the induction of HSP70 during osmotic stress, conferring protection against the high urea concentrations that are present in antidiuresis (Woo et al. 2002) and it also activates the transcription of the heat-shock family member Osp94 (Kojima et al. 2004). Furthermore, it stimulates the transcription of the papillary UT-A1 and UT-A3 urea transporters, which are essential for generating high interstitial urea concentrations during antidiuresis (Nakayama et al. 2000). However, NFAT5 does not appear to play a major role in regulating the transcription of the TauT gene in renal medullary cells *in vivo* (Lopez-Rodriguez et al. 2004), whereas in cultured cardiomyocytes NFAT5 transcriptionally activates a TauT reporter gene (Ito et al. 2007).

The critical importance of NFAT5 within the kidney has been demonstrated by targeted deletion of the NFAT5 gene in mice. Although just approximately 3% of the homozygous knockout progeny does not die in late gestation or perinatally, surviving mice display progressive growth retardation and perinatal lethality associated with

severe renal abnormalities. These mice present kidney hypoplasia and an altered medullary morphology, with marked atrophy of the renal medulla and increased apoptosis. This phenotype is associated with impaired activation of osmoprotective genes such as AR, BGT-1, and SMIT (Lopez-Rodriguez et al. 2004). In accordance, transgenic mice that overexpress a dominant negative form of NFAT5 in the cells of the renal collecting tubules show impairment in their urine concentrating mechanism and develop progressive hydronephrosis (Lam et al. 2004). NFAT5-deficient cells also show reduced proliferation under hyperosmotic culture conditions, and inability to cope with a local hypertonic environment in lymphoid organs has also been reported as a possible cause for T and B cell dysfunction in these animals (Go et al. 2004). Previously, Trama et al. described that transgenic mice expressing an inhibitory form of NFAT5 in T cells present impaired cell growth under hypertonic conditions (Trama, Go & Ho 2002). Moreover, as shown by Kultz and Burg laboratories, osmotic stress can lead to genotoxic stress, causing DNA damage, growth arrest and cell death (Dmitrieva, Burg 2005). Increased apoptosis, as said above, has been found in the inner medulla of NFAT5-deficient mice (Lopez-Rodriguez et al. 2004) and DNA strand breaks and activation of p53 pathway is present in the eye lens of transgenic mice that overexpress a dominant negative NFAT5 mutant in these cells (Wang et al. 2005). A summary of these studies is shown in table 2.

NFAT5 is also involved in the induction of the proimflammatory cytokines TNF-α and LT-β in response to hypertonic conditions (Lopez-Rodriguez et al. 2001), hinting that NFAT5 enhances the proinflammatory immune response under pathologic conditions associated with hypertonic stress.

GENERATION	PHENOTYPE OF MICE
KO Deletion of the DNA binding loop. Lopez-Rodriguez et al. 2004	High late gestational and perinatal lethality. Renal abnormalities: kidney hypoplasia and altered medullary morphology (atrophy of the medulla). Increased apoptosis in the inner renal medulla. Impaired activation of osmoprotective genes (AR, BGT1 and SMIT, but not TauT). Reduced expression of AR in MEFs.
KO Deletion of exons 6 and 7 Go et al. 2004	Late gestational lethality. Abolished transcriptional activity of NFAT5. Diminished proliferation of MEFs. Reduced cellularity of thymus and spleen in heterozygous. Heterozygous present impaired antigen-specific antibody response and reduced IgG levels in serum. Splenocytes from NFAT5$^{+/-}$ mice proliferate >60% less than wild-type under hypertonic conditions.
Dominant negative CD2 promoter: expression in thymocytes and activated T cells Trama et al. 2002	30% reduction of cellularity in thymus and 15% in spleen. 50% reduction in the percentage of CD8$^+$ T cells and 25% in CD4$^+$ cells in spleen. No differences in thymus percentages. Impaired antigen-specific antibody and cytotoxic T cell responses. Reduced proliferation under conditions of medium depletion, probably due to increased osmolarity. Impaired cell viability by aminoacid limitation in isotonic conditions. Reduced viability of ConA blasts and thymocytes under hyperosmotic culture conditions.
Dominant negative Kidney-specific cadherin promoter: expression in epithelial cells of the renal collecting tubules Lam et al. 2004	Progressive bilateral hydronephrosis with partial penetrance. Lower osmolarity of the urine and concentration of ions, slightly higher serum osmolarity and urea concentrations. Decreased expression of AQP2, UT-A1 and UT-A2 transporters.
Dominant negative αA-crystallin promoter: expression in lens fiber cells Wang et al. 2005	Cellular deformities: numerous vacuoles, retarded elongation of fibers. Development of bilateral nuclear cataract. Increased DNA damage, p53 and phospho-Chk2 accumulation in lenses.

Table 2. Summary of transgenic mice expressing dominant negative NFAT5 proteins and knockout for the transcription factor.

4.1. Mechanisms regulating NFAT5 in response to hypertonicity

The activation of NFAT5 by osmotic stress involves its nuclear translocation and an increase of its transcriptional activity and synthesis (Dahl, Handler & Kwon 2001, Lopez-Rodriguez et al. 2001, Miyakawa et al. 1999, Trama et al. 2000, Woo et al.

2000). Nuclear translocation can be observed within 30 minutes of hypertonic stimulation (Dahl, Handler & Kwon 2001), while some hours later NFAT5 mRNA and protein increase (Miyakawa et al. 1999). The rise in the transactivation of NFAT5 is associated with the phosphorylation of its transactivation domain (Ferraris et al. 2002). However, the individual contribution of these mechanisms has not yet been dissected and quantified in different cell types.

Nuclear translocation of NFAT5

NFAT5 is found both in the cytoplasm and nucleus of cells under isotonic conditions whereas exposure to hypertonic conditions causes its complete nuclear translocation (Dahl, Handler & Kwon 2001, Lopez-Rodriguez et al. 2001). Three protein motifs responsible for NFAT5 nucleocytoplasmic shuttling have been characterized: a nuclear export sequence (NES), a putative auxiliary export domain (AED) and a nuclear localization signal (NLS). The NES comprises the sequence FISLLSADLDL and is primarily responsible for the nuclear export of NFAT5 under isotonic conditions by interacting with the exportin Crm1. Since this leucine-rich sequence is only found in the amino-terminal region of one of the three isoforms of NFAT5, the AED also plays an essential role regulating its localization. With the amino acid sequence HPSTPKRHTVLYISPPPEDLLDNSR the AED regulates the nuclear export of NFAT5 under hypotonic conditions independently of Crm1, while NFAT5 nuclear import is regulated by a consensus NLS. The NLS is comprised in the amino acids RKS**RKR**NPKQRPGV**KRR**D, where two clusters of basic amino acids are aligned in tandem, but only the mutation of the first cluster abolished its nuclear translocation under hypertonic conditions. As a general mechanism, it seems that hypertonicity inactivates the NES and AED, allowing full translocation of NFAT5 (Tong et al. 2006). The specific mechanisms that regulate the masking or unmasking of these motifs remain unidentified, even if several studies, sometimes contradictory, have been performed. The proteasome inhibitor MG-132 was shown to slightly inhibit the nuclear translocation of NFAT5 in response to hypertonic stimulation in MDCK cells (Woo et al. 2000) and HepG2 cells (Zhang et al. 2005) while it did not in COS-7 cells (Zhang et al. 2005), so it seems that the proteasome effect on NFAT5 localization is cell type-dependent. Wortmannin was able to inhibit NFAT5 translocation in COS-7 cells when used at concentrations that inhibit PI3-kinase-related kinases (PIKK), but overexpression of ATM in cells lacking the kinase also inhibited NFAT5 nuclear translocation (Zhang et al. 2005). However, in these cells wortmannin also enhanced

NFAT5 nuclear localization, suggesting that other kinase inhibited by wortmannin, such as DNA-PK, PI3K, SmMLCK or mTOR may participate in the regulation of NFAT5 shuttling. Recently, CK1α1L has been involved in the regulation of NFAT5 export, since its inhibition by the chemical compound CK1-7 or siRNA impeded NFAT5 translocation to the cytoplasm in hypotonic conditions, proposing that Ser155 and Ser158 might be phosphorylated by this kinase to induce NFAT5 cytoplasmic localization (Xu et al. 2008).

Regulation of NFAT5 by increased synthesis

Hypertonicity induces a significant increase in the amount of NFAT5 protein (Dahl, Handler & Kwon 2001, Lopez-Rodriguez et al. 2001), which indicates that it can play an outstanding contribution on its activity. In the same direction, overexpression of NFAT5 could activate NFAT5-dependent reporters in absence of hypertonicity in COS-7 (Lee et al. 2003) or HEK293 cells (Irarrazabal et al. 2004). Stimulation of NFAT5 expression by hypertonicity was in part mediated by the stabilization of preexisting mRNA mediated by the 5'UTR, whereas the 3'UTR of NFAT5 had a destabilizing effect under hypertonic conditions of 500 mOsmol/kg (Cai, Ferraris & Burg 2005). Nonetheless, the contribution of these sequences to the whole increase in NFAT5 mRNA by hypertonicity is partial, and other mechanisms involved in mRNA stabilization by tonicity are not known completely yet. In addition, the 3'UTR of NFAT5 had been described as a putative target for micro-RNAs *in silico* and, as micro-RNAs primarily function by repression, it may explain the NFAT5 mRNA destabilization in response to osmotic stress (Asirvatham et al. 2008). Beyond these reports, there are not detailed studies about the regulatory regions that control NFAT5 mRNA or protein synthesis, stability or degradation, as well as the signaling pathways controlling these functions.

Transcriptional activation of NFAT5

NFAT5 is a phosphoprotein with a considerable number of putative target residues for kinases. It contains a high number of serines, threonines and tyrosines, mainly located at its carboxy-terminal domain, which might regulate its activity. Moreover, this domain also contains polyglutamine repeats with a still unknown function.

Kinases

In general terms, NFAT5 includes a large hypertonicity-responsive transactivation domain located in the carboxy-terminal domain of the protein (Lee et al. 2003, Lopez-Rodriguez et al. 2001). More precisely, NFAT5 presents three activation domains that are distinguished from its other two modulation domains by its ability to stimulate transcription independently, even though all of them can act synergistically if combined. The most amino-terminal transactivation domain is just found in one isoform of NFAT5. However, the other two domains are stimulated by hypertonicity and the modulatory regions are able to enhance their activity, achieving the maximal transactivating capacity when the four domains are present. As NFAT5 is phosphorylated in response to osmotic stress in parallel to its increased transactivation capacity, it was thought that both phenomena could be related. In spite of this, phosphorylation correlated with increased transactivation only in some of the carboxy-terminal subdomains, whereas others were activated by hypertonicity without being phosphorylated (Lee et al. 2003).

The signaling pathways that trigger NFAT5 transcriptional activation are not completely known but several kinases had been shown to affect it. A phosphoamino acid analysis of NFAT5 performed by Dahl and colleages showed that hypertonicity induced phosphorylation of serine and tyrosine residues of the transcription factor (Dahl, Handler & Kwon 2001). Briefly, the kinases involved in NFAT5 activation are p38, Fyn, PKA, ATM, PI3K and ERK.

More in detail, a dominant negative construct of either p38 or JNK did not inhibit an NFAT5-dependent reporter under hypertonic conditions in rabbit renal medullary cells PAP-HT25 (Kultz et al. 1997). However, the p38 pharmacological inhibitor SB203580 and a p38 dominant negative construct have been shown to inhibit the activation of NFAT5 in response to tonicity increases in NIH3T3 cells (Ko et al. 2002). Tsai et al confirmed that the expression of a dominant negative p38 construct or the p38 inhibitor SFK86002 inhibited NFAT5-dependent transcription (Tsai et al. 2007). In the study by Ko and colleagues where the function of p38 is described, Fyn was shown as another kinase able to increase NFAT5 transcriptional activation, as cells expressing a dominant negative Fyn construct and fibroblasts lacking the kinase had reduced NFAT5 activation. The inhibition of both p38 and Fyn had a cooperative effect on the decrease of NFAT5 activity (Ko et al. 2002). As Fyn belongs to the Src family of tyrosine kinases and p38 is able to phosphorylate in serine and threonine, they were

suggested as putative candidates to regulate the transcriptional activity of NFAT5, but neither SB203580 nor the Fyn inhibitor PP2 were able to affect the global phosphorylation state of NFAT5 induced by hypertonicity in MDCK cells (Dahl, Handler & Kwon 2001). Nevertheless, the discrepancy in cell types and stimulation times used makes difficult to conclude whether Fyn and p38 are general regulators of NFAT5 and whether they directly phosphorylate it. Moreover, the latest article by the Burg laboratory showed that NFAT5 is regulated differentially by p38 isoforms. p38α, which is inhibited by SB203580, would be a positive regulator of NFAT5 whereas p38δ, insensitive to the pharmacological inhibitor, would be a negative regulator (Zhou et al. 2008).

In addition to p38 and Fyn, PKA has been shown to regulate the activity of an NFAT5-dependent reporter. Under hypertonic conditions in HepG2 cells, its pharmacological inhibitor H89 and a dominant negative of the kinase reduced the activity of the reporter, while a constitutively active construct enhanced its activity. Moreover, NFAT5 and PKA interacted both when cells were exposed to isotonic or hypertonic media (Ferraris et al. 2002).

ATM, which is activated by osmotic stress, is also involved in the activation of NFAT5. Expression of wild-type ATM increased transcriptional and transactivating activity in osmotic stress conditions, but expression of an inactive ATM mutant (S1981A) did not, in HEK293 cells. Moreover, ATM and NFAT5 coimmunoprecipitated both in isotonic and hypertonic conditions, although their interaction was not enhanced by the augment in tonicity. Reporter assays in cells lacking ATM (AT cells) pointed up S1247 as the most probable site of phosphorylation for ATM in NFAT5, even though in HEK293 cells all mutants tested were not able to decrease NFAT5 stimulation by NaCl. Additionally, NFAT5 activity was inhibited by wortmannin at low or high dosis in AT cells, indicating that other wortmannin-inhibitable kinases, such as conventional PI3K or kinases from the PIKK family, may be relevant for its transcriptional activation (Irarrazabal et al. 2004).

Later on, the same group demonstrated that PI3K class IA (PI3K-IA) kinases are also implicated in NFAT5 activation. Hypertonicity increased PI3K-IA lipid kinase activity, and inhibiting this kinase by either expressing a dominant negative of its regulatory subunit, p85, or by small interfering RNA-mediated knockdown of its catalytic subunit, p110α, reduced high NaCl-induced increases in NFAT5 transcriptional activity and

transactivation, while nuclear localization of NFAT5 or its synthesis were not affected. Furthermore, suppression of PI3K-IA inhibited NaCl-dependent ATM activation, pointing that PI3K-IA are necessary for the ATM-dependent activation of NFAT5 in response to osmotic stress (Irarrazabal et al. 2006).

Finally, the latest kinase that has been proposed as a regulator of NFAT5 transcriptional activity under an osmotic environment is ERK, both in nucleus pulposus and HeLa cells. ERK1/2 were phosphorylated and activated under hypertonic conditions and ERK pharmacological inhibitors induced the downregulation of NFAT5 transcriptional activity. The expression of a dominant negative construct or small interfering RNA-mediated knockdown of ERK had the same effect. Upstream activation of the pathway by a constitutive active MEK construct also enhanced NFAT5 transcriptional activity in isotonicity, as constitutively active ERK did (Tsai et al. 2007).

During the last years, some other kinase inhibitors have been tested in order to study the role of other kinases in NFAT5 activation. The transactivation activity of NFAT5 was partially inhibited by herbimycin, a tyrosine kinase inhibitor, and DRB, which inhibits the serine/threonine kinase CK2 (Ferraris et al. 2002). However, the role of these kinases has not been investigated further on. Rottlerin, a PKCδ inhibitor, was also able to inhibit NFAT5 transcriptional activity and expression after hypertonic treatment. However, PKCδ was not activated by osmotic stress and a dominant negative construct had no effect on NFAT5 transcriptional activity, suggesting that rottlerin may inhibit NFAT5 independently of PKCδ (Zhao, Tian & Cohen 2002). Conversely, NFAT5-dependent transcription was not affected by SP600125, a JNK inhibitor (Tsai et al. 2007), or a dominant negative construct that inhibits JNK activity (Kultz et al. 1997).

As a whole, several kinases that can be activated by osmotic stress seem to cooperate to activate NFAT5 (Table 3). Each of them is necessary for full activation of its transcriptional activity by hypertonicity, but individual inhibition of any of them does not fully prevent the activation of this factor. The use of the kinase inhibitor wortmannin has been valuable to point out the role of PI3K and PIKK in NFAT5 transcriptional activation, even if these results should be interpreted carefully, as wortmannin is known to inhibit also DNA-PK, SmMLCK or mTOR, and may inhibit others either directly or indirectly (Brunn et al. 1996, Davies et al. 2000). Interaction studies also showed that NFAT5 may be associated to several kinases in a resting state but no kinase whose interaction is hypertonicity-induced has been described. In addition, it is not known if

they act directly or indirectly. Moreover, putative sites for phosphorylation by ATM have been described, but broader studies are necessary to conclusively define which sites are really phosphorylated *in vivo*.

Despite the fact that quite a few kinases are associated to NFAT5 transcriptional regulation, other mechanisms have also been proposed. As NFAT5 translocation is partially affected by the proteasome inhibitor MG-132 in some cellular lines, the same study also tested if it could affect NFAT5 transcriptional activity, showing that MG-132 partially inhibited an NFAT5-dependent reporter under hypertonic medium (Woo et al. 2000). Ferraris and co-workers also tested the effect of this drug upon the transcriptional activity of NFAT5 using a chimeric NFAT5 transactivation domain fused to the GAL4-DBD, but they saw no inhibition (Ferraris et al. 2002). In conclusion, the role of the proteasome in NFAT5 activation remains controversial.

Other regulators

In resting isotonic conditions, RNA helicase A (RHA) associated with NFAT5 through its E'F loop, and hypertonicity caused its dissociation and transcriptional activation of NFAT5. Overexpression of RHA inhibited NFAT5-dependent transcription independently of its catalytic activity, while it forms parts of the transactivating complex of other transcription factors (Colla et al. 2006).

NFAT5 was also able to interact with Hsp90 and PARP-1 (poly(ADP-ribose) polymerase-1) both under 300 and 500 mOsm/kg conditions. Geldanamycin, an Hsp90 inhibitor (Giannini, Bijlmakers 2004), reduced the transcriptional activity of NFAT5 at both tonicities, without affecting its synthesis or nuclear localization. Although overexpression of PARP-1 also inhibited an NFAT5-dependent reporter, it was independent of the catalytic activity of PARP-1 (Chen et al. 2007), and additional studies are needed to understand the functional relevance of this association.

Moreover, the Ferraris' group noticed that NFAT5 DNA binding sites in some hypertonicity-responsive promoters often presented a consensus AP-1 DNA binding site near them (Chen et al. 2007) and, although before it was already described that the presence or not of the AP-1 consensus site in the aldose reductase enhancer did not affect its induction in response to osmotic stress (Ko et al. 1997) and, even if NFAT5 lacks the consensus AP-1 binding residues present in the other NFAT proteins,

they demonstrated that AP-1, when present in a native context forming a composite site, was necessary for NFAT5-transcriptional response to hypertonicity. AP-1 was activated by osmotic stress, interacted with NFAT5 regardless of medium osmolarity and inhibition of its activity by using dominant negative constructs or small interfering RNAs, partially decreased NFAT5 transcriptional activity (Irarrazabal et al. 2008).

FUNCTION	PATHWAYS INVOLVED	CELLS	REF
Nuclear translocation	Slightly inhibited by proteasome inhibitor MG-132.	MDCK, HepG2	Woo 2000 Zhang 2005
	Insensitive to proteasome inhibitor MG-132.	COS-7	Zhang 2005
	PI3K inhibitor wortmannin at 20 μM inhibits translocation.	COS-7	Zhang 2005
	Overexpression of ATM enhances nuclear localization.	AT cells ($ATM^{-/-}$)	Zhang 2005
	Inhibition of CK1α1L avoids nuclear export in hypotonicity.	HeLa	Xu 2008
Synthesis	Stabilization of mRNA mediated by 5'UTR.	mIMCD3	Cai 2005
Transcriptional activity	NFAT5 is phosphorylated in serine and tyrosine residues.	MDCK	Dahl 2001
	SB203580 and PP2 do not inhibit phosphorylation of NFAT5 in response to hypertonicity.	MDCK	Dahl 2001
	SB203580 and a dominant negative of p38 inhibit NFAT5 transcriptional activity after hypertonicity	NIH3T3	Ko 2002
	Inhibited by a dominant negative construct of Fyn.	NIH3T3	Ko 2002
	Activity inhibited in cells lacking Fyn.	MEF $Fyn^{-/-}$	Ko 2002
	Dominant negative p38α or SFK86002 inhibit NFAT5-dependent transcription.	Nucleus pulposus cells	Tsai 2007
	p38α is a positive regulator of NFAT5 and p38δ a negative one	HEK293	Zhou 2008
	H89 and a dominant negative PKA inhibit NFAT5-dependent transcriptional activity while a constitutive active construct enhances it.	HepG2	Ferraris 2002
	Inhibited by wortmannin (1-20 μM).	HEK293, AT cells	Irarrazabal 2004
	ATM overexpression increases NFAT5 transcriptional activity in response to osmotic stress, while a inactive ATM does not.	HEK293	Irarrazabal 2004
	Dominant negative p85 or small interfering RNA for p110α reduced NFAT5 activity.	HEK293	Irarrazabal 2006
	ERK pharmacological inhibitors, ERK dominant negative contruct or small interfering RNA for ERK downregulate NFAT5 activity. Constitutive active MEK enhances it.	Nucleus pulposus cells	Tsai 2007
	MG-132 partially inhibits NFAT5 activity.	MDCK	Woo 2000
	Overexpression of RNA helicase A inhibits NFAT5-dependent transcription.	HEK293	Colla 2006
	Inhibited by geldanamycin.	HEK293	Chen 2007
	Inhibited by overexpression of PARP-1.	PARP-$1^{-/-}$ cells	Chen 2007
	Dominant negative or small interfering RNA for AP-1 partially inhibits it.	HEK293	Irarrazabal 2008

Adapted from Aramburu et al. (2006)

Table 3. Summary of studies on the regulation of NFAT5 by hypertonicity in different cell types.

5. OTHER FUNCTIONS OF NFAT5

Several evidences imply NFAT5 in cellular processes unrelated, or at least not directly connected, to the osmotic stress response. These processes include embryonic development, integrin-induced cellular migration, muscle differentiation, T cell receptor activation, proliferation, doxorubicin-induced toxicity in cardiomyocytes and HIV replication.

Mice lacking NFAT5 presented dramatically reduced embryonic viability between days E13.5 and E17.5 and perinatal lethality (Go et al. 2004, Lopez-Rodriguez et al. 2004). However, even if mice lacking NFAT5 displayed progressive disruption of kidney morphology and function after birth, these did not explain the abrupt drop in survival, because maintenance of the extracellular milieu of the fetus depends on the placenta, not the fetal kidney. Furthermore, mice with bilateral renal agenesis, such as caused by Pax-2 deficiency, are born at expected Mendelian ratios and succumb only postnatally (Dressler et al. 1993). So, the development of the urinary concentrating ability by the kidney is likely not the cause of perinatal lethality. NFAT5 is expressed in most organs during mouse embryo development (Maouyo et al. 2002) raising the possibility that it might regulate some yet unknown function that could explain the perinatal lethality.

In addition, transgenic mice expressing a dominant-negative NFAT5 specifically in the eye lens presented cellular deformities in them and developed nuclear cataract after birth (Wang et al. 2005), suggesting that this factor is necessary for the proper development of the lens. Although it was discussed that these defects might be caused by an inappropriate response to hypertonic stress, the authors did not confirm such interpretation experimentally.

Clustering of the integrin $\alpha 6\beta 4$ by its extracellular matrix ligands, resulting in enhanced cell migration, also induced the transcriptional activity of NFAT5. Integrins are critical regulators of the invasive phenotype and, specifically, the $\alpha 6\beta 4$ integrin has been linked with epithelial cell motility, cellular survival and carcinoma invasion (Shaw et al. 1997, Trusolino, Bertotti & Comoglio 2001). Besides, NFAT5 expression was high in $\beta 4$-expressing cell lines and a dominant negative NFAT5 significantly inhibited its transcriptional activity when transfected into these cell lines, in correlation with the capacity of this construct to block cellular invasion (Jauliac et al. 2002). This integrin also activated NFAT1 in a calcineurin-dependent manner, controlling migration and

invasion (Jauliac et al. 2002), in part by inducing autotaxin/ENPP2 (Chen, O'Connor 2005). NFAT5 activity, on the other hand, lacks calcineurin-regulated amino-terminal sites (Lopez-Rodriguez et al. 1999) and did not seem to regulate autotaxin (Chen, O'Connor 2005). Not much is known about the signaling pathways regulating NFAT proteins in this context nor the genes targeted specifically by these transcription factors, and increasing this knowledge may reveal important aspects of tumor invasion.

Nevertheless, NFAT5 seems to have a general role in cell migration, as it promotesd cell invasion in cell lines derived from human breast and colon carcinomas (Jauliac et al. 2002) and inhibition of its transcriptional activity caused impaired migration and differentiation in cultured myoblasts (O'Connor et al. 2007). In order to regulate myoblast migration NFAT5 activated the transcription of the secreted cysteine-rich CNN (connective tissue growth factor) matrix protein Cyr61. Besides, $NFAT5^{+/-}$ heterozygous mice presented a defect in muscle regeneration with fewer myofibers formed at earlier times after injury (O'Connor et al. 2007).

NFAT5 expression was also induced by stimulation of T lymphocytes via their T cell receptor (TcR) or with mitogens and was downregulated by calcineurin inhibitors (Lopez-Rodriguez et al. 2001, Trama et al. 2000), distinguishing it from hypertonic stimulation, where calcineurin is not necessary (Trama et al. 2000). The upregulation of NFAT5 by the TcR-calcineurin pathway suggests a role for NFAT5 in T cell activation, although its function is still unclear. Mutant heterozygous mice with reduced NFAT5 activity (25% compared to wild-type) showed no defect in TcR-stimulated proliferation in isotonic conditions, whereas they were much less resistant to hypertonic stress (Go et al. 2004). However, this reduced NFAT5 expression could be sufficient to bypass defects in proliferation, so it is necessary to study these effects in T cells that express no NFAT5 at all.

The role played by NFAT5 in T cell activation is closely related with proliferation itself, as seen above. Although NFAT5 mRNA is expressed ubiquitously in mouse tissues (Lopez-Rodriguez et al. 1999, Trama et al. 2000), protein expression is limited to tissues with a high proliferation rate, such as thymus, testis, lung and brain (Trama et al. 2000), cell lines and TcR-stimulated lymphocytes (Lopez-Rodriguez et al. 2001, Trama et al. 2000). As an exception, some neurons can express relatively high levels of NFAT5 (Maallem et al. 2006a, Maallem et al. 2006b). All these observations suggest that NFAT5 may participate in proliferation processes under isotonic conditions.

Downregulation of NFAT5 in cardiomyocytes by small interfering RNA targeting or overexpression of dominant negative form decreased cell viability. In addition, the anti-tumor agent doxorubicin induced degradation of NFAT5 protein and downregulation of its targets, suggesting that NFAT5 might play a role in cardioprotection under isotonic conditions (Ito et al. 2007).

Finally, NFAT5 interacted with a specific enhancer binding site of HIV and it was involved in the replication of the virus in human primary differentiated macrophages, that express it constitutively (Ranjbar et al. 2006).

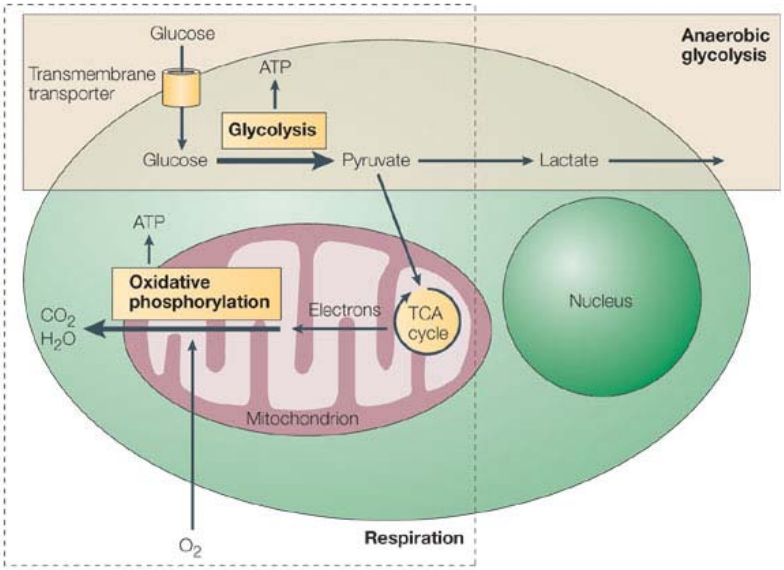
6. ENERGY METABOLISM DURING CELL ACTIVATION

Cell growth, understood as accumulation of mass, is an extensively coordinated process that is regulated in both time and space. To sustain viability, living organisms must regulate their proliferation and growth in response to changes in their environment. Unicellular organisms regulate growth and proliferation according to the availability of nutrients in their environment. By contrast, growth and proliferation of metazoan cells are not regulated according to the availability of nutrients in the extracellular environment because these cells are provided with a relatively constant supply of nutrients. Instead, growth factor receptors and other mechanisms have evolved to regulate growth and proliferation in response to changes in the availability of growth factors. When nutrients and other appropriate growth stimuli are present, cells upregulate macromolecular synthesis and thereby increase in size and mass. Conversely, cells respond to nutrient limitation or other types of stress by restraining macromolecular synthesis and enhancing turnover of excess mass.

The onset of proliferation introduces important challenges in cellular metabolism. Metabolism in proliferating cells differs from quiescent cell metabolism by high rates of glycolysis, lactate production, and biosynthesis of lipids and other macromolecules. Lymphocytes are an excellent model to study differential metabolic processes as these cells can be obtained in a resting state and activated *in vitro*, which explains that a substantial number of the works in this field use this cellular type.

6.1. Generation of cellular energy

Under aerobic conditions, glycolysis coupled to oxidative phosphorylation (collectively known as respiration) is the source of cellular energy. During glycolysis, glucose is converted to pyruvate, which is subsequently transported into the mitochondria, transformed to acetyl-CoA, which then enters the tricarboxylic acid (TCA) cycle. The electrons produced in the TCA cycle reactions are passed through the mitochondrial respiratory chain to oxygen, the terminal electron acceptor. The electrochemical gradient created by the electron-transport pathway is then transformed into ATP through the action of ATP-synthase. The synthesis of ATP in this chain of electron-transport reactions is known as oxidative phosphorylation. When the oxygen supply is insufficient, anaerobic glycolysis takes place, and this results in the accumulation of a large amount of pyruvate, which cannot be used by the TCA cycle. As a result, pyruvate is reduced to lactate and released from the cell. Under aerobic conditions, during glycolysis coupled to oxidative phosphorylation, the total energy yield from one glucose molecule is 30–38 molecules of ATP, of which only 2 of these ATP molecules come from glycolysis (Figure 5).



Adapted from Sitkovsky, Lukashev (2005)

Figure 5. Simplified scheme of glycolysis and oxidative phosphorylation.

During glycolysis, glucose is converted to pyruvate, which enters the tricarboxylic acid (TCA) cycle in mitochondria. There, electrons are released in a series of reactions and they enter the electron-transport chain creating an electrical gradient at the mitochondrial membrane. To equalize charge on both sides of the membrane, protons move across the membrane, and this is coupled to the generation of ATP as a result of oxidative phosphorylation. In the absence of oxygen, anaerobic glycolysis results in the reduction of pyruvate to lactate, which is then released from the cell.

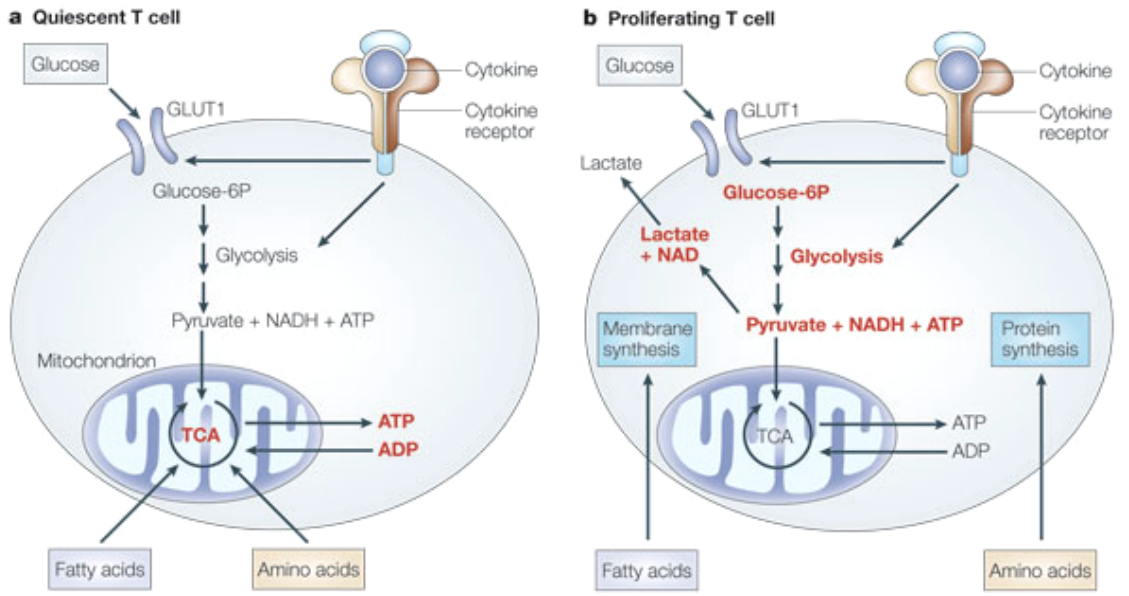
6.2. Metabolism of quiescent cells

Quiescent cells have fewer energetic and biosynthetic demands that proliferating cells and they dedicate the majority of their metabolism to housekeeping functions such as maintaining membrane integrity, volume regulation or ionic transport (Frauwirth, Thompson 2004, Krauss, Brand & Buttgereit 2001). They display a lower rate of nutrient uptake and derive most of their ATP from catabolic processes that fuel oxidative phosphorylation. Hence, in quiescent cells the combined breakdown of glucose, lipids and amino acids results in the synthesis of large amounts of ATP by oxidative phosphorylation.

6.3. Metabolism of proliferating cells

Upon stimulation, anabolic processes such as protein and lipid synthesis are increased, whereas catabolic processes are actively suppressed. Characteristically, despite adequate oxygen levels to undergo complete oxidation of glucose, cellular ATP production switches from oxidative phosphorylation to glycolisys. To distinguish this process from the anaerobic glycolysis, such as the performed in contracting muscle, it has been called aerobic glycolysis or the Warburg effect (Warburg 1956). Rapidly proliferating cells, such as immune cells and also some tumor cells, maintain glycolysis even under aerobic conditions despite being able to use oxidative phosphorylation (Buttgereit, Brand & Muller 1992, Roos, Loos 1973). This implies a high rate of lactate production, which allows the recycling of NADH to NAD^+ to maintain glycolytic flux (Brand et al. 1988). Even though glycolysis is much less effective than respiration, under conditions of unlimited glucose supply, glycolysis produces ATP substantially faster. In addition, glycolysis also generates metabolic intermediates critical for cell growth. Metabolism of glucose through the pentose phosphate shunt generates both ribose-5-phosphate, a key intermediate in nucleotide biosynthesis, and NADPH, which supplies reductive power for both nucleotide and fatty acid biosynthesis. Glycolytic intermediates also contribute to protein and lipid synthesis and, finally, the glycolytic end product pyruvate can be imported into the mitochondria where it can be converted into substrates for the production of additional amino acids or for fatty acid synthesis (DeBerardinis et al. 2008, Fox, Hammerman & Thompson 2005, Jones, Thompson 2007). Furthermore, increased glycolysis might protect cellular DNA from reactive-oxygen species that are generated in the respiratory chain during oxidative phosphorylation (Brand, Hermfisse 1997). The processes of activation and proliferation

of T cells constitute well characterized examples of these types of mechanisms. In mature T cells, the activation of glycolytic metabolism requires ligation of both the T cell receptor and CD28 (Frauwirth et al. 2002). Increased glycolysis precedes increased cell size, known as blastogenesis, and the entry to the cell cycle that occurs after T cell activation.



Adapted from Fox, Hammerman & Thompson (2005)

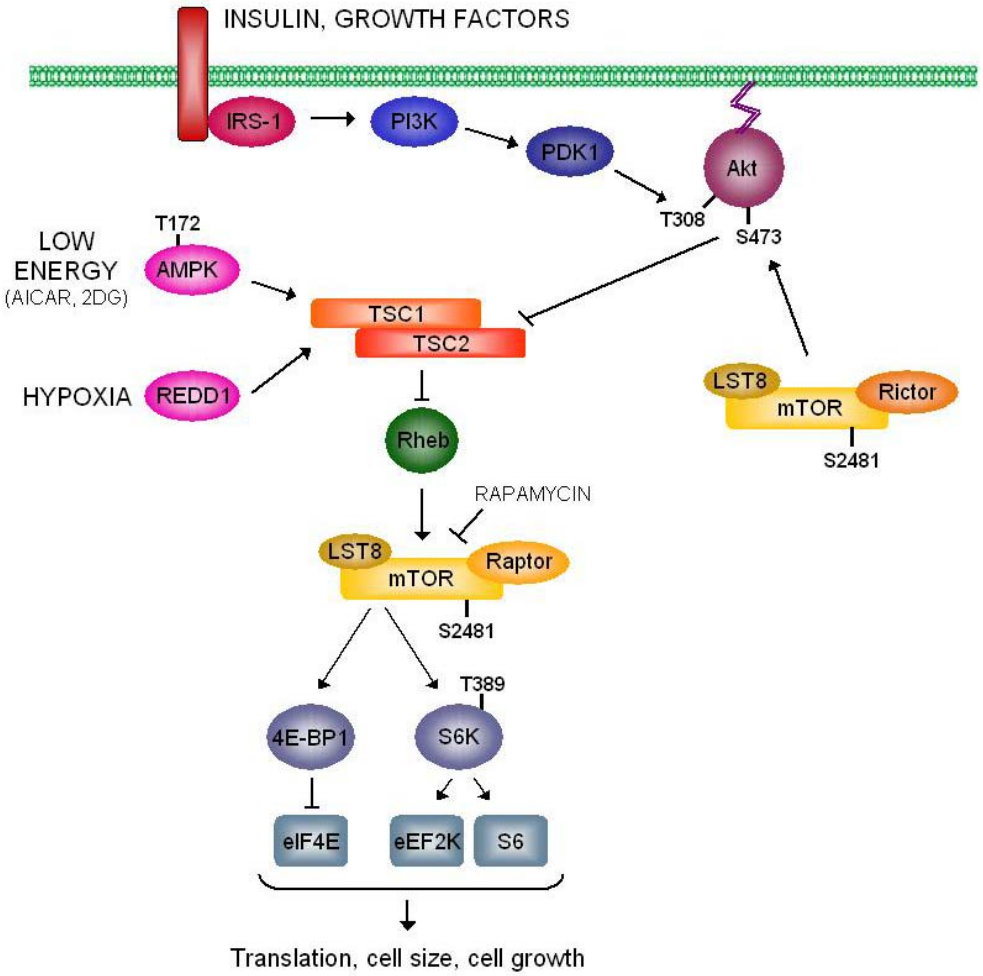
Figure 6. Metabolic changes are induced by cellular activation.

Quiescent cells obtain ATP through oxidative phosphorylation coupled to glycolysis. Stimulation of T cells involves a substantial increase in the glycolytic rate that generates most ATP. Fatty acids and amino acids are shunted away from the TCA cycle into membrane and protein synthesis, respectively.

7. SIGNALING PATHWAYS INVOLVED IN CELL GROWTH AND ENERGETIC STRESS

Several signaling pathways have been involved in the tight regulation of processes that allow cells to grow, proliferate and, therefore, survive. The mTOR pathway is active in proliferating cells and it is switched off when a stressful environment is present, whereas AMPK signaling pathway switches on under the latter conditions (Figure 7). In response to nutrient deprivation, cells respond promoting maintenance of energy production and suppressing cell cycle progression. In cases of severe metabolic stress

it also promotes apoptosis. On the whole, these mechanisms have evolved to maintain the rates of catabolism in balance with the rates of ATP consumption (Hardie 2004, Hardie 2005, Kimball 2006, Yang, Guan 2007).



Adapted from Corradetti, Guan (2006)

Figure 7. mTOR signaling to translation initiation, regulation of cell size and cell growth.

7.1. Mammalian Target of Rapamycin (mTOR) signaling pathway

The mammalian TOR (mTOR) is a serine/threonine protein kinase that belongs to the phosphatidylinositol kinase-related kinase (PIKK) family, with a predicted molecular weight of 290 kDa. The immunosuppressant rapamycin binds a small protein receptor called FKBP12 (FK506-binding protein 12 kDa) and the rapamycin/FKBP12 complex specifically binds to mTOR (Harding et al. 1989, Siekierka et al. 1989a, Siekierka et al. 1989b). Even if the binding of the complex to mTOR blocks some of the physiological

functions of mTOR and it has also been described that mTOR autophosphorylation is blocked by rapamycin, it is not clear whether rapamycin directly inhibits mTOR intrinsic kinase activity, being possible that it prevents mTOR from interacting with its substrates (Edinger et al. 2003, Jacinto et al. 2004, Peterson et al. 2000). While the phosphorylation of Ser2481 in mTOR has been considered the major indicator of mTOR intrinsic kinase activity, the contributions of other phosphorylation sites are not completely understood.

The most studied mTOR downstream factors are S6K1 (p70 ribosomal protein S6 kinase 1), S6K2 (p70 ribosomal protein S6 kinase 2) and 4E-BP1 (eIF4E binding protein 1) (Chung et al. 1992, Gingras, Raught & Sonenberg 1999, Martin et al. 2001). Under basal conditions, S6K1 and 4E-BP1 are bound to eIF3 (eukaryotic initiation factor 3) and remain inactive (Holz et al. 2005). Upon growth stimulating conditions, mTOR binds to eIF3 and phosphorylates S6K1 and 4E-BP1, releasing S6K1 from eIF3 and activating the kinase. 4E-BP1 inhibits cap-dependent mRNA translation by binding to the translation initiation factor eIF4E (eukaryotic translation initiation factor 4E) and the phosphorylation of 4E-BP1 by mTOR frees it, stimulating the initiation of translation (Gingras et al. 1999, Hay, Sonenberg 2004, Khaleghpour et al. 1999). S6K1 is then able to phosphorylate several targets involved in cell size, proliferation, protein synthesis and glucose homeostasis, among others (Ruvinsky, Meyuhas 2006). The ribosomal protein S6 was first described as a main regulator in translation initiation and consequently, protein synthesis, in response to S6K1 phosphorylation. However, S6 knockin mice where all S6K1 phosphorylation sites have been mutated to alanine presented similar or higher rates of protein synthesis and ribosome recruitment into polysomes than wild-type mice (Ruvinsky et al. 2005). Nowadays, the enhanced protein synthesis that is observed when cells are stimulated by mitogens or amino acids is explained by the phosphorylation by S6K1 of the eukaryotic elongation factor 2 kinase (eEF2K). eEF2K negatively regulates translation elongation by phosphorylating and inhibiting the eukaryotic elongation factor 2 (eEF2) and S6K1 phosphorylation relieves the inhibitory effect (Wang et al. 2001).

Upstream mTOR, the TSC1/2 complex (tuberous sclerosis complex 1 and 2) is a major inhibitory regulator of mTOR (Tee et al. 2002). When growth conditions are favorable, the TSC1/2 complex is kept inactive by phosphorylations mediated by Akt, ERK and RSK (Ballif et al. 2005, Inoki, Zhu & Guan 2003, Rolfe et al. 2005, Roux et al. 2004), while under stress conditions it suppresses mTOR activity to restrain cell growth.

mTOR complex 1

mTOR, Raptor and mLST8 form a complex, termed mTOR complex 1 (mTORC1), that is sensitive to rapamycin inhibition (Hara et al. 2002, Kim et al. 2002b, Kim et al. 2003). Raptor is an essential and presumably non-enzymatic subunit of mTORC1 (Hara et al. 2002, Kim et al. 2002a) and it is indispensable for mTOR to phosphorylate S6K1 and 4E-BP1. It has been proposed that Raptor can function as either a positive regulator of mTOR acting as a scaffolding protein (Hara et al. 2002) or a negative regulator when binding to the kinase (Kim et al. 2002a). Amino acid withdrawal or rapamycin treatment enhances or reduces binding to mTOR, respectively (Kim et al. 2002a, Oshiro et al. 2004). mLST8 seems to bind mTOR constitutively and recent data points that it is not essential for mTORC1 function (Guertin et al. 2006).

Growth-promoting stimuli activate the mTORC1 pathway

mTORC1 is a central controller of growth and integrates various signals to regulate it, basically growth factors, nutrients, energy and stress.

mTORC1 most relevant function is to sense growth factors signals to regulate cell growth. Growth factors and hormones, such as insulin, activate the recruitment of PI3K to the membrane, which converts phosphatidylinositol-4,5-phosphate (PIP2) in the membrane to phosphatidylinositol-3,4,5-phosphate (PIP3) (Engelman, Luo & Cantley 2006). PIP3 recruits PDK1 and Akt/PKB to the membrane, enabling the phosphorylation of Akt by PDK1 at the activation loop site threonine 308 (Toker, Newton 2000). Akt then phosphorylates and inactivates TSC1/2, upregulating mTORC1 activity (Dan et al. 2002) and promoting cell growth under growth factor or insulin stimulation.

Upstream mTORC1, TSC2 acts as a GAP (GTPase-activating protein) for the small GTPase Rheb, converting Rheb from the GTP-bound (active) to the GDP-bound (inactive) form (Garami et al. 2003, Inoki et al. 2003, Tee et al. 2003). Rheb binds directly to the kinase domain of mTORC1, preferentially when bound to GTP, stimulating its activity (Long et al. 2005a, Long et al. 2005b). While TSC1/2 relays its inhibition of mTORC1 through Rheb, PRAS40 (proline-rich Akt substrate 40kDa) has been identified as a direct inhibitor of mTORC1 binding to its kinase domain and to Raptor (Sancak et al. 2007, Vander Haar et al. 2007). PRAS40 mediates growth factor

signals to mTORC1 bypassing the TSC-Rheb pathway, by phosphorylation of threonine 246 of PRAS40 by Akt (Kovacina et al. 2003).

Growth factors signal through the PI3K-Akt pathway, as referenced above. Amino acid starvation, in particular the absence of leucine, results in a rapid dephosphorylation of the mTOR targets S6K1 and 4E-BP1, whereas addition of amino acids restores their phosphorylation in an mTORC1-dependent manner (Hay, Sonenberg 2004). Amino acids have been proposed to activate mTORC1 via inhibition of the TSC1/2 complex (Gao et al. 2002) or, alternatively, via stimulation of Rheb (Garami et al. 2003, Long et al. 2005b, Saucedo et al. 2003). However, it has also been proposed that the activity of the class III PI3K hVps34 (human vacuolar protein sorting-34) is necessary for the activation of S6K1 by amino acid stimulation via mTORC1 (Byfield, Murray & Backer 2005, Nobukuni et al. 2005). Thus, the mechanism by which nutrient status is communicated to mTORC1 requires further study.

Cell growth depends on a high rate of protein synthesis and consequently requires a high level of cellular energy. mTORC1 senses the energy status of the cell through AMPK (AMP-activated protein kinase). AMPK is activated in response to low cellular energy and, when activated, AMPK downregulates energetically demanding processes like protein synthesis and stimulates ATP-generating processes such as fatty acid oxidation. Its activation inhibits mTORC1-dependent phosphorylation of S6K1 and 4E-BP1 by directly phosphorylating TSC2 on serine 1345 (Inoki, Zhu & Guan 2003). However, there may be additional AMPK-independent pathways involved in the response to energy depletion via mTOR, as acute treatment of TSC2-deficient cell with the energy depleting agent 2-deoxyglucose inactivates S6K1 (Smith et al. 2005a). Moreover, REDD1 (also know as RTP801/Dig1/DDIT4) is a stress-response factor, induced in chronic energy depletion, which inhibits mTORC1 signaling to S6K1 independently of AMPK and by phosphorylating TSC2 (Corradetti et al. 2004, Sofer et al. 2005). Taken together, these data suggest that there are multiple mechanisms by which energy levels regulate mTORC1 signaling.

Stress inhibits mTOR signaling

Cells respond to environmental stresses by downregulating energy-demanding processes and arresting growth, which involves mTOR pathway inactivation. Several

types of stress, such as hypoxia, radiation, ROS (reactive oxygen species) or osmotic stress, have been described implying mTOR signaling.

Upon hypoxia, a state of low oxygen availability, protein synthesis is limited by PERK-dependent (PKR-like ER kinase) mechanisms in an early phase and by hypophosphorylation of 4E-BP in a late phase (Connolly et al. 2006). Under acute low oxygen, mTOR signaling is downregulated in a HIF-1 and AMPK-independent manner (Arsham, Howell & Simon 2003, Liu et al. 2006). However, even if short-term hypoxia does not seem to affect mTOR activity by transcriptional changes or energy depletion, a prolonged drop of oxygen levels stimulate HIF-dependent and HIF-independent transcriptional recovery mechanisms and promotes the activation of AMPK (Inoki, Zhu & Guan 2003). An essential hypoxia-responsive factor is REDD1, the expression of which is induced in response to hypoxia in a HIF-1-dependent manner. Under hypoxic conditions, REDD1 inhibits the mTOR pathway by releasing TSC2 from its growth factor-induced association with inhibitory 14-3-3 proteins (DeYoung et al. 2008).

The tumor suppressor p53 functions in cell cycle arrest, induction of DNA repair proteins and apoptosis (Harris, Levine 2005) and can be induced and stabilized by several stressors. p53 induction decreases protein synthesis and S6K1 activity (Horton et al. 2002) and addition of etoposide, a DNA damaging agent, downregulates S6K1 activity mediated by AMPK and TSC2 (Feng et al. 2005). The DNA damaging agent ultraviolet (UV) light initially stimulates S6K1 activity in a rapamycin-sensitive manner and, afterwards, S6K1 stimulation decreases and reaches background levels (Huang et al. 2002, Parrott, Templeton 1999). The activating effect can be attributed to the generation of reactive oxygen species that accumulate in response to UV exposure, whereas pretreatment with ROS antioxidants prevents S6K1 activation (Huang et al. 2002). The importance of the initial up-regulation of mTOR pathway upon life-threatening stimuli is still not understood.

In mammalian cells, osmotic stress causes a transient inactivation of the mTOR pathway and inhibition of protein synthesis. This is reflected in a rapid and reversible dephosphorylation of active S6K1 and 4E-BP1 independently of p38 and JNK kinases (Fumarola, La Monica & Guidotti 2005, Morley, Naegele 2002, Parrott, Templeton 1999). S6K1 activity is also decreased in $TSC2^{-/-}$ mouse embryonic fibroblasts (MEF) upon hypertonicity, suggesting that the regulation of S6K1 activity occurs downstream of TSC2 (Smith et al. 2005a). Moreover, this downregulation is also independent of

Rheb, as overexpression of this factor did not block sorbitol-induced S6K1 dephosphorylation (Inoki et al. 2003). It has been proposed that osmotic stress affects a pool of mTOR complexes that localize to the mitochondria, as the mitochondrial proton gradient is perturbed and mitochondrial fragmentation due to this stress (Copp et al. 2005a, Desai, Myers & Schreiber 2002). Reduction in S6K1 phosphorylation suggests that mTORC1 complex is involved in this process, but osmotic stress also triggers adaptative changes of the cytoskeleton (Di Ciano-Oliveira et al. 2006) that may involve mTORC2 activity.

mTOR complex 2

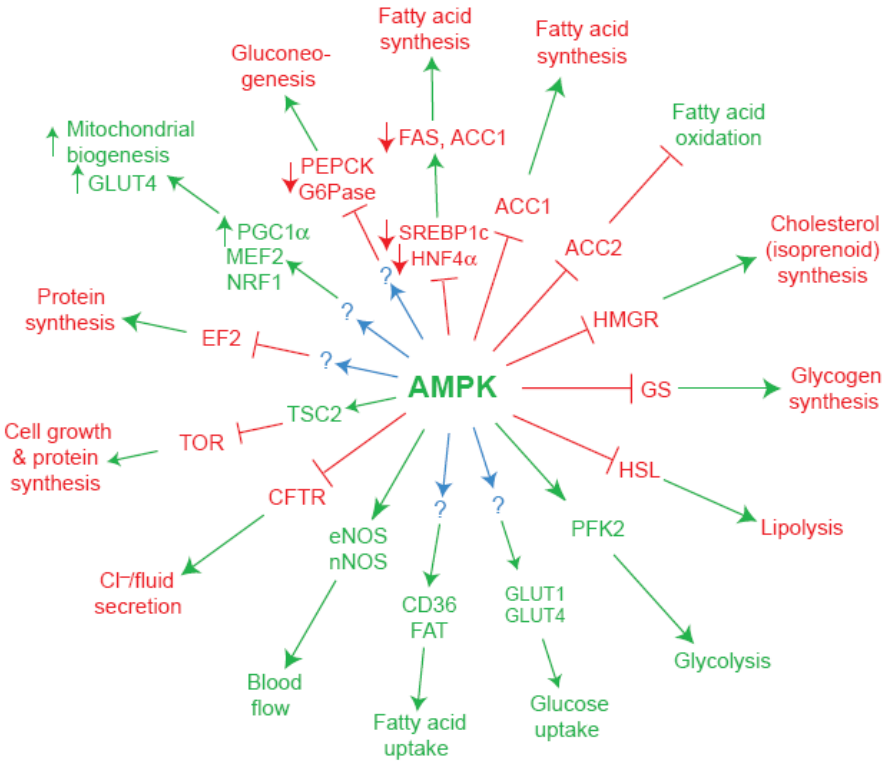
In recent years, progress has been made to identify mTOR-associated proteins, leading to the discovery of mTOR complex 2 (mTORC2), which has distinctive structure and physiological functions compared to mTORC1 (Sarbassov, Ali & Sabatini 2005, Wullschleger, Loewith & Hall 2006). Contrary to mTORC1, mTORC2 was firstly described as insensitive to rapamycin inhibition. However, reduction of mTORC2 has been observed in certain cell types undergoing prolonged rapamycin treatment at higher concentrations than the necessary to inhibit mTORC1 (Sarbassov et al. 2006). One postulation is that some mTORC2 components may block the binding of rapamycin/FKBP12 complex to mTOR and, then, prolonged rapamycin treatment may decrease mTORC2 by competing for newly synthesized mTOR. Not much is known about the mechanisms regulating upstream mTORC2 and, functionally, mTORC2 mainly regulates the actin cytoskeleton, possibly through the Rho small GTPase family and PKC (protein kinase C) (Jacinto et al. 2004, Sarbassov et al. 2004). Moreover, mTORC2 phosphorylates the serine 473 in Akt, serving as a docking site for PDK1, that then phosphorylates Akt threonine 308 (Scheid, Marignani & Woodgett 2002, Scheid, Woodgett 2003), which is necessary to fully activate Akt and, in consequence, contributes to the activation of mTORC1 by inhibiting TSC1/2 complex, as mentioned above.

The components of the mTORC2 complex are mTOR, Rictor (rapamycin-insensitive companion of mTOR), Sin1 and mLST8 (Jacinto et al. 2006, Sarbassov et al. 2004, Yang et al. 2006). The interaction between Rictor and mTOR is neither blocked by rapamycin nor affected by nutrient levels and, in consequence, knockdown of Rictor using siRNA does not change the phosphorylation state of S6K1 and 4E-BP1 (Jacinto et al. 2004). Altogether with Rictor, Sin1 stabilizes through binding the assembly of the

mTORC2 complex (Yang et al. 2006). The constitutively bound in mTORC1, mLST8, seems to play a more relevant function in mTORC2 complex, as reflected in mLST8-deficient mice, whose phenotype resemble Rictor-deficient mice (Guertin et al. 2006). In addition, mLST8-deficient MEF present disminished phosphorylation of Akt serine 473 while they show no defects in the phosphorylation state of 4E-BP1 and S6K1, indicating impaired mTORC2 and a functional mTORC1. In addition, Protor-1 (Protein observed with Rictor-1) has also been described as proteins members of mTORC2 through interaction with Rictor, but their functional role is at present unknown (Pearce et al. 2007).

7.2. AMPK

Living cells normally maintain a high ratio of ATP to ADP, typically around 10:1, which is many orders of magnitude away from the equilibrium ratio for ATP hydrolysis under cellular conditions. This represents a store of energy that can be used to drive energy-requiring processes. Catabolism converts ADP to ATP, whereas most other cellular processes require ATP hydrolysis. Most cells manage to maintain their ATP:ADP ratio within fairly narrow limits, which indicates that the rate of ATP synthesis exactly matches the rate of ATP consumption. The pathways involved in these processes involve AMPK kinase (AMP-activated protein kinase), that is sensitive to the cellular AMP:ATP ratio (which is a more sensitive indicator of the cellular energy status than the ADP:ATP ratio) and, thus, it is activated by metabolic stresses that inhibit ATP production or those that stimulate ATP consumption. Once activated, AMPK switches on catabolic pathways that generate ATP such as the uptake and metabolism of glucose and fatty acids, while switching off ATP-consuming, anabolic pathways such as the synthesis of fatty acids, cholesterol, glycogen and proteins (Hardie, Hawley & Scott 2006). It achieves this by rapid phosphorylation of metabolic enzymes and by phosphorylation of transcription factors and coactivators that regulate later gene expression. AMPK regulates a large subset of genes involved in energy metabolism, cell signaling, cell growth and proliferation, immunity, transcription and apoptosis (McGee, Hargreaves 2008) (Figure 8).



Adapted from Hardie (2004)

Figure 8. AMPK targets.

Target proteins and processes activated by AMPK activation are shown in green, and those inhibited by AMPK activation are shown in red. Where the effect is caused by a change in gene expression, an upward-pointing green arrow next to the protein indicates an increase, whereas a downward-pointing red arrow indicates a decrease in expression.

AMPK is a heterotrimeric complex comprising a catalytic α-subunit and regulatory β- and γ-subunits. The α-subunits (α1 and α2) contain a conventional serine/threonine kinase domain at the amino-terminus whereas the carboxy-terminal region is required for formation of the complex with β and γ (Crute et al. 1998). The β-subunits contain a carboxy-terminal region required for complex formation and a central N-isoamylase domain that causes AMPK complexes to bind to glycogen (Hudson et al. 2003, Polekhina et al. 2003). The γ-subunits contain variable amino-terminal regions followed by four tandem repeats of a sequence known as CBS motif or Bateman domain, where AMP binds (Bateman 1997, Scott et al. 2004). AMP activates the kinase in three distinct ways: (1) allosterically, by binding to the γ-subunit; (2) promoting phosphorylation of the threonine 172 located in the kinase domain by an upstream kinase, being essential for the activation of AMPK (Hawley et al. 1996) and (3) inhibiting the dephosphorylation of this Thr172 by phosphatases (Davies et al. 1995). The kinase acts as an energy sensor: the effects of AMP are antagonized by high

concentrations of ATP (Sanders et al. 2007). Many downstream targets of AMPK were initially identified by activating the kinase in intact cells using the drug AICAR, an adenosine analogue that is taken up into cells via adenosine transporters (Gadalla et al. 2004) and converted to the monophosphorylated nucleotide and AMP mimicking agent, ZMP, by adenosine kinase (Vincent et al. 1991). AICAR is not completely specific for AMPK, because ZMP regulates other AMP-sensitive enzymes such as fructose-1,6-bisphosphatase (Vincent et al. 1991) and muscle glycogen phosphorylase (Longnus et al. 2003). Moreover, AICAR activates other kinases such as p38 (Lemieux et al. 2003), Akt, GSK3 (King, Song & Jope 2006) and the JAK/STAT pathway (Zang et al. 2008) regardless of AMPK. Moreover, a pharmacological inhibitor of AMPK that is widely used in the literature, compound C, is also able to affect cellular responses independently of AMPK (Emerling et al. 2007).

Upstream kinases that phosphorylate threonine 172

LKB1 and CaMKKβ have been identified as the upstream kinases responsible for the phosphorylation of the threonine 172 of AMPK.

LKB1 is a tumor suppressor mutated in the rare autosomal dominant human genetic disorder called Peutz-Jegher Syndrome (PJS) (Hemminki et al. 1998, Jenne et al. 1998). PJS patients develop numerous benign tumors in the gastrointestinal tract and have a higher risk of developing malignant tumours (Giardiello et al. 2000). LKB1 is required for activation of AMPK in response to treatments that elevate AMP or AMP mimetic agents, both in cultured cells (Hawley et al. 2003) and in skeletal muscle *in vivo* (Sakamoto et al. 2006). LKB1 must be bound to the subunits STRAD and MO25 to be functional (Boudeau et al. 2003, Hawley et al. 2003) and this complex itself is not regulated by AMP and seems constitutively active (Lizcano et al. 2004, Sakamoto et al. 2004).

Increases in AMP do not stimulate phosphorylation of threonine 172 by CaMKKβ, which is activated by signals that increase the concentration of cytosolic Ca^{2+} (Hawley et al. 2005, Hurley et al. 2005, Woods et al. 2005). While LKB1 is ubiquitously expressed, CaMMKβ expression is more restricted, mainly to neural tissues (Anderson et al. 1998). It has been involved in the activation of AMPK in response to K^+-induced depolarization in rat brain slices (Hurley et al. 2005), thrombin activation in endothelial

cells (Stahmann et al. 2006) and stimulation of the T cell receptor in T lymphocytes (Tamas et al. 2006).

Metabolic stresses activate AMPK

AMPK is activated by any stress that depletes cellular ATP including metabolic poisons such as inhibitors of the tricarboxylic acid cycle (arsenite) (Corton, Gillespie & Hardie 1994), the respiratory chain (antimycin A, azide) (Witters, Nordlund & Marshall 1991), or the mitocondrial ATP synthase (oligomycin) (Marsin et al. 2000), as well as uncouplers of oxidative phosphorylation such as dinitrophenol (Witters, Nordlund & Marshall 1991). AMPK is also activated by pathological stresses such as glucose deprivation, ischemia, hypoxia (Marsin et al. 2000), oxidative stress and hyperosmotic stress (Fryer, Parbu-Patel & Carling 2002, Woods et al. 2003, Woods et al. 2005). Physiological stimuli that activate AMPK by increasing ATP consumption are exercise (Winder, Hardie 1996) and contraction (Hutber, Hardie & Winder 1997) in skeletal muscle. AMPK is also activated indirectly by metformin (Zhou et al. 2001) and thiazolidinediones (Fryer, Parbu-Patel & Carling 2002), widely used treatments for Type 2 diabetes.

Cell growth and proliferation are energy-intensive processes and activation of AMPK inhibits them. MEF that are deprived of glucose arrest at the G1-S boundary and this effect requires phosphorylation of serine 18 on p53 by AMPK, suggesting that the kinase forms an energy check-point that delays progress through the cell cycle if not enough energy is available (Jones et al. 2005). Furthermore, a number of studies have shown that AICAR treatment induces a cell cycle arrest similar to that caused by glucose starvation in a number of cell types (Imamura et al. 2001, Jones et al. 2005). As well as causing cell cycle arrest, AMPK activation inhibits cell growth by inhibiting lipid synthesis and by switching off protein synthesis by two pathways. First, the activation of eukaryotic elongation factor-2 kinase (eEF2K), which causes inhibition of the elongation step of translation (Horman et al. 2002), and inhibition of the mTOR pathway by phosphorylating TSC2 (Inoki et al. 2006, Inoki, Zhu & Guan 2003), which stimulates the initiation step of protein synthesis by phosphorylation of multiple targets.

There is a discrepancy on whether osmotic stress activates AMPK by decreases in energy status in cells. Although increased AMPK activity after osmotic shock induced by sorbitol was associated with decreased intracellular ATP, phosphocreatine, and

glycogen content in isolated rat epitrochlearis muscles (Hayashi et al. 2000), there was no effect of sorbitol on the ATP/AMP ratio in H-2K^b skeletal muscle cells (Fryer, Parbu-Patel & Carling 2002). Unlike rotenone and hypoxia, but similar to contractions, glucose transport induced by osmotic shock was not affected in muscles from AMPK dominant-negative mice (Fujii et al. 2005). This differs from results in H-2K^b cells, where osmotic shock-stimulated glucose transport is completely abolished by adenoviral mediated overexpression of the AMPKα dominant-negative mutant (Fryer, Parbu-Patel & Carling 2002). In conclusion, AMPK is activated in several cell types by osmotic stress, even if it has not been clearly defined whether it involves a decrease in the ATP/AMP ratio.

OBJECTIVES

The objectives of this thesis were:

1) To determine the tonicity levels at which NFAT5 is activated in different types of primary cells.

2) To study the signaling pathways involved in the activation of NFAT5 by osmotic stress.

RESULTS

Results and methods are described in the following articles:

1) Morancho B, Minguillon J, Molkentin JD, Lopez-Rodriguez C, Aramburu J. *Analysis of the transcriptional activity of endogenous NFAT5 in primary cells using transgenic NFAT-luciferase reporter mice.* BMC Mol Biol. 2008 Jan 25;9:13

2) Morancho B, Drews-Elger K, Viollet B, Laderoute KR, Lopez-Rodriguez C, Aramburu J. *Osmoregulatory effects of the mammalian target of rapamycin (mTOR) and AICAR via NFAT5-dependent and independent mechanisms in mammalian cells.* Submitted

Analysis of the transcriptional activity of endogenous NFAT5 in primary cells using transgenic NFAT-luciferase reporter mice

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Osmoregulatory effects of the mammalian target of rapamycin (mTOR) and AICAR via NFAT5-dependent and independent mechanisms in mammalian cells.

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Running title: regulation of NFAT5 by mTOR and AICAR.

Key words: NFAT5, hypertonicity, osmotic stress, mTOR, rapamycin, AICAR.

SUMMARY

Mammalian cells can adapt to hypertonic conditions by activating the transcription factor NFAT5/TonEBP, which controls the induction of osmoprotective genes. Since hypertonicity can induce cellular responses akin to those caused by energy and nutrient deprivation, activating the energy-sensing kinase AMPK and inhibiting the mTOR pathway, we investigated whether NFAT5 could be regulated by these kinases. The mTOR inhibitor rapamycin reduced the hypertonicity-stimulated transcriptional activity of NFAT5 in HEK293 cells and mouse embryo fibroblasts (MEF), and inhibited the induction of Hsp70.1 in response to hypertonic stress in primary T lymphocytes. The AMPK activator AICAR inhibited the expression of NFAT5 and caused the dephosphorylation of mTOR and S6K1 in hypertonicity-treated HEK293 cells and MEF. These inhibitory effects were also observed in AMPK-null MEF, indicating that they occurred via AMPK-independent mechanisms. In primary T cells, AICAR also inhibited the expression and activation of NFAT5, downregulated the mTOR pathway, and impaired cell proliferation under hypertonic conditions. However, although AICAR inhibited NFAT5 function, it could also enhance the expression of Hsp70.1 in an NFAT5-independent manner. These findings reveal the existence of different layers of regulation of NFAT5 and other osmoresponsive functions by pharmacological modulators of energy and nutrient-sensing mechanisms.

INTRODUCTION

NFAT5/TonEBP belongs to the Rel family of transcription factors, which comprises the NF-κB and NFATc proteins, with which NFAT5 shares a structurally and functionally conserved DNA binding domain (Aramburu et al., 2006). NFAT5 is fundamental in the adaptation of mammalian cells to hypertonic stress (Burg and Ferraris, 2008). Hypertonicity activates the synthesis, nuclear accumulation and transcriptional activation of NFAT5, which in turn induces the expression of genes whose products allow the intracellular accumulation of compatible osmolytes or allow cells to withstand stress conditions. Work from different laboratories has shown that NFAT5 regulates the expression of aldose reductase (AR), Na^+/Cl^- -coupled betaine/γ-aminobutyric acid transporter (BGT1), Na^+-dependent myo-inositol transporter (SMIT), Na^+ and Cl^- -dependent taurine transporter (TauT), UT-A urea transporter, and Hsp70 (reviewed in (Jeon et al., 2006) and (Burg and Ferraris, 2008)). NFAT5-deficient mice suffer severe atrophy of the renal medulla, a naturally hypertonic environment, and impaired lymphocyte function (Lopez-Rodriguez et al., 2004; Go et al., 2004). Overall, the osmoresponsive function of NFAT5 has been documented in diverse cell types, such as kidney cells (Woo and Kwon, 2002; Lopez-Rodriguez et al., 2004), neurons (Loyher et al., 2004; Maallem et al., 2006), embryonic fibroblasts (Lopez-Rodriguez et al., 2004; Go et al., 2004), lymphocytes and macrophages (Lopez-Rodriguez et al., 2001; Trama et al., 2002; Morancho et al., 2008), and cell lines of different lineages (Jauliac et al., 2002; Irarrazabal et al., 2004).

Activation of NFAT5 by hypertonicity requires the activity of several kinases and phosphatases. The stress-activated MAP-kinase p38α (Ko et al., 2002; Zhou et al., 2008), Fyn (Ko et al., 2002), PKA (Ferraris et al., 2002), ERK (Tsai et al., 2007), phosphoinositide 3-kinase (PI3-kinase) (Irarrazabal et al., 2006), the PI3-kinase-related kinase (PIKK) ATM (Irarrazabal et al., 2004), and the calcium-dependent phosphatase calcineurin (Li et al., 2007; Morancho et al., 2008) have been reported to activate NFAT5. The MAP-kinase p38δ has recently been shown to be a negative regulator of NFAT5 (Zhou et al., 2008). Some of these regulators are more relevant in specific cell types. Thus, inhibition of p38 did not impair NFAT5 activity in PAP-HT25 rabbit renal medullary cells (Kultz et al., 1997), while it did in NIH3T3 cells, mouse embryo

fibroblasts (MEF) (Ko et al., 2002), nucleus pulposus cells (Tsai et al., 2007) and T lymphocytes (Morancho et al., 2008). Calcineurin enhanced NFAT5 activity in response to hypertonicity in murine collecting duct cells and primary T lymphocytes (Li et al., 2007; Morancho et al., 2008), but contributed much less in MEF and macrophages (Morancho et al., 2008). Inhibitors of ERK reduced NFAT5 activity in nucleus pulposus cells (Tsai et al., 2007) but not in T cells (Morancho et al., 2008). In addition, we have reported that the responsiveness of NFAT5 to hypertonicity in T cells was lowest in quiescent lymphocytes and was substantially enhanced in cells that had received mitogenic stimulation (Morancho et al., 2008), suggesting that a state of active growth could be a positive regulator of NFAT5.

The mammalian TOR (mTOR) pathway is a major controller of biogenesis and cell growth in response to growth factors and nutrients (reviewed in (Wullschleger et al., 2006; Reiling and Sabatini, 2006)). The central component of the pathway is mTOR, a PI3-kinase-related serine/threonine kinase that can be found in two protein complexes, TORC1 and TORC2 (Sarbassov et al., 2004). TORC1 contains Raptor, mLST8, and the GTP/GDP binding protein Rheb, and is active in cells that are stimulated by growth factors while at the same time having access to energy and nutrient stores (reviewed in (Inoki and Guan, 2006)). TORC1 promotes protein synthesis by enhancing cap-dependent mRNA translation. This function is mediated by the TOR-dependent phosphorylation and activation of the S6 kinases S6K1 and S6K2 (Martin et al., 2001; Holz et al., 2005), and phosphorylation and inactivation of the translation initiation repressor 4EBP1 (Khaleghpour et al., 1999; Gingras et al., 1999). TORC1 is negatively regulated by the hamartin/tuberin complex TSC1/TSC2 (Inoki et al., 2003). The GTPase activity of TSC2 converts the mTORC1 activator Rheb-GTP into Rheb-GDP, inactivating it (Inoki et al., 2003). Under growth-promoting conditions, the TSC1/TSC2 complex is kept inactive by phosphorylations mediated by AKT/PKB, Erk and RSK (Inoki et al., 2002; Roux et al., 2004; Ballif et al., 2005; Rolfe et al., 2005). Under conditions that cause energy stress, such as glucose deprivation or hypoxia, TSC2 can be activated by two major sensors of energy stress, the LKB1-dependent, AMP-activated kinase AMPK (Inoki et al., 2003; Shaw et al., 2004), and the hypoxia-activated protein REDD1 (Corradetti et al., 2004; Sofer et al., 2005).

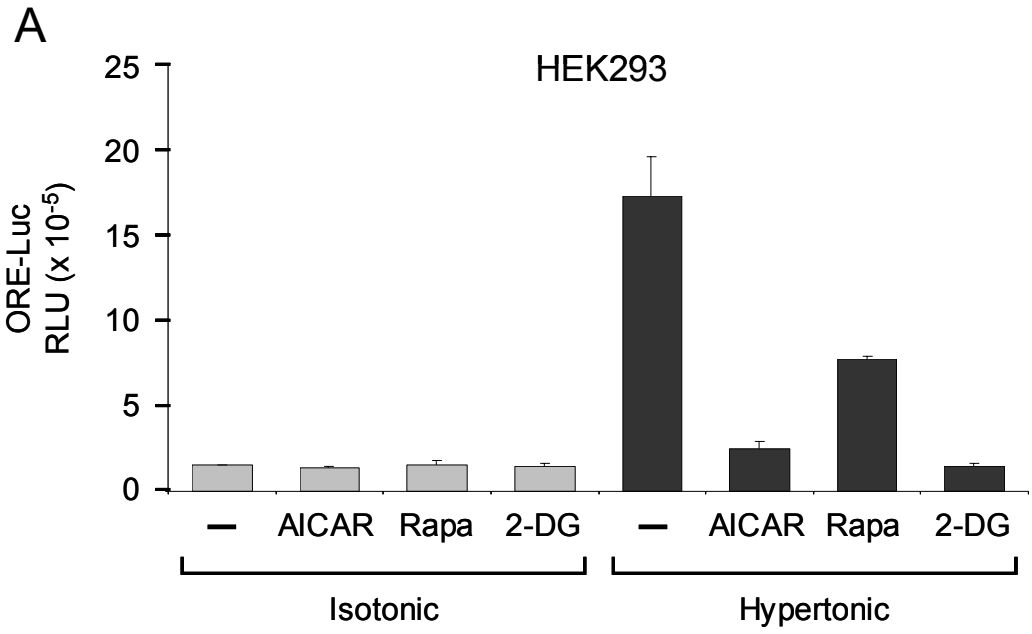
Hypertonic shock can trigger cellular responses similar to those induced by energy and nutrient deprivation, activating the energy-sensing kinase AMPK (Barnes et al., 2002; Fryer et al., 2002), as well as causing a transient inactivation of the mTOR pathway and inhibition of protein synthesis (Chen et al., 1999; Morley and Naegele, 2002). Elegant experiments by Brigotti et al. showed that elevated concentrations of Na^+ or K^+ ions, as occurring in cells during the regulatory volume increase phase that follows exposure to hypertonic media, inhibited translation reactions and the formation of preinitiation complexes in rabbit reticulocyte lysates, whereas increasing the osmolality with the compatible osmolytes betaine or myo-inositol did not inhibit these reactions (Brigotti et al., 2003).

We have investigated the potential involvement of mTOR and AMPK in the activation of NFAT5. Here we report that the mTOR inhibitor rapamycin impaired the hypertonicity-induced activation of NFAT5 dependent on its carboxy-terminal transactivation domain in HEK293 cells and mouse embryo fibroblasts (MEF), but did not inhibit NFAT5 nuclear translocation nor its DNA binding capacity. Rapamycin also inhibited the induction of Hsp70.1 in response to hypertonic stress in primary T cells. Activation of NFAT5 was suppressed by 5-aminoimidazole-4-carboxamide riboside (AICAR), a compound commonly used to activate AMPK. We found that AICAR inhibited the expression and activity of NFAT5 and caused the dephosphorylation of mTOR and S6K1 under hypertonic condtions in several cell types, including AMPK-null MEF, indicating that these effects were AMPK-independent. AICAR also inhibited NFAT5 induction and the mTOR pathway in hypertonicity-treated primary T cells. However, AICAR could enhance the induction of Hsp70.1 in T lymphocytes by NFAT5-independent mechanisms, whereas rapamycin inhibited it. Our findings indicate that NFAT5 is positively regulated by mTOR in diverse cell types, and suggest the existence of different layers of regulation of NFAT5-dependent and independent osmoresponsive pathways by inputs from energy and nutrient-sensing mechanisms and pharmacologic modulators of these responses.

RESULTS

Sensitivity of NFAT5 to inhibition of mTOR and inducers of energy stress.

Since osmotic stress activates NFAT5 but can also induce responses that resemble conditions of energy deprivation, we tested whether the transcriptional activity of NFAT5 was sensitive to pharmacologic modulators of energy stress responses. HEK293 cells transfected with the hypertonicity responsive, NFAT5-dependent, ORE-Luc reporter were exposed to hypertonic conditions (500 mOsm/kg) in the absence or presence of the mTOR inhibitor rapamycin, the glycolysis inhibitor 2-deoxyglucose or the AMPK activator 5-aminoimidazole-4-carboxamide riboside (AICAR). As shown in Figure 1A, these compounds inhibited the activity of the reporter to varying degrees (between 50% -80%) in HEK293 cells. On the other hand, none of them inhibited the nuclear translocation of NFAT5 in response to hypertonic stress and, indeed, AICAR and 2-deoxyglucose moderately enhanced it in isotonic conditions (Figure 1B). To assess whether NFAT5 inhibition might be due to a general shutdown of cellular processes in cells simultaneously exposed to hypertonic stress and these drugs, we tested whether they interfered with the activity of a constitutively active NFAT5 chimaeric construct (DBD5-VP16) that comprised the NFAT5 DNA binding domain (DBD) fused to the VP16 transactivation domain. We performed the experiment in NFAT5-deficient mouse embryo fibroblasts to exclude the contribution of endogenous NFAT5. As shown in Figure 1C, the ORE-Luc reporter did not respond to hypertonicity in NFAT5-deficient MEF, but was activated by osmotic stress in cells transfected with an NFAT5-encoding vector. Similarly to our results in HEK293, the response of the reporter to hypertonicity induced by the recombinant NFAT5 in MEF was inhibited by rapamycin, 2-deoxyglucose and AICAR (Figure 1C). In contrast, activation of the reporter by the constitutively active VP16-DBD5 was not impaired by these drugs either in isotonic or hypertonic conditions. Since the activity of this construct depended on binding to DNA via the NFAT5 DBD, this result indicating that the compounds did not impair NFAT5 DNA binding capacity. Altogether, our results indicated that the downregulation of NFAT5 activity by rapamycin, 2-deoxyglucose and AICAR was not due to a general impairment of transcriptional functions in the cell nor to inhibition of the DNA binding and nuclear translocation capacity of NFAT5, but involved the inactivation of its carboxy-terminal transactivation domain.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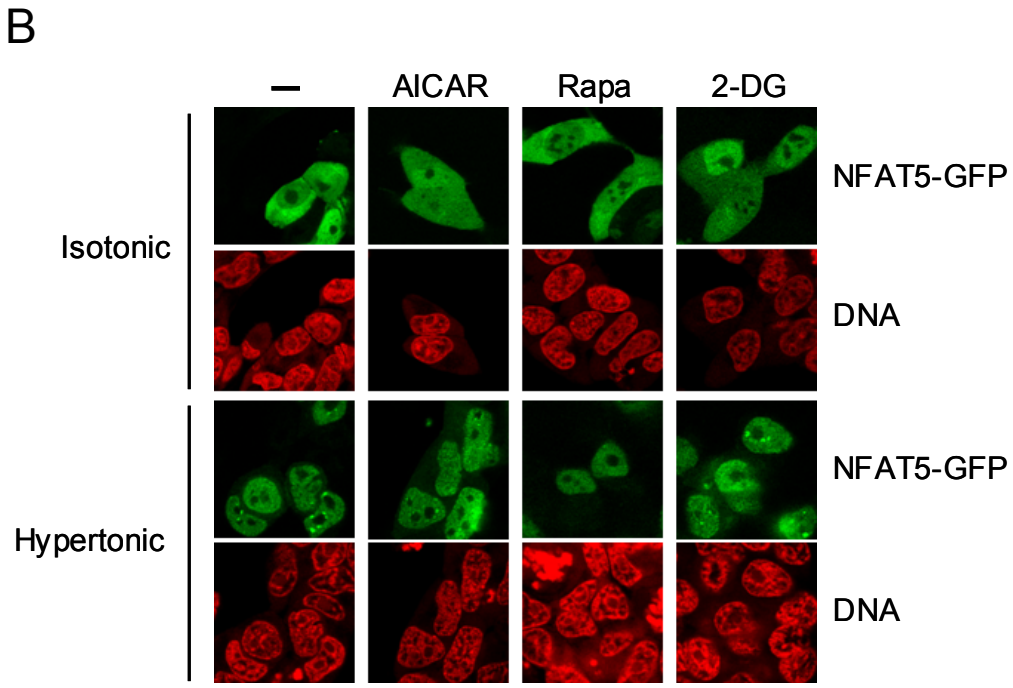
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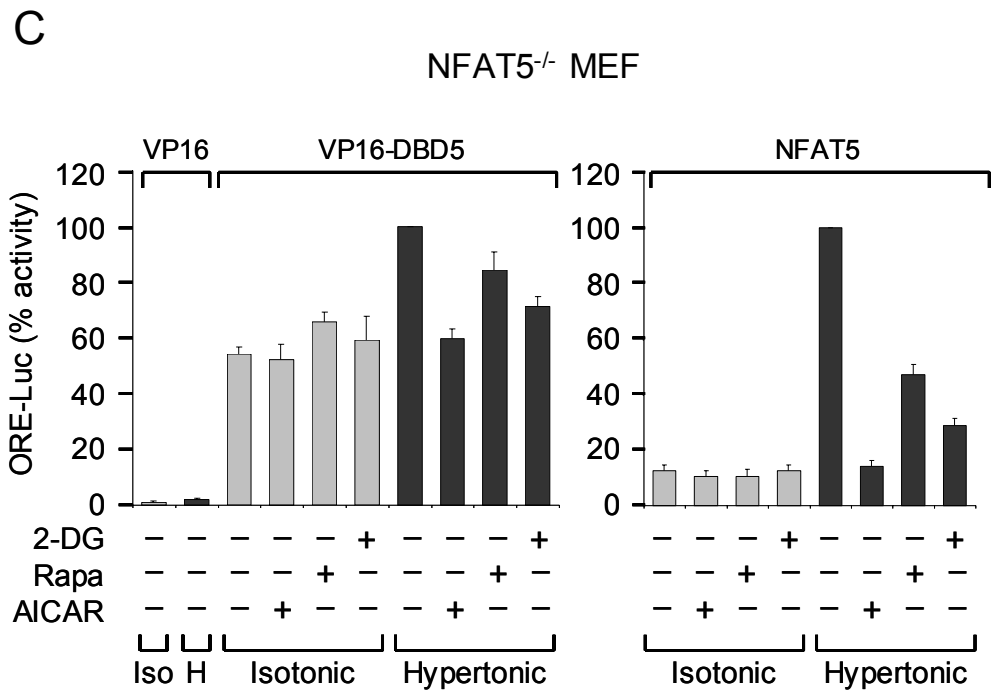


Figure 1. Effect of AICAR, rapamycin and 2-deoxyglucose on the activation of NFAT5 in response to hypertonicity. A) HEK293 cells transfected with the NFAT5-responsive ORE-Luc reporter were cultured in isotonic (300 mOsm/kg) or hypertonic medium (500 mOsm/kg) during 24 hours in the absence or presence of AICAR (1 mM), rapamycin (200 nM) or 2-deoxyglucose (60 mM). Luciferase activity is represented as relative light units per second (RLU) after normalization with Renilla and endogenous lactate dehydrogenase. The graphic shows the mean ± S.D. of three independent experiments. **B)** HEK293 cells expressing Myc-NFAT5-GFP were left untreated or pretreated for 1 hour with AICAR (1 mM), rapamycin (200 nM) or 2-deoxyglucose (60 mM) before culturing them in isotonic (300 mOsm/kg) or hypertonic medium (500 mOsm/kg) during 4 hours. DNA was stained with TO-PRO3. Subcellular localization of NFAT5 was assesed by confocal microscopy. One representative experiment is shown out of three performed independently. **C)** NIH3T3-immortalized NFAT5$^{-/-}$ MEF transfected with the NFAT5-responsive ORE-Luc reporter and expression vectors for the VP16 transactivation domain alone, the DNA binding domain of NFAT5 fused to VP16 (VP16-DBD5), or Myc-NFAT5-GFP were cultured in isotonic (300 mOsm/kg) or hypertonic medium (500 mOsm/kg) during 24 hours in the absence or presence of AICAR (1 mM), rapamycin (200 nM) or 2-deoxyglucose (60 mM). Luciferase activity is shown as the percentage of the activity of VP16-DBD5 or Myc-NFAT5-GFP respectively in hypertonic conditions without drugs (100%). Mean ± S.E.M. of four independent experiments is shown.

Sensitivity of NFAT5 to inhibition of mTOR and modulators of energy stress responses in primary lymphocytes.

In view of the results described above, we extended our analysis to primary, non-transformed T cells, as they induce and activate NFAT5 in response to moderate, pathologically relevant, hypertonic stress (Go et al., 2004; Morancho et al., 2008). We used T cells from 9xNFAT-Luc transgenic mice, since this reporter can be activated by NFAT5 in response to hypertonicity in primary cells (Morancho et al 2008). As shown in Figure 2A, moderate hypertonic conditions (400 mOsm/kg) induced the activation of the reporter by 8 hours in splenocytes that had been prestimulated during 24 hours with concanavalin A (ConA) and IL-2. Most of the activity detected at 8 hours was NFAT5-dependent, whereas after a more prolonged hypertonicity treatment (24 hours), the 9xNFAT-Luc reporter was also activated by NFAT5-independent mechanisms, in agreement with our previous work (Morancho et al., 2008). In these experiments, rapamycin caused a generally modest stimulation of the reporter in most of the cultures in the first 8 hours of hypertonic treatment, but had highly variable effects in individual cultures at 24 hours, inhibiting the reporter by 40-70% in three out of six experiments but stimulating it between 2 to 4 times in the other three. With respect to AICAR, we observed that it suppressed the early, NFAT5-dependent, phase of reporter activation, but strongly enhanced the NFAT5-independent component at 24 hours. AICAR was also able to detectably stimulate the reporter at 24 hours even in isotonic conditions in both wild-type and $NFAT5^{-/-}$ cells (Figure 2A). 2-deoxyglucose did not affect the reporter at 8 hours but enhanced its activity at 24 hours in wild-type as well as in $NFAT5^{-/-}$ T cells, although the magnitude of its stimulatory effect was lesser than that of AICAR. These results indicated that AICAR inhibited the activity of NFAT5 in primary T cells, but could also enhance other hypertonic stress responses that were independent of this factor. With regard to rapamycin, though, the results made it difficult to derive a conclusion on the effect of this inhibitor on the specific activity of NFAT5 in T cells. In order to address this issue by an independent approach, we analyzed the effect of these compounds on the expression of an endogenous NFAT5-regulated gene. We chose *Hspa1b*, whose promoter is regulated by NFAT5 (Woo et al., 2002), since the commonly studied NFAT5 target aldose reductase is poorly induced by osmotic stress in T cells ((Trama et al., 2002) and Morancho B, unpublished work). Upon exposure of mitogen-activated T cells to 400 mOsm/kg, Hsp70.1 was induced between 2.6 to 52

times (average was 17 times) in the first 8 hours (Figure 3). This induction was transient and by 24 hours of hypertonicity treatment, the levels of Hsp70.1 mRNA were on average only 1.5 times higher than in untreated cells (data not shown). We observed that Hsp70.1 induction was reduced by 80% on average in NFAT5-deficient lymphocytes, but was not completely suppressed (Figure 3). Rapamycin inhibited the induction of Hsp70.1 in most experiments (five out of seven) in wild-type cells, although the extent of inhibition (37-80%) varied among individual cultures, and even stimulated it 5 times in one particular experiment. AICAR, however, reproducibly enhanced the induction of Hsp70.1 in hypertonicity-treated wild-type and NFAT5$^{-/-}$ T cells (Figure 3), confirming that this compound could stimulate NFAT5-independent hypertonic stress responses in lymphocytes. Despite this stimulatory effect, AICAR was a potent inhibitor of T cell proliferation both in isotonic as in hypertonic conditions, as were rapamycin and 2-deoxyglucose (supplementary Figure S1).

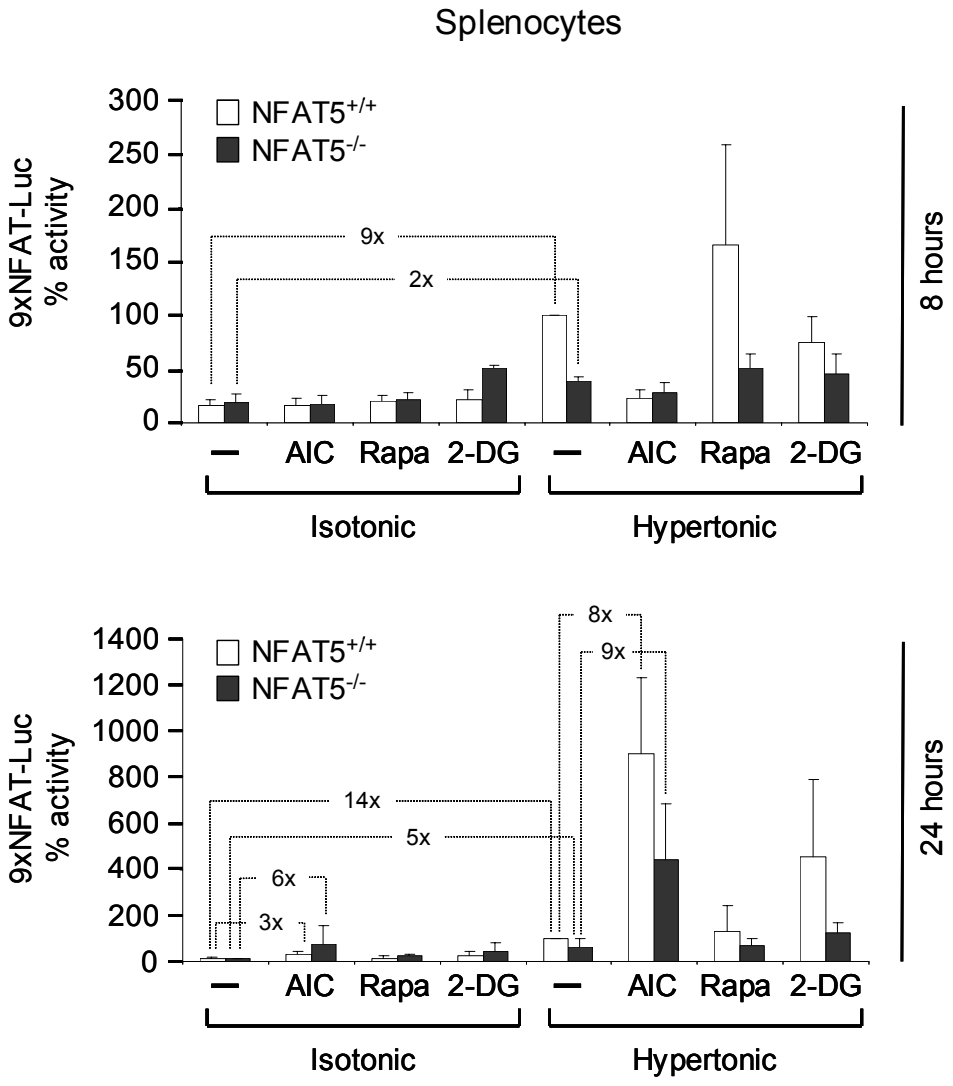


Figure 2. Activity of the NFAT5-responsive reporter 9xNFAT-Luc in transgenic splenocytes in response to AICAR, rapamycin and 2-deoxyglucose. Splenocytes from transgenic 9xNFAT-Luc/NFAT5$^{+/+}$ and 9xNFAT-Luc/NFAT5$^{-/-}$ mice were preactivated with ConA plus IL-2 during 24 hours before culturing them in isotonic (300 mOsm/kg) or moderately hypertonic medium (400 mOsm/kg) during 8 or 24 hours in the absence or presence of AICAR (1 mM), rapamycin (200 nM) or 2-deoxyglucose (60 mM). Luciferase activity after normalization with endogenous lactate dehydrogenase is represented as the percentage of the activity of the reporter in hypertonic conditions without drugs (100%). The graphic shows the mean ± S.D. of six NFAT5$^{+/+}$ and three NFAT5$^{-/-}$ mice.

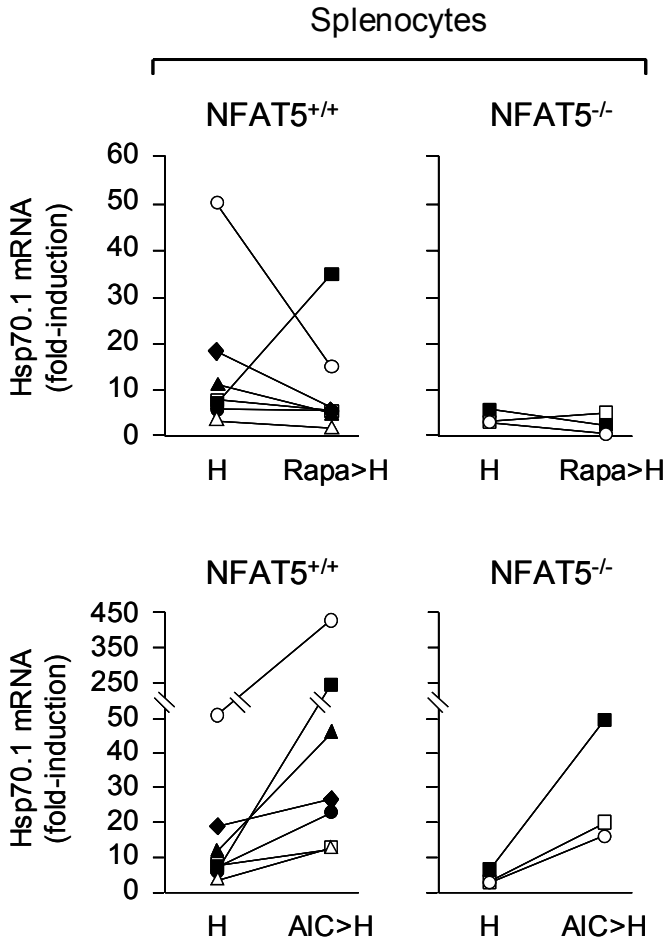


Figure 3. Effect of AICAR and rapamycin on the induction of Hsp70.1 mRNA in response to hypertonicity. Splenocytes derived from NFAT5$^{+/+}$ and NFAT5$^{-/-}$ littermates were preactivated with ConA plus IL-2 during 24 hours before culturing them in isotonic (300 mOsm/kg) or moderately hypertonic medium (400 mOsm/kg) during 8 hours in the absence or presence of AICAR (1 mM) or rapamycin (200 nM). The graphics show the relative induction of

Hsp70.1 mRNA upon hypertonic stimulation with respect to isotonic conditions (which was given an arbitrary value of 1) after normalization to L32 mRNA. Seven individual NFAT5$^{+/+}$ and three NFAT5$^{-/-}$ mice are shown.

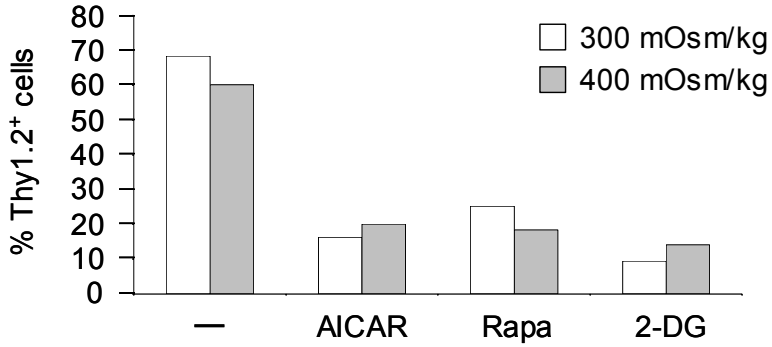
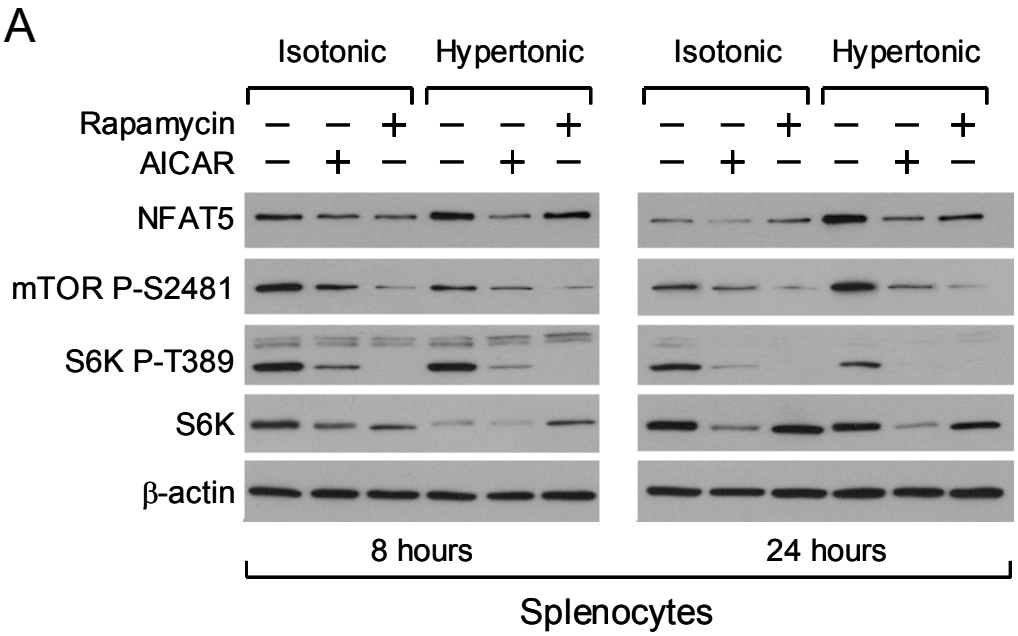


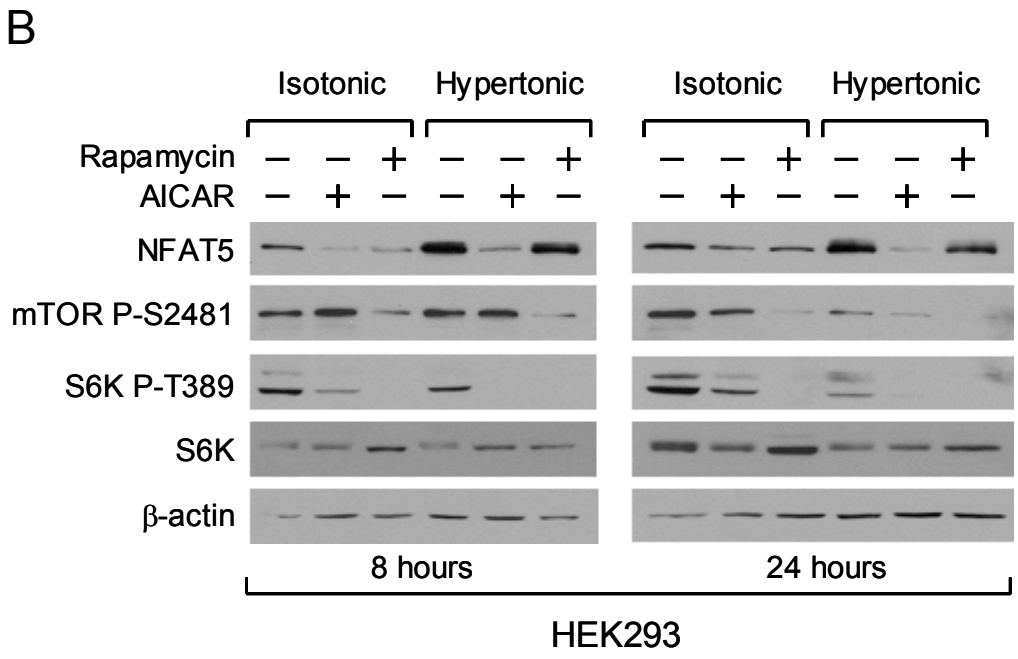
Figure S1. Effect of hypertonicity, AICAR, rapamycin and 2-deoxyglucose on the expansion of T cells in response to mitogens. Splenocytes stimulated with ConA plus IL-2 for 24 hours in the absence or presence of AICAR (1 mM), rapamycin (200 nM) or 2-deoxyglucose (60 mM) were cultured for an additional 48 hours in isotonic (300 mOsm/kg) or moderately hypertonic (400 mOsm/kg) conditions. The proportion of T cells in the cultures was determined by staining with anti-Thy1.2 antibody and flow-cytometry in the population of live cells. Results are representative of two experiments.

Regulation of the mTOR pathway by hypertonic stress and AICAR.

Since acute, elevated hypertonic stress has been shown to inhibit elements of the mTOR pathway and our results showed that mTOR was a positive regulator of NFAT5, we analyzed the sensitivity of the mTOR pathway to prolonged hypertonic conditions in the range required to activate NFAT5. As shown in Figure 4A, exposure of ConA/IL-2-stimulated splenocytes to hypertonic conditions that induced and activated NFAT5 (400 mOsm/kg) did not cause the dephosphorylation of mTOR S2841 and S6K1 T389, while rapamycin did. Rapamycin also inhibited, although moderately, the expression of NFAT5 induced by hypertonicity. In the same experiment, AICAR blocked the induction of NFAT5 by hypertonicity and caused the dephosphorylation of mTOR. Notably, AICAR promoted the dephosphorylation of S6K1 in the first 8 hours, but also caused a decrease in its total amount after longer incubation times. Similar results were obtained in hypertonicity-treated HEK293 cells and an independent NFAT5$^{+/+}$ MEF line (Figure 4B and 4C). In some of these experiments, we noticed that hypertonicity could cause a

limited degree of dephosphorylation of mTOR and S6K1 in the different cell types analyzed, although it was less pronounced than that caused by rapamycin or AICAR (Figure 4B and supplementary Figure S3).





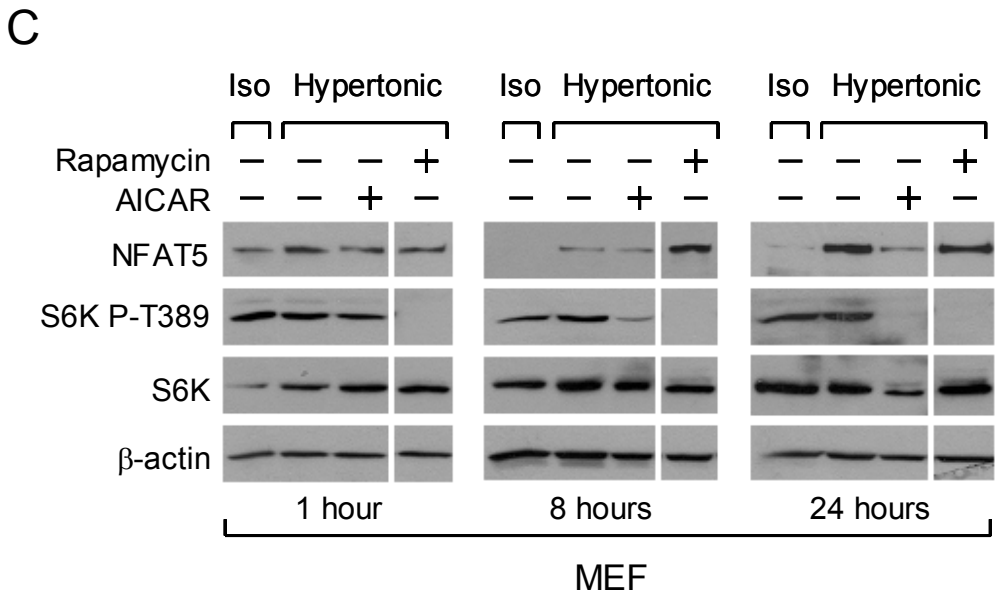
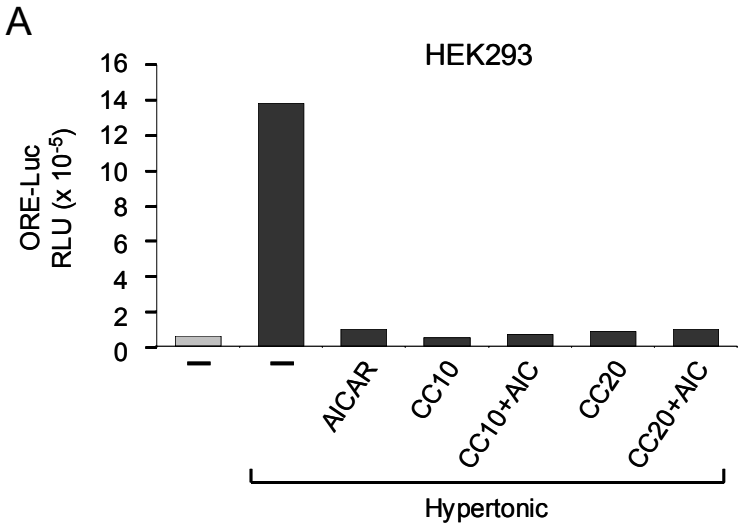


Figure 4. Effect of rapamycin and AICAR on the expression of NFAT5 and the phosphorylation of mTOR and S6K1 in cells exposed to hypertonic stress. Splenocytes activated with ConA plus IL-2 **(A)**, HEK293 cells **(B)**, and MEF **(C)** were cultured in isotonic (300 mOsm/kg) or hypertonic medium (400 mOsm/kg for splenocytes and 500 mOsm/kg for HEK293 cells and MEF) in the absence or presence of AICAR (1 mM) or rapamycin (200 nM) as indicated. NFAT5, S6K1, phospho-S6K1 (Thr389) and phospho-mTOR (Ser2481) were detected by Western blot. β-actin is shown as a loading control. One representative experiment out of three performed for each cell type is shown.



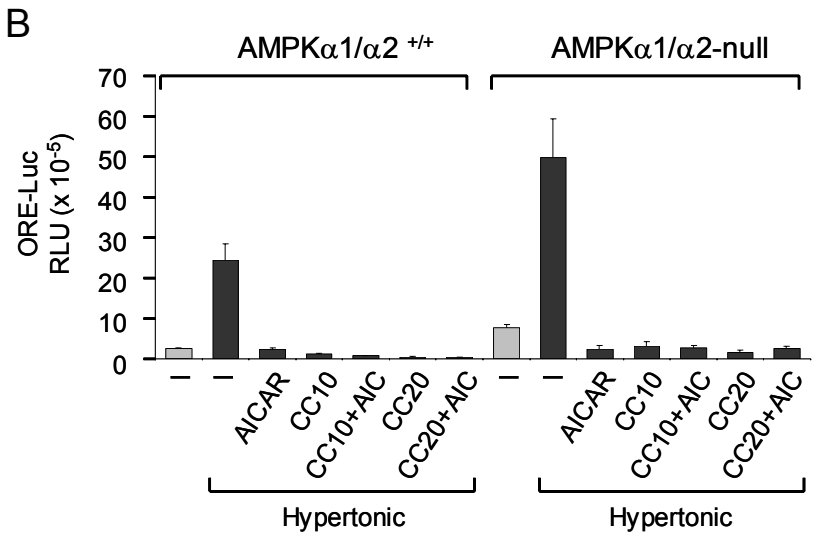
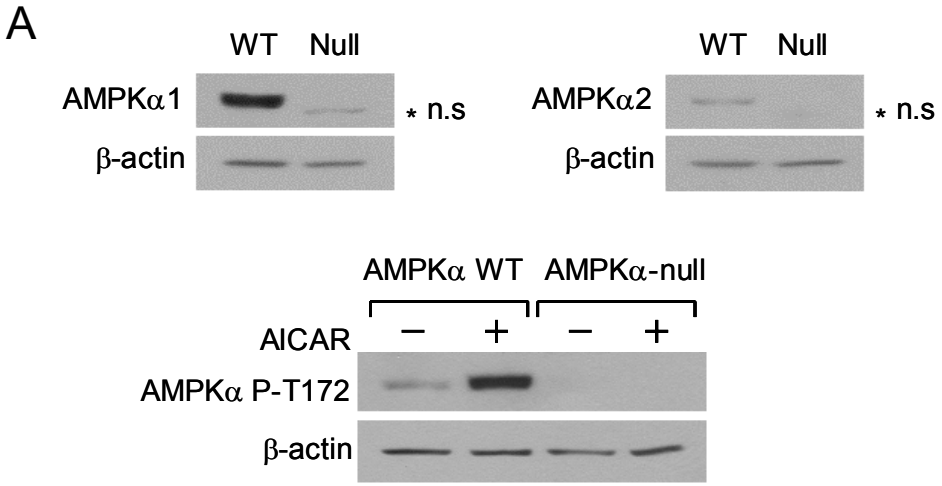


Figure S2. Suppression of NFAT5 activity by the AMPK inhibitor compound C. HEK293 cells **(A)** or wild-type and AMPKα1/α2 double knockout MEF **(B)** transfected with the NFAT5-responsive reporter ORE-Luc were cultured in isotonic (300 mOsm/kg) or hypertonic medium (500 mOsm/kg) during 24 hours in the absence or presence of AICAR (1 mM), compound C (10 and 20 μM), or their combination. Luciferase activity is represented as relative light units per second (RLU) after normalization with Renilla. One representative experiment is shown for HEK293 cells, and the mean ± S.E.M of three independent experiments is shown for MEF.

AMPK-independent inhibition of NFAT5 and mTOR by AICAR.

We next analyzed whether the sensitivity of NFAT5 and mTOR to AICAR reflected a negative regulation of these proteins by AMPK. Experiments with the AMPK inhibitor compound C turned out uninformative since this drug completely inhibited the activity of NFAT5 in HEK293 cells (supplementary figure S2A). We then tested the response of the ORE-Luc reporter to hypertonicity in AMPKα1/α2-double knockout MEF (Figure 5A) treated with AICAR, rapamycin, or 2-deoxyglucose. As shown in Figure 5B, AICAR effectively downregulated NFAT5 transcriptional activity in both cell lines, whereas rapamycin was less inhibitory (40-50% inhibition). This result indicated that AICAR could inhibit NFAT5 in AMPK-null cells. Compound C also inhibited the reporter in the AMPK-null MEF line, confirming that its inhibitory effect on NFAT5 was unrelated to AMPK (supplementary figure S2B). We observed that 2-deoxyglucose did not inhibit the reporter in neither of these MEF lines, whereas in a previous experiment it had inhibited the activity of a recombinant NFAT5 expressed in NFAT5-deficient MEF (Figure 1). This cell line had been derived by the NIH3T3 protocol, which basically

selected for cells with spontaneous long-term proliferative capacity, whereas the wild-type and AMPK-null cell lines were immortalized with a mutant p53. We then tested an independent, NIH3T3-immortalized, wild-type MEF line. As shown in Figure 5C, hypertonicity-induced NFAT5 activation was inhibited by AICAR and rapamycin to the same extent as it was shown for the different MEF lines in Figures 1C and 5B, but was essentially insensitive to 2-deoxyglucose. These results indicated that either the effect of this compound could vary among different MEF lines or, alternatively, that differences between endogenous NFAT5 and the recombinant protein used, which corresponded to human isoform NFAT5a (Lopez-Rodriguez et al., 1999a) made the latter more sensitive to 2-deoxyglucose. The ability of AICAR to inactivate mTOR and inhibit NFAT5 expression was also observed in AMPK-null cells (Figure 6). Although in this particular experiment the dephosphorylation of S6K1 in AICAR-treated cells was more pronounced in AMPK-null MEF than in wild-type ones at 24 hours, both cell lines showed comparable dephosphorylation of S6K1 by AICAR in independent experiments (supplementary Figure S3). Altogether, our results were consistent with the interpretation that mTOR could act as a positive regulator of NFAT5 in several cell types, and that AICAR could inhibit NFAT5 and mTOR by AMPK-independent mechanisms. In T cells, though, whereas rapamycin inhibited the induction of the osmoprotective protein Hsp70.1, AICAR stimulated its induction independently of NFAT5. Nonetheless, such stimulatory effect did not appear to confer any growth or survival advantage under osmotic stress.



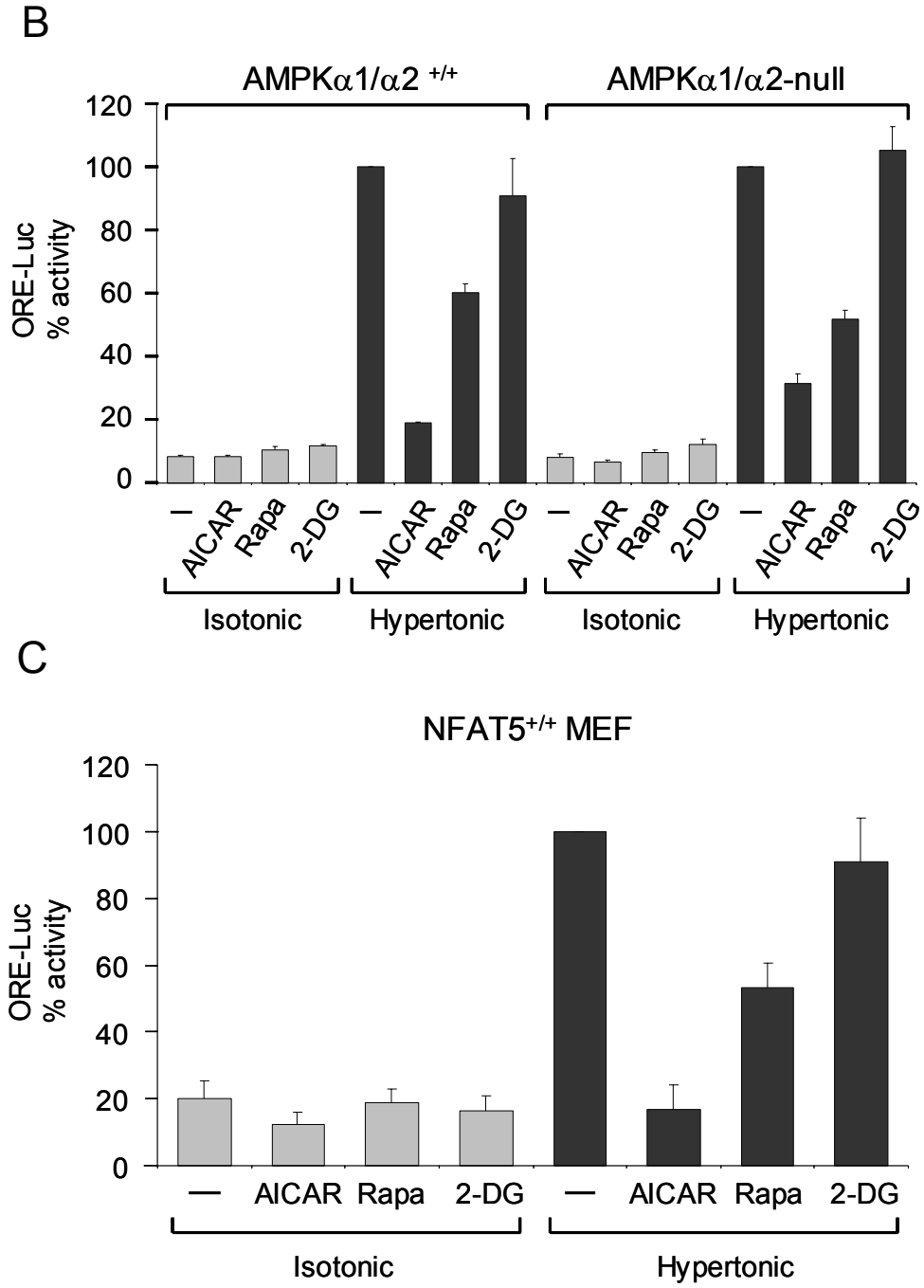


Figure 5. AMPK-independent inhibition of NFAT5 by AICAR. A) Expression and AICAR-induced (1 mM, 1 hour) phosphorylation of AMPKα1 and AMPKα2 in wild-type and AMPKα1/α2 double knockout MEF immortalized with a p53 mutant was monitored by Western blot. A non-specific band is indicated by n.s. β-actin was used as a loading control. **B)** The same cell lines were transfected with the NFAT5-responsive reporter ORE-Luc and cultured in isotonic (300 mOsm/kg) or hypertonic medium (500 mOsm/kg) during 24 hours in the absence or presence of AICAR (1 mM), rapamycin (200 nM) or 2-deoxyglucose (60 mM). Luciferase activity after

normalization with Renilla and endogenous lactate dehydrogenase is represented as the percentage of the activity of the reporter in hypertonic conditions without drugs (100%). Mean ± S.E.M of four independent experiments is shown. **C)** NIH3T3-immortalized NFAT5$^{+/+}$ MEF transfected with the ORE-Luc reporter were cultured in isotonic (300 mOsm/kg) or hypertonic medium (500 mOsm/kg) in the absence or presence of AICAR (1 mM), rapamycin (200 nM) or 2-deoxyglucose (60 mM) during 24 hours. Mean ± S.E.M. of four independent experiments is shown.

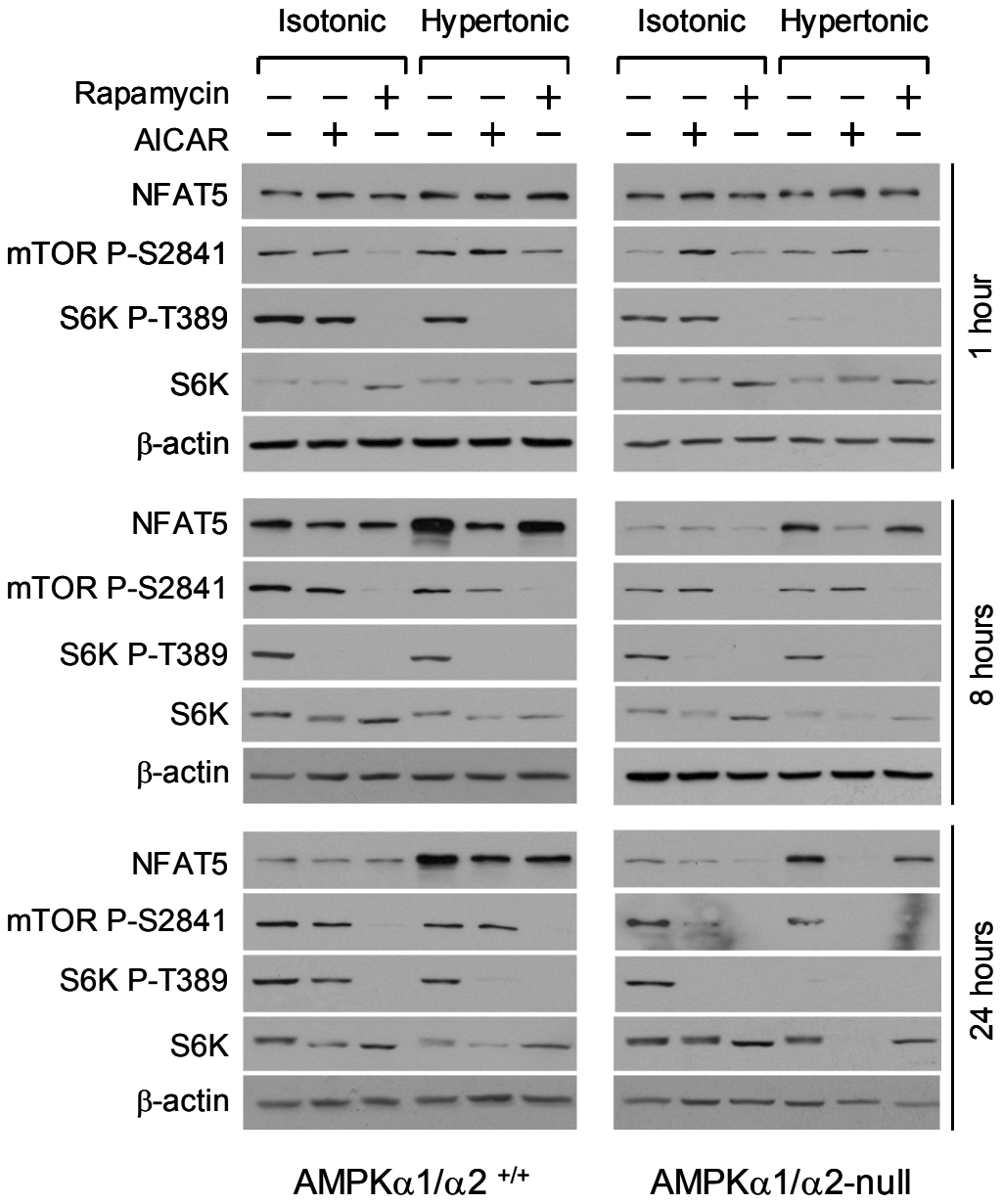


Figure 6. AMPK-independent inhibition of NFAT5 expression and mTOR and S6K1 phosphorylation by AICAR. Wild-type and AMPKα1/α2 double knockout MEF were cultured in isotonic (300 mOsm/kg) or hypertonic medium (500 mOsm/kg) during 1, 8 or 24 hours in the absence or presence of rapamycin (200 nM) or AICAR (1 mM). NFAT5, S6K1, phospho-S6K1 (Thr389) and phospho-mTOR (Ser2481) were detected by Western blot. β-actin is shown as a loading control. One representative experiment out of three performed is shown.

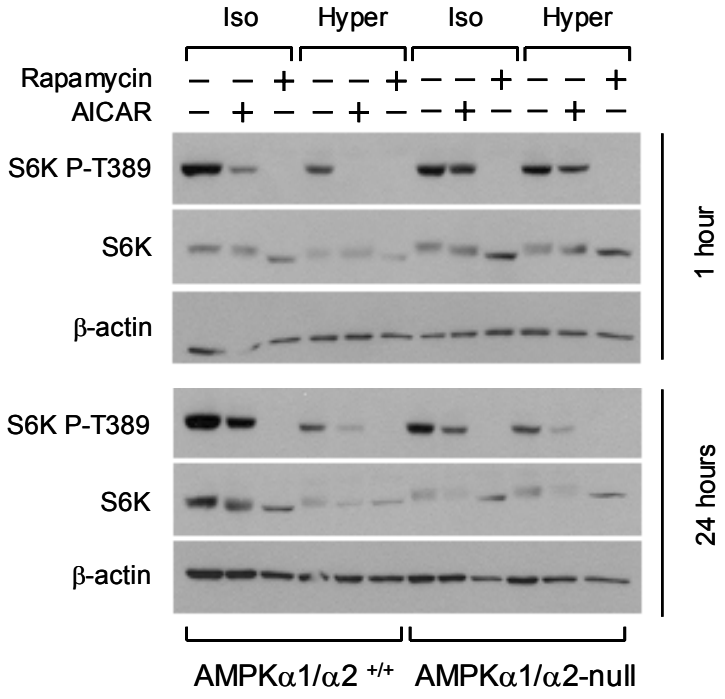


Figure S3. Dephosphorylation of Thr389 of S6K1 induced by AICAR in AMPK-null cells. Wild-type and AMPKα1/α2 double knockout MEF were cultured in isotonic (300 mOsm/kg) or hypertonic medium (500 mOsm/kg) during 1 or 24 hours in the absence or presence of rapamycin (200 nM) or AICAR (1 mM). Total S6K1, phospho-S6K1 (Thr389) and β-actin were detected by Western blot.

DISCUSSION

We report that the rapamycin-sensitive kinase mTOR is a positive regulator of osmoprotective responses in mammalian cells and that it acts in part by stimulating the transcriptional activity of NFAT5. A regulatory role of mTOR on hypertonic stress responses was supported by a) hypertonic conditions in the range required to activate NFAT5, and survivable by most mammalian cell types, were permissive to maintain the phosphorylated state of mTOR and its downstream target S6K1 associated with their active forms, and b) rapamycin, a highly specific mTOR inhibitor (Bain et al., 2007) inhibited the activity of NFAT5 in several cell lines and the induction of Hsp70.1 in T cells exposed to hypertonicity. Our results are consistent with previous reports showing that PI3-kinase, which acts as a positive regulator of the mTOR pathway in response to growth factors, and contributes to hypertonicity-induced NFAT5 activation in the leukemia T cell line Jurkat, HEK293 cells (Irarrazabal et al., 2006), and in non-transformed T cells (Morancho et al., 2008). The latter work also showed that NFAT5 activation by hypertonicity was of a greater magnitude in lymphocytes stimulated with mitogens than in quiescent ones (Morancho et al., 2008), indicating that a state of active growth could facilitate the activation of NFAT5. On the other hand, the sensitivity of NFAT5 to high concentrations of wortmannin and LY294002 (10-30 μM) (Irarrazabal et al., 2004; Irarrazabal et al., 2006; Morancho et al., 2008), commonly interpreted as indicative of its regulation by the PI3-kinase-related kinases (PIKK) ATM and DNA-PKc, might also involve mTOR, itself a PIKK sensitive to both compounds (Brunn et al., 1996).

Although hypertonicity levels in the range of 400-500 mOsm/kg did not generally inhibit the mTOR pathway in the cell types tested in this study, we observed that hypertonicity caused some degree of dephosphorylation of mTOR and S6K1 in some experiments, indicating that osmotic stress can be restrictive for the activity of these pathways. Independent work from other laboratories in diverse cell types has shown that hypertonic shock using 600 mOsm/kg or higher cause an acute dephosphorylation of T389 in S6K1, a general indicator of inactivation of the mTOR pathway (Parrott and Templeton, 1999). Yet other reports have shown that hypertonic shock can induce a transient stimulation of S6K1 activity (Van der Kaay et al., 1999) and the mTOR-

independent ribosomal S6 kinase RSK in mammalian cells (Zhang and Cohen, 1998). Our results in different cell types indicate that mTOR is not essential for the activation of NFAT5 and osmoprotective responses, but rather acts as a modulator. This regulatory role of mTOR might serve to adjust the ability of cells to survive and proliferate to specific growth demands in osmotic stress conditions.

Activation of NFAT5 by hypertonicity can be regulated by several kinases and phosphatases. Our results indicate that mTOR may not be required for NFAT5 nuclear translocation induced by hypertonicity, but acts as an enhancer of its synthesis and of the activity of its large carboxy-terminal transactivation domain. The carboxy-terminal transactivation domain spans 986 amino acids and contains 146 serines and 74 threonines in human isoform NFAT5a (Lopez-Rodriguez et al., 1999a). Although some regions within this domain are phosphorylated in response to hypertonic stress, the role of phosphorylation on NFAT5 activation is incompletely undeerstood (Lee et al., 2003). Despite that several kinases have been shown to regulate NFAT5, the identity of phosphorylation sites in NFAT5, either constitutive or inducible, is unknown. To date, a functional analysis has only been reported for three S/T sites matching potential ATM phosphorylation sequences (Irarrazabal et al., 2004). Mutation of these S/T to alanine reduced the basal activity of NFAT5 in isotonic conditions but did not preclude a robust response to hypertonicity (Irarrazabal et al., 2004). NFAT5 lacks a recognizable TOS motif, which has been described in S6K and 4E-BP1 as a binding site for the Raptor-containing mTORC1 complex that is required for mTOR-mediated phosphorylation of these substrates (Schalm et al., 2003). mTOR can also regulate the phosphorylation of other substrates that lack a TOS motif, such as S78 in eEF2 kinase, via indirect mechanisms (Browne and Proud, 2004). We do not know whether mTOR phosphorylates NFAT5, acts via another regulator of NFAT5, such as one or several of the kinases described to upregulate its activity, or by mechanisms independent of its phosphorylation state, for instance by interfering with NFAT5 inhibitors such as PARP-1 (Chen et al., 2006) or RNA helicase A (Colla et al., 2006). If a phosphorylation-dependent mechanism was involved, it would be expected that mutation of the relevant site(s) in NFAT5 would decrease its responsiveness to hypertonicity while rendering it insensitive to rapamycin. If such site(s) were identified, additional experiments would be required to determine whether they are direct targets of mTOR, S6K1 or another

kinase. However, if the regulatory role of mTOR were found not to involve the phosphorylation of NFAT5, the search for the mechanism could be complex. Future studies addressing these questions should help to understand the precise mode of regulation of NFAT5 by mTOR.

We found that AICAR could regulate NFAT5-dependent and independent responses to hypertonic stress. At least some of these effects, such as inhibition of NFAT5 expression and downregulation of some components of the mTOR pathway could occur independently of AMPK, since they were observed in AMPK-null cells. Another compound able to activate AMPK, the glycolysis inhibitor 2-deoxyglucose, did not have the same effect as AICAR, as it inhibited NFAT5-regulated reporters in HEK293 cells but not in MEF. Unlike AICAR, which is converted to the AMP analogue ZMP that binds to AMPK, activating it, 2-deoxyglucose activates AMPK indirectly, by decreasing the glycolysis-dependent production of ATP and causing a buildup of AMP (Hardie, 2007). Glucose is utilized for ATP production, via glycolysis and oxidative phosphorylation of its end product pyruvate in the mitochondrial tricarboxylic acid (TCA) cycle, but also serves as a precursor for nucleotide synthesis required for cell proliferation (Frauwirth and Thompson, 2004). Whereas limited glucose availability or glycolysis inhibition can inhibit proliferation (Roos and Loos, 1973), cells can use alternative sources such as amino acids to generate ATP (Bauer et al., 2004; DeBerardinis et al., 2007). Thus, the variable sensitivity of NFAT5 to 2-deoxyglucose in different cell lines might result from differences in their dependence on glucose for ATP generation. We also found that compound C, commonly used as an AMPK inhibitor, blunted the activation of an NFAT5-dependent reporter regardless of AMPK. Though it is unclear in which way it does so, this result raises a caution note on the use of compound C as an specific AMPK inhibitor, and other authors have also described AMPK-independent effects of this compound (Emerling et al., 2007). Since AICAR and 2-deoxyglucose can activate AMPK by different mechanisms and also have AMPK-independent effects, we would not entirely rule out the possibility that this kinase might modulate NFAT5 to some degree. However, our observation that NFAT5 is induced and activated normally in AMPK-null cells indicates that this kinase is not essential for NFAT5 function.

A number of findings from different laboratories had shown AMPK-independent effects of AICAR. It has been recently reported that this compound can reduce O_2 consumption rate and mitochondrial oxidative phosphorylation, and decrease the ATP/ADP ratio in hepatocytes and other cell types in an AMPK-independent manner (Guigas et al., 2007). A previous article found that NFAT5 is positively regulated by mitochondrial reactive oxygen species (ROS) during hypertonic stress (Zhou et al., 2006), suggesting that an attenuation of mitochondrial oxidative phosphorylation by AICAR might contribute in part to its inhibitory effect on NFAT5. A decrease in the ATP/ADP ratio could inhibit mTOR in AMPK-null cells and in turn help to downregulate NFAT5 activity. However, AICAR inhibited mTOR less effectively than rapamycin in our assays, yet it caused a greater suppression of NFAT5 expression and activity. AICAR also reduced the amount of total S6K1 in hypertonicity-treated T cells and in wild-type and AMPK-null MEF, while rapamycin did not. These observations indicate that other uncharacterized mechanisms must account for the inhibitory effects of AICAR. Notably, we found that it enhanced some hypertonicity-induced responses, such as Hsp70.1 expression and the activity of the hypertonicity-responsive reporter 9xNFAT-Luc in an NFAT5-independent manner in primary T cells. This reporter comprises 9 copies of an NFAT site in tandem upstream the minimal myosin heavy chain promoter (Wilkins et al., 2004), and we have recently described that it is activated by NFAT5 in response to hypertonicity, but can exhibit some degree of responsiveness to hypertonic stress in an NFAT5-independent manner, at least in T cells, although not in other cells such as macrophages or MEF (Morancho et al., 2008). AICAR is able to activate several transcription factors, such as MEF2 (Al-Khalili et al., 2004), EGR1 (Berasi et al., 2006), E2F1 (Hallstrom et al., 2008), STAT3 (Zang et al., 2008) and various members of the CREB family (Thomson et al., 2008), among others (Hardie, 2007), and thus it is not surprising that it could stimulate the 9xNFAT-Luc reporter and enhance the induction of Hsp70.1 in response to hypertonicity by NFAT5-independent mechanisms. However, it is unclear whether this stimulatory effect might confer any particular advantage to T cells, since AICAR stalled their proliferation in either isotonic or hypertonic conditions. AMPK and mTOR are considered as prime targets for pharmacological intervention in diseases such as diabetes, obesity and some types of cancer. Our results showing that NFAT5 and other osmoregulatory responses are sensitive to inhibitors of mTOR and compounds capable of modulating metabolic processes reveal a novel layer of

interaction between cellular responses to stressors and pathways regulating cell growth, and might help to gain insight into the mechanisms of action and potential side effects of drugs currently used in the clinic or under development.

MATERIALS AND METHODS

Reagents

5-aminoimidazole-4-carboxamide 1-ß-D-ribofuranoside (AICAR) was purchased from BioMol Research Labs (Plymouth Meeting, PA, USA), 2-deoxy-D-glucose was from Sigma-Aldrich (Steinheim, Germany), rapamycin and compound C were from Calbiochem (Darmstadt, Germany). The anti-NFAT5 antibody was from Affinity Bioreagents (Golden CO, USA), anti-pyruvate kinase from Chemicon (Hampshire, UK), anti-β-actin from Sigma-Aldrich (Steinheim, Germany), anti-phospho AMPK (Thr172), anti-phospho p70S6 kinase 1 (Thr389), anti-S6 kinase and anti-phospho mTOR (Ser2481) antibodies were from Cell Signaling Technology (Danvers, MA, USA), and antibodies against AMPKα1 and AMPKα2 from Abcam (Cambridge, UK). The donkey anti-rabbit and sheep anti-mouse secondary antibodies conjugated to horseradish peroxidase (HRP) were purchased from Amersham Biosciences (Buckinghamshire, UK) and HRP-conjugated rabbit anti-goat IgG antiserum was from Dako (Glostrup, Denmark).

Mice

9xNFAT-luc mice (line 15.1) (Wilkins et al., 2004) in FVB background, and $NFAT5^{+/-}$ mice (Lopez-Rodriguez et al., 2004) in 129Sv background were bred and maintained under specific pathogen-free conditions, and handled according to institutional guidelines (PRBB Animal Care and Use Committee).

Cell culture

HEK293 cells were maintained in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine and 50 μM β-mercaptoethanol. AMPKα1/α2 double knockout MEF were obtained from E12.5 mouse embryos from $AMPK\alpha1^{-/-}$, AMPKα2-floxed mice ($AMPK\alpha1^{-/-}$, $AMPK\alpha2^{fl/fl}$) of mixed background (Bl6/CD1) (Guigas et al., 2007). Deletion of AMPKα2 was done by infecting the cells *in vitro* with Adeno-Cre, and cells were immortalized at passage 2 with the carboxy-terminal fragment of p53 (Ossovskaya et al., 1996). $NFAT5^{-/-}$ MEF have been described (Lopez-Rodriguez et al., 2004; Morancho et al., 2008). MEF were cultured in the above supplemented culture medium plus 100 μM non-essential

aminoacids and 1% penicillin-streptomycin (all from Gibco, Pasley, UK). Osmolality of the cell culture medium was measured in a Wescor Vapro 5520 Osmometer (Logan, UT, USA). Since the medium with supplements had an osmolality of 330 mOsm/kg, it was adjusted to 300 mOsm/kg by addition of 10% sterile H_2O (Milli-Q Biocel A10. Millipore, Bedford, MA, USA). Over an isotonic baseline of 300 mOsm/kg, addition of 50 mM NaCl raised the osmolality to 400 mOsm/kg, and 100 mM NaCl raised it to 500 mOsm/kg. Drugs were added 1 hour prior to increasing the tonicity and were maintained throughout the assays.

Splenocytes

Spleens from 9xNFAT-Luc or 9xNFAT-Luc/NFAT5$^{-/-}$ mice (Morancho et al., 2008) were removed from animals of 8-12 weeks of age, and mononuclear cells were isolated by density gradient sedimentation with Lymphoprep (Axis-Shield PoC AS, Oslo, Norway) and stimulated with 2.5 μg/ml concanavalin A (Sigma-Aldrich) plus 25 ng/ml of IL-2 (Proleukin, Chiron; Amsterdam, The Netherlands) for 24 hours in DMEM culture medium with the same supplements as for MEF. The percentage of Thy1.2 positive cells in the cultures was determined with anti-mouse CD90.2-FITC (BD Pharmingen, Erembodegem, Belgium) and flow cytometry (FACScan, BD Biosciences).

DNA constructs

The ORE-Luc reporter, the expression vector for full-length human NFAT5a tagged with 6 copies of a Myc epitope at its amino-terminus and EGFP at its carboxy terminus (Myc-NFAT5-GFP), and pVP16-DBD5 construct have been described (Lopez-Rodriguez et al., 1999b) (Lopez-Rodriguez et al., 2001). The vectors pVP16 (Clontech, Palo Alto, CA, USA), pEGFP-N1 (Clontech), CMV-HA (Clontech) and TK-Renilla (Promega, Madison, WI, USA) are available commercially.

Transfections and luciferase assays

HEK293 cells and MEF were transiently transfected by the calcium-phosphate method in 10 cm-diameter plates (Rodriguez and Flemington, 1999). After transfection, cells were washed, replated in fresh medium, and allowed to grow for 36 hours before being treated as indicated in the respective figures. Luciferase and Renilla were measured with the Dual-luciferase reporter system (Promega) with a Berthold FB12 luminometer

(Berthold, Pforzheim, Germany). When reporters were transfected in cell lines, luciferase activity was normalized to the activity of a cotransfected Renilla vector and to the activity of endogenous lactate dehydrogenase (LDH), which was proportional to the number of viable cells (Minguillon et al., 2005). Luciferase activity in transgenic T cells was normalized to endogenous LDH in the same lysate. LDH activity was measured with the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega).

Cell lysis and immunoblot analysis

Cells were harvested and lysed in buffer lysis (50 mM HEPES pH 7.4, 80 mM NaCl, 5 mM MgCl2, 1 mM EDTA, 5 mM NaPPi, 1 mM sodium-orthovanadate, 20 mM β-glycerophosphate, 1 mM sodium fluoride, 1% Triton X-100, 5 μg/ml leupeptin and aprotinin, 2 mM PMSF, 1 μg/ml pepstatin A and 10 μg/ml DNAse I) for 30 minutes at 4ºC. Lysates were centrifuged at 14,000 rpm for 10 minutes and supernatants were collected. Reducing 5x Laemmli buffer was added to a final 1x concentration and samples were boiled, resolved in SDS-polyacrylamide (7%) gels and transferred to polyvinylidene difluoride membranes (PVDF, Immobilon-P. Millipore, Bedford, MA, USA). Membranes were blocked in Tris-buffered saline (20 mM Tris-HCl pH 7.6, 150 mM NaCl) containing 7.5% non-fat dry milk for 1 hour at room temperature. Membranes were probed with the indicated antibodies, followed by enhanced chemiluminescent detection (Supersignal West Pico Chemiluminescent Substrate, Pierce, Rockford, IL, USA).

RT-qPCR

Total RNA from T cells was isolated with the RNeasy Kit (Qiagen Inc, Valencia, CA, USA) and reversed transcribed using the SuperScript III First Strand Synthesis System (Invitrogen). PCR amplification reactions with primers specific for Hsp70.1 and the L32 housekeeping gene were performed using SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA) in the ABI 7900HT real time analyzer (Applied Biosystems). The following primers were used: L32 (forward primer: 5'-ACCAGTCAGACCGATATGTG-3'; reverse primer: 5'-ATTGTGGACCAGGAACTTGC-3') and Hspa1b (forward primer: 5'-TTGAAGAAGTCCTGCAGCAG-3'; reverse primer: 5'-CTTCTACACATCCATCACGC -3').

Fluorescence confocal microscopy

HEK293 cells transfected with the Myc-NFAT5-GFP expression vector were grown on 35 mm-diameter coverglass dishes coated with 0.01% poly-L-lysine (Sigma-Aldrich). 48 hours post-transfection cells were stimulated with hypertonic medium during 5 hours, fixed with 3% paraformaldehyde and permeabilized with 0.05% Nonidet P-40 in PBS. Fixed cells were stained with the DNA dye TO-PRO3 iodine (642/641, Invitrogen Molecular Probes, Eugene, OR, USA), washed with PBS and mounted on slides with the anti-fading agent Slowfade (Invitrogen Molecular Probes). Images were acquired with a Leica TCS SP2 confocal microscope (Leica, Wetzlar, Germany).

ACKNOWLEDGEMENTS

JA was supported by grants from the Spanish Ministry of Education and Science (BMC2002-00380, BFU2005-02247/BMC), Fundació la Marató TV3 (030231), Generalitat de Catalunya (Distinció per a la Promoció de la Recerca Universitària, and SGR-00478), and Spanish Ministry of Health (Fondo de Investigación Sanitaria, Red HERACLES RD06/0009/1005). CLR was supported by the Ramón y Cajal Researcher Programme and grants of the Ministry of Education and Science of Spain (BMC-2003-00882, SAF2006-04913), Marie Curie International Reintegration Programme, and Fundació la Marató TV3 (030230). BM is the recipient of an FI predoctoral fellowship from the Generalitat de Catalunya. KDE is the recipient of an FI-IQUC predoctoral fellowship from the Generalitat de Catalunya. We thank Verónica Santiago for excellent technical assistance, and Oscar Fornas for advice with flow cytometry.

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DISCUSSION

Diverse signaling pathways and factors regulate NFAT5 in response to hypertonicity. However, its regulation is complex and the processes involved are known partially. Moreover, different observations also reveal that this factor can respond to other hypertonicity-independent stimuli, although this is a poorly explored field. Our results have expanded the information about the regulation of NFAT5 in response to osmotic stress and we have also established the sensitivity of this factor to signaling pathways involved in the control of cell growth and energy metabolism.

1. PRIMARY CELLS AS SENSITIVE RESPONDERS TO PATHOLOGYCAL HYPERTONICITY.

Cells facing a hyperosmotic environment undergo an almost instantaneous reduction in cell volume due to the efflux of intracellular water, which is rapidly corrected by the increase in the intracellular content of ions. This process is referred to as regulatory volume increase (RVI) and is mediated by preexisting ion transport systems. The increase in the intracellular ionic strength has perturbing effects on cellular functions and can cause DNA damage, mitochondrial depolarization, cell cycle arrest or even apoptosis. In order to adapt to these conditions, cells reduce their proliferation by inducing cell cycle arrest (Michea et al. 2000) and inhibiting general transcription and translation (Pederson, Robbins 1970, Robbins, Pederson & Klein 1970, Wengler, Wengler 1972). When hypertonicity exceeds certain levels, cells can not cope and undergo apoptosis. However, at certain tonicities that vary depending on the cell type, they avoid apoptosis by increasing the accumulation of organic osmolytes and heat-shock proteins. This is achieved by activation of a specific gene expression program, of which NFAT5 is a major regulator.

Tonicicity levels around 500 mOsm/kg have been extensively used to analyze NFAT5-regulated processes *in vitro*, even if these concentrations are found *in vivo* only in the renal medulla. In plasma tonicity levels are around 290 mOsm/kg and Go et al. described that thymus, spleen and liver present a slightly higher tonicity, around 330 mOsm/kg, even though in the same work they could not detect the activation of an NFAT5-regulated reporter in MEF until the osmolarity reached at least 500 mOsm/kg (Go et al. 2004), which suggests that either NFAT5 is not active at lower levels of tonicity or that the experimental system was not sensitive enough. However, reports have shown that hypernatremia, causing the elevation of plasma tonicity to 360-430

mOsm/kg and even higher, can result from several disorders in humans and is achieved experimentally in mice deficient in osmoregulatory disorders (Bourdel-Marchasson et al. 2004, Chilton 1995, Cooke et al. 1993, Dogan et al. 2005, Ka et al. 2003, Ma et al. 1998, McDill et al. 2006, Papadimitriou et al. 1997, Paut et al. 1999, Schorn, Manschwetus & Kuhn 1991, Yun et al. 2000). For this reason, we wondered whether NFAT5 activation was possible at lower, pathophysiologically relevant, hypertonicity levels than those in the renal medulla.

In order to achieve it, we needed a system capable of monitoring the activity of the endogenous protein in primary cells, which had not been described before. The transgenic reporter mice used in our experiments (9xNFAT-Luc reporter mice) was generated by Wilkins et al., who make use of it to describe that NFATc factors are involved in pathological but not physiological cardiac hypertrophy, validating the reporter to study the activation of these transcription factors (Wilkins et al. 2004). Our results showed that this reporter is also appropriate to study the transcriptional response to hypertonic stress, which is NFAT5-dependent. Experiments where this reporter was transfected in Jurkat T cells showed that it was able to respond to both PMA plus ionomycin and hypertonicity, but only the former activation was prevented by the calcineurin-inhibitor FK506. In concordance, the VIVIT peptide, which inhibits the NFATc pathway, blocked exclusively the activation by PMA plus ionomycin, while the NFAT5 dominant-negative DD5 inhibited the response to hypertonicity. In view of that, we generated mice with the 9xNFAT-Luc reporter that lacked NFAT5 protein, in order to confirm that the activation caused by NaCl was due to this protein. Impaired activation of the reporter was shown in mouse embryonic fibroblasts and T cells from NFAT5$^{-/-}$ mice, emphasizing that NFAT5 is the main regulator of this reporter in hypertonic conditions.

Our results showed that NFAT5 is sensitive to varying degrees of tonicity depending on the cell type. Activated T lymphocytes are able to induce NFAT5 at low tonicity (330 mOsm/kg) and we could detect transcriptional activity between 360 and 380 mOsm/kg, even if a more remarkable activation was detected at 430 mOsm/kg. The threshold to activate the reporter in T cells varied depending on the state of cellular activation: in fresh splenocytes, as well as thymocytes, the reporter did not respond to hypertonic stimulation, but when cells had been exposed to mitogens for 24 hours it was able to respond to 360 mOsm/kg and, later on, after more that 24 hours of activation, the

magnitude of the response decreased. Low levels of hypertonicity (between 320 and 380 mOsm/kg) enhance IL-2 transcription and secretion in cultured human T cells (Junger et al. 1997, Loomis et al. 2003), while these levels of hypertonicity also increase IL-2 secretion (Junger et al. 1997) and proliferation (Coimbra et al. 1995, Junger et al. 1994, Junger et al. 1997) in human peripheral blood mononuclear cells. Thus, in pathophysiological conditions and at the earlier times of activation, T cells exhibit a higher response to low hypertonicity levels that might be relevant in the immune response. Further work is needed to validate whether in low stress conditions T cells transcribe preferably higher levels of NFAT5 target genes at the first stages of activation. On the other hand, macrophages and mouse embryonic fibroblasts required higher amounts of tonicity to activate the NFAT5-dependent transcriptional response: macrophages at 430 mOsm/kg and MEF at higher osmolarities, around 480 mOsm/kg. In patients with osmoregulatory disorders, tonicity levels in plasma reach 360-430mOsm/kg (Bourdel-Marchasson et al. 2004, Chilton 1995, Cooke et al. 1993, Dogan et al. 2005, Ka et al. 2003, Papadimitriou et al. 1997, Paut et al. 1999, Schorn, Manschwetus & Kuhn 1991), whereas it has been proposed that injecting saline to achieve lower levels of hypertonicity might benefit the immune system of trauma patients (Kramer 2003). In conclusion, this diversity of responses might reflect a cell type-dependent degree of tolerance to osmotic stress. However, the tonicity level reported in thymus, spleen and liver by Go et al. (330mOsm/kg) (Go et al. 2004) was able to induce NFAT5 synthesis in activated T cells but its transcriptional activation was not detected, indicating that either NFAT5 is not active at these conditions or that the reporter is not sensitive enough. Nonetheless, this endogenous reporter is the only system that monitors NFAT5 transcriptional activity *in vivo* and it is sensitive enough to study raises in tonicity that are in the pathophysiological range.

Comparing the already described NFAT5-dependent reporter ORE-Luc (Lopez-Rodriguez et al. 2001) with the 9xNFAT-Luc reporter, we established that both are stimulated by hypertonicity in a similar manner, with the added feature that the 9xNFAT-Luc reporter can also respond to NFATc proteins via other types of stimulation. Though, the ORE-Luc reporter was originated by cloning the enhancer of the NFAT5 target gene aldose reductase, while the 9xNFAT-Luc reporter is an artificial composite site generated by cloning nine copies of an NFAT binding site from the IL-4 promoter upstream of the minimal promoter of the α-myosin heavy chain gene. Thus, both reporters are equally valid to study hypertonic NFAT5-dependent responses,

whereas for NFATc-dependent transcriptional activation only the 9xNFAT-Luc reporter is suitable.

The activation of the 9xNFAT-Luc reporter induced by hypertonicity was decreased by the calcineurin inhibitor FK506 between 20-30% in MEF and 60-70% in primary T cells, while this drug had no effect on macrophages and Jurkat cells, suggesting that calcineurin might modulate hypertonic stress responses in some cells but not others. Hypertonicity can increase intracellular calcium concentrations in HaCaT keratinocytes (Dascalu et al. 2000), hepatocytes (Krumschnabel et al. 2003), endothelial cells (Paemeleire, de Hemptinne & Leybaert 1999) and chondrocytes (Sanchez, Wilkins 2004). This calcium release stimulated NFAT2 and NFAT5 activity in the renal collecting duct cell line mpkCCD$_{c14}$ in a calcineurin-dependent manner, and both factors regulated the expression of aquaporin 2 (AQP2) (Li et al. 2007). These data suggest that the remaining activity of the 9xNFAT-Luc reporter in NFAT5-deficient T cells and MEF in response to hypertonic stress might be caused by calcineurin-dependent NFATc factors or other transcriptional regulators. Calcineurin inhibitors have also been shown to inhibit the expression of NFAT5-target genes such as BGT-1, aldose reductase and HSP70 in kidney medulla, while they inhibited NFAT5 translocation to the nucleus in MDCK2 cells (Sheikh-Hamad et al. 2001). These data suggest that calcineurin might regulate NFAT5 in response to tonicity increases in renal cells, although its contribution is less essential for its function. In addition, our results show that induction of NFAT5 expression by osmotic stress was not inhibited by FK506 in T cells, while its transcriptional activation was partially inhibited. In conclusion, we could conceive a scenario where hypertonicity activates both NFATc and NFAT5 factors and calcineurin might regulate both responses. However, the relative contribution of calcineurin and NFATc proteins to hypertonic stress response appear to vary among different cell types. Even so, the main response is NFAT5-dependent and calcineurin-independent, as shown by the significant reduction in NFAT5 activity in response to hypertonicity in NFAT5-deficient cells and the severe phenotype of defective osmoprotective genes in NFAT5$^{-/-}$ mice.

On the other hand, NFAT5 is induced by PMA plus ionomycin stimulation, and its expression is inhibited by calcineurin inhibitors, in T cells (Lopez-Rodriguez et al. 2001, Trama et al. 2000) and mpkCCD$_{c14}$ cell line (Li et al. 2007). However, NFAT5-deficient and wild-type T cells, as well as MEF, presented comparable activity of the 9xNFAT-

Luc reporter by PMA plus ionomycin and FK506 completely abolished this activation, implying that PMA plus ionomycin dependent transcriptional response is acting through NFATc factors rather than via NFAT5.

NFAT5 function has been studied by using both *in vitro* and *in vivo* models. *In vitro* experiments have allowed to describe the conditions necessary for the synthesis, translocation and transcriptional activation of NFAT5 under hypertonic conditions in several cell types, while the generation of transgenic and knockout mice has confirmed the fundamental function of NFAT5 *in vivo* in organs exposed to high tonicity. The 9xNFAT-Luc reporter mouse is a new tool to study the activity of this transcription factor in primary cells, which allowed us to demonstrate that NFAT5 requires distinct tonicity levels for its activation depending on the cell type, and to analyze its regulation by several kinases, which might be followed by using pharmacological inhibitors. This reporter is then able to respond to both NFATc and NFAT5 proteins and, in consequence, an interesting approach might be studying the activation of them *in vivo* by monitoring the activation of the reporter in transgenic mice. In accordance with our results, it should be necessary to carefully consider the influence of calcineurin inhibitors on NFAT5 activation by performing parallel experiments in cell culture models and in NFAT5-deficient cells. In addition, the use of the calcineurin inhibitors FK506 or cyclosporin A in animal models to inhibit NFATc proteins and track NFAT5 activity *in vivo* might face the problem of side-effects of these compounds, that include nephrotoxicity (Lim et al. 2007), which might affect sodium and water homeostasis and, in consequence, indirectly disturb NFAT5 regulation. In fact, calcineurin inhibitors themselves might downregulate NFAT5 activity in kidney, inhibiting the expression of the osmoregulatory genes that protect renal cells from high levels of tonicity. Thus, tissue-specific conditional knockout for calcineurin and NFATc proteins crossed with 9xNFAT-Luc mice should give more accurate results *in vivo*.

2. SIGNALING PATHWAYS REGULATING NFAT5 IN RESPONSE TO HYPERTONICITY IN DIFFERENT CELL TYPES

Osmotic stress activates several kinases at different time points to regulate cellular volume. Members of the MAPK-SAPK (Mitogen activated protein kinases - Stress activated protein kinases) (Sheikh-Hamad, Gustin 2004), SRC (Cohen 2005) and Ste20-type (Strange, Denton & Nehrke 2006) kinase families have been involved in the

regulation of the hypertonic response. Several kinases among them and other kinase families have been proposed as upstream regulators of NFAT5. Essentially, p38, Fyn, PKA, ATM, PI3K and ERK kinases are NFAT5 regulators, depending on the cellular type and conditions of stress employed. The identification of these kinases has been accomplished by using more or less specific pharmacological inhibitors (Bain et al. 2003, Bain et al. 2007, Davies et al. 2000, Gharbi et al. 2007) and confirming the results with dominant negative constructs and small interfering RNAs. However, it is still unknown if any of these kinases, even the ones that have been shown to interact with NFAT5, are able to phosphorylate it, in which residues and, moreover, which role are playing *in vivo*. As their characterization was done at high tonicity levels, 9xNFAT-Luc reporter mice allowed us to study the role of them upon low stress conditions in primary cells, such as splenocytes and non-transformed mouse embryonic fibroblasts.

Mitogen activated protein kinases (MAPK)

MAPKs are part of a three kinase signaling module composed of the MAPK, a MAPK kinase (MAP2K) and a MAPK kinase kinase (MAP3K). MAP3Ks phosphorylate and activate MAP2K, which in turn phosphorylate and activate MAPK. Hypertonicity stimulates MEK kinase 1 (MEKK1) and activates both c-Jun amino-terminal kinase (JNK) and extracellular signal-regulated protein kinase (ERK1/2) (Yujiri et al. 1998). MEK kinase 2 (MEKK2), which can also be activated by osmotic stress, stimulates ERK5 (Seyfried et al. 2005); and MEK kinase 3 (MEKK3) induces MKK3/6 and p38 activation (Uhlik et al. 2003).

Among them, p38 was the main candidate to regulate NFAT5, as it had been previously involved in the regulation of NFAT5 target genes. SMIT and BGT-1 mRNA were inhibited by the p38 inhibitor SB203580 in human peripheral blood monocytes and human peripheral blood-derived macrophages (Denkert et al. 1998), and the same drug inhibited the hypertonicity-induced expression of BGT-1 and HSP70 (Sheikh-Hamad et al. 1998) in MDCK cells, while aldose reductase mRNA was downregulated in HepG2 cells (Nadkarni et al. 1999). Moreover, the adaptation of yeast to osmotic stress is dependent on the p38 MAPK homolog high-osmolality glycerol (HOG-1) kinase (Brewster et al. 1993). Even though these findings insinuate that p38 might be a putative good candidate to regulate NFAT5, the first report investigating this relation showed that a dominant negative construct for p38 did not inhibit an NFAT5-dependent

reporter after hypertonic stimulation in the cell line PAP-HT25 (Kultz et al. 1997). However, several other reports support the role of p38 in NFAT5 activation in diverse cell lines (Irarrazabal et al. 2008, Ko et al. 2002, Tsai et al. 2007) and our own results also showed that NFAT5 transcriptional activity was inhibited by two independent p38 pharmacological inhibitors, SB202190 and SB203580, which are quite specific (Bain et al. 2007) and are widely used in the literature, including p38-related NFAT5 articles. The balance between p38α and p38δ might explain these variations, as it has recently been shown that the former is involved in NFAT5 activation whereas the latter is able to inhibit this factor (Zhou et al. 2008). We also described that in MEF the expression of NFAT5 was reduced when cells were pretreated with SB203580, in agreement with results from Tsai el al. where NFAT5 expression was inhibited by another p38 inhibitor (SFK86002) (Tsai et al. 2007).

The mechanisms by which p38 kinases might regulate NFAT5 are unknown. In addition to the obvious, yet still unproven, possibility that they might phosphorylate NFAT5 and directly activate its transcriptional activity, as shown for other p38-regulated factors such as ATF2 (Ouwens et al. 2002) and Maf2 (Sii-Felice et al. 2005), other mechanisms might be involved. Physiologically, hypertonicity rapidly causes cell shrinkage by inducing rapid actin polimerization and remodeling of the actin cytoskeleton in a p38-dependent manner (Bustamante et al. 2003), raising the possibility that NFAT5 might be activated by this kinase in response to cytoskeletal rearrangements.

In the same report by Tsai et al., the MEK-1 inhibitor PD98059, which consequently inhibits ERK, and a small interfering RNA targeting ERK also decreased NFAT5 expression and transcriptional activation in nucleus pulposus cells, but we did not observe inhibition in neither splenocytes nor MEF (data not shown). Previously, it had been shown that PD98059 downregulated an NFAT5-mediated reporter and the binding of trans-acting factors to a TonE site in HepG2 cell, but independently of ERK (Nadkarni et al. 1999). Thus, ERK might be regulating NFAT5 in specific cellular types.

Another MAPK activated by osmotic stress is JNK, but its inhibition by using SP600125 did not give us consistent results (data not shown). Furthermore, SP600125 is at present considered a relatively non-specific inhibitor and, in addition to JNK, it also inhibits with similar or greater potency PKD1, CHK2, Aurora B and C, MELK, CK1,

DYRK2, DYRK3 and HIPK3 (Bain et al. 2007). In a previous work, an NFAT5-dependent reporter was inhibited neither by a dominant negative JNK construct (Kultz 1997) nor by the same pharmacological inhibitor (Tsai et al. 2007), suggesting that JNK might probably not be involved in the regulation of NFAT5.

SRC kinase family and Ste20-type kinases

Among SRC family, the nonreceptor protein tyrosine kinase Fyn has been involved in the regulation of NFAT5 activity. Fyn is activated by hypertonicity (Reinehr et al. 2004) and it cooperates with p38 to activate NFAT5 (Ko et al. 2002). Even though, other study showed that the Fyn inhibitor PP2 was not able to affect the global phosphorylation state of NFAT5 (Dahl, Handler & Kwon 2001), but it might reflect differences in the stimulation time and the cell line used. Fyn, as well as other members of the family, such as HCK, FGR and YES are best known for its participation in the regulatory volume increase process than in the transcriptional responses to hypertonicity (reviewed in (Cohen 2005)). Similar is the case of the Ste20-type kinases SPAK and OSR1, which have been associated with the regulation of NKCC channels (Dowd, Forbush 2003, Piechotta, Lu & Delpire 2002) and are activated by cell shrinkage almost immediately (Flatman 2002). Nevertheless, as Fyn participates in the regulation of NFAT5 activity at longer times, it might turn out that other kinases currently known to regulate the first steps of the response to hypertonicity also participate at later phases regulating NFAT5. Furthermore, NFAT5 translocation to the nucleus is seen at short time points and the proteins involved, which are basically unknown, might include these first phase kinases.

Protein kinase A (PKA)

In our experiments, the PKA inhibitor H89 did not affect the expression of NFAT5 but caused a moderate reduction of its transcriptional activity, which agrres with previous data where PKA was shown to interact with NFAT5 (Ferraris et al. 2002, Irarrazabal et al. 2004) and enhance NFAT5 activity in hypertonic conditions in a cAMP-independent manner (Ferraris et al. 2002). Thus, PKA would regulate NFAT5 transcriptional activation without affecting its synthesis.

Phosphatidylinositol 3-Kinase (PI3K)

Hypertonicity-induced NFAT5 transcriptional activation was downregulated in splenocytes by the PI3-kinase inhibitor wortmannin, in agreement with previous work in HEK293 and Jurkat T cells by Irarrazabal et al. (Irarrazabal et al. 2006). The same work showed that the nuclear localization of NFAT5 at 500 mOsm/kg was not affected by a dominant negative PI3K construct (Irarrazabal et al. 2006). In our experiments, LY294002 at low concentration (1 μM) also inhibited PI3-kinase activity and confirmed these results. However, none of the PI3K-inhibitors at low concentrations were able to inhibit the transcriptional activation of NFAT5 in mouse embryonic fibroblasts. In addition, hypertonicity-induced NFAT5 expression was not affected in these conditions in neither MEF nor splenocytes. Altogether, PI3K might participate in the transcriptional activity of NFAT5 in splenocytes, whereas in other cell types, such as MEF, its contribution is less evident.

PI3-kinase related kinases (PIKK) family

However, when LY294002 was used at a higher concentration (25 μM) it inhibited both NFAT5 activation and synthesis in splenocytes and MEF. At this concentration LY294002 inhibits PI3K and members of the PI3-kinase related kinases (PIKK) family of kinases, such as ATM (Ataxia-telangiectasia mutated), DNA-PK (DNA-dependent protein kinase), hSMG-1 (human suppressor of morphogenetic effect on genitalia-1) and mTOR (mammalian target of rapamycin). PIKK mediate responses to diverse stresses and their ability to regulate the cell cycle, cell growth or turn on DNA repair mechanisms under stress conditions are essential to protect cells from damage by allowing them to correct errors in this lapse of time. Concretely, ATM modulates cell cycle progression, DNA repair and apoptosis, DNA-PK is required for the repair of double-strand breaks by non-homologous end-joining repair and (mTOR) is involved in the regulation of cell cycle and translation in response to nutrient availability (Bakkenist, Kastan 2004, Reiling, Sabatini 2006). Another fundamental PIKK is ATR (Ataxia-telangiectasia and Rad3-related), but this kinase is not inhibited by LY294002 nor wortmannin in cells (Knight et al. 2006).

Of these proteins, ATM has been previously involved in the nuclear localization (Zhang et al. 2005) and activation of NFAT5 (Irarrazabal et al. 2004) but not in the expression

of this transcription factor. ATM is also activated by DNA damage and hypertonicity is known to cause double strand breaks (Kultz, Chakravarty 2001), which might activate the kinase and, later on, NFAT5. However, DNA damage itself is not considered an activator of NFAT5, because urea, ultraviolet light and irradiation, which are potent stimulators of ATM, do not activate NFAT5 (Irarrazabal et al. 2004). Nevertheless, the published results do not rule out that other PIKK proteins might regulate NFAT5 transcriptional activity, since an NFAT5-dependent reporter was inhibited by low and high doses of wortmannin in cells lacking ATM (Irarrazabal et al. 2004).

A theme that has emerged, both in the preceding literature and in our work, is the requirement of several kinases to act in concert in order to regulate NFAT5 in response to hypertonic stress. This mechanism might ensure that NFAT5 is only activated by this stress and not by any other stimuli that might affect independently any of the different individual kinases. In addition, the contribution of several kinases might also help to modulate the magnitude of NFAT5-dependent responses and, whereas some kinases such as p38 or PIKK appear to be necessary, others such as PKA, ERK, PI3K or mTOR (our own work) would rather act as modulators. In this context, it could be speculated that inappropriate activation of NFAT5 in the absence of osmotic stress could be deleterious for the cell. This interpretation is supported by reports showing that overexpression of aldose reductase (Cheung et al. 2007) or SMIT (Jiang et al. 2000), which are induced by NFAT5 in response to hypertonicity, can have adverse effects in cells not exposed to osmotic stress.

The combination of 9xNFAT-Luc transgenic with NFAT5$^{-/-}$ mice provides a good control to assay unequivocally the involvement of NFAT5 in responses that are observed using this reporter. Moreover, in combination with other approaches it might make possible to test the contribution of several pathways on NFAT5 activity in some cells of different lineages, tissues and organs.

3. REGULATION OF NFAT5 BY THE mTOR PATHWAY

NFAT5 is expressed in cells undergoing proliferation (Lopez-Rodriguez et al. 2001, Trama et al. 2000) and we have also observed that it is activated more potently in T cells that have been stimulated with mitogens than in quiescent T cells (Morancho et al. 2008). In addition, others and we have observed that NFAT5 is regulated by PI3K,

which is involved in promoting cell growth in response to growth factors and mitogens (Engelman, Luo & Cantley 2006). On the other hand, hypertonicity like other stressful conditions, such as hypoxia, DNA damage, ROS or low nutrient levels, tends to inhibit cell growth and proliferation (Reiling, Sabatini 2006). Altogether these observations led us to ask about the potential involvement of pathways regulating cell growth on NFAT5 activation and, in consequence, we analyzed the regulation of this factor by mTOR, a major regulator of cell growth and proliferation that integrates signals from growth factors with nutrient and energy availability (Corradetti, Guan 2006, Yang, Guan 2007), and by pharmacological modulators of energy stress responses.

Short-term hyperosmolarity at high doses is known to cause dephosphorylation of the mTOR targets S6K1 and 4E-BP1 (Morley, Naegele 2002, Parrott, Templeton 1999) independently of TSC2 (Smith et al. 2005b) and Rheb (Inoki et al. 2003) and also independently of p38 and JNK pathways (Fumarola, La Monica & Guidotti 2005, Parrott, Templeton 1999). In accordance, our results showed that after one hour of treatment, the phosphorylation of S6K1 and also the autophosphorylation of mTOR were reduced, but after long treatments both recovered their active state. In some experiments the levels of phosphorylation at long time points were still decreased compared with the initial situations, but rapamycin treatment inhibited them completely, meaning that the mTOR pathway is active. Liu et al. also observed this biphasic response upon long hypoxic conditions (Liu et al. 2006), characterized by the inhibition of the pathway during acute stress and its subsequent reactivation. Further work is needed to assess whether this is a general mechanism to stress and its functional meaning, but it points out that the mTOR pathway is involved in the regulation of the cellular response to sustained stress. Inhibition of mTOR by the immunosuppressive drug rapamycin was able to decrease the activity of an NFAT5 reporter under hypertonic conditions in HEK293 and NFAT5$^{-/-}$ MEF reconstituted with recombinant NFAT5, without inhibiting its nuclear localization nor its DNA binding capacity. In view of these results, we wanted to study whether NFAT5 was also regulated by mTOR in primary cells under pathophysiological hypertonic stress conditions. The analysis in splenocytes of the 9xNFAT-Luc reporter and the NFAT5 target gene *Hspa1b*, which codes for the heat shock protein Hsp70.1, confirmed that rapamycin was able to inhibit NFAT5 activity, whereas NFAT5 synthesis was slightly decreased by this compound at 24 hours. The effect of rapamycin was reflected in reporter assays at 24 hours in 50% of the experiments, while induction of Hsp70.1 was downregulated by this compound in

around 80% of the mice analyzed. In non-lymphoid cells the effect of rapamycin was more constant, whereas in splenocytes we observed variations in the response to rapamycin in individual experiments. Lymphocyte activation requires mTOR function to avoid anergy (Mondino, Mueller 2007, Zheng et al. 2007) and during this process mTOR regulates the expression of nutrient receptors necessary for blastogenesis, such as CD71 (Galvez et al. 2007, Zheng et al. 2007) and CD98 (Edinger et al. 2003, Kelly et al. 2007), and also controls the transcription of other receptors downregulated during T cell activation, such as CD62L (Sinclair et al. 2008). Therefore, rapamycin is able to inhibit T cell proliferation and activation (Colombetti et al. 2006, Dumont et al. 1990), as we observed in splenocytes after 72 hours in cell culture with this drug, where T cell expansion was arrested. However, for the 9xNFAT-Luc reporter and RT-qPCR experiments, quiescent splenocytes were first stimulated with concanavalin A and IL-2 for 24 hours before adding rapamycin, which can lead to various degrees of T cell activation depending on the culture. mTOR might contribute differentially to NFAT5 activation in different stages of blastogenesis, as NFAT5 transcriptional activity is maximal in the first 24 hours of T cell activation in cells exposed to hypertonicity, suggesting that this factor might present a limited window of activation in splenocytes in which it might be controlled by mTOR. However, more experiments are needed to clarify exactly at which stage mTOR controls NFAT5. Moreover, rapamycin-treated T cells can undergo growth and proliferation by activating Pim kinases (Fox, Hammerman & Thompson 2005), and it might be possible that those kinases might alternate with mTOR in regulating NFAT5 during the active cell growth phase that T cells experience in response to mitogen stimulation. In conclusion, impairment of mTOR pathway diminishes NFAT5 function in response to osmotic stress, suggesting that in different cell types NFAT5 might be a new target of this signaling pathway, while its regulation in lymphocytes appears to be more complex and it might be related to the stage of activation or other factors involved in cell growth or proliferation.

mTOR regulates cell growth and proliferation when forming part of mTOR complex 1 (mTORC1) (Hara et al. 2002, Kim et al. 2002a), and cytoskeletal reorganization when being part of mTORC2 (Jacinto et al. 2004, Sarbassov et al. 2004). Rapamycin is able to regulate S6K activity through inhibition of mTORC1 complex, suggesting that mTORC1 complex might regulate NFAT5. However, long-term rapamycin treatment also inhibits mTORC2 complex, downregulating Akt activity in certain cell types (Sarbassov et al. 2006). Acute hypertonicity has been shown to inhibit Akt (Copp et al.

2005b, Meier, Thelen & Hemmings 1998) but chronic treatment with osmotic stress activates the kinase (Pastukh et al. 2005). Besides, hypertonicity is able to induce reorganization of the cytoskeleton by different mechanisms (Bustamante et al. 2003, Di Ciano et al. 2002, Yamamoto et al. 2006), but it is not known whether these are rapamycin-sensitive. In sum, hypertonicity-induced activation of NFAT5 might require mTORC1, but it might also be influenced by mTORC2-dependent regulation of the cytoskeleton. In both cases, rapamycin treatment during 24 hours would inhibit both complexes and their relative contribution to NFAT5 activity needs to be dissected. In any case, mTOR might be a direct kinase for NFAT5 or its effects on NFAT5 might be dependent on mTOR but not direct. Further experiments are needed to assess whether NFAT5 and mTOR interact and whether NFAT5 is phosphorylated in an mTOR-regulated manner.

Proliferating cells are characterized by displaying high rates of glycolysis, lactate production and biosynthesis of lipids and other macromolecules. These allow the cell to produce ATP from glucose, the most abundant extracellular nutrient. Downregulation of glucose levels and consequent drop in ATP concentration causes activation of AMPK, which limits energy utilization and promotes energy production to ensure cell survival. AMPK regulates proliferation through phosphorylation of TSC2 (Inoki et al. 2006, Inoki, Zhu & Guan 2003), a negative regulator of mTOR signaling, and also through phosphorylation of p53 (Imamura et al. 2001, Jones et al. 2005). Osmotic stress also activates AMPK (Fryer, Parbu-Patel & Carling 2002, Woods et al. 2003, Woods et al. 2005), and the AMPK pharmacological activator AICAR and the glycolysis inhibitor 2-deoxyglucose inhibited a hypertonicity activated NFAT5-dependent reporter in HEK293 cells without affecting the nuclear localization of the protein. AICAR almost abolished the activity of the reporter and also inhibited NFAT5 synthesis at long time points, implying a possible relevant role for this kinase. However, the effects of AICAR were similar in AMPK-null cells when compared to AMPK wild-type ones, suggesting that the effects were independent of the kinase. In the literature there are several emerging articles that describe the unspecific effects of the formerly considered AMPK-specific AICAR. This drug has been shown to reduce oxygen consumption in hepatocytes (Guigas et al. 2007), induce dephosphorylation of Akt and GSK3 (King, Song & Jope 2006), activate p38 (Lemieux et al. 2003) and the JAK/STAT3 pathway (Zang et al. 2008), in an AMPK independent manner. p38 activation is maintained by AICAR at long time points in HEK293 cells (our data not shown), suggesting that at least AICAR

is not inhibiting NFAT5 by downregulating p38. AICAR treatment also reduced the amount of S6K1 in both AMPK wild-type and null MEF, suggesting that a novel mechanism of action of this drug. In addition, the AMPK inhibitor compound C also inhibited the NFAT5-dependent reporter ORE-Luc in HEK293 cells and it did not rescue NFAT5 activation when inhibited by AICAR. Due to its already described unspecific effects (Emerling et al. 2007) we also checked whether compound C had the same effects in AMPK-null cells. Our results showed that compound C was able to inhibit the hypertonicity-induced NFAT5 reporter and it was not capable of rescuing AICAR-mediated inhibition, in both wild-type and AMPK-null MEF. Altogether, these results demonstrate that AICAR and compound C inhibited NFAT5 by AMPK-independent mechanisms. AICAR inhibited the activation of the 9xNFAT-Luc reporter by hypertonicity in splenocytes after 8 hours, but strongly stimulated it at 24 hours. Our analysis of the sensitivity to AICAR of the 9xNFAT-Luc reporter in $NFAT5^{-/-}$ T cells showed that the early inhibitory effect of AICAR was due to the suppression of NFAT5, whereas the later enhancing response was NFAT5-independent. In view of these results, we followed the activation course of the NFAT5-target gene *Hspa1b*. In this case, induction of its mRNA by hypertonicity was upregulated by AICAR, both in $NFAT5^{+/+}$ and $NFAT5^{-/-}$ splenocytes. NFAT5 was shown to control Hsp70.1 transcription by reporter assays (Woo et al. 2002), but it was unknown whether NFAT5 was sufficient to regulate the induction of the endogenous gene. As seen in our results, although $NFAT5^{-/-}$ splenocytes induced Hsp70.1 less efficiently than wild-type cells, they were nonetheless still able to upregulate it, indicating that other factors are involved in its transcription. Likewise, as the 9xNFAT-Luc reporter is under the control of the α-myosin heavy chain promoter, factors regulating it might positively respond to AICAR at longer time points (24 hours). In the literature, other transcription factors such as MEF2 (Al-Khalili et al. 2004), EGR1 (Berasi et al. 2006), E2F1 (Hallstrom, Mori & Nevins 2008), STAT3 (Zang et al. 2008) and CREB (Thomson et al. 2008) have been shown to be upregulated by AICAR and they could be involved in the NFAT5-independent activation of the 9xNFAT-Luc reporter and Hsp70.1. In consequence, AICAR, which mimics ATP depletion in cells, leads to the inhibition of NFAT5 independently of AMPK, while it also promotes the upregulation of hypertonicity-induced genes, such as *Hspa1b*, independently of NFAT5.

Other transcription factors are regulated by mTOR and AMPK pathways in order to regulate cell proliferation. Consistent with the role of mTOR in cell growth via the

modulation of protein synthesis, this kinase regulates the transcription of genes involved in ribosome biogenesis (Mahajan 1994, Powers, Walter 1999, Zaragoza et al. 1998), via the transcription factors TIF-1A (Mayer et al. 2004) and UBF (Hannan et al. 2003). AMPK regulation of cell growth also involves the activation of the transcription factor p53 (Imamura et al. 2001, Jones et al. 2005). Our experiments showed that AICAR, rapamycin and 2-deoxyglucose were able to downregulate NFAT5 activity through the carboxy-terminal domain of NFAT5, where the transactivation domain is located, and that their inhibitory effect is not due to a general shutdown of the transcriptional response. This was seen in NFAT5$^{-/-}$ MEF, where the activity of the NFAT5 reporter ORE-Luc became insensitive to all these drugs when cells were transfected with the NFAT5 DNA binding domain fused to a constitutive active heterologous transactivation domain. In parallel, this reporter was inhibited by these compounds when cells were reconstituted with wild-type full-length NFAT5.

Whereas hypertonicity-dependent activation of NFAT5 was inhibited by rapamycin and AICAR in several cell types, the glycolysis inhibitor and AMPK activator 2-deoxyglucose was also able to downregulate an NFAT5-dependent reporter in HEK293 without affecting NFAT5 subcellular localization, but it had no effect in MEF, indicating that its effect might be cell-dependent. However, 2-deoxyglucose was able to inhibit NFAT5 activity in NFAT5$^{-/-}$ MEF reconstituted with NFAT5 protein, comparably to AICAR and rapamycin. This raises the possibility that the recombinant protein was not able to reproduce the behavior of endogenous NFAT5 in this cell line, perhaps due to an unspecific effect of the tags, the isoform used, or a differential effect of 2-deoxyglucose among different MEF lines. In addition, MEF lacking AMPK presented the same activity as wild-type cells after 2-deoxyglucose treatment, indicating that AMPK is not involved in the regulation of NFAT5. In T cells, 2-deoxyglucose slightly inhibited the 9xNFAT-Luc reporter at 8 hours, but the drug had a stimulatory effect at 24 hours. NFAT5$^{-/-}$ splenocytes were slightly stimulated at both time points in comparison to wild-type, indicating that the effect was independent on NFAT5. The differences among cell lines might be explained by different energy requirements among them. Proliferating T cells, as well as tumors, demand high levels of ATP, and T cell activation induces changes in cellular metabolism, from oxidative phosphorylation to glycolytic metabolism (Roos, Loos 1973). This change to aerobic glycolysis is called the Warburg effect (Warburg 1956) and in T cells happens in order to support growth at the sufficient rate that proliferation demands. Inhibition of glycolysis by 2-deoxyglucose,

which inhibits proliferation in T cells (Miller et al. 1994), was not enough to inhibit NFAT5 activity in these cells. In this regard, previous reports have shown that T cells can maintain ATP production with aminoacids in the absence of glucose, preserving the activity of the mTOR pathways (Bauer et al. 2004, DeBerardinis et al. 2007). In our conditions, thus, we would expect that the mTOR pathway remained active in T cells and, in consequence, NFAT5 transcriptional activity as well.

In conclusion, NFAT5 is also activated in hypertonic conditions by pharmacological modulators of energy and nutrient stress, as it is shown by its sensitivity to AICAR, 2-deoxyglucose and rapamycin. Thus, the mTOR pathway emerges as a new kinase regulating this factor and NFAT5 turns up as new transcriptional regulator whose activity is sensitive to drugs affecting energy stress pathways. Its susceptibility to AICAR, though, is independent of AMPK and it remains to be determined whether the effect of this drug on NFAT5 is related to energy stress.

The sensitivity of NFAT5 in pathological hypertonic conditions to the mTOR pathway inhibitors and pharmacological energy modulators indicates that intact growth and proliferative signaling pathways are required to set up an appropriate response to this stress. This is also reflected in two independent transgenic mice models with reduced NFAT5 activity, which presented impaired cell growth under hypertonic conditions (Go et al. 2004, Trama, Go & Ho 2002) and, besides, hypertonic stress induces cell-cycle arrest and a transient halt in proliferation (Kultz, Madhany & Burg 1998, Michea et al. 2000). Altogether, these observations suggest that osmolytes induced in an NFAT5-dependent manner might be necessary to allow the progression of cellular proliferation and mTOR would be regulating this factor. Thus, our results reveal a new signaling pathway involved in the regulation of NFAT5, which might function as a checkpoint in the progress of proliferation in osmotic stress conditions.

CONCLUSIONS

1) The 9xNFAT-Luc reporter is activated by osmotic stress specifically by NFAT5 in transgenic lymphocytes, macrophages and mouse embryonic fibroblasts (MEF).

2) NFAT5 transcriptional activity is stimulated in primary, non-transformed T cells at pathophysiological tonicity levels reported to occur in plasma in osmoregulatory disorders in patients and animal models (360-430 mOsm/kg).

3) The 9xNFAT-Luc reporter is stimulated at hypertonic conditions of 360-380 mOsm/kg in T cells, at 430 mOsm/kg in bone marrow-derived macrophages and at 480 mOsm/kg in mouse embryonic fibroblasts. The response of the 9xNFAT-Luc reporter to hypertonicity is more effective in mitogen-activated splenocytes than in quiescent cells.

4) Hypertonicity-induced transcriptional activity of NFAT5 in splenocytes and MEF is affected by several kinase inhibitors and pharmacological modulators of energy stress responses: it is partially inhibited by the PI3-kinase inhibitor wortmannin, the PKA inhibitor H89 and the mTOR inhibitor rapamycin, substantially downregulated by p38 inhibitors (SB203580 and SB202190), by inhibition of PI3-kinase-related kinases (PIKK) with LY294002 and by the AMPK activator AICAR, and insensitive to the ERK inhibitor PD98059.

5) Hypertonic levels in the range required to activate NFAT5 do not inhibit the mTOR pathway.

6) Hypertonicity-induced NFAT5 expression is downregulated by LY294002, at concentrations that inhibit PIKK, and AICAR.

7) AICAR, rapamycin and 2-deoxyglucose do not inhibit the nuclear translocation of NFAT5 nor its DNA binding capacity in response to hypertonic stress.

8) AICAR inhibition of NFAT5 induction and activity in response to hypertonicity are independent of AMPK.

9) AICAR inhibits the mTOR pathway in an AMPK-independent manner in cells exposed to hypertonicity.

10) The AMPK activator AICAR inhibits the activity of the 9xNFAT-Luc reporter mediated by NFAT5 in response to hypertonicity at 8 hours but stimulates the reporter independently of NFAT5 at 24 hours in splenocytes.

11) The induction of Hsp70.1 by osmotic stress in primary T cells is regulated by NFAT5, inhibited by rapamycin and activated by AICAR. The activating effect of AICAR on the induction of Hsp70.1 is independent of NFAT5.

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