POMPEU FABRA UNIVERSITY
FACULTY OF HEALTH AND LIFE SCIENCES
DEPARTMENT OF EXPERIMENTAL AND HEALTH SCIENCES

ROLE OF THE TRANSCRIPTION FACTOR NFAT5 IN MAMMALIAN CELL CYCLE REGULATION

KATHERINE DREWS-ELGER

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JOSE ARAMBURU, PhD.
DIRECTOR

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ABBREVIATIONS

AD	Activation domain
ADH	Anti diuretic hormone
AED	Auxiliary export domain
AP-1	Activator protein -1
AQP	Aquaporin
AR	Aldose reductase
ATM	Ataxia-telangiectasia mutated
ATR	ATM and Rad3-related protein
ATRIP	ATR-interacting protein
BGT1	Betaine γ-amino-*n*-butyric acid transporter
CAK	Cdk-activating kinase
Cdk	Cyclin dependent kinases
Chk	Checkpoint kinase
CKI	Cyclin dependent kinase inhibitor
ConA	Concanavalin A
DF	Dermal fibroblasts
DBD	DNA binding domain
DDR	DNA damage response
DNA-PK	DNA-dependent protein kinase
DNA-PKcs	DNA-PK catalytic subunit
DSB	Double strand break
ECF	Extracellular fluid
GPC	Glycerophosphocholine
HR	Homologous recombination
HTS	Hypertonic saline
ICF	Intracellular fluid
IL	Interleukin
JNK	Jun kinase
KAP	Kinase activating protein
KCS	Keratoconjunctivitis sicca
MAPK	Mitogen-activated protein kinase
MD	Modulatory domain

MDCK	Madin-Darby canine kidney cells
MEF	Mouse embryonic fibroblast
mIMCD	Mouse inner medulla collecting duct cells
MRN	Mre11-Rad50-NBS1 complex
mRNA	Messenger RNA
mTOR	Mammalian Target of Rapamycin
NDI	Nephrogenic diabetes insipidus
NES	Nuclear export sequence
NFAT	Nuclear factor of activated T cells
NHEJ	Non-homologous end joining
NLS	Nuclear localization sequence
NTE	Neuropathy target esterase
OREBP	Osmotic response element binding protein
PC	Phosphatidylcholine
PCNA	Proliferation cell nuclear antigen
PI3-K	Phosphatidylinositol 3-kinase
PIKK	Phosphatidylinositol 3-kinase related kinases
PKA	Protein kinase A
PMA	Phorbol myristate acetate
Rb	Retinoblastoma protein
RHR	Rel homology region
ROS	Reactive oxygen species
RVI	Regulatory volume increase
TAD	Transactivation domain
TauT	Taurine transporter
TCR	T cell receptor
TGFβ	Transforming growth factor beta
TNFα	Tumor necrosis factor α
TonEBP	Tonicity-responsive enhancer binding protein
TUNEL	Terminal deoxynucleotidyltransferase-mediated dUTP end-labeling
UT-A	Urea transporter

SUMMARY

SUMMARY

The transcription factor NFAT5/TonEBP belongs to the Rel family, which also comprises NFκB and NFATc proteins. NFAT5 only shares structural and functional homology with other Rel family members at the level of the DNA binding domain, and differs from them considerably in other regions. NFAT5 enables mammalian cells to adapt to and withstand hypertonicity by orchestrating an osmoprotective gene expression program whose products include chaperones as well as transporters and enzymes that increase the intracellular concentration of compatible osmolytes. NFAT5-null mice suffer severe embryonic and perinatal lethality, and surviving adults manifest growth defects, pronounced renal atrophy and lymphocyte dysfunction associated with ineffective responses to hypertonicity. To circumvent the lethality of these mice and study the function of NFAT5 in specific cell types without the possible side effects of generalized defects in the organism, we have produced conditional knockout mice that allow the deletion of NFAT5 in specific cell types. Here we have investigated the hypertonic stress response in wild-type and NFAT5$^{-/-}$ lymphocytes. Proliferating lymphocytes exposed to hypertonic conditions exhibited an early, NFAT5-independent, genotoxic stress-like response with induction of p53, p21 and GADD45, downregulation of cyclins E1, A2 and B1 mRNA, and arrest in S and G2/M. This was followed by an NFAT5-dependent adaptive phase in wild-type cells, which induced osmoprotective gene products, downregulated stress markers, and resumed cyclin expression and cell cycle progression. NFAT5$^{-/-}$ cells, however, failed to induce osmoprotective genes and though they downregulated genotoxic stress markers, they displayed defective cell cycle progression associated with reduced expression of cyclins E1, A2, B1, and aurora B kinase. Finally, T cell receptor-induced expression of cyclins, aurora B kinase, and cell cycle progression were inhibited in NFAT5$^{-/-}$ lymphocytes exposed to hypertonicity levels in the range reported in plasma in patients and animal models of osmoregulatory disorders. Our results support the conclusion that the activation of an osmoprotective gene expression program by NFAT5 enables cells to proliferate under hypertonic stress conditions by maintaining the expression of S and G2/M cyclins and cell cycle progression.

INTRODUCTION

1. NUCLEAR FACTOR OF ACTIVATED T CELLS 5 (NFAT5): discovery and features

The Nuclear Factor of activated T cells (NFAT5), also known as TonEBP/OREBP, belongs to the Rel family of transcription factors, as do the NF-κB and the calcineurin-dependent NFAT proteins NFAT1, NFAT2, NFAT3, and NFAT4, collectively known as NFATc. Members of this family perform a wide variety of functions in the regulation of cellular activation as well as in stress responses. The Rel family of transcription factors is characterized by a single common feature; a conserved DNA binding domain (DBD) called the Rel domain (**Figure 1**) (Hogan *et al.*, 2003).

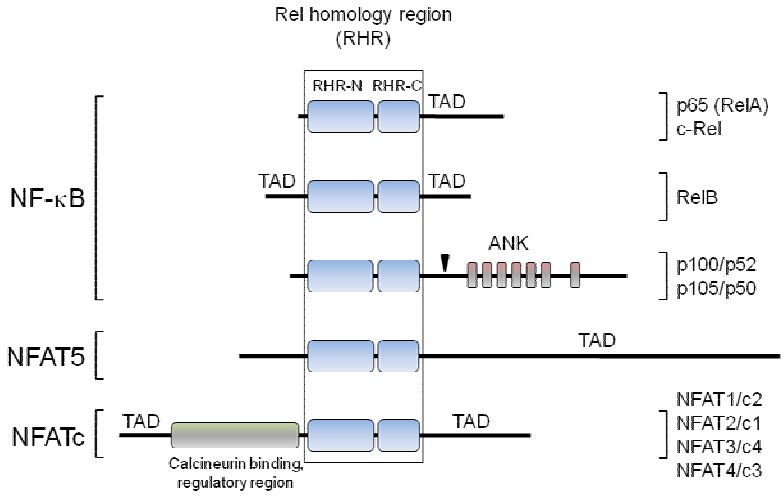

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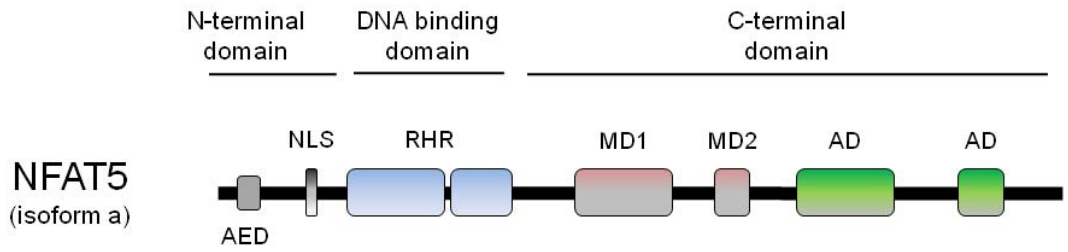
Figure 1. Schematic diagram of members of the mammalian Rel family of proteins. *Rel proteins share a structurally conserved homology region (RHR, shown in blue). Within the RHR are the DNA binding loop (in the RHR-N) and the dimerization domain in the RHR-C. Shown in gray is the calcineurin binding regulatory region present in NFATc proteins. Ankyrin repeats (ANK) present in NF-kB members p100/p52 and p105/p50 and the proteolytic cleavage site (indicated by an arrowhead) are also shown (TAD: transactivation domain)*.

The discovery of NFAT5 was achieved by independent groups undertaking different strategies. Initially, a yeast one-hybrid screening resulted in the identification of the protein binding to the tonicity response element (TonE) of several osmoprotective genes in response to hypertonicity and was termed TonE Binding Protein, TonEBP (Miyakawa *et al.*, 1999). Independently, and by

using database searches for proteins with homology to the DNA binding domain of NFATc proteins, NFAT5 was isolated and classified as a member of the NFAT family based on the greater homology of its DBD with NFATc than with NF-κB (Lopez-Rodriguez *et al.*, 1999b). Other groups have subsequently cloned NFAT5/TonEBP and have called it OREBP (Ko *et al.*, 2000) and NFATL1 (Trama *et al.*, 2000). Hereafter it will be referred to as NFAT5.

NFAT5 is a large protein composed of almost 1500 amino acids and a long transactivation domain of more than 900 amino acids. The C-terminal region of this protein, which is long and glutamine-rich, shows no sequence similarity to the C-terminal regions of NFATc proteins (Lopez-Rodriguez *et al.*, 1999b; Miyakawa *et al.*, 1999). This region contains the transactivation domain (TAD) of NFAT5, which is activated and phosphorylated in response to hypertonicity. Within this domain, there are activation domains that are activated by hypertonicity as well as modulatory domains that potentiate TAD activity but do not actively stimulate transcription on their own (Lee *et al.*, 2003). The amino terminal region is short (177 amino acids) and contains a bipartite nuclear localization sequence, but lacks the calcineurin docking sites found in NFATc proteins (**Figure 2**).

The single NFAT5 gene found in mammals gives rise to three NFAT5 isoforms, named NFAT5a, NFAT5b and NFAT5c (Lopez-Rodriguez *et al.*, 1999a). The first isoform, NFAT5a, is the predominant one and corresponds to the originally isolated cDNA by Lopez-Rodriguez and colleagues (Lopez-Rodriguez *et al.*, 1999b). Transcripts of NFAT5b and NFAT5c are only found in brain and encode 29 and 76 extra amino acids, respectively, in the amino terminal domain (Lopez-Rodriguez *et al.*, 1999a).



Adapted from Lee et al, 2003 and Jeon et al, 2006.

Figure 2. Schematic diagram of NFAT5 regulatory domains. *The amino terminal region of NFAT5 is short and comprises the nuclear localization sequence (NLS) and the auxiliary export domain (AED).*

Isoform NFAT5c also contains a nuclear export sequence (NES) and a transcriptional activation domain (AD1) in its N-terminal region. NFAT5 has a long carboxy-terminal domain (C-terminal domain). Within this region are found the modulatory regions (MD1, MD2) and the activation domains (AD). The RHR is shown in blue.

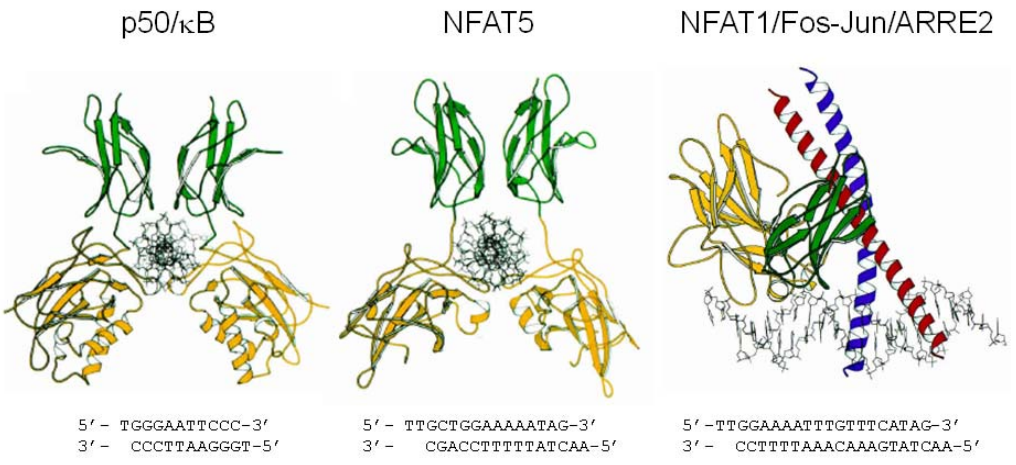
The NFAT5 DNA binding domain (DBD) shows higher amino acid sequence identity with NFATc proteins (41-45%) than with NF-κB (17% identity with p50) (Lopez-Rodriguez *et al.*, 1999b). NFAT5 and NFATc proteins recognize similar DNA binding sites in the regulatory regions of several genes (Lopez-Rodriguez *et al.*, 1999b; Rao *et al.*, 1997); however, NFAT5 binds with lower affinity to DNA than NFATc proteins and has a stricter sequence requirement for its target. NFAT5 shows a preference for the sequence (T/A)GGAAA, while members of the NFATc family prefer the core sequence GGAA (Lopez-Rodriguez *et al.*, 1999b).

On the other hand, the DNA binding domain lacks most of the Jun contact residues found in NFATc proteins (Lopez-Rodriguez *et al.*, 1999a; Lopez-Rodriguez *et al.*, 1999b). This suggests that NFAT5 might not need to bind cooperatively with the AP-1 proteins Fos and Jun to DNA (Lopez-Rodriguez *et al.*, 1999a). Recently however, inhibition of AP-1 was shown to downregulate mRNA levels of NFAT5 targets aldose reductase (AR) and Betaine γ-amino-*n*-butyric acid transporter (BGT1), and AP-1 was shown to associate with NFAT5 in the absence of DNA (Irarrazabal *et al.*, 2008).

NFAT5 differs substantially from NFATc proteins in their biological functions and activation mechanisms. NFATc proteins are all cytoplasmic and highly phosphorylated in resting cells (Rao *et al.*, 1997). Influx of calcium activates calcineurin phosphatase which rapidly dephosphorylates NFATc proteins mediating their nuclear translocation, and full transcriptional activation. These transcription factors form stable, cooperative DNA-binding complexes with dimers of the AP-1 (Fos /Jun) family (Jain *et al.*, 1992; Chen *et al.*, 1998). NFATc proteins play key roles in the immune, nervous and cardiovascular systems (Macian, 2005; Molkentin, 2004; Wu *et al.*, 2007). In contrast, NFAT5 is a predominantly nuclear phosphoprotein activated by osmotic stress, and as previously mentioned, lacks most of the Jun contact residues found in NFATc proteins as well as their characteristic calcineurin binding sites (Lopez-Rodriguez *et al.*, 1999b). At present, the best characterized function of NFAT5 is its sensitivity to hypertonic stress, to which NFAT5 responds by activating the expression of several target genes with

osmoprotective function. Regulation of NFAT5 in the osmotic stress response and other functions of this factor will be discussed in a later section.

NFAT5 forms stable dimers in solution in the absence of DNA, and its dimerization is required for proper DNA binding and transcriptional activity (Lopez-Rodriguez *et al.*, 1999b). Upon binding to DNA, NFAT5 encircles its target, presumably to increase kinetic stability of the transcription factor-DNA complex. This conformation, which structurally resembles that of NF-κB proteins, is known as butterfly conformation (Lopez-Rodriguez *et al.*, 2001; Stroud *et al.*, 2002) (**Figure 3**). Because NFAT5 shares features with NF-κB as well as with NFATc proteins, it is considered a unique member of this family, an outlier, a hybrid. NFAT5 is the most ancient member of the NFAT family. Tests on genomic DNA of several species revealed that homologous sequences were clearly detected in mammalian species (human, pig, and mouse), as well as in chicken, zebrafish, *Xenopus*, and, more faintly, in the model organism *Drosophila* (Lopez-Rodriguez *et al.*, 1999a). The *Drosophila* homolog, called dNFAT, does not exhibit recognizable similarities to neither NFAT5 nor NFATc proteins outside of its DBD (Huang and Rubin, 2000). However, the DBD of dNFAT is more similar to that of NFAT5 than to NFATc, and this protein has been shown to play a role in the survival of insect cells to hypertonic stress (Keyser *et al.*, 2007). Homologous sequences of the NFATc proteins are found only in the genome of vertebrates, and not in *Drosophila* (Hogan *et al.*, 2003).



Adapted from Hogan et al, 2003

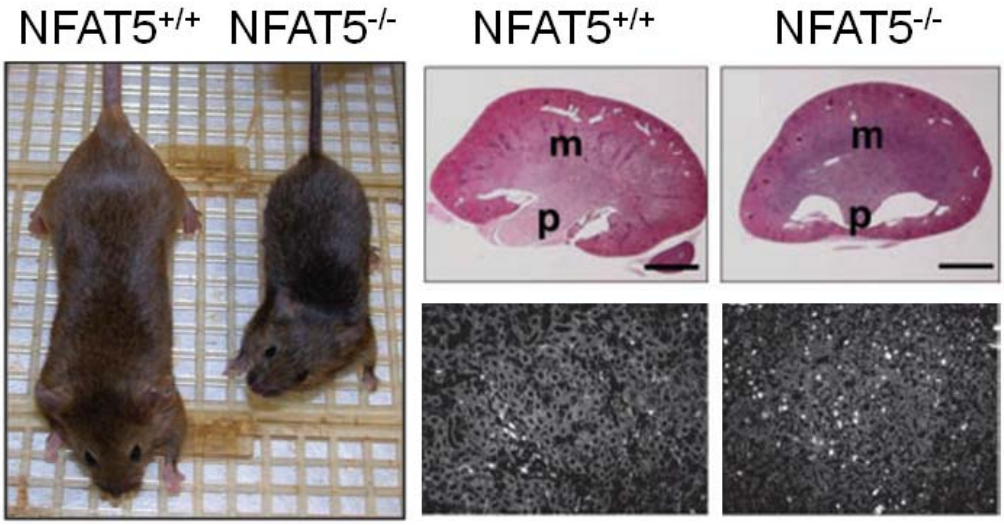
Figure 3. Crystal structures of NFAT5, NFATc and Rel dimeric protein/DNA complexes. *In yellow is shown the RHR-N domain and in green the RHR-C domain. From left to right, NF-κB p50 homodimer is*

shown bound to a κB site; NFAT5 to a tonicity response element and the NFAT1/Fos-Jun complex to the ARRE2 site of the IL-2 promoter. The respective DNA sequences are indicated.

NFAT5 mRNA expression has been detected in a wide range of tissues, such as kidney, spleen, lung, pancreas, colon, testis, ovaries, placenta, thymus, heart, brain, skeletal muscle and peripheral blood lymphocytes (Lopez-Rodriguez *et al.*, 1999b; Miyakawa *et al.*, 1999; Trama *et al.*, 2000). Moreover, expression of NFAT5 mRNA has also been detected in embryonic stem cells and throughout the stages of fetal development (Maouyo *et al.*, 2002). NFAT5 protein has been detected in lymphocytes, fibroblast, skeletal muscle, thymus, testis, embryo, and kidney cell lines (Lopez-Rodriguez *et al.*, 1999b; Trama *et al.*, 2000).

To study the physiological role of NFAT5 *in vivo,* two groups generated NFAT5 knock-out mice (Go *et al.*, 2004; Lopez-Rodriguez *et al.*, 2004) (**Figure 4**). The study by Lopez-Rodriguez and colleagues showed that deletion of NFAT5 gene leads to mid-embryonic lethality, postnatal mortality at day 10, and low survival rate after postnatal day 21 (5%). Surviving NFAT5$^{-/-}$ mice suffer severe renal atrophy and impaired activation of osmoprotective genes as well as growth retardation (Lopez-Rodriguez *et al.*, 2004). Another model generated by the group led by S. Ho found that complete loss of function led to late gestational lethality, and that heterozygous mice suffered lymphoid hypocellularity and impaired antigen specific antibody response (Go *et al.*, 2004). NFAT5-deficient T cells and MEF from these mice proliferated poorly *in vitro* under hypertonic stress. Additionally, three transgenic mouse models expressing dominant negative forms of NFAT5 in specific tissues have been generated. In one of the models, mutant mice expressing a dominant-negative form of NFAT5 in developing and mature T cells showed impaired T cell development, hypocellularity in thymus and spleen, and reduced proliferation *ex-vivo* of T cells upon exposure to hypertonic stress (Trama *et al.*, 2002). The essential role of NFAT5 in the urine concentrating mechanism was proved by the transgenic mouse model overexpressing a dominant negative form of NFAT5 in the renal collecting tubules (Lam *et al.*, 2004). These mice developed polyuria and polidipsia, bilateral hydronephrosis as well as reduced mRNA expression of aquaporin AQP2 and urea transporters UT-A1 and UT-A2. Later on, the same group generated transgenic mice expressing a dominant negative form of NFAT5 in the lens fiber (Wang *et al.*, 2005). These mice exhibited defects in eye lens fiber elongation, markers of DNA damage and/or apoptosis such as induction of p53, phosphorylation of Chk2, increased DNA breaks and developed cataracts soon after birth, suggesting an essential role of

NFAT5 during lens development. However, this study did not determine whether the observed defects were due to an impaired osmotic stress response.



Adapted from Lopez-Rodriguez et al, 2004.

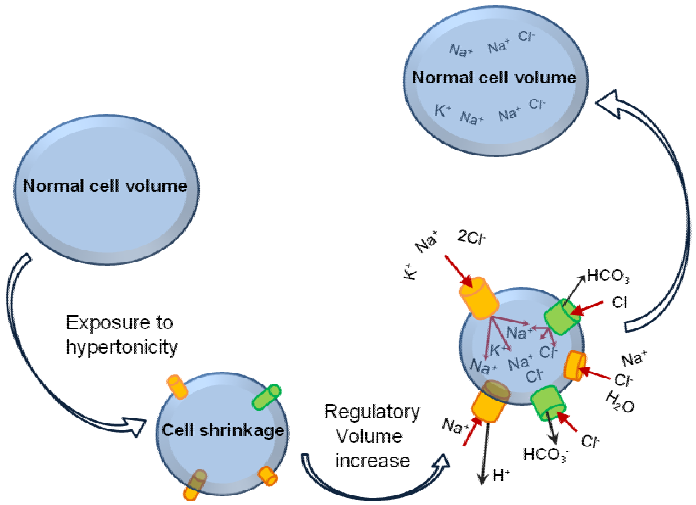
Figure 4. Growth and renal abnormalities in NFAT5-deficient mice. *Comparison of 6 week-old wild-type and NFAT5$^{-/-}$ mice demonstrates growth retardation in the mutant. Renal abnormalities are evidenced by atrophic papillae (**p**) and denser morphology of the renal medulla (**m**) (upper right panel) and by increased number of apoptotic cells shown by terminal deoxynucleotidyltransferase-mediated dUTP end-labeling analysis (TUNEL) shown in the lower right panel.*

2. OSMOTIC STRESS

Membranes of animal cells are highly permeable to water. However, since they do not tolerate nor generate substantial hydrostatic pressure gradients, water moves across the membrane driven by the osmotic pressure gradients, causing changes in cell volume (Lang *et al.*, 1998). The *osmotic pressure* or osmolarity is defined as the opposing hydrostatic force required for preventing water flux in and out of the cell. Osmolarity is dependent on the total concentration of dissolved solute particles. The terms osmolality and osmolarity are a measure of the number of particles present in a kilogram of solvent and a liter of solution respectively, and are independent of the size or weight of the particles. Osmolarity is generally used when referring to fluids outside of the body, while osmolality is used to describe fluids inside the body. However, these terms are interchangeably used, because 1 L of water weights approximately 1 kg

(Strange, 2004). Osmolarity is hence measured by using a property of the solution that is only dependent on the particle concentration such as vapor pressure or freezing point depression. Cellular osmotic stress occurs when the water thermodynamic equilibrium, i.e. osmotic concentration, between intracellular and extracellular fluids is altered. The cellular response generated aims to restore steady-state osmolarity conditions.

The ability of cells to adapt to osmotic imbalances involves an early response, occurring within seconds, and a later response that usually requires hours. Hypotonicity usually causes flow of water into the cell, leading to cell swelling, while hypertonicity forces water out of the cell causing it to shrink (Lang *et al.*, 1998). Exposure of cells to hypertonic media causes alterations in cellular volume by generating flux of water out of the cell, and thus leading to cell shrinkage. The early cellular response is to recover cell volume by uptaking electrolytes. This process is mediated mainly by the pre-existing $Na^+K^+2Cl^-$ cotransporter and the Na^+/H^+ exchanger, and in some cells by Na^+ channels or nonselective cation channels. This process is known as regulatory volume increase, RVI (**Figure 5**). However, electrolyte accumulation is limited, since high ion concentrations interfere with structure and function of macromolecules (Alfieri and Petronini, 2007; Lang *et al.*, 1998).



Adapted from Strange, 2004.

Figure 5. Regulatory volume increase. *Schematic representation of the regulatory volume increase mechanism executed by cells exposed to hypertonic stress.*

To neutralize the perturbing effects of high intracellular ionic strength, in a slow process that can take several hours, cells produce molecules called “compatible osmolytes”. These molecules

create osmolarity without compromising cellular functions (Lang *et al.*, 1998). The term "compatible osmolyte" or "compatible solute" was first introduced by a group of scientists led by A.D Brown, (Brown, 1976; Brown, 1978) while studying microbial water stress. They observed that when certain microbial species were grown in saturated salt solution they accumulated substances, such as polyols, which allowed them to preserve water retention as well as macromolecular function. This adaptation mechanism's osmoprotective effects are remarkably similar between species. It is thought that this mechanism minimizes the requirement for genetic change by allowing a single form of any particular protein to properly function in a wide range of osmotic conditions, hence not requiring that a given form of a protein is generated for every osmotic condition (Burg *et al.*, 1996; Yancey *et al.*, 1982).

Mammalian cells exposed to hypertonic stress accumulate compatible osmolytes of three different groups; polyols such as inositol and sorbitol, methylamines such as betaine and glycerophosphocholine, and neutral amino acids or their derivatives such as taurine, glycine, glutamine, glutamate and aspartate. Use of one group or another, or combination of different molecules seems to be cell type-specific. The accumulation of organic osmolytes is accomplished by stimulated uptake, enhanced formation or decreased degradation. Decrease of these molecules is achieved by degradation or release (Lang *et al.*, 1998).

As previously pointed out, the osmotic balance between the extracellular and intracellular fluids is a key issue for proper cellular function. With the exception of cells of the kidney medulla, most mammalian cells are surrounded by extracellular fluid with well controlled osmolarity and are usually not exposed to acute hypertonicity. Nonetheless, these cells contain a considerable amount of organic osmolytes and are able to accumulate them in the case of tonicity variations. In the kidney medulla, extracellular osmolarity may range from isotonic to extreme hypertonic conditions during the urine concentration process; therefore cells in this tissue have higher concentration of organic osmolytes, mainly sorbitol, betaine, inositol, taurine and glycerophophocholine (GPC) (Burg and Ferraris, 2008; Strange, 2004; Waldegger and Lang, 1998). Organic osmolytes are also intermediate compounds of a variety of biological pathways occurring outside the renal medulla, and not only related to osmoregulation (Burg and Ferraris, 2008).

The **sorbitol** pathway, also known as the polyol pathway, consists on the two step conversion of glucose into fructose. In a reaction catalyzed by aldose reductase (AR), sorbitol is obtained from glucose. To finally produce fructose, sorbitol is oxidized in a reaction catalyzed by sorbitol dehydrogenase. Before the role of sorbitol as a compatible osmolyte was described, it had been previously found to be serving as the source of fructose in seminal vesicles and placentas of sheep (Burg, 1988; Garcia-Perez and Burg, 1991). Under hypertonic conditions, the induction of sorbitol is mainly achieved by increasing the amount and activity of aldose reductase (AR). The increase in AR expression triggered by hypertonicity is achieved rather by greater transcription rate, and not by affecting the mRNA degradation rate (Burg *et al.*, 1996). Sorbitol is accumulated in renal medullary cells for osmoregulation purposes. However, abnormal expression of this osmolyte may be damaging to the cell (Burg and Kador, 1988). Aldose reductase is a member of the keto-aldo reductase family, which are cytosolic, monomeric, and NADPH-dependent enzymes. It is found in various types of mammalian tissues including seminal vesicles, placenta, nerves, kidneys, intestines, brain, red blood cells, ovary, pancreas, blood vessels, eye, but not in other tissues including capillaries throughout the body (Burg, 1988). Corresponding with the large amount of sorbitol in the renal medulla, there is also a high amount and activity of AR. Although activity of AR is needed for proper osmoregulation, over-activity has been shown to be harmful for the cell. Activity of AR is high in diabetes and has been implicated in microvascular complications of this disease, and as a result, great efforts have been made towards the development of AR inhibitors (Burg and Ferraris, 2008).

Betaine is produced by a two step process from choline. Initially, in a reaction catalyzed by mitochondrial choline dehydrogenase, choline is converted to betaine aldehyde. The enzyme betaine-aldehyde dehydrogenase, or choline dehydrogenase, then catalizes the conversion of betaine aldehyde to betaine. Betaine is obtained from the diet and from the oxidation of choline in gut mucosa, liver and kidney (Garcia-Perez and Burg, 1991). Under hypertonic conditions, the rate of betaine synthesis is not significantly affected, but rather the number of the transporters bringing it into the cell. The betaine γ-amino-*n*-butyric acid transporter, BGT1, couples transport of betaine to that of Na^+ and Cl^- (Uchida *et al.*, 1993). Hypertonicity induces transcription of BGT1 gene followed by increase in its mRNA and transport activity, which is in turn driven by the ion gradient (Burg *et al.*, 2007).

Taurine is synthesized from cisteine in the liver and is then released to circulation. Although the enzymes involved in the synthesis of taurine are also found in the kidney, increased taurine uptake in response to hypertonicity is mainly achieved by increased transcription of the taurine transporter TauT and not by a greater synthesis rate of this metabolite (Burg *et al.*, 2007). In addition to its role as a compatible osmolyte, taurine is involved in heart rhythm regulation, energy metabolism, sperm motility, body temperature, blood pressure, bile acid synthesis, cell proliferation and viability (Burg *et al.*, 2007; Schaffer *et al.*, 2000).

Hypertonicity also causes accumulation of **inositol**, which can be absorbed from diet and also synthesized from glucose in the kidney, and by digestion of phospholipids in the gut (Garcia-Perez and Burg, 1991). Following hypertonicity, renal cells accumulate inositol by increased transport rather than by synthesis. The inositol transporter, named sodium *myo*-inositol transporter, SMIT, couples transport of inositol to that of sodium, having as driving force the Na^+ gradient. Under hypertonic stress, SMIT gene transcription rate is elevated, followed by an increase in its mRNA and transport activity (Burg *et al.*, 2007). However, unregulated overexpression of SMIT can be harmful for cells, as shown in mouse eye lens by Jiang and colleagues (Jiang *et al.*, 2000).

Another organic osmolyte accumulated by cells exposed to hypertonic stress is **glycerophosphocholine**, GPC. This methylamine is obtained from phosphatidylcholine (PC) in a reaction catalyzed by neuropathy target esterase (NTE). Regulation of GPC by hypertonicity is achieved by regulation of its synthesis as well as of its degradation, since high NaCl has been shown to raise GPC production from PC and also to inhibit the enzyme converting GPC into choline and α-glycerophosphate (Gallazzini *et al.*, 2006). Interestingly, GPC is not only induced by high NaCl but also by high urea, most likely to counteract its perturbing effects (Burg *et al.*, 1996; Burg and Ferraris, 2008). **Urea** is a permeable solute that coexists in the renal medulla with NaCl. However, an agreement on which solute protects cells from the adverse effect of the other has not been reached (Neuhofer *et al.*, 1998; Santos *et al.*, 1998; Zhang *et al.*, 2004). The majority of urea is generated in the liver as a byproduct of protein metabolism, and because protein intake exceeds its demand for anabolic processes, large amounts of urea are generated is the most abundant solute in (western-type diet) human urine. Specialized mechanisms in the kidney allow the secretion or urea without forcing water excretion (Fenton and Knepper, 2007).

In addition to accumulating organic osmolytes to counteract the deleterious effects of elevated ionic strength, cells also express heat shock proteins (HSPs) in order to protect intracellular macromolecules from unfolding and aggregation (Alfieri and Petronini, 2007; Cohen *et al.*, 1991). Specifically, expression of this chaperone throughout the kidney is much higher in the hypertonic medulla than in the isotonic cortex. HSP70 protects cells by reestablishing protein folding and conformation, and by preventing activation of apoptotic pathways (Neuhofer and Beck, 2005).

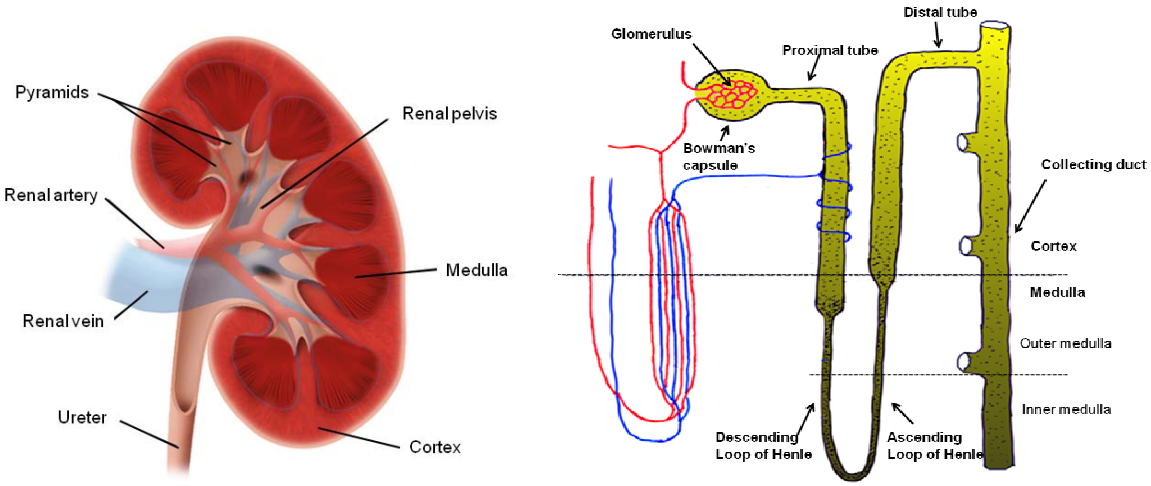
3. WATER AND SODIUM HOMEOSTASIS IN MAMMALS

Total mammalian body water content is approximately 60% of body weight. It is distributed as intracellular fluid (ICF) and extracellular fluid (ECF). The former comprises about 40% of body weight, the latter around 20%. Of the extracellular fluid, 80% is interstitial fluid and 20% is blood plasma, representing 16% and 4% of the body weight, respectively. Interstitial fluid and plasma are very similar in that both contain mostly sodium cations and chloride anions, but there is a higher concentration of protein in plasma. On the other hand, intracellular fluid is rich in potassium cations and organic phosphate anions, with very little sodium and chloride. As cell membranes cannot tolerate large osmotic gradients, ICF and ECF must be thermodynamically balanced. Water movement across the membrane buffers the osmotic pressure on the cell, but does not normalize plasma osmolality. In mammals, the main mechanisms to maintain normal plasma tonicity are thirst, renal excretion of water and release of anti diuretic hormone (ADH) (Shoker, 1994).

The kidney is the organ responsible for controlling water and sodium balance by modulating their excretion and reabsorption. Thus, the kidney plays a critical role in plasma volume and osmolality regulation. Additionally, this organ eliminates metabolic waste, such as urea and creatinine, and exogenous drugs and toxins. The kidney is a major endocrine organ, producing renin, erythropoietin and prostaglandins, and is target of many hormones. It is highly vascularized and receives about 25% of the cardiac output (Cecil *et al.*, 2000).

The human kidney has an inner region called the medulla, and an outer region called the cortex. It has a multipapillary configuration, divided in conical masses called the pyramids (**Figure 6**).

The functional units of the kidney are called nephrons, and around one million of them form a kidney. Each nephron is composed by the glomerulus (renal corpuscle), the proximal tube, thin loops of Henle (descending and ascending) and by the distal tube as shown below in **Figure 6** (Cecil *et al.*, 2000).



Adapted from www.lpch.org and www.udel.edu/biology/Wags

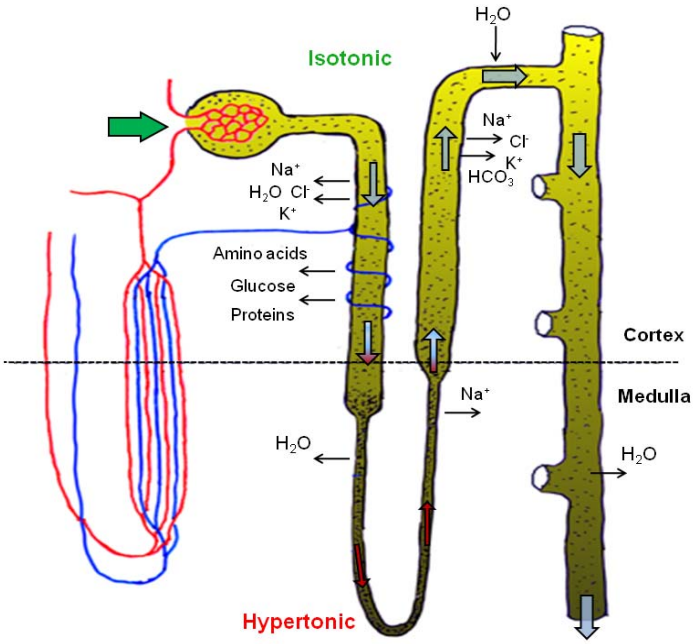
Figure 6. Anatomy of the kidney and the nephron. *On the left side panel, a schematic vertical section of a human kidney and its parts. The nephron and its sections are represented on the right side panel.*

The formation of urine begins with a passive ultrafiltration process, in which the movement of water and associated dissolved small molecules is driven by hydrostatic and colloid osmotic pressures. Most substances filtered through the glomerulus are reabsorbed by the tubules of the kidney into interstitial fluid, and therefore into the blood. Reabsorption conserves substances which are essential to normal function such as water, glucose, amino acids, and electrolytes (Cecil *et al.*, 2000) (**Figure 7**).

In the proximal tube, sodium is reabsorbed and the tonicity of the fluid is thus decreased. As the proximal tubule enters the medulla, which is characterized by having high osmolality, it becomes the thin descending loop of Henle. Due to the presence of water channel aquaporin 1 (AQP1), the descending loop of Henle has a high water and low solute permeability, gradually raising the NaCl and urea concentration in the fluid as it descends until it reaches the tip of the descending

loop, where osmolality is very high. Under severe water restriction, the tonicity of the fluid in the tubule in humans can reach up to 1200 mOsm/kg. In consequence, cells of the renal medulla are constantly exposed to fluctuating and high extracellular solute concentrations (Cecil *et al.*, 2000).

In the ascending limbs of the loop of Henle sodium transport is active, but water does not follow because this portion of the nephron does not express aquaporins. In this section, sodium and chloride ions are reabsorbed from the fluid by the action of $Na^+K^+2Cl^-$ cotransporter, which results in a gradual decrease of the fluid's tonicity as it flows through the ascending loop moving from the medulla to the cortex (**figure 7**).



Adapted from www.udel.edu/biology/Wags

Figure 7. Summary of the urine concentration process. *The green arrow on the left indicates the beginning of the urine concentration process. Arrows indicate flow direction.*

Altogether, the proximal tubule, the descending and ascending loops of Henle account for about 90% of the glomerular ultrafiltrate water reabsorption. In the late distal tubules and collecting ducts, the permeability to water is under the control of ADH, which permits the fine regulation of water balance. Thus, in these regions of the kidney, the reabsorption of salt and water can be controlled independently. The collecting duct is the final site where urine volume and ionic

composition are modified. Throughout the collecting duct water is reabsorbed, mainly by the presence of aquaporins, especially the vasopressin-regulated aquaporin 2 (AQP-2). The collecting duct also mediates acid secretion by its involvement in proton transport, central for pH regulation. Urea is also reabsorbed via urea transporters in the collecting duct, specifically in the inner medulla collecting duct, since the other segments of the collecting duct are greatly impermeable to this solute. By the time urine is formed, the osmolality is essentially returned to its baseline levels (Cecil *et al.*, 2000).

4. PHYSIOLOGICAL IMPACT OF HYPERTONIC STRESS

In an over-simplified way, the urine concentration mechanism can be divided in two steps; sodium concentration in Henle's loop and water transport in the collecting ducts. Proper water balance is a key issue for cellular well-being, as osmotic pressure between extracellular and intracellular fluids must be equilibrated. Therefore, the mechanisms regulating the control of intake and output of water must be finely regulated (Verbalis, 2003).

The two major players of body fluid homeostasis are water and sodium. Sodium is the most abundant impermeable cation in the body and it must be transported out of the cell against an electrochemical gradient by the energy-dependent Na^+/K^+-ATPase pump. Therefore, sodium is the main determinant of plasma tonicity (Shoker, 1994; Porth, 2005). In normal individuals plasma tonicity is kept in a range from 285 to 295 mOsm/L by regulation of urinary concentration. Different physiological events or diseases may potentially disrupt the fine tuning of the water balance control, leading to disorders of body fluids (Verbalis, 2003). Plasma tonicities values above 300 mOsm/kg are generally considered as abnormally elevated. If the ratio of solutes to water in total body is increased, a hypertonic disorder will develop; if the ratio is decreased, the disorder is of hypotonic nature.

Of our particular interest are hypertonic states. Among the hypertonic disorders are hypernatremia and uncontrolled hyperglycemia. Hypernatremia can be caused by water loss or by sodium gain. This condition is more common in children, elderly and hospitalized patients. Although rare in young adults, the outcome of reported cases of severe hypernatremia may

have as outcome mortality or neurological consequences. The most common causes of hypernatremia are listed below (Cecil *et al.*, 2000).

Common causes of hypernatremia

- Decreased thirst perception:
    - coma (unconscious state)
    - essential hypernatremia (conscious state): disorder of the hypothalamus leading to chronic hyperosmolality and inappropriate lack of thirst
- Osmotic diuresis: diabetic keto-acidosis, mannitol administration
- Severe burn
- Excessive loss of water
    - Renal: pituitary or nephrogenic diabetes insipidus
    - Extra renal: excessive sweating, diarrhea, excessive vomiting
- Combined: coma plus hypertonic nasogastric feeding

In pituitary or nephrogenic diabetes insipidus (NDI) there is deficiency or insensitivity to ADH, which leads to excessive water losses and hypernatremia. Other pathological situations in which serum sodium levels are higher than normal include congestive heart failure, liver cirrhosis, Na^+ channel defects, pregnancy, Cushing's Syndrome (hypercorticolism), Bartter's syndrome (mutation in potassium channel) and primary hyperaldosteronism (increased sodium reabsorption) (Adrogue and Madias, 2000; Cecil *et al.*, 2000).

All hypernatremic states are hypertonic, but not all hypertonic disorders are hypernatremic, as is the case of hyperglycemia, where the increase in tonicity is caused a non-sodium solute, glucose. In the presence of insulin, glucose can normally move freely across the cell membrane. However, in diabetic individuals, glucose becomes an effective osmol and creates a hyponatremic but indeed hypertonic state (Cecil *et al.*, 2000).

Considerable water loss may also occur with excessive sweating due to performing exercise in high humidity or too rigorously, which in some cases can favor the development of heat stroke (Cecil *et al.*, 2000). In individuals with diabetes, hypertonicity is known to promote complications leading to blindness, amputations, and cardiovascular disease (Stookey, 2005).

Historically, hypertonic saline (HTS) has been used to treat a variety of conditions. Loss of sodium in patients with cystic fibrosis, hyponatremia, or Leishmaniasis, has been treated with HTS (Johnson and Criddle, 2004). HTS is widely used in patients with trauma in order to reduce swelling after shock, especially in patients with elevated intracranial pressure (Tyagi *et al.*, 2007). If inappropriately handled, hypernatremia-induced brain shrinkage may even lead to vascular rupture, hemorrhage, permanent neurological damage and even death (Adrogue and Madias, 2000).

It is known that the osmolarity of tears increases in keratoconjunctivitis sicca (KCS) patients and therefore could be a sensitive and specific indicator for the diagnosis of KCS (Ogasawara *et al.*, 1996). High plasma tonicity was found to be prevalent in non-disabled adults and predicted incident disability and mortality. It was therefore proposed as a readily measured marker of early frailty (health weakness) in older adults, considering that high plasma tonicity may imply organ deregulation, fluid and food intake deficit and may be associated to anorexia (Stookey *et al.*, 2004).

Under most conditions, plasma osmolality is kept within a narrow range. Nonetheless, several cases have been reported where plasma tonicity in patients suffering of hypothalamic-hypodipsic-hypernatremia (HHH) has reached values as high as 408 mOsm/kg (Soylu *et al.*, 2000). Elevated solute plasma concentrations have been found to be associated to overweight and obesity in free-living adults independently of glucose levels (Stookey *et al.*, 2007). Childhood obesity, an actual pandemic health problem, leads to type 2 diabetes mellitus. This condition is further complicated by the hyperglycemic hyperosmolar syndrome (HHS), in which plasma tonicities are within the 350 mOsm/kg range and cause a greater mortality rate (Cochran *et al.*, 2006).

As mentioned above, the proper regulation and function of the kidney, and thus the urine concentration mechanism, allow adjustments in water and sodium excretion allowing control of plasma tonicity. Great efforts have been made in order to better understand the urine concentration mechanism by generation of transgenic and knockout mice in which expression of specific renal transporters or receptors have been deleted. Such models include deletion of

sodium transporters and channels, aquaporins, urea transporters, potassium and chloride channels, as well as receptor and signaling molecules (Fenton and Knepper, 2007).

Many of these models exhibit defects in urine concentration ability, and specifically, increased in plasma tonicity has been detected in the following mutants:

- AQP-1: Mice deficient in AQP-1 exposed to 36h of water deprivation exhibited serum osmolality values greater than 500 mOsm/kg (Ma *et al.*, 1998; Yun *et al.*, 2000).
- AQP-2: aquaporin-2 mutant mice generated to recreate nephrogenic diabetes insipidus scenario showed plasma hyperosmolarity, reaching values of up to 500 mOsm/kg. Pups died by postnatal day 6 (Shi *et al.*, 2007; Yang *et al.*, 2001).
- Calcineurin Aα: Serine/Threonine phosphatase, shown to be required for proper AQP-2 traficking. Serum osmolality reached values of 350 mOsm/kg (Gooch *et al.*, 2006).
- Cytosolic phospholipase A_2: after 48hr of water deprivation: greater weight loss and greater increase in plasma osmolality (up to 360 mOsm/kg), most likely due to abnormal trafficking and folding of AQP-1 (Bonventre *et al.*, 1997; Downey *et al.*, 2001).
- Vasopressin 2 receptor (V2R): females suffer severe hypernatremia, calculated plasma tonicity is around 380 mOsm/kg. Males died within a week after birth (Yun *et al.*, 2000).
- Prostaglandin E_2 (PGE_2) receptor EP1: mutant mice exposed to 24hr water deprivation showed higher plasma tonicity (320 mOsm/kg) than their wild-type littermates (Kennedy *et al.*, 2007).
- *ROMK* potassium channel: early death associated to dehydration and hydronephrosis. Plasma sodium and chloride levels of 7-9 day-old pups are high, reaching values of 170 mM and 119 mM respectively. Surviving mice suffer from metabolic acidosis, hypernatremia (170 mM Na^+), polyuria, and polydipsia (Lorenz *et al.*, 2002).

5. PERTURBING EFFECTS OF HYPERTONICITY ON CELLS

Cellular changes caused by hypertonicity are numerous and suggest a substantial alteration of the state of cells. The fact that cells not normally exposed to hypertonic environment are prepared to eventually confront it, suggests that hyperosmolarity may be a basic threat on them. Extreme hypertonic stress (>600 mOsm/Kg) usually leads mammalian cells in culture to induce

apoptosis. However, they are able to survive a less severe hypertonic environment (up to 500 mOsm/Kg) by activating the osmo-adaptation process in which cells accumulate organic osmolytes (Burg, 1995; Kultz *et al.*, 1997). Hypertonicity causes activation of a complex response and involves different signaling pathways, concomitant with the broad range of effects it has on cells; from DNA double strand breaks, to cell cycle delay and ultimately apoptosis (Burg *et al.*, 2007).

The impact that hypertonicity has on cellular proliferation depends on the intensity of the stress and the time of exposure as well as on the type of solute. There is a limit to the extent of stress a certain type of cell can withstand; a mild hypertonic stress causes growth arrest and phase delay, while a greater stress on the same type of cell may direct them straight to apoptosis (Michea *et al.*, 2000).

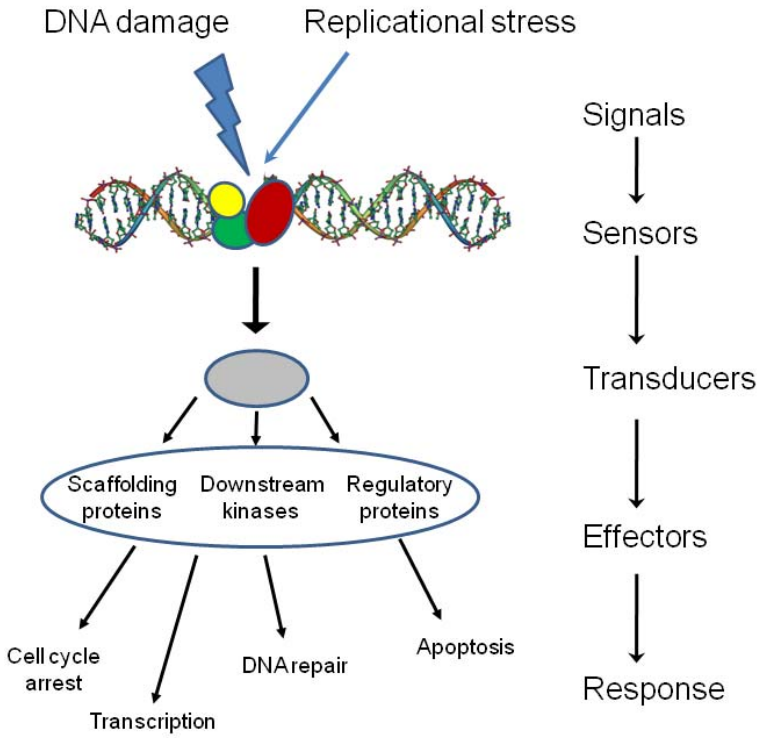
5.1. DNA damage

Upon exposure to DNA damage, cells activate a signaling network that is known as the DNA-damage response pathway (DDR), orchestrated by checkpoint proteins. This pathway comprises DNA damage sensors, signal transducers and effectors that generate a cellular response that allows quick metabolization and repair of DSBs. The coordination of the cell cycle and the DNA-repair pathway is controlled by the activity of cell cycle regulators, such as that of cyclin-dependent kinases, Cdks (Branzei and Foiani, 2008; Zhou and Elledge, 2000).

To repair the lesions, cells rapidly activate signal-transduction pathways that affect cell survival and maintenance of genomic stability. Cellular response to DSBs thus activates signaling cascades that lead to cell cycle arrest -in one or several phases-, activation of DNA repair and/or initiation of apoptosis. The type of lesion and phase of the cell cycle strongly influence the choice of repair mechanism, either the error-free homologous recombination (HR) or the error-prone non-homologous end joining (NHEJ) (Branzei and Foiani, 2008).

Double strand breaks (DSBs) may arise from exogenous or endogenous sources. The former includes events such as exposure to ionizing radiation, UV light, toxic chemical agents, oxidative or hypertonic stress (Branzei and Foiani, 2008; Kultz *et al.*, 1998; Shrivastav *et al.*, 2008). Endogenous events such as replication intermediates, oxidative metabolism, errors in

replication, meiotic recombination, and V(D)J recombination in lymphocytes may also lead to DSBs (Cahill *et al.*, 2006; Shrivastav *et al.*, 2008). These types of lesions may have devastating consequences for the cell and are among the most damaging and difficult to repair. Nonetheless, lesions must be repaired in order to prevent chromosomal rearrangements, senescence or apoptosis. Improper or failed repair enhances genomic instability and may lead to developmental abnormalities or cancer (Branzei and Foiani, 2008; Shrivastav *et al.*, 2008). General outlines of the DNA damage response signaling pathway are shown in **Figures 8 and 9**.



Adapted from Zhou and Elledge, 2000.

Figure 8. General outline of the DNA damage response signal-transduction pathway.

The first step in the cellular response to DSBs is sensing the lesion. Potential candidates to play a role as sensors of DNA damage include the Mre11-Rad50-NBS1 (MRN) complex and chromatin remodeling complex INO80 (Cahill *et al.*, 2006). Another critical event for efficient detection and repair is the amplification of the signal, largely performed by members of the phosphatidylinositol 3-kinase related kinases (PIKK) family Ataxia-telangiectasia mutated

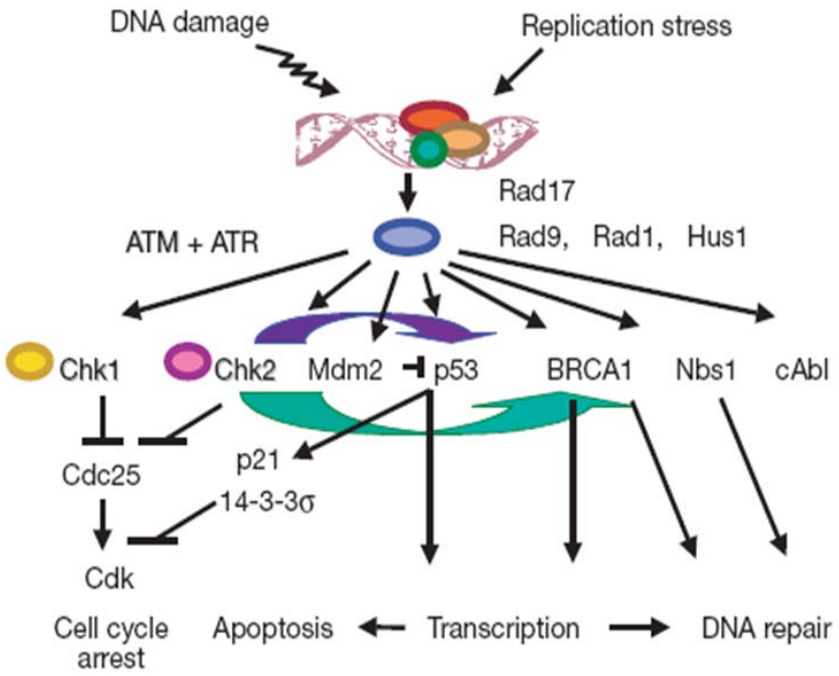
(ATM), ATM and Rad3-related (ATR), and DNA-dependent protein kinase (DNA-PK) (Abraham, 2004; Cahill *et al.*, 2006).

ATM is a serine/threonine protein kinase that is primarily activated by DSBs, such as those caused by ionizing radiation (IR) or radiomimetic drugs (Bakkenist and Kastan, 2003) while ATR responds mainly to replication stress and UV-induced damage. Downstream of these kinases are the checkpoint kinases Chk1 and Chk2. Although structurally unrelated, ATM and ATR share some overlapping specificity in targeting other proteins to induce cell-cycle arrest and allow DNA damage repair and reduction of Cdk activity (Scott and Pandita, 2006; Zhou and Elledge, 2000). An important goal of cell cycle arrest mediated by the checkpoint kinases is to allow time to repair DNA lesions (Branzei and Foiani, 2008).

Once the damage has been detected and the cell cycle checkpoints activated, the DNA repair machinery is recruited to the site of damage. ATM is recruited to the damaged site by binding of the MRN complex, a primary sensor of DSBs. ATR is recruited to the site of damage, RPA-coated single stranded DNA, by ATR-interacting protein (ATRIP). Once in the break, ATM and ATR phosphorylate checkpoint kinases to induce cell cycle arrest (Scott and Pandita, 2006). Inactive ATM dimers are autophosphorylated on Ser-1981 and dissociate, becoming activate. The event leading to ATM autophosphorylation is not yet clear, as it has been proposed to occur either by a chromatin modification event which resulted from the damage or to be promoted by the MRN complex (Bakkenist and Kastan, 2003; Cahill *et al.*, 2006; Zhou and Elledge, 2000). Once activated, ATM is recruited by the MRN complex to the site of damage where it phosphorylates numerous substrates which include checkpoint kinases Chk1 and Chk2, histone 2 variant H2AX, p53, Mdm2, Nbs1, Brca1 and SMC1 (Cahill *et al.*, 2006; Kitagawa *et al.*, 2004). Phosphorylated molecules of H2AX on Ser-139 (γH2AX) flank the damaged region and are thought to serve as signal or scaffolding molecule for repair factors or also to amplify the signal and damage response. p53 is a known tumor suppressor whose accumulation and activation result in cell cycle delay or in apoptosis if the damage is not successfully repaired (Scott and Pandita, 2006).

The DNA-PK complex is a multi subunit serine-threonine kinase composed of a catalytic subunit, the DNA-PKcs, and a DNA binding subunit, the Ku heterodimer, which is formed by the Ku86 and Ku70 proteins. DNA-PK plays an important role in the repair of DNA DSBs by the

error-prone mechanism of NHEJ. The Ku heterodimer binds with high affinity to DNA ends independently of their end sequence or structure. Biochemical analyses suggest that Ku acts as an alignment factor to promote end joining (Walker *et al.*, 2001).



Adapted from Zhou and Elledge, 2000.

Figure 9. Mammalian DNA damage response pathway. *General organization and proteins involved in the DNA damage response pathway. Arrowheads represent positive actions, perpendicular ends represent inhibitory steps.*

Hyperosmolality in the form of elevated NaCl (up to 750 mOsm/kg) causes DNA double-strand breaks (DSB) in mIMCD3 cells (Kultz and Chakravarty, 2001), having a more lethal effect on cells in the S phase (Dmitrieva *et al.*, 2001). Hypertonicity induced DSB have been detected *in vitro* as well as *in vivo* in renal medullary cells. Breaks on the DNA remain as long as there are high levels of NaCl. Amazingly, these cells are able to adapt and proliferate despite having DNA breaks, and show no signs of apoptosis under hypertonic conditions (Dmitrieva *et al.*, 2003; Dmitrieva *et al.*, 2004; Zhang *et al.*, 2002). This phenomenon has been proposed to be owed to the shut-down of the DNA damage response pathway (DDR) under hypertonic conditions (Dmitrieva *et al.*, 2005).

Although hypertonicity caused by high NaCl has been shown to cause DNA breaks in mIMCD3 cells, as well as cell cycle arrest (Dmitrieva *et al.*, 2001; Dmitrieva *et al.*, 2004; Kultz and Chakravarty, 2001; Michea *et al.*, 2000; Santos *et al.*, 1998), there are key differences between the cell cycle arrest caused by hypertonicity and the one caused by other DNA damaging agents. Whereas DNA breaks caused by other genotoxic insults, for example ionizing radiation, are repaired during the cell cycle arrest, while breaks caused by high NaCl remain unrepaired (Dmitrieva *et al.*, 2003).

A study by the Burg Laboratory showed that upon 2 hours of exposure to hypertonic stress, Mre11 exonuclease is forced out of the nucleus in mIMCD3, MEF, dermal fibroblasts (DF) and p2mIME cells (Dmitrieva *et al.*, 2003). In addition, phosphorylation of both H2AX and Chk1 were inhibited, suggesting that the G2/M arrest observed in response to high NaCl is Chk1-independent. Interestingly, they also reported that in the presence of hypertonicity, lesions by UV or IR do not trigger the usual DNA damage response and remain unrepaired until the NaCl concentration is back to normal. It is therefore proposed that the mechanism by which hypertonicity causes DNA DSBs is by interfering with the DNA repair machinery. However, if the amount of extracellular NaCl is lowered, the classical DNA repair machinery is activated and lesions are successfully repaired (Dmitrieva *et al.*, 2003).

On the other hand, an independent study proved the opposite (Sheen *et al.*, 2006). The nuclear exclusion of Mre11 previously reported was not detected by analysis of MRN complex components in hypertonically-stressed HeLa nor in mIMCD3 cells. *In vivo* analysis of Mre11 expression in kidney showed a nuclear subcellular localization in cells of the isotonic cortex as well as of the hypertonic medulla. Additionally, phosphorylation of histone H2AX was in fact observed after hypertonic exposure of HeLa and mIMCD3 cells, reaching a maximum expression after 16 and 4 hours, respectively. Formation of MRN foci in MEF and COS-7 cells was also observed in hypertonically stressed cells. While phosphorylation of checkpoint kinases Chk1 was not affected by high NaCl, phosphorylation on Thr68 of Chk2 was stimulated in response to this stress. However, these authors agreed with previous work by the Burg laboratory in that they found that hypertonicity interferes with DNA repair signaling of other DNA-damaging agent, specifically hydroxyurea (Sheen *et al.*, 2006).

With regard to DNA-PK, a study by Dmitrieva and colleagues showed that MEFs lacking the member of the Ku heterodimer Ku86, but not those lacking DNA-PKcs, have slower growth, aberrant mitosis and increased chromatin fragmentation compared to their wild-type counterpart when exposed to hyperosmotic stress (500 mOsm/kg). In addition, they performed experiments in *C. elegans* which although lacking DNA-PK, are able to adapt to hypertonic environments. They observed that in this model, cells adapted to high NaCl have many DNA breaks, as those found in mammalian cells adapted to hypertonic conditions. Lack of Ku86 in the Ku86 mutant *C. elegans* as well *C. elegans* fed with cku86 dsRNA, led to hypersensitivity to high NaCl, as showed by the reduced number of progeny and the prolonged generation time. They thus propose that the Ku heterodimer maintains chromatin integrity by attaching broken ends of DNA (Dmitrieva *et al.*, 2005).

Other proteins that play a role in the DNA damage response are the growth arrest-and DNA damage-inducible proteins GADD45. In mIMCD3 cells, hypertonicity causes increase in levels of growth arrest and DNA damage-inducible protein (GADD45), whose transcription is p53-dependent (Kultz *et al.*, 1998; Zhan *et al.*, 1998). These proteins aid in the maintenance of DNA integrity and genome stability (Hollander *et al.*, 1999). Up to date, three isoforms have been described; GADD45α, GADD45β, and GADD45γ. They have been shown to play a role in the processes required for cellular response to many stresses, including cell cycle delay, apoptosis and DNA repair (Carrier *et al.*, 1999). Upon exposure of mIMCD3 cells to hypertonicity, the three GADD45 isoforms are induced, reaching a maximum at 16-18 hours after onset of stress. In this system, induction of GADD45γ was stronger (7-fold), while the isoforms GADD45α and GADD45β were induced 2 and 3-fold, respectively. Nonetheless, in p2IME cells, the induction of GADD45γ reached a lesser degree than the one observed in mIMCD3 cells. Although variable to some extent, these transcripts were induced by hypertonicity caused by different solutes, including NaCl, KCl, sorbitol and mannitol, while urea had no effect on them. The induction mechanism is in part based on mRNA stabilization, since treatment of cells with actinomycin D did not prevent the rise of GADD45 transcripts (Chakravarty *et al.*, 2002). However, parallel analysis of GADD45α and GADD45β showed a greater and more sustained increase in protein amount than observed for mRNA levels. Both proteins were induced almost twice as much as their level of mRNA, and were still detected 36 hours after exposure to hypertonicity, while mRNA levels had returned to basal amounts. Taken together, these results suggest that

increase in abundance of these proteins, besides being mediated by mRNA stabilization, is also achieved by additional mechanisms (Chakravarty *et al.*, 2002). It has been shown that in the event of hypertonic stress, GADD45 proteins inhibit mitosis and promote G2/M arrest in renal medulla cells (Mak and Kultz, 2004). However, generation of single, double and triple GADD45 knockout mice showed that their absence does not affect osmotic tolerance of renal medullary cells, and the urine concentration mechanism is functional (Burg *et al.*, 2007).

Altogether, independent studies have shown that a hypertonic environment may evoke a DNA-damage stress-like cellular response and that it may interfere with the DNA damage response to other genotoxic insults. However, the exact nature and number of the high NaCl-induced DNA breaks and the role of the MRN complex this response remains a matter of debate. In this regard, knowledge on the regulation of the DDR by osmotic stress is still far from complete, and for instance, the participation of other components such as PARP and ATR (Schreiber *et al.*, 2006; Yelamos *et al.*, 2008; Shrivastav *et al.*, 2008; Wang *et al.*, 2006), which are known to be important mediators in response to other stresses, has not been reported up to date.

5.2. Cell cycle delay and Apoptosis

Acute hypertonicity caused by elevation of NaCl or urea leads to cell cycle arrest. The cell cycle delay results from delay in the G1 and G2/M phases, and from a slower S-phase transit (Dmitrieva *et al.*, 2001; Michea *et al.*, 2000). The duration of the arrest is also dependent on the degree of hypertonicity (Michea *et al.*, 2000). However, elevation of tonicity *per se* is not the cause of the reduction in cell number and cycling rate, since an equivalent level hypertonicity caused by glycerol affects cell growth to a lesser extent. Both in immortalized cell lines and early passage (2) mouse renal inner medullary epithelial cells, hypertonic stress causes a rapid G2 arrest which, in these cells, is mediated by p38 kinase (Dmitrieva *et al.*, 2001).

Apoptosis increases with increasing tonicity, either by addition of NaCl or urea, usually reaching maximum level at 12 hours and significantly decreasing at 24 hours after exposure to hypertonic stress. Dual-parameter analysis of DNA content and caspase-3 activation showed the presence of apoptotic cells in all phases of the cell cycle, with prevalence in the G1 and S phases (Michea *et al.*, 2000). Acute elevation of osmolality by NaCl addition increases the amount of total and

Ser-15 phosphorylated p53 in mIMCD3 cells. Transcriptional activity of p53 is induced in response to hypertonicity, as shown by changes in mRNA of p53 targets Bcl2 and MDM2, and the activation of a p53-dependent luciferase reporter at 500 mOsm/kg, but not at 700 mOsm/kg, where most cells die by apoptosis (Dmitrieva *et al.*, 2000). Inhibition of p53 by means of specific antisense oligonucleotide caused increased levels of apoptosis in hypertonically stressed cells, and was therefore proposed that activation of p53 protected mIMCD3 cells exposed to hypertonicity. However, elevation of tonicity with urea did not increase levels of p53 (Dmitrieva *et al.*, 2000). Phosphorylation of Ser-15 of p53 occurs mainly in cells in the S phase. Inhibition of p53 with an antisense oligonucleotide or caffeine made the cells elude the hypertonicity-induced cell cycle arrest and DNA replication reduction, and caused greater levels of apoptosis in cells in which the DNA content had increased. Altogether, these results led to the conclusion that p53 activation was likely protective in mIMCD3 cells exposed to hypertonicity by restricting DNA replication and transition from G1 to S (Dmitrieva *et al.*, 2000; Dmitrieva *et al.*, 2001). Nonetheless, $p53^{-/-}$ mice have normal urine concentration ability and histology of renal inner medullas (Cai *et al.*, 2006).

Simultaneous addition of urea and NaCl (1000 mOsm/kg) does not potentiate the amount of stress and has no additional effect than those caused by each of these solutes added individually (Michea *et al.*, 2000).

5.3. Disruption of mitochondrial function

In addition to causing DNA damage or growth arrest, exposure of cells to hypertonicity results in a rapid and reversible decrease in mitochondrial proton gradient. Sorbitol-induced hypertonicity in Jurkat cells caused a decline in the mitochondrial function in a time and dose-dependent manner, which was almost completely reversible. Mitochondrial dysfunction in turn, causes a quick deactivation of p70S6K, a fundamental kinase that regulates cell growth and is the target of mTOR, a member of the phosphatidylinositol kinase-related kinases (PIKKs). The scattering effect caused by hypertonicity on the mitochondrial network was further confirmed by immunofluorescence analysis of cytochrome *c* and live imaging experiments that showed the disappearance of the membrane potential upon exposure to hypertonicity caused by NaCl (Copp *et al.*, 2005).

5.4. Effect of osmotic stress on cell growth

Osmotic stress has been shown to counteract cell growth promoting pathways in mammalian cells. Hypertonicity-induced increase of intracellular ion concentration inhibits translation of most cellular proteins, and as organic osmolytes accumulate, the inhibitory effect diminishes. The onset and ending of the cell cycle arrest coincides temporally with the changes in protein synthesis (Burg *et al.*, 2007).

Morley and Naegele found that hypertonic shock causes a rapid inhibition of protein synthesis and disaggregation of the polysomes. These effects were found to be associated to the dephosphorylation of eIF4G, eIF4E, 4E-BP and ribosomal protein S6 (Morley and Naegele, 2002). Moreover, by using a cell-free system, Briggotti and colleagues showed that initiation and elongation are inhibited by hypertonicity created by inorganic ions Na^+ and K^+ (such as those encountered during RVI after hypertonic shock) but not when the same increase in tonicity was induced by organic osmolytes such as betaine or myo-inositol (Brigotti *et al.*, 2003).

On the other hand, several studies have suggested that under conditions of osmotic stress, growth factor receptors are likely involved in sensing and signaling the cellular stress. For example, exposure of HeLa cells to osmotic stress led to clustering and internalization of several receptors including epidermal growth factor (EGF), interleukin 1 (IL-1) and tumor necrosis factor (TNF) (Rosette and Karin, 1996). It has also been shown that hyperosmolarity caused by sorbitol activates the EGF receptor in human keratinocytes through the p38 stress kinase pathway (Cheng *et al.*, 2002). Furthermore, Copp and colleagues (Copp *et al.*, 2005) found that the activation of MAPK downstream of growth factor receptors is transiently inhibited under hypertonic stress conditions. In addition to activating several stress kinases, hyperosmolarity signaling was also found to be partially mediated through the insulin receptor in CHO cells exposed to hypertonic shock caused by addition of 500 mM NaCl or 700 mM sorbitol (Ouwens *et al.*, 2001). However, these conditions of osmotic stress are extreme and raise the question of whether the results obtained in this study can be extrapolated to other systems.

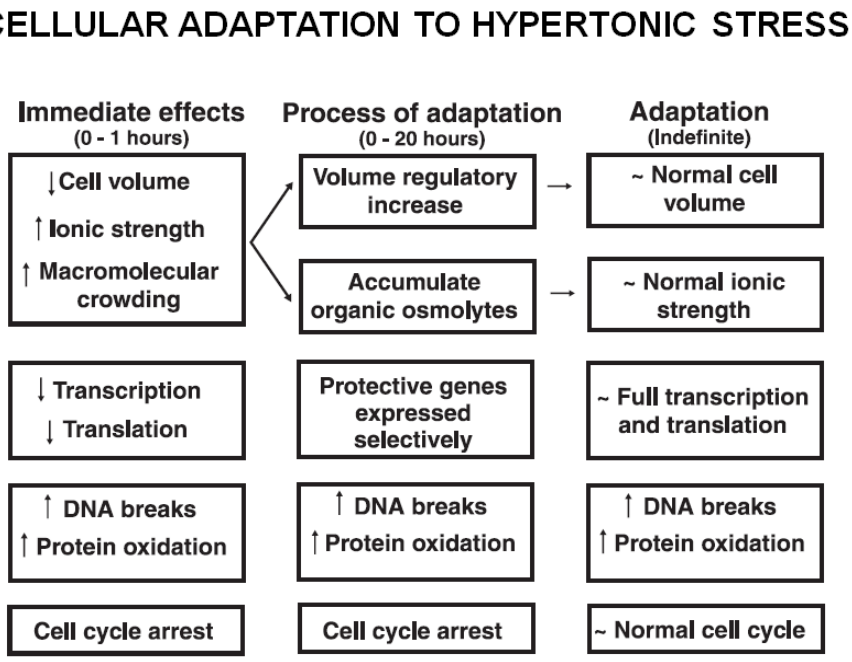
5.5. Cytoskeleton remodeling

Osmotic stress is known to alter the cytoskeleton and to induce changes in F-actin content or organization in different mammalian cells. The cytoskeleton has been suggested to be a volume sensor and a mechanical transducer that may signal ion channels and transporters (Pedersen *et al.*, 2001). Subsequent studies have shown that hypertonicity induces the *de novo* assembly of submembranous F-actin concomitant with key components of the actin nucleation machinery. This same study showed that the signaling triggered by cytoskeleton reorganization upon exposure to hypertonicity stimulates Rac and Cdc42, and is independent of the shrinkage-induced tyrosine phosphorylation of cortactin, a key component of the actin nucleation machinery (Di Ciano *et al.*, 2002).

5.6. Induction of oxidative stress

Reactive oxygen species (ROS) are increased in cultured mIMCD3 cells exposed to high urea or NaCl concentrations. In these cells, high urea causes single strand DNA breaks and lesions caused by the formation of 8-hydroxyguanine in DNA induced by oxidative stress. On the other hand, exposure of mIMCD3 cells to high NaCl, which had been previously shown to suffer double strand DNA breaks (DSBs), does not cause 8-oxoguanine lesions. Both urea and high NaCl significantly induce protein carbonylation, indicative of protein oxidation (Zhang *et al.*, 2004).

Overall, studies have highlighted the fact that hypertonic stress has profound effects on cells, such as the ones mentioned above. By activation of osmoprotective mechanisms, cells are able to survive and function during harsh osmotic conditions, even carrying cellular lesions that in another context may be considered pathological. The osmotic adaptation process, as depicted in **Figure 10,** is one of high complexity and further studies are necessary for it to be fully understood.



Adapted from Burg et al, 2007.

Figure 10. Cellular adaptation to osmotic stress.

6. HYPERTONICITY AND NFAT5

6.1. Regulation of NFAT5 by hypertonicity

The role of NFAT5 in its ability to regulate osmoprotective genes in response to hypertonic stress has been well established. In response to hypertonicity, NFAT5 is subjected to three levels of regulation: phosphorylation and nuclear translocation (Dahl *et al.*, 2001; Ko *et al.*, 2000; Miyakawa *et al.*, 1999) followed by increase in mRNA and protein synthesis (Dahl *et al.*, 2001; Ko *et al.*, 2000) and increased transcriptional activity.

Nuclear translocation

Unlike cytoplasmic NFATc and NF-kB proteins, NFAT5 is predominantly nuclear in various cell types in resting conditions (Lopez-Rodriguez *et al.*, 1999b). Three localization regulatory motifs have been described in the amino-terminal region of NFAT5 by Tong and colleagues (Tong *et*

al., 2006). These motifs include a nuclear localization sequence (NLS), an auxiliary export domain (AED) and a canonical nuclear export sequence (NES). The NLS is formed by two clusters of basic amino acids in tandem, but only the first cluster is required for nuclear import of NFAT5. The AED is found in the three NFAT5 isoforms and regulates nuclear export of this protein under isotonic and hypotonic conditions in a Crm1 exportin-independent fashion. Deletion of this domain results in NFAT5 nuclear retention regardless of the tonicity. On the other hand, the NES which is found only in NFAT5 isoform c, is a Crm1-responsive domain. Disruption of this domain increases NFAT5 in the nucleus under isotonic conditions but not under hypotonic conditions. It thus appears that hypertonicity causes inactivation of the NES and AED, allowing full translocation of NFAT5 (Tong *et al.*, 2006).

In cells in isotonic media, NFAT5 is present in phosphorylated as well as non-phosphorylated forms. Upon exposure to hypertonicity, additional phosphorylation ensues within 30 minutes. This modification occurs mainly in serine and tyrosine residues and precedes the subsequent nuclear translocation and accumulation of the protein. The observation that phosphorylation of NFAT5 upon exposure to hypertonic stress precedes nuclear accumulation, suggests the possibility that this event might be required for nuclear translocation or that it favors nuclear retention of this factor (Dahl *et al.*, 2001).

Hypertonicity-induced nuclear translocation of NFAT5 is essentially undisturbed by chemical inhibitors of p38 α and β isoforms, tyrosine kinases, Src family of tyrosine kinases or MAPK kinases (Dahl *et al.*, 2001). On the other hand, the role of PI3 kinase-related kinases (PIKK) on NFAT5 translocation is unclear. These kinases have been shown to be important for the transcriptional activity of NFAT5 (see below).

Nuclear translocation of NFAT5 in response to hypertonicity is in part signaled by the DNA damage-response protein ATM. Inhibition of ATM kinase by means of the PI3-K inhibitor wortmaninn reduced translocation of NFAT5 to the nucleus of COS-7 cells exposed to hypertonic stress. Subsequent experiments showed that activation of ATM contributes to hypertonicity-induced nuclear translocation of NFAT5 (Zhang *et al.*, 2005) which was decreased in cells expressing functional ATM treated with wortmannin. Surprisingly, in cells lacking functional ATM, wortmannin enhanced the high NaCl-induced nuclear translocation of NFAT5, contradicting what was observed in cells with reconstituted ATM. It is speculated that another

wortmannin-sensitive molecule is in charge of inhibiting the nuclear translocation of NFAT5 in response to high NaCl (Zhang *et al.*, 2005). While it has been shown that under hypertonic stress ATM contributes to NFAT5 nuclear translocation, it is not clear whether the effect of ATM on NFAT5 is a direct or indirect one, since other proteins are sensitive to wortmannin such as DNA-PKc, PI3-K, mTOR and SmMLCK (Brunn *et al.*, 1996; Davies *et al.*, 2000; Zhang *et al.*, 2005).

More recently, nucleoplasmic shuttling of NFAT5 was reported to be regulated by phosphorylation on Ser-155 and Ser-158 under hypotonic conditions. This study also found that phosphorylation on Ser-155 precedes and is required for phosphorylation of Ser-158, which is directed by Casein Kinase 1, CK1 (Xu *et al.*, 2008).

Increased protein synthesis and mRNA accumulation

Hypertonicity increases substantially the amount of NFAT5 (Lopez-Rodriguez *et al.*, 2001; Miyakawa *et al.*, 1999), suggesting that this event is a key step in its activity. In mIMCD3 cells, hypertonicity stabilizes NFAT5 mRNA, facilitating its increase. This stabilization is dependent on the presence of the 5`-UTR and is hindered by the presence of the 3`-UTR (Cai *et al.*, 2005).

Upregulation of transcriptional activity

Evidence shows that multiple kinases are involved in full activation of NFAT5 in response to hypertonic stress, but not one of them is sufficient on its own (Morancho *et al.*, 2008). Among them are the mitogen-activated kinase p38, shrinkage-activated tyrosine kinase Fyn (Ko *et al.*, 2002), cAMP-dependent kinase PKA (Ferraris *et al.*, 2002), ERK (Tsai *et al.*, 2007), PI3-kinase (Irarrazabal *et al.*, 2006) and PI3-K related kinase ATM (Irarrazabal *et al.*, 2004; Zhang *et al.*, 2005). In addition, the calcium-activated phosphatase calcineurin has been shown to be required for the activation of NFAT5 in certain cell types (Li *et al.*, 2007; Morancho *et al.*, 2008).

The activation of p38 MAPK by hypertonicity can involve autophosphorylation as well as different combinations of the MAPK kinases MKK3 and MKK6 (Kang *et al.*, 2006). Activation of NFAT5 by hypertonicity was partially blocked in NIH3T3 cells by the p38 inhibitor SB203580 as well as by the dominant negative p38 mutant. The same report provided similar results for the SRC family member Fyn, which is thought to be activated by hypertonicity-induced cell shrinkage rather than by hypertonicity itself. Inhibition of Fyn by pre-treatment of cells with

tyrosine kinase inhibitor PP2 or by cotransfection of dominant negative mutant reduces hypertonicity-driven NFAT5 transcriptional activity. Moreover, Fyn-deficient fibroblasts exhibit a decreased NFAT5-dependent reporter activity in response to hypertonicity, which was further reduced, but not completely abolished, by pre-treatment of these cells with the p38 inhibitor SB203580 (Ko *et al.*, 2002). In PAP-HT25 rabbit renal medullary cells, a dominant negative of MKK3, the upstream kinase of p38, failed to inhibit an NFAT5-dependent reporter (Kultz *et al.*, 1997). On the other hand, mouse splenocytes and mouse embryonic fibroblasts pre-treated with two pharmacological inhibitors of p38, SB203580 and SB202190 individually, displayed reduced activation of NFAT5 (Morancho *et al.*, 2008). NFAT5 targets Tau-T and Hsp70 promoters were inhibited by a dominant negative p38 in nucleus pulpous cells (Tsai *et al.*, 2007). Overexpression of recombinant active MKK3 in canine kidney MDCK cells increased NFAT5 activity as well as the NFAT5-target BGT1 mRNA levels (Padda *et al.*, 2006). However, expression of dominant negative MKK3 in MDCK cells did not affect the expression or function of NFAT5 in response to hypertonicity (Kultz *et al.*, 1997). More recently, it was proposed that different p38 isoforms, p38α and p38δ, exert opposing effects in response to high NaCl, the former promoting its activity while the latter inhibits it (Zhou *et al.*, 2008).

It is thus thought that the pathway for hypertonic activation of NFAT5 via p38 is cell-type specific and the identity of the molecular mechanisms involved remain uncertain, since for instance, it has not been determined whether p38 phosphorylates NFAT5 and phosphorylation sites in this factor have not been indentified so far. It has been also proposed that activation of p38 by hypertonicity might be dependent on the accompanying hypertonicity-induced increase of ROS (Burg *et al.*, 2007).

NFAT5 has two putative PKA consensus phosphorylation sites, one in the carboxy-terminal and another in the amino-terminal. This serine-threonine kinase regulates many cellular processes, usually responding to increased intracellular cAMP. However, activation of this kinase by high extracellular NaCl seems to be cAMP-independent (Ferraris *et al.*, 2002). The catalytic subunit of PKA, PKAc, co-immunoprecipitates with NFAT5, but this association is not enhanced upon exposure to hypertonicity. In HepG2 cells, chemical inhibition or dominant negative constructs of this kinase reduce hypertonicity-induced NFAT5 transcriptional and transactivational activity, while overexpression of wild-type PKAc increased it (Ferraris *et al.*, 2002). The PKA inhibitor H89 also caused partial inhibiton of NFAT5 activity in splenocytes (Morancho *et al.*, 2008). As

with p38 and Fyn, the activity of PKA in NFAT5-mediated response to hypertonic stress proved to be necessary but not sufficient.

Nucleus pulpous cells were used to study the role of the ERK pathway in hypertonic activation of NFAT5. In these cells, NFAT5 was partially inhibited by a dominant negative construct and the pharmacological ERK inhibitor PD98059 (Tsai *et al.*, 2007). In primary mouse T cells, however, NFAT5 activity was insensitive to that same inhibitor (Morancho *et al.*, 2008).

Initial studies on NFAT5 regulation by calcineurin reported that TCR-dependent activation of NFAT5 was dependent on this phosphatase while independent of NFATc proteins. On the other hand, hypertonic activation of NFAT5 in Jurkat T cells has been shown to be independent of calcineurin (Morancho *et al.*, 2008; Trama *et al.*, 2000). Recently, however, it was reported that calcineurin was a positive regulator of NFAT5 in the activation of the aquaporin-2 promoter by hypertonicity in murine collecting duct cells (Li *et al.*, 2007). Additionally, hypertonic activation of NFAT5 was variably inhibited by the calcineurin inhibitor FK506 in T cells and MEF, but not in macrophages or Jurkat cells (Morancho *et al.*, 2008). Altogether, these results suggest that the contribution of calcineurin to the regulation of hypertonicity-induced NFAT5 activity is cell type-specific and is not generally essential for its function.

The PIKK ATM is a serine-threonine protein kinase activated by DNA damage via intermolecular autophosphorylation on serine 1981. Once activated, ATM phosphorylates several proteins such as p53, BRCA1 and NBS1 to initiate the DNA damage response. Taking into account that high NaCl may cause DNA damage (Dmitrieva *et al.*, 2004; Kultz and Chakravarty, 2001) and that NFAT5 has several putative ATM binding sites within its large carboxy-terminal domain, Irarrazabal and colleagues studied the role of this kinase in the mediation of hypertonicity-dependent NFAT5 activity and showed that high NaCl indeed causes activation of ATM (Irarrazabal *et al.*, 2004). As with PKA, NFAT5 and ATM physically interact as shown by co-immunoprecipitation analysis, but this interaction is not further strengthened under hypertonic stress (Irarrazabal *et al.*, 2004). In addition, pharmacological inhibition of ATM in HEK293 cells by pre-treatment with wortmannin significantly inhibited the increase in NFAT5 transcriptional activity in response to hypertonicity. Similar results were obtained in non-transformed lymphocytes and MEF treated with wortmannin as well as with LY294002 (Morancho *et al.*, 2008). In cells lacking functional ATM (AT cells) overexpression of the wild-type kinase led to

increased transcriptional activation and transactivating activity of NFAT5 in response to hypertonic stress, whereas an inactive version of the kinase was not able to generate such response. However, NFAT5 was able to respond to hypertonicity in AT cells, indicating that ATM can be substituted by other kinases, and pre-treatment of AT cells with wortmannin inhibited as well the high NaCl-induced transcriptional activity, an effect most likely due to the ability of wortmannin to inhibit other members of the PIKK family (Irarrazabal *et al.*, 2004).

The phosphoinositide-3 kinase family of lipid kinases is involved in many cellular responses such as cell growth and survival. They are classified into four classes based on sequence homology and lipid specificity; I_A, I_B, II and III (Koyasu, 2003). The one involved in activation of NFAT5 is class I_A (PI3K-I_A), which generates PIP_3 from PIP_2 (Engelman *et al.*, 2006). Class I_A of PI3K are heterodimers composed by a catalytic and a regulatory subunit and are activated downstream of receptors involved in protein tyrosine kinase signaling (Koyasu, 2003). Inhibition of this class of kinases by overexpression of a dominant negative mutant of its regulatory subunit reduces high NaCl-induced transcriptional activity of NFAT5, and inhibition of PI3K-I_A by siRNA of its catalytic subunit results in the reduction of NFAT5-dependent transcription in HEK293 and Jurkat cells (Irarrazabal *et al.*, 2006). Hypertonicity-induced activation of NFAT5 in primary T lymphocytes was substantially downregulated by PI3-K inhibitors LY294002 (1 μM) and wortmannin (0.5 μM) (Morancho *et al.*, 2008).

Accumulating evidence shows that activation of NFAT5 in response to hypertonicity requires the contribution of different signaling pathways, and that each of these kinases are necessary but not sufficient on their own for full NFAT5 activation. Moreover, the role of each specific kinase varies in different cell types, and some of the kinases not always regulate NFAT5 (Morancho *et al*., 2008). Although studies have identified NFAT5 interacting proteins, and described putative ATM and PKA phosphorylaytion sites, direct evidence of phosphorylation of NFAT5, or identification of phosphorylation sites targeted by any of the above mentioned kinases remains to be provided.

6.2. NFAT5 target genes in response to hypertonicity

Exposure of cells to hypertonicity causes transient disequilibrium of intracellular environment, as a consequence of the water efflux and the subsequent rise in concentration of intracellular components during regulatory volume increase (RVI). All cells have adaptive mechanisms to cope with osmotic stress and restore intracellular inorganic ion homeostasis and volume by accumulating organic osmolytes (Burg and Ferraris, 2008; Kultz, 2005).

In mammalian cells, NFAT5 activates an osmoprotective gene program in response to hypertonic stress. NFAT5 target genes contain at least one osmotic response element (ORE) DNA consensus motif in their regulatory regions (Ferraris *et al.*, 1996). The genes identified up to date include transporters of organic osmolytes such as Na^+/Cl^- coupled betaine/γ-aminobutyric acid transporter (BGT1), Na^+- dependent sodium myo-inositol transporter (SMIT), aquaporin 2 (AQP-2), urea transporter (UT-A2) and amino acid transporters such as System A amino acid transporter and the Na^+ and Cl^- -dependent taurine transporter (Tau-T). Additionally, under hypertonic conditions the aldose reductase (AR) enzyme, some inflammatory cytokines, and molecular chaperones Osp94 and heat shock protein 70 (isoform Hsp70.1) are also regulated by NFAT5, as shown in **Table 1**. Importantly, molecular chaperones are induced during the first hours of exposure to hypertonic stress to counteract the harmful effects of elevated ionic strength by protecting intracellular macromolecules from folding or aggregating (Alfieri and Petronini, 2007; Beck *et al.*, 2000; Bukau and Horwich, 1998). As previously mentioned, deletion of NFAT5 in mice results in renal atrophy and failure to induce osmoprotective genes AR, BGT1 and SMIT, leading to increased apoptosis *in vivo* (Lopez-Rodriguez *et al.*, 2004) .

Exposure to hyperosmotic conditions (330 - 410 mOsm/kg) induces gene expression of the proinflammatory cytokines IL-8, IL1-α and IL1β in human peripheral blood mononuclear cells (PBMC). In addition, PMBC exposed to hypertonic conditions and subsequently stimulated with lipopolysaccharide (LPS) or serum showed synergistic expression of IL-8, IL1-α, IL1β and TNFα (Shapiro and Dinarello, 1997). In osmotically stressed T cells NFAT5 was shown to regulate lymphotoxin-β and TNF-α cytokine genes. Downregulation of NFAT5 by overexpression of a dominant negative that inhibits its dimerization, hypertonicity-induced expression lymphotoxin-β

and TNFα genes is repressed. Moreover, chromatin immunoprecipitation experiments confirmed that NFAT5 binds the TNFα promoter *in vivo* under hypertonic conditions (Lopez-Rodriguez *et al.*, 2001).

TNFα is a key cytokine that regulates immune responses, differentiation and apoptosis by turning on NF-κB and Jun kinase (JNK) signaling (Kucharczak *et al.*, 2003). In response to osmotic stress, TNFα induces the expression of the osmoprotective gene aldose reductase in an NF-κB dependent manner in several human cell types, including liver, lens and retinal pigment epithelial cells (Iwata *et al.*, 1999). NF-κB can also be activated by osmotic stress and has been shown to induce the expression of COX2 and adenosine A1 receptor (A1AR) (Pingle *et al.*, 2004).

Although response to hyperosmolarity has been mainly studied in kidney or kidney-derived cells, as they are physiologically exposed to such environment, studies with immune, cardiac, muscle, neuronal and eye lens cells, have confirmed the need of an adequate osmotic stress response for non-renal cells greatly orchestrated by the transcription factor NFAT5.

Target gene		Role	Reference
Transporters	SMIT	Na^+-dependent sodium myo-inositol transporter	(Rim *et al.*, 1998) (Lopez-Rodriguez *et al.*, 2004) (Navarro *et al.*, 2008)
	BGT-1	Na^+/Cl^- coupled betaine/γ-aminobutyric acid transporter	(Miyakawa *et al.*, 1998) (Lopez-Rodriguez *et al.*, 2004)
	AQP-2	Water channel	(Lopez-Rodriguez *et al.*, 2004) (Kasono *et al.*, 2005)
	UT-A2	Vasopressin regulated urea transporter	(Nakayama *et al.*, 2000)
	SNAT2 (ATA2)	Neutral amino acid transporter	(Trama *et al.*, 2002)
	TauT	Taurine transporter	(Ito *et al.*, 2004)
Enzyme	Aldose reductase	Converts glucose into sorbitol	(Ferraris *et al.*, 1996) (Ko *et al.*, 1997) (Lopez-Rodriguez *et al.*, 2004) (Navarro *et al.*, 2008)
Cytokines	Lymphotoxin β	Involved in organogenesis and apoptosis	(Lopez-Rodriguez *et al.*, 2001)
	TNFα	Pro-inflammatory cytokine	(Lopez-Rodriguez *et al.*, 2001) (Esensten *et al.*, 2005)
Chaperones	HSP70.1	Critical role in cell survival and thermotolerance in response to various stresses	(Woo *et al.*, 2002) (Go *et al.*, 2004) (Heo *et al.*, 2006) (Navarro *et al.*, 2008)
	Osp94	Member of HSP110 family, induced by osmotic stress	(Kojima *et al.*, 2004)

Table 1. NFAT5 target genes in response to hypertonicity.

7. NFAT5 BEYOND OSMOTIC STRESS

The widespread expression of NFAT5 mRNA in numerous tissues suggests that this protein may have other roles in addition to its involvement in orchestrating an osmoprotective gene program in response to hypertonicity. Accumulating evidence suggests that NFAT5 is relevant under *in vivo* isotonic conditions.

In this regard, NFAT5 is induced upon $CD4^+$ T cell activation by pharmacological mitogenic stimulation (ionomycin plus PMA) and TCR-dependent stimulation (ConA and TCR cross-linking). This induction, unlike hypertonic stress-driven induction, proved to be dependent on calcineurin. Intriguingly, lack of NFAT5 did not impair the activation or proliferation of T cells stimulated via TCR and mitogens, and at present it is unknown what role is played by this factor might be in hypertonicity-independent processes in T cells (Go *et al.*, 2004; Trama *et al.*, 2000).

In addition, NFAT5 has a role in promoting cell migration in epithelial carcinomas overexpressing $\alpha 6\beta 4$ integrin, a laminin receptor in normal epithelia. This receptor is involved not only in the mediation of adhesive interactions, but also has a strong impact on signaling pathways influencing epithelial carcinoma cell migration and invasion as is the PI3-K pathway (Mercurio *et al.*, 2001; Shaw *et al.*, 1997). Evidence shows that $\alpha 6\beta 4$ integrin signaling activates NFAT5 in human breast and colon cancer cells, and that this factor plays a functional role in promoting cell migration but not invasion (Jauliac *et al.*, 2002).

Interestingly, the investigation of the role of NFAT5 during development showed that this transcription factor is expressed in murine embryonic stem (ES) cells, early in mouse embryos and throughout fetal development. Curiously, exposure of mouse ES cells to hypertonicity did not further induce the protein's expression (Maouyo *et al.*, 2002). Moreover, disruption of NFAT5 gene in mice results in midembryonic lethality and high postnatal mortality, growth retardation and renal abnormalities (Lopez-Rodriguez *et al.*, 2004).

In addition to the role of NFAT5 in the kidney, several lines of evidence suggest the existence of a biological function for NFAT5 in organs bathed by non-hypertonic environment, such as the heart or skeletal muscle. For example, TauT (an NFAT5 target gene) plays an important role in a large number of biological events such as heart rhythm, contractile function, blood pressure,

cell proliferation and viability, modulation of ion transport, energy metabolism among others (Schaffer *et al.*, 2000). The study of the pathophysiological significance of the down-regulation of the NFAT5 target genes Tau-T and SMIT in cardiomyocytes treated with doxorubicin, an anti-tumor drug with important cardiotoxic side effects, established a cytoprotective role for NFAT5 in doxorubicin-treated cardiomyocytes (Ito *et al.*, 2007). This treatment caused a proteasome-dependent down-regulation of NFAT5 protein levels as well as decrease in transcriptional activity, both in a time dependent manner. However, NFAT5 mRNA levels were not affected by the treatment. Lack of NFAT5 protein led to reduced cardiomyocyte survival. The role of NFAT5 in cellular survival upon exposure to doxorubicin proved to be specific for cardiomyocytes, since treatment of non-cardiac cells with doxorubicin did not affect NFAT5 protein levels (Ito *et al.*, 2007).

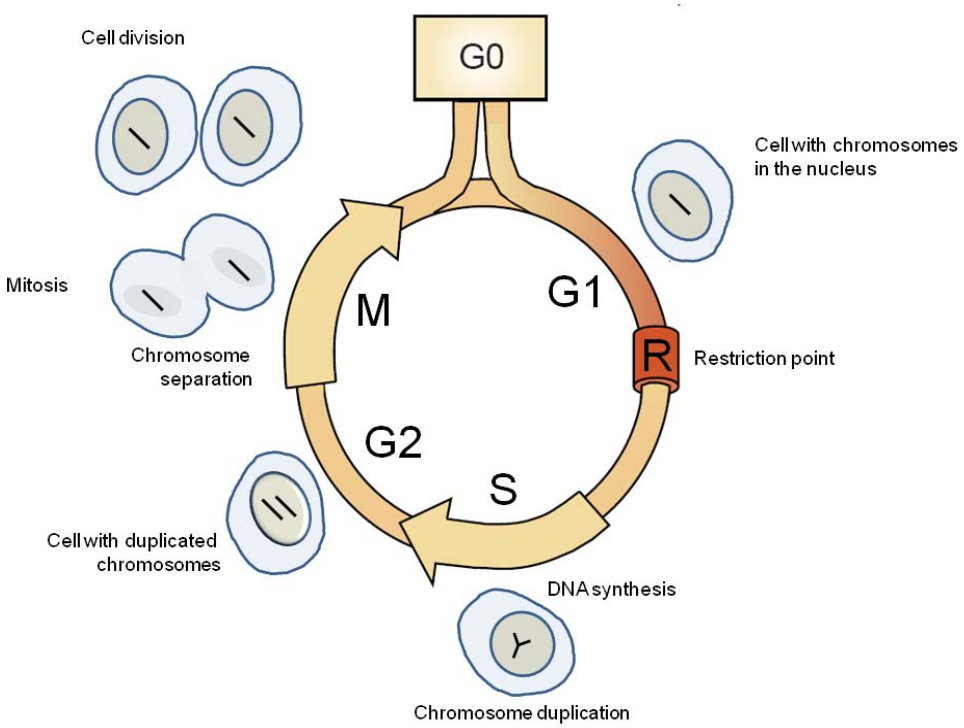
Initial studies reported detection of NFAT5 protein in the myoblast cell line C2C12 and high expression of NFAT5 mRNA in skeletal muscle (Lopez-Rodriguez *et al.*, 1999b). In fact, NFAT5 is expressed and transcriptionally active in skeletal muscle *in vivo*, as well as throughout myogenesis. Impairment of NFAT5 causes a decrease in myoblast migration (O'Connor *et al.*, 2007).

Finally, a recent study has reported that in human primary differentiated macrophages, replication of three major human immunodeficiency virus (HIV) subtypes is NFAT5-dependent (Ranjbar *et al.*, 2006).

8. MAMMALIAN CELL CYCLE

In normal mammalian cells, the cell cycle is a strictly regulated cellular process that is defined as a round of four sequential phases (G1, S, G2 and M) in which two important events take place; DNA replication and partitioning of the newly synthesized DNA and cellular components into two identical daughter cells (Norbury and Nurse, 1992; Obaya and Sedivy, 2002). The first takes place during the synthetic or S phase and the latter during mitosis or M phase (**Figure 11**).

G1, S and G2 phases are collectively known as “interphase”. G1 and G2 are “gap” periods during which the cell prepares and evaluates whether the successful initiation and culmination of DNA replication (during S phase) has been achieved to allow entry into mitosis (M phase) (Norbury and Nurse, 1992).



Adapted from Malumbres and Barbacid, 2005.

Figure 11. Schematic representation of the mammalian cell cycle.

Progression through the cell cycle is driven by the activity of a family of heterodimeric protein kinases known as cyclin-dependent kinases (Cdk). Initially discovered in yeast, Cdks are nuclear serine threonine kinases which are enzymatically activated primarily by binding of cyclins, the most studied activating partners of Cdks (Olashaw and Pledger, 2002). Up to date, 11 genes encoding Cdks (Cdk1-Cdk11) and 9 genes encoding Cdk-like proteins have been identified (Malumbres and Barbacid, 2005). The requirement of a regulatory subunit has only been shown for 10 of these 20 proteins (Cdk1-Cdk9 and Cdk11), and only the partners of Cdk1,

Cdk2, Cdk3, Cdk4 and Cdk6 belong to the cyclin family. Cdk1 drives passage through mitosis while the other four drive interphase (Malumbres and Barbacid, 2005). Other Cdks play auxiliary roles or unrelated roles to cell cycle progression (Morgan, 1997).

It was therefore proposed that mammalian cells require the sequential activation of four interphase Cdks: Cdk4, Cdk6, Cdk3 and Cdk2, and the subsequent activation of Cdk1 to culminate the cell cyle. Recently however, targeted deletion of individual and multiple Cdks in mice showed that the only cyclin-dependent kinase that is able to drive the cell cycle through interphase and mitosis on its own in the absence of all others Cdks is Cdk1, which is also able to bind all cyclins (Santamaria *et al.*, 2007).

To ensure accurate transmission of genetic information, the cell cycle machinery possesses mechanisms to sense the presence of growth factors, inhibitory factors or DNA damage, and is able to take action accordingly, by progressing, slowing or halting the cycle. Together with extracellular events, different checkpoints along the cell cycle conduct the cell's fate; progress, arrest or death (Berthet and Kaldis, 2007). Thus, proper cell cycle progression is essential for genome integrity. Once mitosis and cytokinesis are successfully completed, the new cells have two options; either continue cycling or enter quiescence, a state termed G0 (see **Figure 11**) (Hermeking and Benzinger, 2006; Malumbres and Barbacid, 2005; Obaya and Sedivy, 2002).

The presence of extracellular factors such as growth factors and/or mitogens, trigger cytoplasmic signaling cascades that lead to the successive activation of the cyclin/Cdk protein complexes which conduct proper cell cycle progression (Berthet and Kaldis, 2007; Obaya and Sedivy, 2002). The activity of cyclin/Cdk complexes must be finely tuned and controlled by different mechanisms including binding to positive regulators (complex assembly), phosphorylation and dephosphorylation events, subcellular localization, transcriptional and post-transcriptional regulation, degradation rate and inhibition by binding of Cdk inhibitors (CKIs) (Borriello *et al.*, 2007; Morgan, 1997). In general, Cdk genes are expressed constitutively throughout the cell cycle, while cyclins, as their name suggests, have a periodic expression pattern (Obaya and Sedivy, 2002; Udvardy, 1996). Protein levels of cyclins and CKIs are controlled by their synthesis rate and phosphorylation events leading to ubiquitination and proteasome-mediated degradation (Malumbres and Barbacid, 2005).

An important step in Cdk activation is cyclin binding, which renders a partially active complex. Most of the cyclin/Cdk complexes form with high affinity in the absence of other events (Desai *et al.*, 1995). However, full activation of cyclin/Cdk complexes is positively and negatively regulated by phosphorylation events, and by binding of CKIs as discussed in following sections.

Cdk activity drives the cell cycle progression up to the metaphase stage in mitosis (Peters, 2006). Thereafter, successful culmination of cell cycle progression depends on the dephosphorylation of Cdk substrates and ubiquitination by the ubiquitin-protein ligase termed the anaphase-promoting complex (APC) or cyclosome that labels several regulatory proteins for proteasome-mediated degradation (Peters, 2006).

8.1. Regulation of cell cycle progression by cyclins and Cdks

8.1.1. G1-S transition

The initiation of the G1 phase is dictated by extracellular growth factor signaling. The sequence of events leading to the transition of G1 to S phase begins with the mitogenic signal driven induction of D type cyclins, decrease of the Cdk inhibitor p27 and the involvement of at least three different Cdks; Cdk4, Cdk6 and Cdk2, and possibly Cdk3 (Malumbres and Barbacid, 2005; Pardee, 1974; Ren and Rollins, 2004). Cyclin/Cdk complexes are formed in the following sequential manner (Ekholm and Reed, 2000):

cyclinD/Cdk4 or cyclinD/Cdk6 ⟶ cyclinE/Cdk2 ⟶ cyclinA/Ckd2

Active cyclinD/Cdk4 (or cyclin D/Cdk6) and cyclinE/Cdk2 complexes initiate phosphorylation and inactivation of members of the retinoblastoma family of proteins, also known as “pocket proteins”. This family comprises pRb, p107 and p130 proteins, which act as transcriptional repressors by binding and modulating activity of E2F family of transcription factors, histone deacetylases and chromatin remodeling complexes (Cobrinik, 2005; Harbour and Dean, 2000; Malumbres and Barbacid, 2005).

Phosphorylation of Rb proteins causes the release of E2F proteins, which are now free to form heterodimers with its dimerization partners DP proteins (DP1 and DP2). E2F-DP action drives

the cell towards S phase by actively transcribing genes of molecules required for progression such as cyclin E, cyclin A2, and DNA replication enzymes (DeGregori *et al.*, 1995). It has been proposed that irreversible inactivation of Rb, caused by hyper-phosphorylation, is finally executed by the cyclin E/Cdk2 complex. It has been recently reported that activation of Cdk3 by binding of cyclin C is likely involved in exit from G0 by contributing to Rb phosphorylation. However, Cdk3 is not expressed in many cell types and its precise role in cell cycle regulation has not been fully elucidated (Ren and Rollins, 2004; Santamaria and Ortega, 2006).

This is the point in G1 where the cell is committed to follow through with the cycle. This particular stage during G1 progression has been termed the “restriction point” (Pardee, 1974). Once the cell reaches the restriction point, cell cycle proceeds without the requirement of mitogenic signals, however, extra- and intracellular signals can still order delay or forestall of progression (McGowan, 2003).

8.1.2. S phase: Synthesis

Regulation of entry into S phase, DNA replication initiation and progression, and re-replication control is mainly conducted by two cyclin/Cdk complexes; cyclin E/Cdk2 and cyclin A2/Cdk2. The first complex participates in S phase promotion by taking part in the release of E2F from Rb, by phosphorylating E2F, thus allowing expression of genes required for S phase. Cyclin E/Cdk2 is also involved in phosphorylation of components of the replication complex needed for DNA replication to initiate, proteins involved in histone modification and DNA repair (Moroy and Geisen, 2004; Morris *et al.*, 2000; Obaya and Sedivy, 2002). Moreover, cyclin E/Cdk2 complex also targets $p27^{Kip1}$ (its own inhibitor) and it auto-phosphorylates on the cyclin subunit, thus targeting itself for degradation (Clurman *et al.*, 1996; Hwang and Clurman, 2005; Won and Reed, 1996).

Cyclin A2/Cdk2 complex is involved in the control of DNA replication as shown by experiments in which an accelerated entry of G1 cells into S phase is observed in cells expressing ectopic cyclin A2, and downregulation of this cyclin impedes S phase. Cyclin A2/Cdk2 complex is also involved in limiting replication, by allowing the formation of the replication complex only once per cycle. This complex phosphorylates E2F factors, specifically E2F1, inhibiting its transcriptional activity (Yam *et al.*, 2002).

8.1.3. G2-M transition

Transition from G2 to M phase is directed by cyclin A/Cdk and cyclin B/Cdk1 complexes (Fung and Poon, 2005). Cyclin A mRNA and protein accumulate and diminish at similar times, remaining high from late G1 up to early mitosis (Furuno *et al.*, 1999; Pines and Hunter, 1990). Cyclin A/Cdk complexes drive the initiation of chromosome condensation and proper timing of nuclear envelope breakdown, as shown by RNA interference experiments with HeLa cells (Furuno *et al.*, 1999; Gong *et al.*, 2007). This cyclin is destroyed during prometaphase, ahead of cyclin B1 (den Elzen and Pines, 2001).

Inactive cyclin B1/Cdk1 complexes begin to accumulate at late S phase up to G2/M shuttling between the nucleus and the cytoplasm, favoring the latter. When phosphorylated, thus activated, cyclin B1 is detectable at the centrosomes, probably to trigger their separation (Jackman *et al.*, 1995; Jackman *et al.*, 2003). Next, cyclin B moves to the nucleus and shortly after the nuclear envelope breakdown occurs (Hagting *et al.*, 1998; Jin *et al.*, 1998; Yang *et al.*, 1998). By promoting the chromosome condensation (initiated by cyclin A) and spindle assembly, this complex drives the cells up to metaphase, where cyclin B1 is degraded by the APC complex. The latter, as previously mentioned, is now responsible for successful progression and exit from mitosis.

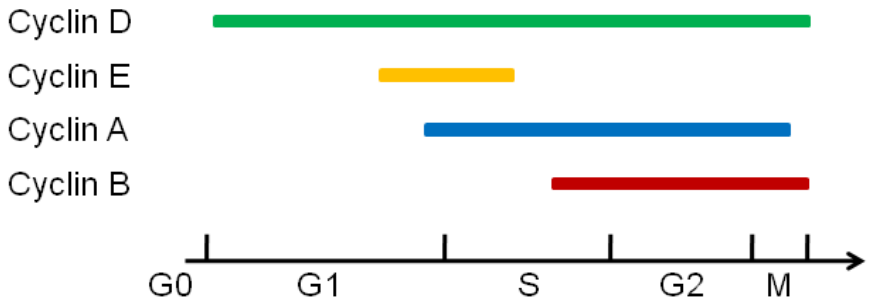
8.2. Positive regulators of Cdk activity: cyclins

Cyclins owe their name to the initial observation that led to their discovery in sea urchin eggs, the fact that they are synthesized and degraded during each cycle (Evans *et al.*, 1983). These proteins play an important part in cell cycle regulation, not only by inducing enzymatic activity of Cdks, but also by directing the substrate specificity of the complex (Norbury and Nurse, 1992; Sherr and Roberts, 2004).

Up to date, about 29 genes have been show to encode related proteins with a “cyclin box” domain, although many of them have not been fully characterized (Berthet and Kaldis, 2007; Evans *et al.*, 1983; Malumbres and Barbacid, 2005). Cyclins are mainly regulated by gene

transcription, post-translational modifications and protein degradation mechanisms, the latter being mediated by the ubiquitination pathway (Morgan, 1997).

Cyclin expression occurs in a coordinated, sequential manner, and upon stimulation of quiescent cells with mitogens or growth factors, they are induced in the following order; cyclin D, cyclin E, cyclin A and cyclin B. Based on the similarities of their amino acid sequence and expression patterns during the cell cycle, cyclins are classified into different families (see below and **Figure 12**). Except for D-type cyclins, which once induced are expressed throughout the cell cycle in proliferating cells; E, A and B type cyclins are short lived, their expression is transient and they are downregulated after they have completed their function (**Figure 13**). The amount of cyclins is regulated by the levels of their respective mRNA, which in turn are controlled by the rate of transcription and their stability during cell cycle phases. Thus, oscillations in cyclin mRNA levels closely parallel those in protein along the cell cycle (Morgan, 1997; Penelova *et al.*, 2005). In addition, cyclins are subjected to regulated degradation mechanisms including ubiquitination and proteasome-mediated degradation, which ensure that their expression is terminated in a timely manner as shown for cyclin E (Clurman *et al.*, 1996; McEvoy *et al.*, 2007; Won and Reed, 1996) , cyclin A (den Elzen and Pines, 2001) and cyclin B (Hagting *et al.*, 2002).



Adapted from Obaya and Sedivy, 2002.

Figure 12. Expression patterns of cyclins during the cell cycle.

8.2.1. D-type cyclins: cyclin D1, cyclin D2 and cyclin D3

Upon exposure to mitogenic stimuli the first type of cyclins to be induced are D-type cyclins. The presence of at least one type of D cyclin is a requirement for G1 phase completion. Levels of D-type cyclins are not detected in quiescent cells, but are greatly induced in response to mitogenic stimulation in early G1 phase. Once induced, D-type cyclins bind and activate Cdk4 and Cdk6. Levels of D-type cyclins are kept relatively constant throughout the cell cycle in the presence of growth factors (Bates *et al.*, 1994; Obaya and Sedivy, 2002).

This family of cyclins has three members; cyclins D1, D2 and D3. In addition to stimulating cyclin D synthesis, growth factors direct their activity, their assembly of complex with Cdks and the subcellular localization of resulting heterodimers (Matsushime *et al.*, 1991; Sherr and Roberts, 1999).

In mouse models, expression of individual D-type cyclins, although overlapping, occurs in a tissue-specific manner throughout embryonic development and in adult tissues (Wianny *et al.*, 1998). Surprisingly, gene knockout studies have shown that all three single knockout mice are viable, which was unexpected based on previous results obtained *in vitro*. The observed defects were tissue-specific, and the existence of a compensatory mechanism cannot be ruled out as an explanation to the mild phenotype observed. Generation of double and triple knockouts has revealed that the tissue specific expression is not maintained, and that the remaining cyclin is ubiquitously expressed (Ciemerych *et al.*, 2002). Mice lacking all D-type cyclins develop through mid to late gestation when they die due to severe anemia and heart defects, suggesting a critical role for this type of cyclins for proper expansion of hematopoietic stem cells. However, triple knockout MEFs are able to propagate normally in culture, but exhibit reduced resistance to oncogenic transformation. Altogether, these results suggest the existence of an alternative mechanism that allows cell cycle progression in the absence of D-type cyclins (Kozar *et al.*, 2004; Santamaria and Ortega, 2006).

Activation of cyclin D/Cdk2 complex in response to mitogenic signals is mediated by induction of cyclin D expression by transcription factors activated by the Ras-Raf-MAP kinase signaling pathway; PI3 kinase and AKT-mediated inhibition of GSK3β to prevent cyclin D degradation, downregulation of the cyclin/Cdk repressor $p27^{Kip1}$, phosphorylation and inactivation of the E2F

repressor Rb by cyclin D/Cdk complexes, and further induction of cyclin D by the transcription factor E2F (Sherr, 2002).

8.2.2. E-type cyclins: cyclin E1 and cyclin E2

Cyclin E is expressed during late G1 phase until the end of S phase, where it binds to and activates Cdk2, regulating processes involved in transcription and DNA replication (Dulic *et al.*, 1992; Hinds *et al.*, 1992; Koff *et al.*, 1992).

Two members of the E-type family of cyclins have been described: cyclin E1 and cyclin E2. These proteins show significant amino acid similarity and are coexpressed in all proliferating cells. Transcription of these cyclins is mainly regulated by members of the E2F family. Cyclin E/Cdk2 complexes phosphorylate members of the Rb family of proteins in different sites than those of cyclin D/Cdk2, most likely to completely inactivate these proteins. Additionally, cyclin E/Cdk2 complex can phosphorylate E2F, thus modulating its own activity. E-type cyclins additionally bind to Cdk1 and Cdk3 (Geisen and Moroy, 2002).
E-type cyclins were thought to be a critical requirement for cellular proliferation. However, generation of single knockout mouse models revealed striking and unexpected results as cyclin E proved to be dispensable for normal mouse development and cyclin E–deficient cells were able to actively proliferate (Geng *et al.*, 2003). Nonetheless, generation of double knockout mice revealed that total lack of E-type cyclins is embryonic lethal. However, while cyclin E is largely dispensable for embryo development, it is essential for development of mammalian placenta. This study also showed that while cyclin E is not essential for continuous cycling, it is required for cell cycle reentry and lack of cyclin E renders the cells resistant to oncogenic transformation (Geng *et al.*, 2003).

8.2.3. A-type cyclins: Cyclin A1 and cyclin A2

After induction of D-type and E-type cyclins, a third group of cyclins, the A-type, is induced at the late G1 to S boundary. Activity of the cyclin E/Cdk2 and cyclin A/Cdk2 complexes dictate S phase entry and initiation of DNA replication. Cyclin A starts to accumulate at late G1 phase throughout G2 phase (Yam *et al.*, 2002).

Cyclin A family is comprised by two proteins; cyclin A1 and cyclin A2. In mammals, cyclin A1 is referred to as the embryonic form, since it is a male-specific cyclin only expressed in meiosis and very early embryos. The only function described up to date for cyclin A1 in adult mice is its involvement in spermatogenesis (Liu *et al.*, 1998). Cyclin A2 on the other hand, is ubiquitously expressed in all cell types and from four-stage embryo forwards. Disruption of this gene is embryonic lethal, as embryos reach the blastocyst stage but die soon after, suggesting that the early cycles can go on without cyclin A2 (Murphy *et al.*, 1997). However, in somatic cells cyclin A2 plays an important role in promoting S and M phase progression by binding to Cdk2 and Cdk1 respectively (Furuno *et al.*, 1999).

The cyclin A2 promoter is activated at S phase entry (Henglein *et al.*, 1994). The messenger RNA (mRNA) of this cyclin begins to accumulate during S phase and decreases in M phase, slightly before cyclin B1 mRNA (see below). Transcription of cyclin A gene is stimulated by cyclins accumulating in G1 phase, specifically cyclins E and D (Schulze *et al.*, 1995; Zerfass-Thome *et al.*, 1997). E2F transcription factors play an important role in cyclin A2 transcription regulation, which can be positively regulated by TAFII250, potentiated by MDM2 or negatively regulated p53 (Yam *et al.*, 2002).

8.2.4. B-type cyclins: cyclin B1 and cyclin B2

Cyclin B1 is expressed in late S through G2 phases, where it associates to and activates Cdk1. However, activation of this complex is only required for transition from G2 to M phase, which heavily relies on cyclin B/Cdk1 complex activity (Clute and Pines, 1999). Expression of cyclin B starts at late S phase and remains high through G2 and early mitosis, slightly behind that of cyclin A2 (Pines and Hunter, 1990; Yam *et al.*, 2002).

Three members of this family have been described; cyclin B1, B2 and more recently cyclin B3 (Evans *et al.*, 1983; Lozano *et al.*, 2002; Nguyen *et al.*, 2002). Cyclins B1 and B2 are ubiquitously expressed, while cyclin B3 is only found in fetal ovaries and adult testes (Lozano *et al.*, 2002; Nguyen *et al.*, 2002). In addition to diverse expression patterns, function and subcellular localization of B-type cyclins are unique to each member. Cyclins B1 and B2 are coexpressed in the majority of dividing cells; however, while cyclin B1 is a microtubule-associted protein which translocates to the nucleus prior to nuclear envelope breakdown, cyclin B2

associates to intracellular membranes and is involved in the Golgi apparatus reorganization that takes place during mitosis (Clute and Pines, 1999; Draviam *et al.*, 2001). Cyclin B3 accumulates in the nucleus of human spermatocytes in testis. It is thought to be degraded in anaphase after cyclin B1, however, its exact role in mitosis remains to be understood (Sullivan and Morgan, 2007). Improper cyclin B3 expression has been reported to lead to aberrant spermatogenesis (Nguyen *et al.*, 2002; Refik-Rogers *et al.*, 2006; Tschop *et al.*, 2006).

Deletion of cyclin B1 in mice is embryonic lethal, while knockout of cyclin B2 does not show any obvious phenotype. These results suggest that cyclin B1 may be able to compensate loss of cyclin B2 (Brandeis *et al.*, 1998). Up to date, no cyclin B3-deficient mouse model has been reported.

8.3. Negative regulators of Cdk activity: CKIs

The activity of many Cdks is controlled by interaction with Cdk inhibitory subunits (CKIs), whose amount, like that of cyclins, varies along the cell cycle. The CKIs contribute to cell cycle regulation by inhibiting cyclin/Cdk activity, thus allowing proper timing of Cdk activation (Besson *et al.*, 2008; Olashaw and Pledger, 2002).

In mammalian cells, two CKIs families have been described; INK4 and Cip/Kip proteins (Lee and Yang, 2001; Sherr and Roberts, 1995). INK4 family is comprised by $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, $p19^{INK4d}$ proteins. Members of this family recognize Cdk4 and Cdk6, but not Cdk2 (Canepa *et al.*, 2007; Lee and Yang, 2001). Their mechanism of action is to interfere with the association of Cdk4 and Cdk6 to D-type cyclins by specifically binding to monomeric Cdk4 and Cdk6, thus inhibiting the enzymatic activity by impeding complex assembly (Borriello *et al.*, 2007; Olashaw and Pledger, 2002). Although apparently structurally redundant, INK4 proteins are differentially expressed during mouse development. $p16^{INK4a}$ and $p15^{INK4b}$ proteins are highly expressed during embryogenesis while the remaining two INK4 family members are undetectable before birth. By 1 month of age, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$ are detected in a variety of tissues, while expression of $p16^{INK4a}$ is limited to spleen and lung of older mice, and expression of the latter becomes greater and more widespread with aging in many human and murine tissues (Zindy *et al.*, 2000). With the exception of $p19^{INK4d}$, INK4 proteins are found to be

deleted or inactivated in many cancer cell lines and tumors (Ortega *et al.*, 2002; Pei *et al.*, 2004; Santamaria and Ortega, 2006).

$p16^{INK4a}$ is a potent tumor suppressor and is highly expressed in senescent cells, and is the most frequently deleted INK4 family member in many cancer cell lines and tumors (Hara *et al.*, 1996; Lee and Yang, 2001). $p16^{INK4a}$ deficient mice develop normally but are cancer prone (Serrano *et al.*, 1996). Expression of $p15^{INK4b}$ is induced by TGFβ treatment (Hannon and Beach, 1994). Similar ability of both $p16^{INK4a}$ and $p15^{INK4b}$ to induce replicative senescence, inhibit telomerase activity and inhibit cell growth has been reported in glioma cells (Fuxe *et al.*, 2000). Loss of $p15^{INK4b}$ in mice leads to extramedullary hematopoiesis and, in a low percentage of animals, formation of tumors in several tissues. Deletion of $p18^{INK4c}$ in mice showed development of gigantism and organomegaly, and later in life, pituitary tumors (Zindy *et al.*, 2000). These mice also showed increased numbers of B and T cells, which underwent accelerated proliferation upon exposure to mitogenic stimuli (Franklin *et al.*, 1998). Deletion of $p19^{INK4d}$ in the mouse did not affect its development and, although displaying testicular atrophy due to increased apoptosis, their fertility was not compromised (Zindy *et al.*, 2000).

The Cip/Kip family is composed by $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ proteins. An important role of these proteins is to potently inhibit Cdk activity by binding to the already formed cyclin/Cdk complexes (Borriello *et al.*, 2007; Olashaw and Pledger, 2002). Specifically, members of this family bind complexes of cyclin D, A and E with their respective Cdk partner. However, $p21^{Cip1}$, $p27^{Kip1}$ binding to cyclin D/Cdk4 complexes does not inhibit their kinase activity, but rather regulates the proper complex assembly and nuclear import (Cheng *et al.*, 1999; LaBaer *et al.*, 1997; McGowan, 2003). Thus, this family of proteins contributes to G1 regulation by promoting the formation of cyclin D/Cdk4/6 complexes and by simultaneously delaying cyclin E/Cdk2 activity (Besson *et al.*, 2008; Lee *et al.*, 1995). Nonetheless, Cip/Kip proteins may also modulate cell cycle progression by inhibiting components of the replication machinery independently of cyclins and Cdks. Based on their ability to block cell proliferation, $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ proteins were initially considered as tumor supressors, however, they have been shown to be involved in processes beyond cell cycle regulation such as transcription, apoptosis and migration (Besson *et al.*, 2008).

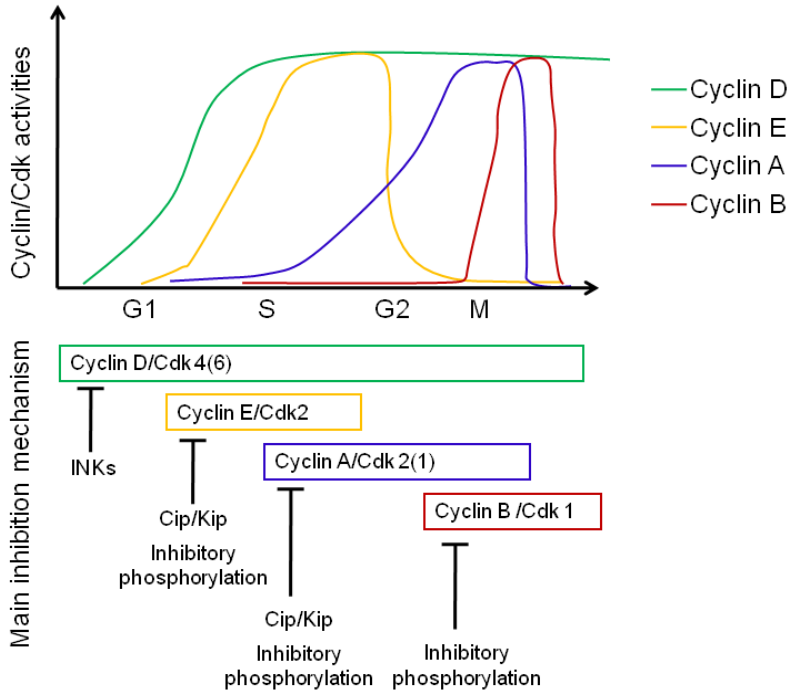
Expression of $p21^{Cip1}$ is greater in cycling cells than in those in a quiescent state. This protein is a transcriptional target of many factors, including p53, BRCA1, STAT, and plays a crucial role in delaying cell cycle progression upon cellular exposure to DNA damaging agents such as ionizing radiation and genotoxic drugs (Gorospe *et al.*, 1999). $p21^{Cip1}$ inhibits cell cycle progression primarily by binding cyclin/Cdk2 complexes (Gartel and Radhakrishnan, 2005). Additionally, $p21^{Cip1}$ is able to effectively inhibit Cdk3, Cdk4 and Cdk6 (Harper *et al.*, 1995). It has also been shown to block DNA synthesis by binding to proliferating-cell nuclear antigen (PCNA) (Luo *et al.*, 1995; Waga *et al.*, 1994). A substantial amount of evidence suggests a role for $p21^{Cip1}$ in protection from apoptosis via cell cycle inhibition, transcriptional regulation and protein-protein interaction. Nonetheless, the ability to modulate apoptosis seems to be dictated by the cellular type and surrounding environment (Besson *et al.*, 2008; Gartel and Tyner, 1999). Through direct binding, $p21^{Cip1}$ is a strong regulator of several transcription factors including E2F1, c-Myc and STAT3 (Besson *et al.*, 2008). Disruption of the $p21^{Cip1}$ gene did not lead to gross abnormalities; however, studies with MEFs lacking $p21^{Cip1}$ showed that this protein has an important role in the regulation of growth arrest upon DNA damage (Brugarolas *et al.*, 1995; Deng *et al.*, 1995). *In vivo* studies have shown that $p21^{Cip1}$ plays a role in regulating keratinocyte and hematopoietic cells renewal, control of T cell proliferation, and balance regulation of hyperplasia and hypoplasia in the kidney (Gartel and Tyner, 2002).

Of all the members of the Cip/Kip family, $p27^{Kip1}$ is thought to be the main modulator of cellular proliferation as it maintains the quiescent cellular state. Consistent with this observation, mice lacking $p27^{Kip1}$ are viable, but they display a larger body size, multiple organ hyperplasia and sterility (Borriello *et al.*, 2007; Kiyokawa *et al.*, 1996; Santamaria and Ortega, 2006). Moreover, mitogenic stimulation of quiescent cultures causes down-regulation of $p27^{Kip1}$ protein, and depending on its cellular localization, $p27^{Kip1}$ may function as a tumor suppressor when in the nucleus, or as an oncogene when in the cytosol (Borriello *et al.*, 2007; Olashaw and Pledger, 2002). In addition to its role as a Cdk inhibitor, $p27^{Kip1}$ has also been implicated in cell motility in some cell types (Borriello *et al.*, 2007) and modulation of transcription factor activity by direct binding, as reported for Neurogenin-2 (Nguyen *et al.*, 2006).

Unlike the widespread presence of $p21^{Cip1}$ and $p27^{Kip1}$, the remaining member of the Cip/Kip family $p57^{Kip2}$ presents a tissue-specific expression (Lee *et al.*, 1995). Specifically, mRNA of $p57^{Kip2}$ is found at relatively high levels in placenta and at lower levels in skeletal muscle, heart,

kidney and pancreas. In view of this restricted expression, $p57^{Kip2}$ is considered to participate in cell cycle regulation in a tissue or cell-type specific fashion (Lee *et al.*, 1995). Importantly, $p57^{Kip2}$ is required for embryonic development, as mice lacking this protein show multiple developmental abnormalities and die at birth (Besson *et al.*, 2008; Yan *et al.*, 1997; Zhang *et al.*, 1997). Moreover, $p57^{Kip2}$ promotes muscle-specific gene transcription by interacting with and stabilizing Myo-D (Besson *et al.*, 2008; Reynaud *et al.*, 2000). Additionally, $p57^{Kip2}$ has also been reported to bind to PCNA, preventing its activity and blocking DNA replication. Accumulating body of evidence supports a tumor suppressor role for $p57^{Kip2}$ but unlike for $p21^{Cip1}$ and $p27^{Kip1}$, no oncogenic activity has been associated yet with $p57^{Kip2}$ up to date (Besson *et al.*, 2008; Watanabe *et al.*, 1998).

As summarized in **Figure 13**, the sequential activation and inactivation of cyclin/Cdk complexes is directed not only by complex assembly, but also involves the activity of CKIs and phosphorylation events (described in the following section).



Adapted from McGowan, 2003

Figure 13. Activity patterns of cyclin/Cdk complexes throughout a normal cell cycle. *In the upper panel, the control of cyclin/Cdk complex activity as a consequence of cyclin protein availability. Cyclin D: green; cyclin E: yellow; cyclin A: blue; cyclin B: red. The lower panel shows the major inhibitory mechanisms of each complex (Phosphorylation events are discussed below).*

8.4. Regulation of Cdk activity: Phosphorylation events

Binding of cyclins to Cdks provides partial activation of the cyclin/Cdk complexes. In addition, Cdks are regulated by activating and inhibitory phosphorylation events. Full activation requires phosphorylation on a conserved threonine residue near the ATP-binding cleft (for Cdk4/6: Thr172; Cdk2: Thr 160; Cdk1: Thr 161), which is located in a loop of amino acids called the T-loop region (Desai *et al.*, 1995; Kaldis, 1999; Morgan, 1997; Obaya and Sedivy, 2002). Phosphorylation of these threonine residues is performed by the Cdk-activating kinase (CAK) which is formed by three subunits: Cdk7, a member of the Cdk family, cyclin H, a cyclin-like unit, and MAT1 (*ménage a trois* 1) (Kaldis, 1999; Lolli and Johnson, 2005). Activity of CAK is constitutive across the cell cycle and the subunits are not regulated by transcription or proteolysis. Together with six other subunits, CAK forms the transcription factor TFIIH, which is involved in promoter clearance and progression of transcription (Lolli and Johnson, 2005; Malumbres and Barbacid, 2005). CAK is antagonized by the Cdk-associated protein phosphatase KAP, which is a dual specificity phosphatase that acts on Thr160 of Cdk2 (Desai *et al.*, 1995; Hannon and Beach, 1994; Morgan, 1997; Obaya and Sedivy, 2002; Yu *et al.*, 2007). KAP also binds Cdk1 and Cdk3, however, direct evidence of regulation of these Cdks by KAP remains to be provided (Yu *et al.*, 2007).

As mentioned above, phosphorylation on Thr160 of Cdk2 is necessary for Cdk2 kinase activity and cell cycle progression, and in addition of the CAK kinase, Cdk2 has been recently reported to be capable of performing autocatalytic phosphorylation on this activating residue (Abbas *et al.*, 2007).

All Cdks are negatively regulated by phosphorylation on sites near their N termini, specifically on Thr14 and Tyr15 for Cdk1 and Cdk2, and Tyr17 for Cdk4 and Cdk6 (Gould and Nurse, 1989; Krek and Nigg, 1991; Morgan, 1997). Phosphorylation on Tyr15 and Thr14 of Cdk1 and Cdk2 is carried out by the Wee1 and Myt1 kinases (**Figure 14**). In consequence, these two kinases serve as negative regulators of mitotic entry (McGowan, 2003; Obaya and Sedivy, 2002). The inhibitory phosphorylation of Cdk4 and Cdk6 on Tyr17 has been reported in response to UV-irradiation induced cell cycle arrest, quiescence and in TGFβ-treated cells. Unlike Cdk1 and Cdk2, Cdk4 and Cdk6 are not *in vitro* substrates of Wee1 tyrosine kinase, and the kinase

performing such phosphorylation has not yet been identified (Bockstaele *et al.*, 2006; Watanabe *et al.*, 1995).

Wee1 is a dual-specificity nuclear protein kinase that preferentially phosphorylates Tyr15 on Cdk, leading to cyclin/Cdk complex inactivation. Specifically, as inactive cyclin B/Cdk1 complexes accumulate along S and G2, they can be rapidly and timely activated by dephosphorylation of those sites by Cdc25 phosphatases (discussed below) (McGowan and Russell, 1993; Mueller *et al.*, 1995; Parker and Piwnica-Worms, 1992). In agreement with its inhibitory role of cyclin B/Cdk1 complexes activity, Wee1 has been found to be regulated by a combination of hyperphosphorylation events and proteolytic degradation after ubiquitination by the Skp-Cullin F box complex (SCF) at the beginning of mitosis (Hoffmann *et al.*, 1993; Watanabe *et al.*, 1995; Watanabe *et al.*, 2005). *In vitro*, Wee1 is able to phosphorylate both Cdk1 and Cdk2, however, *in vivo* phosphorylation of the latter has not yet been proven.

Additionally, Myt1, a membrane associated Wee1-type kinase, phosphorylates Cdk1 on Tyr15 and Thr14 residues (McGowan and Russell, 1993; Mueller *et al.*, 1995; Parker and Piwnica-Worms, 1992). This kinase has been shown to play an important role in gametogenesis in various species (Burrows *et al.*, 2006; Jin *et al.*, 2005). In the somatic cell cycle, Myt1 is thought to function in mitotic entry regulation. Recently however, in HeLa cells, Myt1 has been shown to be dispensable for some mitotic events, but it proved to be an essential player in the control of intracellular membrane dynamics during mitosis (Nakajima *et al.*, 2008).

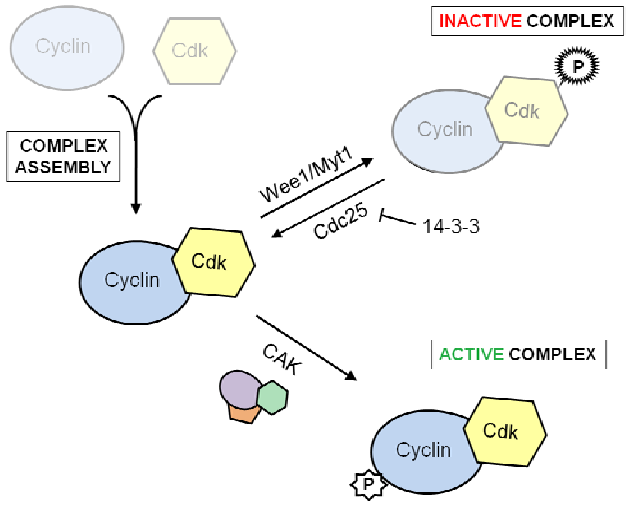
Full activation of Cdk complexes thus requires the removal of the inhibitory phosphorylation. This is conducted by Cdc25 phosphatases, a family of dual-specificity phosphatases (Hoffmann *et al.*, 1993; Izumi *et al.*, 1992; Kumagai and Dunphy, 1992). Additionally, Cdc25 proteins have been also shown to play a role in DNA damage response at the G2/M transition (Mailand *et al.*, 2002; Zhao *et al.*, 2002).

In mammalian cells, three Cdc25 isoforms have been identified (Cdc25A, Cdc25B and Cdc25C). Initially, it was suggested that each isoform functioned during a specific cell cycle phase. However, recent studies have shown that all three Cdc25 isoforms are implicated in regulation of mitosis, and in addition, Cdc25A is involved in G1 to S transition by regulating activity of cyclin E/Cdk2 and cyclinA/Cdk2 complexes (Donzelli and Draetta, 2003; Hoffmann

and Karsenti, 1994; Obaya and Sedivy, 2002). Cdc25A, Cdc25B and Cdc25C shuttle across the nuclear membrane. Cdc25C is cytoplasmic during interphase and translocates to the nucleus concomitantly with cyclin B1 at prophase (Boutros *et al.*, 2006).

Cdc25 isoforms have different expression periods. Cdc25A appears in late G1 and its levels increase throughout mitosis, when the protein is stabilized by phosphorylation mediated by cyclin B1/Cdk1 complex, hence contributing to the activation of that Cdk complex (Donzelli *et al.*, 2002). Overexpression of Cdc25A induces mitotic entry, while inhibition by means of siRNA causes delays in G1 to S and G2/M transitions (Mailand *et al.*, 2002). Cdc25B is detected from S phase until it disappears in mitosis, where its activity peaks before that of Cdc25C. Inhibition of Cdc25B can block cells in the G2 phase. It has been reported that cyclin A2/Cdk2 complexes are important regulators of Cdc25B activity in S phase and of Cdc25C in G2/M (Mitra and Enders, 2004). Cdc25C on the other hand, although present throughout the cell cycle, is only active in mitosis by hyperphosphorylation mediated by the cyclin B1/Cdk1 complex (Donzelli and Draetta, 2003). A study by Lindqvist and colleagues showed that specifically Cdc25B is needed for the activation of the cyclinB1/Cdk1 complex at the centrosomes, where its activity begins (Lindqvist *et al.*, 2005). Interestingly, Cdc25 phosphatases are targets of Cdks, since Cdc25A is phosphorylated and activated by cyclin E/Cdk2, and Cdc25C is activated by phosphorylation by cyclinB/Cdk1 (Boutros *et al.*, 2006; Hoffmann *et al.*, 1993).

Cdc25 phosphatases are bound and negatively regulated by 14-3-3 proteins, a family of highly homologous proteins that function at several key points of the cell cycle, as are G1 to S and G2 to M transitions. Binding by 14-3-3 may lead to different effects on their protein ligands, whose number is in the range of the several hundreds. In most cases, this association takes place after the protein ligand has been phosphorylated by a specific kinase, and the outcome of 14-3-3 binding may be cytoplasmic sequestration, activation or repression of function or enzymatic activity, facilitation or prevention proteins modifications or nuclear retention. In consequence, this family of proteins is involved in many cellular processes, playing pivotal roles in cell cycle regulation, signal transduction, stress response, apoptosis, coordination of cell adhesion and motility, as well as in transcriptional regulation (Hermeking and Benzinger, 2006).



Adapted from Boutros et al, 2006; Malumbres and Barbacid, 2005.

Figure 14. Phosphorylation events regulate cell cycle progression. *Cyclin/Cdk complexes are activated by Cdc25-mediated dephosphorylation. Cdc25 are in turn negatively regulated by 14-3-3 proteins. On the other hand, inactivation of the cyclin/Cdk complexes is achieved by Wee1 and Myt1-dependent phosphorylation.*

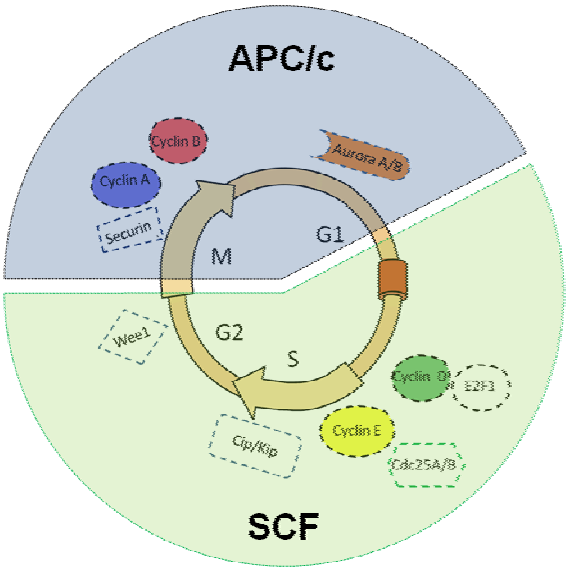
8.5. Protein degradation: key factor in cell cycle regulation

Degradation of proteins throughout the cell cycle is mediated by the ubiquitination pathway, in which two different ubiquitin ligases play important roles at specific points of the cell cycle; Skp-Cullin F box complex (SCF) and the anaphase promoting factor (APC) (Peters, 1998; Peters, 2006; Pintard *et al.*, 2004) (**Figure 15**).

The SCF complex is active throughout the cell cycle, mainly controlling G1/S and G2/M boundaries by inducing degradation of different factors at these points of the cycle. Activity of this ubiquitin-ligase is still controlled by Cdks, as F-box-dependent recognition of most of its target proteins requires phosphorylation by these complexes. Such is the case of cyclin E, where degradation is initiated by a phosphorylation event catalyzed by the cyclin E/Cdk2 complex itself. The latter also phosphorylates Cip/Kip protein $p27^{Kip1}$, labeling it for degradation (Castro *et al.*, 2005; Pintard *et al.*, 2004; Spruck and Strohmaier, 2002; Won and Reed, 1996).

The APC on the other hand, is active at the end of G2 to mediate mitosis transition and exit, ensuring proper chromosome segregation and timing of cytokinesis (Page and Hieter, 1999; Peters, 1998; Pines, 2006). Substrate recognition by this complex was initially thought to be mediated by a short conserved sequence in B-type cyclins called the “destruction box”. However, as the list of APC substrates keeps growing, the understanding of how this complex recognizes its targets has become less clear. Activation of the APC complex is mediated by phosphorylation of two of its subunits, Cdc20 and Cdh1 (called the APC activators) by the Cdk1 and Plk1 kinases (Kotani *et al.*, 1999; Morgan, 1999; Pines, 2006). Among the APC substrates are cyclin A and cyclin B, which are recognized for degradation when phosphorylated by their Cdk partner. Other proteins degraded by APC important for cell cycle regulation include the Cdc25 phosphatases, aurora kinases, securin, Polo-like kinase and Cdc20 itself, among others (Pines, 2006).

Ubiquitin-mediated degradation thus serves as the main pathway that frees cells from cell cycle proteins that have completed their function, and by doing so, it ensures that cell cycle events do not reoccur in an unsuitable manner.



Adapted from Nakayama and Nakayama, 2006

Figure 15. Cell cycle regulation by ubiquitin ligases SCF and APC/c complexes. *Two ubiquitin ligases, the SKP-Cul1-Fbox protein and the anaphase promoting complex/cyclosome (APC/c) are responsible for the specific degradation of many components of the cell cycle machinery. Schematic representation of SCF and APC/c activity timing and some (but not all) substrates of each complex.*

In brief, the mammalian cell cycle is a complex and finely tuned process in which events promoting its progression are carefully regulated at many levels. Essential components of the cell cycle machinery include not only the cyclin-dependent kinases complexes, but also involve phosphatases, kinases and ubiquitin ligases; all of which must function in a proper and timed manner for the successful culmination of one cycle (see **Figure 16**). Over the past years much has been learned about these mechanisms, nonetheless, much remains to be elucidated.

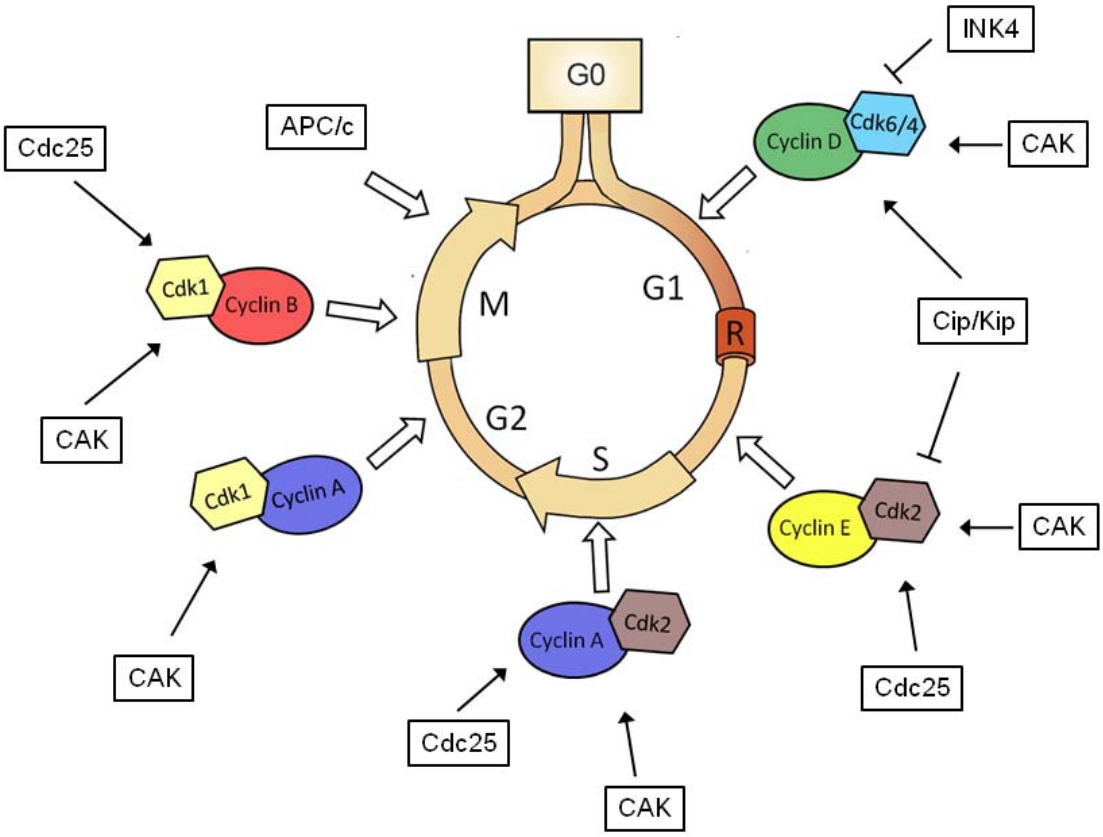


Figure 16. General representation of cell cycle progression.

9. OSMOTIC STRESS AND LYMPHOCYTES

As described in previous sections, the transcription factor NFAT5 is a major contributor to the cell's ability to withstand and adapt to hypertonic stress by orchestrating the expression of gene products that increase the intracellular concentration of compatible osmolytes. Osmotic stress has been shown to impair the survival and ability of mammalian cells to proliferate. These effects have been associated, at least in part, with the activation of genotoxic stress-like responses including cell cycle arrest, induction of p53 and GADD45 (Dmitrieva *et al.*, 2005).

In this regard, NFAT5-deficient cells exhibit viability defects and reduced proliferative capacity when exposed to hypertonic conditions that are well tolerated by wild-type cells, as shown in fibroblasts, T cells (Go *et al.*, 2004) and in the kidney (Lopez-Rodriguez *et al.*, 2004). However, although different studies have reported the ability of NFAT5 to respond to osmotic stress in numerous cell types, as well as that cell death is the endpoint for NFAT5-deficient cells exposed to hypertonicity, little is known about the cellular processes that are sensitive to the lack of this factor.

Furthermore, there is considerable heterogeneity not only in the diversity of cell types analyzed but also in the severity of stress conditions used in the numerous studies in the literature that have addressed the effect of osmotic stress on different aspects of cellular functions (Burg *et al.*, 2007). The latter is not a trivial matter, since some reports on osmotic stress-induced DNA damage or inhibition of signaling pathways have used osmolality of up to 1300 mOsm/kg by addition of NaCl or sorbitol (Reitsema *et al.*, 2005; Desai *et al.*, 2002), which are conditions that have been shown to preclude the activation of NFAT5 (Go *et al.*, 2004).

Most studies addressing the response of mammalian cells to hypertonic stress routinely use osmolality conditions of 500 mOsm/kg or even higher. Indeed, very different mammalian cell types, such as neurons, leukocytes, fibroblasts and others can withstand these conditions and have the ability to induce and activate NFAT5 along with a conserved osmoprotective gene expression program in response to osmotic shock. The fact that so diverse cell types have retained the ability to respond to substantial hypertonic stress is intriguing, and, as previously discussed, has lent support to the notion that this type of stress must be a sufficiently relevant condition for mammalian cells to warrant that an adaptive response to it has been maintained

throughout evolution. However, besides specific cell types, such as those in the renal medulla, which are subjected to acute osmolality changes (Burg and Ferraris, 2008), and the nucleus pulposus of intervertebral discs, which are embedded in a hypertonic matrix at 420-450 mOsm/kg (Ishihara *et al.*, 1997), knowledge on physiopathological scenarios in which mammalian cells might be exposed to severe osmotic stress is limited. Nonetheless, reports in the clinical literature indicate that several disorders can cause hypernatremia and increase the osmolality of plasma to 360-430 mOsm/kg (see Introduction, section 4). As mentioned earlier, elevated plasma osmolality (400 mOsm/kg) can also result from prolonged water deprivation, as shown in camels (Dmitrieva *et al.*, 2005). In addition, constitutively high plasma tonicity (~ 400 mOsm/kg) has been also reported in mice models deficient in molecules important for the urinary concentration mechanism such as aquaporins and vasopressin 2 receptor (see Introduction, section 4).

Available data in the literature show that neurons and lymphocytes are sensitive to moderate hypertonicity levels (around 400 mOsm/kg) in the range reported in plasma in a number of pathologic conditions. Thus, elevation of plasma osmolality to 420 mOsm/kg causes the induction and nuclear accumulation of NFAT5 in neurons (Loyher *et al.*, 2004) and exposure of primary lymphocytes to hypertonicity levels of 380-430 mOsm/kg during the blastogenic phase of their response to mitogens can induce a robust activation of NFAT5 transcriptional activity (Morancho *et al.*, 2008). In addition, hypertonic conditions of 380 mOsm/kg impair the ability of NFAT5-deficient lymphocytes to proliferate in response to T cell receptor stimulation (Go *et al.*, 2004). In contrast to neurons and renal medullary cells, which have a very low turnover rate, lymphocytes grow and proliferate rapidly in response to antigenic stimulation and thus provide a good cellular model to study the effect of pathologic conditions of hypertonic stress and the role of NFAT5 in primary, untransformed cells.

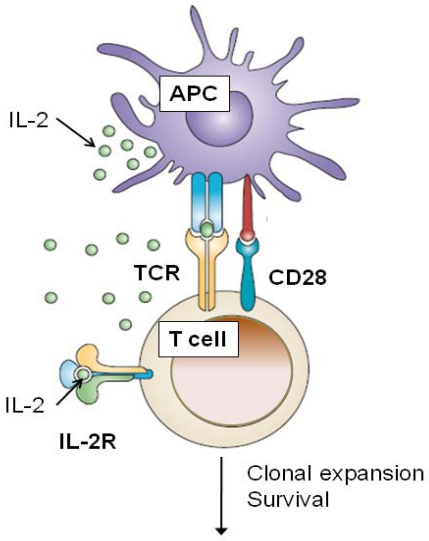
9.1. Regulation of lymphocyte activation by the T cell receptor

T cells provide an accessible and well characterized model for the study of cellular proliferation, since, as mentioned above, their ability to grow and proliferate is an essential part of the immune response. T cell activation and proliferation are triggered by antigens binding to the T cell receptor complex which can be mimicked *in vitro* by antibodies specific to receptors CD3 and CD28, or by mitogens (Diehn *et al.*, 2002). Resting lymphocytes can be isolated form

periphery and then pharmacologically stimulated to enter cell cycle progression (Chitko-McKown and Modiano, 1997). Specifically, the lectin concanavalin A (ConA) has been used since the early 1970's to stimulate splenocytes and trigger T cell proliferation (Dwyer and Johnson, 1981). The extent of the T cell activation process must be strictly regulated, since an overly active immune response may turn into a powerful destructive force attacking the organism (Liu, 2005).

T cell development takes place in the thymus where cells undergo a process of proliferation and differentiation that is regulated by cytokine and antigen receptor signaling. As they develop, they mature through four double-negative (DN) stages before surviving and becoming single positive (SP) thymocytes (Rowell and Wells, 2006). During this process, cells that react too strongly to self-peptides are eliminated, and cells that survive the selection process are released to peripheral circulation as $CD4^+$ or $CD8^+$ naive T cells (Okkenhaug and Vanhaesebroeck, 2003). These cells travel through lymph nodes, spleen and secondary lymphoid organs interacting with specialized antigen-presenting cells (APCs) known as dendritic cells. Upon infection, APCs kill and ingest the pathogenic organism, digest its proteins into small peptides to present some of these in the major histocompatibility complex (MHC) context (Palacios and Weiss, 2004). For a given pathogen there is a specific subset of T cells that can bind this peptide-MHC complex and become activated.

TCR signaling is initiated when the peptide-MHC complex binds to the TCR and simultaneously, other coreceptors, such as CD28, bind to their respective ligands. This dual binding is required to finely control the activation process of a naïve T cell (Liu, 2005; Okkenhaug *et al.*, 2004). Integration of TCR signaling to that of costimulatory receptors initiates gene transcription, secretion of the key autocrine growth factor interleukin 2 (IL-2), induction of the high-affinity IL-2 receptor (IL-2R), and results in vigorous proliferation (Liu, 2005; Okkenhaug *et al.*, 2004; Rowell and Wells, 2006) (**Figure 17**).



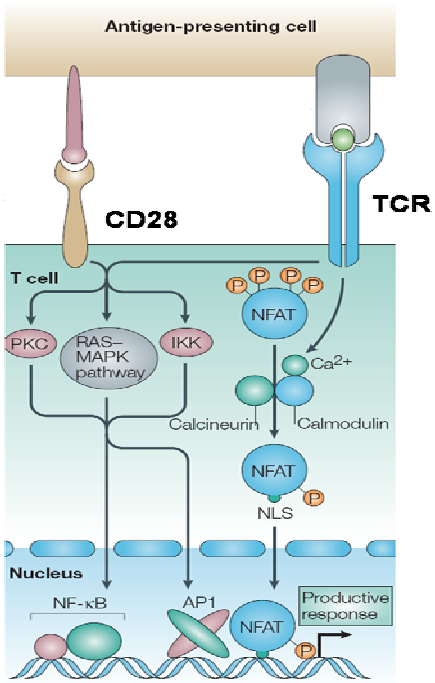
Adapted from Malek and Bayer, 2004.

Figure 17. Three receptors are involved in T cell activation.

The T cell receptor is a membrane bound heterodimer composed by two disulfide linked polypeptide chains (α and β) which have long extracellular parts that fold in a variable and a constant domain. A minor percentage of T cells found mainly in epithelia have γ and δ chains instead of α and β, and they are believed to provide a first line of defense against microorganisms attempting to cross the epithelial sheets of skin or gut. Although the TCR heterodimer allows the cell to recognize antigens presented by APCs, the expression and function of the receptor depends on up to five other transmembrane proteins that associate in a non-covalent fashion with the $\alpha\beta$ heterodimer, and together form the functional TCR. Three of these proteins are the members of the IgG superfamily, called CD3 molecules γ, δ, and ε. Up to 90% of the TCR complexes contain a disulfide linked homodimer of a protein called the ζ chain. The remaining TCR complexes express a heterodimer formed by the ζ chain and a highly homologous FcεRI γ chain (Abbas *et al.*, 2000).

TCR signaling is initiated by cytosolic tyrosine kinases Lck and Fyn (Okkenhaug *et al.*, 2004). These kinases phosphorylate immunoreceptor tyrosine-based motifs (ITAM) in the CD3 invariant chain, initiating the cascade of events leading to phosphorylation of tyrosines essential for signal transmission to the nucleus. TCR signaling activates transcription factors of the

NFATc, NF-κB and AP-1 families which are held inactive and cytosolic in quiescent T cells (**Figure 18**). Along with the T cell activation signal comes an attenuation and termination instruction executed by negative regulators. Some of these regulators are responsible for maintaining the quiescent cellular state before TCR signaling, others, whose transcription is induced by TCR signaling, are part of the feedback inhibition and termination of TCR signaling (Liu, 2005). Among the negative regulators are the autoinhibitory domain of calcineurin and the transcription factors Foxo3a and Foxj1. The first one, by keeping calcineurin inactive, helps prevent the dephosphorylation and nuclear localization of NFATc proteins.



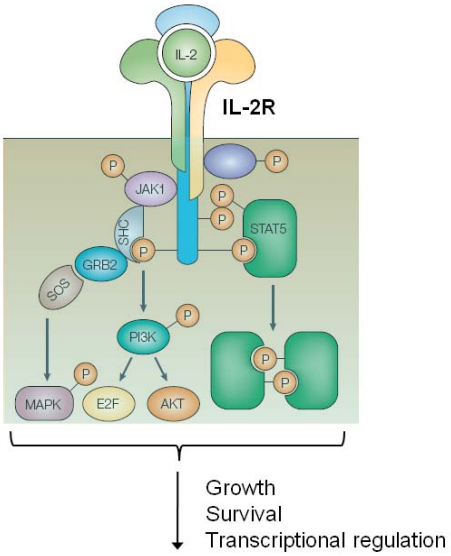
Adapted from Macian, 2005.

Figure 18. Schematic representation of T cell activation.

Target genes of the NFATc family of proteins include many cytokine genes (including IL-2) as well as cell cycle progression and induced cell death genes (Macian, 2005). The forkhead transcription factors Foxo3a and Foxj1 play a role in the maintenance of the cellular quiescent state by activating the expression of IκB, this way maintaining NF-κB inactive (Liu, 2005).

The IL-2 receptor is formed by three subunits called the α, β and γ_c chains. The first one is specific to the IL-2 receptor. The β chain is a common chain to IL-2R and IL-15R, and the γ_c

chain is a shared component of multiple T cell growth factor receptors. IL-2 binding to its receptor signals to at least three mitogenic pathways including the signal transducer and activator of transcription 5 (STAT5) and STAT3, the Ras/MAPK and PI3K/Akt signaling cascades. As represented in **Figure 19**, activation of kinases Jak1 (which is associated to the IL2-R β chain) and Jak3 (which is associated to the IL2-R γ_c chain) leads to phosphorylation and activation of the transcription factor STAT5 and the adaptor protein Shc. STAT5 then translocates to the nucleus and induces genes required for cell cycle progression, as are cyclins D2 and D3, cyclin E and c-myc. Shc is coupled to the Ras/MAPK and PI3K/Akt pathways, which by promoting events such as degradation of $p27^{Kip1}$, hyperphosphorylation of pRb (release of E2F), activation of transcription of cyclins E and A, Cdks and DNA synthetic enzymes, potentiate the expression and activity of cyclin/Cdk complexes (Rowell and Wells, 2006) .



Adapted from Malek and Bayer, 2004.

Figure 19. IL-2 receptor signaling.

9.2. Signaling downstream the TCR

Upon pathogen presence, lymphocytes must quickly go from a resting state to an active, proliferative state. This process, known as blastogenesis, is a considerable energetic challenge for cells, as transcriptional and translational events that promote cell growth, proliferation and function require metabolic substrates from the extracellular environment (Fox *et al.*, 2005). In T

cells, the instruction to initiate metabolite uptake is not an intracellular event, but it is rather signaled by the environment - through the surface receptors mentioned above- in the form of cytokine engagement, and ligand stimulation of antigen and costimulatory receptors (Fox *et al.*, 2005). In quiescent T cells, engagement of the TCR by itself is not enough to drive proliferation or to induce IL-2 gene transcription. CD28 costimulation in the absence of TCR engagement is also unable to produce IL-2 or promote proliferation. Likewise, exogenous IL-2 molecules are not enough to promote cell proliferation unless there is TCR/CD28 stimulation (Rowell and Wells, 2006). Thus, for T cells to successfully enter the cell cycle there must be a proper integration of the combination of signals from these three molecules.

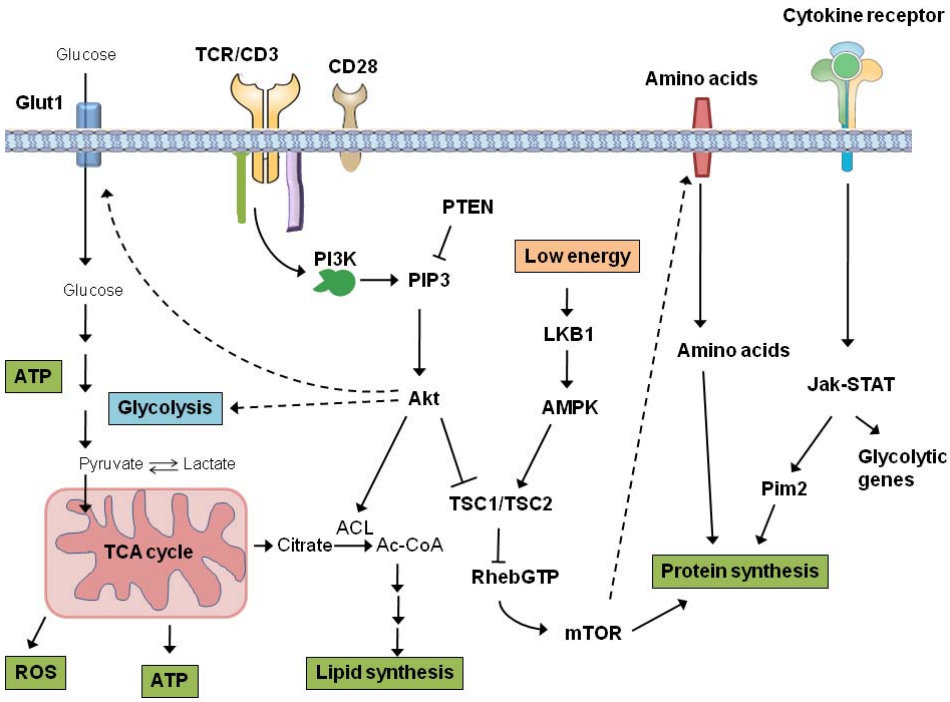
Stimulation through the TCR, the costimulatory receptor CD28 and the IL-2R, as previously mentioned, activates phosphatidylinositol 3'-kinases (PI3Ks), specifically members of class I_A. The PI3K-activated pathway includes regulatory proteins such as TSC1 and TSC2, RHEB, RICTOR and RAPTOR, PTEN and PP2A, as well as the kinases AKT, mTOR and AMPK (Fox *et al.*, 2005; Jones and Thompson, 2007) (**Figure 20**).

Activated T cells present an anabolic metabolism profile which is characterized by increased rates of nutrient uptake, glycolysis and biosynthesis. Glycolysis, in addition to being the primary source of ATP, generates metabolic intermediates important for cell growth, contributing to protein and lipid synthesis (Fox *et al.*, 2005; Jones and Thompson, 2007) (Jones and Thompson, 2007). Upon mitogenic stimulation, naïve T cells increase their glucose uptake up to twenty times in a short amount of time (Fox *et al.*, 2005). The kinase AKT regulates glucose metabolism by stimulating the translocation of the glucose transporter Glut1 to the plasma membrane and increases glycolysis by promoting activity of rate limiting enzymes hexokinase and phosphofructokinase (Jones and Thompson, 2007).

Triggering of the TCR initiates an energy-demanding program that is only successful when cellular energy production satisfies the biosynthetic demands of an immune response. The AMP-activated protein kinase AMPK is a key regulator of cellular energy homeostasis. The net result of AMPK signaling is the engagement of ATP-generating pathways and inhibition of ATP-consuming processes. AMPK plays multiple roles in the metabolic control of proliferating cells including the regulation of protein translation through stimulation of TSC1-TSC2 complex-mediated inhibition of mTOR and through modulation of the cellular rates of glycolysis and β-

oxidation. In T cells, AMPK was recently shown to be activated by TCR-dependent Ca^{2+} signals (Tamas *et al.*, 2006). However, the impact of receptor-mediated AMPK activation on T cell function remains to be elucidated (Jones and Thompson, 2007).

In addition to regulating glucose metabolism, AKT regulates protein metabolism by phosphorylating the mTOR inhibitor TSC2. Phosphorylation exerts an inhibitory effect on TSC2, hence favoring mTOR activation (Fox *et al.*, 2005). The latter is an important player in the regulation of protein synthesis, since its activity couples amino acid uptake to the stimulation of cap-dependent mRNA translation. Important components of the translational machinery such as the translational repressor 4E-BP1, p70S6 kinase and EIF2B are phosphorylated by mTOR when stimulated by exogenous leucine (Fox *et al.*, 2005; Jones and Thompson, 2007). In T cells, mTOR has been proposed to be a key player in the integration of information from the environment when antigen recognition is taking place (signaled in the form of ligation of either cytokine or costimulatory receptors), as well as from nutrients necessary for the high metabolic demands of proliferating cells (Mondino and Mueller, 2007).



Adapted from Jones and Thompson, 2007

Figure 20. Metabolic pathways of growth and proliferation in T cells.

The PI3K/Akt pathway has been shown to play a role in IL-2 induced T cell proliferation and also to lie downstream the costimulatory molecule CD28 (**Figure 20**). Thus, the relationship between PI3K and the cell cycle machinery is likely to be one of critical importance. Treatment of T cells with rapamycin, an mTOR inhibitor, leads to impaired T cell function as progression from G1 to S phase is prevented by this drug (Hleb *et al.*, 2004).

Initial studies showed that PI3K transcriptionally and postranscriptionally regulate expression of D-type cyclins (first G1 cyclins) in T cells. Treatment of cells with LY294002 (PI3K inhibitor) prevented D-type cyclins induction of both mRNA and protein, while rapamycin (mTOR inhibitor) only inhibited protein induction of those cyclins (Breslin *et al.*, 2005). Moreover, the study by Colombetti and colleagues showed that both PI3K and mTOR activities were needed for T cells to proliferate to TCR/CD28-initiated stimuli and for optimal cyclin E expression. Additionally, they reported that either PI3K or mTOR are sufficient for IL-2-driven cell proliferation as they independently mediate cyclin E induction. Treatment of cells with rapamycin, an inhibitor of mTOR, delayed cell cycle entry of IL-2-sufficient T cells, but did not prevent their expansion (Colombetti *et al.*, 2006).

Results from an earlier study suggested that rapamycin interfered with proper activation of Cdk1 and Cdk2 by blocking induction of cyclin A in T cells, leaving cells trapped in late G1 phase, likely due to interruption of the IL-2 signaling cascade (Morice *et al.*, 1993a; Morice *et al.*, 1993b). In agreement with those results, a recent report confirmed the role of mTOR in regulating Cdk1 and Cdk2 activity (Song *et al.*, 2007). In this study, mTOR was found in a complex with aurora B kinase and survivin. As previously reported for survivin, aurora B kinase was also present in all phases of the cell cycle (Song *et al.*, 2005; Song *et al.*, 2007). It is interesting to point out that in T cells, some of the molecules that are widely regarded as having a role in promoting mitosis, as is the case of survivin and aurora B, were shown not only to be expressed in all phases of the cell cycle, but to additionally promote G1 to S progression (Song *et al.*, 2005; Song *et al.*, 2007). Specifically, aurora B kinase mediated pRb phosphorylation, cyclin A induction as well as Cdk1 and Cdk2 activity upon stimulation. In addition, aurora B associates with and regulates phosphorylation of mTOR and its targets p70S6 kinase and 4E-BP1. While induction of aurora B in T cells was not dependent on mTOR, activity of aurora B was indeed affected in the absence of mTOR, as treatment of cells with rapamycin impaired its kinase activity (Song *et al.*, 2007).

As mentioned above, rapamycin blocks IL-2 induced T cell proliferation at the G1 to S phase transition step, suggesting that T cell proliferation is driven via mTOR. To assess whether aurora B was activated by IL-2R signaling, cells were stimulated with IL-2 alone in the absence of TCR signaling or IL-2 and antigen in the absence of CD28 signaling. Treatment with IL-2 alone resulted in expression and kinase activity of aurora B, phosphorylation of survivin, pRb, Cdk1 and Cdk2. These IL-2R induced events were greatly but not completely inhibited by rapamycin, suggesting that aurora B activity partially depends on mTOR. In the second case, treatment with IL-2 led to aurora B, survivin and cyclin A upregulation as well as hyperphosphorylation of pRb, and activation of Cdk1 and Cdk2. However, when these cells expressed a kinase-dead aurora B, they lost the ability to upregulate not only aurora B but also survivin, and exhibited distinctive characteristics of cells blocked in G1 to S phase transition including low cyclin A expression, weak Cdk1 and Cdk2 activities and pRb hypophosphorylation. Notably, by reconstituting aurora B activity, G1 to S phase progression was promoted. These findings point to the existence of a regulatory feedback loop between mTOR and aurora B that controls antigen-induced T cell division at the transition from G1 to S phase of the cell cycle (Song *et al.*, 2007).

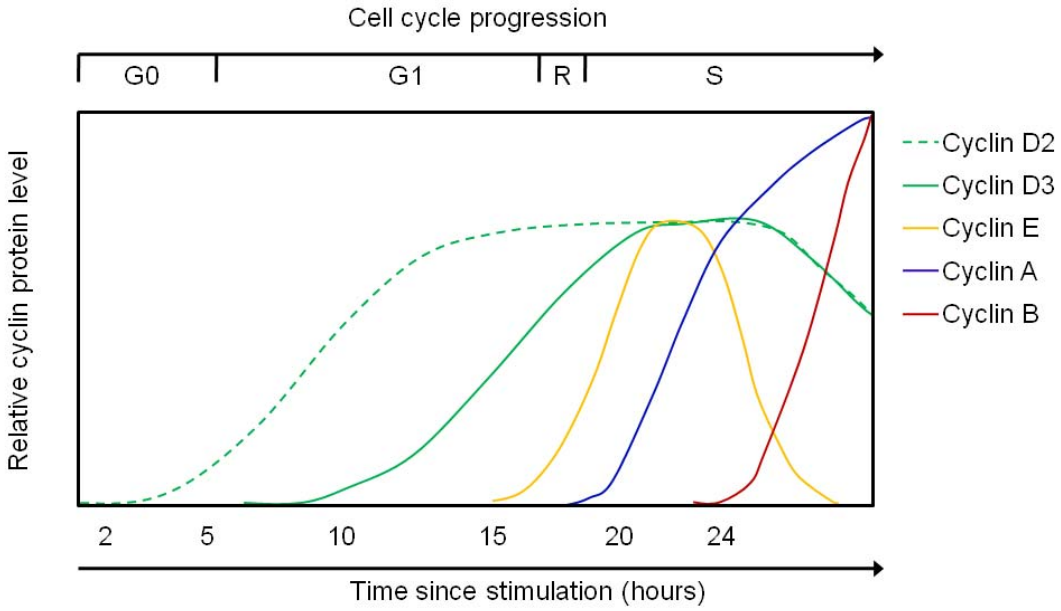
9.3. T lymphocyte proliferation

Naïve T cells represent a model of truly quiescent cells in which a G0 population must integrate extracellular signals into a proliferative response in a unique way, since entry of these cells into the cycling mode requires a combination of signals from antigen, costimulatory and growth factor receptors. The G0 state is in part maintained by the Cdk inhibitor $p27^{Kip1}$. Upon stimulation of the TCR and CD28, the cell cycle block is released and cells enter the cycling mode.

It takes about 30 hours from the moment of antigenic stimulation for them to progress from G0 to S phase, and around 36 hours have passed when they undergo the first mitosis. Nonetheless, subsequent cycles are much faster, as they only take 5 to 10 hours to complete, undergoing mitosis every 5 to 6 hours. This significant reduction in the time needed per cycle is largely due to a shorter G1 phase, allowing an earlier initiation of S-phase (Rowell and Wells, 2006). Several studies in $CD8^+$ and $CD4^+$ T cells suggest that only the first division requires signals from the TCR, while following cycles are more dependent on growth factor signaling.

Therefore, daughter cells are not subjected to the same constraints as the parent cell with regards to TCR requirements, costimulation and contact with APCs (Rowell and Wells, 2006).

In T cells cyclin D1 is not detected. Successful response to activation signaling leads to induction of the other D-type cyclins, D2 and D3. Induction of cyclin D2 mRNA starts within 2 hours from stimulation and plateaus by 24 hours, in a similar kinetic of that of IL2 mRNA. Cyclin D3 mRNA, on the other hand, is detected after 10 hours of stimulation and reaches a maximum at 20 hours post-stimulation, and has been shown to be required for proper lymphocyte development (Ajchenbaum *et al.*, 1993; Sicinska *et al.*, 2003). The protein, however, is detected at about 30 hours after stimulation. Cdk6 and Cdk4 proteins, partners of D-type cyclins, are detected as early as six hours after stimulation. Shortly after, complexes of cyclin D/Cdk6(4) begin to assemble and become active at around 20 hours after stimulation; giving rise to the transcription of cyclins E and A. Some hours later, cyclin E/Cdk2 complexes are able to phosphorylate their substrates and thus promote cell cycle progression (see **Figure 21**) (Rowell and Wells, 2006).



Adapted from Rowell and Wells, 2006.

Figure 21. Graphic representation of cyclin protein kinetics during the first cycle in T cells in response to an activating signal.

As Cdk2 activity rises, levels of $p27^{Kip1}$ (a member of the Cip/Kip family of Cdk inhibitors) are significantly reduced by 24 to 36 hours after stimulation. Levels of a fellow family member, the $p57^{Kip2}$ protein, do not appear to fluctuate in cycling lymphocytes. In contrast to $p27^{Kip1}$, the remaining member of the Cip/Kip family, $p21^{Cip1}$ is induced upon stimulation and reaches a peak of expression near or in S-phase. At the end of the proliferative response, when growth factors become limiting, levels of $p27^{Kip1}$ rise and the cell is forced out of the cycling mode. Genetic deletion of Cip/Kip proteins in mice and its effect on T cell function is summarized in **Table 2.**

Members of the INK4 family, another Cdk inhibitor family, are expressed in T lymphocytes but their regulation in response to extracellular signals has not yet been fully characterized (Rowell and Wells, 2006). Nonetheless, genetic ablation of these proteins in mice has revealed a phenotype in T cell function, as summarized in **Table 2.**

The roles of other cyclins and Cdks (such as cyclins A2, B1, Cdk1) in T cell function are far less studied. Most cyclins, Cdks and CKIs have been successfully ablated in mice and have provided specific information with regards to their contribution to T cell function. As deletion of cyclins A2, B1, E1/E2, $p57^{kip1}$ and Cdk1 are embryonic lethal, many of the studies of the role of these molecules in cell cycle regulation have been performed in MEF -when possible- or transformed cell lines (**Table 2**). Even so, these findings do not necessarily extrapolate to the immune system, as there is lineage-specific expression of some of these proteins (Rowell and Wells, 2006; Santamaria *et al.*, 2007). The following mouse models have no reported defects nor in thymus nor in periphery: cyclin $D1^{-/-}$, cyclin $D2^{-/-}$, double cyclin $D^{-/-}$, cyclin $E1^{-/-}$, cyclin $E2^{-/-}$, cyclin $A1^{-/-}$, cyclin $B2^{-/-}$, $cdk4^{-/-}$, $cdk2^{-/-}$ and $p19^{INK4d-/-}$.

Cell cycle regulator	**Phenotype in thymus**	**Phenotype in peripheral T cells**
Cyclin $D3^{-/-}$	Hypoplastic thymus	Reduced proliferation
Cyclin E Tcell Tg^{+}	Not reported	N/A Earlier S-phase entry, Greater proliferation.
$Cdk6^{-/-}$	Hypoplastic thymus	Hypoplastic spleen Reduced proliferation
$p15^{INK4b-/-}$	Not reported	Extramedullary hematopoiesis Increased proliferation Increase in $CD4^{+}$ memory cells
$p16^{INK4a-/-}$ $p16^{INK4a}$ Tg^{+}	Thymic hyperplasia Block at DN3	Increased proliferation N/A
$p18^{INK4c-/-}$	Thymic hyperplasia	Increased proliferation
$p21^{Cip1-/-}$ $p21^{Cip1}$ Tg^{+}	Not reported Thymic hypoplasia Increased thymocyte apoptosis	Increased memory cells, lupus in some strains N/A
$p27^{Kip1-/-}$ $p27^{kip1}$ Tg^{+}	Thymic hyperplasia, Increased proliferation Thymic hypoplasia, block at DN3	Increased proliferation Reduced proliferation
Cyclin D1/2/$3^{-/-}$ Cyclin E1/$E2^{-/-}$ Cyclin $A2^{-/-}$ Cyclin $B1^{-/-}$ Cdk4/$6^{-/-}$ $p57^{Kip2-/-}$	Embryonic lethal at E16.5. (Megaloblastic anemia and defective fetal hematopoiesis) Embryonic lethal E11.5 (Hematopoietic defects seen by tetraploid conformation) Embryonic lethal at implantation. Embryonic lethal at E10.5 Late embryonic lethal at E14.5-18.5. (defective fetal hematopoiesis and severe anemia) Perinatal lethal.	

Adapted from Rowell and Wells, 2006.

Table 2. Cell cycle regulators in T cell function: Transgenic and knockout phenotypes.

As briefly depicted above, proper T lymphocyte cell cycle regulation is of pivotal importance for the physiological events involved in their cellular function, including thymic development, homeostatic cell division, antigen-driven responses, mature T lymphocyte proliferation, T cell memory and peripheral tolerance (Rowell and Wells, 2006). Thus, the role of cell cycle regulators and the signaling pathways lying upstream of these molecules is a matter in need of further detailed studies in order to achieve a better understanding of T cell function.

OBJECTIVES

OBJECTIVES

NFAT5 induces an osmoprotective gene expression program that allows mammalian cells to adapt to hypertonic conditions. Cellular changes caused by hypertonicity are numerous and may include DNA damage, cell cycle delay and apoptosis. The study of the role of NFAT5 in specific cell types has been hindered by the severe phenotype of NFAT5-deficient mice, since only a small proportion survive after birth, and those that do manifest pronounced renal atrophy and growth defects (Go *et al.*, 2004; Lopez-Rodriguez *et al.*, 2004). To circumvent these problems, we generated NFAT5$^{Flox/Flox}$ mice which could be used to inactivate NFAT5 in a tissue-specific manner. We have crossed the NFAT5$^{Flox/Flox}$ mice to CD4-Cre animals to obtain mice with a selective deletion of NFAT5 in mature T cells, and analyzed the role of this factor in the hypertonic stress response in proliferating T lymphocytes. As shown by us and others, primary, non-transformed T cells regulate NFAT5 comparably to other cell types (Morancho *et al.*, 2008) and display impaired proliferative capacity under hypertonic conditions when lacking this factor (Go *et al.*, 2004). We thus set the following objectives:

General objective

- To study the role of NFAT5 in regulation of the cell cycle and DNA-damage response in mammalian cells exposed to hypertonic conditions.

Specific objectives

- To characterize the role of NFAT5 in the proliferation and survival of primary lymphocytes upon exposure to hypertonic stress and DNA damage.

- To characterize the molecular mechanisms regulated by NFAT5 that impinge on primary T cells exposed to hypertonic shock and pathologic hypertonic conditions.

MATERIALS AND METHODS

MATERIALS AND METHODS

Chemicals. Hoechst 33342 was purchased from Sigma (Steinheim, Germany). A stock solution of 10 mg/ml was prepared, aliquoted and stored at -20ºC. Phorbol 12-myristate 13-acetate (PMA) and the calcium ionophore ionomycin were purchased from Calbiochem (Darmstadt, Germany). LY294002 and SB203580 were purchased from Calbiochem (Darmstadt, Germany), reconstituted at 10 mM in DMSO (Sigma), and stored at -20°C protected from light. Phorbol 12-myristate 13-acetate (PMA) and ionomycin were from Calbiochem (Darmstadt, Germany).

Generation of NFAT5 conditional mice. A targeting vector was designed to flank exon 6 of the *NFAT5* gene with two *loxP* sites (**Figure 1A**). A BamHI fragment of the mouse *NFAT5* genomic locus isolated from a P1 clone was used. A 3.3-kb ApaLI-AvrII fragment was used as 5' homology region, and a 4.8-kb EcoRI-XbaI fragment was used as 3' homology region. One *loxP* site was introduced 5’ to exon 6, in the 1.8-kb AvrII-EcoRI fragment. An *frt* site-flanked selection cassette, with a neomycin resistance gene, the Flpe cDNA cloned under control of the ACE promoter (Sasaki *et al.*, 2004) and the *loxP* site, was inserted into an EcoRI site in the sixth intron of the *NFAT5* gene. The targeting vector also contained a thymidine kinase gene that was used for negative selection of clones with random integration of the targeting vector.

Bruce-4 embryonic stem (ES) cells (Kontgen *et al.*, 1993) derived from C57BL/6 mice were transfected, cultured, and selected as previously described (Lopez-Rodriguez *et al.*, 2004). Of 800 G418 (neomycin) and gancyclovir-resistant colonies, 3 were identified as homologous recombinants with cointegration of the second *loxP* site by Southern blot analysis of BamHI-digested DNA, using a probe spanning the exon 5 as 5' external probe (Fig. 1A) and Neo as a 3' probe. ES clones with the appropriately targeted allele were injected into BALB/c blastocysts to generate chimeric mice, which transmitted the targeted allele to their progeny. All mice were maintained on a pure C57BL/6 genetic background. The *frt*-flanked neomycin resistance cassette was removed through intercrossing with FLPe-deleter mice (Rodriguez *et al.*, 2000). Mice lacking NFAT5 in T cells were obtained afer successive crosses of $NFAT5^{Flox/Flox}$ mice with CD4-Cre transgenic mice, in which the Cre recombinase is under the control of the mouse CD4 promoter/enhancer/silencer (Lee *et al.*, 2001). Mice were bred and maintained in specific pathogen-free conditions, and animal handling was performed according to institutional guidelines approved by the ethical committee (PRBB Animal Care and Use Committee). The

CD4-Cre transgenic mouse strain (Lee *et al.*, 2001) was obtained from the Jackson Laboratory (Bar Harbor, ME).

9xNFAT-Luc mice and p53$^{-/-}$ mice. 9xNFAT-Luc mice (line 15.1) in FVB background were previously described (Wilkins *et al*., 2004; Morancho *et al*., 2008). p53$^{-/-}$ mice were obtained from the Jackson Laboratory and have been previously described (Jacks *et al.*, 1994).

Antibodies. The anti-NFAT5 polyclonal antibody (Cat. PA1-023) was from Affinity Bioreagents (Golden, CO, USA) and recognizes a C-terminus epitope (DLLVSLQNQGNNLTGSF). The anti-NFAT5 polyclonal antibody recognizing the N-terminal region of NFAT5 was previously described (Lopez-Rodriguez *et al*., 1999). Rabbit polyclonal anti-phospho-p53 (Ser15) (Cat. 9284), mouse monoclonal anti-p53 (Cat. 2524) and mouse monoclonal anti-cyclin D3 (Cat. 2936) were from Cell Signaling Technology (Danvers, MA, USA); mouse monoclonal anti-phospho-histone H2AX (Ser139, γH2AX) (Cat. 05-636) was purchased from Upstate Technologies (Lake Placid, NY, USA); mouse monoclonal anti-BrdU antibody (Cat. 555627) was purchased from BD Pharmingen (San Diego, CA, USA). The anti-NFAT1 antibody (anti-NFAT1-C) has been described (Wang *et al.*, 1995). The surface marker-specific antibodies anti-CD3-PE (Cat. 553064), anti-Thy1-PE (Cat. 553090) and anti-B220-PE (Cat. 553006) were from BD Biosciences. Anti-cyclin A2 (Cat. Sc-751), anti-cyclin B1 (Cat. Sc-245) anti-cyclin E1 (Cat. sc-481) and anti p21 (Cat. sc-397) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Hamster anti-mouse CD3 (Cat. 553058) and hamster anti-mouse CD28 (Cat. 553295) were from BD Biosciences; goat-anti hamster IgG was from MP Biomedicals (Cat. 55397). Goat anti-pyruvate kinase (AB1235) was purchased from Chemicon (Hampshire, UK). Anti-β-actin was purchased from Sigma (Cat. A5441)**.** FITC-labeled anti-mouse IgG (Cat. F0313), FITC-labeled anti-rabbit IgG (Cat. F0054), and HRP-labeled anti-goat IgG (Cat.P010.60) were from DAKO (Glostrup, Denmark). HRP-labeled anti–mouse IgG (Cat. NA931V), and HRP-labeled anti-rabbit IgG (Cat. NA934V) were from Amersham (Buckinghamshire, UK). Anti-Hsp70 (Cat. SPA-810) was purchased from Stressgen (Ann Arbor, MI, USA).

Lymphocytes. 9xNFAT-Luc mice, p53$^{-/-}$ mice and NFAT5 conditional mice were bred and maintained under specific pathogen-free conditions, and handled according to institutional guidelines (PRBB Animal Care and Use Committee). Primary mouse T cells were obtained from spleens of CD4-Cre^{+} / NFAT5$^{Flox/Flox}$ mice (hereafter abbreviated as NFAT5$^{-/-}$), littermate

CD4-Cre^{-} / NFAT5$^{Flox/Flox}$ mice (hereafter abbreviated as NFAT5$^{+/+}$ or wild-type), 9xNFAT-Luc mice, p53$^{+/+}$ and p53$^{-/-}$ mice of 8-12 weeks of age. We also confirmed that NFAT5-expressing T cells derived from CD4-Cre^{-} / NFAT5$^{Flox/Flox}$ or CD4-Cre^{+} / NFAT5$^{+/+}$ mice were indistinguishable in their response to the stresses tested (not shown). Splenocytes were isolated by density gradient centrifugation with Lymphoprep^{TM} (Axis-Shield PoC AS, Oslo, Norway). Proliferating T cells were obtained by activating splenocytes (2.5 x 10^6 cells/ml) with 2.5 μg/ml concanavalin A (Cat.C-2010, Sigma. Steinheim, Germany) plus 25 ng/ml recombinant human IL-2 (Proleukin; Chiron, formerly Eurocetus. Amsterdam, The Netherlands) during 72 hours in culture medium (Dulbecco's Modified Eagle Medium (DMEM, Gibco. Pasley, UK)), supplemented with 10% fetal bovine serum (Cat. CH30160.03 Hyclone, Logan, UT, USA), non-essential amino acids (Gibco), 2 mM L-glutamine (Gibco), 50 μM beta-mercaptoethanol (Gibco), 1 mM sodium pyruvate (Gibco) and antibiotics penicillin and streptomycin (Gibco). Cultures were then cleaned of dead cells and debris by centrifugation on Lymphoprep^{TM}, washed and replated in fresh medium supplemented with IL-2 for an additional 24 hours, after which both wild-type and conditional knockout cultures had >95% CD3^{+}, TCRβ^{+} T cells. For **Figures 39 and 40**, fresh splenocytes were induced to proliferate as described above, or stimulated with hamster anti-CD3 (1 μg/10^6 cells) plus hamster anti-CD28 (1μg/10^6 cells) antibodies and seeded onto goat-anti hamster IgG coated plates (0.15 μg/μl). Cells were either grown in isotonic or moderately hypertonic media as indicated in figure legends. Before cell lysing was performed for protein and mRNA analysis, samples were depleted of remaining B-cells by incubation with sheep anti-mouse IgG magnetic beads (Dynal Biotech, Cat. 110.31). Proliferating B cells were obtained by culturing splenocytes (2 x 10^6 cells/ml) with 25 μg/ml of lipopolysaccharide (LPS, Cat. L7261, Sigma) during 7 days.

Cell culture. The human embryonic kidney fibroblast HEK293 cell line, the T cell line Jurkat (Clone E6-1, American Type Culture Collection, #TIB 152, kindly provided by Dr. Jeremy Luban (Columbia University College of Physicians and Surgeons, New York, NY)) and Hsp70.1/3$^{-/-}$ MEFs (kindly provided by Dr. T.K Pandita (Washington University School of Medicine, St. Louis, MO) (Hunt *et al.*, 2004)) were maintained in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 2mM L-glutamine, 1mM sodium pyruvate, and 50 μM β-mercaptoethanol (Gibco, Pasley, UK).

DNA constructs

NFAT5-activated luciferase reporter plasmid ORE-Luc was previously described (Lopez-Rodriguez *et al*., 2001).The luciferase reporter 9xNFAT-Luc was previously described (Wilkins *et al*., 2004; Morancho *et al*., 2008). Cyclin A2-862-Luc was kindly provided by Dr. J.B.P Viola (Division of Cellular Biology, National Cancer Institute (INCA); Rio de Janeiro, Brazil) and has been described (Carvalho *et al*., 2007). Cyclin B1 reporter plasmid was kindly provided by Dr. A. Gewirtz (University of Pennsylvania, Philadelphia, USA) and has been described (Nakata *et al*., 2007). The transfection control plasmid TK-Renilla was from Promega. The GFP-specific shRNA in the pBSU6 vector was previously described (Sui *et al.*, 2002), and the two NFAT5-specific shRNAs were done by inserting the following 21-nucleotide sequences complementary to NFAT5 mRNA in pBSU6: shNFAT5-1, 5'-GGT CAA ACG ACG AGA TTG TGA-3'; and shNFAT5-3, 5'-GGT CGA GCT GCG ATG CCC TCG-3'.

Transfections and reporter assays.

Jurkat T cells (20 x 10^6 cells/ 400 μl serum-free DMEM) were transfected by electroporation (Bio-Rad Gene Pulser. Bio-Rad, Hemel Hampstead, UK) with luciferase reporter plasmids (60 ng/10^6 cells), TK-Renilla (0.1 μg/10^6 cells) and corresponding shRNA (1.8 μg/10^6 cells) as indicated in figure legends and has been previously decribed (Minguillon *et al*., 2005). 36 hours post-transfection, cells were placed in fresh isotonic (300 mOsm/kg) media and stimulated with either 20 nM PMA plus 1 μM ionomycin or subjected to hypertonic conditions as indicated in figure legends. 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 Renilla and endogenous lactate dehydrogenase (LDH), which was proportional to the number of viable cells (Minguillon *et al.*, 2005). Luciferase activity in transgenic cells was normalized to endogenous LDH in the same lysate, measured with the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega).

HEK293 cells were transfected with 100 ng/10^6 cells of the NFAT5-activated luciferase reporter plasmid ORE-Luc (Lopez-Rodriguez et al, 2001) and 250 ng/10^6 cells of pTK-Renilla plasmid (Promega, Madison, WI, USA), using a calcium phosphate protocol (Flemington and Rodriguez, 2004). 36 hours after transfection, cells were subjected to hypertonic stress by addition of NaCl or irradiated as indicated. Cells were harvested 24 hours later, and luciferase activity in cell lysates was measured with the Dual luciferase reporter assay system (Promega). Luciferase

values were normalized to Renilla and lactate dehydrogenase (LDH) to account for cell viability (Minguillon *et al.*, 2005) using the Cytotox-96 assay (Promega).

Hypertonic stress and ionizing radiation. All experiments using T cells were done with proliferating lymphocytes. The osmolality of the culture medium was measured in a Fiske ONE TEN osmometer (Fiske Associates. Norwood, MA, USA). Since the T cell medium with supplements had an osmolality of 330 mOsm/kg, we adjusted it to an isotonic baseline of 300 mOsm/kg by adding 10% sterile H_2O (Milli-Q Biocel A10. Millipore, Bedford, MA, USA). This medium was made hypertonic by adding NaCl from a sterile 4 M stock solution (addition of 100 mM NaCl increased the tonicity to 500 mOsm/kg). Before subjecting T cells to stress conditions (hypertonicity and/or radiation), non-viable cells and debris in the cultures were first removed by centrifugation on a Lymphoprep^{TM} cushion. Then, T cells were adjusted to 0.5 x 10^6 cells/ml in medium supplemented with 25 ng/ml IL-2, and cultured under isotonic or hypertonic conditions as indicated in the figure legends. When kinase inhibitors LY294002 (25 μM) and SB203580 (10 μM) were used, cells were pretreated with them in isotonic medium 1 hour before increasing the tonicity. For ionizing radiation experiments, cells were exposed to 1, 3 or 10 Gy using a ^{137}Cs IBL 437C type H irradiator (CIS Biointernational. Gif Sur Yvette, France), and then placed (0.5 x 10^6 cells/ml) in fresh isotonic or hypertonic medium supplemented with IL-2. HEK293 cells were plated at 3 x 10^4 cells/cm^2 at the beginning of the respective treatment.

Heat shock. Cells were exposed to 43ºC for 30 minutes and allowed to recuperate at 37ºC for 2 to 24 hours, as indicated in figure legends.

Flow cytometry. Flow cytometry analysis of cell cycle distribution, DNA replication, and intracellular staining of γH2AX and phosphorylated p53, were done in cells gated as viable by FSC/SSC. Flow cytometry was done with a BD LSR flow cytometer (Becton Dickinson. San Diego, CA, USA) unless otherwise indicated.

- **Viability, apoptosis, and cell cycle analysis.** For viability and cell cycle analysis, cells were labeled during the last hour of culture with the DNA dye Hoechst 33342 (5 μg/ml, 60 minutes in incubator at 37ºC with 5% CO_2). For the determination of apoptosis, cells were first labeled with Hoechst 33342 and then stained with annexin-V Fluos (Roche. Manheim, Germany) during 30 minutes. Simultaneous analysis of forward and side

scatter parameters (FSC/SSC), together with DNA content and annexin-V staining showed that non-viable cells, that had DNA content < 2N, and were annexin-V^+ in their majority, could be readily identified by their distinct position in the FSC/SSC plots. The proportion of live cells was determined after substracting the population of non-viable cells in the FSC/SSC plots. Flow cytometry analysis of cell cycle distribution, DNA replication, and intracellular staining of γH2AX and phosphorylated p53, were done in cells gated as viable by FSC/SSC.

- **Determination of DNA replication by BrdU incorporation**. Cells were pulse-labeled during 30 min (in a 37ºC, 5% CO_2 incubator) with 10 μM BrdU (Cat. B5002, Sigma) and then fixed in 70% ethanol. BrdU was detected with a monoclonal mouse anti-BrdU antibody after acidic denaturation following the protocol supplied by the manufacturer (BD Pharmingen™). Labeled cells were then stained with propidium iodide in RNase-containing solution to simultaneously analyze DNA replication and cell cycle.

- **Cell sorting**. Cells were labeled with Hoechst 33342 as indicated above, sorted with a FACSvantage flow cytometry system (BD Biosciences) according to their cell cycle phase; G0/G1, S or G2/M, collected and immediately lysed.

- **Intracellular detection of γH2AX and phospho-p53 (Ser-15).** Cells were labeled following the intracellular staining protocol previously described (Huang and Darzynkiewicz, 2006). Briefly, cells were fixed in 1.5% paraformaldehyde (Sigma) on ice for 15 minutes, and permeabilized with 70% ethanol at -20°C for at least 2 hours. Ethanol was removed by centrifugation and two washes with PBS, and cells were incubated with mouse monoclonal anti-γH2AX antibody (1 μg/10^6 cells) or rabbit polyclonal anti-phospho-p53 (Ser15) (1 μg/10^6 cells) for 2 hours. Bound primary antibodies were detected by incubating cells with FITC-labeled secondary antibodies for 1hour at room temperature and protected from light. DNA was stained with 5 μg/ml Hoechst 33342 for 30 minutes at room temperature.

- **Surface marker labeling**. 2x10^5 cells were blocked for 20 minutes in 1x PBS containing 3% fetal calf serum (FCS), 0.1% sodium azide, and 0.2 μg anti-FCγ receptor antibody (BD Biosciences, Cat. 553142). Cells were then incubated with surface marker-specific

antibodies in the same solution (1 μg of antibody for $1x10^6$ cells) and analyzed with a FACScan flow cytometer (Becton Dickinson).

Comet assay. Alkaline comet assay to visualize DNA damage was done using the Trevigen kit (catalog 4250-050-K), according to the manufacturer's instructions (Trevigen, Gaithersburg, MD, USA). In order to assess DNA breaks only in the population of live cells, dead cells were removed by centrifugation on a Lymphoprep™ cushion and excluded from the assay.

Protein sample preparation and Western blot Analysis. Cells were lysed (30 minutes at 4ºC) in 50 mM HEPES (pH 7.4), 80 mM NaCl, 5 mM $MgCl_2$, 10 mM EDTA, 1% Triton X-100, 5 mM sodium pyrophosphate, 20 mM β-glycerophosphate, and protease inhibitors PMSF, leupeptin (Cat.L2884. Sigma), aprotinin (Cat.236624. Roche), and pepstatin A (Cat. P5318. Sigma). Lysates were cleared by centrifugation (15,000g, 15 minutes, 4ºC) and the protein concentration in the supernatants was determined using the BCA Protein Assay (Cat. 23227. Pierce, Rockford, IL, USA). Equal amounts of protein from each sample were separated in SDS-polyacrylamide gels under reducing conditions, transferred to PVDF membranes (Immobilon-P. Millipore, Bedford, MA, USA), and detected with specific primary antibodies followed by HRP-labeled secondary antibodies and enhanced chemiluminescence (Supersignal West Pico Chemiluminescent Substrate, Pierce). Pyruvate kinase or β-actin were used as protein loading control.

Real-time quantitative PCR (RT-qPCR). Total RNA was isolated using the RNeasy kit following manufacturer's instructions (Quiagen, Cat. 74104). 2-3 μg of total RNA was retro-transcribed to cDNA using SuperScript III reverse transcriptase and random primers (Invitrogen). For real-time quantitative PCR (RT-qPCR), Power SYBR Green PCR master mix (Applied Biosystems, Cat. 4367659) and an ABI7900HT sequence detection system (Applied Biosystems) were used following the instructions provided by the manufacturers. Samples were normalized to L32 mRNA levels using the ABI Prism SDS 2.1 software. Primer sequences for the PCR reactions were: 5'-CAG CCA AAA GGG AAC TGG AG-3' (Forward) and 5'-GAA AGC CTT GCT GTG TTC TG-3' (Reverse) for NFAT5; 5'-ACC AGT CAG ACC GAT ATG TG-3' (Forward) and 5'-ATT GTG GAC CAG GAA CTT GC- 3' (Reverse) for L32; 5'-CTT CTA CAC ATC CAT CAC GC-3' (Forward) and 5'-TTG AAG AAG TCC TGC AGC AG- 3' (Reverse) for HSP70.1; 5'-ATG GTT GTC ATC AGC ATA GCA TGG-3' (Forward) and 5'-GGT GGT GTG

AGA AGA CTA ACA ATC-3' (Reverse) for mouse SMIT; 5'-TAC TAT GCA GCT AGT GGT GTA TGC-3' (Forward) and 5'-ACC TGG TCC TAT GAG AAT CTA ACG-3' (Reverse) for mouse TauT; 5' TGA GCT GTG CCA AAC ACA AG-3' (Forward) and 5'-GGA AGA AAC ACC TTG GCT AC-3' (Reverse) for mouse aldose reductase (AR); 5'-CTG GAC TCT TCA CAC AGA TG-3' (Forward) and 5'-CAT CCA CAC TTG CTC ACA AC- 3' (Reverse) for cyclin E1; 5'-GAC CAA GAG AAT GTC AAC CC-3' (Forward) and 5'-CAT CGT TTA TAG GAA GGT CC- 3' (Reverse) for cyclin A2; 5'-AGT TAC TGC TGC TTC CAA GC-3' (Forward) and 5'-GGT AGG GCT TTA ACA GTA CC- 3' (Reverse) for cyclin B1; 5'-AAG AGT CGG ACC TTC GAT GA-3' (Forward) and 5'-CTC CCT GCA GAC CTA ACA GC- 3' (Reverse) for Aurora B kinase; 5'-AGA AGA CCG AAA GGA TGG AC-3' (Forward) and 5'-GAT GTT GAT GTC GTT CTC GC- 3' (Reverse) for Gadd45α; 5'-CTG CTG CGA CAA TGA CAT TG-3' (Forward) and 5'-GAC CCA TTG GTT ATT GCC TC- 3' (Reverse) for Gadd45β; 5'-TGT TCG TGG ATC GCA CAA TG-3' (Forward) and 5'-CTC ATC TTC TTC ATC GGC AG- 3' (Reverse) for Gadd45γ.

Statistical Analysis. Mean, standard deviation, standard error of the mean (SEM) and statistical significance (t-Student test) were calculated using Microsoft Excel software.

RESULTS

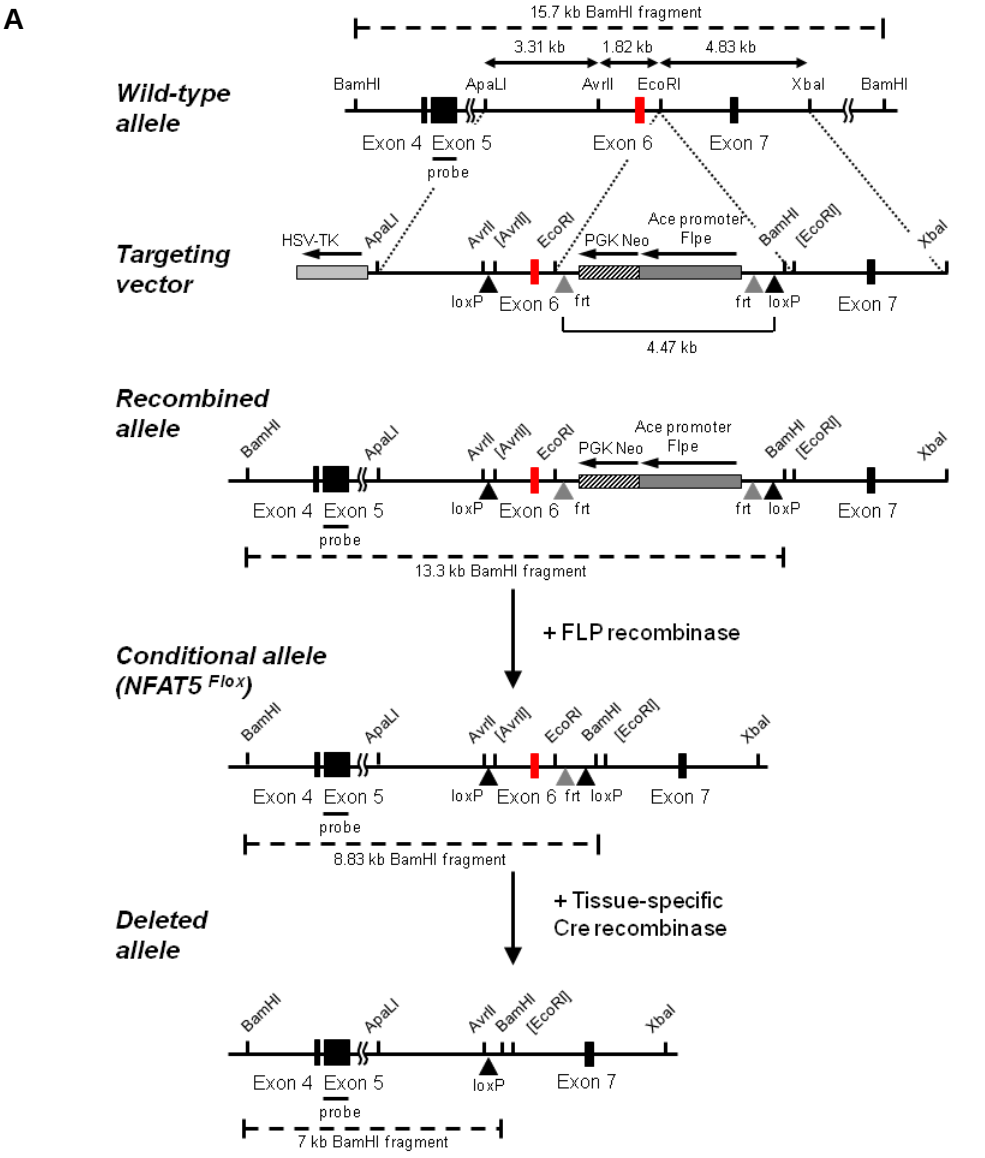
RESULTS

1. Characterization of NFAT5-conditional knockout mice.

Previous studies showed that NFAT5 is regulated in T lymphocytes in a similar manner as in other cell types (Lopez-Rodriguez *et al.*, 2001; Morancho *et al.*, 2008). Moreover, NFAT5-deficient T cells displayed reduced survival capacity with respect to wild-type ones when exposed to hypertonic stress (Go *et al.*, 2004; Trama *et al.*, 2002). As described earlier (see section 9 of Introduction), primary lymphocytes are sensitive to pathologic osmolality levels reported to occur in hypernatremic disorders and animal models that have osmoregulatory defects. Since T cells have the ability to proliferate in response to mitogens or TCR stimulation, we used lymphocytes to study the effect of osmotic stress and the role of NFAT5 in the regulation of the cell cycle. To overcome the severe viability problems encountered with NFAT5-null mice (Go *et al.*, 2004; Lopez-Rodriguez *et al.*, 2004), we generated NFAT5-conditional knockout mice. $NFAT5^{Flox/Flox}$ mice had exon 6 of the *NFAT5* gene, which encodes the DNA binding loop, flanked by *loxP* sites so that it could be deleted by the Cre recombinase expressed in a cell type-specific fashion (See **Figure 22** and materials and methods). These mice were crossed to CD4-Cre transgenic animals (Lee PP *et al.*, 2001), in which Cre is induced during the double positive stage of thymocyte development after cells have rearranged the T cell receptor. T cells from mice with the CD4-Cre^{+} /$NFAT5^{Flox/Flox}$ genotype will be hereafter referred as $NFAT5^{-/-}$.

Lack of NFAT5 in T cells from these mice was confirmed by Western blot analysis with two independent antibodies; one against a C-terminal epitope and another raised against the N-terminal region (**Figure 22D**). As seen in these experiments, and as confirmed by the analysis of NFAT5 mRNA by RT-qPCR with specific primers for NFAT5 exon 6 (**Figure 22D**), NFAT5 was deleted in > 90% of the T cells, although a small proportion of them (below 10%) escaped deletion in some mice. In contrast to NFAT5-null mice (Lopez-Rodriguez *et al.*, 2004), $NFAT5^{-/-}$ knockout mice did not differ from their wild-type littermates in body weight (**Figure 22F**). With regard to the spleen, wild-type and conditional knockout mice displayed comparable spleen weight and cell number, as shown below in **Figure 22F**. To further characterize $NFAT5^{-/-}$ conditional T cells, expression of cell surface receptors was analyzed in spleens of wild-type and NFAT5-deficient mice. NFAT5-deficient mice had a comparable proportion of splenic B and

T cells (**Figure 22F**), and showed no differences in the percentages of TCRα/β, γ/δ, CD4 and CD8 T cell subsets in lymphoid organs (R. Berga and C. Lopez-Rodriguez, unpublished results).



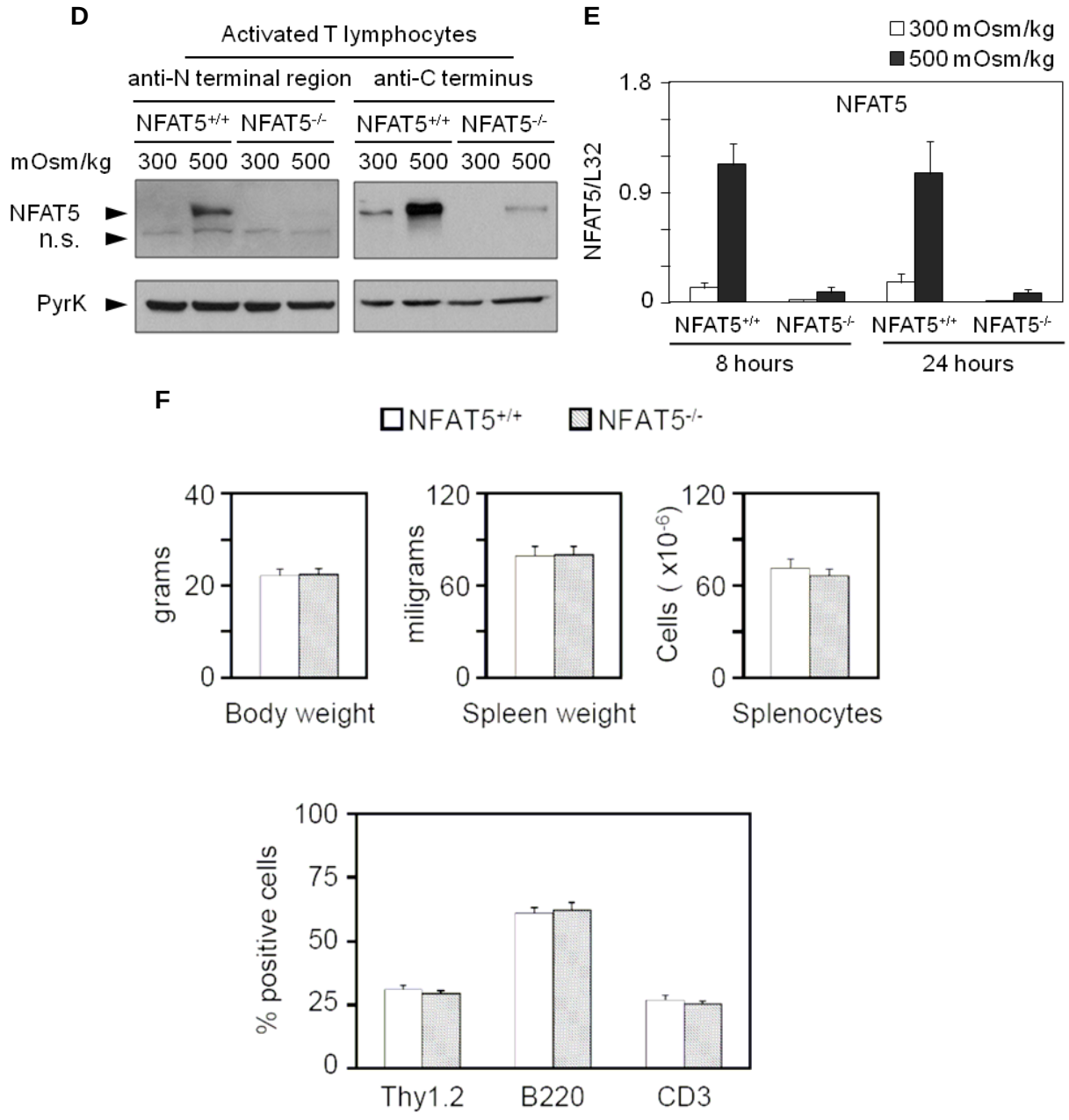
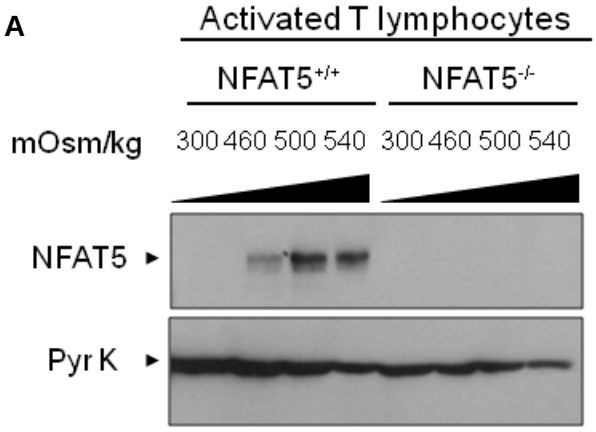


Figure 22. Generation of NFAT5-conditional knockout mice. ***A)*** *Schematic representation of the targeting construct, in which Exon 6, encoding the DNA binding loop in the DBD of NFAT5, was flanked by loxP sites. The vector contained an frt-flanked neomycin-resistance cassette (Neo) inserted at the EcoRI site downstream of Exon 6 and upstream of the 3' loxP site. Restriction sites in brackets indicate that they were inactivated during subcloning. Mouse ES clones with the correctly recombined allele were used to generate mice that were crossed to FLPe-deleter mice to produce NFAT5-floxed mice, without the Neo cassette, and with Exon 6 flanked by loxP sites so that it could be removed by the Cre recombinase*. ***B***) *Southern blot of genomic DNA extracted from T cells of wild-type (WT), NFAT5-floxed (Flox) mice, and mice obtained after crossing them with CD4-Cre mice (CD4-Cre^{+}). Genomic DNA was*

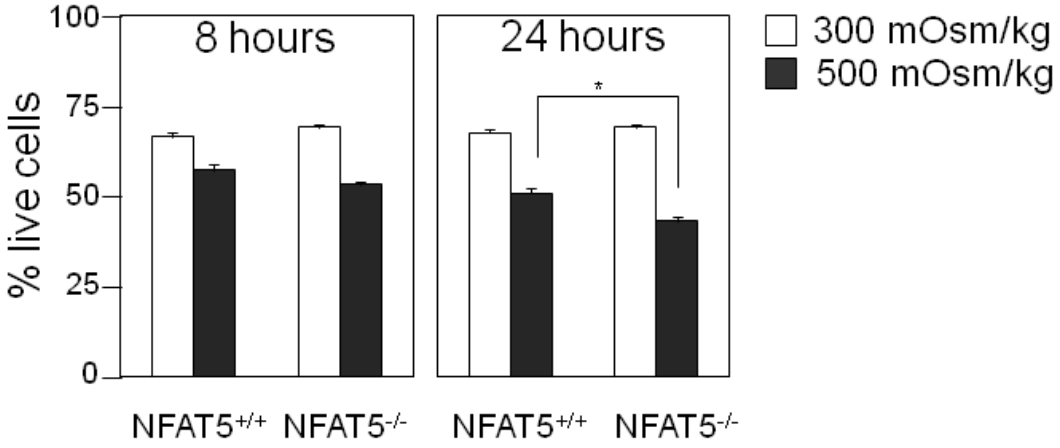
digested with BamHI and hybridized to a probe derived from Exon 5. ***C****) Specific deletion of NFAT5 in T cells, but not in B cells of CD4-Cre^{+} / NFAT5$^{Flox/Flox}$ mice was confirmed by Western blotting with an antibody against a C-terminal epitope. B and T cell-enriched populations were obtained after 4-day culture of splenocytes with mitogens (LPS for B cells, and concanavalin A + IL-2 for T cells). The non-specific crossreacting band (n.s.) above NFAT5 serves as a loading control.* ***D****) Western blot detecting NFAT5 in activated T cells was performed with two different antibodies, specific for a C-terminal epitope and the N-terminal region respectively.* ***E****) NFAT5 mRNA induced by hypertonicity in proliferating T cells was analyzed by RT-qPCR (bars represent the mean of five independent experiments ± SEM).* ***F****) Upper panel: weight of mice and spleens, and splenocyte count after Lymphoprep^{TM} gradient separation (n=8, bars are mean ± SEM). Bottom panel; expression of surface markers CD3, B220 and Thy1.2 in fresh splenocytes was analyzed by flow cytometry (n=5, values are mean ± SEM).*

2. Decreased viability and cell cycle arrest in proliferating NFAT5$^{-/-}$ T cells exposed to hypertonicity.

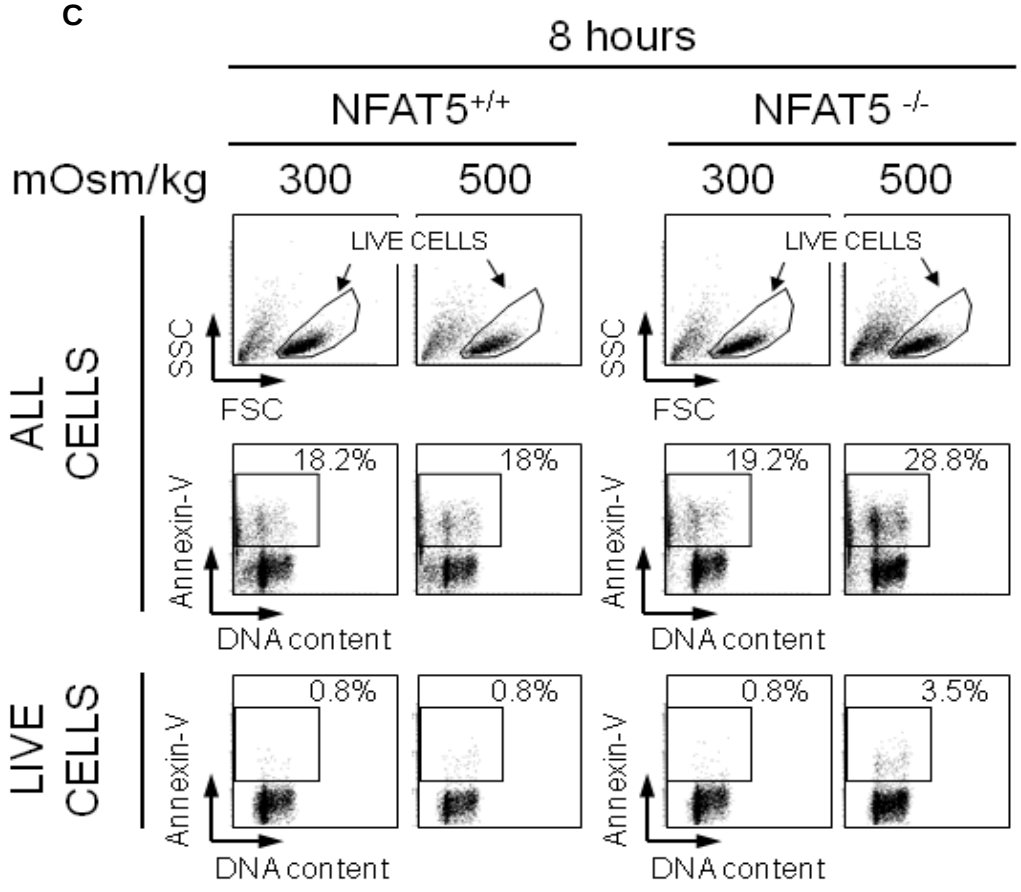
We thus sought to analyze the role of NFAT5 in response to hypertonicity in proliferating lymphocytes isolated from NFAT5$^{-/-}$ mice. Proliferating T cells (>95% CD3^{+}, TCRβ^{+}) were obtained by culturing splenocytes with the T cell mitogen concanavalin A in the presence of IL-2. T cells were then cultured during 8 or 24 hours in isotonic medium (300 mOsm/kg), or subjected to hypertonic conditions (500 mOsm/kg) that promoted maximal induction of NFAT5 (**Figure 23A**). Mitogen-stimulated NFAT5$^{-/-}$ T cells proliferated indistinguishably from wild-type cells in isotonic conditions (see **Figures 23B, 23C, 23D and 24A**), in agreement with previous work (Go *et al.*, 2004). As shown in **Figure 23B**, both wild-type and NFAT5$^{-/-}$ cells had the same viability in isotonic medium and underwent a moderate loss of viability after 8 hours in hypertonic conditions (**Figure 23C**). By 24 hours though, NFAT5$^{-/-}$ cultures exhibited a greater extent of apoptosis and loss of viability (~45%) than wild-type cells (~26%) (**Figures 23B and 23D**).



B


8 hours
24 hours
300 mOsm/kg
500 mOsm/kg
% live cells
100
75
50
25
0
*
NFAT5+/+
NFAT5-/-
NFAT5+/+
NFAT5-/-

C


8 hours
NFAT5+/+
NFAT5 -/-
mOsm/kg
300
500
300
500
ALL CELLS
LIVE CELLS
SSC
FSC
LIVE CELLS
18.2%
18%
19.2%
28.8%
Annexin-V
DNA content
0.8%
0.8%
0.8%
3.5%

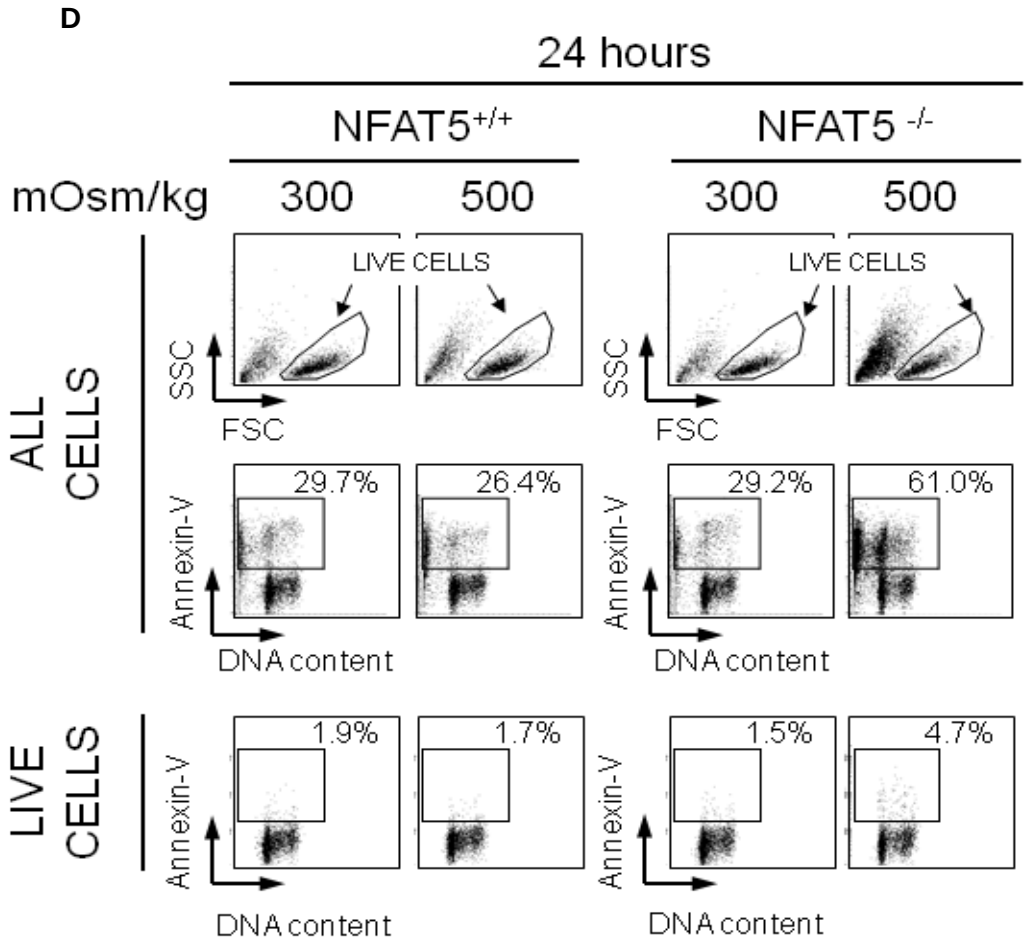
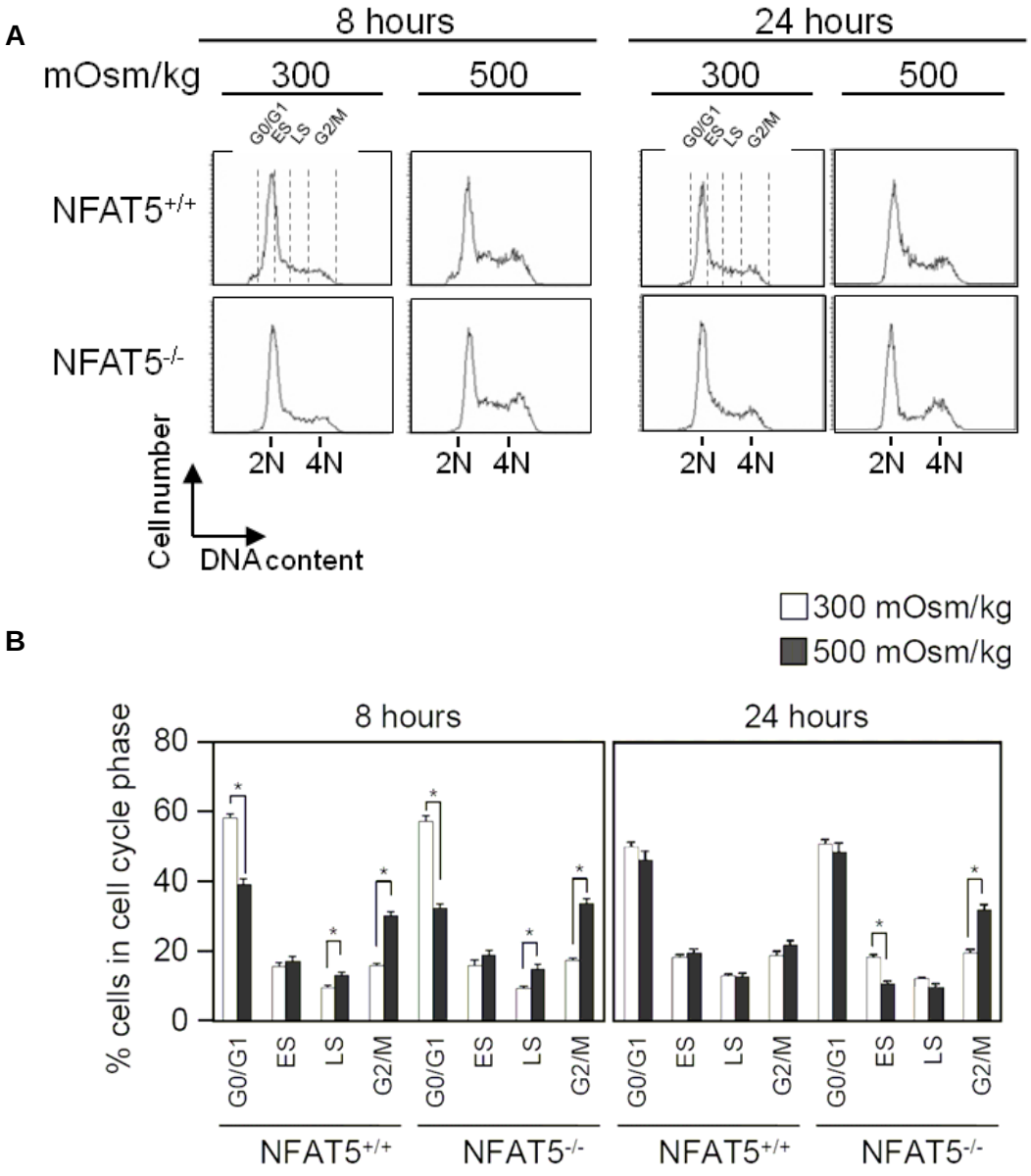


Figure 23. Cellular viability of cells exposed to hypertonic stress. ***A)*** *Induction of the expression of NFAT5 by increasing levels of hypertonicity in NFAT5$^{Flox/Flox}$ (NFAT5$^{+/+}$) and CD4-Cre^{+} / NFAT5$^{Flox/Flox}$ lymphocytes (NFAT5$^{-/-}$) was detected by Western blot with an anti-NFAT5 (C-terminus epitope) antibody.* ***B)*** *T cells grown under isotonic (300 mOsm/kg) or hypertonic conditions (500 mOsm/kg) during 8 or 24 hours were stained with the DNA dye Hoechst 33342 and analyzed by flow cytometry. Viability was assessed by FSC/SSC parameters (as illustrated in C).T cells grown in isotonic or hypertonic conditions during 8 hours (**C**) or 24 hours (**D**) were stained with the DNA dye Hoechst 33342 and annexin-V-Fluos and analyzed by flow cytometry. The experiment shown is representative of three independently performed (* = p < 0.05).*

Analysis of the cell cycle in the population of viable cells revealed that hypertonicity caused a similar arrest at 8 hours in both wild-type and NFAT5$^{-/-}$ lymphocytes, with enhanced accumulation in S and G2/M relative to G0/G1 (**Figure 24A and 24B**). However, whereas wild-type cells recovered from the arrest by 24 hours, NFAT5$^{-/-}$ lymphocytes did not, and exhibited a decreased proportion of cells in early S phase and an increase in the proportion of cells in G2/M

with respect to isotonic conditions (**Figure 24A and 24B**). BrdU-pulse experiments showed that the percentage of replicating cells in NFAT5$^{-/-}$ cultures after 24 hours in hypertonic medium was ~ 50% lower than in isotonic conditions, and the proportion of cells in G2/M increased 3.7 times. In contrast, wild-type cells maintained in hypertonic medium for 24 hours had resumed an apparently normal cycle after the initial arrest (**Figure 24C and 24D**). These results indicated that NFAT5$^{-/-}$ cells displayed ineffective replication and cell cycle progression under hypertonic conditions.



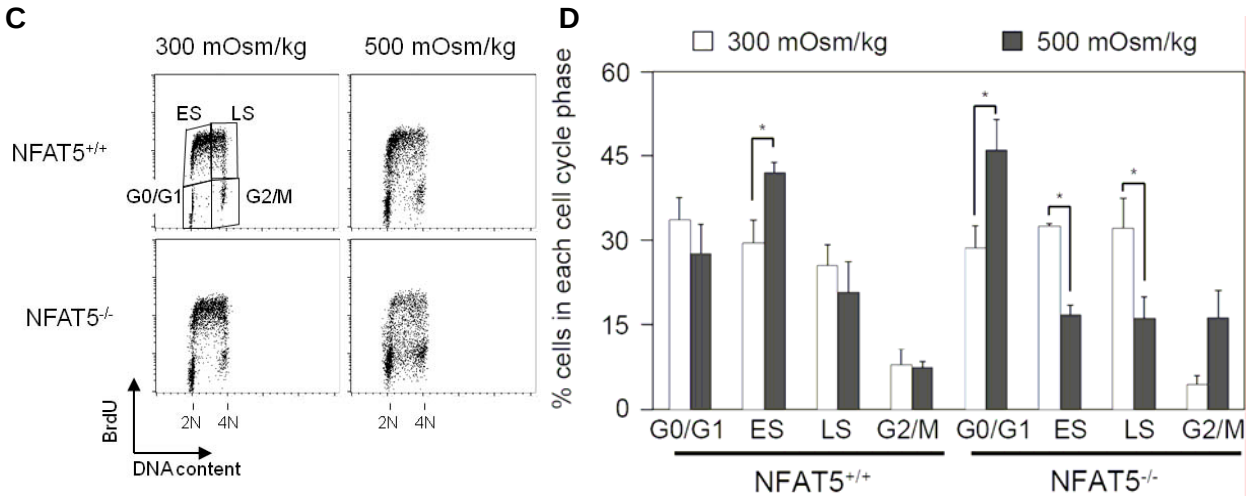


Figure 24. Cell cycle distribution of T cells under hypertonic stress. ***A)*** *DNA content histograms represent the cell cycle distribution in live cells: G0/G1, early S (ES), late S (LS), and G2/M.* ***B)*** *cell cycle distribution in live wild-type and NFAT5$^{-/-}$ cells after 8 hours or 24 hours in isotonic or hypertonic conditions (Values are the mean ± SEM of at least 5 independent experiments). Cells grown in isotonic or hypertonic medium during 24 hours were pulsed for 30 minutes with BrdU, fixed and analyzed. A representative experiment showing BrdU incorporation plotted against DNA content is shown in* ***C)****, and the average values from three independent experiments are represented in* ***D****) (bars represent mean ± SEM, * = p < 0.05).*

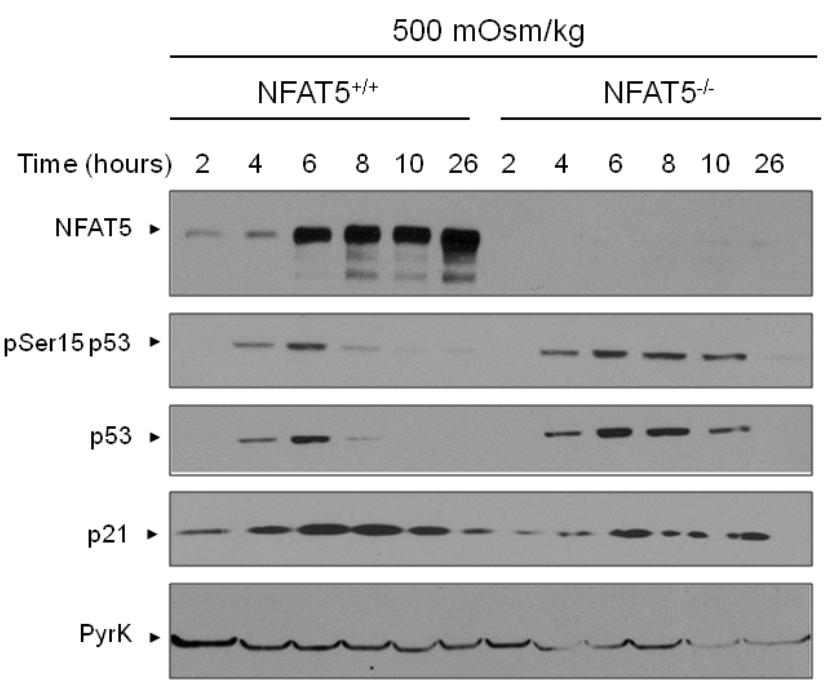
3. Induction of p53 and markers of genotoxic stress in hypertonicity-treated NFAT5$^{-/-}$ cells.

As cell cycle arrest might be an indicator of genotoxic stress, and hypertonicity can cause DNA damage (Kultz and Chakravarty, 2001), we asked whether NFAT5$^{-/-}$ cells exposed to hypertonicity displayed enhanced markers of genotoxic stress. As shown in **Figure 25A**, both cell types displayed a comparable induction and phosphorylation of p53 at 4-6 hours after exposure to hypertonic stress. These parameters were downregulated in wild-type cells by 8 hours, but persisted in NFAT5$^{-/-}$ cells up to at least 10 hours, although eventually they subsided by 24 hours, and only a small proportion (~5%) of NFAT5$^{-/-}$ cells exhibited phospho-p53 after 24 hours, as detected by intracellular staining and flow cytometric analysis (**Figure 25A**). Consistent with the activation of p53, we observed a rapid upregulation of p21^{Cip} in response to hypertonic stress in both wild-type and NFAT5$^{-/-}$ cells. Despite their more prolonged induction of p53, NFAT5$^{-/-}$ lymphocytes did not accumulate substantially greater amounts of p21^{Cip} (**Figure 25A** and supplementary **Figure S1**). Since the induction of p53 preceded the increase in NFAT5

expression (**Figure 25A**), we asked whether p53-deficient cells were competent to express NFAT5. As shown in **Figure 25C**, p53 was not required for the induction of NFAT5 in response to hypertonic stress, whereas it was necessary for the induction of $p21^{Cip}$. Cell cycle analysis of proliferating T cells from wild-type and p53-deficient mice showed a similar response to hypertonic treatment, while etoposide-treatment caused significantly different effects on $p53^{-/-}$ T cells (not shown).

Phosphorylation of Ser15 of p53 in response to DNA damaging agents can be mediated by kinases of the PI3-kinase family such as ATM (Siliciano *et al.*, 1997) and regulated by p38-SAPK (Bulavin *et al.*, 1999). Since both types of kinases are activated by hypertonic stress (Dmitrieva *et al.*, 2002; Irarrazabal *et al.*, 2004), we asked whether they were involved in the induction of p53 in hypertonicity-treated $NFAT5^{-/-}$ cells. As shown in **Figure 25D**, accumulation and phosphorylation of p53 were partially inhibited by the PI3-kinase family inhibitor LY294002, but not by the p38 inhibitor SB203580, indicating that the early induction of p53 in $NFAT5^{-/-}$ cells involved PI3-kinase-related kinases (PIKKs) rather than p38-SAPK.

A



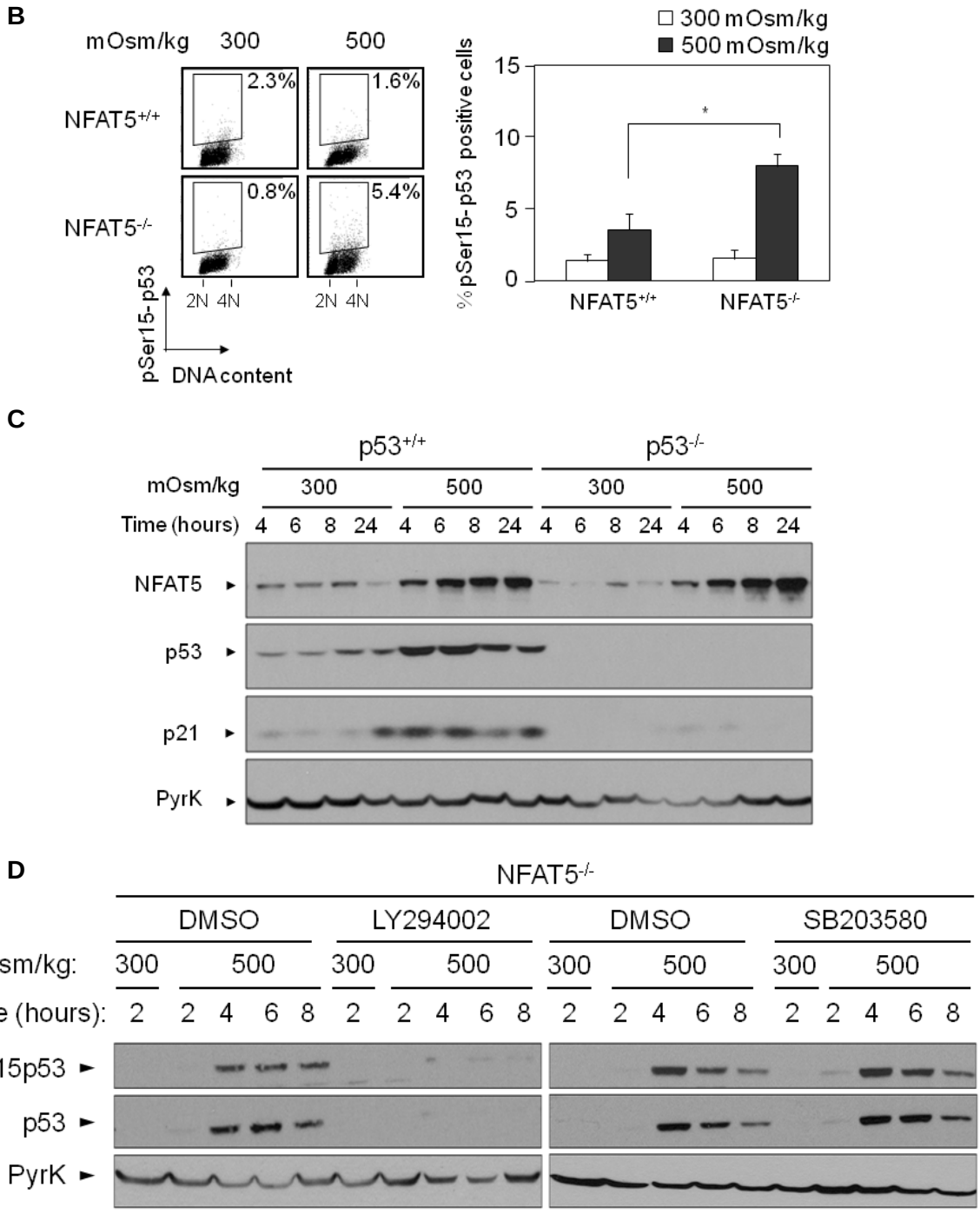


Figure 25. Induction of p53 and p21 in response to hypertonicity. ***A)*** *Western blot shows the time course of p53-Ser15 phosphorylation, accumulation of total p53 and induction of p21 in NFAT5$^{+/+}$ and NFAT5$^{-/-}$ cells in response to hypertonicity. The experiment shown is representative of three independently performed (see* ***supplementary Figure S1****).* ***B)*** *Phospho-p53 (Ser15) was detected by intracellular staining in NFAT5$^{+/+}$ and NFAT5$^{-/-}$ T cells cultured in isotonic or hypertonic medium during 24 hours. Results correspond to cells gated in the live population. Dot plots show one representative*

*experiment. Bars on the right represent the mean ± SEM of 4 independent experiments (* = p < 0.05).* ***C)*** *Time course of NFAT5 and p21 induction in $p53^{+/+}$ and $p53^{-/-}$ cells in response to hypertonicity. The experiment shown is representative of three independently performed.* ***D)*** *The effect of the PIKK inhibitor LY290042 (25 μM) and p38 inhibitor SB203580 (10 μM) on the phosphorylation and accumulation of p53 under hypertonicity in $NFAT5^{-/-}$ cells was analyzed by Western blotting. The result shown is representative of three independent experiments.*

We next determined whether hypertonicity-treated $NFAT5^{-/-}$ cells underwent enhanced genotoxic stress. To analyze whether the population of live $NFAT5^{-/-}$ cells had more DNA damage than wild-type cells under hypertonic conditions, we used the alkaline comet assay and also analyzed the phosphorylation of histone H2AX at Ser139 (γH2AX), a sensitive marker of DNA breaks. The comet assay did not reveal any greater extent of DNA damage in live $NFAT5^{-/-}$ cells compared to wild-type ones after either 6 or 24 hours in hypertonic medium (**Figure 26**). As a control, cells treated with the DNA-damaging agent etoposide showed clear comet tails. However, intracellular detection of γH2AX showed a greater proportion of $\gamma H2AX^{+}$ cells in $NFAT5^{-/-}$ cultures (11.6 %) than in wild-type ones (5.2 %) after 24 hours of hypertonic stress (**Figure 27A and 27B**). Accumulation of γH2AX in $NFAT5^{-/-}$ cells was not evident after 6 hours of neither hypertonic nor isotonic conditions. The relatively small proportion of $\gamma H2AX^{+}$ cells (~ 12%, **Figure 27A**) was consistent with the lack of detection of generalized DNA damage in the comet assay. $NFAT5^{-/-}$ cells had a greater proportion of $\gamma H2AX^{+}$ cells in all phases of the cell cycle as compared to wild-type cells when exposed to hypertonic conditions, but showed a more notable accumulation of γH2AX in S and G2/M (**Figure 27B**), suggesting a possible accumulation of DNA breaks in cells transiting through S and G2/M.

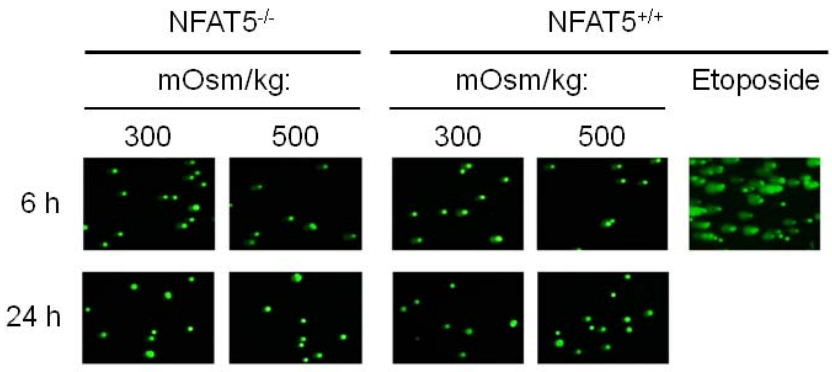
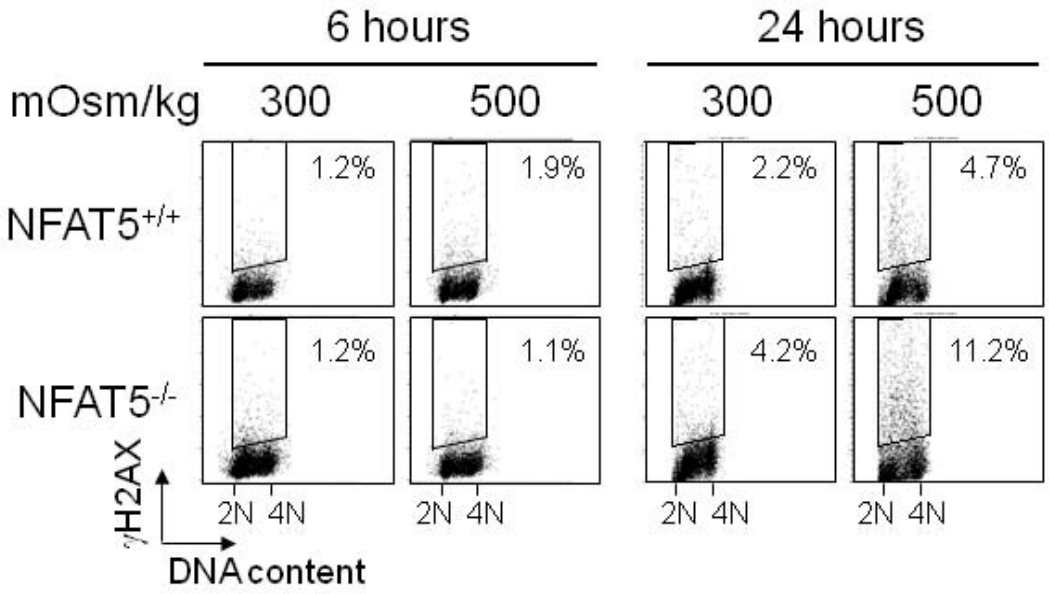


Figure 26. Genotoxic stress markers in NFAT5-deficient cells. *Single-cell alkaline gel electrophoresis assay (comet assay) done on the population of live cells isolated from cultures of $NFAT5^{-/-}$ and wild-type cells after 6 or 24 hours in isotonic or hypertonic conditions. Cells treated with etoposide (1 μM, 6 hours)*

are included as a positive control. The experiment shown is representative of three independently performed.

DNA breaks and phosphorylation of H2AX can also be indicative of apoptosis. As shown earlier (**Figure 23C**), hypertonicity caused considerable apoptosis in NFAT5$^{-/-}$ cells at 24 hours, evidenced by the accumulation of annexin-V^{+} cells and cells with DNA content < 2N. Also, a small proportion (3-5%) of annexin-V^{+} cells was routinely detected after hypertonic treatment in cells gated as alive in NFAT5$^{-/-}$ cultures. The similar percentages of annexin-V^{+}, phospho-p53^{+} and γH2AX^{+} cells in alive cells (**Figures 23C, 25B and 27B**) suggested that at least some of the cells with γH2AX and phosphorylated p53 might already be committed to apoptosis. On the other hand, we noticed that the population of γH2AX-negative cells in the cultures of NFAT5-deficient lymphocytes also displayed features of cell cycle arrest (fewer cells in S phase and accumulation in G2/M) (**Figure 27C**). This result indicated that although a small percentage of NFAT5$^{-/-}$ T cells exhibited signs of DNA damage under hypertonic stress, the majority of cells did not, yet they still underwent cell cycle arrest.

A



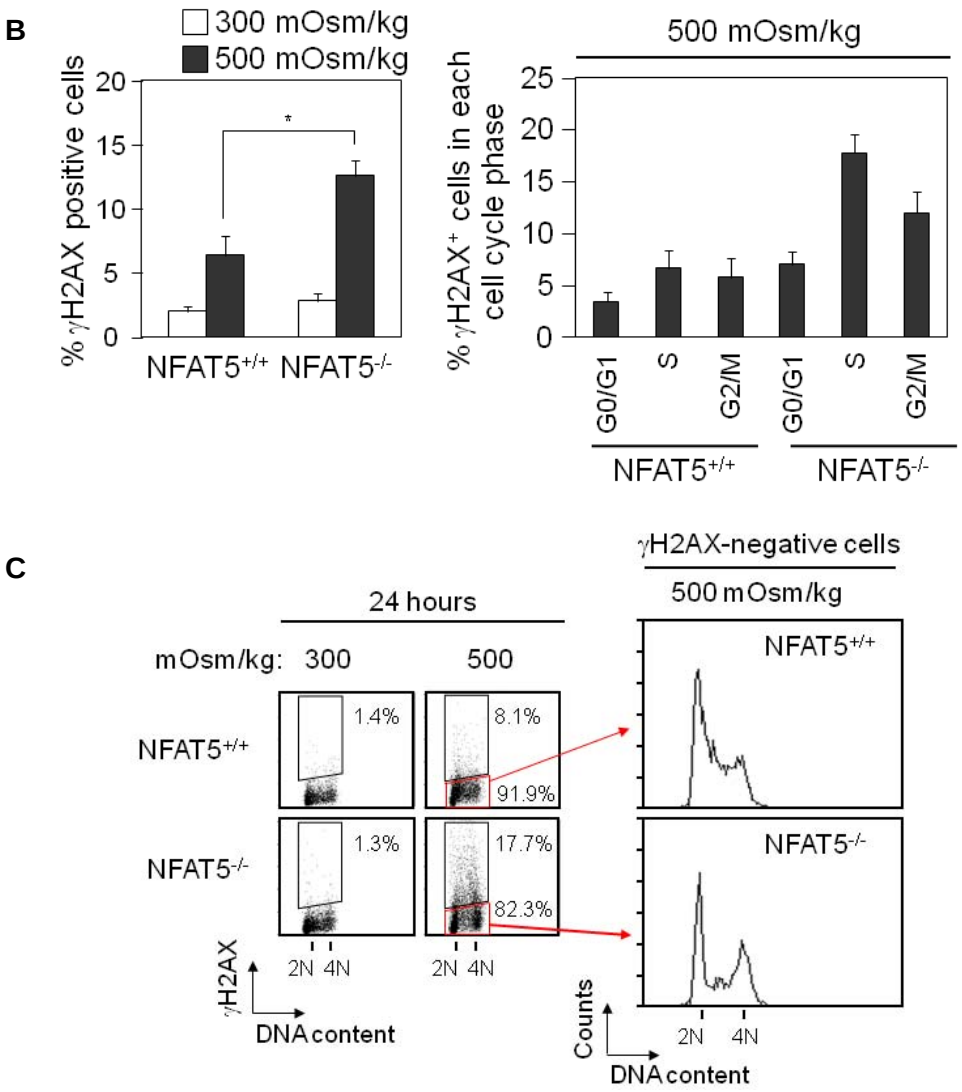


Figure 27. Induction of γH2AX in T cells exposed to hypertonic stress. ***A)*** *Dot plots representing γH2AX and DNA content in live wild-type (NFAT5$^{+/+}$) and NFAT5$^{-/-}$ lymphocytes after 6 and 24 hours in isotonic or hypertonic conditions.* ***B)*** *On the left, bars represent the percentage of γH2AX^{+} cells after 24 hours in isotonic or hypertonic medium (bars express the mean ± SEM of 7 independent experiments, * = p < 0.05). Bars on the right express the percentage of γH2AX^{+} cells within each cell cycle phase in NFAT5$^{+/+}$ and NFAT5$^{-/-}$ lymphocytes after 24 hours in hypertonic medium (values are the mean ± standard deviation of 5 independent experiments, * = p < 0.05).* ***C)*** *Dot plots of dual γH2AX-DNA labeling of a representative experiment. On the right, cell cycle distribution of γH2AX negative T cells exposed to hypertonic stress for 24 hours.*

We also analyzed the induction of GADD45 isoforms, which have been shown to mediate the cell cycle arrest induced by hypertonic shock in mouse renal medullary cells (Mak and Kultz,

2004). As shown in **Figure 28**, GADD45α and β were comparably induced in wild-type and NFAT5$^{-/-}$ T cells after 8 hours of hypertonic stress and were downregulated to basal levels by 24 hours. On the other hand, GADD45γ was downregulated by hypertonic stress in both wild-type and NFAT5$^{-/-}$ T cells, suggesting that it was not contributing to the cell cycle arrest. These results also indicated that NFAT5 did not regulate the transcription of neither GADD45 isoform in T cells. By contrast, the induction of Hsp70.1, whose mRNA accumulated with a similar kinetics in response to hypertonic stress, was in part dependent on NFAT5 (**Figure 28**), in agreement with previous reports in which it was shown that this factor can activate the Hsp1b gene promoter (Woo *et al.*, 2002). Likewise, NFAT5$^{-/-}$ T cells failed to induce the osmoprotective gene products SMIT and TauT in response to hypertonic stress (**Figure 28**). Thus, cell cycle arrest in hypertonicity-treated NFAT5$^{-/-}$ cells could not be fully explained by increased DNA damage, considering that H2AX phosphorylation was detected in a relatively small percentage of the cells (**Figure 27A and 27B**) and even those that did not display DNA damage still exhibited cell cycle arrest, as shown in **Figure 27C**. Moreover, proteins involved in DNA-damage response (such as GADD45) were upregulated and downregulated in a similar fashion in both wild-type and NFAT5-deficient T cells (**Figure 28**).

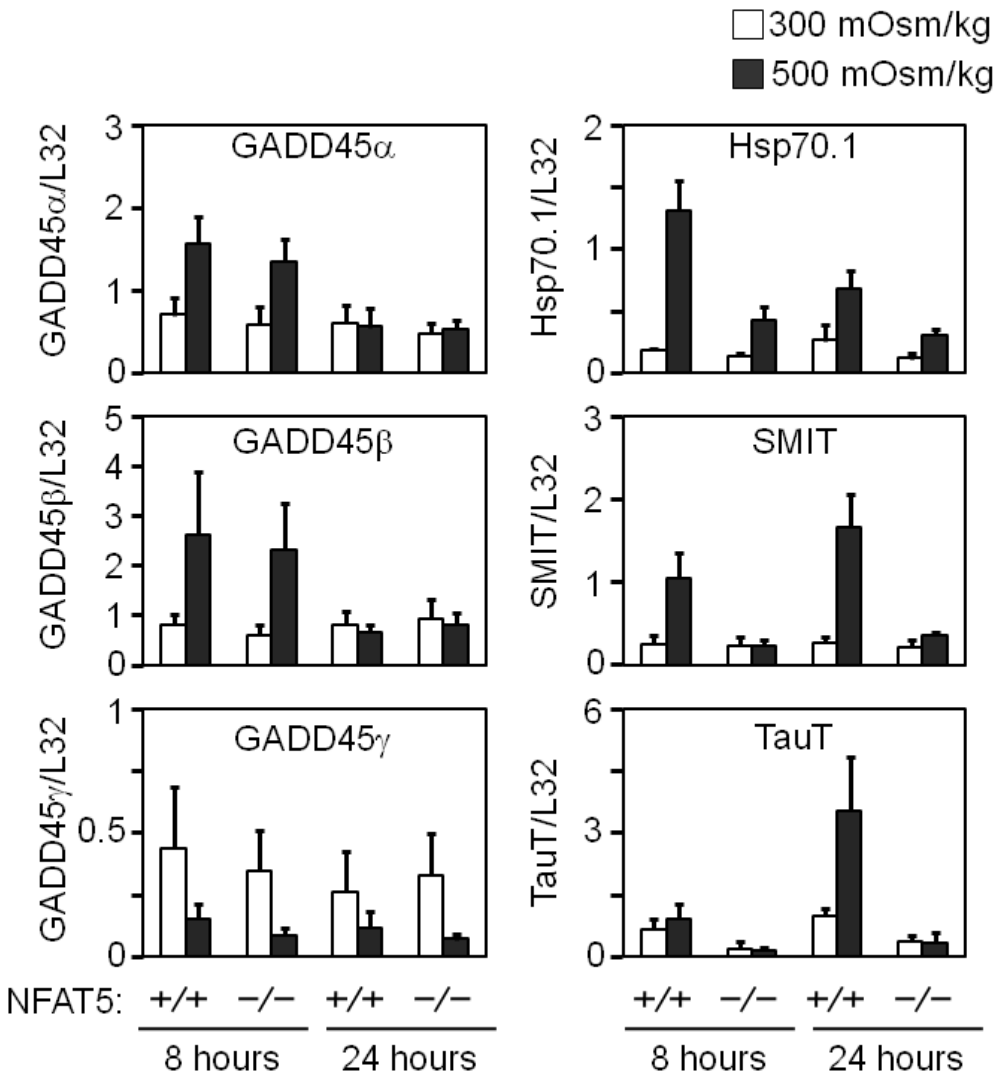


Figure 28. Induction of GADD45 proteins and osmoprotective genes in response to hypertonicity. *NFAT5$^{+/+}$ and NFAT5$^{-/-}$ proliferating T cells were subjected to hypertonicity for 8 and 24 hours, RNA was isolated and analyzed by RT-qPCR. The values were normalized to L32 mRNA levels (bars are mean ± SEM of 4 independent experiments; * = p <0.05).*

Since Hsp70.1-deficient cells have been shown to be sensitive to a variety of stresses, including osmotic stress (Hunt *et al*., 2005; Shim *et al*., 2002), we explored whether the lesser induction of Hsp70.1 (**Figure 28**) could be responsible for the sensitive status in which NFAT5$^{-/-}$ T cells are left after 24 hours of hypertonic treatment. As shown in **Figure 29**, Hsp70.1/3$^{-/-}$ MEFs (Hunt *et al.*, 2004) accumulated in G1 and had impaired progression to S and G2/M phases when exposed to hypertonicity (**Figure 29C**), despite that they could induce NFAT5 (**Figure 29B**).

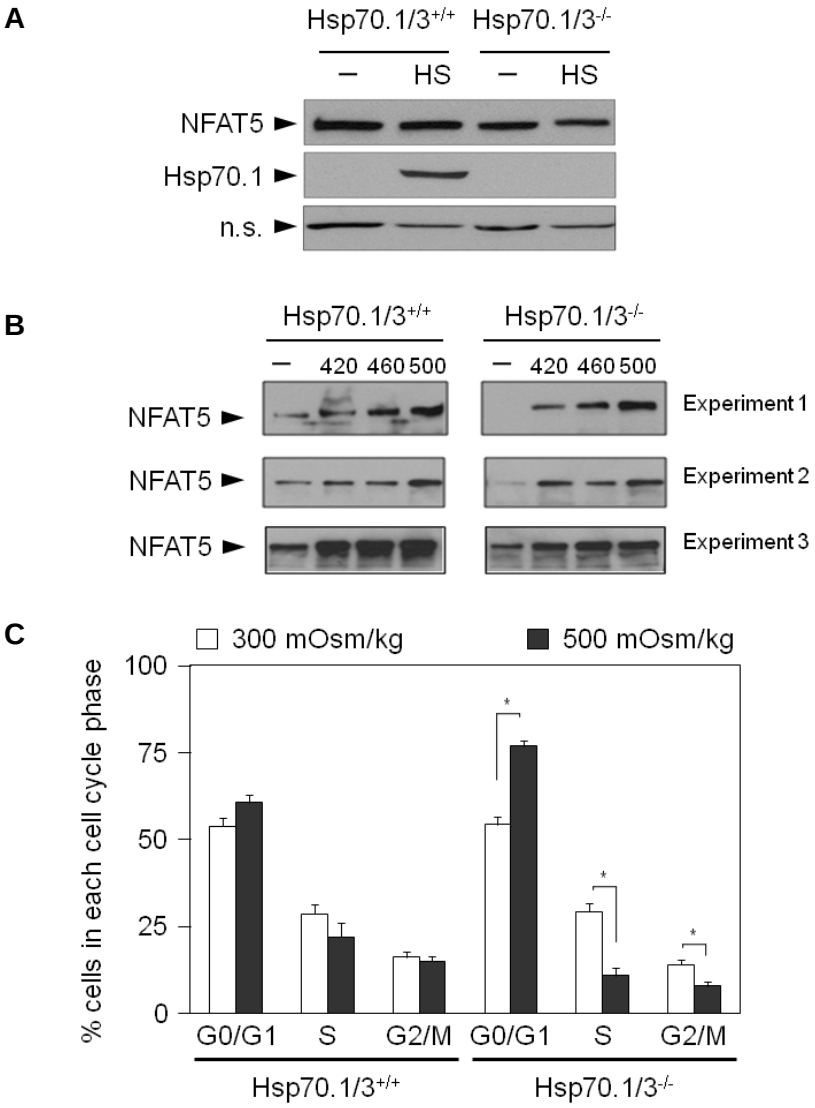


Figure 29. Response of Hsp70.1/3$^{-/-}$ MEF to hypertonicity. ***A)*** *Western blot analysis of Hsp70.1 in wild-type and Hsp70.1/3-null MEF in response to heat shock (HS) (30 minutes at 43^{o}C, 2 hour recovery at 37 ^{o}C).* ***B)*** *Western blot analysis of NFAT5 in wild-type and Hsp70.1/3-null MEF in response to hypertonicity.* ***C)*** *Cells were labeled with Hoechst and analyzed by flow cytometry (Bars express mean ± SEM of 6 independent experiments, * = p <0.05).*

We also treated NFAT5$^{-/-}$ T cells with heat shock to induce Hsp70 and determined whether increasing the expression of this chaperone would protect them from the cell cycle arrest caused by hypertonicity. As shown in **Figure 30,** heat shock induced Hsp70 NFAT5$^{-/-}$ T cells comparably to their wild-type counterpart, as expected since NFAT5 is not required for the heat shock response. However, induction of this chaperone did not prevent NFAT5$^{-/-}$ T cells from

undergoing cell cycle arrest by a subsequent treatment with hypertonicity. Altogether, these results indicated that induction of Hsp70 by itself was not sufficient to restore the cell cycle progression in hypertonically stressed NFAT5$^{-/-}$ T cells.

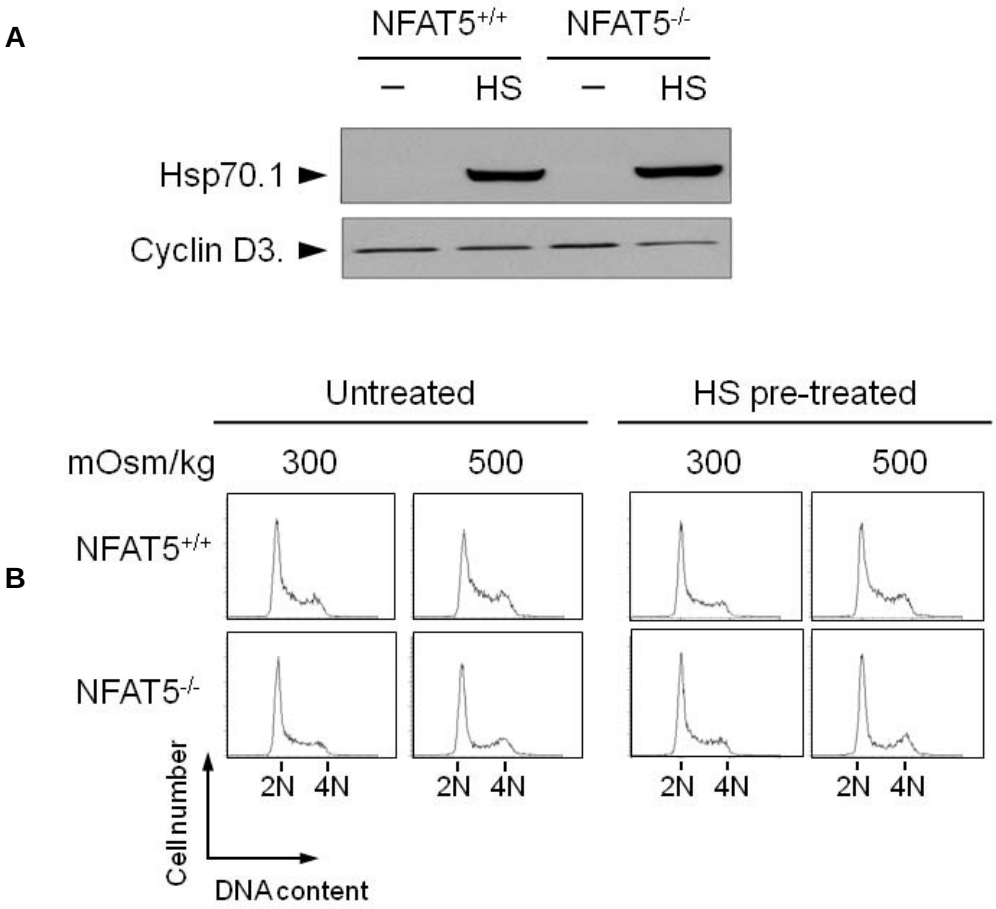


Figure 30. Response of T cells to heat shock and hypertonicity. *Wild-type and NFAT5$^{-/-}$ T cells were pre-treated with heat shock (HS) and allowed to recuperate for 4 hours in isotonic media.* ***A)*** *Western blot analysis of Hsp70.1 protein after a 4 hour recovery. At this time point, T cells were exposed to hypertonicity for 20 hours and analyzed by flow cytometry as shown in* ***(B)****.*

4. Sensitivity of NFAT5$^{-/-}$ T cells to ionizing radiation.

Since hypertonicity has been shown to cause genotoxic stress, and can activate a similar response as other DNA damaging agents, we asked whether NFAT5 might be involved in the response to other genotoxic agents. We examined the response of NFAT5$^{-/-}$ cells to a prototypical genotoxic agent, ionizing radiation (IR). As shown in **Figure 31A**, loss of viability and cell cycle arrest upon exposure to 3 Gy or 10 Gy were very similar in NFAT5$^{+/+}$ and NFAT5$^{-/-}$ cells after 24 hours, indicating that lack of NFAT5 did not confer an obvious disadvantage in response to radiation.

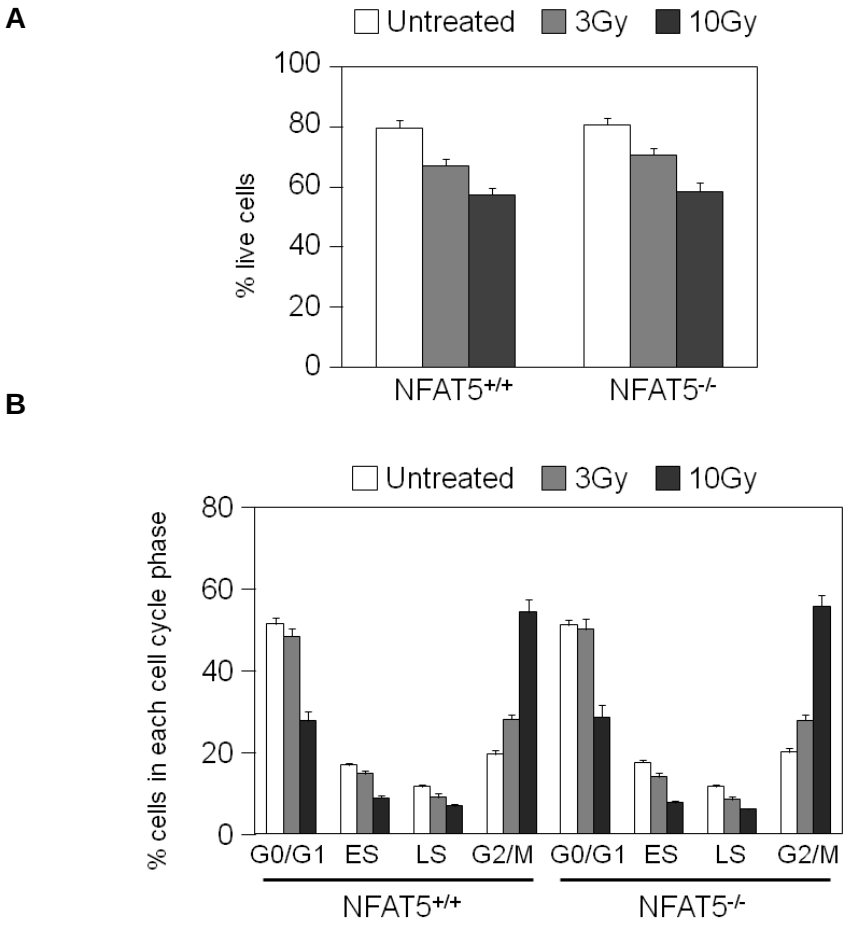


Figure 31. Cellular viability and cell cycle distribution in response to ionizing radiation. *Proliferating T cells were irradiated with 3 or 10 Gy and cultured in fresh isotonic culture medium with IL-2 during 24 hours.* ***A)*** *Cellular viability was determined in the total population and cell cycle* ***(B)*** *was analyzed in the gate of live cells. Results are the mean ± SEM of 5 independent experiments.*

In addition, phosphorylation of p53 in response to IR was comparable in wild-type and NFAT5$^{-/-}$ cells (**Figure 32A**). Ionizing radiation did not induce the expression of NFAT5, nor affected its induction by hypertonicity in normal lymphocytes and HEK293 cells (**Figures 32B, 32C**). Likewise, ionizing radiation did not activate an NFAT5-dependent reporter in HEK293 cells nor affected its activation by hypertonicity, in agreement with previous reports (Irarrazabal *et al.*, 2004) (**Figure 32D**).

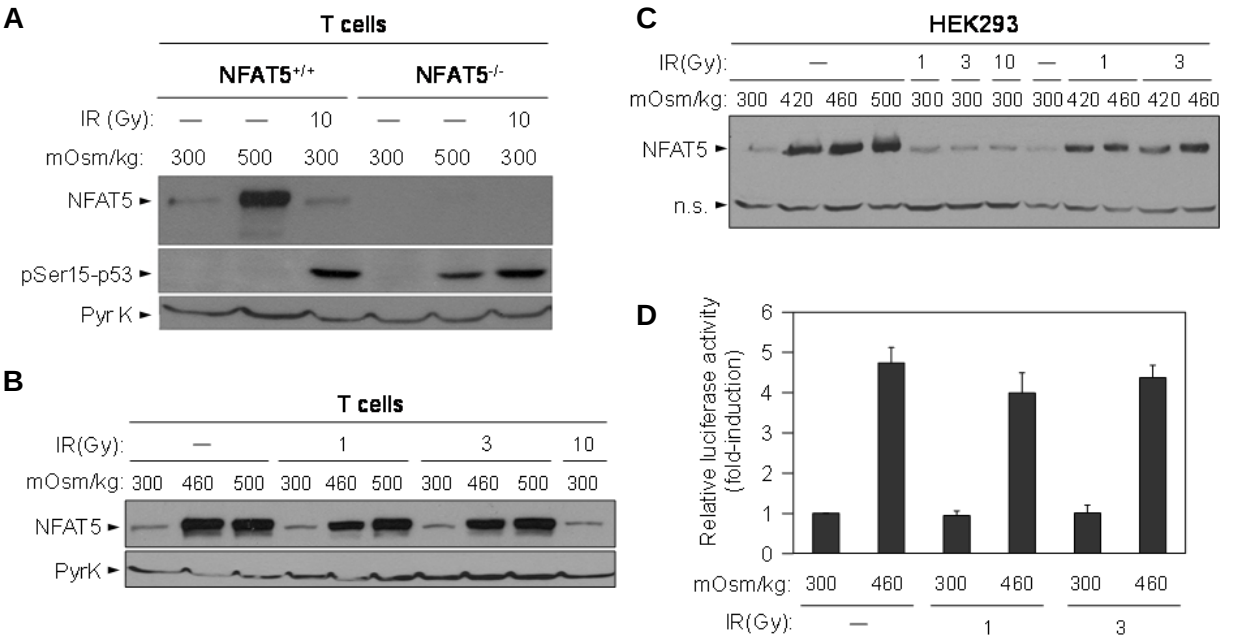


Figure 32. Effect of ionizing radiation on the expression and activity of NFAT5. ***A****) NFAT5 and phospho-p53 (Ser 15) were analyzed by Western blot in NFAT5$^{+/+}$ and NFAT5$^{-/-}$ cells after 6 hours of exposure to hypertonicity or 10 Gy of ionizing radiation (IR). Western blot analysis of the amount of NFAT5 in activated T cells (**B**) and HEK293 cells (**C**) after 24 hours of exposure to hypertonic conditions, ionizing radiation, or combinations of both stressors.* ***D****) Effect of ionizing radiation on the activity of the NFAT5-dependent reporter ORE-Luc. HEK293 cells transfected with ORE-Luc and pTK-Renilla were left untreated or irradiated (1 or 3 Gy) and then cultured in isotonic or hypertonic media. Luciferase activity was measured 24 hours later and normalized to the activity of the Renilla reporter. Results correspond to the mean ± SEM of 3 independent experiments.*

However, when cells were exposed to moderate hypertonic conditions (420 mOsm/kg) combined with a low dose of ionizing radiation (3 Gy), we observed that NFAT5$^{-/-}$ cells suffered a more severe cell cycle arrest than NFAT5$^{+/+}$ cells, while these stresses were well tolerated when individually added, as shown in **Figure 33**. This result shows that moderate conditions of

osmotic and genotoxic stress can have a cooperative deleterious effect on cells, and that NFAT5 is necessary for the stability of the cell cycle under these conditions.

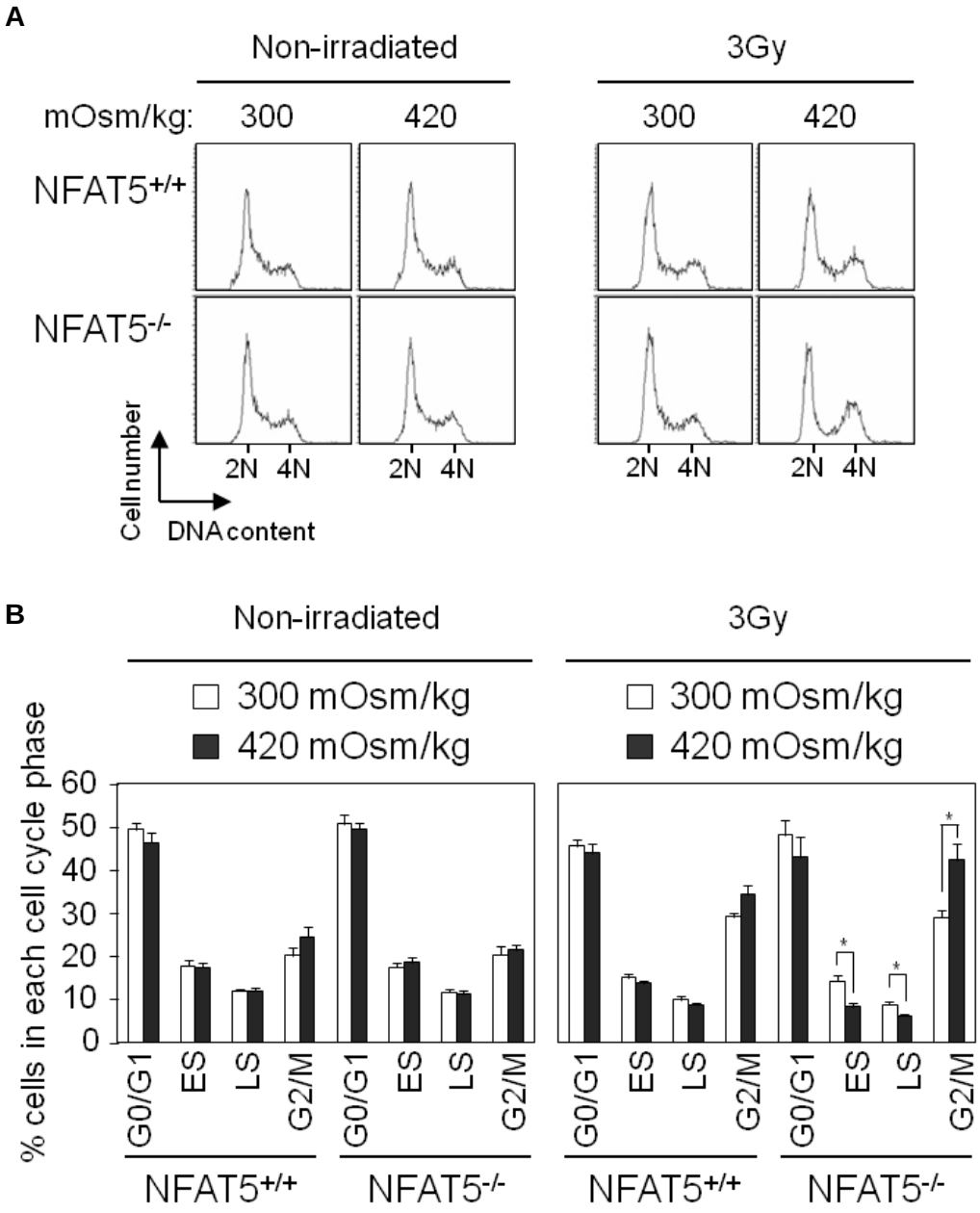


Figure 33. Sensitivity of NFAT5-deficient T cells to the combination of ionizing radiation and hypertonicity. *Effect of the combination of low doses of ionizing radiation and hypertonicity on the cell cycle of wild-type and NFAT5$^{-/-}$ T cells. Proliferating T cells were left untreated or irradiated with 3 Gy and then plated in fresh isotonic (300 mOsm/kg) or moderately hypertonic medium (420 mOsm/kg).* ***A)*** *Cell cycle analysis of NFAT5$^{+/+}$ and NFAT5$^{-/-}$ was done 24 hours later.* ***B)*** *Results shown are the mean ± SEM of 5 independent experiments, * = p<0.05.*

5. Regulation of NFAT5 throughout the cell cycle in hypertonically stressed cells.

Since hypertonicity enhances the synthesis of NFAT5 in lymphocytes (Lopez-Rodriguez *et al.*, 2001; Trama *et al.*, 2002) and, as shown here, its absence caused defects in S and G2/M, we wondered whether the activity of NFAT5 was regulated throughout the cell cycle. T cells proliferating under isotonic or hypertonic conditions were labeled with Hoechst 33342, and sorted according to their cell cycle phase. As shown in **Figure 34**, the expression of NFAT5 was higher in S phase and G2/M in isotonic conditions. Upon hypertonic stimulation, its expression was increased considerably under osmotic stress, with a pronounced accumulation in S and G2/M.

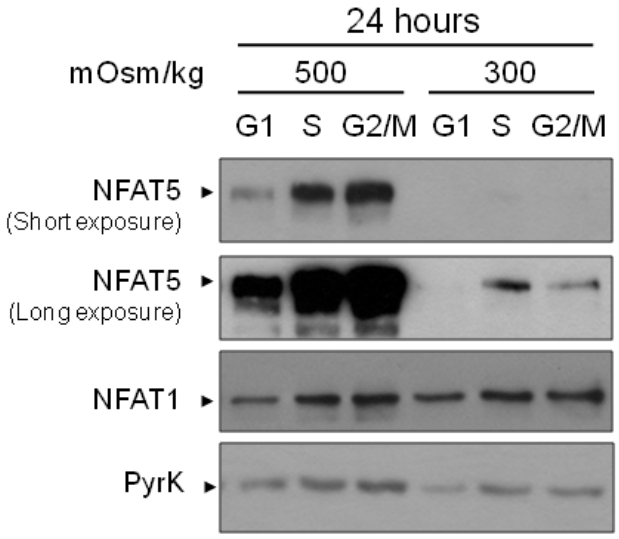


Figure 34. Regulation of NFAT5 throughout the cell cycle in hypertonically stressed cells. *NFAT5$^{+/+}$ T cells were labeled with Hoechst 33342 and sorted according to DNA content as G0/G1, S or G2/M phase. Sorted cells were lysed and equal amounts of protein from each lysate were analyzed by Western blot with anti-NFAT5 antibody. NFAT1 and anti-pyruvate kinase (PyrK) are shown as protein loading controls. The result is representative of three independent experiments.*

In order to determine whether NFAT5 activity was increased in parallel to its expression we used transgenic T cells with an integrated NFAT5-responsive reporter, 9xNFAT-Luc (Morancho *et al.*, 2008). As shown in **Figure 35**, 8 hours after increasing the tonicity NFAT5 was active in all the phases of the cell cycle, with a greater activity in G2/M, and by 24 hours, when cells had recovered a normal cell cycle, NFAT5 activity was highest in S and G2/M, which were the

phases more severely affected by hypertonicity in NFAT5$^{-/-}$ cells. Considering that a proportion of the luciferase measured in the sorted fractions had been already synthesized in the previous cell cycle phase, it seems likely that in the 24 hour samples the peak of NFAT5 activity occurred in S phase and that its activity in G1 was probably lower than what was measured in the experiment.

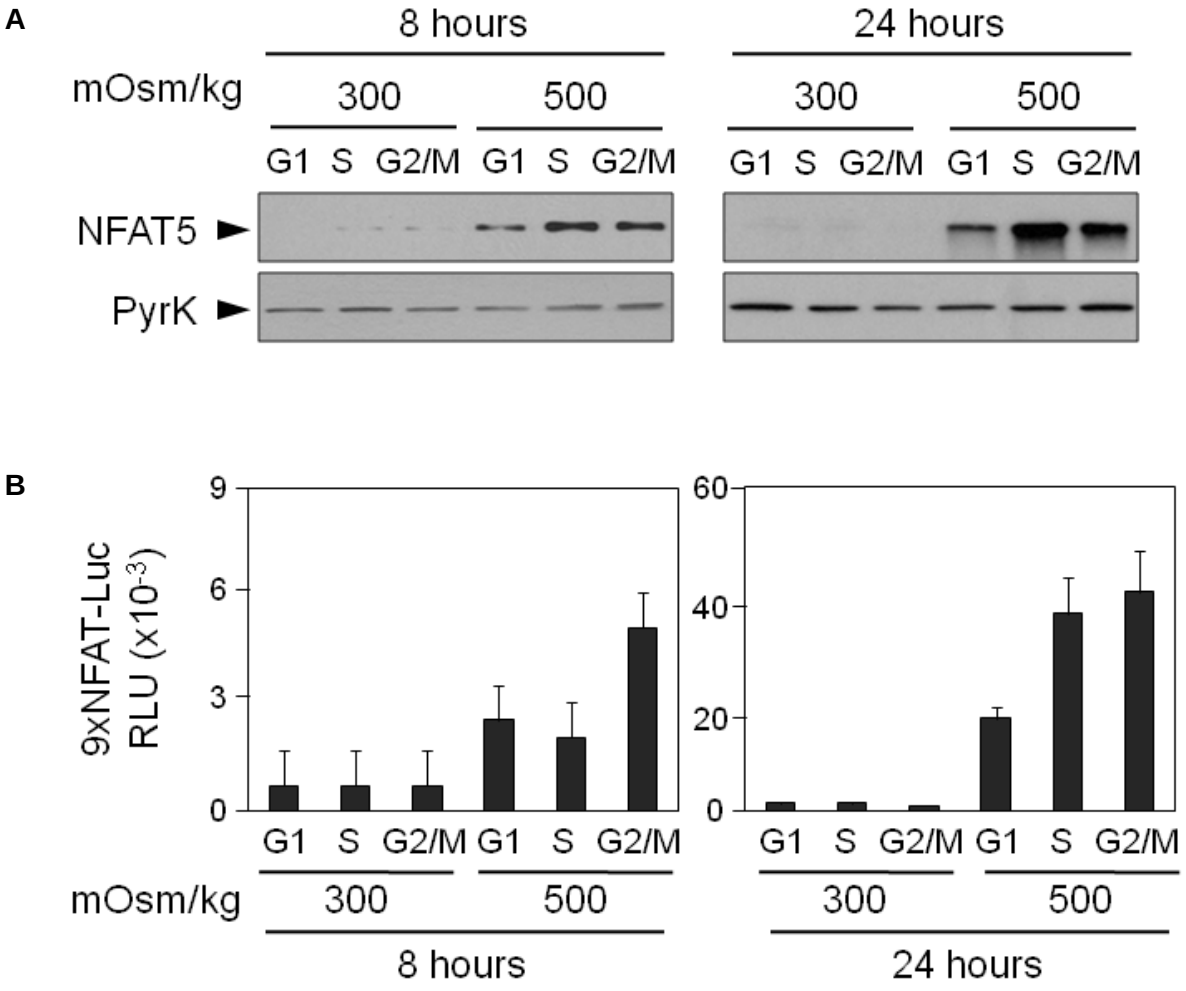


Figure 35. Regulation of NFAT5 throughout the cell cycle in hypertonically stressed T cells. *NFAT5 protein expression along G1, S and G2/M phases of the cell cycle of 9xNFAT-luc proliferating T cells. Cells were labeled with Hoechst 33342 and sorted as described in* ***Figure 34****. Sorted cells were split for Western blot and luciferase activity assays. A representative Western blot analysis (**A**) is shown of three independently performed. Pyruvate kinase (PyrK) is shown as protein loading control. (**B**) 9xNFAT-Luc reporter activity in sorted cells is shown. Results are the mean ± SEM of 3 independent experiments.*

6. Defective expression of cyclins in hypertonicity-treated NFAT5$^{-/-}$ T cells.

In summary, the results described above suggested that hypertonic shock induced an early, transient genotoxic stress-like response that was comparable in both wild-type and NFAT5$^{-/-}$ cells. This response was followed by an adaptive phase in wild-type cells, which induced and activated NFAT5, downregulated stress markers and resumed a normal cell cycle. In contrast, NFAT5$^{-/-}$ lymphocytes exhibited a greater viability loss and surviving cells displayed cell cycle defects. Although NFAT5$^{-/-}$ lymphocytes still presented some stress markers by 24 hours, such as phosphorylated H2AX and p53 in a small proportion of cells, they had downregulated other genotoxic stress-associated responses such as p21 and GADD45α and β as much as wild-type lymphocytes, suggesting that their cell cycle arrest might not be attributable to a sustained genotoxic stress-like response. We thus analyzed the expression of several cyclins known to be relevant in successive phases of the cell cycle.

A short exposure to hypertonic stress (8 hours) had similar effects on the expression of cyclins in wild-type and NFAT5$^{-/-}$ lymphocytes. Cyclin D3 was downregulated in both cell types in six out of six independent experiments, cyclin E1 in two out of five, and cyclin B1 in three out of seven independent cultures tested for each wild-type and NFAT5$^{-/-}$ T cells, whereas cyclin A2 was not affected after 8 hours in the majority of experiments (**Figure 36A and supplementary Figure S2**). However, after 24 hours in hypertonic medium, wild-type and NFAT5$^{-/-}$ cells displayed distinct differences. Both cell types upregulated cyclin D3, but whereas wild-type lymphocytes maintained or upregulated cyclins E1, A2 and B1 levels to similar ones of cells growing in isotonic medium, NFAT5$^{-/-}$ cells had substantially reduced levels of these cyclins (**Figure 36A and supplementary Figure S2**). Furthermore, the inability of NFAT5$^{-/-}$ proliferating T cells to maintain or reinduce cyclins A2 and B1 levels under hypertonic stress occurred in both S and G2/M (**Figure 36B)**.

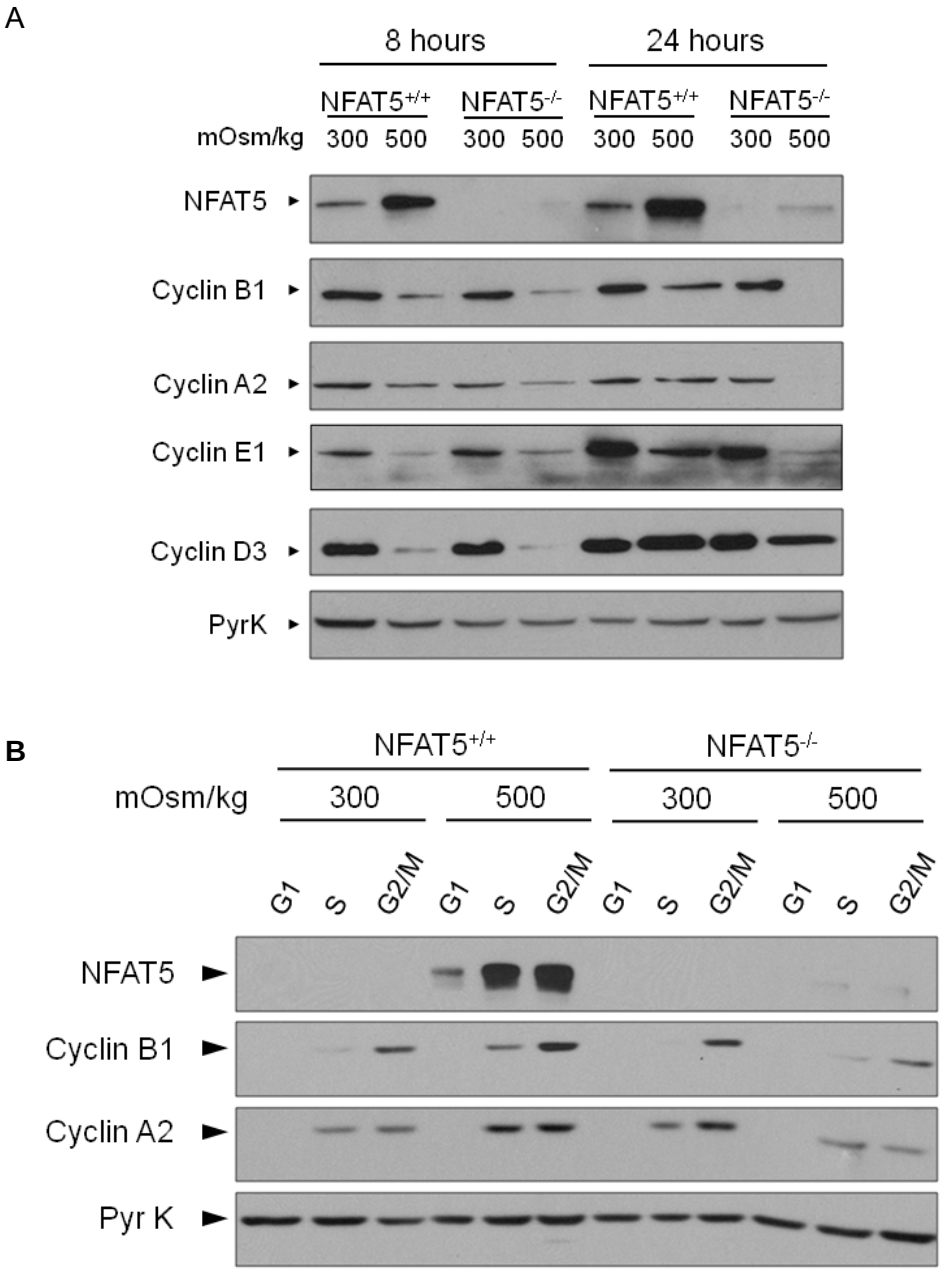


Figure 36. Expression of cyclins under hypertonic conditions. ***A)*** *Expression of cyclins D3, E1, A2 and B1 was analyzed by Western blot in lysates of proliferating NFAT5$^{+/+}$ and NFAT5$^{-/-}$ T cells after 8 and 24 hours of hypertonicity treatment. Pyruvate kinase (PyrK) is shown as protein loading control. The result is representative of at least four independent experiments (see* ***supplementary Figure S2****).* ***B)*** *Wild-type and NFAT5$^{-/-}$ T cells were exposed to hypertonicity for 24 hours, labeled with Hoechst 33342 and sorted as described in Figure 34. Expression of NFAT5, cyclin B1 and cyclin A2 was analyzed by Western blot. Pyruvate kinase (PyrK) is shown as protein loading control. The result is representative of two independent experiments.*

We analyzed whether cyclin downregulation correlated with a decrease in their mRNA levels. The amount of mRNA for cyclins A2 and B1 was substantially reduced (by 60-70%) in both wild-type and NFAT5$^{-/-}$ lymphocytes after 8 hours of hypertonicity treatment (**Figure 37**). By 24 hours, wild-type lymphocytes had recovered similar cyclin mRNA levels as those of cells grown in isotonic medium, whereas NFAT5$^{-/-}$ cells did not. Despite that the amount of cyclin E1 protein was considerable lower in hypertonically stressed NFAT5$^{-/-}$ cells than in wild-type ones, hypertonic stress caused a lesser decrease in its mRNA abundance than it did for cyclins A2 and B1, indicating that the downregulation of cyclin E1 might involve defective synthesis and/or enhanced degradation of the protein. In these experiments, we also observed that the abundance of aurora B kinase mRNA, which has been recently shown to regulate cell cycle progression in T cells (Song *et al.*, 2007), was reduced in hypertonicity-treated NFAT5$^{-/-}$ T cells.

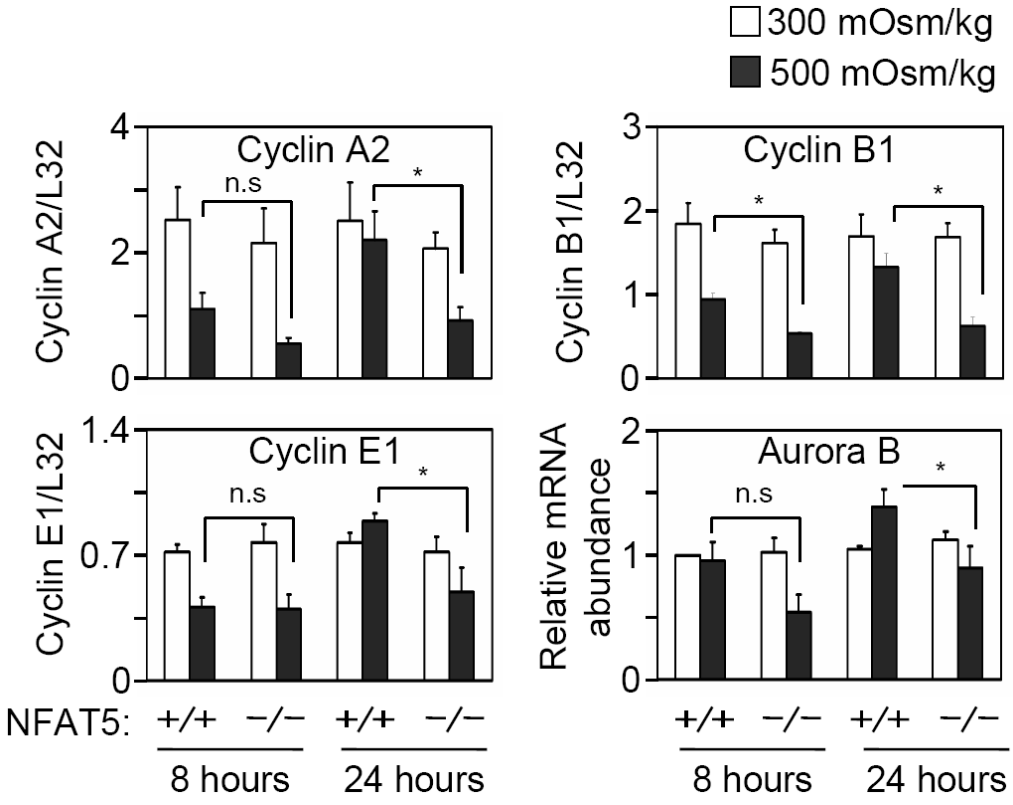


Figure 37. Expression of cyclins and aurora B kinase in response to hypertonicity. *mRNA abundance of cyclins and aurora B kinase was analyzed by RT-qPCR in proliferating NFAT5$^{+/+}$ and NFAT5$^{-/-}$ T cells subjected to hypertonicity for 8 and 24 hours. Aurora B kinase mRNA levels are represented as relative to wild-type cells in isotonic conditions. All the values were normalized to L32 mRNA levels in each respective sample (bars are mean ± SEM of 4 independent experiments; * = p <0.05; n.s = not statistically significant).*

We next analyzed whether the lack of NFAT5 affected the activity of the promoters of cyclins A2 and B1 in Jurkat T cells exposed to hypertonic stress. Two independent NFAT5-specific shRNA inhibited the hypertonicity-induced activation of the NFAT5-responsive reporter 9xNFAT-Luc (Morancho *et al.*, 2008), without affecting the activation of the same reporter via NFATc proteins in response to PMA and ionomycin (**Figure 38**). In the same experiment, suppression of NFAT5 did not inhibit the activity of cyclin A2 and B1 promoters in hypertonic conditions, indicating that NFAT5 was not required to maintain the activity of these promoters in hypertonically stressed cells.

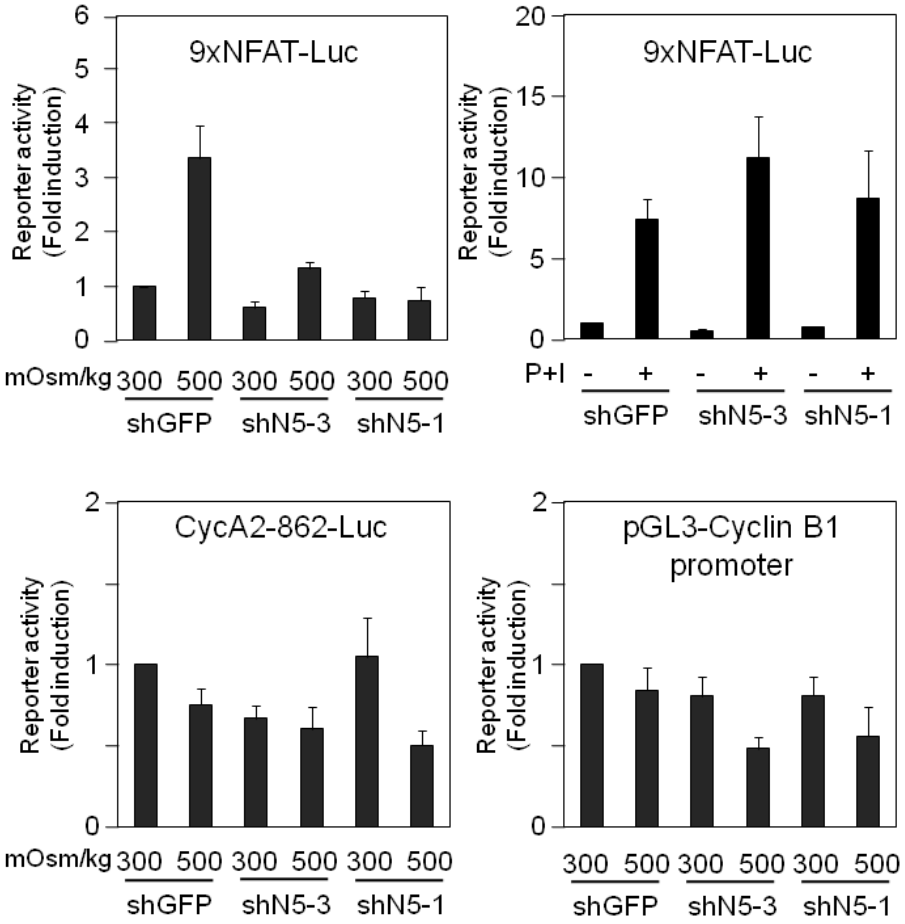


Figure 38. Effect of hypertonicity on cyclin A2 and cyclin B1 promoters. *Jurkat T cells were cotransfected with the indicated vectors and with pTK-Renilla. 36 hours post-transfection, cells were placed in fresh media and were either left untreated, stimulated with hypertonicity or with PMA plus ionomycin (P+I). Luciferase activity was measured 24 hours later and normalized to the activity of the Renilla reporter. Results are expressed as the mean fold induction ± SEM of 4 independent experiments*

7. Defective induction of cell cycle regulators in primary NFAT5$^{-/-}$ T cells exposed to pathologic hypertonic conditions.

In the experiments shown above we had used hypertonic conditions of 500 mOsm/kg, which have been routinely utilized throughout the literature to analyze NFAT5-dependent responses in diverse cell types. However, switching cell cultures from an isotonic medium of 300 mOsm/kg to 500 mOsm/kg constitutes a rather severe osmotic shock that might not reflect pathophysiological situations. Several anisosmotic disorders described in patients as well as in mouse models lacking osmoregulatory proteins (see Introduction, section 4) have been reported to cause hypernatremia with plasma osmolality values of 360-430 mOsm/kg. These conditions are likely to expose different cell types, including lymphocytes, to a hypertonic millieu. Previous work by Go et al. (Go *et al.*, 2004) had shown that the proliferative capacity of fresh NFAT5-deficient lymphocytes stimulated via T cell receptor was reduced by 50% when stimulated in culture media of 370 mOsm/kg, and our recent work showed that NFAT5 was activated at a lower hypertonicity threshold (~380-400 mOsm/kg) in T cells during their early phase of mitogen stimulation than when they were already actively proliferating (480 mOsm/kg) (Morancho *et al.*, 2008). We thus tested whether fresh NFAT5$^{-/-}$ T cells induced to proliferate in moderately hypertonic medium (380-420 mOsm/kg) exhibited cell cycle and cyclin expression defects. Induction of cyclins A2 and B1 by concanavalin A stimulation in T cells was more severely impaired by hypertonicity in NFAT5$^{-/-}$ T cells than in wild-type ones (**Figure 39A**). In this experimental setting, cyclin expression was maintained in wild-type cells grown in 380 mOsm/kg medium but was partially inhibited at 400 mOsm/kg. The threshold for hypertonicity-mediated inhibition was lower in NFAT5$^{-/-}$ T cells, and cyclin expression was detectably reduced at 380 mOsm/kg and severely inhibited at 400 mOsm/kg. Similar results were obtained in cells stimulated with anti-CD3 plus anti-CD28 antibodies (**Figure 39B**). These experiments also showed that the proportion of cells entering the cell cycle upon T cell receptor stimulation was significantly lower in NFAT5$^{-/-}$ T cells exposed to 420 mOsm/kg than in wild-type ones (**Figure 39C**).

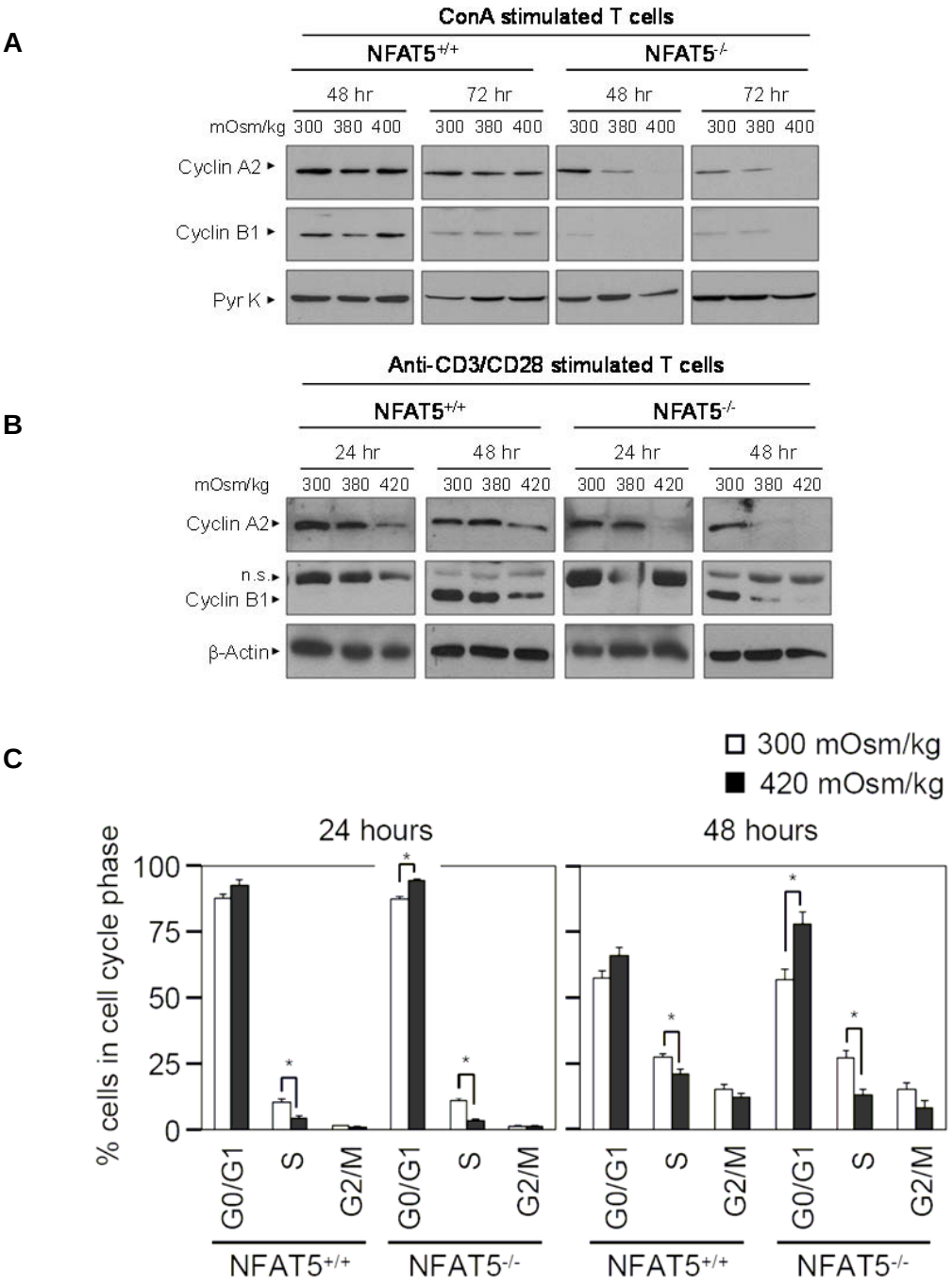
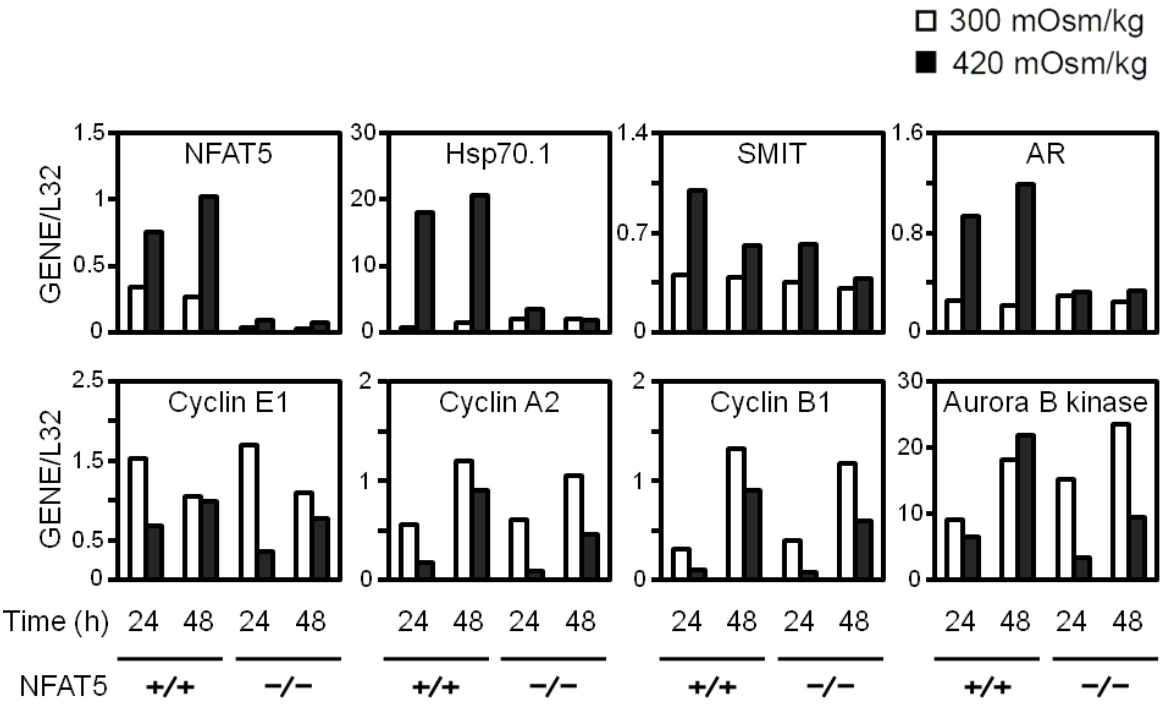


Figure 39. Effect of hypertonicity on cyclin induction and cell cycle entry induced by mitogens or T cell receptor activation. *Expression of cyclins A2 and B1 was analyzed by Western blot in lysates of splenocytes isolated from NFAT5$^{+/+}$ and NFAT5$^{-/-}$ mice and induced to proliferate with (**A**) ConA plus IL-2 or (**B**) anti-CD3/CD28 antibodies plus IL-2 in isotonic or moderately hypertonic media. Cells were collected at the indicated time points and depleted of B cells before lysing. Pyruvate kinase (PyrK) or β-Actin were used as protein loading controls. Each panel shows a representative experiment of three independently performed. **C)** Cell cycle distribution of CD3/CD28-stimulated splenocytes. Cultures were*

*labeled with Hoechst 33342 and analyzed by flow cytometry. Values express mean ± SEM of three independent experiments (* = p <0.05).*

Finally, we analyzed the mRNA abundance of osmoprotective gene products and different cell cycle regulators in wild-type and NFAT5-deficient lymphocytes in response to CD3/CD28 stimulation under isotonic or moderately hypertonic conditions (420 mOsm/kg). NFAT5$^{-/-}$ T cells exhibited an impaired osmoprotective response, as shown by the poor expression of Hsp70.1, SMIT and aldose reductase (AR) (**Figure 40A**). In addition, induction of cyclins A2, B1 and aurora B kinase was more severely inhibited in NFAT5$^{-/-}$ T cells than in wild-type ones (**Figure 40A and 40B**).

A



B

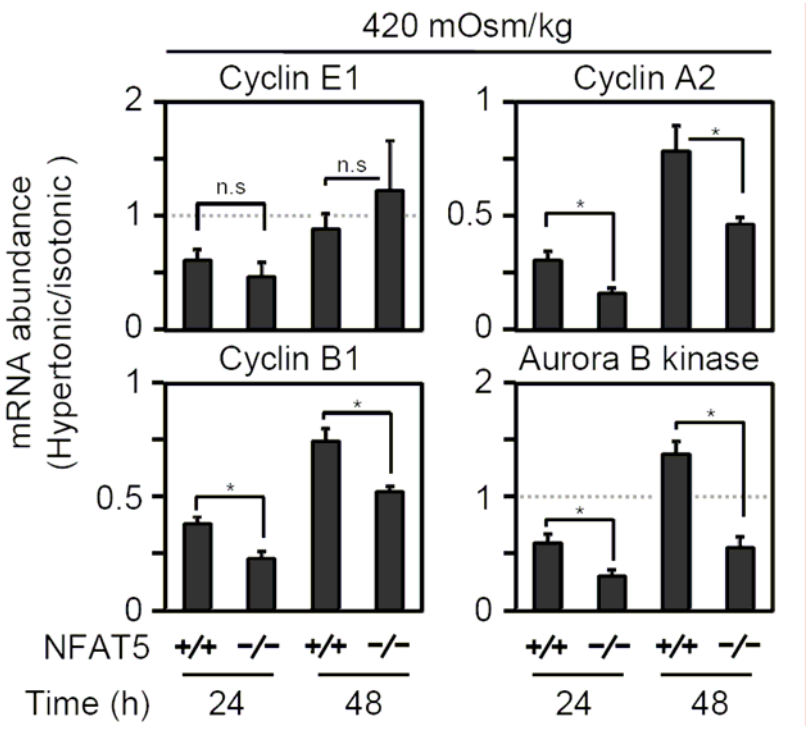


Figure 40. Effect of hypertonicity on cyclin induction and cell cycle entry induced by mitogens or TCR activation. *NFAT5$^{+/+}$ and NFAT5$^{-/-}$ splenocytes were induced to proliferate with anti-CD3/CD28 antibodies plus IL-2 in isotonic or moderately hypertonic media. Cultures were harvested at the indicated time points and depleted of B cells. RNA was isolated and analyzed by RT-qPCR.* ***A)*** *A representative experiment of three independently performed is shown.* ***B)*** *mRNA abundance for the indicated genes in hypertonic relative to isotonic conditions at the same time point. All values were normalized to each respective L32 mRNA level. Values are mean ±SEM of three independent experiments (* = p <0.05; n.s = not statistically significant).*

SUPPLEMENTARY FIGURES

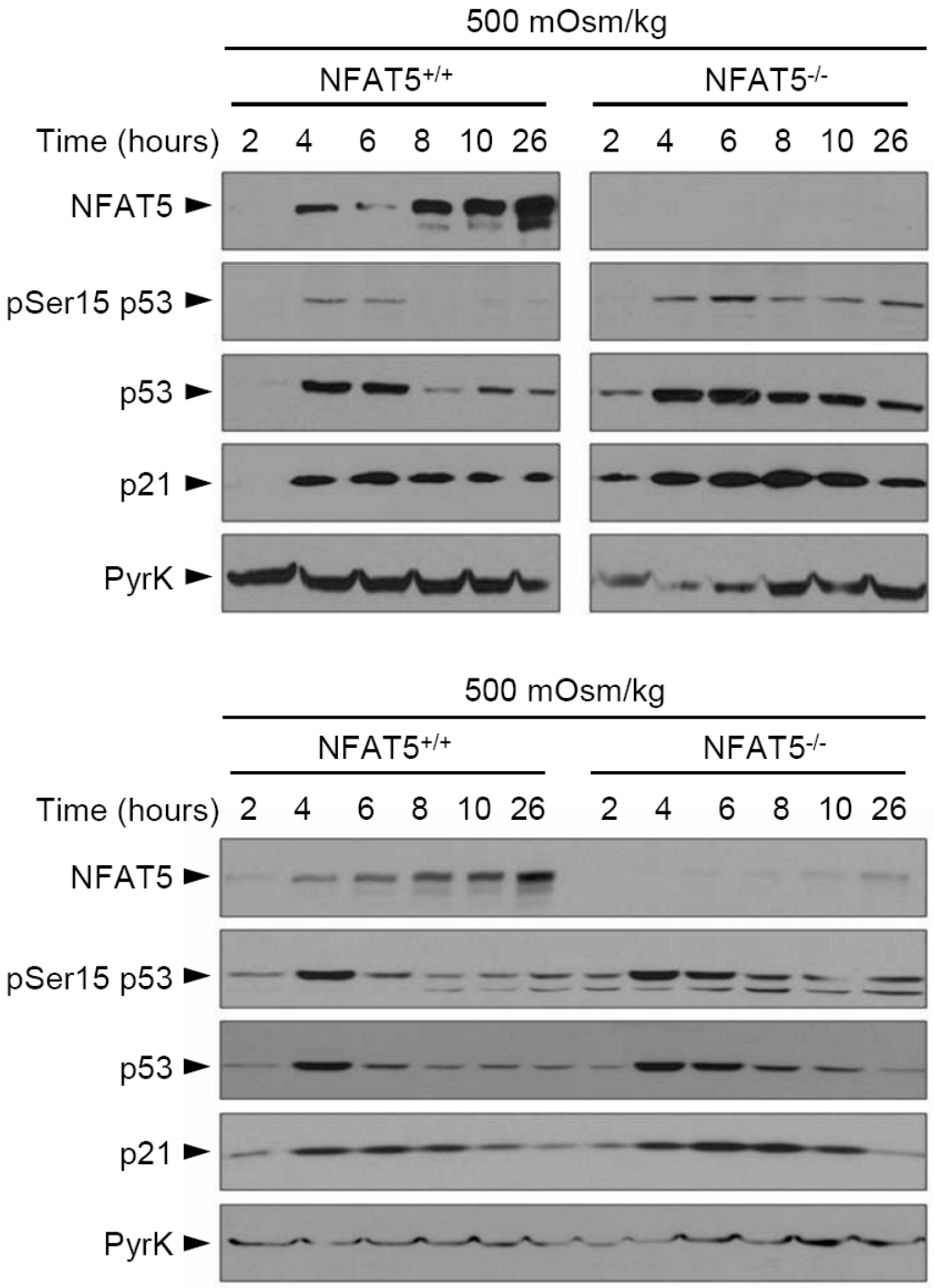


Figure S1. Induction of p53 and p21 in response to hypertonicity. *Time course of p53-Ser15 phosphorylation, accumulation of total p53 and p21 in NFAT5$^{+/+}$ and NFAT5$^{-/-}$ cells in response to hypertonicity were analyzed by Western blot. Pyruvate kinase (PyrK) is shown as protein loading control*.

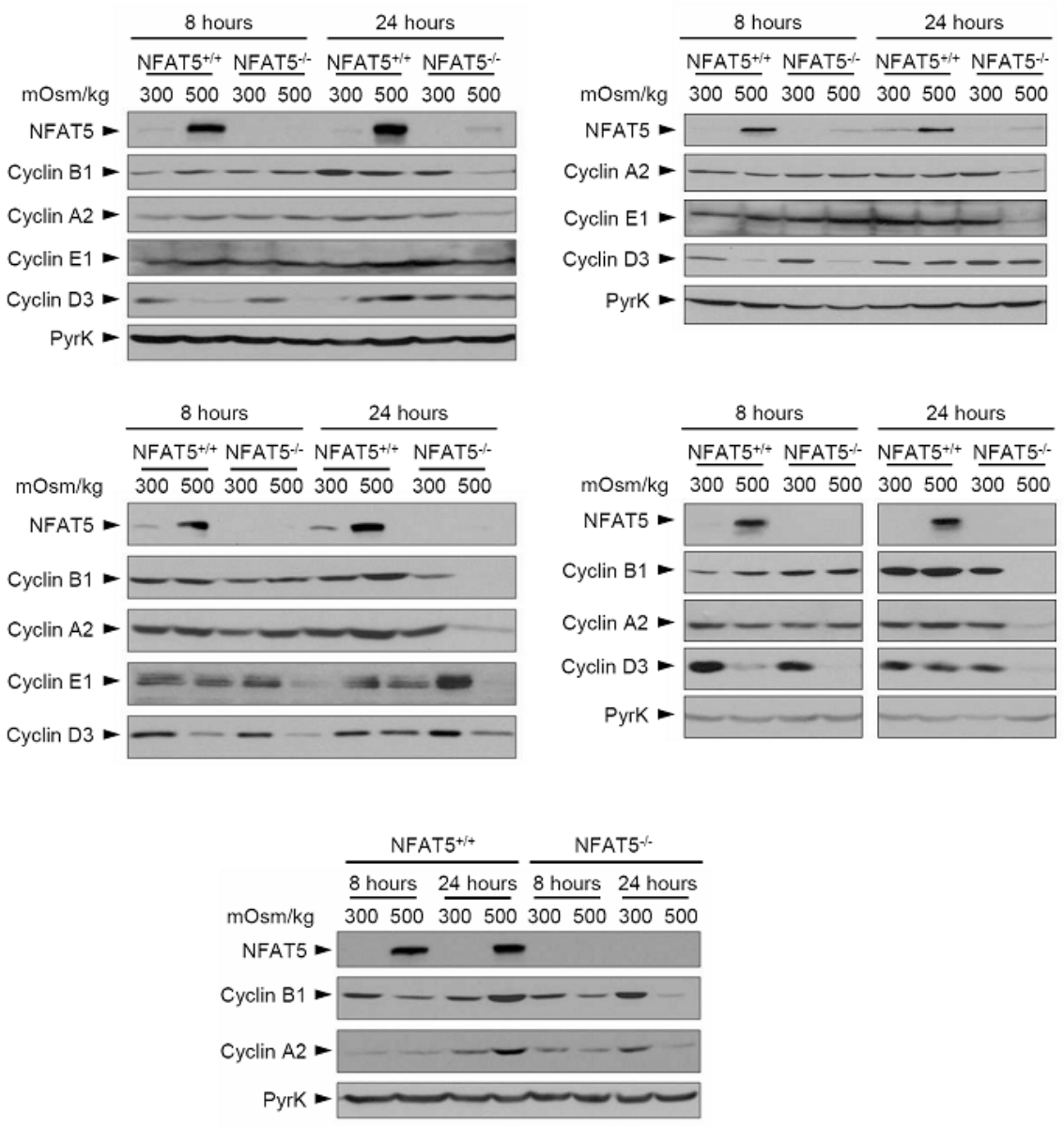


Figure S2. Expression of cyclins in proliferating T cell upon exposure to hypertonic conditions. *Expression of cyclins D3, E1, A2 and B1 was analyzed by Western blot in lysates of proliferating NFAT5$^{+/+}$ and NFAT5$^{-/-}$ T cells after 8 and 24 hours of hypertonicity treatment. Pyruvate kinase (PyrK) is shown as protein loading control.*

DISCUSSION

DISCUSSION

Work from different laboratories has shown that hypertonicity elicits a stress response that is recognizably conserved in different mammalian cell types, triggering the activation of NFAT5, which in turn induces osmoregulatory genes that allow cells to restore their intracellular osmotic equilibrium.

Here we have analyzed the regulation of the cellular response to hypertonicity in wild-type and NFAT5-deficient lymphocytes. Our results show that T cells exhibit an overall stress response, with induction of osmoprotective gene products, genotoxic stress markers and cell cycle arrest, which is remarkably similar to that of cells from the renal medulla (Burg *et al.*, 2007; Lopez-Rodriguez *et al.*, 2004). The fact that different types of mammalian cells activate highly conserved mechanisms in response to osmotic shock should not be surprising since components of this response including stress signaling pathways, cell cycle control and upregulation of compatible osmolytes are found in cells from as distant organisms as vertebrates and yeast, as recently reviewed (Burg *et al.*, 2007).

One of our main findings, which had not been addressed in previous studies, is the dissection of the role of NFAT5 in the course of the hypertonic stress response. Our results show that this response develops in two phases and draw a clear distinction between NFAT5-regulated and NFAT5-independent events in the osmotic stress response of proliferating mammalian cells. In addition, we describe that NFAT5 is necessary for the appropriate expression of cell cycle regulators and for cell cycle progression in primary T cells when exposed to pathologic hypertonic conditions.

1. Advantages of using conditional-NFAT5 knockout mouse model.

Genetic ablation of NFAT5 in mice leads to mid-embryonic lethality, growth abnormalities, severe renal defects and impaired T cell responses *in vivo* (Go *et al.*, 2004; Lopez-Rodriguez *et al.*, 2004). In order to avoid the severe viability defect of NFAT5-null mice we generated conditional knockout mice in which selective deletion of NFAT5 was achieved by crossing these mice with CD4-Cre animals to obtain mice lacking NFAT5 specifically in mature T cells. This

mouse model allowed us to obtain NFAT5-deficient ($NFAT5^{-/-}$) T cells whose function was not biased by generalized defects in the whole organism. $NFAT5^{-/-}$ T cells proliferated indistinguishably from their wild-type counterpart *in vitro* in response to mitogens (**Figure 24**) or TCR stimulation (**Figure 39**) in isotonic media. However, when exposed to hypertonic stress, $NFAT5^{-/-}$ T cells exhibited viability and proliferation defects (**Figures 24 and 39**). These results are in agreement with those reported by the Ho laboratory (Go *et al.*, 2004). However, these authors also showed that NFAT5-deficient mice had fewer T cells than wild-type littermates, and reduced T cell-dependent responses *in vivo*. As shown here, conditional deletion of NFAT5 in T cells did not affect the proportion of mature T cells *in vivo* (**Figure 22**), nor their ability to mount adaptive responses to alloantigens (C. Lopez-Rodriguez, unpublished data). On the other hand, recent results from our laboratory show that conventional NFAT5-null mice do have reduced proportion of T cells and impaired responses to antigens *in vivo*. Altogether, our results indicate that whereas mice lacking NFAT5 in the whole organism have defective T cell function, these are not attributable to the lack of NFAT5 in mature lymphocytes. In this regard, our analysis of plasma biochemical parameters of NFAT5-null mice has shown that these mice exhibit significantly higher levels of sodium in plasma, creating a hypertonic disorder (hypernatremia) which is likely due to the renal dysfunction suffered by these mice (C. Lopez-Rodriguez, unpublished data). Thus, as the few surviving NFAT5-null mice suffer from chronic hypernatremia (average plasma tonicity of 390 mOsm/kg compared to 316 mOsm/kg in wild-type and 336 mOsm/kg in heterozygous animals), the lymphoid cells and tissues and possibly other organs in this model are surrounded by a hypertonic environment. Therefore, the defect in T cell function observed *in vivo* in NFAT5-null mice and the lack of such defect in the conditional NFAT5 knockout mice described here, might be explained by the hypertonic disorder in the former. In the study by Go and colleagues, they explain the *in vivo* T cell defect of NFAT5-null mice as being caused by the slight hyperosmolarity that naturally occurs in lymphoid organs, which ranges from 310-330 mOsm/kg compared to 290-300 mOsm/kg found in plasma. However, this explanation does not fully support their own results, as they showed that a tonicity level of 340 mOsm/kg did not inhibit proliferation of NFAT5-deficient T cells *in vitro*. In addition, as they did not measure plasma tonicity in their NFAT5-deficient model, they might have overlooked the possibility that the *in vivo* defects were caused by a much greater pathological elevation of tonicity in the plasma of their mice.

In this study we have shown that exposure of NFAT5$^{-/-}$ T cells to moderately hypertonic media in the range of 380 - 420 mOsm/kg, as reported to occur in plasma of patients with anisosmotic disorders (see Introduction, section 4) and in NFAT5-null mice (unpublished observations), impairs their proliferative capacity and expression of cell cycle regulators such as cyclins and aurora B kinase. These findings will be discussed in a following section.

2. Role of NFAT5 in the response of proliferating T cells to hypertonic shock.

Our first set of experiments were done using mitogen-activated, proliferating T cells (T cell blasts) and stressing cells with hypertonic media of 500 mOsm/kg. This experimental setup was initially chosen since these conditions are widely used in the literature to study the osmotic stress response and the regulation of NFAT5 in diverse type of mammalian cells, and additionally because they triggered a maximal induction of NFAT5 in proliferating T cell blasts (**Figure 23A**).

One of our main findings, which had not been addressed in previous studies, is the dissection of the role of NFAT5 in the course of the hypertonic stress response. The early phase (6 to 10 hours after onset of hypertonicity) followed a similar course in both NFAT5$^{+/+}$ and NFAT5$^{-/-}$ T cells, resembling a genotoxic stress-like response. The early response evolved into an NFAT5-regulated adaptive phase. This phase began as early as 6 hours after exposure to hyperytonic stress as shown in **Figure 25A**, in which the correlation between the rapid extinction of p53 and the induction of NFAT5 in wild-type cells indicated that this factor rapidly activates an adaptive response that allows cells to withstand the stress. Furthermore, NFAT5-dependent gene expression was clearly detected by 8 hours (**Figures 28 and 35**). After 24 hours of exposure to hypertonicity wild-type cells had adapted and resumed proliferation. In contrast, NFAT5$^{-/-}$ cells manifested greater viability loss, ineffective replication and G2/M arrest. Key differences in the adaptation process of wild-type and NFAT5-deficient T cells came to light, as discussed below.

During the early phase of the hypertonic stress response, both cell types suffered a similar viability loss and acute cell cycle arrest through S and G2/M phases. In addition, this phase was characterized by a sharp induction of genotoxic stress markers such as p53, p21, GADD45α and GADD45β, and downregulation of GADD45γ (**Figure 28**). In view of these results, we addressed whether the sustained cell cycle arrest observed in hypertonicity-treated NFAT5$^{-/-}$

cells was due to a greater occurrence of DNA damage. Direct analysis of DNA breaks did not provide any compelling evidence of a substantial increase in DNA damage in NFAT5$^{-/-}$ cells compared to wild-type. However, NFAT5$^{-/-}$ cells had a more prolonged induction and phosphorylation (Ser15) of p53 than wild-type cells in the first 10 hours, which was PIKK-dependent (**Figure 25D**), consistent with the described activation of ATM by hypertonicity (Irarrazabal *et al.*, 2004; Irarrazabal *et al.*, 2006). The fact that NFAT5$^{-/-}$ T cells showed a more prolonged upregulation of p53 than wild-type cells could indicate a stronger genotoxic stress-like response as well as enhanced pro-apoptotic signaling. However, after 24 hours in hypertonic conditions, viable NFAT5$^{-/-}$ did not express higher amounts of the p53 target p21 and only showed slightly higher levels of phosphorylation of Ser15-p53 and γH2AX than wild-type cells. In addition, they had downregulated other markers of genotoxic stress, such as GADD45 proteins, in a similar fashion to NFAT5$^{+/+}$ lymphocytes. This indicated that their genotoxic stress-like response had abated by 24 hours after the onset of hypertonicity, despite of which they still exhibited a persistent cell cycle arrest.

As sensors of several kinds of stressors, expression of GADD45 proteins has been described to be affected by hypertonicity (Cai *et al.*, 2006; Chakravarty *et al.*, 2002; Kultz *et al.*, 1998). Induction of GADD45α and GADD45β are associated with growth arrest, differentiation and apoptosis. In contrast, GADD45γ is strongly induced by IL-2 (Liebermann and Hoffman, 2007). Our results show that during the early response to hypertonicity, T cells exhibited induction of GADD45α, GADD45β and downregulation of GADD45γ mRNAs, which is consistent with their inducible expression patterns. Importantly, our data also indicate that these events are not mediated by NFAT5. Nonetheless, we do not rule out that these proteins may play a role in the cellular response to hypertonicity, as generation of GADD45 isoforms α and β knockout mice indicate that these proteins are important players in protecting hematopoietic cells from apoptosis induced by DNA damaging agents such as UV radiation and some anticancer drugs (Gupta *et al.*, 2006; Liebermann and Hoffman, 2007). However, as recently shown by the Burg laboratory, mice lacking GADD45 proteins did not display defects in the morphology of the inner renal medulla nor in their urine concentration ability (Cai *et al.*, 2006). On the other hand, while GADD45γ is strongly induced by IL-2, generation of GADD45γ$^{-/-}$ mice suggests that it is dispensable for normal T cell proliferation (Hoffmeyer *et al.*, 2001).

Members of the heat shock family of proteins Hsp70 play an essential role in the enhancement of cellular survival under several stresses, including high NaCl (Shim *et al.*, 2002), by acting as molecular chaperones that prevent misfolding and aggregation of newly synthesized proteins (Bukau and Horwich, 1998). Hsp70, specifically isoform Hsp70.1 (Hsp70-2), is induced by hypertonicity in an NFAT5-dependent manner (Woo *et al.*, 2002). As expected, our experiments showed that NFAT5-deficient T cells induced this chaperone to significantly lower levels than $NFAT5^{+/+}$ cells did, as assessed by RT-qPCR analysis. However, we were not able to compare the induction of mRNA with its protein, as the antibody that we used (Stressgen, Cat. SPA-810) did not detect Hsp70.1 induced by hypertonic stress, while it clearly detected that induced by heat shock (**Figure 29**). We thus addressed whether lack of Hsp70.1 impaired the cellular response to hypertonicity. As a model we used MEF that lack both Hsp70.1 and Hsp70.3 isoforms (Hunt *et al.*, 2004) and exposed them to increasing amounts of hypertonic stress. As the wild-type cells, Hsp70.1/3-deficient MEF were able to induce NFAT5 protein upon exposure to hypertonic stress cells, but became more arrested at the G1 phase and had fewer cells in S and G2/M (**Figure 29**) under these conditions than wild-type cells. On the other hand, induction of Hsp70 by heat shock in $NFAT5^{-/-}$ T cells prior to exposure to hypertonic stress did not prevent their cell cycle arrest. Altogether, these results suggest that defective expression of Hsp70.1 might contribute to the cell cycle defects observed in hypertonically stressed $NFAT5^{-/-}$ T cells, although restoring its expression does not appear to be sufficient to overcome this defect. In this regard, we observed that $NFAT5^{-/-}$ T cells had a generalized impairment to induce other osmoprotective genes such as SMIT, aldose reductase and TauT (**Figures 28 and 40**). Impairment of this response might be the responsible for the defective proliferative capacity of hypertonically-stressed $NFAT5^{-/-}$ lymphocytes.

In addition to the effects discussed above, our studies showed that another important part of the early stress response is the downregulation of cyclins. Both protein and mRNA levels of G1, S and G2/M cyclins are downregulated in a similar fashion during the first hours of the response to hypertonicity in both wild-type and $NFAT5^{-/-}$ T cells (**Figures 36 and 37**). These cell cycle regulators showed to be important in the adaptation process and will be discussed in a following section.

3. Regulation of NFAT5 throughout the cell cycle and expression of cyclins under hypertonic stress.

We were interested in determining whether the impairment in cell cycle progression owed to defects in cell cycle regulators in NFAT5-deficient cells. Consistent with the cell cycle arrest observed during the early response (8 hours), most cyclins tested (cyclin D3, E1, A2, and B1) and aurora B kinase were comparably downregulated in wild-type and NFAT5$^{-/-}$ T cells, as shown by protein and mRNA levels (**Figures 36 and 37**). However, by 24 hours wild-type cells recovered the expression of these cyclins whereas NFAT5-deficient T cells only induced cyclin D3 but could not maintain the expression of cyclins E1, A2 and B1. The expression and activity of NFAT5 were progressively increased with the duration of the stress, and by 24 hours these parameters were substantially enhanced in S and G2/M (**Figure 34, 35 and 36B**). This finding is consistent with the observation that in S and G2/M phases NFAT5$^{-/-}$ cells were highly sensitive to hypertonic stress as indicated by the cell cycle arrest (**Figure 24**) and defect in maintenance or upregulation of S and G2/M cyclins when challenged by hypertonicity (**Figures 36 and 37**). Our results also indicate that lack of NFAT5 affects these regulators at different levels, since downregulation of cyclins A2 and B1 correlated with a reduction in their mRNA abundance (**Figure 37**) whereas the decrease in cyclin E1 did not appear to be attributable to the loss of its mRNA and might be due to reduced protein stability and/or synthesis rate. In this regard, there are two possible interpretations to explain the correlation between the observed cell cycle arrest and cyclin downregulation; the first would be that the decreased levels of phase-specific cyclins would lead to the arrest or slower progression through each phase. Alternatively, inability to maintain cyclin E1 levels would impair the G1-S transition leading to fewer cells progressing to S and G2 and thus the reduced levels of cyclin A2 and B1 would be a consequence of there being fewer cells in those phases in asynchronous cultures. These possibilities are not mutually exclusive. However, our results in fresh lymphocytes (**Figures 39 and 40**) show that while cyclin E1 is comparably induced in wild-type and NFAT5$^{-/-}$ T cells, induction of cyclins A2 and B1, which preceeds the entry of cells into a cycling mode, is greatly impaired in NFAT5-deficient cells, indicating that defective cell cycle progression results from ineffective cyclin induction.

The observed defect in expression of cyclins and aurora B kinase in actively proliferating NFAT5$^{-/-}$ lymphocytes subjected to an osmotic stress reveals a novel mechanism of cell cycle control by hypertonic stress and NFAT5. Downregulation of cyclins A2 and B1 can impair the

cell cycle in different cell types (De Boer *et al.*, 2008; Yuan *et al.*, 2004), and aurora B kinase has been shown to be required for cell cycle progression of antigen-stimulated T cells (Song *et al.*, 2007). Cyclin B1 is a major coordinator of S phase progression with cell division (Lukas *et al.*, 1999), and it has been shown that suppression of cyclin B1 in cell lines can cause inhibition of proliferation, arrest in G2/M and apoptosis (Yuan *et al.*, 2004). Proper timing of mitotic entry is greatly regulated by the activity of cyclin B1/Cdk1 complexes. In addition to being regulated by complex assembly and phosphorylation events, cyclin B1/Cdk1 complex activity has been recently reported to be regulated by an additional inhibitory mechanism of cyclin B1/Cdk1 mediated mitotic entry. While the well-described phosphorylation events controlling the activity of cyclin B1/Cdk1 complex take place in the cytoplasm, the newly described inhibitory mechanism is a nuclear event mediated by the death-effector domain containing protein (DEDD) by which mitotic entry is dictated by proper cellular growth before division (Miyazaki and Arai, 2007). Inhibition of cyclin A2 expression can cause cell cycle arrest in S and G2/M phases (De Boer *et al.*, 2008). Loss of cyclin A2 could also be contributing to the subsequent downregulation of cyclin B1, as several reports indicate several functions for cyclin A2 in the regulation of the timing of cyclin B1/Cdk1 complexes activity. Downregulation of cyclin A2 was shown to promote inhibitory phosphorylation of Cdk1, inducing a G2 phase arrest through a checkpoint-independent mechanism, and cells were not able to follow through mitosis in the absence of cyclin B1 (Fung *et al.*, 2007). Other reports have shown that cyclin A2 directs the nuclear accumulation of cyclin B1/Cdk1 complexes (Gong *et al.*, 2007) and coordinates the activation of this complex at the centrosome and the nucleus (De Boer *et al.*, 2008). Although we have not explored whether hypertonically stressed NFAT5$^{-/-}$ T cells have additional defects in the regulation of cyclins and Cdks, it is possible that such defects might be found.

Upon exposure to hypertonic stress, cells initially shrink and in order to restore cellular size they undergo a process known as regulatory volume increase (RVI), in which the intracellular concentration of inorganic ions is increased (see introduction, section 2). RVI is followed by an osmoadaptation process in which NFAT5 activates the expression of several enzymes and transporters whose collective function is the accumulation of intracellular compatible osmolytes that prevent a harmful buildup of inorganic ions. It also induces heat shock proteins and chaperones, which might protect sensitive proteins from the deleterious effect of water stress (Burg and Ferraris, 2008). It is thus plausible that if NFAT5$^{-/-}$ T cells have not been able to recover appropriate cellular size or restore their intracellular ionic environment after 24 hours of

exposure to hypertonicity, this would affect not only cyclin expression but also other regulatory events such as subcellular distribution and activity of cyclin/Cdk complexes along the cell cycle.

Our findings here are consistent with previous articles and suggest that inhibition of NFAT5 in cells exposed to hypertonicity could lead to different outcomes, with a proportion of the cells undergoing apoptosis, possibly associated with an enhanced genotoxic stress-like response, whereas surviving cells had reduced proliferative capacity. The proliferation defect of NFAT5-deficient lymphocytes did not correlate with the presence of conspicuous symptoms of DNA damage, but rather with a substantially reduced expression of cyclins and aurora B kinase. In addition, inhibition of NFAT5 did not compromise the activity of the promoters of cyclins A2 and B1 in Jurkat T cells subjected to hypertonic shock, suggesting that NFAT5 is not among the transcription factors required for the function of these promoters. Nonetheless, we do not rule out that transcription of the endogenous cyclin gene might be altered in NFAT5$^{-/-}$ T cells as other regions not included in the limited promoter tested here could be affected.

4. Role of NFAT5 in DNA damage response in proliferating T cells.

We have shown that failure to induce NFAT5 during hypertonic stress causes cell death or severe cell cycle arrest in surviving cells, of which a small proportion also displayed symptoms of genotoxic stress. We thus addressed whether NFAT5 was involved in the response to genotoxic agents independent of hypertonicity. It has been long suggested that exposure to hypertonic medium results in increased radiosensitivity (Raaphorst *et al.*, 1977; Zhu *et al.*, 2008). However, in most of these studies cells were subjected to extremely high tonicity levels, as final NaCl concentration ranged from 500 mM up to 1.5 M, which leads to tonicity levels of over 1000 mOsm/kg.

Our results showed that ionizing radiation did not activate NFAT5 nor affected its activation by hypertonicity, indicating that the engagement of a DNA damage-response pathway was not sufficient *per se* to induce or activate NFAT5, in agreement with Irarrazabal *et al* (Irarrazabal *et al.*, 2004). On the other hand, NFAT5-deficient cells did not appear to be more sensitive than wild-type ones to ionizing radiation (**Figure 31**) nor to the DNA damaging drugs etoposide and hydroxyurea (not shown).

We exposed T cells to a low dose of ionizing radiation (3 Gy) and then cultured them in a moderately hypertonic environment, one that on its own had no greater effect on NFAT5$^{-/-}$ than on wild-type T cells. Our data showed that NFAT5$^{+/+}$ T cells were able to tolerate each stress individually or combined, while NFAT5$^{-/-}$ T cells were only able to tolerate these stresses individually, as culture in moderately hypertonic media (420 mOsm/kg) resulted in enhanced radiosensitivity as shown by the cell cycle arrest observed. This finding was not unexpected, since some of the genes that can be induced by NFAT5 in response to hypertonicity also participate in the survival to other types of stress (Kultz, 2003). For instance, Hsp70 is important in the resistance to ionizing radiation (Hunt *et al.*, 2004), and downregulation of TauT and SMIT has been linked to the toxicity of the radiomimetic drug doxorubicin in cardiac myocytes (Ito *et al.*, 2007). Our results also agree with the study by Dmitrieva and colleagues, who reported that elevated levels of hypertonicity impaired the cell cycle and survival to UV and ionizing radiation (Dmitrieva *et al.*, 2003).

Our findings thus highlight the importance of NFAT5 in protecting cells from the detrimental effect and of even moderate levels of hypertonic stress, which can cross-sensitize cells to other DNA-damaging agents.

5. Physiological relevance

Most studies addressing the response of mammalian cells to hypertonic stress routinely use conditions of 500 mOsm/kg or even higher. Indeed, very different mammalian cell types, such as neurons, leukocytes, fibroblasts and others can withstand these conditions and have the ability to induce and activate NFAT5 along with a conserved osmoprotective gene expression program in response to osmotic shock (Burg *et al.*, 2007). The observation that so diverse cell types have retained the ability to respond to substantial hypertonic stress is intriguing. However, besides specific anatomical niches, such as the renal medulla, which is subjected to acute osmolality changes (Burg *et al.*, 2007) and the nucleus pulposus of intervertebral discs, which is embedded in a hypertonic matrix of 420-450 mOsm/kg (Ishihara *et al.*, 1997), knowledge on physiopathological scenarios in which mammalian cells might be exposed to severe osmotic stress is limited. Nonetheless, reports in the clinical literature indicate that several disorders can cause hypernatremia and increase the osmolality of plasma to 360-430 mOsm/kg (See

introduction, section 4). Elevated plasma osmolality (400 mOsm/kg) can also result from prolonged water deprivation, as shown in camels (Dmitrieva *et al.*, 2005). In addition, constitutively high plasma tonicity (~ 400 mOsm/kg) has been also reported in mice deficient in molecules important for the urine concentration (see introduction, section 4). Hypertonic levels around 400 mOsm/kg are sufficient to mobilize NFAT5 in neurons (Loyher *et al.*, 2004) and primary lymphocytes (Morancho *et al.*, 2008).

Unpublished results from our laboratory showed that NFAT5-null mice had hypernatremia that led to hypertonic plasma -in the range of anisosmotic disorders in human patients and animal models- and may be a likely cause of the impaired *in vivo* T cell response of NFAT5-null mice. We thus analyzed whether the cell cycle defects observed in NFAT5$^{-/-}$ T cell blasts also occurred in fresh T cells induced to proliferate via TCR-engagement when grown in media with moderate tonicity levels that corresponded to documented pathological conditions.

As in T cell blasts, we observed a greater defect in cyclin and aurora B kinase expression in fresh NFAT5$^{-/-}$ T cells that were induced to proliferate in moderate hypertonic conditions (380-420 mOsm/kg) (**Figures 39 and 40**). The results shown here are consistent with previous work from others and our group, showing that NFAT5 is required for T cells to proliferate optimally in response to TCR stimulation under pathologic hypertonic conditions (~370 mOsm/kg) (Go *et al*., 2004), and that NFAT5 can be induced and activated by pathologically relevant hypertonicity levels (360 - 420 mOsm/kg) in T cells during their early phases of activation by mitogenic signals (Morancho *et al.*, 2008). Once T cells have been activated and are in a proliferative state, they require much higher tonicity levels to activate NFAT5 (~480 mOsm/kg) (Morancho *et al.*, 2008) and are relatively resistant to moderate hypertonic stress even in the absence of this factor, at least with regard to their capacity to maintain a functional cell cycle as shown here in **Figure 33**. Altogether, our findings help to explain the T cell immunodeficiency reported in NFAT5-null mice (Go *et al.*, 2004) and which, according to our observations, suffer constitutive plasma hypernatremia leading to plasma hypertonicity (C. Lopez-Rodriguez, unpublished results).

Collectively, our observations suggest that the early phase of T cell activation, when cells are acquiring proliferative competency, is highly sensitive to alterations of ambient tonicity, in the range reported to occur in a number of pathological conditions, and that NFAT5 is crucial to

allow cells to enter the cell cycle. The notion that NFAT5 contributes to maintain diverse cellular functions by controlling the intracellular tonicity is simple and is supported by our results. Nonetheless, future work is needed to dissect the mechanisms by which specific cellular processes are impaired under restrictive tonicity conditions and to address whether the main role of NFAT5 is to control an osmoprotective program or it might have additional functions in the regulation of the cell cycle. Although we have not explored other T cell functions, such as cytokine production or ability to differentiate toward effector populations, we would hypothesize that these functions are probably affected by hypertonic stress and might be altered in NFAT5-deficient T cells exposed to hypertonicity.

Diverse types of stressors can threaten the genetic integrity of the cell, and stress responses are often associated with cell cycle arrest, which allows cells to activate adaptive programs that enable them to withstand the stress and resume proliferation. Failure to adapt or irreparable damage might cause permanent arrest and cell death. In this context, the stress-activated p38-MAPK/MK2 kinase pathway can activate cell cycle checkpoints (Manke *et al.*, 2005; Reinhardt *et al.*, 2007), and DNA damage-activated kinases such as ATM and other PIKKs can regulate stress-responsive transcription factors such as p53, NF-κB, c-Jun, or ATF2 and ATF3 (Siliciano *et al.*, 1997; Panta *et al.*, Piret *et al.*, 1999; Foray *et al.*, 2003; 2004; Bhoumik *et al.*, 2005; Fan *et al.*, 2002). NFAT5 is activated by p38-MAPK and PIKK in response to hypertonic stress (Ko *et al.*, 2002; Irarrazabal *et al.*, 2004; Morancho *et al.*, 2008), and is required for cell survival and proliferation under sustained hypertonic conditions (Go *et al.*, 2004; Lopez-Rodriguez *et al.*, 2004). Our results show that NFAT5 is required to enable the expression of cyclins and the progression of the cell cycle, thus playing a major role in the adaptive phase of the cellular response to both osmotic shock and pathologic hypertonic conditions.

CONCLUSIONS

CONCLUSIONS

1. Proliferating T cells respond to hypertonic stress in two phases; an early NFAT5-independent phase, and a subsequent adaptation phase orchestrated by NFAT5.

2. The early adaptation phase of proliferating T cells subjected to osmotic shock resembles a DNA damage response as it involves upregulation of genotoxic stress markers p53, p21 and GADD45 proteins (isoforms α and β). During this phase, cell cycle is arrested along S and G2/M phases, and cyclins A2 and B1 mRNA levels are downregulated. This phase is mainly NFAT5-independent.

3. In proliferating T cells, NFAT5 plays a major role in the adaptive phase of the cellular response to osmotic shock by enabling the expression of osmoprotective gene products such as Hsp70.1, TauT and SMIT. NFAT5 is also necessary for proper expression of cyclins, aurora B kinase, and cell cycle progression.

4. NFAT5 protein expression and transcriptional activation in hypertonically-stressed lymphocytes are greater in the S and G2/M phases than in G1.

5. The cell cycle arrest observed in NFAT5$^{-/-}$ lymphocytes after 24 hours after the onset of hypertonicity does not correlate with greater DNA damage but rather with the downregulation of cyclins A2, B1 and aurora B kinase protein and mRNA levels.

6. NFAT5 is not activated by general DNA damage responses and does not appear to regulate cell cycle in cells exposed to ionizing radiation and other genotoxic stressors. Nonetheless, lack of NFAT5 enhances the sensitivity to low doses of ionizing radiation when combined with moderate hypertonic conditions, which could be individually tolerated by NFAT5$^{-/-}$ T cells.

7. NFAT5 is required for proper T lymphocytes cell cycle entry under pathological hypertonic conditions found in plasma of patients and animal models of osmoregulatory disorders. In this scenario, NFAT5 was required for induction of osmoprotective genes Hsp70.1, SMIT and aldose reductase, as well as for that of cyclin A2, cyclin B1 and aurora B kinase.

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ANNEX: SUBMITTED ARTICLE

The transcription factor NFAT5/TonEBP is required for cyclin expression and cell cycle progression in lymphocytes exposed to hypertonic stress.

Running title: cell cycle regulation in NFAT5-deficient cells.

Katherine Drews-Elger[1], M. Carmen Ortells[1], Anjana Rao[2], Cristina López-Rodriguez[1¶*] and Jose Aramburu[1¶*].

[1] Departament de Ciències Experimentals i de la Salut, Universitat Pompeu Fabra, and Barcelona Biomedical Research Park (PRBB), 08003 Barcelona, Spain.
[2] Immune Disease Institute and Department of Pathology, Harvard Medical School, Boston, 02115 MA, USA.

[¶] Both authors contributed equally.
[*] To whom correspondence should be addressed:

Jose Aramburu,
Departament de Ciències Experimentals i de la Salut, Universitat Pompeu Fabra.
Carrer Dr Aiguader 88, 08003 Barcelona, Spain.
Tel: +34 93 3160809. Fax: +34 93 3160901. Email: jose.aramburu@upf.edu

Cristina López-Rodríguez,
Departament de Ciències Experimentals i de la Salut, Universitat Pompeu Fabra.
Carrer Dr Aiguader 88, 08003 Barcelona, Spain.
Tel: +34 93 3160810. Fax: +34 93 3160901. Email: cristina.lopez-rodriguez@upf.edu

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Abstract

The transcription factor NFAT5/TonEBP enables cells to adapt to hypertonicity by inducing osmoprotective gene products. Under hypertonic conditions, NFAT5-deficient cells exhibit survival and proliferation defects, as shown for kidney medullary cells and lymphocytes. Here we have investigated the hypertonic stress response in wild-type and NFAT5$^{-/-}$ lymphocytes. Proliferating lymphocytes switched to hypertonic conditions exhibited an early, NFAT5-independent, genotoxic stress-like response with induction of p53, p21 and GADD45, downregulation of cyclins E1, A2 and B1 mRNA, and arrest in S and G2/M. This was followed by an NFAT5-dependent adaptive phase in wild-type cells, which induced osmoprotective gene products, downregulated stress markers, and resumed cyclin expression and cell cycle progression. NFAT5$^{-/-}$ cells, however, failed to induce osmoprotective genes and though they downregulated genotoxic stress markers, they displayed defective cell cycle progression associated with reduced expression of cyclins E1, A2, B1, and aurora B kinase. Finally, T cell receptor-induced expression of cyclins, aurora B kinase, and cell cycle progression were inhibited in NFAT5$^{-/-}$ lymphocytes exposed to hypertonicity levels in the range reported in plasma in patients and animal models of osmoregulatory disorders (380-420 mOsm/kg). We conclude that NFAT5 regulates cell proliferation under pathologic hypertonic stress by contributing to maintain the expression of cell cycle regulators.

Keywords: NFAT5, osmotic stress, cell cycle, cyclins, lymphocytes.

Introduction

NFAT5/TonEBP belongs to the Rel family of transcription factors, which also comprises NF-κB and the calcineurin-dependent NFATc proteins (NFAT1/NFATc2, NFAT2/NFATc1, NFAT3/NFATc4, NFAT4/NFATc3) (40, 31, 35, 1). The homology between NFAT5 and other Rel proteins is limited to the DNA binding domain, with no recognizable similarities outside of it. The DNA binding domain (DBD) of NFAT5 is considered a hybrid between that of NFATc and NF-κB proteins, since it has NFATc-like DNA sequence specificity but is a constitutive dimer, structurally similar to NF-κB (30, 55). NFAT5 allows mammalian cells to withstand hypertonic conditions by inducing an osmoprotective gene expression program whose products include Hsp70 (63), the chaperone Osp94 (26), and enzymes and transporters that increase the intracellular concentration of compatible osmolytes, such as aldose reductase (AR), Na^+/Cl^--coupled betaine/γ-aminobutyric acid transporter (BGT1), Na^+/ myo-inositol cotransporter (SMIT), Na^+ and Cl^--dependent taurine transporter (TauT), and UT-A urea transporter (reviewed in (23)).

Hypertonicity can cause double-strand DNA breaks, activate mechanisms involved in genotoxic stress responses, cause cell cycle arrest, and induce apoptosis (4). Hypertonic stress can also exacerbate the cell cycle arrest and viability loss induced by other genotoxic agents such as UV and ionizing radiation (13). Different laboratories have shown that cells exposed to hypertonic conditions display features characteristic of genotoxic stress responses, such as accumulation of Nbs1 in nuclear foci (52), activation of ATM and checkpoint effectors p53 and Chk2 (11, 52), and enhanced expression of GADD45 (36). Studies in mouse inner medullary cells have revealed that hypertonicity causes a transient inhibition of replication and cell cycle arrest in G1 and G2/M, involving p53 (12), the stress-activated protein kinase p38 (14) and GADD45 (36). However, cells recover after several hours and resume proliferation despite being kept in hypertonic medium (5, 49).

Our previous work had shown that NFAT5-null mice suffered severe atrophy and cellular loss in the renal medulla due to deficient expression of osmoprotective genes (29). The renal medulla of NFAT5-null mice contained a greater proportion of TUNEL-positive cells than the medulla of control animals, which suggested increased apoptosis in $NFAT5^{-/-}$ cells. Work by Wang et al. showed similar results in differentiated eye lens fiber cells that overexpressed a dominant negative NFAT5 transgene (60). A proportion of these cells were TUNEL-positive and accumulated markers associated with DNA damage such as p53 and phospho-Chk2, although it was not determined whether these effects were due to

hypertonic stress. Consistent with its role in the hypertonic stress response, NFAT5 can be activated by DNA-damage responsive and stress-activated kinases such as the PI3-kinase-related kinases (PIKK) ATM and DNA-PKc (21, 7) and p38 (25, 3) in different cell types, including primary lymphocytes (41). Overall, the osmoprotective function of NFAT5 has been reported in diverse cell types, as this factor has been shown to be induced and activated by hypertonicity in lymphocytes (58, 30, 41), macrophages (41), embryonic fibroblasts (29, 19), kidney cells (62), neurons (34), and cell lines of different lineages (21).

Although it is known that NFAT5 is important for the survival and proliferation of cells subjected to sustained hypertonic conditions, less is understood about the stepwise regulation of the cell cycle and stress response in primary cells lacking this factor. The study of the role of NFAT5 in specific cell types has been hindered by the severe phenotype of NFAT5-deficient mice, since only a small proportion survive after birth, and those that do manifest pronounced renal atrophy and growth defects (29, 19). To circumvent these problems, we generated $NFAT5^{Flox/Flox}$ mice which could be used to inactivate NFAT5 in a tissue-specific manner. We have crossed the $NFAT5^{Flox/Flox}$ mice to CD4-Cre animals to obtain mice with a selective deletion of NFAT5 in mature T cells, and analyzed the role of this factor in the hypertonic stress response in proliferating T lymphocytes. As shown by us and others, primary, non-transformed T cells regulate NFAT5 comparably to other cell types (41) and display impaired proliferative capacity under hypertonic conditions when lacking this factor (19).

In this work we show that lack of NFAT5 did not alter the proportions of mature T cells in vivo, and NFAT5-deficient lymphocytes showed no apparent defects in viability and proliferative capacity when stimulated with mitogens or via T cell receptor in isotonic medium. Exposure of proliferating wild-type T cells to hypertonic shock induced an early stress response that lasted about 6-8 hours, and consisted on a transient accumulation of cells in S and G2/M, rapid induction and phosphorylation of p53, accumulation of the cyclin-dependent kinase inhibitor p21, induction of GADD45α and β, and downregulation of cyclins E1, A2 and B1 mRNA. These cells induced osmoprotective gene products in an NFAT5-dependent manner and after 24 hours in hypertonic conditions, they had recovered from the cell cycle arrest with only a moderate viability loss, expressed normal levels of cyclins, and maintained elevated levels of transcriptionally active NFAT5 in S and G2/M. $NFAT5^{-/-}$ T cells underwent a comparable early stress phase within the first 8 hours, albeit with a more prolonged induction of p53. By 24 hours, though, they exhibited a greater loss of viability than wild-type lymphocytes, and surviving cells displayed sustained inhibition of replication and defective cell cycle progression. This correlated with a persistent downregulation of cyclins E1, A2, B1 and aurora B kinase. Defective induction of cyclins, aurora B

kinase, and cell cycle progression were also observed in fresh NFAT5$^{-/-}$ T cells when they were induced to proliferate via T cell receptor under moderate hypertonic conditions, in the range measured in plasma in patients and animal models of osmoregulatory disorders. We conclude that the osmoregulatory role of NFAT5 under hypertonic conditions is necessary to maintain the expression of cell cycle regulators, such as cyclins and aurora B kinase, and cell cycle progression.

Materials and Methods

Generation of NFAT5 conditional mice. A targeting vector was designed to flank exon 6 of the mouse NFAT5 gene with two loxP sites (Figure 1A). A BamHI fragment of the mouse NFAT5 genomic locus isolated from a P1 clone was used. A 3.3-kb ApaLI-AvrII fragment was used as 5' homology region, and a 4.8-kb EcoRI-XbaI fragment was used as 3' homology region. One loxP site was introduced 5' to exon 6, in the 1.8-kb AvrII-EcoRI fragment. An frt site-flanked selection cassette, with a neomycin resistance gene, the Flpe cDNA cloned under control of the ACE promoter (50), and the second loxP site, was inserted into an EcoRI site in the sixth intron of the NFAT5 gene. The targeting vector also contained a thymidine kinase gene was used for negative selection of clones with random integration of the targeting vector.

Bruce-4 embryonic stem (ES) cells (27) derived from C57BL/6 mice were transfected, cultured, and selected as previously described (29). Of 800 G418 (neomycin) and gancyclovir-resistant colonies, 3 were identified as homologous recombinants with cointegration of the second loxP site by Southern blot analysis of BamHI-digested DNA, using a probe spanning the exon 5 as 5' external probe (Fig. 1A) and Neo as a 3' probe. ES clones with the appropriately targeted allele were injected into BALB/c blastocysts to generate chimeric mice, which transmitted the targeted allele to their progeny. All mice were maintained on a pure C57BL/6 genetic background. The frt-flanked neomycin resistance cassette was removed through intercrossing with FLPe-deleter mice (48). Mice lacking NFAT5 in T cells were obtained afer successive crosses of $NFAT5^{Flox/Flox}$ mice with CD4-Cre transgenic mice, in which the Cre recombinase is under the control of the mouse CD4 promoter/enhancer/silencer (28). Mice were bred and maintained in specific pathogen-free conditions, and animal handling was performed according to institutional guidelines approved by the ethical committee (PRBB Animal Care and Use Committee). The CD4-Cre transgenic mouse strain (28) was obtained from the Jackson Laboratory (Bar Harbor, ME).

9xNFAT-Luc mice and $p53^{-/-}$ mice. 9xNFAT-Luc mice (line 15.1) in FVB background were previously described (61, 41). $p53^{-/-}$ mice were obtained from the Jackson Laboratory and have been previously described (22).

Antibodies. The anti-NFAT5 polyclonal antibody (Cat. PA1-023) was from Affinity Bioreagents (Golden, CO, USA) and recognizes a carboxy-terminal epitope (DLLVSLQNQGNNLTGSF). The anti-NFAT5 polyclonal antibody recognizing the N-terminal region of NFAT5 was previously described (31). Rabbit polyclonal anti-phospho-p53 (Ser15) (Cat. 9284), mouse monoclonal anti-p53 (Cat. 2524) and

mouse monoclonal anti-cyclin D3 (Cat. 2936) were from Cell Signaling Technology (Danvers, MA, USA); mouse monoclonal anti-phospho-histone H2AX (Ser139, γH2AX) (Cat. 05-636) was purchased from Upstate Technologies (Lake Placid, NY, USA); mouse monoclonal anti-BrdU antibody (Cat. 555627) was purchased from BD Pharmingen (San Diego, CA, USA). The anti-NFAT1 antibody (anti-NFAT1-C) has been described (59). Anti-CD3-PE (Cat. 553064), anti-Thy1.2-PE (Cat. 553090) and anti-B220-PE (Cat. 553006) were from BD Biosciences. Anti-cyclin A2 (Cat. sc-751), anti-cyclin B1 (Cat. sc-245), anti-cyclin E1 (Cat. sc-481) and anti p21 (Cat. sc-397) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Hamster anti-mouse CD3 (Cat. 553058) and hamster anti-mouse CD28 (Cat. 553295) were from BD Biosciences; goat anti-hamster IgG (Cat. 55397) was from MP Biomedicals (Illkirch, France). Goat anti-pyruvate kinase (AB1235) was purchased from Chemicon (Hampshire, UK). Anti-β-actin (Cat. A5441) was from SIGMA (Steinheim, Germany). FITC-labeled anti-mouse IgG (Cat. F0313), FITC-labeled anti-rabbit IgG (Cat. F0054), and HRP-labeled anti-goat IgG (Cat.P010.60) were from DAKO (Glostrup, Denmark). HRP-labeled anti–mouse IgG (Cat. NA931V), and HRP-labeled anti-rabbit IgG (Cat. NA934V) were from Amersham (Buckinghamshire, UK).

Lymphocytes. Primary mouse T cells were obtained from spleens of CD4-Cre^{+} / NFAT5$^{Flox/Flox}$ mice (hereafter abbreviated as NFAT5$^{-/-}$), littermate CD4-Cre^{-} / NFAT5$^{Flox/Flox}$ mice (wild-type), 9xNFAT-Luc mice, p53$^{-/-}$ and p53$^{+/+}$ mice of 8-12 weeks of age. We confirmed that NFAT5-expressing T cells derived from CD4-Cre^{-} / NFAT5$^{Flox/Flox}$ or CD4-Cre^{+} / NFAT5$^{+/+}$ mice were indistinguishable in their response to the stresses tested (not shown). Splenocytes were isolated by density gradient centrifugation with Lymphoprep^{TM} (Axis-Shield PoC AS, Oslo, Norway). Proliferating T cells were obtained by activating splenocytes (2.5 x 10^6 cells/ml) with 2.5 μg/ml concanavalin A (Cat. C-2010, SIGMA) plus 25 ng/ml recombinant human IL-2 (Proleukin; Chiron, formerly Eurocetus. Amsterdam, The Netherlands) during 72 hours in culture medium (Dulbecco's Modified Eagle Medium DMEM, Gibco. Paisley, UK), supplemented with 10% fetal bovine serum (Cat. CH30160.03 Hyclone, Logan, UT, USA), non-essential amino acids (Gibco), 2 mM L-glutamine (Gibco), 50 μM beta-mercaptoethanol (Gibco), 1 mM sodium pyruvate (Gibco) and antibiotics penicillin and streptomycin (Gibco). Cultures were then cleaned of dead cells and debris by centrifugation on Lymphoprep^{TM}, washed and replated in fresh medium supplemented with IL-2 for an additional 24 hours, after which both wild-type and conditional knockout cultures had >95% CD3^{+}, TCRβ^{+} T cells (not shown). Before subjecting T cells to hypertonic conditions, non-viable cells and debris in the cultures were first removed by centrifugation on a Lymphoprep^{TM} cushion. Then, T cells were adjusted to 0.5 x 10^6 cells/ml in medium supplemented with 25 ng/ml IL-2, and cultured under isotonic or hypertonic conditions as indicated in the figure legends. Proliferating B cells were obtained by

culturing splenocytes (2 x 10^6 cells/ml) with 25 μg/ml of lipopolysaccharide (LPS, Cat. L7261, SIGMA) during 7 days. For Figure 7, fresh splenocytes were induced to proliferate with concanavalin A and IL-2 as described above, or stimulated with hamster anti-mouse CD3 (1 μg/10^6 T cells) plus hamster anti-mouse CD28 (1 μg/10^6 T cells) antibodies and seeded onto goat-anti hamster IgG coated plates (0.6 μg/cm^2). Cells were either grown in isotonic or moderately hypertonic media as indicated in figure legends. Before lysing cells for protein and mRNA analysis, samples were depleted of remaining B cells by incubation with sheep anti-mouse IgG magnetic beads (Dynabeads Cat. 110.31. Dynal Biotech, Invitrogen. Paisley, UK).

Surface marker analysis. 2 x 10^5 cells were blocked for 20 minutes in 1x PBS containing 3% fetal calf serum (FCS), 0.1% sodium azide, and anti-Fcγ receptor antibody (1 μg/10^6 cells) (BD Biosciences, Cat. 553142). Cells were then incubated with surface marker-specific antibodies in the same solution (1 μg/10^6 cells) and analyzed with a FACScan flow cytometer (BD Biosciences) and Cellquest software.

Hypertonic stress. The osmolality of the culture medium was measured in a Fiske ONE TEN osmometer (Fiske Associates. Norwood, MA, USA) or by using a VAPRO 5520 vapor pressure osmometer (Wescor. Logan, UT, USA). Since the T cell medium with supplements had an osmolality of 330 mOsm/kg, we adjusted it to an isotonic baseline of 300 mOsm/kg by adding 10% sterile H_2O (Milli-Q Biocel A10. Millipore, Bedford, MA, USA). This medium was made hypertonic by adding NaCl from a sterile 4 M stock solution. Over an isotonic baseline of 300 mOsm/kg, addition of 40 mM NaCl raised the osmolarity to 380 mOsm/kg, 50 mM NaCl to 400 mOsm/kg, 60 mM NaCl to 420 mOsm/kg, and 100 mM NaCl to 500 mOsm/kg.

Viability, apoptosis, and cell cycle analysis. Flow cytometry was done with a BD LSR flow cytometer (BD Biosciences). For viability and cell cycle analysis, cells were labeled during the last hour of culture with the DNA dye Hoechst 33342 (SIGMA) (5 μg/ml, 60 minutes in incubator at 37ºC with 5% CO_2). For the determination of apoptosis, cells were first labeled with Hoechst 33342 and then stained with annexin-V Fluos (Roche. Manheim, Germany) during 30 minutes on ice. Simultaneous analysis of forward and side scatter parameters (FSC/SSC), together with DNA content and annexin-V staining showed that non-viable cells, that had DNA content < 2N, and were annexin-V^+ in their majority, could be readily identified by their distinct position in the FSC/SSC plots (illustrated in supplementary Figure S1). The

proportion of live cells in Figure 2B was determined after substracting the population of non-viable cells in the FSC/SSC plots. Flow cytometry analysis of cell cycle distribution, DNA replication, and intracellular staining of γH2AX and phosphorylated p53, were done in cells gated as viable by FSC/SSC.

Determination of DNA replication by BrdU incorporation. Cells were pulse-labeled during 30 min (in a 37ºC, 5% CO_2 incubator) with 10 μM BrdU (Cat. B5002, SIGMA) and then fixed in 70% ethanol. BrdU was detected with a monoclonal mouse anti-BrdU antibody after acidic denaturation following the protocol supplied by the manufacturer (BD Pharmingen). Labeled cells were then stained with propidium iodide in RNase A-containing solution to simultaneously analyze DNA replication and cell cycle.

Intracellular detection of γH2AX and phospho-p53 (Ser-15). Cells were labeled following an intracellular staining protocol previously described (20). Briefly, cells were fixed in 1.5% paraformaldehyde (SIGMA) on ice for 15 minutes, and permeabilized with 70% ethanol at -20°C for at least 2 hours. Ethanol was removed by centrifugation and two washes with PBS, and cells were incubated with mouse monoclonal anti-γH2AX antibody (1 μg/10^6 cells) or rabbit polyclonal anti-phospho-p53 (Ser15) (1 μg/10^6 cells) for 2 hours. Bound primary antibodies were detected by incubating cells with FITC-labeled secondary antibodies for 1 hour at room temperature and protected from light. DNA was stained with 5 μg/ml Hoechst 33342 for 30 minutes at room temperature.

Cell sorting. Cells were labeled with Hoechst 33342 as indicated above, sorted with a FACSvantage flow cytometry system (BD Biosciences) according to their cell cycle phase; G0/G1, S, or G2/M, collected at 4ºC and lysed immediately after sorting.

Protein sample preparation and Western blot Analysis. Cells were lysed (30 minutes at 4ºC) in 50 mM HEPES (pH 7.4), 80 mM NaCl, 5 mM $MgCl_2$, 10 mM EDTA, 5 mM sodium pyrophosphate, 1% Triton X-100, 20 mM β-glycerophosphate, and protease inhibitors PMSF, leupeptin (Cat. L2884. SIGMA), aprotinin (Cat. 236624. Roche), and pepstatin A (Cat. P5318. SIGMA). Lysates were cleared by centrifugation (15,000g, 15 minutes, 4ºC) and the protein concentration in the supernatants was determined using the BCA Protein Assay (Cat. 23227. Pierce, Rockford, IL, USA). Equal amounts of protein from each sample were separated in SDS-polyacrylamide gels under reducing conditions,

transferred to PVDF membranes (Immobilon-P. Millipore, Bedford, MA, USA), and detected with specific primary antibodies followed by HRP-labeled secondary antibodies and enhanced chemiluminescence (Supersignal West Pico Chemiluminescent Substrate, Pierce). Pyruvate kinase or β-actin were used as protein loading control.

Real-time quantitative PCR (RT-qPCR). Total RNA was isolated using the RNeasy kit (Cat. 74104. Quiagen. Qiagen Iberia S.L., Madrid, Spain) following manufacturer's instructions. 2-3 μg of total RNA was retro-transcribed to cDNA using SuperScript III reverse transcriptase and random primers (Invitrogen). For real-time quantitative PCR (RT-qPCR), Power SYBR Green PCR master mix (Applied Biosystems, Cat. 4367659) and an ABI7900HT sequence detection system (Applied Biosystems) were used following the manufacturer's instructions. Samples were normalized to L32 mRNA levels using the ABI Prism SDS 2.1 software. Primer sequences for the PCR reactions were: 5'-CAG CCA AAA GGG AAC TGG AG-3' (Forward) and 5'-GAA AGC CTT GCT GTG TTC TG-3' (Reverse) for mouse NFAT5; 5'-ACC AGT CAG ACC GAT ATG TG-3' (Forward) and 5'-ATT GTG GAC CAG GAA CTT GC- 3' (Reverse) for mouse L32; 5'-CTT CTA CAC ATC CAT CAC GC-3' (Forward) and 5'-TTG AAG AAG TCC TGC AGC AG- 3' (Reverse) for mouse Hsp70.1; 5'-ATG GTT GTC ATC AGC ATA GCA TGG-3' (Forward) and 5'-GGT GGT GTG AGA AGA CTA ACA ATC-3' (Reverse) for mouse SMIT; 5'-TAC TAT GCA GCT AGT GGT GTA TGC-3' (Forward) and 5'-ACC TGG TCC TAT GAG AAT CTA ACG-3' (Reverse) for mouse TauT; 5' TGA GCT GTG CCA AAC ACA AG-3' (Forward) and 5'-GGA AGA AAC ACC TTG GCT AC-3' (Reverse) for mouse aldose reductase (AR); 5'-CTG GAC TCT TCA CAC AGA TG-3' (Forward) and 5'-CAT CCA CAC TTG CTC ACA AC- 3' (Reverse) for mouse cyclin E1; 5'-GAC CAA GAG AAT GTC AAC CC-3' (Forward) and 5'-CAT CGT TTA TAG GAA GGT CC- 3' (Reverse) for mouse cyclin A2; 5'-AGT TAC TGC TGC TTC CAA GC-3' (Forward) and 5'-GGT AGG GCT TTA ACA GTA CC- 3' (Reverse) for mouse cyclin B1; 5'-AAG AGT CGG ACC TTC GAT GA-3' (Forward) and 5'-CTC CCT GCA GAC CTA ACA GC- 3' (Reverse) for mouse Aurora B kinase; 5'-AGA AGA CCG AAA GGA TGG AC-3' (Forward) and 5'-GAT GTT GAT GTC GTT CTC GC- 3' (Reverse) for mouse GADD45α; 5'-CTG CTG CGA CAA TGA CAT TG-3' (Forward) and 5'-GAC CCA TTG GTT ATT GCC TC- 3' (Reverse) for mouse GADD45β; 5'-TGT TCG TGG ATC GCA CAA TG-3' (Forward) and 5'-CTC ATC TTC TTC ATC GGC AG- 3' (Reverse) for mouse GADD45γ.

DNA constructs. The luciferase reporter 9xNFAT-Luc was previously described (61). Cyclin A2-862-Luc was kindly provided by Dr. J.B.P Viola (Division of Cellular Biology, National Cancer Institute

(INCA); Rio de Janeiro, Brazil) and has been described (6). Cyclin B1 reporter plasmid was kindly provided by Dr. A. Gewirtz (University of Pennsylvania, Philadelphia, USA) and has been described (42). The transfection control plasmid TK-Renilla was from Promega (Promega Biotech Ibérica, Madrid, Spain). The GFP-specific shRNA in the pBSU6 vector was previously described (56), and the two NFAT5-specific shRNAs were done by inserting the following 21-nucleotide sequences complementary to NFAT5 mRNA in pBSU6: shNFAT5-1, 5'-GGT CAA ACG ACG AGA TTG TGA-3'; and shNFAT5-3, 5'-GGT CGA GCT GCG ATG CCC TCG-3'.

Transfections and reporter assays. The human T cell line Jurkat (Clone E6-1, American Type Culture Collection, #TIB 152) was kindly provided by Dr. J. Luban (Columbia Universtity College of Physicians and Surgeons, New York, NY) and maintained in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate and 50 μM beta-mercaptoethanol (Gibco. Pasley, UK). Cells (20 x 10^6 cells/ 400 μl serum-free DMEM) were transfected by electroporation (Bio-Rad Gene Pulser. Bio-Rad, Hemel Hampstead, UK) with luciferase reporter plasmids (60 ng/10^6 cells), TK-Renilla (0.1 μg/10^6 cells) and shRNA vectors (1.8 μg/10^6 cells) as indicated in figure legends. 36 hours post-transfection, cells were placed in fresh isotonic media (300 mOsm/kg) and subjected to hypertonic conditions (500 mOsm/kg) or stimulated with 20 nM phorbol 12-myristate 13-acetate (PMA) plus 1 μM ionomycin (Calbiochem. Darmstadt, Germany) as indicated in figure legends. Luciferase and Renilla were measured with the Dual-luciferase reporter system (Promega) with a Berthold FB12 luminometer (Berthold, Pforzheim, Germany). Luciferase activity was normalized to Renilla and endogenous lactate dehydrogenase (LDH), measured with the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega), which was proportional to the number of viable cells (39). Luciferase activity in transgenic 9xNFAT-Luc lymphocytes was normalized to endogenous LDH in the same lysate (41).

Statistical Analysis. Mean, standard error of the mean (SEM) and statistical significance (t-Student test) were calculated using Microsoft Excel software.

Results

1) Decreased viability and cell cycle arrest in proliferating NFAT5$^{-/-}$ T cells exposed to hypertonicity.

As NFAT5 deficiency impairs the survival and proliferation of T cells (57, 19), and lymphocytes regulate NFAT5 comparably to other cell types (30, 41), we sought to analyze the role of NFAT5 in response to hypertonicity in proliferating T lymphocytes. We generated NFAT5-conditional knockout mice to overcome the severe viability problems encountered with NFAT5-null mice (29, 19). NFAT5$^{Flox/Flox}$ mice had exon 6 of the NFAT5 gene, which encodes the DNA binding loop, flanked by loxP sites so that it could be deleted by the Cre recombinase expressed in a cell type-specific manner (Figure 1A). These mice were crossed to CD4-Cre transgenic animals (28), in which Cre is induced during the double positive stage of thymocyte development after cells have rearranged the T cell receptor. Lack of NFAT5 in T cells from these mice was confirmed by Western blot with two independent antibodies, one against a carboxy-terminal epitope and another raised against the amino-terminal region (Figures 1B to 1D). The efficiency of the deletion was assessed by the analysis of NFAT5 mRNA by RT-qPCR with primers specific for exon 6 (Figure 1E). NFAT5 was generally absent in the majority of the T cell cultures obtained from CD4-Cre^{+} / NFAT5$^{Flox/Flox}$ mice, although in some experiments we could detect a small proportion of cells (below 10%) that had escaped deletion (Figures 1D and 1E, see also Figure 6A and supplementary Figures S2 and S3). Mice lacking NFAT5 in T cells had no apparent defects as compared to wild-type animals, and had normal proportions of T cells in spleen (Figure 1F) and thymus (not shown).

Proliferating T cells (>95% CD3^{+}, TCRβ^{+}) were obtained by culturing splenocytes with the T cell mitogen concanavalin A plus IL-2. Mitogen-stimulated NFAT5$^{-/-}$ T cells proliferated indistinguishably from wild-type cells in isotonic conditions (Figure 2), in agreement with previous work (19). T cells were then cultured during 8 or 24 hours in isotonic medium (300 mOsm/kg), or subjected to hypertonic conditions (500 mOsm/kg) that promoted maximal induction of NFAT5 (Figure 2A). As shown in Figure 2B, both wild-type and NFAT5$^{-/-}$ cells had the same viability in isotonic medium and underwent a moderate loss of viability after 8 hours in hypertonic conditions. By 24 hours, though, cultures of NFAT5$^{-/-}$ cells exhibited a greater loss of viability (~ 45%) than wild-type ones (~ 25%) and a higher proportion of apoptotic cells (Figure 2B and supplementary Figure S1). Analysis of the cell cycle in the population of viable cells revealed that hypertonicity caused a similar arrest at 8 hours in both wild-type and NFAT5^{-}

$^{-/-}$ cells, with enhanced accumulation in S and G2/M relative to G0/G1 (Figure 2C). However, whereas wild-type cells recovered from the arrest by 24 hours, NFAT5$^{-/-}$ cells exhibited a decreased proportion of cells in early S phase and an increase in the proportion of cells in G2/M with respect to isotonic conditions (Figure 2C). BrdU-pulse experiments showed that the percentage of replicating NFAT5$^{-/-}$ cells after 24 hours in hypertonic medium was ~ 50% lower than in isotonic conditions, and the proportion of cells accumulated in G2/M increased about 3 times (Figure 2D). In contrast, wild-type cells maintained in hypertonic medium for 24 hours had resumed cell cycle progression after the initial arrest. These results indicated that NFAT5$^{-/-}$ cells displayed ineffective replication and G2/M arrest under hypertonic conditions.

2) Induction of p53 and markers of genotoxic stress in hypertonicity-treated NFAT5$^{-/-}$ cells.

As cell cycle arrest might be an indicator of genotoxic stress, and hypertonicity can cause DNA damage, we asked whether NFAT5$^{-/-}$ cells exposed to hypertonicity displayed enhanced markers of genotoxic stress. As shown in Figure 3A, both cell types displayed a comparable induction and phosphorylation of p53 at 4-6 hours. These parameters were downregulated in wild-type cells by 8 hours, but persisted in NFAT5$^{-/-}$ cells up to at least 10 hours, although eventually they subsided by 24 hours, and only a small proportion (~5%) of NFAT5$^{-/-}$ cells exhibited phospho-p53 after 24 hours, as detected by intracellular staining and flow cytometry (Figure 3B). Consistent with the activation of p53, we observed a rapid upregulation of p21 in response to hypertonic stress in both wild-type and NFAT5$^{-/-}$ cells. Despite their more prolonged induction of p53, NFAT5$^{-/-}$ lymphocytes did not accumulate substantially greater amounts of p21 (Figure 3A and supplementary Figure S2). Since the induction of p53 preceded the increase in NFAT5 expression (Figure 3A), we asked whether this factor was induced in a p53-dependent manner. As shown in Figure 3C, p53 was not required for the induction of NFAT5 in response to hypertonic stress. In contrast, induction of p21 by hypertonic stress was p53-dependent. We also analyzed the induction of GADD45 isoforms, which have been shown to mediate the cell cycle arrest induced by hypertonic shock in mouse renal medullary cells (36). GADD45α and β were comparably induced in wild-type and NFAT5$^{-/-}$ T cells after 8 hours of hypertonic stress and were downregulated to basal levels by 24 hours (Figure 3D). On the other hand, GADD45γ was downregulated by hypertonic stress in both wild-type and NFAT5$^{-/-}$ T cells, suggesting that it was not contributing to the cell cycle arrest. These results also indicated that NFAT5 did not regulate the transcription of neither GADD45 isoform in T cells. Besides reacting to hypertonicity by activating an NFAT5-independent genotoxic stress-like

response, lymphocytes also induced the sequential expression of the osmoprotective gene products Hsp70.1, SMIT and TauT, and this response was NFAT5-dependent (Figure 3D).

We also assessed whether the population of NFAT5$^{-/-}$ cells that remained viable under hypertonic conditions exhibited a greater extent of DNA damage than wild-type cells. To this end, we analyzed the phosphorylation of histone H2AX at Ser139 (γH2AX), a sensitive marker of DNA breaks. Intracellular detection of γH2AX by flow cytometry showed that cultures of NFAT5$^{-/-}$ lymphocytes contained a greater proportion of live γH2AX^{+} cells (11.6%) than wild-type ones (5.2%) after 24 hours of hypertonic stress (Figure 4A). However, the percentage of γH2AX^{+} cells was small, which suggested that NFAT5$^{-/-}$ T cells did not suffer generalized DNA damage. Moreover, NFAT5-deficient lymphocytes that did not display phosphorylated H2AX also exhibited features of cell cycle arrest (fewer cells in S phase and accumulation in G2/M) (Figure 4B), indicating that hypertonicity inhibited the cell cycle in NFAT5$^{-/-}$ lymphocytes even if they did not display evident signs of DNA damage.

3) Regulation of NFAT5 throughout the cell cycle in hypertonically stressed cells.

Since hypertonicity enhances the synthesis of NFAT5 in lymphocytes (30, 57) and, as shown here, its absence caused defects in S and G2/M, we wondered whether the activity of NFAT5 was regulated throughout the cell cycle. T cells proliferating under isotonic or hypertonic conditions were labeled with Hoechst 33342, and sorted by cell cycle phase. As shown in Figure 5A, the expression of NFAT5 was higher in S phase and G2/M in isotonic conditions. Its expression was increased considerably under osmotic stress, with a pronounced accumulation in S and G2/M. In order to determine whether NFAT5 activity was also increased, we used transgenic T cells with an integrated NFAT5-responsive reporter, 9xNFAT-Luc (41). As shown in Figure 5B, 8 hours after increasing the tonicity NFAT5 was active in all the phases of the cell cycle, with a greater activity in G2/M, while by 24 hours, when cells had resumed proliferation, NFAT5 activity was highest in S and G2/M, which were the phases more severely affected by hypertonicity in NFAT5$^{-/-}$ cells. Considering that a proportion of the luciferase measured in the sorted fractions had been already synthesized in the previous cell cycle phase, it seems likely that in the 24 hour samples the peak of NFAT5 activity occurred in S phase and that its activity during G1 was probably lower than what was measured in the experiment.

4) Defective expression of cyclins in hypertonicity-treated NFAT5$^{-/-}$ cells.

In summary, the results described above suggested that hypertonic shock induced an early, transient genotoxic stress-like response that was comparable in both wild-type and NFAT5$^{-/-}$ cells. This response was followed by an adaptive phase in wild-type cells, which activated NFAT5, induced osmoprotective gene products, downregulated stress markers and resumed a normal cell cycle. In contrast, NFAT5$^{-/-}$ lymphocytes exhibited a greater viability loss and surviving cells displayed cell cycle defects. Although NFAT5$^{-/-}$ lymphocytes still presented some stress markers by 24 hours, such as phosphorylated H2AX and p53 in a small proportion of cells, they had downregulated other genotoxic stress-associated responses such as p21 and GADD45α and β as much as wild-type lymphocytes, suggesting that their cell cycle arrest might not be attributable to a generalized genotoxic stress-like response. We thus analyzed the expression of several cyclins known to be relevant in successive phases of the cell cycle. A short exposure to hypertonic stress (8 hours) had similar effects on the expression of cyclins in wild-type and NFAT5$^{-/-}$ lymphocytes. Thus, after 8 hours of hypertonic stress, cyclin D3 was downregulated in both cell types in six out of six independent experiments, cyclin E1 in two out of five, and cyclin B1 in three out of seven independent cultures tested for each wild-type and NFAT5$^{-/-}$ T cells, whereas cyclin A2 was not affected in the majority of experiments (Figure 6A and supplementary Figure S3). However, after 24 hours in hypertonic medium, wild-type and NFAT5$^{-/-}$ cells displayed distinct differences. Both cell types upregulated cyclin D3, but whereas wild-type lymphocytes maintained cyclins E1, A2 and B1 at levels similar to those of cells growing in isotonic medium, NFAT5$^{-/-}$ cells had substantially reduced levels of these cyclins (Figure 6A and supplementary Figure S3). We analyzed whether this downregulation correlated with a decrease in cyclin mRNA levels. The amount of mRNA for cyclins A2 and B1 was substantially decreased (by 60-70%) in both wild-type and NFAT5$^{-/-}$ lymphocytes after 8 hours of hypertonicity treatment (Figure 6B). By 24 hours, wild-type lymphocytes had recovered similar cyclin mRNA levels as those of cells grown in isotonic medium, whereas NFAT5$^{-/-}$ cells did not. With regard to cyclin E1, hypertonic stress caused a lesser decrease in its mRNA abundance than it did for cyclins A2 and B1, despite of which the amount of cyclin E1 protein was considerable lower in hypertonically stressed NFAT5$^{-/-}$ cells than in wild-type ones, indicating that the downregulation of cyclin E1 might involve defective synthesis and/or enhanced degradation of the protein. In these experimemts, we also observed that the abundance of aurora B kinase mRNA, which has been recently shown to regulate cell cycle progression in T cells (54), was reduced in hypertonicity-treated NFAT5$^{-/-}$ T cells. We next analyzed whether the lack of NFAT5 affected the activity of the promoters of cyclins A2 and B1 in Jurkat T cells exposed to hypertonic stress. Two independent NFAT5-specific shRNA inhibited the hypertonicity-induced activation of the NFAT5-responsive reporter 9xNFAT-Luc (41), without affecting the activation of the same reporter via NFATc proteins in response to PMA and ionomycin (Figure 6C).

In the same experiment, suppression of NFAT5 did not inhibit the activity of cyclin A2 and B1 promoters in hypertonic conditions, indicating that NFAT5 was not required to maintain the activity of these promoters in hypertonically stressed cells.

In the experiments shown above we had used hypertonic conditions of 500 mOsm/kg, which have been routinely utilized throughout the literature to analyze NFAT5-dependent responses in diverse cell types. However, switching cell cultures from an isotonic medium of 300 mOsm/kg to 500 mOsm/kg constitutes a rather severe osmotic shock that might not reflect pathophysiological situations. Several anisosmotic disorders described in patients (8, 45, 9, 44, 24, 51, 16), as well as mouse models lacking osmoregulatory proteins (65, 38) have been reported to cause hypernatremia with plasma osmolality values of 360-430 mOsm/kg. These conditions are likely to expose different cell types, including lymphocytes, to a hypertonic millieu. Previous work by Go et al. (19) had shown that the proliferative capacity of fresh NFAT5-deficient lymphocytes stimulated via T cell receptor was reduced by 50% when stimulated in culture media of 370 mOsm/kg, and our recent work showed that activation of NFAT5 required a lower hypertonicity threshold (~380-400 mOsm/kg) in T cells during their early phase of mitogen stimulation than when they were already actively proliferating (480 mOsm/kg) (41). We thus tested whether fresh NFAT5$^{-/-}$ T cells induced to proliferate in moderately hypertonic medium (380-420 mOsm/kg) exhibited cell cycle and cyclin expression defects. Induction of cyclins A2 and B1 by concanavalin A stimulation in T cells was more severely impaired by hypertonicity in NFAT5$^{-/-}$ T cells than in wild-type ones (Figure 7A). In this experimental setting, cyclin expression was maintained in wild-type cells grown in 380 mOsm/kg medium but was partially inhibited at 400 mOsm/kg. The threshold for hypertonicity-mediated inhibition was lower in NFAT5$^{-/-}$ T cells, and cyclin expression was detectably reduced at 380 mOsm/kg and severely inhibited at 400 mOsm/kg. Similar results were obtained in cells stimulated with anti-CD3 plus anti-CD28 antibodies (Figure 7B). These experiments also showed that the proportion of cells entering the cell cycle upon T cell receptor stimulation was significantly lower in NFAT5$^{-/-}$ T cells exposed to 420 mOsm/kg than in wild-type ones (Figure 7C). Finally, analysis of osmotic stress-induced gene products and cell cycle regulators showed that NFAT5-deficient lymphocytes exhibited an impaired osmoprotective response, with poor expression of Hsp70.1, SMIT and aldose reductase, and that induction of cyclins A2, B1 and aurora B kinase mRNA in response to CD3/CD28 stimulation was also more severely inhibited in NFAT5$^{-/-}$ T cells than in wild-type ones (Figure 7D).

Discussion

Work from different laboratories has shown that hypertonicity elicits a stress response that is recognizably conserved in different cell types, triggering the activation of NFAT5, which then induces osmoregulatory genes that enable cells to restore their intracellular osmotic equilibrium. Here we have analyzed the hypertonic stress response in wild-type and NFAT5-deficient lymphocytes. Our results showed that T cells exhibited an overall stress response, with induction of genotoxic stress markers and cell cycle arrest, that was remarkably similar to that described in renal medullary cells and other cells (15, 3). In addition, T cells induced various osmoregulatory molecules such as SMIT, TauT, aldose reductase and the chaperone Hsp70.1 in response to hypertonicity in an NFAT5-dependent manner. That very different types of mammalian cells activate highly conserved mechanisms in response to osmotic shock may not be surprising since, as summarized in a recent review (3), basic components of this response including stress signaling pathways, cell cycle control and upregulation of compatible osmolites, are found in cells from as distant organisms as vertebrates and yeast.

One of our main findings, which had not been addressed in previous studies, has been to draw a clear distinction between NFAT5-regulated and NFAT5-independent events in the osmotic stress response of proliferating mammalian cells. Our results show that this response developed in two phases. The early phase was similar in wild-type and NFAT5-deficient cells, indicating that it was NFAT5-independent, and was characterized by a sharp induction of p53 and other genotoxic stress markers (p21, GADD45), downregulation of cyclin mRNA, and acute cell cycle arrest in S and G2/M. This initial response changed into an NFAT5-regulated adaptive phase in wild-type cells, which rapidly induced the expression of osmoprotective gene products, and resumed cyclin expression and a functional cell cycle. In contrast, $NFAT5^{-/-}$ cells showed poor induction of osmoprotective genes, manifested an extended early stress phase with prolonged accumulation of p53, and exhibited increased apoptosis and a greater viability loss than wild-type cells by 24 hours. Surviving $NFAT5^{-/-}$ cells had downregulated stress markers but displayed a reduced replication rate and defective cell cycle progression, associated with the inability to maintain cyclin expression. The finding that S and G2/M phases were highly sensitive to hypertonic stress in NFAT5-deficient cells was consistent with the observation that the expression and activity of NFAT5 were substantially increased in these phases in wild-type cells proliferating under sustained hypertonic conditions.

In this work we have identified the expression of cyclins and at least other regulators such as aurora B kinase, as a novel mechanism of cell cycle control by hypertonic stress and NFAT5. We had previously reported that the renal medulla of NFAT5-null mice contained a greater proportion of apoptotic cells than the medulla of wild-type littermates (29). A later work by Wang et al. described that eye lens fiber cells overexpressing a dominant negative NFAT5 accumulated abundant DNA breaks, p53 and phospho-Chk2, although it was not determined whether these effects were due to hypertonic stress (60). On the other hand, the Ho laboratory reported that NFAT5-deficient T cells exhibited reduced proliferative capacity when cultured in hypertonic media (19). Our findings here are consistent with these previous articles and suggest that inhibition of NFAT5 in cells exposed to hypertonicity could lead to different outcomes, with a proportion of the cells undergoing apoptosis, possibly associated with an enhanced genotoxic stress-like response, whereas surviving cells had reduced proliferative capacity. The proliferation defect of NFAT5-deficient lymphocytes did not correlate with the presence of conspicuous symptoms of DNA damage, but rather with a substantially reduced expression of cyclins and aurora B kinase. Downregulation of cyclins A2 and B1 can impair S and G2/M progression in different cell types (64, 10), and aurora B kinase has been shown to contribute to the G1/S transition and cell cycle progression through S, G2 and M phases in antigen-stimulated T cells (54). Defective expression of these regulators might underlie the cell cycle defects of NFAT5$^{-/-}$ cells under hypertonic stress. Our results also indicate that lack of NFAT5 affects cyclins at different levels, since downregulation of cyclins A2 and B1 correlated with a reduction in their mRNA abundance whereas the decrease in cyclin E1 did not appear to be solely attributable to the loss of its mRNA and might be due to reduced protein stability and/or synthesis rate. In response to hypertonic stress, NFAT5 activates the expression of several enzymes and transporters whose collective function is the accumulation of intracellular compatible osmolites that prevent a harmful buildup of inorganic ions. It also induces heat shock proteins and chaperones, which might protect sensitive proteins from the deleterious effect of water stress (3). We have shown that NFAT5 induces the expression of osmoprotective gene products in T cells, which is consistent with the notion that this factor contributes to maintain diverse cellular functions by stabilizing the intracellular tonicity.

We observed defective expression of S and G2/M cyclins in actively proliferating NFAT5$^{-/-}$ lymphocytes subjected to an osmotic shock of 500 mOsm/kg, as well as in fresh T cells that were induced to proliferate in moderate hypertonic conditions (380-420 mOsm/kg). While 500 mOsm/kg has been routinely used in the literature to study osmotic stress responses and activation of NFAT5 in cell types as diverse as neurons, leukocytes and fibroblasts, it is unknown whether such elevated tonicity levels can occur out of the renal medulla. On the other hand, 360-430 mOsm/kg is in the range reported in plasma of patients

with hypernatremic disorders (8, 45, 9, 44, 24, 51, 16). Plasma osmolality levels of 400 mOsm/kg can occur in mammals after prolonged water deprivation (15), and have been reported in mice deficient in V2 vasopresin receptor (65) and aquaporin-2-mutant mice with congenital progressive hydronephrosis (38). In aquaporin-1-deficient mice, plasma tonicity reached 517 mOsm/kg after 36 hours of water deprivation, after which they could survive if water was administered to them again (33). Tonicity levels around 400 mOsm/kg are sufficient to mobilize NFAT5 in neurons (32) and primary lymphocytes (41).

We also found that the cellularity and proportion of T cells in lymphoid organs were not altered in vivo in our conditional knockout mice. The Ho laboratory had reported that a transgenic mouse which overexpressed a dominant negative NFAT5 DNA binding domain since the early stages of T cell ontogeny had reduced thymic cellularity and decreased proportions of T cells in spleen (57). A later work by the same group showed a similar phenotype in NFAT5-deficient mice, which had substantially lower numbers of thymocytes and splenocytes than wild-type mice (19). It is possible that inhibiting NFAT5 already since the beginning of thymocyte development, as occurs in both mouse models from the Ho laboratory, might be detrimental for T cell maturation in the moderately hypertonic millieu (~ 330 mOsm/kg) of the thymus (19). In our conditional knockout mice, however, deletion of NFAT5 occurs at a later phase, after Cre is expressed during the double positive stage of thymocyte ontogeny, and our results show that loss of NFAT5 at that point does not impair the subsequent T cell maturation nor affects the number and proportion of T cells in periphery. Nonetheless, mature T cells from our conditional knockout mice and those from the NFAT5-deficient mice developed by Go et al. (19) exhibited a comparable sensitivity to pathologic tonicity levels.

Diverse types of stressors can threaten the genetic integrity of the cell, and stress responses are often associated with cell cycle arrest, which allows cells to activate adaptive programs that enable them to withstand the stress and resume proliferation. Failure to adapt or irreparable damage might cause permanent cell cycle arrest and cell death. In this context, the stress-activated p38-MAPK/MK2 kinase pathway can activate cell cycle checkpoints (37, 47), and DNA damage-activated kinases such as ATM and other PIKKs can regulate stress-responsive transcription factors such as p53, NF-κB, c-Jun, or ATF2 and 3 (53, 46, 43, 18, 2, 17). NFAT5 is activated by p38-MAPK and PIKK in response to hypertonic stress (25, 21, 41), and is required for cell survival and proliferation under sustained hypertonic conditions (19, 29). Our results show that NFAT5 is required to enable the expression of cyclins and the progression of the cell cycle, thus playing a major role in the adaptive phase of the cellular response to both osmotic shock and pathologic hypertonic conditions.

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Figure legends

Figure 1. **Generation of NFAT-conditional knockout mice. A)** Schematic representation of the targeting construct, in which Exon 6, encoding the DNA binding loop in the DBD of NFAT5, was flanked by loxP sites. The vector contained an frt-flanked neomycin-resistance cassette (Neo) inserted at the EcoRI site downstream of Exon 6 and upstream of the 3' loxP site. Restriction sites in brackets indicate that they were inactivated during subcloning. Mouse ES clones with the correctly recombined allele were used to generate mice that were crossed to FLPe-deleter mice to produce NFAT5-floxed mice, without the Neo cassette, and with Exon 6 flanked by loxP sites so that it could be removed by the Cre recombinase. **B)** Southern blot of genomic DNA extracted from T cells of wild-type (WT), NFAT5-floxed (Flox) mice, and mice obtained after crossing them with CD4-Cre mice (CD4-Cre^{+}). Genomic DNA was digested with BamHI and hybridized to a probe derived from Exon 5. **C)** Specific deletion of NFAT5 in T cells, but not in B cells of CD4-Cre^{+} / $NFAT5^{Flox/Flox}$ mice was confirmed by Western blotting with an antibody against a C-terminal epitope. B and T cell-enriched populations were obtained after culture of splenocytes with mitogens (LPS for B cells, and concanavalin A + IL-2 for T cells). The non-specific crossreacting band (n.s.) above NFAT5 serves as a loading control. **D)** Western blot detecting NFAT5 in activated T cells was performed with two different antibodies, specific for a C-terminal epitope and the N-terminal region respectively. **E)** NFAT5 mRNA was analyzed by RT-qPCR (bars represent the mean of five independent experiments ± SEM). **F)** Upper panel: weight of mice and spleens, and splenocyte count after ficoll gradient separation (n = 8, bars are mean ± SEM). Bottom panel; expression of surface markers CD3, B220 and Thy1.2 in fresh splenocytes was analyzed by flow cytometry (n = 5, values are mean ± SEM).

Figure 2. Viability and cell cycle profile of proliferating T cells under hypertonic stress. **A)** Induction of the expression of NFAT5 by increasing levels of hypertonicity in $NFAT5^{Flox/Flox}$ ($NFAT5^{+/+}$) and CD4-Cre^{+} / $NFAT5^{Flox/Flox}$ lymphocytes ($NFAT5^{-/-}$) was detected by Western blot with an anti-NFAT5 (C-terminus epitope) antibody. **B)** Proliferating T cells were grown under isotonic (300 mOsm/kg) or subjected to hypertonic conditions (500 mOsm/kg) during 8 or 24 hours. Viability was assessed by FSC/SSC parameters (as illustrated in **supplementary Figure S1**). Cells were labeled with Hoechst 33342 and DNA content was analyzed by flow cytometry. Columns represent mean ± SEM of 5 independent experiments, (* = $p < 0.05$). **C)** DNA content histograms represent the cell cycle distribution in live cells: G0/G1, early S (ES), late S (LS), and G2/M. On the lower panel, cell cycle distribution in live wild-type and $NFAT5^{-/-}$ cells after 8 and 24 hours in isotonic or hypertonic conditions. Values are the

mean ± SEM of 5 independent experiments and 9 independent experiments for 8 and 24 hours respectively. **D)** Cells grown in isotonic or hypertonic medium during 24 hours were pulsed during 30 minutes with BrdU, fixed and analyzed. The upper panel depicts one representative experiment showing BrdU incorporation plotted against DNA content, and the lower panel represents the results (mean ± SEM) from three independent experiments (* = $p < 0.05$).

Figure 3. Induction of p53 and p21 in response to hypertonicity. A) Western blot shows the time course of p53-Ser15 phosphorylation, accumulation of total p53 and induction of p21 in $NFAT5^{+/+}$ and $NFAT5^{-/-}$ cells in response to hypertonicity. The experiment shown is representative of three independently performed (see **supplementary Figure S2**). **B)** Phospho-p53 (Ser15) was detected by intracellular staining in $NFAT5^{+/+}$ and $NFAT5^{-/-}$ T cells cultured in isotonic or hypertonic medium during 24 hours. Results correspond to cells gated in the live population. Dot plots show one representative experiment. Bars on the right represent the mean ± SEM of 4 independent experiments. **C)** Time course of NFAT5 and p21 induction in $p53^{+/+}$ and $p53^{-/-}$ cells in response to hypertonicity. The experiment shown is representative of three independently performed. **D)** $NFAT5^{+/+}$ and $NFAT5^{-/-}$ proliferating T cells were subjected to hypertonicity for 8 and 24 hours, RNA was isolated and analyzed by RT-qPCR. mRNA values for each gene and condition were normalized to their respective L32 mRNA levels (bars are mean ± SEM of 4 independent experiments).

Figure 4. Induction of γH2AX in response to hypertonicity. A) Dot plots representing γH2AX and DNA content in live $NFAT5^{+/+}$ and $NFAT5^{-/-}$ lymphocytes after 24 hours in isotonic or hypertonic conditions. Bars on the right represent the percentage of $\gamma H2AX^{+}$ cells after 24 hours in isotonic or hypertonic medium (values are the mean ± SEM of 7 independent experiments; * = $p < 0.05$). **B)** Cell cycle distribution of viable, γH2AX-negative cells after 24 hours in hypertonic medium (representative of 7 independent experiments).

Figure 5. Expression and activity of NFAT5 throughout the cell cycle. A) $NFAT5^{+/+}$ T cells were labeled with Hoechst 33342 and sorted according to DNA content as G0/G1, S or G2/M phase. Sorted cells were lysed and equal amounts of protein from each lysate were analyzed by Western blot with anti-NFAT5 antibody. NFAT1 and anti-pyruvate kinase (PyrK) are shown as protein loading controls. The result is representative of three independent experiments. **B)** Transgenic 9xNFAT-Luc T cells were labeled with Hoechst 33342 and sorted as described above after 8 and 24 hours of exposure to hypertonicity. Sorted cells were split for Western blot and luciferase activity assays. A representative

Western blot analysis (upper panel) is shown of three independently performed. Pyruvate kinase (PyrK) is shown as protein loading control. 9xNFAT-Luc reporter activity in sorted cells is shown in the lower panel. Results are the mean ± SEM of 3 independent experiments.

Figure 6. Expression of cyclins in proliferating T cells upon exposure to hypertonic conditions. A) Expression of cyclins D3, E1, A2 and B1 was analyzed by Western blot in lysates of proliferating $NFAT5^{+/+}$ and $NFAT5^{-/-}$ T cells after 8 and 24 hours of hypertonicity treatment. Pyruvate kinase (PyrK) is shown as protein loading control. The result is representative of at least four independent experiments (see **supplementary Figure S3**). **B)** mRNA abundance of cyclins and aurora B kinase was analyzed by RT-qPCR in proliferating $NFAT5^{+/+}$ and $NFAT5^{-/-}$ T cells subjected to hypertonicity for 8 and 24 hours. Aurora B kinase mRNA levels are represented as relative to the 8 hour time point of wild-type cells in isotonic conditions (which was given an arbitrary value of 1). All the values were normalized to L32 mRNA levels in each respective sample (bars are mean ± SEM of 4 independent experiments; * = $p < 0.05$; n.s = not statistically significant). **C)** Effect of hypertonicity on cyclin A2 and cyclin B1 promoters. Jurkat T cells were cotransfected with the indicated vectors and with pTK-Renilla. 36 hours post-transfection, cells were placed in fresh media and were either left untreated, stimulated with hypertonicity or with PMA plus ionomycin (P+I). Luciferase activity was measured 24 hours later and normalized to the activity of the Renilla reporter. Results are expressed as the mean fold induction ± SEM of 4 independent experiments.

Figure 7. Effect of hypertonicity on cyclin induction and cell cycle entry induced by mitogens or T cell receptor activation. Expression of cyclins A2 and B1 was analyzed by Western blot in lysates of splenocytes isolated from $NFAT5^{+/+}$ and $NFAT5^{-/-}$ mice and induced to proliferate with (**A**) ConA plus IL-2 or (**B**) anti-CD3/CD28 antibodies plus IL-2 in isotonic or moderately hypertonic media. Cells were collected at the indicated time points and depleted of B cells before lysing. Pyruvate kinase (PyrK) or β-Actin were used as protein loading controls. Each panel shows a representative experiment of three independently performed. **C)** Cell cycle distribution of CD3/CD28-stimulated splenocytes. Cultures were labeled with Hoechst 33342 and analyzed by flow cytometry. Values express mean ± SEM of three independent experiments (* = $p < 0.05$). **D)** $NFAT5^{+/+}$ and $NFAT5^{-/-}$ splenocytes were induced to proliferate with anti-CD3/CD28 antibodies plus IL-2 in isotonic or moderately hypertonic media. Cultures were harvested at the indicated time points and depleted of B cells. RNA was isolated and analyzed by RT-qPCR. A representative experiment of three independently performed is shown on the upper panel. The bottom panel shows the mRNA abundance for the indicated genes in hypertonic relative to isotonic

conditions at the same time point. All values were normalized to each respective L32 mRNA level. Values are mean ±SEM of three independent experiments (* = $p < 0.05$; n.s = not statistically significant).

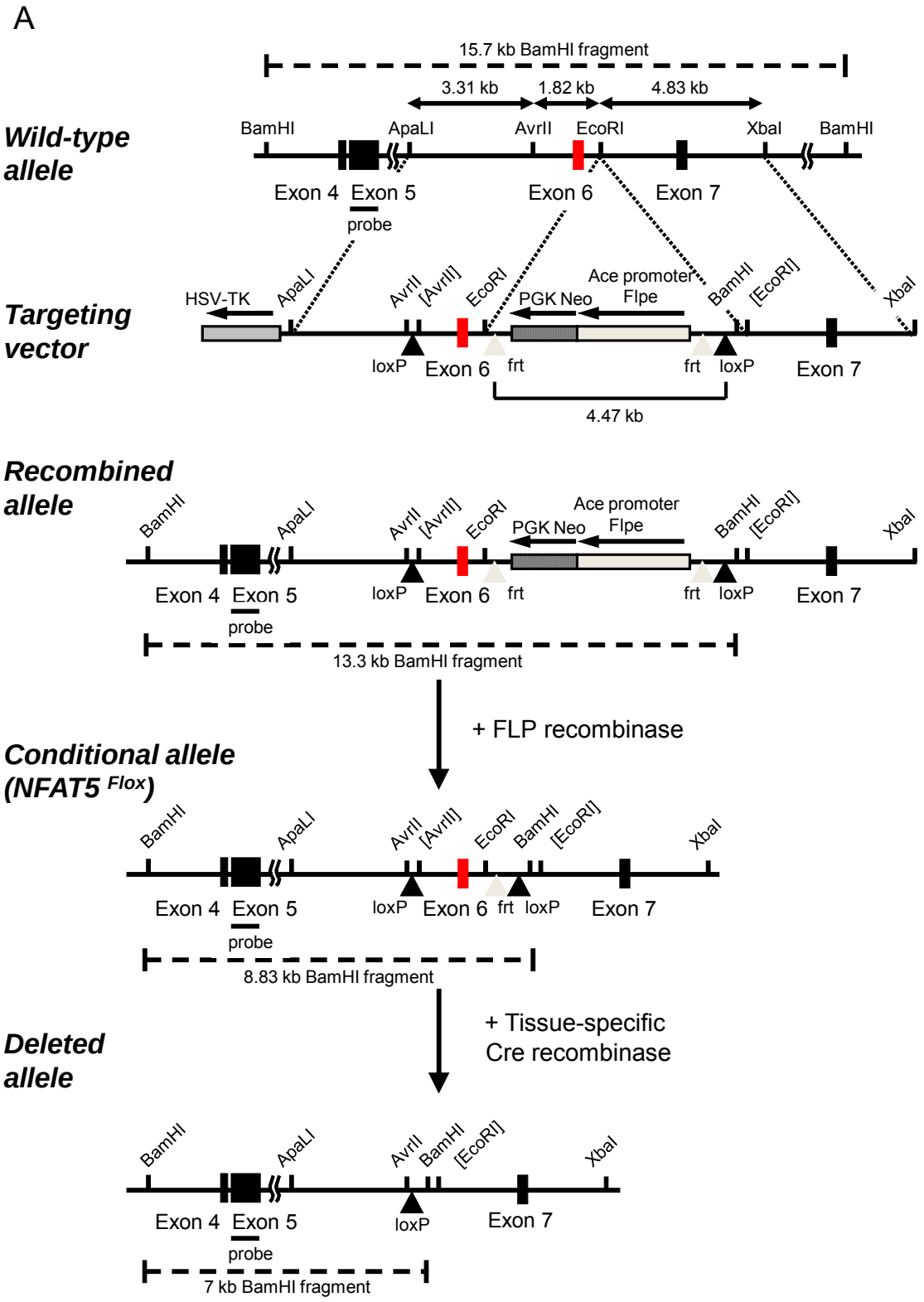
Supplementary figures

Figure S1. Viability of proliferating T cells under hypertonic stress. T cells grown in isotonic or hypertonic conditions during 24 hours were stained with the DNA dye Hoechst 33342 and annexin-V-Fluos and analyzed by flow cytometry. The experiment shown is representative of three independently performed.

Figure S2. Induction of p53 and p21 in response to hypertonicity. Time course of p53-Ser15 phosphorylation, accumulation of total p53 and p21 in $NFAT5^{+/+}$ and $NFAT5^{-/-}$ cells in response to hypertonicity were analyzed by Western blot. Pyruvate kinase (PyrK) is shown as protein loading control.

Figure S3. Expression of cyclins in proliferating T cell upon exposure to hypertonic conditions. Expression of cyclins D3, E1, A2 and B1 was analyzed by Western blot in lysates of proliferating $NFAT5^{+/+}$ and $NFAT5^{-/-}$ T cells after 8 and 24 hours of hypertonicity treatment. Pyruvate kinase (PyrK) is shown as protein loading control.

Drews-Elger et al., Figure 1



Drews-Elger et al., Figure 1

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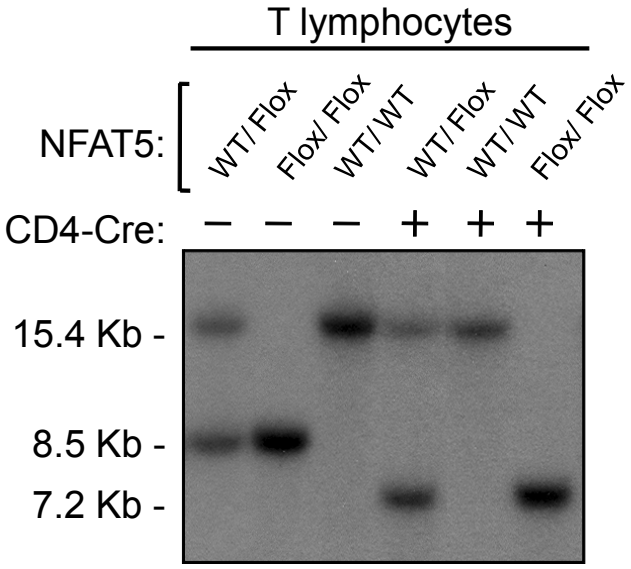

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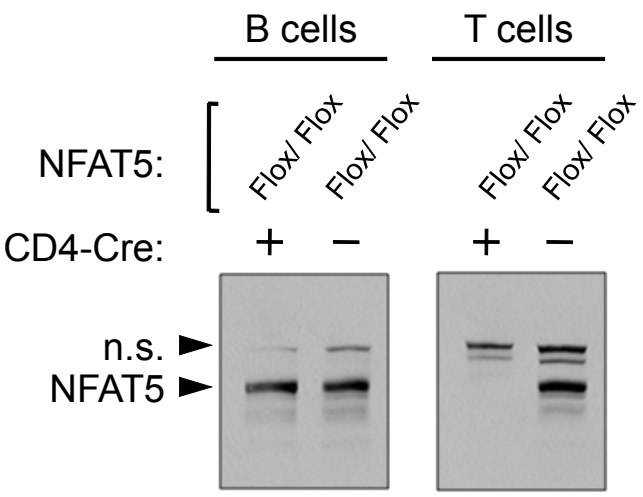

Drews-Elger et al., Figure 1

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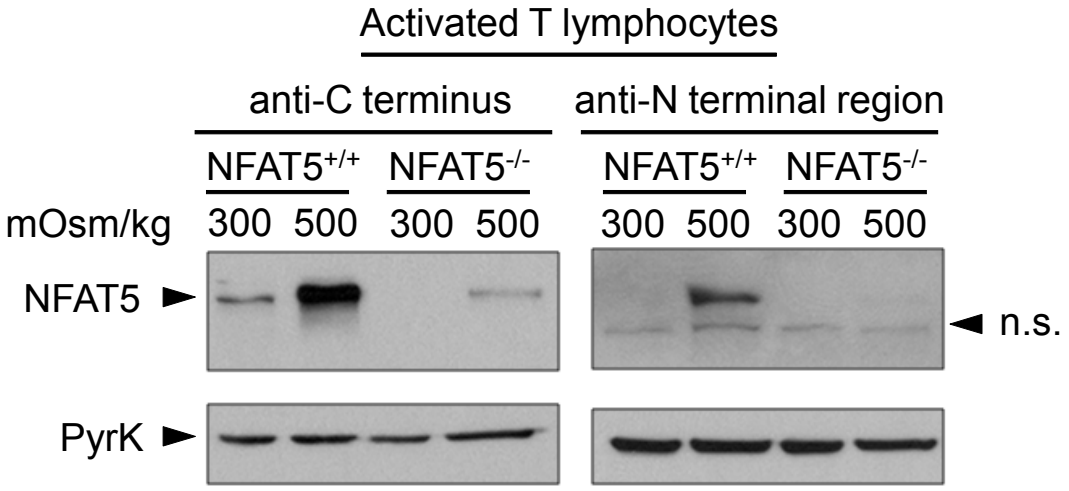

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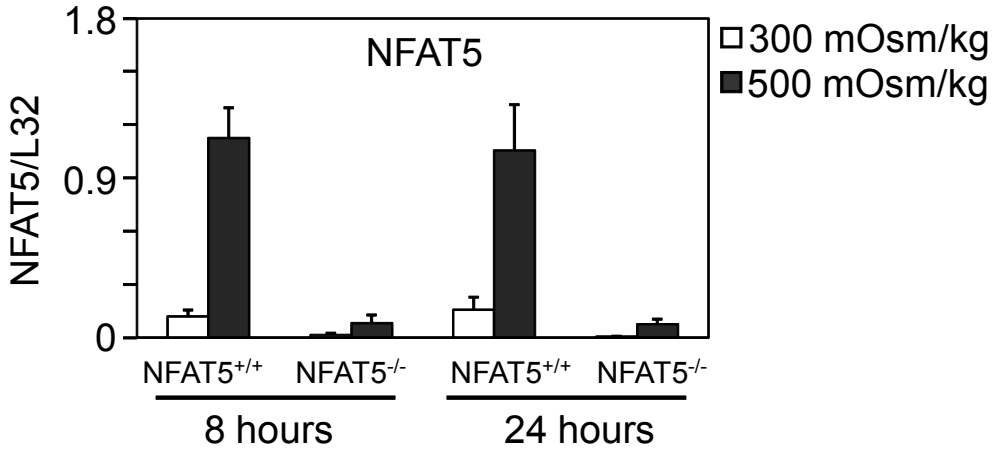

Drews-Elger et al., Figure 1

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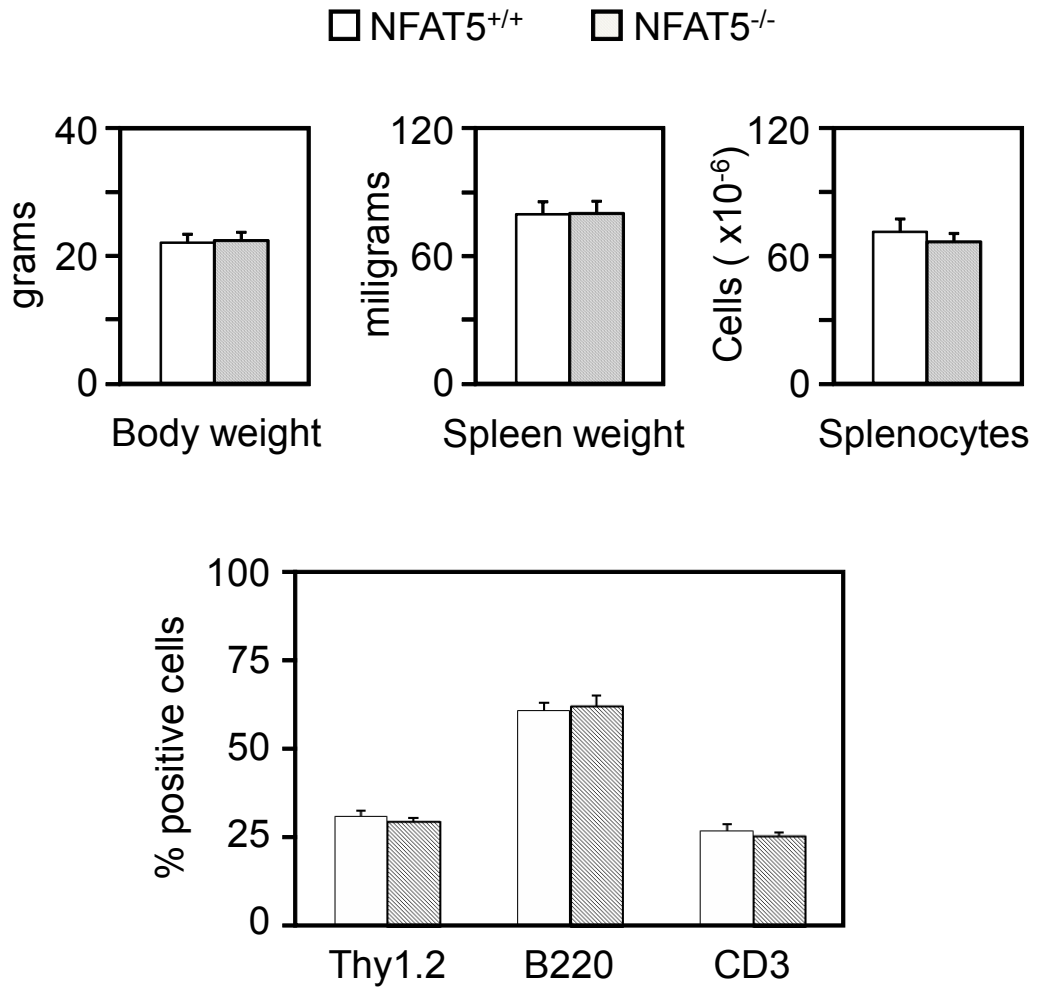

Drews-Elger et al., Figure 2

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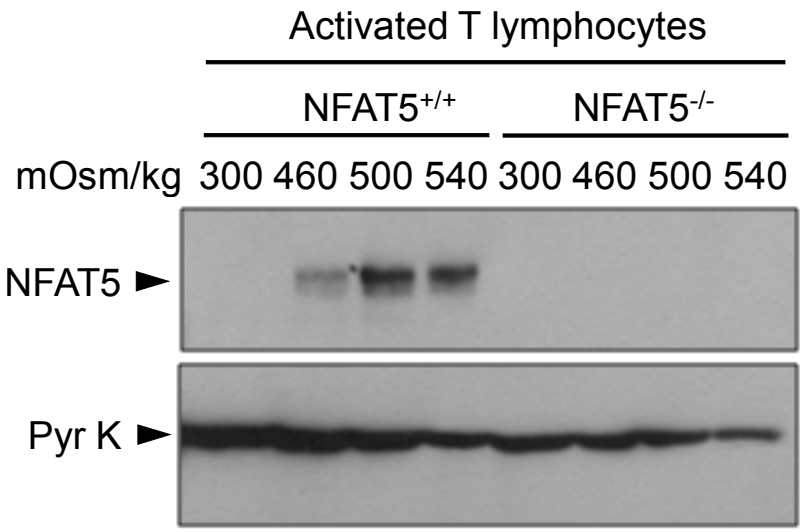

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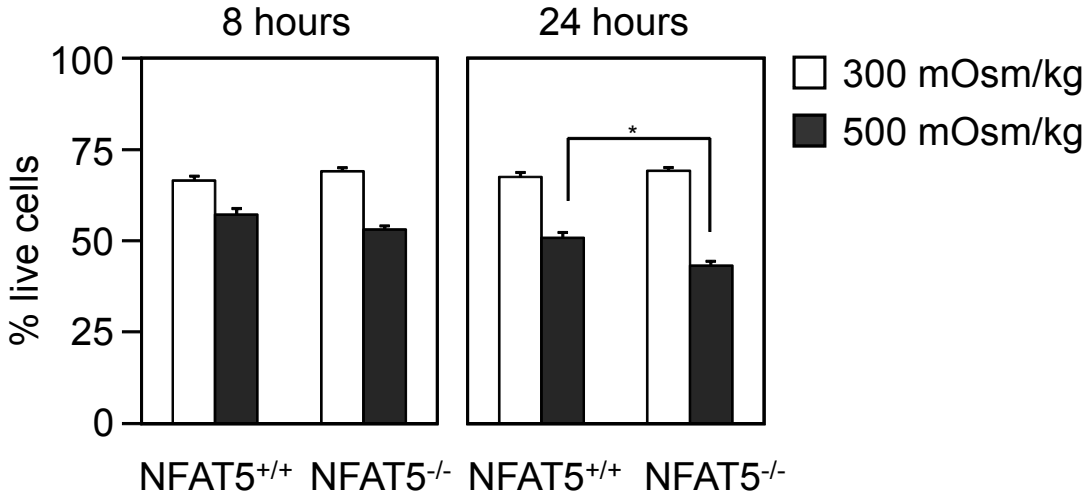

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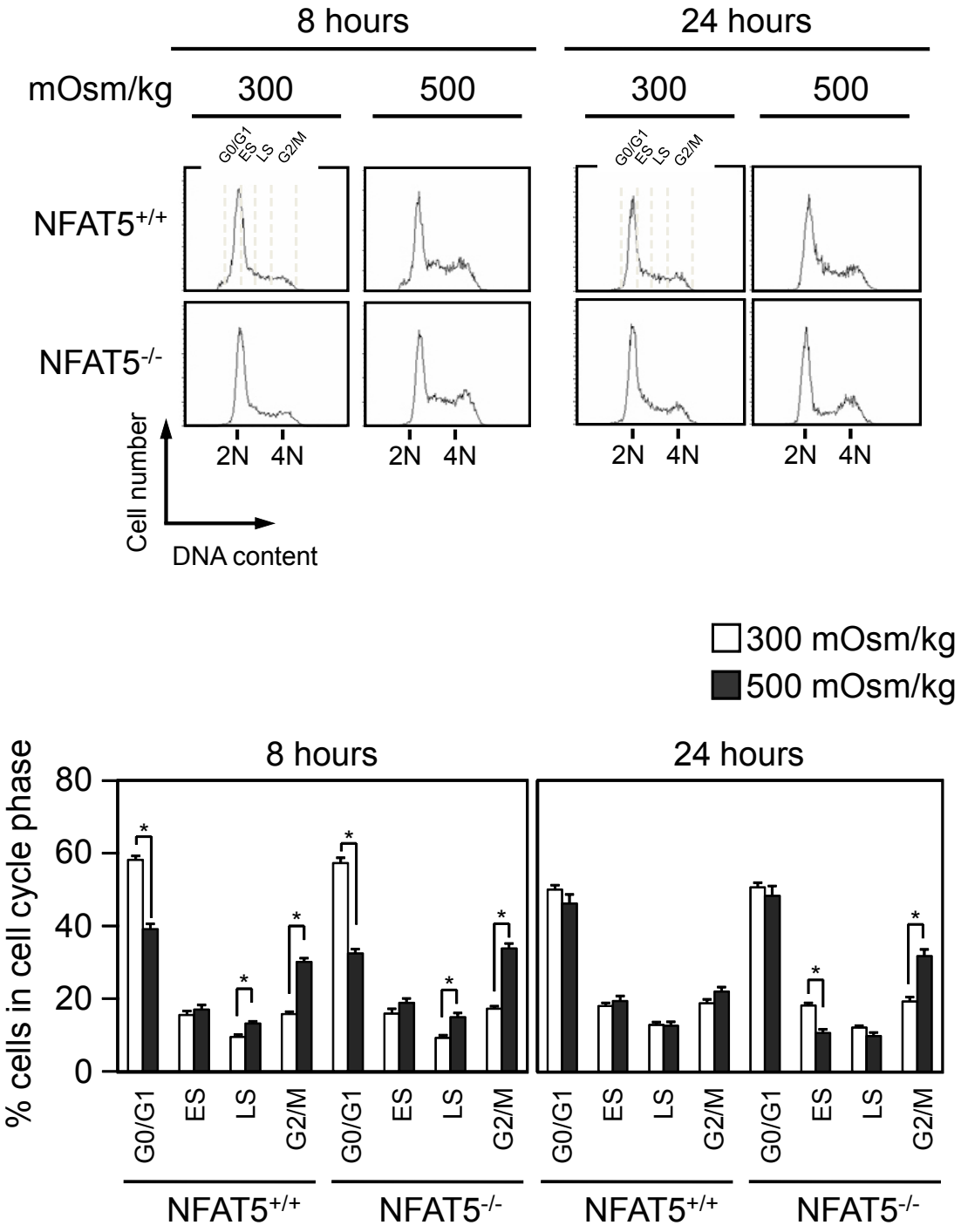

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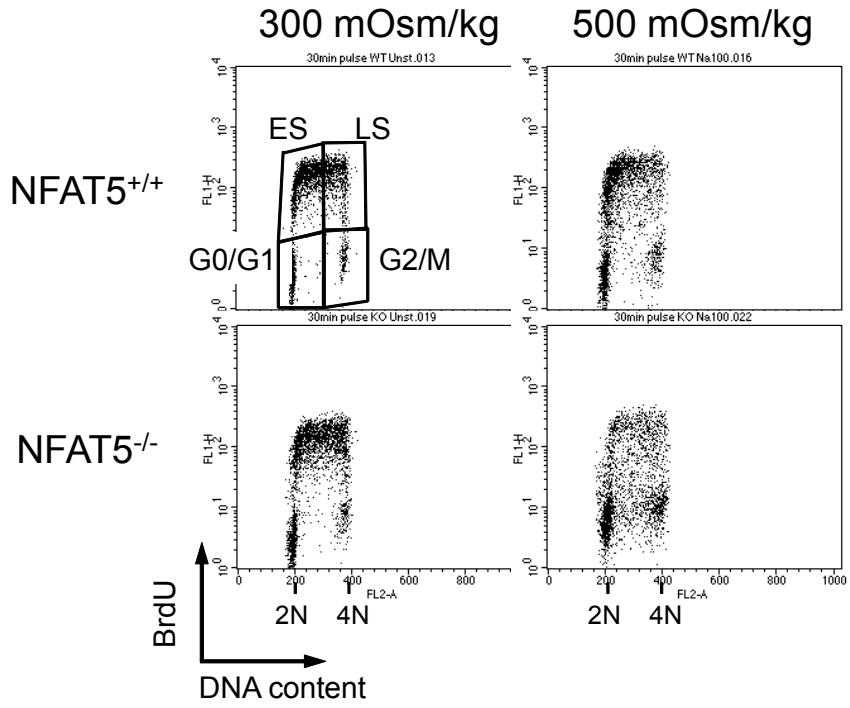

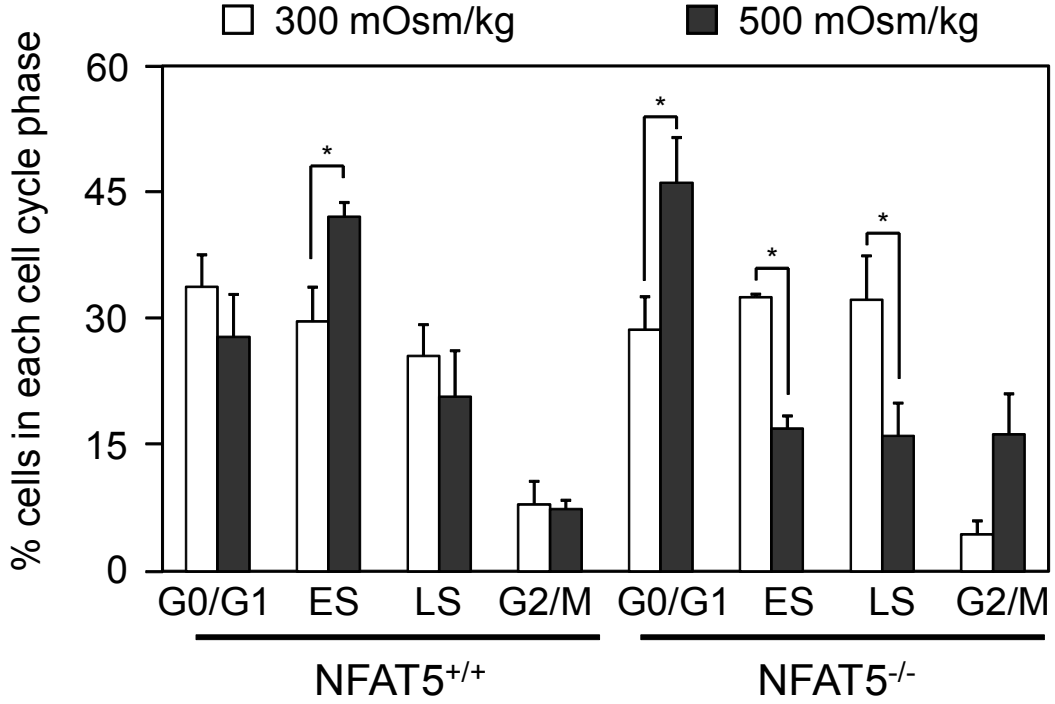

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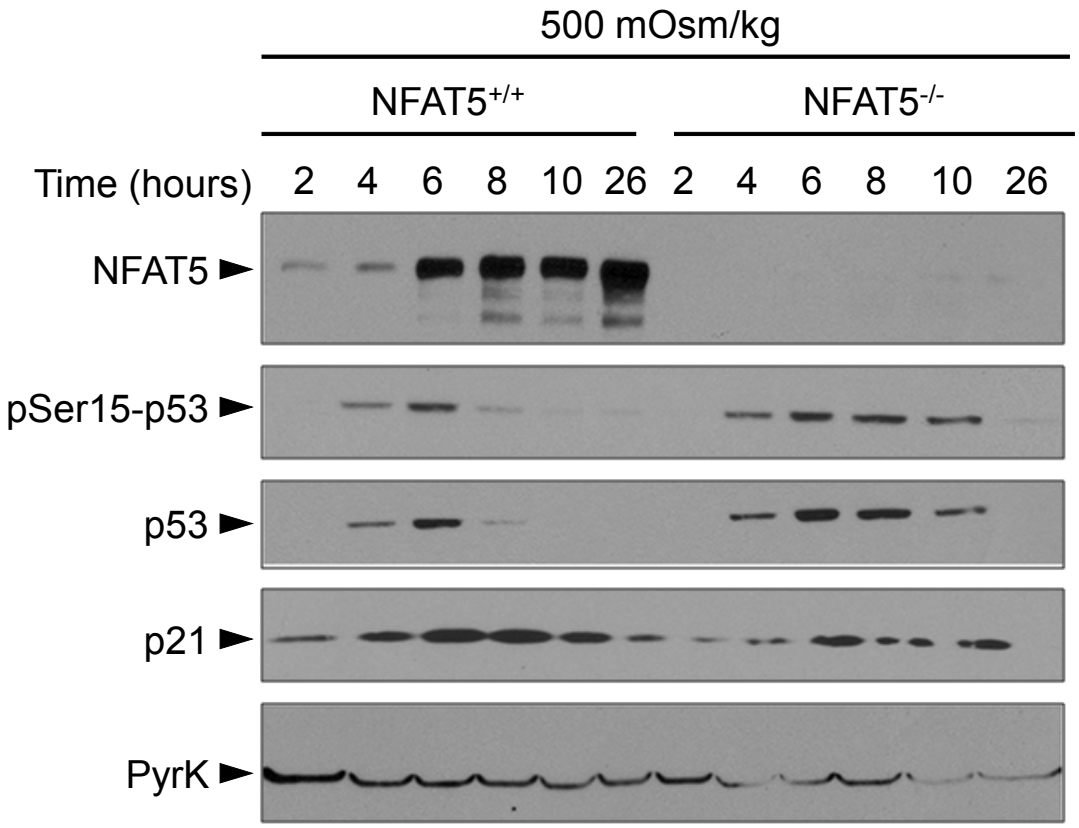

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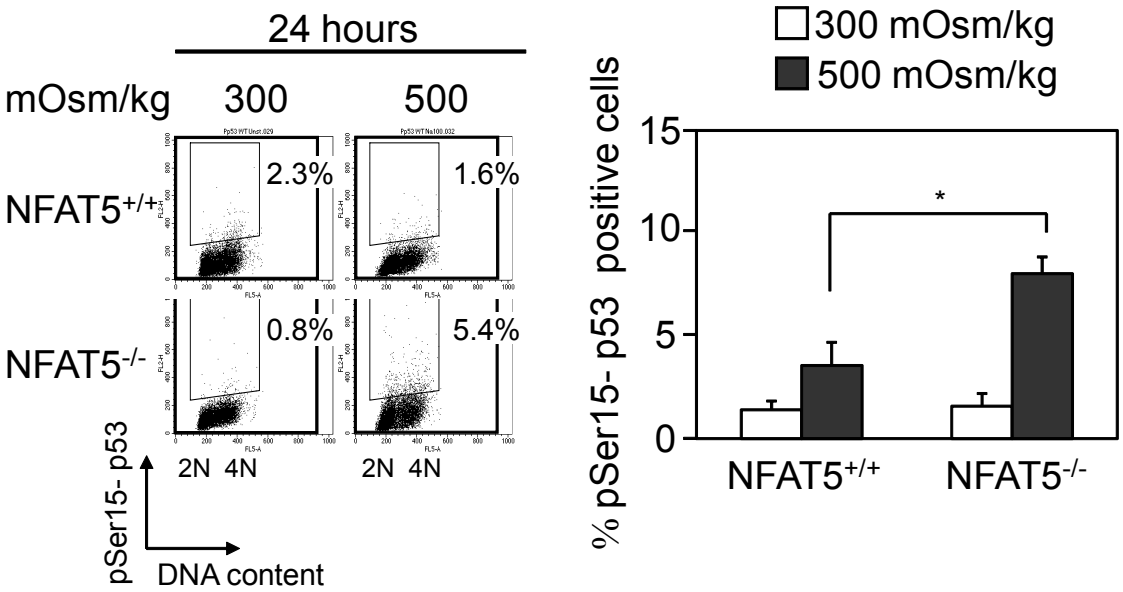

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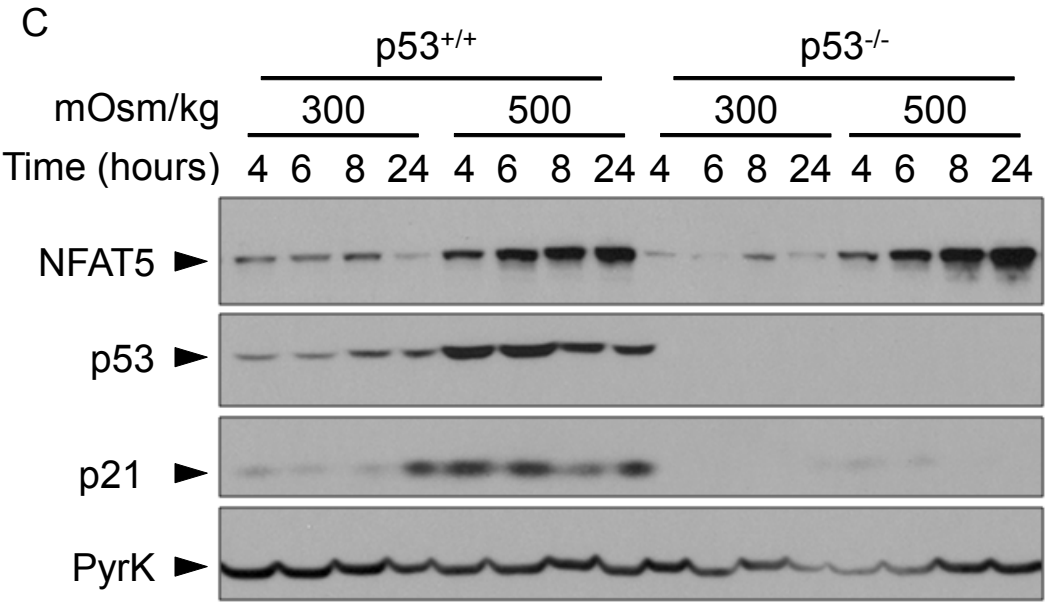

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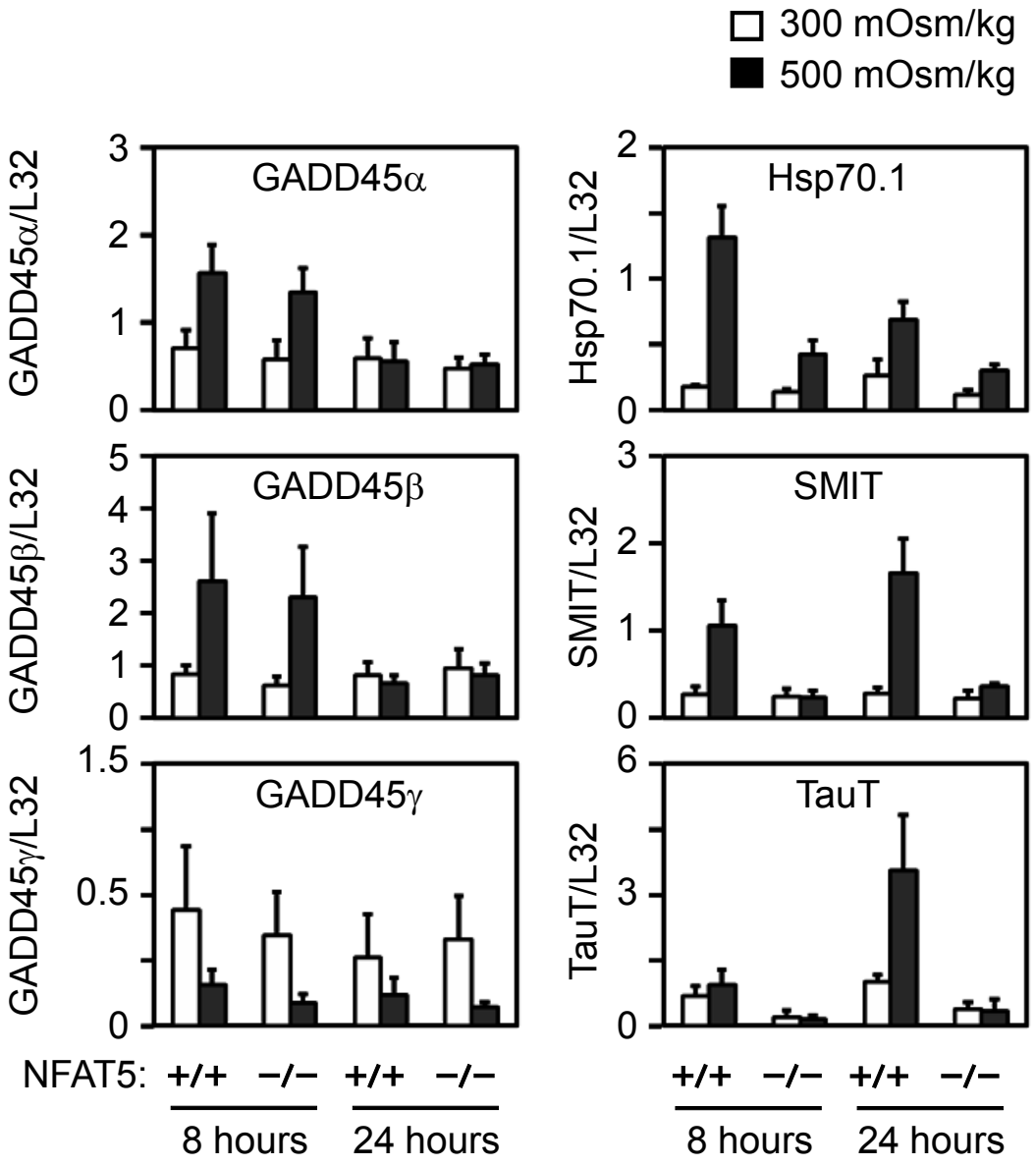

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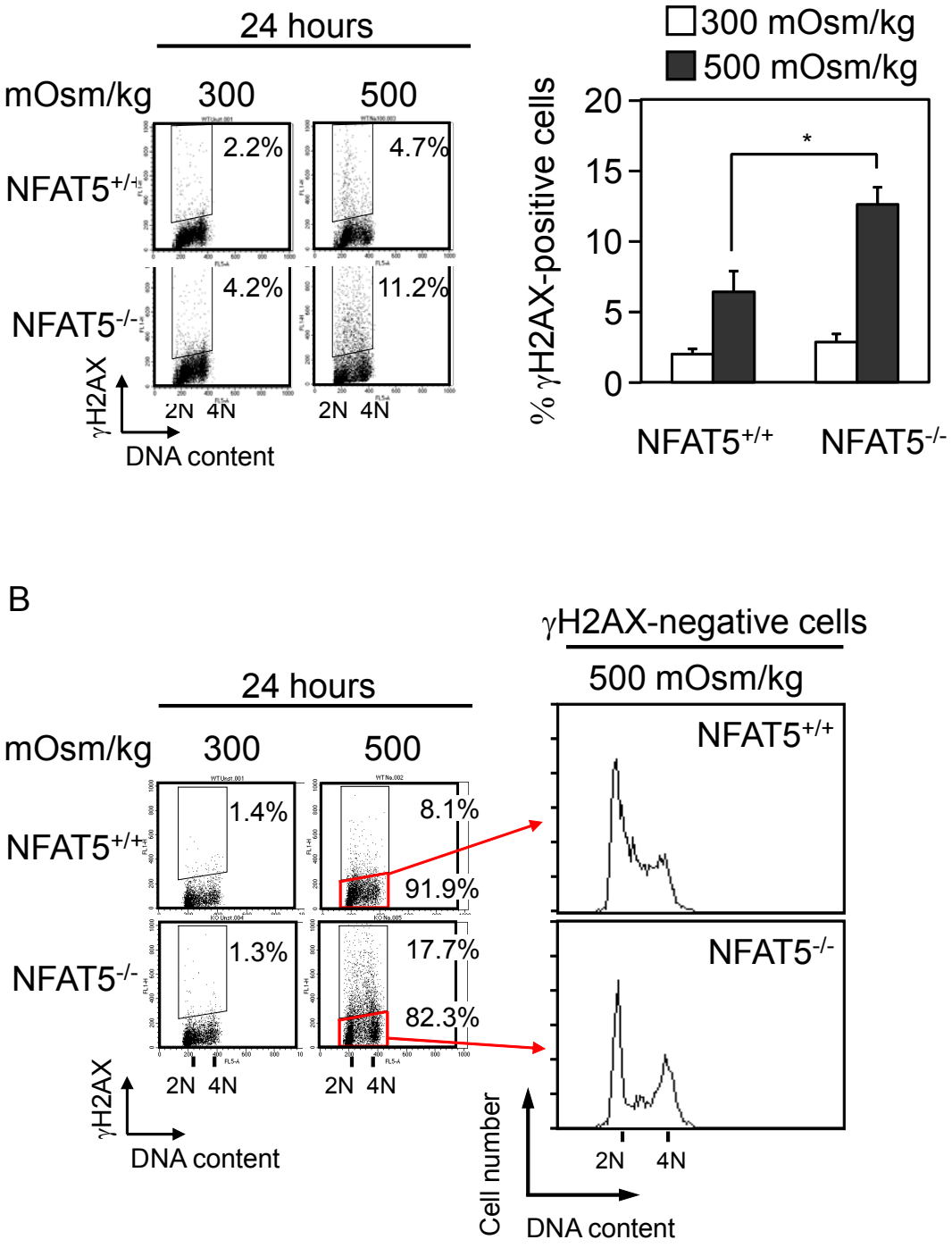

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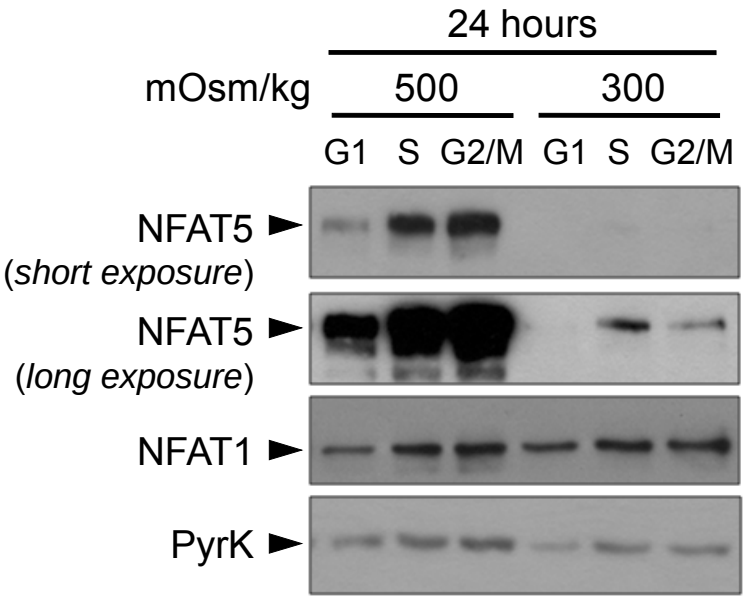

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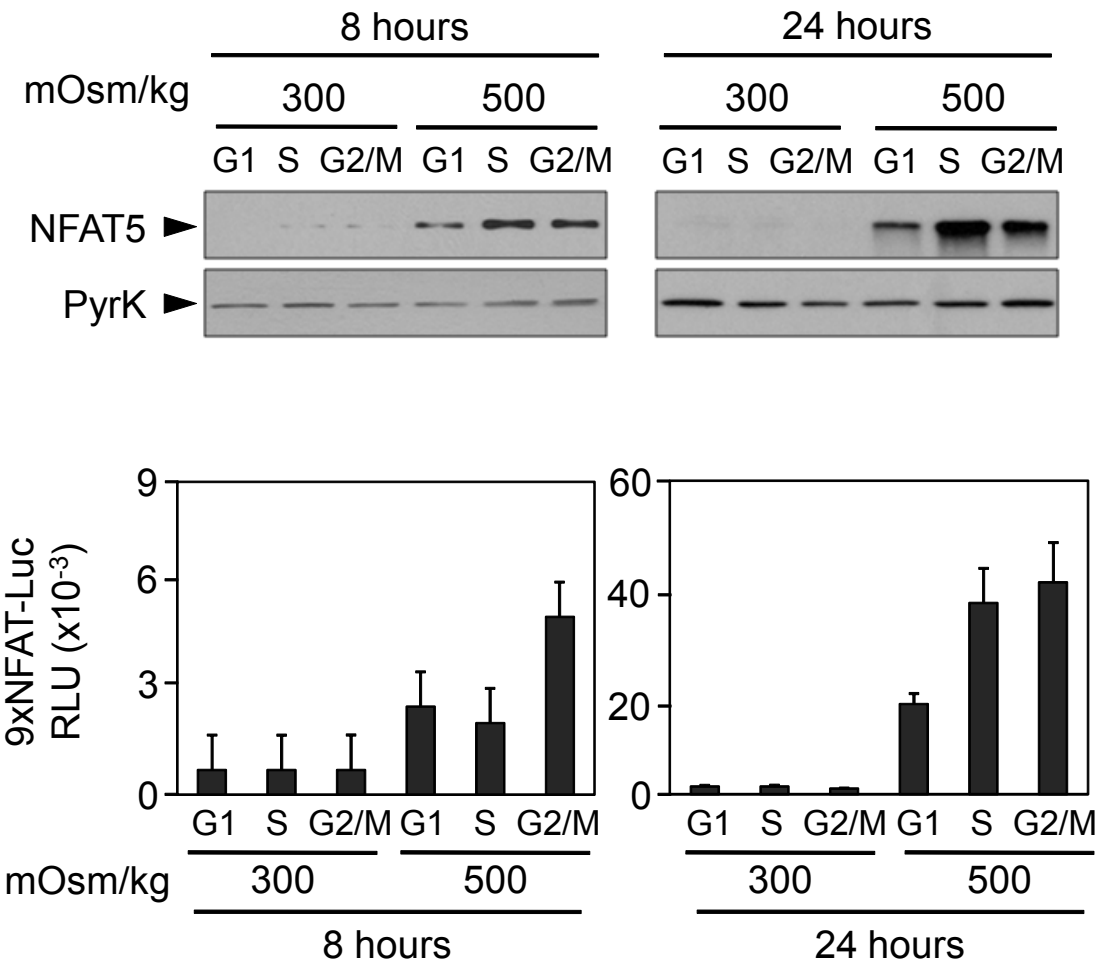

Drews-Elger et al., Figure 6

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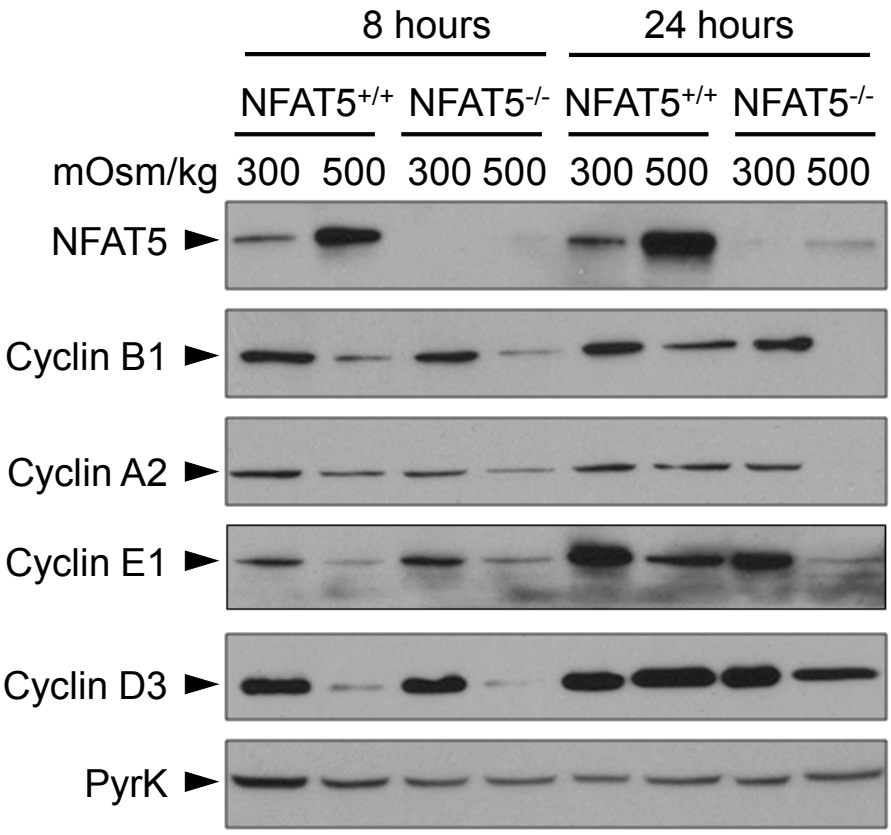

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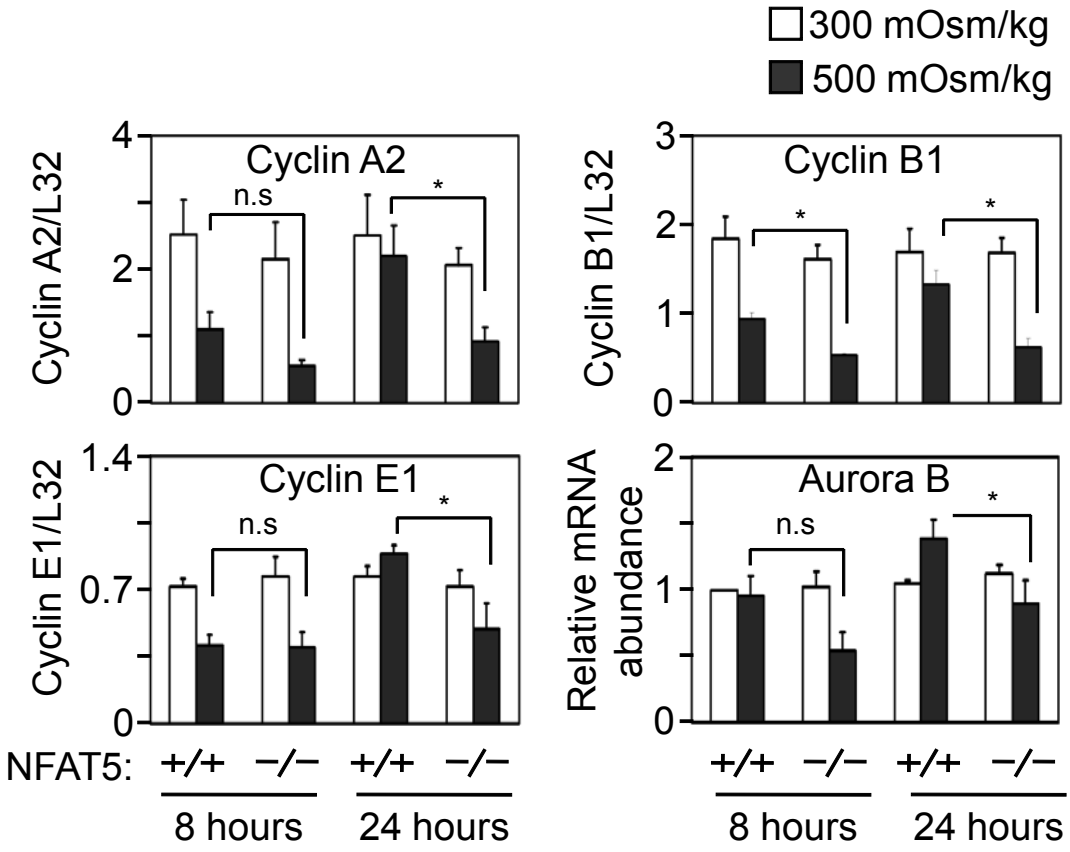
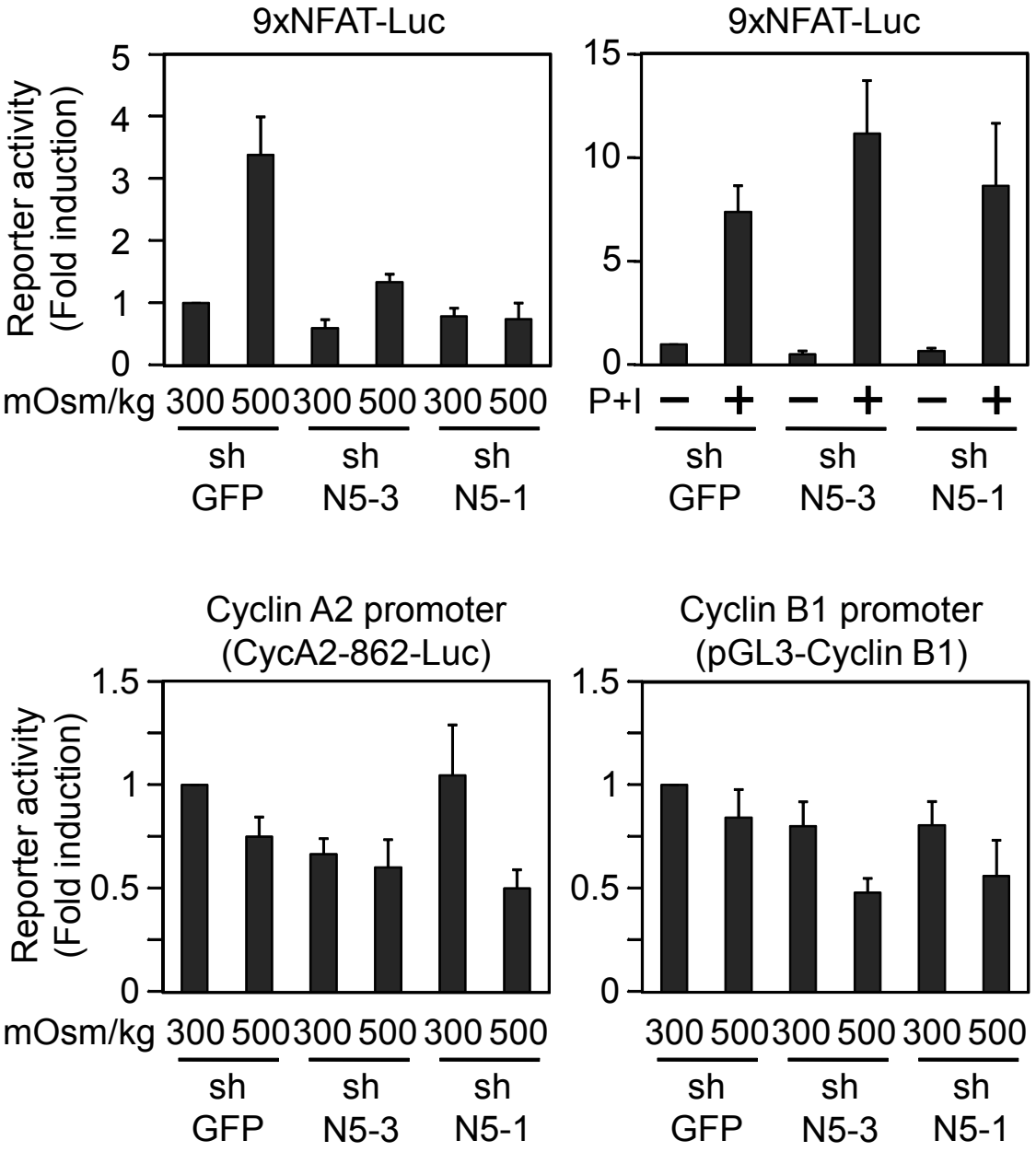
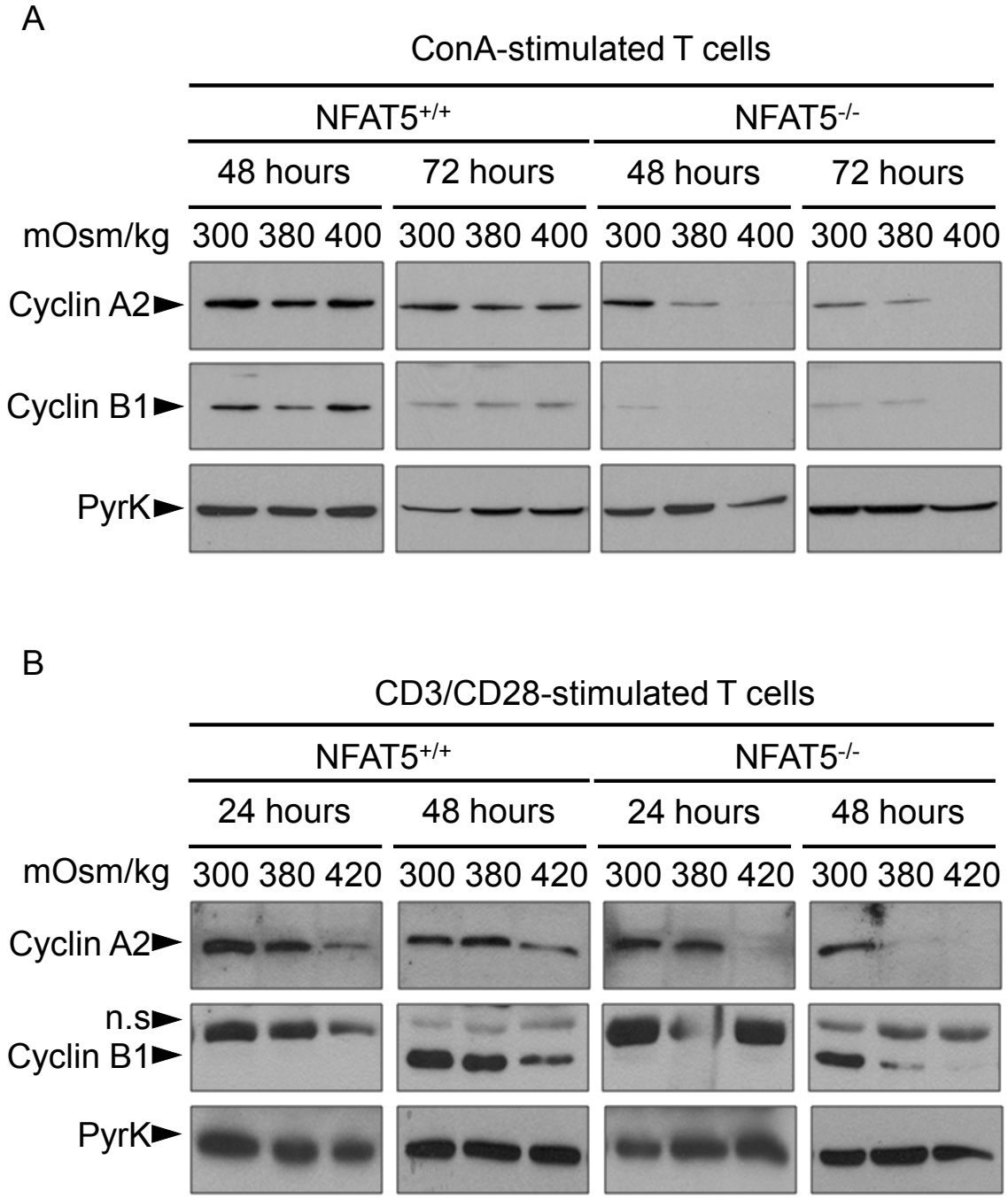


Drews-Elger et al., Figure 6

C

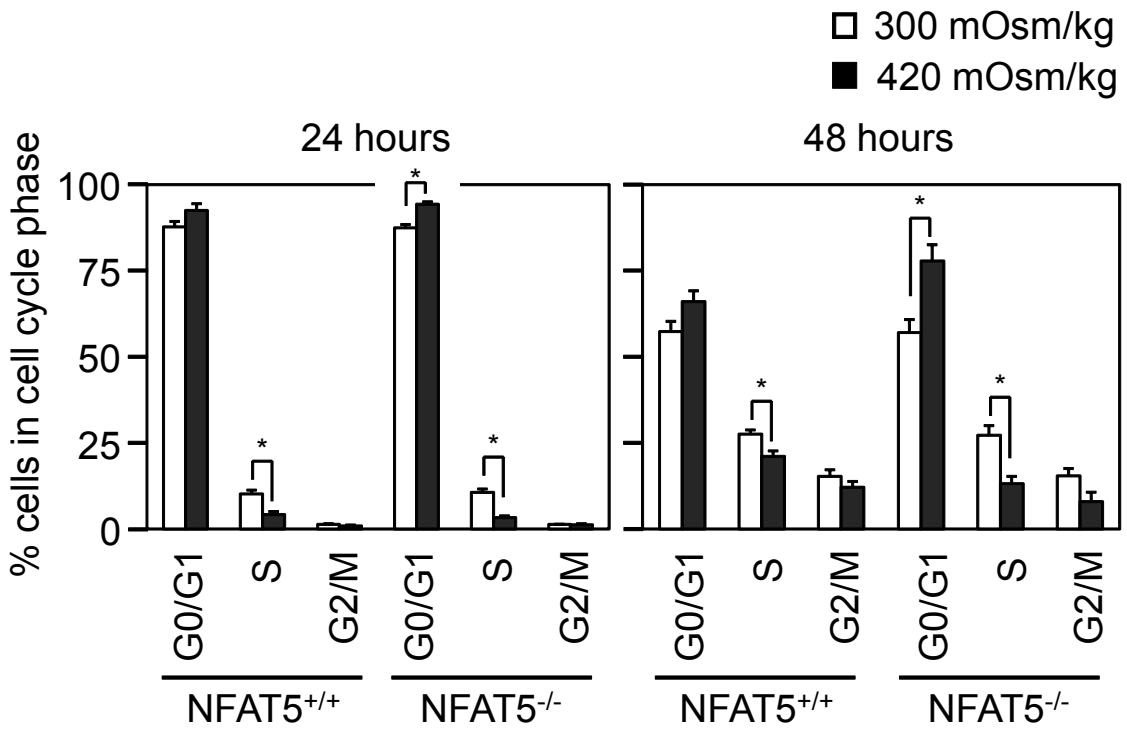


Drews-Elger et al., Figure 7



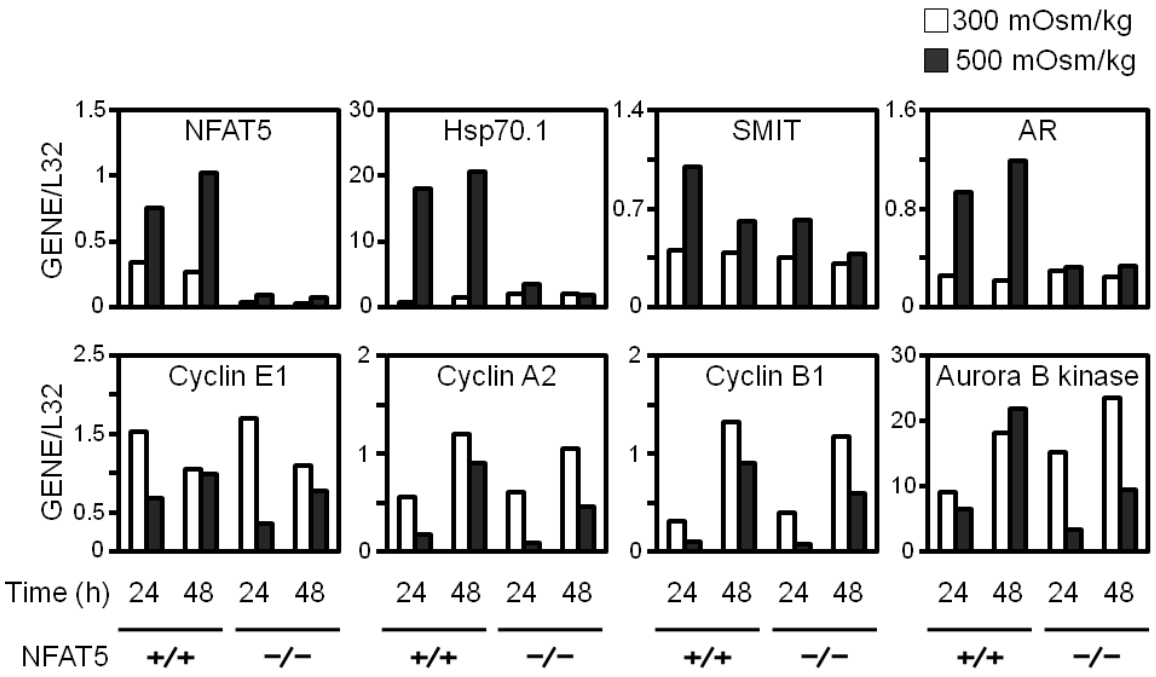
Drews-Elger et al., Figure 7

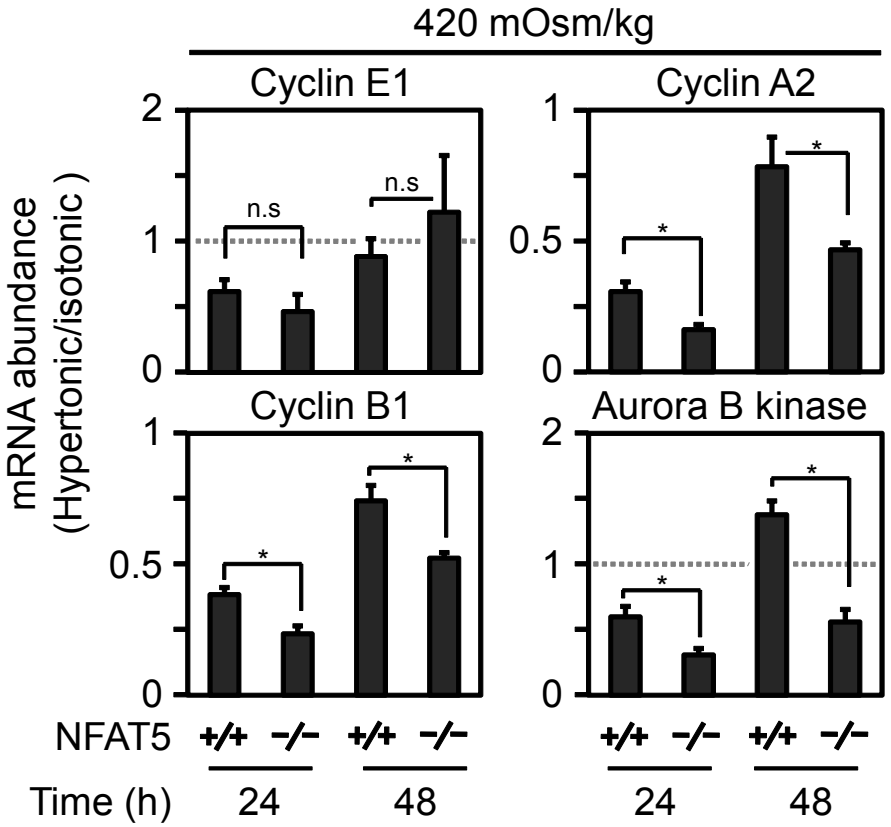
C



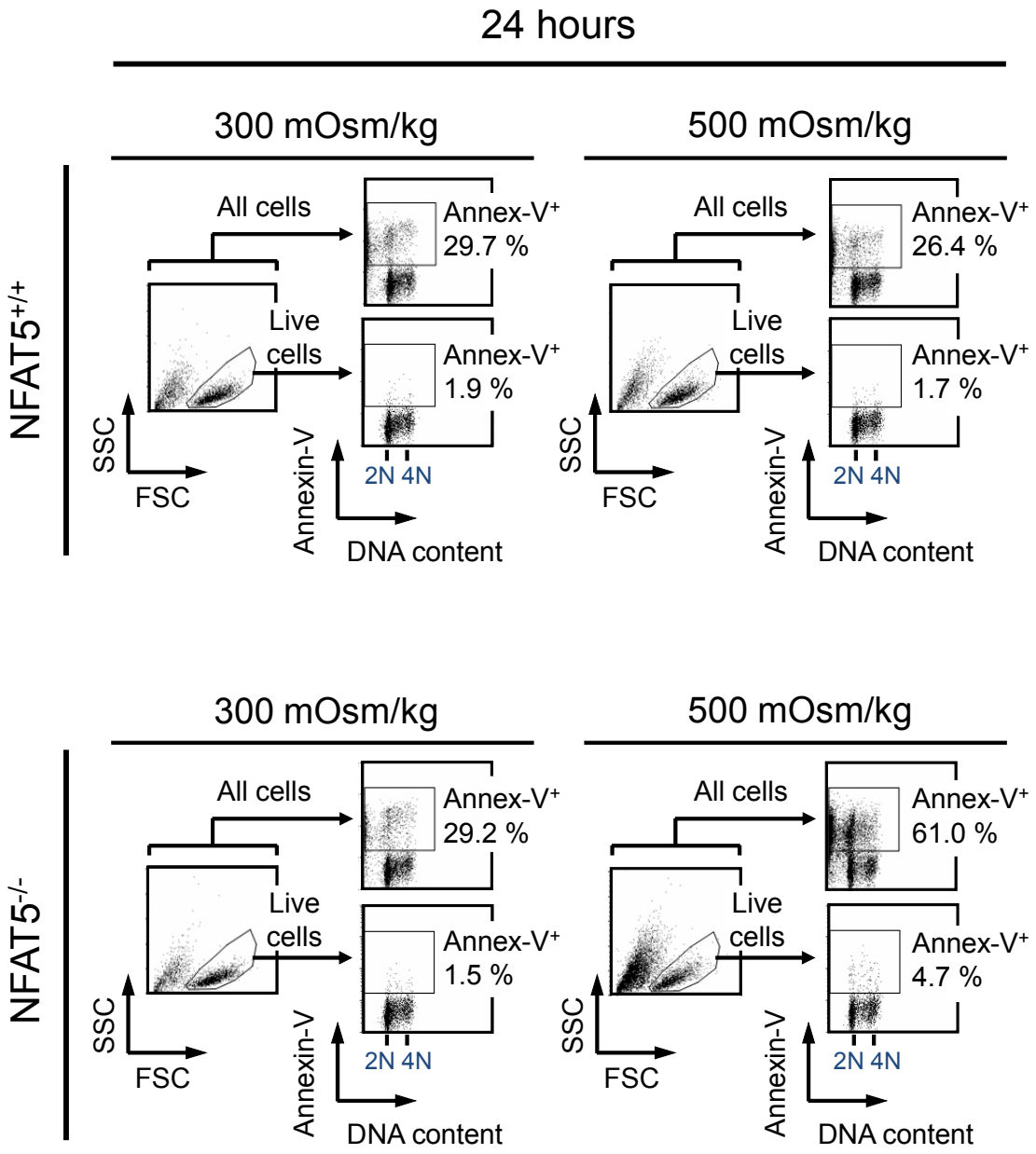
Drews-Elger et al., Figure 7

D

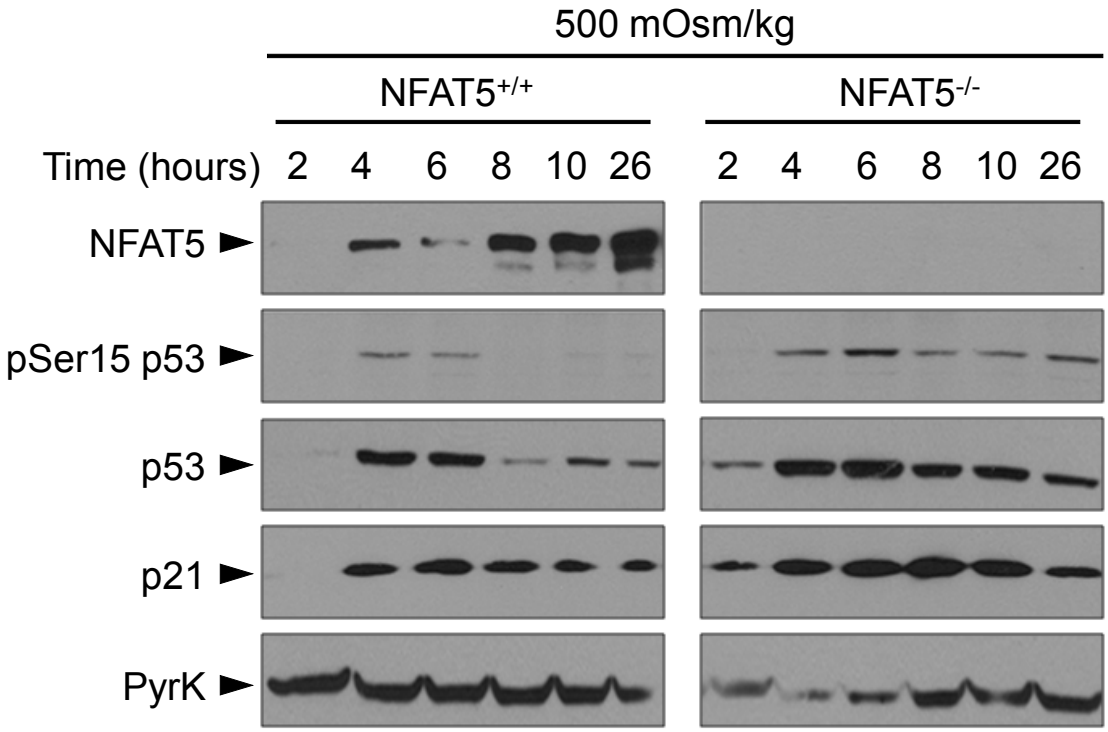


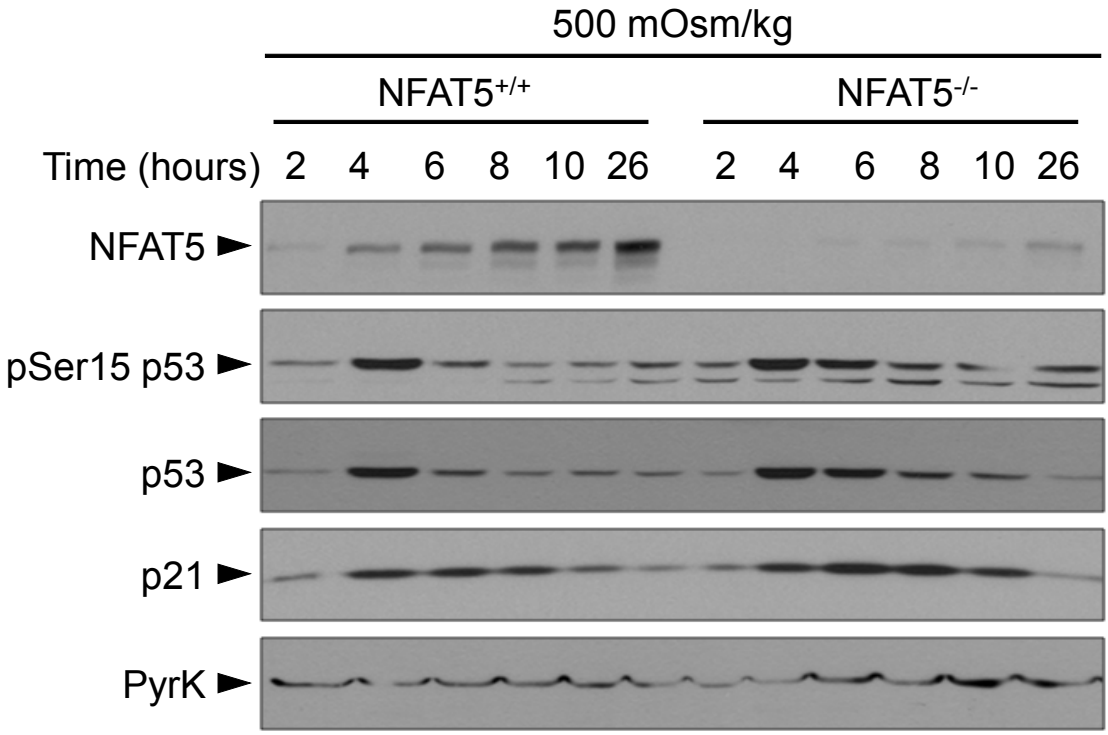


Drews-Elger et al., supplementary Figure S1

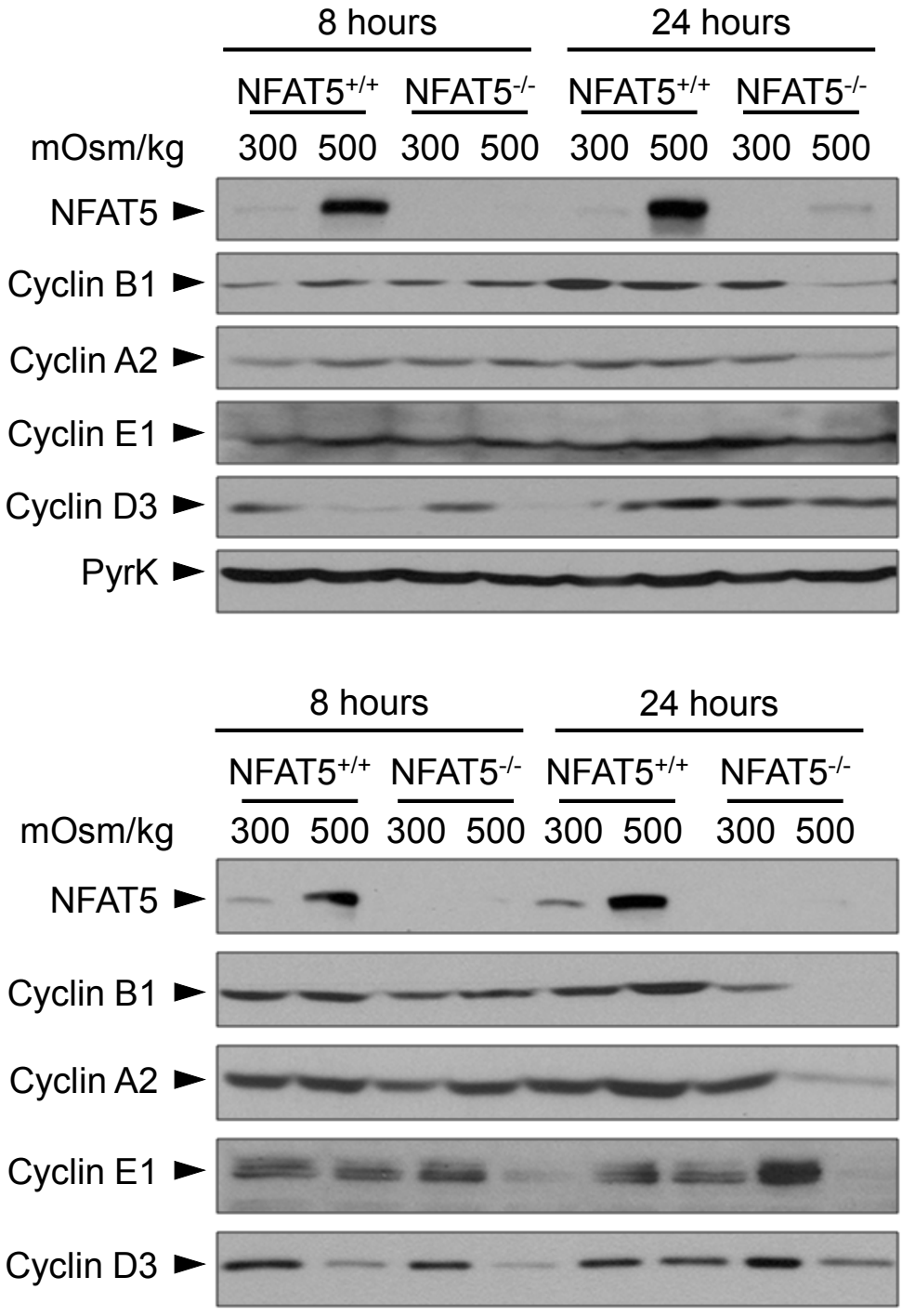


Drews-Elger et al., supplementary Figure S2

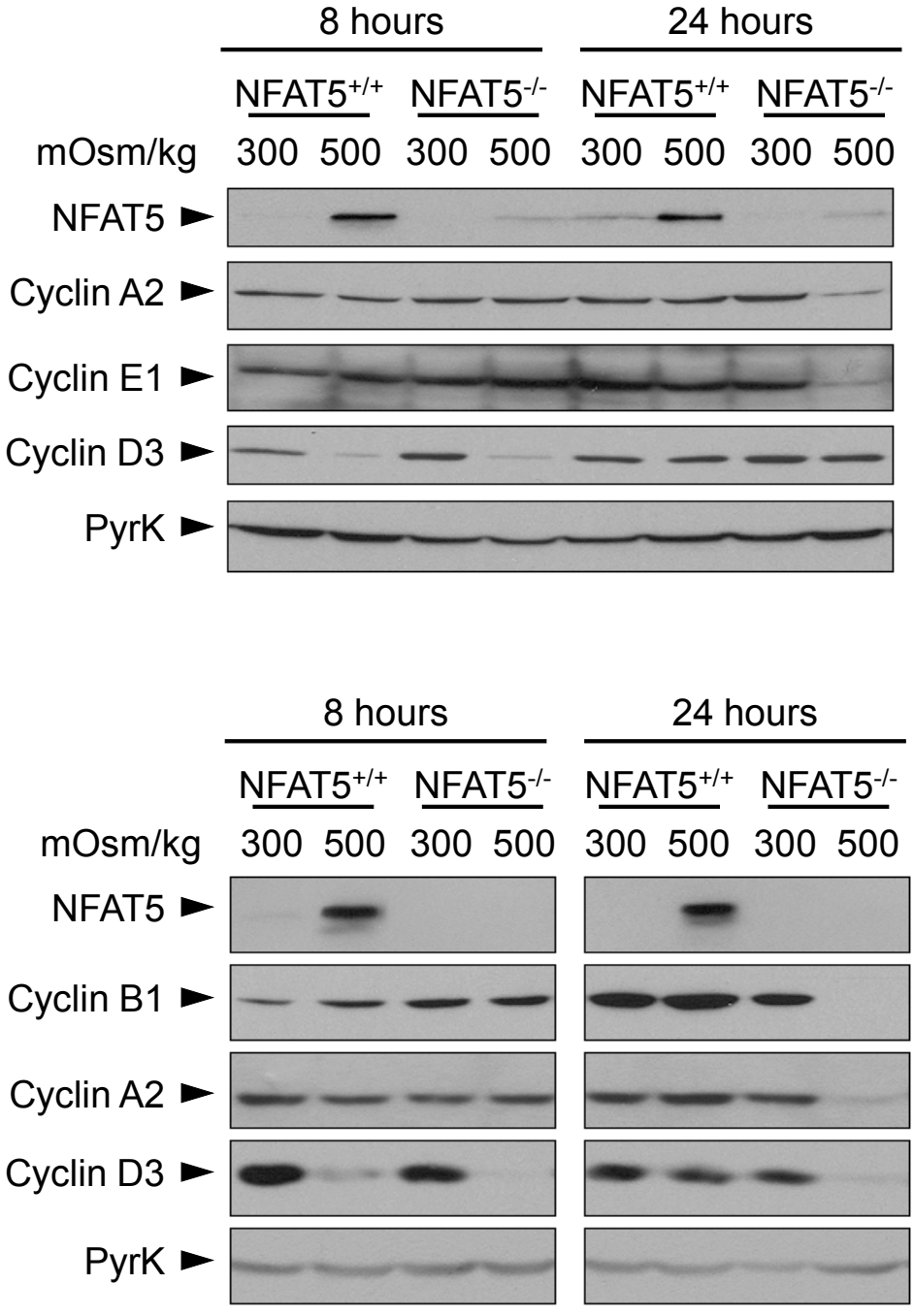




Drews-Elger et al., supplementary Figure S3



Drews-Elger et al., supplementary Figure S3



Drews-Elger et al., supplementary Figure S3