REGULATORY ROLE OF THE MECHANISTIC TARGET OF RAPAMYCIN (mTOR) ON THE EXPRESSION OF OSMOTIC STRESS RESPONSE GENES IN MAMMALIAN CELLS

Mª Carmen Ortells Campos

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DIRECTOR

José Aramburu Beltrán, Ph.D

DEPARTMENT OF EXPERIMENTAL AND HEALTH SCIENCES
(CEXS-UPF)

"Si buscas resultados distintos, no hagas siempre lo mismo"

"Todos somos muy ignorantes. Lo que ocurre es que no todos ignoramos las mismas cosas"

Albert Einstein (1879 – 1955)

A Rosa y a Giulia por tantos momentos juntas

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ABSTRACT

Adaptive responses allow cells to maintain their growth as well as their proliferative potential under diverse stress conditions. It is known that, growth and proliferation can be suppressed by intense stress, but maintained under tolerable stress conditions under which cells can induce compensatory responses. The kinase mTOR is a central regulator of proliferative and growing capacity in mammalian cells, and has been shown to be sensitive to diverse stressors. However, little is known about the role played by mTOR in the adaptive responses that cells utilize to resist stress and maintain their growth capacity. We addressed this question in the context of osmotic stress, to which cells can adapt by inducing the transcription of specialized genes. We showed that mTOR is active under moderate osmostress conditions and regulates the induction of a set of genes by mechanisms dependent and independent of NFAT5, the main transcription factor involved in the transcription of genes upon hypertonic stress. In addition, we observed that the overall set of genes whose induction was sensitive to mTOR activity is enriched in regulators of growth and proliferation. We also have identified REDD1 and REDD2 as two osmostress and mTOR-dependent induced genes, which previously had been characterized in other stress contexts acting as negative regulators of the mTORC1 pathway. We observed that mTOR promoted changes in chromatin predisposing it towards a transcriptional permissive configuration, with higher levels of acetylated histone H4 and increased recruitment of active RNA-pol II to promoters as well as transcribed regions. Altogether, the results described in this thesis reveal a new role for the mTOR kinase in the regulation of gene expression to facilitate the cellular adaptive response upon osmostress.

RESUMEN

Las respuestas adaptativas frente al estrés permiten a las células mantener su crecimiento así como su potencial proliferativo. Aunque se ha establecido que el crecimiento y la proliferación celular pueden inhibirse en respuesta a un estrés intenso, en situaciones de estrés tolerable las células pueden mantener su crecimiento y proliferación mediante la inducción de respuestas compensatorias. La quinasa mTOR es una proteína clave para el mantenimiento de la capacidad proliferativa y del crecimiento en las células de mamífero; además se ha descrito que es sensible a varios estreses. Sin embargo, poco se sabe acerca del papel que juega en las respuestas de adaptación que son utilizadas por las células para resistir el estrés y mantener así su capaciad de crecimiento. Nuestro trabajo se ha centrado en el ámbito del estrés osmótico, en cuyo caso las células pueden adaptarse mediante la transcripción de diversos genes especializados. Nuestro estudio demuestra que mTOR se encuentra activo en condiciones moderadas de estrés osmótico y regula la indución de un conjunto de genes mediante mecanismos dependientes e independientes de NFAT5, el principal factor de transcripción responsable de la transcripción de genes en respuesta a un estrés hipertónico. Además, observamos que la mayoría de los genes cuya inducción es sensible a la actividad de mTOR tienen funciones en la regulación del crecimiento y de la proliferación. También hemos identificado a REDD1 y REDD2 como genes que se inducen en respuesta a estrés osmótico dependientes de mTOR, y que con anterioridad se habían caracterizado en otros escenarios de estrés actuando como reguladores negativos de la ruta de señalización de mTORC1. Por último hemos observado que mTOR origina cambios en la cromatina, promoviendo una configuración permisiva para la transcripción, con un incremento de la acetilación de la histona H4 y un aumento en el reclutamiento de la forma activa de la RNA-polimerasa II en los promotores y regiones transcritas de

ciertos genes. En resumen, los resultados descritos en esta tesis muestran un nuevo papel de la quinasa mTOR en la regulación de la expresión génica facilitando así la respuesta de adaptación celular frente al estrés osmótico.

ABBREVIATIONS

2-DG	2-deoxyglucose
4E-BP1	Eukaryotic initiation factor 4E binding protein 1
ACC	Acetyl-CoA carboxylase
AED	Auxiliary export domain
AICAR	5-aminoimidazole-4-carboxamide1-β-D ribofuranoside
Akt/PKB	Serine/Threonine kinase Akt
AMPK	AMP-activated protein kinase
AQP1/*Aqp1*	Aquaporin 1
AR/*Akr1b3*	Aldose reductase
ARNT	Aryl hydrocarbon receptor nuclear translocator
ATA2	Sodium-coupled neutral amino acid transporter 2
ATF	Activating transcription factor
ATG13/101	Autophagy-related gene 13 and 101
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3-related protein
BGT1/*Bgt1*	Betaine-GABA transporter *or* sodium/chloride/ betaine cotransporter
BNIP3	Bcl2/adenovirus E1B 19 kDa protein-interacting protein 3
Bpgm/*Bpgm*	2,3-bisphosphoglycerate mutase
C	Carboxy
Cdk5	Cyclin-dependent kinase 5
CFDA-SE	Carboxyfluorescein diacetate succinimidyl ester
ChIP	Chromatin immunoprecipitation
CK-1	Casein kinase-1
CTD	Carboxy-terminal domain
DBD	DNA-binding domain
DEPTOR	DEP domain-containing mTOR-interacting protein

DNA-PK	DNA-dependent protein kinase
DR	Dietary restriction
eEF-2K	Eukaryotic elongation factor 2 kinase
eIF4B	Eukaryotic initiation factor 4B
eIF4E	Eukaryotic translation Initiation Factor 4E
ERK1/2	Extracellular signal-regulated kinase 1 and 2
FIP200	Focal adhesion kinase family-interacting protein of 200 kDa
FKBP12	FK506-binding protein, MW of 12 kD
FOXO1/3	Forkhead box O1 and O3
FRB	FKBP12-rapamycin binding domain
GADD45/153	Growth arrest and DNA damage 45 and 153
GSK3	Glycogen synthase kinase 3
H4	Histone H4
HEK293	Human embryonic kidney 293 cells
HIF-1	Hypoxia-inducible factor-1
HM	Hydrophobic motif
hnRNA	heterogeneous nuclear RNA
Hspa70.1/*Hspa1b*	Heat-shock protein 70
IGF	Insulin-like growth factor
IGF-1R	Insulin-like growth factor-1 receptor
IR	Ionizing radiation
IRES	Internal ribosome entry site
IRS1	Insulin receptor substrate 1
IRS-1	Insulin receptor substrate-1
JNK	C-Jun NH_2-terminal kinase
KO	Knockout
L32	Ribosomal protein L32
LKB1	Serine/threonine kinase 11
LTR	Long terminal repeats
MDCK	Madin-Darby Canine Kidney Cells

MEFs	Mouse embryonic fibroblasts
mIMCD3	mouse Inner medullary collecting duct cells 3
mLST8	mammalian lethal with Sec13 protein 8
MRN	Mre11/Rad50/Nbs1
mRNA	Messenger RNA
mSIN1	mammalian stress-activated protein kinase interacting protein
mTOR	mammalian Target of rapamycin/mechanistic Target of rapamycin
mTORC1	mammalian Target of rapamycin complex 1
mTORC2	mammalian Target of rapamycin complex 2
NES	Nuclear export signal
NFAT5	Nuclear factor of activated T cells 5
NFATc	Nuclear factor of activated T cells
NF-κB	Nuclear factor of kappa B
NLS	Nuclear localization signal
Nrf2	NF-E2-related factor 2
ORE	Osmotic response element
OREBP	Osmotic responsive element binding protein
p38 MAPK	Mitogen-activated protein kinase p38
p70S6K/S6K1	p70 ribosomal S6 kinase
p90 RSK	Serine/threonine protein kinase p90-kDa ribosomal S6 kinase
PARP	Poly (ADP-ribose) polymerase
PDCD4	Programmed cell death 4
PDK1	Phosphoinositide-dependent kinase-1
PDK4/*Pdk4*	Pyruvate dehydrogenase kinase 4
PGC1-α	PPAR-γ coactivator 1-α
PI3K	Phosphoinositide-3-kinase
PIKK	Phosphatidyl inositol 3' kinase-related kinases
PIP2	Phosphatidylinositol (4,5)-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate

PKA	Protein kinase A
PKC	Protein kinase C
PLC-γ	Phospholipase C-gamma1
PML	Promyelocytic leukemia tumor suppressor
PPARγ	Peroxisome proliferator-activated receptor γ
PRAS40	Proline-rich Akt substrate 40 kDa
Protor-1/2	Protein observed with Rictor-1 and -2
PTEN	Phosphatase and tensin homolog deleted on chromosome 10
Rag	Ras-related GTP binding protein
Raptor/RAPTOR	Regulatory associated protein of mTOR
REDD1/*Ddit4*	Transcriptional regulation of DNA damage response 1
REDD2/*Ddit4l*	Transcriptional regulation of DNA damage response 2
Rheb	Ras homolog enriched in brain
RHR	Rel homology region
Rictor/RICTOR	Rapamycin-insensitive companion of mTOR
RNA-pol I/II/III	RNA-polymerase I, II and III
ROS	Reactive oxygen species
rRNA	Ribosomal RNA
RT-qPCR	Real time-quantitative polymerase chain reaction
RVD	Regulatory volumen decrease
RVI	Regulatory volumen increase
S6	ribosomal protein S6
SA-β-Gal	Sensescence-associated β-galactosidase
SGK	Serum- and glucocorticoid-induced protein kinase
siRNA	Small interfering RNA
SKAR	S6K1 aly/REF-like target
SMIT/*Smit1*	Sodium myo-inositol cotransporter
SREBP1/2	Sterol regulatory element binding protein 1 and 2

STAT1/3	Signal Transducer and Activator of Transcription 1 and 3
TAD	Transactivation domain
TauT	Taurine transporter
TFIIIB	Transcription Factor III B
TIF-IA	Tripartite motif-containing protein 24
TLR	Toll like receptor
TM	Transmembrane motif
TonEBP	Tonicity enhancer binding protein
TOR	Target of rapamycin/mechanistic Target of rapamycin
TORC1	Target of rapamycin complex 1 (yeast)
TORC2	Target of rapamycin complex 1 (yeast)
tRNA	Transfer RNA
TSC1/2	Tuberous sclerosis complex 1 and 2
TSS	Transcription Start Site
ULK1/2	Unc-51-like kinase 1 and 2
UT-A1/A3	Vasopressin-regulated urea transporter
UTR	Untranslated region
VEGF	Vascular Endothelial Growth Factor
YY1	Ying-Yang 1

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INTRODUCTION

1. CELLULAR STRESS

When environmental stress, independently of its underlying origin, exceeds a specific threshold is sensed by cells as a cellular stress causing macromolecular damage as well as affecting different cellular processes disturbing the correct cellular homeostasis. This cellular stress response comprises mechanisms evolutionary conserved from prokaryotes to eukaryotes that involve the different defense reactions, as well as damage sensors, that cells have towards a damaging agent (Kultz, 2005). This cellular stress response involves adaptation mechanisms, common for different stressors, as well as stress-specific mechanisms guaranteeing the re-establishment of cellular homeostasis (Kultz, 2003). However, when stress exceeds a certain threshold that cannot be managed by this cellular adaptation response cells can undergo death (Kultz, 2005), thus restricting the cellular adaptation of cells to certain limits depending on the type and intensity of the stresses applied.

2. OSMOTIC STRESS

Osmotic stress

"A change in state or activity of a cell or an organism (in terms of movement, secretion, enzyme production, gene expression, etc.) as a result of a stimulus indicating an increase or decrease in the concentration of solutes outside the organism or cell "

Gene Ontology site

Hyperosmotic stress

"Increase in the concentration of solutes outside the organism or cell causing a consequent efflux of water from the cell"

The bulk movement of water across a semipermeable membrane is termed osmosis (Strange, 2004). Two different terms are used to define osmolyte concentrations, osmolality and osmolarity. Osmolality indicates the total number of particles present in one kilogram of solvent and is typically expressed as mOsm/kg. Osmolarity indicates the total number of particles present in one liter of solution and is typically expressed as mOsm/L. One osmole is 1 mol of particle, which are equivalent to 6.023×10^{23} individual particles. When referring to the relatively dilute solutions of the body osmolality and osmolarity are used interchangeably, assuming that 1 liter of water weights 1 kg. So, in a physiological context, when we calculate the plasma osmolality, we have to keep in mind that is unlikely to be affected by changes in water content. Because the weight of the solute in plasma is

negligible compared to the weight of the solvent in this physiological scenario, plasma osmolarity and osmolality could be considered as equals. The term 'osmotic stress' usually refers to hyperosmotic stress, or the condition in which the concentration of solutes outside the cell is higher than the concentration inside the cell.

2.1 Cellular response to osmotic stress:

Cellular homeostasis is a process tightly regulated by the cell and it is essential for proper cell function and survival. This cellular homeostasis can be disturbed by osmotic pressure gradients, immediately causing changes in cell volume mediated by gain or loss of water.

The ability of cells to adapt to osmotic perturbations involves an early response, initiated few seconds to minutes after the osmotic imbalance sensing, in which water and ions move across cell membranes, having changes in cell volume, and a late response also called adaptation phase, that usually requires hours, characterized by increased synthesis of either membrane transporters essential for uptake of compatible organic osmolytes, or of enzymes involved in their synthesis. The goal of these responses is to return the cell to its normal size and restore the intracellular ion homeostasis.

With only few exceptions (Greger, 1985), the membranes of animal cells are highly permeable to water, compared with their permeability to Na^+, K^+ or Cl^-. Animal cell membranes cannot tolerate substantial hydrostatic pressure gradients, and water movement across those membranes is in large part dictated by osmotic pressure gradients. Thus, any imbalance of intracellular and extracellular osmolarity is paralleled by respective water movement

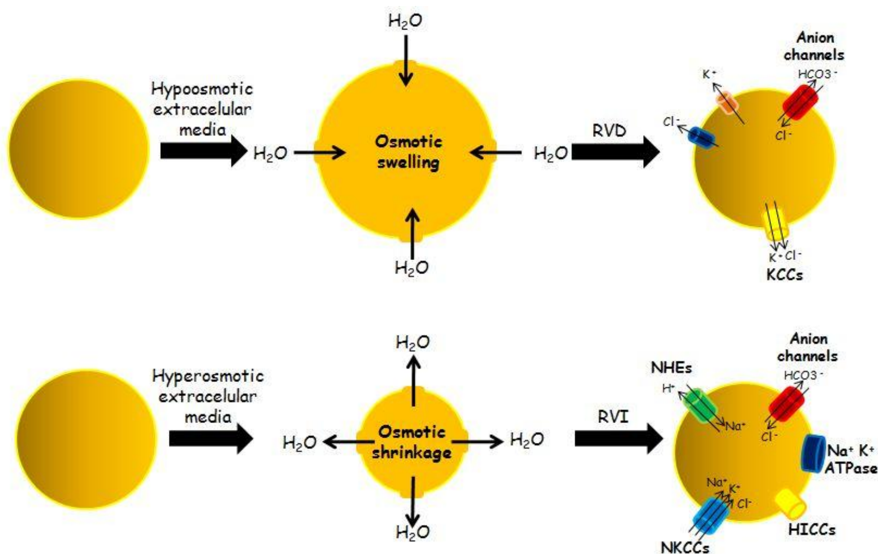
across cell membranes and subsequent alterations of cell volume (Lang et al., 1998).

All cells face constant challenges to their proper volume either through changes in intracellular solute content, caused by changes in intracellular metabolic pathways, or by changes in extracellular solute content, experienced in a variety of diseases. Those perturbations in cell volume are counteracted by activating membrane transporters and/or metabolic processes that result in net solute loss or gain and the return of cell volume to its normal resting state. This process is known as maintenance of cellular homeostasis, and can be initiated with such as small changes of only 3% in cell volume (Lohr and Grantham, 1986).

Water is the direct agent affecting cellular volume and it is in thermodynamic equilibrium across the plasma membrane. In other words, the osmotic concentration of cytoplasmic and extracellular fluids are equal under steady-state conditions. Because cell membranes are freely permeable to water, with fewer exceptions (Greger, 1985), any such gradient results in the immediate flow of water into or out of the cell until equilibrium is again achieved. Since animal cell membranes are unable to generate or sustain significant hydrostatic pressure gradients, water flow causes cell swelling or shrinkage (Strange, 2004).

The processes by which swollen and shrunken cells return to normal volume are collectively termed regulatory volume decrease (RVD) and regulatory volume increase (RVI), respectively. Cell volume can only be regulated by the gain or loss of osmotically active solutes, in the early response phase mediated by accumulation of inorganic ions such as Na^+, K^+, Cl^-, and in the late phase also is mediated by small organic molecules termed organic osmolytes.

Extracellular hypotonicity causes cell swelling, thus triggering RVD, and extracellular hypertonicity causes cell shrinkage, activating RVI that leads to accumulation of ions. The process known as RVD is characterized by the influx of water into the cell and the activation of several ion channels, such as the swelling K^+ channels, the swelling Cl^- channels and the K^+/Cl^- co-transporters (KCCs) (Hoffmann et al., 2009). In contrast, the process known as RVI is characterized by the efflux of water out of the cell and the activation of other ion channels such as the Na^+/H^+ exchangers (NHEs), the Na^+, K^+, $2Cl^-$ cotransporters (NKCCs) and in some cell types by the hypertonicity-induced cation channels (HICCs) (Hoffmann et al., 2009) (**Figure I-1**).



Adapted from Hoffman et al. (2009) and Strange et al. (2004)

Figure I-1. Regulatory volume decrease and regulatory volume increase and the different channels involved in both responses. Upon exposure to hypoosmotic extracellular media, cells swell by influx of water and require a regulatory volume decrease mechanism to restore their initial volume. The main channels involved in the RVD mechanism are K^+/Cl^- symport (KCCs), and

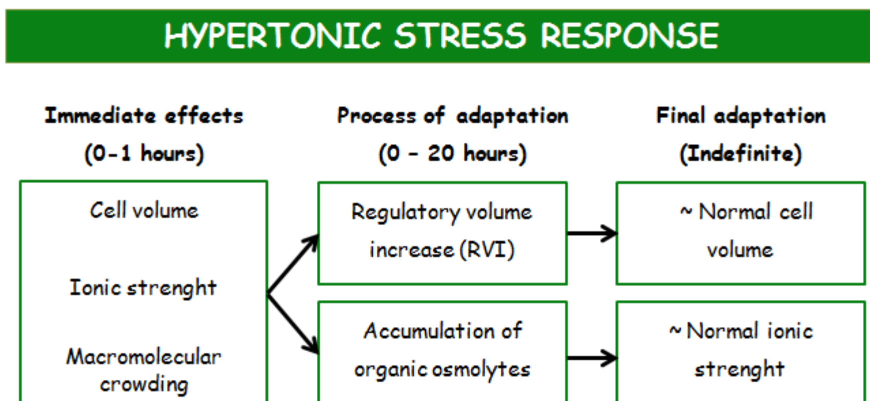
the swelling K^+ and Cl^- channels among others. Upon exposure to hyperosmotic extracellular media, cells shrink by an efflux of water and require a regulatory volume increase mechanism to restore the initial volume. The main channels involved in the RVI mechanism are the Na^+/H^+ exchangers (NHE), the $Na^+/K^+/2Cl^-$ cotransporters (NKCCs) and the hypertonicity induced cation channels (HICCs), among others.

Hypertonicity, in the early response phase, is characterized by two main processes. First an efflux of water out of the cell leading to a reduction in cell volume -shrinkage-, macromolecular crowding, and uptake of inorganic ions causing an increase in ionic strength in the cell. This perturbing and rapid compensatory mechanism has to be counteracted additionally by the accumulation of compatible organic osmolytes, in the phase of adaptation, requiring transcriptional activity (**Figure I-2**).

The usual explanation for this phenomenon is the need to replace the early cellular accumulation of inorganic ions with small organic molecules that do not affect cell function even at relatively high intracellular concentrations (Alfieri and Petronini, 2007), (Burg et al., 2007).The mechanisms by which hypertonic stress induces increased expression of genes encoding organic osmolyte transporters and enzymes involved in their synthesis have been studied extensively in the kidney (Burg, 1996).

Organic osmolytes comprise polyols such as sorbitol and myo-inositol, methylamines such as glycerophosphorylcholine and betaine, and amino acids such as taurine, glutamic acid, and s-alanine amongst others (Wehner et al., 2003). Intracellular accumulation of organic osmolytes involves mainly two processes. The first represents uptake across the cell membrane by specific transporter systems, the second intracellular generation of the specific osmolyte by different metabolic reactions. Examples of the former are sodium-cotransport systems for myo-inositol (SMIT), taurine (TauT),

and betaine (BGT1) which use the sodium gradient across the plasma membrane as driving force. Metabolic reactions are primarily responsible for the generation of sorbitol from D-glucose using the aldose reductase (AR) enzyme and glycerophosphorylcholine (GPC) from phosphatidylcholine (PC) (Wehner et al., 2003). The release of organic osmolytes from the cell involves channel-like proteins and in some instances organic and inorganic osmolytes share the same transporter. Thus, a complex picture emerges in which inorganic and organic osmolyte levels have to be controlled in a well coordinated manner (Burg, 1996).



Adapted from Burg et al. (2007)

Figure I-2. Cellular response to extracellular hypertonic stress. The response to a hyperosmotic insult is classified in the immediate effects, the process of adaptation and the final adaptation. The immediate effects, occurring within a few seconds after the hyperosmotic insult, are characterized by a decrease in cell volume, an increase in the intracellular ionic strength and an increase in the macromolecular crowding. The process of adaptation, that stretches up to twenty hours, is characterized by a RVI and by accumulation of compatible organic osmolytes. After that period, the cell restores its normal cell volume and its normal ionic strength.

2.2 Physiological and pathological scenarios of hyperosmotic stress:

The adaptive responses to hypertonicity have been studied mainly in epithelial cells (derived from the kidney medulla) as these cells are physiologically exposed to marked variations in osmolarity during their normal function in vivo (Burg, 1996). However, similar responses have been detected in several other cell models exposed to hypertonicity such as monocytes and macrophages (Denkert et al., 1998), chondrocytes (de Angelis et al., 1999), mesothelial cells (Matsuoka et al., 1999), endothelial cells (Petronini et al., 2000), T cells (Lopez-Rodriguez et al., 2001), (Go et al., 2004b), fibroblasts (Sanguinetti et al., 2003), astrocytes (Olsen et al., 2005), nucleus pulposus cells (Tsai et al., 2006) and neurons (Maallem et al., 2006) among others.

In mammals, the normal plasma osmolality ranges between 285 and 300 mOsm/kg, by the regulation of urinary concentration (Dmitrieva et al., 2004). When plasma osmolality is below this value is considered as hypoosmolar and when is higher is considered hyperosmolar. Because body water is the primary determinant of extracellular fluid osmolality, disorders of body water balance can lead to hypoosmolar and hyperosmolar disorders depending on the presence of an excess or a deficiency of body water relative to body solute (Adler and Verbalis, 2006). Because sodium is the main impermeable cation in the extracellular fluid, the concentration of serum sodium (and of its accompanying anion) is the main determinant of the effective plasma tonicity. Except in conditions of increased blood glucose or the presence of exogenous impermeable substances, hyponatremia and hypernatremia are synonymous with hypotonicty and hypertonicity, respectively (Shoker, 1994).

The kidney is the principal organ that is subjected to high tonicities in the body, as high as 1700 mOsm/kg due to extraordinary high levels of NaCl and urea. These two major solutes differ in their osmotic action. Hyperosmotic salt is hypertonic, causing cell shrinkage due to osmotic water efflux. On the other hand, hyperosmotic urea is not hypertonic due to its high membrane permeability, and therefore, urea equilibrates across the plasma membrane, causing high hyperosmolarity but not increased hypertonicity.

The kidney is the major body organ that experiences a physiologically hypertonic environment. It has been described also that lymphoid tissues like thymus and spleen (Go et al., 2004a) and other organs like the liver (Go et al., 2004a) are moderately hypertonic, reaching osmolalities around 335 mOsm/kg. Also cells in the nucleus pulposus of intervertebral discs (Tsai et al., 2006) can be exposed to osmolalities that reach 450 mOsm/kg (Urban et al., 1993). In the skin it is also reported that during feeding on a high salt diet, interstitial tonicity may exceed that of plasma, and that water-free Na^+ storage may occur as Na^+ proteoglycan-binding in the skin, causing local hypertonicity (Machnik et al., 2009). Additionally it is also interesting to point out that little is known about whether hypertonicity can occur naturally in the different organs of our body. This lack of information leads to the question of why so many cell types can respond and adapt to hypertonic stress.

Beyond physiologic hypertonic stress scenarios, there are different pathological processes in which hypertonic conditions in plasma or tissues can be found. The different pathologies are due principally to the disruption of water homeostasis. The most documented hypertonic disorders, by its importance and influence, are the hypernatremic states. Hypernatremia is defined as a rise in the serum sodium concentration to a value exceeding 145

mmol per liter and is considered a common electrolyte disorder. Normally, the extracellular-fluid and intracellular-fluid compartments account for 40 and 60 percent of total body water, respectively (Adrogue and Madias, 2000). Hypernatremia can be manifested by a deficit of water in relation to the body sodium stores, which can result from a net water loss –the majority of the cases- or a hypertonic sodium gain (**Table I-1**) (Adrogue and Madias, 2000).

Because sustained hypernatremia can occur only when thirst or access to water is impaired, the highest risk groups are patients with altered mental status (Macdonald et al., 1989), intubated patients (Hans et al., 2001), infants (FINBERG and HARRISON, 1955), and elderly persons (Borra et al., 1995). Hypernatremia in infants usually results from diarrhea, whereas in elderly persons it is usually associated with febrile illness due to bacterial infections and thirst impairment. Hospitalized patients are prone to hypernatremia because they depend on others for their water requirements.

The range of osmolalities observed in the plasma of some hypernatremic disorders described in the literature is the followings:

- Adypsic hypernatremia in infancy by defects in osmo-regulation of thirst and the secretion of arginine vasopressin: 430 mOsm/kg (Papadimitriou et al., 1997)
- Poliyuria secondary to chronic interstitial nephritis: 320 mOsm/kg (Shoker, 1994).
- Chronic alcohol liver disease with diabetes insipidus: 360 mOsm/kg (Shoker, 1994).
- Diabetes ketoacidosis: 315 mOsm/kg (Shoker, 1994).

Moreover, apart from pathologies of water homeostasis it has been described that inflammatory disorders like Crohn's disease in the intestine and corneal

inflammation in dry eye syndrome in the cornea can cause local hyperosmolality (Neuhofer, 2010).

CAUSES of HYPERNATREMIA (I)

NET WATER LOSS			
	PURE WATER LOSS	**Dehydra-tation**	Unreplaced insensible losses (dermal and respiratory) Hypodipsia (Abnormalities of thirst appreciation)
		Diabetes insipidus	Neurogenic diabetes insipidus: Post-traumatic Caused by tumors, cysts, histiocytosis, tuberculosis, sarcoidosis Idiophatic Caused by aneurysms, meningitis, encephalitis, Guillain-Barré syndrome Caused by ethanol ingestion (transient)
			Congenital nephrogenic diabetes insipidus
			Acquired nephrogenic diabetes insipidus: Caused by renal disease (e.g. medullary cystic disease) Caused by hypercalcemia or hypokalemia Caused by drugs (lithium, demeclocycline, foscarnet, methoxyflurane, amphotericin B, vasopressin V_2-receptor antagonists)
	HYPO-TONIC FLUID LOSS	**Renal causes**	Loop diuretics Osmotic diuresis (glucose, urea, mannitol) Post-obstructive diuresis Polyuric phase of acute tubular necrosis Intrinsic renal disease
		Gastro-intestinal causes	Vomiting Nasogastric drainage Enterocoutaneous fistula Diarrhea Use of osmotic cathartic agents (e.g. lactulose)
		Cutaneus causes	Burns Excessive sweating

CAUSES of HYPERNATREMIA (II)

HYPERTONIC SODIUM GAIN	
	Hypertonic sodium bicarbonate infusion
	Hypertonic feeding preparation
	Ingestion of sodium chloride
	Ingestion of sea water
	Sodium chloride-rich emetics
	Hypertonic saline enemas
	Intrauterine injection of hypertonic saline
	Hypertonic sodium chloride infusion
	Hypertonic dialysis
	Primary aldosteronism (Conn syndrome)
	Cushine's syndrome (Hypercorticosteirodism)
	Bartter syndrome

Table I-1. Causes of hypernatremia by net water loss or hypertonic sodium gain.

2.3 Perturbing effects of osmotic stress:

In the literature it is described that hyperosmotic stress can cause a broad range of disturbing effects in cells (**Figure I-3**). These effects can be discriminated in two different groups: those that can be caused at high intensity of hyperosmotic stress and those observed during more moderate conditions, more likely occurring in physiopathological scenarios. When considering how a particular cell type responds to osmostress it is important to determine whether the stress conditions used permit cellular adaptation or, on the contrary, are so extreme that they will be lethal.

It is important to keep in mind that the level of osmotic stress that cells can sense *in vivo* it is not known. Normally, the cells in the renal medulla are surrounded of osmolalities as high as 1700 mOsm/kg. but these high values of Na^+ ions can be partially compensated by a high local concentration of urea. It is for this reason that using concentrations of NaCl higher than 150 or 200 mM (equivalent to osmolalities between 600-700 mOsm/kg) to mimic the osmotic stress in the renal medulla may not reflect the real stress unless urea is also added *in vitro*.

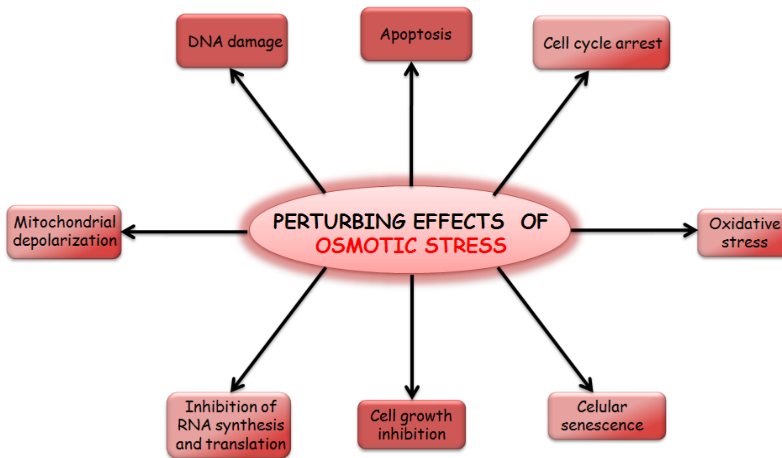


Figure I-3. Perturbing effects of osmotic stress. Osmotic stress can produce a broad range of cellular effects among which we can find DNA damage, apoptosis, cell cycle arrest, oxidative stress, cellular senescence, cell growth inhibition, inhibition of transcription and translation and mitochondrial depolarization. The two-color graded boxes represent effects observed at lower osmolalities -physiologic- and the single color boxes represent those effects observed at high osmolalities -artificial-.

The following section describes several effects that different intensities of osmotic stress can have in different cell types. A graphic summary is provided in **Figure I-4**.

Apoptosis:

Cell death by apoptosis results when acute hypertonicity exceeds a certain stress threshold that cannot be restored by the adaptive stress response. The level differs between cell lines, but is rather sharply defined in each. Above that level, massive cell death occurs within several hours (Burg et al., 2007). Hyperosmotic stress has been shown to induce apoptosis in cell lines such as human neuroblastoma cells (Matthews et al., 1997), Jurkat T lymphocytes (Juo et al., 1997), rat alveolar type II cells (Edwards et al., 1998), cardiomyocytes (Hoover et al., 2000), (Morales et al., 2000), cardiac fibroblasts (Mockridge et al., 2000) and chondrocytes (Racz et al., 2007). As an example of the different intensities of hyperosmotic stress and mechanism of apoptosis in those different cell types, cell culture chondrocytes subjected to 600 mOsm/kg induced the activation of proapoptotic signaling factors such as JNK, p38 and caspase-3 and also led to an increased level of ERK1/2 (Racz et al., 2007). In contrast, cultured cardiomyocites subjected to 300 mM sorbitol, which would increase osmolality to 600 mOsm/kg, induce strong and early increase of apoptosis, indicated by PARP (Poly (ADP-ribose) polymerase*)* degradation, DNA fragmentation, and an increase in Bcl-X_S proapoptotic protein (Hoover et al., 2000), (Morales et al., 2000).

DNA damage and cell cycle arrest:

An increase in the osmolality by addition of NaCl to 500 – 600 mOsm/kg to mIMCD3 cells (mouse Inner Medullary Collecting Duct Cells 3) induces double-strand DNA breaks rapidly (Kultz and Chakravarty, 2001), (Dmitrieva et al., 2004), and those DNA breaks persist even after the cells become adapted to the high NaCl added. The adapted cells proliferate rapidly in cell culture and function adequately in *vivo* despite these DNA breaks (Dmitrieva et al., 2004). Both in cell culture and *in vivo* the breaks are

rapidly repaired if the NaCl concentration is lowered. Also acute hyperosmolality causes transient cell cycle arrest in all the phases of the cell cycle (Michea et al., 2000), promoting a G0/G1 and G2/M delays and a prolongation of the S phase of the cell cycle, all of them mediated by p38 kinase (Dmitrieva et al., 2002), (Goloudina et al., 2003).

The DNA breaks caused by osmostress cannot be repaired because high NaCl impairs activation of several components of the classical DNA damage response such as Mre11, H2AX and Chk1 leading to the inhibition of DNA repair (Dmitrieva and Burg, 2005). Nevertheless, some other regular participants in the DNA damage response, such as Gadd45 (Mak and Kultz, 2004), (Kultz et al., 1998), Gadd153 (Kultz et al., 1998), p53 (Dmitrieva et al., 2000), ATM (Sheen et al., 2006), Nbs1 (Sheen et al., 2006) and Chk2 (Sheen et al., 2006) are still upregulated or activated by high NaCl. Others, however, described that in Hela cells, as well as in mIMDC3 cells, DNA breaks caused by osmolalities between 500 - 600 mOsm/kg can be repaired (Sheen et al., 2006) thus leading to conflictive observations between these two different groups in this specific process.

The DNA damage response of renal inner medullary cells under hyperosmotic stress has been well studied. These cells are constantly exposed to a high osmolality as a consequence of their participation in the urinary concentrating mechanism. It is described that these cells exhibit many DNA breaks *in vivo* yet they do not mount noticeable DNA repair, nor do they suffer noticeable consequences (Dmitrieva et al., 2004), but these effects are mainly seen with osmolarities greater than 600 mOsm/kg, only measured *in vivo* in the inner medulla.

With regard to the cell cycle arrest in other stress scenarios such as DNA damage induced by a variety of agents including UV light, ionizing radiation

(IR), reduced DNA ligase activity, and camptothecin (CPT) the protein kinase Chk1 becomes rapidly phosphorylated after the induction of DNA breaks, initiating the transduction signaling that converges in cell cycle arrest (Capasso et al., 2002). In hyperosmolar stressed cells Chk1 is not phosphorylated and it is not in the nucleus, indicating that the signal that triggers the cell cycle arrest induced upon hyperosmotic stress conditions is different from that observed in others genotoxic scenarios (Dmitrieva et al., 2004). Consequently, the Burg's group proposed that the cell cycle arrest observed under acute elevation of NaCl has some additional functions than fixing the damage and providing a pause in DNA replication to repair the DNA breaks. In contrast, other group disagreed with this interpretation as they have observed that the MRN complex is fully active upon exposure of cells to high NaCl and in a long time -of 8-20 hours- cells can adapt to this DNA damage (Sheen et al., 2006).

Data from our lab showed that in T cells hypertonicity triggered an early, NFAT5 independent, genotoxic stress-like response with induction of p53, p21 and GADD45, downregulation of cyclins, and cell cycle arrest followed by an NFAT5-dependent adaptive phase which induced an osmoprotective gene expression program, downregulated stress markers, resumed cyclin expression and proliferation, and displayed enhanced NFAT5 transcriptional activity in S and G2/M (Drews-Elger et al., 2009).

<u>Oxidative stress</u>:

Increase in osmolality to 600 mOsm/kg by urea or NaCl can also cause oxidative stress with increase in the ROS (reactive oxygen species) production in mIMCD3 (Zhang et al., 2004). Oxidative stress occurs when the generation of ROS overshoots the ability of a cellular system to neutralize and eliminate them. All free radicals derived from O_2 are called ROS -superoxide anion, hydrogen peroxide, hydroxyl, among others- and are

highly unstable molecules that can react with proteins, lipids and DNA causing the oxidation of these biomolecules, disturbing their normal functions and may contribute to a variety of disease states, such as neurological disorders like Alzheimer and Parkinson diseases (Jomova et al., 2010). Some perturbing effects that can cause oxidative stress, induced by hyperosmotic stress, on the cell are the formation of 8-oxoguanine (or hydroxyguanine) in the DNA, causing mutations (Greenberg, 2004) and the carbonylation of proteins affecting protein stability and function (Levine et al., 2000).

Also, oxidative stress has been proposed as important in the pathogenesis of hypertension, and hypertension has been related to high salt intake, indicating a direct relationship between osmotic stress, ROS and hypertension. In fact, numerous experimental studies have documented the induction or intensification of oxidative stress by NaCl (Kitiyakara et al., 2003), (Kushiro et al., 2005), (Zhou et al., 2005).

Cellular senescence:

Cells become senescent after serial passages *in vitro*, a mechanism called replicative senescence. The mechanism that leads to this type of senescence is mediated by telomere shortening produced in each replication cycle (Harley et al., 1990), (Allsopp et al., 1992). Stress also leads to senescence. Among these types of stress we can identify the DNA damage and oxidative stress, producing premature senescence in cells that have not reached their state of replicative senescence. Because the senescence is observed before the ordinary replicative limit is reached, the process has been termed “stress-induced premature senescence” (Toussaint et al., 2000). The cellular functional and morphological features that characterize the process of senescence, independently of its origin, are the unresponsiveness to

mitogenic stimulation, senescence-associated β-galactosidase (SA-β-Gal) activity, changes in chromatin and gene expression, enlargement and flatness of cells, weakness of cell to cell contact, telomere shortening and non-dividing state (Toussaint et al., 2000).

Given that high NaCl causes DNA damage and oxidative stress and that these conditions can induce senescence, it is not strange to think that high NaCl could mediate cellular senescence. For example, Hela cell cultures subjected to osmolalities of 400 to 500 mOsm/kg for 18 days have fewer cells and are smaller and more auto-fluorescent than those cultured at 300 mOsm/kg (Dmitrieva and Burg, 2007). Moreover, it has been shown that mouse embryonic fibroblasts (MEF) exposed to 500 mOsm/kg for 10 days have an increase in SA-β-Gal activity (Dmitrieva and Burg, 2007), all of them hallmarks of cellular senescence.

Cell growth inhibition:

The mammalian target of rapamycin (mTOR) pathway has been described as a central controller of cell growth and it was described as sensitive to a broad range of environmental stresses such as nutrient availability, amino acid insufficiency, hypoxia, DNA damage and osmotic stress (Reiling and Sabatini, 2006).

It has been shown that high NaCl or high sorbitol between the ranges of 600 to 900 mOsm/kg can inhibit the mTOR pathway (Kruppa and Clemens, 1984), (Saborio et al., 1974), (Parrott and Templeton, 1999), (Desai et al., 2002), (Patel et al., 2002), (Naegele and Morley, 2004), (Fumarola et al., 2005). This inhibition is transient and is completely reversed after returning cells to isotonic culture conditions. However, other authors have reported that mTOR can be activated transiently by intense osmostress (>600

mOsm/kg) (Bae et al., 2008), (Kwak et al., 2012). An extended description of these observations is provided in the section titled *mTOR pathway: signaling pathway involved in growth and proliferation.*

Inhibition of RNA synthesis and translation:

High osmolality leads to inhibition of RNA and protein synthesis. This inhibition is reversible upon returning of cells to isotonic conditions, or when cells have adapted to the conditions of hypertonic stress. The final outcome of the hypertonic stress response on RNA synthesis and translation depends on the intensity and duration of the stress.

It has been shown that Hela cells display a total suppression of heterogeneous nuclear RNA (hnRNA), transcribed by RNA-pol II, at 450 mOsm/kg after 20 minutes treatment, a total inhibition of the 45s RNA, transcribed by RNA-pol I, after exposure of 600 mOsm/kg for 20 minutes treatment and the total inhibition of synthesis of the tRNA, transcribed by RNA-pol III, after exposure to 840 mOsm/kg for 20 minutes treatment (Pederson and Robbins, 1970), indicating that hyperosmotic stress affects differentially all three RNA-polymerases in Hela cells at different specific intensities.

Protein translation and polyribosome disaggregation were shown to be reduced in Hela cells, only 10 minutes after the addition of NaCl up to 840 mOsm/kg (Robbins et al., 1970), (Saborio et al., 1974). Also, sorbitol-induced hyperosmotic stress (700 mOsm/kg) affects overall protein synthesis (Patel et al., 2002). Additionally, it is described that hypertonic stress selectively inhibits the initiation of peptide chain formation and not the elongation and termination steps of translation (Saborio et al., 1974).

It is important to keep in mind that although hypertonicity -at high osmolalities- inhibits general translation, the specific translation of some other proteins, such as enzymes for the synthesis of compatible osmolytes or heat-shock proteins is increased. Diverse stresses limit protein synthesis, mostly via inhibition of translation initiation. The exact mechanism by which high hypertonicity inhibits translation is not well understood, but several possibilities have been proposed in the literature. The Chen's and Hatzoglou's groups described that possibly the inhibition of eIF2alpha caused this selective translation inhibition observed in IRES containing mRNAs (Lu et al., 2001) (Bevilacqua et al., 2010). Others, have shown that leader length and secondary structure are determinants on the translation efficiency upon hyperosmotic stress, showing that mRNAs like that of the heat shock protein, which is induced upon hypertonic stress, have a long 5'UTR sequence, facilitating its translation (Kozak, 1988).

The best described function of the mTOR kinase is its regulation of translation, by directly phosphorylation of some key translation regulators such as the p70 ribosomal S6 kinase (p70S6K) and the eukaryotic initiation factor 4E binding protein 1 (4EBP1). It has been known since more than thirty years ago that high hypertonic stress induced by NaCl or sorbitol treatment in mouse myeloma cells, Hela cells or HEK293 cells reversibly decreases the phosphorylation of S6 (Kruppa and Clemens, 1984), (Saborio et al., 1974), (Naegele and Morley, 2004) a direct target of p70S6K1-, as well as the overall translational output of the cell (Saborio et al., 1974), (Kruppa and Clemens, 1984). Just in the last ten years the possible mechanisms have been described. There are several hypotheses for the decrease of translation at high osmolalities. One of them is explained by the disintegration of the mitochondrial proton gradient (Desai et al., 2002). Others postulate that hyperosmotic stress induces a phosphatase that dephosphorylates p70S6K1, then decreasing the overall translation of the cell (Parrott and Templeton,

1999). It must be noticed that the effects on translation are observed at high osmolalities, between 600 to 900 mOsm/kg, are detected at short time points and are completely reversible when the culture medium is restored to normal tonicities.

Mitochondrial depolarization:

High hyperosmotic stress greatly perturbs mitochondrial function in a few seconds. There are studies in different cell lines describing effects in mitochondria, but with varying results depending on the strength and time of the hyperosmotic stimuli. In kit 225 cells (T-cell derived from chronic lymphocytic leukemia), the decline in mitochondrial function in response to osmotic stress is time-dependent, dose-dependent, almost completely reversible and dependent on mTOR activity (Desai et al., 2002). In contrast, in Vero cells (cells derived from kidney epithelial cells of the African Green Monkey) as little as 30 mM NaCl (equivalent to 360 mOsm/kg) cause a transient and reversible reduction in the mitochondrial membrane potential just after 30 seconds of the addition of NaCl of which cells recover after 10 minutes (Copp et al., 2005). However, in mIMCD3 it is necessary to apply high doses of osmotic stress (as high as 700 mOsm/kg) that in Vero cells to induce mitochondrial depolarization (Michea et al., 2002).

In summary, osmotic stress can interfere with diverse cellular processes that underlie basic functions such as growth, genomic and protein stability, survival and aging. In this regard, the effects of osmotic stress can be similar to those observed in the context of other stressors, such as DNA damaging agents, nutrient deprivation, oxidative stress or hypoxia.

Finally, it is important to take into account that the cellular response to osmotic stress, and ultimately its ability to adapt and survive will depend on

the intensity and duration of the stress. Therefore, when interpreting the experimental results on the effect of osmostress, or any other form of stress, one has to know whether the particular stress conditions used are tolerable for the particular cell line used, allowing cellular adaptation and survival, or on the contrary, are too intense to cause cell death. As an example, results from the Burg laboratory show differences in p53 phosphorylation, transcriptional activity and apoptosis in mIMCD3 cells subjected to 500-600 mOsm/kg. or 700-800 mOsm/kg (Dmitrieva et al., 2000).

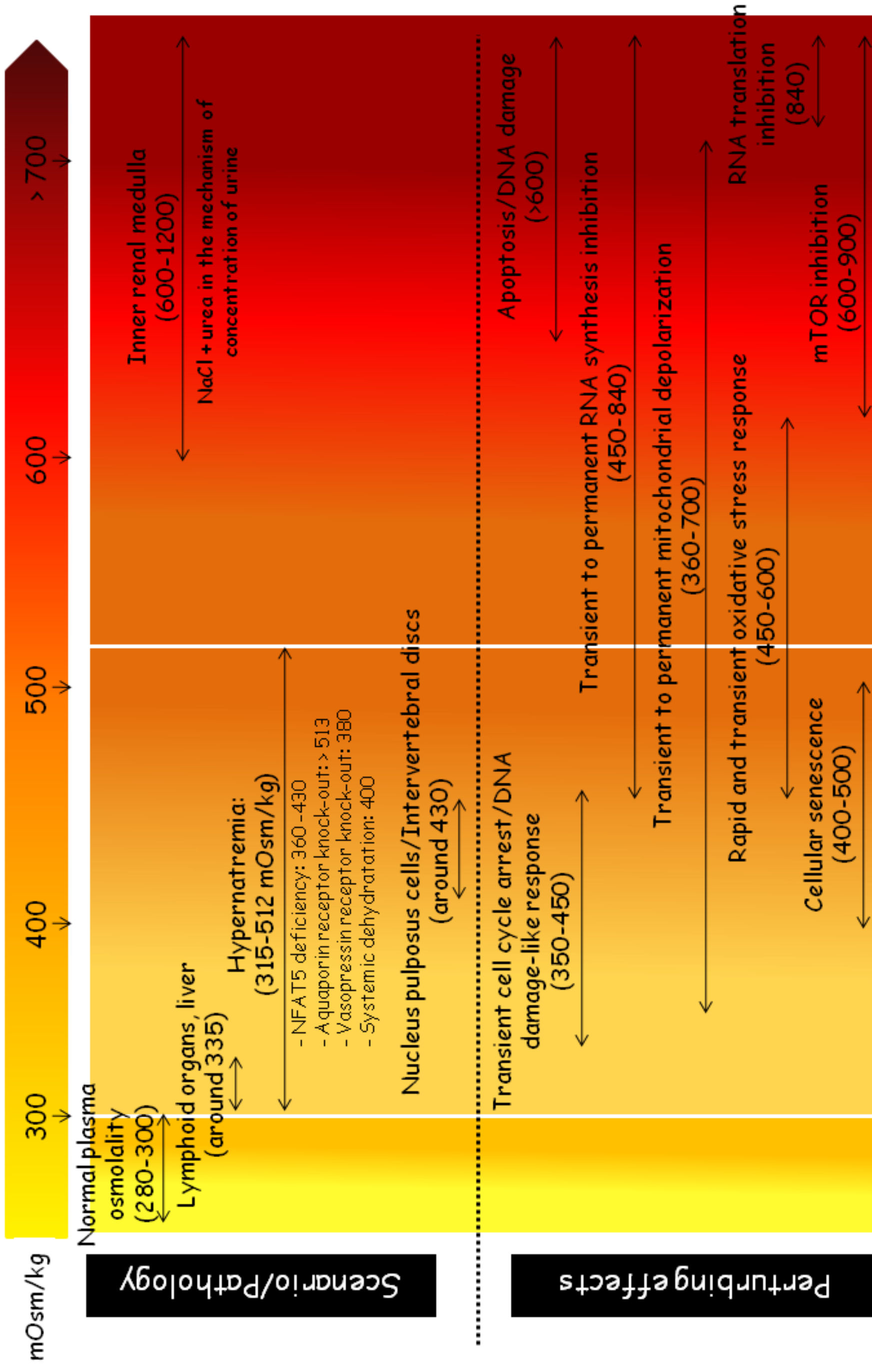


Figure I-4. Scenarios associated with different intensities of osmotic stress and its consequent perturbing effects.

3. THE TRANSCRIPTION FACTOR NFAT5

Specific cellular adaptation to diverse environmental stressors needs an active change in gene expression patterns. Cells have several transcription factors that are activated upon those stress conditions. Some of them are specifically involved in some specific stress responses like HIF-1 upon hypoxia (Semenza, 2012), Nrf2 upon oxidative stress (Florczyk et al., 2010) or NFAT5 upon osmotic stress (Aramburu et al., 2006). Others are activated in response to a broad range of stressors like p53, ATF or c-Jun. In this section we will describe in detail NFAT5, a key transcription factor necessary for cellular adaptation to osmotic stress.

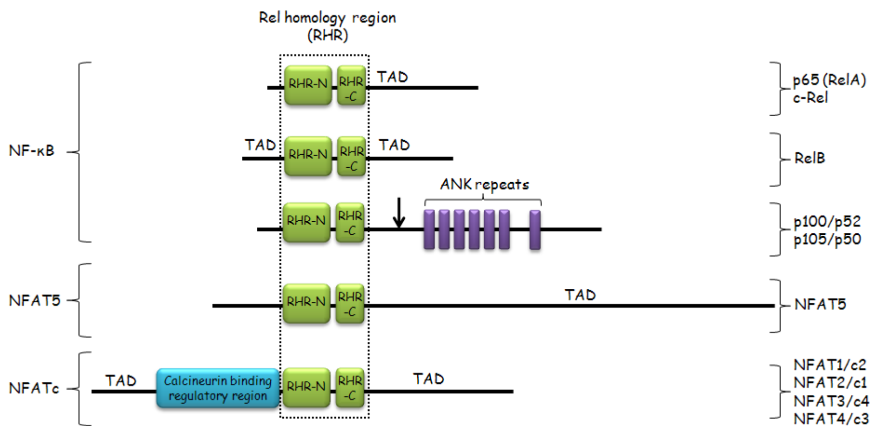
NFAT5 (Nuclear factor of activated T cells 5)

"NFAT5 belongs to the Rel family of transcription factors, comprising the NF-κB and the calcineurin-dependent NFAT proteins (NFATc) sharing with them the Rel homology domain. NFAT5 is regulated by hyperosmotic stress and contributes to the cellular response of adaptation to this stress"

3.1 General characteristics:

NFAT5 was cloned in 1999 by two different groups (Miyakawa et al., 1999), (Lopez-Rodriguez et al., 1999b) and received two different names: TonEBP -for tonicity enhancer binding protein- (Miyakawa et al., 1999) and NFAT5 -for nuclear factor of activated T cells 5- (Lopez-Rodriguez et al., 1999b). NFAT5 is also known as OREBP -for osmotic responsive element binding protein- (Ko et al., 2000) or NFATL1 (Trama et al., 2000).

NFAT5 is the largest member of the Rel family of transcription factors in vertebrates, with almost 1500 amino acids and a long carboxy (C)-terminal transactivation domain of more than 900 amino acids. NFAT5 shares the DNA binding domain, called the Rel homology region, which is about 200 amino acids long, with the other members of this family, the NF-κB and the NFATc proteins (**Figure I-5**). However, NFAT5 differs from them by having exclusive features. NFAT5 has two long glutamine regions in the C-terminal region, so far with unknown function, and is the only member of the Rel family that is strongly activated upon hypertonic stimulation.



Adapted from Aramburu et al. (2006)

Figure I-5. Schematic diagram of the Rel family of transcription factors. All the members of the Rel family share the Rel homology region (RHR). The RHR-amino (N) region comprises the DNA binding loop and the RHR-C region contains the dimerization domain. The NF-κB family comprises five members p65 (RelA), c-Rel, RelB, p100/p52 and p105/p50. RelA and c-Rel have short N-terminal regions and their transactivation domain (TAD) is located in the C-terminal region. Rel B has two different TADs located at N- and C-terminal regions. p100/p52 and p105/p50 contain ankyrin repeats in its C-terminal region; their active forms are produced by proteolytic cleavage (indicated by an arrow). NFAT5 has a large C-terminal region where is TAD is located. NFATc proteins (NFAT1-4)

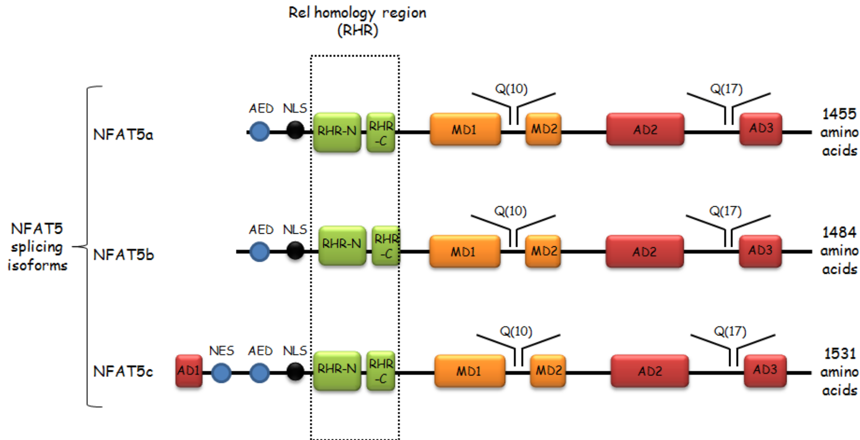
contain a calcineurin binding regulatory region in their N-terminal region and a TAD in their N- and C-terminal regions.

The mouse *Nfat5* gene encodes for three main NFAT5 isoforms: NFAT5a, b and c, which have identical amino acid composition except in their amino (N)-terminal region. The shortest isoform is NFAT5a with 1455 amino acids and is broadly expressed. The NFAT5b and c isoforms contain 29 and 76 extra amino acids respectively before the first methionine of the NFAT5a isoform (Lopez-Rodriguez et al., 1999a).

The amino acid sequence and gene structure of NFAT5 are highly homologous between humans and mice (Maouyo et al., 2002). NFAT5 contains three major functional regions: a Rel-like DNA-binding domain (RHR or DBD) flanked by a short amino-terminal region (N-terminal region) and a large carboxy-terminal domain (C-terminal region) (Lopez-Rodriguez et al., 2001). The N-terminal region contains several sequences that regulate its cytoplasmic-nuclear translocation (Tong et al., 2006), (Estrada-Gelonch et al., 2009) and a transactivation domain (TAD) in the long NFAT5c isoform. The C-terminal region includes its different TADs besides other modulatory domains (Lee et al., 2003), (Jeon et al., 2006) (**Figure I-6**).

The NF-κB proteins dimerize through their RHR-C domain, bind DNA as dimers and can combine between them to form different types of homo and heterodimers (Sengchanthalangsy et al., 1999) acquiring a similar "butterfly" conformation. Like NF-κB proteins, but unlike the calcium-regulated NFAT proteins, NFAT5 is constitutively dimeric, and dimerization is essential for DNA binding and transcriptional activity (Lopez-Rodriguez et al., 1999b) (**Figure I-7**). Although NFAT5 dimerizes through the same region as NF-κB proteins, the sequence homology of the RHR between both is minimal

and they only share 20% homology. In addition, NFAT5 binds DNA by encirclement (Stroud et al., 2002).



Adapted from Lee et al. (2003), Jeon et al. (2006), Tong et al. (2006), Xu et al. (2008) and Kwon et al. (2008)

Figure I-6. Schematic diagram of the 3 described NFAT5 isoforms. Schematic structure of NFAT5a (1455 amino acids), NFAT5b (1484 amino acids) and NFAT5c (1531 amino acids). The Rel-like DNA-binding domain (or RHR) and the C-terminal region (with two modulatory domains: MD1 and MD2; two activation domains: AD2 and AD3 and two stretches of glutamines Q10 –ten glutamines in tandem- and Q17 –seventeen glutamines in tandem-) are equal for all of them. The N-terminal region differs between them: its nuclear translocation in response to hypertonicity is mediated by a nuclear localization sequence (NLS) common to the three NFAT5 isoforms, whereas its export in the absence of hypertonic stress is regulated by two distinct motifs, a CRM1-dependent nuclear export signal (NES) found only in the long isoform NFAT5c, and a CRM1-independent, CK1-regulated, auxiliary export domain (AED) found in the three isoforms.

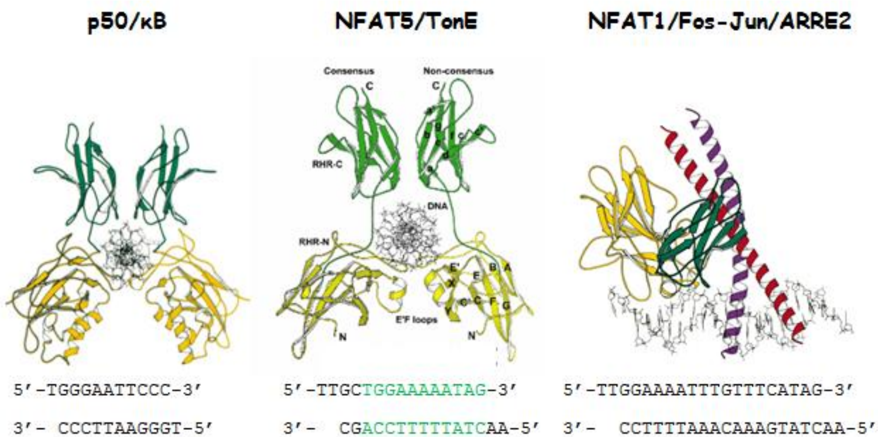
In contrast, NFAT5 binds DNA sequences similar to those recognized by NFATc proteins (NFATc1-NFATc4). Thus NFAT5 is capable of binding a putative consensus sequence like TGGAAANN(C/T)N(C/T) -where N

represents any nucleotide-. This sequence also comprises the NFATc binding core GGAA (**Figure I-7**), but differs from the palindromic elements bound by NF-κB proteins (GGGRNNYYCC) (Lopez-Rodriguez et al., 1999b). The four calcineurin-regulated transcription factors of the NFATc family are monomeric in solution and act synergistically with AP-1 (Fos and Jun) proteins (Chen et al., 1998) on composite DNA elements which contain adjacent NFAT and AP-1 binding sites, where they form highly stable ternary complexes to regulate the expression of diverse inducible genes. The NFAT component is activated by Ca^{2+}/calcineurin signaling, while the AP-1 heterodimer is activated by PKC/MAP kinase signals (Hogan et al., 2003). Although NFAT5 has a Rel homology domain similar to that of other members of the NFATc family, with a mean 43% homology (Lopez-Rodriguez et al., 1999b), it does not have calcineurin-docking sites in its N-terminal region nor interacts with AP-1. Therefore, it is not activated by the same regulatory pathways as NFATc1-4 (Lopez-Rodriguez et al., 1999b). Moreover, the NFATc proteins as well as NFAT5 protein can be found in nearly all tissues (Lopez-Rodriguez et al., 1999b).

Evolutionarily, NFAT5 is the most ancient member of the NFAT family. The single NFAT-like protein encoded in the *Drosophila* genome, called dNFAT, has a DBD closely related to that of NFAT5, suggesting that NFAT5 was the first to diverge from Rel family proteins early in evolution (Adams et al., 2000). Also, it is interesting to note that, like NFAT5 in mammals, also the *Drosophila* dNFAT is involved in salt stress tolerance (Keyser et al., 2007). Genome databases show that the Rel-like transcription factors -dNFAT and NFAT5- are only present in *Drosophila* and mammals but not in *C.elegans*, yeast and plants (Riechmann et al., 2000).

The mRNA of NFAT5 is expressed in a broad range of human and mouse tissues in the adult body: kidney, heart, brain, lung, liver, skeletal muscle,

pancreas, spleen, thymus, testis, ovary, small intestine and colon (Lopez-Rodriguez et al., 1999b), (Miyakawa et al., 1999), (Trama et al., 2000). A great number of these tissues are bathed in isotonic milieu suggesting a possible function of NFAT5 outside hypertonic stress scenarios. In fact, NFAT5 plays important roles in processes unrelated to osmotic stress. These include embryonic development (Maouyo et al., 2002), integrin-mediated carcinoma invasion (Jauliac et al., 2002), binding to LTR of retrovirus in hepatocytes (Yamaguchi et al., 2003), proliferation (Go et al., 2004), HIV replication (Ranjbar et al., 2006), muscle differentiation (OConnor et al., 2007), doxorubycin-induced toxicity in cardiomyocites (Ito et al., 2007) and TLR-induced macrophage innate immune responses (Buxade et al., 2012).



Adapted from Stroud et al. (2002) and Hogan et al. (2003)

Figure I-7. Crystal structure of the DNA binding domains of p50/κB site, NFAT5/TonE site and NFAT1/AP-1/ARRE2 site on the IL-2 gene. The proteins are shown in ribbon style, with the RHR-N domain in green and the RHR-C domain in yellow, and the DNA is drawn in stick model. The DNA sequence that bound each dimer is below each structure represented.

3.2 NFAT5-dependent gene expression under osmostress:

In mammalian cells, upon exposure to hyperosmotic conditions, NFAT5 activates an osmoprotective gene program to compensate the stressful environmental conditions achieved. Cells can survive in a hypertonic environment mainly by increasing the transcription of genes whose products ensure the cellular accumulation of compatible osmolytes. In this regard, NFAT5 plays a key role in stimulating the transcription of several genes that encode for transporters of organic osmolytes like the sodium/chloride/betaine cotransporter (*Bgt1*), the sodium myo-inositol cotransporter (*Smit1*), aquaporins (*Aqp1* and *Aqp2*), the vasopressin-regulated urea transporter (*UT-A1/A3*); and the amino acids transporters or their precursors like sodium-coupled neutral amino acid transporter 2 (*ATA2*) and taurine transporter (*TauT*). Moreover, NFAT5 stimulates the transcription of others genes encoding enzymes like aldose reductase (*Akr1b3*) enzyme or heat shock proteins like *Hspa70.1* and *Hspa4l*. Some cytokines and chemokines are also NFAT5-induced like lymphotoxin β (*Ltb*), tumor necrosis factor-α (*Tnf*) and monocyte chemoattractant protein-1 (*Ccl2*) **(Table I-2)**.

Gene (mouse nomen-clature)	Cell line /Tissue	Osmolality	References
Bgt1 (Slc6a12)	MDCK cells	Not described	(Miyakawa et al., 1998)
	p2mIME cells	640 to 1,640 mOsm/kg	(Cai et al., 2004)
	Kidney	Natural kidney osmolality	(Lopez-Rodriguez et al., 2004)
	MDCK cells	500 mOsm/kg	(Woo et al., 2002)
Smit1 (Slc5a3)	Hela cells	500 mOsm/kg	(Na et al., 2003)
	Kidney	Natural kidney osmolality	(Lopez-Rodriguez et al., 2004)

	MDCK cells	500 mOsm/kg	(Woo et al., 2002)
Aqp1	mIMCD3	500 mOsm/kg	(Lanaspa et al., 2010)
Aqp2	Renal epithelial cells	Not described	(Lam et al., 2004)
	Kidney	Natural kidney osmolality	(Lopez-Rodriguez et al., 2004)
	MDCK cells	450 mOsm/kg	(Kasono et al., 2005)
UT-A1/A3 (Slc14a2)	Renal epithelial cells	Not described	(Lam et al., 2004)
	MDCK cells	600 mOsm/kg	(Nakayama et al., 2000)
ATA2 (Slc38a2)	T cell blasts	400-500 mOsm/kg	(Trama et al., 2002)
	Forebrain	400-426 mOsm/kg	(Maallem et al., 2008)
TauT (Slc6a6)	Kidney	Natural kidney osmolality	(Lopez-Rodriguez et al., 2004)
	HepG2 cells	632-732 mOsm/kg	(Ito et al., 2004)
	Nucleus pulposus cells	330 mOsm/kg.	(Tsai et al., 2006)
Akr1b3 (AR)	MEF	500 mOsm/kg	(Lopez-Rodriguez et al., 2004)
	Hela cells	500 mOsm/kg	(Na et al., 2003)
	Kidney	Natural kidney osmolality	(Lopez-Rodriguez et al., 2004)
	MDCK cells	500 mOsm/kg	(Woo et al., 2002)
Hspa1b (Hsp70.1)	Hela cells	500 mOsm/kg	(Na et al., 2003)
	mIMCD cells	500 mOsm/kg	(Woo et al., 2002)
	Nucleus pulposus cells	450 mOsm/kg	(Tsai et al., 2006)
Hspa4l (Osp94)	p2mIME cells	640 to 1,640 mOsm/kg	(Cai et al., 2004)
Ltb	Jurkat cells	340-650 mOsm/kg	(Lopez-Rodriguez et al., 2001)
Tnf	Jurkat cells	340-650 mOsm/kg	(Lopez-Rodriguez et al., 2001)
Ccl2 (MCP-1)	NRK-52E	500 mOsm/kg	(Kojima et al., 2010)
Kcnj1	Kidney medullary thick ascending limb cells	450 mOsm/kg	(Gallazzini et al., 2006)

Table I-2. Hypertonically NFAT5-induced genes described in the literature.

3.3 NFAT5-deficient mouse models:

Different mouse models have been used to study NFAT5 function. Between them there are two of them are total knockout (KO) mouse models. The first total NFAT5 KO mouse was generated by López-Rodriguez and coworkers by deletion of the sixth exon on NFAT5, causing a premature stop codon that leads to a lack of the protein product. These NFAT5-null mice showed embryonic and perinatal lethality and the small proportion of them that survived to adulthood failed to thrive. These mice have half the weight of wild-type littermates, show kidney abnormalities and cellular apoptosis in their renal medulla (Lopez-Rodriguez et al., 2004) and are hypernatremic as well as immunodeficient (Berga-Bolaños et al., 2010). The other total KO mouse model was generated by Go and coworkers by deletion of NFAT5 sixth and seventh exons leading to a mutant protein containing an internal deletion of amino acid residues encoded by exons six and seven. This strategy produced a truncated NFAT5 polypeptide that was transcriptionally inactive in homozygosis, but in heterozigosis functioned as a dominant negative protein, capable of binding to the wild-type protein, thus inhibiting NFAT5 transcriptional function. These mutant mice in S129/SvJ background present late gestational or perinatal lethality when both alleles are deleted and the heterozygous mice suffered lymphoid hypocellularity and impaired antigen specific antibody response (Go et al., 2004). It is important to note that the heterozygous mutant mice described in Go's paper only have 25% of the total NFAT5 activity and this might cause a more severe phenotype than in the case of the heterozygous mice described by López-Rodriguez's work where heterozygous mice have 50% of NFAT5 activity in their tissues.

In addition, three different transgenic mouse models expressing dominant negative forms of NFAT5 in specific cells -drived by tissue-specific

promoters- have been described. The first of these models were mutant mice that expressed a dominant-negative form of NFAT5 in thymocytes and activated T cells driven by the CD2 promoter. These mice showed reduced cellularity in thymus and spleen, altered percentages of CD4 and CD8 cells only in spleen, and reduced T cell growth *ex vivo* under conditions of osmostress (Trama et al., 2002). The second described mouse model, was done using an NFAT5 dominant negative form controlled by the kidney-specific cadherin promoter that has been shown to direct the expression of heterologous genes specifically in the epithelial cells of the renal collecting tubules. These mice showed impairment in their urine concentrating mechanism, most likely due to reduced expression of the aquaporin Aqp2 and the urea transporter UT-A mRNAs, and developed progressive hydronephrosis soon after weaning (Lam et al., 2004). Another mouse model, generated by the same group as above, was created with the same NFAT5 dominant negative form under the control of the mouse α A-crystallin promoter, being expressed only in the fiber lens. These mice showed defects in lens fiber cells elongation and developed nuclear cataract due to an increase in DNA breaks and activation of the DNA damage-response pathway (Wang et al., 2005).

More recently, our group described one targeted conditional KO mice in T cells. These conditional KO mice are generated by intercrosses of mice that expressed the Cre-recombinase under the control of the CD4 promoter with mice that have the exon six of NFAT5 gene flanked with two *loxP* sites, recognized by this Cre-recombinase. These conditional KO mice showed a normal T cell phenotype *in vivo* and *in vitro*, but their T cells exhibited decreased viability, cell cycle arrest mainly in the G2/M phase and induction of a DNA damage-like response upon exposure to hypertonicity (Drews-Elger et al., 2009).

3.4 <u>NFAT5 regulation in response to hyperosmotic stress</u>:

The activation of NFAT5 under hyperosmotic stress conditions involves three main processes: nuclear translocation, upregulation of its transcriptional activity and an increase in its protein abundance. These three processes are coordinated to achieve the full activation of NFAT5 in response to hypertonicity, but the individual contribution of each individual process to the full activation of NFAT5 in different cell types is still not well studied.

Regarding the induction times of these processes, the nuclear translocation is initiated rapidly after the hyperosmotic insult and then the NFAT5 transcriptional activity and its protein synthesis are enhanced.

<u>Nuclear translocation</u>:

In isotonic conditions, NFAT5 can be found in the cytoplasm, in the nucleus or distributed in both the cytoplasm and the nucleus, depending on the cell type tested, whereas in response to hypertonic stress it accumulates in the nucleus, binds to regulatory regions in target genes, and activates their transcription. As an example, in isotonic conditions in the MDCK cell line, NFAT5 protein expression is observed in both compartments (Woo et al., 2000), (Dahl et al., 2001). In contrast, in neurons or in T cells is mainly located in the nucleus (Maallem et al., 2006), (Lopez-Rodriguez et al., 1999b) and in Hela cells is mostly found in the cytoplasm (Tong et al., 2006).

Three sequence motifs in the N-terminal region were found in NFAT5 protein regulating its nucleo-cytoplasmic distribution: a nuclear export sequence (NES), an accessory export domain (AED) and a nuclear

localization signal (NLS). The NES motif is only found in the long NFAT5c isoform, comprising the MPSDFISLLSADLDLESPK sequence responsible for the export of NFAT5 in isotonic conditions, mediated by the nuclear exportin Crm-1. The AED is found in all three described NFAT5 isoforms, is also involved in the nuclear export process in isotonic and hypotonic conditions and is independent of Crm-1. On the other hand, the NLS is also found in all three NFAT5 isoforms, is formed by a consensus bipartite sequence like RKSRKRNPKQRPGVKRRD, in which only the first basic triplet is important for regulating its nuclear translocation upon exposure of cells to hypertonicity (Tong et al., 2006).

Regarding kinases affecting NFAT5 nuclear translocation, it has been described that casein kinase-1 (CK-1) phosphorylates the NFAT5 residues Ser155 and Ser158 and mediates the nuclear exclusion of the NFAT5c isoform upon hypoosmostic stress. The phosphorylation of Ser-155 primes the later phosphorylation of Ser158, both observed in hypotonic conditions. Inhibiting CK-1 precluded phosphorylated Ser158 and consequently NFAT5 isoform c could not be exported from the nucleus to the cytoplasm (Xu et al., 2008). More recently, the Ferrari's group has shown that cyclin-dependent kinase 5 (Cdk5) phosphorylates the Thr135 on NFAT5c isoform and its mutation to alanine or inhibition of Cdk5 cause reduced nuclear accumulation of NFAT5 upon hyperosmotic stress conditions (Gallazzini et al., 2011). Besides kinases, it has been shown that dephosphorylation of Tyr143 of NFAT5 by SHP-1 phosphatase causes a lower nuclear accumulation of NFAT5 upon hyperosmotic stress conditions (Zhou et al., 2010).

Transcriptional activity:

NFAT5 has a large hypertonicity-responsive transactivation domain (TAD) in its C-terminal region and another short TAD, not activated by

hypertonicity, in the NFAT5c isoform located at the beginning of the N-terminal region (Lee et al., 2003). The C-terminal region of NFAT5 is enriched in Ser, Thr and Tyr residues that are candidate amino acids to be phosphorylated or dephosphorylated by several kinases or phosphatases in response to changes in tonicity. In this regard, there are many studies addressing the complex regulation of NFAT5 by multiple kinases and a few others have studied regulatory phosphatases. However, despite that several kinases and phosphatases have been shown to influence the activity of NFAT5, only few residues in this transcription factor has been conclusively identified as being able to regulate NFAT5 depending on their phosphorylation status. In this regard, it is largely unknown which of these kinases affecting NFAT5 activity do so by directly modifying NFAT5 phosphorylation or by acting on other targets, such as chromatin regulators, or modifying potential NFAT5 transcriptional partners.

Kinases involved in NFAT5 transcriptional activity

The literature about the activation of NFAT5 by several kinases suggests that NFAT5 activation by hypertonicity requires a combination of multiple kinases. The increasing list of kinases studied by their implication in the transcriptional activity of NFAT5 includes the followings: p38 (Nadkarni et al., 1999), (Ko et al., 2002), (Zhou et al., 2008), (Kojima et al., 2010), MEK1 (Nadkarni et al., 1999), Fyn (Ko et al., 2002), Src (Chen et al., 2011), PKA (Ferraris et al., 2002), ATM (Zhang et al., 2005), PI3K-IA (Irarrazabal et al., 2006), ERK (Gallazzini et al., 2006), (Tsai et al., 2007), c-Abl (Gallazzini et al., 2010) and Cdk5 (Gallazzini et al., 2011).

The involvement of the stress-activated kinase p38 in the transcriptional activity of NFAT5 has been assayed by the use of some kinase inhibitors like the p38 inhibitors SB203580 (Nadkarni et al., 1999) and SB202190 (Morancho et al., 2008), a dominant negative form of MKK3 (Kultz et al.,

1997), an upstream activator of p38, and a specific dominant negative form of p38 (Ko et al., 2002). These approaches have been tested in different cell types with contradictory results, suggesting that the involvement of p38 in NFAT5 transcriptional activity may be cell-dependent. The activity of an ORE-driven reporter gene –containing the enhancer of the aldose reductase gene that was activated by osmotic stress- and the mRNA expression of several NFAT5 target genes like aldose reductase (*Akr1b3/AR*), the betaine/GABA transporter-1 (*Bgt1/Slc6a12*) and the sodium myoinositol transporter (*Smit1/Slc5a3*) have been shown to be abrogated by SB203580 in HepG2 cells (Nadkarni et al., 1999). Also, this same inhibitor and a p38 dominant negative construct were shown to inhibit the activation of NFAT5 in response to hypertonicity in NIH3T3 cells (Ko et al., 2002), and other studies extended these results by using a p38 inhibitor, in nucleus pulposus cells (Tsai et al., 2007), mouse splenocytes and mouse embryonic fibroblasts (Morancho et al., 2008). In contrast, the Kültz laboratory showed that a p38 dominant negative construct did not inhibit an NFAT5-dependent reporter under hypertonic conditions in derived rabbit renal medullary PAP-HT25 cells (Kultz et al., 1997).

Adding further complexity to the regulation of NFAT5 by p38, it has been also described that two different isoforms of p38, the alpha and delta isoforms, both activated by hypertonic stress, having opposite effects on the high NaCl-induced NFAT5 activity, the former being activating and the latter inhibitory (Zhou et al., 2008), (Zhou et al., 2011). However, there are no papers that show specific phosphorylated residues on NFAT5 by p38 or even demonstrate a direct phosphorylation of NFAT5 by p38, raising the question of whether p38 regulates NFAT5 directly phosphorylating it or via an indirect mechanism. In this regard, the increase in NFAT5 phosphorylation induced by osmotic stress in MDCK cells was shown to be insensitive to p38 inhibition by SB203580 (Dahl et al., 2001).

MEK1 kinase also was suggested as a possible NFAT5 modulating kinase since the addition of PD098059, a MEK1 specific inhibitor, inhibited the hypertonic induction of aldose reductase mRNA, an ORE-driven reporter gene expression –containing the enhancer of the aldose reductase gene- and the binding of trans-acting factors to the ORE region in HepG2 cells (Nadkarni et al., 1999).

In another study, the Ko laboratory described that the tyrosine Fyn kinase, a member of the Src family of protein kinases, was also a regulator of the NFAT5 transcriptional activation upon hypertonicity. This study showed that NIH3T3 cells transfected with an ORE-luc reporter plasmid had a reduction in the transcriptional activity in hypertonic conditions upon the addition of PP2, a potent and specific inhibitor of the Src family of protein kinases to which Fyn belongs, or by a dominant negative Fyn construct. Moreover, Fyn KO fibroblasts displayed a smaller increase in the transcriptional activity of an ORE-luc reporter plasmid (Ko et al., 2002). More recent works have shown that NFAT5 expression is increased in a subset of cancer cell lines and that Src inhibition, during hypertonic stress conditions, reduces NFAT5 transactivation and also its protein levels (Chen et al., 2011). The involvement of the Fyn kinase in NFAT5 transcriptional activity is also controversial because others described that the phosphorylation status of NFAT5 in the MDCK cell line was not affected by the addition of PP2 upon hypertonicity (Dahl et al., 2001), indicating that Fyn requirement might be, like that of p38, cell type-dependent.

Another kinase that has been proposed to be modulated by hypertonicity and to mediate the transcriptional activation of NFAT5 is the c-Abl kinase (Gallazzini et al., 2010). By using overexpressed NFAT5 and c-Abl in HEK293 cells, it was shown that this kinase directly associated with NFAT5 and phosphorylated its Tyr143, enhancing its transcriptional activity and

nuclear localization. Moreover, silencing c-Abl by siRNAs or transfecting kinase dead constructs slightly reduced the transcriptional activity of an ORE-luc reporter. In other contexts ATM acts upstream of c-Abl whereas in the case of hypertonicity c-Abl is necessary for activation of ATM, not *vice versa* (Gallazzini et al., 2010).

cAMP-dependent protein kinase (also kwon as PKA) was also shown to enhance NFAT5-mediated transcriptional activity in HepG2 cells in response to hypertonicity, and to co-immunoprecipitate with it. However, PKA activation of NFAT5 does not appear to be cAMP dependent. This increase in NFAT5 activity is inhibited by H89, a relatively nonspecific inhibitor of PKA. Moreover, H89 reduces native NFAT5-driven reporter activity as well as tonicity-dependent induction of *Akr1b3* and *Bgt1* genes (Ferraris et al., 2002). These results, though, might not entirely be due to specific inhibition of PKA since H89 has been shown to be a relatively nonspecific inhibitor (Davies et al., 2000).

Several studies from the Burg, Ferraris and Irarrazabal group showed that ATM could be involved in NFAT5 transcriptional activation. The NFAT5 protein sequence contains ATM consensus phosphorylation sites at Ser1197, Ser1247, and Ser1367 (Irarrazabal et al., 2004). They showed that ATM was activated by hyperosmotic stimulation in HEK293 cells, followed by ATM Ser1981 autophosphorylation, and this activation correlated with an increase in the activity of an NFAT5-dependent reporter plasmid. Moreover, treatment of the same cells with wortmannin, in a dose that also could inhibit other PIKK besides ATM, reduced the activity of the same NFAT5-dependent reporter plasmid and also reduced the *Bgt1* mRNA levels (Irarrazabal et al., 2004). However, the inhibition with wortmannin in AT cells, that have an inactive ATM, also had similar effects indicating this effect was ATM-independent and could be mediated by other proteins of the

PIKK family. In this scenario, experiments of our group done in splenocytes and mouse embryonic fibroblasts showed that LY294002, at doses of 1 and 25 μM (that could inhibit all PIKK family members) inhibited the activation by osmotic stress of an NFAT5-dependent reporter (Morancho et al., 2008).

The phosphoinositide-3 kinase family PI3K can be divided into three main classes on the basis of their *in vitro* lipid substrate specificity, structure, and mode of regulation. PI3K-IA, which has both lipid and Ser-protein kinase activity, has been proposed by Ferraris and coworkers as an NFAT5 regulator. PI3K-IA phosphorylation and the phosphorylation of downstream substrates were upregulated upon the addition of NaCl to the medium. Reporter assays in Jurkat and HEK293 cells with a dominant negative form of the PI3K-IA regulatory subunit p85 and with siRNA against the p110 catalytic subunit, showed that both constructs caused a decrease in the hyperosmotic induction of the transcriptional activity of an NFAT5-dependent reporter in both cell lines. Furthermore they also showed that PI3K-IA activity was necessary for the NaCl-dependent activation of ATM (Irarrazabal et al., 2006).

The first paper that described the involvement of ERK in NFAT5 transcriptional activity used nucleus pulposus cells and HeLa cells (Tsai et al., 2007). They showed that NFAT5 was partially inhibited by a dominant negative construct and the pharmacological ERK inhibitor PD098059. Later, two different groups also used PD98059 in kidney medullary thick ascending limb cells (Gallazzini et al., 2006) and in *NRK-52E* rat renal proximal tubular cells (Kojima et al., 2010) and observed the abrogation of the NaCl-induced mRNA and protein stimulation of *Kcnj1* and *Ccl2*, both NFAT5 regulated genes. In primary mouse T cells, however, NFAT5 activity was insensitive to this same inhibitor (Morancho et al., 2008).

Other recently identified kinase that modulates, in a narrow time range, NFAT5 transcriptional activity is Cdk5. Cdk5 is involved in the initial increase of the transcriptional activity of NFAT5 because their requirement was only seen at 4 hours but not at 16 hours after addition of the hyperosmotic insult (Gallazzini et al., 2011).

The latest complex, so far, that affected the transcriptional activity of NFAT5 was Rac1/OSM. Ferrari's group showed that knocking-down Rac1 or OSM reduces high NaCl-induced increase of NFAT5 transcriptional activity (by reducing its transactivating activity but not its nuclear localization), independently of p38 (Zhou et al., 2011).

Phosphatases involved in NFAT5 transcriptional activity

As commented before, NFAT5 lacks the calcineurin regulatory binding region characteristic of the NFATc family proteins, being *a priori* not regulated by calcineurin. However, one study on NFAT5 regulation reported that TCR-dependent activation of NFAT5 was dependent on calcineurin in Jurkat T cells, while independent of NFATc proteins (Trama et al., 2000). In contrast, hypertonic activation of NFAT5 in Jurkat T cells was shown to be independent of calcineurin in an independent study (Morancho et al., 2008). Hypertonic activation of NFAT5 was variably inhibited by the calcineurin inhibitor FK506 in mouse T cells and MEF, but not in macrophages or Jurkat cells (Morancho et al., 2008). Moreover, experiments done in $mpkCCD_{c14}$ cells (immortalized murine collecting duct principal cells) showed dependence on calcineurin to activate NFAT5-dependent Aqp2 expression (Li et al., 2007). However, nucleus pulposus cells treated with cyclosporine and FK506, that antagonized calcineurin activity, failed to change the promoter activity of the NFAT5 target gene *TauT* (Gajghate et al., 2009). However, ionomycin addition to calcineurin deficient cells, that raise only the intracellular calcium levels, enhanced the NFAT5

transcriptional activity. Altogether, these results suggest that the contribution of calcineurin to the hypertonicity-mediated activation of NFAT5 might be cell type-specific and possibly gene-specific.

Regarding NFAT5 regulation by phosphatases, the last identified in a genome wide RNA interference phosphatase screening was SHP-1. High NaCl, inhibits SHP-1 phosphatase activity towards Tyr143 on NFAT5 enhancing its transcriptional activation and nuclear localization (Zhou et al., 2010).

Phospholipase C-gamma1 (PLC-γ) is also involved in regulating NFAT5 transcriptional activation. In PLC-γ null MEFs cells or in HEK293 cells in which PLC-γ was knocked down by siRNAs the transcriptional activity induced by high NaCl was reduced. The authors proposed that high NaCl causes phosphorylation of NFAT5 in the Tyr143 in the cytoplasm causing the PLC-γ binding to NFAT5-Tyr143 through its SH2-C domain. Then, NFAT5-phospho-Tyr143 together with PLC-γ accumulates in the nucleus increasing its transcriptional activity towards its regulated genes (Irarrazabal et al., 2010). The same group, in a more recent paper, concluded that Rac1 mediates the increase on the transcriptional activity of NFAT5 via PLC-γ1 (Zhou et al., 2011).

<u>Increased synthesis</u>:

Hypertonicity causes a substantial increase in NFAT5 protein levels (Miyakawa et al., 1999), (Lopez-Rodriguez et al., 1999b), but not as much in its mRNA levels, suggesting that enhanced protein synthesis is not only due to increased transcription. Little is known about the mechanism that regulates this increase in protein levels, but at least one report showed that was mediated in part by the stabilization of preexisting mRNA mediated by the 5'UTR in mIMCD3 cells (Cai et al., 2005). Also, in an *in silico* study has

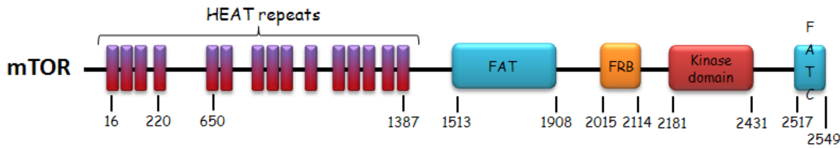
been found that the long 3'UTR of NFAT5 is a putative target for miRNAs, suggesting a negative repressive function upon hyperosmotic stress conditions on NFAT5 mRNA stability (Asirvatham et al., 2008). So far, only one miRNA has been identified as capable of regulate NFAT5 expression. In melanoma cells growing in an isotonic culture, NFAT5 mRNA levels were downregulated by overexpression of miRNA-211 (Levy et al., 2010).

4. mTOR PATHWAY: SIGNALING PATHWAY INVOLVED IN GROWTH AND PROLIFERATION

mTOR

"The mammalian (or mechanistic) target of rapamycin (mTOR) is an evolutionary conserved serine-threonine kinase that is known to sense the environmental, nutritional and energetic status of the cell"

mTOR is a 289 KDa Ser/Thr kinase belonging to the family of PI3-kinase-related kinases (PIKK) class IV along with ATM, ATR, DNA-PK, and SMG1. It is a central regulator of cell growth and proliferation due to its ability to activate the biosynthesis of proteins, nucleic acids and lipids in response to growth-promoting signals (Fingar and Blenis, 2004). mTOR protein structure comprises several HEAT repeats (for domains found in these different proteins: Huntingtin, elongation factor 3 (EF3), protein phosphatase 2A (PP2A), and the yeast PI3-kinase TOR1) in its N-terminal half that seem to mediate the interaction with other proteins, a FAT domain (FRAP-ATM-TTRAP), a FRB domain (FKBP12-Rapamycin Binding domain), a kinase domain and another FAT domain in its C-terminal end called FATC (**Figure I-8**). The pool of mTOR molecules is distributed into two main protein complexes, called complex 1 (mTORC1) and complex 2 (mTORC2), composed by different accessory proteins and mediating different cellular functions.



Adapted from Laplante and Sabatini, 2009

Figure I-8. Schematic diagram of mTOR protein structure. mTOR protein shows different domains in its structure: several HEAT domains in its N-terminal half (repeats of ~47 residues that form two anti-parallel α-helices and two turns arranged about a common axis. HEAT motifs have conserved Asp and Arg residues at positions 19 and 25, respectively), a FAT domain and a FATC domain (intramolecular interactions between FAT and FATC have been postulated to modulate kinase activity) (Gingras et al., 2001), a FRB domain (where complexes FKBP12/rapamycin and FKBP38 bind to inhibit kinase activity) and its kinase domain, that is subjected to different phosphorylation events.

4.1 mTORC1:

mTORC1 is composed by Raptor (Kim et al., 2002), mLST8 (also known as GβL) (Kim et al., 2003), PRAS40 (Vander Haar et al., 2007), (Wang et al., 2007), (Oshiro et al., 2007) and DEPTOR (Peterson et al., 2009). Raptor is a core component of the mTORC1 complex, and has different functions, such as regulating mTORC1 assembly and recruiting mTOR substrates, such as 4E-BP1 or S6K1, through its HEAT domains (Hara et al., 2002), (Kim et al., 2002). Regarding mLST8, despite that it is a core component of mTORC1, its exact role remains unclear because its deletion has not been found to affect mTORC1 activity *in vitro* or *in vivo* (Guertin et al., 2006). PRAS40 and DEPTOR are components as well as substrates of mTORC1, and in their dephosphorylated state, they appear to repress mTORC1 activity (Vander Haar et al., 2007), (Wang et al., 2007), (Oshiro et al., 2007), (Sancak et al., 2007), (Peterson et al., 2009). The mTORC1 complex controls the activity of

two main classes of regulators of protein translation, the ribosomal S6 subunit kinases 1 and 2 (S6K1 and S6K2), which are phosphorylated and activated by mTORC1 (Lee-Fruman et al., 1999), and the [eIF4E (eukaryotic Initiation Factor 4E)-Binding Proteins 1 and 2] (4E-BP1 and 2) that act as translational repressors, and are inactivated by mTORC1-mediated phosphorylation (Hara et al., 1997).

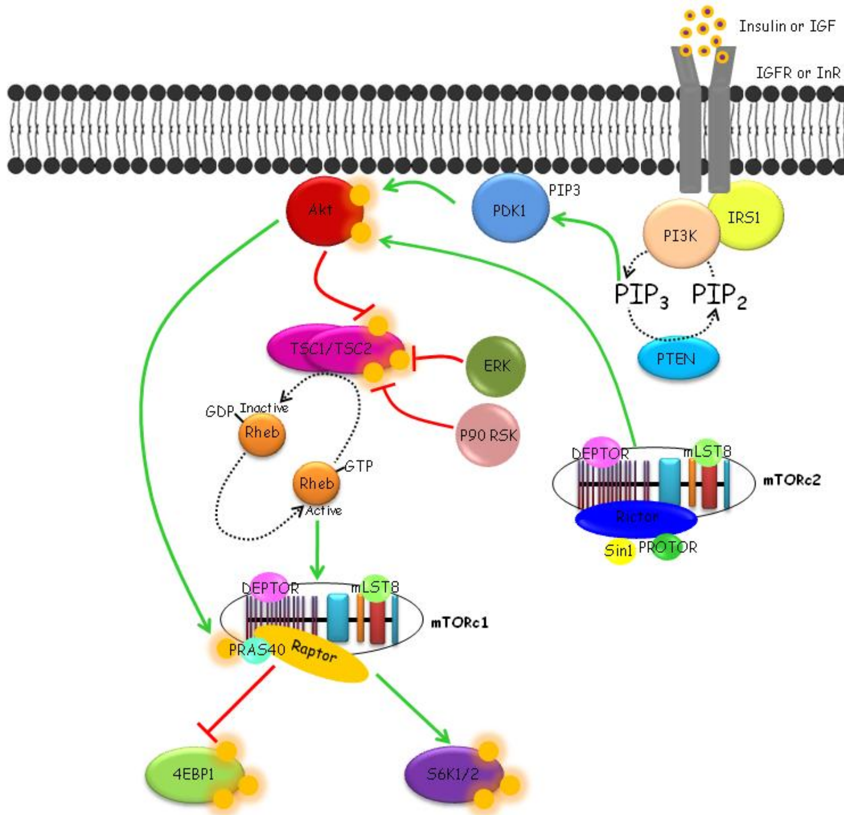
Upstream signals:

A variety of upstream signals regulate mTORC1 activity, including growth factors, amino acids, glucose, energy levels, oxygen levels and several stressors. Regulation of mTORC1 by most signals occurs primarily through two types of mechanisms: the direct modification of mTORC1 components or the regulation of Rheb, a small GTP-binding protein, that when bound to GTP, directly interacts with and activates mTORC1 (Garami et al., 2003), (Inoki et al., 2003).

Growth factors

Growth factor (insulin or IGF -insulin growth factor-) regulation of mTORC1 can be mediated by two different mechanisms depending on TSC2 (also called tuberin). When insulin or IGF bind to their receptors, PI3K kinase is activated and phosphorylates phosphatidylinositol (4,5)-bisphosphate (PIP2) to generate phosphatidylinositol (3,4,5)-trisphosphate (PIP3). PIP3 recruits Akt to the plasma membrane, where it becomes phosphorylated by PDK1 and mTORC2. In turn, activated Akt, ERK and p90 RSK phosphorylate TSC2 that together with TSC1 (also called hamartin) constitutes the TSC1/TSC2 complex (Inoki et al., 2002), (Manning et al., 2002), (Potter et al., 2002). This complex exerts its GAP (GTPase activating protein) activity towards the small G-protein Rheb (Ras homologue enriched in brain), acting as a negative regulator of mTORC1 activity. When TSC2 is phosphorylated by Akt, ERK and p90 RSK, its GAP activity is inhibited,

enhancing GTP loading of Rheb (Rheb-GTP) and allowing mTORC1 activity. However, when TSC2 is not phosphorylated by its upstream regulators, Rheb is GDP-loaded and mTORC1 is inhibited (**Figure I-9)**.



Adapted from Sengupta et al. (2010)

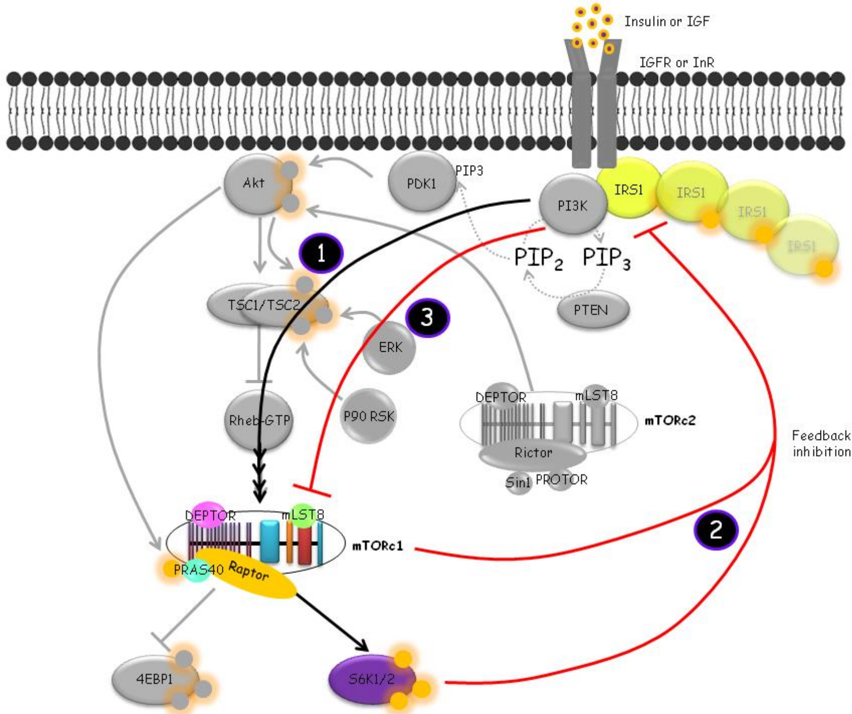
Figure I-9. Growth factor signaling to mTORC1. For description *see the main text.* Green lines indicate an activating interaction, and when accompanied with a phosphorylated mark it indicates that the activation is mediated through phosphorylation. Red lines indicate an inhibitory interaction and when accompanied with a phosphorylated mark it indicates that the inhibition is mediated through phosphorylation.

Akt can also activate mTORC1 activity independently of TSC2 as observed in TSC2-null cells. This involves the direct phosphorylation of PRAS40, an mTORC1 repressor, by active Akt, thus bypassing the TSC1/TSC2 complex and causing the activation of mTORC1 (Vander Haar et al., 2007), (Wang et al., 2007), (Oshiro et al., 2007) (**Figure I-9**).

Upon being activated by growth factors, mTORC1 modulates the extent of growth factor signaling via an 'auto-regulatory feedback loop' (**Figure I-10**). This feedback loop auto-modulates mTORC1 activity by itself when PI3K signaling is chronically active or when TSC2 is inactivated (or absent) inducing a high mTORC1 activity. When PI3K signaling is highly active S6K1 is capable to inhibit the activity and expression of IRS1 –by direct phosphorylation- interfering with its binding to the insulin receptor and promoting its protein degradation, as well as decreasing its mRNA levels by suppression of gene transcription dependent on S6K (Harrington et al., 2004), (Shah and Hunter, 2006), (Tremblay et al., 2007). Additionally mTORC1 can also interact with IRS1 through Raptor and phosphorylate it at critical residues preventing its association with PI3K (Shah and Hunter, 2006), (Tzatsos and Kandror, 2006). Both events lead finally to the inhibition of mTORC1 signaling, creating an auto-regulatory feedback loop.

This 'auto-regulatory feedback loop' explains the low level of malignancy observed in TSC1- or TSC2-deficient patients displaying the hamartoma syndrome tuberous sclerosis. The disruption of *TSC1* or *TSC2* genes gives rise to cells and tumors with an mTORC1 hyperactivation (Goncharova et al., 2002), (Kwiatkowski, 2003) and mice models deficient for TSC proteins developed tumors that were highly sensitive to rapamycin and its analogs. However, the development of malignancy in these tumors was very infrequent (Kwiatkowski, 2003). This low growing potential and malignancy

was due to this PI3K inactivation caused by the chronic activation of mTORC1 caused by TSC1 or TSC2 deficiency.



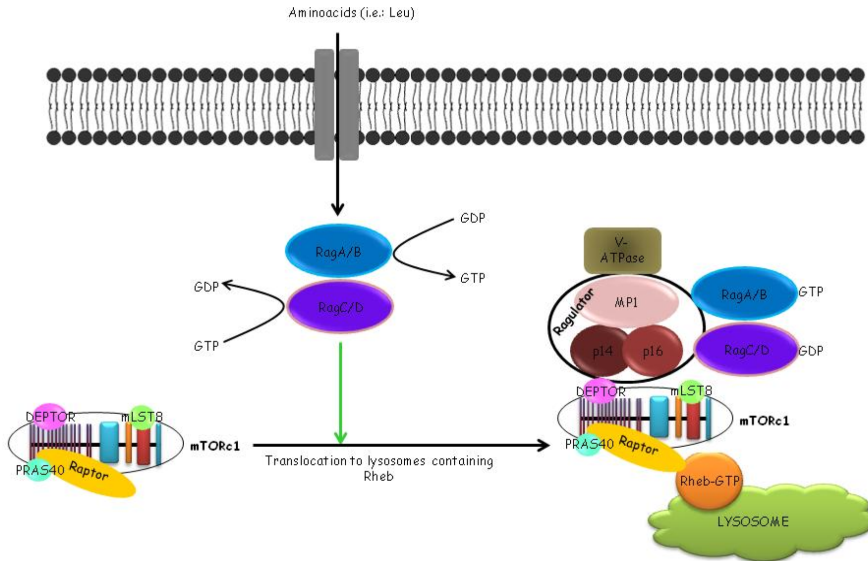
Adapted from Harrington et al. (2004), Shah et al. (2006), Tzatsos et al. (2006) and Tremblay et al. (2007)

Figure I-10. mTORC1 auto-regulatory feedback loop. For description *see the main text*. Numbers inside circles indicate the sequence for mTORC1 auto-regulatory feedback loop. Red lines indicate an inhibitory interaction and when accompanied with a phosphorylated mark it indicates that the inhibition is mediated through phosphorylation.

Amino acids

Low levels of amino acids inhibit mTORC1 signaling in a wide range of organisms from yeast to mammals. By contrast, a high intracellular amino acid content causes mTORC1 to be recruited to lysosomal membranes

where it can interact with a pool of Rheb that resides at this compartment leading to mTORC1 activation (Sancak et al., 2008), (Sancak et al., 2010) (**Figure I-11)**.



Adapted from Sancak et al. (2008 and 2010) and Zoncu et al. (2011)

Figure I-11. Amino acid signaling to mTORC1. *For description see the main text.* The green line indicates an activating interaction.

Lysosomal membranes contain a multimeric complex termed Ragulator consisting of MP1, p14, and p18 (Sancak et al., 2010). This complex acts as a scaffold for a heterodimer of Rag GTPases of which there are four (RagA, RagB, RagC, and RagD). The loading of the Rags with GTP or GDP appears to be regulated by amino acids. In cells deprived of amino acids, RagA/B is bound to GDP and RagC/D to GTP. Stimulation of cells with amino acids leads to a flipping, via an unknown mechanism, of the bound nucleotide so that RagA/B and RagC/D become GTP and GDP-bound, respectively. This is the active state of the Rag heterodimer, which, through its interaction with

Raptor, serves as a docking site for mTORC1 on the lysosomal surface (Sancak et al., 2008), thus, activating mTORC1 independently of TSC2 status (Smith et al., 2005) **(Figure I-11)**.

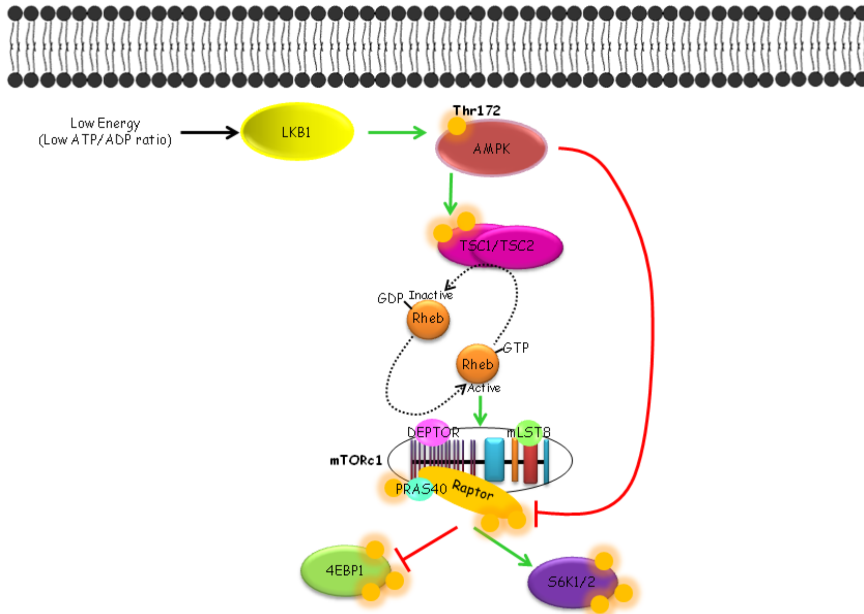
In a more recent paper, it has been postulated that the vacuolar H^+-adenosine triphosphatase ATPase (v-ATPase) is necessary for amino acids to activate mTORC1, engaging the interactions between Ragulator complex and Rag GTPases to the lysosome (Zoncu et al., 2011) (**Figure I-11**).

Energy

Glucose consumption by the cell is necessary to generate sufficient energy, in the form of ATP, for the correct function of almost all the cellular processes. When cells are energetically deprived, i.e by glucose deprivation, the AMP/ATP ratio builds up, turning on the master energy sensor and signal transducer, the AMP-activated protein kinase (AMPK). Binding of AMP to AMPK generates a conformational switch that elicits the phosphorylation in Thr172 by its upstream kinase, LKB1, leading to fully active AMPK (Shaw et al., 2004). This AMPK activation permits to slow down cell growth and switch off those energy consuming processes (biosynthetic or anabolic metabolism) while fueling those ATP-producing processes (catabolic metabolism) like autophagy, mitochondrial biogenesis or glucose uptake (Hardie, 2004).

The main mode of action by which AMPK inhibits cell growth under energy deprivation is via mTORC1 inhibition. AMPK, in fact, can directly phosphorylate TSC2 in two activating serine residues, Ser1227 and Ser1345, (Inoki et al., 2003), (Corradetti et al., 2004) as well as phosphorylate Raptor at Ser722 and Ser792 inhibiting mTORC1 function (Gwinn et al., 2008). These two independent mechanistic events, occurring after AMPK activation, lead to mTORC1 inhibition under a broad range of energy

depleting processes such as glucose deprivation, mitochondrial dysfunction, exercise or hypoxia, among others (**Figure I-12**).



Adapted from Shaw et al. (2009)

Figure I-12. Energy signaling to mTORC1. *For description see the main text.* Green lines indicate an activating interaction and when accompanied with a phosphorylated mark they indicate that the activation is mediated through phosphorylation. Red lines indicate an inhibitory interaction and when accompanied with a phosphorylated mark they indicate that the inhibition is mediated through phosphorylation

Stresses

The mTORC1 pathway is sensitive to a variety of stresses like DNA damage, heat shock, oxidative stress, UV radiation, mechanical stress, hypoxia and osmotic stress (Reiling and Sabatini, 2006). DNA damage and hypoxia, the two most studied stresses affecting mTORC1 signaling, beside osmotic stress

are described more in detail in the section *"Stress and mTORC1 regulation: DNA damage, hypoxia and osmotic stress"*.

Downstream effector functions and substrates:

Initially, mTORC1 was mainly regarded as a regulator of protein synthesis -by acting upon cap-dependent mRNA translation, translation elongation, mRNA biogenesis and ribosome biogenesis- (Ma and Blenis, 2009), but at present it has been shown to play wider functions in different cellular processes that contribute to enhance cell growth, proliferation and maintaining cellular homeostasis. In addition to mRNA translation, mTORC1 is involved in autophagy, cell cycle progression, apoptosis inhibition, cellular metabolism and mRNA transcription.

In the process of protein synthesis there are two main mTORC1 direct downstream targets: 4E-BP1 and p70S6K1 proteins, both regulating several aspects of mRNA translation in a phosphorylation-dependent fashion. 4E-BP1 when hypophosphorylated suppresses mRNA translation; however, when hyperphosphorylated by mTORC1 at multiple Ser and Thr residues (Brunn et al., 1997), (Fadden et al., 1997) 4E-BP1 dissociates from the eukaryotic translation Initiation Factor 4E (eIF4E), allowing eIF4E to recruit the translation initiation factor eIF4G to the 5' end of most mRNAs and enhancing translation (Hara et al., 1997). It is known that mTORC1 directly phosphorylates 4E-BP1 at Thr36 and Thr45 (Brunn et al., 1997), (Fadden et al., 1997) having both a positive impact on mRNA translation **(Figure I-9)**. p70S6K1 is also phosphorylated by mTORC1 at Thr389 (Burnett et al., 1998). This phosphorylation also allows mRNA translation through several direct S6K1 substrates like SKAR (S6K1 aly/REF-like target), PDCD4 (programmed cell death 4), eEF-2K (eukaryotic elongation factor 2 kinase) and eIF4B (eukaryotic initiation factor 4B) (Ma and Blenis, 2009). S6

ribosomal protein is also a direct substrate of S6K1 (Ferrari and Thomas, 1994), (Jefferies et al., 1997), and is normally used as a readout for mTORC1 activity. However, although it was initially thought to enhance translation, it is currently known that S6K1-mediated phosphorylation of S6 does not play any obvious role in this process.

mTORC1 also plays an active role in suppressing autophagy, a process by which intracellular proteins as well as organelles are degraded into their constituent components in lysosomes to obtain nutrients as well as energy when the cell is in a period of starvation. Under rich nutrient conditions the complex composed by ULK1-2/ATG13/ATG101/FIP200 is maintained continuously suppressed by a direct mTORC1-mediated phosphorylation on ULK1 and ATG13 proteins, thus inhibiting the process of autophagy (Jung et al., 2010).

Rapamycin, an mTORC1 inhibitor, blocks the G1 to S transition, indicating that mTORC1 is involved in cell cycle progression. This G1 arrest induced by rapamycin is mainly mediated by the upregulation of p27 *de novo* protein expression and by its mRNA stabilization (Luo et al., 1996), (Leung-Pineda et al., 2004), p21 mRNA stabilization (Leung-Pineda et al., 2004) and inhibition of the expression of several G1 cyclins like cyclin D1, D3, A and E (Leung-Pineda et al., 2004), (Decker et al., 2003), (Hashemolhosseini et al., 1998).

It has been also shown that mTORC1 controls several aspects of cellular metabolism by controlling specific genes through mRNA transcription. This mTORC1 transcriptional regulation is mainly mediated by modulation of some key transcription factors or transcriptional regulators involved in lipid and mitochondrial metabolism, adipogenesis as well as glycolisis. In this scenario, the nuclear mTORC1/PGC1-alpha/YY1 complex (Cunningham et

al., 2007) mediates the transcription of mitochondrial genes to enhance mitochondrial biogenesis. SREBP1 (Düvel et al., 2010), SREBP2 (Düvel et al., 2010) and PPARγ (Kim and Chen, 2004) are also regulated by mTORC1 at different levels and are all involved in the transcription of specific genes that enhance lipid biosynthesis. In addition, activation of mTORC1 signaling increases HIF1-α mRNA specific translation as well as its protein levels, promoting the transcription of glucose transporters and some key glycolytic enzymes enhancing glycolisis under normoxic conditions (Semenza et al., 1994), (Düvel et al., 2010).

Not only the regulation of key metabolic transcription factors has been shown to impact on gene expression. It is known that nuclear TOR enhances the transcription of rRNAs and tRNAs through direct binding to DNA promoter regions regulated by RNA-pol I and RNA-pol III in yeast (Li et al., 2006), (Wei et al., 2009) as well as in mammals (Mayer et al., 2004), (Woiwode et al., 2008), (Kantidakis et al., 2010). This regulation of RNA-pol I and -pol III-dependent genes is also extended through modulation of several regulators of RNA-pol I and -III polymerases. In this way, mTORC1 binds and directly phosphorylates Maf1, a transcriptional repressor for RNA-pol III-dependent transcription, (Kantidakis et al., 2010) and influences de binding of the TFIIIB complex to the transcriptional machinery (Woiwode et al., 2008), enhancing in both cases the transcription of those RNA-pol III dependent genes. In the case of RNA-pol I-dependent genes mTOR can positively control the activity of TIF-IA, a regulatory factor needed for RNA-pol I dependent transcription (Mayer et al., 2004). Also in yeast, it has been described that TORC1 inhibition influences chromatin configuration, consequently influencing transcription. In this way, when TORC1 is inactivated by rapamycin, yeast Rpd3 histone deacetylase is recruited to chromatin inhibiting RNA-pol I-dependent transcription by deacetylating

histone H4 at their respective bound promoters (Rohde and Cardenas, 2003), (Tsang et al., 2003).

Also, it has been shown that mTORC1 inhibition by rapamycin disturbs the activity of other transcription factors involved in apoptosis, inflammation and angiogenesis such as STAT1, STAT3, NF-κB and NFATc4. In this way, mTORC1 activity inhibits constitutive STAT1 nuclear localization and abrogates STAT1 dependent apoptotic gene transcription (Fielhaber et al., 2009); regulates STAT3 phosphorylation status, enhancing its specific transcriptional activity (Yokogami et al., 2000); stimulates NF-κB activity, allowing the transcription of some anti-apoptotic genes and other inflammatory genes and furthermore, together with ERK5 activity, inhibits NFATc4 transcriptional activity thus inhibiting the transcription of some NFATc4-dependent genes like VEGF (Yang et al., 2008).

To keep in control this wide range of functions mTORC1 has been described to be located at different cellular compartments such as mitochondria (Desai et al., 2002), endoplasmic reticulum (Drenan et al., 2004), Golgi (Drenan et al., 2004), lysosomes (Sancak et al., 2010) and nucleus (Zhang et al., 2002), (Mayer et al., 2004), (Woiwode et al., 2008), (Kantidakis et al., 2010), playing different functions depending on its location. The mTORC1 nuclear localization and the nucleo-cytoplasmic shuttling are essential for mTORC1 functioning as a controller of gene expression, associating with some transcriptional complexes in the nucleus and influencing mRNA translation in the cytoplasm.

4.2 mTORC2:

mTORC2 is composed by Rictor (also known as mAVO3) (Sarbassov et al., 2004), (Jacinto et al., 2004), mLST8 (Sarbassov et al., 2004), (Jacinto et al., 2004) and DEPTOR (Peterson et al., 2009), the last two also found in the mTORC1 complex, but also contains several exclusive components in addition to Rictor, like mSIN1 (mammalian stress activated protein kinase interacting protein), Protor-1 (protein observed with Rictor-1) (also known as PRR5) and Protor-2 (also known as PRR5-like) (Thedieck et al., 2007). Rictor, like Raptor in mTORC1, serves to recruit substrates and is absolutely required for the mTORC2 catalytic activity (Sarbassov et al., 2004). Rictor and mSIN1 help stabilize each other, and thus may provide structural integrity to mTORC2 (Frias et al., 2006), (Jacinto et al., 2006). Protor binds Rictor, but is not required for mTORC2 catalytic function (Woo et al., 2007), (Pearce et al., 2007). mLST8, in contrast to what happens in mTORC1, is required for mTORC2 kinase activity *in vitro* and its function *in vivo* (Guertin et al., 2006). Finally, DEPTOR expression is regulated by mTORC1 as well as mTORC2, at the transcriptional and post-translational level. When bound to these two complexes DEPTOR acts as a negative regulator of mTORC1 and mTORC2 kinase activities. However, when greatly overexpressed, as found in a subset of multiple myelomas, leads to mTORC1 inhibition, and, unexpectedly, to the activation of mTORC2 by relieving the negative feedback loop transmitted from mTORC1 to the insulin growth factor 1 receptor/insulin receptor substrate-1/phosphatidylinositol 3-kinase cascade (IGF-1R/IRS-1/PI3K) and favoring survival by anti-apoptotic signals transmitted by the mTORC2 signaling (Wan et al., 2007), (Peterson et al., 2009).

While much is known about the regulation and functions of mTORC1 due to the availability of a natural compound rapamycin, capable to inhibit this

complex, studies that define mTORC2 cellular functions and signaling have lagged behind. The present availability of pharmacological inhibitors that inhibit both mTORC1 and mTORC2 complexes along with the generation of animal models that disrupt mTORC2 in specific tissues have allowed more detailed characterization of mTORC2 upstream signals, downstream effectors and regulated cellular functions.

Upstream signals:

Growth factors can also activate the mTORC2 complex. Cells activated by growth factors from a starved state acquire the phosphorylation on the hydrophobic motif (HM) site Ser473 of Akt (Sarbassov et al., 2005). Moreover PIP3 addition to cell cultures enhances this same phosphorylation, which is exclusively dependent on mTORC2 (Gan et al., 2011).

On the other hand, it is established that insulin-activated PI3K signaling can also stimulate mTORC2 activity. This activation permits that mTORC2 physically associates with ribosomes facilitating the direct phosphorylation of the transmembrane motif (TM) site Thr450 of Akt (Oh et al., 2010), (Zinzalla et al., 2011). This event is exclusively observed during the synthesis of nascent Akt polypeptides bound to ribosomes. Moreover, inhibitors of translation do not perturb this activation, suggesting that ribosomes by themselves are also capable of activating mTORC2 (Zinzalla et al., 2011).

Finally, there are some articles that propose that mTORC2 could also be regulated by amino acids in some specific nutrient conditions (Tato et al., 2011), by the TSC1/TSC2 complex (Huang et al., 2008) and by the GTPase Rac1 (Saci et al., 2011) but further investigation is needed to define the specific regulation of these upstream signals.

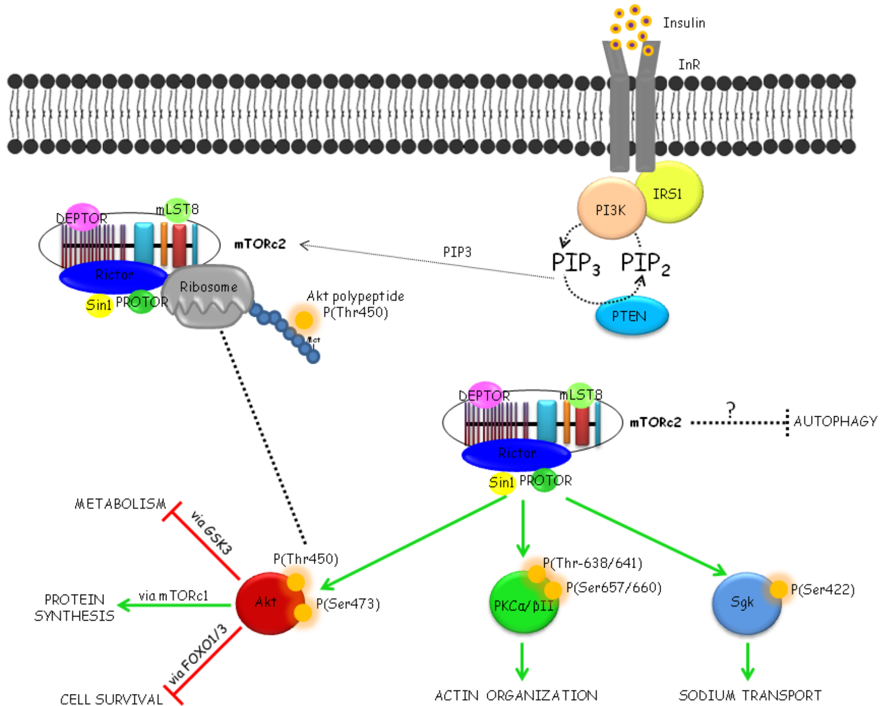
Downstream effector functions and substrates:

The most described and referenced function for mTORC2 is its role on actin cytoskeleton reorganization. Moreover, there are some evidences that report that it also participates in other processes like protein synthesis and maturation, metabolism and autophagy **(Figure I-13)**. This last process is still in discussion because some studies employing Torin1 in Rictor-null MEFs showed the same levels of autophagy than wild-type MEFs indicating that mTORC2 is not involved in this specific process (Thoreen et al., 2009).

Regarding the substrates of this complex, there are three known mTORC2 direct substrates: Akt (also known as PKB), PKC and SGK. Regarding Akt kinase, it can be phosphorylated sequentially at three different residues: first on Thr308 by PDK1, then on Thr450 by mTORC2 and finally on Ser473 by mTORC2 as well (Toker and Newton, 2000), (Hresko and Mueckler, 2005). This last event is the most important one because it defines the full activation of Akt in addition to the verification of the switch-on activity of mTORC2. However, in mTORC2-deficient cells wherein Ser473 phosphorylation is absent, indicative of not fully Akt activation, some Akt substrates, such as GSK3 and TSC2 proteins, remain phosphorylated whereas others, such as FoxO1 and FoxO3, become dephosphorylated (Jacinto et al., 2006), (Guertin et al., 2006), suggesting additional requirements or compensatory mechanisms for GSK3 and TSC2 activity regardless of phosphorylation of Ser473 on Akt **(Figure I-13)**.

mTORC2 is also involved in the maturation and stability of PKC, primarily the conventional (cPKC) and novel (nPKC) PKC isotypes. Phosphorylation of the turn motif (TM) Thr638/641 of PKCα/βII and the hydrophobic motif (HM) Ser657/660 sites of all cPKC and nPKC induces their activation

and requires mTORC2 (Facchinetti et al., 2008), (Ikenoue et al., 2008) **(Figure I-13)**.



Adapted from Oh WJ et al. (2010), Zinzalla et al. (2011) and Oh WJ et al. (2011)

Figure I-13. mTORC2 signaling, downstream substrates and functions. mTORC2 activated either by PI3K signaling or PIP3 can be associated with ribosomes where nascent Akt polypeptides can be phosphorylated by mTORC2 at Thr450. Also mTORC2 can phosphorylate Akt at Ser473 in response to growth factors. PKCα/βII and SGK are also phosphorylated by mTORC2 in different residues. mTORC2 signaling can regulate several cellular functions such as metabolism, protein synthesis, cell survival, actin organization and sodium transport. *For more details see text.* Green lines indicate an activating interaction and when accompanied with a phosphorylated mark they indicate that the activation is mediated through phosphorylation. Red lines indicate an inhibitory interaction and when accompanied with a phosphorylated mark they indicate that the inhibition is mediated through phosphorylation.

SGK (Serum and Glucocorticoid-induced protein kinase) is stimulated by growth factors and is upregulated in response to osmotic stress (Garcia-Martinez and Alessi, 2008), (Chen et al., 2009). In this case, mTORC2 is required for SGK activation by direct phosphorylation of the HM site Ser422 on SGK. Phosphorylation of several substrates of SGK is also affected by the disruption of several components of mTORC2 like RICTOR, mSIN1, mLST8 or Protor-1 (Garcia-Martinez and Alessi, 2008). It is described that mTORC2 could also phosphorylate other residues on SGK, but for the moment these residues are unknown (**Figure I-13**).

4.3 mTOR drug inhibitors:

Due to its ability to regulate cell growth and metabolism, mTOR is emerging as a relevant player in several human pathologies such as cancer and diabetes as well as involved in the process of ageing. For this reason, there is an increasing interest in the development of new mTOR inhibitors.

Rapamycin:

This allosteric inhibitor, a lipophilic macrolide, was isolated from a strain of *Streptomyces hygroscopicus* found in a soil sample from Easter Island (known by the natives as Rapa Nui) (Vezina et al., 1975), then acquiring its current name. This compound potently inhibits yeast cell growth, as well as the proliferation of several types of mammalian cells, including B and T lymphocytes. Because of its inhibitory effects on lymphocyte proliferation, rapamycin is a potent immunosuppressant, and effectively prevents allograft rejection. Rapamycin is a highly specific inhibitor of mTOR function when complexed with FKBP12 (FK506-binding protein, MW of 12 kD), an abundant and ubiquitously expressed protein, being the primary intracellular rapamycin receptor. Rapamycin/FKBP12 complex bound near the TOR

kinase domain (FRB) inhibits the downstream signaling. Although rapamycin was widely assumed to completely inhibit mTORC1 activity, recently it has been described that not all mTORC1 substrates are equally sensitive to rapamycin in all cell types studied (Choo et al., 2008), (Thoreen et al., 2009), (Feldman et al., 2009a). As an example, Choo *et al.* showed that in a subset of cultured cancer and primary cells 4E-BP1 phosphorylation is initially inhibited by rapamycin but recovers within a few hours despite continued rapamycin exposure and S6K1 hypophosphorylation (Choo et al., 2008). Moreover, they proved that this rephosphorylation was dependent on *de novo* protein synthesis, mTORC1 activity and did not depend on the negative feedback loop between mTORC1 and the PI3K-Akt axis. This combination of phenomena suggests that mTORC1 has still activity when rapamycin is added and therefore some rapamycin-resistant functions in mTORC1 subsist (Thoreen et al., 2009), (Feldman et al., 2009a), (Dowling et al., 2010).

Although initially mTORC2 was described as a rapamycin-insensitive complex, nowadays it is well accepted that, depending on the cell line studied and dose used, rapamycin could also inhibit mTORC2 kinase activity (Sarbassov et al., 2006), (Zeng et al., 2007), (Delgoffe et al., 2011). This phenomenon has been well studied by Sabatini's group showing that prolonged exposure to rapamycin can, after all, inhibit mTORC2 in some but not all cell types, apparently by interfering with the assembly of new mTORC2 complexes (Sarbassov et al., 2006). Moreover, the rapamycin-dependent inhibition of mTORC1 suppresses the negative feedback loop that normally dampens growth factor signaling to IGF-IR/IRS-1/PI3K signaling leading to a final increase in mTORC2 activity as well as Akt Ser473 phosphorylation (Wan et al., 2007), (Wang et al., 2008). However, this phenomenon is not observed in all the scenarios where rapamycin, or its derivatives, are used. The study of Wang *et al.* shows that low concentration of rapamycin (nanomolar) activates this negative feedback loop towards Akt

whereas more high doses (micromolar) cause mTOR signaling inhibition of both complexes mTORC1 and mTORC2 in different cell lines (Wang et al., 2008), (Shor et al., 2008), (Chen et al., 2010).

Torin1 and Torin2:

Two other kind of inhibitors that have been used to inhibit mTORC1 activity were Torin1 (Thoreen et al., 2009) and Torin2 (Liu et al., 2011). These two catalytic site ATP-competitive inhibitors have been recently produced by the Sabatini and Gray laboratories in 2009 and 2011, respectively. Both drugs are very efficient to inhibit both complexes - mTORC1 and mTORC2- at low concentrations (2 nM) and do not inhibit PI3K or other PIKK, as observed with other inhibitors. Unlike rapamycin, Torin1 and Torin2, inhibit the mTORC1-dependent phosphorylations on p70S6K1 and 4E-BP1 regardless of cell context (Thoreen et al., 2009), (Liu et al., 2011). At much higher doses (>1μM) Torin1 could also bind to ATR, ATM and DNA-PK., but *in vitro* below 1 μM concentrations it did not reveal any off-target activities (Thoreen et al., 2009), (Liu et al., 2012). In addition to translation, these catalytic site ATP-competitive inhibitors also potently regulate other known outputs of mTORC1 signaling, such as autophagy and proliferation, to a greater degree than rapamycin does.

Other inhibitors:

There are a variety of inhibitors, besides those enumerated before, that have been used in molecular biology to inhibit mTOR activity, however none of them are strictly selective for mTOR because they can also inhibit other related kinases. These inhibitors are the followings: PI-103 (Fan et al., 2006), LY294002 (Brunn et al., 1996) and wortmannin (Brunn et al., 1996), all three ATP-competitive and dual PI3K and mTOR inhibitors. WYE354 and KU-63794 are two highly potent and selective mTOR inhibitors at low doses (1

μM) compared with other kinases (Garcia-Martinez et al., 2009), however at higher doses (10 μM) can also bound to p38 kinases and PI3K isoforms. PP242 and PP30, both ATP-competitive inhibitors, can inhibit mTOR activity but also other related kinases (Feldman et al., 2009b). It has been shown that below 1 μM concentrations PP242 efficiently inhibited RET receptor (EC50: 42 nM) and JAK1/2/3 kinases (EC50: 780 nM) (Liu et al., 2012).

The next step for understanding the functional processes regulated by mTORC1 or mTORC2 by themselves needs to achieve an alternative strategy to develop new inhibitors that selectively target mTORC2 without directly affecting mTORC1.

4.4 Stress and mTORC1 regulation:

The existence of TOR homologs in unicellular organisms whose growth is actively affected by environmental factors, such as temperature, nutrients, pH and osmolarity, suggests an ancient role for the TOR signaling network in the surveillance of stress conditions. In fact, most of the cellular and environmental stresses like DNA damage, oxidative stress, hypoxia and osmotic stress can modulate the mTORC1 pathway.

DNA damage:

The central coordinator of the DNA damage response is p53. In this regard, p53 is capable of inducing the expression of PTEN, TSC2, AMPK and REDD1 (regulated in development and DNA damage responses 1) (Ellisen et al., 2002a), (Feng et al., 2005), (Feng et al., 2007), which all act to inhibit mTORC1 activity. p53 also induces Sestrin 1 and Sestrin 2, which can

repress mTORC1 via AMPK-dependent regulation of the TSC1/TSC2 complex (Feng et al., 2005), (Budanov and Karin, 2008). All these events leading to mTORC1 inhibition are important for the cellular response to DNA damage because several reports showed that mTORC1 hyperactivation caused by TSC1 or TSC2 deficiency sensitizes cells to the effects of DNA damage, making them more sensitive to the mTOR-dependent accumulation of p53 and cell death (Ghosh et al., 2006), (Lee et al., 2007), (Choo et al., 2010).

Hypoxia:

Low oxygen levels set a scenario of hypoxia. Many mechanisms are involved in mTORC1 pathway perturbation under hypoxia. Among them we find the activation of AMPK as in the case of glucose deprivation leading to inhibition of mTORC1 via TSC2 and Raptor; the transcription, synthesis and action of REDD1 and REDD2 (Regulated in development and DNA damage responses 1 and 2) proteins (*for a more extended description see "stress and REDD1 and REDD2 proteins" section below*), and other types of mechanisms still not fully characterized that also lead to mTORC1 inactivation, such as mTORC1 sequestration in nuclear bodies by PML (promyelocytic leukemia tumor suppressor) (Bernardi et al., 2006) or mTORC1 inhibition by BNIP3 (Bcl2/adenovirus E1B 19 kDa protein-interacting protein 3) (Li et al., 2007).

Osmotic stress:

In unicellular organisms, like *S.cerevisiae* and *S.pombe*, TOR signaling is needed to sustain growth under high salt conditions (Crespo et al., 2001), (Weisman and Choder, 2001). In mammalian cells, however, it has been known for decades that high hypertonic stress induced by sorbitol or NaCl addition to cultured cells reversibly decreases the phosphorylation of S6K1 and S6 –the inhibition of phosphorylation and consequent rephosphorylation are seen in

less than 1 hour after treatment-, as well as the overall translational output of the cell (Martini and Kruppa, 1979), (Kruppa and Clemens, 1984), (Naegele and Morley, 2004). Different mechanisms for this phenomenon have been suggested: high osmotic stress induced by high sorbitol treatment causes a rapid and reversible disintegration of the mitochondrial proton gradient -500 mOsm/kg. caused 10% loss of mitochondrial membrane potential and 900 mOsm/kg. caused a 50% loss in 30 min-. This reduction observed in the mitochondrial function was due to the downregulation of mTOR activity directly sensed by the pool of mTOR molecules localized in the outer mitochondrial membrane (Desai et al., 2002).

Another mechanism suggests that the reduction of mTORC1 activity and consequent S6K1 dephosphorylation by high hyperosmotic stress upon sorbitol addition (to 700 mOsm/kg) may be due to the activation of a phosphatase sensitive to calyculin A (Parrott and Templeton, 1999). Moreover there are different data showing that the rapid dephosphorylation seen on S6K1 by high hypertonic stress (between 600 mOsm/kg and 900 mOsm/kg during 30 min treatments) is independent of TSC2 (Smith et al., 2005) and Rheb (Inoki et al., 2003). Moreover, the reduction in mTOR activity in response to high hyperosmotic stress was also shown to be independent of p38 MAPK (Parrott and Templeton, 1999), (Patel et al., 2002). Other work done in 3T3-L1 adipocytes, showed that high hyperosmotic stress (900 mOsm/kg for 40 min) promote insulin desensitization through an mTOR-dependent phosphorylation of IRS-1 on Ser307 (Gual et al., 2003). Additionally there are some studies that report that intense osmotic stress conditions (>600 mOsm/kg) can activate AMPK in some cell types, thus consequently leading to mTORC1 inhibition (Barnes et al., 2002), (Hawley et al., 2010).

Similar results were also observed in plants. *Arabidopsis* plant cultured in hyperosmotic stress conditions induced by mannitol showed reduced phosphorylation status of S6K1. This reduction on S6K1 was reversible by overexpression of Raptor, and interestingly, transgenic plants expressing high levels of S6K1 were rendered hyperresponsive to osmotic stress-mediated cell shrinkage and grow worse (Mahfouz et al., 2006). This phenomenon supports the hypothesis that one of the main mechanisms employed by the plants to sustain harsh environmental conditions is to have mTORC1 activity slowed down under osmotic stress conditions.

However, there are also some studies in mammalian cells that postulate the contrary effects of those commented before. One of them argued that hyperosmotic treatment (between 600 and 900 mOsm/kg) enhances S6K1 activation and IRS-1 phosphorylation (Bae et al., 2008) and the other that hyperosmotic stress enhances S6K1 phosphorylation and mTORC1 kinase activity via JNK-mediated Raptor phosphorylation (Kwak et al., 2012).

Nearly all the studies describing the effects of osmotic stress on mTOR signaling are done in extreme stress conditions (>500 mOsm/kg) and not under a more physiopathological stress scenario (≤500 mOsm/kg). Moreover, all of them describe short time effects of hyperosmotic stress (between the first minutes to 1 hour) and neither of them analyzed the process of adaptation that can be as long as 20 hours for the osmotic stress response. This high hyperosmotic stress leads to a "DNA damage-like stress" phenotype. This effect was corroborated by studies done in mIMCD3 cells by Burg and coworkers showing that adding NaCl to a total osmolality of 700 mosmol/kg kills >90% of the cells by apoptosis within 24 hours whereas conditions of 500 mOsm/kg are well tolerated (Dmitrieva et al., 2000).

Intriguingly, not all studies coincide in presenting osmotic stress as an mTOR inhibitor, and at least two articles have shown that high osmolality can transiently enhance mTOR activity (Bae et al., 2008), (Kwak et al., 2012).

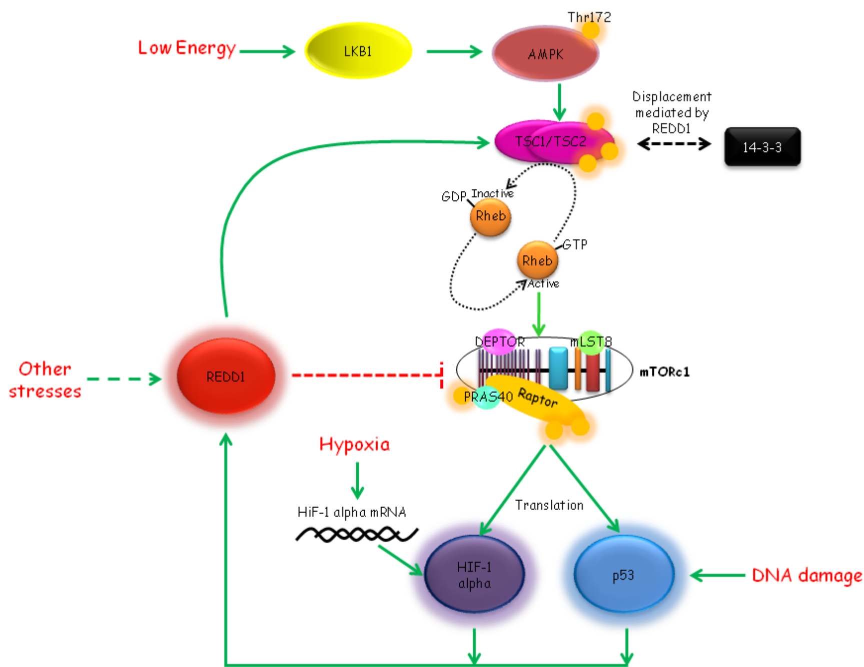
Finally, it is important to keep in mind that cells can adapt to hypertonic stress and maintain their growth and proliferative capacity under prolonged hypertonic conditions. This implies that they must be able to maintain mTOR activity under conditions of osmostress and suggests that even if the acute early stress phase may perturb mTORC1 function, its activity will be restored later.

4.5 Stress and REDD1 and REDD2 proteins:

REDD1 and 2 proteins are both stress response genes. More is known about the regulation of REDD1. REDD1 (also known as DDIT4, RTP801 or Dig-2) encodes a 232 amino acid cytosolic protein with no recognizable functional domains. REDD1 was initially described in response to hypoxia and later was shown to be a direct transcriptional target of HIF1-α under hypoxia (Shoshani et al., 2002) and of p53 under DNA damage (Ellisen et al., 2002a).

In a hypoxic scenario, low oxygen levels stabilize HIF1-α protein, allowing the formation of a heterodimer with the aryl hydrocarbon receptor nuclear translocator (ARNT). The HIF1-α/ARNT dimer activates the transcription of hypoxic response genes, among them REDD1. It has been shown that REDD1 induction by hypoxia occurred concurrent with downregulation of mTOR substrate phosphorylation, like S6K1 (Reiling and Hafen, 2004), (Brugarolas et al., 2004); moreover MEFs deficient in REDD1 are defective in hypoxia-mediated inhibition of S6K1, supporting its role as a negative

modulator of mTORC1 (Brugarolas et al., 2004), (Corradetti et al., 2005). In fact, the effect of REDD1 expression on mTORC1 activity is mediated through the TSC1/2 complex. When REDD1 is expressed, it is capable of disrupting the inhibitory interaction between TSC1/2 complex and the 14-3-3 proteins thus enhancing the TSC1/2 GTPase activity and leading to mTORC1 inhibition (DeYoung et al., 2008) (**Figure I-14**).



Adapted from Corradetti et al. (2006) and Vadysirisack et al. (2011)

Figure I-14. Energy and cellular stress signaling coupled to mTOR. Under diverse cellular stress conditions cell maintained the cellular homeostasis by directly modulating mTORC1 activity. Under DNA damage mTORC1 regulates the translation of p53, which in turn induces the transcription and expression of REDD1. In the same way, under low oxygen levels, HIF1-α is highly translated by the action of mTORC1 leading to the same REDD1 induction and effect on mTORC1 activity. Not only hypoxia and DNA damage induce REDD1 expression, and low energy, starvation and oxidative stress are also capable of inducing REDD1

expression and consequently regulate mTORC1 activity. Green lines indicate an activating interaction and red lines indicate an inhibitory interaction.

More recently, the Ellisen's group has been studying the contribution of endogenous REDD1 to the DNA damage response *in vivo*. They find that *Redd1*$^{-/-}$ tissues and cells exhibit slightly increased sensitivity to ionizing radiation and chemotherapy treatment, both *in vitro* and *in vivo*. Remarkably, they show that this increased sensitivity in the absence of REDD1 is due to slightly increase in p53 protein levels, an effect which is due to abnormally elevated mTORC1-dependent translation of p53 itself (Vadysirisack et al., 2011) (**Figure I-14**).

As an example of mTORC1 regulation by REDD1, it is known that down-regulation of mTOR activity by hypoxia requires *de novo* mRNA synthesis and correlates with increased expression of the hypoxia-inducible REDD1 gene. Disruption of REDD1 abrogates the hypoxia-induced inhibition of mTOR, and REDD1 overexpression is sufficient to downregulate S6K1 phosphorylation in a TSC1/TSC2-dependent manner (Brugarolas et al., 2004).

In fact, now it is known that besides hypoxia and DNA damage, this protein is also induced and stabilized in other types of stress like energy stress (Sofer et al., 2005), glucocorticoids (Wang et al., 2003), starvation (McGhee et al., 2009), oxidative stress (Ellisen et al., 2002b), ROS (Lin et al., 2005) and also osmotic stress -described in our study- (**Figure I-15)**.

There is little information related to REDD2 (also known as DDIT4L or RTP801L), but it has been shown that it is predominantly expressed in skeletal muscle and acts as inhibitory modulator of mTORC1 signaling

(Miyazaki and Esser, 2009), suggesting that both proteins REDD1 and REDD2 could perform similar functions.

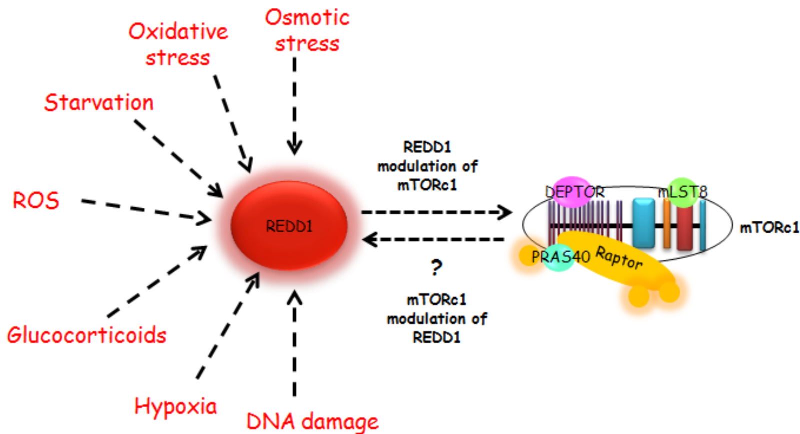


Figure I-15. REDD1 as an integrator of stress responses to mTORC1. A variety of stress stimuli could finally lead to REDD1 expression. It is established that under DNA damage and hypoxia REDD1 is expressed and its expression correlates with less activity of mTORC1 pathway being in these two cases a negative regulator of mTORC1 activity. It is necessary more effort in studying how other stresses could interfere with mTORC1 activity and how REDD1 is involved in this process.

4.6 mTOR, stress, ageing and senescence:

It is not surprising that all the stresses previously shown converge in mTOR inhibition. In fact, this decrease in mTOR activity in cells exposed to stress serves to slow down or arrest growth and proliferating-promoting processes when cells face conditions that can damage critical components on the cell.

In this regard, it is interesting to keep in mind that there are many evidences, in different model organisms like *C.elegans*, *Drosophila* and mammals, showing that transient mTOR suppression or decreased mTOR signaling is associated with increased resistance to stress, suggesting that the modulation of the activity of this pathway –mainly mediated by the activity of mTORC1- is important for the outcome of a broad range of stress responses (Holzenberger et al., 2003), (Broughton et al., 2005), (Tettweiler et al., 2005), (Powers et al., 2006). Also, it is observed that key transcription factors involved in mediating specific stress responses are regulated by mTORC1 signaling (Shoshani et al., 2002), (Ellisen et al., 2002a) and mTORC1 signaling is one actor in mediating lifespan extension (Blagosklonny, 2010).

Expanding this thought, it was shown that strong inhibition of mTORC1 early in life (embryogenesis or larval stages) drastically slows or even stops development but in contrast, inhibition of this pathway during adulthood extends lifespan. This is described in a study of Harrisson and collaborators showing that the administration of rapamycin late in life (mice aged 600 days) was sufficient to cause a lifespan extension in mice in both sexes, although having sex-specific differences (males >9%, females >14% comparing with untreated mice) (Harrison et al., 2009) and improving the onset of age-related pathologies and consequently organism aging.

In this regard, cell senescence has been proposed as one causal mechanism for tissue and organism aging. The senescence concept is used to define a cell that has the cell cycle blocked but has the mTOR pathway still activated (Demidenko and Blagosklonny, 2008). In fact, when cells have their cell cycle arrested but maintain the mTOR signaling pathway active they become large and with flat morphology because of growth without division, and acquire β-galactosidase staining, losing their proliferative potential. Rapamycin, by inhibiting mTORC1, suppresses the conversion from

quiescence to senescence (Demidenko and Blagosklonny, 2008), (Leontieva and Blagosklonny, 2010), (Korotchkina et al., 2010) because although it is capable to inhibit the cell cycle, it can also stop cell growth.

As a summary, cells can prevent its normal way to aging by limiting its growth by suppressing the mTORC1 pathway, to avoid becoming senescent and undergoing a "quiescent-like" state. The same happens when a cell is encountering a stress scenario, slowing down its growth by mTORC1 transient inactivation, allowing better survival. So having mTORC1 under a tight control –active when is necessary to be active and shut down when is necessary to be inactive- favors correct cellular outcomes.

OBJECTIVES

OBJECTIVES

Under high hyperosmotic stress (600-900 mOsm/kg) growth promoting signaling pathways are inhibited to avoid lethal and irreversible cell damage. Moreover, under the same conditions, the transcriptional activity of NFAT5, a central transcription factor involved in the induction of several osmoregulatory and osmoadaptive genes, is inhibited. By contrast, under more tolerable hyperosmotic stress conditions (500 mOsm/kg) cells are still capable to proliferate, maintain cell growth and NFAT5 can induce properly the expression of genes involved in osmoadaptation. This phenomenon lead us to consider that one of the main signaling pathways involved in growth and proliferation, the mTOR pathway, could be a modulator of the outcome of the osmotic stress response under tolerable osmostress conditions.

Specific objectives of our work were as follows:

1. To determine the effect of physiopathological osmostress conditions (500 mOsm/kg) on the mTOR signaling pathway.

2. To explore whether mTOR could regulate a specific osmostress gene expression network.

3. To characterize the molecular mechanisms by which mTOR modulates specific osmostress genes.

MATERIALS and METHODS

MATERIALS AND METHODS

Cells

The human embryonic kidney cell line HEK293 was obtained from the American Type Culture Collection (ATCC) (ATCC number CRL-1573). The human embryonic kidney cell line HEK293T was kindly provided by Josep Lluís Parra (Vall d'Hebron Institut of Oncology) and was from the ATCC (ATCC number CRL-11268). AMPK wild-type and knock-out mouse embryonic fibroblasts immortalized by a carboxy-terminal fragment of p53 (C-terminal p53 MEFs/MEFs AMPK wt) were kindly provided by Benoit Viollet (INSERM, Institut Cochin, CNRS, and Université Paris Descartes) and Tomi Makela (Institute of Biotechnology, University of Helsinki). Briefly, MEFs were obtained from 12.5-day mouse embryos from mice of mixed background (Bl6/CD1) and were immortalized with a fragment of the carboxy-terminus of p53 (Vaahtomeri et al., 2008). NFAT5 wild-type and NFAT5-deficient MEFs (MEFs NFAT5$^{+/+}$ and NFAT5$^{-/-}$) were prepared from 13.5-day littermate embryos (129sv background) using the NIH3T3 protocol to obtain spontaneously immortalized cells (Lopez-Rodriguez et al., 2004). SV40 immortalized mouse embryonic fibroblasts (SV40 MEFs/MEFs RAPTOR control) were obtained from 12.5-day mouse embryo from mice of mixed background (129S6/C57BL6) containing a homozygous floxed raptor allele using the SV40 large T antigen protocol to obtain spontaneously immortalized cells (Cybulski et al., 2012). Splenocytes were obtained from 8-12-weeks old *Nfat5*$^{-/-}$ and littermate *Nfat5*$^{+/+}$ (Lopez-Rodriguez et al., 2004) from 129/Sv background mice. Spleen was disaggregated and splenocytes were isolated by density gradient sedimentation with Lymphoprep (Axis-Shield PoC AS) and proliferating T cells, were obtained by activating splenocytes (2,5x 10^6 cells/ml) with 2.5 μg/ml concanavalin A plus 25 ng/ml of human IL-2 for 24 or 48 hours.

Cell culture

HEK293 cells, HEK293T cells, C-terminal p53 MEFs, SV40 MEFs were maintained in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 50 µM β-mercaptoethanol , 2 mM L-glutamine and 1 mM sodium pyruvate. NFAT5$^{+/+}$ and NFAT5$^{-/-}$ MEFs were cultured in the above medium although using 4 mM L-glutamine in contrast of 2 mM. Splenocytes and proliferating T cells were cultured in the same medium as HEK293T cells plus 100 µM non-essential amino acids and 1% antibiotics penicillin and streptomycin and then cultured under isotonic or hypertonic conditions as indicated in the figure legends.

Hypertonic stress

The osmolarity of the culture medium was measured in a VAPRO 5520 vapor pressure osmometer (Wescor). Culture medium with supplements had an osmolality of 330 mOsm/kg, and was adjusted to 300 mOsm/kg by adding 10% sterile H_2O (Milli-Q Biocel A10, Millipore). Media were made hypertonic by adding NaCl from a sterile 4 M stock solution in water. Over an isotonic baseline of 300 mOsm/kg, addition of 50 mM NaCl raised the osmolarity to 400 mOsm/kg and 100 mM NaCl to 500 mOsm/kg. In some experiments, sorbitol (200 mM final concentration) was used to induce hyperosmotic stress of an intensity of 500 mOsm/kg.

Reagents and drug inhibitors

5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR) was from BioMol Research Labs, and rapamycin was from Calbiochem. Torin1 was kindly provided by David M. Sabatini (Whitehead Institute, Massachusetts Institute of Technology and Howard Hughes Medical Institute) and Nathanael S. Gray (Dana-Farber Cancer Institute and Harvard Medical School). Formaldehyde, sodium chloride, sorbitol, Trizma base, glycine,

EDTA, magnesium chloride, manganesium chloride, potassium chloride, iodoacetamide, sodium pyrophosphate (NaPPi), sodium orthovanadate, β-glycerophosphate, phenylmethanesulfonyl fluoride (PMSF), leupeptin, pepstatin A, aprotinin, sodium dodecyl sulphate (SDS), Tween-20, glycine, methanol, Triton X-100, Nonidet P-40, CHAPS and sodium deoxycholate were from Sigma-Aldrich. Sodium fluoride (NaF) was from Merck. Dynabeads sheep anti-mouse IgG magnetic beads were from Dynal Biotech (Invitrogen). To-PRO3 was from Molecular Probes (Invitrogen). Hoechst 33342 was purchased from Sigma. IL-2 was from Proleukin (Chiron Corp). Lymphoprep was from Axis-Shield. DMEM, β-mercaptoethanol, L-glutamine, sodium pyruvate, non-essential amino acids and antibiotics penicillin and streptomycin were from Gibco.

Antibodies

Total anti-mTOR (Cat. 2972), total anti-TSC2 (Cat. 4308), anti-phospho AMPK (Thr 172) (Cat. 2531 and 2535), anti-phospho ACC (Ser79) (Cat. 3661), total anti-S6 kinase 1 (Cat. 9202), anti-phospho S6 kinase 1 (Thr 389) (Cat. 9205), total anti-Akt (Cat. 9272), anti-phospho AKT (Ser 473) (Cat. 9271), total anti-S6 (Cat. 2317), anti-phospho S6 (Ser 235/236) (Cat. 2211), and anti-4E-BP1 (Cat. 9452) antibodies were from Cell Signaling. Antibodies against anti-AMPKα1 (Cat. ab3759), anti-AMPKα2 (Cat. ab3760) and rabbit anti-H3 (Cat. ab85869) were from Abcam. Anti-REDD1 antibody (Cat. 10638-1-AP) was from ProteinTech. Goat anti-pyruvate kinase (AB1235) was from Chemicon (Hampshire, UK). The donkey anti-rabbit (Cat. NA934V) and sheep anti-mouse (Cat. NA931V) secondary antibodies conjugated to horseradish peroxidase (HRP) were from Amersham Biosciences and HRP-conjugated rabbit anti-goat IgG antiserum (Cat. P010.60) was from Dako. The NFAT5 polyclonal antibody specific for the carboxy-terminal region of NFAT5 (C5 antibody) used in the Western blots (Cat. PA1-023) was from Affinity Bioreagents. The two rabbit anti-NFAT5

polyclonal antibodies used in chromatin immunoprecipitation (ChIP) experiments have been described previously (Lopez-Rodriguez et al., 1999), (Lopez-Rodriguez et al., 2001) and are specific for the amino-terminal (N-terminal) and DNA binding domain regions (DBD) of the protein. Preimmune rabbit serum served as control. The polyclonal antibody to acetylated lysines 5, 8, 12 and 16 in histone H4 (AcH4) was from Millipore (Cat. 06-866) and the anti-histone H4 antibody was from Abcam (Cat. ab7311). Antibodies specific for the carboxy-terminal domain heptad repeat (CTD) of the largest subunit of RNA polymerase-II (Cat. ab817), and phosphorylated forms of the CTD at Ser5 (Cat. ab5131) or Ser2 (Cat. ab5095) were also from Abcam. Normal rabbit IgG used for chromatin immunoprecipitations was from Santa Cruz (Cat. SC-2027). Normal mouse IgG used for chromatin immunoprecipitations was from Santa Cruz (Cat. SC-2025). Anti-β-actin (Cat. A5441) was from Sigma-Aldrich and anti-tubulin (Cat. SC-32293) was from Santa Cruz.

DNA constructs

The luciferase reporter ORE-Luc was previously described (Lopez-Rodriguez et al., 2001). The vectors pCMV-HA (Clontech) and pTK-Renilla (Promega) are available commercially.

Sequence of primers

- Primers used for quantifying the expression of the different mRNAs were:

L32:
forward 5'-ACC AGT CAG ACC GAT ATG TG-3'
reverse 5'-ATT GTG GAC CAG GAA CTT GC-3'

Gapdh (glyceraldehyde-3-phosphate dehydrogenase):
forward 5'- TGC GAC TTC AAC AGC AAC TC-3'
reverse 5'-TCT CTT GCT CAG TGT CCT TG-3'

Hspa1b (heat shock protein 1b):
forward 5'-AGG TGC AGG TGA ACT ACA AG-3'
reverse 5'-TTG ATG ATC CGC AGC ACG TT-3'

Akr1b3 (aldose reductase):
forward 5'-TGA GCT GTG CCA AAC ACA AG-3'
reverse 5'-GGA AGA AAC ACC TTG GCT AC-3'

Slc5a3 (solute carrier family 5 (inositol transporter), member 3, SMIT):
forward 5'-ATG GTT GTC ATC AGC ATA GCA TGG-3'
reverse 5'-GGT GGT GTG AGA AGA CTA ACA ATC-3'

Slc6a6 (solute carrier family 6 (neurotransmitter transporter, taurine), member 6):
forward 5'-TAC TAT GCA GCT AGT GGT GTA TGC-3'
reverse 5'-ACC TGG TCC TAT GAG AAT CTA ACG-3'

Ddit4 (DNA-damage-inducible transcript 4/REDD1):
forward 5'-GAC TTT GAG CTG CTC AGT GA-3'
reverse 5'-TCT TGC CTT GCT CCA CAC ACA-3'

Ddit4l (DNA-damage-inducible transcript 4-like/REDD2):
forward 5'-TTG TTG GAC GGT GGC TAT CA-3'
reverse 5'- AGG GAC CAA GAC CTT AGA GCA A-3'

Slc1a3 (sodium-dependent glutamate/aspartate transporter 1):
forward 5'-GAG ATG GAA GAC ATG GGT GTG A-3'
reverse 5'-GCA GCA ACC CTC CAA TGA AA-3'

Mrps6 (mitochondrial ribosomal protein S6):
forward 5'-CGC TGC TGC TTT GAA ACG TA-3'
reverse 5'-CAC AGC ACT TGT CGG AGC ATA A-3'

Aqp1 (aquaporin 1):
forward 5'-TGT ACA TCA TCG CCC AGT GTG T-3'
reverse 5'-CTG CAG AGT GCC AAT GAT CTC A-3'

Prrg4 (proline rich Gla (G-carboxyglutamic acid) 4 (transmembrane)):
forward 5'-AGG ACC AAC CAC AGG ATC AGA T-3'
reverse 5'-CTT GGA TAT GGC CGC CTT TT-3'

Kcnj2 (potassium inwardly-rectifying channel, subfamily J, member 2):
forward 5'-AGC AAA GCA TGC GTG TCA GA-3'
reverse 5'-GCA GCC TAC GAT TGA CTG GAA T-3'

Hist1h1d (histone cluster 1, H1d):
forward 5'-AGA GGA CAA CCT GCA ACA GTC A-3'
reverse 5'-ACA CAG CTC TTG AGG CTC GAA T-3'

Amd1 (S-adenosylmethionine decarboxylase 1):
forward 5'-GCC AGA TCA AAC CCT GGA AA-3'
reverse 5'-CAT CGA GTA GCC ACA AGG ATT G-3'

Bpgm (2,3-bisphosphoglycerate mutase):
forward 5'-GCC AGA TCA AAC CCT GGA AA-3'
reverse 5'-CAT CGA GTA GCC ACA AGG ATT G-3'

Slc19a2 (solute carrier family 19 (thiamine transporter), member 2):
forward 5'-ATG GAA CGT TAC GCC CTT GT-3'
reverse 5'-TGC CAT TCG CCA GGA AAA-3'

Slc39a10 (solute carrier family 39 (zinc transporter) member 10):
forward 5'-GCG AGA AGC ACC ACA TGT TAG A-3'
reverse 5'-CGT GAA TAG ATC CGG TGA GAT G-3'

Itga3 (integrin alpha 3):
forward 5'-CCT TCC AGA CAC CTC CAA CAT T-3'
reverse 5'-CCA TGT TGA TGG TAG GGA TGC T-3'

Fosl1 (fos-like antigen 1):
forward 5'-CCC CAA AGC TGC TCA CTG TT-3'
reverse 5'-AGT GAC GTG GTG TGG GAA AGT-3'

Prl2c3/Prl2c5 (prolactin family 2, subfamily c, member 3 and member 5):
forward 5'-TCA ACC ATG CTC CTG GAT ACT G-3'
reverse 5'-TGA GAC AAA CTG CCG GCT AA-3'

Pdk4 (pyruvate dehydrogenase kinase 4):
forward 5'-TGA TGG ATT TGG TGG AGT TCC-3'
reverse 5'-GAG CCA TTG TAG GGA CCA CAT T-3'

- The primers used to analyze the immunoprecipitated chromatin were:

Intergenic region of *Akr1b3* gene at -3.43 kb from the transcription start site (TSS) (designated as AR-a):
forward 5'-ACT TTG GCT CTC TTG GGA CA-3'
reverse 5'-AAG CAA GGC CAC GCA TCA C-3'

Akr1b3 enhancer (which contains osmotic response element (ORE) NFAT5 binding sites) at -1.13 kb (designated as AR-b):
forward 5'-CAC CAG AAT TTC CAC ATG CC-3'
reverse 5'-AGG GAC AAC TGC ATC TGC AA-3'

Akr1b3 proximal promoter and TSS region at -94 bp (designated as AR-c):

forward 5'-CCT AGG GAA AGA AGC ATC CT-3'

reverse 5'- CCA ACG GCC TGT AGA AAG AA-3'

Region between the *Akr1b3* intron 1 and exon 2 (designated as AR-d):

forward 5'-GGC TTA TAC TCC TCC CTT TC-3'

reverse 5'-CTT CTC ATT CTG GTA CAC CTG G-3'

Bpgm distal promoter at -1.65 kb from the TSS (designated as BPGM-a):

forward 5'-AGC AGC CCA CAA ACA TTG C-3'

reverse 5'-CAG ACG CAC GGA TAG ACC AA-3'

Bpgm proximal promoter and TSS at -8 bp (designated as BPGM-b):

forward 5'-AGG GAC TTG AGA AAC CGG AAA-3'

reverse 5'-CGA TAG CGC TGG GTA TTG GA-3'

Bpgm exon 2 at +11.2 kb (designated as BPGM-c):

forward 5'-GTA GTT CAG CCA CCA GCA TGT C-3'

reverse 5'-CTG GTC CAC CCA ACT ACA GAA TC-3'

Ddit4l distal promoter at -1.5 kb from the TSS (designated as DDIT4L-a):

forward 5'-GAC CCA TTC TAA CCG GCT TA-3'

reverse 5'-AGC CAG CTT GAA GGT CCA TT-3'

Ddit4l proximal promoter at -0.47 kb (designated as DDIT4L-b):

forward 5'-GAA CAT GGG CAT AAA GGA TGC T-3'

reverse 5'-TCC AGG TCC TGT TAG GCA TGA-3'

Ddit4l exon 3 at +2.46 kb (designated as DDIT4L-c):

forward 5'-GGG TTG TGT TAT GCA CGT GAA-3'

reverse 5'-CAC AAG CGT GAG CTC AAA GG-3'

Real time quantitative PCR (RT-qPCR)

Total RNA was isolated using the High Pure RNA isolation kit (Cat. 11 828 665 001, Roche) following manufacturer's instructions. 1 to 2 µg of total RNA were retrotranscribed to cDNA using SuperScript III reverse transcriptase and random primers (Invitrogen). For real-time quantitative PCR, LightCycler 480 SYBR Green I Master Mix (Cat. 11608521, Roche) and a LightCycler480 system apparatus (Roche) were used following the instructions provided by the manufacturers. Samples were normalized to *L32* mRNA levels using the LightCycler 480 SW 1.5 software (Roche).

Microarray experiments and analysis

NIH3T3-immortalized wild-type and NFAT5-deficient MEFs (Lopez-Rodriguez et al., 2004) from passages 30-35 were plated (175,000 cells in 60 mm dishes) in isotonic medium and two days later were either left untreated or treated for 8 hours with 500 mOsm/kg. When indicated, the mTOR catalytic inhibitor Torin1 was added 1 hour before hypertonicity (500 mOsm/kg) treatment. Cells were lysed in RLT buffer (300 µl, RNeasy system, QIAGEN Cat. 74104) and total RNA was isolated using the manufacturer protocol. The RNA integrity was assessed using Agilent 2100 Bioanalyzer (Agilent), and only samples with high integrity (RNA integrity number (RIN) >7.5) were subsequently used in microarray experiments. Amplification, labelling and hybridizations were performed according to protocols from Ambion and Affymetrix. Briefly, 250 ng of total RNA were amplified using the Ambion® WT Expression Kit (Ambion/Applied Biosystems), labeled using the WT Terminal Labeling Kit (Affymetrix Inc), and then hybridized to Mouse Gene 1.0 ST Array (Affymetrix) in a GeneChip® Hybridization Oven 640. Washing and scanning were performed using the Hybridization Wash and Stain Kit and the GeneChip® System of Affymetrix (GeneChip® Fluidics Station 450 and GeneChip® Scanner 3000 7G). Three independent microarray hybridizations were

performed for each experimental condition: cells maintained at 300 mOsm/kg or exposed to 500 mOsm/kg (8 hours) without or with 100 nM Torin1 in the case of wild-type MEFs, or 300 mOsm/kg and 500 mOsm/kg for *Nfat5*$^{-/-}$ MEFs.

Microarray data analysis was performed as follows: after quality control of raw data, it was background-corrected, quantile-normalized and summarized to a gene-level using the robust multi-chip average (RMA) (Irizarry et al., 2003) obtaining a total of 28853 transcript clusters, excluding controls, which roughly correspond to genes. Core annotations (version NetAffx 30, human genome 18) were used to summarize data into transcript clusters. Linear Models for Microarray (LIMMA) (Smyth, 2004), a moderated t-statistics model, was used for detecting differentially expressed genes between the conditions in study. Correction for multiple comparisons was performed using false discovery rate. Genes with an adjusted p-value lower than 0.05 or with a p-value lower than 0.01 for those comparisons with few results after adjusting p-values were selected as significant. Hierarchical cluster analysis was also performed to analyze how data aggregated and linear model for regression purposes. All data analysis was performed in R (version 2.11.1) with packages aroma.affymetrix, Biobase, Affymetrix, LIMMA and genefilter. The microarray data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE27485.

RNA interference assays

ON-TARGETplus SMARTpool small interfering RNA (siRNA) pools were purchased from Dharmacon: nontargeting scramble (D-001810-10), mouse *Ddit4*/REDD1 (L-056656-01), mouse *Ddit4l*/REDD2 (L-056952-00), mouse TSC2 (L-047050-00) and mouse RAPTOR (L-058754-01). siRNA (40 nM) was transfected using Lipofectamine 2000 (Invitrogen) or Lipofectamine

RNAiMax (Invitrogen) in a six well-plate format following the manufacturer's protocol.

Transfections and luciferase reporter assays

HEK293 cells and mouse embryonic fibroblasts (MEFs) were transiently transfected by the calcium-phosphate method (Rodriguez and Flemington, 1999). After transfection, cells were washed, replated in fresh medium, and allowed to grow for 36 hours before being treated as indicated in the respective figures. Firefly-luciferase and Renilla-luciferase were measured with the dual-luciferase reporter system (Promega) with a Berthold FB12 luminometer (Berthold). Luciferase activity was normalized to the activity of a cotransfected Renilla vector. Luciferase and Renilla units were in the range of 100,000-200,000 units for basal ORE-Luc and 20,000-60,000 units for the normalization vector Renilla in isotonic conditions for MEFs, and about 200,000 units for ORE-Luc activity and 600,000 units for Renilla in HEK293 cells. Background readings in lysates from non-transfected cells were below 500 units.

Flow cytometry

Flow cytometry analysis of cell cycle, cell size and proliferation were done always in cells gated as viable by FSC/SSC. For viability all population was used. Flow cytometry was done with a BD LSR flow cytometer (Becton Dickinson Biosciences).

- **Cell viability and cell size determination**

  Cells were labeled with 5 μg/ml of the DNA dye Hoechst 33342 (SIGMA) in suspension in cytometry tubes for 1 hour at 37°C in a water bath. Viability was determined by forward and side scatter parameters (FSC/SSC) in the total population of cells. Nonviable cells were readily identified by their distinct position in the FSC/SSC plots.

Cell size (FSC parameter) was analyzed always in cells gated as viable by FSC/SSC plot and only in the G1 phase population of cells in the cell cycle distribution.

- **Cell proliferation**

  Cells were labeled with CFDA-SE. Briefly, MEFs were labeled with 5 μM carboxyfluorescein diacetate succinimidyl ester (CFDA-SE, Molecular Probes, Invitrogen) at day 0 and then analyzed at 48 and 72 hours after labeling. The decrease in CFDA-SE fluorescence intensity in cells, which was proportional to the number of cell divisions, was analyzed by two-color flow cytometry in all the population of live cells (gated as viable by FSC/SCC plot and also excluding those cells stained with Hoechst 33342 (5 μg/ml) with a sub-G0/G1 DNA content).

Fluorescence confocal microscopy

Immortalized MEFs were grown on sterile glass coverslips coated with 0.01% poly-L-lysine (Sigma-Aldrich). 48 hours after plating, cells were exposed to 500 mOsm/kg hypertonicity for the indicated times, without or with pretreatment (1 hour) with mTOR inhibitors. Cells were then processed for microscopy as described (Estrada-Gelonch et al., 2009). Briefly, cells were fixed with 3% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and permeabilized with 0.5% Nonidet P-40 in PBS. Fixed cells were washed with PBS, and endogenous NFAT5 was detected with a rabbit polyclonal anti-NFAT5 antibody specific for its DNA binding domain (Lopez-Rodriguez et al., 1999) as described (Estrada-Gelonch et al., 2009). Cell nuclei were stained with the DNA dye TO-PRO3 iodide (Invitrogen), and preparations were mounted on slides with the anti-fading agent Slowfade (Invitrogen Molecular Probes). Images were acquired with a Leica TCS SP2 confocal microscope (Leica). For phase contrast photographs of non-fixed

live cultures, cells were plated in six- or twelve-well format at day 0, and then left in isotonic medium (300 mOsm/kg) or exposed to osmotic stress (500 mOsm/kg), with or without rapamycin or Torin1 during 48 hours.

Total protein cell extracts preparation and Western blot analysis

Before lysis, cells were always washed once with cold PBS adjusted to the same osmolarity of the culture medium and then lysed for 30 minutes at 4ºC. In the experiments were mTOR complex integrity is not important, like total protein extracts cells were lysed in Triton X-100 lysis buffer (40 mM HEPES pH 7.4, 120 mM NaCl, 1 mM EDTA, 10 mM NaPPi, 1% Triton X-100, 10 mM β-glycerophosphate, 0,5 mM sodium-orthovanadate, 50 mM sodium-fluoride and protease inhibitor cocktail set III, EDTA-free (Cat. 539134, Calbiochem)). Lysates were pre-cleared by centrifugation (18,000 g for 8 minutes at 4°C) and supernatants were collected. Protein concentration in the supernatants was determined using the BCA Protein Assay (Cat. 23227; Pierce). Equal amounts of protein in 1x reducing Laemmli buffer were resolved in SDS-polyacrylamide (5-12.5%) gels and transferred to polyvinylidene difluoride membranes (PVDF, Immobilon-P. Millipore). Membranes were blocked in Tris-buffered saline (20 mM Tris-HCl pH 7.6, 150 mM NaCl) containing 5% non-fat dry milk for 1 hour at room temperature. Membranes were probed with the indicated primary antibodies (diluted in Tris-buffered saline containing 5% non-fat dry milk/5% BSA), followed by enhanced chemiluminescent detection (Supersignal West Pico Chemiluminescent Substrate, Pierce). β-actin or tubulin were used as protein loading controls.

Chromatin and soluble protein fractionation

Cells growing in 10 cm dishes were rinsed twice with cold PBS (adjusted to the same osmolarity of the culture medium) and lysed at 4ºC for 15 min with occasional tapping in buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5

mM $MgCl_2$, 0.34 M sucrose, 10% glycerol, 1 mM iodoacetamide, 5 mg/ml leupeptin, 5 mg/ml aprotinin, 1 mg/ml pepstatin A, 10 mM β-glycerophosphate, 1 mM PMSF, 10 mM NaF, 10 mM sodium orthovanadate and 0,1% Triton X-100. Nuclei were precipitated by low-speed centrifugation (at 1,300 g, 4°C for 4 min), and the supernatant (soluble nucleo-cytoplasmic fraction) was separated for protein quantification and boiled in reducing 1x Laemmli buffer for 10 min at 100°C. Nuclei were washed twice in buffer A, and then were either boiled in 1x Laemmli buffer for 1 hour min at 100°C, or incubated in buffer A plus DNase I (Worthington, 0.2 mg/ml, room temperature, 15 min in orbital shaker) to extract chromatin-associated proteins. Samples containing proteins released from chromatin were centrifuged (13,000 g, 4°C, 10 min) to sediment debris and the supernatants were boiled for 10 min at 100°C in 1x Laemmli reducing buffer.

Chromatin immunoprecipitation

Two days before stimulation 0.4 x 10^6 MEFs (wild-type-AMPK) were plated in 10 cm-diameter dishes, and one day before applying the stimulus the medium was replaced by fresh isotonic one (300 mOsm/kg). Cells were treated during the indicated times with 500 mOsm/kg with or without Torin1 (100 nM) pretreatment. Cells were fixed with 1% formaldehyde for 10 minutes at room temperature and with continuous agitation. Formaldehyde was then quenched with glycine (final concentration of 125 mM) for 5 minutes. After washing the plates twice with cold PBS and once with cold PBS + PMSF, cells were lysed in SDS lysis buffer (50 mM Tris-HCl pH 8.1, 1% SDS, 10 mM EDTA, and protease inhibitor cocktail set III, EDTA-free (Cat. 539134, Calbiochem)) for 5-10 minutes and then stored at -80ºC for at least 24 hours. Lysates were sonicated in 1.5 ml polypropylene tubes with a bath sonicator (Diagenode Bioruptor) for 8 cycles of 30 seconds on and 30 seconds off on high power for cells stimulated for ≤ 4 hours and

6 cycles for cells stimulated 8 hours, to obtain DNA fragments between 500-1000 bp, and centrifuged 10 minutes at 18,000 g to remove insoluble debris. Supernatants were collected and 10% of each sample was separated to use as a measure of chromatin input for normalization. The rest of the sample was diluted ten times in ChIP dilution buffer (16.7 mM Tris-HCl pH 8.1, 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 167 mM NaCl and protease inhibitor cocktail set III, EDTA free) for immunoprecipitation. Samples were precleared with protein A-sepharose beads (Amersham, cat. 17-0780-01) that had been preadsorbed with 57 μg/ml salmon sperm DNA (Roche, cat. 11 467 140 001) and 0.1 μg/μl of bovine serum albumin (BSA) (New England Biolabs) by rocking for 1 hour at 4ºC. After removing the preclearing beads, the specific antibodies were added to the lysates and incubated overnight at 4º C in rotation. Protein A-sepharose beads pre-adsorbed with 60 μg/ml salmon sperm DNA and 0.1 μg/μl of BSA were then added, incubated for 3-4 hours at 4ºC, and then washed once with low salt wash buffer (20 mM Tris-HCl pH 8.1, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl), once with high salt wash buffer (20 mM Tris-HCl pH 8.1, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl), once with LiCl immune complex wash buffer (10 mM Tris-HCl pH 8.1, 250 mM LiCl, 1% NP40, 1% sodium-deoxycholate, 1 mM EDTA) and twice with 1x TE (10 mM Tris-HCl pH 8.1 and 1 mM EDTA). To elute the DNA, beads were incubated with 200 μl elution buffer (1% SDS and 100 mM $NaHCO_3$) for 20 minutes at room temperature with shaking. To reverse the crosslinking, samples were incubated overnight at 65º C with 1 μg RNase per sample (Roche, cat. 11 119 915 001) and 200 mM NaCl final concentration. DNA was purified using the QIAGEN PCR purification system (Cat. 28104). DNA was then subjected to real time quantitative PCR (RT-qPCR) with the primers described above. Immunoprecipitated DNA from each sample was normalized to its respective chromatin input.

Western blotting protein quantification

Band quantification was determined using ImageJ software. To quantify each Western blot (blotted with one specific antibody) the following commands were executed:

1. Open the image file
2. Go to *Analyze>Gels>Gel Analyzer Options* and click into the boxes for label with percentages and invert peaks.
3. Choose the rectangular selection tool and draw a rectangle around the first band.
4. Press the 1 button. A new window will pop up with a copy of the image and a label over the first rectangular selection.
5. With arrow keys move the rectangle over the next band. Press 2 to place a selection around the following band. Repeat this for each band on the Western blot, moving the box and pressing 2 to place each new selection.
6. When finished, press 3, which pops up a new window with a profile plot of each band.
7. Choose the straight line selection tool. At the base of each peak, draw a line from one side of the peak to the other. This encloses the area of the peak. The tails to either side of the peak are the background signal.
8. When each peak has been closed off at the base with the straight line tool, choose the wand tracing tool from the tool palette.
9. With the wand, click inside the peak. Repeat this for each peak as you go down for each profile plot.
10. When each peak has been selected, go to *Analyze>Gels>Label Peaks*. This labels each peak with its size expressed as a percentage of the total size of all the measured peaks.

To analyze the data:

1. Go to the Results window (that has pop up) and choose *Edit>Copy All* to copy the results.
2. Place the data in a spreadsheet. One of the peaks should be the standard.
3. In a new column next to the Percent column, divide the Percent value for each sample by the Percent value for the standard.
4. The resulting column of values is a measure of the relative density of each peak, compared to the standard, which will obviously have a relative density of 1.
5. Multiply the relative density of each peak by 100 to obtain as a percentage.

Statistical analysis

The statistical significance of the experimental data was determined with paired Student's t-test.

RESULTS

The main body of this thesis has been published in the following article:

ARTICLE:

Transcriptional regulation of gene expression during osmotic stress responses by the mammalian target of rapamycin.

M. Carmen Ortells, Beatriz Morancho, Katherine Drews-Elger, Benoit Viollet, Keith R. Laderoute, Cristina López-Rodríguez and Jose Aramburu

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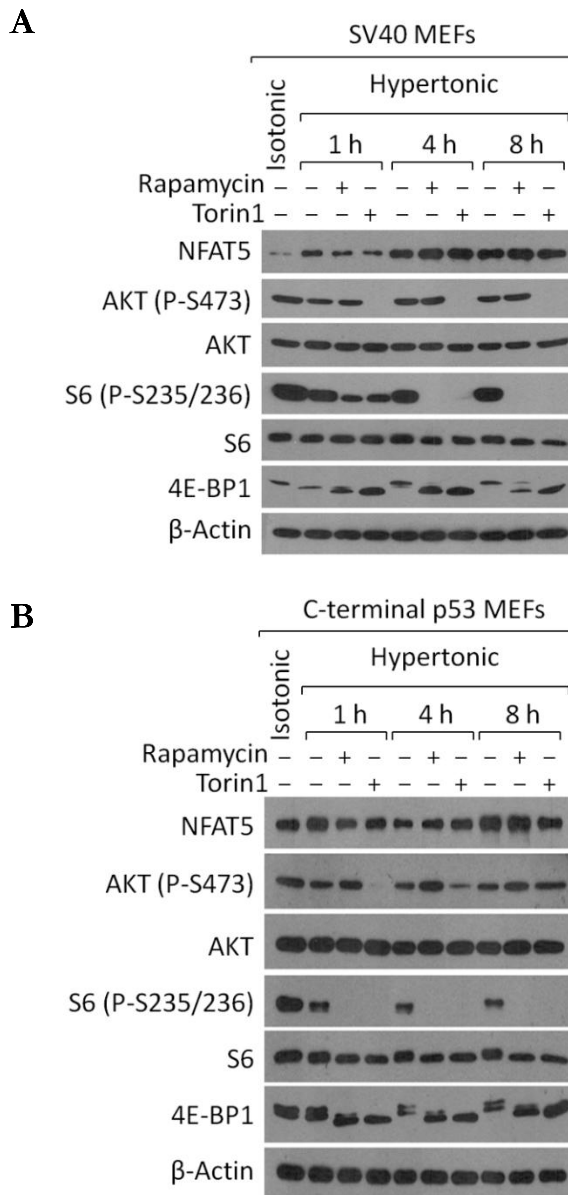
SECTION I: mTOR remains active under sustained osmotic stress and regulates the induction of osmostress response genes

The existing literature about the effects that osmotic stress can cause to the mTOR pathway and processes controlled by it is still confusing, with some publications showing totally inhibitory effects (Martini and Kruppa, 1979), (Kruppa and Clemens, 1984), (Parrott and Templeton, 1999), (Desai et al., 2002), (Inoki et al., 2003), (Naegele and Morley, 2004), (Smith et al., 2005) whereas others reported transiently activatory effects (Bae et al., 2008), (Kwak et al., 2012). One possible explanation for these apparent discrepancies could be that different studies used different experimental approaches and intensities of hypertonic stress. In this context, we and others have shown that different types of mammalian cells such as mouse embryonic fibroblasts (MEFs) and T cells can resist moderate osmotic stress, in the range of 400 to 500 mOsm/kg, and induce an adaptation program to maintain their proliferative capacity (Cai et al., 2004), (Go et al., 2004), (Drews-Elger et al., 2009), (Berga-Bolaños et al., 2010). These observations strongly suggested that the mTOR pathway must remain functional under moderate stress conditions. Therefore we tested whether this pathway was active during hypertonic stress conditions of 500 mOsm/kg, which are strong enough to induce a robust osmoadaptive gene expression response but not so high that they would suppress cell growth and proliferative capacity.

To determine the overall mTOR pathway status we analyzed some key mTORC1 downstream substrates as well as an mTORC2 substrate in two different immortalized MEFs obtained by different immortalization protocols. Thereby we use two different readouts for mTORC1 activity status, the phosphorylation of Ser235/236 of the ribosomal S6 (S6 (P-S235/236)) protein and the 4E-BP1 electrophoretic mobility shift. To follow

the mTORC2 activity we used the phosphorylation of Ser473 on Akt (AKT (P-S473)). Our results showed that although osmostress caused a partial inhibition of both mTOR complexes, mainly in the first hour of stimulation, they retained substantial mTOR activity under hypertonic stress conditions, induced by hypernatremia by NaCl addition (**Figure 1A**, **1B and 1C**).



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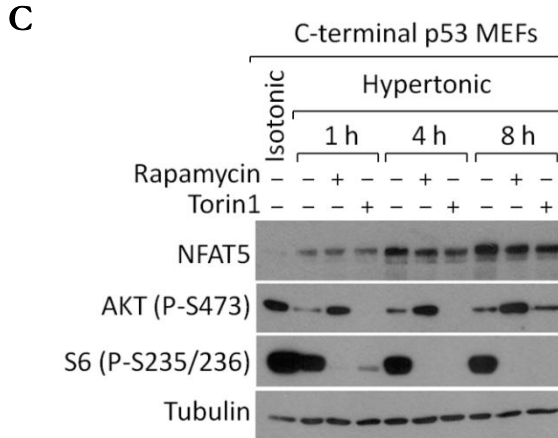


Figure 1. Effect of hypertonic stress on mTOR signaling pathway. Wild-type MEFs immortalized with a p53-carboxy-terminal fragment **(A)** or by SV40 large T antigen **(B and C)** were cultured in isotonic (300 mOsm/kg) or hypertonic medium (500 mOsm/kg, upon addition of 100 mM NaCl) without or with rapamycin (50 nM) or Torin1 (100 nM). NFAT5, phospho-S6 (Ser 235/236), S6, phospho-AKT (Ser 473), AKT, 4E-BP1, and β-actin or tubulin (as loading controls) were detected by Western blotting.

The induction of NFAT5 protein expression observed in this same experiment served as an indicator of the ongoing osmostress response, being more expressed at longer time points under hypernatremic conditions. This experiment also showed that rapamycin specifically inhibited mTORC1 complex activity, but not that of mTORC2 complex, in MEFs exposed to osmotic stress, whereas both mTOR complexes were efficiently inhibited by Torin1, an ATP-competitive mTOR inhibitor whose mechanism of action is unrelated to that of rapamycin (Thoreen et al., 2009).

Previous experiments done in our laboratory and mainly presented in the PhD thesis report of Beatriz Morancho (http://www.tdx.cat/handle/10803/77906) showed that HEK293 cells transfected with the NFAT5-responsive ORE-Luc reporter and exposed to hypertonic conditions (500

mOsm/kg) in the presence of rapamycin had a significant inhibition of the reporter activity compared to the hypertonic condition alone (**Figure 2; from B. Morancho PhD thesis**). Furthermore, she showed that mitogen-stimulated splenocytes exposed to hypertonic conditions (400 mOsm/kg) during 8 hours and pretreated with rapamycin had defects in the induction of some NFAT5-dependent osmostress response genes (**Figure 3; from B. Morancho PhD thesis**).

Taking into account these previous results we wanted to confirm if these defects observed in HEK293 and splenocytes are also observed in MEFs. For this purpose we transfected MEFs with the same NFAT5-responsive ORE-Luc reporter and stimulated them with hypertonic stress (500 mOsm/kg) alone or with a pretreatment with rapamycin. Our results confirmed that the reduction of the reporter activity was of a similar magnitude as in HEK293 cells (**Figure 4)**.

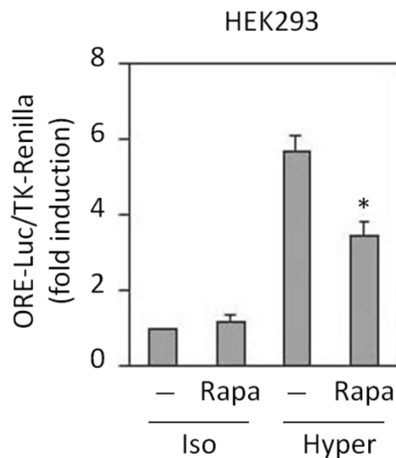


Figure 2. Effect of rapamycin on activation of the NFAT5-regulated reporter ORE-Luc in HEK293 cells (*from previous experiments*). HEK293 cells cotransfected with the ORE-Luc reporter and a Renilla luciferase vector were cultured in isotonic (300 mOsm/kg) or hypertonic medium (500

mOsm/kg) during 24 hours without or with rapamycin (200 nM). Luciferase activity was normalized to that of Renilla. The graphic represents the mean ± S.E.M of three independent experiments (* $p < 0.05$).

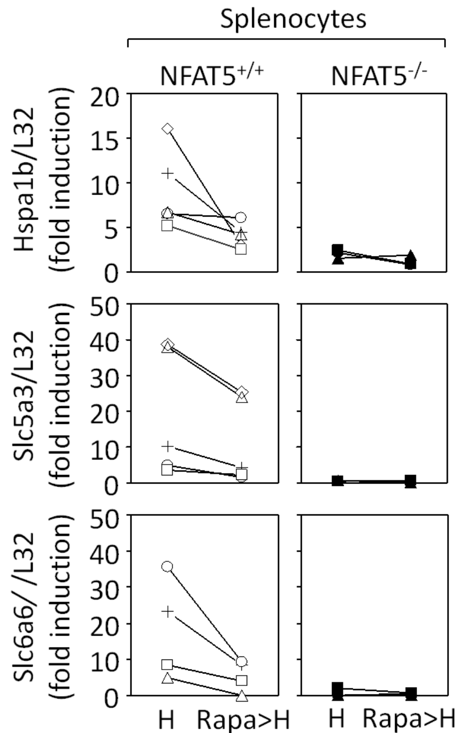


Figure 3. Effect of rapamycin on the expression of osmostress response genes in mitogen-stimulated splenocytes (*from previous experiments*). RNA was isolated from *Nfat5*$^{+/+}$ and *Nfat5*$^{-/-}$ splenocytes that had been stimulated with 2.5 μg/ml concanavalin A plus 25 ng/ml of IL-2 during 24 hours and then cultured in isotonic (300 mOsm/kg) or hypertonic medium (400 mOsm/kg) during 8 hours in the absence or presence of rapamycin (200 nM). Relative mRNA abundance for *Hspa1b*, *Slc5a3* and *Slc6a6* were determined by RT-qPCR, normalized to *L32* mRNA levels, and represented relative to isotonic conditions in wild-type cells, which was given an arbitrary value of 1. Four to five individual experiments are shown, each represented by a different symbol.

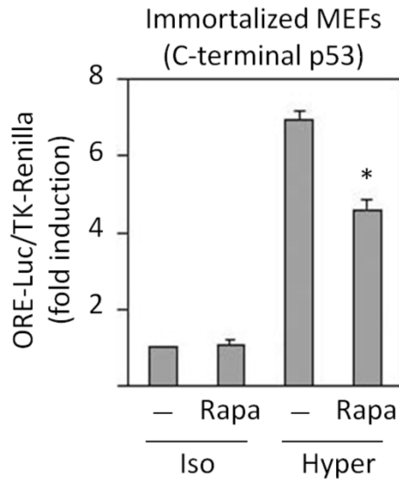


Figure 4. Effect of rapamycin on the activation of the NFAT5-regulated reporter ORE-Luc in wild-type MEFs. C-terminal p53 immortalized MEFs cotransfected with the ORE-Luc reporter and a Renilla luciferase vector were cultured in isotonic (300 mOsm/kg) or hypertonic medium (500 mOsm/kg) during 24 hours without or with rapamycin (200 nM). Luciferase activity was normalized to that of Renilla. The graphic represents the mean ± S.E.M of three independent experiments (* $p < 0.05$).

In the same way we tested the effect of rapamycin and Torin1 on the expression of the endogenous mRNAs of several osmoresponsive genes in response to hypertonic stress (500 mOsm/kg) induced by either hypernatremia or sorbitol in MEFs. Comparison of both inhibitors on the induction of *Akr1b3* (aldose reductase), *Aqp1* (aquaporin 1), *Hspa1b* (Hsp70.1) and *Slc5a3* (sodium/myoinositol cotransporter, SMIT) showed that Torin1 effect was moderately stronger than rapamycin (**Figure 5).**

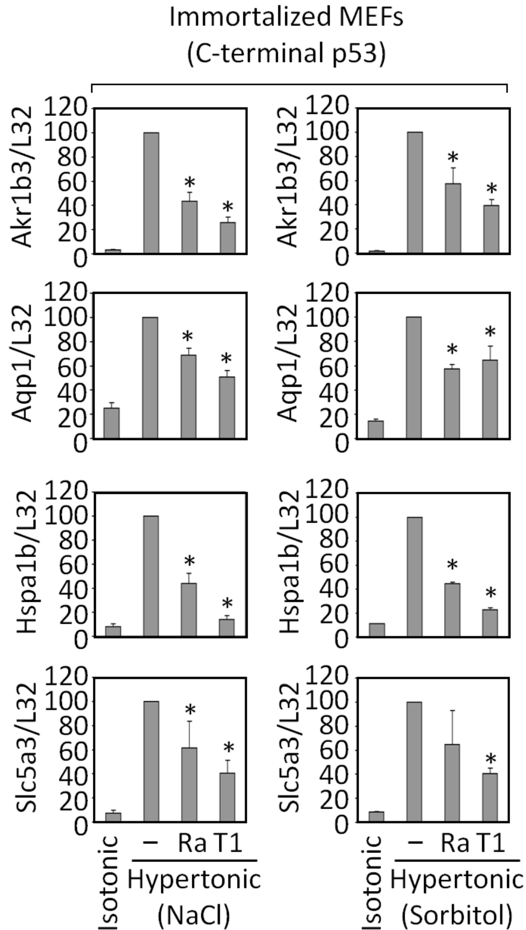


Figure 5. Effect of rapamycin and Torin1 on the mRNA expression of some osmoresponsive genes. RNA was isolated from C-terminal p53 immortalized MEFs cultured in isotonic (300 mOsm/kg) medium or medium made hypertonic (500 mOsm/kg) by addition of 100 mM NaCl or 200 mM sorbitol, during 8 hours without or with 50 nM rapamycin (Ra) or 100 nM Torin1 (T1). mRNA abundance for *Akr1b3*, *Aqp1*, *Hspa1b* and *Slc5a3* normalized to *L32* mRNA is represented relative to hypertonic conditions (100%). Bars represent the mean ± S.E.M. of five independent experiments (* $p < 0.05$).

Moreover, we confirmed that hypertonic stress induced by sorbitol addition was also capable of increasing NFAT5 protein levels, as NaCl does **(Figure**

6). These results show that not only hypernatremia induced by NaCl addition but also hypertonicity induced by sorbitol addition can initiate an osmoprotective response, with a subsequent NFAT5 increased synthesis and mTOR-sensitive induction of mRNA expression of these osmoresponsive genes.

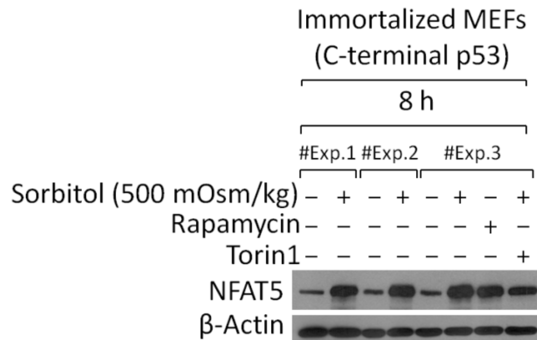


Figure 6. NFAT5 expression under hypertonic stress induced by sorbitol. C-terminal p53 immortalized MEFs were cultured in isotonic (300 mOsm/kg) or hypertonic medium (500 mOsm/kg, upon addition of 200 mM sorbitol) without (-) or with (+) rapamycin (50 nM) or Torin1 (100 nM). NFAT5 and β-actin (as loading control) were detected by Western blotting. Figure shows three independent experiments (Experiment 1 (#Exp.1), Experiment 2 (#Exp.2) and Experiment 3 (#Exp.3)).

Because mTOR can regulate the synthesis and subsequent processing of some ribosomal RNAs (White, 2005) as well as translation of the mRNAs encoding ribosomal proteins (Meyuhas, 2000) and we normally use *L32* mRNA as a normalization control we wanted to be sure that the expression of *L32* mRNA, our normalization control in mRNA experiments, was not affected by pretreatments with rapamycin or Torin1. mRNA levels of *L32* were compared with another usual normalization control, *Gapdh*, and confirmed as insensitive to that mTOR inhibitors (**Figure 7**).

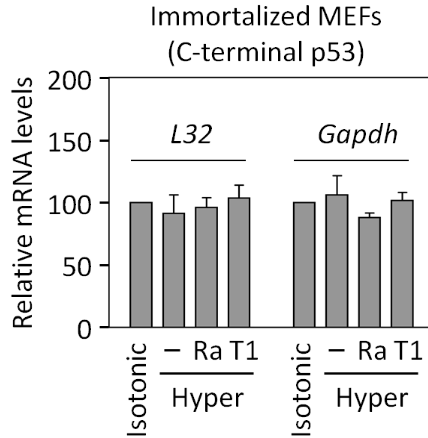


Figure 7. Effect of rapamycin and Torin1 on the mRNA expression of housekeeping genes used as normalization controls. RNA was isolated from C-terminal p53 immortalized MEFs cultured in isotonic (300 mOsm/kg) or hypertonic medium (500 mOsm/kg upon addition of 100 mM NaCl) without or with rapamycin (Ra, 50 nM) or Torin1 (T1, 100 nM). mRNA abundance for *L32* and *Gapdh* is shown. Bars represent the mean ± S.E.M. of three independent experiments.

Experiments also showed that rapamycin and Torin1 caused a stronger inhibition of gene expression at later time points (**Figure 8)**, in parallel with the progressive inactivation of mTOR activity (**Figure 1A).**

The finding that mTORC2 was still active, and in some cases even up-regulated due to the inhibition of the mTORC1-mediated negative feed-back loop (Wan et al., 2007), in rapamycin-treated MEFs exposed to osmostress indicated that the downregulation of gene expression caused by rapamycin was due to the inhibition of mTORC1, and suggested that the greater potency of Torin1 likely reflected its ability to inhibit mTORC1-dependent functions better than rapamycin (Thoreen et al., 2009). This interpretation was also supported by the observation that Torin1 caused a complete dephosphorylation of the mTORC1 substrate 4E-BP1 (**Figure 1A and 1B)**

whereas rapamycin had a partial effect, in agreement with previous publications (Choo et al., 2008), (Thoreen et al., 2009), showing that rapamycin had a partial effect on some of the processes and phosphorylations relying on mTORC1.

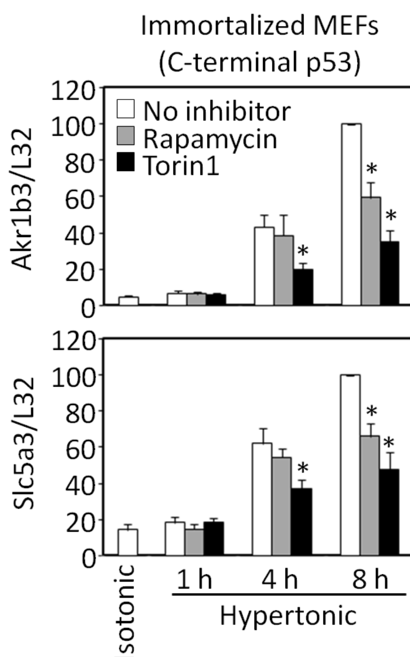


Figure 8. Effect of rapamycin and Torin1 on the mRNA expression of osmoresponsive genes during a time course experiment. RNA was isolated from C-terminal p53 immortalized MEFs cultured in isotonic (300 mOsm/kg) or hypertonic medium (500 mOsm/kg, upon addition of 100 mM NaCl) without or with rapamycin (50 nM) or Torin1 (100 nM). mRNA abundance for *Akr1b3* and *Slc5a3* normalized to *L32* mRNA is represented relative to the 8-hours time point (100%). Bars represent the mean ± S.E.M. of four independent experiments (* $p < 0.05$).

We had observed that although mTORC1 was functional in cells exposed to a hypertonicity of 500 mOsm/kg, its activity was partially decreased. Since it

has been reported that AMPK can be transiently activated by intense osmotic stress conditions (>600 mOsm/kg) (Barnes et al., 2002), (Hawley et al., 2010), we tested whether the lower osmostress levels used in our assays activated AMPK, which could contribute to cause a partial downregulation of mTORC1 activity. Our results showed that hypertonic conditions of 500 mOsm/kg had minimal or no effect on the LKB1-mediated phosphorylation of AMPKα in T172 (P-AMPKα) in MEFs, in contrast with the strong phosphorylation induced by the AMPK activator AICAR and the inhibition of phosphorylation of S6 at Ser235/236 caused by this drug in this same experiment **(Figure 9)**.

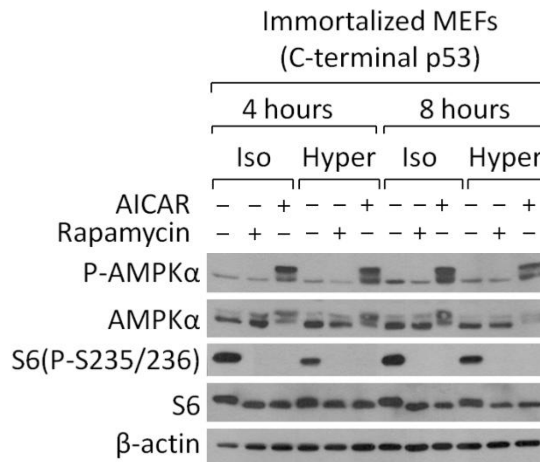


Figure 9. Effect of osmotic stress on the activation of AMPK. (A) C-terminal p53 immortalized MEFs were cultured in isotonic (Iso, 300 mOsm/kg) or hypertonic medium (Hyper, 500 mOsm/kg, upon addition of 100 mM NaCl) without (-) or with (+) AICAR (1 mM) or rapamycin (50 nM). Phospho-AMPKα (Thr 172), AMPKα1 and α2, phospho-S6 (Ser 235/236), S6 and β-actin (loading control) were detected by Western blotting. This figure is representative of three independent experiments.

<u>SECTION II</u>: Identification of mTOR-regulated genes under an osmostress response

The involvement of mTORC1 in promoting protein synthesis have been extensively studied, however, over the past years several studies revealed that mTORC1 also plays an important role in controlling specific transcriptional programs, through activation of key transcription factors. The results above showed that mTOR was active in proliferating cells exposed to physiopathologic osmostress conditions and contributed to enhance the expression of several NFAT5-dependent genes upon osmostress conditions **(Section I results)**. In this way, as our results indicated that known NFAT5-dependent genes were affected by mTOR inhibition we decided to determine if the effect seen on the NFAT5-dependent transcriptional response was mediated by direct regulation of NFAT5 by mTOR.

For this purpose, we did several co-immunoprecipitation assays with some of the main protein components of mTORC1 and mTORC2 complexes to asses if NFAT5 was bound to any of those mTOR complexes. In parallel, because most of the mTOR interacting proteins can be also mTOR substrates (Yokogami et al., 2000), (Land and Tee, 2007), (Hong et al., 2008), and NFAT5 is phosphorylated when activated by hyperosmotic stress by several signaling kinases, we decided to analyze whether mTOR could also directly phosphorylate NFAT5 regulating its activity. However, although we tried to elucidate this putative interaction as well the direct phosphorylation of NFAT5 by mTOR we finally did not obtain conclusive results.

Nonetheless, in order to get a broader picture of genes whose expression was regulated by osmostress and sensitive to mTOR, covering now both NFAT5-regulated genes as well as NFAT5-independent ones, we decided to

perform RNA microarray experiments under hypertonic conditions, giving us helpful results for our study.

For this general analysis of gene expression we used spontaneously immortalized MEFs derived from mice of 129sv background (NIH3T3 protocol for MEF immortalization). RNA from three independently performed experiments was analyzed with the Affymetrix Mouse Gene 1.0 ST array. Cells were exposed to hypertonic stress for 8 hours, since our previous experiments had shown that this time point was sufficient for a robust induction of osmotic stress response genes **(Figure 8)**, but not so long that cells had fully adjusted to a hypertonic environment (Lee et al., 2011) nor to the inhibition of mTOR kinase activity (Düvel et al., 2010). For these assays we used Torin1 instead of rapamycin because it had a stronger effect in downregulating osmostress response genes and has a greater potency to inhibit mTORC1 activity (Thoreen et al., 2009). For these reasons we considered that the use of Torin1 would allow a clearer identification of mTOR-regulated genes when comparing independent samples in the microarray analysis. These experiments included a parallel analysis of osmoresponsive genes in NFAT5-deficient MEFs to identify NFAT5-dependent genes.

The microarray analysis revealed that Torin1 pretreatment significantly affected the expression of numerous genes in cells exposed to osmotic stress **(Figure 10)**. We identified 107 genes that were induced by osmotic stress ≥ 2 times with a p value < 0.01, of which 24 (22%) were repressed by Torin1 by at least 35% **(Table 1)**. This analysis also showed that another 74 genes were repressed by ≥ 50% by osmotic stress, and the inhibition of 8 of them (11%) was attenuated by Torin1 **(Table 2)**. Moreover, we detected very few mRNAs that were consistently inhibited by Torin1 at 8 hours, and none upregulated, in non-stressed cells **(Table 3)**. This modest effect likely

reflected that inhibition of mTOR for the last 9 hours in cells that until then had been growing at normal rates had a limited impact on the representation of preexisting mRNAs. Furthermore, our analysis showed that the combination of osmotic stress and Torin1 downregulated the expression of 12 genes, and increased the expression of 2, that were not significantly affected by either osmotic stress or Torin1 alone compared to basal conditions **(Table 4)**. As an overview of this analysis, it can be observed that mTOR regulated the induction and repression of a selective pattern of osmostress responsive genes.

Comparison of wild-type and NFAT5-deficient MEFs showed that 74% (79 of 107) of the genes induced by osmostress were regulated by NFAT5, of which 17 (22%) were Torin1-sensitive **(Table 1)**. Of 28 NFAT5-independent genes, Torin1 inhibited the induction of 7 (25%) of them. Therefore, although a majority of the genes strongly induced by osmostress were NFAT5-regulated, there was no association between the dependence on NFAT5 and sensitivity to mTOR inhibition, excluding NFAT5 as a main requisite for those genes that are mTOR-sensitive upon osmotic stress conditions.

After analyzing the microarray we decided to validate by RT-qPCR a sample of 14 new genes detected in our microarray experiments chosen by their robust induction by osmostress, and also illustrative of Torin1-sensitivity and insensitivity, as well as NFAT5 dependence and independence **(Figure 11)**. As an internal control we also included 4 genes that in our previous experiments (**Figures 3 and 5**) had been validated as NFAT5-dependent and mTOR-sensitive (*Akr1b3, Hspa1b, Slc5a3* and *Aqp1*) **(Figure 11)**.

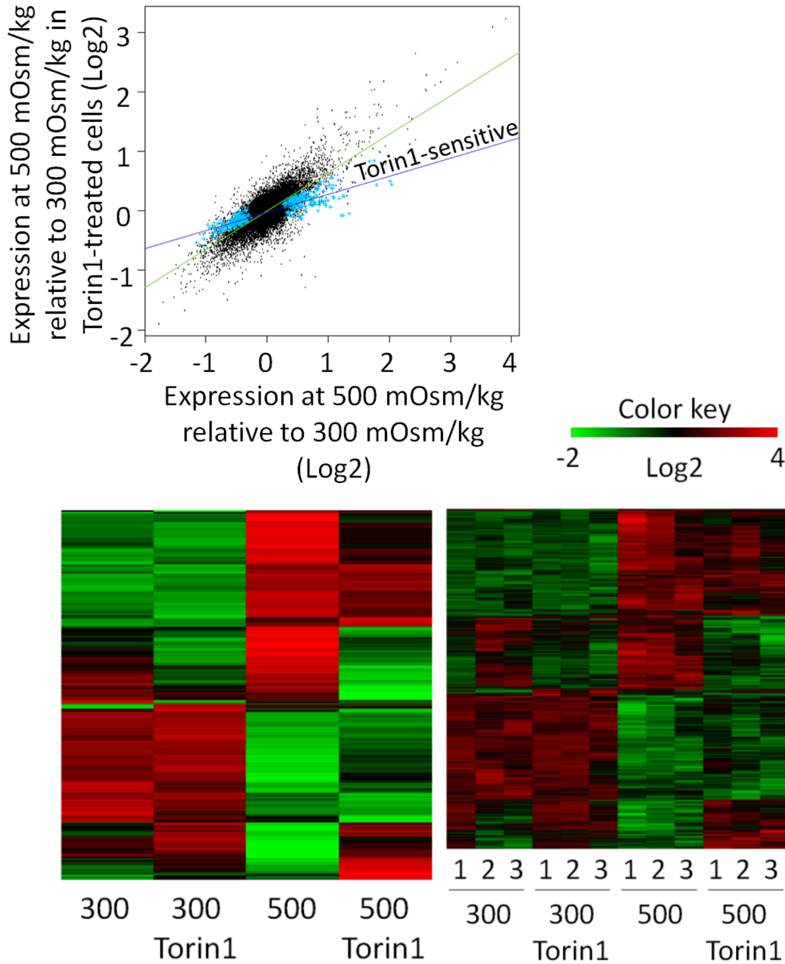


Figure 10. Gene expression and RNA microarray data analysis. Scatter plot in which the black dots represent the expression pattern of the entire dataset and the blue crosses the genes whose induction by osmostress was repressed by Torin1 or whose repression by osmostress was attenuated by Torin1. The bottom panels show the heat maps of the gene probes found to be regulated by osmostress and their sensitivity to Torin1. Expression levels correspond to the log2 of the mean of three independent samples per condition (left panel) and for each individual sample (right panel). The brightness of red (induced) and green (downregulated) represents the magnitude of the change in the expression of each gene. Gene names and values are provided in **Tables 1 to 4**.

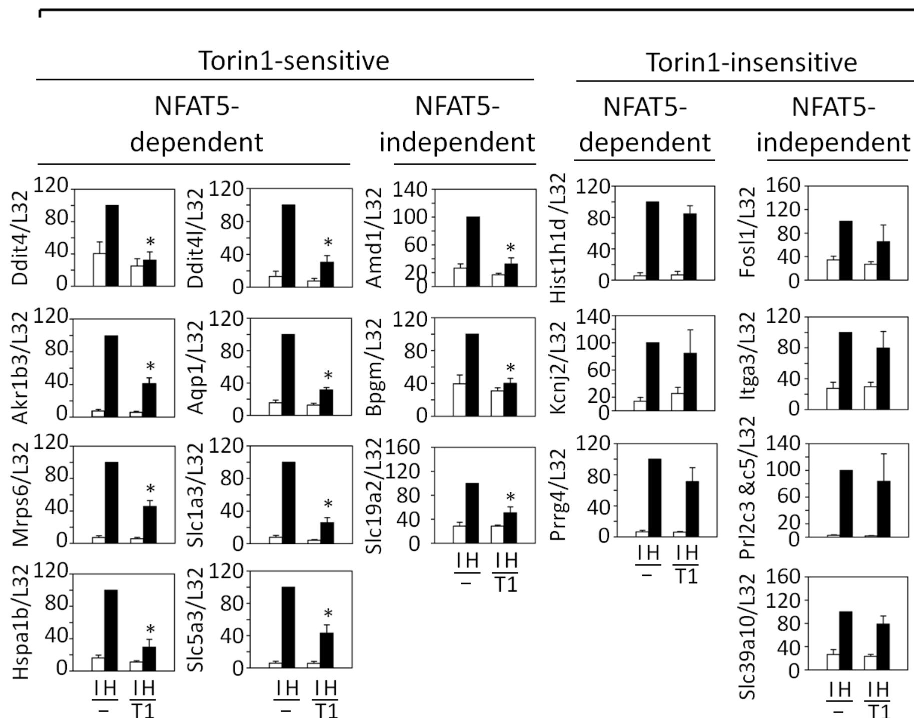


Figure 11. Identification of osmostress-regulated genes and their sensitivity to mTOR inhibition. RT-qPCR validation of Torin1 sensitivity of osmoresponsive genes identified in the microarray analysis. RNA from the three individual samples used for the microarray analysis plus one additional experiment were analyzed by RT-qPCR for the expression of the indicated genes and normalized to *L32*. Samples correspond to wild-type MEFs (MEFs NFAT5 wild-type, spontaneously immortalized by the NIH3T3 protocol) cultured in isotonic (I, white bars) (300 mOsm/kg) or hypertonic (H, black bars) medium (500 mOsm/kg) during 8 hours without or with Torin1 (T1, 100 nM). RNA levels are represented relative to the amount of mRNA in hypertonic conditions, which was given an arbitrary value of 100. Bars represent the mean ± S.E.M. of four independent experiments (* $p < 0.05$).

This analysis confirmed the sensitivity to Torin1 of the NFAT5-dependent genes *Ddit4*, *Ddit4l*, *Slc1a3* and *Mrps6* and the NFAT5-independent *Amd1*, *Bpgm* and *Slc19a2* **(Figure 11).** Also consistent with the microarray data, Torin1 had minimal or no effect on the induction of the NFAT5-dependent *Hist1h1d*, *Kcnj2* and *Prrg4* and the NFAT5-independent genes *Fosl1*, *Itga3*, *Prl2c3/c5*, and *Slc39a10* **(Figure 11)**. Moreover, although we observed that *Akr1b3*, *Aqp1* and *Mrps6* had not scored as Torin1-sensitive with sufficient statistical significance in the microarray analysis, they showed to be inhibited by Torin1 when analyzed by RT-qPCR. This result likely reflects differences in sensitivity between both techniques, and suggests that the number of osmoresponsive genes that could be affected by mTOR inhibition might be greater than detected in the microarray experiments.

Furthermore, to rule out specific gene expression patterns from one immortalized MEF cell line, validation by qRT-PCR was done using several genes found in the microarray analysis being representative of those Torin1-sensitives (*Amd1*, *Bpgm*, *Ddit4*, *Ddit4l* and *Mrps6*) in an independent MEF cell line (C-terminal p53 immortalized MEFs). This analysis confirmed that these genes were also inhibited with Torin1 as well as by rapamycin **(Figure 12)**, similarly to what we had previously observed with our internal controls *Akr1b3*, *Aqp1*, *Hspa1b*, and *Slc5a3* **(Figures 5 and 11)**.

Besides genes with already known osmoprotective functions, such as *Akr1b3*, *Aqp1*, *Hspa1b*, and *Slc5a3* (Miyakawa et al., 1999), (Ma et al., 1998), (Shim et al., 2002), (Haussinger, 1996), mTOR regulated the expression of others that had not been previously shown to respond to osmostress, although they had been described in the context of other stress responses: *Ddit4* and *Ddit4l* in hypoxia, DNA damage and oxidative stress (Corradetti et al., 2005), *Figf/VEGF-D* (Nilsson et al., 2004) in hypoxia, and *Slc19a2* in DNA damage (Lo et al., 2001) **(Tables 1 to 5)**. Some of the Torin1-inhibited

genes encoded for positive regulators of proliferation: *Amd1* (Nishimura et al., 2002), *Figf/VEGF-D* (Liu et al., 2008) and *Tacstd2/Trop2* (Cubas et al., 2010) **(Table 1)**. The set of genes repressed by osmostress and whose repression was attenuated by Torin1 included *Atg10*, a regulator of autophagosome formation (Boya et al., 2005), and *Gstz1*, which participates in cellular responses to oxidative stress (Blackburn et al., 2006) **(Table 2)**. Another gene found in this analysis was *Sesn2*, whose basal expression was not affected by osmostress, but was reduced by Torin1 in isotonic and hypertonic conditions **(Table 3)**. *Sesn2* can be induced by DNA damage, and its product Sestrin 2 has been shown to inhibit mTOR via TSC2 (Budanov and Karin, 2008). Torin1 also enhanced the expression of *Pdk4* and *Pim1* in cells exposed to osmostress **(Table 4)**. These genes have been described in the context of cellular responses to starvation or inhibition of growth signaling (Wu et al., 2000), (Fox et al., 2005). The stress-related function of the different genes is summarized in **Table 5**. It is important to note that the expression of several of these genes was affected to a greater or lesser degree, but not eliminated, upon inhibiting mTOR **(Tables 1 to 4)** suggesting that this pathway regulated their magnitude of induction but was not an absolute requirement.

The finding that mTOR regulated the expression of a complex pattern of genes raised the question of how they might contribute to cell functions under stress. However, since the contribution of mTOR to their magnitude of expression varied depending on the particular gene, we considered that trying to reproduce the effect of mTOR inhibition by suppressing or overexpressing combinations of genes would be complex. Nonetheless, because mTOR contributes to growth and proliferation we tested whether mTOR activity was important to sustain these processes in cells exposed to osmostress.

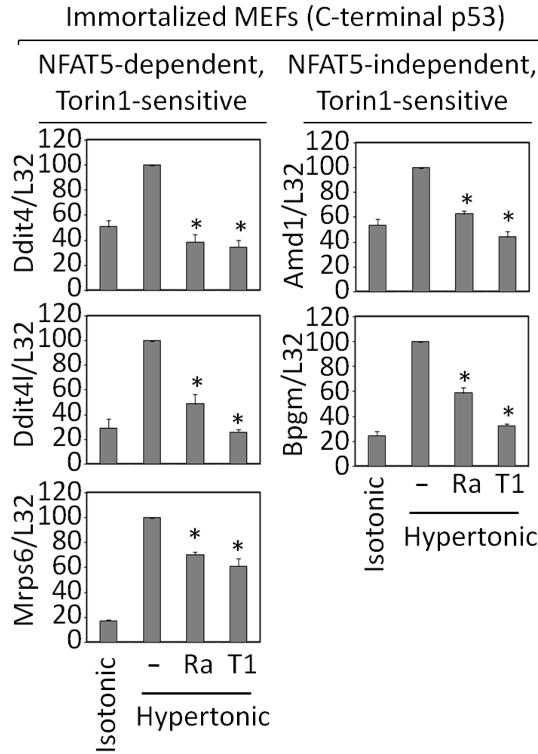


Figure 12. Microarray validation by qRT-PCR in an independent MEF cell line. RNA was isolated from C-terminal p53 immortalized MEFs cultured in isotonic (300 mOsm/kg) or hypertonic medium (500 mOsm/kg) during 8 hours in the absence or presence of rapamycin (Ra, 50 nM) or Torin1 (T1, 100 nM). Relative mRNA abundance was determined by RT-qPCR and normalized to *L32* mRNA. RNA levels are represented relative to the amount of mRNA in hypertonic conditions, which was given an arbitrary value of 100. Bars represent the mean ± S.E.M. of four independent experiments (* $p < 0.05$).

For this approach, MEFs were labeled with the fluorescent dye CFDA-SE and then cultured for 48 or 72 hours in isotonic (300 mOsm/kg) or hypertonic medium (500 mOsm/kg) with or without rapamycin or Torin1. As cells proliferate, the concentration of CFDA-SE per cell decreases as the initial label is distributed to daughter cells in successive rounds of division.

Besides proliferation capacity, we monitored the overall viability and cell size by flow cytometry, using the FSC and SSC parameters. MEFs exposed to osmotic stress during 48 and 72 hours proliferated more slowly and are slightly larger than those maintained in isotonic conditions, but showed comparable viability **(Figure 13A and 13B)**. Inhibition of mTOR impaired cell proliferation and caused a decrease in their size, clearly evident in Torin1-treated cells, in both isotonic and hypertonic conditions **(Figure 13B)**.

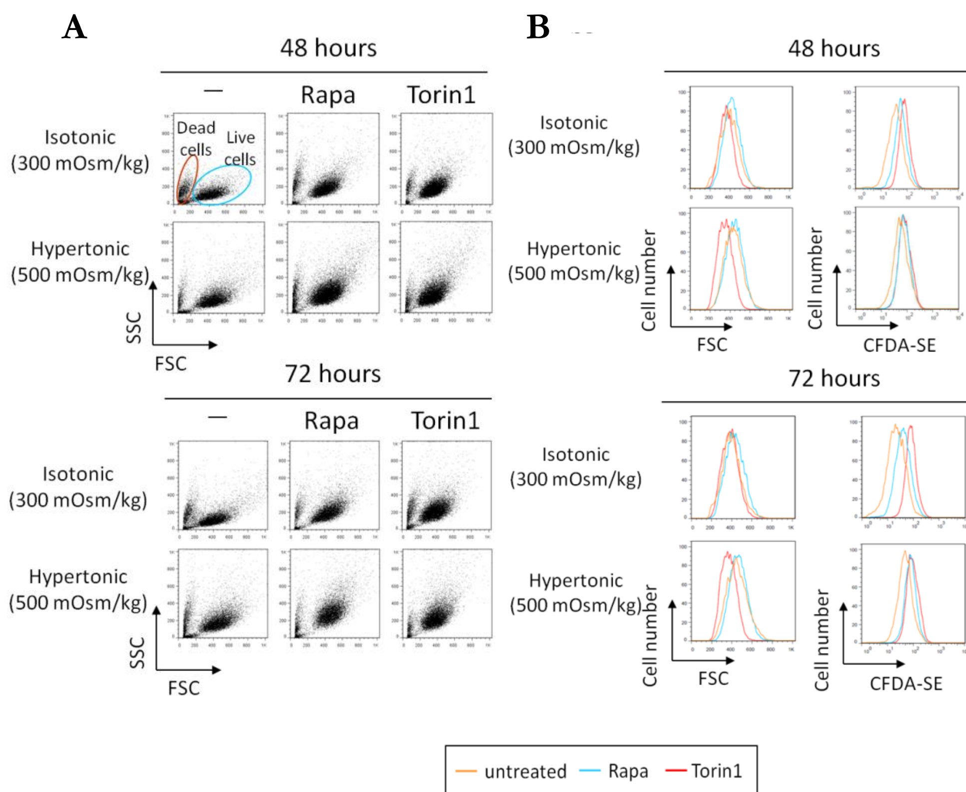


Figure 13. Effect of osmotic stress and mTOR inhibitors on cell growth and proliferative capacity. C-terminal p53 immortalized MEFs were labeled with CFDA-SE and cultured during 48 or 72 hours in isotonic (300 mOsm/kg) or hypertonic medium (500 mOsm/kg, upon addition of 100 mM NaCl) without or with rapamycin (Rapa, 50 nM) or Torin1 (100 nM). **(A)** Cell viability

(FSC/SSC dot plots), **(B)** size (FSC parameter) and relative proliferation (proportional to dilution of CFDA-SE signal) were analyzed by flow cytometry. The FSC parameter in **(B)** was analyzed in cells in G1, gated by staining the culture with the DNA dye Hoechst 33342. Flow cytometry graphics are representative of three independent experiments with similar results.

Moreover visual examination of the cells indicated no obvious signs of toxicity under the conditions tested **(Figure 14)**. Altogether, these results indicate that mTOR was required to sustain growth and proliferative capacity in cells exposed to osmostress.

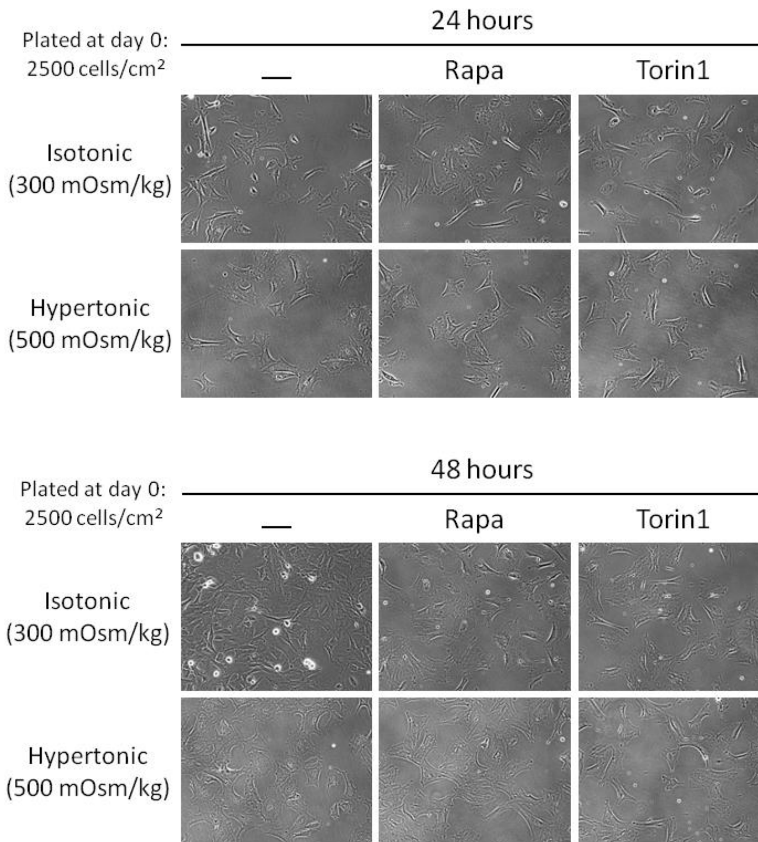


Figure 14. Effect of osmotic stress and mTOR inhibitors on cellular morphology and growth. Immortalized MEFs (C-terminal p53 immortalized)

were labeled with CFDA-SE and cultured during 24 and 48 hours in isotonic (300 mOsm/kg) or hypertonic medium (500 mOsm/kg, upon addition of 100 mM NaCl) without or with rapamycin (Rapa, 50 nM) or Torin1 (100 nM). Phase contrast microphotographs are representative of three independent experiments with similar results.

TABLE 1: Genes that were upregulated ≥ 2-fold ($p < 0.01$) by osmotic stress (500 mOsm/kg) in wild-type MEFs, their sensitivity to Torin1 in wild-type MEF, and response to osmotic stress in NFAT5-deficient MEF.

(1) Fold-induction with respect to isotonic conditions (300 mOsm/kg). Values correspond to the mean of 3 independent experiments.
(2) Inhibition by Torin1 in wild-type MEFs exposed to hypertonic conditions (500 mOsm/kg). Values correspond to the mean of 3 independent experiments.
(3) Fold-induction in NFAT5-deficient MEFs with respect to isotonic conditions in the same cells. Values correspond to the mean of 3 independent experiments.
(4) Not inhibited indicates that Torin1 did not inhibit significantly the mRNA induction by osmotic stress.
(5) Not induced indicates that osmotic stress did not cause a significant increase in the abundance of the mRNA in NFAT5-deficient MEF.

Legend. WT: wild-type MEF; KO: NFAT5-deficient MEF; OS: osmotic stress; N.I.: not induced; N.Ih.: not inhibited.

Affimetrix ID	Gene Name	Δ O.S vs. iso in WT(1)	*p*-value	% Inh. by Torin1 (2)	*p*-value	Δ O.S vs. iso in KO (3)	*p*-value		
10501244	Ampd2	2.104	6.60E-006	35.2	1.46E-003	0.650	8.27E-005	Inhibited by Torin1 >35%	NFAT5-dependent induction
10469066	Ccdc3	2.047	1.53E-003	47.6	3.33E-003	N.I. (5)			
10355205	D630023F18Rik	2.493	4.52E-008	46.2	6.43E-006	N.I.			
10369290	Ddit4	2.151	7.49E-003	61.2	1.64E-003	N.I.			
373	Ddit4l	3.857	6.10E-005	61.8	1.42E-003	N.I.			
00485	Dkc1	2.076	2.60E-004	41.2	3.69E-003	N.I.			
03099	Figf	2.090	2.91E-004	40.6	4.85E-003	N.I.			
50189	Gm10679	3.462	3.33E-004	59.3	4.55E-003	N.I.			
10550208	Gm3994	4.038	1.69E-004	62.9	3.18E-003	N.I.			
10522585	Gm7682	2.002	9.75E-004	42.1	5.90E-003	N.I.			
10450369	Hspa1a/b	2.261	1.61E-008	45.6	7.83E-007	1.358	1.18E-003		
10593449	Layn	2.367	4.20E-006	35.2	3.28E-003	N.I.			
10382300	Map2k6	2.347	1.47E-005	36.8	4.40E-003	0.401	6.62E-006		
10427590	Slc1a3	3.334	4.70E-009	50.1	6.19E-006	0.660	1.14E-003		
10436945	Slc5a3	7.178	2.80E-008	43.8	9.27E-003	0.351	6.58E-005		
10545168	Tacstd2	2.521	4.93E-010	48.0	6.41E-008	N.I.			
10549972	Zscan4c	2.373	6.48E-005	55.5	1.26E-004	N.I.			
10368670	Amd1	2.631	5.67E-007	64.2	2.61E-007	2.705	7.89E-007		Induction not dependent on NFAT5
537179	Bpgm	2.114	6.93E-005	48.3	2.45E-004	1.791	7.55E-004		
10587266	Gclc	2.095	2.55E-005	35.3	3.32E-003	4.062	7.52E-009		
10351259	Slc19a2	2.292	3.48E-005	40.9	2.34E-003	2.566	8.28E-006		
10432176	Snora34	2.123	8.83E-005	46.1	5.78E-004	4.205	3.46E-008		
10564207	Snord116	2.497	1.28E-004	42.3	8.19E-003	3.353	9.63E-006		
10378572	Tlcd2	2.058	9.61E-005	48.2	2.43E-004	4.096	2.79E-008		
10577641	1810011O10Rik	3.275	1.10E-003	N.Ih. (4)		N.I.			
10393751	2900052L18Rik	2.109	1.91E-003	N.Ih.		N.I.			
10419082	5730469M10Rik	5.146	4.52E-005	N.Ih.		N.I.			
10601421	A630033H20Rik	5.401	1.34E-004	N.Ih.		N.I.			
10354768	Akr1b3	3.312	5.71E-006	N.Ih.		N.I.			
10407435	Akr1c18	2.447	6.46E-004	N.Ih.		N.I.			

10577315	Angpt2	2.720	2.17E-004	N.Ih.		N.I.	
10538459	Aqp1	3.609	3.05E-003	N.Ih.		N.I.	
10396831	Arg2	2.502	4.37E-006	N.Ih.		N.I.	
10434934	Bdh1	2.322	3.98E-004	N.Ih.		N.I.	
10362896	Cd24a	2.078	4.00E-003	N.Ih.		N.I.	
10392796	Cd300lb	3.365	2.05E-003	N.Ih.		N.I.	
10521678	Cd38	2.476	8.17E-003	N.Ih.		N.I.	
10435712	Cd80	2.179	4.21E-003	N.Ih.		N.I.	
10571312	Dusp4	2.315	2.10E-003	N.Ih.		N.I.	
10509965	Epha2	2.122	4.00E-003	N.Ih.		N.I.	
10503659	Epha7	2.479	2.76-004	N.Ih.		N.I.	
10523175	Ereg	2.721	3.85E-003	N.Ih.		N.I.	
10395457	Etv1	2.943	2.98E-004	N.Ih.		N.I.	
10351623	F11r	2.107	7.87E-004	N.Ih.		N.I.	
10531944	Gm10047	2.071	1.93E-005	N.Ih.		0.687	6.86E-003
10533725	Gpr81	2.248	9.68E-003	N.Ih.		N.I.	
10404059	Hist1h1c	3.200	4.60E-005	N.Ih.		N.I.	
10404033	Hist1h1d	6.078	2.95E-007	N.Ih.		N.I.	
10408220	Hist1h2ac	6.012	2.70E-009	N.Ih.		N.I.	
10404036	Hist1h2bg	2.506	7.97E-007	N.Ih.		N.I.	
10404038	Hist1h3d	3.004	2.63E-006	N.Ih.		N.I.	
10494407	Hist2h2bb	2.041	1.16E-004	N.Ih.		N.I.	
10500327	Hist2h3c2	2.126	1.03E-004	N.Ih.		N.I.	
10376455	Hist3h2a	2.301	6.43E-005	N.Ih.		N.I.	
10491780	Hspa4l	3.819	1.00-004	N.Ih.		N.I.	
10382316	Kcnj16	4.992	1.48E-005	N.Ih.		N.I.	
10382321	Kcnj2	5.058	3.63E-004	N.Ih.		N.I.	
10544108	Klrg2	2.505	4.88E-003	N.Ih.		N.I.	
10403945	LOC100041230	2.041	2.18E-004	N.Ih.		N.I.	
10429568	Ly6c1	2.811	1.59E-003	N.Ih.		N.I.	
10429573	Ly6c2	2.597	1.92E-003	N.Ih.		N.I.	
10436662	mmu-mir-155	2.537	3.21E-004	N.Ih.		N.I.	
10436941	Mrps6	6.064	9.29E-008	N.Ih.		N.I.	
10367591	Myct1	3.556	8.62E-006	N.Ih.		N.I.	
10576639	Nrp1	2.468	1.56E-006	N.Ih.		N.I.	
10352867	Plxna2	2.058	1.19E-004	N.Ih.		N.I.	
10418898	Ppyr1	2.095	1.37E-003	N.Ih.		N.I.	
10407797	Prl2c3	14.956	8.46E-009	N.Ih.		6.701	9.32E-007
10485624	Prrg4	8.659	1.21E-008	N.Ih.		N.I.	
10494978	Ptpn22	4.912	2.59E-004	N.Ih.		N.I.	
10585703	Rpp25	3.468	4.11E-004	N.Ih.		N.I.	
10349157	Serpinb2	6.695	3.20E-003	N.Ih.		N.I.	
10587503	Sh3bgrl2	2.454	3.11E-005	N.Ih.		N.I.	
10582719	Sipa1l2	2.560	2.08E-005	N.Ih.		N.I.	
10459866	Slc14a1	2.111	1.76E-003	N.Ih.		N.I.	
10541333	Slc6a12	3.189	9.70E-004	N.Ih.		N.I.	
10507500	Slc6a9	2.435	5.83E-003	N.Ih.		N.I.	
10382328	Sox9	2.728	1.12E-003	N.Ih.		N.I.	
10499899	Sprr1a	5.388	2.03E-003	N.Ih.		N.I.	
10411459	Tmem171	2.773	4.52E-003	N.Ih.		N.I.	
10445241	Tnfrsf21	2.237	4.88E-004	N.Ih.		N.I.	
10569494	Tnfrsf22	3.687	1.12E-004	N.Ih.		N.I.	

Not inhibited by Torin1 or inhibited <35%

NFAT5-dependent induction

10569504	Tnfrsf23	3.365	3.94E-005	N.Ih.		N.I.		
10396952	Ttc9	2.352	9.79E-003	N.Ih.		N.I.		
10348632	Twist2	2.437	6.08E-005	N.Ih.		N.I.		
10505163	Zkscan16	2.984	1.33E-008	N.Ih.		N.I.		
10455550	4833403I15Rik	2.285	2.17E-007	N.Ih.		1.810	1.29E-005	Induction not dependent on NFAT5
10424667	4930572J05Rik	3.559	1.42E-005	N.Ih.		1.988	4.16E-003	
10375313	Ccnjl	2.128	6.88E-006	34.0	2.32E-003	1.557	1.42E-003	
10551025	Cd79a	2.257	5.14E-007	N.Ih.		4.017	3.16E-010	
10413338	Dnahc12	2.226	4.56E-004	N.Ih.		3.766	1.97E-006	
10449284	Dusp1	2.331	3.14E-005	N.Ih.		1.890	5.30E-004	
10605874	Eda2r	2.243	2.48E-003	N.Ih.		3.985	1.51E-005	
10461365	Eef1g	2.072	3.04E-003	N.Ih.		2.323	9.64E-004	
10460585	Fosl1	2.532	6.75E-005	N.Ih.		2.273	2.32E-004	
10578874	Gm4975	2.016	4.77E-005	N.Ih.		N.I.		
10404053	Hist1h2bc	2.453	8.06E-006	N.Ih.		1.686	1.68E-003	
10463737	Ina	2.073	3.52E-004	N.Ih.		1.867	6.27E-004	
10390117	Itga3	2.557	5.69E-005	N.Ih.		2.104	5.47E-004	
10540227	Kbtbd8	2.260	2.90E-006	34.0	2.42E-003	1.449	5.40E-003	
10395925	Mia2	2.717	5.64E-008	27.5	6.99E-003	4.127	4.13E-010	
10553897	Mtmr10	2.385	2.28E-006	N.Ih.		1.942	5.03E-005	
10493640	Nup210l	3.184	3.11E-009	N.Ih.		2.884	3.24E-008	
10403579	Prl2c5	7.595	1.11E-006	N.Ih.		4.058	7.81E-005	
10493803	S100a3	2.144	5.35E-005	N.Ih.		1.885	3.35E-004	
10354389	Slc39a10	2.718	2.31E-006	N.Ih.		2.931	9.36E-007	
10346150	Tmeff2	2.297	6.87E-003	N.Ih.		2.556	2.98E-003	

TABLE 2: Genes that were downregulated ≥ 50% ($p < 0.01$) by osmotic stress in wild-type MEF, their sensitivity to Torin1 in wild-type MEF, and response to osmotic stress in NFAT5-deficient MEFs.

(1) Relative expression with respect to isotonic conditions (300 mOsm/kg). Values correspond to the mean of 3 independent experiments.
(2) Expression in Torin1-treated cells exposed to hypertonic conditions (500 mOsm/kg). Values correspond to the mean of 3 independent experiments.
(3) Expression in NFAT5-deficient MEFs under osmostress with respect to isotonic conditions (300 mOsm/kg) in the same cells. Mean of 3 independent experiments.
(4) NA: not affected indicates that osmotic stress did not cause a significant decrease in the abundance of the mRNA in NFAT5-deficient MEFs.
(5) NP: not protected indicates that Torin1 did not prevent significantly the repression by osmotic stress.
(6) Expression in Torin1-treated cells exposed to osmostress was ≥ 1.5-fold higher than in cells not treated with Torin1.
(7) Genes whose downregulation in NFAT5-deficient MEF was ≥ 2-fold greater than in wild-type MEFs.
(8) Genes whose repression by osmotic stress was ≥ 2-fold greater in wild-type MEF than in NFAT5-deficient MEFs.

Legend: WT: wild-type MEF; KO: NFAT5-deficient MEF; OS: osmotic stress; Tor1 (Torin1).

Affimetrix ID	Gene Name	Expression in WT+OS (1)	*p*-value	Expression in WT+OS +Tor1 (2)	*p*-value	Expression in KO+OS (3)	*p*-value		
10410959	Atg10	0.427	5.11E-005	0.688	7.28E-003	0.433	6.02E-005	Repression ameliorated by Torin1 (6)	Repression independent of NFAT5
10520842	Bre	0.468	1.28E-006	0.715	6.78E-004	0.592	8.67E-005		
10503376	Gm11818	0.465	7.24E-006	0.702	2.82E-003	0.479	1.15E-005		
10397507	Gstz1	0.369	3.80E-007	0.687	9.46E-005	0.379	5.56E-007		
10506658	Hspb11	0.466	1.48E-008	0.717	2.05E-005	0.579	1.25E-006		
10593799	Scaper	0.411	3.15E-005	0.753	1.25E-003	0.339	3.32E-006		
10524020	Tmem175	0.485	1.09E-004	0.809	2.35E-003	0.199	5.28E-009		(7) NFAT5
10372324	Syt1	0.471	8.75E-005	0.784	2.79E-003	NA (4)			(8) NFAT5-repressed
10397416	1700019E19Rik	0.498	6.60E-007	NP (5)		0.565	7.43E-006		
10406364	2210408I21Rik	0.452	2.11E-006	NP		0.601	2.74E-004		
10402073	2610021K21Rik	0.495	2.49E-005	NP		0.410	1.53E-006		
10527649	6330406I15Rik	0.456	4.20E-006	NP		0.342	7.82E-008		
10600235	Abcd1	0.480	2.28E-004	NP		0.277	3.93E-007		
10593591	Acat1	0.499	4.45E-004	NP		0.471	2.09E-004		
10344725	Adhfe1	0.404	2.35E-003	NP		0.384	1.54E-003		
10368356	Akap7	0.475	4.03E-006	0.699	2.56E-003	0.455	2.01E-006		
10401473	Aldh6a1	0.484	2.03E-005	NP		0.578	3.55E-004		
10570483	Arhgef10	0.495	8.85E-009	0.658	4.22E-004	0.524	2.87E-008		
10357833	Atp2b4	0.438	6.81E-007	NP		0.699	3.41E-003		

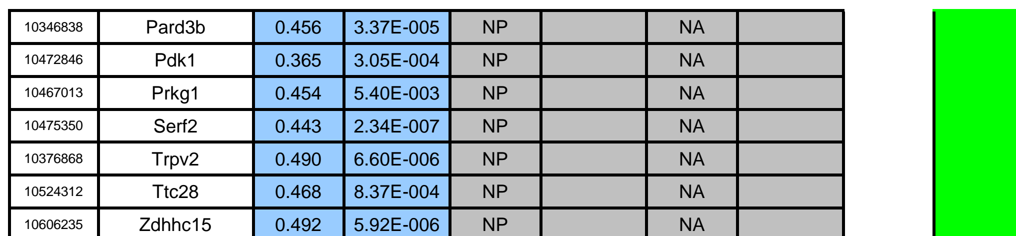
10541670	C1rl	0.499	2.34E-003	NP		0.444	6.68E-004
10569203	Chid1	0.442	3.35E-006	NP		0.642	1.64E-003
10364712	Cirbp	0.475	1.59E-004	NP		0.261	1.63E-007
10417167	Clybl	0.419	8.96E-005	NP		0.574	4.25E-003
10449955	Cyp4f13	0.378	1.17E-006	NP		0.418	4.46E-006
10474825	D2Ertd750e	0.491	1.64E-005	NP		0.488	1.44E-005
10511679	Decr1	0.445	8.80E-007	NP		0.471	2.14E-006
10449225	Decr2	0.394	7.26E-008	0.536	6.70E-003	0.414	1.47E-007
10506767	Echdc2	0.471	3.07E-006	NP		0.578	1.09E-004
10604451	Enox2	0.494	6.50E-008	NP		0.446	1.02E-008
10522060	Fam114a1	0.353	1.45E-006	NP		0.558	7.06E-004
10446351	Fert2	0.478	1.36E-003	NP		0.447	6.53E-004
10373902	Gatsl3	0.485	4.18E-005	NP		0.313	1.34E-007
10469609	Gm13375	0.443	7.17E-005	NP		0.407	2.47E-005
10418747	Hacl1	0.497	1.09E-004	NP		0.405	6.35E-006
10548761	Hebp1	0.473	3.78E-004	NP		0.484	4.98E-004
10574676	Nol3	0.486	1.42E-006	NP		0.610	1.04E-004
10600082	Nsdhl	0.454	0.11E-003	NP		0.557	9.22E-003
10413874	Ogdhl	0.484	2.39E-006	0.706	1.82E-003	0.605	1.46E-004
10518781	Per3	0.387	1.31E-005	NP		0.422	3.81E-005
10602925	Phka2	0.481	1.72E-005	NP		0.509	4.14E-005
10470959	Phyhd1	0.387	1.52E-005	NP		0.552	1.46E-003
10514275	Ptplad2	0.470	2.25E-006	NP		0.657	1.02E-003
10488797	Pxmp4	0.488	4.40E-005	NP		0.374	1.11E-006
10552824	Rras	0.435	3.64E-010	0.612	3.91E-005	0.488	3.04E-009
10514956	Scp2	0.471	5.00E-004	NP		0.521	1.67E-003
10571567	Sorbs2	0.414	1.96E-004	NP		0.581	8.98E-003
10508436	Sync	0.380	1.25E-005	NP		0.502	4.15E-004
10451736	Tbc1d5	0.472	2.29E-006	0.693	2.01E-003	0.455	1.26E-006
10507479	Tmem53	0.425	5.62E-003	NP		0.216	3.37E-005
10405343	Tspan17	0.424	6.19E-006	NP		0.454	1.62E-005
10399588	Zfp125	0.490	2.18E-003	NP		0.308	1.89E-005
10471058	Cstad	0.447	1.66E-003	NP		0.202	1.35E-006
10398432	mmu-mir-377	0.401	1.12E-06	NP		0.151	4.70E-011
10386775	Prpsap2	0.490	3.31E-006	NP		0.228	1.65E-010
10489985	Atp9a	0.478	3.26E-004	NP		NA	
10414269	Bnip3	0.322	1.04E-004	NP		NA	
10418300	Cacna2d3	0.386	1.77E-003	NP		NA	
10348451	Cxcr7	0.493	2.53E-003	NP		NA	
10438639	Dgkg	0.408	1.79E-006	NP		NA	
10605392	F8	0.483	2.82E-004	NP		NA	
10511258	Fam132a	0.491	1.52E-004	NP		NA	
10505998	Fggy	0.307	3.89E-008	NP		0.667	3.39E-003
10602896	Gpr64	0.449	1.19E-003	NP		NA	
10467766	Loxl4	0.480	1.17E-003	NP		NA	
10492890	Lrba	0.493	5.86E-006	NP		NA	
10497490	Naaladl2	0.292	8.94E-005	NP		0.732	3.88E-003
10395733	Npas3	0.483	9.58E-007	NP		NA	

Repression not prevented by Torin1

Repression independent of NFAT5

(7) NFAT5

(8) NFAT5-repressed



10346838	Pard3b	0.456	3.37E-005	NP		NA			
10472846	Pdk1	0.365	3.05E-004	NP		NA			
10467013	Prkg1	0.454	5.40E-003	NP		NA			
10475350	Serf2	0.443	2.34E-007	NP		NA			
10376868	Trpv2	0.490	6.60E-006	NP		NA			
10524312	Ttc28	0.468	8.37E-004	NP		NA			
10606235	Zdhhc15	0.492	5.92E-006	NP		NA			

TABLE 3: **Genes downregulated ≥ 35% ($p < 0.01$) by Torin1 in isotonic conditions in wild-type MEFs.**

Affimetrix ID	Gene Name	Expression in WT + Torin1	*p*-value
10562905	Atf5	0.614	6.19E-003
10562563	Ccne1	0.585	8.93E-003
10588836	Gmppb	0.595	8.91E-003
10573626	Gpt2	0.621	7.94E-003
10532954	Mlec	0.645	2.08E-003
10545672	Mthfd2	0.367	1.94E-003
10578916	Sc4mol	0.473	5.13E-003
10516932	Sesn2	0.527	7.59E-003
10424349	Sqle	0.532	8.56E-003

TABLE 4: Genes whose expression did not change upon osmotic stress alone in wild-type MEFs, but was downregulated or upregulated ≥ 2-fold under the combination of osmotic stress and Torin1.

(1) Expression in Torin1-treated cells exposed to osmotic stress relative to cells not treated with Torin1.
(2) No Change indicates that osmotic stress did not affect significantly the mRNA expression.

Legend. WT: wild-type MEF; OS: osmotic stress; Tor1 (Torin1).

Affimetrix ID	Gene Name	Expression in WT + OS	Expression in WT+OS+Tor1 (1)	*p*-value	
10444883	Cdsn	No Change (2)	0.432	8.76E-003	Inhibited by Torin1 > 50%
10503023	Cth	No Change	0.434	5.69E-003	
10351039	Gas5	No Change	0.391	9.75E-004	
10373452	Gm129	No Change	0.383	7.24E-003	
10516064	Mfsd2	No Change	0.386	4.09E-003	
10545672	Mthfd2	No Change	0.375	2.28E-003	
10606436	Nsbp1	No Change	0.494	7.61E-003	
10516932	Sesn2	No Change	0.430	1.01E-003	
10384539	Slc1a4	No Change	0.485	2.50E-003	
10535852	Slc7a1	No Change	0.431	5.55E-004	
10391985	Taf1d	No Change	0.462	5.19E-003	
10435075	Tfrc	No Change	0.357	3.28E-003	
10543017	Pdk4	No Change	2.064	4.57E-003	Induced by Torin1 > 2 fold
10443527	Pim1	No Change	2.479	4.67E-003	

Table 5: Summary of stress, cell survival and proliferation-related genes regulated by osmotic stress and sensitive to Torin1.

Sensitivity to osmostress and Torin1	Gene name	Full name	GO number	GO Biological Process	Function	References
Upregulated by osmostress and inhibited by Torin1	*Amd1*	S-adenosylmethionine decarboxylase 1.	GO:0006595	polyamine metabolic process	Required for cell proliferation in blastocysts.	(Nishimura et al., 2002)
	Ddit4	DNA-damage-inducible transcript 4. REDD1.	GO:0009968	negative regulation of signal transduction	Induced by hypoxia, energy stress, oxidative stress and DNA damage. Inhibitor of mTOR.	(Ellisen et al., 2002) (Brugarolas et al., 2004) (Corradetti et al., 2005)
	Ddit4l	DNA-damage-inducible transcript 4-like. REDD2.	GO:0009968	negative regulation of signal transduction	Induced by hypoxia, energy stress, oxidative stress and DNA damage. Inhibitor of mTOR.	(Corradetti et al., 2005)
	Dkc1	Dyskeratosis congenita 1.	GO:0009451	RNA modification	Mutations in *Dkc1* cause impairment of proliferation and enhanced DNA damage responses in MEFs.	(Gu et al., 2008)
	Figf	c-fos induced growth factor. VEGF-D.	GO:0008283	cell proliferation	Stimulates the expansion of vascular endothelial cells exposed to hypoxia.	(Nilsson et al., 2004)
	Gclc	glutamate-cysteine ligase, catalytic subunit.	GO:0045454	cell redox homeostasis	Catalyzes the formation of the cellular antioxidant glutathione (GSH). Induced by oxidative stress. Protects cells from oxidative stress and inducers of apoptotic death.	(Krejsa et al., 2010)
	Hspa1b	heat shock protein 1B. Hsp70.1.	GO:0006281	DNA repair	Induced by osmotic stress, heat stress, ionizing radiation and other stressors. Facilitares cell survival under diverse stresses.	(Go et al., 2004) (Hunt et al., 2004)
	Map2k6	mitogen-activated protein kinase kinase 6. MKK6.	GO:0000187	activation of MAPK activity	Activates p38 in response to diverse stressors and cause the inhibition of cell proliferation.	(Sturchler et al., 2010) (Brancho et al., 2003) (Takekawa et al., 1998) (Chen et al., 2000)
	Slc5a3	solute carrier family 5 (inositol transporters), member 3. SMIT.	GO:0006020	inositol metabolic process	Induced by hypertonic stress. Osmoprotective function.	(Kwon et al., 1992)
	Slc19a2	solute carrier family 19 (thiamine transporter), member 2.	GO:0071934	thiamine transmembrane transport	Its expression and activity are induced by DNA damage. Expression induced by p53.	(Lo et al., 2001)
	Tacstd2	tumor-associated calcium signal transducer 2. Trop2.	GO:0008150	biological process	Trop2 can promote cell proliferation and cell cycle progression.	(Cubas et al., 2010)
	Zscan4c	zinc finger and SCAN domain containing 4C.	GO:0008150	biological process	Zscan4 is involved in telomere maintenance and long-term genomic stability in ES cells. Zscan4 knockdown slows down cell proliferation.	(Zalzman et al., 2010)

Not affected by short-term osmostress alone, but repressed by the combination of osmostress and Torin1	*Cth*	cystathionase (cystathionine gamma-lyase)	GO:0008285	negative regulation of cell proliferation	Its overexpression inhibits cell proliferation via sustained activation of ERK1/2 and p21Cip/WAK-1 activities.	(Yang et al., 2004)
	Gas5	growth arrest specific 5	non coding RNA	non-coding RNA	Gas5 is abundant in cells whose growth has been arrested because of lack of nutrients or growth factors. It can induce growth arrest and sensitize cells to apoptosis.	(Kino et al., 2010) (Mourtada-Maarabouni et al., 2009)
	Mfsd2	major facilitator superfamily domain containing protein 2	GO:0055085	transmembrane transport	Induced in brown adipose tissue during fasting and cold-induced thermogenesis. Overexpression in tumor cells can induce G1 arrest.	(Angers et al., 2008) (Spinola et al., 2010)
	Nsbp1	high-mobility group nucleosome binding domain 5 (Hmgn5).	GO:0006357	regulation of transcription from RNA polymerase II promoter	Dysregulation of the cellular levels of Nsbp1 alters the transcription level of numerous genes.	(Rochman et al., 2009)
	Sesn2	Sestrin 2.	GO:0007050	cell cycle arrest	DNA damage and oxidative stress induce Sestrin 2 expression mediated by p53. Hypoxia induces Sestrin 2 expression via HIF-1α. Sestrin 2 can inhibit mTOR via AMPK activation.	(Budanov and Karin, 2008) (Essler et al., 2009)
	Slc1a4	Slc1a4 solute carrier family 1 (glutamate/neutral amino acid transporter), member 4. ASCT1.	GO:0015175	neutral amino acid transporter activity	Inducible by cysteine deprivation.	(Lee et al., 2008)
	Slc7a1	cationic amino acid transporter, y+ system (CAT-1).	GO:0003333	amino acid transmembrane transport	Induced during amino acid starvation and endoplasmatic reticulum stress.	(Bhattacharyya et al., 2006) (Fernandez et al., 2002)
	Tfrc	Transferrin receptor.	GO:0006879	cellular iron ion homeostasis	Downregulation of Tfrc expression causes the reduction of cell proliferation. Oxidative stress increases protein levels of transferrin receptor.	(Trowbridge and Lopez, 1982) (Jones et al., 2006) (Chirasani et al., 2009)
	Taf1d	TATA box binding protein (Tbp)-associated factor, RNA polymerase I, D.	GO:0045449	regulation of transcription	Resides at the rDNA promoter in the nucleolus and is a positive regulator of RNA Pol I transcription.	(Gorski et al., 2007)
Downregulation in response to osmostress was attenuated by Torin1	*Atg10*	autophagy related 10	GO:0006914	autophagy	Necessary for the process of autophagy.	(Boya et al., 2005)
	Bre	brain and reproductive organ- expressed protein.	GO:0006281	DNA repair	Bre knockdown increases cell proliferation, and concomitantly decreases p53 expression. Anti-apoptotic in cells treated with TNF and other inducers.	(Tang et al., 2006) (Li et al., 2004)
	Gstz1	glutathione transferase zeta 1.	GO:0006749	glutathione metabolic process	Deficiency of Gstz1 causes oxidative stress and activation of antioxidant response pathways.	(Blackburn et al., 2006)
	Syt1	synaptotagmin I	GO:0006810	transport	Necessary for the release of FGF in response to several stresses: hypoxia, heat shock and serum starvation.	(LaVallee et al., 1998)

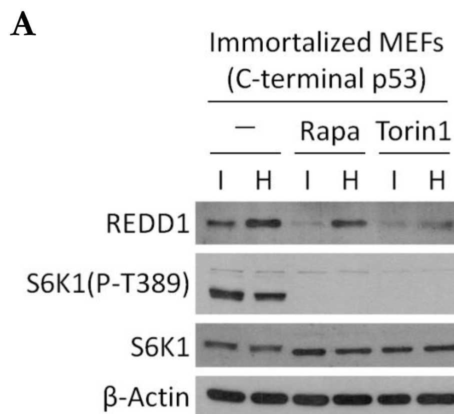
	Scaper	S phase cyclin A-associated protein in the ER	GO:0008150	biological process	Interacts with cyclin A/Cdk2 complex. Its ablation results in a delayed G1/S transition upon cell cycle reentry from G0.	(Tsang et al., 2007)
Not affected by short-term osmostress alone, but enhanced by Torin1 plus osmostress	*Pdk4*	pyruvate dehydrogenase kinase, isozyme 4	GO:0006086	acetyl CoA biosynthetic process from pyruvate	PDK4 expression is increased in tissues in response to starvation.	(Wu et al., 2000) (Kwon and Harris, 2004) (Kwon et al., 2004)
	Pim1	proviral integration site 1 / Pim1 oncogene	GO:0008283	cell proliferation	Pim1 expression increases proliferation, survival and activation in T lymphocytes. Pim1 can promote rapamycin-resistant survival of lymphocytes.	(Moroy et al., 1993) (Wingett et al., 1996) (Fox et al., 2005) (Zhang et al., 2009)

<u>SECTION III</u>: The mTOR regulators REDD1 and REDD2 are induced upon osmotic stress in an mTOR-sensitive manner

After the analysis of the microarray data we were very intrigued by the finding that hypertonic stress upregulated in a mTOR-dependent manner the expression of *Ddit4*/*REDD1* and *Ddit4l*/*REDD2* mRNAs, two related genes -with 50% of sequence identity- whose products had been described to be expressed and inhibit mTOR activity in certain contexts of stress, such as hypoxia (Shoshani et al., 2002), (Brugarolas et al., 2004), (Corradetti et al., 2005), energy stress (Sofer et al., 2005) or DNA damage (Ellisen et al., 2002).

Upon osmotic stress, REDD1 protein, like its mRNA **(Figures 11 and 12)**, was induced in a rapamycin and Torin1-sensitive manner **(Figure 15)** in two different cell types, MEFs **(Figure 15A)** and HEK293T **(Figure 15B)**. We did not asses the induction of REDD2 protein due to the lack of antibodies validated in the literature at the moment of doing these experiments. For this reason, we focused on REDD1 analysis and tested its effect on the activity of mTOR and induction of some osmoresponsive genes.

A



B

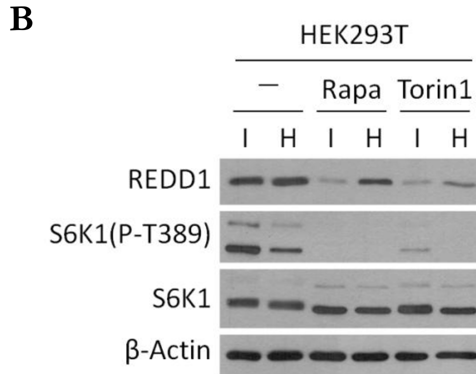


Figure 15. mTOR-sensitive induction of REDD1 by osmotic stress. **(A)** MEFs immortalized with a p53-carboxy-terminal fragment or **(B)** HEK293T cells were cultured during 8 hours in isotonic (I, 300 mOsm/kg) or hypertonic medium (H, 500 mOsm/kg, upon addition of 100 mM NaCl) without or with rapamycin (Rapa, 50 nM) or Torin1 (100 nM). REDD1, phospho-S6K1 (Thr 389), S6K1 and β-actin (loading control) were detected by Western blotting.

As described in the literature, upon energy stress induced by 2-deoxyglucose (2-DG) REDD1 mRNA and protein levels were induced **(Figure 16A and 16B)**. Interestingly, in isotonic conditions, although 2-DG can efficiently stimulate a productive energy stress response activating AMPK and it downstream substrate ACC, the induction of REDD1 mRNA and protein levels were unable to suppress mTORC1 activity **(Figure 16B)**. However, in hypertonic stress conditions, REDD1 mRNA and protein levels were higher compared to the energy stress condition alone **(Figure 16A and 16B)** and mTORC1 signaling seemed to be less active than in isotonic conditions. Upon combination of both stresses the effect on REDD1 induction, both at mRNA and protein level, was additive to that observed under osmotic or energy stress alone (**Figure 16A and 16B)**. Moreover, the NFAT5 mRNA and protein expression observed in this same experiment were not suppressed by energy stress alone (**Figure 16A and 16B)**.

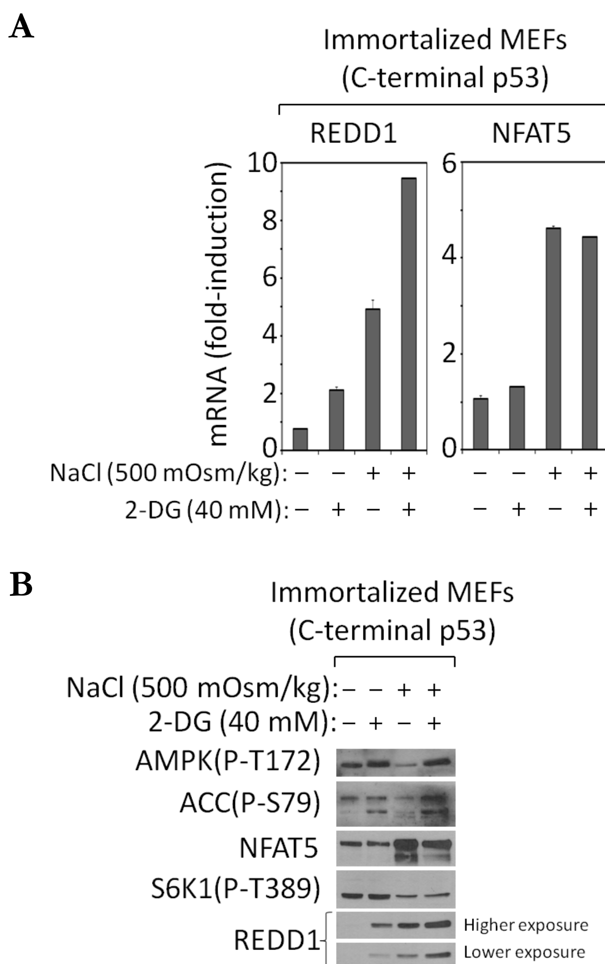


Figure 16. Induction of REDD1 by energy stress. (A) RNA was isolated from immortalized MEFs (C-terminal p53) cultured during 8 hours in isotonic (-; 300 mOsm/kg) or hypertonic medium (+; 500 mOsm/kg upon addition of 100 mM NaCl) without (-) or with (+) 2-deoxyglucose (2-DG, 40 mM) during the last 4 hours. mRNA abundance for REDD1 (*Ddit4)* and NFAT5 normalized to *L32* mRNA is represented by fold induction relative to isotonic condition alone (fold; arbitrary units = 1). **(B)** C-terminal p53 immortalized MEFs were cultured during 8 hours in isotonic (-; 300 mOsm/kg) or hypertonic medium (+; 500 mOsm/kg, upon addition of 100 mM NaCl) without (-) or with (+) 2-deoxyglucose (2-DG, 40 mM) during the last 4 hours. Phospho-AMPK (Thr 172), phospho-ACC(Ser 79), NFAT5, phospho-S6K1(Thr 389) and REDD1 were detected by Western blotting.

We also analyzed the mTOR dependence of these two different stresses in REDD1 mRNA expression. As validated before, upon osmotic stress the induction of REDD1 mRNA was sensitive to mTOR inhibition by rapamycin and Torin1. Additionally, the REDD1 mRNA induction observed under energy stress is also mTOR-sensitive **(Figure 17)**.

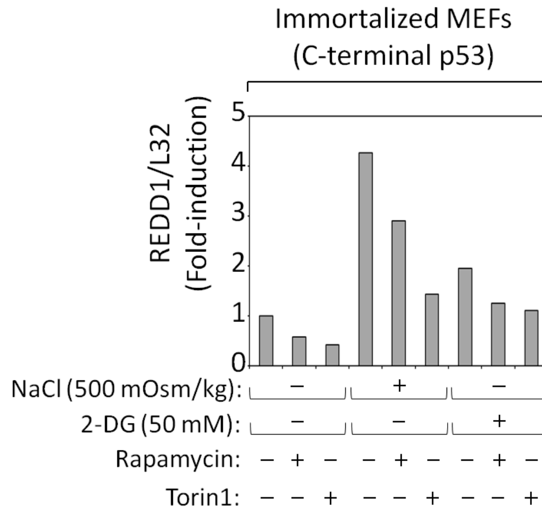


Figure 17. mTOR-sensitive induction of REDD1 mRNA by energy stress. RNA was isolated from immortalized MEFs (C-terminal p53) cultured during 8 hours in isotonic (-; 300 mOsm/kg) or hypertonic medium (+; 500 mOsm/kg upon addition of 100 mM NaCl) without (-) or with (+) rapamycin (50 nM) or Torin1 (100 nM). To induce energy stress cells were cultured during 8 hours with 50 mM 2-deoxyglucose (2-DG) and treated without (-) or with (+) rapamycin (50 nM) or Torin1 (100 nM). mRNA abundance for REDD1 *(Ddit4)* was normalized to *L32* mRNA and is represented by fold induction relative to isotonic condition alone (fold; arbitrary units = 1).

We had previously shown that in our hypertonic stress conditions (500 mOsm/kg) there was a slight decrease of phosphorylation of the mTORC1 substrate S6K1 (**Figure 1 and Figure 16B)**, that was independent of AMPK

activation **(Figure 9)**. Since, REDD1 was described, in other stresses contexts, as a negative regulator of mTORC1 signaling (Reiling and Hafen, 2004), (Brugarolas et al., 2004), (Corradetti et al., 2005), (Vadysirisack et al., 2011), we speculated whether REDD1 may be negatively regulating mTORC1 in cells exposed to osmostress.

To analyze if REDD1 could also be a negative regulator of mTOR under hypertonic stress we use a specific siRNA pool targeting REDD1 mRNA. Suppression of REDD1 with siRNAs did not affect the phosphorylation on Thr389 of the mTORC1 substrate S6K1 **(Figure 18),** which suggested that induction of endogenous REDD1 by osmotic stress did not inhibit mTORC1, in contrast to its inhibitory effect observed in other stress responses.

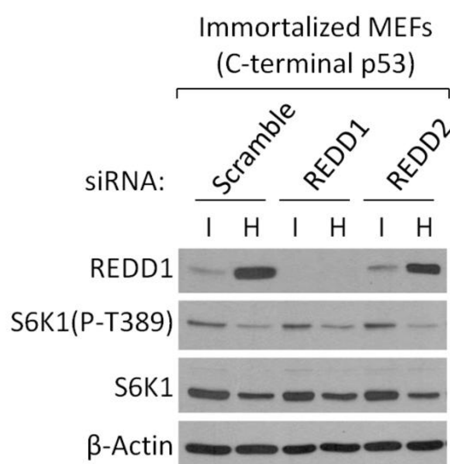


Figure 18. REDD1 suppression cannot restore S6K1 activity. Immortalized MEFs (C-terminal p53) were transfected with control non-targeting siRNA (scramble) or siRNA specific for REDD1 or REDD2. 24 hours later, they were cultured during 8 hours in isotonic (I, 300 mOsm/kg) or hypertonic medium (H, 500 mOsm/kg, upon addition of 100 mM NaCl). Phospho-S6K1(Thr 389), S6K1, REDD1 and β-Actin –as loading control- were detected by Western blotting.

Although in our setting experiments suppression of REDD1 protein expression did not restore S6K1 phosphorylation, when we tested the induction of some mTOR-sensitive genes under hyperosmotic stress conditions like *Akr1b3* and *Hspa1b* we observed a significant decrease in their respective inductions **(Figure 19)**. This observation was in line with a recent paper where it has been described that REDD1 can influence gene expression via an mTORC1 independent pathway, that include increased ROS production and HIF-1α destabilization (Horak et al., 2010).

To further explore the REDD1-mediated regulation of gene expression we took advantage of the microarray data of REDD1$^{-/-}$ MEFs presented by Horak et al. in their recent work. Since *Pdk4* was scored as a gene repressed by REDD1 in MEFs (Horak et al., 2010), and our microarray analysis showed that inhibition of mTOR in cells exposed to osmostress enhanced the expression of *Pdk4* (pyruvate dehydrogenase kinase 4) **(Table 4)** we tested whether mTOR-mediated induction of REDD1 during osmostress was involved in the inhibition of *Pdk4*. We found that REDD1 suppression, prevented the downregulation of PDK4 by osmotic stress to a similar extent as Torin1 did **(Figure 19)**. However, suppression of REDD1 did not have any effect on REDD2 induction, indicating that REDD1 can affect only the expression of a subset of those osmoregulated genes.

In contrast, in the same set of experiments, downregulation of REDD2 with a specific siRNA pool did not cause significant changes in the induction of osmostress response genes nor in the activity of mTORC1 **(Figure 18 and 19)**. However, since we have not confirmed the induction of REDD2 protein, and we cannot determine if the increased mRNA levels of REDD2 are translated to increased levels of REDD2 protein under hypertonic stress conditions, we are cautious about the interpretation of this result regarding the potential lack of effect of endogenous REDD2.

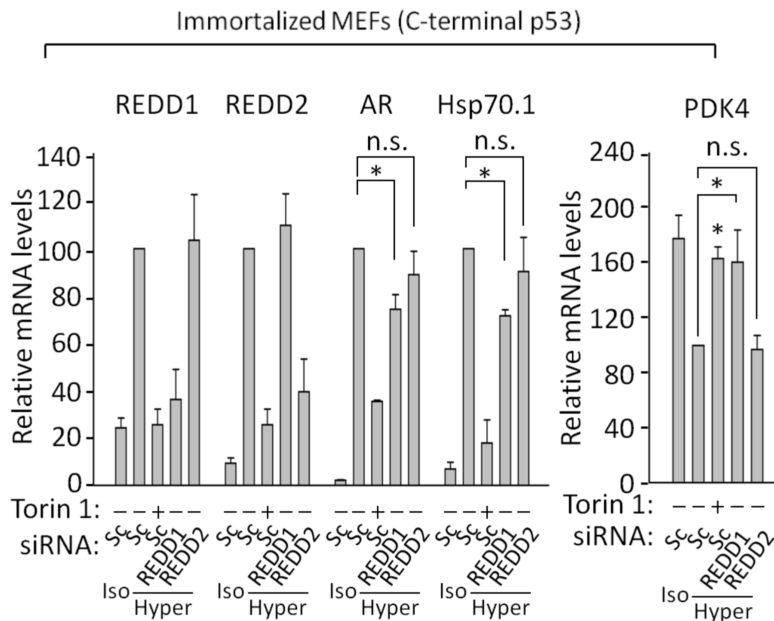


Figure 19. Effect of REDD1 and REDD2 suppression on some mTOR-sensitive genes under hypertonicity. Immortalized MEFs (C-terminal p53 immortalized) were transfected with control non-targeting siRNA (scramble) or siRNA specific for REDD1 or REDD2. Effect analyzed on REDD1 (*Ddit4*), REDD2 (*Ddit4l*), AR (*Akr1b3*), Hsp70.1 (*Hspa1b)*, and PDK4 *(Pdk4)*. RNA levels for each gene and condition were normalized to *L32* mRNA and are represented relative to the amount of mRNA in hypertonic conditions in cells transfected with control scramble siRNA, which was given an arbitrary value of 100. Bars represent the mean ± S.E.M. of three independent experiments (* $p < 0.05$).

Altogether, these results indicated that although the effects of REDD1 were moderate, it could modulate without affecting mTORC1 status, both positively (*Akr1b3* and *Hspa1b*) and negatively (*Pdk4*), the expression of several Torin1-sensitive osmoresponsive genes.

SECTION IV: mTOR regulates osmostress-induced changes in chromatin configuration and RNA-pol II recruitment to osmoresponsive genes

Next, we analyzed the effect of mTOR inhibition on transcriptional mechanisms potentially involved in the induction of osmostress responsive genes. We observed that Torin1 did not inhibit the initial recruitment of NFAT5 to the *Akr1b3* (aldose reductase) enhancer (at 1 hour) but caused its partial dissociation from chromatin at later time points (≥ 4 hours) **(Figure 20)**. We also observed this behavior after 8 hours of hypertonic stimulation through an independent technique in wild-type MEFs (**Figure 21A, 21B and 21C)** and HEK293T cells **(Figure 21D and 21E)** using total chromatin extracts.

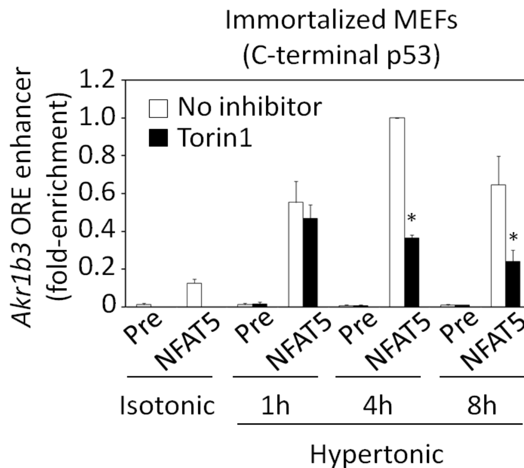


Figure 20. Effect of Torin1 on the recruitment of NFAT5 to the *Akr1b3* (aldose reductase) gene. (A) Chromatin from immortalized MEFs (C-terminal p53) cultured in isotonic (300 mOsm/kg) or hypertonic medium (500 mOsm/kg, upon addition of 100 mM NaCl) without or with Torin1 (100 nM) was immunoprecipitated with preimmune rabbit serum (Pre) or a mixture of two rabbit polyclonal antibodies specific for NFAT5 (NFAT5). Immunoprecipitated chromatin was amplified with specific primers for a genomic region located at -1.13 kb

upstream of the transcription start site that contains three osmotic responsive elements (ORE), and normalized to its respective total chromatin input. Results are represented relative to the sample of 4 hours of hypertonic treatment (arbitrary value of 1). Bars represent the mean ±S.E.M. of four independent experiments (*p< 0.05).

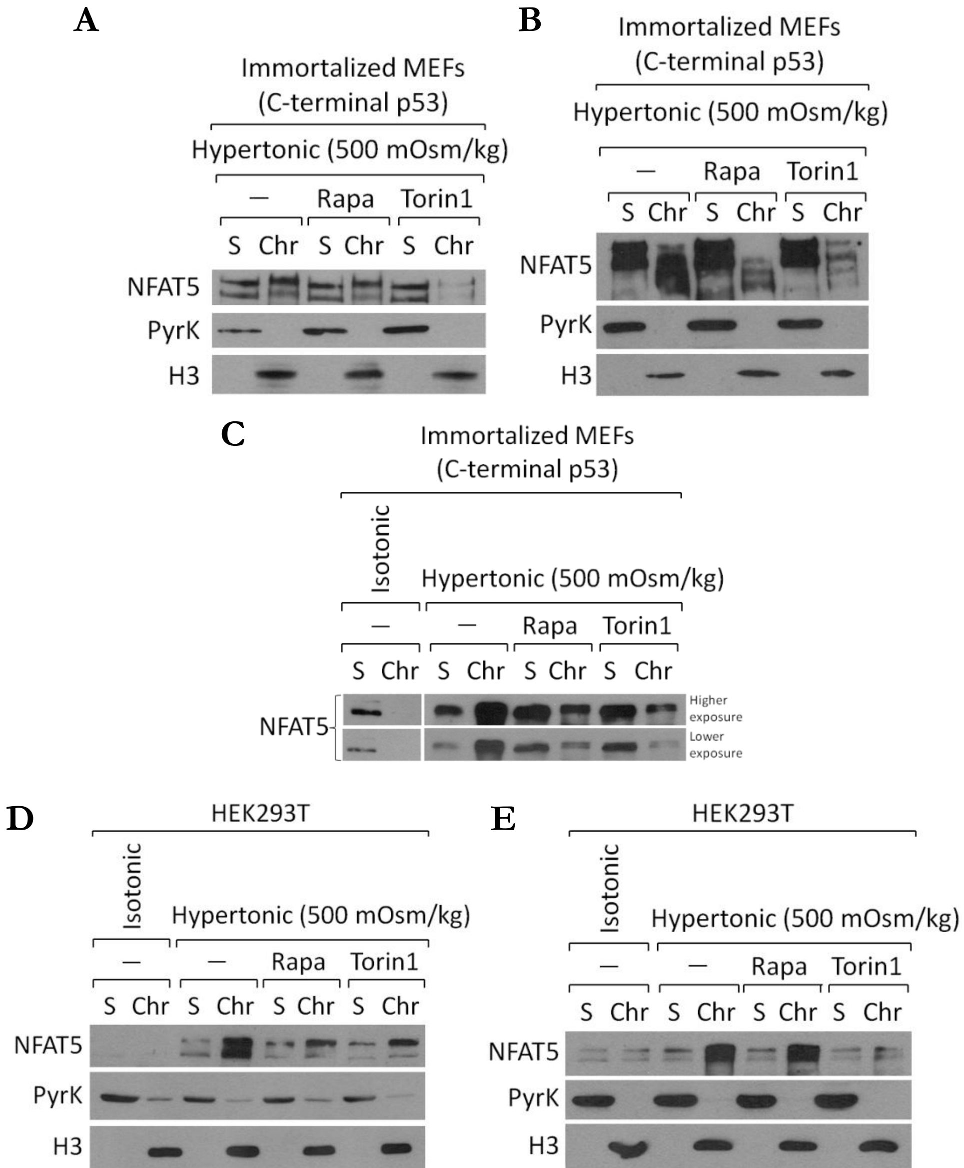


Figure 21. Effect of rapamycin and Torin1 on the osmostress-induced recruitment of NFAT5 to chromatin. Soluble and chromatin fractions from

(A, B and C) C-terminal p53-immortalized MEFs (from three independent experiments) or **(D and E)** HEK293T cells (from two independent experiments) were cultured in isotonic (300 mOsm/kg) or hypertonic medium (500 mOsm/kg, upon addition of 100 mM NaCl) for 8 hours without or with rapamycin (Rapa, 200 nM) or Torin1 (100 nM). NFAT5, pyruvate kinase (to differentiate soluble fractions) and histone H3 (to differentiate chromatin fractions) were detected by Western blotting.

We also observed that Torin1, and rapamycin to a lesser extent, reduced the basal constitutive nuclear accumulation of NFAT5 at 8 hours, but did not impair its translocation induced by osmotic stress **(Figure 22).**

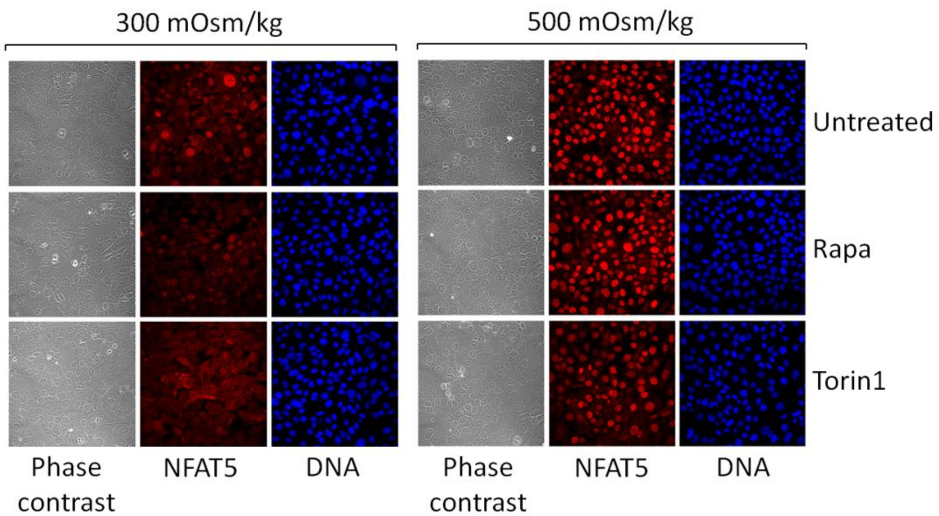


Figure 22. Effect of rapamycin and Torin1 on the subcellular localization and osmostress-induced nuclear translocation of NFAT5. Immunofluorescence images show the subcellular distribution of endogenous NFAT5 in C-terminal p53-immortalized MEFs that were cultured in isotonic (300 mOsm/kg) or hypertonic medium (500 mOsm/kg) for 8 hours in the absence or presence of rapamycin (Rapa, 50 nM) or Torin1 (100 nM). Nuclei stained with TO-PRO3 iodide and phase contrast images of the cultures are shown.

These results indicated that the activity of NFAT5 was reduced upon prolonged mTOR inhibition. However, seeing that Torin1 inhibited the induction of only a set (22%) of NFAT5-dependent genes, as well as a similar proportion (25%) of NFAT5-independent ones, we considered that NFAT5 might not be a primary target of mTOR, and wondered whether Torin1 might affect other transcription regulatory mechanisms.

Prompted by a recent work showing that hypertonic stress induced rapid changes in the chromatin configuration of the aldose reductase gene (Tong et al., 2009), we asked whether mTOR could regulate this processes in some NFAT5-dependent and independent osmostress-responsive genes. For this reason we decided to analyze the chromatin configuration pattern of three different induced as well Torin1-sensitive genes upon osmostress. Two of them were NFAT5-dependent: *Akr1b3*, a well characterized NFAT5 target gene, and *Ddit4l* (REDD2), identified in our study **(Figure 11**). The other one, *Bpgm* (2,3-bisphosphoglycerate mutase), was also induced by osmostress but its induction was NFAT5-independent.

To determine the appropriate time required to analyze changes in chromatin under hyperosmotic stress conditions we took advantage of our time course mRNA expression experiments **(Figure 8 and 23)**. Regarding *Bpgm* and *Ddit4l* genes, they showed similar patterns of mRNA expression of those observed in *Akr1b3* gene **(Figure 8)**, presenting a stronger inhibition by Torin1 at later time points **(Figure 23)**. However their mRNA inducibility from baseline conditions (300 mOsm/kg) upon hyperosmotic treatment were lower compared with *Akr1b3* **(**compare **Figure 8** with **Figure 23)**. Because we observed significant differences in mRNA expression after 4 and 8 hours of hyperosmotic stimulation between cells not treated or treated with Torin1 in all three genes, and chromatin conformational changes must

occur prior to their mRNA induction, we decided to analyze chromatin configuration after 1 and 4 hours of hyperosmotic stress treatment.

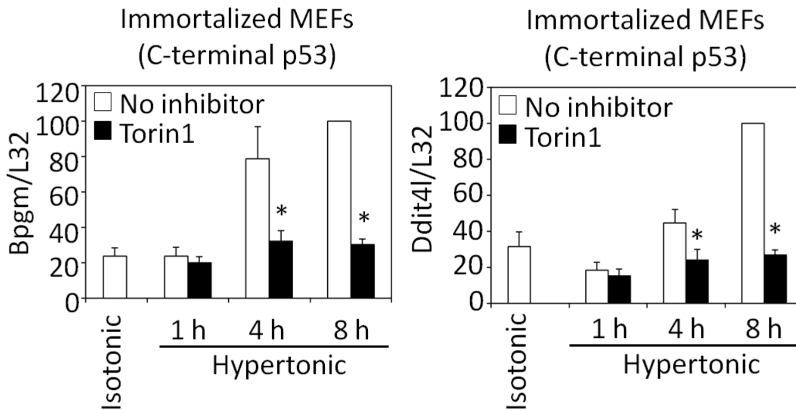


Figure 23. Effect of Torin1 on the mRNA expression of *Ddit4l* and *Bpgm* during a time course experiment. RNA was isolated from immortalized MEFs (C-terminal p53) cultured in isotonic (300 mOsm/kg) or hypertonic medium (500 mOsm/kg, upon addition of 100 mM NaCl) without or with Torin1 (250 nM). mRNA abundance for *Ddit4l* and *Bpgm* normalized to *L32* mRNA is represented relative to the sample of 8 hours of hypertonic stimulation (100%). Bars represent the mean ± S.E.M. of three independent experiments (* $p < 0.05$).

In agreement with Tong et al. (Tong et al., 2009), our analysis of the *Akr1b3* (aldose reductase) gene showed that hypertonic stress induced extensive acetylation of histone H4 at different promoter and transcribed regions, with different degrees depending on the region studied **(Figure 24A and 24B)**, since the first hour of stimulation **(Figure 24A and 25)**, followed by eviction of nucleosomes from the transcription start site (TSS) and upstream regions at later time points **(Figure 25)**. Torin1 inhibited the acetylation of H4 throughout an extended region of ~7 kb clearly evident after 4 hours of hypertonic stimulation **(Figure 24B and 25)**, but did not affect the nucleosome eviction **(Figure 25)**.

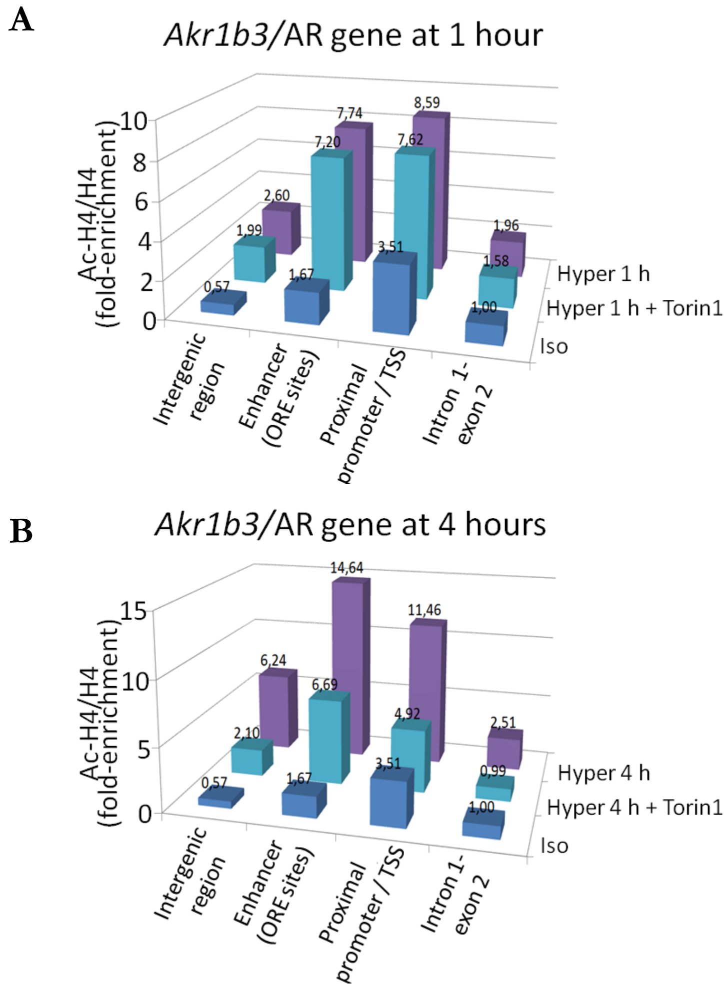


Figure 24. Effect of Torin1 on the relative acetylation of *Akr1b3* (aldose reductase) gene in response to osmostress. Chromatin from immortalized MEFs (C-terminal p53) cultured in isotonic (300 mOsm/kg) or hypertonic medium (500 mOsm/kg) without or with Torin1 (100 nM) was immunoprecipitated with an acetylated histone H4-specific rabbit antibody or a histone H4-specific rabbit antibody. Immunoprecipitated chromatin was normalized to its respective total chromatin input. Bars show the ratio of acetylated H4 to total H4 at **(A)** 1 hour and **(B)** 4 hours of stimulation for the different regions of the *Akr1b3* gene relative to the sample of untreated cells (300 mOsm/kg, at time 0) on the intron 1-exon 2 region, arbitrary value of 1. Results represent the mean of four independent experiments.

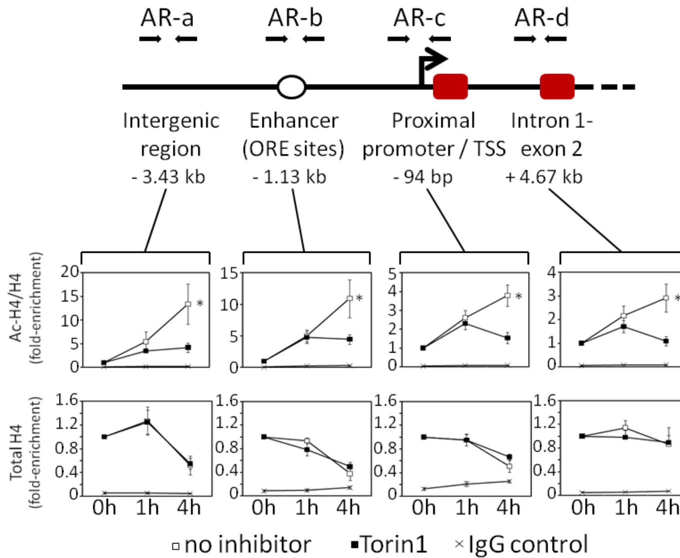


Figure 25. Effect of Torin1 on the chromatin configuration of the *Akr1b3* (aldose reductase) gene in response to osmostress. Schematic representation of the *Akr1b3* gene showing the location of the primers used for the different regions analyzed. Chromatin from C-terminal p53-immortalized MEFs cultured in isotonic (300 mOsm/kg) or hypertonic medium (500 mOsm/kg) for 1 and 4 hours without (white squares) or with (black squares) Torin1 (100 nM) was immunoprecipitated with a control IgG, an acetylated histone H4-specific antibody or a histone H4-specific antibody. Immunoprecipitated chromatin was normalized to its respective total chromatin input. Upper graphics show the ratio of acetylated H4 to total H4 at different time points and conditions relative to a sample of untreated cells (300 mOsm/kg, time 0, arbitrary value of 1) for each pair of primers used. Lower panels correspond to the immunoprecipitation with the H4-specific antibody in the same samples. Results represent the mean ±S.E.M. of four independent experiments (* $p < 0.05$).

We then tested the effect of osmostress and Torin1 on H4 acetylation and nucleosome eviction in the NFAT5-dependent *Ddit4l*, and the NFAT5-independent *Bpgm*. When we analyzed the same chromatin parameters we observed that the increase in H4 acetylation induced by osmotic stress in these genes was rather modest in comparison with *Akr1b3*, but was still

Torin1-sensitive **(Figure 26A, 26B, 27A and 27B).** Similarly to what we had observed in the *Akr1b3* gene, osmotic stress caused a progressive nucleosome eviction in *Ddit4l* and *Bpgm* that was not affected by Torin1 **(Figure 28A and 28B)**.

We also analyzed whether inhibition of mTOR under osmostress conditions affected the recruitment and phosphorylation of RNA polymerase II (RNA-pol II), a more direct indicator of transcriptional activity. The carboxy-terminal domain (CTD) of the largest subunit of RNA-pol II (RPB1) is a flexible scaffold allowing the recruitment of a variety of nuclear factors which facilitates mRNA processing, transcription elongation, or termination and whose binding is enhanced by phosphorylation of this CTD (Phatnani and Greenleaf, 2006). The CTD of RNA-pol II comprises tandem repeats (between 25 to 52, depending on the organism studied) of the heptad (YSer(2)PTSer(5)PSer(7)) sequence. CTD repeats can be phosphorylated on Ser5 when RNA-pol II is initiating transcription and in Ser2 (with concomitant Ser5 phosphorylated) when RNA-pol II is transcriptionally elongating (Sims et al., 2004), (Phatnani and Greenleaf, 2006), thus coupling RNA-pol II phosphorylation status to the transcriptional process. Osmotic stress induced a rapid and substantial recruitment of RNA-pol II to the TSS and transcribed region (exon 2) of *Akr1b3* gene after 1 hour of hypertonic stimulation **(Figure 29)**. Inhibition of the osmostress-induced RNA pol II recruitment by Torin1 was already detected in 1 hour at the transcribed region, and preceded the partial dissociation of NFAT5 from the *Akr1b3* enhancer **(Figure 20)**, indicating that inhibition of RNA-pol II was not due to the dissociation of NFAT5 from upstream regions. Further analysis showed that both rapamycin and Torin1 were significantly effective at inhibiting the binding of RNA-pol II to the TSS of *Akr1b3* gene at 4 hours of hyperosmotic treatment **(Figure 30A)**, as well as the recruitment to the TSS and exon 2 of active forms of RNA-pol II **(Figure 30B and 30C)**.

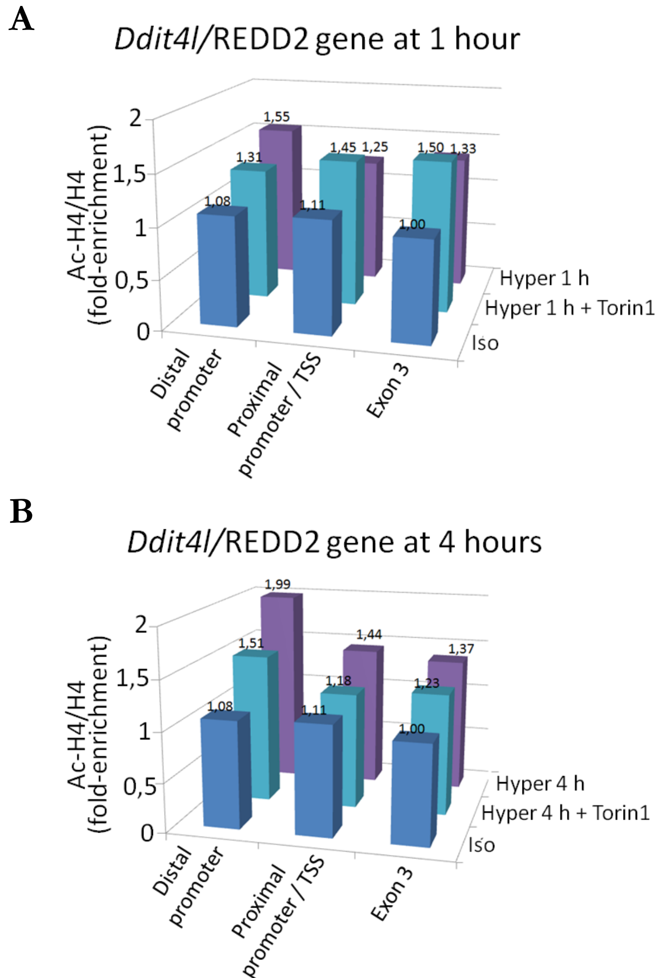


Figure 26. Effect of Torin1 on the relative acetylation of the *Ddit4l*/REDD2 gene in response to osmostress. Chromatin from immortalized MEFs (C-terminal p53) cultured in isotonic (300 mOsm/kg) or hypertonic medium (500 mOsm/kg) without or with Torin1 (100 nM) was immunoprecipitated with an acetylated histone H4-specific rabbit antibody or a histone H4-specific rabbit antibody. Immunoprecipitated chromatin was normalized to its respective total chromatin input. Bars show the ratio of acetylated H4 to total H4 at **(A)** 1 hour and **(B)** 4 hours of stimulation relative to the sample of untreated cells (300 mOsm/kg, at time 0) on the exon 3 region, arbitrary value of 1. Results represent the mean of five independent experiments.

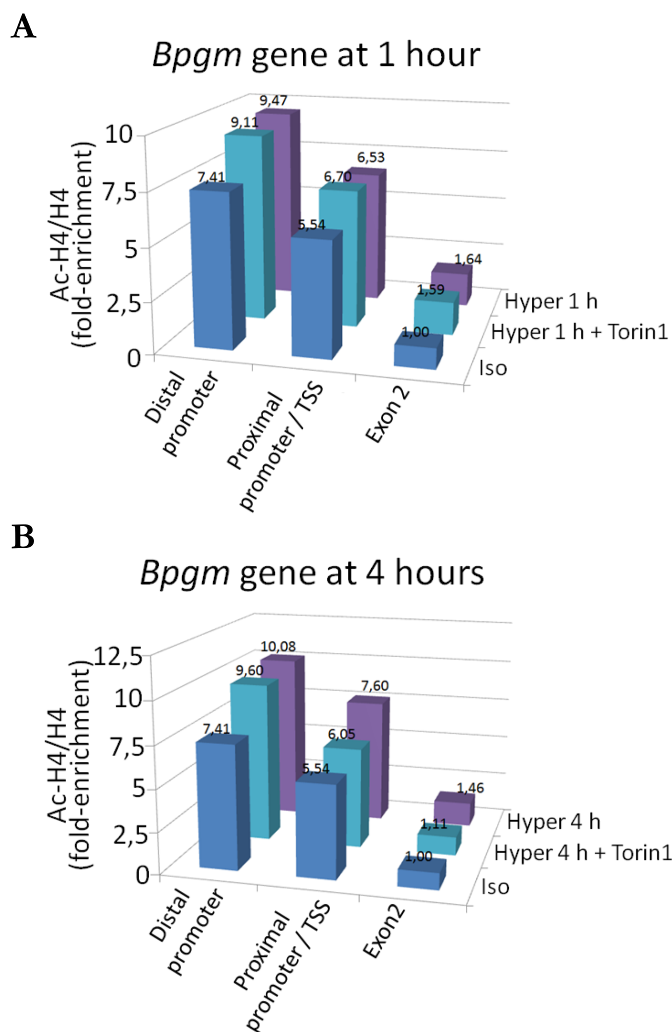


Figure 27. Effect of Torin1 on the relative acetylation of *Bpgm* gene in response to osmostress. Chromatin from immortalized MEFs (C-terminal p53) cultured in isotonic (300 mOsm/kg) or hypertonic medium (500 mOsm/kg) without or with Torin1 (100 nM) was immunoprecipitated with an acetylated histone H4-specific rabbit antibody or a histone H4-specific rabbit antibody. Immunoprecipitated chromatin was normalized to its respective total chromatin input. Bars show the ratio of acetylated H4 to total H4 at **(A)** 1 hour and **(B)** 4 hours of stimulation for the different regions of the *Bpgm* gene relative to the sample of untreated cells (300 mOsm/kg, at time 0) on the exon2 region, arbitrary value of 1). Results represent the mean of five independent experiments.

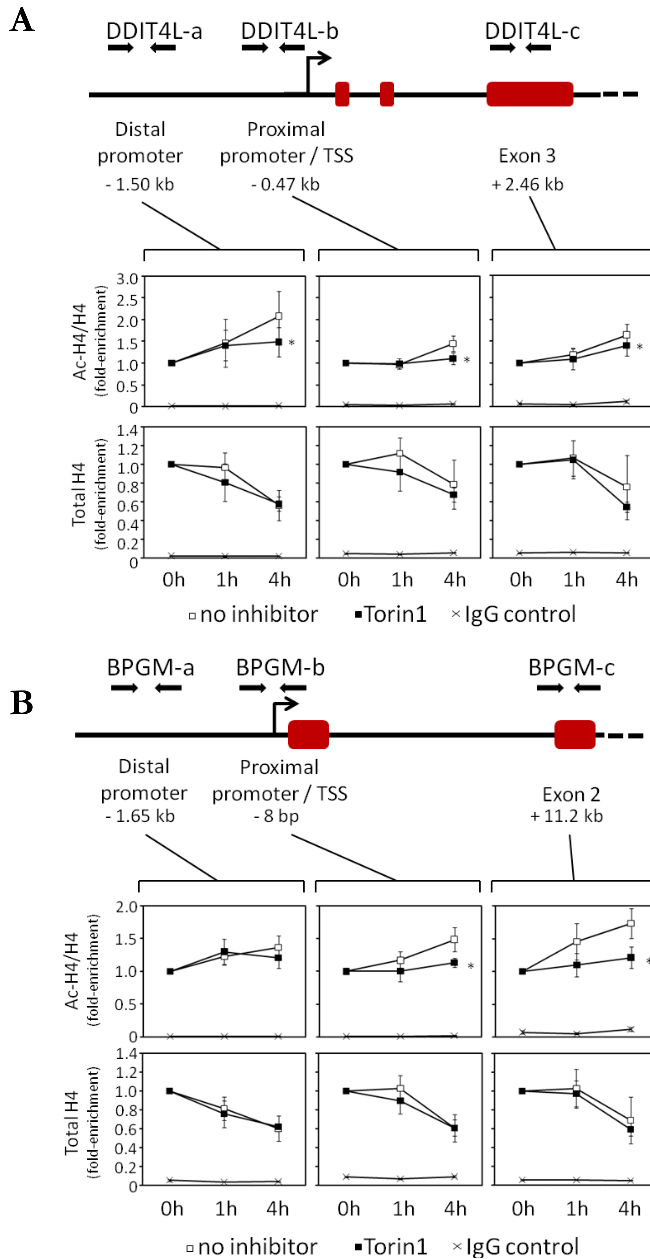


Figure 28. Effect of Torin1 on the chromatin configuration of *Ddit4l* and *Bpgm* in cells exposed to osmotic stress. Schematic representation of **(A)** *Ddit4l* and **(B)** *Bpgm* genes showing the location of the primers used to analyze chromatin configuration. Chromatin from C-terminal p53-immortalized MEFs cultured in isotonic (300 mOsm/kg) or hypertonic medium (500 mOsm/kg, upon

addition of 100 mM NaCl) without or with Torin1 (100 nM) was immunoprecipitated with a control IgG, an acetylated-histone H4-specific antibody or a histone H4-specific antibody. Immunoprecipitated chromatin in each sample was normalized to its respective total chromatin (input). Upper graphics in **(A)** and **(B)** show the ratio of acetylated H4 to total H4 at different time points and conditions relative to a sample of untreated cells (300 mOsm/kg, time 0) for each pair of primers, which was given an arbitrary value of 1. Lower panels show the enrichment in chromatin immunoprecipitated by the total H4-specific antibody in the same samples. Results represent the mean ±S.E.M. of five independent experiments (* $p<0.05$).

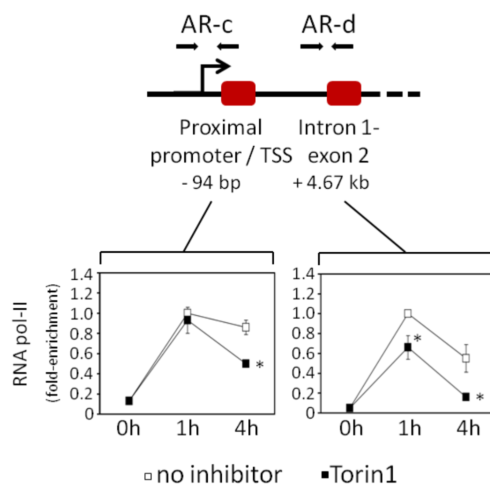


Figure 29. Effect of Torin1 on the time course recruitment of RNA pol-II to the *Akr1b3* gene in cells exposed to osmotic stress. Diagram shows a schematic representation of the *Akr1b3* gene showing the location of the primers used for different regions in this analysis. Formaldehyde-crosslinked chromatin from immortalized MEFs (C-terminal p53) cultured in isotonic (300 mOsm/kg) or hypertonic medium (500 mOsm/kg, upon addition of 100 mM NaCl) without or with Torin1 (100 nM) during 1 and 4 hours was immunoprecipitated with an RNA pol-II specific rabbit antibody. Immunoprecipitated chromatin in each sample was normalized to its respective total chromatin (input). Results are shown relative to the sample of 1 hour of hypertonic treatment (arbitrary value of 1), and represent the mean ± S.E.M. of three independent experiments (* $p < 0.05$).

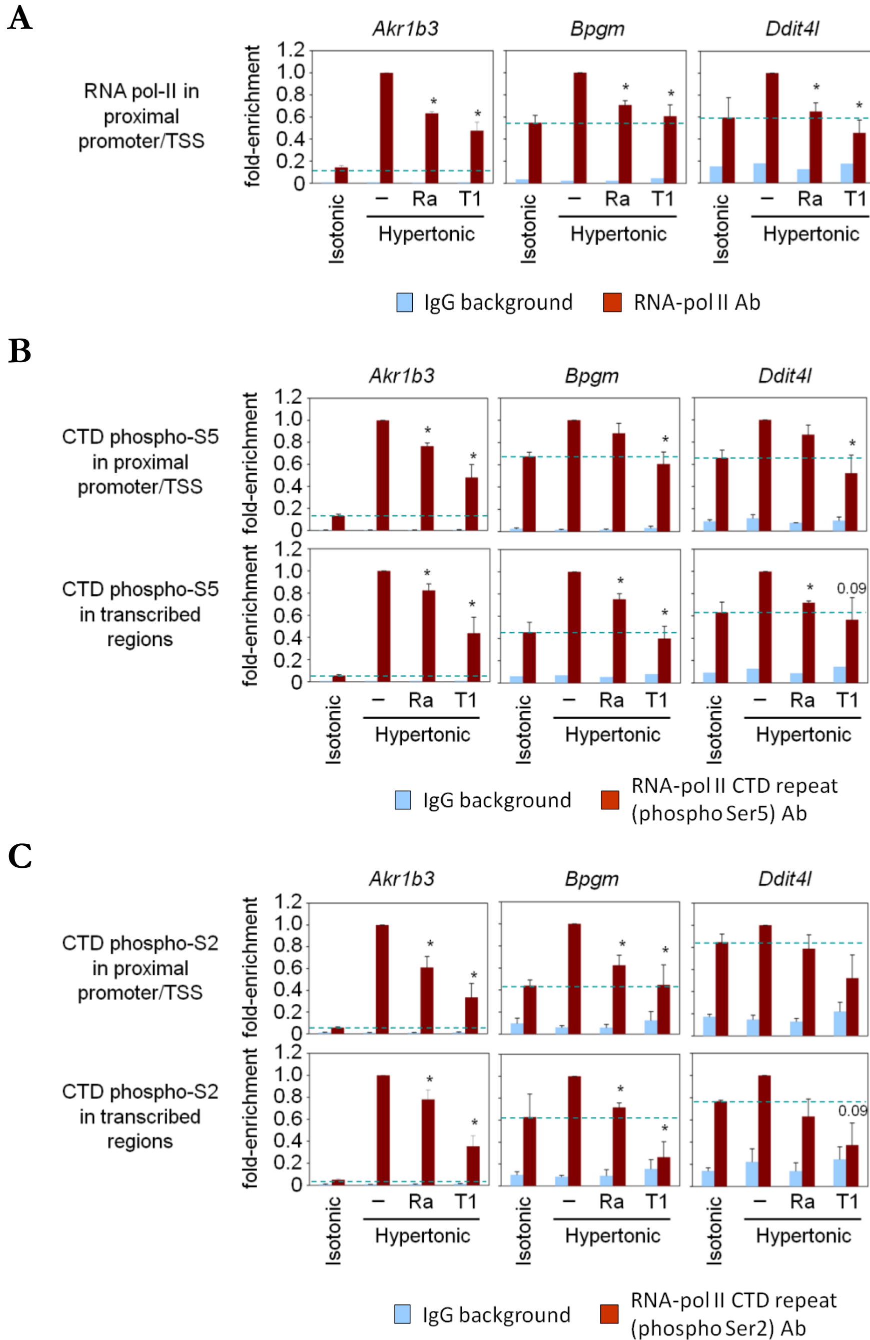


Figure 30. Effect of rapamycin and Torin1 on the recruitment of RNA-pol II to osmostress responsive genes. Chromatin from C-terminal p53-immortalized MEFs cultured during 4 hours in isotonic (300 mOsm/kg) or

hypertonic medium (500 mOsm/kg, upon addition of 100 mM NaCl) without or with 50 nM rapamycin (Ra) or 100 nM Torin1 (T1) was immunoprecipitated with a control rabbit IgG antibody or specific antibodies for **(A)** RNA-pol II, **(B)** phosphorylated Ser5 or **(C)** phosphorylated Ser2 in its carboxy-terminal domain heptad repeat (CTD). Immunoprecipitated chromatin was analyzed with primers corresponding to the proximal promoter/TSS and transcribed exonic regions of *Akr1b3* (intron 1 - exon 2), *Bpgm* (exon 2) and *Ddit4l* (exon 3) genes, and normalized to its respective total chromatin input for each sample. Results are shown relative to the sample of 4 hours of hypertonic treatment (arbitrary value of 1), and represent the mean ± S.E.M. of three independent experiments (* $p < 0.05$).

Regarding *Bpgm* gene, we observed a moderate effect of osmostress in enhancing the recruitment of RNA-pol II and its phosphorylated Ser5 and Ser2 forms compared to the *Akr1b3* gene, but nonetheless this effect was significant and inhibited by either rapamycin or Torin1 **(Figure 30A, 30B and 30C)**. For *Ddit4l* gene, recruitment of RNA-pol II to its TSS and binding of active forms to its TSS and transcribed regions were minimally increased by osmostress compared to *Akr1b3* and *Bpgm* genes **(Figure 30A, 30B and 30C)**. However Torin1, and in a lesser extent rapamycin, although they did not lead to a significant reduction in all the activation readouts analyzed for RNA-pol II, could suppress the modest RNA-pol II recruitment and activation induced by osmostress on the *Ddit4l* gene **(Figure 30A, 30B and 30C)**.

SECTION V: mTOR signaling pathway perturbation by siRNAs in mammalian cells

Although some drug inhibitors, like rapamycin, have been commonly used for years in basic research as a tool to study mTORC1 signaling, now it is known that rapamycin does not inhibit the function of all its substrates equally (Choo et al., 2008), (Thoreen et al., 2009), (Feldman et al., 2009). Others recently described synthetic inhibitors, such as Torin1, that efficiently inhibit both mTORC1 and mTORC2 complexes (Thoreen et al., 2009), (Liu et al., 2012).

As our results showed, rapamycin and Torin1 pretreatment only differed in the stronger effect of Torin1 in inhibiting the different osmostress studied processes like induction of gene expression **(Figure 5, 8 and 12)**, cell proliferation and cell size **(Figure 13)**, NFAT5-chromatin recruitment **(Figure 21)** and RNA-pol II gene-specific recruitment **(Figure 30).** Importantly, our results showed that rapamycin inhibited partially the phosphorylation of 4E-BP1, a well-established mTORC1 target, comparing with Torin1 treatment **(Figure 1)**, and did not inhibit the phosphorylation of the well-characterized mTORC2 substrate Akt in Ser473 (**Figure 1)**, indicating that mTORC1 was partially active and mTORC2 was not being inhibited by rapamycin during the overall duration of our assays. These data strongly suggested the involvement of mTORC1 rather than mTORC2 in the osmostress responses, and established Torin1 as a more potent and efficiently inhibitor of mTORC1 than rapamycin, in agreement with a previous work published by Thoreen et al. (Thoreen et al., 2009).

To revalidate the data obtained by these two mechanistically independent mTOR inhibitors, and focusing now only on mTORC1, we decided to analyze the effect of specifically modulate mTORC1 signaling in some

osmostress-induced genes by an independent approach. Although MEFs knockout for TSC2 (TSC2$^{-/-}$) having a constitutively hyperactive mTORC1 signaling have been generated (Zhang et al., 2003), the isolation of MEFs lacking mTORC1-specific components was problematic, because mTOR, mLST8 and RAPTOR are essential and knocking-out these genes in mice results in developmentally delayed embryos that die by embryonic day 5.5 to 11.5 post-implantation depending on the gene deleted (Gangloff et al., 2004), (Guertin et al., 2006), complicating the isolation of viable knockout MEFs. For this reason, we decided to determine the involvement of mTORC1 in the osmostress response using RNA interference (RNAi) mediated knockdown experiments using transient transfection of siRNA pools targeting TSC2 –to obtain cells with hyperactive mTORC1 signaling- or RAPTOR –to achieve specific mTORC1 inhibition without perturbing mTORC2 complex-.

Under osmostress conditions, the suppression of TSC2 in wild-type MEFs was confirmed by the decrease of its own mRNA and protein levels **(Figure 31 and 32)**. When an efficient TSC2 protein knockdown was achieved there was an increase in the mTORC1 activity -being Thr389 of S6K1 and Ser235/236 of S6 its specific readouts- in cells subjected to osmostress **(Figure 32A and 32B)**. However, this increase in mTORC1 activity did not correlate with an enhancement of the expression of the different osmostress-induced genes studied such as *Akr1b3, Hspa1b, Bpgm, Ddit4 and Ddit4l* **(Figure 33)**, suggesting that the mTORC1 activity in these cells was already sufficient to achieve the maximal induction of these genes.

On the other hand, the suppression of RAPTOR in wild-type MEFs yielded an unexpected result. Although in almost all our experiments we achieved a substantial suppression of RAPTOR mRNA and protein levels **(Figure 31 and 32)** we found that mTORC1 signaling perturbation was more difficult to achieve **(Figure 32)**. In different experiments we found that cells in

which RAPTOR expression had been substantially reduced with its specific siRNA, the residual RAPTOR protein levels were sufficient to maintain mTORC1 still active (i.e. ≤65% RAPTOR protein suppression only caused ≤40% reduction on Thr389 phosphorylation of S6K1) **(Figure 32C)** and to maintain the mTORC1-dependent induction of some of the genes tested *(data not shown)*, indicative of being highly steady against cellular perturbations. However, when we were able to suppress more extensively RAPTOR protein levels (between 75 and 85% protein reduction) we observed a greater inhibition of mTORC1 activity and also a substantial decrease in the induction of osmoresponsive genes (**Figure 32A and 32B** *and data not shown***)**.

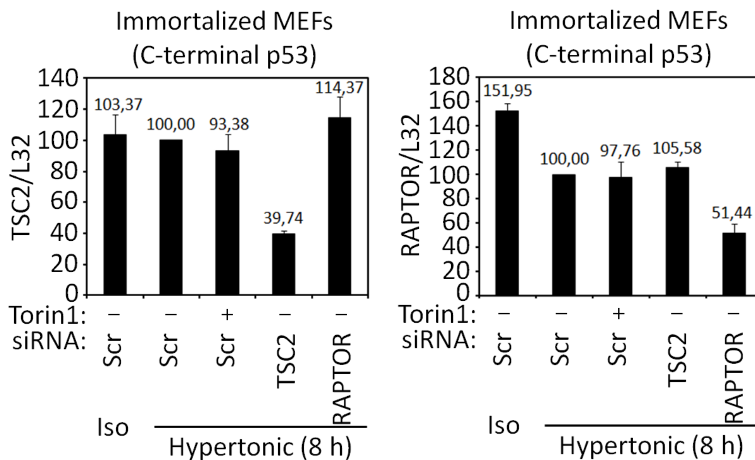
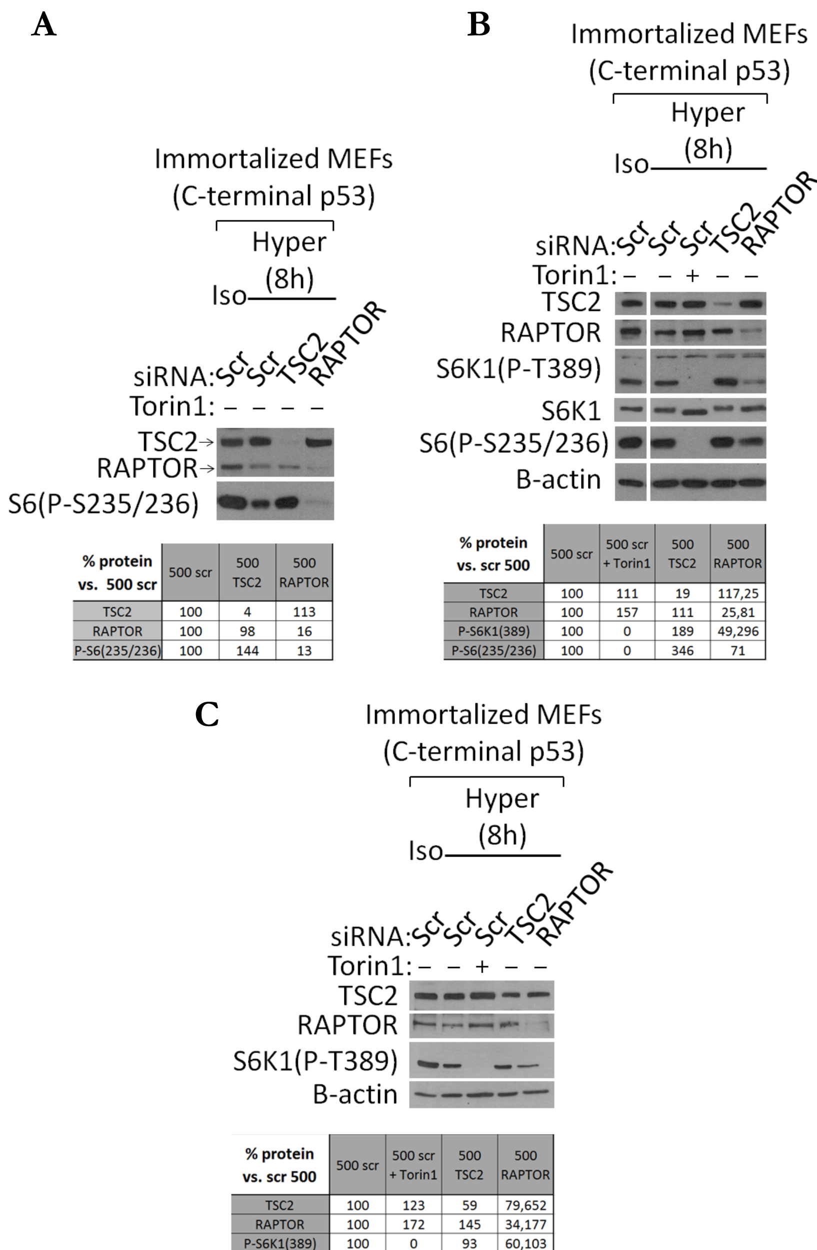


Figure 31. Effect of TSC2 and RAPTOR siRNA knockdown on its own mRNAs. Immortalized MEFs (C-terminal p53) were transfected with control non-targeting siRNA (scramble; Scr) or siRNA specific for TSC2 or RAPTOR and maintained in isotonic conditions (Iso, 300 mOsm/kg) or hypertonic conditions (500 mOsm/kg, upon addition of 100 mM NaCl) for 8 hours. Torin1 (100 nM) was added 1 hour before hypertonic treatment when indicated (+). RNA levels for each gene and condition were normalized to *L32* mRNA and are represented relative to the amount of mRNA in hypertonic conditions in cells transfected with control scramble siRNA, which was given an arbitrary value of 100. Bars represent the mean ± S.E.M. of four independent experiments.



% protein vs. 500 scr	500 scr	500 TSC2	500 RAPTOR
TSC2	100	4	113
RAPTOR	100	98	16
P-S6(235/236)	100	144	13

% protein vs. scr 500	500 scr	500 scr + Torin1	500 TSC2	500 RAPTOR
TSC2	100	111	19	117,25
RAPTOR	100	157	111	25,81
P-S6K1(389)	100	0	189	49,296
P-S6(235/236)	100	0	346	71

% protein vs. scr 500	500 scr	500 scr + Torin1	500 TSC2	500 RAPTOR
TSC2	100	123	59	79,652
RAPTOR	100	172	145	34,177
P-S6K1(389)	100	0	93	60,103

Figure 32. Effect of TSC2 and RAPTOR siRNA knockdown on its own protein levels and on the mTORC1 signaling pathway activity. Immortalized MEFs (C-terminal p53) were transfected with control non-targeting siRNA (scramble; Scr), siRNA specific for TSC2 or for RAPTOR. Between 15 to 48 hours after transfection. Cells were cultured during 8 hours in isotonic (Iso, 300

mOsm/kg) or hypertonic medium (500 mOsm/kg, upon addition of 100 mM NaCl). Torin1 (100 nM) was added when indicated (+). TSC2, RAPTOR, S6K1(P-T389), S6K1 total, S6(P-S235/236) or β-actin (as loading control) were detected by Western blotting. Figure **(A), (B),** and **(C)** show three independent experiments performed. Bottom panels of each figure show the protein quantification under hypertonic conditions of TSC2 and RAPTOR –as indicators of efficiency of siRNA protein knockdown- and S6K1(P-T389) and S6(P-S235/236) –as readouts of mTORC1 activity- for each experiment relative to the condition of 500 mOsm/kg scramble transfected (which was given an arbitrary value of 100%).

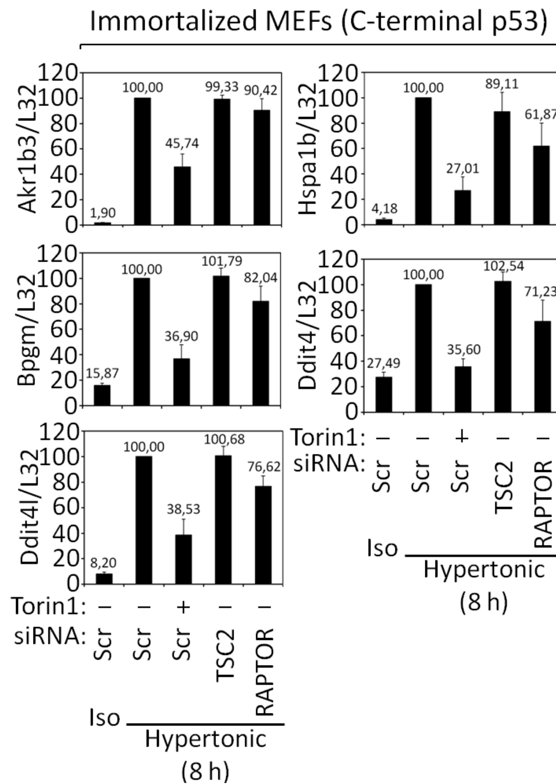


Figure 33. Effect of TSC2 and RAPTOR siRNA knockdown on AR (*Akr1b3*), Hsp70.1 (*Hspa1b*), BPGM (*Bpgm*), REDD1 (*Ddit4*) and REDD2 (*Ddit4l*) mRNAs. Immortalized MEFs (C-terminal p53) were transfected with control non-targeting siRNA (scramble; Scr), siRNA specific for TSC2 or for RAPTOR. Between 15 to 48 hours after transfection, they were

cultured during 8 hours in isotonic (Iso, 300 mOsm/kg) or hypertonic medium (500 mOsm/kg, upon addition of 100 mM NaCl). Torin1 (100 nM) was added 1 hour before of hypertonic treatment when indicated (+). RNA levels for each gene and condition were normalized to *L32* mRNA and are represented relative to the amount of mRNA in hypertonic conditions in cells transfected with control scramble siRNA, which was given an arbitrary value of 100. Bars represent the mean ± S.E.M. of three independent experiments.

While our results suggested that in immortalized MEFs the induction of osmoresponsive genes could be slightly decreased (between 25 and 50% depending on the gene tested) when RAPTOR siRNA was capable of decreasing RAPTOR protein availability to an extent sufficient to greatly limit mTORC1 activity **(Figure 32A and 32B)**, we did not consistently achieve sufficient inhibition of mTORC1 activity to observe a great reduction in the inducibility of the different genes tested, compared to that Torin1 treated cells under hypertonicity. Despite this, we observed that cells transfected with siRNAs for RAPTOR had a tendency to induce less the mRNAs of all the genes analyzed **(Figure 33)**.

Interestingly, our results are not an exception because other works that used RAPTOR specific siRNAs to downregulate mTORC1 activity in different cell types also showed that after a substantial downregulation of RAPTOR protein, mTORC1 activity is still sufficient to maintain mTORC1-dependent functions and processes (Yang et al., 2006), (Wang et al., 2008), (Breuleux et al., 2009), (Nyfeler et al., 2011). Moreover, we have also found in the literature that suppression of mTORC1 activity by specific RAPTOR siRNA was more efficient in cells that had low mTORC1 activity levels as under poor nutritional media conditions –i.e. serum deprived cells- or with low cell proliferation rates–i.e. stem cells- because similar levels of RAPTOR knockdown were sufficient to suppress mTORC1 activity completely (Sarbassov and Sabatini, 2005), (Goncharova et al., 2011), (Valli et al., 2010).

However, other works that used a more efficient molecular approach for protein knockdown, such as infection of cells with recombinant viruses expressing mTOR and RAPTOR RNAi cassettes, that lead to a more potent downregulation of the targeted protein of interest, observed a highly evident reduction on mTORC1 activity (Sarbassov et al., 2004), (Sarbassov et al., 2006), (Dowling et al., 2010).

Until the synthesis of more potent mTOR inhibitors, mainly ATP-competitive, the study of mTOR function in cultured cells has relied on rapamycin treatment or RNA interference (RNAi) mediated knockdown of several mTOR signaling components (Cybulski et al., 2012). However, as we confirmed with our results, neither of those two approaches is optimal because neither of them causes the complete inhibition of mTOR signaling. In our study, protein knockdown mediated by siRNA silencing, was never complete and the remaining protein levels were enough to provide at least partial mTORC1 complex function.

DISCUSSION

DISCUSSION

Cells and organisms are continuously exposed to environmental as well as intracellular insults that perturb their cellular homeostasis. The adequate cellular adaptation towards damaging agents is essential to allow cells to survive. Adaptive stress responses are mechanisms conserved through evolution that are capable of integrating these extracellular as well as intracellular perturbations to different cellular functions, like growth and proliferation, thus minimizing further perturbing outcomes (Kultz, 2005). In fact, many reports have addressed the question of how stress impacts on growth, survival and proliferative capacity of the cell (Liu et al., 2006).

In the last decade, the knowledge about the mTOR signaling pathway has been growing exponentially and it has been established as a central effector pathway in cell growth and proliferation influenced by the nutritional as well as the energetic status of the cell (Sengupta et al., 2010). It has been shown that different extracellular cues like growth factors, glucose, amino acids and stresses can influence the activity of mTOR kinase. In this regard, many works have addressed the question of how stress impacts on mTOR signaling (Inoki et al., 2003), (Brugarolas et al., 2004), (Parrott and Templeton, 1999), (Horton et al., 2002). However, how the mTOR pathway influences cellular adaptive stress responses is still largely unexplored. In this work we have analyzed how mTOR modulate the specific adaptive response to tolerable osmostress.

Although nearly all studies regarding mTOR and osmotic stress have been done in intense hyperosmotic stress conditions (600 – 900 mOsm/kg) and short time points (few minutes to 1 hour), most argue that upon osmostress the mTORC1 signaling pathway is inhibited, detected by complete inhibition of S6K1 phosphorylation (Parrott and Templeton, 1999), (Martini and

Kruppa, 1979), (Kruppa and Clemens, 1984), (Naegele and Morley, 2004), (Desai et al., 2002). However, this acute elevation of osmolality leads to a "DNA damage-like stress response" phenotype, characterized by some irreversible changes that cannot be repaired in the process of adaptation to stress. Thereby, studies done in mIMCD3 cells by Burg and co-workers showed that adding NaCl to a total osmolality of 700 mOsm/kg kills >90% of the cells by apoptosis within 24 h (Dmitrieva et al., 2000). By contrast our study, done at more moderate hypertonic stress conditions (500 mOsm/kg) and at longer time points (between 1 hour to 8 hours), shows that although a partial inhibition of both mTOR complexes can be observed from the first hour of osmostress induction, they retained substantial activity to be capable of effectively phosphorylate their respective downstream substrates, S6K1 at Thr389 for mTORC1 and AKT at Ser473 for mTORC2. Additionally, these cells maintained their growth and proliferative potential after 72 hours of osmostress induction, indicative of having a functional and active mTOR signaling under moderate osmostress conditions.

Other studies have shown that intense hyperosmotic stress conditions (>600 mOsm/kg) can activate AMPK (Barnes et al., 2002), (Hawley et al., 2010), thus consequently inhibiting mTORC1, as observed in other stress scenarios like hypoxia or glucose deprivation. As we did not notice substantial changes in AMPK phosphorylation at Thr172, an AMPK activation mark, in our experimental settings, we could argue that the partial inhibition that we observed on the mTOR signaling is not due to the inhibitory effect of AMPK towards mTORC1.

Another important issue to take into account regarding stress and mTOR is the relationship that exists between mTOR hyperactivation and cell sensitization to stress. Several reports have shown that the constitutive mTOR hyperactivation observed in TSC1 or TSC2 knockout MEFs leads to

reduced survival and increased apoptosis (Ng et al., 2011) upon challenge with different types of stress, mainly due to p53 stabilization (Choo et al., 2010), (Lee et al., 2007) or reduced NF-κB signaling (Ghosh et al., 2006). Moreover, they showed that mTORC1 inhibition by rapamycin during stress improves survival as well as higher resistance to stress (Ghosh et al., 2006), (Ng et al., 2011). This protective effect can explain why cells subjected to moderate intensities of osmotic stress have reduced activity of mTOR while stress persists, conferring better resistance during their process of adaptation. However, additional work is needed to characterize the specific mechanism that mediates this slightly reduced activity of mTOR during the course of stress but also to elucidate the possible mechanisms that restore mTOR signaling once cells have adapted to it.

The finding that rapamycin suppressed mTORC1, but not mTORC2, in cells exposed to osmostress, together with the comparable inhibitory effect of rapamycin and Torin1 on gene expression indicate that at least mTORC1 is capable to influence the transcription of some osmoresponsive genes. Nonetheless, our results do not rule out that mTORC2 might also regulate the expression of some of the genes analyzed in our study because most of the osmoresponsive genes analyzed in our microarray are inhibited better with Torin1, that could inhibit both mTOR complexes, than with rapamycin. The precise involvement of each of the two mTOR complexes in the induction of the different osmostress response induced genes awaits future studies.

One of our main findings is that mTOR modulates a subset of all osmostress induced genes. Our microarray data showed that inhibition of mTOR by Torin1 significantly reduced the expression of 22% of all the genes induced, at 8 hours, by hypertonic stress, but did not suppress the whole osmostress response. In this way, the mTOR signaling is shown not only to be required

to regulate the induction of this subset of osmostress induced genes but also has been shown to modulate gene expression in different ways. As commented before, some genes required mTOR to be optimally induced, others were not particularly responsive to hypertonicity but needed an active mTOR signaling to maintain their expression and even a few more were downregulated during osmostress in an mTOR-dependent manner. Our findings suggested that the overall impact of mTOR signaling under osmostress is to regulate a subset of genes that later on sustain the growth and proliferative capacity of cells during their adaptation process to stress. Inhibition of mTOR under moderate osmostress makes cells grow and proliferate more slowly. However, mTOR inhibition under hypertonic stress conditions did not decrease cellular viability, supporting the idea mentioned above in which mTOR inhibition, by slowing down growth and proliferative capacity of the cells, confers a protective effect under stress.

On the other hand, very few genes were detected to be affected by mTOR inhibition under isotonic conditions in continuously growing MEFs. In this regard, independent microarray studies have shown that mTOR inhibition by rapamycin has a greater impact on specific gene expression programs (Peng et al., 2002), (Düvel et al., 2010). Conversely, another work showed a more reduced set of genes affected by mTOR suppression, as in our study (Grolleau et al., 2002). Our results, however, can be reconciled with these other works considering that different cellular systems, culture conditions and time point analysis were used in the different mentioned works. Düvel *et al.* used serum-starved TSC1 or TSC2 deficient MEFs, having a constitutively hyperactive mTOR signaling independently of growth factor supplies, and consequently amplifying the magnitude of the response observed after mTOR inhibition. Moreover, as shown in Peng *et al.*, the specific time point used for the analysis on changes in gene expression could also be determinant to get these diverse experimental results. As they

showed, the longest time points after mTOR suppression, between 12 and 24 hours, had the highest impact on gene expression (Peng et al., 2002). Thereby, in the report of Grolleau *et al.*, after 4 hours of mTOR suppression they observed a reduced subset of genes affected by mTOR inhibition (Grolleau et al., 2002).

In the last years, apart from its well known role in translation, mTOR has been suggested as a modulator of specific gene expression programs through its regulation of key transcription factors like HIF-1α, SREBP1, PPARγ, p53, NF-κB, NFATc4, STAT1 or STAT3 (Semenza et al., 1994), (Yokogami et al., 2000), (Ellisen et al., 2002), (Kim and Chen, 2004), (Feng et al., 2005), (Yang et al., 2008), (Dan et al., 2008), (Fielhaber et al., 2009), (Düvel et al., 2010). Moreover, it has been shown that both mTOR complexes could directly associate with, as well as phosphorylate some cellular proteins modifying thus their activities (Yokogami et al., 2000), (Hong et al., 2008), (Shor et al., 2010), (Jung et al., 2010). In this line, previous studies had suggested that NFAT5 nuclear translocation as well as NFAT5 transcriptional activity was enhanced by several signaling kinases (reviewed in Aramburu et al., 2006). Specifically, results of our group and others, have shown that NFAT5 is more expressed in cells undergoing proliferation (Trama et al., 2000), (Lopez-Rodriguez et al., 2001), and its expression stimulated by mitogens (Morancho et al., 2008). Moreover, an active PI3K signaling has been shown to enhance NFAT5 transcriptional activity (Irarrazabal et al., 2006). All these evidences, together with our data indicating that NFAT5 transcriptional activity as well as its direct recruitment to promoter regions of some of its target genes were rapamycin-sensitive, led us to speculate that mTOR could directly bind and phosphorylate NFAT5 protein regulating thus its activity or its nuclear translocation capacity. Although we invested considerable effort in elucidating this putative interaction we did not obtain any conclusive results. However, although we

cannot fully rule out possible interactions between NFAT5 and mTOR we have described some evidences indicating that NFAT5 by itself may not the primary determinant in the sensitivity of the different osmoresponsive genes to mTOR inhibition. In this regard, as observed in the microarray analysis, not all the NFAT5-dependent genes are sensitive to mTOR inhibition, although the majority of the osmostress induced genes were NFAT5-dependent. Furthermore, we have observed that mTOR inhibition by rapamycin or Torin1 did not affect NFAT5 synthesis nor its nuclear translocation, although it impairs its sustained binding to NFAT5-regulated promoters.

It was remarkable that mTOR enhanced the induction of *Ddit4* and *Ddit4l*, two NFAT5-dependent genes, in response to hypertonic stress. Both gene products have been shown to be induced in other stress contexts like hypoxia or DNA damage by key transcription factors like HIF-1α and p53, respectively, and to inhibit mTORC1 activity, acting as negative regulators of the mTORC1 signaling (Reiling and Hafen, 2004), (Brugarolas et al., 2004), (Ellisen et al., 2002). However, our data indicated that under hypertonic conditions REDD1 expression did not seem to inhibit mTORC1 signaling, even the slightly decrease observed in the phosphorylation of S6K1 in the Thr389. In this regard, another study has shown that REDD1 expression can be induced by other stimuli without disturbing mTORC1 signaling, such as insulin (Regazzetti et al., 2010). Moreover, under normal growth conditions, REDD1 deficient MEFs did not show higher levels of mTORC1 signaling than wild-type ones (Horak et al., 2010). This set of observations suggests that REDD1 induction and expression in different scenarios is not always associated with mTORC1 signaling inhibition.

In addition to the results discussed above, preventing the induction of REDD1 protein in a hyperosmotic stress scenario leads to an altered

expression of some osmoresponsive genes, suggesting an mTOR-independent role in the control of gene expression mediated by REDD1. Our findings here are in line with a previous article by Horak et al. showing also some mTOR-independent effects of REDD1 on gene expression. In this work they described that REDD1-deficient MEFs under normoxia have increased levels of HIF-1α protein, with a higher expression of several glycolytic genes and an enhanced glycolytic metabolism. In fact, HIF-1α stabilization in REDD1-deficient MEFs was mediated by derepression of mitochondrial ROS production (Horak et al., 2010). Understanding the complexity of these autoregulatory loops between common stress transcriptional targets like REDD1, and master stress transcription factors like HIF-1α in hypoxia, p53 in DNA damage and NFAT5 under osmotic stress, will be helpful to elucidate the common molecular mechanisms acting in the different adaptive stress responses.

One important event in gene transcription regulation is to dynamically change some epigenetic marks of core histones towards a permissive chromatin configuration state, allowing thus transcription (Mellor, 2005), (Smith and Shilatifard, 2010). Histone acetylation is one of the best characterized permissive chromatin marks that positively enhance gene transcription. Our findings here are consistent with a previous article by Tong. et al showing that upon hypertonic stress the NFAT5-dependent *Akr1b3* gene was nucleosome depleted and histone hyperacetylated at its promoter as well as transcribed regions (Tong et al., 2009). Interestingly, the data provided in our study support this fact by adding two more genes, *Bpgm* and *Ddit4l,* to this hypertonicity-dependent regulation.

Besides the modulation of gene expression by mTOR through regulation of several transcription factors, several works in yeast have studied the involvement of TOR in controlling RNA-pol dependent transcription as well

as chromatin dynamics (Mayer et al., 2004), (Li et al., 2006), (Woiwode et al., 2008), (Wei et al., 2009), (Kantidakis et al., 2010). In contrast, very little is known about the role of mTOR in these transcriptional events in mammalian cells. Since we observed that only a fraction of all osmostress response genes was affected by mTOR inhibition and not all of them were NFAT5-dependent we tried to find common epigenetic as well as RNA-pol II transcriptional events, independently of NFAT5 requirement, governing the specific dependence of this subset of genes to mTOR activity. In this regard, our data show that active mTOR signaling is needed to enhance histone acetylation at promoters and transcribed regions upon osmostress, which possibly facilitates RNA-pol II recruitment to transcriptional start sites and allows transcription. However, the three genes analyzed in this work exhibited differences in their magnitude of induction of acetylation as well as RNA-pol II recruitment and activation that might be explained by the following individual arguments. Regarding *Akr1b3* gene, the basal acetylation (at 300 mOsm/kg) of all the regions studied is comparable to an irrelevant region that should not be affected by chromatin dynamics and consequently must have low levels of acetylation. Under osmostress induction (500 mOsm/kg), when *Akr1b3* gene begins to be transcribed chromatin needs to change to a permissive conformation that elicits the productive transcriptional process, which comprises an increase in acetylation. In the case of *Akr1b3* gene, the hyperacetylation of histones under stress is evident from the first hour of stimulation, in all the regions studied, due to the low level of acetylation observed under basal conditions. In contrast, in *Bpgm* gene, the basal acetylation in their distal promoter and proximal promoter/TSS regions seems to be very high compared to a putatively unaffected region like the exon2, that was far away from the TSS (+11,2 kb), leading to a slightly detectable increase in acetylation under hypertonic stress conditions that was still Torin1-sensitive. On the other hand, the case of *Ddit4l* gene is the more intriguing. Although *Ddit4l* chromatin configuration

has not been studied by any published work, it seems to behave as a primary response gene. This type of genes are characterized by its rapid expression after stimulus, the presence of CpG islands in their promoters, and to be constitutively assembled into a permissive chromatin structure similar to that found in active genes (Hargreaves et al., 2009). Thereby, the DNA content of *Ddit4l* gene near its TSS shows a very high CG-content (69,1%), presence of CpG islands, and according to our results, seems to have high levels of acetylated histone H4 in basal conditions and moreover presented minimal changes in acetylation after an hyperosmotic stimulus, although still being Torin1-sensitive. Altogether, these evidences suggest that although chromatin dynamics are affected by mTOR inhibition in *Ddit4l* gene, the main regulatory mechanism by which it is induced upon osmostress must include other processes besides histone H4 acetylation and nucleosome eviction. Different possibilities and hypothesis can be considered, which would require future work for its proper validation.

An interesting effect of mTOR inhibition in whole organisms is to increase lifespan. Recent studies have been shown that suppressing mTOR signaling improves ageing in a variety of model organisms such as yeast (Powers et al., 2006), drosophila (Broughton et al., 2005) and mice (Harrison et al., 2009). Dietary restriction (DR) is one of the best characterized forms to slow down the process of ageing, by reducing mTOR signaling, and it has been shown that it also enhances the resistance towards diverse environmental stresses (Masoro, 2003). Additionally, the modulation of stress pathways has been recognized to extend lifespan and mTORC1 activity has been shown to be affected by several stresses. These evidences lead us to speculate whether experiencing different forms of stress through our life, thus maintaining reduced mTOR activity, equally to that observed under DR, allows cells as well as organisms to increase their viability and extend their lifespan.

The idea that mTOR can be an integral component of adaptive stress responses has been overlooked under the consideration that stress generally inhibits growth and proliferation, both mTOR regulated functions. In fact, as our work supports, at least under moderate osmostress conditions the mTOR pathway is still active and can favor the subsequent cellular adaptation to stress. In this way, is not surprising to speculate that mTOR could likely coordinate similar long-term responses to other different stresses it senses. In summary, our work described here reveals a novel role of mTOR in the modulation of the adaptive osmotic stress response through regulation of different transcriptional mechanisms controlling gene expression.

CONCLUSIONS

CONCLUSIONS

1. The mTOR signaling pathway remains active under sustained hypertonic stress conditions of 500 mOsm/kg.

2. mTOR was required to sustain growth and proliferative capacity in cells exposed to hypertonic stress conditions of 500 mOsm/kg.

3. AMPK is not activated by hypertonic stress conditions of 500 mOsm/kg in mouse embryonic fibroblasts.

4. Hypertonic stress induced by either NaCl or sorbitol enhances the mRNA expression of several NFAT5-dependent osmoresponsive genes (*Akr1b3, Aqp1, Hspa1b and Smit1*) in an mTOR-dependent manner.

5. mTOR inhibition by Torin1 under hypertonic stress conditions of 500 mOsm/kg significantly affected the induction of a proportion of all osmostress-responsive genes, including NFAT5-dependent and -independent target genes in mouse embryonic fibroblasts.

6. Most osmostress-responsive genes induced after 8 hours of hypertonic stimulation were NFAT5-dependent, but only a subset of them was sensitive to mTOR activity in mouse embryonic fibroblasts.

7. REDD1 and REDD2, previously characterized as regulators of mTORC1 in other stress contexts, are induced upon hypertonic stress conditions of 500 mOsm/kg in an mTOR-sensitive and NFAT5-dependent manner.

8. REDD1 does not act as an inhibitor of the mTORC1 signaling in cells exposed to hypertonic stress conditions of 500 mOsm/kg.

9. REDD1 could modulate independently of mTORC1 status, both positively (for *Akr1b3* and *Hspa1b*) and negatively (for *Pdk4*), the expression of several osmoresponsive genes.

10. Inhibition of mTOR does not affect the initial recruitment of NFAT5 to the *Akr1b3* enhancer, but causes its partial dissociation from chromatin at larger time points.

11. mTOR inhibition, by rapamycin or Torin1, does not impair NFAT5 nuclear translocation induced by hypertonic stress conditions of 500 mOsm/kg.

12. mTOR regulates the hyperosmotic stress-induced acetylation of histone H4 on promoter and transcribed regions of the NFAT5-dependent genes *Akr1b3* and *Ddit4l* and the NFAT5-independent gene *Bpgm*.

13. mTOR enhances the hyperosmotic stress-induced recruitment of RNA-polymerase II to the transcriptional start site of the NFAT5-dependent genes *Akr1b3* and *Ddit4l* and the NFAT5-independent gene *Bpgm*.

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ANNEX

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