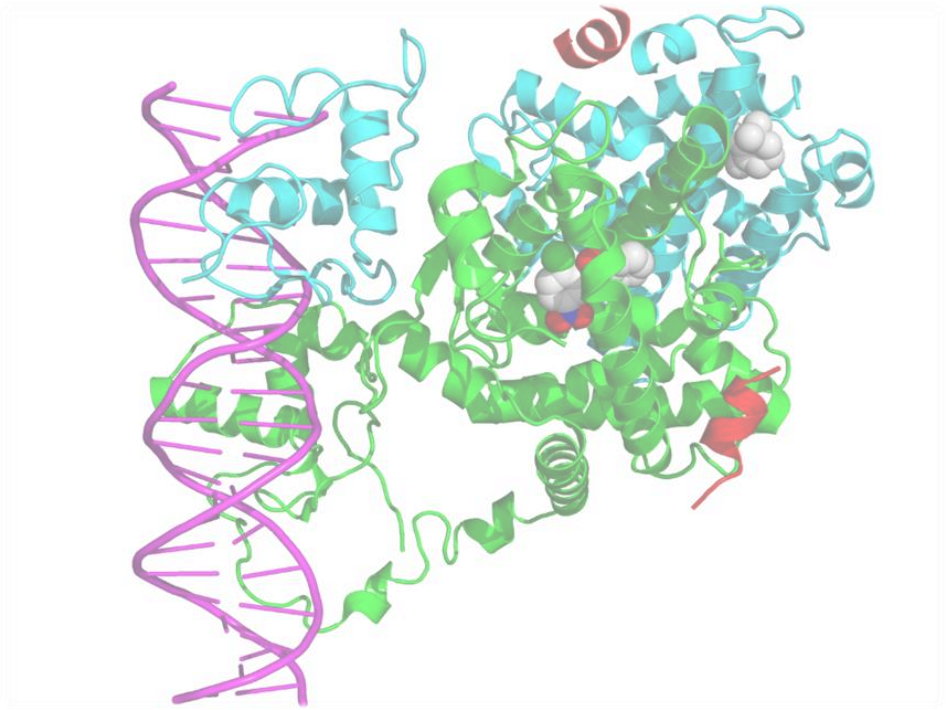
Universidad de La Laguna

Master en Biomedicina

Instituto de Tecnologías Biomédicas
Universidad de La Laguna



POSSIBLE FEEDBACK MECHANISM BETWEEN CORTICOSTEROIDS RECEPTORS AND SGK1

POSIBLE MECANISMO DE RETROALIMENTACIÓN ENTRE LOS RECEPTORES DE CORTICOESTEROIDES Y SGK1

Máster en Biomedicina

Curso 2020-2021

Trabajo de fin de máster

Tutorizado por: Dr. Diego Álvarez de la Rosa Rodríguez

Miguel Eduardo Martin Zurita

Septiembre 2021

Dr. Diego Alvarez de la Rosa, componente/coordinador del Grupo de Investigación Fisopatología celular y molecular de receptores nucleares y canales iónicos, adscrito a la Titulación Máster en Biomedicina por la ULL

CERTIFICO /CERTIFICAN

- Que el Trabajo Fin de Máster (TFM) titulado POSSIBLE FEEDBACK MECHANISM BETWEEN CORTICOSTEROIDS RECEPTORS AND SGK1 ha sido realizado bajo mi supervisión por D. Miguel Eduardo Martín Zurita, matriculado en el Máster en Biomedicina, durante el curso académico 2020-2021.
- Que una vez revisada la memoria final del TFM, doy/damos mi/nuestro consentimiento para ser presentado a la evaluación (lectura y defensa) por el Tribunal designado por la Comisión Académica de la Titulación.

- Que se ha indicado expresamente, a través del mecanismo de entrega de la memoria TFM por sede electrónica ULL, el deseo de hacer público o no los resultados del TFM.

Para que conste, se firma el presente certificado en La Laguna a siete de septiembre de 2021

Dr. Diego Alvarez de la Rosa

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

CO_2: carbon dioxide
CTD: C-terminal domain
DBD: DNA binding domain
DMEM: Dulbecco´s Modified Eagle Medium
DMEM-CS: Dulbecco´s Modified Eagle Medium charcoal-stripped FBS
EDTA: ethylenediaminetetraacetic acid
eGFP: enhanced green fluorescent protein
eYFP: enhanced yellow fluorescent protein
FBS: fetal bovine serum
GR: glucocorticoid receptor
HR: hinge region
HRE: hormone response element
KCl: potassium chloride
kDa: kilodalton
KH_2PO_4: monopotassium phosphate
LB: Luria Bertani broth
LBD: ligand binding domain
$MgCl_2$: magnesium chloride
MR: mineralocorticoid receptor
mTORC2: mammalian target of rapamycin complex 2
Na_2HPO_4: disodium phosphate
NaCl: sodium chloride
NCoR: nuclear receptor corepressor
NEAA: non-essential amino acids
NEDD4-2: neural precursor cell expressed developmentally-down regulated 4-like
ng: nanograms
nM: Nanomolar
NTD: N-terminal domain
O. n.: overnight
P/S: penicillin-streptomycin
PBS: phosphate buffered saline
PDK1: phosphoinositide dependent kinase-1
PI3K: phosphoinositide 3-linase
PIK2: phosphoinositide kinase-2
PLL: poly-L-lysine
PVDF: polyvinylidene difluoride
SDS-PAGE: sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SGK1: serum and glucocorticoid-regulated kinase
SOC: super optimal broth with catabolite repression
SR: steroid receptors

SRC-1: nuclear receptor coactivator 1
TBS-T: Tris-buffered saline + Tween 20
TENT: Tris-HCl-NaCl-EDTA-Triton

LIST OF FIGURES

ABSTRACT

Mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) are two nuclear receptors that regulate transcription when they bind to their preferred corticosteroid hormone. The regulation of MR and GR have been intensely studied since they are involved in key physiological tasks including modulating ion and water transport or in energetic homeostasis. To this day we are still discovering new functions of both receptors which have been shown to overlap or even interact between them. In this work we propose that the serum and glucocorticoid-regulated kinase (SGK1) an MR and GR target activated by the phosphoinositide 3-linase pathway (PI3K) may regulate MR and GR function, providing a feedback loop in corticosteroid receptor signalling. To answer our hypothesis, we performed MR and GR dependent transactivation assays using firefly luciferase as a reporter in N2a SGK1 knockout cells and HEK293T cells transfected with two SGK1 mutants, S422D (constitutively active) and K127A (catalytically inactive). In addition, we also studied the phosphorylating activity of the enzyme using one of its known target as a readout, the neural precursor cell expressed developmentally-down regulated 4-like (NEDD4-2).

Keywords: Mineralocorticoid receptor, glucocorticoid receptor, aldosterone, SGK1, regulation.

RESUMEN

El receptor de mineralocorticoides (MR) y el receptor de glucocorticoides (GR) son dos receptores nucleares capaces de regular la transcripción cuando se unen con sus hormonas corticoesteroides preferidas. La regulación de MR y GR ha sido intensamente estudiada, ya que, están involucrados en procesos fisiológicos claves, en los que se incluye la modulación iónica y el transporte de agua o en la homeostasis energética. Hasta el día de hoy seguimos descubriendo nuevas funciones de ambos receptores, que pueden solaparse e incluso interactuar entre ellos. En este trabajo proponemos que la quinasa regulada por suero y glucocorticoides (SGK1), una diana de MR y GR activada por la via de la fosfoinositida 3-quinasa (PI3K) podría regular la función de MR y GR proporcionando un bucle de retroalimentación en la señalización de los corticoesteroides. Para responder a nuestra hipótesis, realizamos análisis dependientes de la transactivación de MR y GR usando la luciferasa de luciérnaga como reportero en células N2a SGK1 knockout y células HEK293T transfectadas con dos mutantes de SGK1, S422S (constitutivamente activo) y K127A (catalíticamente inactivo). Además, estudiamos la actividad fosforilante de la enzima usando una de sus dianas conocidas, el precursor de célula neuronal expresado durante el desarrollo regulado corriente abajo-4 (NEDD4-2).

Palabras clave: Receptor de mineralocorticoides, receptor de glucocorticoides, aldosterona, SGK1, regulación.

1 INTRODUCTION

1.1 NUCLEAR RECEPTORS

Nuclear receptors have been evolving since the Cambrian, more than 500 million years ago along with the firsts vertebrates (Cavalier-Smith, 2017; Swalla & Smith, 2008). During this time nature has exploited this intricate system of ligand and receptor, which is now involved in all kinds of process that can go from sexual development to homeostasis and growth, or act in response to stress and hunger.

Nuclear receptor bind directly to DNA and regulate the transcription of surrounding genes, hence working as transcription factors when they bind their preferred ligand (Evans, 1988; Olefsky, 2001). There are four characterized classes of nuclear receptors. For the purpose of this assignment, we are going to focus on class I, which includes steroid receptors (SR) (Mangelsdorf et al., 1995; Porter et al., 2019). All nuclear receptors have a common molecular architecture that includes a variable N-terminal domain (NTD), a DNA binding domain (DBD), a hinge region (HR), a ligand binding domain (LBD) and a variable C-terminal domain (CTD) (Figure 1) (Porter et al., 2019). The clustering of class I receptors is based on their activation by ligands derived from cholesterol (steroids). After ligand binding there is a conformational change allowing homodimerization of the receptor and binding to a specific DNA sequence, known as hormone response element (HRE). This site consist of palindromic repeats of variable length (Figure 1) (Mangelsdorf et al., 1995; Porter et al., 2019).

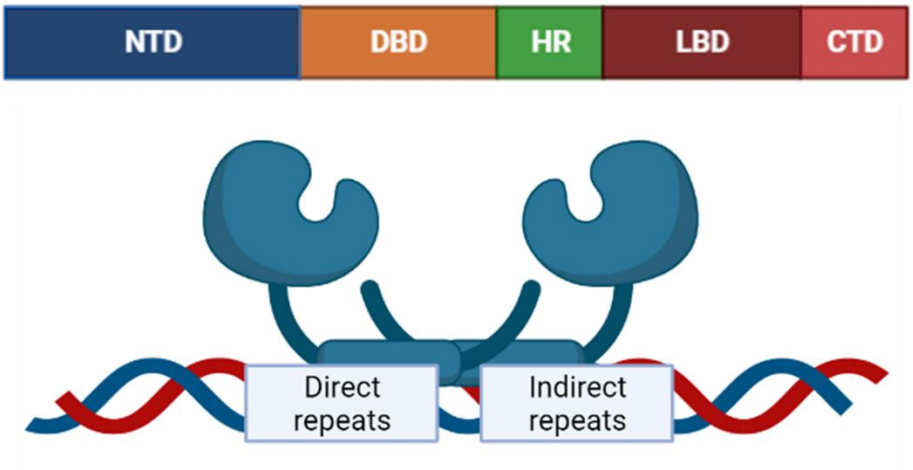


Figure 1: Scheme of class I receptor (also known as steroid receptor). Class I receptors form a homodimer upon ligand binding to bind DNA and reach their active conformation. NTD: N-terminal domain, DBD: DNA binding domain, HR: Hinge region, LBD: Ligand-binding domain. CTD: Variable C-terminal domain. Modified form (Porter et al., 2019)

Another important concept related to nuclear receptors is the existence of coregulators that can potenciate or inhibit transcripcional responses (McKenna & O'Malley, 2000). These coregulators can act in different ways. For instance, SRC-1 (nuclear receptor coactivator 1) recruits histone acetylation complexes to modify chromatin to a more open conformation and potentiate transcription initiation (Hultman et al., 2005; Meijer et al., 2006). NCoR (nuclear receptor corepressor) does the opposite, recruiting histone deacetylases, closing chromatin and hindering transcripcion (Wang et al., 2004). These are only two simple examples taken from more than 400 coregulators operating through different mechanism, adding more layers of complexity to the nuclear receptors machinery (O'Malley, 2016).

1.2 MINERALOCORTICOID RECEPTOR AND GLUCOCORTICOID RECEPTOR

Research on steroid hormones began in the late 1800s, and at first the scientific community thought that steroid hormones were involved in the catalysis of enzymatic reactions (Heitzer et al., 2007). Discoveries made in the next century, including the isolation of aldosterone (Simpson., et al 1953) and cortisol (Ward et al., 1951), the development of the receptor theory to explain hormone actions (Tata, 2005) and the molecular cloning of mineralocorticoid receptor (MR) (Arriza et al., 1987) and glucocorticoid receptor (GR) (Hollenberg et al., 1985) notoriously expanded basic and applied research on the biology of hormone receptors. Nowadays, we have reached the point of having atomic structures of nuclear receptor domains, including the DBD or the LBD in complex with relevant ligands (Hudson et al., 2014; Luisi et al., 1991)

Mineralocorticoids (e.g., aldosterone) and glucocorticoids (e.g., cortisol, corticosterone) are two classes of adrenal hormones, collectively known as corticosteroids, that are mainly involved in facilitating adaptation to deprivation of food and water, acute injury, or other situations of stress. Corticosteroids are the preferred ligands of the mineralocorticoid receptor (MR) and glucocorticoid receptor (GR), two closely related class I nuclear receptors. MR is activated with high affinity not only by mineralocorticoids but also by glucocorticoids. (Arriza et al., 1987; Hudson et al., 2014). On the other hand, GR activation occurs mainly by glucocorticoids, while mineralocorticoids are weak ligands (Hellal-Levy et al., 1999). In addition, MR and GR control overlapping sets of genes. This is in part explained due to the similarity between their DNA binding domains (Hudson et al., 2014), thus recognizing overlapping HREs. To further complicate the picture, MR and GR can physically interact, forming

heterodimers with yet-to-be-determined functions (Bigas et al., 2018; Porter et al., 2019). Both receptors have been intensively targeted by research since they are involved in physiological process of great importance for the correct functioning of the body. MR most prominent functions are modulating ion and water transport across epithelia, being key to controlling osmotic and hemodynamic homeostasis, among others (Gomez-Sanchez & Gomez-Sanchez, 2014). Inappropriate activation of MR in the kidney and heart can cause hypertension, in addition to inflammation, cardiovascular and renal disease (Gomez-Sanchez & Gomez-Sanchez, 2012). This has generated generating a great interest in obtaining MR antagonists for therapeutic use. GR on the other hand is essential for energy homeostasis, responses to stress and inflammation (Gomez-Sanchez & Gomez-Sanchez, 2014). Poor regulation of GR can cause glucose homeostasis unbalance that can lead to metabolic syndrome and other metabolism-related pathologies like diabetes and obesity (Fallo et al., 2006). GR expression is ubiquitous. However MR expression and activation by aldosterone is mainly associated to tight epithelia, such as the distal nephron or distal colon, where it is involved in regulating mineral homeostasis and blood pressure (Gomez-Sanchez & Gomez-Sanchez, 2014). To avoid inappropriate activation of MR by glucocorticoids, which circulate at much higher concentrations than aldosterone, tight epithelia express the 11β-HSD2 enzyme that metabolizes glucocorticoids to inactive forms (Bocchi et al., 2003; Galigniana et al., 2004). This provides a selectivity mechanism, allowing specific activation of MR by aldosterone. However, we now know that many tissues not directly related to mineral and fluid homeostasis express MR but not 11β-HSD2, indicating that MR can also function as a high-affinity glucocorticoid receptor or, alternatively, that there are additional specificity mechanisms allowing for aldosterone action in the absence of the enzyme (Gomez-Sanchez & Gomez-Sanchez, 2014). Thus, MR could participate in process traditionally considered to be regulatory targets of GR, such as energy homeostasis or stress responses in the brain. This overlap between both receptors can results in potentiation or antagonisms in glucocorticoid responses (Gomez-Sanchez & Gomez-Sanchez, 2014). Thus, it is essential to identify MR and GR common and unique gene targets and understand their function in glucocorticoid responses.

1.3 SGK1, A PI3K-DEPENDENT KINASE REGULATED BY MR/GR

The serum and glucocorticoid-regulated kinase 1 (SGK1) is part of the AGC family of the serine-threonine kinases (Pearce et al., 2010). It was initially described as an early gene transcript induced by glucocorticoids (Webster et al., 1993). Additional studies showed that SGK1 can be quickly induced by other steroids hormones, including aldosterone (Wang et al., 2011, 2014). Another realisation was that SGK1 protein and mRNA have a short half-life, indicating that SGK1 expression is regulated and inducible via various stimuli: serum, glucocorticoids, insulin, mineralocorticoids, growth factors and oxidative stress being remarkable (Leong et al., 2003; Mizuno & Nishida, 2001; Náray-Fejes-Tóth et al., 1999). SGK1 activation depends on regulatory phosphorylation controlled by the phosphoinositide 3-kinase (PI3K) pathway (Kobayashi & Cohen, 1999). PI3Ks are a family on enzymes that phosphorylate the 3´-OH of phosphatidylinositol (Martini et al., 2014). Over the years, it has become clear that the PI3K pathway plays a fundamental role in regulating different processes such as glucose metabolism, cell proliferation, inflammation, cell survival and cancer, among others (Vanhaesebroeck et al., 2010). PI3K pathway downstream effectors include phosphoinositide dependent kinase-1 (PDK1), AKT and SGK (Burgering & Coffer, 1995; Pearce et al., 2010). SGK1 activation depends on PDK1, which phosphorylates SGK1 at Thr256, followed by a second phosphorylation event at Ser422 catalyzed by the mammalian target of rapamycin complex 2 (mTORC2) (previously associated with AKT), (Figure 2) (García-Martínez & Alessi, 2008). This pathway had been previously shown to be essential to activate AKT, raising the possibility that at least some of the roles ascribed to this kinase are actually performed by SGK1. Both AKT and SGK1 share a common consensus phosphorylation motif and overlapping targets like GSK3β and FOXO3 (Figure 2) (Brunet et al., 2001; Kobayashi & Cohen, 1999). It has been suggested that the specificity of action between AKT and SGK1 is mainly provided by subcellular compartmentalization of the kinases and their targets (Gleason et al., 2019)

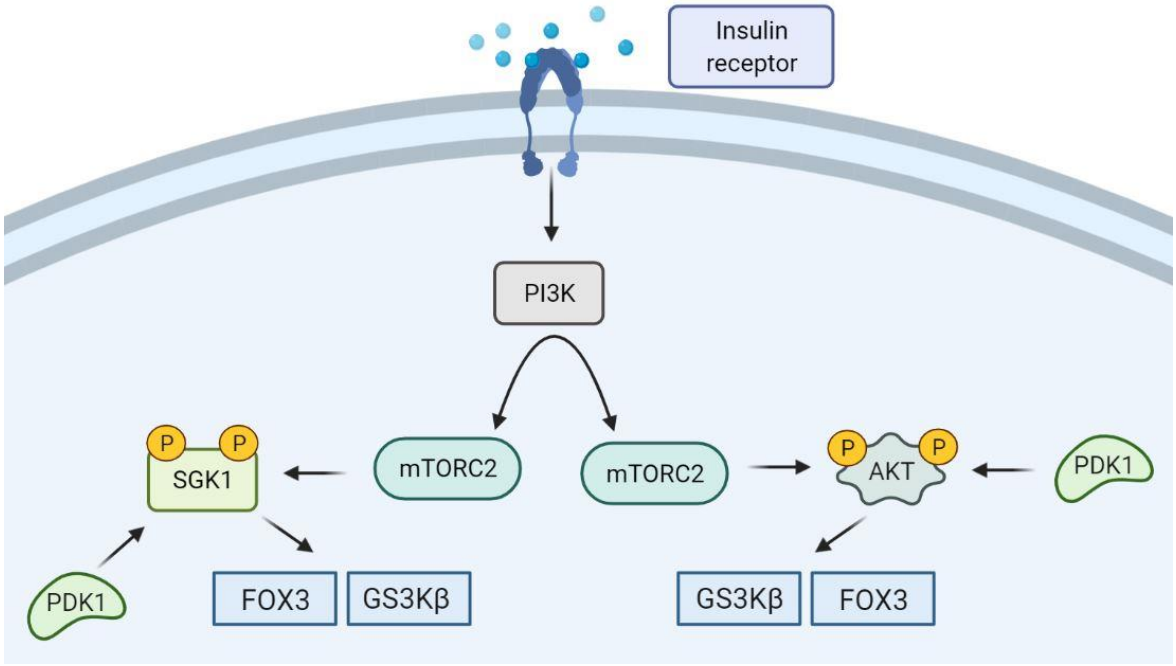


Figure 2: Scheme representation of PI3K insulin activation. Detail in pathways leading to SGK1 and AKT activation and common targets of SGK1/AKT. PDK1 activity depends on phosphoinositide kinase-2 (PIK2) which was not included to not overcomplicate the figure. SGK1 needs double phosphorylation to be fully active. Modified from (Di Cristofano, 2017)

MR role in ion homeostasis and blood pressure regulation is well known, as we mentioned above. However with the "rediscovery" of MR in different tissues where there is no 11β-HSD2 expression has open the possibility that this receptor has new additional functions, including its possible participation in energy homeostasis, metabolism and glucose utilization, similar to GR (Gomez-Sanchez & Gomez-Sanchez, 2014; Jaisser & Farman, 2016). It is clear that SGK1 plays an important role in energy metabolism, with possible implications in obesity, insulin resistance and comorbidities such as hypertension and cardiovascular and renal damage (Gleason et al., 2019; Hills et al., 2008; Sierra-Ramos et al., 2021). In addition, unpublished results from our laboratory suggested a possible link between SGK1 and MR or GR activity. After MR/GR-mediated increased SGK1 transcription, the kinase could provide a feedback loop, regulating the receptors. This could be accomplished by different direct or indirect mechanisms, including chromatin remodelling via histone methylation by phosphorylation of the lysine methyltransferase KMT2D, as recently demonstrated for the estrogen receptor (Toska et al., 2019). Uncovering possible SGK1-mediated feedback loops that differentially affect MR and GR could have important consequences to explain the physiological roles of these receptors and their differences as drug targets in metabolic and cardiorenal disorders (Figure 3).

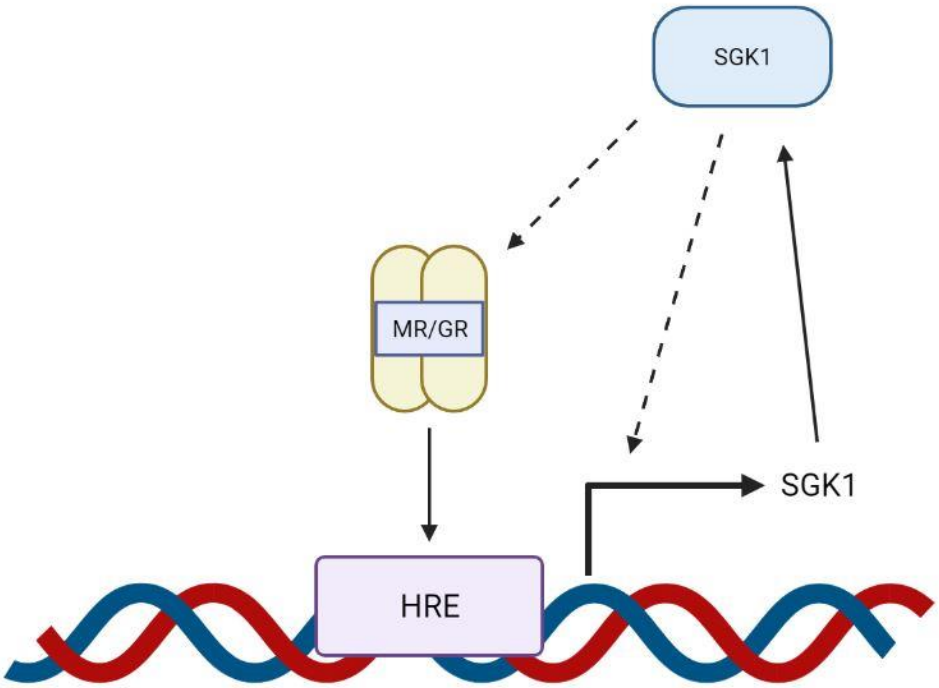


Figure 3: Possible mechanism of SGK1-mediated regulation of MR or GR. SGK1 could alter transcription via coregulator recruitment and chromatin remodelling or directly affect receptor function by altering its phosphorylation status, subcellular localization, stability, or activity as transcription factors.

2 HYPOTHESIS

Our hypothesis is that SGK1 regulates MR and GR function, providing a feedback loop in corticosteroid receptor signalling.

2.1 OBJECTIVES

The general aim of this study is to determine whether SGK1 alters corticosteroid receptor function.

Specific aims:

1. To test the effect of SGK1 expression and activity on MR- and GR-mediated transactivation.
2. To study the mechanisms involved in the potential regulation of MR and GR by SGK1.

3 MATERIAL AND METHODS

3.1 PLASMIDS USED

Plasmid	Description and origin
pcDNA3.1	Plasmid for mammalian expression of cDNAs under the control of a CMV promoter (Invitrogen)
pcDNA4-mMR147GFP	Plasmid encoding for mouse MR with GFP inserted after amino acid position 147 (Aguilar-Sánchez et al., 2012)
pEYFP-SGK1 S422D	Plasmid encoding mouse SGK1 with constitutively activating mutation S422D and fused in the C-ter to eYFP (our laboratory, unpublished).
pEYFP-SGK1 K127A	Plasmid encoding mouse SGK1 with dominant negative mutation K127A and fused in the C-ter to eYFP (this work).
pmCherry-C3-mGR	Plasmid encoding mouse GR fused in the N-terminus to with mCherry (our laboratory, unpublished).
pCMV-Ren	Plasmid expressing luciferase from *Renilla reniformis* under the control of a CMV promoter (kind gift of F. Gebauer, CRC, Barcelona).
pGRE2X-luc	Plasmid expressing a firefly luciferase under the control of two glucocorticoid response elements (kind gift of Rainer Lenz, Baylor College, Houston).
pcDNA3.1-NEDD4-2	Plasmid encoding NEDD4-2 isoform (kind gift of Cecilia Canessa, Yale University).

3.2 HEAT SHOCK TRANSFORMATION

Heat shock was used to introduce foreign DNA into *Escherichia coli* (Top10 strain). After defrosting an aliquot of competent cells, the necessary amount of plasmid DNA to obtain a concentration of 100-200 nanograms (ng) (1-2 µL of DNA) was added, after thawing softly the mix was kept in ice for 15-20 minutes. Next, we applied the heat shock, 42º C for 45 seconds, then we incubated the cells in ice for 2 minutes and add 300 µL of SOC (super optimal broth with catabolite repression: 2% w/v tryptone, 0,5 w/v yeast extract, 8.56 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$ and 20 mM glucose) preheated at 37º C. After that, we incubated the cells for 1 hour at 37º C with agitation at 250 rpm. Later 100-150 µL were spreaded onto Luria Bertani broth agar plates (LB-agar: 1% tryptone, 0,5% yeast extract 1% NaCl and 0,75% agar) containing the appropriate antibiotic for selection.

Plates were incubated overnight at 37º C and isolated colonies were selected for plasmid purification.

3.3 PLASMID PURIFICATION

Plasmid DNA extraction was done using a commercial system (NucleoSpin Plasmid EasyPure, Macherey-Nagel) using the manufacturer´s protocol. Briefly, bacteria grown overnight (o.n.) in LB media supplemented with the appropriate selection antibiotic were centrifuged at 4100 rpms for 10 minutes, the supernatant was discarded and 350 µL of A1 buffer were added. The mix was vortexed, and the resultant suspension was transferred to a new Eppendorf tube. After that, 250 µL of buffer A2 was added, mixed by carefully inverting the Eppendorf tube. After 2 minutes incubation at room temperature, 350 µL of A3 buffer were added and the mixture mixed by inversion. Next, tubes were centrifuged at 12.000 xg for 3 minutes, supernatant was transferred to a column with a collection tube and centrifuged at 2.000 xg 30 seconds. The flow through was discarded and 450 µL of AQ buffer were added. Tubes were then centrifuged at 12.000 xg for 1 minute twice and the flow though was discarded each time. Collection tube was substituted for a new Eppendorf tube, 50 µL of elution buffer (previously incubated at 70º C) was added, and after a 1-minute incubation at room temperature, tubes were centrifuged at 12.000 xg.

In the events of our plasmids having low concentration, we repurified them following the NuceloSpin Gel and PCR Clean-up protocol (Macherey-Nagel). Fist, the DNA binding conditions were adjusted adding 2 volumes of NTI buffer for each volume of sample. Then, the mix was placed into a column with a collection tube and centrifuged for 30 seconds at 11.000 xg, the flowthrough was discarded. Next, the silica membrane was washed with 700 µL of NT3 buffer and centrifuged 30 seconds at 11.000 xg, the flowthrough was discarded. After that, the column was centrifugated again for 30 seconds at 11.000 xg to make sure all NT3 buffer is washed. Finally, the DNA was eluted by placing the column under a microcentrifuge tube in which we added 50 µl of NE buffer, and incubated at room temperature for 1 minute, then the tubes were centrifuged for 1 minute at 11.000 xg.

3.4 cDNA MUTAGENESIS

A point mutation in K127A inactivating SGK1 catalytic activity and generating a dominant negative kinase (Kobayashi et al. 1999) was generated using Quickchange Lightning Multi Site-Directed Mutagenesis Kit (Agilent) using pEYFP-SGK1 S422D as a template (Figure 4).

Figure 4: Full sequence map for pEYF-N1-SGK1. Image created with SnapGene software (from Insightful Science; available at snapgene.com)

DNA was denatured so the mutagenic primers could anneal to it (Figure 5B**)**. Next, *Pfu* polymerase extended the primers with high fidelity so double stranded DNA chains with the mutation of interest were generated (Figure 5C). To eliminate the plasmid template, the reaction mix was then treated with the *Dpn I* endonuclease. The reason behind this was that most *E. coli* strains methylate their DNA product. When we used *Dpn I* endonuclease we were making sure that the parental DNA would be digested, and our products will contain the desired mutation. The following step was the transformation of XL10-Gold ultracompetent cells. After transformation bacteria were seeded in LB-agar supplemented with kanamycin (Figure 5D). A single bacterial colony was used to inoculate a liquid culture of LB-kanamycin medium (1% tryptone, 0.5% yeast extract and 1% NaCl). After o.n. incubation under agitation at 37º C, plasmid DNA was purified using a commercial system (NucleoSpin Plasmid EasyPure, Macherey-Nagel). The presence of the desired mutation was verified by DNA sequencing (Figure 5E).

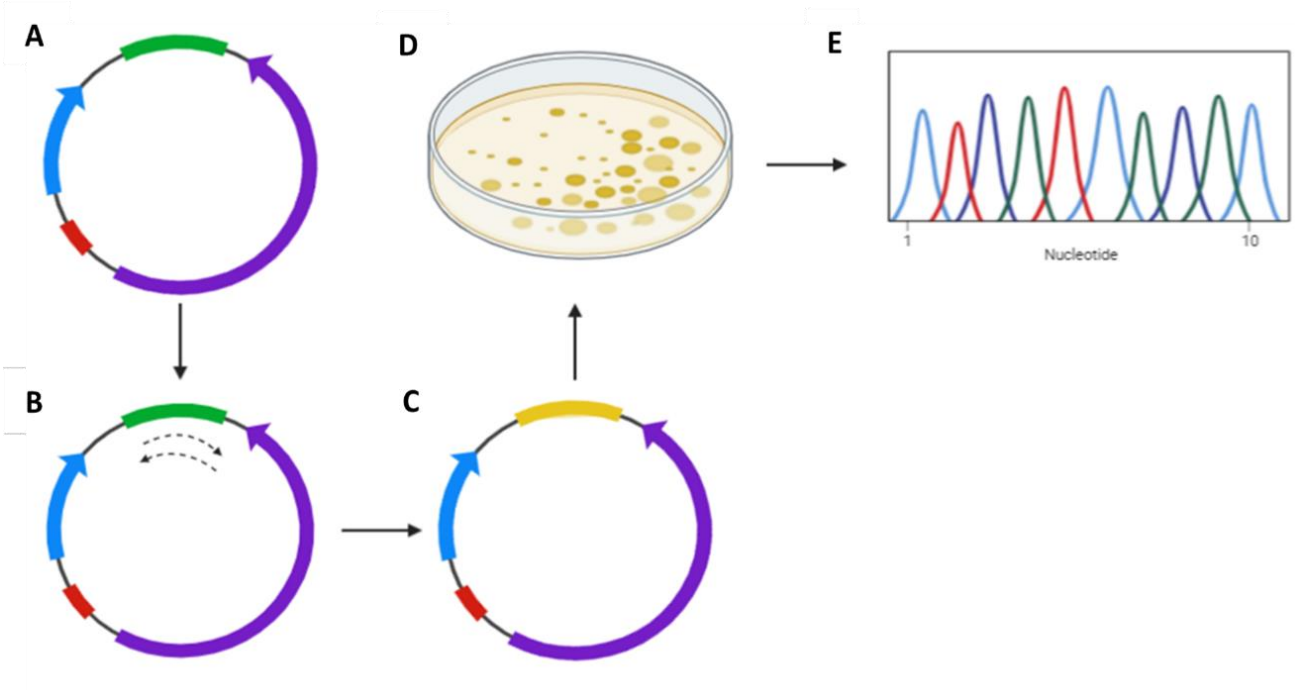


Figure 5: Scheme of the mutagenesis process.

Samples were sequenced by EZ-Seq service (Macrogen). For this, 100 ng of sample DNA were mixed with 1 µL of a 10 pmol/µL primer up to a final concentration of 125 ng. Sequencing results were confirmed using Basic Local Alignment Search Tool (BLAST) (Figure 6).

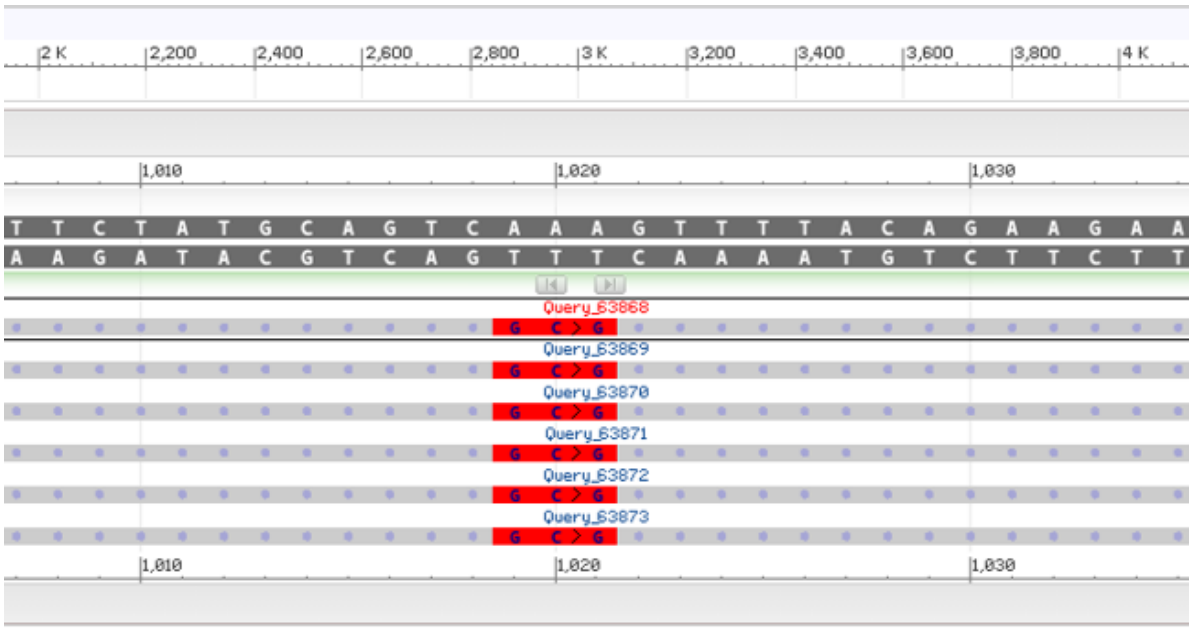


Figure 6: Sequence alignment results. In red we can see the outcome of the mutagenesis process, codon GCG codifies for alanine (image taken from the Basic Local Alignment Search Toll, BLAST).

3.5 CELL CULTURE

Cell lines used in this study:

- **HEK293T** (CRL-3216): human embryonic kidney cells, deriving from HEK293 strain, contains the SV40 T-antigen.
- **N2a SGK1 KO Clone 16 CCL-131**: neuro-2a cells derived from neuroblastoma from mice *(Mus musculus)* and modified by CRPR-Cas9 to obtain a stable knockout cell line without SGK1.

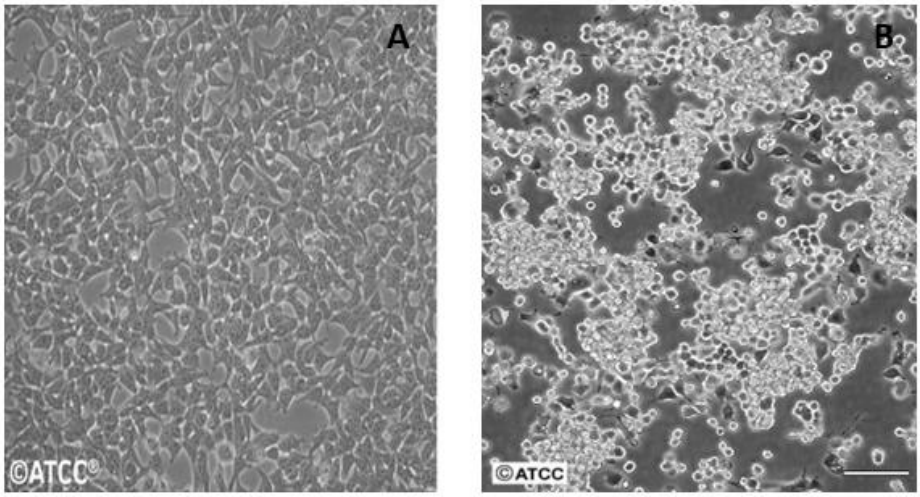


Figure 7: Microscopic detail of cell lines used. (A) HEK293T cells (B) N2a cells. Differences in morphology and confluency can be appreciated. (Modified from ATCC)

Cell were grown in Dulbecco´s Modified Eagle Medium (DMEM, Sigma- Aldrich), enriched with 10% fetal bovine serum (FBS, Sigma-Aldrich), 1% penicillin-streptomycin (P/S, Thermo-Fisher Scientific), 1% non-essential amino acids (NEAA, Sigma-Aldrich) and Mycozap (Lonza). Cells were maintained in an incubator at 37º C under an atmosphere of 5% carbon dioxide (CO_2) with 90-95% of relative humidity. Cell culture was performed working under a class II laminar flow hood.

When cells reached the desired confluency old medium was aspired with vacuum and cells were washed with 5 mL of PBS 1x (phosphate buffered saline: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4 at 7.4 pH). To detach the cells from the flask they were treated with 2 mL of trypsin-EDTA solution (trypsin-ethylenediaminetetraacetic acid, at 37º C in a 5 % CO_2 atmosphere, for varying amounts of time, depending on the cell line used (2 minutes for HEK293T (Figure 7A) and N2a cells (Figure 7B). Afterwards, trypsinization was stopped by adding fresh complete DMEM medium. Cells were then centrifuged at 300 xg for 5 minutes, medium was removed, and the pellet was resuspended in fresh complete DMEM medium.

Depending on the experiment, cells were seeded on different types of plates. All experiments performed with HEK293T and N2a cells needed the plate to be treated with poly-L-lysine (PLL) to promote cell attachment. PLL treatment for plates consisted of adding enough PLL solution (0.1% w/v, Sigma-Aldrich) to cover the bottom of the well. The plate was then incubated at 37º C for 30 minutes, and then washed thrice with PBS 1x.

3.6 TRANSIENT TRANSFECTION

For every experiment performed in this project we needed the cells to express different combinations of plasmids. To achieve this, we used a transient transfection method. The transfection reactive used was jetPrime (Polyplus-transfection) following the manufacturer´s protocol described in Figure 8. For each plate size, the quantities of cells/well, total DNA, jetPrime buffer and jetPrime reagent vary, but the transfection protocol is the same: first, DNA was diluted in jetPrime buffer® and mixed by vortexing; jetPrime reactive was added, the mix was vortexed and pulled down by brief centrifugation; the mix was incubated for 10 minutes at room temperature; finally, transfection mix was put onto each dwell, after 4 hours media is changed, then cells were incubated at 37º C for 24 or 48 hours.

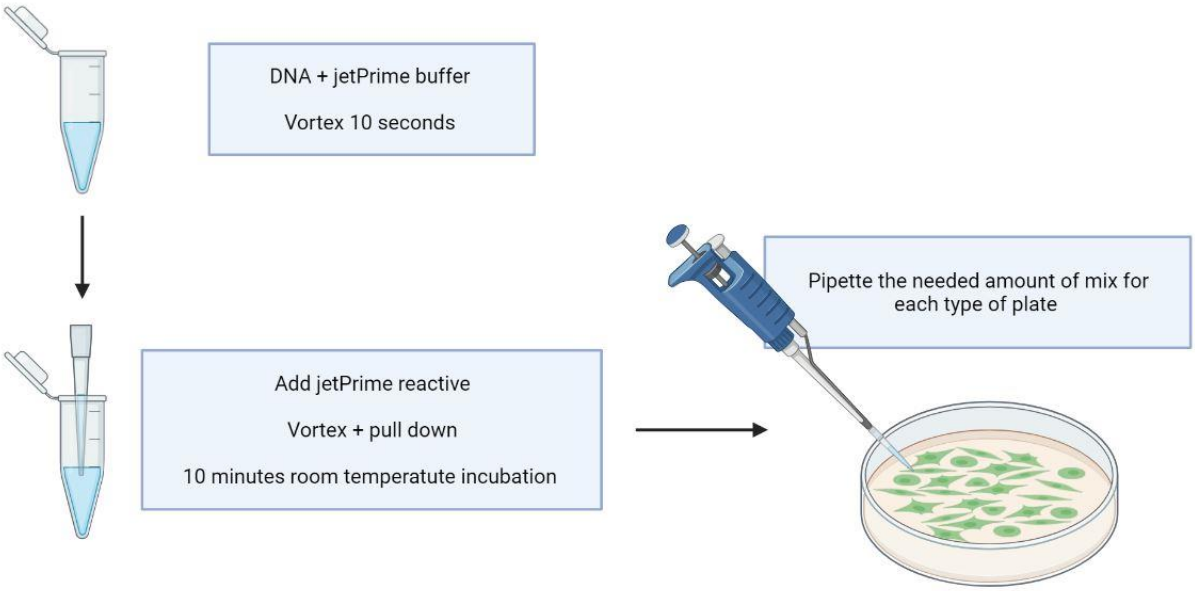


Figure 8: JetPrime protocol for DNA transfection. (https://www.polyplus-transfection.com/products/jetprime/)

3.7 REPORTER GENE ASSAY

Receptor-mediated transactivation assays were performed using firefly luciferase as reporter gene. Luciferase assays allow us to measure if our protein of interest, have an effect in the transcription of the luciferase gene, thus seeing an increase in luciferase activity measured in cell extracts. To achieve this, we needed to transfect a construct that had the DNA regulatory region of our protein of interest (in this case, an HRE) placed next to a basal promoter controlling the luciferase cDNA insert (Figure 9) (Carter & Shieh, 2015).

Assays were performed in 96-well plates treated with PLL. In each well there were approximately 10.000 cells. One day after seeding, cells were transfected following the protocol explained above. At the time of transfection, medium was switched to DMEM supplemented with charcoal-stripped FBS (DMEM-CS) to eliminate steroids and avoid basal activation of MR or GR. The next day, we added the treatment, aldosterone 10 nanomolar (nM) or dexamethasone 100 nM, in DMEM media without FBS. In wells where no hormone was added, cells were treated with vehicle (EtOH 100% at 1:1000 dilution). Once this was done and after an incubation of approximately 16 hours, we performed the luciferase assay. First, 70 µL of medium were taken out each well and 30 µL of luciferase reactive was added. After 10 minutes at room temperature with agitation at 110 rpm, luciferase activity was measured using the Dual-Glo luciferase assay reagent

(Promega). To normalize our data, *Renilla* luciferase was used. Following the same steps as before, 30 µL of *Renilla* buffer mix were added and enzymatic activity was measured after 10 minutes incubation under agitation at 110 rpm.

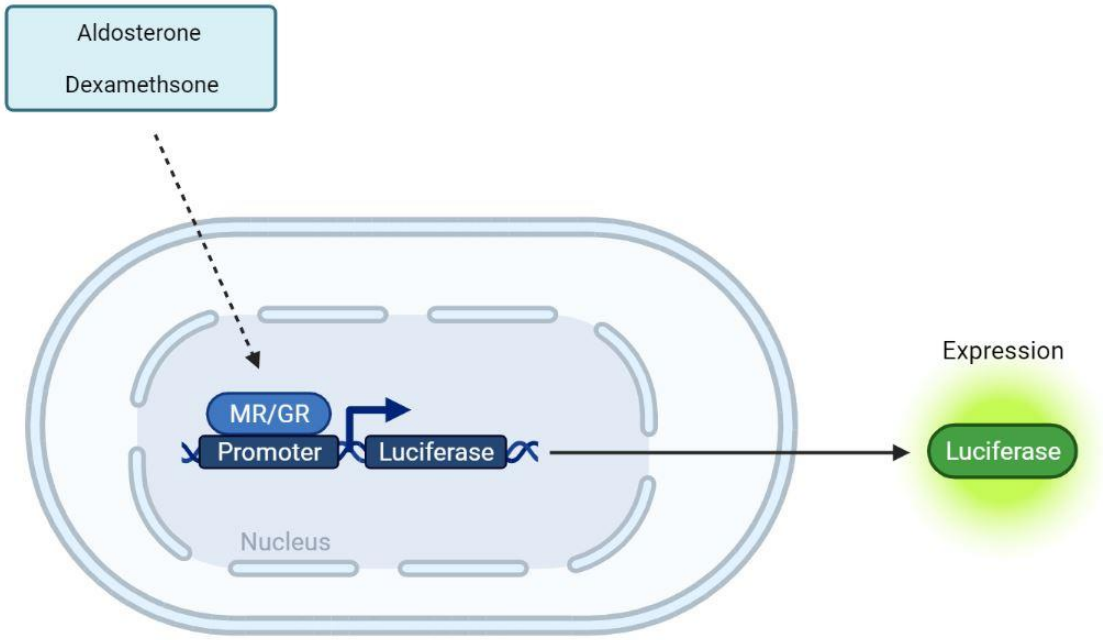


Figure 9: Luciferase transactivation assay. When the receptor is stimulated with the respective hormone or synthetic ligand, transcription starts. Next, we can then correlate transcription with the amount of luciferase activity detected in the assay.

3.8 WESTERN BLOT

Protein extracts from transfected cells were obtained using the following protocol. First, cell medium was aspirated, and each well was washed with cold PBS 1x. Then we added 30 µL of TENT (10 mM Tris-HCl, 250 mM NaCl, 1 mM EDTA, 1% v/v Triton X-100) supplemented with protease and phosphatase inhibitor cocktails (Complete and PhosSTOP, Roche). The cell extract was then transferred to an Eppendorf tube and incubated for 5 minutes on ice. Finally, the extract was centrifuged for 10 minutes at 14.000 xg. The supernatant was then transferred to a new Eppendorf tube and kept at -80º C until use.

Protein samples were resolved in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). For this, protein samples were prepared by mixing the protein extract with 6X Laemmli Buffer (4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.004% bromophenol blue and 0.125 M Tris-HCl, pH 6.8). After this, samples were incubated for 5 minutes at 95º C and cleared by centrifugation. Next, SDS-

PAGE was performed using Mini-Protean TGX Stain-Free Precast Gel (Bio-Rad). Running buffer was already prepared (25 mM of Tris, 192 mM of Glycine, 0.1% of SDS). Precision Plus Protein™ Dual Color Standards (Biorad) were used as molecular mass markers. Samples were loaded into the corresponding well (12.5 µL of sample or 8 µL of marker). Electrophoresis was performed at 120 V for approximately 1 hour. Proteins were then transferred by electroblotting to a polyvinylidene difluoride (PVDF) membrane using a liquid transfer system for 1 hour at 350 mA. After that, membranes were blocked for 1 hour in blocking solution (5 % w/v non-fat dried milk in TBS-T 1x: 20 mM of Tris-HCl, 150 mM of NaCl and 0.1% of Tween20). The membranes used to measure protein phosphorylation were incubated in a blocking solution containing PhosphoBLOCKER reagent (Cell Biolabs). The membranes were then incubated o.n. at 4º C with the primary antibody (Table 1) in TBS-T with 0.5% blocking reagent. The following day, membrane was washed three times for 5 minutes with TBS-T and then incubated with the secondary antibody (Table 1) in 0.5% non-fat dried milk in TBS-T with agitation at room temperature. When the incubation was over, membranes were washed three times for 5 minutes in TBS-T and incubated with the detection reagent (Clarity Western ECL Substrate, Bio-Rad), prepared by mixing 1:1 of luminol-enhancer reagent with peroxide reagent. Membranes were incubated for 4 minutes with the detection reagent. Chemiluminescence was captured with a CCD camera using an ImageQuant LAS 500 apparatus (GE Healthcare Bio-Sciences AB).

Primary antibodies	Dilution	Secondary Antibodies	Dilution
Mouse Anti-MR 1-18 1d5 (Gómez Sánchez)	1:1000	Anti-Mouse (Dako)	1:10000
Ab 43606 Rabbit Anti-SGK1 (Abcam)	1:1000	Ab 6721 Goat Anti-Rabbit (Abcam)	1:10000
Rabbit Anti-GR (H-300) sc-8922 (Santa Cruz)	1:1000		
Ab 290 Rabbit Anti-GFP (Abcam)	1:1000		
Rabbit Anti-GFP (FC) sc-8334 (Santa Cruz)	1:2000		
Rabbit P-NEDD4 (Cell Signalling)	1:2500		
Rabbit tNEDD4 (Cell Signalling)	1:2500		

Table 1: Primary and secondary antibodies used for western blot and working concentration

In some experiments, PVDF membranes were reused and incubated several times with new antibodies. Antibody stripping was performed by incubating the membranes in stripping solution (4% SDS, 6.25 mM Tris-HCl pH 6.7 and 0.1 M β-mercaptoethanol) at 50º C for 30 minutes under agitation, followed by extensive washing in TBS-T. Next, the membranes were exposed to detection reagent to make sure the stripping process was successful, and then used again for detection or stored once they were dry.

4 RESULTS AND DISCUSSION

4.1 SGK1 INHIBITS MR ON N2A SGK1 KO CELLS

In other to measure the effects of SGK1 on MR and GR we decided that the best approach was to perform gene transactivation assays using luciferase as a reporter. Most cell lines express endogenous SGK1, potentially interfering with our measurements of the effects of the kinase on MR or GR. Therefore, we took advantage of the recent generation of a SGK1 knockout cell line in our laboratory using a CRISPR-Cas9 approach on the mouse neuroblastoma cell line N2a (**N2a SGK1 KO Clone 16 CCL-131,** B. Rivero et al. unpublished). In addition, we included in the assay a catalytically inactive mutant SGK1 (K127A) to control for possible effects of the kinase independent of its phosphorylating activity.

For all experiment, transfections were done in triplicate and several independent replicates including all conditions were performed in order to ensure reproducibility

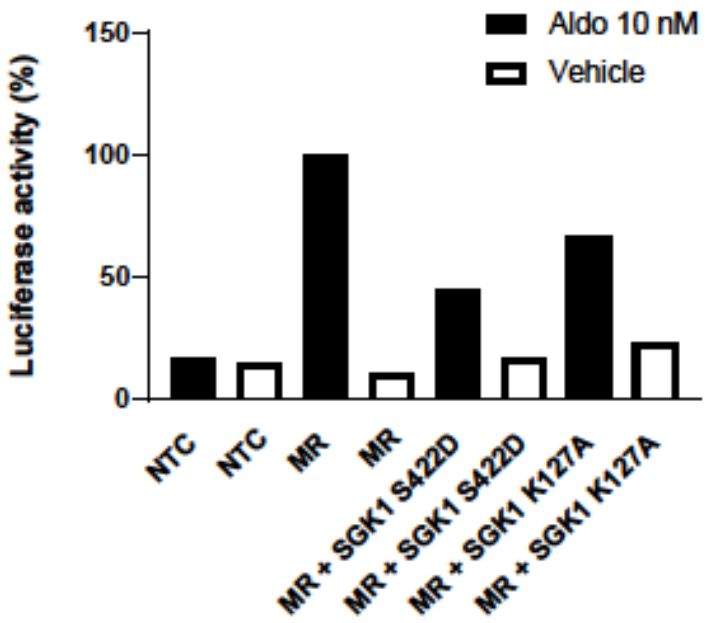


Figure 10: MR-dependent transactivation assay in N2a SGK1 KO. Cells were with the indicated plasmid combination or with empty plasmids (NTC). One day after transfection cells were treated o.n with 10 nM aldosterone or vehicle and *Renilla* and firefly luciferase activities were assayed consecutively in the same cells extracts. Plots represent average ratios of firefly/*Renilla* activities ± SD (n=3) firefly.

This cell line displayed low transfection efficiency and high mortality, resulting in low signal to noise ratio. However, after analysing the data obtained, there was a clear and significant induction of MR activity by aldosterone (Figure 10). Co-transfection of both SGK1 mutants appeared to reduce MR activity, with SGK1 S422D displaying a stronger

inhibitory effect. Even though these results were promising, we could not reproduce them in new experiments due to problems related to the number of cells at the end of the transfection, resulting in low luciferase activities. Thus, we decided to switch model to HEK293T cells.

4.2 EFFECT OF SERUM ON SGK1

The first assay was done in HEK293T cells, keeping in mind that these cells express endogenous SGK1. We transfected constitutively active mutant SGK1 S422D mutant, which has been reported to produce a 10-fold increase in activity (Kobayashi et al. 1999) to surpass the activity of the endogenous SGK1.

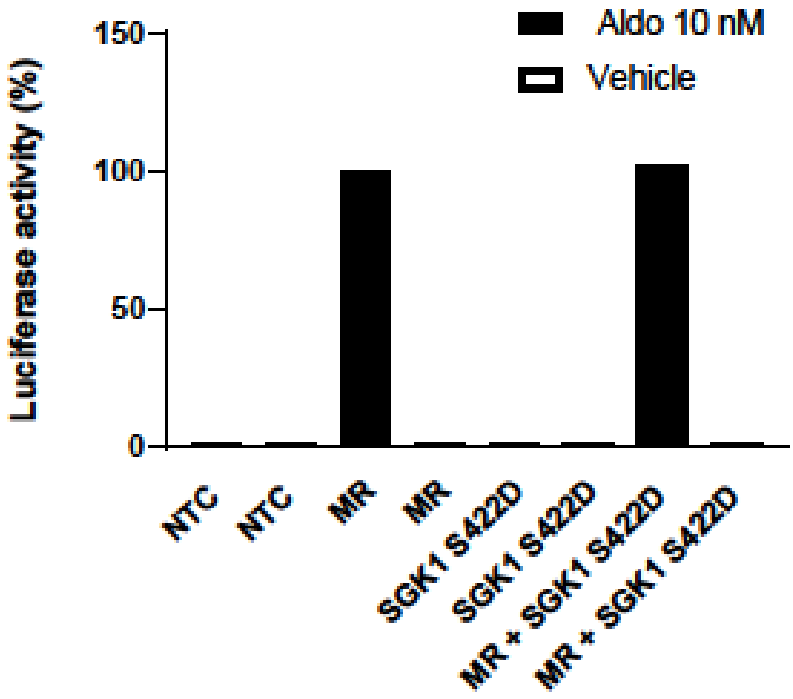


Figure 11: MR-dependent transactivation assay in HEK293T. Cells were transfected with the indicated plasmid combination or with empty plasmids (NTC). For this experiment medium contained FBS. One day after transfection cells were treated o.n. with 10 nM aldosterone or vehicle and *Renilla* and firefly luciferase activities were assayed consecutively in the same cell extracts. Plots represent average ratios of firefly/*Renilla* activities ± SD (n=3 firefly)

As seen in Figure 11, transfection of MR induced a potent induction of firefly luciferase activity above basal conditions. Co-transfection of constitutively active SGK1 did not show any significant difference in MR-mediated transactivation. This result may be explained by the presence of FBS in the medium, which is a potent activator of the endogenous SGK1 (Leong et al., 2003; Mizuno & Nishida, 2001; Náray-Fejes-Tóth et al., 1999) and may mask any additional effect induced by the transfected kinase.

4.3 EFFECTS OF FBS FREE MEDIUM INCUBATION ON HEK293T CELLS

Due to the results described above, we decided to incubate HEK293T cells in medium free of FBS to avoid activation of the endogenous SGK1. To prove that HEK293T cells survived the o.n. treatment with FBS-free medium, we observed cells under the microscope at different times after media change looking for signals of cellular stress.

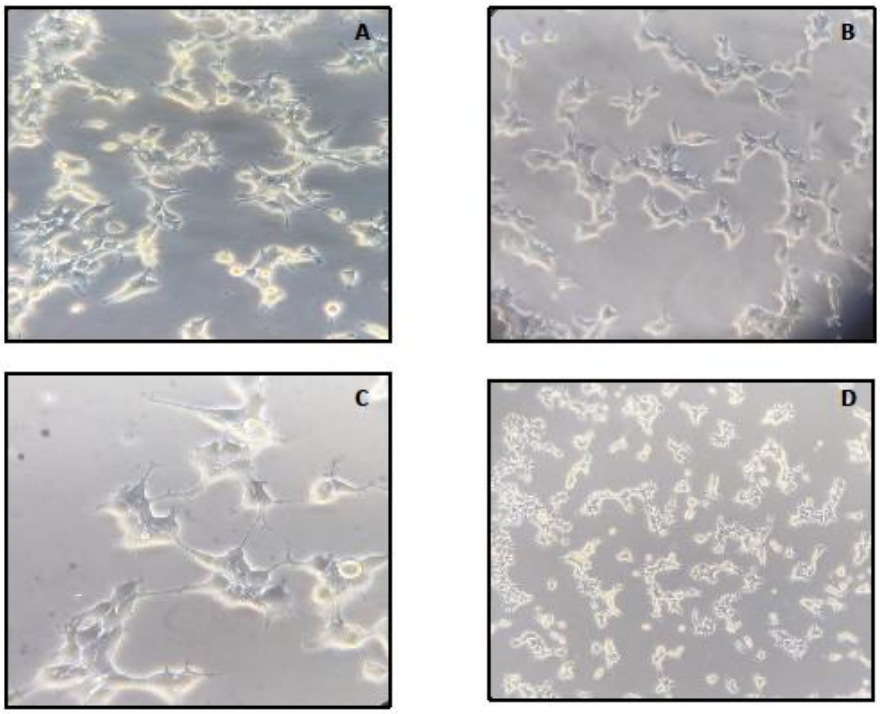


Figure 12: HEK293T cell morphology before and after treatment with serum-free medium. (A) Cells after +24 hours incubation in FBS containing medium 20x magnification. (B) Cells after +24 hours incubation in FBS free media 20x magnification, (C) 40x magnification and (D) 10x magnification

HEK293T cells maintained normal morphology up to 24h after switching to serum-free medium (Figure 12) and therefore were used for all subsequent experiments despite the presence of endogenous SGK1.

4.4 SGK1 REGULATES MR AND GR ACTIVITY

Results in the assays of SGK1 on MR-mediated transactivation showed similar inhibitions as the one done with N2a SGK1 cells, in this case both mutants seemed to inhibit MR activity in the same way (Figure 13).

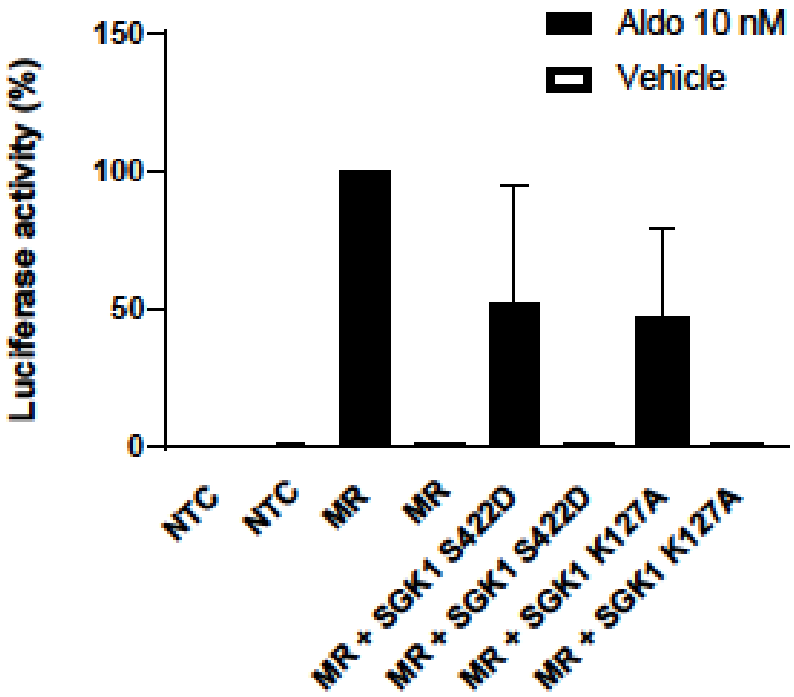


Figure 13: MR-dependent transactivation assay in HEK293T. Cells were transfected with the indicated plasmid combination or with empty plasmids (NTC). One day after transfection cells were treated o.n with 10 nM of aldosterone or vehicle and *Renilla* and firefly luciferase activities were assayed consecutively in the same cell extracts. Plots represent average ratios of firefly/*Renilla* activities ± SD (N=3)

These experiments appear to show that SGK1 inhibited MR activity and enhanced GR activity, independently of its catalytic activity. However, several potential confounding factors could be affecting our results, including SGK1 effects on the constitutive expression of *Renilla* luciferase, the enzyme used to normalize our experiments, or unexpected behaviour of our transfected SGK1 mutants. To assess the first factor, we analysed *Renilla* luciferase activities separately.

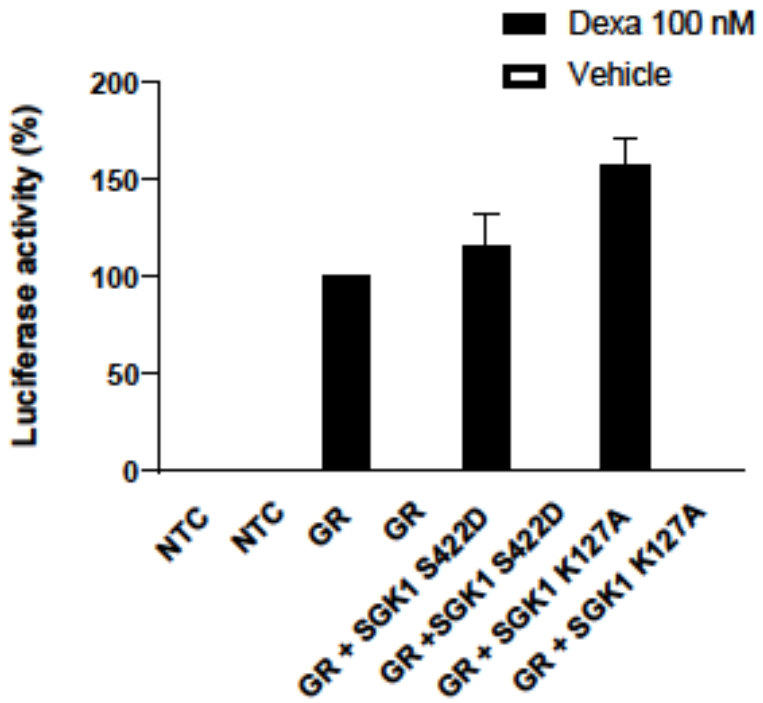


Figure 14: GR-dependent transactivation assay in HEK293T. Cells were transfected with the indicated plasmid combination or with empty plasmids (NTC). One day after transfection cells were treated o.n with 100 nM of dexamethasone or vehicle and *Renilla* and firefly luciferase activities were assayed consecutively in the same cell extracts. Plots represent the average ratios of firefly/*Renilla* activities ± SD (N=2)

Surprisingly, when we tested the effects of SGK1 on GR-mediated transactivation we found that the kinase potently enhanced GR activity. Additionally, it seemed clear that SGK1 K127A was eliciting near 50% more GR activity more than SGK1 S422D (Figure 14)

4.5 *RENILLA* STATISTICAL ANALYSIS

Renilla luciferase is used to normalize firefly luciferase experiments, since it was expressed under the control of a CMV promoter, whether or not there was a hormonal stimulus present. Therefore, the values obtained should adjust to a normal distribution, depending only on the amount of transfected plasmid. We used GrahpPad Prism software to test for normality in *Renilla* values when it was co-transfected with MR, GR or SGK1 mutants.

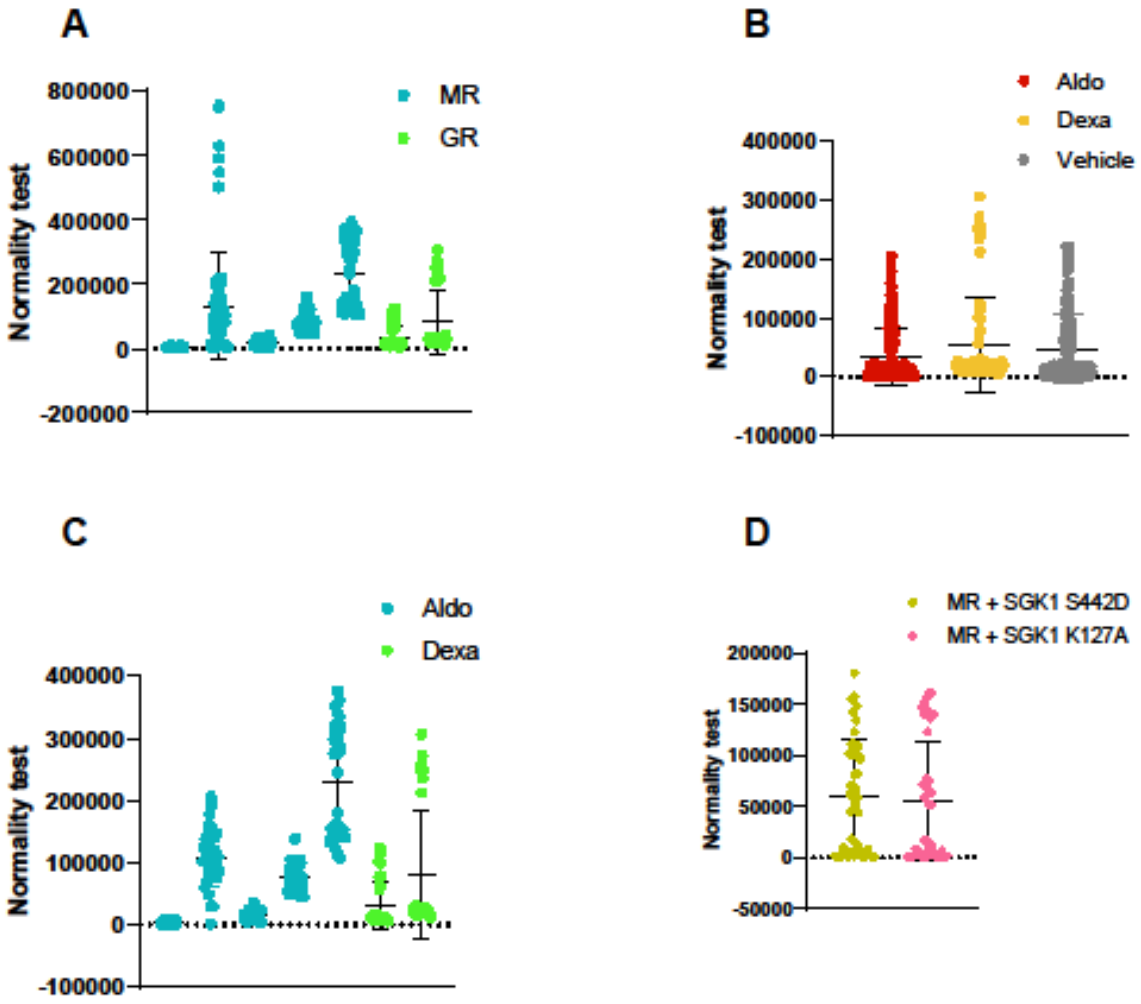


Figure 15: *Renilla* luciferase normality analysis. Normality test on *Renilla* values obtained from luciferase experiments. Values were tested for D´Agostino & Pearson normality and lognormality test. (A) Grouped *Renilla* from all experiments. (B) Grouped *Renilla* values from the conditions containing aldosterone, dexamethasone, and vehicle. (C) Grouped *Renilla* values of hormone conditions in each experiment. (D) Grouped *Renilla* values from the conditions with MR + SGK1 S422D and K127A. Dots represent individual *Renilla* values.

Despite not adjusting to a normal distribution, values adjusted better to a lognormal distribution. In all groups formed there was no indicative of an artifact when *Renilla* was in the presence of hormone, vehicle or co-transfected with MR, GR or SGK1 since all groups formed did not show any differences when compared (Figure 15).

4.6 SGK1 PHOSPHORYLATION ON NEDD4-2

To ensure that the activity of the SGK1 mutants is correct, we measured phosphorylation of one of its known targets the neural precursor cell expressed developmentally-down regulated 4-like (NEDD4-2) (Bhalla et al., 2005), which is a member of the E3 ubiquitination enzymes (Harvey & Kumar, 1999; S Kumar et al.,

1997; Sharad Kumar et al., 1992). To that end, we co-transfected NEDD4-2 with different combinations of MR and GR and the SGK1 mutants.

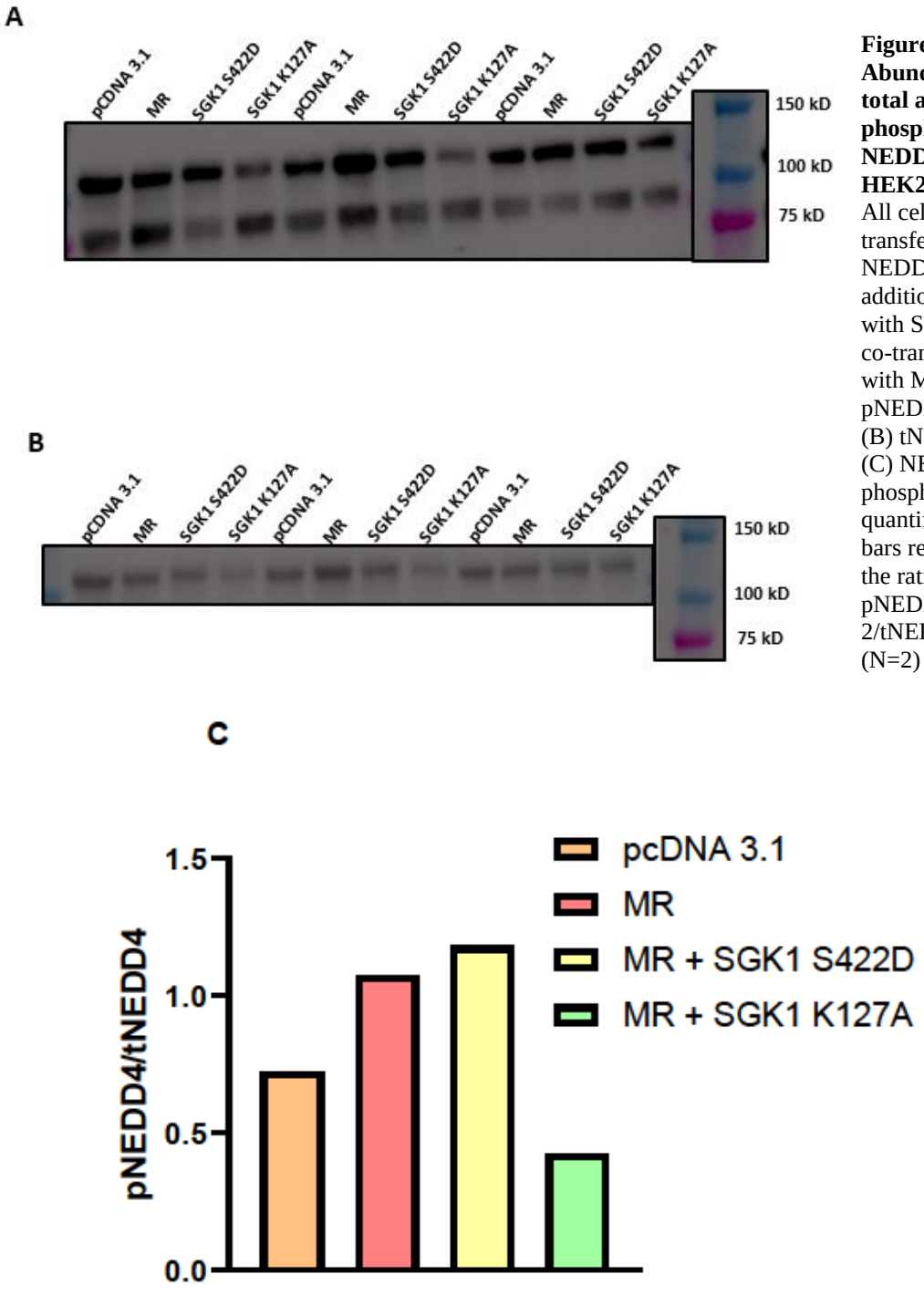


Figure 16: Abundance of total and phosphorylated NEDD4-2 in HEK293T cells. All cells were transfected with NEDD4-2, in addition lanes with SGK1 were co-transfected with MR. (A) pNEDD4-2 and (B) tNEDD4-2. (C) NEDD4-2 phosphorylation quantification, bars represent the ratio of pNEDD4-2/tNEDD4-2, (N=2)

Previous results from our laboratory showed that HEK293 cells do not express endogenous NEDD4-2 (Armas-Capote et al., 2020). Cell extracts were analysed by western blot using antibodies against total NEDD4-2 or a phospho-specific antibody that recognizes only proteins phosphorylated at residue S448.

The western blots using the phospho-specific antibody showed two bands (Figure 16A, 17A), one migrating at approximately 115 kilodaltons (kDa), which was expected, and the other one, at approximately 90 kDa. In contrast, the antibody detecting total NEDD4-2 detected only the expected band (Figure 16B, 17B). For this reason, we quantified the signals taking into account only the 115 kDa band. Results are shown as the ratio between the signals corresponding to the phosphorylated form and the total protein (Figure 16C, 17C).

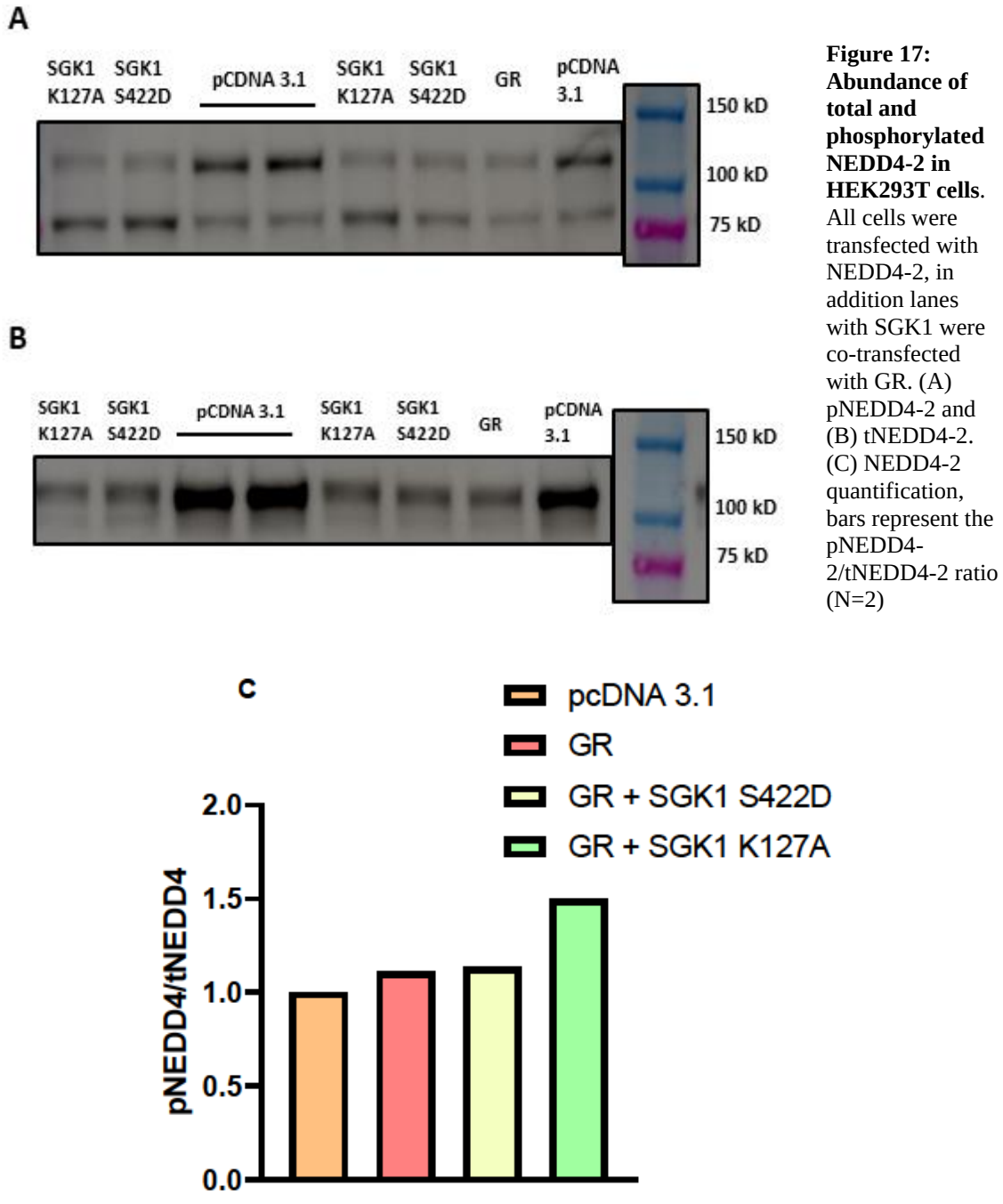


Figure 17: Abundance of total and phosphorylated NEDD4-2 in HEK293T cells. All cells were transfected with NEDD4-2, in addition lanes with SGK1 were co-transfected with GR. (A) pNEDD4-2 and (B) tNEDD4-2. (C) NEDD4-2 quantification, bars represent the pNEDD4-2/tNEDD4-2 ratio (N=2)

The results of SGK1 phosphorylation on NEDD4-2 were surprising. SGK1 S422D did not significantly increase NEDD4-2 phosphorylation. The presence of MR alone appeared to slightly change NEDD4-2 phosphorylation, but this effect appears to be minor. On the other hand, when SGK1 K127A was co-transfected with MR the catalytically inactive mutant showed reduced phosphorylation of NEDD4-2 (Figure 16C). Since this mutant has dominant-negative activity (Kobayashi et al. 1999), this may be explained by residual endogenous SGK1 activity. However, when it was co-transfected with GR, SGK1 K127A appears to show increased phosphorylation of NEDD4-2 (Figure 17C). It is important to note that all phosphorylation changes appear minor. Further experimental replicas would be needed to demonstrate if these changes are reproducible and statistically significant. A possible explanation for these results could be that our mutants were not working as expected, so we decided to do further western blots to confirm that our transfection was successful and that the antibody detecting NEDD4-2 is specific.

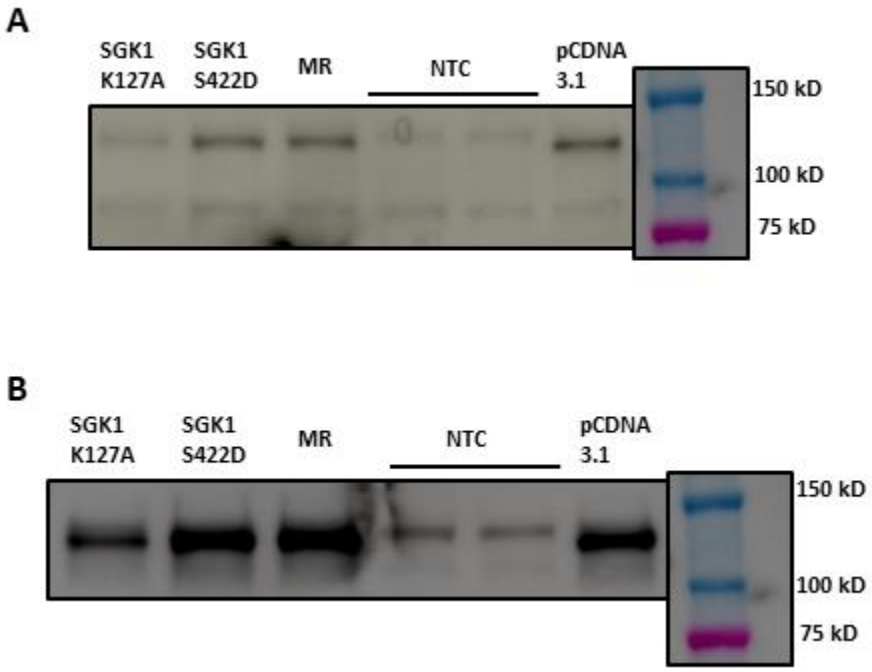
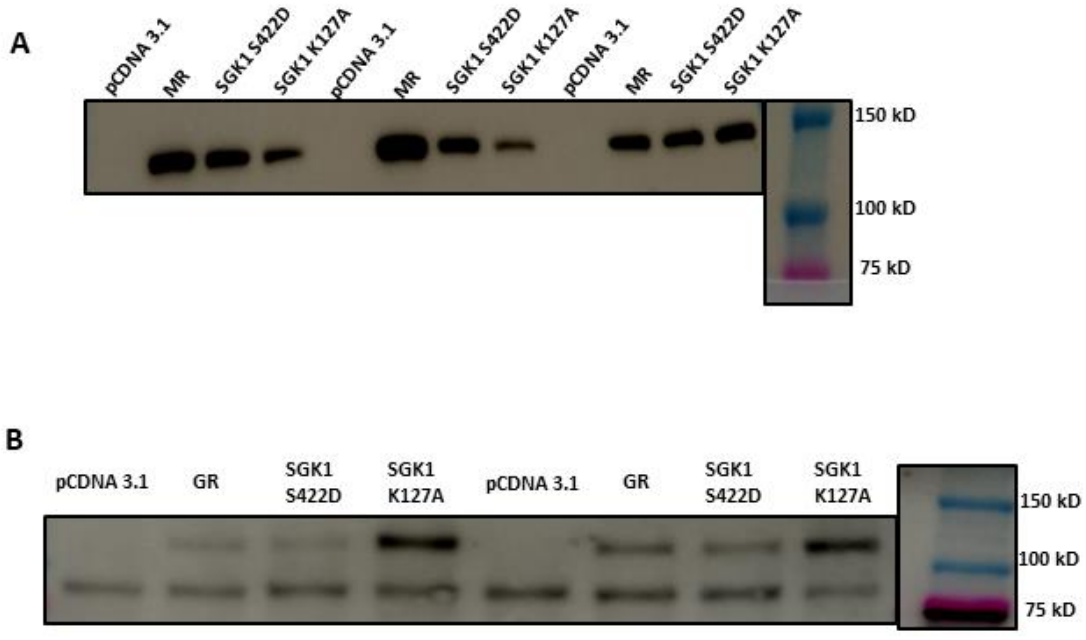


Figure 18: NEDD4-2 antibody specificity. All cells were transfected with NEDD4-2, except for two non-transfected controls (NTC), additionally lanes with SGK1 were co-transfected with MR. (A) pNEDD4-2 and (B) tNEDD4-2.

As seen in Figure 18A the NTC control showed the same two bands we detect in transfected cells, but with decreased intensity. In addition, the heavier band appears to migrate slightly slower than the band detected in transfected cells. It is possible that that

HEK293T cells express a distinct NEDD4 isoform (Fotia et al., 2006; Harvey et al., 2001), although the signal intensity level is unlikely to interfere with our analysis.

To prove that our transfection was correct we performed a set of western blots targeting MR, GR and SGK1. We next tested whether the expression of SGK1 and the corticosteroid receptors was correct. When we transfected MR, we detected the protein only in the conditions where it was included in the plasmid mix. MR migrated at near 130 kDa, corresponding to the expected weight of the fusion protein between the receptor and the enhanced green fluorescent protein (eGFP) (Figure 19A). GR on the other hand is expressed endogenously in HEK293T cells. However, the transfected form was tagged with eGFP, giving an additional 27 kDa that allowed for detection of two clear bands, one of them at 90-95 kDa corresponding to endogenous GR and the other one 120 kDa being the transfected GR (Figure 19B). We repeated the process for SGK1. However, the western blot only showed one band corresponding to the endogenous SGK1 present in HEK293T cells at 49 kDa (Figure 19C). In the event that the antibody used would be defective, we performed another western blot targeting this time the enhanced yellow fluorescent protein tag (eYFP) fused to SGK1. This experiment showed only the fluorescently tagged GR, with a faint band visible even in NTC cells migrating around the expected size for SGK1-eYFP (Figure 19D). In the end we could not confirm that SGK1 was being transfected correctly despite having differences in the luciferase assays.



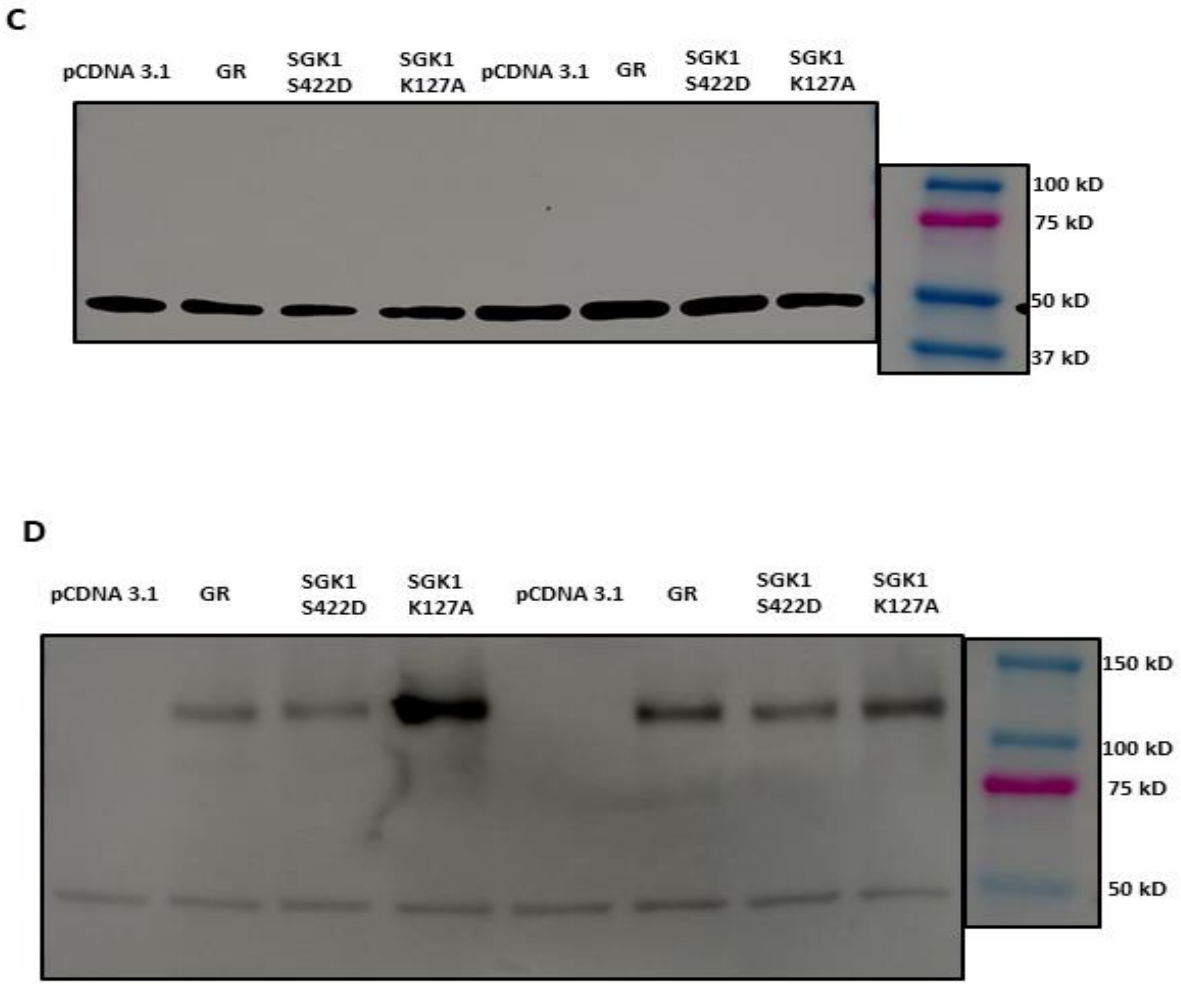


Figure 19: Western blots of HEK293T cells. Targeting (A) MR, lanes with SGK1 were co-transfected with MR. (B) GR, lanes with SGK1 were co-transfected with GR. (C) SGK1, lanes with SGK1 were co-transfected with GR and (D) eYFP, lanes with SGK1 were co-transfected with GR.

5 CONCLUSIONS

- Co-expression of SGK1 appears to affect MR and GR activity in opposite ways, regardless of the catalytic activity of the kinase.
- Analysis of a SGK1 phosphorylation target was not conclusive and suggests that the expression of SGK1 mutants in our experiments may below or defective

6 BIBLIOGRAPHY

Aguilar-Sánchez, C., Hernández-Díaz, I., Lorenzo-Díaz, F., Navarro, J. F., Hughes, T. E., Giraldez, T., & Alvarez De La Rosa, D. (2012). Identification of permissive insertion sites for generating functional fluorescent mineralocorticoid receptors. *Endocrinology*, *153*(7), 3517–3525. https://doi.org/10.1210/en.2012-1210

Armas-Capote, N., Maglio, L. E., Pérez-Atencio, L., Martin-Batista, E., Reboreda, A., Barios, J. A., Hernandez, G., Alvarez De La Rosa, D., Lamas, J. A., Barrio, L. C., & Giraldez, T. (2020). SGK1.1 Reduces Kainic Acid-Induced Seizure Severity and Leads to Rapid Termination of Seizures. *Cerebral Cortex*, *30*(5), 3184–3197. https://doi.org/10.1093/cercor/bhz302

Arriza, J. L., Weinberger, C., Cerelli, G., Glaser, T. M., Handelin, B. L., Housman, D. E., & Evans, R. M. (1987). Cloning of human mineralocorticoid receptor complementary DNA: Structural and functional kinship with the glucocorticoid receptor. *Science*, *237*(4812), 268–275. https://doi.org/10.1126/science.3037703

Bhalla, V., Daidié, D., Li, H., Pao, A. C., LaGrange, L. P., Wang, J., Vandewalle, A., Stockand, J. D., Staub, O., & Pearce, D. (2005). Serum- and glucocorticoid-regulated kinase 1 regulates ubiquitin ligase neural precursor cell-expressed, developmentally down-regulated protein 4-2 by inducing interaction with 14-3-3. *Molecular Endocrinology*, *19*(12), 3073–3084. https://doi.org/10.1210/me.2005-0193

Bigas, J., Sevilla, L. M., Carceller, E., Boix, J., & Pérez, P. (2018). Epidermal glucocorticoid and mineralocorticoid receptors act cooperatively to regulate epidermal development and counteract skin inflammation article. *Cell Death and Disease*, *9*(6). https://doi.org/10.1038/s41419-018-0673-z

Bocchi, B., Fagart, J., Cluzeaud, F., Fay, M., Rafestin-Oblin, M. E., & Farman, N. (2003). Glucocorticoid metabolism by 11-β hydroxysteroid dehydrogenase type 2 modulates human mineralocorticoid receptor transactivation activity. *Journal of Steroid Biochemistry and Molecular Biology*, *84*(2–3), 239–244. https://doi.org/10.1016/S0960-0760(03)00036-0

Brunet, A., Park, J., Tran, H., Hu, L. S., Hemmings, B. A., & Greenberg, M. E. (2001). Protein Kinase SGK Mediates Survival Signals by Phosphorylating the Forkhead Transcription Factor FKHRL1 (FOXO3a). *Molecular and Cellular Biology*, *21*(3), 952–965. https://doi.org/10.1128/mcb.21.3.952-965.2001

Burgering, B. M. T., & Coffer, P. J. (1995). Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature* (Vol. 376, Issue 6541, pp. 599–602). https://doi.org/10.1038/376599a0

Carter, M., Shieh, J. C. Chapter 15 - Biochemical Assays and Intracellular Signaling, Editor(s): Matt Carter, Jennifer Shieh, Guide to Research Techniques in Neuroscience (Second Edition), Academic Press, (2015), Pages 311-343, ISBN 9780128005118, https://doi.org/10.1016/B978-0-12-800511-8.00015-0.

Cavalier-Smith, T. (2017). Origin of animal multicellularity: Precursors, causes, consequences—the choanoflagellate/sponge transition, neurogenesis and the Cambrian explosion. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *372*(1713). https://doi.org/10.1098/rstb.2015.0476

Di Cristofano, A. (2017). SGK1: The Dark Side of PI3K Signaling. In *Current Topics in Developmental Biology* (1st ed., Vol. 123). Elsevier Inc. https://doi.org/10.1016/bs.ctdb.2016.11.006

Evans R. M. (1988). The steroid and thyroid hormone receptor superfamily. *Science (New York, N.Y.)*, *240*(4854), 889–895. https://doi.org/10.1126/science.3283939

Fallo, F., Veglio, F., Bertello, C., Sonino, N., Della Mea, P., Ermani, M., Rabbia, F., Federspil, G., & Mulatero, P. (2006). Prevalence and characteristics of the metabolic syndrome in primary aldosteronism. *Journal of Clinical Endocrinology and Metabolism*, *91*(2), 454–459. https://doi.org/10.1210/jc.2005-1733

Fotia, A. B., Cook, D. I., & Kumar, S. (2006). The ubiquitin-protein ligases Nedd4 and Nedd4-2 show similar ubiquitin-conjugating enzyme specificities. *International Journal of Biochemistry and Cell Biology*, *38*(3), 472–479. https://doi.org/10.1016/j.biocel.2005.11.006

Galigniana, M. D., Pilipuk, G. P., Kanelakis, K. C., Burton, G., & Lantos, C. P. (2004). Molecular mechanism of activation and nuclear translocation of the mineralocorticoid receptor upon binding of pregnanesteroids. *Molecular and Cellular Endocrinology*, *217*(1–2), 167–179. https://doi.org/10.1016/j.mce.2003.10.041

García-Martínez, J. M., & Alessi, D. R. (2008). mTOR complex 2 (mTORC2) controls hydrophobic motif phosphorylation and activation of serum- and glucocorticoid-induced protein kinase 1 (SGK1). *Biochemical Journal*, *416*(3), 375–385. https://doi.org/10.1042/BJ20081668

Gleason, C. E., Oses-Prieto, J. A., Li, K. H., Saha, B., Situ, G., Burlingame, A. L., & Pearce, D. (2019). Phosphorylation at distinct subcellular locations underlies specificity in mTORC2-mediated activation of SGK1 and Akt. *Journal of Cell Science*, *132*(7). https://doi.org/10.1242/jcs.224931

Gomez-Sanchez, E., & Gomez-Sanchez, C. E. (2014). The multifaceted mineralocorticoid receptor. *Comprehensive Physiology*, *4*(3), 965–994. https://doi.org/10.1002/cphy.c130044

Gomez-Sanchez, E. P., & Gomez-Sanchez, C. E. (2012). Central regulation of blood pressure by the mineralocorticoid receptor. *Molecular and Cellular Endocrinology*, *350*(2), 289–298. https://doi.org/10.1016/j.mce.2011.05.005

Harvey, K. F., Dinudom, A., Cook, D. I., & Kumar, S. (2001). The Nedd4-like Protein KIAA0439 is a Potential Regulator of the Epithelial Sodium Channel. *Journal of Biological Chemistry*, *276*(11), 8597–8601. https://doi.org/10.1074/jbc.C000906200

Harvey, K. F., & Kumar, S. (1999). Nedd4-like proteins: An emerging family of ubiquitin-protein ligases implicated in diverse cellular functions. *Trends in Cell Biology*, *9*(5), 166–169. https://doi.org/10.1016/S0962-8924(99)01541-X

Heitzer, M. D., Wolf, I. M., Sanchez, E. R., Witchel, S. F., & DeFranco, D. B. (2007). Glucocorticoid receptor physiology. *Reviews in Endocrine and Metabolic Disorders*, *8*(4), 321–330. https://doi.org/10.1007/s11154-007-9059-8

Hellal-Levy, C., Couette, B., Fagart, J., Souque, A., Gomez-Sanchez, C., & Rafestin-Oblin, M. E. (1999). Specific hydroxylations determine selective corticosteroid recognition by human glucocorticoid and mineralocorticoid receptors. *FEBS Letters*, *464*(1–2), 9–13. https://doi.org/10.1016/S0014-5793(99)01667-1

Hills, C. E., Squires, P. E., & Bland, R. (2008). Serum and glucocorticoid regulated kinase and disturbed renal sodium transport in diabetes. *Journal of Endocrinology*, *199*(3), 343–349. https://doi.org/10.1677/JOE-08-0295

Hollenberg, S. M., Weinberger, C., Ong, E. S., Cerelli, G., Oro, A., Lebo, R., Brad Thompson, E., Rosenfeld, M. G., & Evans, R. M. (1985). Primary structure and expression of a functional human glucocorticoid receptor cDNA. *Nature*, *318*(6047), 635–641. https://doi.org/10.1038/318635a0

Hudson, W. H., Youn, C., & Ortlund, E. A. (2014). Crystal structure of the mineralocorticoid receptor DNA binding domain in complex with DNA. *PLoS ONE*, *9*(9), 1–9. https://doi.org/10.1371/journal.pone.0107000

Hultman, M. L., Krasnoperova, N. V., Li, S., Du, S., Xia, C., Dietz, J. D., Lala, D. S., Welsch, D. J., & Hu, X. (2005). The ligand-dependent interaction of mineralocorticoid receptor with coactivator and corepressor peptides suggests multiple activation mechanisms. *Molecular Endocrinology*, *19*(6), 1460–1473. https://doi.org/10.1210/me.2004-0537

Jaisser, F., & Farman, N. (2016). Emerging roles of the mineralocorticoid receptor in pathology: Toward new paradigms in clinical pharmacology. *Pharmacological Reviews*, *68*(1), 49–75. https://doi.org/10.1124/pr.115.011106

Kobayashi, T., & Cohen, P. (1999). Activation of serum- and glucocorticoid-regulated protein kinase by agonists that activate phosphatidylinositide 3-kinase is mediated by 3-phosphoinositide-dependent protein kinase-1 (PDK1) and PDK2. *Biochemical Journal*, *339*(2), 319–328. https://doi.org/10.1042/0264-6021:3390319

Kumar, S, Harvey, K. F., Kinoshita, M., Copeland, N. G., Noda, M., & Jenkins, N. A. (1997). cDNA cloning, expression analysis, and mapping of the mouse Nedd4 gene [published erratum appears in Genomics 1997 Aug 15;44(1):156]. *Genomics*, *40*(0888-7543 SB-M), 435–443.

Kumar, Sharad, Tomooka, Y., & Noda, M. (1992). Identification of a set of genes with developmentally down-regulated expression in the mouse brain. *Biochemical and Biophysical Research Communications*, *185*(3), 1155–1161. https://doi.org/10.1016/0006-291X(92)91747-E

Leong, M. L. L., Maiyar, A. C., Kim, B., O'Keeffe, B. A., & Firestone, G. L. (2003). Expression of the serum- and glucocorticoid-inducible protein kinase, Sgk, is a cell survival response to multiple types of environmental stress stimuli in mammary epithelial cells. *Journal of Biological Chemistry*, *278*(8), 5871–5882. https://doi.org/10.1074/jbc.M211649200

Luisi, B. F., Xu, W. X., Otwinowski, Z., Freedman, L. P., Yamamoto, K. R., & Sigler, P. B. (1991). Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. In *Nature* (Vol. 352, Issue 6335, pp. 497–505). https://doi.org/10.1038/352497a0

Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schütz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., & Evans, R. M. (1995). The nuclear receptor superfamily: The second decade. *Cell*, *83*(6), 835–839. https://doi.org/10.1016/0092-8674(95)90199-X

Martini, M., De Santis, M. C., Braccini, L., Gulluni, F., & Hirsch, E. (2014). PI3K/AKT signaling pathway and cancer: An updated review. *Annals of Medicine*, *46*(6), 372–383. https://doi.org/10.3109/07853890.2014.912836

McKenna, N. J., & O'Malley, B. W. (2000). From ligand to response: Generating diversity in nuclear receptor coregulator function. *Journal of Steroid Biochemistry and Molecular Biology*, *74*(5), 351–356. https://doi.org/10.1016/S0960-0760(00)00112-6

Meijer, O. C., Van Der Laan, S., Lachize, S., Steenbergen, P. J., & De Kloet, E. R. (2006). Steroid receptor coregulator diversity: What can it mean for the stressed brain? *Neuroscience*, *138*(3), 891–899. https://doi.org/10.1016/j.neuroscience.2005.07.004

Mizuno, H., & Nishida, E. (2001). The ERK MAP kinase pathway mediates induction of SGK (serum- and glucocorticoid-inducible kinase) by growth factors. *Genes to Cells*, *6*(3), 261–268. https://doi.org/10.1046/j.1365-2443.2001.00418.x

Náray-Fejes-Tóth, A., Canessa, C., Cleaveland, E. S., Aldrich, G., & Fejes-Tóth, G. (1999). sgk Is an aldosterone-induced kinase in the renal collecting duct. Effects on epithelial Na+ channels. *Journal of Biological Chemistry*, *274*(24), 16973–16978. https://doi.org/10.1074/jbc.274.24.16973

O'Malley, B. W. (2016). Origins of the field of molecular endocrinology: A personal perspective. *Molecular Endocrinology*, *30*(10), 1015–1018. https://doi.org/10.1210/me.2016-1132

Olefsky, J. M. (2001). Nuclear Receptor Minireview Series. *Journal of Biological Chemistry*, *276*(40), 36863–36864. https://doi.org/10.1074/jbc.R100047200

Pearce, L. R., Komander, D., & Alessi, D. R. (2010). The nuts and bolts of AGC protein kinases. *Nature Reviews Molecular Cell Biology*, *11*(1), 9–22. https://doi.org/10.1038/nrm2822

Porter, B. A., Ortiz, M. A., Bratslavsky, G., & Kotula, L. (2019). Structure and function of the nuclear receptor superfamily and current targeted therapies of prostate cancer. *Cancers*, *11*(12). https://doi.org/10.3390/cancers11121852

Sierra-Ramos, C., Velazquez-Garcia, S., G. Keskus, A., Vastola-Mascolo, A., Rodríguez-Rodríguez, A. E., Luis-Lima, S., Hernández, G., Navarro-González, J. F., Porrini, E., Konu, O., and Alvarez de la Rosa., D (2021) Increased SGK1 activity potentiates mineralocorticoid/NaCl-induced kidney injury *American Journal of Physiology-Renal Physiology* 320:4, F628-F643

Simpson, S.A., Tait, J.F., Wettstein, A. *et al.* (1953) Isolierung eines neuen kristallisierten Hormons aus Nebennieren mit besonders hoher Wirksamkeit auf den Mineralstoffwechsel. *Experientia* **9,** 333–335. https://doi.org/10.1007/BF02155834

Swalla, B. J., & Smith, A. B. (2008). Deciphering deuterostome phylogeny: Molecular, morphological and palaeontological perspectives. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *363*(1496), 1557–1568. https://doi.org/10.1098/rstb.2007.2246

Tata, J. R. (2005). One hundred years ofhormones A. *EMBO Reports*, *6*(6), 490. doi: 10.1038/sj.embor.7400444. PMID: 15940278; PMCID: PMC1369102.

Toska, E., Castel, P., Chhangawala, S., Arruabarrena-Aristorena, A., Chan, C., Hristidis, V. C., Cocco, E., Sallaku, M., Xu, G., Park, J., Minuesa, G., Shifman, S. G., Socci, N. D., Koche, R., Leslie, C. S., Scaltriti, M., & Baselga, J. (2019). PI3K Inhibition Activates SGK1 via a Feedback Loop to Promote Chromatin-Based Regulation of ER-Dependent Gene Expression. *Cell Reports*, *27*(1), 294-306.e5. https://doi.org/10.1016/j.celrep.2019.02.111

Vanhaesebroeck, B., Guillermet-Guibert, J., Graupera, M., & Bilanges, B. (2010). The emerging mechanisms of isoform-specific PI3K signalling. *Nature Reviews Molecular Cell Biology*, *11*(5), 329–341. https://doi.org/10.1038/nrm2882

Wang, Q., Anzick, S., Richter, W. F., Meltzer, P., & Simons, S. S. (2004). Modulation of transcriptional sensitivity of mineralocorticoid and estrogen receptors. *Journal of Steroid Biochemistry and Molecular Biology*, *91*(4–5), 197–210. https://doi.org/10.1016/j.jsbmb.2004.04.007

Wang, Y., Zhou, D., & Chen, S. (2014). SGK3 Is an androgen-inducible kinase promoting prostate cancer cell proliferation through activation of p70 S6 kinase and up-regulation of cyclin D1. *Molecular Endocrinology*, *28*(6), 935–948. https://doi.org/10.1210/me.2013-1339

Wang, Y., Zhou, D., Phung, S., Masri, S., Smith, D., & Chen, S. (2011). SGK3 is an estrogen-inducible kinase promoting estrogen-mediated survival of breast cancer cells. *Molecular Endocrinology*, *25*(1), 72–82. https://doi.org/10.1210/me.2010-0294

Ward E, Slocumb CH, Polley HF, Kendall EC, Hench PS. (1951) Clinical effects of cortisone administered orally to 100 patients with rheumatoid arthritis. *Ann Rheum Dis*;10:477–84.

Webster, M. K., Goya, L., Ge, Y., Maiyar, A. C., & Firestone, G. L. (1993). Characterization of sgk, a novel member of the serine/threonine protein kinase gene family which is transcriptionally induced by glucocorticoids and serum. *Molecular and Cellular Biology*, *13*(4), 2031–2040. https://doi.org/10.1128/mcb.13.4.2031-2040.1993