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Doctoral thesis presented by Estefania Alcaraz for the degree of PhD in Biochemistry, Molecular Biology and Biomedicine from the Universitat Autònoma de Barcelona.

Thesis performed in the Department of Biochemistry and Molecular Biology supervised by Dr. Emilio Itarte Fresquet.

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LIST OF ABBREVIATIONS

4E-BP1	*Eukaryotic translation initiation factor 4E-binding protein 1*
AII	*angiotensin II*
AJCC	*American Joint Commitee on Cancer*
AMPK	*AMP-activated protein kinase*
AQP1	*Aquaporin-1*
AR	*amphiregulin*
ASK-1	*apoptosis signalling kinase 1*
AURKB	***Aurora kinase B***
BAD	*Bcl-2-Associated Death promotor*
BHD	*Birt-Hogg-Dube*
BTC	*betacellulin*
Card9	*Caspase recruitment domain-containing protein 9*
CBC	*Complete Blood Count*
ccRCC	*clear cell Renal Cell Carcinoma*
CDK1	*Cyclin-dependent kinase 1*
ChRCC	*Chromophobe Renal Cell Carcinoma*
CIL	*Contact Inhibition of Locomotion*
CK	*Citokeratin*
CK2	*Protein kinase CK2*
COL4A2	*(collagen IV α2 chain),*
Cul-2	*Cullin-2*
CX-4945	*CK2 inhibitor*
CYT	*C-terminal cytoplasmatic tails*
Dbox	*Destruction Box*
DMEM	*Dulbecco's Modified Eagle's Medium*
DMSO	*Dimethyl Sulfoxide*
ECM	*ExtraCellular Matrix*
EGF	*epidermal growth factor*
EGFR	*Epithelial Growth Factor Receptor*
eIF2β	*Eukaryotic translation initiation factor 2 subunit β*
EMT	*Epithelial-Mesenchymal Transition*
EPK	*Eukaryotic Protein Kinase Family*

EPR	*epiregulin*
ERK	*Extracelular-signal-regulated kinase 1, 2 (ERK).*
FBS	*Fetal Bovine Serum*
FITC	*fluorescein isothiocianate*
FOXO	***Forkhead box protein O***
GAP	*gamma-activated sites*
GNB2L1	*Guanine nucleotide-binding protein subunit beta-2-like 1*
GSK	***Glycogen synthase kinase***
HB-EGF	*heparin-binding growth factor*
HGF receptor	***Hepatocyte growth factor receptor***
HGFR	*Hepatocyte Growth Factor Receptor*
HIF	*Hypoxia Inducible Factor*
HPRCC	*Hereditary Papillary Renal Cell Carcinoma*
HPV-16	*human papilloma virus 16*
HRE	*Hypoxia Response Elements*
HSPGs	*heparan-sulfate proteoglicans*
ICD	*intracellular domain*
IHC	*Immunohistoquímica*
IKKα	*kappa B kinase-α*
IL-2	*Interleukin-2*
IL-6	*Interleukin-6*
INF-α	*interferon-α*
INPP4B	*Inositol polyphosphate-phosphatase type II*
IP	*Immunoprecipitation*
IRS-1	*Insulin receptor substrate 1*
JAKs	*janus kinases*
JM	*juxtamembrane domains*
JNKs	*Jun amino-terminal kinases*
KSR	*kinase suppressor of Ras,*
lyso-PC	*lyso-phosphatidylcholine*
MAPK	*Mitogen-activated protein kinase 1*
MDM2	*murine doble minute 2*
MDR-1	*Multidrug Resistance -1*
MEK	*Mitogen-activated protein kinase ERK kinase*
MKI	*Multityrosine kinase Inhibidor*
MLST8	*Target of rapamycin complex subunit LST8*
MMP	*Extracel·lular matrix metalloproteinases*
MRI	*Magnetic Resonance Imaging*
mTOR	*(the mammalian target for rapamycin)*
MTT	*3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide*
NF-κB	***NF-kappaB1***
NFQ	*Non-Fluorescent Quencher*
NHS	*Normal Horse Serum*
NLS	*Nuclear Localizating Signals*
PAI-1	***Plasminogen activator inhibitor 1***
PARP	***Mono [ADP-ribose] polymerase PARP***

PDGF	*Plateled Derived Growth Factor*
PFA	*Paraformaldehide*
PH	*Plekstrin Homology Domains*
PI3K	*phosphatidilinositol 3-kinase*
PIKKs	*PI3K related protein kinase*
PKC	***Protein kinase C***
PLK1	*polo-like kinase 1*
POMs	*polyoxometalates*
PRAS40	*Proline-rich AKT1 substrate 1*
PRCC	*Papillary Renal Cell Carcinoma*
PTB domains	*phosphotyrine-binding domains*
PTC	*proximal convolute tubule*
PtdIns	*phosphatidylinositol*
PtdIns(3,4,5)P3	*phosphatidylinositol 3,4,5-trisphosphate*
PtdIns(4,5)P2	*phosphatidylinositol 4,5- bisphosphate*
PTEN	*phosphatase and tensin homolog*
PTH	*parathyroidal hormone*
pVHL	*VHL protein*
Raf	*RAF proto-oncogene*
Rbx1	***E3 ubiquitin-protein ligase RBX1***
RCC	*Renal Cell Carcinoma*
REDD1	*DNA damage-inducible transcript 4 protein*
Rheb	*Ras homologue enriched in the brain*
RKIP	*Raf kinase inhibitory protein*
RKTG	*(signal transducers and activators of transcription)*
RQ	*Relative quantification*
RTKs	*Tirosine Kinase Receptors*
RT-PCR	*Reverse Transcription-Polymerase Chain Reaction*
RT-qPCR	*Real Time- quantitative Polymerase Chain Reaction*
S6K1	***Ribosomal protein S6 kinase beta-1***
SAPKs	*stress-activated protein kinases*
SGK1	*erine/threonine-protein kinase Sgk1*
SH2	*Src homology region 2*
shRNA	*short hairpin RNA*
Síndrome VHL	*Síndrome de Von Hippel-Lindau*
siRNA	*small interfering RNAs*
Sistema TNM	*Sistema de Tumor, Nòduls i Metàstasi*
STAT	*signal transducers and activators of transcription)*
TCEB1	*Transcription elongation factor B polypeptide 1*
TfR	***Transferrin receptor protein 2***
TGF-α	*Tumoral Growth Factor-α*
TGF-β	*tumoral Growth Factor-β*
Tm	*melting temperature*
TMAs	*Tissue MicroArrays*
TSC1	*Hamartin*
TSC2	***Tuberin***

TβRII	*TGF-β receptor*
UICC	*International Union Against Cancer*
VEGF	*VasoEndotelial Growth Factor*
WHO	*World Health Organisation*
WWOX	*WW domain-containing oxidoreductase*
ZEB2	*Zinc finger E-box-binding homeobox 2*

Summary

Protein kinase CK2 is a Serine/Threonine kinase widely expressed in all eukaryotic organisms. To date, more than 300 substrates for this kinase have been discovered, most of them essential for cell viability. For that reason, CK2 has been considered as a protein kinase decisive for the viability of all eukaryotic cells.

CK2 plays pivotal roles in cell survival, proliferation and anti-apoptotic mechanisms, and its dysregulation is associated with human malignancies. Furthermore, the pleiotropic effects of this protein have connected CK2 with other pathways that are crucial in several processes involved in tumorigenesis. However, little is known on the potential cross-talk between CK2 and these signalling pathways in clear cell renal cell carcinoma (ccRCC) cells.

The purpose of this work has been to study the involvement of CK2 in the molecular basis of ccRCC, analysing the effect of CK2 inhibition on Akt and ERK1/2 signalling pathways in response to heparin-binding EGF-like growth factor (HB-EGF), as well as the connection between CK2 and ErbB4, which has been found downregulated in ccRCC. In order to assess this gkoal, CK2 activity has been targeted by pharmacological inhibitors, and the regulatory and catalytic CK2 subunits have been independently silenced by *short hairpin RNA* (shRNA), in tubular proximal cells derived from normal kidney (HK-2), and cells derived from a primary clear cell adenocarcinoma (786-O).

The most striking result is that CK2 inhibition, either by chemical inhibitors or shRNA downregulation, impairs the activation of Akt and ERK1/2 in response to HB-EGF, which are decisive signalling pathways involved in the process of cell death, proliferation and autophagy. Likewise, downregulation of regulatory subunit CK2β is accompanied by changes in the expression of Epithelial-Mesenchymal-Transition (EMT) markers, such as E-cadherin and Snail1. Interestingly, the results of this study suggest that CK2β downregulation induces HIF-α expression and STAT3 phosphorylation which may contribute to E-cadherin and Snail1 regulation in ccRCC cells. On the other hand, overexpression of ErbB4 in 786-O cells alters cell proliferation as well as enhances HB-EGF-induced Akt and ERK1/2 activation. In addition, CK2 inhibition by CX-4945 and downregulation of CK2β by siRNAs results in a significant reduction of ErbB4 levels.The results of this research show that CK2 affects key components of signalling pathways, such as ErbB4, Akt, ERK1/2, HIF-α, Snail 1 and STAT3 in renal cells, supporting the potential involvement of CK2 in ccRCC.

Introduction

1. RENAL CELL CARCINOMA (RCC)

1.1. Introduction

Renal cell carcinoma (RCC), constitutes the 2-3% of all malignant diseases in adults. It is the commonest solid lesion within the kidney and accounts for approximately 90% of all kidney malignancies [1]. There is a 1.5:1 predominance in men over women, appearing in the sporadic form between 60 and 70 years old [2]. Hereditary forms appear earlier, at 40's and constitutes only the 5% of total kidney cancers. Cigarette smoking, obesity, hypertension and/or related medications are an established risk factor for renal cell carcinoma [3]–[5]. Also renal cell carcinoma seems to be more common in patients with end-stage renal failure, acquired renal cystic disease, and tuberous sclerosis than in the general population [6]–[8]. Approximately 2-3% of RCC are hereditary and several autosomal dominant syndromes are described. Most notable is von Hippel-Lindau syndrome that is characterised by the development of several vascular tumours including clear cell renal cell carcinoma, haemangioblastomas of the central nervous system and pheochromocytoma [9]. Currently, RCC is heterogeneous and comprises several histological cell types with different genetics, biology and behaviour. The identification of many of the oncogenes and tumour suppressor genes that are mutated leading to pathway dysregulation in RCC remain to be elucidated, but could led to substantial clinical advances in management of the disease.

The most common histological type is clear cell carcinoma, which represents 75-80% of RCC. Other histological types are papillary RCC (10-15%), chromophobe (5%), collecting duct (1%) and unclassified (4%). ccRCC arises from the epithelium of the proximal tubule, mine while chromophobe RCC, oncocytoma, and collecting duct RCC are believed to arise from the distal nephron, probably from the epithelium of the collecting tubule. [10]

1.1.1. Clear cell renal cell carcinoma (ccRcc)

Clear cell RCC, is the most common type of RCC. It usually presents as a solitary, golden yellow mass, which reflects the high lipid content of its cells, well circumscribed an with distinct areas of haemorrhage and necrosis. Microsopically, the ccRCC cells are classified by their nuclear grade, evaluated by the Furman nuclear system (Table 1)[11]. The neoplastic cells of low nuclear grade clear cell RCC have water-clear and agranullar cytoplasm, however high nuclear grade clear cell RCC typically has more granular and eosinophilic cytoplasm [12].

Table 1. Fuhrman nuclear grading system used in ccRCC			
Grade	**Nuclei**		**Nucleoli**
	Size	**Shape**	
1	10 µm	Round, uniform	without nucleoli visible
2	15 µm	Slightly irregular	Conspicuous and evident at high power (x400 magnification)
3	20 µm	Obviously irregular	Prominent, large at low power (x100 magnification)
4	>20 µm	Pleomorphic and hyperchromatic	Macronucleoli
Content adapted by authors from Fuhrmann et al., [11]			

CcRCC may have sporadic (>96%) or inherited/genetic origin (<4%) [13]–[15] . Almost all familiar clear cell RCC, comes from an inherited mutation in the von Hippel-Lindau (VHL) tumour suppressor gene, located on chromosome 3p. Patients with VHL syndrome show kidney cysts and multiple bilateral clear cell RCC at an average 37 years of age. The second allele of VHL has been shown to be inactivated by deletion and less commonly by promoter hypermethylation or rearrangemen[16]–[18]. The sporadic form of ccRCC appears at an average of 60 years and it usually presents as a solitary tumour of several centimetres in size.

Recent studies of ccRCC gene expression have detected 725 genes deregulated in tumoral tissue [19]. In general the downregulated genes tend to represent biological pathways related to tissue remodelling and wound repair, blood cotting, vasodilatation and energy metablosim. The proteins that they codify can be assigned to three specialized cellular compartments: (1) Extracellular Matrix (ECM), playing an important role in the regulation of numerous cellular functions, such as cell morphology and polarity, adhesion, proliferation, differentiation, migration, apoptosis and wound healing processes; (2) integral membrane proteins, serving as entry and exit routes for many ions, nutrients, waste products, hormones, drugs, and large molecules ; (3) membrane vesicles (endosomes and lysosomes) implicated in signal transduction process, as well as in morphogenetic aspects of normal physiology adhesion and migration. On the other hand, the upregulated genes are classified into pathways generally deregulated in cancer: immune system response, inflammatory response, DNA damage response, mitogenic signalling, angiogenesis, and apoptosis. In that case, the localization of the proteins codified by these genes are mainly cytoplasmic, but also in the cellular membranes and vesicles.

1.1.2. Diagnosis

Patients with this cancer, present with local or systemic symptoms, although more than 50% of RCCs cases are detected incidentally when non-invasive imaging is using to investigate a variety of nonspecific symptoms and other abdominal diseases. Local signs and symptoms include haematuria, flank pain or a palpable abdominal mass are rare and correlates with aggressive histology and advanced disease. Systemic syndromes can be due to a several paraneoplastics syndromes (hypertension, cachexia, weight loss, pyrexia, neuromyopathy, amyloidosis, elevated erythrocyte sedimentation rate, anemia, abnormal liver function, hypercalcemia, polycythemia) [20]. Despite of an increased detection of incidental renal masses has led to a detection of this cancer at early stages and subsequent removal with surgery, mortality has increased.

1.1.2.1 Molecular markers of renal carcinoma

Although image techniques have been allowed to improve incidentally diagnostic in early stages, they do not determinate the type of tumour, the stage, and the best treatment to use. Thus, is necessary a biopsy since in ccRCC there are not no invasive procedures such as urine or blood samples to detect, as well as no markers have been found yet allowing its detection in early stages. It is obvious that new diagnostic and prognostic biomarkers are needed to diagnose and monitor renal cancers. Recently it has been described that the cell molecule HAVCR/KIM-1 is upregulated in ccRCC. This molecule activates IL-6/STAT-3/HIF-1A cascade in ccRCC-derived cell lines. In addition it was suggested that pSTAT-3 S727 levels represented an independent prognostic factor [21].

2. MOLECULAR FEATURES OF CLEAR CELL RENAL CELL CARCINOMA

The main molecular alterations in ccRCC are related with hypoxia induced pathway, due to inactivation of VHL gene, which is involved in oxygen and energy sensing. It is clear that loss of pVHL results in the activation of cellular processes that are strongly associated with tumour initiation and progression. However, ccRCC tumours exhibit substantial mutation heterogeneity. Recent studies have implicated novel mutations in genes such as TCEB1 (which encodes Elongin C), mTOR, TSC1, PIK3CA and PTEN, which are involved in other signalling pathways that can also co-operate with pVHL in the development of the tumour [22], [23] (**Figure 1**).

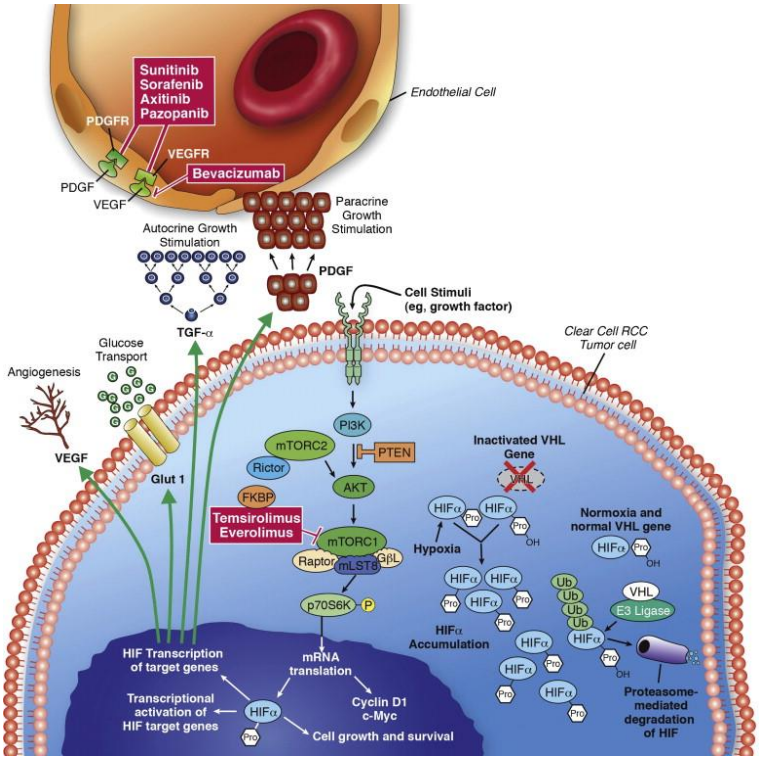


Figure 1. Most significant signalling pathways involved in ccRCC, included approved agents that act on this pathway. Adapted from B.Shuch et al.,[24].

2.1. VHL/HIF pathway in ccRCC

The familiar cancer syndrome, von Hippel-Lindau (VHL) disease, occurs as a result of inheriting a mutation in the VHL tumour-suppressor, leading to the development of haemangio-blastomas of the central nervous system and retina, renal cysts and ccRCC in Kidney and pancreatic cysts and tumours [17].

The VHL gene encodes two different proteins with alternative start codons, pVHL30 and pVHL19. Collectively are referred to as pVHL, because both behave similarly in many biochemical and cell-based processes [25], [26]. This protein has several functions in the cell; however the best-characterised role of pVHL is its paper in polyubiquitination. Specifically, pVHL is the substrate recognition subunit of an ubiquitin ligase complex that also contains elongin C, elongin B, Cul-2 and Rbx1. This complex targets proteins for ubiquitin-mediated degradation by the 26S proteasome. pVHL contains two functional domains. Residues 155-213 comprise the α domain which binds to elongin C, elongin B and p53 protein. The first 154 residues of the VHL protein comprise the β domain that acts as the substrate-docking interface for target proteins [27]–[29]. Diverse substrates of this E3 ubiquitin ligase complex have been identified but the best-known target is the α-subunit of the HIF (Hypoxia Inducible Factor) family members, HIF-1α, HIF-2α and HIF-3α [30].

HIF is a heterodimeric transcription factor composed of an α-subunit (HIF-α) which is sensitive to oxygen, and β-subunit (HIF-β) which is constitutively expressed. Under normoxia conditions, HIF-α is hydroxylated on key proline residues by HIF-prolyl hydroxylase (PHD). This process requires molecular oxygen, 2-oxoglutarate, ascorbate and Fe2+ as cofactors, facilitating HIF- α binding to pVHL, and its proteasomal degradation by the E3 ubiquitin ligase complex. Under hypoxic conditions, PHD cannot hydroxylate HIF-α, therefore the pVHL/elonginC/elonginB/cullin complex does not recognize HIF-α, inducing its accumulation. Then, HIF-α protein translocates to the nucleus where it dimerizes with HIF-1β and activates the transcription of target genes[31], [32]. Similarly, if loss of functional pVHL occurs due to genetic or epigenetic events in normoxia, HIF-α is not targeted for degradation and consequently is up-regulated.

In humans, three HIF genes have been identified. HIF-1α and HIF-2α are well characterized and over 100 genes have been identified downstream of HIF. The HIF induced genes are involved in stimulating angiogenesis, erythropoiesis, glucose uptake and metabolism, regulation of extracellular pH, matrix metabolism, cell proliferation and survival, and apoptosis. However, little is known about the regulation and function of HIF-3α. Interestingly, HIF-2α and HIF-3α appear to be expressed in a cell specific manner when compared with the ubiquitously expression of HIF-1α. Moreover, has been interest in distinguishing the roles of HIF-1α and HIF-2α (reviewed in [33]).

2.1.1. HIF1α and HIF2α contribution to ccRCC

Several evidences suggest different contribution of HIF-1α and HIF-2α to renal tumorigenesis. Analysis of patient renal tissue samples, which lacked to functional VHL, has shown to have up-regulated HIF-2α [34]. This is in agreement with the result obtained in a number of VHL-defective RCC cell lines, where only is expressed the HIF-2α isoform [35]. Accordingly, in a wild-type pVHL ccRCC, normoxic stabilization of HIF-1α fails to promoted tumorigenesis *in vivo*. Contrary, normoxic stabilization of HIF-2α overrides tumour suppression by pVHL and promotes tumorigenesis [36]. Additionally, siRNA knockdown of HIF-2α in VHL-defective cell lines suppressed tumour formation in vivo [37]. Both isoforms also differ respect to their transcriptional genes. Thus, in RCC cells HIF-2α up-regulated a number of tumorigenic genes such as TGF-α, cyclin D1, VEGF and GLUT-1 or up-regulating TGF-α/EGFR (epidermal growth factor receptor) pathway. On the other hand, HIF-1α up-regulated pro-apopotic genes (BNip3, REDD1, TXNIP, and ZAC1). Therefore, some genes are regulated HIF-2α and HIF-1α in antagonic manner; for example, HIF-2α increased c-Myc activity

whereas HIF-1α suppresses c-Myc activity. At the same way, HIF-1α enhances p53 functions while HIF-2α decreases its levels [38], [39]. These results suggest that ccRCC requires a shift from expression of HIF-1α to HIF-2α and that HIF-2α plays a pathogenic role in ccRCC.

2.1.2. Other pathways altered by VHL in ccRCC

Although HIF-2α is involved in renal carcinogenisis, downregulation of it does not recover the alterations associated with pVHL loss. It is known that pVHL interacts with other proteins several of which may contribute to the tumorigenic phenotype.

2.1.2.1. Extracellular matrix and matrix metalloproteinases regulated by pVHL

It is known that VHL disease have impaired extracellular matrix (ECM) assembly capabilities and sporadic ccRCC cases show low stain of fibronectin [40]. The mechanism of how pVHL controls the assembly of ECM is not yet to be proposed, but it seems to be independent of HIFs. Recently, diverse studies have been described that pVHL binds directly COL4A2 (collagen IV α2 chain), and coimmuniprecipitation experiments, biochemical fractionation and immunofluorescence studies have shown interaction between pVHL and fibronectin. The interaction of pVHL with these component of ECM is important to the correct assembly of the ECM [41].

Moreover, loss of pVHL up-regulates the expression of metalloproteinases, particularly MMP-2, MMP-9 ant MT1-MPP, promoting EMC destruction. Loss of ECM integrity could promote and maintain tumour angiogenesis by providing a route for blood vessels to infiltrate tumours[42], [43].

2.1.2.2. Role of pVHL in an intracellular cell junctions.

The appropriate formation of intracellular junctions is essential to maintain a regular cell shape and a polarized epithelial structure. Several studies have disclosed a link between the reduction of their integrity and tumour initiation and progression. Adherent and tight junctions are the most important functional complexes in the kidney epithelium. Adherent junctions are comprised by a family of transmembrane glycoproteins called cadherins, which bind intracellularly with the actin cytoskeleton and other signalling proteins of catenin family. Tight junctions consist of transmembrane components, such as the claudins and occludin, which interact with the actin cytoskeleton via other proteins. VHL inactivation triggers a down-regulation of both adherent junctions and tight junction which results in a damage of intracellular

junctions, alterations in cell polarity and epithelial-mesenchymal transition (EMT) [44]. Moreover some of transcriptional repressors of EMT such as Snail 1, ZEB2, E47 and Twist are up-regulated via HIF in VHL-defective cells, which result in attenuation of E-cadherin level [45]–[47]. E-cadherin is an established tumour suppressor, observing a correlation between the loss of this protein and the tumour aggressiveness, associated also a worsening of prognosis. Reexpression of E-cadherin suppresses tumour development and invasion in various *in vitro* and *in vivo* tumour model systems [48]. It has been observed that the loss of VHL provokes the stabilization of HIF-α leadding the up-regulation of transcriptional repressors of E-cadherin. All these results suggest that the mechanism linking VHL to different aspects of EMT involves either HIF-dependent or HIF-independent processes.

2.1.2.3. Influence of pVHL on primary cilium formation and microtubule stabilization.

The primary cilium is a structure on the cell surface that regulates the transduction of both chemical and mechanical signals, and has been proposed to be a negative regulator of cell proliferation. The ciliary axoneme is composed of microtubules organized in nine peripheral doublets that are templated from the basal body or mother centriole [49]. pVHL and GSK3β function together in maintain ciliar signalling network, disruption of which enhances the vulnerability of cells to lose their cilia, promoting cyst formation. GSK3β phosphorylates pVHL at Ser 68 and thereby inhibits the microtubule stabilizing function of pVHL [50], [51].

2.1.2.4. Regulation of apoptosis

Clear cell renal cell carcinomas are notable for their insensitivity to conventional cytotoxic chemotherapies. It is known that the efficacy of chemotherapy is highly linked to p53-mediated apoptosis. However, ccRCC do not appear to have loss or p53 mutations, suggesting either mechanism of p53 activity regulation or activation of other anti-apoptotic pathways. Previous reports have proposed that p53 interacts with pVHL and this interaction enables the stabilization of p53 by suppressing Mdm2 – mediated ubiquitination, resulting in an increase in its transcriptional activity [52], [53]. Moreover, it has been shown that HIF-1α can directly bind to and modulate p53 activity [54], and promote p53-dependent apoptosis [55], [56]. A novel mechanism for the stabilization of p53 has been described recently, in which upon hypoxia or VHL loss, the programmed cell death 5 (PDCD5) translocates to the nucleus, leading to p53 acetylation and

thereby increasing the affinity of p53 for its targets sites [57]. By contrast, other studies have reported that HIF-1α antagonizes p53-mediated apoptosis by triggering homeodomain-interacting protein kinase-2 (HIPK2) degradation [58]. One possible explanation is that this discrepancy can be due to cancer cell type [59]. More research is required to interpret fully the connexion within pVHL and p53.

It has been described that NF-κB acts as a pro-survival factor of cancer cells, triggering activation of anti-apoptotic pathways that mediated resistance to chemotherapy. Accumulating evidences suggest that in the absence of a functional pVHL, the expression and activity of NF-κB is enhanced, at least partially dependent upon HIF signalling [60]. However, pVHL can modulate NF-κB activity by a HIF-independent mechanism in which pVHL binds to CK2 and it inhibits phosphorylation of the NF-κB agonist Card9. Failure to phosphorylate Card9 leads to increased NF-κB activity and decreased apoptosis. Moreover down-regulation of Card9in *VHL*−/− cancer cells delayed tumor growth [61].

Jade-1 is a short-lived protein mainly expressed in renal proximal tubules that is stabilized directly by pVHL. Loss of Jade-1 stabilization by pVHL correlates with renal cancer risk. The Jade-1 gene resides at chromosome 4q26-27, and 4q loss may occur in as many as 50 % of clear-cell renal cancers. Thus, Jade-1 is a candidate renal tumour suppressor and promotes apoptosis affecting cell anchoring or mitochondrial metabolism by down-regulating Bcl-2 [62], [63]. Moreover, pVHL down-regulates β-catenin in a Jade-1 dependent manner and inhibits Wnt tumorigenesis pathway. Low Jade-1 and high beta-catenin levels have been linked to poor prognosis in renal cancer [64]. Recently Jade-1 has been described as a modulator of Akt pathway. Reintroduction of pVHL into renal cancer cells increases Jade-1 expression and it inhibits Akt1 phosphorylation. Hyeractivation of Akt pathway could facilitate the survival and proliferation, and make cells less sensitive to apoptotic signals [65].

Although, it is clear that loss of pVHL can result in the activation of different cellular pathways, which are tightly associated with tumour initiation and progression, there are evidences suggesting that, other deregulating processes, co-operate with pVHL in the development of a tumour.

2.2. mTOR pathway

Growth factors, nutrients, energy, stress signals and essential signalling pathways like PI3K, MAPK and AMPK activate mTOR pathway in order to controls cell growth, proliferation and survival [66]. mTOR pathway is dysregulated in many cancers,

including RCC, and activation of this pathway has been suggested to correlate with agressive behaviour and poor prognosis in RCC tumours [67]. mTOR (the mammalian target for rapamycin) is a 289 kDa serine/threonine kinase that belongs to the PI3K related protein kinase (PIKKs) family. mTOR comprise two distinct protein complexesmTORC1 and mTORC2 [68].

mTORC1, which is sensitive to rapamycin, is composed by mTOR, raptor, MLST8, PRAS40 and DEPTOR. Raptor acts as a scaffold for recruiting mTORC1 substrate. A recent report suggests that the activity of mTORC1 can be regulated by the phosphorylation status of raptor. MLST8 binds to the kinase domain of mTOR, and regulates positively the mTOR kinase activity. It seems essential to maintain a nutrient and rapamycin-sensitive interaction between raptor and mTOR. It is also necessary to sustain the rictor-MTOR interaction, also in mTORC2, being important for the consistent equilibrium of the both complexes in mammalian cells. PRAS40 associates with mTORC1 through raptor and blocks its activity. DEPTOR seems to be mTOR-interacting protein. It associates both mTORC1 and mTORC2, negatively inhibiting their activities. mTORC2 is resistant to rapamycin, however long-term treatment with rapamycin disrupt mTORC2 assembly and function. It includes the mTOR, rictor, Mlst8 protein, Msin1 and recently have been described new components, Protor, Hsp70 and DEPTOR. Rictor is an mTOR-associated protein exclusive from mTORC2. mSin1 is essential for mTORC2 integrity and mTOR activity against Akt Ser473 phosphorylation. Protor-1 interacts with rictor, although it is not important for the assembly of other mTORC2 subunits. Hsp70 is required for kinase activity of mTORC2 under basal conditions and following heat shock. And Mlst8 and DEPTOR are components of both complexes (reviewed in [66], [68], [69]).

mTORC1 is activated by PI3K/Akt and Ras/Raf/MAPK pathway and inhibited by the TSC1/TSC2 complex. Stimulation of Akt, phosphorylates TSC2 (tuberous sclerosis complex 2), leading to dissociation of the TSC1/TSC2 heterodimer complex. TSC2 loses its GAP activity when is not associated with TSC1, and can no longer hydrolyze Rheb-GTP, resulting in a continued activation of mTORC1. Akt also phosphorylates and inhibits PRAS40, which negatively regulates mTORC1 by antagonizing its activation by Rheb. Moreover mTOR integrates other stimuli including nutrients, cellular energy level and cellular stress. Activation of this pathway, triggers numerous down-stream biological effects, including regulation of ribosomal biogenesis and protein synthesis by phosphorylating 4E-BP1 and p70S6 Kinase [70], [71]. mTORC2 is activated in response to growth factors, and phosphorylates PKC-α, Akt (on Ser473), Paxilin (focal adhesion associated protein) and regulates the activity of FOXO and

SGK1 and small GTPases Rac and Rho, proteins associated to cell survival, migration and regulation of the actin cytoskeletion [72]–[74]. Interestingly, HIF2α expression depends upon the activity of mTORC2, whereas HIF1α levels depend upon both complexes mTORC1 and mTORC2 [75].

2.2.1. mTOR pathway in carcinoma renal

A negative feedback loop links VHL with mTORC1 pathways. HIF-1α and HIF-2α induce REDD1 (DNA damage response) expression which is sufficient to inhibit mTORC1. REDD1 is consistently upregulated in most ccRCC, however, mTORC axis is often hyperactivated in the majority of ccRRCC [76]. This may be accomplished by alterations or mutations in other proteins involve in mTOR pathway. PI3KCA, PTEN, TSC1, TSC2, RHEB and FAT domain mTOR mutations haven been shown in ccRCC,, and represent a mechanism to activate mTORC1 [16].

2.3. PI3K/Akt axis

The phosphatidylinositol 3-kinase/protein kinase-B / mammalian target of rapamycin (PI3K/Akt) signalling axis regulates cell proliferation, differentiation, cellular metabolism and cytoskeletal reorganization. Hyperactivation of this signalling cascade has a crucial role in promoting tumour development as well as resistance to antitumoral therapies [77].

2.3.1. PI3K protein

PI3K is a family of lipid kinases that phosphorylate the 3- hydroxyl group of the inositol ring of phosphatidylinositol (PtdIns) lipids in the plasma membrane. PI3K is divided into three classes according to their different structures and lipid substrate preferences [78]. The kinase proteins are heterodimers which consist of a regulatory subunit p85 and a catalytic subunit p110. The best studied is class I PI3K because it plays an important function in cancer. There are four p110 catalytic isoforms produced from different genes, and seven p85 regulatory subunits made by a combination of alternative start codons and different genes in mammals. The p85 regulatory subunit stabilizes and protects the p110 subunit from degradation and also inhibits its catalytic activity under basal conditions, thereby negatively regulating signal generation [78].

PI3K is stimulated by extracellular signals, including growth factors such as epidermal growth factors (EGF), heparin binding EGF-like growth factor (HB-EGF), platelet-derived growth factor (PDGF), or insulin-like growth factor [79], [80]. These factors bind to their corresponding receptor tyrosine kinases, leading to the autophosphorylation of tyrosine residues on the cytoplasmic regions of the RTKs and in linker molecules. PI3K

is recruited and translocated to the plasma membrane where is activated. Besides to RTKs, G protein coupled receptors such as activated Ras can stimulate PI3K through its catalytic subunit as well as by signal adapters proteins, such as IRS-1 and IRS-2. Then functional PI3K phosphorylates phosphatidylinositol 4,5- bisphosphate [PtdIns(4,5)P2] to form phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P3] [81].

The PtdIns (3,4,5)P3 ,generated by PI3K, activate 3-phosphoinositide-dependent protein kinase-1 (PDK-1), which initiates Akt phosphorylation at Thr308 [82]. This Akt modification is sufficient to activate mTORC1 by the direct phosphorylation and activation of PRAS40 and TSC2. Additionally, mTORC2 phosphorylates Akt at Ser473, resulting in optimal activation [83]. Complete Akt activation leads to substrate-specific phosphorylation in both cytoplasm and nucleus. In mammals there are three isoforms of Akt (Akt1, Akt2 and Akt3) encoded by three genes locate on different chromosomes, but with more than 80% structural homology. While Akt1 and Akt2 are ubiquitously expressed, Akt3 is found mainly in brain, kidney and heart. In addition to Thr308 and Ser473 phosphorylation, other residues have been identified using mass spectrometry or site-specific approaches as potential sites for phosphorylation of Akt. The regulation, stoichiometry, and functions of these phosphorylation sites are only beginning to be elucidated [84]. For example, co-translational phosphorylation at Thr450 is required for proper folding and stability of Akt [85]. Moreover, phosphorylation at S129 by CK2 may be required for maximal activation of Akt [86].

Akt plays a key role in promoting cell growth, inhibiting TSC2/TSC1 complex and inducing mTORC1 activation by RHEB [87]. Furthermore, Akt activation delivers anti-apoptotic signals by inhibiting the pro-apoptotic Bcl-2 family members Bax and Bad, as well as phosphorylating members of FOXO family that are required for the expression of proteins that promotes apoptosis such as Fas-ligand [88], [89]. Akt increases cell survival phosphorylating apoptosis signalling kinase 1 (ASK-1), or kappa B kinase-α (IKKα), which in turns phosphorylates and promotes the degradation of the inhibitory cofactor of NF-κB, I-κB, allowing NF-κB translocates to the nucleus where it stimulates transcription of pro-survival genes [90]. In addition, Akt stimulation induces Murine double minute 2 (MDM2) phosphorylation which antagonize p53-mediated apoptosis [91]. Regarding to cell proliferation, Akt phosphorylates members of Cip/Kip family of cyclin-dependent kinase (CDK) inhibitors, promoting cell cycle progession [92]. Moreover, Akt phosphorylates and inactivates glycogen synthase kinase (GSK) 3β. It plays a key role in the regulation of numerous signalling pathways including cellular process such as cell cycle, inflammation and cell proliferation. GSK3β phosphorylates cyclin D1 and Myc which promotes their degradation and facilitates the G1/S

progression of the cell cycle. Likewise, GSK3β inhibits tumour migration and invasion regulating the expression of several epithelial mesenchymal transition repressors such as Snail 1, slug, or β-catenin [93].

2.3.2. Regulation of PI3K/Akt pathway

Activatin of the PI3K pathway is regulated by different manners. The first is the activation of the p85 regulatory subunit, which maintains p110 in an inactive state. The second is the constitutively active negative repressor, phosphatase and tensin homolog (PTEN). PTEN is a lipid phosphatase that dephosphrylates PtdIns(3,4,5)P3 to PtdIns(4,5)P2, and thus reverses PI3K functionality in signal propagation. PTEN is composed of an N-terminal specific phosphatase-like enzyme domain and a C-terminal regulatory domain, which binds to phospholipid membranes. The C-terminal region is important in the regulation of the stability and half-life of the molecule. Also, the C-terminal PTEN amino acid sequence possesses a putative PDZ binding motif, which has been proposed to modulate PTEN functions by association to PDZ domain-containing proteins [94]. Phosphorylation of several serine/threonine residues in this C-terminal fragment of PTEN is important for the regulation of its stability [95]. Despite CK2 and GSK3 are the dominant kinases responsible for PTEN C-terminal phosphorylation in several cell types [96], other kinases are able to phosphorylate PTEN efficiently *in vitro* and may contribute to the regulation of PTEN functions.

PI3K signalling is also attenuated by inositol polyphosphate-phosphatase type II (INPP4B) that converts PtdIns(3,4)P2 to PtdIns(3)P. Other critical mechanism to control PI3K activation is a downstream target of mTORC, S6K1 which is able to phosphorylate and inhibit IRS-1, inducing a feedback inhibition of PI3K [87].

Akt is inactivated through the action of protein phosphatases. The residue Thr308 is dephoshorylated by protein phosphatase 2A (PP2A) [97], whereas the Ser473 residue is targeted by pleckstrin homology domain leucine-rich repeat protein phosphatase (PHLPP) family of isozymes [98]. There are different isoforms of PHLPP which are involved in the dephosphorylation of specific Akt. The protein phosphatase that target Akt Ser129 remain unknown so far.

2.3.3. The PI3K/AKT pathway in ccRCC

In ccRCC genetic components of PI3K-Akt axis are frequently altered including guanine nucleotide binding protein (GNB2L1) amplification (6%), PIK3CA amplification or mutations (5%), PIK3R1 down-regulation specially in advanced and metastatic RCC, PTEN deletions or mutations (5%), as well as the recurrent mutations in RHEB,

consistent with the activation of the PI3K/Akt pathway. Although the overall mutation rate of PI3K/AKT pathway in ccRCC is low compared with other cancer types, the activation of Akt is high as indicated by high phosphorylation levels of Akt and Akt substrates. This effect can be elucidated by the cross talk between VHL/HIF and PIK3/Akt/mTOR pathway. HIF upregulation due to VHL loss promotes expression of several growth facotrs, including EGF, PDGF and VEGF, which can activate PI3K/Akt axis through RTKs. The subsequent activation of mTORC1 and mTORC2 promotes HIF expression, inducing a positive feed-back loop resulting in constitutive activation of the signaling pathway. Moreover, deregulation of cytokines such as Interleukin-6, or growth factors like transforming growth factor α (TGF-α), have been suggested to function as autocrine factors in renal cell carcinoma and is a possible mechanism to induce Atk activation [77].

2.4. Ras / Raf / MAPK pathway

The Ras/Raf/MAPK pathway has a crucial role in intracellular signal transduction, regulating multiples processes such as changes in patterns of gene expression, mitosis, cell survive, proliferation and apoptosis.

Ras is a small GTP-binding proteins which is the upstream molecule of Raf/MEK/ERK and PI3K/Akt pathways. There are four different Ras proteins that show diverse abilities to activate Raf/MEK/ERK and PI3K/Akt cascades. Post-translational modifications of Ras are required to target it to the cell membrane. These modifications are sites for therapeutic intervention. Several factors such as cytokines or growth factors bind to their appropriate receptors and activate Shc/Grb2/SOS. Upon stimulation by Shc/Grb2/SOS, inactive Ras exchanges GDP for GTP and undergoes a conformational change and becomes active. The Ras active bound to GTP and can recruit Raf to cell membrane [99], [100].

Raf kinases are serine/threonine protein kinases that function in this pathway as downstream effector molecules of Ras. The Raf kinase family is composed of three members: A-Raf, B-Raf and Raf-1 (C-Raf), which share three domains, CR1 is the Ras-binding domain, CR2 is the regulatory domain, and CR3 is the kinase domain. The activation of Raf kinases comprise a series events including: (i) recruitment to the plasma membrane mediated by an interaction with Ras; (ii) dimerization of Ras proteins; (iii) phosphorylation/dephosphorylation on different domains; (iv) diassociation from the Raf kinase inhibitory protein (RKIP) and (v) association with scaffolding complexes (kinase suppressor of Ras, KSR). Raf kinase activity is controlled by multiple pathways which utilize phosphorylation as a key mechanism of regulation.

Ras, Src and PKC are activators of Raf [101], [102], whereas Akt and PKA phosphorylates the CR2 regulatory domain and inhibit its activity [103], [104]. On the other hand, PP2A, and others unknown phosphatases, dephosphorylated the activators sites which allows Raf to be phosphorylated [105].

Three Raf family members are able to phosphorylate and activate Mitogen-activated protein kinase ERK kinase (MEK). This protein is a tyrosine and S/T – dual specificity protein kinase, itspredominate down-stream target is Extracelular-signal-regulated kinase 1, 2 (ERK).

ERK1/ERK2 are a multifunctional serine/threonine kinase S/T kinases that belongs to the mitogen-activated protein kinases family (MAPKs).The MAPK ca be classified into three groups, ERKs (Extracelular-signal-regulated kinase), JNKs (Jun amino-terminal kinases), and p38/SAPKs (stress-activated protein kinases). This family can be activated by a wide variety of different stimuli, but in general, ERK1 and ERK2 are preferentially activated in response to growth factors and phorbol esters, whereas the JNK and p38 kinases are more responsive to stree stimuli including osmotic shock, ionizing radiation or cytokine stimulation. The activity of ERK1/2 is positively regulated by phosphorylation mediated by MEK1 and MEK2. ERK1/2 phosphorylate a broad array of substrates localized in all cellular compartments which determine their effects on phosphorylation and nuclear transolaction of a key kinases (Cdk2), cyclins (cyclin E), transcription factors (Elk, c-Myc and CREB). ERK1/2 activation is involved in cell proliferation and play a role in translational control and cell growth, through phosphorylation of RSK1 as well as phosphorylation of TSC2, disrupting TSC1/TSC2 complex formation and activating mTOR signalling. Other mechanism by which ERK1/2 my impact on global protein synthesis is through direct regulation of ribosomal gene transcription and in pyrimidine biosynthesis pathway [99], [100].

2.4.1. Role of Ras / Raf / MAPK pathway in ccRCC

Several studies have demonstrated a shift in the c-raf-1 locus in RCCs as well as Ras gene mutations. In addition, constitutive activation of MAPKs occurs in a majority of RCCs, and this activation show a significant correlation with the histological grade of RCC [106]. Moreover there are other mechanisms by which this cascade is involve in carcinogenesis of RCCs. For example some studies have revealed that Raf kinase trapping to Golgi (RKTG) acts as a spatial regulator of Raf kinase by sequestering Raf to the Golgi apparatus, thereby antagonizing the mitogen-activated protein kinase (Raf/MEK/MAPK signalling pathway). Protein and mRNA levels of RKTG are downregulated in clinical ccRCC tumor samples and are inversely correlated with an

elevated level of VEGF. Enhanced Raf/MEK/ERK signalling cascade, owing to the downregulation of RKTG, may potentiate the autocrine VEGF signalling and synergize with loss of VHL to increase HIF-1α protein level, thereby promoting the highly vascularized features of ccRCC [107]. Morevoer, exist cross-talk between Raf/MEK/MAPK signalling cascade and other signalling pathways such as mTOR and PI3K/Akt axis. These connections between Raf/MEK/MAPK-Mtor-PI3K/Akt pathways may be a crucial mechanism of resistance to therapeutic agents. Therefore, inhibition mTORC1 by rapamycin or analogues, increases IRS-1 and subsequently PI3K activity toward Ras and MAPK, promoting both Akt activation and MAPK phosphorylation. For all these reasons new strategies for combined inhibition of PI3K/mTOR and MAPK are being developed [108].

2.5. JAK/STAT axis

Cytokines mediated their responses through activation of the JAK/STAT signalling pathway. STAT (signal transducers and activators of transcription) family comprise seven members (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6). JAKs (janus kinases) represent a family of four non-receptor tyrosine kinases, Jak1, Jak2, Jak3 and Tyk2. These kinases selectively phosphorylate tyrosine residue of STATs at position 705. STATs transduce signals for large numerous of molecules including IL-6, EGF, S1P, inflammatory OSM, Src family members and growth factor receptors that possess intrinsic tyrosine-kinase activity such EFGRs, HGF receptor (c-Met) and PDGFR. Several members of the G-protein-coupled receptors have been also shown to signal through STATs.

Signalling through the JAK/STAT pathway is initiated when a cytokine binds to its corresponding receptor, inducing the activation of protein JAK family kinases which mediate phosphorylation at the specific receptor tyrosine residues. Activated STATs forms dimers, translocates into the nucleus , and binds to gamma-activated sites (GAP), a family of STAT-specific DNA-response elements [109].

2.5.1. Role of STAT singalling in ccRCC

STATs have been detected in a wide variety of human cancer cell lines and primary tumours. Many studies have suggested that STAT proteins could participate in tumorigenesis through up-regulation of genes encoding apoptosis inhibitors (Mcl-1, Bcl-xL), cell-cycle regulators (cyclin D1/D2, c-Myc), and inducers of angiogenesis (VEGF) [110].

STAT3 plays an important role in tumour growth, invasion and angiogenesis in RCC. Activated STAT have been observed in RCC tumour tissues and phosphorylated form have been enhanced in ccRCC, papillary RCC and urothelial carcinoma [111]. Increase of pSTAT3 levels and nuclear active form of STAT3 have been found in human renal carcinoma cells, under hypoxia conditions. Other reports have been demonstrated that STAT3 is a novel and essential component of HIF-1 regulatory machinery. Under hypoxic conditions pSTAT3 directly binds HIF-1α and up-regulates HIF-1α stability through delaying protein degradation and accelerating protein synthesis in human renal carcinoma cell [112]. Phosphorylated STAT3 in response to IL-6, whose protein levels are increased in RCC, is associated with overexpressed HIF-1α under normoxic conditions. Moreover STAT3 activity up-regulates VEGF expression in renal carcinoma cells [113].

Based on these finding, blocking STAT3 is expected to inhibit VEGF expression that is induced in ccRCC by loss of VHL and subsequently accumulation of HIF. Therefore, STAT3 inhibition may be a target for clinical therapy by reducing HIF levels and VEGF expression.

2.6. Epidermal growht factor receptor signalling

The ErbB family of receptors tyrosine kinases comprises four members: the EGFR (also known as ErbB1/HER1), ErbB2 (neu, HER2), ErbB3 (HER3) and ErbB4 (HER4). All receptors are anchored in the cytoplasmic membrane and share similar structure that is composed of an extracellular domain that is divided into four parts: domain I and III that participate in ligand binding, and domains II and IV, which participate in disulfide bond formation. In addition domain II participates in homo and heterodimer formation. The extracellular domain is followed by a single transmembrane segment of 19-25 amino acid residues and an intracellular portion of about 550 amino acid residues that contains a juxtamembrane segment, a protein kinase domain and a carboxyterminal tail. The intracellular tyrosine kinase domain of ErbB receptors is highly conserved, although ErbB3 have substitutions of critical amino acids and therefore lacks kinase activity. Contrary, the extracellular domains are less conserved, suggesting that they show different specificity in ligand bind. In fact EGF-related growth factors can be divided into three groups. The first group bind specifically to the EGFR and includes epidermal growth factor (EFG), transforming growth factor α (TGF-α) and amphiregulin (AR). The second group includes betacellulin (BTC), heparin-binding growth factor (HB-EGF) and epiregulin (EPR), which show dual specificity by binding both EGFR and ErbB4. The third group is divided in two subgroups neuregulin 1 and 2 which bind

ErbB3 and ErbB4 and neuregulin 3 and 4 that bind only ErbB4. It should be noted that neither growth factors nor soluble ligands bind to ErbB2, however it readily forms heterodimers. On the other hand, ErbB3 has an inactive kinase but can be activated through heterodimerization by the other members of the family for signalling.The epidermal growh factors family of ligand and members of ErbB are shown in Figure 2. (reviewed in [114]).

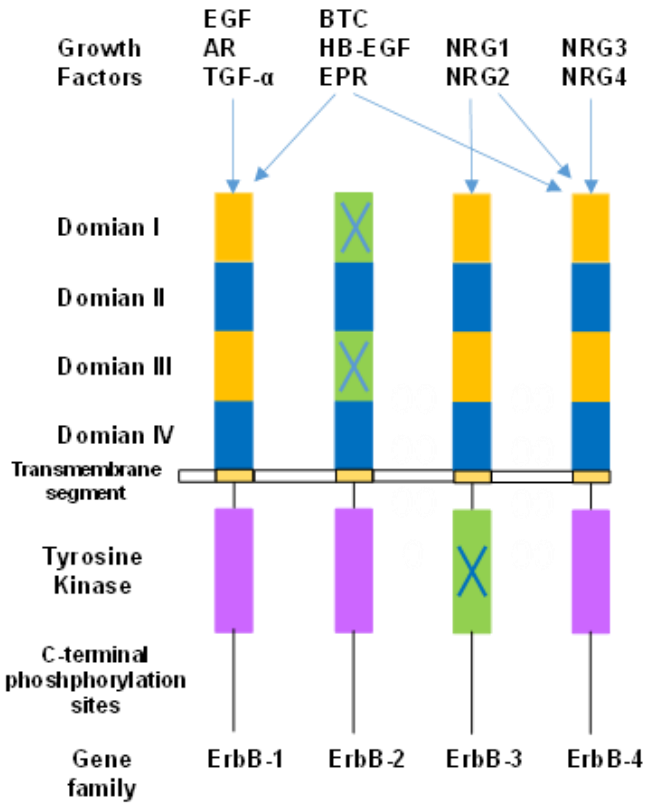


Figure 2. ErbB family receptors and their ligands. The inactive ligand-binding of ErbB2 and the inactive kinase domain of ErbB3 are denoted with a X.

Binding of ligands, produced by the same cell that expressed the recept, or by sorroundig cells, to the extracellular domain induces the formation of receptor homo- or hetero- dimers, and subsequent activation of intrinsic tyrosine kinase domain, which serve as docking sites for signalling molecules that contain SH2 (Src homology region 2) and PTB (phosphotyrine-binding) domains. Heterodimerization of two different memeber of the ErbB family increases the diversity of ligands recognized by individual receptors. ErbB2 has enchanced capacity of heterodimerization and it is the favorite dimerization partner for the other activated ErbB receptors. Moreover the transforming potential and signaling pathway activated by different dimers are distinctive. Thus, ErbB-/ErbB2 heterodimers are associated with a more robust signal than ErbB1/ErbB1

homodimers and ErbB2/ErbB3 heterodimers represented the most potent signalling module in terms of cell proliferation and in vitro tansformation [114].

All four memebers of the ErbB family have the potential to stimulate the Ras/Ras/MAPK cascade, but only ErbB3 and ErbB4 have specific phosphotyrosines that binds to the regulatory subunit of PI3K, leading to Akt activation. Furthermore, ligand binding also induces receptor clustering and endocytosis in clathrin-coated pits on the cell surface, followed by either recycling back to the cell surface or moving through a series of endosomes to lysosome for degradation. After endocytosis, nuclear translocation of the receptors may also occur and may regulate gene expression [114], [115].

2.6.1. Role of ErbB4 in ccRCC

ErBb4 is a 180 kDa transmembrane glycoprotein that serves as a receptor for the neuregulin family of growth factors as well as for HB-EGF, BTC, and ER, which also activate ErbB1 receptor. ErbB4 and its ligands have a crucial role in normal cardiovascular and neural development, differentiation of the mammary gland and in pathological condition such as heart diaseases and cancer.

2.6.1.1. ErbB4 isoforms

ErbB4 is expressed as alternatively four spliced isoforms that differ either in their extracellular juxtamembrane domains (JM) or in their C-terminal cytoplasmatic tails (CYT) (**Figure 3**). The JM domain of type a (JM-a) includes 23 amino acids that confer a proteinase cleavage site that is missing from the alternative 13 amino acids in the JM domain of type b. Two additional JM splice variants (JM-c and JM-d) have identified with isoforms lacking or including, respectively, both JM-a and JM-b sequences. In response to binding of ligands or PKC activation, both JM-a and JM-d isoforms can be cleaved by metalloproteae ADAM17 (TACE), releasing a 120-kDa ectodomain fragment into the extracellular environment and generating a fragment of 80-kDa which remains associated to the membrane and can be degraded by proteasome activity after polyubiquitilination or can be further cleaved by γ-secretase. This cleavage releases the intracellular domain (ICD) from the membrane and allows nuclear translocation. The biological significance of the generated soluble ICD is currently not fully understood. It is know that ErbB4 ICD stimulate cell death [116], [117] and increase p53 levels through ubiquitylation of Hdm2. However, ectopic expression of ICD stimulates mammary epithelial cell differentiation [118]. In addition, ICD generation is associated with cell proliferation and survival. In vivo, ErbB4 ICD epitope is frequently observed in the nuclei of breast cancer cells, and it is associated with poor

survival when compared to the presence of ErbB4 at the cell surface [119]. Interestingly, interaction on ErbB4 with the WW domain-containing oxidoreductase (WWOX), a tumour suppressor, prevents nuclear localization of ErbB4 ICD [120]. Cytoplasmic isoforms differ by containing (CYT-1) or not containing (CYT-2) a sequence of 16 amino acids within their cytoplasmic tails. These 16 amino acids serve as a binding site for the PI3K SHK-2 domain (YTPM) and putatively for WW-domain containing signalling molecules (**Figure 3**). Some studies have reported differential coupling and activation of PI3K signalling between these CYT-1 and CYT-2 alternative isoforms [121].

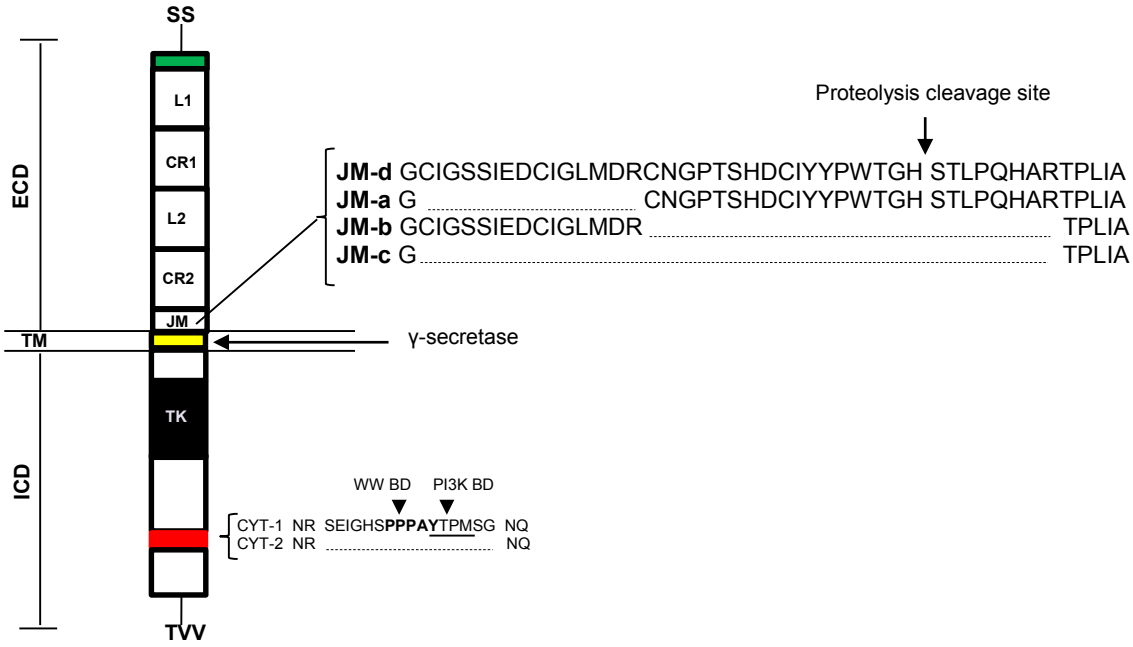


Figure 3. ErbB4 structure isoforms. All the isoforms contain extracellular ligand binding domain (ECD), juxtamembrane (JM), transmembrane (TM) and intracellular domain (ICD). ECD contains a signal sequence (SS), two ligand-interacting domains (L1, L2) and two cysteine-rich domains (CR1 and CR2). Different JM are shown, with (JM-d, JM-a) or without (JM-b, JM-c) a photolytic cleavage site for TACE. ICD contains a tyrosine kinase domain (TK), a regulatory domain with autophosphorylation sites and sequences related to the CYT-1 and CYT-2 isoforms. Dashes represent deletions.

Moreover other signalling differences between ErbB4 CYT1 and ErbB4 CYT2 have been detected. Hence, ICD2 is targeted more efficiently than ICD1 into the nucleus due to the enhanced tyrosine kinase activity in comparison with the impaired activity of ICD1. This differential targeting to the nucleus is associated with a functional difference. Moreover, the 80 KDa fragment cleaved from ErbB4 CYT 2 has been suggested to be metabolically more stable because is less ubiquitilynated than CYT1,

suggesting that the 16 amino acids present in the CYT1 isoform are involve in ErbB4 ubiquitylation that may regulate degradation. Both, greater stability of the membrane-anchored 80 kDa fragment if CYT2, as well as the more efficient nuclear translocation of ICD2, suggest that JM-a CYT2 could be more active in signalling into the nucleus [122]. Consistent with these findings, JM-a CYT2 is overexpressed in a subset of breast cancer cases and it is associated with more oncogenic potential [123]. However, the significance of ErbB4 as an oncogenic factor has remained controversial, there are observations that support oncogenicity, whereas others support anticonogenic or differentiation-inducing role. This controversy could be attributed to the existence of functionally dissimilar and differentially regulated isoform.

2.6.1.2. Expression of ErbB4 isoforms is tissue-specific

Distribution of the ErbB4 isoforms is tissue specific. For exemple, ErbB4 JM-b is expressed mainly in the heart, and ErbB4 JM-a principally in kidney, but both ,JM-a and JM-B, are expressed in various neural tissues. On the other hand, heart expresses CYT-1 isoform, whereas neural tissues and kidney predominantly ErbB4 CYT-2 [124]. The specificity of ErbB4 expression isoforms suggest that might exist an isoforms specific function in the cell. ErbB4 has been described to play an important role in cardiac and neural development. ErbB4 knockout mice die at midembryogenesis before the inception of nephrogenesis, due to impaired cardiac muscle differentiation and defects in pathfinding by cranial neural crest cells and in the migration of cerebellar granule cells along radial glial fibres. Moreover ErbB4 receptor and their ligands, HB-EGF and HRG-β1, are also involved in the control of proliferation and polarization of kidney epithelial cells in the nephrons and collecting ducts during kidney development [125].

2.6.2. ErbB4 ligand: Heparin-binding epidermal-growth-factor like growth factor (HB-EGF).

HB-EGF is 22 kDa O-glycosylated protein, which belongs to the EGF receptor family that activates the EGFR as well as ErbB4, and binds heparan-sulfate proteoglicans (HSPGs) present on the cell surface and in the extracellular matrix. It has been shown that HB-EGF plays a role in cancer, wound healing, cardiac hypertrophy and heart development and function.

2.6.2.1. HB-EGF structure

HB-EGF is synthesized as a transmembrane precursor (pro HB-EGF). This precursor is composed by several domains including a signal peptide, propeptide, mature HB-EG, juxtamembrane, transmembrane and cytoplasmic domains (**Figure 4**).

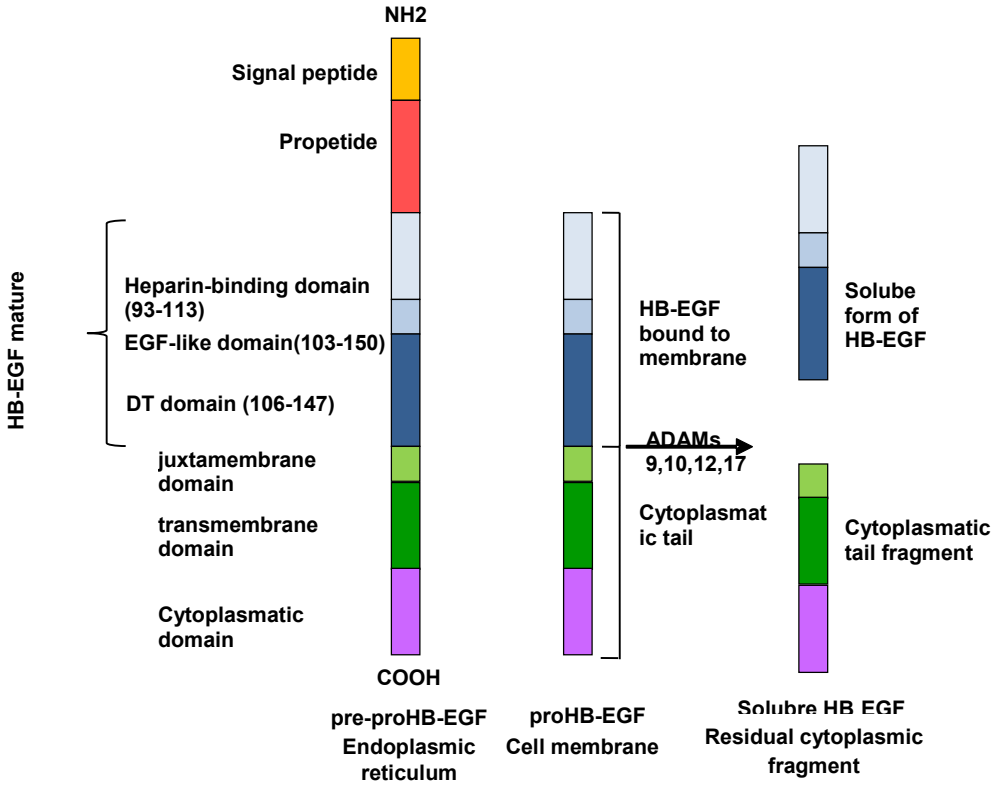


Figure 4. HB-EGF protein structure. The structure of the primary translation product of HB-EGF in the endoplasmic reticulum includes signal peptide, propeptide, mature HB-EGF (that contains the EGF-like domain, heparin-binding domain and the DT-binding domain), juxtamembrane, transmembrane and cytoplasmic domains. The propeptide domain is cleaved rapidly producing pro-HB-EGF which is cleaved in the cell surface by metalloproteases belonging to the ADAM family, releasing the soluble form of HB-EGF and cytoplasmic fragment.

There is also an EGF- like domain, heparin binding domain and DT-binding domain within the mature HB-EGF. The propeptide domain is cleaved rapidly after synthesis of the precursor. Protein ectodomain shedding of pro-HB-EGF by metalloproteases releases a soluble form of HB-EGF (**Figure 4**). Pro-HB-EGF is biologically active in its membrane-anchored form, however, only acts as a juxtacrine mode causing cell growth inhibition and in some cases apoptosis [126]. After proteolytic cleavage, HB-

EFG can diffuse and activate receptors in autocrine or paracrine mode, leading to cell proliferation and migration. Additionally, the cytoplasmatic remnant of proHB-EGF produced after ectodomain shedding, plays a role in growth factor receptor signalling. Therefore, ectodomain shedding is a critical step in regulating the biological action of HB-EGF [127].

The expression of the HB-EGF gene is tightly regulated and a number of regulatory factors have been described. For example, HB-EGF mRNA levels are increased in vascular smooth muscle cells (SMCc) by factors associated with vessel injury and atherosclerosis including TNF-α, lyso-phosphatidylcholine (lyso-PC), thrombin and angiotensin II (AII) [128]. Other regulatory factors that control HB-EGF levels are α-adrenergic agonist and electrical stimuli in cardiac myocits, oxidative stress in gastric epithelial cells or mercury chloride and HB-EGF itself in kidney tubular epithelium.

2.6.2.2. HB-EGF biology

The ability of HB-EGF to produce effects on a variety of cell types and its expression in a large number of tissues suggests that a wide variety of potentials roles may exist for HB-EGF. This growth factor plays an important role in many normal development processes such as SMC growth [129], skeletal muscle myogenesis, gastrointestinal tract mucosa maintenance, embryo implantation, wound healing and injury repair. But it may also be relevant to the progression of many disease states such as atherosclerosis, diabetes and carcinogenesis [130], [131].

HB-EGF expression is altered in many tumours, including hepatocarcinoma, colon, melanoma, breast, prostate and bladder tumours. However, how the membrane bound fragment (HB-EGF-C) or the soluble form of HB-EGF contributes to tumorigenic processes is unknown. It is well established that HB-EGF can promote proliferation of a wide range of cells, through its binding to EGFR leading to downstream signalling that converges on the Ras/Raf/MAPK and PI3K/Akt activation which promote survival and proliferation [132]. Moreover the C-terminal fragment of HB-EGF that remains after ectodomain cleavage, has been shown to inhibit the transcription-repressing capabilities of PLZF and Bcl16, resulting in enhanced expression of cyclin A and cyclin D2 with increased cell cycle progression [133]. Besides, HB-EGF, is a potent inducer of several oncogenic behaviours including growth and migration as well as xenograft growth and angiogenesis in vivo inducing the expression of VEGF [134]. Likewise, it has been reported that HB-EGF released by TACE, stimulates EGFR in an autocrine manner, which in turn activates invasion activity via MMP-9 upregulation [129]. In addition, HB-EGF-C nuclear translocation plays a critical role in gastric cancer invasion

[135]. HB-EGF is also found to participate in the epithelial-mesenchymal transition (EMT) in some types of cancer cell such as gastric, ovarian or thyroid cells, reducing E-cadherin levels and upregulating expression of snail1, a key regulator of EMT [136], [137]. HB-EGF is known to participate in additional processes linked to development and progression of cancer such as inflammation through NFκB pathway [138].

2.6.3. Role of ErbB1, ErbB4 and HB-EGF in ccRCC

The role of ErbB1 receptors and some of their ligands in the pathogenesis of human carcinomas is confirmed by a number of studies that have shown overexpression of these proteins in the majority of solid neoplasms.

ErbB1 (EGFR) plays a crucial role in the control of both normal and malignant cell growth. Several studies have reported increased EGFR expression in ccRCCb but it is no not due by amplification or mutation of the gene. A recent study proposes that an increased copy number of chromosome 7 could be the possible reason for EGFR overexpression [139]. However, the prognostic significance of EGFR overexpression is controversial. Also, multiple reports have documented the existence of an overexpression of EGFR ligands such as EGF and TGF-α. Both induce VEGF expression via activation of EGFR [140]. The angiogenic phenotype of ccRCC is regulated also by up-regulated EGFR-driven signalling pathways. Moreover, it has been described that loss of VHL in ccRCC induces HIF accumulation and it the expression of pro-angiogenic factors such as VEGF. In addition, hypoxia also enhances the expression and the activity of HB-EGF-related molecules, such as ADAM17 [141]. ErbB4 activation induces different effects in different types of cancer due to the expression of functionally distinct isoforms. ErbB4 deletion in cpk mice accelerates polycystic kidney disease progression [142]. In ccRCC, it has been demonstrated that ErbB4 expression is downregulated, suggesting that this receptor might function as a tumour suppressor in RCC [143], [144].

According to all these results EGFR pathway provide attractive molecular targets for therapy. A variety of anti EGFR drugs are currently tested in clinical trials, including small inhibitors molecules such as gefitinib and erlotinib, as well as antibodies such as ABX-EGF or bevacizumab. However, clinical results with these antibodies and inhibitors have failed to demonstrate activity in patients with advanced renal carcinoma. Despite poor results with single-agent treatment, preclinical data suggest that treatment with an EGF receptor tyrosine kinase inhibitor in the presence of VEGF blockade may result in a different outcome. Furthermore, HB-EGF have identified as a key mediator of treatment resistance in several tumours [145]. Exposure of cancers cells to either

conventional chemotherapy or treatment with small molecule inhibitors, upregulates HB-EGF expression, releases and activates EGFR, thereby enhancing survival signalling. For this reason HB-EGF inhibition is using as a therapy in many tumors types [146], [147].

3. PROTEIN KINASE CK2

3.1. General features of CK2

One of the mechanism for the regulation of a wide array of fundamental cellular processes is the reversible phosphorylation of proteins. Given this importance, the human genome encodes several hundred different protein kinases and on-third of all cellular proteins appear to be phosphorylated, many of them at several distinct phosphorylation sites [148]. There are protein kinases, such as wee1 or MEK, which are specific and are present in cells to phosphorylate only a limited number of distinct proteins targets. However, many other protein kinases exhibit a much broader specificity and are able to phosphorylate hundreds of different proteins within cell. Protein Kinase CK2 belongs to a small family of protein kinases and phosphorylates more than 300 physiological targets. As the number of targets phosphorylated by CK2 continues to grow, it becomes evident that CK2 has a decisive role in the regulation of a diverse cellular processes, including differentiation, proliferation and survival [149]. CK2 is a serine/ threonine kinase distributed ubiquitously in eukaryotic organism, where it most often appears to exist in tetrameric complexes consisting of two catalytic subunits (CK2α and CK2α') and two regulatory subunits (CK2β). However, during the last years, significant evidence has emerged to challenge the view of CK2 to another idea more flexible and complex [150].

CK2 is highly conserved in evolution, showing its decisive role in cellular life. Although CK2 has been traditionally described as a stable tetramer, several reports indicate that CK2 subunits have functions independently of CK2 tetramer. In humans, two catalytic isoforms CK2α and CK2α' have been well characterized, while a third isoform designated CK2α'' have been identified [151].On the other hand, only one regulatory subunit, CK2β, has been identified. Contrary, multiple forms of CK2β have been identified in other organisms such as *Saccharomyces cerevisiae*, or in plants where the genome contain multiple genes for each subunit [152]. In mammalian, the combination of different catalytic subunits with CK2β regulatory subunit gives different active holoenzyme, thereby CK2 complexes can contain identical (CK2α/α) or (CK2α'/α') or non-identical (CK2α/α') catalytic subunits [150]. Although the catalytic subunits CK2α and CK2α' (42 and 38 kDa) exhibit approximately 90% of identity, providing an explanation for the fact that they display similar enzyme properties in vitro, CK2α is encoded by the gene CSNK2A1 located on chromosome 20, and CK2α' is encoded by the gene CSNK2A2 located on chromosome 13 [153], [154]. On the other hand, the C-

terminal domains of CK2α and CK2α' are completely different. Very little is known about CK2α''. On the basis of its amino acid sequence CK2α'' is most closely related to CK2α and probably is the results of alternative splicing. However, many studies have not made a distinction between the different isoenzymes of CK2. It has been known that CK2α and CK2α' show differences in distribution, CK2α' is mainly expressed in brain and testicles. Also the affinity between CK2α' and CK2β is significantly lower than that between CK2α and CK2β and both holoenzymes have also different aggregation [150]. CK2α' misses the phosphorylation sites in C-terminal domain that contains CK2α and which are phosphorylated in a cell cycle-dependent manner, suggesting that CK2α and CK2α' are differentially regulated during the cell cycle [155]. In mammalian cells several proteins including PP2A, CKP-1 or Pin-1 that are isoform-specific interactors partner for CK2α [156], [157]. Recently it has been reported that CK2α′ exhibits a striking preference for caspase-3 phosphorylation in cells as compared to CK2α [158]. All these evidences give further support for the existence of functional differences in CK2α and CK2α'.

CK2 has traditionally been classified as a protein serine / threonine kinase that phosphorylates its substrates by using either ATP or GTP as a phosphate donor. It has been described a minimal consensus sequence for phosphorylation by CK2 (i.e. Ser-Xaa-Xaa-Acidic, where the acidic residue may be Glu, Asp, pSer or pTyr). However there are limitations to the use this consensus sequence for the identification of targets. Furthermore, it has become apparent that CK2 may be a dual-specificity kinase phosphorylating Tyr residues in yeast. The ability of CK2 to phosphorylate tyrosine residues in vitro was confirmed with recombinant human CK2. However, the kinetic parameters for tyrosine phosphorylation are much less favourable than those for serine or threonine.

CK2 has not required to be phosphorylated for activation, it is consider as constitutively active with or without CK2β [159]. No consistent, or general mechanisms responsible for the regulation of CK2 in cells so far. In fact it seems that a number of distinct mechanisms contribute to the physiological regulation of CK2. These include regulation by covalent modification, regulated expression and assembly, phosphorylation or interaction with other proteins and subcellular localization.

3.1.1. Genetic manipulation of CK2

Different approaches overexpressing or downregulating the subunits of CK2 have been performed to obtain information about the function of each subunit of CK2 in different mammalian cell lines or yeast. Thus, in the yeast *S.cerevisiae*, disruption of one of the

catalytic subunits (CKA1 or CKA2) results in cell death, while disruption of both CKA1 and CKA2 is synthetic lethal, suggesting that two isoforms of CK2 can compensate for each other in the context of viability [160]. Knockout of the gene encoding CK2α' in mice results in viable offspring, but it is sterile and displays a defect in spermatogenesis [161]. This result demonstrate that CK2α has the capacity to compensate for CK2α' in the context of viability however, the ability of CK2α is not absolute. Although knockout of CK2α have not been reported, stable downregulation of CK2α inhibits migration and invasion in hepatocellular carcinoma HepG2 cells [162]. On the other hand, knocking out the regulatory beta subunit of protein kinase CK2 in mice leads to early embryonic lethality, suggesting the important role of CK2β in the viability [163]. Nevertheless recent studies have proposed that depletion of regulatory subunit in tumoral cell lines does not affect the viability [164] .

3.2. Molecular structure of CK2

The analysis of amino acids sequences as well crystallography studies have allowed to elucidate the biochemical features of CK2, such as its constitutive activity, its heterotetrameric quaternary structures, and interactions between subunits and its substrates. 40 crystal structures corresponding to CK2, including both subunits and tetramer form, have been published in the last decade. The main models of CK2β structure are hsCK2β$^{1-182}$ (high resolution but It lacks to C-terminal domain) and hsCK2β$^{1-193}$(less resolution with C-terminal tail). These structures provide information about the structure of regulatory subunit. Crystal structures have been published for recombinant maize CK2α. For the CK2 holoenzyme a model derived from the crystal structure of maize CK2α and a short peptide of human CKβ was first established and more recently, the crystal structure of a complete CK2 tetramer, has been described using recombinant human CK2 [165] (**Figure 5**).

In the CK2 complex, the regulatory subunit form a stable dimer linking the two catalytic subunits, which make no direct interaction with one another. The catalytic subunits contact with both regulatory chains, predominantly via C-terminal tail of the regulatory subunit. The structure of CK2α is subdivided in a different regions, which are responsible of the main functional characteristics of that subunit, such as its constitutive activity, structure of substrate recognition as well as the basis of dual-cosubstrat specificity. In the structure of CK2β the three regions more important are the zinc-binding motif, the CK2α-binding interface of CK2β and the acidic loop [166].

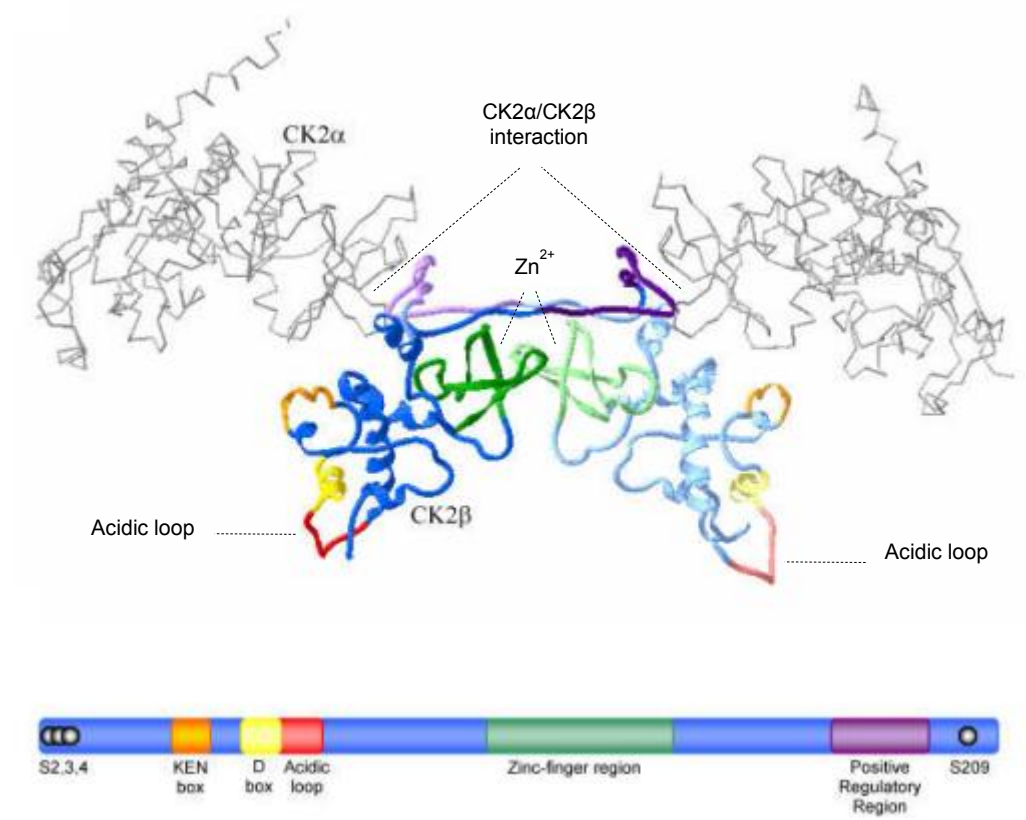


Figure 5. The regulatory CK2 subunit. (A) Crystal structure of the CK2 holoenzyme. The CK2β dimer forms the core of the enzyme and is represented by blue drawings. Important motifs of CK2β are coloured as indicated in the schematic diagram (B). The catalytic subunits are represented as an alpha carbon trace. (B) Schematic representation of the more relevant regions of CK2β. The phosphorylation sites are represented by black spheres, the putative degradation motifs are drawn in orange and yellow, the acidic loop involved in modulation of catalytic subunit activity is represented in red and zinc-finger motif which mediates CK2β dimer formation and CK2α coordination is illustrated in green. Figure adapted from A.Bibby et al,.[166]

3.2.1. Functional regions of CK2β

CK2β presents several important features .For example, the N-terminal domain of CK2β contains a region of autophosphorylation site comprising Ser^2, Ser^3 and possibly Ser^4 [167]. This process was initially classified as an intramolecular process, however, the recent high-resolution structure of tetrameric CK2 show that the N-terminus of CK2β is located far from the active site of either catalytic subunits within the tetrameric CK2 tetramer. It is possible that the mechanism for autophosphorylation may reside in the formation of higher-order CK2 structures by interaction of CK2 tetramers [168]. CK2β exhibits another phosphorylated site at Ser209, which is phosphorylated in a cell cycle-dependent manner in cells and in vitro by $p34^{cdc2}$ [169]. It has been also described that CK2 contains a sequence that very closely resembles the destruction box that plays an important role in the specific degradation of cyclin B at the end of

mitosis [170].This motif is situated on a surface exposed α-helix where is available for recognition by the cellular degradation machinery. Moreover CK2β contains a similar KEN box that is present in other proteins including cyclins, Aurora A and B, and Nek2A. However neither putative destruction motif nor KEN boxes of CK2β have been characterized yet [166].

Although the role of CK2β has been usually considered as restricted to form the tetrameric CK2 complexes, nowadays this view has changed and CK2β is now accepted to have both, CK2 dependent and independent functions of CK2.

3.2.2. CK2β interactor partners

Several proteins have been identified that bind to the tetrameric form of CK2 through binding sites on CK2β, thus CK2β appears to be a substrate docking protein. A couple of proteins including Cdc25B, $p27^{KIP1}$, Nopp140 and $p21^{WAF1/CIP1}$ have been shown to bind to the extreme N-terminal region of CK2β [166]. Topoisomerase II and p53 phosphorylate CK2 prior interaction with CK2β [171], [172]. Other molecules such as FGF-2 binds to CK2β, stimulating CK2 activity against nucleolin *in vitro* [173]. All these results provide alternative mechanism by which CK2β modulates the ability of CK2 to phosphorylate specific cellular targets (**Table 2**).

3.2.3. CK2 independent binding partners of CK2β

There are proteins that bind to CK2β in the absence of CK2α, including A-raf, c-Mos and Chk1 serine/threonine protein kinases [174]–[176].These proteins contain a sequence of amino acids in the extreme N-terminal that resemble to the sequence present in the catalytic subunits CK2α and CK2α'. Accordingly, other proteins, holding these appropriate structural elements, might be identified (**Table 2**).

Taula 2. CK2 interaction proteins Interaction partner	Function	Detected
CK2 dependent binding partners of CK2β		
p90^{Rsk}	S/T protein kinase	in vitro
PKCζ	S/T protein kinase, mediate NF-κB activation	in vitro
Topoisomerase II	DNA remodelling, essential during mitosis and meiosis	in vitro
p53	Tumor supressor gene product	in vitro/in vivo
p27^{KIP1}	CDK inhibitor, cell cycle progression	in vitro
p21$^{WAF1/CIP1}$	CDK inhibitor, cell cycle progression	in vitro/in vivo
Cdc25B	Phophatase, CDK activator, cell cycle progression	in vitro/in vivo
CD5	Cell surface receptor, thymocytes, T-cells, some B1a B-cells	in vitro
FGF-2	Fibroblast growth factor 2, cell proliferation	in vitro
Nopp 140	Nucleolar protein, potential chaperone for nucleolar transport	in vitro
L5	Ribosomal protein	in vitro/in vivo
L41	Ribosomal protein	in vitro
HHV-6 IE2	Human herpesvirus 6 immediate-early 2 protein, gene promoter transactivator	in vitro/in vivo
CK2 independent binding partners of CK2β		
c-Mos	S/T protein kinase, MAPK activation, cell cycle progression	in vitro/in vivo
Chk1	S/T protein kinase, regulator of DNA damage induced G2 arrest, checkpoint control	in vitro/in vivo
A-Raf	S/T protein kinase, mitogenic signalling, cell proliferation	in vitro/in vivo

Table 2. CK2 specific interacting proteins. Adapted of Bibby et al.,[166]

3.2.4. CK2 regulation in cells

The regulation of CK2 is the largest area of discussion. Over the years it has been believed that CK2 has been constitutively active or unregulated, but more recent studies have reported that CK2 respond to diverse stimuli, and that a number of different mechanisms contribute to its regulation including regulation of the expression and subunits assembly, regulation by covalent modification, and regulatory interactions with protein and /or non-protein molecules.

Expression and assemblity

The regulatory subunit controls the catalytic activity and substrate specificity of CK2 as well as the assembly of CK2 subunits. However, there are evidences suggesting that the catalytic subunits of CK2 also exist in a free CK2 form and bind and phosphorylate specific proteins independent of the CK2β subunit. The fact that CK2β contains putative destruction boxes, is ubiquitinated and degradated through a proteosomoal pathway [166], strengthen the notion that CK2β share similarities with cyclines. Thus, it has been reported that CK2 activity and expression oscillates during the cell cycle. However, little is known about the regulation of CK2α and CK2β expression. It has been observed that in several types of cells CK2β is synthesized in a large ecxess of catalytic subunit and the fraction of β subunit that do not enters the tetramer is rapidly degradated [177]. Moreover, it was reported that CK2α may regulate the trancription of regulatory subunit, binding to a sequence upstream of the human CK2β gene, positively regulating its trancription . This notion is supported by more recent evidences that the CK2 catalytic subunits stimulated the expression of CK2α and CK2β genes, although the direct binding of CK2α and CK2β to these promoter DNAs has not been proved a [178].

Protein-protein regulatory interactions

Protein-protein interactions is a major mechanism for the regulation of specific protein kinases. It is known that directly or indirectly CK2 is regulated by interacting proteins. There are diferent proteins that are indentified as CK2-interacting partners. eIF2β inhibits the activity of CK2α on calmodulin and β-casein, but it has a little effect on CK2 activity holoenzyme [179]. Other protein whose interaction decrease CK2α activity *in vitro* is Nopp140 [180].It is also known that CK2 interacts with other proteins inluding FGF-1, Hsp90 that alter or stabilize its catalytic activity. CK2 binds other proteins, such as tubulin, CKIP-1 , which are involved in the targeting of CK2 to determinated sites or structures in the cell [166]. Moreover exist several proteins that interact specifically with CK2 in response to a stimuli modulating its activity. For instance, Pin-1 interacts with CK2 in a phoshorylation-dependent manner [181],and CK2 interacts with FACT in response to UV [182].

CK2 phosphorylation

Although phosphorylation is not a requirement of CK2 activity, it has been clearly shown that this mechanism participates in the modulation of CK2 activity. In mammalian cells CK2β and CK2α are phosphorylated in a cell cycle-manner at

different sites. Moreover phosphorylation in the C-terminal of CK2α allows CK2α to interact with Pin-1, interaction which do not appear to influence the activity of CK2, but modulate the activity of CK2 towards topoisomerase IIα. Other protein kinases are also able to phosphorylated CK2 including Src-family protein tyrosine kinase [183].

3.2.5. The localization of CK2 subunits

CK2 is present at different sites within cells and CK2α/α' subunits and CK2β subunits are not exclusively co-localized. For example, in mammalian cells, immunofluorescence studies have been demonstrated that the three subunits of CK2 were localized to the smooth endoplasmic reticulum and the Golgi complex, whereas only CK2α and CK2α' subunits could be detected in the rough endoplasmic reticulum [184]. These studies reveal the asymmetric distribution of the individual CK2 subunits. Through other techniques, it has been shown that the CK2 subunits are independently imported to the nucleus and highly mobile within the nucleus. This subcellular localization of CK2 subunits is highly regulated and may be a key to its function and modulation towards specific substrates that are distribute at different cellular compartments [166].

4. CK2 AND CANCER

CK2 participates in the phosphorylation and regulation of a wide variety of cellular targets due to its ubiquitously expression within the cell. It has been reported to be a key role in embryonic development, circadian rhythms, in the differentiation of pre-adipocytes into adipocytes, in the cell metabolism as well as linked to disease, including cardiac hypertrophy, multiple sclerosis and inflammation. However, it appears to be most prominently involved in cancer. CK2 controls a number of cellular processes that are characteristic to cancer development, including the regulation of cell growth, cell proliferation, cell survival, cell morphology, cell transformation, and angiogenesis and is a kinase that affects numerous pathways vital in cancer. Abnormally high levels of mRNA and protein expression of CK2 have been observed in a multiple cancers as well as deregulation of CK2 subunits expression [185], [186]. However, the role of CK2 in ccRCC are rather scarce. A previous report, published two decades ago, indicated that the catalytic subunit CK2α protein levels in ccRCC tumour samples were moderately higher than those present in the normal renal tissue whereas the regulatory subunit (CK2β) showed more marked increases in tumour samples [187]. In previous studies in our group, moderate increases in CK2α content in G2 and more advanced tumour grades, whereas the most remarkable changes were detected with CK2α' subunit whose content increased consistently in all ccRCC tumour samples. CK2β expression also increased in all G1 and G2 tumour samples, but decreased in more advanced tumours. This disclosed a second feature of the more advanced and aggressive ccRCC tumours, as it is the marked increase in the ratio of the CK2 catalytic/regulatory subunits (Vilardell J. PhD Thesis, 2013 UAB).

The molecular basis by which CK2 is involved in the development of tumorigenesis remain incompletely understood, which it is clearly that a number of tumour suppressor proteins, pro-apoptotic proteins and oncogens are target or protein interactors of CK2.

4.1. Role of CK2 in cell cycle and cell division

It has been widely known that CK2 phosphorylates and controls proteins that have important role in the cell cycle progression including $p34^{cdc2}$, cdc^{34} and topoisomerase II [155]. Phosphorylation of CK2α and CK2β in a cell cycle manner indicate that these proteins are regulatory part of events related with cell cycle progression. Moreover, CK2 is associated with the mitotic spindle and with centrosomes. Antisense oligonucleotides directed against CK2α or CK2β as well as CK2 inhibitors block the cell cycle progression [188].

4.2. Function of CK2 in cell survival, apoptosis and autophagy

The loss of tumor suppressor activity in cells has been related with many cancers, suggesting the important role of these proteins in cell viability. It has been observed that CK2 phosphorylates some tumor suppressors, increasing or decreasing the affinity for their substrates. For example, CK2 phosphorylates p53 at Ser392 in response to DNA damage and this promote DNA-binding activity and transcriptional activity of p53 [189]. Another way by which CK2 affects the activity of tumor suppressor proteins is through the regulation of proteasome degradation pathway. An example for this is the phosphorylation of PML (tumor suppression that controls cell growth, apoptosis and senescence). CK2 phosphorylates PML, promoting its degradation through proteasome pathway and protecting cells from apoptosis [190]. Also CK2 phosphorylates PTEN, what facilities the phosphorylation of GSK3, inducing its destabilization. In addition, a number of proto-oncogenic products, including c-Myc , c-Myb, and c-Jun, as well as transcriptional activators NF-κB, β-catenin, and Max, have been observed to be affected by CK2 phosphorylation [150].

It has been widely reported that downregulation of CK2 activity by antisense RNAi, overexpression kinase inactive CK2, or chemical inhibition induces apoptosis in cancer cells, supporting the role of CK2 in cell survival. The molecular basis of linking CK2 inhibition and apoptosis is not fully understood. A robust similarity exist between the sequence of recognition to caspase degradation and the CK2 consensus phosphorylation motif, thus CK2 phosphorylate several proteins such as BID, Max and HS1 and PTEN, in a position close to the caspase recognition sequence inhibiting the caspase cleavage [150]. Moreover CK2 phosphorylation also affects directly proteins that are component of apoptotic machinery. For example, CK2 phosphorylates procaspase-2 preventing caspase activity by blocking the dimerization of procaspase-2 [191]. Interesting, example of this is the phosphorylation of caspase-3. In that case, CK2α' exhibits a preference for caspase-3 phosphorylation in cells as compared to CK2α and the presence of CK2β abolishes caspase-3 phosphorylation [158].

Autophagy is a cellular catabolic mechanism in response to starvation or stress where cellular proteins, organelles and cytoplasm are engulfed, digested and recycled to maintain cellular metabolism. Generally, tumour cells in response to cellular stress and/or high metabolic demand related to cell proliferation, activate autophagy and it blocks the induction of apoptosis. However sometimes autophagy or autophagy-relevant proteins may help to induce apoptosis, leading to 'autophagic cell death'. It has been described that CK2 is a key protein involved in the formation and clearance

of aggresomes, and thus in cell viability in response to misfolded protein stress [192]. Moreover it has been observed that CK2 inhibition by, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), stimulate ROS production and senescence in MCF-7 [193]. Recently it has been reported that CK2 downregulation by DRB facilitates TNF-α-mediated chondrocyte death through apoptosis in cartilage obtained from osteoarthritis (OA) model rats and human OA patients [194]. More recently, it has been described that siRNA-mediated downregulation of CK2 leads to autopaghic cell death in human glioblastoma cells [195]. All these data support the involvement of CK2 in autophagic pathway.

4.3. CK2 and epithelial-mesenchymal transition

Epithelial-mesenchymal transition (EMT) is a biological process in which epithelial cells transform to mesenchymal cells, acquiring invasive and migratory characteristics, and elevated resistance to apoptosis. EMT is a necessary mechanism in embryonic development, but also contributes to cancer progression and tissue fibrosis. Although little is known about CK2 in EMT mechanism, there is increasingly interest in its role in cell invasion and migration. Recently, it has been suggested that CK2 regulates cell proliferation and cell invasion in colorectal cancer cells by modulation of genes that are related to EMT. CK2 downregulation decreases snail1 and smad2/3 levels and it decreased E-cadherin levels which is a well-studied EMT repressor [196]. Other study has reported that CK2β downregulation exhibit EMT characteristics such as morphological changes, and anchorage-independent growth, increased cell migration as well as the induction of EMT-related markers including Snail1 [197]. All these studies support the notion that CK2 is a protein which may play an important role in process related with EMT, for example cancer metastasis.

4.4. CK2 in angiogenesis

Angiogenesis is a biological mechanism by which new blood vessels arise from existing ones. This phenomenon is important in embryogenesis, skeletal growth, wound healing as well as it has a critical role in pathological processes including tumour growth. Angiogenesis provides oxygen and nutrients to the newest tumour cells. One of the most important extracellular factors that are required in this process is VEGF which regulates the function of vascular and non-vascular cells and modulate different steps of angiogenesis. Proline-Rich-Homeo domain protein (PRH) inhibits genes that encodes for VEGF –signalling components. Phosphorylation of PRH by CK2 blocks the DNA binding activity of PRH and it provokes the inhibition of VEGF components [198]. Besides VEGF, other factor are important in angiogenesis, such as

FGF which is a binding partner of CK2β subunit, or PDGF that also is phosphorylated by CK2. In addition to the interaction between CK2 and some of the extracellular factors, which are important in angiogenesis, CK2 can phosphorylated many signalling proteins, including Akt, p38 MAPK and HIF1α , that regulate the effect angiogenic of these growth factors. There are some studies that support the notion that CK2 is a key protein in angiogenesis. Therefore, it has been observed that inhibition of this protein kinase reduces endothelial cell proliferation, survival and migration, tube formation in vitro and supresses preretinal neovascularization in a mouse model of retinopathy , and it has also been described that combination of a CK2 inhibitor with other anti-angiogenic compound (octreotide) inhibit retinal neovascularization stronger than any of these agents alone [199]. More recently, it has been described that CK2 inhibition by quinalizarin blocks vascularization of developing endometriotic lesions (Dilu Feng 2012), and a new small molecule, SRPIN803, which inhibits CK2 as well as SRPK1, suppresses VEGF production in ARPE-19 cells and choroidal neovascularization in model mice [200].

4.5. CK2 and signalling pathways

In cell survival regulation, apoptosis, autophagy, EMT, and angiogenesis there are involved multiple signalling networks in which CK2 assume an important role, though the mechanism by which CK2 can affect these signalling pathways are not still fully understood (**Figure 6**).

4.5.1. CK2 in NF-κB pathway

NF-κB is one of the signalling pathway that are linked with CK2 pro-survival effects, and are involved in cell proliferation, inflammation, migration and angiogenesis. Phosphorylation of IκB, inhibitor of κappa bete, in its PEST region by CK2, induces IκB desgradation. NF-κB released from the inhibitory complex IκBs, translocates to the nucleus where regulates expression of different antiapoptotic genes. In addition CK2 also phosphorylate the NF-κB family member RelA/p65 after TNF-α stimulation, enchancing NF-κB activity [201] .

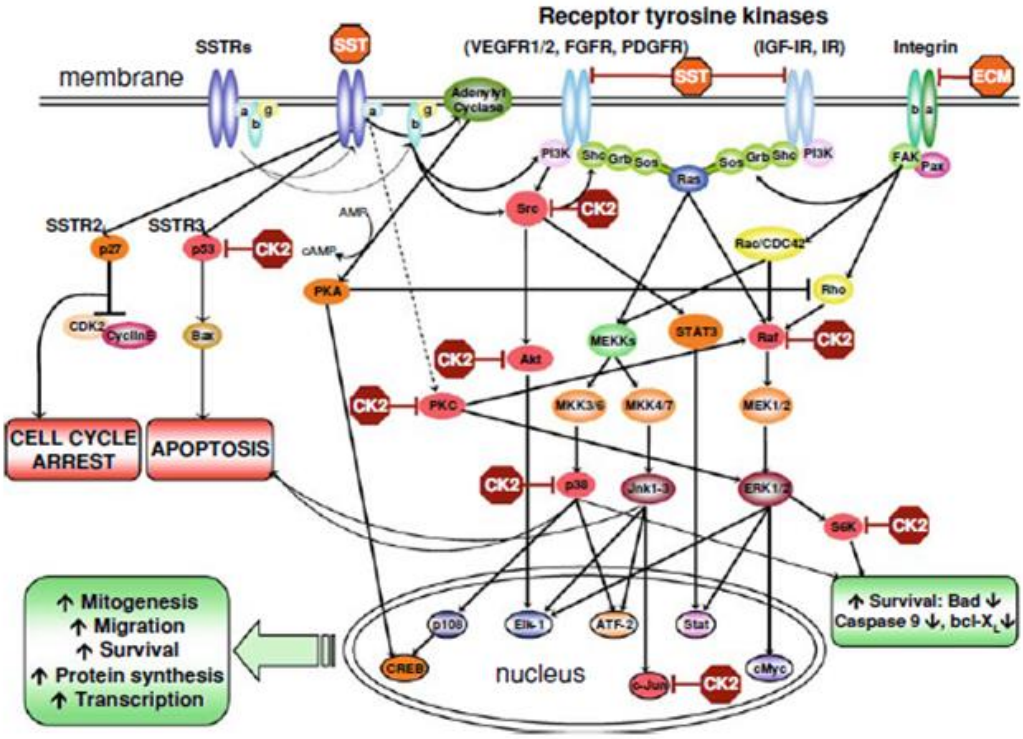


Figure 6. Schematic of intracellular signalling pathways connected with CK2.CK2 modulate different pathways involved in cell growth, cell proliferation, resistance to apoptosis, angiogenesis, as well invasiveness and migration. Therefore, CK2 phosphorylates and interacts with different proteins including Akt, PTEN, S6K, Raf, ERK, IκB, NF-κB, β-catenin, pVHL and others, all of them with important roles in signalling pathways engaged in tumor development and progression.

4.5.2. Connection between CK2 and PTEN/PI3K/Akt/mTORC cascade

NF-κβ activation is not the only mechanism by which CK2 promotes pro-survivale effects. It has also been described that PTEN/PI3K/Akt pathway is involved in cell proliferation, resistance to apoptosis and EMT. CK2 phoshorylates Akt1 in Ser129, a residue situated in the linker region within the pleckstrin homology (PH) and the catalytic domains [86], but not the equivalent AKT2 Ser131 [202]. The phosphorylation of Akt at Ser129 facilities its association with Hsp90, thereby preventing Thr308 dephosphorylation and maintaining Akt in its active form[203] . Moreover it has been observed that CK2α and CK2β isolated subunits interact with Akt and it increases Akt kinase activity in vitro as well in vivo, while an effect to the same characteristics is not detected in thecase of CK2 holoenzyme [204]. These results suggest that Akt is also modulated by CK2 through protein-protein interaction and is not exclusively dependent on site-specific phosphorylation. Besides, CK2 is also involved in the regulation of this pathway through phosphorylation of PTEN. Phosphorylation of several serine/threonine residues in the C-terminal fragment of PTEN is important for the regulation of its

stability. It is known that CK2 is one of the kinases that phosphorylate these residues. Moreover GSK3β phosphorylates PTEN at Thr366, and it has been shown that previous phosphorylation of PTEN at Ser370 by CK2 facilitates the phosphorylation at Thr366 by GSK3β [96]. The phosphorylation of PTEN induce its destabilization and it is also responsible of hyperactivation of PI3K/Akt pathway [95].

Another important protein kinase involved in cell growth and energy metabolism is Ribosonal S6 kinase (S6K), which is activated through PI3K and mTORC1 pathway. It has been described that S6K1 is phosphorylated by CK2 as well as interacts with the regulatory subunit of CK2. Phosphorylation of S6K1 at Ser-17 by CK2 enhances its nuclear export. However, the importance of nucleocytoplasmic shuttling of S6K1 induced by CK2 remains unclear [205].

4.5.3. CK2 interacts with Wnt pathway

Phosphorylation of β-catenin by CK2 leads its stabilization and protection from proteosome degradation. It appears to be crucial in the development of tumorigenesis because high levels of β-catenin induce the activation of pro-survival proteins including c-Myc, c-Jun and cyclin D1 . CK2 interacts at different points with Wnt pathway. When GSK3β is inactive, CK2 is able to phosphorylate β-catenin promoting the dissociation of the complex formed by chaperones, APC and Axin, and β-catenin translocates to the nucleus promoting the activation of pro-survival genes. In addition CK2 phosphorylates UBC3 and UBC3, which are E2 ubiquitin-conjugating enzymes, and influences the recognition of certain proteins, such as β-catenin, that are predestined to degradation by proteasomal pathway [206]. Moreover CK2 phosphorylates and interacts with proteins that are components of Wnt axis, including Dishevelled-2 (Dvl-2), Dishevelled-3, the tumor suppressor APC, altering their function. It has also been described that overexpression of CK2α lead to the increase of survivin whose transcription is regulated by β-catenin, inducing resistance to apoptosis. However it has been reported that this effect is inverted in cells with an AKT mutant deficient in Ser129 phosphorylation by CK2, suggesting that the enhancement of β-catenin transcriptional activity and survival expression is regulated by the hyperactivation of Akt pathway [207]. Taken together these results, CK2 as crosslink between different pathways, appears to be indispensable for the Wnt pathway function in transformation and tumorigenesis.

4.5.4. Role of CK2 in Raf/Ras/MAPK signalling pathway

It is known that Raf/Ras/MAPK cascade has an important role and modulates many cellular processes, such as cell survival, cell growth, cell proliferation and differentiation. For Raf, MEK, ERK spatial and temporal modulation, is required a molecular scaffold named Kinase Supressor of Ras (KSR). CK2 has been described as a component of KSR complex, and KSR/CK2 interaction has been shown to be necessary to maximally facilitate ERK casacade and the regulation of Ras kinase activity. Consequently CK2 phosphorylates the activating serine site in the N-region of B-Raf and C-Raf, however both B-Raf and C-Raf are differentially regulated by CK2. B-Raf is a constitutive target of CK2, while C-Raf S338 phosphorylation needs to be phosphorylated prior by Src family quinases. The decrease in Raf N-fragment phosphorylation is related with a reduction in Raf, MEK and ERK activation [208]. Other connections between ERK pathway, CK2 and other signalling pathways have been described. Thus, it has been observed that upon stimulation ERK2 binds to and phosphorylates CK2α, enhancing CK2α activity against α-catenin phosphorylation. This effect induces β-catenin transactivation and promotes tumor invasion [209]. Lately, it has been observed that CK2 directly phosphorylates ERK at Ser244 and Ser246 and promotes its nuclear import where ERK regulates several targets that are involved in cell proliferation and oncogenic transformation [210].

4.5.5. CK2 and the hypoxia pathway

It is known that the HIF pathway has an important role in the development of tumour agiogenesis, and there are increasing evidences that connected CK2 and HIF stability. It has been observed that under hypoxia conditions, CK2 subunits appear to be relocalizate in the cell, CK2β translocates to the plasma membrane and catalytic subunits translocate to the nucleus. In addition, an increase of CK2 activity is also detected under hypoxia. Blocking CK2 by DRB, TBB (4,5,6,7-tetrabromotriazole) and apigenin, inhibit HIF-1α activity but it does not affect HIF-1α protein level [211]. TBB, another CK2 inhibitor, increase stability of VHL and contributes to the decrease of HIF-activity and degradation of HIF-1α [212], and newly specific inhibitor of CK2, E9, which exerts a strong anti-tumour activity, has reported to induces HIF-1α degradation [213]. Although the process by which CK2 affects HIF-1α activity and stability is not understood yet, some studies have shown that other intermediates, such as p53 and histone deacetylases (HDACs), are involved in the CK2-dependent regulation of HIF-1α activity [214].

4.6. CK2 as a therapeutic target

CK2 has emerged as a new therapeutic agent due to the growth of evidence that supports the involvement of CK2 in several biological process such as cancer development and progression. Over last years several inhibitors of CK2 have been described. These molecules can be classified in diverse groups depending on the ability to bind to the ATP site (ATP-competitive inhibitors), or to other different regions (non-competitive inhibitors), as well as other alternatives strategies which target the assembling of the tetrameric holoenzyme complex or the regulatory subunit of CK2.

4.6.1 ATP competitive inhibitors

These agents mimic adenine, binding to the ATP pocket and blocking the interaction of substrates that bind to the ATP site. Several chemical compounds have been developed as a competitive inhibitors. The first one was the nucleoside analog 5,6-1-(β-D-ribofuranosyl) benzimidazole (DRB). Subsequently, a novel class of inhibitors were synthetized with higher specificity, 4,5,4,7-tetrabromo-1H-benzotriazole (TBB) and 2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole (DMAT). In addition it has been observed that natural compounds such as emodin and flavonoids (apigenin) are also potent inhibitors of CK2 [215].

Although many ATP-competitive inhibitors have been characterised, only CX-4945 (5-(3-chlorophenylamino)-benzo[c] naphthyridine-8-carboxylic acid) has entered in Phase II clinical trials as an anticancer target. CX-4945, also known as a Silmitasertib, is a potent specific inhibitor against CK2α and CK2α' with an IC_{50} of 13 nM. Crystal structure of human CK2α with CX-4945 has disclosed the interaction between these molecules is through an extensive combination of direct and water-mediated hydrogen bonds as well as van der Walls contacts. These interactions allows CX-4945 to binds with a high affinity to CK2α [216]. Several studies have been demonstrated that effect of CX-4945 as an anti-proliferative agent. Thus it has been described that CX-4945 exerts anti-proliferative effects in breast cancer cell lines (BT-474), in BxPC-3 pancreatic xenografts [217] as well as in leukemias and [218]. Also, this inhibitor blocks epithelial-to-mesenchymal transition induced by TGF-β1 in A549 human lung adenocarcinoma cells [219]. Moreover, CX-4945 has been described as a good agent for combined therapy due to role of CK2 in many processes that are important in drug resistance. Thereby, it has been shown that CX-4945 blocks the DNA repair response induced by gemacitabine and cisplatin in ovarian cancer [220], and combination of CX-4945 with EGFR tyrosine kinase inhibitor (erlotinib), results in an increase in apoptosis *in vitro* and an improved antitumor efficacy *in vivo* [221].

4.6.2. Non-ATP-Competitive CK2 inhibitors

There are some compound that inhibit CK2 without binding to the ATP pocket. Among these types of inhibitors there are peptides which bind to the acidic phosphoacceptor site on CK2 substrates and inhibit CK2-calatyzed phosphorylation. Also there are other types of molecules that bind to an allosteric region in the β4/β5 fragment, such as DRB which have a competitive and non-competitive behaviour. Other non-competitive inhibitors are hematin, benzothiazole derivates and polyoxometalates (POMs) [215].

4.6.3. Alternative approaches

The catalytic and regulatory subunit are subjected to different connections that provides a dynamic molecular structure, allowing CK2 inhibition diverse. A possible alternative is to block the interaction between CK2β and specific substrates. By two-hybrid screening has been discovered a peptide (P1) which binds to the N-terminal domain of CK2β without disrupting CK2 holoenzyme formation [222]. It has been shown that this peptide triggers apoptosis through the recruitment of a p53. On the other hand, disrupting the holoenzyme formation could be a strategy to inhibit CK2, due to CK2β and CK2α interactions is sometimes necessary to bring CK2α into proximity with its substrates.

Objectives

Protein Kinase CK2 is a ubiquitous eukaryotic ser/thr protein kinase involved in a wide variety of cellular processes. Alterations in the expression of this protein have been reported in a several diseases including cancer, where has been found an increase in its activity. Although a large and growing body of literature has investigated the role of CK2 in a different cancer, little is known in clear cell renal cell carcinoma (ccRCC). Previous studies from our group have been demonstrated that in ccRCC the unbalanced expression of CK2 subunits are involved in increased proliferation, cell migration and acquisition of mesenchymal phenotype. However do not exist many studies describing the role of CK2 in the modulation of signalling pathways involve in proliferation, migration and invasiveness in ccRCC. Considering all these aspects we set out the following objectives/aims:

1. Determine the role of CK2 in the modulation of Akt and ERK1/2 pathways in response to HB-EGF by pharmacological inhibitors of CK2 and by stable-silencing of CK2 subunits with shRNAs.
2. Study the effect of the CK2 inhibitors and CK2 downregulation in the proliferation and cell survival of renal cells.
3. Determine if CK2 downregulation is sufficient to drive epithelial-mesenchymal transition affecting other proteins that are involved in this process such as VHL, HIFs or STAT3 activation.
4. Explore if there is a connection between CK2 and ErbB4 stability and processing in ccRCC cell lines.

Material and Methods

1. CELL LINES

1.1. Characteristics of cell lines

The human cell lines used in this present work were obtained from ATCC (*American Type Culture Collection, Rockville, MD, USA*) and are derived from epithelial renal cells.

- **786-O (CRL-1932^{TM}): BSL-1**

Cell line derived from a primary clear cell adenocarcinoma, obtained from a 58 years old Caucasus man. They are hypertriploid cell lines and only the 60% of the cells examined present the chromosome Y. The line is characterized by its epithelial morphology, presence of microvilli and desmosomes, and even though are adherent cell lines, can also grow in soft agar. It has been also determined their tumorigenic properties in immunodepressed mice. The line expresses also *Parathiroidal like peptides* (PTH), expressed in other tumours such as breast and lung cancers.

- **HK-2 (CRL-2190^{TM}): BSL-2**

Human cell line obtained from the proximal convolute tubule (PTC) from a healthy kidney of an adult man. The cells were immortalized by transduction with human papilloma virus 16 (HPV-16) E6/E7 genes. The cell line has been obtained from a monoclonal cell, and presents a characteristic epithelial morphology and anchorage-depend cell growth, preventing their expansion in suspension, soft agar and in a methylcellulose matrix. The cell line maintains some functional properties of the tubular epithelium, like Na^+-dependent glucose transporters, response to parathyroidal hormone (PTH) as well as the capability to made gluconeogenesis and glycogen storage.

1.2. Stable transfection of cells

- **Cellular transduction of HK-2 and 786-O cell lines using lentiviral particles to obtain silenced cells for the subunits of CK2**

The renal cell lines stable silenced for the different subunits of CK2 (CK2α or CK2β) were generated by other member of the group (Vilardell J. Thesis, UAB 2013). For this purpose, it was used a commercial shRNA against the CK2 subunits cloned inside an integrative vector which was previously packaged in a lentiviral particles (MISSION®

Lentiviral Transduction Particles, Sigma Aldrich). The shRNA for the CK2 subunits and for the control were cloned inside two different types of vectors (pLKO.1-puro vector and TRC2-pLKO-puro vector). Both vectors possess integrative capacity, a constitutive promoter for the shRNA expression (*U6 promoter*) and the puromycin resistance gene (*puroR*) to select the cells that have integrated the plasmid. The shRNA used as control does not interact with any mammalian mRNA.

The properties of the shRNA used for the silencing of CK2 subunits, are detailed in the table below.

Table 3. Characteristics of shRNA used for the silencing of the different CK2 subunits	
shRNA against CK2β	
Reference	NM_001320.x-823s1c1
Clone number	TRCN0000003796
Gene	CSNK2B
Vector	pLKO.1-puro (U6, cppt, hPGK, puroR, SIN/3' LTR, f1 ori, ampR, pUC ori, 5' LTR, Psi, RRE)
Sequence	5'-CCGGTGGTTTCCCTCACATGCTCTTCTCGAGAAGAGCATGTGAGGGAAACCATTTTT-3'
Target	CDS (nucleotides 823-844 of CSNK2B mRNA)
shRNA against CK2α	
Reference	NM_177559.2-1895s21c1
Clone number	TRCN000032085
Gene	CSNK2A1
Vector	TRC2-pLKO-puro (U6, cppt, hPGK, puroR, SIN/3' LTR, f1 ori, ampR, pUC ori, 5' LTR, Psi, RRE, WPRE)
Sequence	5'-CCGGCAATCCCGGCTGCTGCATTTACTCGAGTAAATGCAGCAGCCGGGATTGTTTTTG-3'
Target	3'UTR de CSNK2A1 mRNA
Control shRNA (does not target any known mammalian genes)	
Reference	SHC202V
Clone number	n.a
Gene	Non target
Vector	TRC2-pLKO-puro (U6, cppt, hPGK, puroR, SIN/3' LTR, f1 ori, ampR, pUC ori, 5' LTR, Psi, RRE, WPRE)
Sequence	n.a (information is not available)
Target	Non target

The renal cell lines were transduced using lentiviral particles (MISSION® Lentiviral Transduction Particles) following manufacturer instructions

Materials and reagents:

- 6-well plates
- PBS 1X (137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2PO_4 \cdot 2H_2O$, 1.8 mM KH_2PO_4, pH 7.4)
- Trypsin
- Complete medium DMEM
- Complete medium DMEM + 1 µg/mL puromycin
- Polybrene (hexadimethrine bromide)

Procedure:

Day 1:

- The HK-2 and 786-O cells were seeded in a complete DMEM medium 24 hours prior to the transduction procedure. Cells should be between 50-80% of confluence the day of the transfection with lentiviral particles. Likewise, for each cell line was seeded a control plate at the same cellular density.

Day 2:

- While the stock of lentiviral particles is thawing on ice, add 8µg/mL of Polybrene in each well of the plate.
- Add the volume of lentiviral particles required to obtain the required transduction efficiency. In our case, a MOI (Multiplicity of infection) of 2 was used.
- Cells were incubated O/N at 37ºC in an incubator.

Day 3:

- The medium containing the lentiviral particles is replaced by a new complete fresh medium.

Subsequent days:

- Cells that have incorporated the vector in their genome must be selected. For this purpose, the medium is replaced by new complete DMEM medium containing 1 µg/ml of puromycin. The amount of puromycin used for the selection depends on the sensitivity of the cells to the antibiotic, and was previously determined with an antibiotic kill curve
- The medium is replaced every 2-3 days for fresh prewarmed complete DMEM with 1µg/mL of puromycin. Once cells have been selected (5-6 days) they must be subcultured in a new plate with fresh medium to be expanded for different experimental purposes and preservation.

- **Stable 786-O/VHL30 and 786-O/VHL167 cells**

786-O cells were transfected with pCMV-HA-VHL and pCMV-HA-VHL167 vectors to express VHL30 protein and their truncated form VHL167. Cells were stably selected

using G418. These cells were generated by other member of the group (Garcia A., Master Thesis, UAB 2010).

- **Stable 786-O/ErbB4 cells**

786-O cells were transfected with pYRES-hygro/ErbB4 vector to express the ErbB4 receptor. After that, cells were stably selected using Hygromycin during 1 month.

Materials and reagents:

- 10 mm dishes
- Pyres-hygro/ErbB4
- Fugene HD
- PBS 1X Trypsin
- Complete DMEM medium
- T75 flask

Procedure:

- Plate cells in a 10 mm dishes 24 h before transfection to reach the 60-80 % of confluence at the day of transfection.
- Leave one 10 mm culture dish culture untransfected. This will serve as a control and is used as a reference during the selection process.
- Per dish, dilute 3 µg of ErbB4 DNA in 200 µl of complete DMEM medium. Vortex gently and spin down briefly. Add 6 µl of Fugene HD to the DNA solution. Vortex gently and incubate at RT 15 minutes.
- Per dish, add the 200 µl Fugene HD/DNA mix to the cells. Return the plates to the cell culture incubator.
- At 48 h post transfection the medium is replaced for complete DMEM medium containing 300 µg/µl of Hygromicin.
- Change media containing 300 µg/µl of Hygromicin every 2-3 days. Examine the cells for visual toxicity daily. Cells that have not integrated the transfected plasmid will die while the cells that have undergone plasmid integration will survive by 9 days post-transfection.
- Surviving cells should expand in the T75 flask to ensure that the selected clones are not unstable.
- Keep replacing media containing selection antibiotic twice a week until the cells rise high confluence. At this moment, the cells can be frozen down as a polyclonal cell line.

1.3. TRANSIENT TRANSFECTION OF SUBUNITS OF CK2

786-O/HK-2 cells were transiently transfected with pCMV-HA empty plasmid, pCMV-CK2α or pCMV-CK2β to express CK2α and CK2β subunit, or with siRNA CK2α and siRNA CK2β to transiently downregulate CK2 subunits. Due to the difficulty of transfection we use Metafectane Pro, a highly efficient transfection reagent for eukaryotic cells.

Materials and reagents:

- 6-well plate
- pCMV-HA, pCMV-CK2α or pCMV-CK2β
- Metafectane Pro
- Complete medium DMEM

Procedure for a 6-well plate:

- Plate cells in a 6-well plate to obtain 24 h after, a confluence of 90-100%.
- Temperate at room temperature the METAFECTENE PRO stock solutions and the DNA. Agitate the stock solutions gently before use to homogenate them.
- Dilute 1 µg of DNA in 50 µl of PBS for each well
- Add 2 µl of METAFECTANE PRO in 50 µl of PBS.
- Mix the solutions gently by pipetting them carefully.
- Combine the two solutions, mix gently by pipetting up and down and after, incubate at room temperature for 15 min.After the incubation time, add DNA dropwise to the cells. Incubate the plate at 37°C in a CO_2 incubator. 48 h after, collect cells by centrifugation to obtain cell extracts.

For siRNA CK2α or CK2β transfection the assay can be performed at the same conditions, but replacing the DNA by 25 nM final concentration of each siRNA diluted in PBS RNase-free, and incubate the plates 65 h for protein analysis.

1.4. GENOMIC DNA EXTRACTION

For this purpose, we have used the commercial GenElute Plasmid Miniprep Kit for plasmid DNA extraction from recombinant E. coli cultures.

Materials and reagents:

- GenElute Plasmid Miniprep Kit (containing the Resuspension, Lysis, Neutralitzation, Column preparation and Wash solutions).
- Sterile H_2O
- Eppendorf

Procedure:

- Pellet 5 ml of an overnight recombinant E.Coli culture by centrifugation at 5000g for 10 min at 4ºC.
- Discard the supernatant and resuspend the bacterial pellet with 200 µl of the *Resuspension Solution*.
- Lysis the resuspended cells by adding 200 µl of the Lysis Solution. Mix by gentle inversion and incubated for 5 minutes.
- Precipitate by adding 350 µl of the *Neutralization/Binding Solution*. Invert the tube 4-6 times. Pellet the cell debris by centrifuging at 12.000g for 10 minutes.
- Insert the column into a provide tube, add 500 µl of the *Column Preparation Solution* and centrifuge at 12.000g for 1 minute.
- Transfer the lysate to the column tube and centrifuge at 12.000g for 1 minute.
- Add 500 µl of the *Wash Solution* and centrifuge at 12.000g for 1 minute.
- Add 750 µl of the diluted Wash Solution to the column. Centrifuge at 12.000g for 1 minute.
- Transfer the column to a new collection tube. Add 50-100 µl of sterile H_2O and centrifuge at 12.000g for 1 minute to elute the DNA. DNA concentration/purity is measured with a Nanodrop 200 Spectrophotometer.

1.5. MAMMALIAN CELL LINE MAINTANCE , TREATMENTS AND STORAGE

Cell maintenance

The cell lines used during this work were maintained in a T75 flask until 80-90% of confluence, then were subcultured in a new flask. The split ratios are useful to ensure cells should be ready for an experiment or just to keep the cell culture running for future use. Depending on the growth of the cell line and the experiment the split ratio used will change. Moreover, the passage number of these cells are recommended not get too high (<30). This is to prevent use of cells undergoing genetic drift and other variations.

All renal cell lines used in this work, were grown in completed DMEM (*Dulbecco's Modified Eagle's Medium*). Nevertheless, in some cases (as in the case of the cells silenced for the different CK2 subunits) the presence of antibiotics in the media is required to select and ensure that the lines maintain silencing of our protein.

Materials and Reagents:

- Complete DMEM
- Trypsin
- PBS 1x sterile
- Pasteur pipettes
- Serological pipettes
- T75 flasks

Procedure in a T75 flask:

- Remove the medium and wash the T75 flask with 10 ml PBS 1x sterile.
- Add 2 ml of trypsin and incubate the flask at 37ºC for 3 minutes in the incubator to detach the cells.
- Add 8 ml of a complete DMEM to inactivate the trypsin.
- Resuspend carefully the cells and add 2 ml of this resuspension to a new T75flask and completed with fresh medium to reach a final volume of 15 ml.
- The flask is incubated at 37ºC, 5% CO_2 in humid atmosphere.

Treatments

- For the experiments, cells were counted using a *Neubauer chamber* or the automatic *Cell Counter* [Bio-Rad]. Both, are used to precisely count the number of cells present in the culture. To determinate the effect of inhibitors in the HB-EGF response, the cells were seeded at 2×10^5 cells/ml and after 24 hours with complete DMEM medium, cells were starved for 16 hours in medium supplemented with 0.5% FBS. HB-EGF was dissolved in PBS and the pharmacological inhibitors were in DMSO. In all cases controls were carried out with the corresponding vehicle. HB-EGF, TBB and TBCAII were from Calbiochem, CX-4945 was from Selleckhem, PI3K inhibitors were from Ascent and Apigenin and other pharmacological agents used such as PMA, chloroquine and proteases inhibitors were from Sigma Aldrich.

The table below illustrates media used depending on the cell line.

Table 4. Mediums used for cell line growing

Medium	Composition	Cell line	Condition
Complete DMEM	DMEM (10% FBS, 1 mM Sodium pyruvate, 1% (v/v) Glutamin, 1% (v/v) Penicilin, Streptomycin)	HEK293T 786-O HK-2	Growth and maintenance of wild type cell lines
Complet DMEM + selection antibiotics	Complete DMEM supplemented with 1µg/mL puromycin	786-O shCK2α 786-O shCK2β 786-O shCV HK-2 shCK2α HK-2 shCK2β HK-2 shCV	Growth, maintenance and selection of CK2-silenced cell lines and control cell lines
Complet DMEM + selection antibiotics	Complete DMEM supplemented with 1µg/mL G418	786-O/VHL30 786-O/VHL167	Growth, maintenance and selection of stably-expressed VHL cells
Completed DMEM + selection antibiotics	Complete DMEM supplemented with 300µg/mL Hygromicin	786-O/ErbB4	Growth, maintenance and selection of stably-expressed VHL cells
Starving medium	DMEM (0.5% FBS, 1 mM Sodium pyruvate, 1% (v/v) Glutamin, 1% (v/v) Penicilin, Streptomycin)		

Table 5. Volumes used for the different flasks and plates

Flasks and plates	PBS washes	Trypsin	Resuspension Medium	Medium final volume
24 well plates	0,5 mL	0,1 mL	0,250 mL	0,5 mL
12 well plates	1 mL	0,2 mL	0,5 mL	1 mL
6 well plates	2 mL	0,4 mL	1 mL	2 mL
35 mm diameter well plates	2 mL	0,4 mL	1 mL	2 mL
60 mm diameter well plates	6 mL	1 mL	2 mL	6 mL
T25 Flasks	5 mL	1 mL	2 mL	5 mL
T75 Flasks	15 mL	3 mL	5 mL	15 mL

Freezing cell lines

Eukaryotic cell lines can be cryopreserved in liquid nitrogen to create a working banks with some vials. When the bank is used up, a new working bank can be cultured and created from one vial from the original bank. The protocol used to freeze the cells is indicated below:

Materials and Reagents:

- Sterile PBS 1X
- Trypsine
- DMSO [Sigma-Aldrich]
- Cryovials of 1,5 ml [Nunc]
- FBS (*Fetal Bovine Serum*) ([Reactiva], 04-001-A)
- Special box for freezing cells

Procedure:

- Remove medium from one flask, wash cells with sterile PBS 1X and trypsinize for 3 minutes at 37ºC.
- Once cells are detached, add 8 ml of complete DMEM medium and transfer to a centrifuge tube.
- Count cells with the cell counter.
- Spin down at 1500 rpm for 5 minutes and remove medium
- Resuspend the cells in enough freezing medium (FBS with 5% DMSO) to generate a cell suspension of $1x10^6$ cells /ml.
- Aliquot about 1 ml into cryovials.
- Transfer cells immediately to -20ºC for one hour, afterwards overnight at -80ºC and finally storage in liquid nitrogen.

Thawing cell lines

This step must to be done very quickly because DMSO is toxic for cells at room temperature and should be removed immediately.

Materials and Reagents:

- Complete DMEM medium
- Culture Flasks T25 or T75

Procedure:

- Prepare pre-warmed complete DMEM medium

- Remove cryovials from liquid nitrogen and immediately place in 37ºC water bath until about 80% has thawed.
- Pipette into a flask, add the appropriate amount of medium and place in the incubator.
- After 24 hours change culture media to remove non-adherent cells and DMSO residues. **In the case that the cell line needs a selection antibiotic this must be added in this moment**.

1.6. PROLIFERATION MTT ASSAY

Reduction of tetrazolium salts is a method widely accepted to determine cell proliferation and viability. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay described for the first time by Mosmann (Mosmann, 1983), is based on the ability of the mitochondrial dehydrogenases (Succinate dehydrogenase) of living cells to reduce rings of MTT to purple formazan crystals, allowing its accumulation inside healthy cells. To solubilize the crystals DMSO is added. Using a spectrophotometer is possible to determine the quantity of formazan formed, which will be directly related to the number of viable cells.

Materials and Reagents:

- 96-well plates
- Complete DMEM medium
- MTT (Sigma M-2128)
- PBS 1X
- DMSO
- Victor3

Procedure:

- 100 µl/well from a 10.000 cells/ml cell suspension were added to a 96-well plates per tetraplicate. Some wells containing just medium without cells were used as blanks.
- Cells are incubated with vehicle (control) or drugs at the appropriate concentration and for the desired time.
- MTT is dissolved in PBS 1X at a concentration of 5 mg/ml (Stock solution). MTT is then diluted to a final concentration of 1 mg/ml (Working solution) and 50 µl are added in each well.
- Plates are incubated at 37ºC for 30 minutes.
- Aspirate the medium and add 100 µl DMSO per well and shake.

- Read the plate with the *Victor3 Multilabel Counter* at two wavelengths: 560 nm and 620 nm.
- First measurement (T=0 hours), is read 5 hours after cells seeding. Next measurements are done 24 h after T=0.

1.7. PREPARATION OF WHOLE CELL EXTRACTS

For total protein extraction we use a *Lysis buffer* composed by: 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 % Triton-X-100, 1 mM DTT, 1 mM PMSF, 1 mM EDTA, 25 mM NaF, 0,2 mM Na_2VO_3, 2 mM PPi, 1 µg/mL protease inhibitors (leupeptin, benzamidin, aprotinin, pepstatin).

Materials and reagents:

- Serological pipettes
- Pasteur pipettes
- Eppendorf tubes
- Pre-chilled cell *Lysis buffer*.
- Refrigerated microcentrifuge

Procedure:

- Wash cells with PBS1x two times and lyse them with 750 µL of pre-chilled *Lysis buffer* (for a dish of 10 cm diameter). Depends on the plate or dish surface, the volume of *Lysis buffer* will vary.
- Kept the cells with the lysis buffer on ice for 10 minutes. The cells are taken off with the help of a scrapper, and recovered in an Eppendorf tube.
- Samples are centrifuged at 4ºC at 14.000 x g for 10 minutes.
- The supernatant obtained contains the whole cell extract.

1.8. SOFT AGAR COLONY FORMATION ASSAY

Soft agar colony formation assay, is a method used to test the anchorage-independent growth of the cells *in vitro*, to detect the tumorigenic potential of this cells. This assay also allows for semi-quantitative evaluation of this capability, in response to different treatment conditions. In this assay, the cells are cultured in soft agar for 21 days. After this period, the colonies formed are analysed and quantified.

Materials:

- 6% sterile noble agar al 6% (previously diluted in H_2O) [BD-DIFCO](Ref. 214220)
- 3% or 4% of sterile noble agar (previously diluted in H_2O)
- Water bath
- 6-well dishes plates
- Nitroblue tetrazolium chloride monohydrate [Sigma-Aldrich](Ref. N15405)

Procedure:

Day 1: Preparation of bottom layer (0.6% of agar)

- Melt 6% agar in a sterile glass bottle by microwave and keep it warm in a 50°C water bath for 15 minutes, and then at 42-44ºC for 30 minutes.
- Dilute the agar 10 times in pre-warmed complete medium to obtain a final concentration of 0.6%
- Add 4 ml of the 0.6% agar in each well.
- Wait 30 minutes until agar solidifies. Once the agar is solid the plates can be conserved at 4ºC.

Day 2: Preparation of top layer (0.4% agar)

- 6-well plates with the bottom layer 0.6% agar are preincubated at 37ºC.
- Melt agar 4% in a microwave, keep it warm in a 50°C water bath for 15 minutes, and then at 42ªC for 30 minutes.
- Preparation of the top layer. It contains 5000 cells in 2 ml of complete DMEM medium with 0.4% of agar. Seeding process should be done quickly and carefully to avoid generation of bubbles.
- Do not shake the plate. Leave the plate at room temperature for 30 minutes and put the plate at 37ºC incubator for 3 weeks.
- Next day add 0.5 ml of complete DMEM medium with 0.1 % DMSO to the control wells and 0.5 ml of complete DMEM medium with the CK2 inhibitor, CX-4945, at a final concentration of 10 µM to treat cells.
- Every 3-4 days replace the media for fresh medium containing 0.1% DMSO or 10 µM of CX-4945.
- The plates are incubated at 37ºC until colonies can be observed. The colonies are stained with 1mg/ml of nitroblue tetrazoliummonohydrate (500 µl/well) and incubated

overnight at 37ºC. Photographs of the stained colonies have been taken and analysed using the ImageJ software after scanning the plate.

1.9. IMMUNOFLUORESCENCE

The immunofluorescence allows detection and quantification of proteins of interest in cultured cells (immunocitofluorescence) or in tissue cells (immunohistofluorescence) using a system of specific antibodies conjugated with a fluorescent molecule. We used an indirect immunofluorescence, in which primary unlabelled antibody binds to the target, after, a fluorophere-labeled secondary antibody is used to detect the primary antibody.

Materials and Reagents:

- Round coverslip
- Slides
- 24-well plates
- Complete DMEM medium
- PBS 1X DAPI
- Fluoroprep mounting medium
- PFA (Paraformaldehyde) 4% in PBS
- Petri dish
- Aluminium paper
- Wet tissue
- Parafilm
- Primary antibody: rabbit anti-ERbB4
- Secondary antibody: goat anti-rabbit IgG Alexa 488 [Sigma-Aldrich](Ref. AP132JA4)
- *Buffer A* (0,02% Saponin in PBS)
- *Buffer B* (0,01% Saponin, 10 mM Glycine in PBS)
- *Buffer C* (0,01% Saponin, 10 mM Glycine, 5% BSA in PBS)
- *Buffer D* (0,01% Saponin, 1% BSA in PBS)

Procedure:

- Dispose sterile round coverslips in a 24 well plate
- Cells (5.000cells/well) are seeded in 250 µl of complete DMEM medium in the 24 well plate. The cells are incubated at 37ºC until the next day.
- Remove the medium and wash 2 times with PBS 1X.

- Fix the cells with PFA 4% in PBS for 20 minutes at room temperature.
- Wash 3 times for 5 minutes with PBS 1x at room temperature.
- Permeabilize the cells by adding 450 µL of *Buffer A*, incubate for 7 minutes at room temperature without agitation. It is important to respect the time to avoid the destruction of membranous structures in the cells.
- Wash the cells for 5 minutes in PBS 1x with gently agitation.
- Add 450 µL of *Buffer B*, and incubate for 15 minutes at room temperature without agitation.
- Block for 1 hour by adding 450 µL of *Buffer C* at room temperature.
- For each coverslip, we add the primary antibody ErbB4 (1:250) diluted in *Buffer D*. For this purpose, the coverslips must be disposed in a petri dish with a wet tissue. The final volume required of primary antibody is 12 µL. Incubate with the primary antibody overnight at 4ºC.
- Wash with PBS 1X 3 times for 5 minutes with gently agitation.
- After, coverslips are incubated with 12 µL of the secondary antibody *anti-rabbit IgG Alexa 488* (1:500) diluted in *Buffer D*, for 1 hour at room temperature, protected from light and without agitation.
- Wash coverslips 4 times for 5 minutes in PBS 1X.
- Stain the nuclei of the cells with 400 µL of DAPI (0.1 µg/ml PBS) for 5 minutes at room temperature without agitation.
- Wash the samples 4 times for 5 minutes with PBS 1x and mount the coverslips with the cells facing down on the glass slides, adding *Fluoroprep mounting medium*.
- Seal the coverslips with polish nail.
- The samples can be conserved at 4ºC protected from light until the moment of their analysis under a confocal microscope or fluorescence microscope.
- The microscopes used were: *Leica TCS SP5 microscope*

1.10. PULL-DOWN ASSAY

This methodology is used to determine the physical interaction between proteins. In this study we analyse the possible association between CK2 and ErbB4 receptor in 786-O/ErbB4 cells.

Materials and Methods:

- Lysis Buffer (EBC) (50 mM Tris-HCl pH 8, 120 mM NaCl, 0.5% Nonidet NP-40, protease inhibitors, 50 mM Imidazole)

- Cellular extract lysate
- Ni-Sepharose High Performance
- Recombinant-His-CK2α or CK2β
- PBS 1X

Procedure:

- Apply 20 µg of the recombinant-His-CK2α or CK2β protein to a 1 ml resin *Ni-Sepharose High Performance* column.
- Add 500 µl of PBS 1X and leave gently shaking at 4ºC for 1 hour.
- Wash the column with 1 ml of PBS 1X, centrifuge at 735 x g for 1 minute and repeat this step with EBC.
- Apply up to 500 µg of cell extract lysate in buffer EBC and incubate at 4°C for 2 hours.
- Wash with 1 ml of EBC and centrifuge at 735 x g for 1 minute. Repeat the same procedure 2 times more.
- Add 60 µl of Elution buffer (Laemmli Buffer)Boil the samples at 100ºC for 3 minutes and save the flowthrough. Samples are analysed using conventional SDS-PAGE and western blot.

1.11 SUBCELLULAR FRACTIONATION

This methodology allows the analysis of a protein of interest based on its subcellular distribution.

Materials and Reagents:

- *Homogenized buffer*: 20 mM HEPES (pH 7,4), 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 250 mM sucrose and 1µg/ml of protease inhibitors.
- *Resuspension nuclei Buffer*: 20 mM HEPES (pH 7,4), 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 150 mM NaCl, 1% Tritó X-100 i 1µg/ml of protease inhibitors.
- Dounce homogenizer
- Scraper

Procedure:

- Cells are resuspended in 800 µl of Homogenized Buffer with the help of a scraper and are kept on ice for 10 minutes.
- Cells are homogenized for 1 minute on ice using a Dounce homogenizer.
- The homogenate is centrifuged at 800 x g for 10 minutes at 4ºC.

- Pelletcorresponds to the nuclei fraction and is resuspended in 200 µl of *Resuspension nuclei Buffer*. The supernatant, is then centrifuged at 13000 x g for 30 minutes at 4ºC.
- Pelletcorresponds to the mitochondrial fraction and is homogenized in 30 µl of *Homogenized Buffer*. This pellet also contains endoplasmatic reticulum (heavy membranes). The supernatant instead is centrifuged at 100.000 x g for 1 hour at 4ºC.
- The pellet resultant of this last centrifugation is resuspended in 30 µl of Homogenized Buffer and corresponds to plasma membrane, while the supernatant is the cytosolic fraction.

Additionally, a commercial cell fractionation kit designed to separate nuclei and cytoplasmic fractions was used. [Pierce Biotechnology, *NE-PER nuclear and cytoplasmic extraction kit*](Ref. 78833).

2. WESTERN BLOT

2.1. Bradford

To determine the protein concentration of the cell extracts, we use the *Bradford's colorimetric method*. This procedure was initially described at 1976 by *M.Bradford*, and it is based in the use of the colorant Comassie Brilliant Blue G-250.

2.2 Polyacrilamide Gel Electrophoresis in denaturising conditions (SDS-PAGE)

Polyacrilamide Gel Electrophoresis (PAGE) is a methodology used for the separation and visualization of the proteins by its molecular weight, although it has been applied for separating small DNAs and RNAs. When these gels contains sodium dodecylsulfate (SDS), the electrophoresis is done under denaturating conditions, designating itself as SDS-PAGE.

Materials and reagents:

- Acrylamide andbis acrylamide (Acryl-bis acryl 30%) [CONDA]
- *Buffer B*:Tris/HCl 1 M, 0,4% SDS, pH 8,8
- *Buffer C*:Tris/HCl 0,5 M, 0,4% SDS, pH 6,8
- H_2O milliQ

- TEMED
- Ammonium persulfate 10% (APS)
- Laemmli buffer 5X (TMR 5X) (350 mM Tris, 10% SDS, 50 % glycerol, 0,0125 % bromofenol blue, 0,5% β-mercaptoetanol)
- *Running Buffer:* 25mM Tris, 192mM glicina, 0,1% SDS
- Mini Protean 3

Procedure:

- To prepare the gels two glasses of 10x7 cm and 1,5 mm of thickness are used. The stacking gel has a constant concentration of acrylamide (3,5%) and the running gel vary (in our case of 10% due to our proteins under study). The preparation of each gel is indicated in the table below.

Table 6. SDS-PAGE composition

	Running Gel			Stacking Gel
Acrylamide Composition	**10%**	**12%**	**15%**	**3,5%**
Acrylamide (mL)	2,5	3	3,75	0,7
Buffer B (mL)	1,875	1,875	1,875	1,5
H_2OmQ (mL)	3,125	2,625	1,875	3,8
TEMED (µL)	10	10	10	10
APS 10% (µL)	100	100	100	100

- Samples are diluted in Laemmli buffer 5X (TMR 5X), boiled at 100ºC for 5 minutes, vortexed and centrifuged for 1 minute.
- 25-30 µl of prepared samples are loaded into wells. As a molecular weight marker we use *Precision Plus Protein Dual Color (Bio-Rad)*.
- Run the electrophoresis at constant amperage ((30-35 mA/gel) until the forehead of the samples arrive to the final of the gel).

2.3. Immunological analysis by western blot

Western blot is a semi-quantitative methodology that allows identifying proteins that have been separated based on size by gel electrophoresis, using specific antibodies. The gel is placed next to a PDVF membrane and application of an electrical current induces the migration of the proteins from the gel to the

membrane. This membrane is processed with antibodies specifics for the protein of interest and visualized using secondary antibodies conjugated with an enzymatic reporter.

2.4. Electrotransfer of proteins:

Materials and reagents:

- *Transfer Buffer*: 192mM Glycine, 25mM Tris, 10% methanol
- Absolute Methanol
- PVDF Immobilion-P membranes
- Mini-Protean 3 transference system
- Ponceau-S red (0,1% (p/v) in 5% acetic acid)
- *Blocking solution*: TTBS 1X + 5% non-fat powdered milk

Procedure:

- The PVDF (immobilon P) membrane is activated for 1 minute in methanol and rinsed with distilled water and after, with transfer buffer before preparing the stack.
- The transfer cassette is assembled following the manufacturer's instructions and the proteins are transferred at a constant voltage of 100 v for 1 hour.
- After 1 hour, rinse the membrane with H_2O for some minutes and check the transfer of proteins using Ponceau S before blocking step. To eliminate the Ponceau S, wash the membrane with water and *Transfer Buffer*.

To determine some specific proteins (i.e. Akt Ser129) a different process was used. A semi-dry transfer without a buffer tank and gel cassettes. In this case, before starting the transfer, the membrane is rinsed in the different buffers and is placed within Whatmann papers previously rinsed in buffers. Proteins are transfer for 17 minutes at 24V.

2.5. Inmunodetection:

Materials and Reagents:

- TTBS 1X: 50mM Tris-HCl, 150 mM NaCl, 0,1% Tween-20, pH 7.4
- *Blocking buffer*: TTBS 1X + 5% non-fat powdered milk
- *Primary antibody buffer*: TTBS 1X, 5% BSA, 0.02% sodium azide

- Primary antibody: see the table of primary antibodies used, part 3.4
- Secondary HRP-antibody: see the table of secondary antibodies used, part 3.4
- Chemiluminescent kit Clarity Bio Rad
- Amersham Hyperfilm ECL [GE-Healthcare]
- Chemidoc MP Image System [Bio-Rad]

Procedure:

- Block the membrane for 1 hour at room temperature using *Blocking Buffer*.
- Wash the membrane 3 times for 10 minutes with TTBS 1X.
- Incubat the membrane with appropriate dilutions of primary antibody in the primary antibody buffer overnight at 4ºC.
- The day after, retrieve the primary antibody and wash 3 times with TTBS 1X, 10 minutes each.
- Incubate the membrane with the dilution of conjugated secondary antibody (1:5000) in *Blocking buffer* at room temperature for 1h in constant agitation.
- After incubation with the secondary, wash the membranes 3 times with TTBS 1X, 10 minutes each.
- For signal development is used the chemioluminescence kit *Clarity* of Bio-Rad. Add 1 ml per membrane, remove excess reagent and cover the membrane in transparent plastic wrap.
- The image is acquired in a darkroom using a photographic film (Amersham Hyperfilm ECL) or using normal image scanning methods *Chemidoc MP Image System*

2.6. List of antibodies used in Western blot

Table 8. List of primary antibodies used in WB

Antibody	Reference	Molecular Weight (KDa)	Dilution	Producer Host
CK2α (1AD9)	05-1431	38-44	1:500	Mouse
CK2α'	A300-199A	38	1:1000	Rabbit
CK2β home	*homemade*	28	1:4000	Rabbit
CK2β (6D5)	sc-12739	28	1:125	Mouse
β-Actin	sc-47778	43	1:1000	Mouse
N-Cadherin	610920	130	1:1000	Mouse
E-Cadherin	610181	120	1:2000	Mouse
Snail1	A5228	42	1:1000	Mouse
PTEN	sc7974	54	1:1000	Rabbit
p-Akt Ser473	9271	60	1:1000	Rabbit

p-Akt Thr308	9275	60	1:1000	Rabbit
p-Akt Ser129	33458	60	1:1000	Rabbit
Akt1	2938	60	1:1000	Rabbit
Akt2	3063	60	1:1000	Rabbit
Akt1/2/3	9272	60	1:1000	Rabbit
p-GSK3β	9336	57	1:1000	Rabbit
GSK3β	610201	57	1:1000	Mouse
p-ERK1/2	9101	42/44	1:1000	Mouse
ERK1/2	610129	42/44	1:1000	Mouse
HIF-1α	Ab82832	97	1:500	Mouse
HIF-2α	Ab199	97	1:500	Mouse
VHL	556347	30	1:500	Mouse
p-STAT3 Tyt705	4113	80	1:1000	Mouse
p-STAT3 Ser727	9134	86	1:1000	Rabbit
STAT3	9139	80	1:1000	Mouse
ErbB4	4797	180	1:500	Rabbit
EGFR	4267	175	1:1000	Rabbit
TfR	13113	90	1:1000	Rabbit
IκBα	Sc-371	35	1:500	Goat
LC3-II	48394	17/15	1:500	Rabbit
PARP cleaved	5625	89	1:1000	Rabbit

Table 9. List of secundary antibodies used in Western Blot

Antibody	Reference	Dilution	Producer Host
IgG Goat Anti-Rabbit IgG (H+L)-HRP conjugate	170-6515 (Bio-Rad)	1:3000	Goat
IgG Goat-Anti-Mouse IgG (H+L)-HRP conjugate	170-6516 (Bio-Rad)	1:3000	Goat

3. Assay of CK2 activity in biological samples

CK2 activity can be determined in whole cell extracts. As CK2 is quite soluble therefore, no drastic conditions are necessary to solubilize this protein. Only are required treatments that respect the native conditions of the protein, without affect the kinase activity. Lysates of cells can be stored at -20ºC and used several times for CK2 assays. *Lysis buffer* (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.5% (v/v) Triton X-100, 2 mM DTT, protease inhibitor cocktail,10 mM NaF, 1 mM Na3VO4, 1 mM okadaic acid) must be kept ranges of temperature 0-4ºC, and

inhibitors of proteases and protein phosphatases should be always freshly added. CK2 is present in the cell as holoenzyme but also as isolated subunits. Both forms are catalytically competent, however show differences in their specificity to different substrates and can be used to distinguish between them. For this purpose we use a CK2 peptide substrate derived from the sequence of eiF2β that is phosphorylated only for the holoenzyme, whereas CK2-tide it can be phosphorylated also for CK2α free subunits.

Table 7. Specific CK2 peptide substrates		**CK2 $\alpha_2\beta_2$**		**CK2α**	
Peptide	**Sequence**	**K_m**	**V_{max}**	**K_m**	**V_{max}**
CK2-Tide	RRRADDSDDDDD	14	56.5	25	18.9
eiF2β-Tide	MSGDEMIFDPTMSKKKKKKKKP	10	71.4	660	5.0

The values are reported from Poletto *et al.* (2008)

Material and Reagents:

- 0.5M M Tris–HCl pH 7.4
- 0.1 M $MgCl_2$
- NaCl 0.1
- ATP 0.1 mM
- ATPγ33 (1-2x10^6 cpm/ reaction).
- CK2-tide 1 mM
- eiF2β-tide 1 mM
- Phosphocellulose paper
- 0.5% orthophosphoric acid
- Scintillator fluid
- Scintillator counter (Perkin Elmer)

Procedure:

- For a final volume of 20 µl, add: H_20, 0.5M M Tris–HCl pH 7.4, 0.1 M $MgCl_2$, NaCl 1 M, ATP 0.1 mM, 1 or 2 µg of protein and 1 mM of CK2-tide or eiF2β-tide. To determined CK2α instead of $\alpha_2\beta_2$, NaCl should be avoided in the buffer.
- When all tubes are prepared, start the reaction by adding ATPγ33 (1-2x10^6 cpm/ reaction). Place at 30º C for 30 min.
- Spot the volume of the tube on a phosphocellulose filter to stop the reaction.
- Wash 3 times the filters 5 min in 0.5% (v/v) phosphoric acid.

- Once the papers are dried, put them in vials and add 3mL of scintillation liquid.
- Incubate the samples for 30 min at RT and proceed to the lecture in a Liquid Scintillation Analyzer (Tri-Carb 2810TR, Perkin Elmer)

3.1. In-gel assay of CK2 catalytic subunit activity

This method allows the separation of cellular proteins in SDS-PAGE, according to their molecular weight in denaturing conditions, and the detection of the kinase activity in the position where the subunit migrates. CK2 is able to recover its active conformation after SDS removal, therefore it is a sensible assay that allows to determine the activity of catalytic subunits of CK2. To perform this assay, a well-known CK2 substrate, β-casein, is incorporated into a polyacrylamide–SDS gel prepared for protein separation. The cellular extract of proteins are run and then the gel is washed and incubated in a renaturing buffer. Afterwards, the gel renatured is incubated in a radioactive phosphorylation mixture and washed with buffer before be analysed by autoradiography.

Material and Reagents:

- Acrylamide-bisacrylamide (Acryl-bis acryl 30%
- Buffer B: Tris/HCl 1 M, 0,4% SDS, pH 8,8
- Buffer C: Tris/HCl 0,5 M, 0,4% SDS, pH 6,8
- H_2O milli-Q
- TEMED
- Ammonium persulfate 10%
- Laemmli buffer (0.625 M Tris, 2% SDS, 50 % glycerol, 0,0125 % bromofenol blue, 5% β-mercaptoetanol)
- *Running Buffer*: 25mM Tris, 192mM glicina, 0,1% SDS
- Mini Protean 3
- Tris 50 mM pH 8.0
- *Buffer A* : Tris 50 Mm pH 8.0 + 2-propanol 20 %
- *Buffer B*: Tris 50 mM pH 8.0 + β-mercaptoehtanol 5mM
- *Buffer C*: Tris 50 mM pH 8.0 + β-mercaptoehtanol 5mM+ Guanidin 6 M
- Buffer C: Tris 50mM + β-mercaptoehtanol 5Mm + Tween 20 0.04%
- *Washing buffer*: TCA 5%+ Pirophosphat 1%
- Radioactive phosphorylation mixture: Tris 0.5 M pH 7.5, $MgCl_2$ 0.1M, ATP 1mM, [γ-^{33}P]ATP 1-2x10^6 cpm/reaction and H_2O.
- Comassie
- Ciclone

Procedure:

- Prepare a 11% polyacrimalide gel adding casein (0.5 mg/ml) in the running and the stacking gel.
- Prepare the samples mixing 40u µg of total protein with Laemmli buffer. Load the samples in the gel and run the electrophoresis at 25 mA.
- Afterwards, remove the SDS present in the gel washing 2 times for 30 min with buffer A at room temperature (RT).
- Then, rapidly rinse the gel in 50 mM Tris HCl pH 8.0
- Incubate the gel for 1 hour at room temperature in Buffer B
- Incubate the gel for 1 hour at room temperature in Buffer C
- Incubate overnight at 4ºC in Buffer D.
- Incubate the gel in a phosphorylation mixture for 1 hour with slight shaking.
- Wash the gel with the *Washing Buffer* 4 times for 20 minutes.
- The gel is stained with Comassie, dried and analysed by autoradiography with the instrument Cyclone Plus, Perkin Elmer. The time of exposition can vary from hours to days depending of the case.

3.2. Phosphorylation in vitro of Akt1 by CK2

The *in vitro* phosphorylation assay of a protein is a rapid method to know if a protein is phosphorylated by a specific kinase. In this study, we wanted to determine whether Akt1, was phosphorylated by different forms of CK2. For this purpose, purified recombinant Akt1 active, previously phosphorylated at Ser473 and Thr308 or inactive recombinant Akt1 form were incubated with different recombinant forms of CK2 (CK2α, CK2α', $CK2\alpha_2\beta_2$ and $CK2\alpha'_2\beta_2$) as well as with ATPɣ33. Afterwards, samples were run on an 11% SDS/PAGE, and radioactivity was detected by autoradiography. Before performing the assay, $CK2\alpha'_2\beta_2$ were reconstituted incubating 50 pmols of CK2α' and 50 pmols of CK2β in a buffer containing 50 mM Tris, pH 7,5, 150 mM NaCl i 0,1 % (1mg/ml) de BSA. It must be kept at 0-4ºC. Place at 30ºC for 5 min and then incubate O/N at 4ºC. The CK2 activity of $CK2\alpha'_2\beta_2$ was determined previously by activity kinase assays with the Ts-peptide and the eiF2β peptide.

Purified recombinant Akt (0.2-0.5 µg) was incubated with 10-20 units of recombinant CK2 (α or α2β2) where 1 unit is the amount of enzyme necessary to transfer 1 pmol of Pi to the peptide substrate in 1 minute. The recombinant Akt was incubated in the presense of 50 mM Tris-HCl, pH 7.5, 12 mM $MgCl_2$, 10 µM [γ-^{33}P]ATP (aprox. 1500 cpm/pmol), 0.1 M NaCl in a final volume of 20 µL, for 10 min at 30ºC.

Purified recombinant Akt (0.2–0.5 μg) was incubated with recombinant CK2 (α or $\alpha_2\beta_2$, 10–20 units, where 1 unit is the amount of enzyme transferring 1 pmol of Pi to the peptide substrate per min) in the presence of 50 mM Tris-HCl, pH 7.5, 12 mM $MgCl_2$, 10 μM [γ-^{33}P]ATP (~1500 cpm/pmol), 0.1 M NaCl, in a total volume of 20 μl, for 10 min at 30°C.

Materials and Reagents:

- TrisHCl (0.5 M, pH 7.4)
- $MgCl_2$ (0.1M)
- ATP (0,1mM)
- NaCl (0.1M)
- ATPγ33 (1-2x10^6 cpma/ reaction)
- Recombinant Akt1 active 0.3μg
- Recombinant Akt1 inactive 0.3μg
- Recombinant CK2α (1.25, 2.5 or 5 ng), CK2α' (40, 80 or 160 ng) $CK2\alpha_2\beta_2$ (2.5, 5 10 ng) and $CK2\alpha'_2\beta_2$ (25 ng).
- CK2-tide (1mM)

Procedure:

- Prepare the mix solution (50mM TrisHCl, 10mM $MgCl_2$, 0.1 mM ATP, 1M NaCl 0.1mM Peptide CK2-tide). It must be kept on ice.
- Add 0.3 μg of Akt1 active or inactive as well as different amount of recombinant CK2α (1.25, 2.5 and 5 ng), CK2α' (40, 80 and 160 ng), $CK2\alpha_2\beta_2$ (2.5, 5 and 10 ng) or 40 ng of $CK2\alpha'_2\beta_2$.
- Add ATPγ33 (1-2x10^6 cpma/ reaction) at the mix solution.
- Add 15 μL of mix solution in each tube and incubate at 30°C for 20 minutes.
- Add Laemmli buffer and heat at 100ºC to stop the reaction.
- Separate samples by SDS–PAGE (polyacrylamide gel electrophoresis).
- Stain the gel with Comassie and then dry the gel to analyse by autoradiography with the instrument Cyclone Plus, Perkin Elmer. Time of ranges from hours to days.

Results

Chapter 1

Protein Kinase CK2 modulates the response to HB-EGF

1.1. CK2 inhibitors affect Akt pathway activation in response to HB-EGF.

It is well known that PI3K/Akt and MAPK/ERK1/2 signal transduction pathways play important roles in cell growth and cell survival. Previous reports have shown that some of the proteins involved in Akt pathway are phosphorylated by CK2. Among them, PTEN is phosphorylated by CK2 what reduces its stability and results in Akt activation [223]. Moreover Akt activity is modulated by mTORC2 and PDK1, which phosphorylate Akt at Ser473 and Thr308 respectively, as well as by CK2, which phosphorylates Akt at Ser129 [86]. In addition, other protein kinases including Raf and p38 MAPK, which bind to or are phosphorylated by CK2, might also modulate Akt [224]. A wealth of evidences support the role of CK2 in Akt signaling pathway activation in response to different stimuli. However, no studies on the involvement of this protein kinase in the response to HB-EGF have been reported so far. The expression of HB-EGF is increased in the rat kidney after acute ischemic insult and HB-EGF is expressed predominantly in the ureteric bud during rat metanephric development [225]. These results suggest that HB-EGF would stimulate cell proliferation during kidney development and regeneration. Previous data from our group showed that HB-EGF activates Akt and ERK1/2 signaling pathways in HK-2 cells, in a similar or even greater way than EGF (Galeano N, Master Thesis UAB 2011).

The present study was aimed to determine the effect of CK2 in the activation of Akt and ERK1/2 signalling pathways in response to HB-EGF. As a first approach, several pharmacological inhibitors of CK2 and PI3K were used. HK-2 renal cells were pre-treated with Wortmannin (a specific PI3K inhibitor), LY294002 (an inhibitor of PI3K which also inhibits CK2) or Apigenin (a CK2 inhibitor which also inhibits PI3K), and, HB-EGF was added then. As observed in **Figure 7**, in the HK-2 cell line, Wortmaninn and LY294002 completely blocked both constitutive Akt Ser473 phosphorylation (pAkt-Ser473), and its inducible activation by HB-EGF. On the contrary, Apigenin only produced a partial inhibition of basal and activated pAkt-Ser473. Similar results obtained concerning the inhibition of GSK3β-Ser9 phosphorilation by these three compounds. In contrast, only Apigenin induced slight inhibition of ERK1/2 phosphorylation in response to HB-EGF.

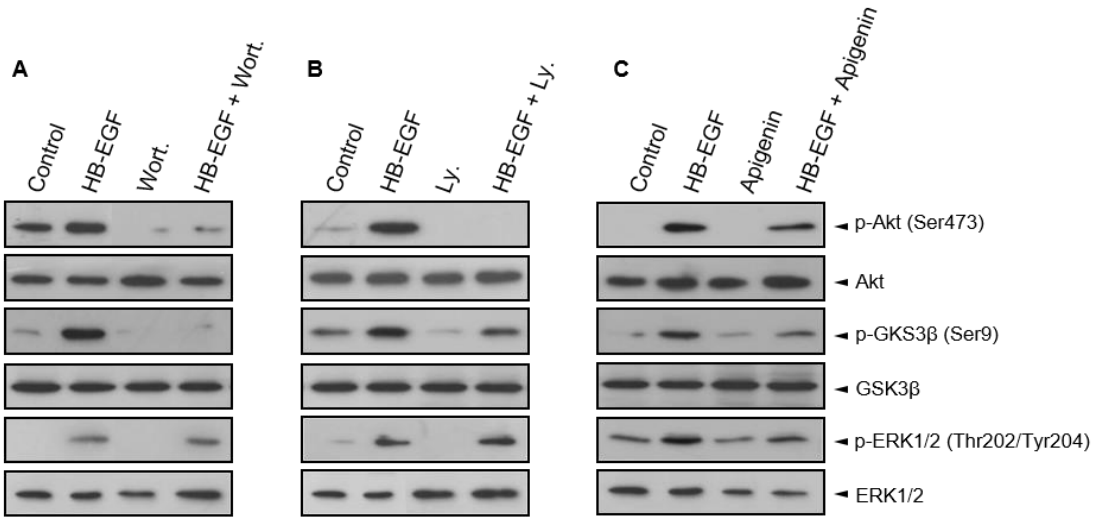


Figure 7. Effect of PI3K and CK2 inhibitors in the response to HB-EGF in Akt and ERK1/2 pathways in HK-2 cell line. HK-2 cells were seeded and after 24 h medium was replaced by serum starved medium for 16 h. Afterwards, cells were incubated for 30 min with different inhibitors (A) Wortmannin 100 nM, (B) LY294002 40 μM, and (C) Apigenin 40 μM and subsequently treated with HB-EGF (20 ng/ml) was added for 20 min. ERK and PKB activation were analysed by Western Blot using phospho-specific antibodies, as described in Materials and Methods.

To further understand the contribution of CK2 to these signal transduction pathways, we decided to test more specific CK2 inhibitors such as TBB and TBCAII. As can be seen from **Figure 8A**, cells treated with either TBB or TBCA showed a marked reduction in the p-Akt-Ser473 ,induced by HB-EGF, similar to that caused by Wortmannin or LY294002 (**Figure 7).**

Most of the studies on Akt response to different stimuli use only pAkt-Ser473 as an indicator of its activation. However, it is widely known that full activation of Akt requires both phosphorylation of Akt Ser473 and Akt Ser308. Due to that reason, we wondered whether HB-EGF induced phosphorylation of Akt at Thr308, and whether this process was affected by the treatment with CK2 inhibitors. As **Figure 8A** shows, HB-EGF induces Akt Thr308 activation, which is inhibited by TBB and TBCAII. It is interesting to remark that the inhibition is more marked in p-Thr308, than in p-Ser473. Moreover, the inhibition caused by TBB on p-AktSer473 and p-GSK3βSer9 is stronger the one observed with TBCAII. On the other hand, neither TBB nor TBCAII inhibits basal ERK1/2 phosphorylation and caused only a slight inhibition in response to HB-EGF.

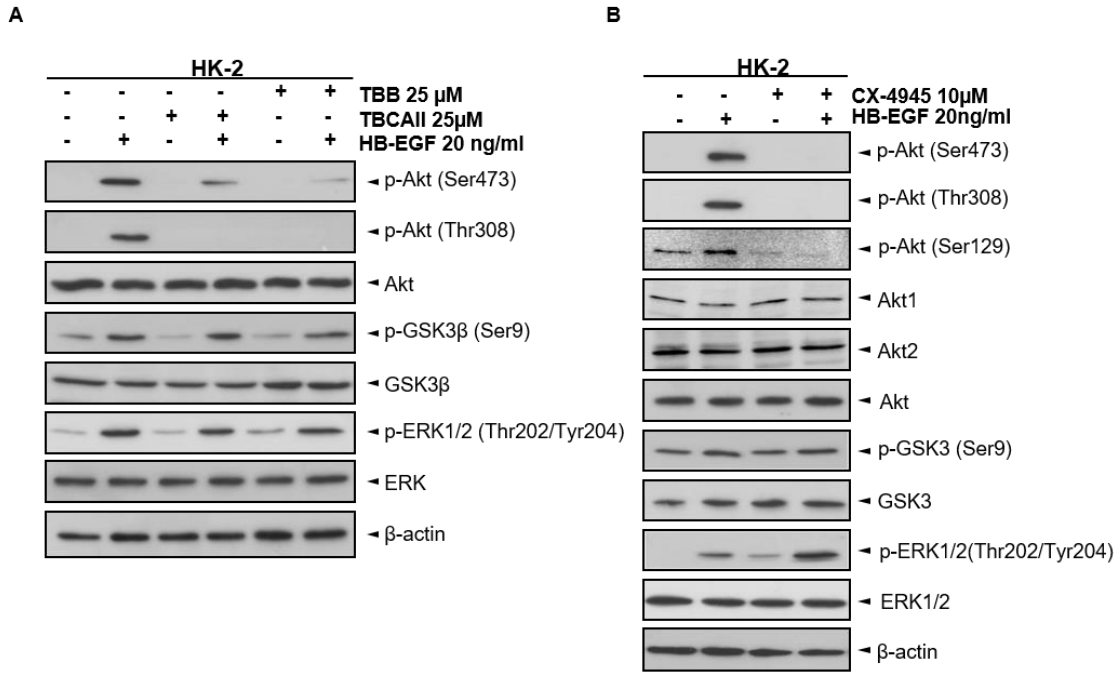


Figure 8. Effect of specific CK2 inhibitors in the response to HB-EGF in PI3K/Akt and ERK1/2 signalling pathway. HK-2 cells were seeded, and after 24 h medium was replaced by starving medium for 16 h. Cells were incubated with (A) TBB 25 μM or TBCAII 25 μM 30 min, or (B) CX-4945 for 8 h, and subsequently HB-EGF 20 ng/ml was added for 20 min. ERK and Akt stimulation were analysed by Western Blot using anti-phospho specific antibodies described in Materials and Methods.

While our studies were in progress, a new CK2 inhibitor was described,CX-4945, which shows a higher specificity for this kinase and is being used in pioneer phase I clinical trials with promising results in cancer therapy [217]. As observed with the other CK2 inhibitors, CX-4945 causes a blockage of Akt Ser473 and Akt Thr308 phosphorylation in response to HB-EGF. Recent reports have emphasised the role of CK2 in Akt Ser129 phosphorylation what had been shown to up-regulate its activity [86], [203].We wondered whether HB-EGF also affected Akt Ser129 phosphorylation and if CX-4945 blocked this response. **Figure 8B** shows that HB-EGF induces phosphorylation of Akt at Ser129 which is blocked by CX-4945. Moreover constitutive pAkt-Ser129 is also attenuated in response to CX-4945. On the other hand, CX-4945 has little effect on GSK3β Ser9 phosphorylation. Intriguingly, and in contrast to the inhibitory effects on Akt phosphorylation, CX- 4945 was found to cause an increase in both constitutive and HB-EGF-induced phosphorylation of ERK1/2.

HK-2 cell line was established from normal renal proximal convoluted tubular (PCT) cells and are non-tumorigenic. Several reports have shown that deregulation of Akt and ERK1/2 signalling pathways contributes to cancer progression. Therefore, we wondered whether the effects of the chemical inhibitors detected in HK-2 would also occur in the tumorigenic renal cell line 786-O. PI3K and CK2 inhibitors (Apigenin, TBB and TBCAII) affect Akt and ERK activation in response to HB-EGF in a similar way to that detected in HK-2 cells. The main differences between the two cell lines are the increased basal levels of pAkt-Ser473, and the higher resistance to inhibitors detected in 786-O cells with respect to HK-2 cells (**Figure 9 and 10B**). The most remarkable effects are observed with CX-4945. In 786-O cells, pAkt-Ser473 is little affected by CX-4945 whereas pAkt-Thr308 is strongly blocked by this inhibitor (**Figure 10B**). One of the most striking results emerging from our data is that 786-O cells show negligible Akt-Ser129 phosphorylation, and very low levels of Akt1 in contrast to those of Akt2. These results support the notion that Akt1, but not Akt2, is phosphorylated in the linker region by protein kinase CK2 [202].

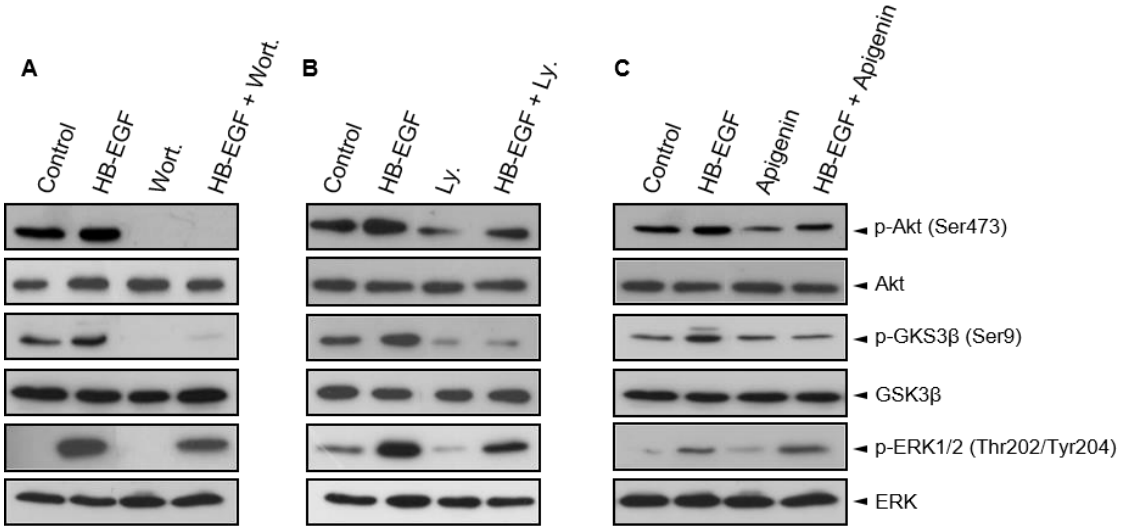


Figure 9. Effect of different inhibitors in the response to HB-EGF in Akt and ERK pathways in 786-O cell line. 786-O cells were seeded as indicated in methods, and after 24 h medium was replaced by starving medium for 16 h. Cells were pre-treated with (A) Wortmannin 100 nM (B) LY294002 40 μM and (C) Apigenin 40 μM for 30 minutes, and they were then incubated with HB-EGF 20 ng/ml for 20 min. ERK and PKB activation were analysed by Western Blot, using phospho-specific antibodies described in Materials and Methods.

Regarding the ERK1/2 pathway, the effects of the different chemical inhibitors tested were quite similar in both 786-O and HK-2 cell lines. Once again, CX-4945 increased basal phosphorylation as well as HB-EGF dependent ERK stimulation.

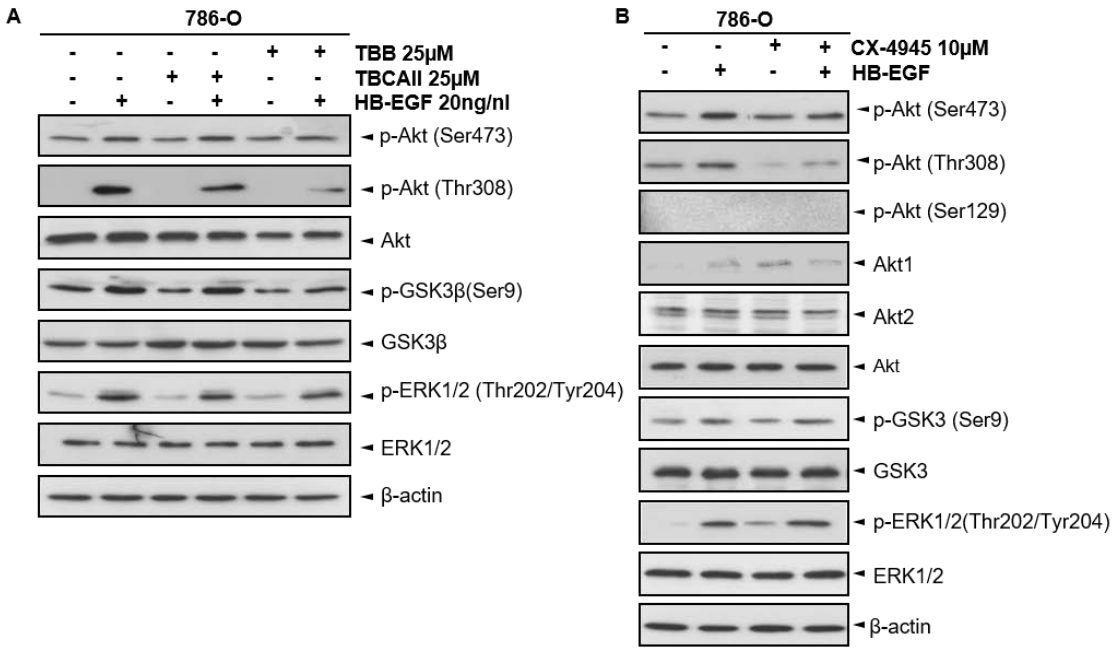


Figure 10. Effect of specific inhibitors of CK2 in response to HB-EGF in PI3K/Akt and ERK1/2 signalling pathway.786-O cells were seeded as indicated in methods, and after 24 h medium was replaced by starving medium for 16 h. Cells were incubated with (A) TBB 25 μM or TBCAII 25 μM for 30 min, or with (B) CX-4945 for 8 h. Afterwards, HB-EGF 20 ng/ml was added for 20 min. ERK1/2 and Akt stimulation were analysed by Western Blot using anti-phospho specific antibodies described in Material and Methods.

Taken together these results suggest that in HK-2 cells, CK2 inhibitors affect mainly Akt activation in response to HB-EGF. Moreover, the results in HK-2 cells support the idea that the changes in Akt Thr308 phosphorylation are influenced by alterations in pAkt-Ser129 levels in cell lines expressing Akt1 [203]. In 786-O cells, in contrast, the modulation of Akt Thr308 phosphorylation cannot be explained by changes in pAkt-Ser129, since these cells do not express Akt1, what suggests that, in the 786-O cell line, the decrease of p-Akt Thr308, caused by CK2 inhibitors, is mediated by different mechanisms.

1.2. Levels of CK2α, CK2α' and CK2β subunits expression and CK2 activity in renal cells HK-2 and 786-O.

This section progress to exploring the involvement of CK2 on the Akt activation induced by HB-EGF, using stable HK-2 and 786-O cell lines that express reduced levels of CK2α or CK2β. These cell lines were generated to express shRNA anti-CK2α or anti-CK2β and characterised by another member from our group (Vilardell J. Thesis UAB 2013). The first set of analysis was to determine the levels of CK2α, CK2α' and CK2β through Western Blot analysis. As observed in **Figure 11A,** in both cell lines, stable expression of shCK2α provokes a robust reduction of CK2α subunit, which is accompanied by a decrease of CK2β subunit. These results are in agreement with those obtained by other groups, which showed that, in other cell lines, depletion of both catalytic subunits cause a reduction of CK2β levels [197]. Stable expression of shCK2β in both HK-2 and 786-O cell lines, promotes a marked decrease of regulatory subunit CK2β. It is noteworthy that CK2 downregulation causes a strong reduction of CK2α', but not CK2α levels, as observed previously by Deshiere *et al.,* [197]. It is also interesting to note that all these effects are more evident in HK-2 cells than in 786-O cells.

It has been reported that CK2 substrates can be classified in three categories. Substrates which are phosphorylated both by CK2α/α' free subunits or by CK2 holoenzyme, substrates which are phosphorylated by the holoenzyme, but not by the free catalytic subunits, and substrates which are phosphorylated by the isolated catalytic subunits, but not by the holoenzyme [215]. Therefore, we performed kinase activity assays using two different peptides: Ts_peptide, which is specific for catalytic subunit (either free or being part of the holoenzyme), and eiF2β peptide, which is specific for CK2 holoenzyme. As shown in **Figure 11B**, downregulation of CK2α in HK-2 cells reduces the activity of CK2 against the Ts_peptide by almost 50%, whereas in 786-O cells the decrease is less marked. Moreover, the depletion of CK2β causes little effect on Ts_peptide phosphorylation, in both HK-2 and 786-O cells. On the other hand, the activity of CK2 on eiF2β peptide is noticeably affected in CK2β depleted cells, especially in HK-2, since the downregulation of CK2β subunit in these cells was stronger than in 786-O cells. (**Figure 11A**). The most interesting result emerging from this study is that, in both cell lines, depletion of CK2α does not affect significantly the activity on eiF2β peptide. This result suggests that CK2α', which remains in the cell after CK2α downregulation, must be part of CK2 holoenzyme.

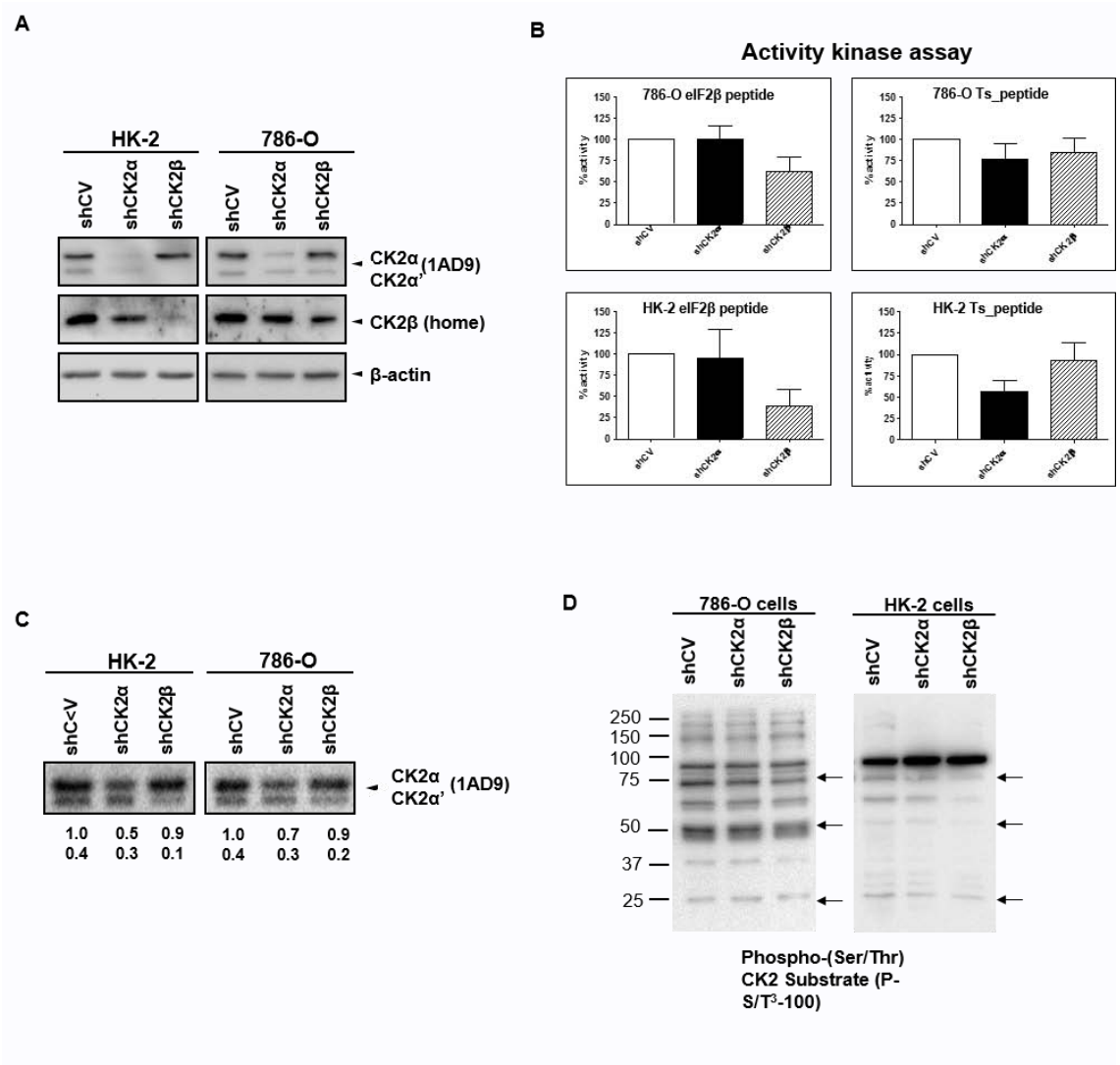


Figure 11. Measurement of CK2 expression and activity in 786-O and HK-2 cells. (A) Levels of CK2α and CK2β were analysed in HK-2 and 786-O stably silenced cells by Western Blot, using specific antibodies anti CK2α/CK2α' and CK2β. Antibody anti-βactin was used as loading control**.** (B) As detailed in Materials and Methods, 1μg of cell lysate proteins was analysed for CK2 activity against the specific peptide (Ts_peptide) and towards eiF2β peptide specific of the holoenzyme. The values are means ± SEM of 3 separate experiments. (C) The protein kinase activity of monomeric CK2α/α' was tested by an in-gel kinase assay. 40 μg of cell lysate protein of silenced cells were loaded on a polyacrylamide gel containing casein 0.5mg/ml. CK2α activity was detected as detailed in Materials and Methods. (D) 786-O and HK-2 cell lysates were analysed by Western blot using the Phospho-(Ser/Thr) CK2 Substrate (P-S/T^3-100) antibody**.** With arrows are highlighted the bands that are more affected by CK2β downregulation.

As an additional CK2 activity assay, we performed an in-gel kinase assay (**Figure 11C**). This technique consists on co-polymerization of β-casein, a CK2 type I substrate, within the gel matrix, followed by detection of the enzymatic activity in situ. The results obtained in this assay are in good agreement with those obtained by Western Blot and by activity kinase assay on the Ts_peptide. Moreover we analysed the pattern of substrates phosphorylated by CK2 in silenced cells, using an antibody that recognizes endogenous proteins containing a pS/pTDXE motif, which is a CK2 phosphorylation consensus sequence. As can be seen from **Figure 11D**, substrates phosphorylated by CK2 in 786-O cells were higher than in HK-2. Concerning the silencing of CK2, was evident that the depletion of CK2α, especially CK2β downregulation, decreased the phosphorylation of CK2 substrates, suggesting the requirement of CK2 holoenzyme or CK2α' for the phosphorylation of most substrates present in the cell. The effect was more evident in HK-2 cells, since this cell line showed lower levels of CK2 subunits (**Figure 11A**).

1.3. Effect of CK2α and CK2β depletion on Akt pathway in asynchronous growing cells

Our preliminary studies showed that CK2 inhibitors blocked Akt pathway, though the strength of this inhibition depend on the cell type. In order to go deeper into the study of the modulation of Akt by CK2, stable silenced cells were used. To this purpose, cells were seeded and grown in the complete medium containing 10% serum until 70-80% of confluence and then, different components of Akt pathway were analysed by Western Blot. As can be seen from **Figure 12**, control HK-2 cells (transfected with control vector or shCV-cells) showed detectable levels of Akt phosphorylation at Ser129, Thr308 and Ser473, likely due to the presence of growth factors in the serum.

Surprisingly, HK-2 cells silenced for CK2β subunit (shCK2β HK-2 cells) showed higher p-Akt Ser473 and p-GSK3β Ser9 levels, compared to control HK-2 cells. This effect was not evident in HK-2 cells silenced for CK2α (shCK2α HK-2 cells), which present similar levels of p-Akt Ser473 and p-GSK3β Ser9, or even slightly lower levels than those observed in control HK-2 cells. This unexpected result prompted us to study whether changes in p-Akt473 and p-GSK3β levels were directly consequence of CK2β silencing or might involve alterations in other proteins, such as PTEN. As observed from **Figure 12**, shCK2β HK-2 cells show lower levels of PTEN than control and shCK2α HK-2 cells. This result could explain the increase of Akt Ser473 and GSK3β Ser9 phosphorylation detected in HK-2 cells. On the other hand, in shCK2α HK-2 cells, and even more in shCK2β HK-2 cells, p-Akt Thr308 and p-Akt Ser129 levels are lower

than in control HK-2 cells. Similar studies were performed in 786-O silenced cells. As expected, in 786-O cells the levels of p-Akt Ser473 and p-GSK3β Ser9 are markedly higher than in HK-2 cells and they are not further increased after silencing of either CK2α or CK2β. In addition these cells exhibit high levels of p-AktThr308 even express low levels of p-Akt Ser129 and Akt1. The results obtained are likely to be related to the lack of PTEN in 786-O cells (**Figure 12**) [226].

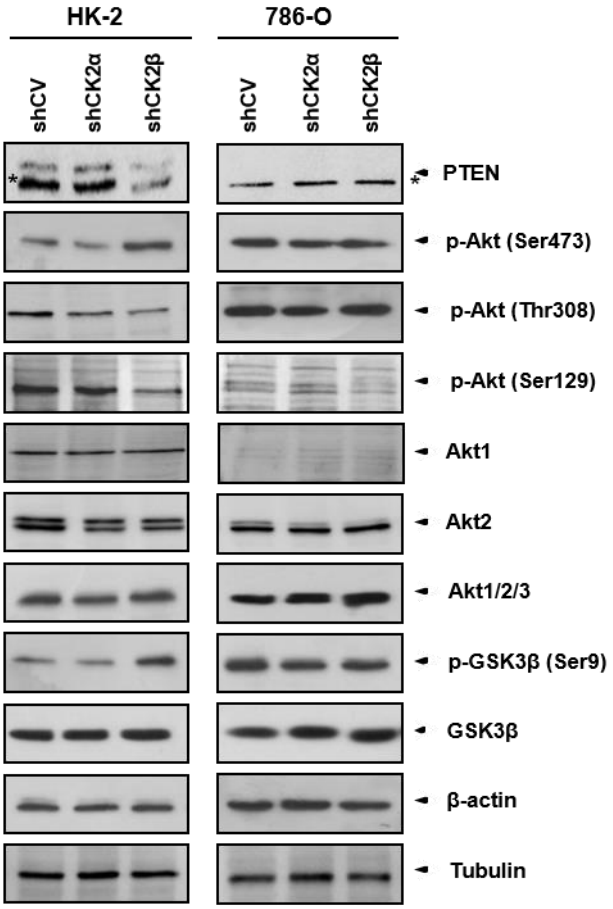


Figure 12. Effect of CK2 subunits downregulation in Akt/GSK3β pathway. HK-2 (left panel) and 786-O (right panel) control and silenced cells, were seeded and grown until 70-80 % confluence. Then cells were lysated and protein extracts were analysed by Western Blot to determine levels of components of Akt/GSK3β pathway. The antibodies used were indicated in *Material and Methods*. Tubulin and β-actin were used as protein loading control. Asterisks indicate non-specific bands.

1.4. Effect of CK2α and CK2β depletion on Akt pathway in response to HB-EGF

The next question in this research sought to determine the contribution of CK2 subunits in the activation of Akt in response to HB-EGF. In order to assess it, silenced cells were starved for 16 h and then treated with HB-EGF at different times. As it can be seen from **Figure 13A,** in HK-2 cells the strength and length of phosphorylation of Akt Ser473 and Akt Thr308 was weaker and shorter in silenced cells, this effect being particular evident in CK2β depleted cells. By contrast, in 786-O cells, downregulation of CK2 subunits caused only a slight inhibition of Akt Ser473 phosphorylation in response to HB-EGF whereas that the activation of Akt Thr308 was clearly decreased by CK2 downregulation, especially in CK2β depleted cells (**Figure 13B**)

As we mentioned above, Akt is phosphorylated by CK2 at Ser129. Thus we also studied the phosphorylation of Akt Ser129 in CK2 stable silenced HK-2 cells treated with HB-EGF. The first observation was that downregulation of CK2β decreased basal Akt Ser129 phosphorylation (**Figure 14A)**. In addition, HB-EGF induces phosphorylation in Akt Ser129 at 20 minutes, though this activation is attenuated by CK2 subunits depletion, mainly by CK2β downregulation. Similarly (**Figure 14B**), the response of Akt Ser129 to HB-EGF is lower and less sustained in CK2 downregulated cells, and this effect is particularly marked in CK2β silenced cells.

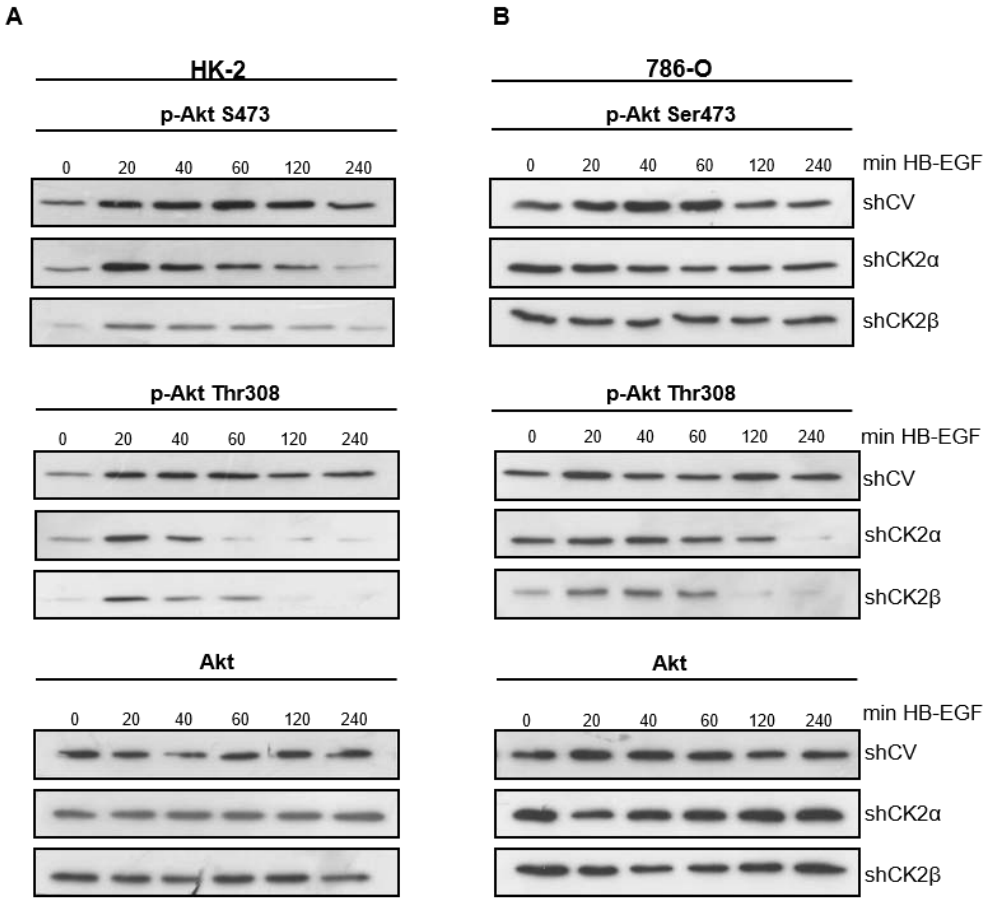


Figure 13. Involvement of CK2 subunits in the response of Akt in response to HB-EGF. (A) As described in Methods and Methods, HK-2(A) and 786-O (B) control (shCV) and silenced cells (shCK2α and shCK2β) were seeded and after 24 h the medium was replaced by starving medium for 16 h. Then cells were treated with HB-EGF 20 ng/ml at indicates time and then p-Akt Ser473, p-Akt Thr308 and Akt were analysed by Western Blot using specific antibodies as indicated in Materials and Methods. Figure is representative of 3 separate experiments.

Regarding 786-O cells, little phosphorylation of Ser129 was observed (**Figure 12)** due to the low expression of Akt1 in these cells. In order to improve the detection of Ser129 phosphorylation, we increased the exposure time of the blot by 10-fold as compared to HK-2 cells. Although a weak activation was then detected in control 786-O cells, the tendency is that CK2β depletion blocked the Akt Ser129 phosphorylation in response to HB-EGF in a similar way to that evidenced in HK-2 cells. (**Figure 14C**).

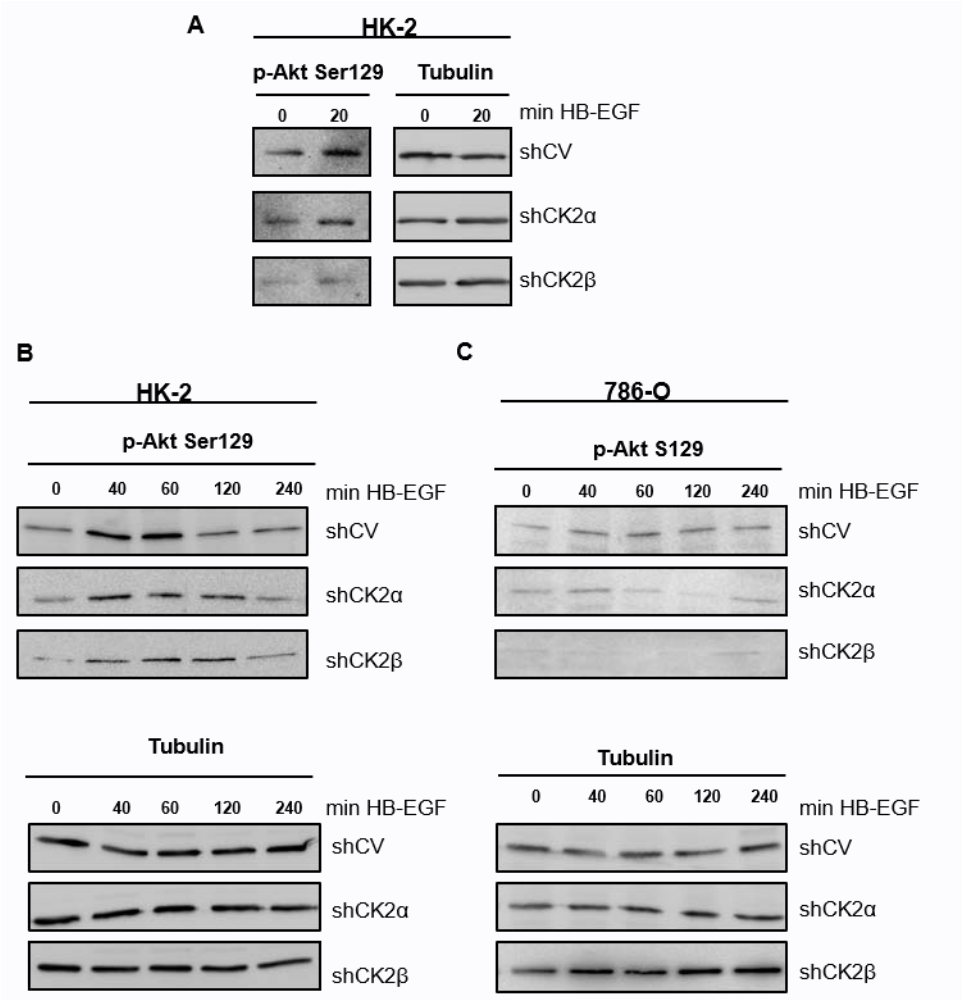


Figure 14. Involvement of CK2 subunits in Akt Ser129 activation in response to HB-EGF. Control (shCV) and silenced cells (shCK2α and shCK2β) were seeded and after 24 h medium was replaced by starving medium for 16 h, as described in Methods and Methods. Following it, HK-2 cells were treated with HB-EGF 20 ng/ml for (A) 20 minutes or (B) or 40, 60, 120 and 240 minutes or (C) 786-O cells were treated with HB-EGF 20 ng/ml for 20, 40, 120 and 240 minutes. Then p-Akt (Ser129) was analysed by Western Blot using specific antibody as described in Materials and Methods. Antibody anti-tubulin was used as loading control. The Figure is representative of 2 separate experiments.

1.5. Akt phosphorylation *in vitro* assays

The results obtained indicated that CK2β downregulation affected Akt phosphorylation. We wondered if this effect was due to a CK2β requirement for Akt phosphorylation, to differences in the ability of CK2α and CK2α' to phosphorylate it or to other factors. In a previous report, Ruzzene et al. (2005) indicated that Akt was phosphorylated *in vitro* by $CK2\alpha_2\beta_2$ and by isolated CK2α. However, no attempts were made to determinate which one of the CK2 forms (CK2α or CK2α', $CK2\alpha_2\beta_2$ or $CK2\alpha'_2\beta_2$) was more active on Akt. Thus, Akt1 *in vitro* phosphorylation assays were performed. As it can be seen from

Figure 15A, the active form of Akt1 (previously phosphorylated at Ser473 and Thr308) is phosphorylated by CK2α and CK2α' more efficiently than by CK2 holoenzyme. Under the same conditions, β-casein, which can be phosphorylated either by the free catalytic subunits or the holoenzyme, is similarly phosphorylated by CK2α, CK2α' and even greater by $CK2\alpha_2\beta_2$. To discard the possible auto-phosphorylation of activated Akt1, similar experiments were performed with inactive unphosphorylated Akt1. **Figure 15B** shows that CK2α is more efficient phosphorylating inactive Akt1 than CK2 holoenzyme, as occurred with active Akt. Then we decided to test the phosphorylation of active Akt1 by CK2 in the presence of NaCl to emulate the physiological conditions of cultured cells.

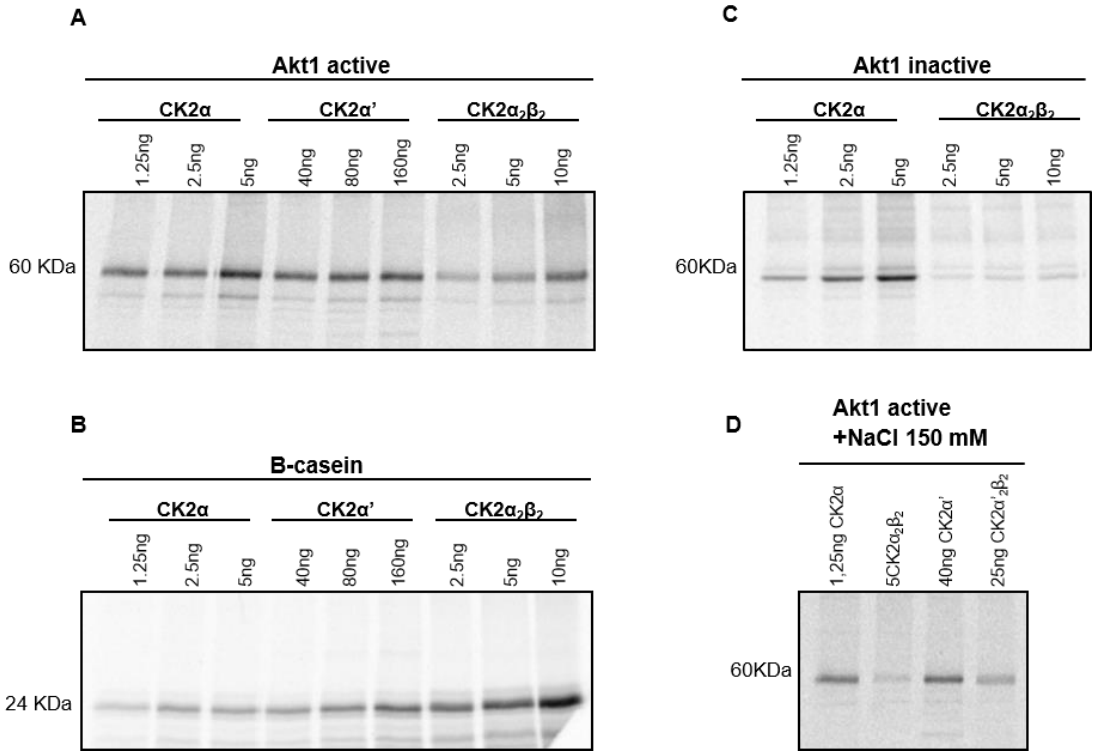


Figure 15. Phosphorylation *In vitro* assay of Akt by different forms of CK2. As described in Materials and Methods, Akt and the indicated form of protein kinase CK2, were incubated for 20 min in the radioactive phosphorylation mixture. (A) Different amounts of CK2α (1.25, 2.5 and 5 ng), CK2α' (40, 80 and 160 ng) or $CK2\alpha_2\beta_2$ (2.5, 5 and 10 ng) were incubated with 0.3 µg of active Akt1. (B) Under the same conditions, CK2α (1.25, 2.5 and 5 ng), CK2α' (40, 80 and 160 ng) or $CK2\alpha_2\beta_2$ (2.5, 5 and 10 ng) were incubated with 1 µg of β-casein. (C) 0.3 µg of inactive Akt1 was incubated with CK2α (1.25, 2.5 and 5 ng) or $CK2\alpha_2\beta_2$ (2.5, 5 and 10 ng). (D) 0.3 µg of active form of Akt was incubated with CK2α (1.25 ng), $CK2\alpha_2\beta_2$ (5 ng), CK2α' (40 ng) or $CK2\alpha'_2\beta_2$ (25 ng) in presence of NaCl 150Mm.The amount of the enzyme used was equally active against the specific peptide Ts_peptide and was determined by activity kinase assay. In all cases Akt phosphorylation was detected by autoradiography.

As observed in **Figure 15D**, the pattern of Akt phosphorylation is not altered by the presence of 150mM NaCl since either CK2α or CK2α' still phosphorylate Akt in a way greater than the corresponding holoenzyme ($CK2\alpha'_2\beta_2$ or $CK2\alpha_2\beta_2$). The results of this study indicate that the decrease in Akt1 Ser129 phosphorylation is not due to ability of the free CK2α to use it as a substrate.

1.6. The response to HB-EGF in ERK pathway is impaired by CK2 downregulation in renal cells

Different potential connections between CK2 and MAPK pathway have been reported previously by other groups (ref). We wondered if downregulation of CK2α and CK2β subunits in HK-2 and 786-O cells provoked alterations in HB-EGF-induced ERK activation. As observed in **Figure 16** in both cell lines the short-term activation induced by HB-EGF is slightly altered by CK2 downregulation. The most remarkable effect was detected in shCK2α HK-2 and 786-O cells whose ERK phosphorylation in response to HB-EGF is significantly less sustained than in control cells.

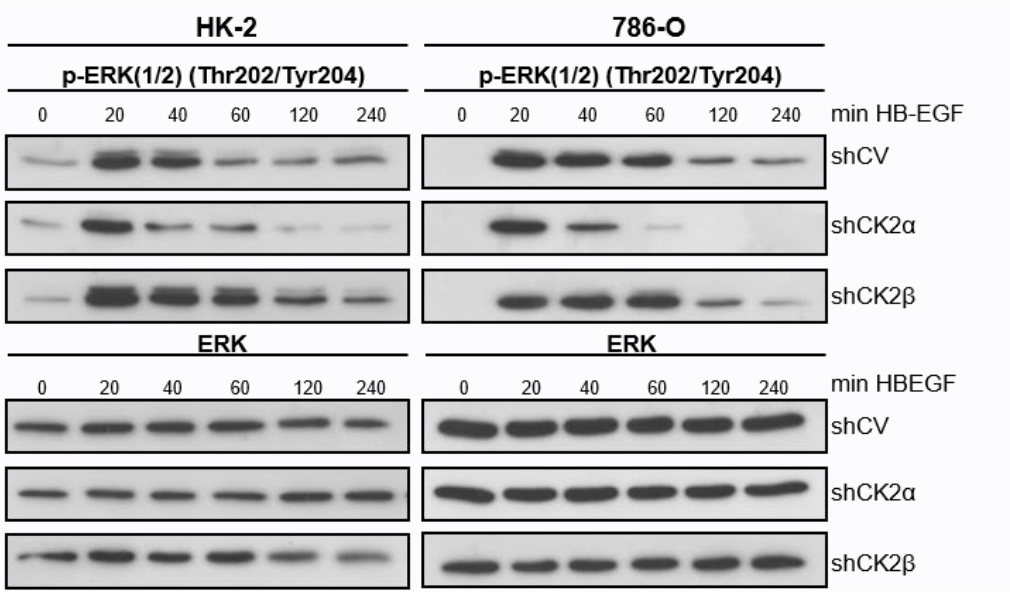


Figure 16. Involvement of CK2 subunits in the time response to HB-EGF in ERK1/2 signalling transduction. As described in Material and Methods, control (shCV) and silenced cells (shCK2α and shCK2β) were seeded and after 24 h medium was replaced by starving medium for 16 h. Following it, cells were treated with 20 ng/ml of HB-EGF for the indicated times. Then p-ERK and ERK were analysed by Western Blot, using specific antibodies as indicated in Materials and Methods. Figure is representative of 3 separate experiments.

In order to better define the time-course of the ERK response we treated the cells with HB-EGF at short times (5-20 minutes). Then, the levels of p-ERK1/2 were evaluated by Western Blot running the samples from HK-2 and 786-O cells in the same gel to allow

a direct comparison of the effects in both cell lines. **Figure 17A** shows that, in both cases the maximum response is achieved at 5 minutes, although the stimulation is more marked in 786-O than in HK-2 cells. Differences in ERK activation in response to HB-EGF between these cell lines can be explained by the fact that HK-2 cells express the Von Hippel-Lindau protein (VHL) whereas 786-O cells are VHL null. VHL is part of an E3 ubiquitin ligase complex that poly-ubiquitylates its targets and promotes their degradation. Several studies have described that loss of VHL prolongs the activation of epidermal growth factor receptor that is attributable to lengthened receptor half-life and retention in the endocytic pathway and consequently phospho-ERK signals last longer [227]. To verify whether VHL affected the response to HB-EGF we reconstituted VHL in 786-O cells as indicated in Material and Methods. As observed in **Figure 17B**, in 786-O/VHL cells the EGFR receptor is downregulated in response to HB-EGF whereas in 786-O/VHL null cells, EGFR receptor remains rather stable after HB-EGF stimulation. In agreement with this, pERK signal last longer and is more robust in in 786-O VHL null cells than in VHL reconstituted cells.

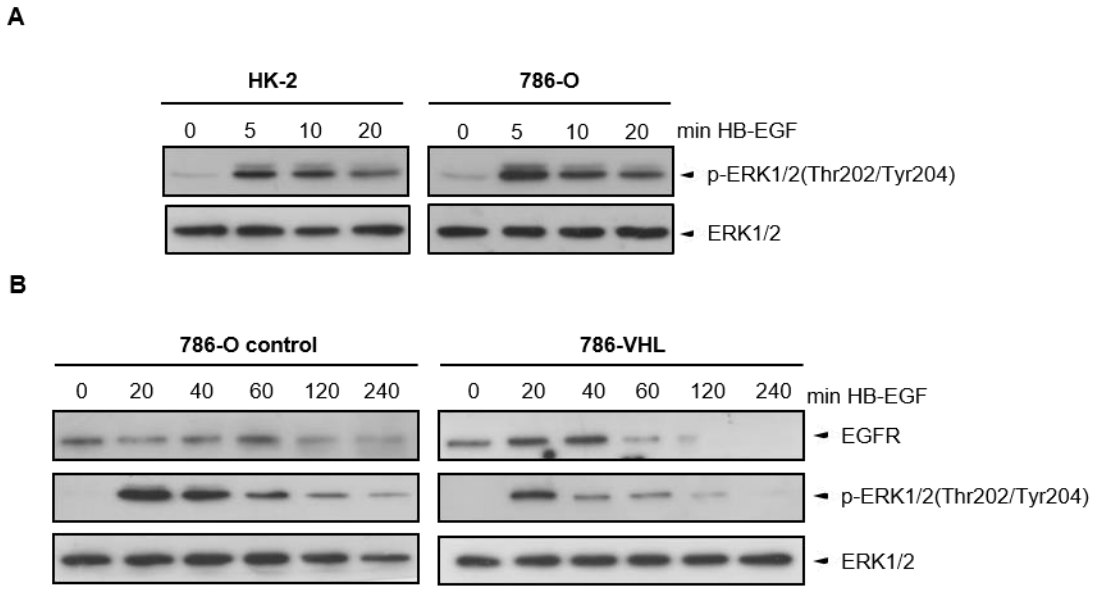


Figure 17. Connection within CK2 and other proteins are involved in ERK response to HB-EGF. (A) HK-2 and 786-O cells were seeded and after 24 h medium was replaced by starving medium for 16 h more. Following it, cells were treated with 20 ng/ml of HB-EGF for the indicated times. Then p-ERK and ERK levels were analysed by Western Blot, using specific antibodies. (B) 786-OVHL-null and 786-OVHL (reintroduced as described in Material and Methods) were seeded and after 24 h medium was replaced by starving medium for 16 h. Following it, cells were treated with 20 ng/ml of HB-EGF for the indicated times. EGFR, p-ERK and ERK levels were detected by Western Blot assay, using specific antibodies.

Chapter 2

Protein Kinase CK2 inhibition or downregulation compromises cell viability and induces autophagy

2. CK2 inhibition affects the viability of renal cells

It has been widely described that high levels of CK2 are associated with increased cell growth and proliferation as well as with suppression of apoptosis. Several studies have reported that inhibition of CK2 by chemical agents such as Apigenin or TBB, decreases cell viability in different cell lines [228], [229]. It was therefore interesting to determine the effect of TBB and CX-4945 in the viability of 786-O and HK-2 renal cells lines. As observed in **Figure 18**, MTT assays showed that TBB induces almost 50% of cell death at 8 h in both cell lines. Intriguingly, in 786-O and HK-2 cells the treatment with CX-4945, which has been reported to show broad spectrum anti-proliferative activity in multiple cancer cell lines, results only in a slight reduction of cell viability. An interesting observation in this study was the development of intracellular vacuoles induced by these inhibitors (**Figure 19**). Surprisingly, CX-4945 induced more and larger vacuoles than TBB and this effect was more evident in HK-2 cell line.

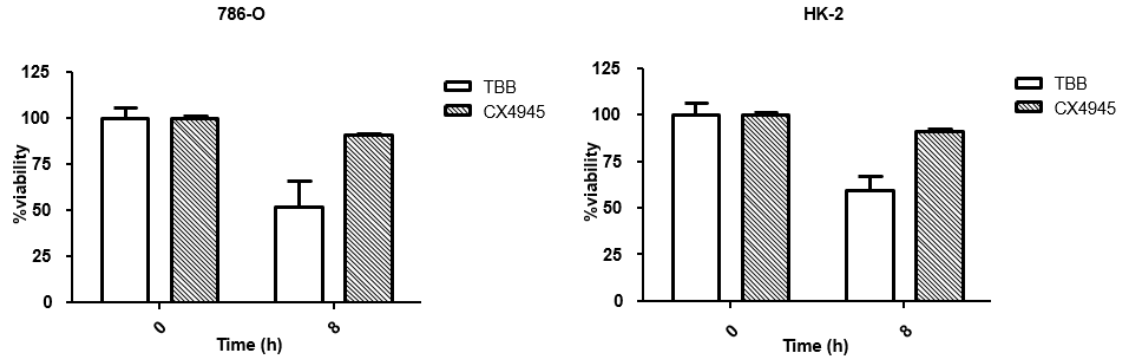


Figure 18. Toxicity of different CK2 inhibitors. 786-O (left) and HK-2 (right) cells were plated in 96-well plate (2.5 x 10^4 per well), and treated with TBB and CX-4945 for 8 h. Viability of 786-O and HK-2 cells was determined by MTT assay, and expressed as a percentage of inhibition of cell proliferation. The results are the mean from two independent experiment performed in triplicate, with errors bars representing the SEM.

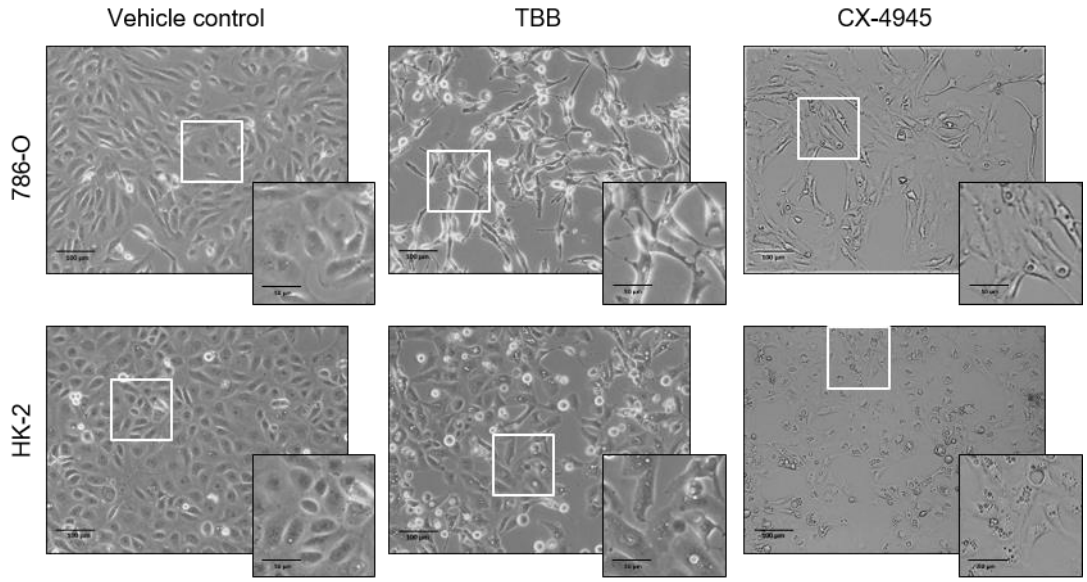
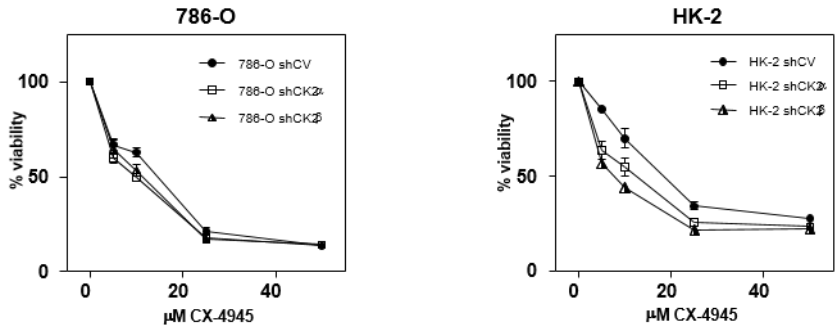


Figure 19. Morphologic alteration induced by CK2 inhibitors in HK-2 and 786-O cells. 786-O (upper pannel) and HK-2 (lower pannel) cells treated with CX-4945 10 μM or TBB 25 μM for 24 h. Images were captured by an inverted microscope. Scale bar = 100μm.

To further investigate the contribution of CK2 in cell viability, we performed proliferation assays in HK-2 and 786-O cell lines stably silenced for CK2α or CK2β subunits, in the absence and in the presence of CX-4945. As a first approach, we determined the IC50 of this inhibitor in these cell lines. To this purpose cells were treated with 4 doses of CX-4945 for 72 h. As observed in **Figure 20A and 20B** the concentration of CX-4945 required for 50% inhibition (IC50) is within 10-18μM**.** We decided, then, to use 10 μM CX-4945 to perform MTT proliferation assays.

A



B

786-O	shCV	shCK2α	shCK2β
IC50	17,11	13,54	13,11

HK-2	shCV	shCK2α	shCK2β
IC50	18	11,89	10,36

Figure 20. Toxicity of CX-4945 in 786-O and HK-2 CK2α and CK2β silenced cells. (A) Cells were plated in 96-well plate (2,5 x 10^4 cells per well) and treated at different concentrations of CX-4945 (5, 10, 25 and 50 µM) for 72h. Toxicity of CX-4945 was determinate by MTT assay as a percentage of inhibition of cell proliferation. The results are the mean of an independent experiment performed in quadruplicate, with error bars representing the SEM. (B) IC50 of CX-4945 in 786-O and HK-2 silenced cells was determinate through GraphPad Prism.

As observed in **Figure 21A,** downregulation of CK2α or CK2β in 786-O cells does not affect significantly their proliferation. Similarly (**Figure 22A**), no effect on proliferation was detected in HK-2 cells after CK2β downregulation. We wondered afterwards, whether downregulation of CK2 subunits potentiated the effect of CX-4945. As can be seen from **Figure 21A**, in 786-O cells, downregulation of CK2α or CK2β sensitizes cells to the effect of CX-4945, inducing a noticeable reduction in cell growth when compared to control cells (786-O shCV). Similar results are obtained in HK-2 cells, whose proliferation rate is drastically decreased in the presence of CX-4945(**Figure 22A**).

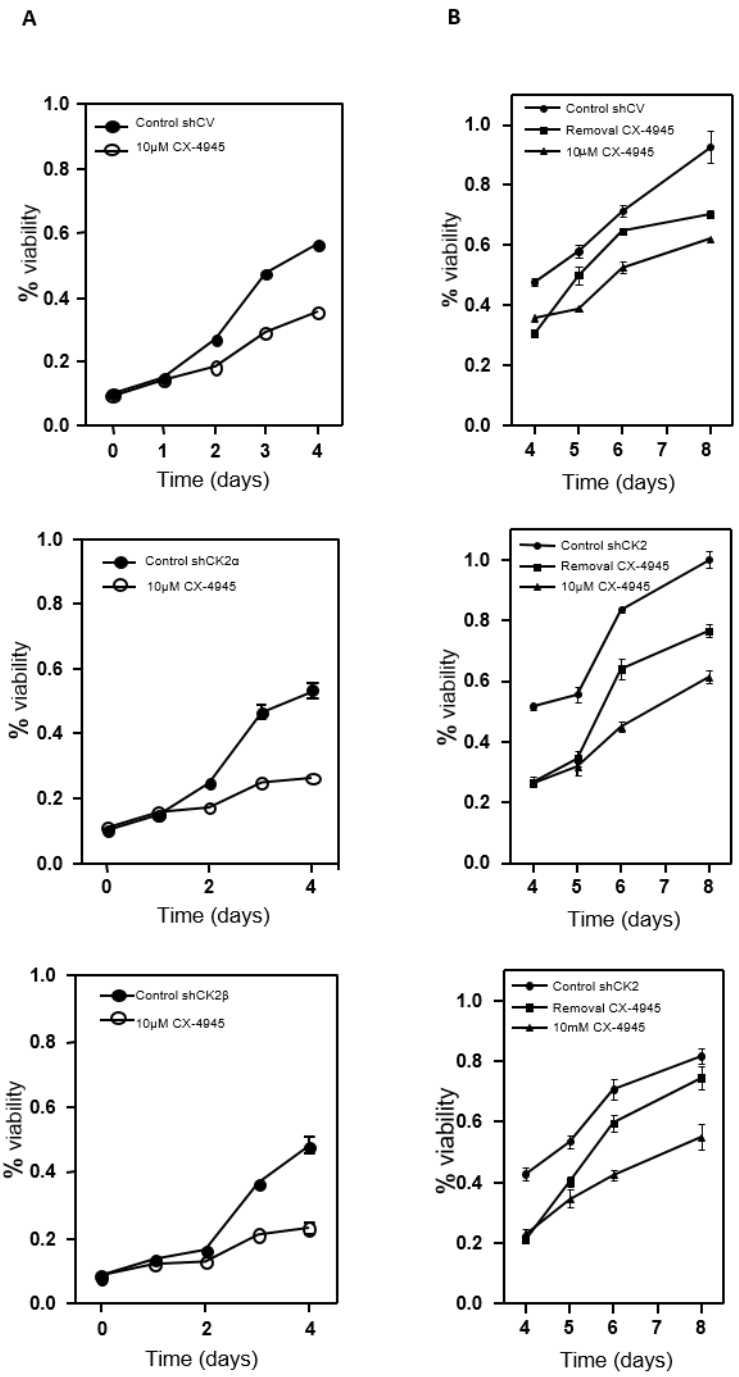


Figure 21. Toxicity of CX-4945 in 786-O cells downregulated for CK2 subunits. (A) 786-O control and silenced cells were plated in 96-well plate (2,5 x 10^4 cells per well), and treated with 10 μM of CX-4945, or vehicle control (DMSO) for 4 days. (B) After 4 days, the media was replaced by fresh medium supplemented with either 10μM CX-4945, or vehicle control (DMSO) for 4 additional days. The toxicity of CX-4945 was determined by MTT proliferation assays. The results are the mean from two experiment performed in quadruplicate, with error bars representing the SEM.

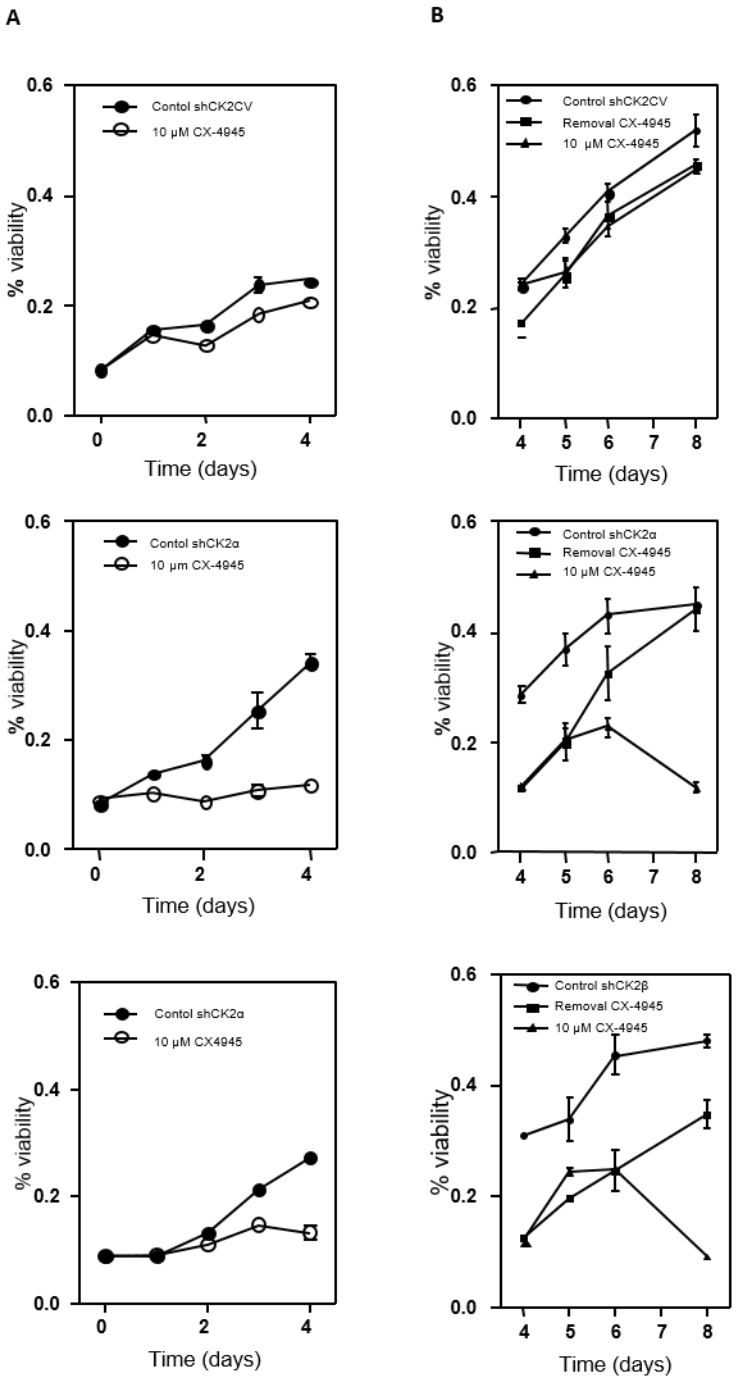


Figure 22. Toxicity of CX-4945 in HK-2 cells downregulated for CK2 subunits. (A) HK-2 control and silenced cells were plated in 96-well plate (2,5 x 10^4 cells per well), and treated with 10 µM of CX-4945, or vehicle control (DMSO), for 4 days. (B) After 4 days, the media was replaced by fresh medium, supplemented with 10 µM CX-4945, or vehicle control (DMSO), for 4 additional days. The toxicity of CX-4945 was determined, by MTT proliferation assays. The results are the mean from two experiment performed in quadruplicate, with error bars representing the SEM.

We decided afterwards, to test whether removal of CX-4945 after 4 days of incubation with this inhibitor would restored the cell proliferation rates. As observed in **Figure 21B**, 786-O cells recovered normal growth after CX-4945 removal, whereas those cells incubated in a medium still containing CX-4945 showed low proliferation rates. A similar tendency was detected in HK-2 cells (**Figure 22B**), although in CK2α and CK2β silenced cells long-term exposure to CX-4945 significantly reduced the percentage of viable cells, probably triggering cell death.

2.1. CK2 inhibition induces autophagy in renal cells

In recent years, the role of CK2 in the autophagy process has been described. Guerra B. *et al.* (2012) reported that CK2α downregulation induces autophagic cell death via mTOR and MAPK signaling pathways in human glioblastoma cells. Moreover, CX-4945 has been shown to induce autophagy in HeLa and LNCaP cells down-regulating Akt-mTOR-S6K signaling (ref). However, more research on this topic needs to be undertaken before the association between CK2 and autophagy is clearly understood. The observation that CX-4945 caused growth inhibition, and the formation of intracellular vacuoles in renal cells may support the hypothesis that CK2 is involved in autophagy mechanism. In order to test it, we further analyzed vacuole formation in renal cell lines silenced for different CK2 subunits. As observed in **Figure 23**, in 786-O cells neither CK2α nor CK2β downregulation induce vacuole formation. However, the treatment with CX-4945 generates small vacuoles in all these three cell lines, which was more evident in CK2α or CK2β depleted cells than in the control cell line.

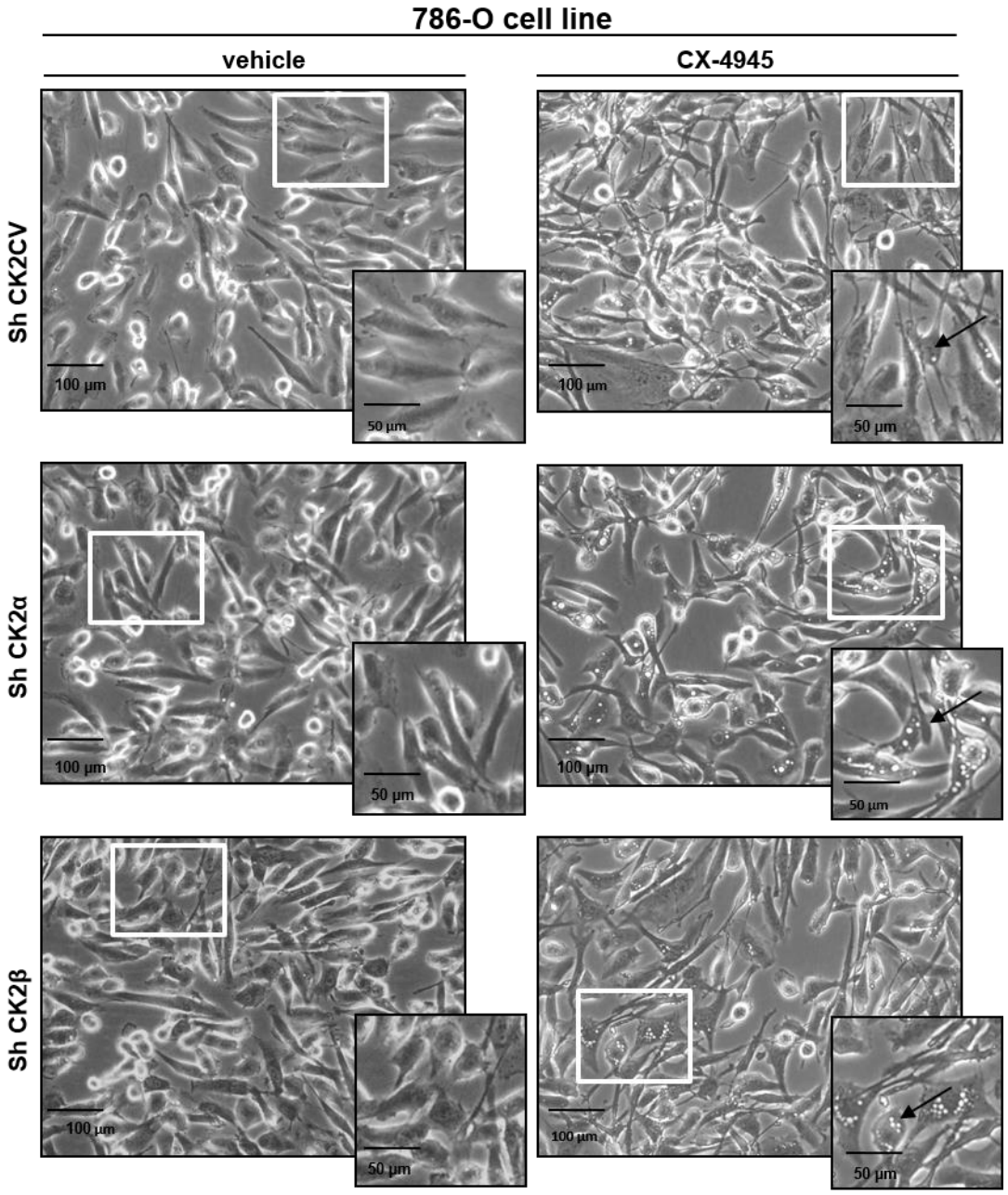


Figure 23. Morphological changes induced by CX-4945 in 786-O cells silenced for CK2α and CK2β subunits. Representative inverted microscopy images of control and CK2α and CK2β silenced 786-O cell line. Cells were seeded in a 6-well plate at 10 x 10^6 cells per well, and were treated with vehicle (DMSO) (left panel) or 10 µM CX-4945 for 8 h (right panel). Scale bar = 100 µm.

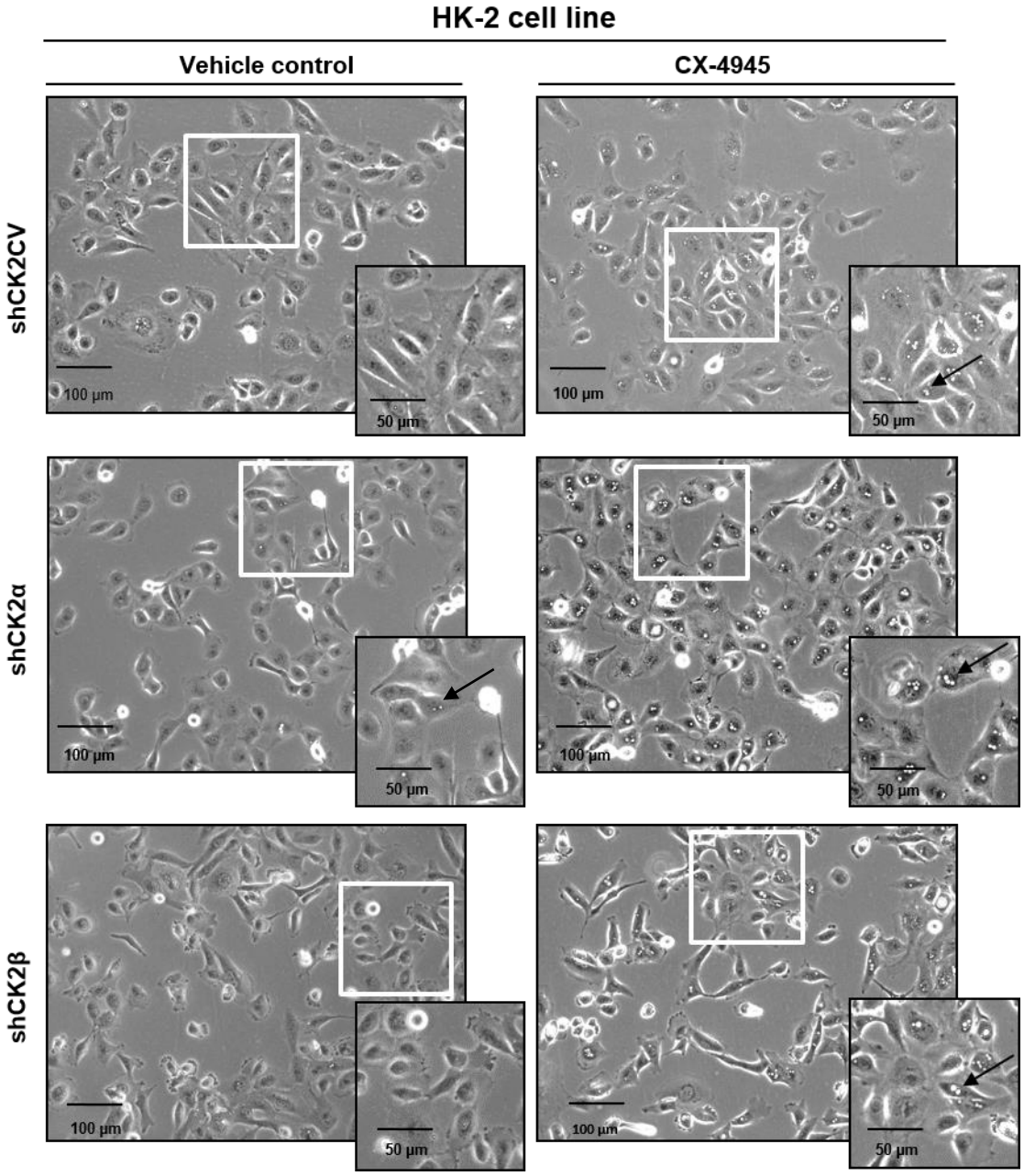


Figure 24. Morphological changes induced by CX-4945 in cells silenced for CK2α and CK2β subunits. Representative inverted microscopy images of control and silenced HK-2 cells**.** 10 x 10^5 of control and silenced cells, were seeded in a 6-well plate, and were treated with vehicle (DMSO) (left panel) and 10 µM CX-4945 for 8 h (right panel). Scale bar = 100 µm.

Exposure to CX-4945 also induced formation of vacuoles in HK-2 control and silenced cell lines, and this effect was more marked in CK2α silenced cells (**Figure 24**, right panel). In fact, the sole downregulation of CK2α already induces tiny vacuoles in HK-2 cells (**Figure 24**, left panel). The results obtained in both cell lines, indicated that downregulation of CK2α sensitized the cells to the effects of CX-4945. Moreover, CX-

4945 promotes more vacuole formation in HK-2 than in 786-O cells. The high sensibility of the CK2 silenced HK-2 cells to CX-4945 was confirmed in further studies using different doses of this inhibitor. As **Figure 25** shows**,** 1 μM of CX-4945 was enough to induce the formation of tiny vacuoles in CK2-silenced cell lines, in particular in CK2α, but not in control cells (shCV). The same tendency was observed in cells treated with 5 μM of this inhibitor, confirming that downregulation of CK2, especially CK2α, sensitized cells to the effect of CX-4945.

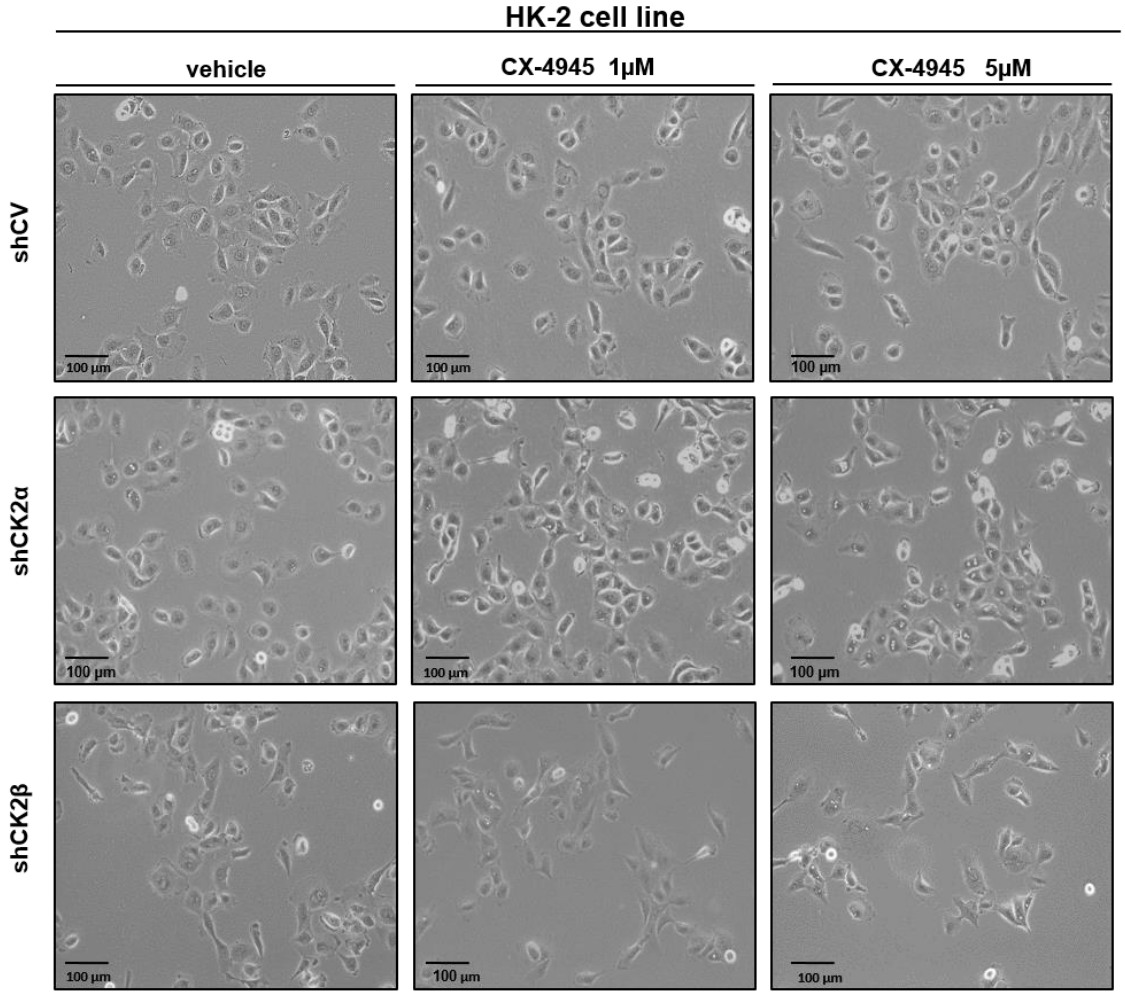


Figure 25. Differential effects induced by CX-4945 on vacuole formation in control and silenced HK-2 cells. Control, CK2α and CK2β silenced cells were seeded (10 x 10^5 cells per well) and then treated with vehicle (DMSO), 1 μM and 5 μM of CX-4945 for 8 h. Cells were then visualized by phase-contrast microscopy. Scale bar = 100 μm.

Cumulative evidences support lipogenesis as a part of the malignant process of RCC [230], as well as in many other cancers [231]. In fact, the histological appearance of clear-cell RCC is derived from the cellular accumulation of vesicles, containing glycogen and lipids. To further study the origin of these vacuoles, we stained the cells

with nile red (a colorant used for the detection of lipids in cells). As observed in **Figure 26**, the colorant stained the cellular membrane, as well as the membrane of the vacuoles, but it did not accumulate inside the vesicles. This observation indicates that the vesicles formed upon CX-4945 exposure are not filled with lipids, and suggest the idea that CX-4945 induces autophagy.

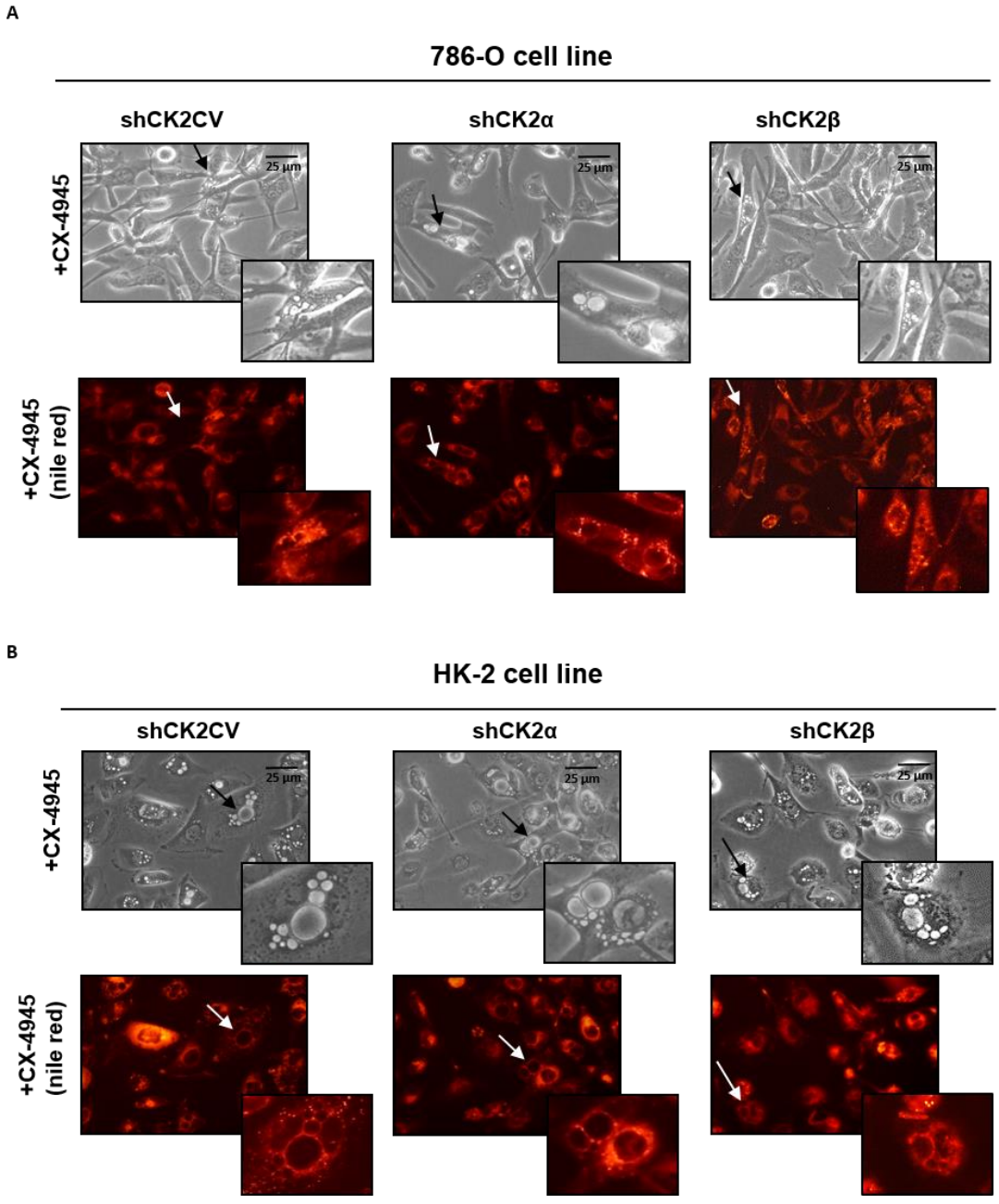


Figure 26. Nile red fluorescence of 786-O and HK-2 cells. Control, CK2α and CK2β silenced cells (786-O upper panel, and HK-2 lower panel), were seeded and treated with 10 µM CX-4945. Afterwards, cells were treated with nile red (0.1 µg/ml) for 10 minutes. The fluorescence was viewed at Ex/Em =552/636 nm with a fluorescence microscope. Scale bars = 25 µM.

To discern whether the CX-4945 induced formation of vacuoles was due to autophagy, we determined the autophagasomal marker Microtubule-associated protein 1A/1B-light chain 3 (LC3). LC3 is a soluble protein distributed ubiquitously in mammalian tissues and cultured cells. During autophagy, autophagosomes engulf cytoplasmic components, and the cytosolic form of LC3 (LC3-I) is conjugated to phosphatidylethanolamine and the LC3-phosphatidylethanolamine form (LC3-II) is recruited to autophagosomal membranes. LC3-II is next degraded in the autolysosomal lumen, as well as the intra-autophagosomal components. As can be seen from **Figure 27**, CK2α but not CK2β depletion, causes a slight increase in LC3-II in the presence of lysosomal inhibitors. Moreover, the LC3-I conjugation to form LC3-II is always more evident after CX-4945 treatment. Interestingly, LC3-II is more clearly detected in HK-2 than in 786-O cells, as it occurred with the vacuole formation seen in our previous studies (**Figure 24**). Differences between cell lines can be explained through divergences in Akt pathway. As **Figure 27** shows, in HK-2 cells, CX-4945 exposure reduces Akt Ser473 and Thr308 phosphorylation, whereas in 786-O this inhibitor does not block Akt Ser473 phosphorylation, and produces only a slight reduction of Akt Thr308 phosphorylation. It has also been reported that ERK1/2 pathway regulate autophagy [232]. In order to understand whether ERK1/2 is involved in CX-4945 mediated vacuole formation, we analyzed pERK1/2 levels. As shown in **Figure 27,** CX-4945 does not affect ERK phosphorylation in 786-O. By contrast, CX-4945 increases moderately p-ERK1/2 levels in control and CK2 silenced HK-2 cells. Taken together, these results give further support to the idea of the involvement of CK2 controlling autophagy, by acting, either directly, or through alterations in Akt and ERK1/2 signaling pathways.

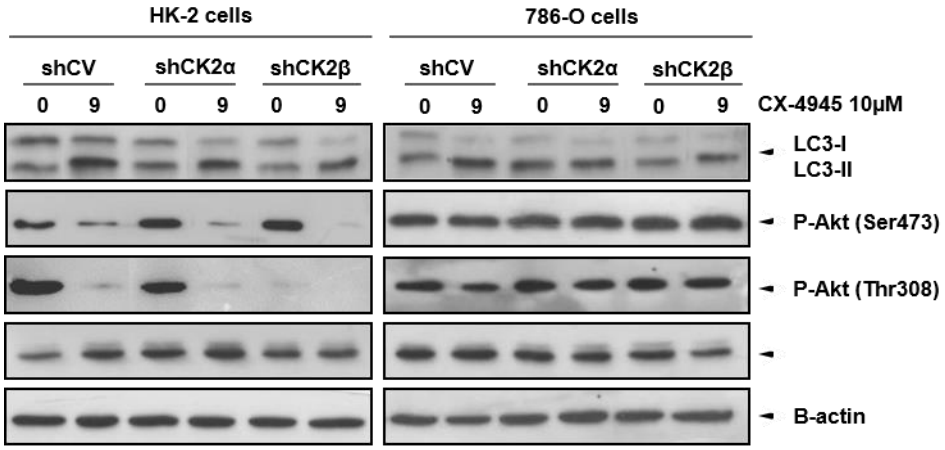


Figure 27. Effect of CX-4945 in LC3-II accumulation. HK-2 (left panel) and 786-O (right panel) control and CK2α and CK2β silenced cells were plated and pre-treated with lysosomes inhibitors for 1 h (Leupeptin 20 μM and Pepastin A 20 μM). After that, 10 μM CX-4945 was added for 9 h. LC3-II accumulation, ERK and Akt inhibition were then analyzed by Western Blot using specific antibodies which have been described in Materials and Methods.

Chapter 3

Role of CK2 in Epithelial Mesenchymal Transition

3. CK2α- or CK2β-silencing alters the expression of E-cadherin and snail 1 in HK-2 and 786-o cells.

Recent reports have shown that CK2β silencing induces an epithelial to mesenchymal transition (EMT) phenotype in breast cell lines [197]. E- and N-cadherin are well-known EMT markers. Control HK-2 cells express significant levels of E-cadherin whereas tumoral 786-O cells express very low levels of this protein. Due to the low expression of E-cadherin, we increased the exposure time of the blot by 5-fold as compared to HK-2 cells. In both cell lines, CK2β-silencing causes a marked decrease in E-cadherin giving support to their acquisition of a mesenchymal phenotype. Moreover, CK2α-silencing also promoted a decrease in E-cadherin, but the effects were less marked that those of CK2β-silencing. CK2α-silencing also results in a marked decrease in CK2β levels, what might promote the decrease in E-cadherin (**Figure 28**). The acquisition of a mesenchymal-like phenotype, with a decrease in E-cadherin levels, has been shown to occur in MCF10A cells after treatment with CK2αα' siRNA [197], what also promoted a marked decrease in CK2β levels.

Snail 1 is a well-known repressor of E-cadherin expression and a substrate for CK2. We wondered whether Snail 1 levels correlated with the changes in E-cadherin expression observed in HK-2 and 786-O cells after CK2 silencing. In both cells lines, CK2β silencing causes an increase in Snail 1 protein levels, although both basal and stimulated levels are more robust in 786-O than in HK-2 cells. Intermediate increases in Snail 1 are detected in both cell lines after.

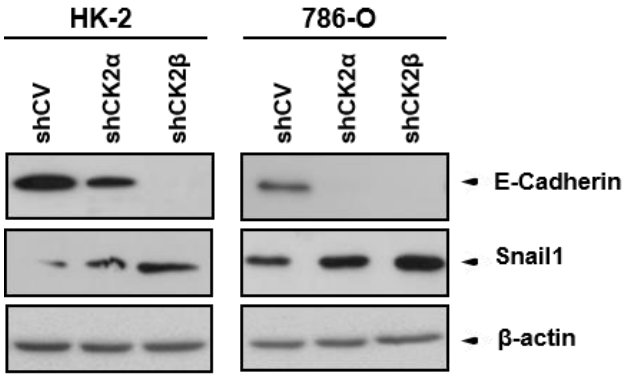


Figure 28. Expression of EMT markers in downregulated CK2α and CK2β cells. HK-2 (left panel) and 786-O (right panel) control (shCV) and silenced cells (shCK2α and shCK2β) were grown until 80-90% confluence and then E-cadherin and Snail1 were analysed by Western Blot using the indicated antibodies described in *Material and Methods*. β-actin was used as a loading control.

3.1. GSK3β connects CK2β silencing with Snail1

Several studies have reported that GSK3β plays an important role in EMT through regulating transcription factors involved in this process. An example of this is the report showing that GSK3β regulates Snail1 and β-catenin expression in gastrointestinal cancer [233]. Deshire *et al.,*[197] have shown that in MCF10A cells the stability of Snail 1 protein was controlled through hierarchical phosphorylation by CK2 and GSK3β, a process that depended strongly on CK2β levels. These observations and the results obtained in Chapter 1 on the increased phosphorylation of GSK3β in CK2β downregulation HK-2 cells, encouraged us to explore the potential link between CK2β/GSK3β/snail1/E-cadherin. We decided to test the effect of increasing CK2β levels in HK-2 cells. In the aim to reduce the effect of the shCK2β expressed in HK-2/shCK2β cells, we transfected the cells with a vector containing a synthetic CK2β, coding sequence optimized for its expression in human cells and carrying base mutations that would weaken its recognition by the shCK2β without altering the primary structure of the expressed protein. As observed in **Figure 29A**, forced expression of CK2β in control and shCK2α HK-2 cells do not significantly alter CK2α and CK2α' levels and causes only small changes in snail1 and E-cadherin.

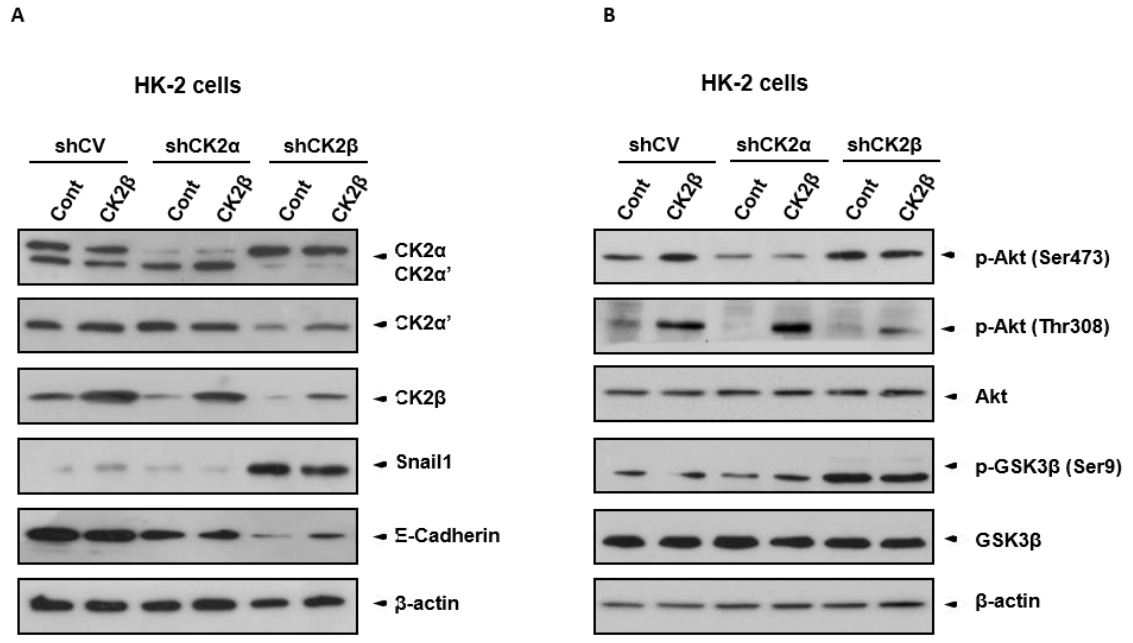


Figure 29. Study of CK2β overexpression in HK-2 control and silenced cells. HK-2 shCV (control) and shCK2α and CK2β (silenced cells), were transfected with a synthetic vector CK2β as indicated in *Material and Methods*. After 65 h, cell lysates were examined for (A) CK2α/α', CK2α', CK2β, Snail1, E-cadherin, and (B) p-AKT Ser473, AKT, p-GSK3β Ser9 and GSK3β by Western Blot using specific antibodies as indicated in *Material and Methods*. β-actin was used as the protein loading control.

A positive effect of CK2β on CK2α' level is evidenced in transfected HK-2/shCK2β cells in spite of the lower CK2β expression levels attained in these cells. Interestingly, a light decrease of p-Akt Ser473 levels and an increased in Akt Thr308 phosphorylation is evident in transfected shCK2β. Moreover, decreases in p-GSK3β and Snail1 together with a small increase in E-cadherin levels are detected in the CK2β transfected HK-2/shCK2β cells (**Figure 26B**).

Exposure of cells to the PI3K pathway inhibitor LY294002 is known to decrease p-GSK3β Ser9 levels [234]. As expected, exposure of HK-2 or 786-O to this inhibitor cause a decrease in their p-GSK3β Ser9 levels and this is accompanied by a marked decrease in Snail1 levels. Surprisingly, the decrease in Snail1 levels is also detected in cells whose CK2β levels were reduced as a consequence of CK2β-silencing (**Figure 30A and 30B**). These unexpected results question the requirement of CK2β for the control of snail stability in this type of renal cell lines. On the other hand, the decreases in Snail1 detected after exposure to LY294002 are not reflected in increases in E-cadherin expression, suggesting the involvement of other transcription factors in the control of E-cadherin expression in these renal cell lines.

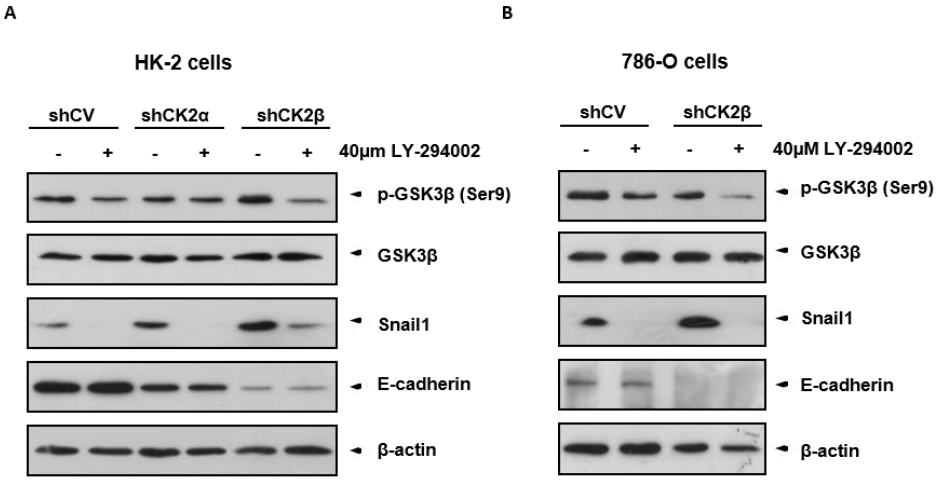


Figure 30. Effect of LY-294002 inhibitor in the expression of EMT markers altered by CK2 downregulation. (A) ShCV, shCK2α and shCK2β HK-2 cells (left panel) and (B) shCV and shCK2β 786-O cells (right panel), were treated with LY-294002 at the concentration of 40 μM for 8 h. Then protein extracts were examined for Snail1, E-cadherin, p-GSK3β Ser9 and GSK3β by Western Blot using specific antibodies as indicated in *Material and Methods*. β-actin was used as the protein loading control.

3.2. CK2β-silencing up-regulates the expression of HIF-1α in HK-2 and of HIF-2α in 786-O cells.

Although it is known that GSK3β controls epithelial-mesenchymal transition (EMT), the lack of increases in p-GSK3β Ser9 in CK2β down-regulated 786-O cells (**Figure 12**) suggested that other factors, besides GSK3β, would contribute to the effects detected after CK2 silencing (**Figure 28**). Previous reports have shown that the expression of EMT markers are upregulated by HIFα in 786-O and other renal cell lines [235], [236]. Therefore we decided to explore the potential effects of CK2α and CK2β-silencing on HIFα expression in HK-2 and 786-O cells under the normoxic conditions used throughout our studies. As observed in **Figure 31A,** increase in HIF-1α expression is detected in HK-2 silenced cells, in particular in CK2β downregulated cells. Similarly, downregulation of CK2 subunits, especially CK2β, increase the expression of HIF-2α in 786-O cells (**Figure 31B**).

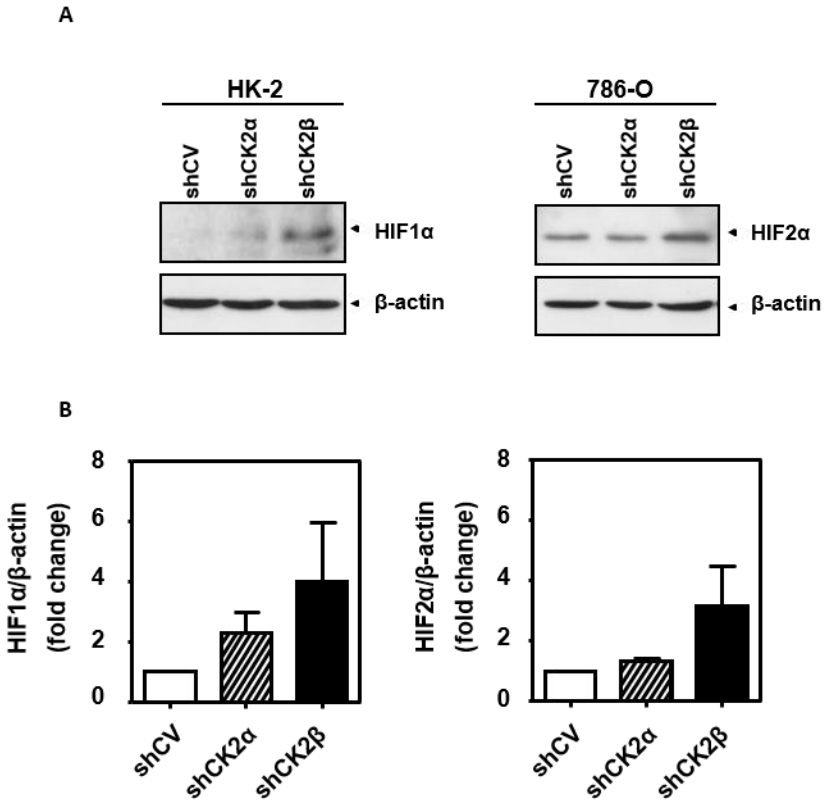


Figure 31. Effect of CK2 downregulation on HIF expression. (A) HK-2 and 786-O, control and silenced cells, were grown in normoxic conditions until 80-90% confluence. Then, HIF levels were determined by Western Blot, using specifics antibodies anti HIF2α in 786-O cells, and anti HIF1α in HK-2 cells. (B) The graph shows densitometric quantification of western blot bands of HIF1α and HIF2α levels normalized to β-actin. Data is shown as the media ± SD of 2 different experiments.

To ensure that these effects were caused by downregulation of the $\alpha_2\beta_2$ form of CK2 or the increase in free CK2α, we overexpressed CK2 subunits in 786-O cells. As can be seen from **Figure 32A,** transfection of control 786-O cells with the CK2α expression vector increased the levels of CK2α but unexpectedly this was accompanied by an increment of CKβ levels. Therefore, the form of CK2 overexpressed in these cells would probably correspond to $\alpha_2\beta_2$. Interestingly, under this conditions a notably reduction of HIF-2α expression was detected. However, this result did not clarify if CK2β was required for the regulation of HIFs. We decided to overexpress CK2β in control and CK2β-silenced cells. As observed in **Figure 32B,** CK2β overexpression results in a reduction of HIF-2α levels in 786-O CK2β silenced cells. Overall, these results indicate that CK2β (as CK2 holoenzyme) might contribute to the regulation of HIFs which may influence the induction of EMT.

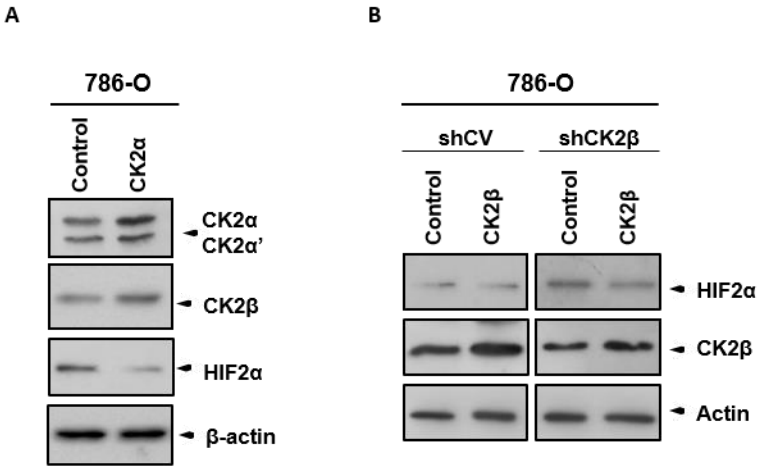


Figure 32. Effect of CK2 overexpression on HIF expression. (A) 786-O cells were transfected with a CK2α plasmid as indicated in Material and Methods. After 65 h, protein extracts were examined by Wester Blotting to analyse CK2α/α', CK2β and HIF2α expression, using specific antibodies as indicated. (B) shCV and shCK2β 786-O cells, were transfected with a CK2β plasmid as indicated in *Material and Methods*. After 65 h, protein extracts were examined by Wester Blotting to analyse CK2β and HIF2α expression, using specific antibodies as described in *Material and Methods*. In all experiments β-actin was used as the protein loading control.

3.3. VHL does not block the CK2 downregulation effects on HIF2-α expression

In the majority of clear cell renal cell carcinomas (ccRCC), HIF is constitutively active by inactivation of the von Hippel-Lindau gene (*vhl*). Previous studies have described an association between CK2α and VHL protein (VHL30). Indeed, CK2α subunit phosphorylates *in vitro* VHL30 in its N-terminal acidic domain. We asked if reintroduction of VHL30 wild type interfered with or abolished the HIF2-α response to downregulation of CK2 subunits. To this purpose we made use of 786-O cell lines stably transfected to express VHL30 wt or a non- functional VHL-truncated form corresponding to residues 1-167 which harbors the CK2 phosphorylation sites but lack the elongin-binding domain. These cell lines were generated in our laboratory by another member of the group (Garcia-Madrid A. Master Thesis, UAB 2011). As observed in **Figure 33**, HIF2-α expression levels were considerable lower in cells transfected with VHL30 than in cells expressing VHL167. In addition, reintroduction of pVHL30 recovers E-cadherin levels, whereas reintroduction of VHL167 is not sufficient to rescue E-cadherin expression.

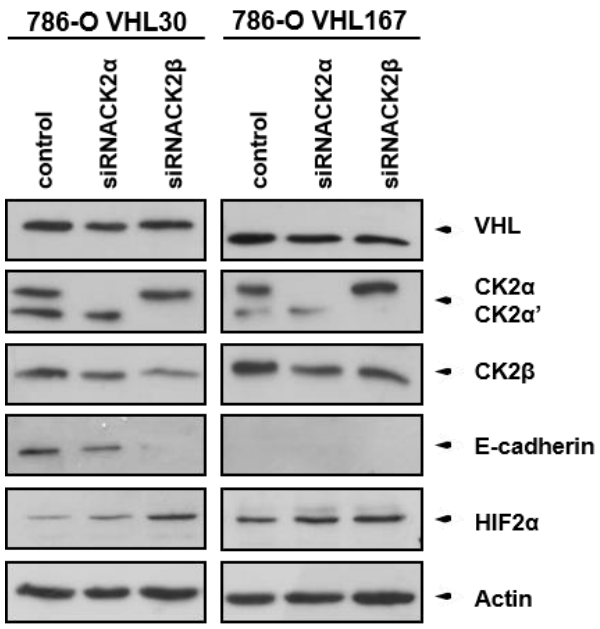


Figure 33. Effect of CK2 downregulation by siRNAs in VHL expression. VHL30 (left panel) and VHL167 (right panel) proteins were re-expressed in 786-O cells as described in *Material and Methods*. Cells were grown until 80-90 % confluence and were transfected with 25 nM of siRNACK2α and 25nM of siRNACK2β. After 65 h cells lysates were collected and analysed by Western Blot to examine the levels of VHL, CK2α/α', CK2β, E-cadherin and HIF2α. β-actin was used as a protein loading control.

Then, we decided to explore the effects of siRNA mediated CK2 subunits downregulation in these cell lines. As expected from the results with stably CK2 silenced cells, transient CK2α downregulation also reduces CK2β expression. On the other hand, transient CK2β downregulation decreases CK2α' but not CK2α levels (**Figure 33**). As can be seen from **Figure 33**, transient downregulation of both subunits, in particular CK2β decreases HIF2-α expression levels either in cells that express VHL30 or in cells that express non-functional VHL167. It is also evident that 786-O/VHL30-CK2 silenced cells, in particular CK2β downregulated cells, express lower levels of E-cadherin than control 786-O/VHL cells, supporting the results obtained with stably silenced cells. Taken together, these results support the notion that the effects of CK2 downregulation on HIF-2α changes are independent of the VHL protein.

An interesting observation from this study was that the decreased expression of CK2 subunits did not significantly alter the VHL30 and VHL167 expression levels. A previous report indicated that exposure of HeLa cells to the CK2 inhibitor TBB abolished the decrease in VHL expression induced by hypoxia, but it had no effect on VHL levels under normoxic conditions [214] . On the other hand, Ampofo *et al.,* [237] reported that under normoxic conditions TBB increased VHL protein stability in HEK293T cells which had been transiently transfected to express HA-VHL30. Therefore, we decided to test the effect of CK2 inhibition by chemical agents (TBB and CX-4945) on VHL stability in 786-O/VHL30 cells. The results in **Figure 34** demonstrates that no significant differences in VHL expression levels were found at short-term exposure to either TBB or CX-4945. What agrees with previous reports. Intriguingly, long-term exposure to CX-4945, but not to TBB, lead to undetectable levels of VHL (**Figure 34**). It is interesting to note that the CX-4945 induced effect on VHL stability was dose dependent and particularly marked in serum-starved cells. Overall, these results suggest that the mechanism by which the stability of VHL is affected might involve other processes altered by the long-term exposure to CX-4945, what adds a word of caution on the use of this inhibitor,

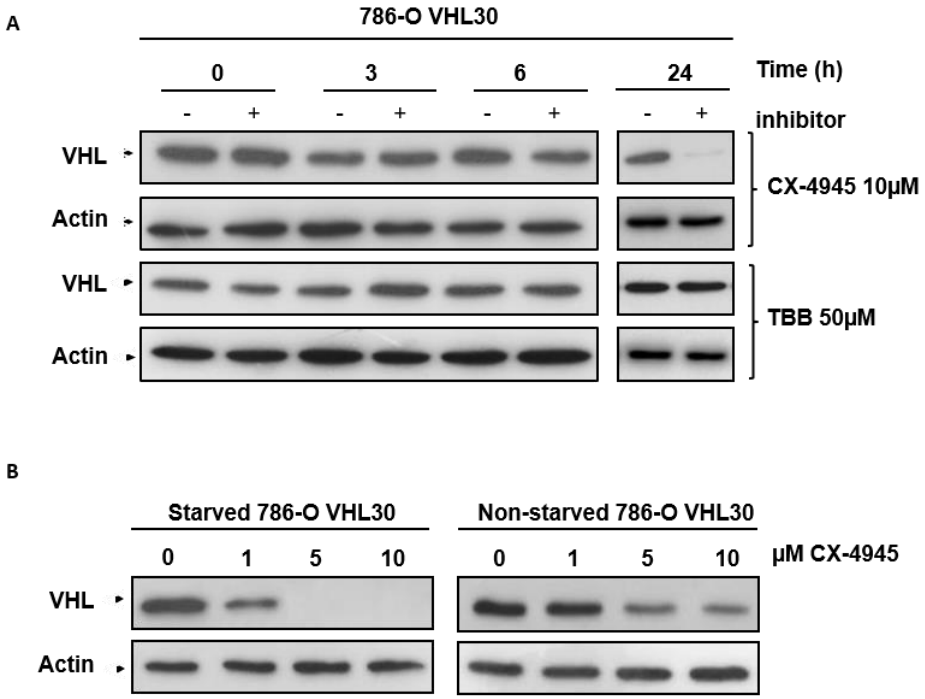


Figure 34. Effect of CK2 inhibitors in VHL stability. (A) 786-O/VHL30 cells were plated in a 6-well plate (10x10^5 cells per well), and then treated with CX-4945 10 μM or TBB 50 μM for different times (3,6 and 24h). Then the cell lysate was collected and was analysed by Western Blot to assess the levels of VHL protein. (B) 786-O/ VHL30 cells were plated in a 6-well plate (10x10^5 cells per well). After 24 h medium from 1 plate was replaced by starving medium. After 16 h cells from both plates were lysate and VHL levels were analysed by Western Blot using a specific antibody as indicated in *Material and Methods*. β-actin was used as protein loading control.

To verify that CK2 did not affect VHL levels and that the effects of CX-4945 on VHL stability were mediated by other processes altered, we determined the levels of this protein in HK-2 silenced cells and in the HEK293T cell line known to express VHL (**Figure 35**). Established cell lines and cancer cells shown a preferential expression of the 19KDa form of VHL, together with several different band in the range of 37-50KDa corresponds to post-translational modification of VHL such as ubiquitylation. Moreover, silencing of CK2 subunits does not significantly affect the levels of VHL. The pattern of VHL detected in HEK293T and HK-2 cells, fits quite well with that reported for other cell lines.

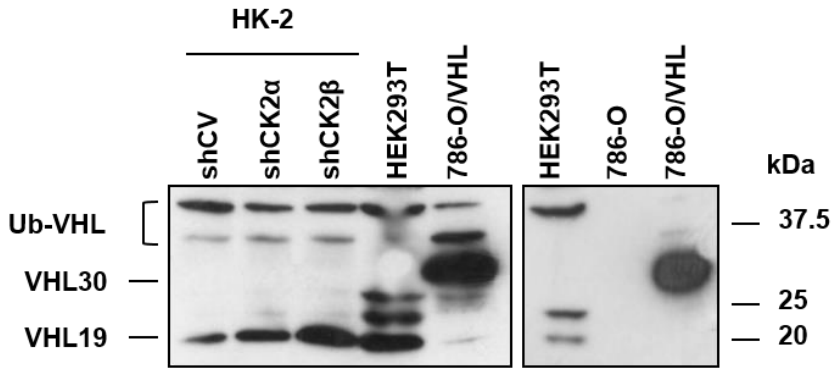


Figure 35. Expression of endogenous VHL in a different cell lines. Cell were seeded at 2x10^5 cells/ml under normoxic conditions. After 24 h, were lysated and total protein were analysed by Western Blot to determinate VHL levels using the specific anti-VHL antibody described in Material and Methods.

3.4. STAT3 pathway is altered by CK2

Activator of transcription STAT-3 is a potent regulator of HIF-1α and HIF-2α expression [238] as well as a positive modulator of HIF-1α activity [112], [239]. STAT-3 activity is reflected through its phosphorylation at Tyr-705 and Ser-727 residues, both of which are increased in ccRCC tumours. Previous reports have shown that exposure of cells to CK2 chemical inhibitors (which block the activity of both CK2 holoenzyme and free catalytic subunits) diminished STAT-3 phosphorylation in response to different stimuli.

These evidences and our previous results, prompted us to investigate whether the changes in HIFs expression may involve alterations in STAT3 phosphorylation. **Figure 36A** shows that in HK-2 cells, downregulation of CK2 subunits increase pSTAT-3 Tyr705 levels but the strongest increment was detected in CK2β-silenced cells. A similar effects were observed in 786-O cell line. In contrast, difference response were observed in both cell line concerning STAT-3 Ser727 phosphorylation. In HK-2 cells, which are not tumorigenic, phosphorylation of STAT3 at Ser727 decreased after CK2α and CK2β silencing. On the other hand, in the tumorigenic 786-O cell line, downregulation of CK2α, and, in particular CK2β, induced a slight increment in STAT3 Ser727 phosphorylation. These results are consistent with the previous report from T. Cuadros *et al.*,[21] showing that STAT3 Ser727 phosphorylation plays an important role in STAT3 activation and is an independent prognostic factor in ccRCC.

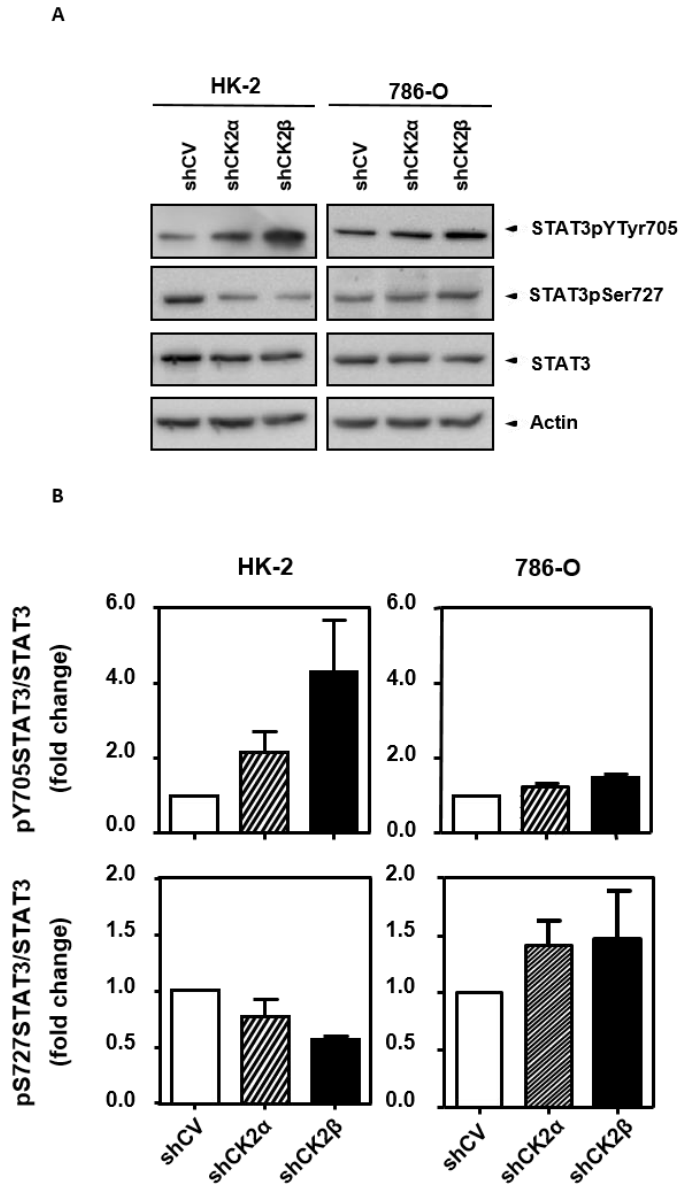


Figure 36. Study of STAT3 pathway in downregulated CK2 cells. (A) shCV, shCK2α, shCK2β HK-2 and 786-O cells, were grown until 70-80% confluence. Then cell lysates were analysed by Western Blot to examine STAT3 pathway (STATpTyr705, STATpSer727 and STAT3), using specific antibodies described in Material and Methods. (B) Quantification of STAT3pTyr705 and STAT3pSer727 normalized to STAT3. The results were quantified using ImageJ software and are expressed as the mean ± SEM of 3 different experiments.

Chapter 4

ErbB4 receptor in the response to HB-EGF and its connection with CK2.

4.1. ErbB4 over expression promotes Akt activation in 786-O cells

It is well known that HB-EGF binds to and activates Epidermal Growth Factor Receptor (EGFR) and Erb-b2 receptor tyrosine kinase 4 (ErbB4/HER4). ErbB4 activation can promote a wide range of cellular responses, including proliferation, differentiation, migration, cell survival and growth arrest, depending on the cell type, the isoform of ErbB4 expressed and the experimental conditions [240]. The aim of this study was to explore the relationship between CK2 and ErbB4. To this purpose we generated a 786-O cell line stably transfected to express ErbB4 JM-a/Cyt2, the predominant ErbB4 isoform expressed in kidney. As a first approach, we wanted to examine the effect of ErbB4 JM-a/Cyt-2 expression on Akt pathway. As observed in **Figure 37**, 786-O cells express no detectable endogenous level of ErbB4 compared with stable transfected 786-O-erbB4 cell line. Moreover p-Akt Ser473, p-Akt Thr308 and p-GSK3β Ser9 levels are higher in cells that express ErbB4. In addition,the low E-cadherin levels that express 786-O cells are totally abrogated and Snail1 levels markedly increased by the expression of ErbB4 receptor. These results give support to a correlation between the Akt/GSK3β activation and E-cadherin / Snail1 expression.

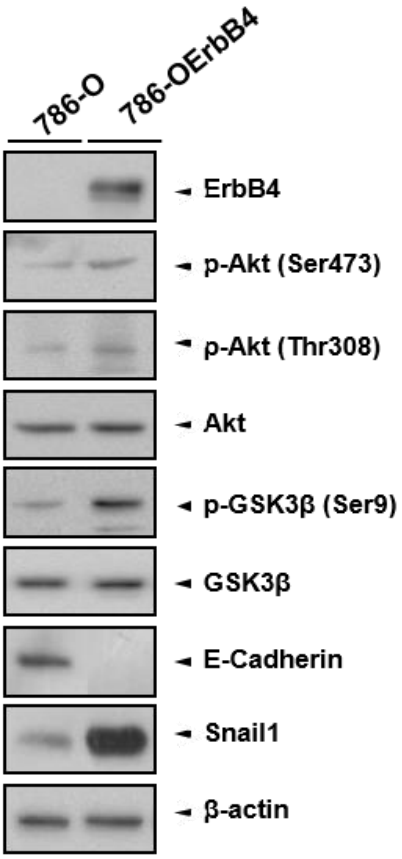


Figure 37. Study of Akt pathway and EMT markers in 786-O cells that express ErbB4 receptor. Cells were seeded at 10 x 10^5 cells per well. After 24 hours cell were lysated, and cell extracts were analyzed by Western Blot, using specific antibodies to detect ErbB4, p-Akt Ser473, p-Akt Thr308, Akt, p-GSK3β, GSK3β, E-cadherin, and Snail1. β-actin was used as a protein loading control.

4.2. ErbB4 potentiates HB-EGF-induced Akt and ERK activation in 786-O cells

Then, we decided to examine the potential effect of HB-EGF on the proliferation of transfected and non-transfected 786-O/ErbB4 cells. Growth curves were obtained for control 786-O and 786-O/ErbB4 cells maintained in DMEM 0.5% FBS, by MTT proliferation assays.

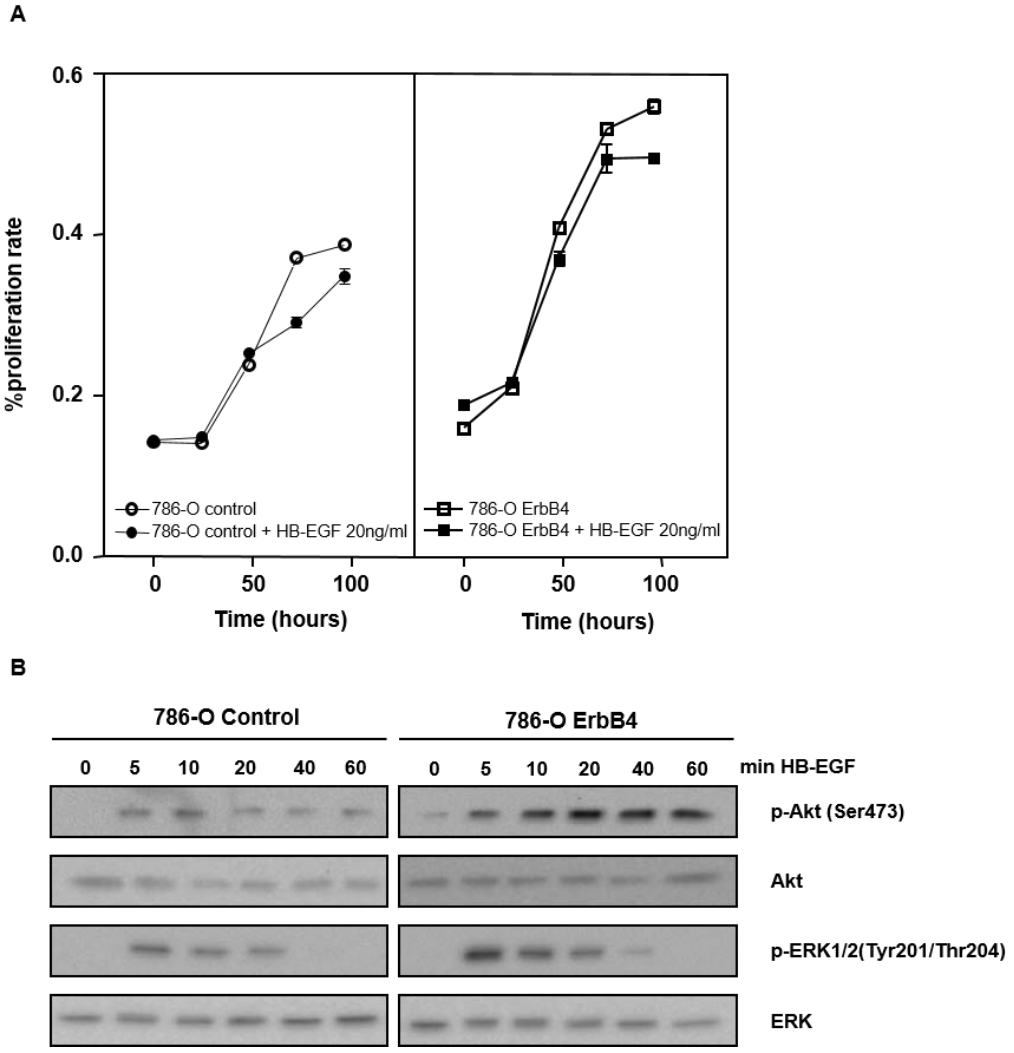


Figure 38. Effect of HB-EGF in cell proliferation and Akt and ERK pathways. (A) 786-O and 786-O/ErbB4 were seeded at 2,5 x 10^4 cells per well in a 96-well plate. Then cells were treated with HB-EGF 20 ng/ml. Every day, the media was replaced by fresh medium supplemented with 20 ng/ml HB-EGF. Proliferation was determined my MTT assay. (B) Time response to HB-EGF in 786-O cells and 786-O-ErbB4 cells. Cells were treated at different time with 20 ng/ml HB-EGF. Then, p-Akt (Ser473), Akt, p-ERK1/2 and ERK levels were analyzed by Western Blotting using the specific antibodies described in Material and Methods.

As observed in **Figure 38A**, a clear increase on the proliferation rates was observed in 786-O cells that express ErbB4 compared with non-transfected cells. However, HB-EGF does not increase, but rather induces a slight decrease in the proliferation of both cell lines (**Figure 38A)**. The next question we addressed was if the expression of ErbB4 receptor fluenced the activation of ERK1/2 and Akt triggered by HB-EGF. As observed in **Figure 38B** p-AKT Ser473 and p-ERK1/2 activation in response to HB-EGF was more potent and sustained in cells that express ErbB4 than in control cells. This suggest that although lacking a PI3K-binding site, ErbB4 JM-a/Cyt2 is able to promote the activation of Akt signaling pathway. Similar observations linking the stimulation of ErbB4 JM-a/Cyt2 to PI3K and Akt activation have been reported to occur in response to other ErbB4 ligands [241]–[243].

As we observed that the mere expression of ErbB4 induced changes in the EMT markers (**Figure 32**), we decided to analyzed if HB-EGF altered the expression of E-cadherin and Snail. **Figure 39** shows that in 786-O cells HB-EGF attenuated E-cadherin levels, which are already low in 786-O cells, and lighty increased Snail 1 expession. 786-O/ErbB4 cells do not express E-cadherin levels and Snail 1 expression is littel affected by HB-EGF.

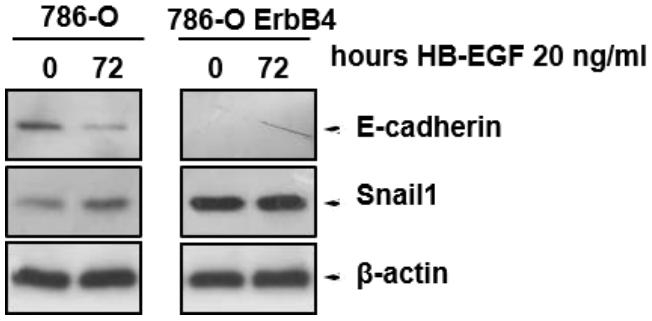


Figure 39. Alteration in EMT markers induced by HB-EGF. 786-O and 786-O/ErbB4 were seeded at 2x10^5 cells/ml and treated with HB-EGF 20 ng/ml for 72 hours. Then, E-cadherin and Snail1 were analyzed by Western Blot using the specific antibodies described in Material and Methods. β-actin was used as a protein loading control.

4.3. CX-4945 induces toxicity in 786-O ErbB4 cell line

The results obtained in previously chapters suggest that CK2 downregulation (Chapter 1) as well as ErbB4 expression affected the activation of Akt and ERK pathways in response to HB-EGF. However, the possible connection between CK2 and ErbB4 has not been studied so far. For this purpose we examined the effect of CK2 inhibitor CX-4945 on the viability of control 786-O and 786-O/ErbB4 cells.

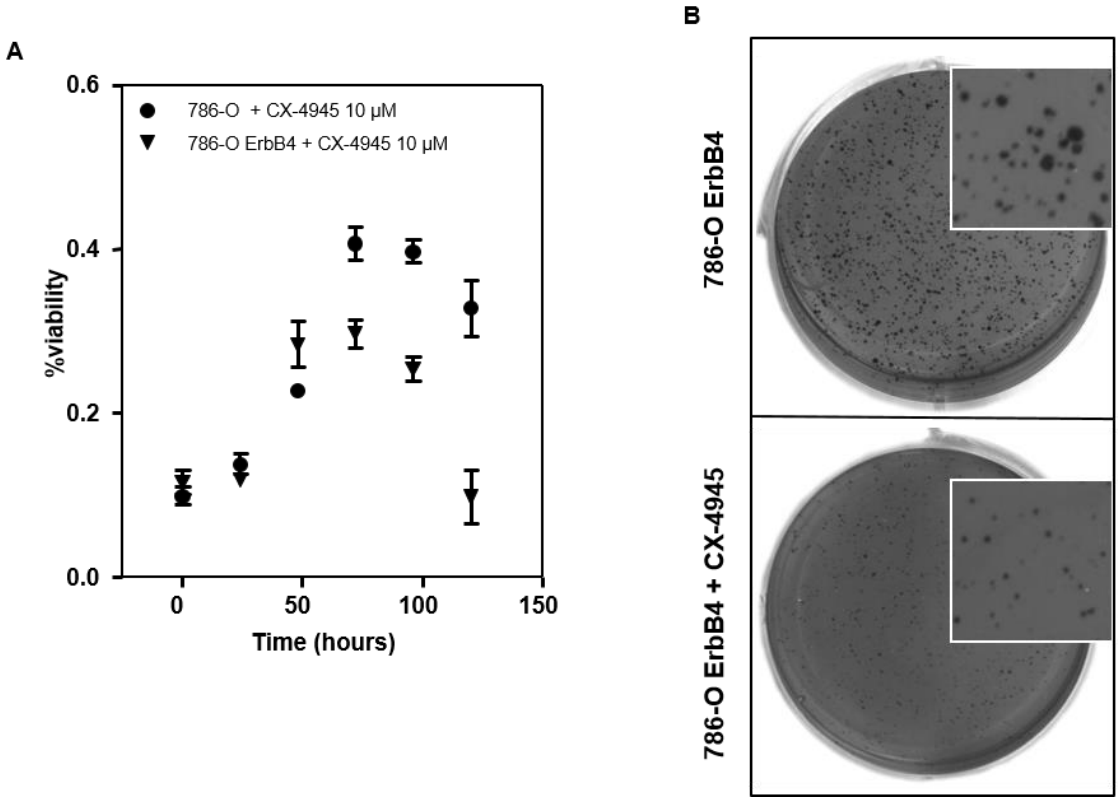


Figure 40. Effect of CX-4945 in proliferation and colony formation in soft agars in 786-O-ErbB4 cells. (A) Cells were seeded in a 96-well plate at 2, 5 x 105 cells per well. Then were treated with 10 µM of CX-4945 for 5 days. Viability was determined by MTT proliferation assay. The result was the mean of one experiment performed in quadruplicate. (B) 5 x 10^4 cells expressing ErbB4, were seeded in triplicate in 6-well plates in growth medium containing 4% agar and treated with 10µM CX-4945. Every 4 days the medium containing CX-4945 was replaced by fresh medium with CX-4945 10µM. After 15 days, colonies formed in soft agar were stained with Nitroblue tetrazolium, and visualized by an inverted microscopy.

The result, shown in **Figure 40A**, indicates that in the presence of CX-4945 the initial cell proliferation rates of control and ErbB4 expressing cells are no longer significantly different up to 48 h of culture. Thereafter, 786-O/ErbB4 cells started to die whereas control 786-O cells grew until 72 h and then also started to die. Interestingly, the decline of the number of living cells was more marked in 786-O/ErbB4 cells than in

control 786-O cells. These results suggest that overexpression of this receptor sensitizes the cell to the effect of CX-4945. Previous studies from different groups including ours (Vilardell J. PhD Thesis UAB 2013) have shown that 786-O cells are able to grow independently of a solid surface. In agreement with this we observed that 786-O/ErbB4 cells grew fast in soft agar media forming large colonies. We wondered if exposure to CX-4945 affected this growth. As shown in **Figure 40B**, CX-4945 reduced both the number as well as the size of the colonies formed by 786-O/ErbB4 cells.

Next we asked if the CX-4945 effects on cell viability were via apoptosis or autophagy. First, we analyzed the morphological changes induced by CX-4945. As observed in **Figure 41A**, CX-4945 exposure resulted in a fibroblast-like shape in both cells lines. Moreover, the formation of vacuoles in response to CX-4945 was lower in 786-O/ ErbB4 cells than in control 786-O cells (**Figure 41B**). Consistently with this, accumulation of LC3-II was observed at 24 h in 786-O cells, whereas in 786-O/ErbB4 cells it was not detected until 48 h (**Figure 41C**). Interestingly, a strong activation of PARP was observed at 48 hours in 786-O/ErbB4 cells (**Figure 41C**). By contrast, in 786-O cells PARP cleavage was less evident and occurred later (72 hours). These data are in agreements with the results observed in the MTT proliferation assays (**Figure 38A**). Taken together, the results suggest that ErbB4 expression renders the 786-O cells more sensitive to the CK2 inhibitor, probably by decreasing autophagy and inducing apoptotic cell death.

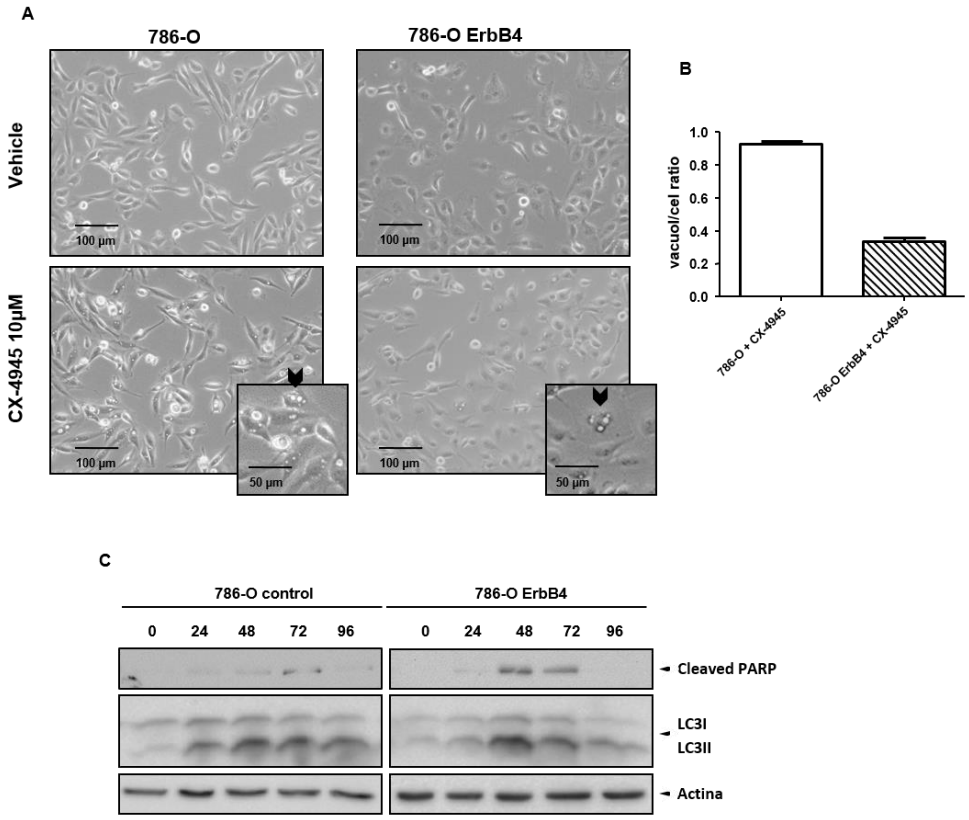


Figure 41.Toxicity of CX-4945 in 786-O cells that express ErbB4 receptor. (A) Cells were seeded at 10 x 105 cells per well, and were treated with 10 µM CX-4945 for 8 hours. Images were obtained by an inverted microcopy. (B) Quantification of the number of vacuoles, represented as a vacuole per cell ratio. (C) Cells were seeded in a 6-well plate and treated with 10 µM CX-4945 at different time (0, 8, 24, 48, 72, 96 hours). PARP cleavage and LC3-II accumulation wasdetected by Western Blotting, using the specific antibodies described in Material and Methods. β-actin was used as a loading control.

4.4. CX-4945 affects stability of ErbB4 in 786-O cell line

To investigate the connection between CK2 and ErbB4 we examined the expression and localization of ErbB4 and CK2 subunits in 786-O/ErbB4 cells by subcellular fractionation. ErbB4 is located in the light membrane fraction (P100, which contains plasma membranes) and in the heavy membrane fraction (P13, which contains the endoplasmic reticulum membranes). These membrane fractions contain CK2 (detected as CK2α and CK2β) which is also present in the cytosolic fraction (S100). Treatment with CX-4945 markedly reduced ErbB4 receptor levels in the endoplasmic reticulum membranes (P13) and in particular in the plasma membrane fraction (P100) (**Figure 42**). In contrast, CX-4945 did not significantly decrease total CK2 levels, causing only small alterations in its subcellular localization.

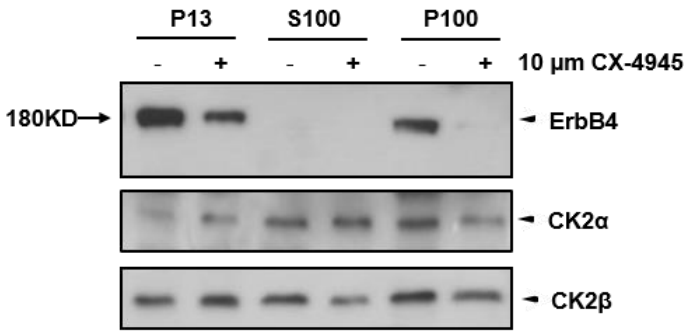


Figure 42**. Subcellular fractionation of 786-O-ErbB4 cells.** Cells were seeded and treated with 10 µM CX-4945 for 24 hours. Then subcellular fractionation was as described in Material and Methods. P13, 13.000 x g pellet, S100 after 100.000 x g centrifugation, P100, 100.000 x g pellet.

Next, we determined ErbB4 receptor intracellular distribution by immunofluorescence staining. In non-treated cells, ErbB4 is detected in the cell membrane and the endoplasmic reticulum (**Figure 43A**). In agreement with the results obtained by Western blot, CX-4945 treatment reduced significantly total receptor levels. In addition, the cell morphological alterations induced by CX-4945 described above (spindle and fibroblast-like shape) were more clearly observed (**Figure 43B**).

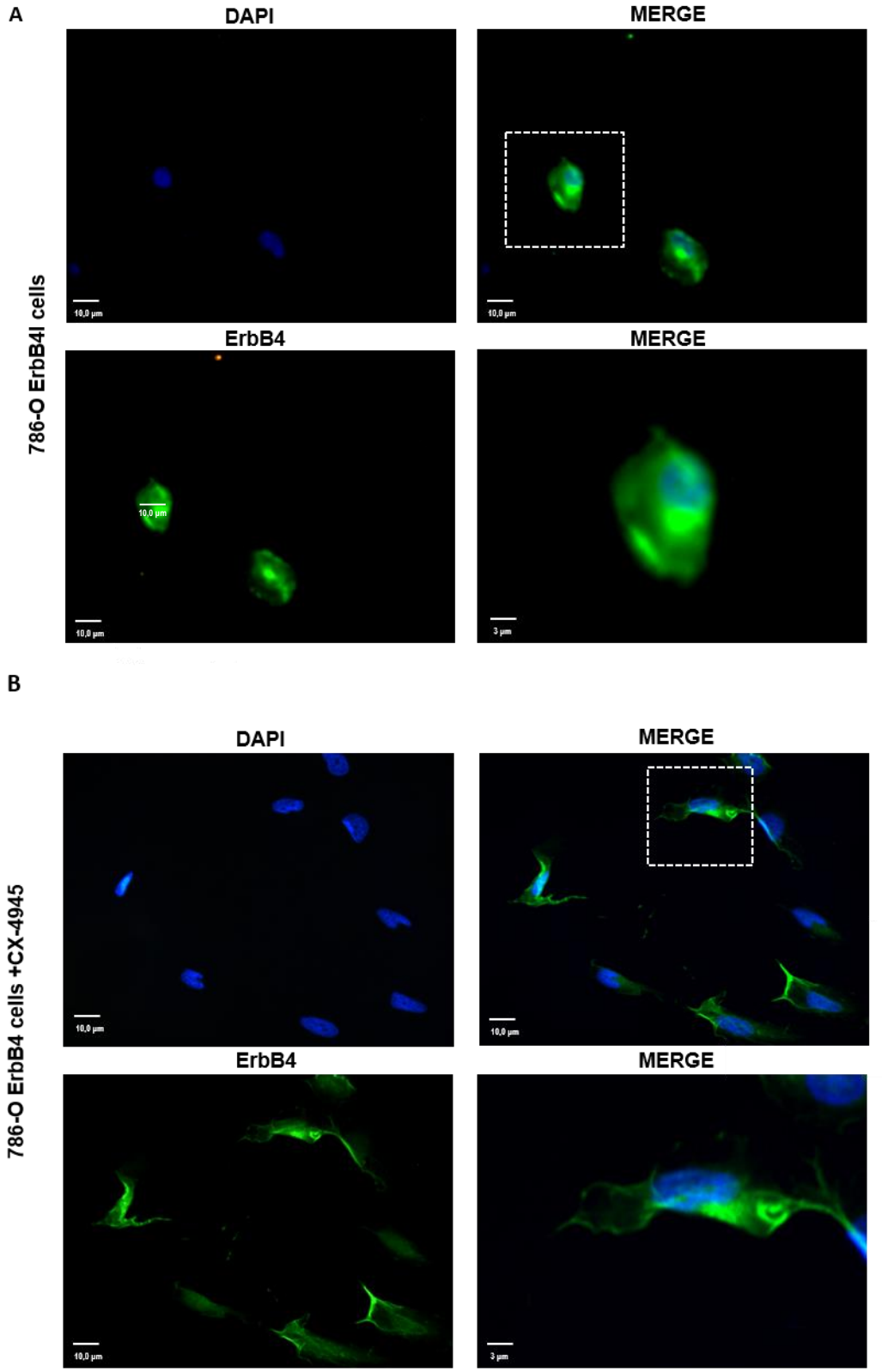


Figure 43. Immunofluorescence staining of ErbB4 in 786-O cells. Representative images acquired of non-treated 786-O cells (A) and 786-O cells treated with 10 μM CX-4945(B). DNA was stained with DAPI (upper panels) and ErbB4 detected with the specific antibody as indicated in Material and Methods. Merged images are shown in the right panels.

It has been recently described that CX-4945 induces autophagy in PC-9/GR and PC-9/ER cells and it increases the translocation of EGFR from the cell surface into the autophagosome, subsequently leading to decrease of EGFR [244]. We wondered if CX-4945 caused similar effects on the ErbB4 receptor. As can be seen from **Figure 44**, treatment of 786-O/ErbB4 cells with CX-4945 at different times, results in a marked reduction of both ErbB4 and EGFR levels. Under the same conditions, CX-4945 does not affect the levels of other membrane proteins such as the transferrin receptor (TfR) or cytosolic proteins such as IκBα.

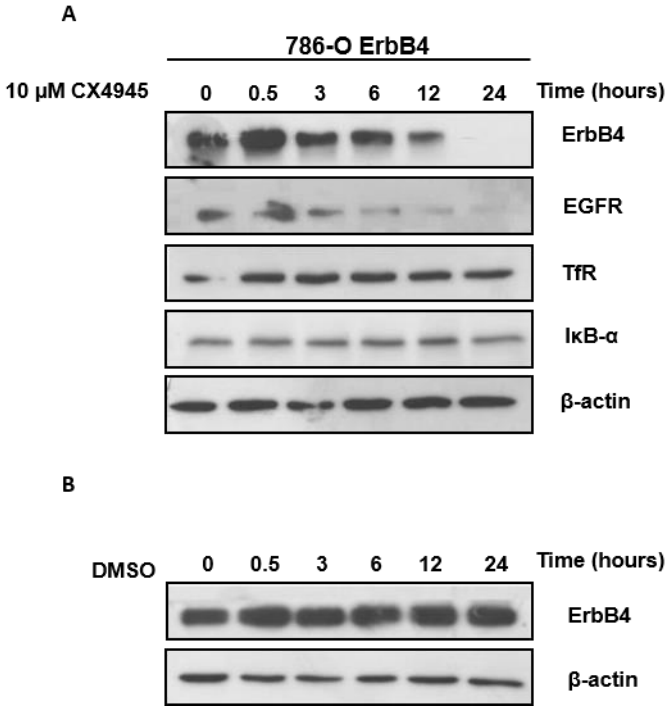


Figure 44. Effect of CX-4945 in ErbB4 stabilization. Cells were seeded at 10 x 10^5 cells per well and then were treated with 10 μM CX-4945 at different time (0, 0.5, 3, 6, 12, 24 h). Stabilization of ErbB4, EGFR, transferrin receptor (TfR) and IκB-α were analysed by Western Blotting, using the specific antibodies described in Material and Methods. β-Actin was used as a loading control.

4.5. CX-4945 does not induces CYT2-ICD accumulation in 786-O cell line

As indicated above, CX-4945 induced autophagy. Therefore, we asked if the destabilization of ErbB4 was a consequence of this effect. For this purpose, we treated 786-O/ErbB4 with chloroquine which inhibits the late phase of autophagy (autophagosome with lysosome fusion) [245]. Accumulation of LC3-II is observed in cells treated with chloroquine, what means that LC3-II turnover is inhibited. However,

chloroquine treatment does not block the decrease in ErbB4 induced by CX-4945 (**Figure 45**).

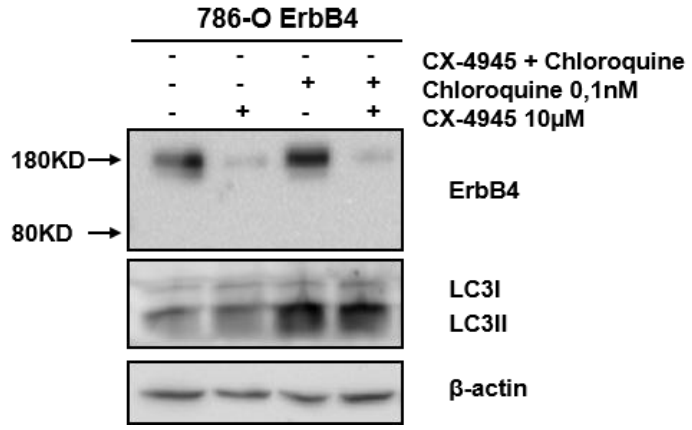


Figure 45. ErbB4 degradation is not affected by chloroquine. 786-O-ErbB4 cells were seeded at 10 x 10^5 cells per well, and pre-treated with chloroquine 50 μM and after 1 h, CX-4945 10 μM was added for 24 h. Western blot of cell lysates using the specific antibodies described in Material and Methods was used to determine the levels of ErbB4, LC3-I/LC3-II. β-actin was used as a loading control.

To further study the mechanism by which CX-4945 induced ErbB4 degradation, we treated cells with lysosomal proteinases inhibitors (leupeptin and pepstatin A). As observed from **Figure 46A**, the presence of these inhibitors lead to the detection of the 80 kDa fragment which corresponds to the processed ErbB4 form Cyt-2 ICD described in previous reports [241], in addition to the intact ErbB4 receptor. The addition of CX-4945 to cells pre-treated with the lysosomal inhibitors caused a marked increase in the Cyt-2 ICD fragment concomitantly with the decrease in the intact ErbB4 receptor.

In the absence of lysosomal inhibitors, exposure to HB-EGF also lead to a moderate increase in the presence of the Cyt-2 ICD fragment. However, the presence of this fragment was hardly detected in cells pretreated with CX-4945 before exposure to HB-EGF, although a decrease in the intact ErbB4 receptor was observed in response to this growth factor. (**Figure 46 B, C**). It has been previously reported that in addition to HB-EGF, the ErbB4 signaling is also activated by phorbol-myristate acetate (PMA). Moreover, it is known that PMA stimulates the cleavage of ErbB4, leading to the appearance of the Cyt-2 ICD fragment [246]**,** and the degradation of IκBα [247]**.**

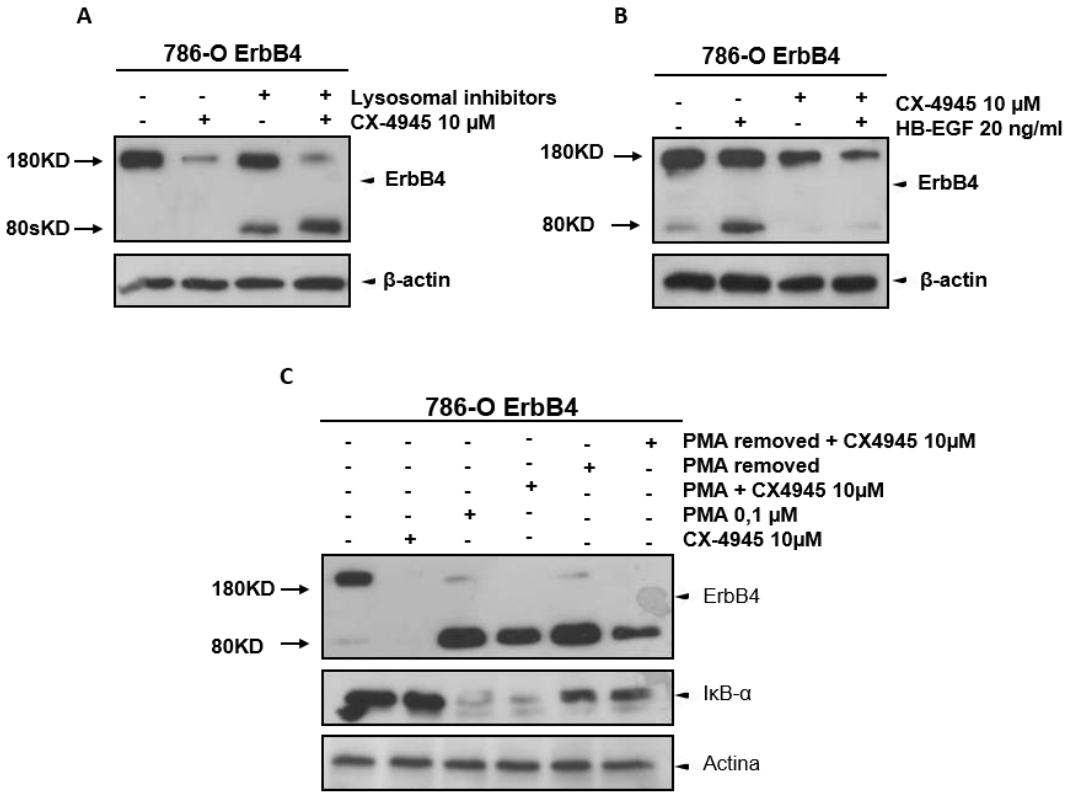


Figure 46. CX-4945 does not induces Cyt2 ICD accumulation as promote HB-EGF and PMA. (A) Cells were seeded and pre-treated with lysosomal inhibitors (Leupeptin 20 μM, and Pepstatin A 20 μM). After 1 h CX-4945 10 μM was added. (B) Cells were treated with CX-4945 10μM 8 hours. 20 minutes before finishing CX-4945 treatment, HB-EGF 20 ng/ml was added. (C) Cells were plated in a 6-well plate as described in Material and Methods, and were treated with PMA (0.1 μm), CX-4945 (10 μM) as well as PMA (0.1 μM) + CX-4945 (10 μM). After 24 h, PMA was removed from the medium, and cells were incubated 40 minutes more. Levels of ErbB4, ErbB4 Cyt2 ICD fragment and IκB-α were analyzed by Western Blotting using specific antibodies, described in Material and Methods.

In agreement with this, exposure of 786-O/ErbB4 cells to PMA decreases full-lenght ErbB4 receptor, promoting Cyt-2 ICD fragment accumulation as well as a strong decrease of IκBα levels **(Figure 46C)**. The concomitant treatment with CX-4945 slightly reduced the effect of PMA on Cyt-2 ICD fragment generation without significantly affecting IκBα degradation. Removal of PMA lead to a rapid, partial recovery of IκBα levels but not of the intact ErbB4 receptor. In fact, the persistence presence of CX-4945 after PMA removal tended to decrease the levels of Cyt-2 ICD fragment without affecting IκBα levels. Overall, these results suggest that CK2 is required for stabilizing ErbB4 receptor.

4.6. CK2 interacts with ErbB4 receptor

Although CX-4945 is one of the most specific and potent inhibitors of CK2 (ref), we asked if ErbB4 downregulation is a direct result of CK2 inhibition or might involve unknown potential side effects of this inhibitor. To better understand the potential involvement of CK2 on the stability of ErbB4 we analyzed the changes in its expression in 786-O/ErbB4 cells treated with CK2α- or CK2β-siRNAs (**Figure 47A**).

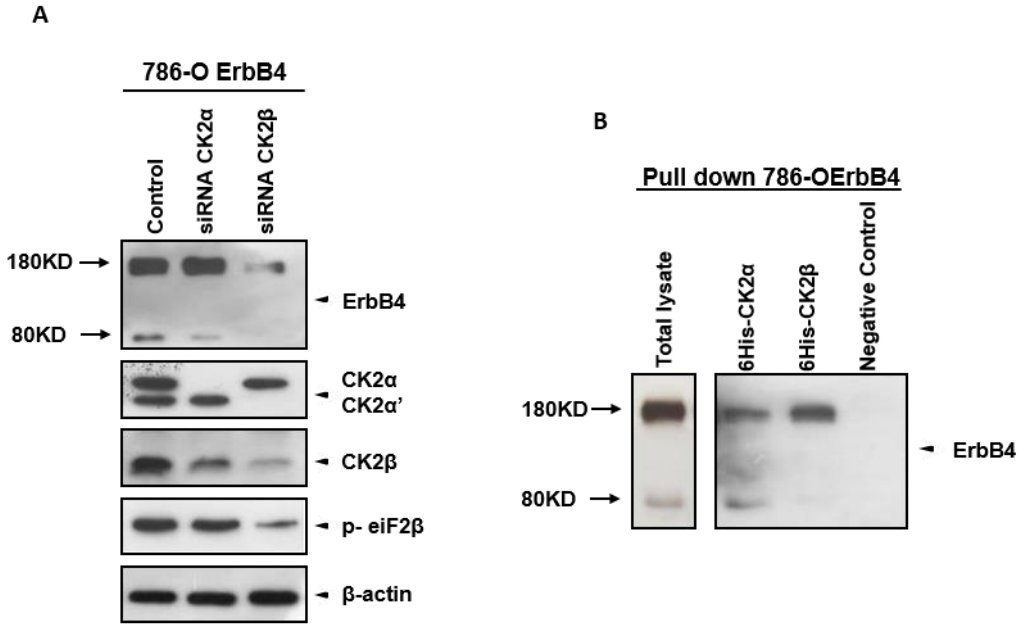


Figure 47. Stability of ErbB4 receptor using CK2α and CK2β siRNAs. (A) Cells were seeded and transfected with CK2α and CK2β siRNAs as described in Material and Methods. After 65 h, cells were lysated and levels of ErbB4, CK2α/α', CK2β, and β-actin, were determined by Western Blotting using specific antibodies described in Material and Methods as well. (B) Pull down assay was carried out using recombinant His6-CK2α and His6-CK2β as described in Material and Methods. Pull-down samples were subjected to immunoblotting with an anti-ErbB4 specific antibody.

As observed in previous results with stable silenced cell lines, CK2α-siRNA promotes a reduction on CK2β levels, and CK2β-siRNA decreased CK2α' but not CK2α levels. Under this conditions, siRNA CK2β but not siRNA CK2α strongly reduces the expression of ErbB4 (**Figure** 47A). Moreover interaction between CK2α and in particular CK2β with ErbB4 receptor was detected by pull down analysis (Figure 47**B**). All these results suggest that CK2 holoenzyme most likely containing CK2α' ($\alpha\alpha'/\beta_2$ or α'_2/β_2) is required for ErbB4 stability.

Discussion

1.1. CK2 inhibitors affect Akt activation in response to HB-EGF in renal cells

Despite several studies have been described the role of CK2 in the activation of signalling pathways triggered by different growths factor in cancer, very little is known about the involvement of CK2 in HB-EGF signalling in clear cell renal cell carcinoma (ccRCC). The initial objective of this present work was addressed to investigate the effect of CK2 in the activation of Akt/GSK3β and ERK1/2 signalling in response to HB-EGF in renal cells using CK2 inhibitors and comparing their effects with those of the PI3K inhibitors. The results obtained indicate that CK2 inhibitors, block constitutive Akt phosphorylation as well as those induced by HB-EGF in a similar way to that PIK3 inhibitors, in both HK-2 and 786-O cells. Interestingly, CK2 inhibitors do not affect ERK1/2 phosphorylation in HK-2 and 786-O cells except the CX-4945, which induces an activation of ERK1/2 phosphorylation in response to HB-EGF and inhibits more markedly the activation of Akt. Differences between CK2 inhibitors can explained by their selectivity and potency. It has been reported that apigenin is a specific CK2 inhibitor however displays rather high IC50 values (around 20 μM) and inhibits CK1 and possibly other protein kinases with comparable efficiency [248].

By contrast, TBB discriminates between CK2 and CK1, and shows a remarkable selectivity toward CK2. However, other protein kinases tested displayed significant inhibition by TBB; two of these, CDK2 and GSK3β, are kinases belonging to the same protein kinase subfamily (the CMGC group), while the other, PHK, belongs to another subfamily (the CaMK group) [249]. The importance of CK2 as a molecular target in cancer has increased the interest in developing new specific inhibitors. CX-4945 is a potent and selective orally bioavailable ATP-competitive inhibitor of CK2α and CK2α′ catalytic subunits with known antitumor efficacy in breast, pancreatic and prostate xenograft mouse models [250]. Compared to the other CK2 inhibitors CX-4945 disclose a low IC50 and only 7 of the 238 kinases tested were inhibited by more than 90% in vitro. In a cell –based functional assay CX-4945 was inactive against three of these kinases (FLT3, PIM1 and CDK1) [217]. In clinical trials, CX-4945 induced stable disease in 20% of patients with different solid tumors, positioning it for phase II combination trials. This compound has opened the door to an entirely new class of therapeutics for cancer patients such as TDB, which is a selective, cell-permeable inhibitor of CK2 and PIM-1, which has been reported to maintain kinase activity inhibited for long periods than CX-4945 and subsequently perpetuates the effect on cell survival and migration [251].

One of the main differences between HK-2 and 786-O cell lines is that 786-O cells are less sensitive to the effect of CK2 inhibitors than HK-2, supporting the idea that tumorigenic cells are more resistant to chemical agents. Moreover, in 786-O cells the phosphorylation of Akt Ser129 is negligible due to the low expression of Akt1, whereas HK-2 cells express considerable levels of Akt1 and consequently Akt Ser129 phosphorylation. Both cell lines show considerable levels of Akt2. These results are consistent with those results published by Girardi C. et al, 2014 that suggest that Akt1 and Akt2 are differently phosphorylated by CK2 *in vivo* [202]. Different expression of Akt isoforms would affect the biological characteristics of these cells. Indeed, it has been reported that Akt2 overexpression increased metastasic features and invasion of human ovarian, human breast cancer cell lines, and colorectal cancer [252], [253] . On the other hand, Akt1 up-regulation has been shown to accelerate cell proliferation and to suppress apoptosis but not always correlate with invasiveness [254].

It is also described that Akt1 is phosphorylated in Ser129 by CK2 prevents the dephosphorylation of Akt Thr308 and therefore induces Akt activation [203]. The results in p-Akt Thr308 and p-Akt Ser129 in HK-2 cells would agree with this hypothesis. By contrast, in 786-O cells the correlation between these two phosphorylation sites is not as evident since this cell line exhibit high levels of p-Akt Thr308 which are reduced by CX-4945, but does not express Akt1. These findings suggest that in 786-O cell line the CX-4945, might affect directly the phosphorylation of Akt Thr308 by other protein kinase or might alter other protein substrates of CK2 which might also regulate Akt activation.

1.2. Donwregulation of CK2α or CK2β affects the expression of the other CK2 subunits

Debate continues about the best strategies for CK2 inhibition. As we noted CK2 inhibitors displayed varying abilities to block Akt pathway suggesting a distinct mechanism of action. Besides, several studies have reported that RNAi or antisense strategies may sometimes be inefficient requiring continual treatment of cells to achieve effective CK2 depletion. For that reason we made used of stable cell lines that expressed shRNAs anti CK2α or CK2β and showed reduced levels of these subunits. The stable transfected cell lines were generated by another member of the laboratory who also carried out their preliminary characterisation (Vilardell J. PhD Thesis UAB 2013). Downregulation of catalytic or regulatory subunits reduced the expression of CK2α or CK2β respectively, in both cell lines. However, the most surprising results

were that the downregulation of CK2α was accompanied by a little decrease of CK2β expression, and that CK2β downregulation strongly reduced CK2α' expression levels. These effects were in agreement with studies described by Deshiere *et al*.[197], and provided further information about the possible inter-regulation of CK2 subunits expression which is one of the major current areas of discussion. Since the initial studies by Litchfield *et al.,* (1994)[177], showing that CK2β was synthesized in a large excess than the catalytic subunits and that the fraction of CK2β subunit which did not enters the tetramer was rapidly degradate, some authors have considered that the regulation of CK2 subunits is mediated by the stability of the protein. CK2β is phosphorylated at serine 209 in a cell-cycle-depedent by $p34^{cdc2}$*in vitro* and in mammalian cells[255], [256], and is autophosphorylated at serines 2 and 3 by CK2 catalytic subunits. These phosphorylations enhance the stability of CK2β. On the other hand, recent studies have shown that CK2 catalytic subunits stimulated the expression of CK2α and CK2β genes, although no direct binding of CK2α and CK2β to their promoter DNA have been found. Moreover, the transcription of CK2 subunits was attenuated after inhibition of CK2 activity suggesting that the activity of CK2 is required for transcriptional regulation of its subunits [178].However, the mechanism linking CK2 activity to transcriptional regulation of CK2 subunits remain obscure.

1.3. CK2 downregulation affects Akt activation

Our results obtained with HK-2 and 786-O cell lines show that the silencing of CK2 subunits promotes diverse changes in Akt phosphorylation which are cell-type dependent. In the tumorigenic 786-O cell line, downregulation of CK2 does not significantly alter the basal phosphorylation of Akt Ser473, Thr308 and GSK3β Ser9. This result may be explain by the fact that 786-O cells lack PTEN and consequently express high constitutive levels of phosphorylation of Akt and GSK3β. On the other hand, in HK-2 cells downregulation of CK2, in particular CK2β, alters phosphorylation of Akt and GSK3β in a divergent manner. The increase observed in the phosphorylation of Akt Ser473 and GSK3β Ser9 may partly be attributed to the destabilization of PTEN detected in CK2β silenced cells. It is important to keep in mind that in these cells downregulation of CK2β does not affect CK2α levels and therefore, it is tempting to speculate that this free catalytic subunit might phosphorylate PTEN at Ser-370 that increased the phosphorylation at Thr-366 by GSK3beta [96] and promotes PTEN destabilization. Although no single study has explored which form of CK2 phosphorylates PTEN so far, it is known that the inhibition of Thr366 phosphorylation

by the CK2 inhibitor DMAT or GSK3β inhibition led to the stabilization of the PTEN protein [95].

On the other hand, the simultaneous decrease in the phosphorylation of Akt Thr308 and Ser129 observed in CK2β silenced HK-2 cells would give further support the hypothesis of Ruzzene *et al*.,[203], who suggested that the reduction of phosphorylation of Akt1 Ser129 causes a concomitant decrease in Akt1 Thr308. Moreover, our results would suggest that CK2 holoenzyme is required for Akt Ser129 phosphorylation.

1.4. Downregulation of CK2β affects Akt activation in response to HB-EGF

We have observed that the activation of Akt is altered by the mere downregulation of CK2. One of our main goals was to explore the effect of CK2 inhibition in this signalling pathway in response to HB-EGF. In HK-2 cells, the stable silencing of CK2 decreases the length of the HB-EGF-induced response in Akt phosphorylation. This effect was noticeably marked on Akt Thr308 and Akt1 Ser129 activation, supporting once again the link between these two residues. Moreover, these effects were observed in CK2α silenced cells and became more marked in CK2β silenced cells. Interestingly both silenced cell lines have in common a decrease in CK2 holoenzyme, which is particularly evident in CK2β depleted cells. In the same vain, in 786-O cells the downregulation of CK2β subunit also caused a less sustained response to HB-EGF, especially detected in Akt Thr308 phosphorylation. Even though 786-O cells show low levels of Akt1 and consequently of Akt Ser129, it is still possible to observe that the downregulation of CK2β decreased the length of p-Akt Ser129 activation in response to HB-EGF.

Summing up, the reduction of Akt Ser129 and Akt Thr308 basal phosphorylation together with the results obtained in the short-term and long-term response to HB-EGF in CK2 silenced cells, suggested that free CK2α is not sufficient to maintain the duration of Akt activation in response to HB-EGF, pointing to a requirement of CK2 holoenzyme for this effect. Interestingly, Ruzzene *et al.,* [86] described that *in vitro,* CK2α phosphorylated Akt1 more efficiently than CK2 holoenzyme ($\alpha_2\beta_2$)*,* but the contribution of other forms of CK2 was not analysed. Our results show that *in vitro* phosphorylation of Akt1 by CK2α or CK2α' is even greater than by the CK2 holoenzyme containing CK2α or CK2α', and that this effect is not altered by the presence of 150mM NaCl. Taken together, the results suggest that the decrease in Akt Ser129 phosphorylation occurring in CK2β silenced cells is not due to a decreased

intrinsic ability of free CK2α to phosphorylate Akt1. In an interesting way, Guerra B *et al.*, [204], showed that Akt in complex with CK2β has a higher kinase activity in vivo, what could explain ours results. However, it is important to keep in mind that Akt interacts with Hsp90 what prevents Akt Thr308 dephosphorylation mediated by PP2A. Phosphorylation of Akt at Ser129 increases its affinity for Hsp90 binding. Interestingly, treatment of HEK293T cells with geldanamycin (which inactivates Hsp90) led to a decrease in Akt phosphorylation at Thr308 and also at Ser129, although the effect was more marked on the first one [203]. On the other hand, Cdc37, a co-chaperone, has been reported to participate in the Akt/Hsp90 complex [257]. This co-chaperone is a substrate of CK2 and overexpression of CK2β in COS7 cells enhanced Cdc37 Ser13 phosphorylation, the unique phosphorylation site of Cdc37 in vivo [258]. Phosphorylation by CK2 on Ser13 is essential for Cdc37 optimal binding activity toward Akt (Yoshihiko Miyata 2004). Taken together, it is tempting to speculate that CK2β would be necessary for the formation of Akt/Hsp90 complex either affecting the affinity of Akt for Hsp90 binding or phosphorylating Cdc37 at Ser13, what would result in preventing the dephosphorylation of Akt by phosphatases. However we could exclude other possibilities Other possible explanation such as the CK2α remaining in the cell after CK2β silencing, would associate to other proteins resulting in a reduction of its kinase activity [259], and preventing the phosphorylation of Akt1. More research on this topic needs to be undertaken.

1.5 CK2α downregulation affects the ERK response induced by HB-EG

Even though some connections between CK2 and ERK1/2 pathway have been reported previously by other groups, no study has been published describing the role of CK2 in the activation of ERK1/2 in response to HB-EGF. Interestingly, we found that the most remarkable effect was on the length of ERK1/2 activation induced by HB-EGF which is impaired by downregulation of CK2α in both cell lines. It has been described that upon activation by extracellular signals, the scaffold protein Kinase Suppressor of Ras (KSR) recruits CK2 to phosphorylate RAF and enhance its activity against MEK which in turn phosphorylates ERK1/2 (REF). Our results suggest that the form of CK2 involved in these effects would be CK2α or CK2αβ holoenzyme since the CK2α' or CK2α'β holoenzyme still present in CK2α silenced cells were not able to maintain ERK1/2 activation whereas CK2α remaining in CK2β silenced cells, which show concomitant depletion in CK2α', is enough to sustain ERK1/2 response to HB-EGF (**Figure 48**).

It has been reported that the temporal control of ERK1/2 is a key modulator for inducing proliferation versus differentiation. Sustained activation of ERK1/2 has been linked to differentiation whereas transient activation would increase cell proliferation. However, discrepancy exist concerning this idea since the intensity and length of the effect depends largely on the cell type and the stimuli that induces the response [260], [261].

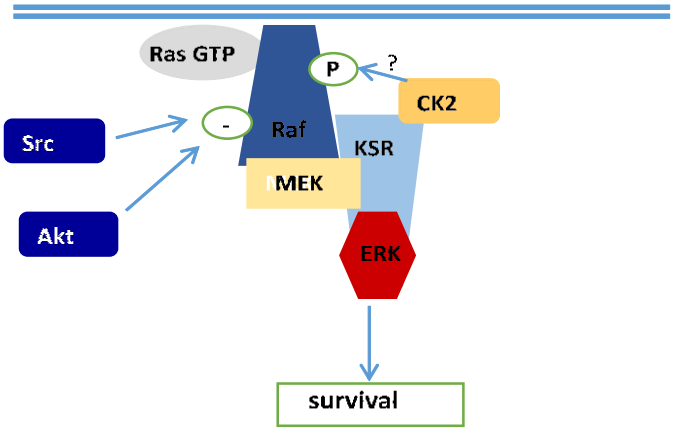


Figure 48. Proposed model for CK2 effect on ERK1/2 signalling. CK2α is recruited to the KSR scaffolding complex where phosphorylates Raf (B-Raf S446 is a constitutive target of CK2, whereas C-Raf requires prior phosphorylation of N-region Y341 by Src family kinases). Active Raf phosphorylates MEK, which phosphorylates ERK and this activation modulates diferent cellular processes such as proliferation.

Activation of ERK1/2 in response to HB-EGF in the tumorigenic 786-O cell line is more intense and sustained than in normal HK-2 cell line. It is known that HK-2 cells express VHL whereas 786-O are VHL-deficient cells. Reintroduction of VHL in 786-O cells promotes a decrease in the ERK1/2 response to HB-EGF due to rapid EGFR downregulation as compared with 786-O VHL-deficient cells. These findings suggest that the loss of VHL enhances and prolongs the activation of EGFR and consequently phospho-ERK signals lasted longer. These data are in agreement with those results published by [227] who described that the activation of EGFR induced by EGF had higher stability in VHL-deficient cells than in VHL-expressing ccRCC cells.

The role of free CK2 subunits, their assembly into the tetramer as well as interaction with other protein which may affect its activity, are major areas of debate concerning CK2 regulation. Overall, the results of this part of our study strengthens the idea that CK2 is involved in the sustainment of the activation of Akt and ERK1/2 mediated by

HB-EGF. Moreover, they show both that signalling pathways are differently affected by alteration in the expression of CK2 subunits since depletion of CK2β impacts on Akt activation without affecting ERK1/2 phosphorylation, whereas CK2α depletion impinges mainly on ERK1/2 response. More generally, the phosphorylation status of CK2 substrates that are involved in Akt and ERK1/2 pathway will be dictated by the active free-catalytic or holoenzyme present in the cell.

2.1 CK2 inhibitors affect the viability of renal cells

A large number of studies have described that in many tumours examined CK2 is dysregulated with elevated total content, unbalanced expression of catalytic and regulatory subunits or alteration in its subcellular localization [262]–[264]. Moreover, CK2 has been described as a pro-survival kinase and a therapeutic target in the treatment of several types of cancer. A considerable amount of literature has been published describing the cytotoxic and anti-proliferative effects of CK2 inhibitors likely due to the negative consequences of CK2 inhibition on multiple pro-survival signalling pathways, including those studied in the present work, such as PI3K/Akt and ERK1/2 pathway [265]. Therefore, as a part of the thesis we decided to determine the effect of CK2 inhibition either by chemical agents or by stably silencing of CK2 subunits, on cell viability and cell proliferation. We observed that TBB and CX-4945 affect differentially the viability of 786-O and HK-2 cell lines. Indeed, TBB induces cell death at 8 h whereas CX-4945 does not essentially affect the viability of renal cells. These results were unexpected since previous studies have described that CX-4945 induces cytotoxicity and apoptosis in other cell lines [265], [266].

2.2 The effect of CX-4945 in the proliferation of CK2α and CK2β silenced cells

To further study the effect of CX-4945 on the viability of renal cells, we have used the stably CK2α and CK2β silenced cells. The first observation is that in HK-2 cells the sole downregulation of CK2β does not significantly compromise the proliferation whereas CK2α downregulation slightly increases the proliferation rates of HK-2 cells. This result also agree with those observed in previous studies in the group (Vilardell J. PhD Thesis 2013). It must be kept in mind that CK2α' could play an important role in cell proliferation. It has been described that CK2α′ knock-out mice exhibited a hyper-apoptotic phenotype in spermatocytes [161], forced expression of kinase-inactive CK2α′ in U2-OS cells resulted in proliferation defects [267], and Orlandini *et al.* (1999) [268], highlighted the oncogenic activity of CK2α′ in experiments that showed co-

operativity with Ras in the transformation of rat fibroblast. Furthermore, in recent years it has been described that CK2α' displays a preference over CK2α for caspase-3 phosphorylation (Litchfield 2013). In this respect, although speculative at this stage, in CK2α silenced HK-2 cells the CK2α' would impact in supporting rapid cell proliferation. In the 786-O tumorigenic cell line the effect of CK2 downregulation slightly reduces the proliferation of cells, in particular CK2β depletion. It is known that knocking out CK2β in mice leads to early embryonic lethality, however the effect of CK2β overexpression on cell proliferation yielded conflicting results. It is important to keep in mind that in the most of the studies reported so far, CK2 is transitorily downregulated with siRNAs instead of stably transfected what would might explain the differences. However the most relevant result obtained is that the downregulation of CK2 subunits sensitize the cells to the effect of CX-4945 although differences exist between both cell lines. Thus, in our conditions in 786-O silenced cells the inhibitor only partial decrease the proliferation but does not induce cell death, what suggests that CX-4945 might activate mechanism of resistance that hamper cell death. It must be emphasized that in this cell line this inhibitor only partially blocks Akt and GSK3β and has no effect in ERK1/2 activation. In contrast, in CK2α and CK2β silenced HK-2 cells CX-4945 strongly reduces the proliferation and induces cell death at long-term exposure (8 days). Concordant with this, Akt and GSK3β phosphorylation is inhibited and ERK1/2 phosphorylation is increased by CX-4945. In addition, in both cell lines, but in particular in HK-2 cells, the decrease of proliferation is reversible after removal of CX-4945, what give further support to the idea that this inhibitor activates survival mechanisms before inducing cell death.

Another interesting result from our study is the evidence that CX-4945 induces the formation of large intracellular vacuoles, which are not filled with lipids, in both cell lines. This effect is particularly noticeable in HK-2 cells, where the mere downregulation of CK2α induces the formation of tiny vesicles. Taken together, these results indicate that CX-4945 induced vacuole formation and that this effect was more evident in CK2α downregulated cells. While this study was in progress another group reported that CK2α downregulation promoted autophagy cell death via mTOR and MAPK signalling pathways in human glioblastoma cells [195]. Since our results were pointed to the same line of evidence, we decided to further study the autophagy mechanism by checking LC3-II accumulation. A slight accumulation of LC3-II is already detected in CK2α but not in CK2β silenced HK-2 cells. Moreover, in all cell lines, in particular in HK-2 cells, the exposure to CX-4945 induces a significant accumulation of this autophagic marker. The differences between both cell lines can be explained by

distinct effect of CX-4945 in Akt and ERK1/2 phosphorylation. CX-4945 cannot counteract the prominent Akt basal phosphorylation and has no effect on ERK1/2 activation in 786-O cells growing in complete medium with 10% FBS. In contrast, CX-4945 completely inhibits Akt Ser473 and Thr308 phosphorylation, and increases p-ERK1/2 levels in HK-2 cells, especially in CK2α silenced cells, which show higher basal p-ERK1/2 levels than control or CK2β silenced cells. Overall, the current data highlight the importance of CK2 in controlling autophagy by acting either directly or through alterations in Akt and ERK1/2 signalling pathways. It is known that the inhibition of the kinase mammalian target of rapamycin (mTOR) triggers the autophagic process (ref). Since mTOR activity is modulated by PIK3K/Akt it is tempting to hypothesize that in HK-2 cells the process of autophagy would be more striking than in 786-O due to the inhibition of Akt activation detected in HK-2 cells.

Autophagy has a dual role in cancer as a tumor suppressor by hampering the accumulation of damaged proteins or as a mechanism of cell survival that can promote growth. In HK-2 cells, CX-4945 promotes autophagy and when this mechanism is unable to mitigate cell stress it induces cell death (**Figure 49**). Little is known about the involvement of CK2 in autophagy and senescence related processes that have multiple overlapping and complementary functions in cancer. S. Ryu *et al,*. [269] reported that CK2 downregulation was associated with senescence and more recently it has been described that in PTEN-deficient cells CK2 enhances the senescence trough regulating Pml stability [270]. Other reports indicate that CX-4945 induces autophagy in HeLa and LNCaP in cells down-regulating Akt-mTOR-S6K signalling. Interestingly, it has been described that concomitant treatment of CX-4945 with a MEK inhibitor overcomes the drug resistance that induces CX-4945, decreasing p-ERK1/2 levels, proliferative markers and inducing apoptosis, suggesting that development of combination strategies with CX-4945 would be effective to modulate the mechanisms of resistance for therapeutic advantages [271].

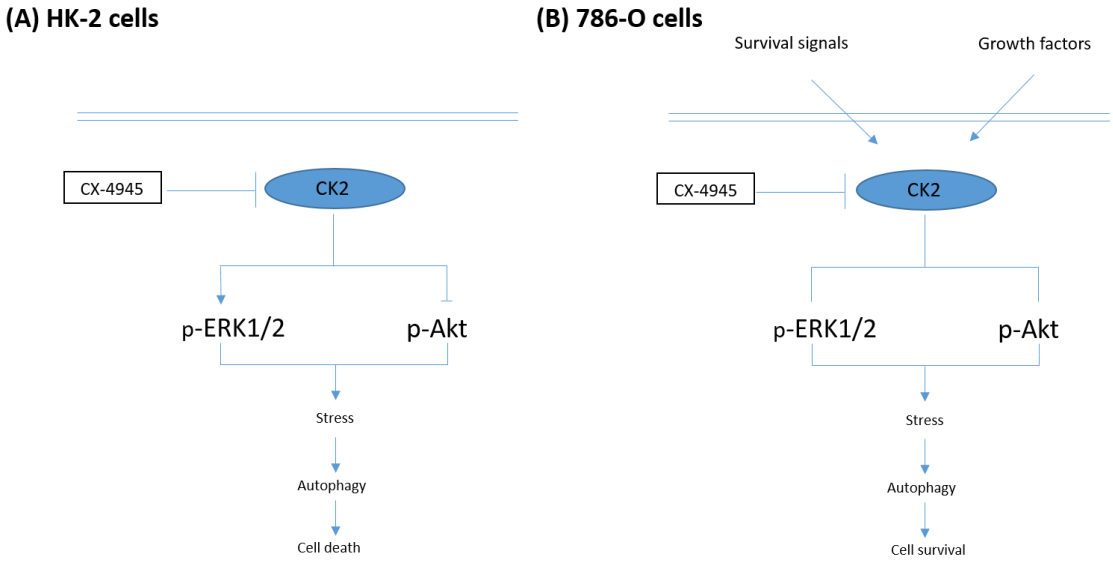


Figure 49. Schematic effect of CX-4945. (A) In HK-2 cells CX-4945 blocks p-Akt473 and induces activation of ERK1/2, likely inducing stress that promotes autophagy and cell death. (B) On the contrary, in 786-O cells the Akt and ERK pathways are hyperactivated probably due to the lack of PTEN and VHL or survival signals and growth factors secreted by tumorigenic cells. Under this conditions the CX-4945 induces autophagy as a mechanism of survival.

3.1 CK2 downregulation induces EMT

Epithelial–mesenchymal transition (EMT) is a conserved cellular processes that confers a more mesenchymal and invasive phenotype. As well as facilitating metastasis, EMT is thought to generate cancer stem cells and contribute to the acquired resistance of some cancers to chemo- and radiotherapy. EMT is characterized by the loss of cell to cell adhesions and apicobasal polarity, and the transition to a cell type with a more spindle-like morphology. Moreover EMT involves the downregulation of epithelial-type proteins and the acquisition of mesenchymal markers [272]. In the past years, it has become clear that EMT reprogramming normal and tumor epithelial cells is regulated at transcriptional, post-transcriptional, translational and post-translational levels. Recent reports have highlighted the role of various post-translational modifications in regulating Snail 1 that are controlled by protein kinases including CK2 [197].

Recently, have increased the notion that deregulation of CK2 subunits induces an EMT-like phenotype. In human breast epithelial cells (MCF10A cells) [197] depletion of CK2β induces EMT-related markers, whereas in human colorectal cell lines CK2α is overexpressed and modulates cell proliferation and migration via regulating EMT-related genes [196]. Our present results are in agreement with those that show that

down-regulation of CK2β increased the expression of EMT markers. On the contrary, in our conditions, downregulation of CK2α also altered the expression of EMT-related markers. This result was unexpected since previous reports have indicated that CK2α-silencing or chemical inhibition of CK2 activity reverts EMT in other types of cancer cell lines and blocks EMT induction in response to TGF-β [219]. However, we cannot conclude whether the induction of EMT markers in this cell line is due to the altered CK2α'/CK2α ratios or triggered by the concomitant decreases in CK2β.

This discrepancy could be explained considering the presence in the HK-2 and 786-O renal cell lines of repressors of EMT induction whose stability is negatively regulated by CK2 holoenzyme. E-cadherin repressor snail 1 stability has been shown to be controlled trough hierarchical phosphorylation by CK2 and GSK3β, what targets it for degradation by the proteasome [197]. This process would require CK2 holoenzyme and, consequently, both the catalytic and regulatory subunits would be essential for initiating it. Snail 1 has been reported as a major regulator of EMT in renal cancer cell lines [273] and increased snail 1 levels have been found associated with cancer invasion and poor prognosis in human RCC [274]. The increases in snail 1 observed in CK2β-depleted HK-2 cells would agree with this hypothesis. However, our data also show a rise in p-GSK3β Ser9 levels in these CK2β-depleted HK-2 cells and restoration of low p-GSK3β Ser9 levels by exposure to the PI3K inhibitor LY-294002 is sufficient to decrease snail 1 protein levels in spite of the almost complete depletion of CK2β. In fact, our results would suggest that CK2β effects on snail 1 are indirect and exerted likely through changes in the phosphorylation state of GSK3β Ser9. The decreases in p-GSK3β Ser9 and snail 1 levels detected after reintroduction of CK2β in HK-2/shCK2β cells would support this hypothesis. In this context, we have observed that downregulation of CK2β in HK-2 cells induces a destabilization of PTEN that might affect the activation of Akt Ser473, and it may ultimately result in changes in p-GSK3β Ser9.

The lack of PTEN, and its functional consequences in the PI3K-Akt-GSK3β pathway, might also support the higher p-GSK3β Ser9 basal levels detected in 786-O cells as compared to those in HK-2 cells. It may also explain the differential response to CK2β depletion, which leads to increased p-GSK3β Ser9 levels in HK-2 but is without significant effect in 786-O cells. In the same vein, the effects of LY-294002 in 786-O cells also support the concept of GSK3β as a main regulator of snail 1 protein levels. On the other hand, the changes in HIF-1α detected in HK-2 as well as those of HIF-2α in 786-O cells indicates that probably CK2 holoenzyme might also affect snail 1 levels

through increases in the expression of HIFs. In this context, it is interesting to note that the effects of CK2-silencing on HIFs are exerted in HK-2 cells, known to be VHL positive, in 786-O/VHL cells, where VHL has been reintroduced but also occur in 786-O cells which expresses non-functional VHL167 or in VHL- deficient 786-O cells, what indicates that the effect of CK2 are exerted, at least in part, through VHL-independent mechanisms.

Activator of transcription STAT-3 is a potent regulator of HIF-1α and HIF-2α expression [238] as well as a positive modulator of HIF-1α activity [112], [239]. STAT-3 activity is reflected through its phosphorylation at Tyr-705 and Ser727. However the role and regulation of STAT-3 Ser727 phosphorylation in cancer have not been clearly understood. Recently, a study in ccRCC tumours has described that both STAT-3 Tyr705 and Ser727 residues are increased in ccRCC and that Ser727 is an independent prognostic factor in ccRCC [21]. These results are in agreement with those obtained in 786-O cells, where CK2 downregulation, in particular CK2β, increase the phosphorylation of STAT-3 at Tyr705 and Ser727. However in HK-2 cells a negative relationship exist between both residues, increasing the phosphorylation of STAT-3 at Tyr705 but decreasing at Ser727. This negative correlation has been detected in previous reports which described an enhancement in pSTAT-Ser727 levels associated with decreased pSTAT-Tyr705 levels and reduced transcriptional activity [275]. Moreover, other groups have shown that exposure of cells to CK2 chemical inhibitors (which block the activity of both CK2 holoenzyme and free catalytic subunits) diminished STAT-3 Ser727 phosphorylation. A possible explanation for this may be that in our silences cells CK2 activity is not abolished, as detected through kinase activity assays. Indeed we have different cell lines that express distinct ratios of CK2α/CK2α', CK2α/CK2β and CK2α'/CK2β, and these ratios might affect differently CK2 substrates. Thus in CK2β silenced cells, the catalytic subunits would remain "free", and therefore could interact and phosphorylate other proteins. It is tempting to speculate that CK2α free would associate with PP2A, which is a well-known interaction partner of CK2α [276], inducing its activity and dephosphorylating STAT-3 at Ser727. Then, this decrease in Ser727 would increase p-STAT3 Tyr705 levels and consequently HIFs levels would also increase.

On the other hand, the data obtained with LY-294002 in HK-2 and 786-O cells showed that reversion of snail 1 increases is not sufficient to restore E-cadherin levels, what suggest the contribution of other E-cadherin repressors in the CK2 mediated effects. It is interesting to note that re-expression of VHL30 but not VHL167 in 786-O cells decreases HIF-2α levels and restores E-cadherin expression. In addition this

expression is affected by CK2 downregulation which, surprisingly, does not cause a significant effect on the levels of VHL30 protein. These results suggest that in 786-O/VHL cells, VHL protein might control E-cadherin levels, and also further give support to the effect caused by CK2 downregulation on HIFs expression is through VHL-independent mechanisms. Usually, loss of VHL function or hypoxia has been postulated to produce identical alterations in gene/protein expression and function. Nevertheless, it has been reported differences in the VHL and hypoxia-induced pathways [277]. These include VHL's ability to regulate apoptosis and senescence [278]. Moreover, in VHL(-/-) renal carcinoma A498 and p53-/- HCT116 cells, reovirus infection was able to reduce HIF-1α levels cell indicating that the decrease of HIF-1alpha mediated by reovirus requires neither VHL nor p53 proteins [279].

In agreement with the results detected by the downregulation of CK2 by siRNAs, CK2 inhibitors do not affect VHL levels at short-term. Surprisingly, at long-term exposures (24 h) CX-4945, but not TBB, decrease dramatically the levels of VHL. This unexpected results is not in concordance with either the previous studies or other reports that have described that the decrease in VHL expression induced by hypoxia in HeLa cells are abolished by TBB [280] or with those that have described that under normoxic conditions TBB increase VHL protein stability in HEK293T transiently transfected to express HA-VHL30 [237]. The results obtained suggest that the effects observed at long-term with CX-4945 might not directly involved the sole of CK2 inhibition.

The pleiotropic effects of CK2 and its connection with the components of other pathways that have prominent impact on EMT such as TGFβ, WNT and Notch signalling pathways [281], [282] suggest the potential contribution of diverse factors in the EMT-like induction promoted by alterations in CK2 subunits.

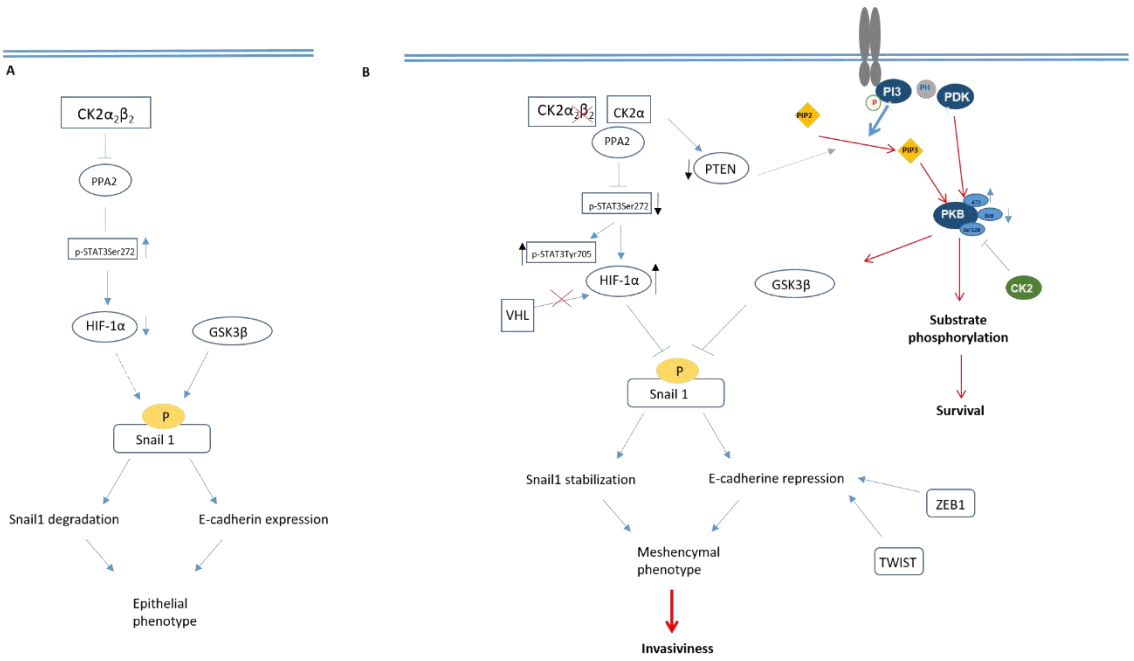


Figure 50. Proposal diagram of CK2β downregulation in HK-2 cells. (A) In HK-2 cells, Snail1 degradation is mediated by GSK3β and CK2 holoenzyme through preventing HIF-1α expression, inducing expression of E-cadherin. (B) In CK2β depleted cells, CK2α free phosphorylates PTEN at Ser370 inducing its destabilization and activation of Akt and GSK3β pathway. Consequently, Snail 1 is not phosphorylated byeGSK3β. Moreover in this cells, CK2α can also induce the activity of PP2A, which in turns dephosphoylates STAT3 at Ser727. Reduction of p-STAT3 Ser727 leads to an increase of p-STAT3 Tyr705 and enhance expression of STAT3 target genes such as HIF-1α which induces Snail1 stabilization. These changes are associated with expression of EMT markers, enhanced cell migration and invasiveness, leading to more aggressive tumor progression.

4.1. ErbB4 expression provokes Akt activation

Previous studies have revealed that down-regulation of ErbB4 occurred in all analyzed RCC types, and it is significantly lower in ccRCC in comparison to papillary RCC, chromophobe RCC, renal oncocytoma and normal renal tissue. Thus ErbB4 is not likely to be an oncogen, instead this receptor might function as a tumour suppressor in ccRCC [283]. The tumorigenic 786-O cell line shows undetectable levels of ErbB4. We decided to re-express ErbB4 JM-a Cyt2, which is the major isoform present in kidney [284], and to explore the effect of this expression on Akt response. In 786-O/ErbB4 cells the basal phosphorylation of Akt and GSK3β is increased as well as the response of Akt to HB-EGF that is more intense and sustained than in 786-O. These results were unexpected since JM-a Cyt-2 does not contain the PI3K binding site. However, as

mentioned in the introduction, specific ligands bind to the extracellular domain of the receptor resulting in the formation of homo- and heterodimeric receptor complexes with subsequent activation of intracellular pathways. It has been reported that upon neuregulin1 (NG1β1) stimulation ErbB4 JM-a Cyt2 forms heterodimeric complexes with ErbB3, which contains multiple PI3K-binding sites and has been shown to be a major recruiter of PI3K. This heterodimerization promotes the activation of Akt pathway [246]. It is also described that ErbB4 forms heterodimeric complexes with other EGFR-family members including the EGFR which is up-regulated in ccRCC [283]. Therefore it is not surprising that the intensity of the response of ERK1/2 to HB-EGF is higher in 786-O/ErbB4 cells.

4.2. ErbB4 expression promotes proliferation and invasiveness features

Contrary to what expected, HB-EGF, which binds to ErbB4 and EGFR, does not increase proliferation either in 786-O control cells or in 786-O/ErbB4 cells. Instead, this growth factor slightly reduces the proliferation in both cell lines. Although it is known that HB-EGF increases proliferation in different cell types [285],[286], Stefan W.Stoll *et al,.*(2012) [287] showed that expression of soluble HB-EGF or full-length transmembrane HB-EGF promotes cell migration and inhibits cell proliferation in human keratinocytes as well as promotes changes in the expression of several epithelial markers including E-cadherin and Snail1. In agreement with this, treatment of 786-O with HB-EGFs significantly reduced E-cadherin levels and increased Snail1 expression. The discrepancy in the results obtained by different groups can be explained by the fact that diverse factors may influence the type of ErbB signaling. As mentioned above, activation of EGFR-family members results in the formation of homo- and heterodimeric receptor complexes that will determine the engagement of specific signalling pathways. It is important to bear in mind, that the different isoforms of ErbB4 present in the cell also affect the type of the cellular response. In fibroblast, expression of ErbB4 JM-a showed increased growth and cell survival whereas ErbB4 JM-b overexpression underwent starvation-induced death [288]. In agreement with this, 786-O/ErbB4(JM-a Cyt-2) cells showed increased proliferation rates and hyper-activation of Akt/GSK3β pro-survival pathway. Moreover, overexpression of ErbB4 completely abolished E-cadherin levels and increased dramatically Snail1 expression in 786-O cells what is in agreement with the reported effect of the ErbB4 JM-a Cyt2 isoform in promoting anchorage free growth [288].

4.3. ErbB4 sensitizes cells to the effect of CX-4945

It has been reported that ErbB4 JM-a CYT-2 promotes resistance whereas JM-b CYT-2 sensitizes to cell death [288].We wanted to explore if CK2 influenced the ErbB4 effects in 786-O cells. Surprisingly, we observed that the expression of ErbB4 sensitizes the 786-O cells to the effects of CX-4945, inducing more rapid cell death. Moreover CX-4945 inhibits the anchorage-independent cell growth in soft agar. These results can be explained by the fact that in 786-O/ErbB4 the activation of PARP takes place at 48 h whereas in control 786-O cells it is not as evident. It is also interesting to note that the expression of ErbB4 reduces LC3-II accumulation and decrease the number of vacuoles as compared to control 786-O cells. These data indicate that the expression of this receptor might overcome the autophagy survival mechanism induced by CX-4945 in 786-O cells, which we have previously discussed, inducing cell death by a mechanism of apoptosis.

As we observed that the inhibition of CK2 by CX-4945 induced different cellular response in cells that express ErbB4, we studied the possible connection between CK2 and ErbB4. The receptor is mainly located in/at the membrane fractions (the fraction that contains the endoplasmic reticulum membranes and that contains plasma membranes), and CK2α and CK2β are distributed among all fractions. The effect provoked by CX-4945 on ErbB4 receptor was quite remarkable since it dramatically reduces ErbB4 expression in all membrane fractions without significantly altering the levels of CK2 subunits.

4.4. CX-4945 affects the ErbB4 processing

It is known that after the ligand-mediated activation and internalization, ErbB4 is either recycled back to the cell surface or transported for lysosomal degradation [244]. Alteration of this balance can change the levels of ErbB4 at cell surface. CX-4945 induces LC3-II accumulation as well as the formation of tiny vacuoles in 786-O/ErbB4, what suggest that this inhibitor would increase the fusion of endosomes, leading to the ErbB4 degradation by autolysomes and lysosome fusion. However, the treatment with chloroquine, which inhibits the late step in autophagy, excludes this possibility since the levels of ErbB4 also decrease whereas LC3-II levels are increased. These results differ from an earlier study that suggest that CX-4945 induces autophagy in PC-9/GR and PC-9/ER cells leading to increased EGFR degradation and that inhibition of autophagy by 3MA or Atg7 siRNA treatment restored the EGFR levels. Interestingly, CX-4945

reduces ErbB4 levels but does not affect membrane proteins such as the transferrin receptor (TfR) or cytosolic proteins such as IκBα whereas also decreases the EGFR. This effect also support the idea that the downregulation of ErbB4 is not due to a mechanism of autophagy, instead other processes are involved in its stability which might also be shared by other EGFR-family members.

It has been shown that overexpression of JM-a Cyt-2 ErbB4 isoform in MCF-7 human breast cancer cell, promotes ErbB4 phosphorylation even in the absence of ligand stimulation by a mechanism involving both ligand-independent tyrosine phosphorylation and proteolytic generation of a soluble ICD [246]. Consisting with this finding, we detected a 80-KDa band in 786-O/ErbB4 cells non-treated with ligand, which was comparable with the band that appeared upon stimulation by HB-EGF or PMA, which is an activator of the PKC. This result supports that JM-a Cyt-2 ErbB4 isoform is constitutive activated and processed. On the other hand concomitant treatment with HB-EGF or PMA and CX-4945 enhances the cleavage of the full-length receptor and accumulation of the Cyt-2 ICD KDa fragment. These preliminary results suggest that CX-4945 induces ErbB4 processing. In addition this inhibitor ultimately induces degradation likely via lysosomal, since treatment with lysosomal inhibitors did not restore full-length ErbB4 expression but promoted the accumulation of the Cyt-2 ICD fragment.

The biological significance of the generated soluble Cyt-2 ICD is currently not fully understood and it has been described that induces divergent effects. Ectopic ICD expression induces mammary epithelial cell differentiation [118], stimulates cell death [117], and it associates with Hdmd2 leading to Hdm2 ubiquitination and increased p53 levels [289]. In contrast, the presence of ErbB4 ICD in the nuclei of breast cancer cells associates with poor prognosis when compared to the presence of intact ErbB4 at the cell surface [290]. Our results indicate that treatment with CX-4945 reduces the levels of Cyt-2 ICD, besides decreasing full length receptor levels. Therefore, it would be plausible that downregulation of ErbB4 and also EGFR by CK2 inhibition with CX-4945 influences the potency and duration of effector pathways, decreasing survival and inducing cell death, as observed in 786-O/ErbB4 cells. It would be a promising finding in cancers where upregulation of ErbB-family members or their mutants constitutive forms, are present.

On the other hand, downregulation of CK2 subunits by siRNA, especially of CKβ, decreases ErBb4 levels. But the most striking result is that ErbB4 interacts with CK2β

even greater than with CK2α, suggesting that CK2β or CK2 holoenzyme is required for the stability of ErBb4 at the cell surface. In a previous study in the group, it was shown that CK2β was downregulated in ccRCC samples corresponding to advanced tumours ,what would suggest a positive correlation between ErbB4 and CK2β expression in ccRCC. Furthermore, in renal cells downregulation of CK2β induced an EMT-like phenotype, downregulating epithelial-type proteins and increasing mesenchymal markers. This EMT-like phenotype induced by CK2β downregulation is accompanied by an increase of the activation of pro-survival Akt/GSK3β pathway, the expression of HIF-2α and the activation of STAT3 at Tyr705 and Ser727. The current data highlight the importance of CK2β downregulation in the EMT-like process in the non-malignant human kidney PTC cell line HK-2 and enhances it in the human ccRCC cancer cell line 786-O.

Conclusions

1. Protein Kinase CK2 inhibitors affect the phosphorylation of Akt in response to HB-EGF, but their effectiveness is cell dependent. Among the CK2 inhibitors, TBB and CX-4945 are the most effective in blocking Akt phosphorylation in response to HB-EGF.

2. CK2 inhibitors do not significantly affect the ERK1/2 activation in response to HB-EGF, except for CX-4945, which increases basal phosphorylation and HB-EGF induced activation of ERK1/2.

3. The normal PTC derived HK-2 cells show significant expression of both Akt1 and Akt2, whereas 786-O cells express Akt2 almost exclusively. In cells, Akt1 is phosphorylated at which is a CK2 target site. Moreover HB-EGF promotes the phosphorylation of Akt1 Ser129 which is also inhibited by CX-4945.

4. In HK-2 cells there is a correlation between changes in the phosphorylation state of Akt Ser129 and Akt Thr308, but this association is not observed in 786-O cells. In addition, CX-4945 inhibits the activation of Akt Thr308 in 786-O cells what indicates that CK2 may affect Akt Thr308 phosphorylation by mechanisms independent of its direct targeting of Akt Ser129.

5. The silencing of CK2 subunits significantly reduced the activity of CK2. In CK2α downregulated cells the CK2α' is likely part of the holoenzyme since the CK2 activity assayed with the eiF2β peptide is hardly affected. By contrast, the CK2 activity on Ts_peptide is reduced. Conversely, in CK2β downregulated cells the CK2 activity on eiF2β peptide is dramatically decreased whereas CK2 activity on Ts_peptide is slightly altered, confirming the presence of free CK2α.

6. The effects of Akt signalling transduction by stable downregulation of CK2 are more remarkable in HK-2 cells, where depletion of CK2β increases p-Akt Ser473 and p-GSK3β Ser9 levels and decreases PTEN levels and Akt Ser129 and Akt Thr308 phosphorylation.

7. Both, HK-2 and 786-O cells the regulatory subunit of CK2 is required for sustaining Akt Thr308 and Akt Ser473 phosphorylation in response to HB-EGF.
8. *In vitro* CK2α and CK2α' exhibit a striking preference for Akt1 phosphorylation as compared to CK2 holoenzyme either $\alpha_2\beta_2$ or $\alpha'_2\beta_2$. By contrast, in cells the activity of CK2 holoenzyme is required for the basal activation of Akt as well as for sustaining the response to HB-EGF.

9. In both 786-O and HK-2 cells the duration of ERK1/2 pathway activation in response to HB-EGF is dramatically decrease by the downregulation of CK2α subunit. Moreover the ERK1/2 activation induced by this growth factor, is attenuated by the expression of VHL protein, which destabilize EGFR receptor.

10. CX-4945 does not affect the viability of HK-2 and 786-O cells at 8 hours whereas TBB decreases it. CX-4945 induces the formation of large intracellular non-lipidic vacuoles and accumulation of LC3-II. This effect is particular in CK2α silenced HK-2 cells and it is accompanied by a stimulation of ERK1/2 and inhibition of Akt, pointing to the involvement of CK2 in preventing autophagy.

11. The regulatory subunit of CK2 plays an important role in the modulation of EMT marker, Snail1, through the regulation of Akt/GSK3β activation.

12. E-cadherin expression in 786-O cells is regulated by the VHL/HIF-2α axis.

13. RNAi-mediated silencing of CK2α and in particular of CK2β, up-regulates HIF-1α in HK-2 cells and HIF-2α in 786-O cells by a VHL-independent mechanism.

14. In HK-2 cells downregulation of CK2, in particular CK2β, increases p-STAT-3 Tyr705 levels and decreases p-STAT3 Ser727 levels. Conversely, in 786-O cells increases p-STAT3 Tyr705 as well as p-STAT3 Ser727, supporting a crucial role of STAT Ser727 in ccRCC and link with CK2β loss.

15. ErbB4 expression in 786-O cells induces activation of the pro-survival Akt/GSK3β pathway and alterations in the expression of EMT markers, promoting an increase of cell proliferation.

16. The overexpression of ErbB4 sensitizes 786-O cell to the effect of CX-4945, overcoming the mechanism of resistance and promoting apoptotic cell death.

17. Both CX-4945 and the downregulation of CK2β affect the stability of ErbB4 and other EGFR-family members, inducing its processing and degradation via lysosomal pathway. Furthermore, CK2β interacts with ErbB4 more efficiently than CK2α, pointing to a requirement of CK2 holoenzyme for ErbB4 stabilization.

18. Summing up, in renal cells unbalanced expression of CK2 subunits alters signalling pathways. Especially, downregulation of CK2β induced an EMT-like phenotype, downregulating epithelial-type proteins and increasing mesenchymal markers. This EMT-like phenotype induced by CK2β downregulation is accompanied by an increased activation of the pro-survival Akt/GSK3β pathway, the expression of HIFs and alteration of STAT3 phosphorylation at Tyr705 and Ser727. The current data highlight the importance of CK2β in the regulation of multiple pathways that have a crucial role in tumoral progression.

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