A-Kinase Anchoring Protein-based compartmentalization of kinases in the control of canonical Wnt signaling and Aquaporin-2 trafficking

DISSERTATION

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I hereby declare that the following work was performed by me alone, only with the use of literature and materials listed. I further declare that to the best of my knowledge this work is novel and does not conflict with any earlier published dissertations.

Berlin, 24/02/2017

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Summary

Protein kinase anchoring by scaffolding proteins is a cornerstone in the regulation of molecular pathways. One of the best studied examples is anchoring of Protein Kinase A (PKA) by A-Kinase Anchoring Proteins (AKAPs). The present study investigates the involvement of PKA-containing signalosomes in two distinct contexts.

The first part of this thesis relates to the role of AKAPs in the regulation of canonical Wnt signaling. Canonical Wnt signaling plays a role in a plethora of molecular mechanisms, including cell proliferation and fate determination, with important clinical implications in several pathologies, including cancer. Wnt signaling inhibition in basal state cells relies on the proteolysis of β-catenin. This depends on Glycogen Synthase Kinase 3β (GSK3β) phosphorylation of β-catenin, and is antagonized by PKA. The present study addresses the role of the cytosolic AKAP Glycogen Synthase Kinase Interaction Protein (GSKIP), and reveals how this scaffold scavenges both PKA and GSK3β away from β-catenin. The result is that GSKIP promotes and inhibits Wnt signaling, highlighting the importance of the GSKIP-mediated PKA-GSK3β crosstalk in the control of Wnt signaling.

The second part of the thesis describes novel findings about the translocation of the water channel Aquaporin-2 (AQP2) into the plasma membrane of renal principal cells. AQP2 insertion increases water permeability of the collecting duct, fine-tuning water homeostasis. This process is not yet entirely understood. Deregulations of AQP2 translocation result in either excessive water excretion or retention, and are clinically relevant in both cases. Previous research established an automated immunofluorescence-based screening and identified the poorly characterized Cyclin Dependent Kinase 18 (CDK18) involvement in AQP2 regulation. In the second part of the thesis it is described how CDK18 is able to control AQP2 localization, abundance and phosphorylation. It was also discovered that the E3 ubiquitin ligase STIP1 Homology And U-Box Containing Protein 1 (STUB1) is interacting with CDK18 and mediates the CDK18-driven AQP2 ubiquitination. STUB1 is additionally responsible for the binding of PKA in proximity with CDK18 and AQP2, behaving in an AKAP-like way. The identification of a novel kinase involved in the control of AQP2 translocation could pave the way to a causal pharmacological window for AQP2-related pathologies.

Zusammenfassung

Die räumliche und zeitliche Kontrolle von Proteinkinasen durch Gerüstproteine ist ein Meilenstein der Regulation molekularer Signalwege. Eines der bisher am besten untersuchten Beispiele ist die intrazelluläre Lokalisation von Protein Kinase A (PKA) durch A-Kinase Ankerproteine (AKAP). Diese Arbeit untersucht die Rolle von PKA-basierten Signalkomplexen in zwei unterschiedlichen Modellen.

Der erste Teil der Dissertation befasst sich mit der Rolle von AKAPs in der Regulation des kanonischen Wnt-Signalweges, der eine Vielzahl von molekularen Mechanismen reguliert. Unter anderem ist er für Zellproliferation und –differenzierung entscheidend und spielt daher eine wichtige Rolle bei der Entstehung von Tumorerkrankungen. Im Grundzustand der Zelle wird das Wnt-Signal durch Proteolyse des Effektorproteins β-catenin inhibiert. Der Abbau wird durch Glycogen Synthase Kinase 3β (GSK3β)-Phosphorylierung von β-catenin induziert; umgekehrt führt eine Phosphorylierung durch PKA zur Stabilisierung des Proteins. In dieser Arbeit wurde das AKAP Glycogen Synthase Kinase Interaction Protein (GSKIP) untersucht, das sowohl PKA als auch GSK3β binden kann, und gezeigt, wie es den Zugang beider Kinasen zu β-catenin regulieren kann. Somit kann GSKIP den Wnt-Signalweg sowohl fördern als auch inhibieren, was die Bedeutung von GSKIP für das PKA-GSK3β-Zusammenspiel im Wnt-Signalwegs hervorhebt.

Im zweiten Teil der Dissertation werden neue Mechanismen beschrieben, die eine Umverteilung des Wasserkanals Aquaporin-2 (AQP2) in die Plasmamembran von renalen Hauptzellen regulieren. Der Einbau von AQP2 in die Plasmamembran erhöht die Wasserpermeabilität des renalen Sammelrohrs und trägt zur Feinregulation des Wasserhaushalts bei. Dieser Prozess unterliegt einem komplexen Zusammenspiel von Enzymen und Gerüstproteinen, das im Ganzen noch nicht verstanden ist. Fehlregulationen der AQP2-Umverteilung führen zu einem exzessiven Wasserverlust über den Primärharn bzw. zu einer abnormalen Wasserretention.

In einer vorangegangenen Studie wurde ein automatisiertes Immunfluoreszenzmikroskopie-basiertes Verfahren entwickelt, das die bisher wenig charakterisierte Kinase CDK18 (Cyclin Dependent Kinase 18) als entscheidend für die AQP2 Umverteilung identifizierte. In dieser Arbeit wird beschrieben, wie CDK18 die Lokalisierung, Proteinmenge und den Phosphorylierungsstatus von AQP2 kontrolliert. Die E3 Ubiquitin-Ligase STUB1 (STIP1 Homology And U-Box Containing Protein 1) interagiert mit CDK18 und vermittelt über CDK18 die Ubiquitinierung von AQP2. STUB1 hält PKA in räumlicher Nähe zu CDK18 und AQP2 und weist damit eine AKAP-ähnliche Funktion auf. Die Identifizierung einer neuen, für die AQP2-

Umverteilung relevanten Kinase könnte neuartige pharmakologische Ansätze für die kausale Behandlung von AQP2-basierten Krankheiten liefern.

Abbreviations

β-TrCP: β-Transducin repeat Containing E3 ubiquitin Protein ligase

Aa: Amino Acid

ACE: Angiotensin Converting Enzyme

AngII: angiotensin II

ANOVA: analysis of variance

AMFR: Autocrine Motility Factor Receptor

AMP: Adenosine Monophosphate

AKAP: A-Kinase Anchoring Protein

AKB: A-Kinase Binding

APC: Adenomatous Polyposis Coli

AQP2: Aquaporin-2

ATP: adenosine triphosphate

AVP: arginine-vasopressin

BIG: Brefeldin A-Inhibited Guanine nucleotide-exchange factor

BRE1B: RING-finger protein 40

C: PKA catalytic subunit

cAMP: 3',5'-cyclic Adenosine Monophosphate

CDK18: Cyclin Dependent Kinase 18

CHIP: Carboxy Terminus Of Hsp70-Interacting Protein

CK: Casein Kinase

CUL5: cullin-5

Cyc A2: cyclin A2

D/D: dimerization and docking

Dvl: Dishevelled

ENaC: Epithelial Sodium Channel

$G_{\alpha S}$: stimulatory G protein α

GID: GSK3 Interaction Domain

GPCR: G Protein-Coupled Receptor

GSK3: Glycogen Synthase Kinase 3

GSKIP: Glycogen Synthase Kinase Interaction Protein

Fsk: forskolin

Fzd: Frizzled

HA: hemagglutinin

HEK293: Human Embryonic Kidney 293 cells

ILK: Integrin-linked Kinase

ITCH: Itchy E3 Ubiquitin Protein Ligase

IF: immunofluorescence

IP: immunoprecipitation

JNK: c-Jun associated N-terminal Kinase

KAIB: Kinase Assay Incubation Buffer

KALB: Kinase Assay Lysis Buffer

LIP: Lyst Interacting Protein

LRP5/6: Low-density lipoprotein Receptor-related Protein 5/6

MAP: Mitogen Activated Protein

MAPK: Mitogen Activated Protein Kinase

MDC4: Murine Collecting Duct 4 cells

MDCK: Madin-Darby Canine Kidney

MLB: mild lysis buffer

MVB: Multi Vesicular Body

NEDD4: neural precursor cell expressed, developmentally down regulated gene 4, E3 ubiquitin protein ligase

NEDD4L: NEDD4-like

NKCC: Na^+-K^+-Cl^- cotransporter

P: phosphate

p70RSK: p70 Ribosomal S6 Kinase

p90RSK1: p90 Ribosomal S6 Kinase

PBS: Phosphate Buffered Saline

PDE: Phosphodiesterase

PI3K: Phosphatidylinositol-4,5-bisphosphate 3-kinase

PK: Protein Kinase

PKA: Protein Kinase A

pNP: para-Nitrophenol

pNPP: para-Nitrophenylphosphate

PP: Protein Phosphatase

PP2R: regulatory subunit of the protein phosphatase 2

R: PKA regulatory subunit

Rab: Rab, Member Of RAS Oncogene Family Like

RAD9: DNA repair exonuclease Rad9 homolog A

Rb: retinoblastoma

Rho: Ras homolog

RIPA: Radio Immuno Precipitation Assay buffer

RNAi: RNA interference

ROCK: Rho-associated, coiled-coil-containing protein kinase 1

SIAD: Syndrome of Inappropriate Antidiuretic Hormone secretion

siRNA: small interfering RNA

SLB: standard lysis buffer

sq-PCR: semi-quantitative polymerase chain reaction

STUB1: STIP1 Homology And U-Box Containing Protein 1

TBS: Tris Buffered Saline

TCF/LEF: T Cell Factor/Lymphoid Enhancer-Binding Factor

Ubq: Ubiquitin

UT: Urea Transporter

V1/2R Vasopressin Receptor 1/2

VACM1: vasopressin-activated calcium mobilizing

WB: Western Blot

Wt: wild type

ZFPL1: Zinc Finger Protein Like 1

Amino acid residues are indicated in the three-letter code unless stated otherwise.

Preamble

This thesis consists of two distinct projects. In the first part, an analysis of the role of the small AKAP GSKIP in the context of Wnt signaling will be presented; this project was concluded and published in September 2016 (1). In the second part, recent advances towards the characterization of the kinase network controlling the translocation of the water channel AQP2 will be presented.

Introduction

The study of the rapid and precise transmission of extracellular signals into the intracellular environment is one of the milestones of cellular biology. Exploiting this ability, cells, either as single entities or as parts of an organism, are able to adapt themselves to the altered challenges imposed by a variable microenvironment and, ultimately, ensure the survival either for themselves or for their host. Various molecular mechanisms are employed to ensure the transmission of the stimuli coming from the extracellular space. Among these, addition of a negative charge via phosphorylation of a specific amino acidic residue on a polypeptide chain can result in a rapid change of protein folding. This dramatically influences protein behaviour in terms of binding ability and enzymatic activity. Regulation of kinase target recognition and phosphorylation is one of the most complexly regulated aspects of the sub-cellular milieu. Especially when treating with an ubiquitous kinase, or one that is diffused through several sub-cellular compartments, the issue of precision in both spatial and temporal control is of crucial importance.

GSK3 and GSK3β biology

GSK3 offers an example of a highly conserved protein kinase, expressed throughout the *eukaryota* and localized in the cytosol, nuclei and mitochondria (2). GSK3 is present in most vertebrates as two distinct but highly homologous isoforms, GSK3α and GSK3β, and is among the few known kinases that are constitutively active (3). This means that, unless it is actively inhibited, it is constantly able to phosphorylate its substrates, even if it shows a strong preference for pre-phosphorylated “primed” ones (4). The increased propensity of GSK3β for a substrate pre-phosphorylated 4 amino acids in the carboxy-terminal direction (respect to the intended target residue) increases the specificity of the kinase (5).

The extent to which the two isoforms are functionally redundant is not fully clear, suggesting a high dependence the context (6). GSK3β is the most fully characterized and studied of the two.

GSK3β is widely involved in cellular signaling pathways, such as Wnt, NFkB, Hedgehog, Notch, Hippo, NFAT, PI3K and insulin signaling (7). It is able to phosphorylate around 20% of the human proteome (8); its activity has therefore to be tightly regulated to ensure correct cellular signaling. GSK3β activity is modulated in several ways, including endosome sequestration (8) and inhibitory phosphorylation, mainly at position Ser9, by several protein kinases, including the Protein Kinases (PK) B (9), PKG (10), different PKC isoforms (11), p70 ribosomal S6 kinase (p70RSK) (12), p90 ribosomal S6 kinase (p90RSK/RSK1) (13), serum/glucocorticoid regulated kinase 1 (SGK1) (14), integrin-linked kinase (ILK) (15) and the cAMP-dependent PKA (16,17). Additionally, an activating phosphorylation at Tyr216 is known, but, due to the high basal activity of GSK3β, a greater attention in the literature is paid to the inhibitory Ser9 phosphorylation (18). This one causes a pseudo-substrate inhibition of the substrate-recognition site of GSK3β, so strongly reducing its activity (19).

GSK3β and Wnt signaling

A well-studied example of a GSK3β-involving pathway is the canonical Wnt, or Wnt/β-catenin pathway (Fig. 1) (20,21). In this pathway, GSK3β is part of a multiprotein complex, known as the “destruction complex”, comprising also Casein Kinase (CK), the scaffold protein Axin, Dishevelled (Dvl), the Adenomatous Polyposis Coli (APC) protein, the β-Transducin repeat Containing E3 ubiquitin Protein ligase (β-TrCP), and β-catenin, that is the main substrate of the complex (20). Cytosolic β-catenin is bound by the destruction complex and phosphorylated first by CK at position Ser45, acting as a priming event for GSK3β, that is then able to serially phosphorylate the positions Thr41, Ser37 and Ser33, with Thr41 priming for Ser37 and Ser37 priming fro Ser33. This prompts the poly-ubiquitination of β-catenin and its subsequent proteasomal degradation (22). Under resting condition in cells hosting an intact canonical Wnt pathway, β-catenin is constantly synthesized and degraded in the cytosol. Ligands of the Wnt family binds the cognate transmembrane receptor Frizzled (Fzd) and its co-receptor Low-density lipoprotein Receptor-related Protein 5/6 (LRP5/6, also known as Arrow). This causes their dimerization and the recruitment of the destruction complex on Fzd-LRP5/6 cytosolic domains close to the plasma membrane (23). This recruitment determines the inactivation of the destruction complex by dissociation of β-TrCP, causing a halt of the ubiquitination of β-catenin (24). This does not necessarily involve an inhibition of the kinase activity of GSK3β; even if sequestration of the kinase in late endosomes might be involved, the Ser9 phosphorylation of GSK3β seems unaffected by canonical Wnt stimulation (8).

Because of the slowed degradation of β-catenin, newly synthesized β-catenin accumulates first in the cytosol and finally translocates into the nucleus, where it directs the transcriptional activation of the canonical Wnt signaling target genes (24). There, β-catenin binds the transcription factor belonging to the family T Cell Factor/Lymphoid Enhancer-binding Factor (TCF/LEF), leading to the decreased interaction of TCF with the transcriptional inhibitor Groucho and the formation of a transcriptional activator complex (25,26). A widely reported target gene of the β-catenin/TCF-organized complex is *Axin2*; its expression can be used to monitor Wnt/β-catenin activity (27). Additional genes whose transcription is activated by the canonical Wnt pathway include, but are not limited to, the transcription factor Myc and the Cyclin D1, that contribute to the Wnt-induced cell proliferation (28). Transcriptional control mediated by the canonical Wnt pathway has been described to positively modulate stem cells self-renewal in several organs ranging from the intestine to the stomach, the skin, the hair follicle, and the mammary gland (29-32). Since its pro-proliferative activity, the canonical Wnt pathway has also important implications in several types of tumour, most prominently, but not only, colon cancer (20,33-35).

A second role of GSK3β in Wnt signaling has opposite effects compared to the one just described. In fact, GSK3β is able to phosphorylate the cytosolic domain of LRP6 at Ser1490, increasing its affinity for Axin, and so for the β-catenin destruction complex (36,37). This is associated with an increased Wnt output through decreased β-catenin degradation rate, and can be considered as a positive effect of GSK3β on Wnt signaling (36).

It is also important to note that also β-catenin has a second, Wnt-unrelated, signaling role, especially in epithelia, where it binds to the cytoplasmic tails of various cadherins in a separate pool with respect to the cytosolic, Wnt-relevant one. The Cadherin-associated β-catenin shows a longer half life than the cytosolic, Wnt-related one, and movement of β-catenin between the two pools is a slow process, leading them to orchestrate biologically distinct roles (38). Compartmentalization of Wnt signaling-relevant proteins is not completely elucidated, and has been in part analysed in the present work (1).

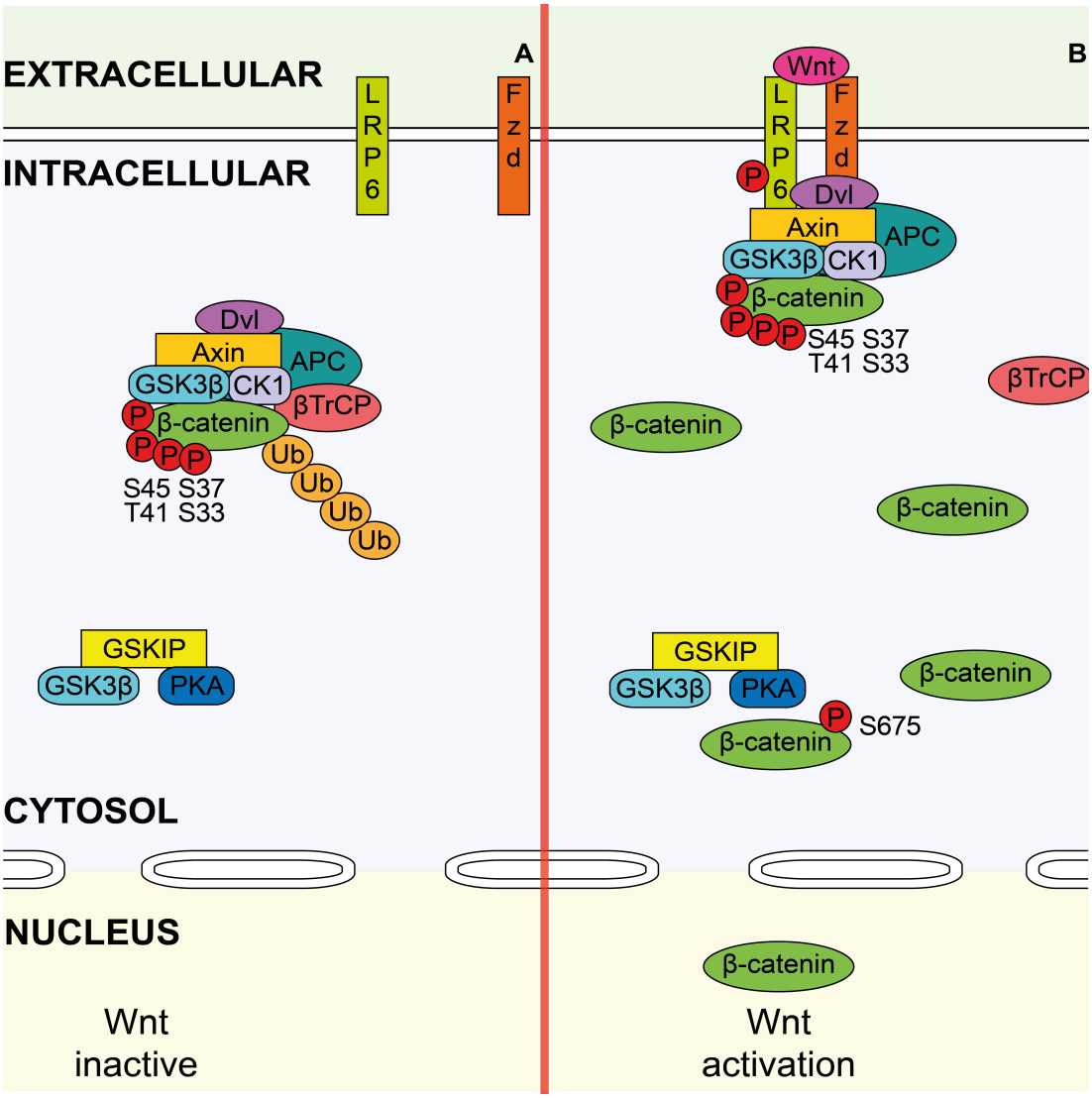

Fig. 1: Scheme of the canonical Wnt signaling in (A) resting state and (B) under Wnt activation. Depicted are the receptor/co-receptor complex for the Wnt ligand (Fzd: Frizzled, LRP6: Low-density lipoprotein Receptor-related Protein 6), the destruction complex components (Dvl: Dishevelled, Axin, APC: Adenomatous Polyposis Coli, GSK3β: Glycogen Synthase Kinase 3β, CK1: Casein Kinase 1, β-TrCP: β-Transducin repeat Containing E3 ubiquitin Protein ligase) and GSKIP (Glycogen Synthase Kinase 3 Interaction Protein) and its interactors (PKA: cAMP-dependent Protein Kinase A and GSK3β: Glycogen Synthase Kinase 3β). Under Wnt stimulus, cytosolic β-catenin ubiquitination and degradation stop, prompting its translocation into the nucleus where it stimulates transcription.

Further abbreviations: cAMP: 3', 5'-cyclic monophosphate Ub, Ubiquitin, S, Ser; T, Thr (20).

PKA biology

Another kinase which has an impact on Wnt signaling is the cAMP-dependent serine/threonine PKA. A PKA holoenzyme is a tetramer consisting of an homodimer of regulatory (R) subunits, each of them binding one catalytic (C) subunit and thereby maintaining the kinase in an inactive state. The catalytic subunits can comprise Cα, Cβ, Cγ or PrKX, and the regulatory ones, that in resting conditions bind and inhibit the catalytic ones, are RIα, RIβ, RIIα or RIIβ.

Various extra-cellular stimuli (e.g. hormones) activate a highly conserved and almost ubiquitous cascade: they stimulate their cognate G protein-coupled receptors (GPCR), which in turn activate the stimulatory G protein α ($G_{\alpha s}$) (Fig. 2A). Active $G_{\alpha s}$ causes the activation of Adenylate Cyclase (AC) proteins, which convert adenosine triphosphate (ATP) into 3′,5′-cyclic adenosine monophosphate (cAMP), the main activator of PKA. The binding of cAMP to the regulatory subunits enables the catalytic ones to phosphorylate the nearby targets (39-46). The PKA-mediated phosphorylation can either activate the substrates, such as the water channel AQP2 (47) or inactivate them such as the small GTPase RhoA (48). As it is generally accepted for most kinases, after phosphorylation by PKA a substrate can revert to its basal state through dephosphorylation by protein phosphatase (49).

AKAPs as regulators of PKA activity

Fine tuning of PKA activity is achieved both by spatial and temporal control; among the principal regulatory elements involved the AKAP family holds a preeminent position (Fig. 2A and B) (45,50-52).

The AKAPs are a heterogeneous group of proteins comprising around fifty members, defined by the presence of one or more A-Kinase Binding domains (AKB), that typically recognize the R subunits of PKA with various grades of specificity, allowing the distinction between RI-, RII- or dual-specific AKAPs (45,46). Distinct AKAPs are responsible for the allocation of PKA to different pools harboured near the various cell organelles, including nucleus, cytosol, mitochondria, plasma membrane, cytoskeleton and the Golgi apparatus (46,51,53-55). The same AKAP localized in different subcellular compartments may organize different interactomes, and can influence different signaling pathways (50,56,57).

Canonical AKAPs interact with the conserved Dimerization and Docking (D/D) domain of regulatory subunits of PKA *via* their structurally conserved AKB domains. AKAPs form amphipathic helixes that establish hydrophobic interactions with a hydrophobic pocket formed by the D/D domain (58-61). Non-canonical AKAPs are

characterized by a different mode of interaction with PKA that can be unique for each protein (50).

AKAPs constitute hubs for the control of cAMP signaling and its interaction with multiple signaling pathways, and organize multi-protein complexes fundamental for fine regulation of cAMP signaling. For example, simultaneous AKAP binding to PKA, its substrates and Phosphodiesterases (PDEs) causes the generation of a directional gradient of cAMP, that is degraded into AMP by the PDEs, so establishing a space-specific phosphorylation of the target proteins. Moreover, the presence of phosphatases causes a tight control of the amount of time the target stays phosphorylated, since it can be quickly de-phosphorylated back to its basal state (50,51). Taken together, the result is a tight spatially and temporally controlled system, necessary for the precise regulation of physiological processes such as the renal inner medullary collecting duct principal cells water reabsorption, mediated by PKA-driven phosphorylation of the water channel AQP2 (see later in the text) (47).

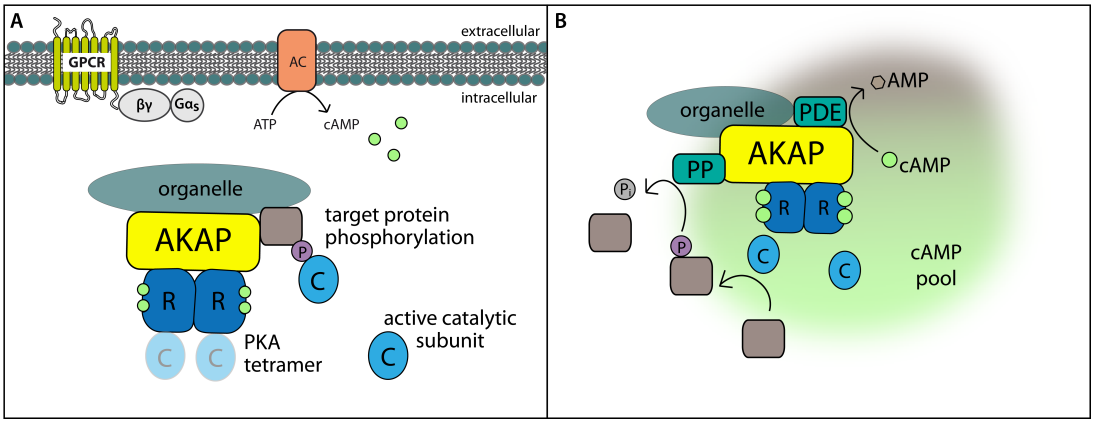

Fig. 2: Representation of the role of A-Kinase Anchoring Protein (AKAP) in Protein Kinase A (PKA) signaling: (A) signal initiation and (B) termination; adapted from (50).

A- The binding of various ligands to membrane receptors, like G Protein-Coupled Receptors (GPCR) determines the activation of the Stimulatory G protein α ($G_{\alpha S}$), followed by Adenylate Cyclase (AC) activation. This determines an increased adenosine triphosphate (ATP) into 3′,5′-cyclic adenosine monophosphate (cAMP) conversion, that in turn binds to the Regulatory (R) subunits of the cAMP dependent PKA tetramer, determining the activation of the catalytic (C) subunits, that are then free to phosphorylate the nearby targets.

B- The phosphorylations mediated by active PKA are limited by two AKAP-dependent mechanisms: first, binding of Phosphodiesterases (PDE) to the AKAP signalosome insures rapid conversion of cAMP to AMP. Second, the presence of Protein Phosphatases (PP) bound to the AKAP permits a rapid de-phosphorylation of the target proteins.

PKA and AKAPs involvement in Wnt signaling

In the context of Wnt signaling, PKA is known to phosphorylate β-catenin at position Ser675, enhancing its stability and nuclear translocation (Fig. 1) (62,63). Among the AKAPs involved in PKA-mediated β-catenin phosphorylation is the Brefeldin A-Inhibited Guanine nucleotide-exchange factor 2 (BIG2), an AKAP interacting with the β-catenin binder BIG1. This enhances β-catenin phosphorylation at Ser675, increasing its stabilization and promoting Wnt-induced transcription (64). Moreover, PKA is know to phosphorylate GSK3β at the crucial, Wnt-independent regulatory position Ser9 (16), inhibiting its activity, and thus potentially promoting Wnt signaling both in a direct and an indirect way. At least two AKAPs are known to facilitate the inhibitory PKA-driven Ser9 phosphorylation of GSK3β: AKAP220 (also known as AKAP11, or Protein Phosphatase 1, Regulatory Subunit 44) (65,66) and GSKIP (67,68).

GSK3β and PKA interacting AKAPs: AKAP220 and GSKIP

AKAP220 is an ubiquitously expressed membrane-bound protein, able to bind several enzymes including PKA, GSK3β and PP1 (65,69-71), and to reorganize the actin apical cytoskeleton, so influencing vesicular trafficking (72). GSK3β binding to AKAP220 at the residue Thr1132 is dependent on a phosphorylation of the same residue mediated by GSK3β itself, even if a previous study demonstrated binding of kinase dead GSK3β to AKAP220, so hinting at the presence of further, still uncharacterized contact points between the two (65,71).

GSKIP is a small (16 kDa) cytosolic AKAP able, in an overexpression system, to increase the β-catenin-dependent proliferation of SHSY-5Y neuroblastoma cells via inhibition of GSK3β (73). It was also implicated in mitochondrial elongation (74) and duplication of the GSKIP containing genomic region predisposes to familiar myeloid malignancies (75). GSKIP null mice show perinatal mortality due to failure in breathing initiation, possibly because of enhanced GSK3β activity (76). Moreover, the *null* animals show a highly penetrant phenotype of cleft plate, reminiscent of several GSK3β transgenic mouse models (7). How, and if, GSKIP binding to GSK3β is influencing Wnt signaling was unknown at the beginning of the present work (1).

Since the possibility of exploiting anchor protein-kinase interactions as drug targets (51), the first part of this thesis aimed at unravelling the complex protein interplay caused by GSK3β compartmentalization by AKAPs, taking as a focus the role of GSKIP and comparing it to AKAP220. Specifically, the main object of this study was

the Wnt canonical signalling, that could represent an alluring target for pharmacological modulation (20).

AQP2 translocation

Another physiological process in which cAMP-driven phosphorylation plays a crucial role is the regulation of the translocation of the water channel AQP2, central for the fine control of water reabsorption form the primary urine. AQP2 translocation happens in the terminal part of the nephron, the collecting duct.

The nephron

In an adult healthy human, the kidneys filter the entire amount of blood several times per day, producing around 180 l of pre-urine, largely constituted of water. Of this massive amount, around 99% is recovered in a multistep process in the nephron, the functional unit of the kidney. One human normal kidney contains 800,000 to 1.5 million nephrons, that are organized as a series of differentially permeable sections (77).

The first filtering unit of the nephron is the glomerulus, responsible for the production of a large volume of filtrate. After the glomerulus the filtrate enters the proximal tubule and the descending loop of Henle. Here, a large part of the water (around 90%) is reabsorbed constitutively through AQP1, a water channel functionally distinct from AQP2 (78). The ascending limb of Henle and the distal convoluted tubules, the following parts of the tubular system, are impermeable, but there sodium and chloride are reabsorbed to maintain the osmotic gradient that drives water recovery (79).

The final step in regulation of water permeability is operated in the collecting duct, the terminal part of the nephron; it accounts for the reabsorption of 10 to 20 litres of water per day, and is subjected to hormonal control (Fig. 3). In the collecting duct, the final amount of urine is produced before it is collected in the bladder (80). The collecting duct epithelium comprises two distinct cell types: principal and intercalated cells. The last ones are responsible for secretion of proton or bicarbonate, and maintain the acid-basis homeostasis. The principal cells are the ones directly responsible for the water reabsorption, and express the water channels AQP2, 3 and 4.

Major contribution to water retention is due to the Renin-Angiotensin-Aldosterone-System (RAAS), responsible for increasing blood pressure both by inducing vasoconstriction and increasing sodium and water retention in the kidney (81). Renin is synthesized in the juxtaglomerular apparatus, a specialized organelle close to the distal collecting tubule, in response to a decrease of arterial blood pressure. Renin hydrolyses angiotensinogen to angiotensin I that is successively cleaved by the

Angiotensin Converting Enzyme (ACE) into angiotensin II (AngII). AngII stimulates the secretion of aldosterone from the adrenal gland, and together, AngII and aldosterone activate the epithelial sodium channel (ENaC) in the collecting duct, so leading ultimately to increased water reabsorption (82-84). The maximal water reabsorption, however, requires the synergistic activation of AngII, aldosterone and arginine-vasopressin (AVP, also known as antidiuretic hormone, ADH) (85).

AVP and AQP2-mediated antidiuresis

AVP is a nona-peptide produced in the hypothalamus and then collected in the pituitary gland *via* axonal transport; in case of decreased blood plasma volume or increased blood electrolyte concentration, it is released into the bloodstream (86).

AVP binds and signals mainly *via* two GPCRs, the Vasopressin Receptor type 1 (V1R) and type 2 (V2R), both consisting of seven transmembrane domains, an extracellular amino- and an intracellular carboxy-terminus. V1R is mainly localized in the brain, liver and peripheral vasculature, and its collecting duct localization seems to be limited to the intercalated cells (87,88); V2R is expressed in the ear, in the sperm and in the kidney thick ascending limb and collecting duct, where the receptor localizes on the basolateral membrane of the principal cells (89-91).

After the binding of nona-peptide AVP to V2R, the bound $G_{\alpha s}$ induces the activation of the AC3 (92) and 6 (93-95), resulting in the cytosolic increase of cAMP and the following activation of PKA (96). Beside its effect on AQP2, AVP promotes NaCl reabsorption by activating the Na^+-K^+-Cl^--cotransporter (NKCC) in the thick ascending limb of Henle, decreasing the renal sodium excretion and increasing the medullary osmotic gradient (97,98). In addition, AVP increases the cell surface expression of urea transporter 1 (UT1) and ENaC, improving the medullary reabsorption of urea and sodium (97,99). Moreover, AVP elevates the intracellular Ca^{2+} concentration, possibly influencing water retention (100). AVP has been suggested to possess an anti-apoptotic effect, to promote Wnt signalling and to influence several mitogen-activated protein (MAP) kinases (101,102). However, this work focuses on the effect of AVP on AQP2.

In the kidneys, AQP1 to 8 and 11 are expressed; AQP1 mediates water reabsorption in the descending loop of Henle, while AQP3 and 4 are localized into the basal plasma membrane of the primary collecting duct cells, AQP5 and 6 were detected in the intercalated cells, and AQP7, 8 and 11 are expressed in the proximal tubule (103-116). AQP2 is an integral membrane proteins assembled in homotetramers, with each monomer forming a water pore (117). The importance of AQP2 is underlined by the early postnatal death of AQP2 *null* mice (118). AQP2

shows high level of protein sequence conservation among the mammals, and can be glycosylated at Asn 123 (119-121). Although the role of this post-translational modification is poorly understood, it does not seem to affect its water conductivity (119-124). The membrane abundance of AQP2 is influenced by both its phosphorylation (Ser256, Ser261, Ser264 and Ser269) and ubiquitination (Lys270), taking place at the carboxy-terminus of the protein, that regulate the balance between its exocytosis-like insertion into the plasma membrane and endocytic internalization (125-128).

Despite considerable attention, the trafficking and biosynthetic pathway of AQP2 still presents some poorly understood points. After folding, homo-tetramerisation and high mannose glycan attachment in the endoplasmic reticulum, AQP2 is sent to the Golgi apparatus, where further processing results in complex N-glycosylation of the glycans attached to one or two molecules of the homotetramer (121,124). A study performed on Madin-Darby Canine Kidney (MDCK) cells revealed exposure of the freshly synthesized AQP2 to the basolateral plasma membrane, followed by clathrin-mediated internalization, microtubule-mediated transcytosis and finally positioning on Ras-related protein Rab11 (Rab11)-positive vesicles, partially co-localizing with PKA. The trafficking of AQP2 to the basal plasma membrane could be involved in cell migration and tubulogenesis (129).

Upon the AVP-dependent cAMP increase, a sub-pool of PKA, localized on AQP2-bearing vesicles by AKAPs (130-132), phosphorylates AQP2 at Ser256, triggering the AQP2 redistribution to the apical plasma membrane (Fig. 3) (133-135). Three out of four monomers of AQP2 need to be phosphorylated at the Ser265 position to achieve translocation (136). However, phosphorylation of Ser256 of AQP2 alone is not able to tether AQP2 to the plasma membrane, and AQP2 internalization can occur even if Ser256 is still phosphorylated (137-139).

The additional phosphorylation of Ser264 and Ser269 by yet to be defined kinases is also increased after AVP stimulation; AQP2 phosphorylated at Ser264 is localised at the plasma membrane and endocytic retrieval compartments, while AQP2 phosphorylated at Ser269 is only detectable at the apical plasma membrane, hinting at this phosphorylation preventing AQP2 internalisation (140-142). The phosphorylation of Ser264 and 269 seems to be also possible in the absence of Ser256 phosphorylation (143). Moreover, AVP enhances AQP2 total protein abundance by stimulating its transcription and translation and preventing its degradation (102,111,126,144-150).

After PKA phosphorylation, AQP2 dissociates from G-actin and assembles with tropomyosin 5b (TM5b), which induces a destabilisation of the F-actin network (151).

In parallel PKA phosphorylates and thus inhibits Ras HOmolog A (RhoA), leading to reduced binding of RhoA to ROCK (152,153). As a result, the F-actin cytoskeleton depolymerises promoting AQP2 translocation to the plasma membrane (154,155). The SNARE proteins-mediated (156) insertion of the AQP2-bearing vesicle on the plasma membrane of the principal cell allows the collecting duct to increase its water permeability (157). The plasma membrane localization of AQP2 is maintained by its interaction with myelin- and lymphocyte-associated protein (MAL), present on the apical plasma membrane of collecting duct cells; MAL interacts with phospho-mimicking Ser256 AQP2 and increases its cell surface expression, probably by reducing its internalisation (158,159).

In contrast to phosphorylation of Ser256, the phosphorylation of Ser261 of AQP2 is diminished upon AVP challenge; this site can be phosphorylated by several kinases, comprising Mitogen-Activated Protein Kinase (MAPK) 1 and 3 (also known as Extracellular Regulated Kinase or ERK 1 and 2 respectively) (160), MAPK8 and 9 (also known as c-Jun N-terminal Kinase or JNK1 and 2 respectively) (161), Cyclin Dependent Kinase (CDK) 1 and 5 (156), and MAPK14 (p38 MAPK). This phosphorylation is associated with AQP2 ubiquitination and endocytosis, being characterized as a functional opposite of the Ser256 phosphorylation (102,162).

The internalization of AQP2 can be increased by the activation of the PKC inducing the short-chain ubiquitination of AQP2, even if a direct AQP2 phosphorylation by PKC seems unlikely (125,126,139,163,164). Removal of AQP2 form the plasma membrane occurs in a clathrin-mediated way (165) and can be increased by short-chain ubiquitination at Lys270 (126). Several E3 ubiquitin ligase are suggested to play a role in AQP2 Lys270 ubiquitination, comprising RING-finger protein 40 (BRE1B), cullin-5 (CUL5, alternative name vasopressin-activated calcium mobilizing, or VACM1), neural precursor cell expressed, developmentally down regulated gene 4, E3 ubiquitin protein ligase (NEDD4), NEDD4-like (NEDD4L), Autocrine Motility Factor Receptor (AMFR), STIP1 Homology And U-Box Containing Protein 1 (STUB1, also known as Carboxy Terminus Of Hsp70-Interacting Protein, or CHIP), Itchy E3 Ubiquitin Protein Ligase (ITCH) and Zinc Finger Protein Like 1 (ZFPL1) (166-168). According to bioinformatic prediction, the ubiquitin ligases most likely to ubiquitinate AQP2 are NEDD4 and NEDD4L, even if *in vitro* evidence is still missing (168,169).

Thanks to the direct interaction of AQP2 with the endocytic machinery, clathrin-coated vesicles are formed and AQP2 is internalised into early endosomes, that mature and coalesce into multi vesicular bodies (MVB) (170,171). From there, AQP2 can be directed to lysosomal degradation via the interaction with lyst-interacting protein 5 (LIP5) (172-175), or AQP2 can be de-ubiquitinated and enter the Rab11-

dependent recycling pathway (176-180), or be targeted by poly-ubiquitination for proteasomal degradation (102). Another possibility is the fusion of the MVB with the apical plasma membrane and release of the AQP2 exosomes into the lumen (181,182); these processes are antagonized by the AVP-driven PKA activity (102).

Thus the net AQP2 contribution to water reabsorption of the collecting duct is dependent on the balance between AQP2 exo- and endocytosis.

AQP2 shuttling dysfunction is pathologically relevant both in case of an excessive plasma membrane localization, resulting in water retention, or defective trafficking, that leads to water loss. AQP2-related excessive water retention is linked to heart failure, liver cirrhosis or the syndrome of inappropriate antidiuretic hormone secretion (SIADH), while reduced insertion of AQP2 into the plasma membrane causes diabetes insipidus (183,184).

Despite longstanding interest on AQP2 translocation many points regarding this process are still obscure, prompting the investigation detailed in the second part of this thesis.

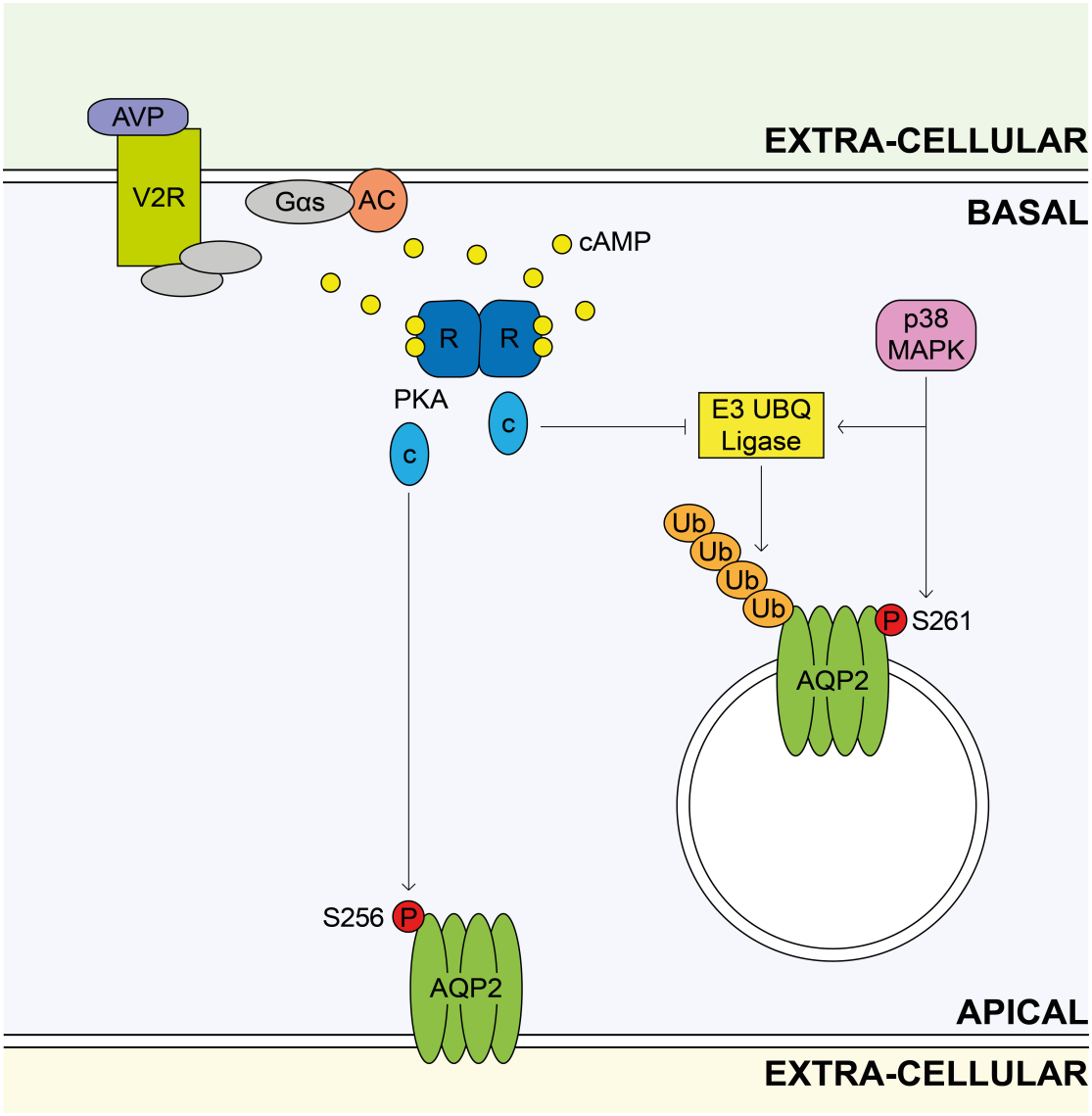

Fig. 3: Scheme of the molecular pathway regulating AQP2 in collecting duct principal cells. Binding of antidiuretic hormone (AVP) to the V2 receptor (V2R) at the basal plasma membrane determines the activation of Adenylate Cyclase (AC) and increase of cAMP levels, followed by activation of the cAMP-dependent tetrameric Protein Kinase A (PKA), constituted by regulatory (R) and catalytic (C) subunits. The resulting phosphorylation of the water channel Aquaporin-2 (AQP2) at the Ser256 (S256) provokes its translocation to the apical plasma membrane, where it increases collecting duct water permeability. The Mitogen Activated Protein Kinase 14 (p38 MAPK/MAPK14) is one of the responsible for the phosphorylation of AQP2 at Ser261 (S261), that is associated with intracellular localization of AQP2 and its ubiquitination via a still undefined E3 Ubiquitin Ligase. This E3 enzyme can be inhibited by PKA (78,102).

CDK18 biology

A newly found candidate influencing AQP2 trafficking is CDK18, part of the group of the highly homologous PCTAIRE kinases, comprising PCTAIRE1/CDK16, PCTAIRE2/CDK17 and PCTAIRE3/CDK18 itself. CDK18 was pinpointed as the result of a kinome-wide assay for new AQP2 trafficking modulators, but the lack of molecular details prompted the second half of this study (185).

Role and activity of this kinase are still largely unexplored, although a few studies suggest that it may be involved in the pathogenesis of Alzheimer disease (186,187). In another publication (188), CDK18 was demonstrated to be dependent on both PKA phosphorylation at the amino-terminus, and specifically at Ser12. Upon binding to the cofactor Cyclin A2, CDK18 reaches full kinase activity. In a recent article, the minor nuclear CDK18 pool was demonstrated to be involved in the ATR-mediated response to single strand DNA and the related cell cycle blockade. This defective checkpoint resulted in CDK18 depleted cells showing an higher abundance of mitosis aberrations, leading ultimately to slower cell proliferation (189). Although the authors identified CDK18 as responsible for DNA Repair Exonuclease Rad9 Homolog A (RAD9) chromatin localization and phosphorylation *in cellulo*, they could not pinpoint CDK18 as the direct responsible for the phosphorylation itself, so a clear kinase target for CDK18 remains elusive. Interestingly, CDK18 has been suggested by mass-spectrometry analysis to interact with the ubiquitin ligase STUB1, one of the enzymes possibly responsible for AQP2 ubiquitination (190). Clarifying the relationship of CDK18, STUB1 and AQP2 was one of the crucial points of the present study.

Results part 1: GSKIP orchestrates a dual kinase complex involved in β-catenin regulation

GSKIP and Axin1 compete for the binding to GSK3β

As a starting point, immunoprecipitations (IP) of GSK3β were performed from lysates of Human Embryo Kidney (HEK293) cells and analysed by Western Blotting (WB) to quantitate the interaction of GSK3β with Axin1. As expected, it was possible to detect the binding of the two endogenous proteins (Fig. 4A). When GSKIP was knocked down with RNA interference (RNAi), it was observed an increase in the GSK3β-bound Axin1 levels (Fig. 4A and B). A similar effect was also seen using as a model system the cervical cancer-derived HeLa cell line (Fig. 4C). Since the crucial role of Axin1 as a central scaffold for the GSK3β-including β-catenin destruction complex (191), these results led to investigate further the influence of GSKIP on the control of Wnt/β-catenin signaling.

Fig. 4: GSKIP downregulation increases GSK3β-Axin1 interaction; adapted from (1).

A, C- HEK293 (A) cells or (C) HeLa cells (n=3) were transfected with siRNA against GSKIP or control non-targeting (NT), and subjected to immunoprecipitations (IP) with antibodies against endogenous GSK3β or control (IgG). Representative Western Blot (WB) analysis are shown.

B- Semi-quantitative densitometric evaluation of the experiment presented in A and replicates; n=4, Student's t test, *p<0,05.

GSKIP reduction increases β-catenin transcriptional activity

To elucidate the function of GSKIP on canonical Wnt-mediated transcription the TOP/FOP flash luciferase reporter assay was employed. In these vectors multiple β-catenin responsive element (in the TOP constructs) or mutated sequences (for the FOP constructs, that acts as a negative control) are cloned upstream of a luciferase encoding gene. This permits a direct readout of the β-catenin-induced transcriptional activity (29). A small, non-significant down regulation of basal Wnt/β-catenin-mediated transcription in resting HEK293 cells was observed when GSKIP was knocked down. Under stimulation with Wnt-conditioned medium, the transcriptional activity increased markedly. Surprisingly, GSKIP knockdown caused a further increase in the transcriptional activity compared to the control (Fig. 5A). This finding was apparently conflicting with the previous observation of an increased GSK3β-Axin1 interaction in the absence of GSKIP. Such an increased interaction would be expected to increase the GSK3β amount in the destruction complex, causing a higher β-catenin degradation-inducing phosphorylation and decrease of canonical Wnt signaling.

To further strengthen this result, the same assay was repeated using colon carcinoma-derived SW480 cells. This cell line harbours an inactivating mutation in the APC gene, so rendering the β-catenin destruction complex inactive and mimicking a constitutive Wnt signal even in the resting state. In this system too, the GSKIP RNAi increased the Wnt signaling-induced transcriptional activity (Fig. 5B). Since the destruction complex is not operational in the SW480 cells, and GSKIP is still able to induce its effect, the results suggest an effect of GSKIP independent of the interaction between GSK3β and the destruction complex.

Fig. 5: GSKIP downregulation increases Wnt signaling transcriptional activity independently of LRP6 and GSK3β phosphorylation; adapted from (1).

A, B- GSKIP downregulation increases Wnt signaling transcriptional activity in (A) HEK293, (B) SW480 cells. Top: TOP/FOP luciferase transcriptional activity assay to monitor β-catenin transcriptional activity in cells transfected with siRNA against GSKIP or control non-targeting (NT) and stimulated with Wnt3a-enriched or control medium; (A) n=5, ANOVA, (B) n=3, Student's t test, *p<0,05, **p<0,01. Bottom: representative Western Blot (WB) to assess GSKIP knockdown.

C- GSKIP RNAi does not influence LRP6 or GSK3β phosphorylation. HEK293 cells were transfected with siRNA against GSKIP or control, and stimulated with Wnt3a-enriched or control medium. Top: semi-quantitative densitometric evaluation of the experiment presented on the bottom panel and replicates; n=6, ANOVA, *p<0,05, ***p<0,001. Bottom: representative Western Blot analysed in the top panels.

GSKIP RNAi increases canonical Wnt signaling through β-catenin stabilization mediated by PKA phosphorylation

It was assayed if the positive action of GSK3β on the Wnt/β-catenin pathway was affected by GSKIP. Analysis of the GSK3β-mediated phosphorylation of the Wnt co-receptor LRP6 at Ser1490 showed no consequences of GSKIP RNAi, nor on resting state nor on Wnt-stimulated HEK293 cells. This suggested that the recruitment of the destruction complex to the plasma membrane was not interrupted. Moreover, in the same samples, the inhibitory phosphorylation at Ser9 of GSK3β was unaffected by both Wnt stimulation or GSKIP RNAi (Fig. 5C).

To assay β-catenin abundance via Western Blot, and be able to distinguish the Wnt-relevant cytosolic pool from the membrane-associated Wnt-independent pool, a cytosolic extraction protocol based on digitonin semi-permeabilization was adopted (192). Several cell lines, including HEK293, HeLa and pulmonary alveolar carcinoma-derived A549, were used. The purity of the cytosolic fractions was routinely assessed by Western Blot analysis, confirming the absence of plasma membrane and nuclear contaminants (Fig. 6A).

It was then performed the analysis of the phosphorylation of cytosolic β-catenin at Ser33/Ser37/Thr41, that is catalysed by GSK3β and responsible for β-catenin degradation (193). HEK293 cells were transfected with siRNA against GSKIP or control, then treated either with vehicle control or with the proteasomal blocker MG-132, in order to permit detection by Western Blot of the degradation-prone poly-phosphorylated β-catenin. Although it was observed an increase in the abundance of total β-catenin in MG-132 treated cells subjected to GSKIP RNAi, the phosphorylated Ser33/Ser37/Thr41 β-catenin abundance was unaffected by the GSKIP knockdown both in unstimulated and MG-132 treated HEK293 cells (Fig. 6B). This suggested that GSKIP RNAi was not affecting GSK3β function on β-catenin, but left open the possibility of PKA involvement.

To test this, HEK293 cells transfected with siRNA against GSKIP or control were stimulated with Wnt conditioned medium in the absence or presence of a short peptide encoding the PKA-inhibitor sequence of the naturally occurring Protein Kinase (cAMP-Dependent, Catalytic) Inhibitor (PKI) protein, rendered cell-permeable by stearate coupling. Once again, it was possible to show an increased cytosolic level of β-catenin in the sample stimulated with Wnt. When GSKIP was knocked down, the effect was further increased, but inhibition of PKA with the peptide abolished the difference caused by GSKIP downregulation. To additionally verify the PKA specificity the analysis of β-catenin Ser675 phosphorylation was performed,

finding that this one, too, was increased (Fig. 6C). Even in the case of this phosphorylation, PKA inhibition was able to rescue the effect caused by GSKIP RNAi.

To further strengthen this point, a similar experiment was performed with HeLa and A549 cells. Congruent effects were observed also in the two cell lines if another PKA inhibitor, the small molecule H89, was used (Fig. 6D and E). Taken together, the results demonstrated the role of PKA in the GSKIP RNAi-mediated Wnt up-regulation.

Fig. 6: legend on the next page

Fig. 6: GSKIP downregulation increases cytosolic abundance of β-catenin and modulates its PKA-mediated phosphorylation; adapted from (1).

A- HEK293, stimulated with Wnt-enriched or control medium, were used to prepare total-cell lysates and purified cytosolic fractions. Representative Western Blot (WB) analysis is shown; the absence of plasma membrane (LRP6, pan-Cadherin) and nuclear (Lamin A/C) markers in the cytosolic samples confirmed the purity of the fractions.

B- GSKIP downregulation increases cytosolic β-catenin. Left: representative Western Blot analysis of purified cytosolic fractions from HEK293 cells transfected with siRNA against GSKIP or control non-targeting (NT) and treated with the proteasomal blocker MG-132 or vehicle control. Middle and right: densitometric semi-quantitative evaluation of the experiment presented on the left panel and replicates; n=6, ANOVA, ***p<0,001.

C, D, E- the GSKIP downregulation-mediated increase in cytosolic β-catenin can be counteracted by PKA blockade in (C) HEK293, (D) cervical cancer-derived HeLa or (E) pulmonary alveolar cancer-derived A549 cell lines.

Top: The cell lines indicated above were transfected with siRNA against GSKIP or control, stimulated with Wnt-enriched or control medium and treated with a peptide derived from the PKA inhibitor PKI, the small molecule PKA inhibitor H89 or the appropriate vehicle control. Cytosolic fractions were purified and analysed via Western Blot. Middle and bottom: densitometric semi-quantitative evaluation of the experiment presented on the top panel and replicates; (A) n=8, (B and C) n>5, ANOVA, **p<0,01, ***p<0,001.

PKA- and GSK3β-interaction-deficient variants of GSKIP: generation and characterization

The results so far left unexplained if and how the increased Axin1-GSK3β interaction that was observed in Fig. 4A-C was influencing Wnt signaling. In fact, such an increased protein binding would be expected to suppress Wnt signaling, providing more GSK3β available to poly-phosphorylate β-catenin and start its degradation cascade. However, such an effect can effectively be masked by the impact of GSKIP on PKA activity. The generation of single kinase interaction-deficient mutants of GSKIP for each GSK3β and PKA allowed to tackle the question from another perspective and to elucidate the GSKIP/GSK3β effect. Based on previously published data (67,194), the Leu130 into Pro (L130P) substitution to abolish GSKIP-GSK3β interaction and the Asn42 into Ile (N42I) substitution to disrupt GSKIP-PKA binding were generated via PCR-directed mutagenesis, using as a template a GSKIP-CFP plasmid. The so-obtained mutants were then sub-cloned into the pCMV6 vectors, obtaining the CFP-untagged, FLAG versions of GSKIP-Wild type (Wt), -L130P and -N42I (see Material and Methods section).

To characterize the PKA-binding activity of the GSKIP mutants, a Far-Western Blot strategy was adopted. HEK293 cells were transfected with plasmids encoding for GSKIP-Wt, GSK3β- or PKA-interaction-deficient mutants or control, and the resulting cell lysates were blotted on Polyvinylidene fluoride (PVDF) membranes. Incubation with recombinant RIIα subunits of PKA followed, and the interaction between RIIα and the blotted proteins was measured after successive incubations with anti-RIIα murine and anti-mouse Peroxidase-coupled antibodies. The substitution N42I indeed reduced drastically the interaction between the RIIα subunit of PKA and GSKIP, while the L130P mutant behaved in a similar way as the Wt (Fig. 7A). To reproduce this data with an independent technique, cAMP pull-down (PD) experiments were performed. HEK293 were transfected with plasmids encoding for GSKIP-Wt, GSK3β- or PKA-interaction deficient or control, and the resulting cell lysates expressing the various GSKIP-FLAG forms and the appropriate vector control were incubated with Sepharose-conjugated cAMP beads. The affinity of R subunits of PKA for cAMP allows to isolate them by centrifugation, co-purifying the bound protein complex. Comparably with the Far-Western Blot approach, the N42I substitution was shown to inhibit the GSKIP-RIIα interaction. This was instead unaffected by the L130P mutation (Fig. 7B).

To test the GSK3β interaction, HEK293 cells were transfected with plasmids encoding for GSKIP-Wt, GSK3β- or PKA-interaction-deficient or control, then lysed

and subjected to immunoprecipitation with anti-GSK3β antibody. This approach revealed a reproducible and dramatic decrease in the interaction of the GSKIP L130P for GSK3β compared to both the Wt and the N42I forms (Fig. 7C).

Immunoprecipitations with anti-FLAG antibodies were also performed using similar lysates; this confirmed the pattern established with the GSK3β immunoprecipitation. Compared to the immunoprecipitation of the FLAG-tagged Wt GSKIP, the co-immunoprecipitated GSK3β was reduced only in the presence of the FLAG-tagged L130P form, but not for the FLAG-tagged N42I substitution (Fig. 7D).

Fig. 7: legend on the next page.

Fig. 7: Characterization of GSKIP GSK3β- and PKA-binding deficient mutants; adapted from (1).

A, B- GSKIP Asn42 Ile (N42I) mutation abolishes GSKIP-PKA interaction. Left: HEK293 cell expressing the indicated constructs were lysed and subjected to (A) RII overlay and control Western Blot (WB) or (B) Western Blot analysis of cAMP Pull-Down (PD). Right: semi-quantitative densitometric evaluations of the experiments presented on the left panel and replicates; (A) n>5, (B) n=5, ANOVA, **p<0,01, *p<0,05.

C, D- GSKIP Leu130 Pro (L130P) mutation abolishes GSKIP-GSK3β interaction. Left: HEK293 cell expressing the indicated constructs were lysed and subjected to Western Blot analysis of (C) GSK3β immunoprecipitation (IP) or IgG control immunoprecipitation or (D) FLAG immunoprecipitation. Right: semi-quantitative densitometric evaluations of the experiments presented on the left panel and replicates, n=3, ANOVA, **p<0,01; *p<0,05.

E, F- GSKIP Wild type (Wt) overexpression increases canonical Wnt signaling transcriptional output. HEK293 cells were transfected with the indicated constructs and transcriptional TOP/FOP luciferase activity assay to monitor β-catenin transcriptional activity was performed (E) in resting conditions or (F) after stimulation with the indicated quantities of recombinant Wnt; (E) n=4, ANOVA, (F) n=3, Student's t test, ***p<0,001, **p<0,01, *p<0,05.

G- GSKIP does not bind to β-catenin. HEK293 cells expressing either FLAG-GSKIP-Wt or a vector control were subjected to FLAG immunoprecipitation and Western Blot analysis. A representative experiment is shown, n=3.

GSKIP requires binding to both PKA and GSK3β to elicit the upregulation of β-catenin

The Wnt/β-catenin-mediated transcriptional activity was evaluated using the TOP/FOP Flash luciferase reporter assay in resting HEK293 cells in which the GSKIP variants, the Wt or a vector control, were expressed. The results showed that GSKIP Wt was able to increase the Wnt/β-catenin-mediated transcription even in the absence of a Wnt ligand. Both PKA- and GSK3β-binding-deficient GSKIP were unable to do so, suggesting that scaffolding of both kinases is required for the positive action of GSKIP on Wnt signaling (Fig. 7E).

In a separate set of experiments, it was furthermore confirmed the positive role of GSKIP by stimulating HEK293 cells, either transfected with a control plasmid or with a GSKIP encoding one, with increasing amounts of purified Wnt3a and monitoring Wnt/β-catenin-mediated transcription (Fig. 7F). The increased transcriptional activation for every Wnt3a concentration analysed suggests an additive role for GSKIP and Wnt3a in the activation of the Wnt/β-catenin signaling pathway. The presence of the same phenotype of Wnt promotion both in conditions of GSKIP deficiency (as seen before with the RNAi experiments) and of GSKIP abundance, led to hypothesize an activity of GSKIP outside the β-catenin destruction complex, but distinct and opposite to the PKA-mediated effect that was previously unravelled with the RNAi of GSKIP.

To exclude a possible direct effect of GSKIP on β-catenin, GSKIP immunoprecipitations were performed finding no β-catenin in the precipitate, suggesting that the effects mediated by GSKIP are mainly due to its activity as a scaffold for the two protein kinases involved in Wnt signaling (Fig. 7G).

To further asses how GSKIP influences the canonical Wnt pathway, HEK293 cells transfected with plasmids encoding for GSKIP-Wt, GSK3β- or PKA-interaction-deficient or vector control were stimulated with Wnt-conditioned medium and the Wnt/β-catenin-mediated transcription was monitored *via* the TOP/FOP assay (Fig. 8A). Consistently with the previous results (Fig. 7E), GSKIP Wt expressing cells showed an higher Wnt/β-catenin-mediated transcription. Cells expressing the GSK3β-binding deficient GSKIP mutant behaved as the control, while the cells expressing the PKA-binding-deficient GSKIP mutant showed an intermediate phenotype, confirming the necessity for a contemporary binding of GSK3β and PKA to GSKIP to elicit the full GSKIP effect.

In order to differentiate between the two GSKIP-binding kinases' contribution, it was evaluated the abundance of cytosolic protein after treatment with Wnt conditioned medium and expression of GSKIP, Wt or binding-deficient (Fig. 8B). The levels of total cytosolic β-catenin followed a similar pattern to the one showed by the TOP/FOP assay: a robust increase in the control sample after Wnt stimulation, that was even more increased in presence of high levels of Wt GSKIP, but failed to reach the same magnitude with both the mutants. The levels of the PKA-mediated phosphorylation at Ser675 of β-catenin were affected by the Wnt stimulation, but did not show any difference between control and GSKIP Wt or mutant expressing samples. Moreover, the GSKIP-mediated PKA-driven inhibitory phosphorylation of GSK3β Ser9 was investigated by transfecting HEK293 cells with plasmids encoding for GSKIP-Wt, GSK3β- or PKA-interaction-deficient or vector control. It was shown that the published GSKIP-dependent increase in the phosphorylation (67,68) requires the simultaneous binding of both PKA and GSK3β to GSKIP to show its full effect, since both the GSKIP mutants are unable to elicit it at the same levels of the Wt (Fig. 8C).

Fig. 8: GSKIP requires binding to both PKA and GSK3β to elicit a full inhibitory effect on GSK3β; adapted from (1).

A, B, C- Both GSK3β- (L130P) and PKA- (N42I) deficient-interaction GSKIP forms do not provoke the full increase of Wnt signaling elicited by the Wild type (Wt). HEK293 cells were transfected with the indicated constructs, stimulated with Wnt3a-enriched or control medium and (A) transcriptional TOP/FOP luciferase activity assay to monitor β-catenin transcriptional activity was performed (n=4, ANOVA, *p<0,05), or (B, C, left) cytosolic fractions were purified and Western Blot (WB) analysis was performed; a representative experiment is shown. (B, C, right) semi-quantitative densitometric evaluation of the experiment presented on the left panel and replicates, (B) n=5, (C) n=13, ANOVA, ***p<0,001, **p<0,01, *p<0,05.

D, E: GSKIP inhibits the GSK3β-dependent poly-phosphorylation of β-catenin. Left: HEK293 cells were transfected with the indicated constructs, treated with the proteasomal blocker MG-132 or vehicle control, cytosolic fractions were purified and Western Blot analysis was performed; representative experiments are shown. Middle and right: Semi-quantitative densitometric evaluation of the experiments presented on the left panel and replicates; (D) n=5, (E) n=8, ANOVA, ***p<0,001, **p<0,01.

GSKIP influences Wnt signaling positively by inhibition of GSK3β-mediated β-catenin poly-phosphorylation

To investigate the role of GSK3β, control and GSKIP-Wt expressing HEK293 cells were treated with the proteasomal inhibitor MG-132. This was necessary in order to evaluate the degradation-inducing phosphorylation of β-catenin at the positions Ser33, Ser37 and Thr41, mediated by the β-catenin destruction complex-contained GSK3β. In the presence of GSKIP, β-catenin cytosolic levels were increased and the GSK3β-mediated phosphorylation of β-catenin was reduced, thus demonstrating a GSKIP scavenger role towards the destruction complex-contained GSK3β (Fig. 8D).

When a similar experiment was repeated with HEK293 cells expressing either GSKIP-Wt, deficient for GSK3β or PKA interaction or a control, it was possible to detect a decreased phosphorylation at Ser33, Ser37 and Thr41 of β-catenin in the presence of Wt GSKIP, that was absent in the presence of both the mutants (Fig. 8E). This argues for a collaborative action of GSKIP and PKA that scavenge GSK3β away from the destruction complex and inactivate it; the positive effect on the Wnt/β-catenin pathway requires the presence of a fully functional, dual-kinase binding GSKIP.

GSKIP, Axin and AKAP220 compete for GSK3β binding

It was then investigated whether the observed effect of Wnt bolstering was specifically due to GSKIP or was possibly obtained also with another protein able to scaffold both PKA and GSK3β. AKAP220, which stimulates the GSK3β inactivation by PKA-mediated phosphorylation at Ser9 (65), was chosen as candidate. When the analysis started, the AKAP220's GSK3β interaction domain (GID) was incompletely characterized (71). Bioinformatics analysis of the AKAP220 human protein sequence, comparing it to the well-known GSK3β-binder Axin1 and to GSKIP, revealed that the GID conserved between GSKIP and Axin1 is also remarkably similar to a sequence found on AKAP220 in the portion between the amino acids 1155-1176. The same region is strongly conserved among mammalian ortologs of AKAP220, and is predicted by the software PSIPRED to fold into an α-helix (Fig. 9A).

It was then investigated if this portion is able to bind to GSK3β. Peptides encoding the protein sequence of human AKAP220 were spot-synthesised with 5 amino acid offset to cover the full-length protein, and were overlaid with purified recombinant GST-GSK3β. As a control were performed incubations with GST alone or GST-GSK3β in the presence of the GSKIP-derived peptide GSKIPtide, that is encoding for the GSK3β-binding region of GSKIP (68).

The interaction of GSK3β was revealed incubating the spots with an anti-GST Peroxidase-coupled secondary antibody; both negative controls showed decreased interaction (Fig. 9B). A specific binding to peptides representing amino acids 1162-1176 was detected; in this sub-portion of the putative GID is evident a conserved FLL domain, present both in human Axin1, human GSKIP and mammalian AKAP220 ortologs. To identify the amino acidic determinants of the GSK3β-AKAP220 interaction, the sequence of the human AKAP220 was peptide-spotted mutating sequentially each residue in Ala, so performing an alanine scanning to pinpoint the crucial residues for the interaction. After GST-GSK3β overlay, the reduced binding of GSK3β on the spots where the Phe1162, Leu1166, or Leu1170 were mutated showed that these amino acids are the crucial ones for the kinase docking (Fig. 9C).

Fig. 9: AKAP220 harbours a conserved GSK3β Interaction Domain (GID) ; adapted from (1), adapting data from (195).

A- Top: protein sequence alignment of human (Hs) Axin1 and 2, GSKIP and AKAP220 from human (Hs), rat (Rn) and mouse (Mm) highlighting the conserved FLL motif. Bottom: 3D folding prediction of human AKAP220 based on the software PSIPRED (http://bioinf.cs.ucl.ac.uk).

B- Overlay of recombinant GST alone (bottom) or GST-GSK3β (top and middle panel) with or without the peptide GSKIPtide (interrupting GSKIP GID and GSK3β interaction (68)) or GSKIPtide-L130P (negative control peptide) on membrane-spotted AKAP220 human sequence. The binding in the region 1162-1176 is highlighted.

C- Overlay of recombinant GST-GSK3β on membrane-spotted sequential alanine scan of the AKAP220 human sequence. The first sequence is the non-mutated Wild type (Wt) control, and the position where the residues Phe 1162, Leu 1166 and Leu 1170 were substituted with Ala are highlighted.

GSKIP-derived peptides inhibit the interaction between spotted AKAP220 and recombinant GSK3β

To investigate if the GSK3β binding to the three proteins is competitive, immunoprecipitation experiments were performed in HEK293 cells, expressing a combination of YFP-GSK3β, HA-AKAP220, FLAG-Axin1, myc-GSKIP or appropriate controls. In the case of the expression of GSK3β and AKAP220 alone, GSK3β was immunoprecipitated via the YFP tag and AKAP220 was co-immunoprecipitated. Upon the co-expression of either Axin1 or GSKIP, the amount of GSK3β-bound AKAP220 was significantly reduced (Fig. 10A). Similarly, GSKIP was normally co-immunoprecipitated by means of GSK3β immunoprecipitation but it was possible to reduce the GSK3β-bound amount of GSKIP by co-expression of Axin1 (Fig. 10B). This shows that GSK3β binding is competitively shared by Axin1 and the two AKAPs.

Fig. 10: GSKIP, AKAP220 and Axin1 compete for GSK3β binding; adapted from (1), adapting data from (195).

A, B- Top: HEK293 cell were transfected with the indicated constructs, lysed and subjected to YFP immunoprecipitation (IP). Representative Western Blot (WB) analysis is shown. Bottom: Semi-quantitative densitometric evaluation of the experiment presented on the top panel and replicates; (A) n=6, (B) n=5, ANOVA, *p<0,05, **p<0,01.

The increase mediated by GSKIP on Wnt signaling is not commonly caused by AKAPs

Since Axin1 is the scaffold around which the β-catenin destruction complex is assembled and GSK3β binding is competitively shared by GSKIP, Axin and AKAP220, the last one was a candidate to influence the canonical Wnt pathway, too. Analysis of the Wnt-induced transcriptional activity with the TOP/FOP assay in HEK293 cell lysates showed that AKAP220 expressing cells behaved in a similar way as the control ones both in resting and Wnt-stimulated conditions (Fig. 7E and 8A).

To further address this point, HEK293 cells were transfected with plasmid encoding for GSKIP or AKAP220, then the purified cytosolic fractions were analysed by Western Blot (Fig. 11A). β-catenin revealed a robust elevation following stimulation of the cells with Wnt-enriched medium compared to unstimulated control, and it was, as expected, additionally increased in cells expressing GSKIP, while the ones expressing AKAP220 showed no difference from the control (Fig. 11A and B). Analysis of β-catenin PKA-mediated phosphorylation at Ser675 showed no significant differences in neither GSKIP or AKAP220 expressing cells (Fig. 11A and C).

Additionally, cytosolic Axin1 showed a decrease in the Wnt stimulated samples, concordantly with the recruitment of the destruction complex to the plasma membrane upon Wnt signaling activation. In resting condition Axin1 shows higher cytoplasmic levels when GSKIP is expressed, hinting at a possible feedback negative mechanism compensating for the GSK3β-recruiting effect of GSKIP in the absence of the Wnt ligand (Fig. 11A and D).

Taken together, the data did not show an influence of AKAP220 on Wnt signaling, thus highlighting the importance and the specificity of the GSKIP role.

Fig. 11: GSKIP, but not AKAP220, increases cytosolic β-catenin; adapted from (1).

A- HEK293 cells were transfected with the indicated constructs and stimulated with Wnt3a-enriched or control medium; cytosolic fractions were purified. A representative Western Blot (WB) analysis is shown.

B, C, D- Semi-quantitative densitometric evaluation of the experiment presented in A and replicates; n=7, ANOVA, ***p<0,001, *p<0,05.

Results part 2: CDK18 is a novel kinase involved in AQP2 trafficking

CDK18 controls AQP2 translocation and abundance

In order to identify new kinases controlling AQP2 trafficking, a siRNA screen was performed by knocking down the kinome of AQP2-expressing Murine Collecting Duct (MCD) 4 cells (Fig. 12A) (185). A super-physiological increase of cAMP *via* Forskolin (Fsk) administration was induced, so activating PKA, and the AQP2 trafficking was monitored via automated immunofluorescence and image analysis. Among several kinases whose down-regulation prevented the cAMP-induced redistribution of AQP2, it was identified a poorly characterized candidate, CDK18/PCTAIRE-3 (185). Smaller scale repetition of the immunofluorescence assay confirmed that MDC4 cells where CDK18 was knocked down by siRNA transfection, compared to control non-targeting siRNA-transfected cells, were unable to translocate AQP2 from the perinuclear region to the cell periphery after Fsk administration (Fig. 12B). The knockdown of CDK18 hardly affected the localisation of AQP2 in resting cells. To evaluate AQP2 transcription, MDC4 cells were transfected either with siRNA control, targeting CDK18 or left untransfected, and stimulated with Fsk or vehicle. The mRNA expression of AQP2 (Fig. 12C) was unaffected, but Western Blot analysis of similarly treated cells revealed an increase in the protein abundance (Fig. 12D and E). On the same samples, an effect on the PKA-catalysed phosphorylation of Ser256 was not observed; however, the knockdown decreased the phosphorylation of Ser261 in resting cells (Fig. 12D and E).

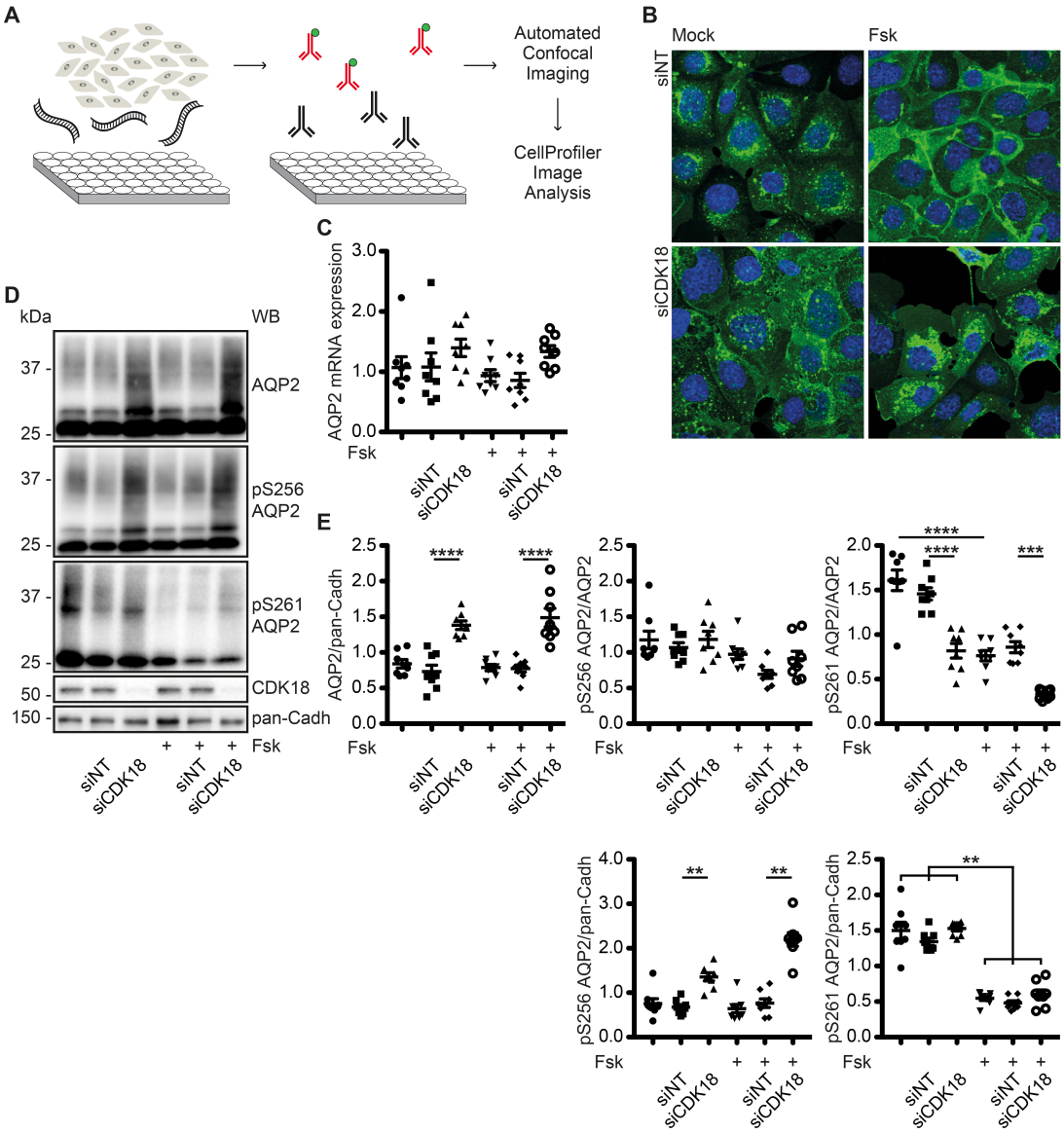

Fig. 12: CDK18 regulates AQP2 translocation, abundance and phosphorylation; adapting data from (185).

A- Scheme of the siRNA kinome-wide screen performed on Murine Collecting Duct 4 (MCD4) cells.

B- AQP2 shuttling is dependent on CDK18. Representative immunofluorescence microscopic analysis of MCD4 cells transfected with either control non-targeting (NT) or CDK18-targeting siRNA, treated with Forskolin (Fsk) or vehicle. AQP2 is stained in green with an appropriate antibody and nuclei are counter-stained in blue with DAPI; n=3.

C, D, E: CDK18 decreases the protein abundance and promotes the phosphorylation of AQP2 at Ser261 (S261). MCD4 cell were left untransfected or transfected with control or CDK18 siRNA and treated with Fsk or vehicle control. They were subjected to (C) semi-quantitative PCR analysis for AQP2 mRNA abundance or (D) Western Blot analysis (a representative experiment is shown) and (E) relative densitometric semi-quantitative evaluation; n=8, ANOVA, ****p<0,0001, ***p<0,001, **p<0,01.

Abbreviation: pS261, phospho-Ser261; pS256, phospho-Ser256.

CDK18 phosphorylates AQP2 at the residue Ser261

It was then investigated whether CDK18 itself could have a direct role in the phosphorylation of AQP2, taking advantage of the previously published mutants of CDK18 Lys150 into Arg (kinase dead) and Ser12 into Asp (mimicking activation by PKA-mediated phosphorylation) (188). An *in vitro* non-radioactive kinase assay approach on the widely used substrate Retinoblastoma (Rb) was established. Human embryonic kidney-derived HEK293 cells were transfected with a combination of plasmids encoding FLAG-tagged CDK18 as Wt, kinase dead or phospho-mimic forms, the CDK18 co-activator Cyclin A2 tagged with Hemagglutinin (HA) and appropriate vector controls. The resulting various CDK18 variants in the cell lysates were purified by immunoprecipitation with beads against the FLAG tag. The fragment of Rb comprising amino acids 792-818 of the human protein was spot-synthesised and overlaid with the immuno-purified CDK18 in the presence of ATP, adapting the protocol described (188). The CDK18-mediated phosphorylation was monitored by incubation of the spots with an antibody recognizing the phosphorylated CDK consensus sequence, followed by incubation with peroxidase-coupled secondary antibody and detection by standard Western Blot technique (Fig. 13A). CDK18 showed an activity pattern consistent with published data (188) that is, weak to undetectable activity in the basal state, that could be increased by the phospho-mimic substitution Ser12 into Asp, and enhanced additionally by the concomitant expression of the co-activator Cyclin A2 (Fig. 13B).

To evaluate if CDK18 is able to phosphorylate Ser261 of AQP2, HEK293 cells were transfected as in the previous experiment, and CDK18 was immuno-purified *via* its FLAG tag. AQP2 was instead isolated by immunoprecipitation from untransfected, unstimulated MCD4 cells. Immuno-purified CDK18 in its Wt, kinase dead or phospho-mimic forms was incubated with immunoprecipitated AQP2. The resulting samples were analysed by Western Blot and the phosphorylation of AQP2 was detected using a specific anti-phosphorylated Ser261 AQP2 antibody. Analysis of the AQP2 phosphorylation profile revealed that only the phospho-mimicking CDK18 form, but not the Wt or the kinase dead, phosphorylates the Ser261 of AQP2 (Fig. 13C and D).

To further confirm this point, immuno-purified CDK18 Wt, kinase dead or phospho-mimic forms were incubated on peptides encoding for human AQP2 carboxy-terminus, comprising the Ser261 position. The peptides were then analysed by Western Blot as in the previous experiment, with similar results (Fig. 13 E and F).

Fig. 13: legend on the next page.

Fig. 13: CDK18 phosphorylates AQP2 at Ser261 (S261).

A, B: CDK18 kinase dead and phospho-mimic mutant validation on Retinoblastoma protein (Rb).

A- HEK293 cells were transfected with a combination of vectors encoding for CDK18-FLAG in the wild type (Wt), kinase dead Lys150 into Arg (K150R), phospho-mimic Ser12 into Asp (S12D) forms, in addition to Hemagglutinin-tagged CDK18 co-activator Cyclin A2 (Cyc A2) or vector control. CDK18 was purified by anti-FLAG immunoprecipitation and incubated on spot-synthesised peptides corresponding to the 792-818 fragment of human Rb. The incubation was as follows: spots 1 and 5, vector control; 2 and 6, CDK18 Wt; 3 and 7, CDK18 K150R, 4 and 8, CDK18 S12D. On spots 5-8, incubation was performed in the presence of co-immunoprecipitated Cyc A2. Rb phosphorylation was revealed by incubation with an anti-phosphorylated CDK substrate antibody, followed by incubation with a peroxidase-coupled secondary antibody and standard Western Blot (WB) procedure. A representative kinase assay is shown.

B- Semi-quantitative densitometric evaluation of the experiment presented in A and replicates; n=5, ANOVA, ***p<0,001; *p<0,05. Due to the very low signal in the spots 1-4, just the spots 5-8 were analysed.

C, D, E, F: CDK18 phosphorylates AQP2 at Ser261.

C, E- HEK293 cells were transfected and CDK18 purified as in (A). CDK18 was incubated with (C) immunoprecipitated AQP2 from MCD4 cells, or (E) peptides derived from the AQP2 carboxy-terminus (amino acids 243-271). AQP2 phosphorylation was revealed by incubation with an anti-phosphorylated Ser261 AQP2 antibody, followed by incubation with a peroxidase-coupled secondary antibody and standard Western Blot procedure. Representative kinase assays and input controls for the immunoprecipitations are shown.

D, F- Semi-quantitative densitometric evaluation of the experiment presented in (D) C and replicates, n>3, (F) E and replicates, n=4; ANOVA; ***p<0,001; **p<0,01; *p<0,05.

CDK18 regulates AQP2 ubiquitination

It was previously shown that AQP2 phosphorylated at Ser261 is ubiquitinated and directed for proteasomal degradation (102). In order to verify if CDK18 is involved in AQP2 ubiquitination, MCD4 cells were transfected with control or CDK18 siRNA, and AQP2 was immunoprecipitated. According to the literature (102), ubiquitinated AQP2 above 50 kDa was considered poly-ubiquitinated, while below it, oligo-ubiquitinated. MDC4 cells were CDK18 was knocked down showed decreased poly-ubiquitination of AQP2, explaining the increase in its abundance (Fig. 14A and B) and suggesting a connection to the reduced Ser261 phosphorylation. Since p38 MAPK is known to phosphorylate AQP2 at Ser261 (102), its activity was investigated by evaluating its phosphorylation at Thr180-Tyr182, its main activating site (196). Surprisingly, p38 MAPK phosphorylation increased in MCD4 cells where CDK18 was knocked down (Fig. 14C and D).

This phenotype can be explained either as an increased phosphorylation, or as a decreased dephosphorylation of p38 MAPK. In order to evaluate if the phosphatases activity in MCD4 cells is dependent on CDK18, after CDK18 downregulation and Fsk stimulation, para-Nitrophenylphosphate (pNPP)-based phosphatase assays were performed in MDC4 cells. pNPP is a chemical that can be converted non-specifically by phosphatases into para-Nitrophenol, a chromogenic product with absorbance at 405 nm, allowing the quick determination of phosphatases activity (197).

In basal conditions, the downregulation of CDK18 did not influence the phosphatases activity in a significant way; however, in the Fsk stimulated samples, after CDK18 knockdown the total phosphatase activity of MCD4 was significantly lower than the appropriate control (Fig. 14E and F), suggesting the involvement of a CDK18-dependent phosphatase regulating p38 MAPK activity. Interestingly, phosphatases have been implicated in the regulation of AQP2 trafficking (198,199). Since the activity of several immunoprecipitated phosphatase subunits was not detectable with pNPP assays, it was not possible to pinpoint the precise protein involved (data not shown). The results so far suggested that CDK18 is able to inhibit the responsible for Ser261 phosphorylation, p38 MAPK, presumably via regulation of phosphatases activity, and to phosphorylate the same target residue itself; thus the crosstalk between CDK18 and p38 MAPK could control Ser261 phosphorylation of AQP2 and thereby its ubiquitination and stability.

Fig. 14: legend on the next page.

Fig. 14: CDK18 regulates AQP2 ubiquitination and affects p38 MAPK/MAPK14 (p38) and Protein Phosphatase (PP) activity.

A, B: CDK18 RNAi decreases CDK18 ubiquitination.

A- MCD4 cells left untransfected or transfected with either control (Non-Targeting, NT) or CDK18 siRNA were subjected to immunoprecipitation (IP) with antibodies anti-AQP2 or control (IgG). A representative Western Blot (WB) analysis is shown.

B- Semi-quantitative densitometric evaluation of the experiment presented in A and replicates; n=4, Student's t test, *p<0,05. The signal of the immunoprecipitation of AQP2 was analysed to evaluate both the oligo- (less than 50 kDa) and the poly- (more than 50 kDa) ubiquitinated (Ubq) AQP2 as a ratio to the immunoprecipitated AQP2 as described (102).

C, D: CDK18 RNAi increases the activatory Thr180/Tyr182 phosphorylation (pTY) of p38.

C- MCD4 cells left untransfected or transfected with either control or CDK18 siRNA, and stimulated with forskolin (Fsk) or vehicle, were analysed by Western Blot. A representative experiment is shown.

D- Semi-quantitative densitometric evaluation of the experiment presented in C and replicates; n=5, ANOVA, *p<0,05.

E, F: CDK18 RNAi decreases global phosphatases activity.

E- MCD4 cells transfected with either control or CDK18 siRNA, and stimulated with Fsk or vehicle, were subjected to para-Nitrophenylphosphate (pNPP)-based phosphatase activity assays. General phosphatases activity was evaluated by the conversion of pNPP into para-Nitrophenol (pNP), a chromogenic product with absorbance at 405 nm. pNPP to pNP conversion was evaluated spectrophotometrically; n=4, ANOVA, *p<0,05.

F- Representative Western Blot analysis for the experiment presented in E, to evaluate CDK18 knockdown.

STUB1 RNAi causes similar effects as CDK18 knockdown

A recent mass-spectrometry study of the interactome of CMGK kinases revealed interaction of CDK18 with the E3 ubiquitin ligase STUB1 (190). In MCD4 cells, the knockdown of CDK18 decreased the protein abundance of STUB1 (Fig. 15A and B) but not the mRNA (Fig. 15C), suggesting that CDK18 could have a role in controlling STUB1 post-transcriptionally. AQP2 staining in MCD4 cells showed that STUB1 knockdown impaired the Fsk-induced AQP2 trafficking to the plasma membrane, as observed for CDK18 RNAi (Fig. 15D), thus hinting at the two proteins being in the same pathway.

Since STUB1 protein sequence encodes for a putative CDK consensus site at theamino acids 20-24 (Ser-Pro-Asp-Lys), kinase assay experiments were performed to investigate if STUB1 is a CDK18 substrate. HEK293 cells were transfected to express FLAG-tagged CDK18 Wt, kinase dead or phospho-mimic together with Cyclin A2. CDK18 was purified via anti-FLAG beads and incubated on the spot-synthesised sequence of STUB1 comprising amino acids 9-34, which harbours the putative phosphorylation site. Phosphorylation of the peptides on the spots was revealed by incubation with an antibody against phospho-CDK substrate. Analysis of several replicates suggested that STUB1 is not a direct target for CDK18 phosphorylation (Fig. 15E).

Mass-spectrometry analysis of cAMP PD revealed the presence of STUB1 in the purified protein complexes (200). MDC4 cells stimulated with Fsk or with vehicle were lysed and immunoprecipitation for STUB1 was performed; it was revealed the co-immunoprecipitation of CDK18 and AQP2 in a Fsk-independent (so cAMP insensitive) way. Moreover, together with STUB1 co-immunoprecipitated the RIIα subunit of PKA, still in a Fsk-insensitive way, and the catalytic subunit of PKA. The binding of the latter subunit is, as expected, strongly reduced upon Fsk treatment (Fig. 15F and G). Taken together, this indicates that STUB1, AQP2, CDK18 and PKA are organized in a multi-protein complex, reminiscent of an AKAP signalosome.

When MDC4 cells transfected with siRNA against STUB1 or control were lysed and AQP2 immunoprecipitation was performed, Western Blot analysis revealed the co-immunoprecipitation of CDK18, that was decreased by STUB1 RNAi (Fig. 15H and I). When, on the same samples, AQP2 poly-ubiquitination was assayed, it was found decreased in the STUB1-depleted samples, as for CDK18 RNAi, suggesting that STUB1 is one of the E3 ubiquitin ligases responsible for AQP2 ubiquitination (Fig. 15H and I).

Surprisingly, STUB1 downregulation did not affect AQP2 protein abundance, nor phosphorylation at Ser256 or Ser261 of AQP2, nor CDK18 abundance (Fig. 16A and

B). It was, however, possible to detect an increase in the p38 MAPK Thr180-Tyr182 phosphorylation in the STUB1 depleted, Fsk treated condition, similarly to the CDK18 RNAi (Fig. 16A and B).

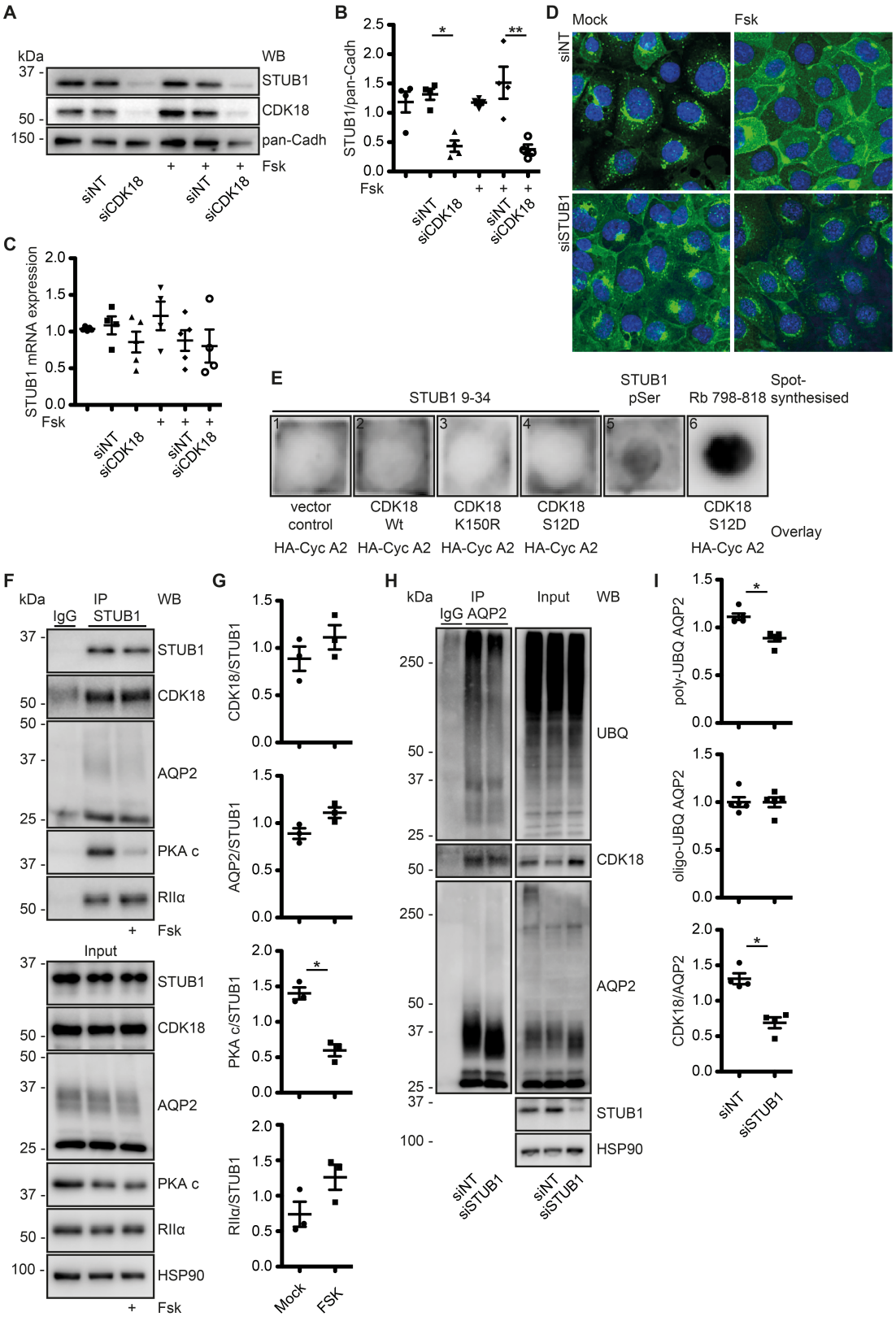

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Fig. 15: STUB1 RNAi mimics CDK18 RNAi.

A, B, C: CDK18 RNAi causes post-transcriptional decrease of STUB1. MCD4 cell were left untransfected or transfected with control (non-targeting, NT) or CDK18 siRNA and treated with forskolin (Fsk) or vehicle control. They were then analysed by (A) Western Blot (WB) and (B) semi-quantitative densitometric evaluation of the experiment presented in A and replicates, n=4, or (C) semi-quantitative PCR for STUB1 mRNA abundance, n>3, ANOVA, **p<0,01; *p<0,05.

D- STUB1 RNAi blocks the Fsk-induced AQP2 translocation. Representative immunofluorescence microscopic analysis of MCD4 cells transfected with either control or STUB1-targeting siRNA, treated with Forskolin (Fsk) or vehicle. AQP2 is stained in green with an appropriate antibody and nuclei are counter-stained in blue with DAPI; n=3.

E- CDK18 does not directly phosphorylate the amino-terminus of STUB1. HEK293 cells were transfected with a combination of vectors encoding for CDK18-FLAG in the wild type (Wt), kinase dead Lys150 into Arg (K150R), phospho-mimic Ser12 into Asp (S12D) forms, in addition to Hemagglutinin-tagged CDK18 co-activator Cyclin A2 (HA-Cyc A2) or vector control. CDK18 was purified by anti-FLAG immunoprecipitation and incubated on peptide-spotted STUB1 murine protein fragment 9-34 (spots 1-4), or on the 792-818 fragment of human Rb (spot 6, positive control). The incubation was as follows: spot 1, vector control; 2, CDK18 Wt; 3, CDK18 K150R; 4 and 6, CDK18 S12D; 1-4 and 6, HA-Cyc A2. On spot 5, where a pre-phosphorylated STUB1 was spot-synthesised, a mock overlay was performed (no kinase was added) as a positive control for the experiment. STUB1 or Rb phosphorylation was revealed by incubation with an anti-phosphorylated CDK substrate antibody, followed by incubation with a peroxidase-coupled secondary antibody and standard WB procedure. A representative kinase assay is shown; n=4.

F, G: STUB1 binds CDK18, AQP2 and PKA.

F- MCD4 cell were treated with Fsk or vehicle control and subjected to immunoprecipitation (IP) with antibodies anti- STUB1 or control (IgG). A representative Western Blot analysis is shown; PKA c: PKA catalytic subunit; RIIα: PKA Regulatory IIα subunit.

G- Semi-quantitative densitometric evaluation of the experiment presented in F and replicates; n=3, Student's t test, *p<0,05.

H, I: STUB1 RNAi decreases AQP2 ubiquitination and AQP2-CDK18 interaction.

H- MCD4 cells left untransfected or transfected with either control or STUB1 siRNA were subjected to immunoprecipitation (IP) with antibodies anti-AQP2 or control. A representative WB analysis is shown.

I- Semi-quantitative densitometric evaluation of the experiment presented in H and replicates; n=4, Student's t test, *p<0,05. The signal of the immunoprecipitation of AQP2 was analysed to evaluate both the oligo- (less than 50 kDa) and the poly- (more than 50 kDa) ubiquitinated (Ubq) AQP2 as a ratio to the immunoprecipitated AQP2 as described (102).

STUB1 organizes a PKA pool

MDC4 cells lysates were treated with the AKAP13-derived peptide Ht31, which disrupts AKAP-PKA interaction (201), or with a control PP-Ht31, where the insertion of two Pro residues renders the peptide unable to interfere with the AKAP-PKA interaction. Subsequent immunoprecipitations for STUB1 and Western Blot analysis revealed that the interaction between STUB1 and PKA can be effectively inhibited by the Ht31 peptide, suggesting an AKAP-dependent mechanism for the binding between STUB1 and PKA (Fig. 16C and D). The identity of this AKAP, that could actually be STUB1 itself, is still elusive, but this finding prompted to investigate the relationship between PKA and STUB1.

To evaluate if STUB1 is able to ubiquitinate PKA, leading to its degradation, MCD4 cells were transfected with siRNA against STUB1 or control in combination with Fsk or vehicle. Western Blot analysis revealed no alteration of the abundance of PKA catalytic or regulatory subunits (Fig. 16E and F), suggesting that STUB1 is not necessary for the degradation of PKA. Under the same conditions of STUB1 downregulation and/or Fsk stimulation, general PKA activity was measured by the PepTag assay. This method is based on the shift in charge of a PKA peptidic substrate after phosphorylation (202). Phosphorylated and non-phosphorylated peptides can be easily separated by electrophoresis on an agarose gel, and the total PKA activity estimated as the ratio of the phosphorylated peptide on the non-phosphorylated one. The assay revealed a robust increase in PKA activity after Fsk stimulation of MDC4 cells. MDC4 cells transfected with siRNA against STUB1 and control exhibited similar levels of PKA activity (Fig. 17A and B). Furthermore, the well-established PKA-dependent CREB Ser133 phosphorylation (203) was analysed in MCD4 transfected with siRNA against STUB1 or control and stimulated with Fsk or vehicle. The Fsk stimulated MDC4 cells displayed an increase in CREB phosphorylation. Comparing MDC4 cells transfected with siRNA against STUB1 and control revealed no differences in CREB phosphorylation (Fig. 17C and D).

These observations, although not showing an impact of STUB1 on global PKA activity, still left open the possibility of a modulation of PKA at the level of the STUB1-organized pool.

Fig. 16: legend on the next page.

Fig. 16: STUB1 organizes PKA in an A-Kinase Anchoring Protein (AKAP)-dependent way

A, B: STUB1 RNAi does not modify AQP2 abundance or phosphorylation, but increases MAPK14/p38 MAPK (p38) activatory phosphorylation at Thr180 and Tyr182 (pTY).

A, E- MCD4 cell were left untransfected or transfected with control (non targeting, NT) or CDK18 siRNA, treated with forskolin (Fsk) or vehicle control and analysed by Western Blot (WB). A representative experiment is shown. Abbreviations: pS256, phospho-Ser256; pS261, phospho-Ser261.

B, F- Semi-quantitative densitometric evaluation of the experiment presented in (B) A and replicates, n=6, (F) E and replicates, n=5, ANOVA, ***p<0,001, **p<0,01, *p<0,05.

C, D: STUB1 interaction with PKA is AKAP-dependent.

C- MCD4 cell were treated with the AKAP-PKA interaction disruptor peptide Ht31, the negative control peptide PP-Ht31 or vehicle control and subjected to immunoprecipitation (IP) with antibodies anti-STUB1 or control (IgG). A representative Western Blot analysis is shown.

D- Semi-quantitative densitometric evaluation of the experiment presented in C and replicates; n=4, ANOVA, **p<0,01, *p<0,05.

E, F: STUB1 RNAi does not alter PKA subunit abundance.

Fig. 17 (on the next page): CDK18 is phosphorylated by PKA.

A, B, C, D: STUB1 RNAi does not influence general PKA activity.

A, C- MCD4 cell were left untransfected or transfected with control (non targeting, NT) or STUB1 siRNA, treated with forskolin (Fsk) or vehicle control and subjected to (A) PepTag assay to determine PKA activation. The ratio between phosphorylated (negatively charged) and unphosphorylated (positively charged) peptide allows to estimate PKA activity. In alternative, (C) Western Blot (WB analysis was performed with antibodies against phosphorylated Ser133 cAMP responsive element binding protein (pS133 CREB), compared to total CREB (203). Representative experiments are shown.

B, D- Semi-quantitative densitometric evaluation of the experiment presented in (B) A and replicates; n=5, or (D) C and replicates, n=7, ANOVA, ****p<0,001, *p<0,05.

E, F, G, H: CDK18 is phosphorylated by PKA in MCD4 cells and in isolated nephric murine inner medullae (mIM).

E, G- MDC4 cells (E) were stimulated with Fsk or a peptide derived from the PKA inhibitor protein PKI, rendered cell-permeable by stearate coupling (PKI), and (G) mIM were stimulated with the cell-permeable activator of PKA Dibutyryl cAMP (DBcAMP). For both, immunoprecipitations (IP) with anti-CDK18 or control (IgG) antibodies were performed, and Western Blot analysis with a phospho-PKA substrate (pSub PKA) antibody assayed the amount of PKA phosphorylation of CDK18. Representative experiments are shown.

F, H- Semi-quantitative densitometric evaluation of the experiment presented in (F) E and replicates, n=6, ANOVA, or (H) G and replicates, n=4, Student's t test, ***p<0,001, *p<0,05.

Fig. 17 (legend on the previous page): CDK18 is phosphorylated by PKA.

CDK18 influences PKA activity

Since the initial step in the Fsk/cAMP induced redistribution of AQP2 is PKA activation (102) and CDK18 can be phosphorylated by PKA, increasing CDK18 kinase activity (188), it was investigated whether PKA phosphorylates CDK18 in the MCD4 cell system. An antibody able to recognize the phosphorylated form of CDK18 is not available. CDK18 was then immunoprecipitated from MCD4 lysates and analysed by Western Blot with an antibody against phospho-PKA substrates. The amount of phosphorylated CDK18 was increased upon Fsk stimulation, and the Fsk-mediated increase in phosphorylation could be prevented by pre-incubation with the cell-permeable peptidic PKA inhibitor, stearate-coupled PKI (Fig. 17E and F), so confirming that PKA is the kinase responsible for CDK18 phosphorylation. To further elaborate this point, inner medullae isolated form murine kidney were stimulated with N^6,2′-O-Dibutyryladenosine 3′,5′-cyclic monophosphate (DBcAMP), a cell-permeable analogue of cAMP able to activate PKA. CDK18 was then immunoprecipitated and

the PKA-driven phosphorylation analysed by Western Blot with the phospho-PKA substrate antibody, replicating the previous findings in a more physiological system (Fig. 17G and H). This suggests that the PKA-driven phosphorylation of CDK18, presumably at Ser12, could be responsible for CDK18 kinase activation in MCD4 cells and in murine kidney inner medullae.

MDC4 cells were transfected with siRNA for CDK18 or control and stimulated with Fsk or vehicle; the PepTag system was used to assay PKA activity. As the knockdown of CDK18 did not affect the PKA-mediated phosphorylation of AQP2 Ser256 (Fig. 12D and E), it was surprising to find that CDK18 depletion increased maximal PKA activity in MCD4 cells in the presence of Fsk (Fig. 18A and B), hinting at a possible negative feedback mechanism involving PKA and CDK18.

To shed some light on the STUB1-CDK18-PKA complex activity, it was investigated if the CDK18 activating phosphorylation by PKA was mediated by STUB1. MDC4 cells were transfected with STUB1 or control siRNA and stimulated with Fsk or vehicle. CDK18 was immunoprecipitated and analysed by Western Blot for CDK18 itself, to confirm the immunoprecipitation, and with the phospho-PKA substrate antibody, to detect CDK18 phosphorylation by PKA. As expected, in the siRNA control transfected samples, Fsk induced a sharp increase in the amount of PKA-phosphorylated CDK18. In the absence of STUB1 Fsk was still able to increase the phosphorylation of CDK18, suggesting that STUB1 could be dispensable for the PKA phosphorylation of CDK18. This indicates that other AKAPs can take over the STUB1 role of bringing together CDK18 and PKA, or that the AKAP-free pool of PKA could account for CDK18 activation.

Fig. 18: CDK18 modulates PKA activity.

A, B: CDK18 RNAi increases the maximal PKA activity under Forskolin (Fsk) stimulation.

A, C- MCD4 cell were left untransfected or transfected with control (non-targeting, NT), (A) CDK18 or (C) STUB1 siRNA and treated with Fsk or vehicle control. Analysis with (A) PepTag assay determined PKA activation; the ratio between phosphorylated (negatively charged) and unphosphorylated (positively charged) peptide allows to estimate PKA activity. In alternative, (C) immunoprecipitations (IP) with anti-CDK18 or control (IgG) antibodies were performed, and Western Blot (WB) analysis with a phospho-PKA substrate (pSub PKA) antibody assayed the amount of PKA phosphorylation of CDK18. Representative experiments are shown.

B, D- Semi-quantitative densitometric evaluation of the experiment presented in (B) A and replicates, or (D) C and replicates, n=4, ANOVA, ***p<0,001, *p<0,05.

C, D: STUB1 RNAi does not affect CDK18 phosphorylation by PKA.

Collectively, these data suggest the formation of a multi-protein complex which regulates AQP2 protein abundance by controlling phosphorylation and ubiquitination of AQP2 (Fig. 19).

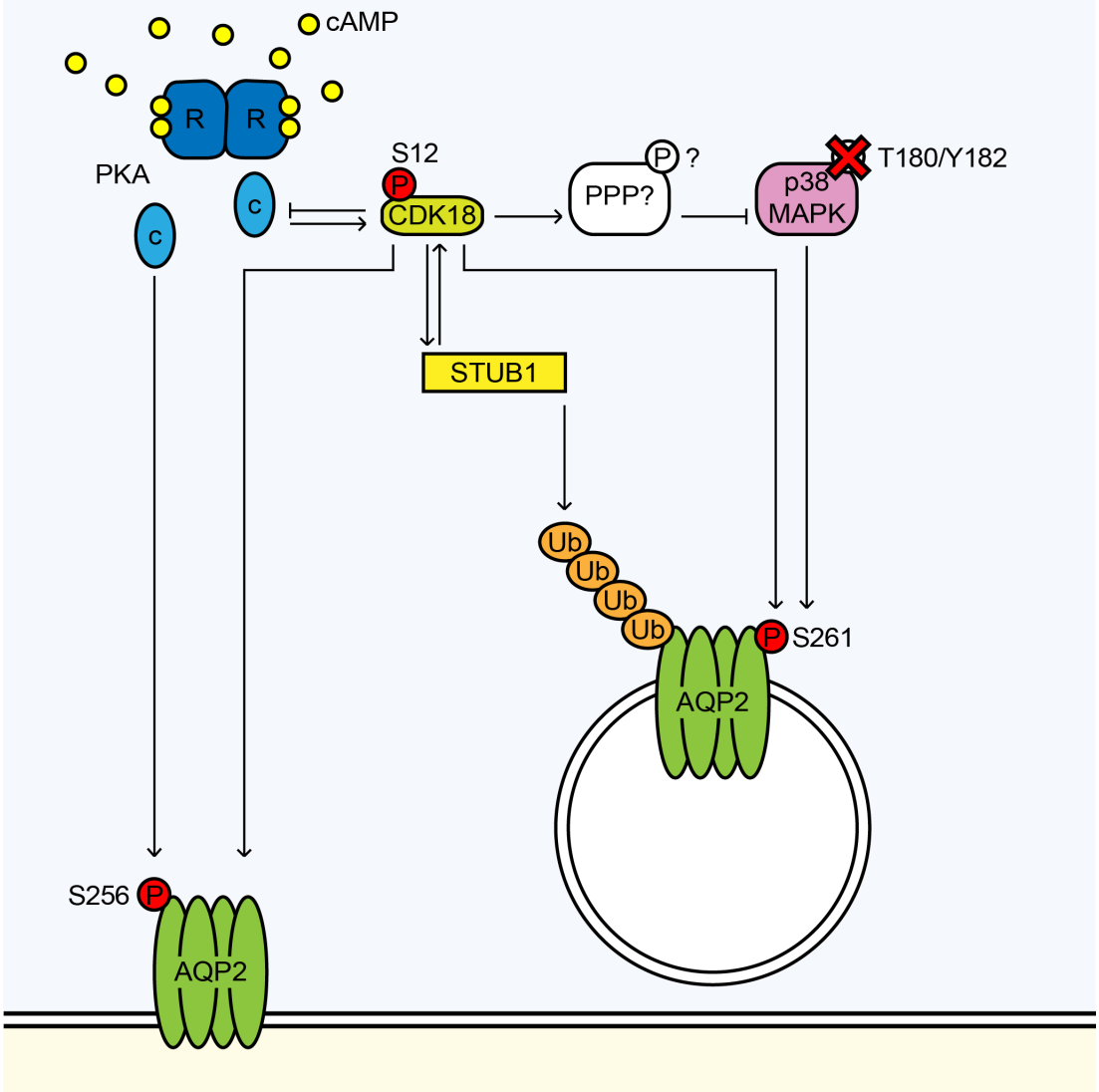

Fig. 19: CDK18 is a central kinase in the AQP2 regulatory network.
Abbreviations: PPP, protein phosphatase; S, Ser; T, Thr; Y, Tyr, Ub, ubiquitin.

Discussion

Specific enzymatic inhibition of a kinase is a potent way to influence molecular pathways to treat pathological conditions. Another arising possibility to achieve the same is interrupting the protein-protein interactions between the kinase and the scaffolds that hold the second in place (50,51). This approach, although less practised than the first one, provides the opportunity to specifically target only the subpopulation of the enzyme binding to the scaffold of interest, so potentially delivering a precise inhibition of the pathway of interest.

Both the research lines presented here lead into this direction. In the first part, the GSKIP-mediated interplay of PKA and GSK3β on Wnt/β-catenin signaling was elucidated; in the second part, it was uncovered the role of CDK18 in the control of AQP2 translocation.

GSKIP regulates Wnt signaling by binding both GSK3β and PKA

The first part of the present analysis investigated how GSKIP, through binding of both GSK3β and PKA, regulates Wnt signaling without interacting directly with the β-catenin destruction complex components (1). GSKIP rather establishes an independent pool for both the kinases. This study suggests that GSKIP exerts its function by subtraction of GSK3β and PKA from the kinase pools that are available to modulate the canonical Wnt signaling. This results in the apparent paradox of an increased Wnt signaling both when GSKIP is abundant (i.e., overexpressed) and when it is absent (e.g. after downregulation by siRNA). Binding to GSK3β and withholding it from the destruction complex-associated pool, GSKIP prolongs β-catenin cytosolic half-life and increase the Wnt signaling transcriptional output. Interacting with PKA, GSKIP is able to scavenge a positive modulator of the Wnt signaling away from β-catenin, reducing its stabilising phosphorylation. The net result is a buffering action on both of the kinases, presumably modulating Wnt signaling. A GSKIP-dependent effect on canonical Wnt signaling was observed in four different cell lines, and since PKA, GSKIP and GSK3β are ubiquitous, this ternary complex could influence Wnt signaling in a broad range of developmental and pathological contexts in which the pathway is implicated (20).

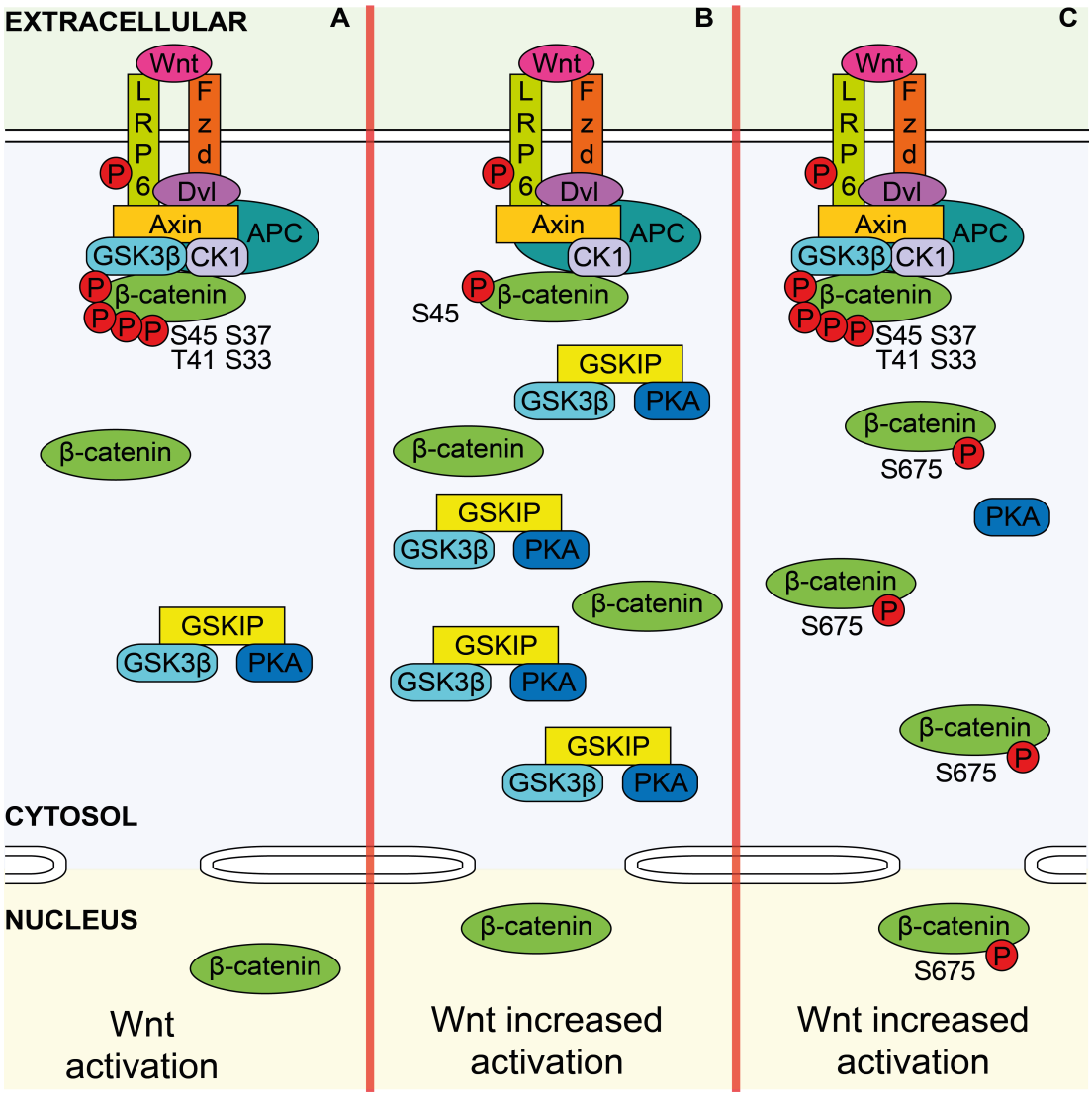

Fig. 20: Enhancing the effect of GSKIP on Wnt signaling depends on both its interactions with PKA and GSK3β.

Schematic representation of active Wnt signaling in normal conditions (A), with high-levels of GSKIP (B) or in case of GSKIP depletion (C).

A: After binding of the Wnt ligand to the Fzd/LRP6 receptor/co-receptor complex, the β-catenin destruction complex slows the proteolysis of β-catenin. This allows the accumulation of the latter in the cytosol, and its eventual translocation into the nucleus, where it promotes transcription.

B: Excessive GSKIP displaces GSK3β from the Axin-comprising destruction complex, increasing Wnt signaling by reducing β-catenin phosphorylation at Ser33, Ser37, Thr41. Phosphorylation of β-catenin Ser45 is dependent on CK1, that is not directly affected by GSKIP (204).

C: Knockdown of GSKIP increases the availability of PKA, stabilising β-catenin *via* phosphorylation at Ser675 and so enhance Wnt signaling.

Abbreviations: Fzd: Frizzled, LRP6: Low-density lipoprotein Receptor-related Protein 6; Dvl: Dishevelled, Axin; APC: Adenomatous Polyposis Coli; GSK3β: Glycogen Synthase Kinase 3β; CK1: Casein Kinase 1; β-TrCP: β-Transducin repeat Containing E3 ubiquitin Protein ligase; GSKIP: Glycogen Synthase Kinase 3 Interaction Protein; PKA: cAMP dependent Protein Kinase A; GSK3β: Glycogen Synthase Kinase 3β; S, Ser; T, Thr.

This study highlights the importance of GSKIP for Wnt signaling control by comparing it to AKAP220, another PKA and GSK3β-binding scaffold protein. Both GSKIP and AKAP220 enhance the inhibitory phosphorylation of PKA on the Ser9 of GSK3β, but the two AKAPs have different localizations: AKAP220 was found at the plasma membrane and on peroxisomes (69,71,205,206), while GSKIP is mainly cytosolic (67,68). Most AKAPs harbour specific domains responsible for the targeting of a PKA pool to a specific subcellular localization, including membranes, cytoskeletal components, or other structures (45,55,207,208). Some AKAPs, however, lack canonical targeting domains and are shown to change their subcellular localization, in some cases after specific stimuli. For example the AKAP Gravin is released from the plasma membrane into the cytosol in response to an elevation of intracellular calcium in HEC-1A endometrial cancer cells (209). Another example is AKAP18δ, a cytosolic protein, that, through positively charged amino acids distributed across its surface, associates with membrane lipids in renal collecting duct principal cells (210,211). GSKIP, instead, does not show relocalisation to cellular organelles after retinoic acid (73), cAMP (67) or Wnt3a stimuli (1), so representing an example of a constitutively cytosolical AKAP. Since the membrane-associated AKAP220 did not show the same influence as GSKIP on Wnt signaling, this study assesses for the first time a function to a purely cytosolical AKAP. AKAP220 is interacting directly with the plasma membrane associated β-catenin, that could compete with the cytosolical, transcriptionally-relevant pool (205,212). However, AKAP220 does not seem to alter Wnt signaling, so suggesting that AKAP220-β-catenin binding is not an efficient mean to displace β-catenin from the cytosol. This observation is agreeing with literature data assessing the independence of the AKAP220-cadherin complex in endothelial barrier from Wnt signaling (205). The different effect of the two AKAPs on Wnt signaling can be explained by their different localization, that could lead to the organization of distinct pools of kinases, regulated in distinct ways.

Part of the present analysis regarded the characterization of the GSK3β Interacting Domain (GID) of AKAP220. A binding region for GSK3β was recently discovered in the 1126-1145 fragment of AKAP220, identifying the phosphorylation of Thr1132 by GSK3β itself to permit the interaction (71). However, a previous study demonstrated binding between kinase-dead GSK3β and AKAP220 (65), which suggests the existence of further points of contact between AKAP220 and GSK3β (71). In the present study it was revealed that a portion of the human AKAP220 carboxy-terminus is highly homologous to the GIDs present on GSKIP and Axin, in addition to being highly conserved among the mammalian AKAP220 ortologs. The structure of GSK3β

bound to the Axin GID was characterized by crystallography studies (18), allowing to identify the molecular determinants of the GSK3β-Axin interaction, and in particular the Phe-Leu-Leu (FLL) motif that is also present in AKAP220. The conservation of the GID explains the competition for GSK3β binding that was found in the co-immunoprecipitation experiments.

Adding a new level to Wnt signaling control, the findings reported could have important clinical implications. Increased Wnt signaling activity plays a role in various cancers, neurological diseases, fibrosis, and other diseases (213,214). Of further interest, duplication of the chromosomal region encoding GSKIP predisposes to myeloid lymphoma, presumably due to increased GSKIP protein, inhibition of GSK3β and increased Wnt signaling (75). A general challenge in targeting the Wnt signaling pathway for therapeutic purposes is represented by its complex crosstalk with MAPK, BMP, Hedgehog, Notch, cAMP/PKA, and other pathways (214-217). Targeting GSK3β itself is equally difficult as it would likely result in broad side effects due to its involvement in several additional pathways (218). Better characterization of the AKAP scaffolds involved in canonical Wnt signaling could permit to specifically target the critical pools of GSK3β (and PKA) involved in β-catenin phosphorylation, without affecting the other multiple pathways depending on both kinases.

CDK18 and STUB1 organize an AKAP-like signaling complex crucial for AQP2 abundance, phosphorylation and trafficking

In the second part of the present analysis, it was uncovered the importance of the CDK18-STUB1 complex for the control of AQP2 translocation (Fig. 21).

The approach chosen for this project was to establish a siRNA-based screening of the murine kinome, i.e. the portion of the genome encoding for protein kinases. The choice of this portion of genes allowed to focus on a class of enzymes widely known for their extensive role in molecular pathway modulation and representing alluring pharmacological targets.

Also very important was the selection of the appropriate cell type to establish the screening. While primary murine Inner Medullary Collecting Duct (mIMCD) cells would have been the most physiological option, relying on freshly isolated primary cells would also presented several disadvantages. Primary cells are difficult to isolate and handle in the large amounts needed for this kind of screening, require the sacrifice of high numbers of mice, are not always comparable from one preparation to the next, and are resistant to lipidic vector-mediated transfection of siRNA, requiring the establishment of a viral library, with all the added costs in time and material that this implies. The choice of an immortalized cell line allows to circumvent all these issues. The Murine Collecting Duct 4 (MCD4) cell line offers the possibility to easily and economically transfect a siRNA library in a robust artificial system able to recapitulate the translocation of AQP2 after PKA activation. The main drawback inherent in the MDC4 cells is the absence of the V2 receptor, that renders these cells AVP-insensitive. However, this is no longer an issue when Fsk is used to directly activate the AC proteins, increase cAMP production, and activate PKA. This stimulates the translocation of AQP2 from the perinuclear region of these cells into the plasma membrane, recapitulating the AVP effect.

The screening revealed as hits several protein kinases, previously not related to AQP2 trafficking; among those, CDK18 was chosen as the most robust candidate. CDK18 is a poorly described kinase, possibly involved in the pathogenesis of Alzheimer disease (187), and the nuclear CDK18 pool is involved in the regulation of DNA damage-dependent signaling (189). Among the few other details known about this kinase, it was published that phosphorylation at the amino-terminus by PKA, more specifically at the Ser12, increases the kinase activity of CDK18, that can be further enhanced by binding of the Cyclin A2 cofactors (188).

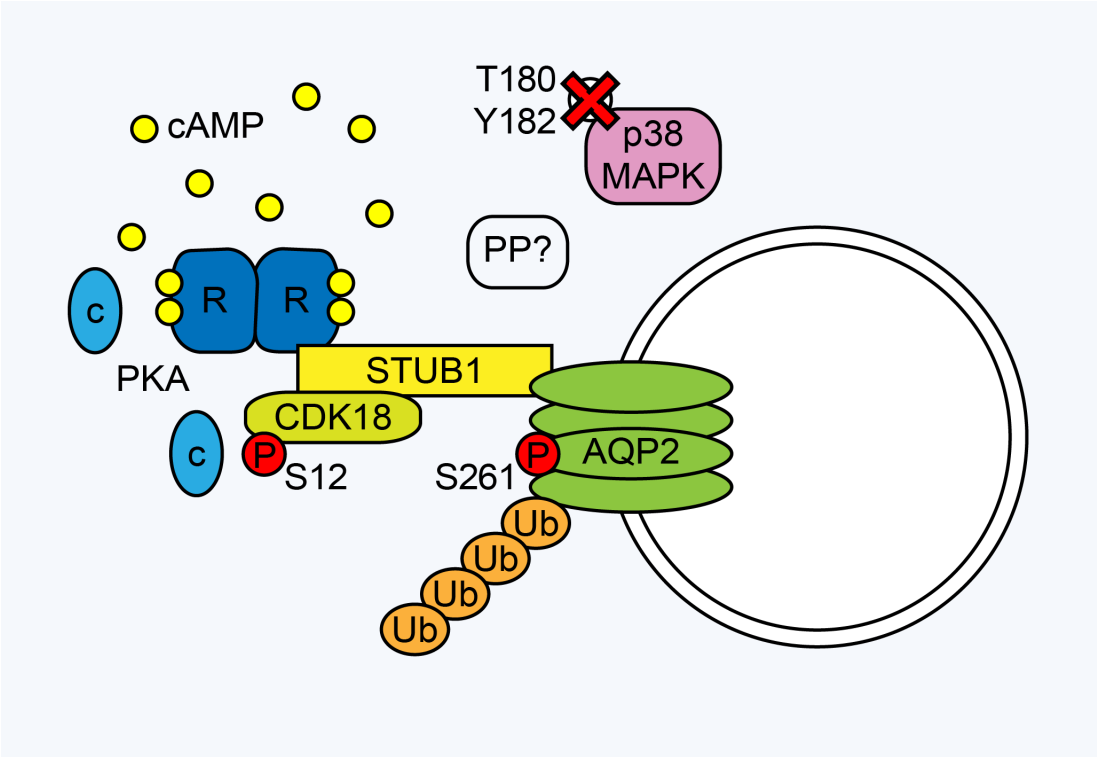

Fig. 21: CDK18 and STUB1 are part of a multiprotein complex regulating AQP2 localization, abundance and phosphorylation.

Schematic representation of the protein complex described in this study, involving the cAMP-dependent Protein Kinase A (PKA) tetramer (formed by two regulatory and two catalytic subunits, R and C, respectively) bound to STIP1 Homology And U-Box Containing Protein 1 (STUB1) that is able to associate with Cyclin Dependent Kinase 18 (CDK18) and the water channel aquaporin2 (AQP2).

The complex may involve also not yet identified Protein Phosphatase (PP) responsible for the de-phosphorylation at Thr180-Tyr182 (T180 Y182) of Mitogen Activated Protein Kinase 14 (p38 MAPK). Furthermore, PKA is responsible for the activating phosphorylation of CDK18 at Ser12 (S12), while CDK18 catalyses the phosphorylation of AQP2 at Ser261 (S261), possibly directing it for STUB1-mediated poly-ubiquitination (Ub).

The present study describes the involvement of CDK18 in the control of the PKA-mediated AQP2 trafficking. MCD4 cells deficient for CDK18 are not only unable to correctly target AQP2 to the plasma membrane upon cAMP increases, but also exhibit an increment of AQP2 protein level. This is not due to increased AQP2 mRNA abundance, but is most likely caused by the decreased phosphorylation of AQP2 at the Ser261. Phosphorylation of this site is linked to AQP2 ubiquitination and degradation (102,162). The decrease in Ser261 phosphorylation in CDK18-deficient MDC4 cells leads to the reduced AQP2 poly-ubiquitination and proteasomal degradation that was observed. Moreover, the present study established that CDK18 is directly responsible for AQP2 phosphorylation at Ser261. This is the first time, at

the best of our knowledge, that a CDK18-mediated phosphorylation can be identified, and that physiological relevance can be attributed to it.

CDK18-deficient MCD4 cells show increased p38 MAPK activation by phosphorylation at the Thr180-Tyr182 site. p38 MAPK is a kinase involved in AQP2 translocation regulation (102) and its activity can be regulated by protein phosphatases, for example downstream prolactin signaling (219). Moreover, recent literature suggest the involvement of phosphatases in the control of AQP2 trafficking (198). The increase in p38 MAPK phosphorylation correlates with the decreased phosphatases activity measured after CDK18 knockdown, suggesting a dependency between the two phenotypes. The decrease in the p38 MAPK-targeted phosphorylation of AQP2 at Ser261, despite the higher activation levels of p38 MAPK when CDK18 is downregulated, underlines the importance of CDK18-mediated phosphorylation of AQP2.

CDK18 binds to the ubiquitin ligase STUB1, predicted to be involved in AQP2 ubiquitination. CDK18 knockdown demonstrated that CDK18 is required to stabilize the STUB1 protein, as to degrade AQP2. STUB1 knock down in MCD4 cells was able to reproduce the effects of CDK18 RNAi in terms of p38 MAPK phosphorylation, AQP2 trafficking and ubiquitination. However, STUB1-deficient MDC4 cell did not show altered AQP2 abundance, suggesting the involvement of further, not yet characterized, ubiquitin ligases. NEDD4 and NEDDL have been predicted to ubiquitinate AQP2 by bioinformatic means, highlighting them in the list AQP2-binding E3 ubiquitin ligases (168,169). NEDD4 protein abundance is reduced in MDC4 cells after CDK18 RNAi (data not shown), hinting that NEDD4 could be responsible for the STUB1-independent, but CDK18-related, AQP2 ubiquitination. STUB1 knockdown does not induce AQP2 protein increase as the CDK18 RNAi does. This suggests that different thresholds of AQP2 ubiquitination are involved in the degradation or the translocation processes, and that STUB1-mediated AQP2 ubiquitination is required for AQP2 translocation, but not for its degradation.

STUB1 was revealed to be part of a CDK18-AQP2-PKA protein complex, the role of which is still largely unexplored. Treatment with the AKAP-PKA interaction disruptor peptide Ht31 demonstrated that STUB1 binding to the RII subunit of PKA depends on AKAPs. AKAP220 and AKAP18δ are responsible for anchoring of PKA on AQP2-bearing vesicles (130-132). These results suggest that STUB1 could either interact with an AKAP or directly with PKA, behaving like an AKAP itself. The role of the STUB1-bound pool of PKA in AQP2 translocation is currently under investigation.

The presence of PKA in the STUB1-bound protein complex leads to careful reconsideration of the influence of CDK18 over PKA and *vice versa*. PKA

phosphorylates CDK18 at the amino-terminus both in MDC4 cells and isolated murine medullae. This phosphorylation, especially at Ser12, activates CDK18 (188). In isolated murine medullae stimulated with the endogenous agonist AVP the protein abundance of CDK18 increased (data not shown) resulting in a non-quantitative immunoprecipitation. This frustrated the efforts to investigate if CDK18 is phosphorylated by PKA in a purely endogenous context. CDK18 knockdown increases in turn the maximal PKA activity, suggesting that CDK18 and PKA establish a negative feedback loop. STUB1, instead, seems not to be involved in PKA abundance or in the control of general PKA activity, and is not required to ensure the PKA-mediated CDK18 phosphorylation.

Understanding the still incompletely characterized scaffold and kinase network regulating AQP2 translocation is crucially important to establish causal treatments for both nephrogenic diabetes insipidus and excessive water retention-related diseases. In this perspective, unravelling the role of the PKA-CDK18-STUB1 complex in the control of AQP2 translocation and abundance highlights exciting possibilities for drug development. In fact, the data not only suggest that CDK18 could influence AQP2 translocation via its kinase activity, but even that pharmacological targeting of the interaction between STUB1 and CDK18 (and possibly, PKA itself) could achieve the same modulation. Such an objective could be achieved by multiple means, for example with peptides or peptidomimetics agents competing with the binding of the ternary complex (50,51). Both alternatives could become relevant to block the undesired reabsorption of water that is so dramatic in late-stage heart failure patients, psychiatric patients undergoing lithium treatment or subjects suffering from SIADH.

Outlook

As always, new findings come together with new questions waiting for an answer.

How does the CDK18-driven phosphorylation influence AQP2 trafficking? Despite the extensive investigations conducted in the present study, it is not absolutely clear, if the CDK18-mediated phosphorylation is causative of the ubiquitination of AQP2. Analysis of *in vitro* ubiquitination of AQP2 mutated at the position Ser261, either in a non-phosphorylatable or phospho-mimicking sense, could demonstrate the importance of this phosphorylation for STUB1-dependent ubiquitination. However, obtaining high amounts of recombinant membrane-embedded proteins, like AQP2, it's notoriously challenging, presenting a serious obstacle for this experiment.

Is the CDK18-mediated inhibition of p38 MAPK dependent on PPs? The decrease of phosphatases activity that accompanies CDK18 downregulation could be linked to the increased activating phosphorylation at Thr180-Tyr182 of p38 MAPK that was also observed. However, verification of this hypothesis will require the characterization of the phosphatases involved in this mechanism in MDC4 cells.

Which is the role of the STUB1-bound pool of PKA? Depletion of STUB1 does not lead to decreased phosphorylation of CDK18, but this process could be AKAP-independent. The phosphorylation of AQP2 by PKA, instead, involves AKAPs, e.g. AKAP18δ (210,220) and AKAP220 (132). Investigating primary cells after depletion of STUB1 is necessary to determine to which extent the STUB1-organized PKA pool overlaps with the one responsible for AQP2 phosphorylation and translocation.

Overall, this work extends the knowledge about the importance of kinase compartmentalization in sub-pools in different physiological context, underlining its general and widespread nature.

Material & Methods

Mutagenesis and cloning

Mutagenesis was carried out using a pECFP-GSKIP plasmid as template (67) and the QuickChange II Site Directed Mutagenesis Kit (Agilent, Santa Clara, USA) according to the manufacturer's recommendation. For preventing GSKIP's interaction with PKA Asn 42 was replaced by Ile. For the generation of a GSKIP variant deficient for binding of GSK3β, Leu 130 was replaced by Pro (194), thus generating the plasmids pECFP-GSKIP-N42I and pECFP-GSKIP-L130P, respectively. The inserts were amplified by PCR and cloned with the inserted restriction sites NheI (5') and XhoI (3') into pCMV6ev (Origene, Rockville, USA) to generate the vectors pCMV6-GSKIP-FLAG, pCMV6-GSKIP-L130P-FLAG and pCMV6-GSKIP-N42I-FLAG (Fig. 22).

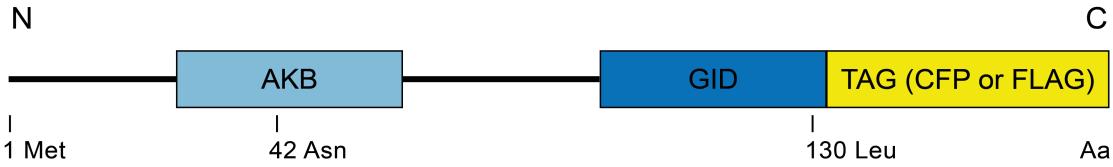

Fig. 22: Scheme of the GSKIP insert used for cloning as detailed in text; AKB: PKA binding domain; GID: GSK3β Interaction Domain.

The pCMV6ev (Origene) backbone was also used to clone the cDNA of CDK18 Wt ordered as a gene art string (Thermo Fisher Scientific, Waltham, USA) using the restriction sites HindIII (5') and XhoI (3'). Mutagenesis in order to obtain the Ser 12 into Asp and Lys150 into Arg mutations was carried on using the Quick Change II system (Agilent).

The following primers were used:

N42I Fwd: CTCGAAGCTGAAGCAGTTGTAAATGATGTTCTCTTTGCT;

N42I Rev: TCGTTTCTCTTGTAGTAAATGTTGACGAAGTCGAAGCTC;

L130P Fwd: CGCACTGCTTCAAAGACCGGAAGCTTTGAAAAGAG;

L130P Rev: CTCTTTTCAAAGCTTCCGGTCTTTGAAGCAGTGCG;

S12D F: GGGGCACGGACAGGTCAAGCCTTCGCTTGAAG;

S12D R: CTTCAAGCGAAGGCTTGACCTGTCCGTGCCCC;

K150R F: AGAACCTCGTGGCCCTGAGGGAGATCCGG;

K150R R: CCGGATCTCCCTCAGGGCCACGAGGTTCT.

Buffers

Phosphate Buffer Saline (PBS): NaCl 137 mM, KCl 3.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 1.8 mM

TBS: 50 mM Tris-Cl, pH 7.5. 150 mM NaCl.

Mild Lysis Buffer (MLB): PBS supplemented with 0.2% Triton X-100, 2 mM EDTA, 2 mM EGTA.

Standard Lysis Buffer (SLB): 10 mM K_2HPO_4, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 1% Triton X-100, 0.2% Na-deoxycholate, pH 7.4.

Radio Immuno Precipitation Assay buffer (RIPA): 150 mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 50 mM Tris, pH 8.0.

Kinase Assay Incubation Buffer (KAIB): 50 mM Tris-HCl pH 7.5, 20 mM Magnesium Acetate, 50 µM ATP.

Colorimetric Assay Buffer (CAB): 20 mM Tris, pH 7.5, 5 mM $MgCl_2$, 1 mM EGTA, 0.02% β-mercaptoethanol, 0.1 mg/ml bovine serum albumin (BSA).

Cell culture, transfection and fractionation

HEK293, SW480, A549 and HeLa cells were grown in DMEM (GlutaMAX, 10 % Foetal Calf Serum, Thermo Fisher Scientific, Waltham, USA) and transfected with the above-mentioned plasmids, and/or pECFP, pEYFP, pEYFP-GSK3β, pΔECFP-myc-GSKIP (CFP deleted), pcDNA3.1-Flag-Axin1, pcDNA3.1-Flag-Axin2, TOPflash, FOPflash and pRL-SV40 (kindly provided by Prof. W. Birchmeier, MDC-Berlin, Germany), pCGN-HA-AKAP220 (kindly provided by K. Taskén, The Biotechnology Centre of Oslo, University of Oslo, Norway), SMARTpool siGENOME GSKIP siRNA and siGENOME Non-Targeting siRNA #2 (GE Healthcare, Chalfont St Giles, UK) using Lipofectamine2000 (Thermo Fisher Scientific, Waltham, USA) (67).

MCD4 cells were cultured in 5% Foetal Calf Serum, 5 µM Dexamethasone F12-DMEM GlutaMAX (Thermo Fisher). For siRNA transfection, cells were transfected in suspension with Lipofectamine 2000 (Thermo Fisher) according to manufacturer's instructions. For DNA transfection, Lipofectamine 3000 (Thermo Fisher) was used according to the supplier's protocol. The following plasmids were used for transfection: pCMV6ev (empty vector control), pCMV6 CDK18 FLAG Wt, pCMV6 CDK18 FLAG K150R (kinase dead), pCMV6 CDK18 FLAG S12D (phospho-mimicking), pCIneo-3HA-mCycA2 (kindly provided by Dr. Lüder Wiebusch, Charité Campus Virchow-Klinikum, Berlin).

The siRNA against CDK18, STUB1 and NT2 (against firefly luciferase) were purchased as siGENOME SMART pools (Thermo Fisher).

For cell fractionation and cytosol purification a digitonin semi-permeabilization protocol was adapted (192). Briefly, cells were treated with Wnt-enriched or control medium for 4 h, washed twice with PBS, and semi-permeabilized with Digitonin. The lysis buffer containing the cytosol was cleared by centrifugation (supernatants; 700 x g, 5 min). The adherent, permeabilized cells were washed twice with PBS and lysed in RIPA buffer. Purity of the cytosolic fractions was routinely confirmed by Western Blotting with antibodies directed against nuclear- (Lamin A/C), cytosolic- (GAPDH, HSP90) and plasma membrane-specific (pan-Cadherin, LRP6) antigens.

Cell treatment and manual immunofluorescence

Wnt-conditioned medium was obtained following the ATCC guidelines for L-Wnt cells cultivation and harvesting; 50% diluted Wnt-conditioned medium was added to the cells for 4h (for cytosolic purification) or for 24h (for luciferase assays) before samples harvesting.

MCD4 cells were treated with Fsk (30 µM) for 30 min before fixation for immunofluorescence or lysis for Western Blot experiments. Ht31 or PP-Ht31 peptides were added to lysates of MCD4 cells at 30 µM concentration for 30 min at 30 °C. PKI-stearate (10 µM) or H89 (30 µM) were added as a pre-treatment to the cells 30 min before the Fsk treatment to inhibit PKA. DBcAMP was added to the freshly isolated murine inner medullary homogenates at 1 mM concentration for 30 min at 37 °C.

Immunofluorescence was performed as described (102) with the H27 antibody against AQP2; nuclei were stained with DAPI (Hoffmann-La Roche) and imaged using a LSM 780 microscope (Zeiss, Oberkochen, Germany).

mRNA abundance evaluation

mRNA was extracted using the Universal RNA purification kit (Roboklon, Berlin, Germany) and cDNA was retro transcribed with the peqLAB Quanta kit (VWR, Darmstadt, Germany). sqPCRs were performed with the Power SYBR Green system (Thermo Fisher) and analysed with the $2^{-\Delta\Delta Ct}$ method using GAPDH as a control.

Cell lysis and tissue isolation

For standard Western Blot analysis, cells were lysed using SLB (1) supplemented with protease and phosphatase inhibitors (Roche).

For immunoprecipitations, cells and organs were scraped in MLB supplemented with protease and phosphatase inhibitors (Roche) and lysed by repeated passage through a 0.4 G needle.

For murine inner medullae collection, mice were sacrificed by cervical dislocation, the kidneys collected in PBS and the medullae excised. After incubation in DMEM medium and treatment with DBcAMP, the tissues were washed twice in PBS, moved into mild lysis buffer supplemented with protease and phosphatase inhibitors (Roche) and disrupted mechanically with a tight-fitting pestle. Cell and tissue lysates were cleared by centrifugation at 4 °C; 5,000 x g, 5 min.

Western Blotting and antibodies

WB was carried out as described (67,210) with the following antibodies.

HSP90 mouse (AC88; Enzo Life Sciences, Lörrach, Germany);

GFP mouse (JL-8; Takara Bio Inc., Shiga, Japan);

GFP-01, rabbit (custom-made (221));

GSKIP, rabbit, (custom-made (67));

LaminA/C, goat, CDK18/PCTAIRE3 rabbit, C-17, AQP2, goat, C-17 (N-18; Santa Cruz Biotechnology);

HA High Affinity, rat (3F10; Roche);

Flag (M2), mouse and pan-Cadherin, mouse (Sigma-Aldrich, St Louis, USA);

Axin1 (C7B12), rabbit; Axin2 (76G6) rabbit; Myc-tag (9B11), rabbit; GAPDH (H-12), rabbit; GSK3β (27C10), rabbit; phospho-GSK3β (Ser9), rabbit; phospho-β-catenin (Ser675), rabbit; β-catenin (#9562), rabbit; phospho-β-catenin (Ser33/Ser37/Thr41), rabbit, STUB1/CHIP rabbit, #2080; p38a MAPK rabbit, #2371; phospho p38 MAPK (Thr180/Tyr182) rabbit, #4511; Ubiquitin rabbit, #3936; phospho PKA substrate rabbit, #9624 (all Cell Signaling, Cambridge, UK);

PKA RIIα mouse, PKA catalytic mouse, PKA RIIβ mouse, PKA RIβ mouse (BD, Franklin Lakes, USA);

STUB1/CHIP mouse, ab180038, APQ2 pSer261 rabbit, ab72383, AQP2 pSer256 rabbit, ab111346, PKA RIα mouse ab60064 (Abcam);

phospho AQP2 (S256) rabbit ZDE12147_0798 (Eurogentec, Köln, Germany).

Signals were detected using EMD Millipore Immobilon Western Chemiluminescent HRP Substrate (Thermo Fisher) and an Odyssey Fc Imaging System (LI-COR Biotechnology, Lincoln, USA). The quantification was then performed with the Image Studio Lite software (LI-COR biotechnology).

Immuno- and cAMP-agarose precipitations

For the GSKIP part, immunoprecipitations were carried out as described (67,210). In brief, cells were lysed in MLB and proteins were immunoprecipitated with anti-GFP (180,221) or anti-GSK3β (L-17, Goat, Santa Cruz Biotechnology, Dallas, USA) antibodies, and protein A-conjugated agarose (Sigma-Aldrich) or anti-FLAG coupled magnetic beads (Sigma-Aldrich) and eluted with Laemmli sample buffer.

As previously described (67), for cAMP-agarose precipitations, the lysates were incubated with 8-AHA-cAMP agarose (4 °C, 3 h; Biolog, Bremen, Germany); agarose-bound proteins were washed four times with lysis buffer and eluted with Laemmli sample buffer. cAMP (15 mM) competitive treatment was used to abolish the binding of PKA to the beads, in order to generate negative control pull-downs.

For immunoprecipitations relative to the CDK18 part, cells were lysed in mild lysis buffer, and 500 µg aliquots of cell lysate were incubated for 1-3 h at 4 °C with 50 µl of Protein A-conjugated magnetic Dynabeads (Thermo Fisher), loaded with the appropriate antibody. After 3 washes in MLB, the precipitated immunocomplexes were separate by the beads by acidic elution via the addition of 0,1 M Glycine pH 2.4, neutralized with Tris HCl 1 M pH 10.6, diluted in Lämmli buffer and analysed with standard Western Blot procedures.

For immunoprecipitations, the following antibodies were used: AQP2 H27 serum, rabbit, custom-made (222); STUB1 rabbit, ab134064 (Abcam); CDK18/PCTAIRE3 rabbit, C-17 (Santa Cruz Biotechnology).

PKA activity assay and pNPP phosphatases activity assay

PKA activity was monitored by PepTag Assay (Promega, Madison, USA) according to manufacturer instruction.

For pNPP-based phosphatase evaluation, cells were lysed in MLB without phosphatase inhibitors, and triplicate aliquots of 100 µg of lysates were diluted in Colorimetric Assay Buffer containing 10 mM pNPP as described (197). After 30 min incubation at 30 °C, the absorbance at 405 nm was determined with an xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad, Hercules, USA), and phosphatase activity was then calculated.

Kinase Assay

For non-radioactive evaluation of CDK18 kinase activity, a modified protocol from (188) was used. In brief, HEK293 cells were transfected with CDK18- and Cyclin A2-encoding plasmids, and after 48h the overexpressed proteins were purified via

immunoprecipitation for 1 h at 4 °C using anti FLAG coupled magnetic beads (Sigma-Aldrich). In parallel, the indicated peptides were peptide-spotted on a nitrocellulose membrane as described (67,223-225). The CDK18 loaded beads were washed and incubated for 1 h at 30 °C on single spots, in KAIB. The spots were washed three times in TBS containing 0,02 % Tween to remove the beads, then incubated with primary antibody overnight (anti Phospho-Ser CDKs Substrate, rabbit #2324 CST). Standard Western Blotting procedure followed up to the detection and quantification.

In alternative, the CDK18-loaded beads were mixed with the AQP2 eluate obtained as described in the immunoprecipitation section, diluted in KAIB, and the CDK18-loaded beads were removed after 1 h at 30 °C. The preparate was then analysed by standard Western Blot.

For kinase assay on peptides, aliquots of 50 µg of AQP2-derived peptide were incubated with one immunoprecipitation each in KAIB for 1 h at 30 °C. The supernatants were then analysed by standard Western Blot.

Peptide spot synthesis and overlay with recombinant GSK3β

With the technical help of Dr. Zülke, 25mer peptides were spot-synthesized as described using an Intavis ResPep SL spot synthesizer (Intavis, Cologne, Germany) and incubated with a recombinant fusion protein of GSK3β and glutathione S transferase (GST; Cell Signaling; 1 µg/ml), or GST alone (67,223-225).

Luciferase Assays

HEK293 cells were transfected with plasmids encoding the GSKIP variants together with pRL-SV40 and TOPflash or FOPflash vectors (29) and stimulated with recombinant human Wnt3a (R&D Systems, Minneapolis, USA) or Wnt3a-conditioned medium 24 h after transfection (226). The cells were lysed 24 h later and luciferase activity was measured using the Dual Luciferase Assay Kit (Promega) and a Centro XS3 LB 960 luminometer (Berthold technologies, Bad Wildbad, Germany). Luciferase activity is induced by binding of β-catenin to the reporter system. Activity of the TOPflash and FOPflash vectors was normalized to activity of the pRL-SV40 vector and normalized TOPflash activity was divided by the normalized FOPflash activity to obtain a TOPflash/FOPflash ratio. For siRNA experiments, 24 h after knockdown of GSKIP the cells were transfected a second time with the reporter vectors. After additional 24 h the cells were stimulated for further 24 h with Wnt3a-conditioned medium.

Statistical analysis

GraphPad Prism 7.0 (GraphPad Software) was used to perform statistical analyses, and results indicated as: * $p<0,05$; ** $p<0,01$; *** $p<0,001$; **** $p<0,0001$. ANOVA statistical testing was used to analyse experiments containing more than two groups, while Student's t test was used to compare experiments containing only two conditions.

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Relevant publications

Dema *et al.*, The AKAP GSKIP regulates beta-catenin through both its interactions with PKA and GSK3beta, August 2016, J Biol Chem, 10.1074/jbc.M116.738047

Dema *et al.*, Pharmacological targeting of AKAP-directed compartmentalized cAMP signalling (Review) December 2015, J Cell Sign, 10.1016/j.cellsig.2015.09.008

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