An Exploration of the Structure and Function of Calcium/calmodulin-dependent kinase kinase 2 (CaMKK2) in Prostate Cancer

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Thesis for the degree of *Philosophiae Doctor*

by

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ABSTRACT

Previous work on the calcium/calmodulin-dependent kinase kinase 2 (CaMKK2) has focussed, in particular, on the contribution of CaMKK2 as an androgen receptor target gene to the development and progression of prostate cancer. Despite the very significant volume of research on the functional contribution of CaMKK2 to disease there has been limited progress in understanding the structure and biophysical properties of the enzyme. To advance drug development it is vital to make progress in these latter areas.

The work presented in this thesis focused on structural and functional studies of CaMKK2 in prostate cancer. Firstly, we set out to improve the expression and purification of CaMKK2 in complex with calmodulin with the intention to crystallise the complex. Since our crystallisation attempts were unsuccessful we decided to pursue other avenues, and instead successfully established a new biophysical assay based on the interaction between CaMKK2 and the CaMKK2 inhibitor, STO-609. In a parallel cellular approach we studied the CaMKK2 interactome using co-immunoprecipitation and mass spectrometry to identify novel interacting partners of the kinase. Furthermore, we went on to evaluate the contribution of CaMKK2 to autophagy using functional assays.

In summary, we report a detailed co-expression and co-purification protocol for the CaMKK2:calmodulin complex, that has led to the development of a novel *in vitro* binding assay for CaMKK2 by exploiting the intrinsic fluorescence of STO-609. Novel phosphorylation sites in the activation segment of the kinase were detected. Furthermore, we identified a direct interaction between CaMKK2 and Gemin4, a subunit of the multiprotein SMN complex, and discovered that CaMKK2 does not regulate autophagy in prostate cancer cells, whilst STO-609 might enhance autophagy.

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ACKNOWLEDGEMENTS

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Figure A. Acknowledgments in pictures. A collection of images of the people who joined and supported me during my PhD, and some events that marked the journey. All the pictures were taken between November 2010 and August 2016; the collection is not intended to be exhaustive.

LIST OF PUBLICATIONS

I. Data for the co-expression and purification of human recombinant CaMKK2 in complex with calmodulin in *Escherichia coli*

Gerner L, Munack S, Temmerman K, Lawrence-Dörner AM, Besir H, Wilmanns M,. Jensen JK, Thiede B, Mills IG, Morth JP; Data in Brief, Volume 8, September 2016, Pages 733-740, ISSN 2352-3409, http://dx.doi.org/10.1016/j.dib.2016.06.033

II. Using the fluorescent properties of STO-609 as a tool to assist structure-function analyses of recombinant CaMKK2

Gerner L, Munack S, Temmerman K, Lawrence-Dörner AM, Besir H, Wilmanns M,. Jensen JK, Thiede B, Mills IG, Morth JP; Biochemical and Biophysical Research Communications 2016 Jul 22;476(2):102-7. doi: 10.1016/j.bbrc.2016.05.045. Epub 2016 May 11; PMID: 27178209

III. CaMKK2 is a novel interacting partner of Gemin4

Gerner L, Stewart LM, Köhler C, Thiede B, Mills IG; Manuscript in preparation

IV. CaMKK2 does not function as a central regulator of autophagy in prostate cancer cell

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ADDITIONAL PUBLICATIONS NOT INCLUDED IN THIS THESIS:

V. Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition)

Klionsky DJ, […] Gerner L, […] *et al.*, Autophagy. 2016 Jan 2;12(1):1-222, DOI: 10.1080/15548627.2015.1100356; PMID: 26799652

VI. A simple cargo sequestration assay for quantitative measurement of non-selective autophagy in cultured cells

Luhr M, Szalai P, Sætre F, Gerner L, Seglen PO, Engedal KN; Methods in Enzymology 2016, Book chapter (re-submitted)

ABBREVIATIONS

3-UTR	3'-untranslated region
aa	amino acid
ADME	absorption, distribution, metabolism, and excretion
ADT	androgen-deprivation therapy
AGO1	Argonaute 1
AID	autoinhibitory domain
Akt	Akt or protein kinase B
AMP	adenosine monophosphate
AMPK	5'-AMP-activated protein kinase
API	active pharmaceutical ingredient
aPK	atypical protein kinase
AR	androgen receptor
ATP	adenosine triphosphate
BBB	blood brain barrier
BPH	benign prostatic hyperplasia
BRCA	breast cancer gene
Ca^{2+}/CaM	calcium/calmodulin
CaM	calmodulin
CaMK	calcium/calmodulin-dependent kinase
CaMKK	calcium/calmodulin-dependent kinase kinase
CBD	calmodulin-binding domain
CDK	cyclin-dependent kinase
CFP	cyan fluorescent protein
C-lobe	C-terminal lobe
co-IP	co-immunoprecipitation
CQ	chloroquine
CRPC	castration-resistant prostate cancer
CT	computed tomography
DDX20	probable ATP-dependent RNA helicase; DEAD box protein 20; Gemin3
CYP17	cytochrome P17
DHT	dihydrotestosterone
DNA	deoxyribonucleic acid
DRE	digital rectal examinations
EMBL	European Molecular Biology Laboratory
ePK	eukaryotic protein kinase
ERK	extracellular signal–regulated kinase
ETS	E26 transformation-specific
FDA	food and drug administration
FRET	fluorescent resonance energy transfer
FSH	follicle-stimulating hormone
GLUT1	glucose transporter 1
GnRH	gonadotropin-releasing hormones
GSK3	glycogen synthase kinase 3
GST	glutathione S-transferase
GTP	guanosine-5'-triphosphate
HEK293	human embryonic kidney cell line
HMS	Harvard Medical School
HTS	high-throughput screening
HT-X	high-throughput crystallisation
IND	investigational new drug
KD	kinase domain

K_D	dissociation constant
kDa	kilo Dalton
K_m	Michaelis constant
LC3	microtubule-associated protein 1A/1B-light chain 3
LDH	lactate dehydrogenase
LH	luteinizing hormone
LHRH	luteinizing hormone-releasing hormones
LKB1	liver kinase B1
LLPD	long-lived protein degradation
LN	lymph node
LUTS	lower urinary tract symptoms
mCRPC	metastatic castration-resistant prostate cancer
MEK	mitogen-activated protein kinase kinase
miR-224	microRNA-224
MRI	magnetic resonance imaging
mTOR	mammalian target of rapamycin
mTORC1	mTOR complex 1
NADPH	nicotinamide adenine dinucleotide phosphate - reduced form
NCMM	Centre for Molecular Medicine Norway
NHR	nuclear hormone receptor
N-lobe	N-terminal lobe
nm	nanometre
NMR	nuclear magnetic resonance
NPC	nuclear pore complex
Nup62	nucleoporin 62
PD	pharmacodynamics
PDB	protein database
PEG	polyethylene glycol
PET	positron emission tomography
PFK	phosphofructokinase
PI3K	phosphoinositide 3-kinase
PK	pharmacokinetics
PKA	protein kinase A
PKB	protein kinase B or Akt
P-loop	phosphate-binding loop
PPP	pentose phosphate pathway
PrCa	prostate cancer
PSA	prostate-specific antigen
PTEN	phosphatase and TENsin homolog
RNA	ribonucleic acid
RP site	arginine-proline-rich site
SHBG	sex-hormone-binding globulin
siRNA	small interfering RNA
SMN	survival of motor neurons
snRNP	small nuclear ribonucleoprotein
STAMPEDE	Systemic Therapy in Advancing or Metastatic PrCa: Evaluation of Drug Efficacy
STK	serine/threonine protein kinase
TCA	tricarboxylic acid cycle
TNM	tumour-node-metastasis
TRUS	transrectal ultrasonography
TSC2	tuberous sclerosis 2
TURP	transurethral resection of the prostate
YFP	yellow fluorescent protein

1 INTRODUCTION

1.1 THE PROSTATE GLAND

The prostate gland is a male organ that is involved in a man's reproductive and urinary systems. The oval shaped prostate gland sits anterior of the rectum and distal to the bladder and its two lobes (right and left lobe) are surrounding the proximal part of the urethra that carries urine and semen out of the body (**Fig. 1A**). Between birth and adulthood, the prostate gland doubles in size through growth spurts during adolescence and young adulthood. Its size varies from man to man and can range from that of a walnut to that of a small apple, but is approximately 4 cm wide and 3 cm thick. The prostate gland is covered in a layer of connective tissue, called the prostatic capsule, and can be divided into three zones; peripheral, central, and transition zone (**Fig. 1B**). The peripheral zone is the area of the prostate that is closest to the rectum and is the largest zone of the prostate gland. The central zone is the part of the prostate furthest away from the rectum and lies in front of the transition zone. This zone is the middle area of the prostate, between the peripheral and central zones. It surrounds the urethra as it passes through the prostate. This zone makes up about 20 % of the prostate gland until the age of 40.

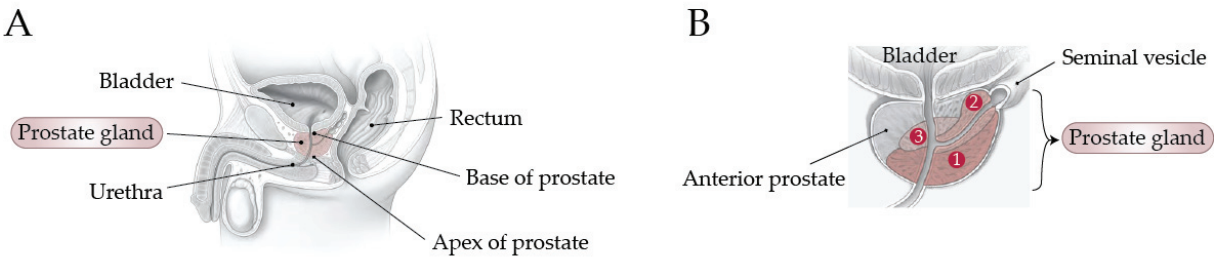

Figure 1. Prostate gland (A) Location. **(B)** Peripheral (1), central (2), and transition zone (3). Adjusted from 2016 Annual Report on Prostate Cancer, HMS [1].

The main function of the prostate is to produce the fluid portion of semen. The testicles produce sperm, but the prostate gland helps supply the semen that is so important for nourishing and protecting the sperm cells during their travels. The prostate's contribution to semen is alkaline [1] which helps the sperm survive in the acidic environment of the vagina. This fluid is made continuously and the excess passes from the body in the urine. When a man is sexually aroused, the prostate produces larger amounts of this fluid which then mixes with sperm and is ejaculated as semen. Seminal vesicles, glands that sit on either side of the prostate, also contribute secretions to the semen that is actually greater in volume compared to the prostate gland.

The prostate also plays a part in controlling the flow of urine. The urethra runs from the bladder, through the prostate, and out through the penis. The muscle fibres of the prostate are wrapped around the urethra and are under involuntary nervous system control. These fibres contract to slow and stop the flow of urine.

1.2 PROSTATE CANCER

Worldwide, prostate cancer (PrCa) is the second most common cancer in men, just behind lung cancer. In Europe, PrCa has the highest incident rates for male cancers with an average of 85.8 cases per 100,000 men (**Fig. 2A**), whereas Norway presents worldwide the second highest number with 129.7 cases per 100,000, only topped by Martinique (**Fig. 2B**) [2, 3]. However, compared to the most lethal lung cancer, the mortality rates for PrCa are only a third (**Fig. 2C**) with a death rate of 17.9 for Norway (**Fig. 2D**) and 10.7 in Europe (**Fig. 2C**). **Figure 2E** highlights the discrepancy for PrCa incidence between Norway and Western Europe, which may be explained by increased opportunistic testing for prostate specific antigen (PSA) in Norway [4] (see also **1.2.1**).

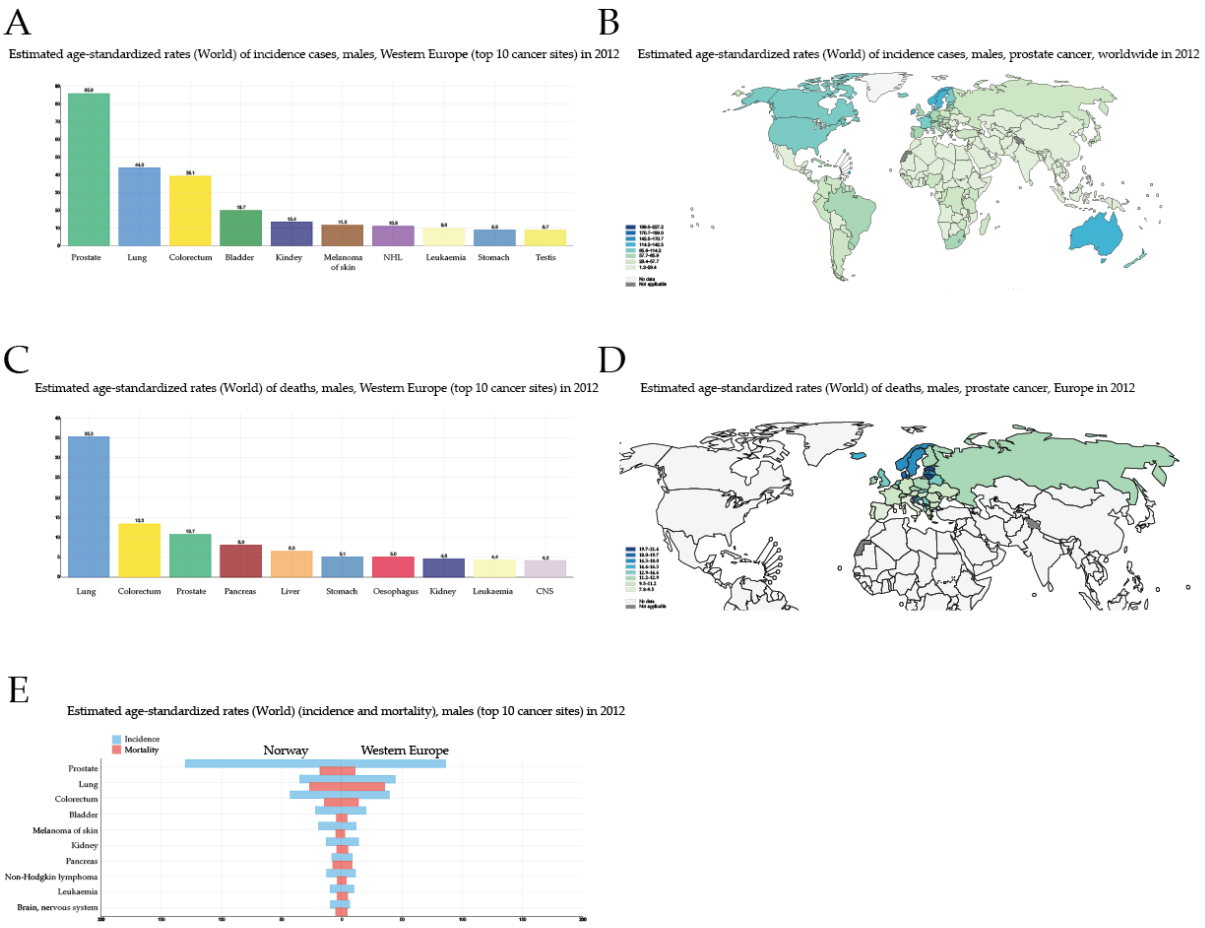

Figure 2. Graphic illustration of cancer incidence (A, B, E) and mortality rates (C, D, E). Data source: GLOBOCAN 2012, Graph production: Cancer today (http://gco.iarc.fr/today, accessed [19/07/2016]), World Health Organisation [2, 3].

What causes PrCa? As of 2015, there are more than 100 genetic variants linked to PrCa and most PrCa patients carry at least a few of these variants [5, 6]. A clear link has been made between defects in the genes breast cancer 1 and 2 (*BRCA1* and *BRCA2*) and aggressive, hard-to-treat Prostate cancers that affect younger men. Defects in these DNA repair genes have been found in about a quarter of all men who have aggressive PrCa [7, 8]. Another common gene mutation leads to a boost of sensitivity to testosterone, allowing PrCa cells to grow more aggressively [9, 10].

In addition to the causes of PrCa, there are numerous risk factors, increasing the odds of getting the disease. These include age (see below), family history (PrCa runs in families) [11-13], race (African American have a 60 % higher incidence and more than double the death rate compared to white men in the US) [14], nationality (highest rates in Australia, New Zealand, Western Europe, Canada and the US, while the lowest rates are found in Thailand and India and northern Africa [15]), diet (western diet high in red and processed meats, fat, and dairy products [16]), ejaculation frequency (frequent ejaculation is protective [17]), and other factors such as sexually transmitted diseases, prostatitis (inflammation of the prostate gland due to bacteria, viruses, urine reflux, dietary factors, or physical trauma), and vasectomy [18-20]. Whether and how prostatitis or vasectomies correlate with the development of PrCa is currently unclear, and remains a field of extensive research.

In summary, age is the greatest risk factor for PrCa. Almost all (97 %) prostate cancers occur in men above 50 years of age [21]. However, it is normal for the prostate to start growing again when men are in their late 40s and 50s. This natural enlargement, which mainly happens in the transition zone until it becomes the largest area in the prostate, is called benign prostatic hyperplasia (BPH). Whereas 50-60 % of men with PBH may never develop

symptoms, many men become first aware of their prostate once it puts pressure on the urethra or bladder due to this enlargement, which can cause lower urinary tract symptoms (LUTS) [1]. Why BPH occurs is still not exactly known. Hormonal changes due to age, abdominal obesity, diabetes, lack of physical activity, race and diet seem all to be risk factors for BPH. It is noteworthy that BPH is a benign condition that does not cause PrCa, even though it often coexists with it. Even though many men with PrCa never experience any symptoms, the enlargement of the prostate gland in both BPH and PrCa can cause similar symptoms.

1.2.1 Diagnosis and staging of prostate cancer

Digital rectal examination (DRE) is a physical examination of the prostate gland. The peripheral zone can easily be felt through the rectal wall in which the majority of prostate tumours (approximately 75 %) are located [1]. However, as only parts of the gland can be examined, and small tumours easily been overlooked, a blood test for PSA is often combined with DRE. The PSA tests have led to the discovery of cancers at earlier, and therefore more treatable stages. This has led to overdiagnosis, the detection of slow growing (indolent) cancers that would not have been diagnosed in the patient's lifetime in the absence of screening, and this accounts to 23 to 42 % of screen-detected prostate cancers [22]. PSA testing gives also many false positive results as PSA levels, since levels can be raised for many other reasons (including BPH and prostatitis). This not only psychologically alarms 'patients' but in addition often leads to further diagnostic examinations such as prostate biopsies which can cause pain, ongoing bleeding problems, infections, and erectile dysfunction [23, 24]. Prostate biopsies are routinely used as a next step triggered by elevated

PSA levels or abnormal DRE. Biopsies are often guided by imaging techniques, such as transrectal ultrasonography (TRUS) or magnetic resonance imaging (MRI). An alternative to the most common and standard transrectal biopsy is the transperineal biopsy, which has a lower risk for infections and does better at detecting high-grade cancers [1]. If a pathologist confirms cancer in the biopsied tissue, further examinations will follow in order to precisely determine the location and size of the tumour, but also if the cancer has spread to other parts of the body. TRUS, MRI, positron emission tomography (PET) scans, computed tomography (CT) can be used to evaluate the spread of the malignant cells and determine the stage of the disease.

There are different ways to stage PrCa, but the most widely used one is the tumour-node-metastasis (TNM) system [25], see **Table 1** and **Figure 3**. The prognosis of PrCa patients depends on whether the tumour is still confined to the prostate. If so, the five-year survival rate is nearly 100 %, whereas metastases to distant lymph nodes, bones, or other organs give substantially lower survival rates. A recent meta-study combined patient data of nine phase III clinical trials in which the median overall survival in men with metastatic castration-resistant PrCa (mCRPC) is associated with specific sites of metastases. Dependent on the site of metastases (lymph node (LN) only, bone with or without LN (with no visceral metastases), any lung metastases (but no liver), and any liver metastases) the median survival is 19 months for lung, 14 months for liver, 21 months for bone, and 32 months for lymph node metastases [26].

Table 1. TNM staging system of PrCa. It describes the extent of the primary tumour (T category), whether the cancer has spread to nearby lymph nodes (N category), and whether it has spread to distant sites (M category) [25]. Illustration of the stages are presented in **Fig. 3**.

TX		Tumour stage could not be assessed
T0		No evidence of primary tumour
T1		Tumour cannot be felt by DRE or detected by TRUS
	T1a	Cancer was incidentally found during transurethral resection of the prostate (TURP) for BPH and represented less than 5% of the resected tissue and is usually of low grade
	T1b	Cancer was incidentally found during TURP procedure and represented more than 5% of the resected tissue or is of high-grade
	T1c (not shown)	Cancer was found by needle biopsy, which was performed due to elevated PSA levels
T2		Tumour can be felt by DRE and is confined to the prostate
	T2a	Tumour involves a maximum of 50% of a single lobe
	T2b	Tumour involves more than 50% of a single lobe but not the other
	T2c (not shown)	Tumour involves both lobes
T3		Tumour extends through the prostate capsule
	T3a	Tumour extends outside the prostate but does not involve the seminal vesicle
	T3b	Tumour extends to the seminal vesicle
T4		Tumour is fixed or invades adjacent structures, such as external sphincter, rectum, bladder, levator muscles or pelvic wall

NX		Nearby lymph nodes were not assessed
N0		Tumour has not spread to nearby lymph nodes
N1		Tumour has spread to nearby lymph nodes

M0		No distant metastases could be detected
M1		Distant metastases are present
	M1a	Metastases in non-regional lymph nodes
	M1b	Bone metastases
	M1c	Metastases in other sites, such as lungs, with or without bone involvement

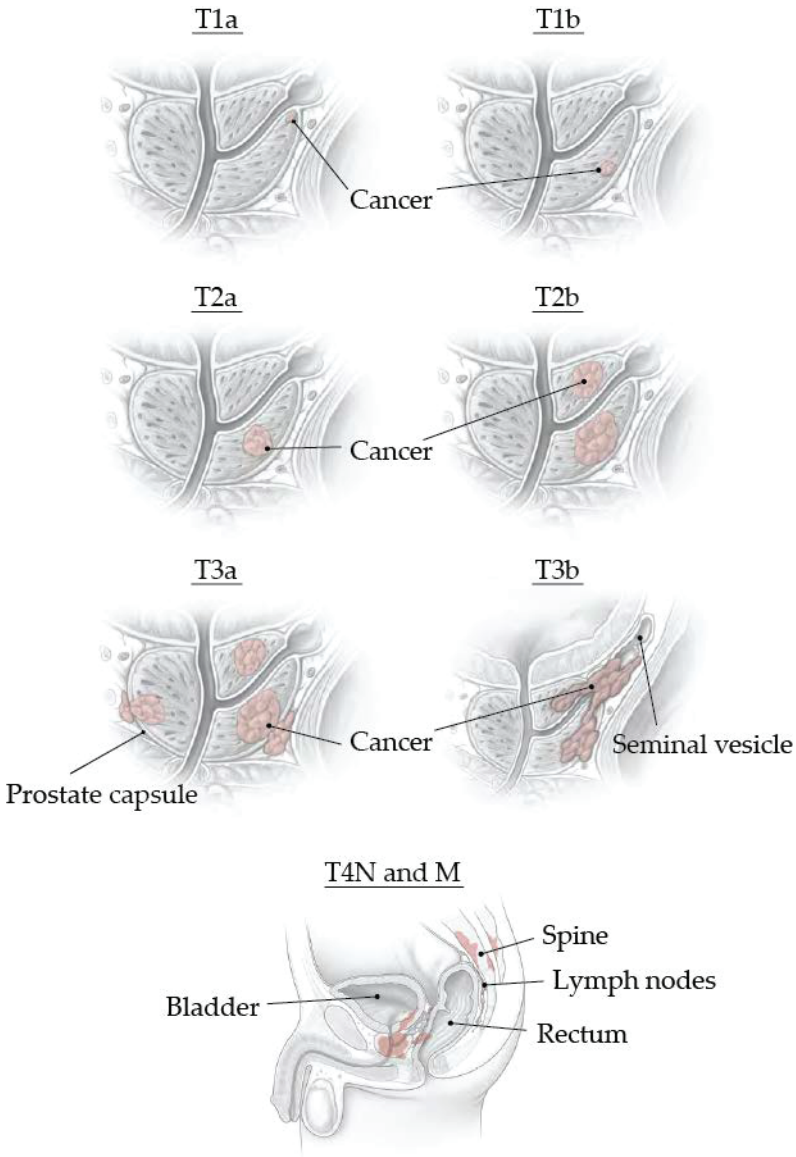

Figure 3. TNM staging system of PrCa - illustrated. Compare with **Table 1.** Adjusted from 2016 Annual Report on Prostate Cancer, HMS [1].

To assess the behaviour of prostate cancer in terms of aggressiveness and potential to spread, a histological evaluation of the biopsied tissue is used. The two most prevalent cell types in the biopsy specimen are assessed and get assigned grades from 1 to 5, based on differentiation (**Fig. 4**).

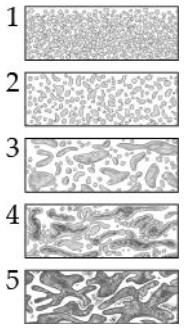

Figure 4. Gleason grades. Cells with grade **1** or **2** are well differentiated. In **1**, cells are small, of fairly uniform shape, and tightly packed together. In **2**, cells display more varied and irregular shapes and are loosely packed. Cells with grade **3** are moderately differentiated, are even more irregular in size and shape and are more dispersed. Some cells are fused and cell borders less distinct. Cells grading **4** and **5** are poorly differentiated. In grade **4**, many cells are fused into irregular masses. Some cells have started to invade the connective tissue that separates cells. In **5**, most of the tumour consists of irregular masses that have invaded connective tissue. Adjusted from 2016 Annual Report on Prostate Cancer, HMS [1].

Combined, the two Gleason grades yield in the Gleason score, ranging practically from 2 to 10. However, pathologists hardly ever use grade 1 and 2 and therefore a Gleason score of 6 only describes a low-risk form of PrCa [1].

Only recently, pathologists proposed a new system to rank PrCa tumours, ranging from grade 1, the least dangerous, to 5, the highest-grade cancer [27] (**Table 2**). This system is also based on histological assessment, but the grade stratification is more accurate, and is less susceptible to over-treatment.

Table 2. Tumour ranking according to: A Contemporary PrCa Grading System: A Validated Alternative to the Gleason Score [27].

Grade group 1 (Gleason score 3 + 3 = 6)	Only individual discrete well-formed glands
Grade group 2 (Gleason score 3 + 4 = 7)	Predominantly well-formed glands with lesser component of poorly formed/fused/cribriform glands
Grade group 3 (Gleason score 4 + 3 = 7)	Predominantly poorly formed/fused/cribriform glands with lesser component of well-formed glands *
Grade group 4 (Gleason score 8)	- Only poorly formed/fused/cibriform glands *or*
	- Predominantly well-formed glands and lesser component lacking glands **
	- Predominantly lacking glands and lesser component of well-formed glands **
Grade group 5 (Gleason scores 9–10)	Lack of gland formation (or with necrosis) with or without poorly formed/fused/cribriform glands *

* For cases with >95% poorly formed/fused/cribriform glands or lack of glands on a core or at radical prostatectomy, the component of <5% well-formed glands is not factored into the grade.

** Poorly formed/fused/cribriform glands can be a more minor component

.

1.2.2 Treatment of prostate cancer

The decision making process regarding PrCa treatment is qualitative. It is not only dependent on the stage of the cancer, but also on the man's age, lifestyle, risk of side effects, other diseases, and anxiety levels. Case-by-case decisions need to be made.

Some men diagnosed with PrCa do not need any treatment. Approximately 40 % of men diagnosed with PrCa have such small and slow-growing tumours that they do not meet the criteria for active treatment. Many prostate cancers can take 15 to 20 years or more to grow, and cause little harm. Especially in elderly men above 75, it is more likely that they die of other conditions before the PrCa becomes dangerous. But even for younger men, the best

therapeutic option is often to be vigilant and 'active surveillance' is now the norm [28-31]. This strategy involves monitoring the cancer closely (regular DREs, periodic PSA tests, biopsies every one to three years) and only undergoing treatment once the cancer progresses or shows evidence of increasing activity.

On the other side are younger men, those aged 50-60, with an aggressive form of PrCa in the early stages and potentially a family history of PrCa. Black men show a higher risk for faster progression from low-grade cancer to more serious forms [14, 32]. For all those, treatment of the PrCa is required, but also men with slow-growing cancer might prefer treatment instead of waiting and worrying.

The initial treatment options for PrCa are surgery and radiotherapy, whereas hormonal and chemotherapy are second-line treatments, usually upon biochemical recurrence. Complication rates in all cases remain high despite tremendous efforts during the last decades. Depending on the therapy, sexual complications, long-term urinary incontinence, impotence, and recently an increased risk of Alzheimer [33] are associated with treatment.

Radical prostatectomy has long been the standard surgical treatment for PrCa and describes the surgical removal of the prostate gland and any surrounding tissue that might be affected. In most cases that includes beside the prostate gland also seminal vesicles, slender saclike glands, and pelvic lymph nodes. Traditionally that has been performed as a radical retropubic prostatectomy, but has been more and more replaced in the last decade by laparoscopic surgery assisted by robotics. Regardless of the method used, the most experienced surgeons achieve the best outcomes [34, 35]. Most common complications involve urinary incontinence and erectile dysfunction, which account for a great percentage of all men undergoing surgery.

Focal therapy, also called targeted therapy, describes the removal of only the cancerous mass while sparing the surrounding tissue [36, 37]. The biggest benefits are less side-effects, such as erectile dysfunction and urinary incontinence, but studies with long-term follow up are missing. As this therapy is only recommended for small, localised tumours, it is especially important to balance against active surveillance.

Radiotherapy is a reasonable alternative to surgery for early-stage PrCa. Two methods are used to target the cancer: external beam of radiation or surgically implanting small radioactive pellets, or 'seeds' into the prostate gland, which is called brachytherapy. Permanent brachytherapy, in which the seeds stay in the prostate and emit less and less radiation over time, is usually recommend for low-risk cancers, whereas more advanced cancers are treated with either high-dose-rate brachytherapy, in which the high intensity seeds are inserted in the prostate but removed several days later, or more standardly with external beam radiation combined with hormonal therapy (see below). Radiotherapy can be used instead of surgery, combined with surgery, or for recurrent cancer after prostatectomy, depending on the PrCa stage, general health of the patient and age [38-40]. The most common side effects of radiotherapy are bowel problems.

Whereas early PrCa with localised tumours confined to the prostate (stages T1 and T2) are usually treated with surgery and/or radiotherapy as described above, the cancer cannot be cured once it has spread beyond the prostate. The five-year survival rate for distant cancers is only 28 % compared to almost 100 % for localised PrCa [41]. In advanced PrCa, parts of the tumour metastasize through the blood and lymph to distant parts of the body, including lymph nodes and bones. In addition to standard therapies, these patients receive hormonal and chemotherapy. Hormonal therapy includes luteinizing hormone-releasing hormones

(LHRH) agonists, gonadotropin-releasing hormones (GnRH) antagonists and anti-androgens. Together, these treatments are collectively called androgen-deprivation therapy, as they all aim on reducing testosterone and other androgen levels and thereby slow growths of PrCa by suppressing the growth supporting androgens (**Fig. 5**). LHRH agonists inhibit the production of luteinizing hormone (LH) in the pituitary gland. Because LH stimulates testosterone secretion in the testicles, inhibiting it decreases testosterone levels. GnRH antagonists work similar to LHRH agonists, blocking the release of LH and thereby lowering testosterone levels. Testosterone is secreted primarily by the testes, but is also formed by peripheral conversion of adrenal steroids. It is the main circulating androgen in the blood, where it is found bound to albumin and sex-hormone-binding globulin (SHBG), with a small freely dissolved serum fraction. When free testosterone enters prostate cells, the majority is converted to dihydrotestosterone (DHT), a more active hormone with a fivefold higher affinity for the androgen receptor (AR) than testosterone [42]. Activation of the AR leads to transcription of many prostate growth supporting genes, and thereby boosting cancer cell growth if they are present. Anti-androgens, such as bicalutamide (Casodex), work by preventing the interaction of DHT with the AR, and thereby suppressing PrCa growth [42, 43].

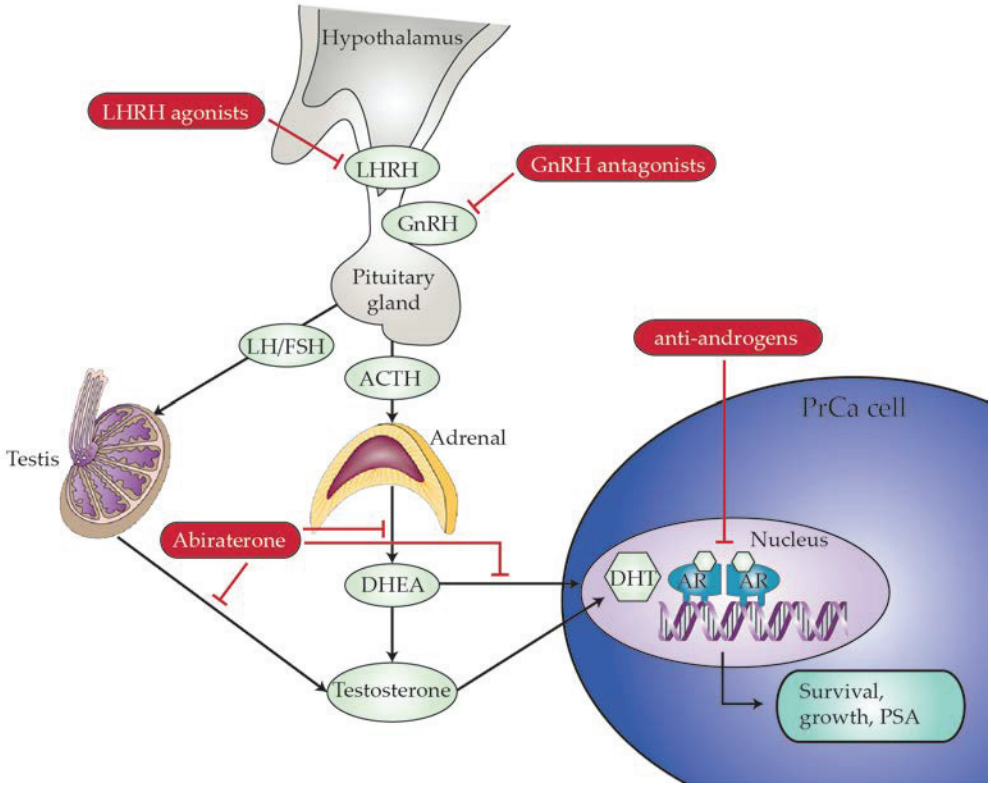

Figure 5. Androgen synthesis and signalling pathways: drugs used for androgen deprivation therapy and to block androgen action. The hypothalamic-pituitary-testicular axis controls testicular androgen synthesis via the actions of LH on the testes. Furthermore, the hypothalamic-pituitary-adrenal axis controls androgen precursor synthesis via the actions of ACTH on the adrenals. Once synthesized, the androgens testosterone or DHT then bind to the AR in the prostate; activation of the AR regulates expression of androgen-dependent target genes that include the control of prostate survival, growth and PSA secretion. Drugs interfering with this mechanism are clinically used in the treatment of PrCa patients. These drugs include GnRH antagonists and LHRH agonists, both targeting the LH production/secretion, and anti-androgens preventing DHT-binding to the AR. Abbreviations: ACTH, adrenocorticotropic hormone; AR, androgen receptor; DHEA, dehydroepiandrosterone; DHT, dihydrotestosterone; FSH, follicle-stimulating hormone; GnRH, Gonadotropin-releasing hormone; LH, luteinizing hormone; LHRH, luteinizing hormone-releasing hormone; PSA, prostate-specific antigen. Illustration redrawn and adjusted from [44].

If PrCa is responsive to that treatment option, it is called castration-sensitive, but tumours can become resistant. PrCa actually becomes hypersensitive to the stimulatory effects of even extremely low levels of androgens and exhibits persistent signalling-mediated growth through the AR [45]. The treatment options for such advanced, castration-resistant prostate cancers (CRPC) are second-line hormonal therapy or chemotherapies. The newest types of hormonal therapy include Abiraterone and Enzalutamide. Abiraterone is a medication

approved for advanced PrCa and it works by inhibiting cytochrome P17 (CYP17), an enzyme essential to testosterone synthesis [46]. Enzalutamide prevents the binding of AR to the DNA, but also co-activators binding to the AR [47].

For PrCa patients, stage T3 and T4, combined hormonal/radiation therapy is now standard. But hormonal therapy may also be combined with chemotherapy for patients with high-extent metastatic PrCa. As PrCa is typically slow-growing, chemotherapy is rarely used to treat early-stage PrCa. However, the UK-led STAMPEDE (Systemic Therapy in Advancing or Metastatic PrCa: Evaluation of Drug Efficacy) trial reported recently that adding Docetaxel chemotherapy (Docetaxel stabilizes microtubules leading to apoptotic cell death) to standard hormonal therapy improved survival for men with newly diagnosed locally advanced, non-metastatic PrCa not previously treated with hormone therapy (hormone-naïve) [48]. Prostate cancers resistant to the already discussed treatments will be treated with chemotherapy. Metastatic advanced PrCa spreads in 70 % to the bones causes the majority of the morbidity and mortality associated with PrCa [49]. Radium-223 (Xofigo) is an approved drug targeting PrCa cells located in the bone tissue by high-energy alpha radiation [50]. The alpha particles travel less than 100 μm, enough to destroy cancer cells but limiting effect on nearby bone marrow. Non-responding prostate cancers can also be treated by immunotherapy. However, the only approved cancer vaccine, sipuleucel-T (Provenge) is costly, and extends life by only a few months, and another one, Prostvac-VF, is currently in clinical trials but has not been approved yet [51].

As there is a lack of effective treatment for recurrent, advanced-stage and metastatic prostate cancers, new therapeutic modalities are currently tested in clinical trials. Oncolytic virotherapy is such a treatment in which replication-competent viruses are used that

specifically infect, replicate in and lyse malignant tumour cells, while minimizing harm to normal cells [52]. Between 2001 and 2014, there have been four trials investigating adenovirus-based [53-56] and two trials investigating reovirus-based [57, 58] treatments in men with PrCa. All trials have promising outcomes with reduced PSA levels and/or reduction in biopsy positivity. However, after assuring the low toxicity of the viral treatment it becomes more important now to enhance the efficiency of the oncolytic viruses. One strategy are the so-called armed oncolytic viruses that express regulatory factors for critical check-points involved in tumour-induced immune suppression which is especially high in PrCa. By intratumoural viral infection this might prime the tumour microenvironment for a synergy between viral oncolysis and immune-mediated tumour killing [52].

In summary, once PrCa has developed into CRPC, the disease becomes fatal and the average survival is less than two years [59]. Although a variety of drugs have been approved in recent years which might be beneficial for specific cases, no curative treatment for CRPC is currently available. This underlines the relevance of finding new treatments for CRPC, and only supports the need for further research on a molecular level.

1.3 MOLECULAR ASPECTS OF PROSTATE CANCER

1.3.1 Metabolic dysregulation in cancer cells

The hormone-dependency of PrCa has long been a focus of research. While targeting hormone synthesis and associated signalling pathways has improved treatment options for PrCa significantly, CRPC remains poorly understood and is still a fatal disease. On a molecular level, intensive research in the last decades has been highlighting certain mechanisms essential for cancer progression.

Metabolic changes are a well-described hallmark of cancer and as PrCa is often a late-onset disease it has been potentially associated with the metabolic syndrome (increase in body mass index, triglycerides, blood pressure). In Nordic cohorts which are genetically uniform, the metabolic syndrome has been associated with an increased risk of PrCa [60], whereas the association in other studies varied or failed [61-65]. Notably, a link between metabolic syndrome and PrCa is more often found in European populations and especially with more aggressive cancers. This link also suggests that certain drugs targeting metabolic diseases might be preventive for PrCa. The diabetes drugs insulin and metformin have both been associated with a change in susceptibility towards PrCa. Insulin makes patients more susceptible to PrCa, whereas metformin lowers the risk. Statins which are used to prevent cardiovascular diseases and lower blood cholesterol levels have also been associated with lower risk of developing PrCa [66-68]. While the primary risk factors of PrCa have been identified, the specific molecular mechanisms remain obscure [69].

However, to adjust to the uncontrolled cell growth and rapid proliferation, cancer cells must change their metabolic regulation to provide macromolecules and energy to fuel cancer cell growth and division. In the 1920's Otto Warburg was the first to observe that cancer cell metabolism differs dramatically from normal cells [70]. The so-called Warburg effect describes the 'aerobic glycolysis' by which the cancer cells, even in the presence of oxygen, dramatically increase their glycolysis to generate lactate in the cytosol for a limited adenosine triphosphate (ATP) production (two molecules ATP per molecule glucose) and an increased macromolecule production, thereby shuttling glucose-6-phosphate into the pentose phosphate pathway (PPP) for NADPH exchange allowing ribose-5-phosphate to increase nucleotide synthesis which is essential for the high proliferative rate of cancer cells (**Fig. 6**). In normal cells, glucose is usually converted via glycolysis into pyruvate, which is then transported to the mitochondria to undergo oxidative phosphorylation through the tricarboxylic acid cycle (TCA), yielding 36 molecules ATP per molecule glucose. This makes glycolysis 18 times less efficient than mitochondrial oxidative phosphorylation in terms of ATP production which initially seems counterintuitive for rapidly dividing cells. However, increased glycolysis is required to generate intermediates as building blocks for biosynthetic pathways that are essential for the rapidly dividing cancer cells. Pyruvate that enters the TCA is mainly utilized for biosynthetic pathways rather than being converted into lactate. Citrate is exported from the mitochondria to the cytosol for lipid synthesis due to a truncated TCA cycle, which is a hallmark of cancer. Other intermediates provide substrates for protein and nucleotide synthesis [71]. In addition, cancer cells drastically increase their glucose uptake by overexpressing the glucose transporter 1 (GLUT1). Glucose and glutamine are abundant nutrients absolutely required for cell growth and survival [72]. Glutamine is a

major source for energy and nitrogen for biosynthesis, and a carbon substrate for anabolic processes in cancer [73]. A meta-analysis of clinical gene expression data reveals that differential expression of genes linked to metabolism are a hallmark of localised PrCa whereas differential expression of cell cycle-associated genes become significant in late-stage/metastatic disease [74, 75].

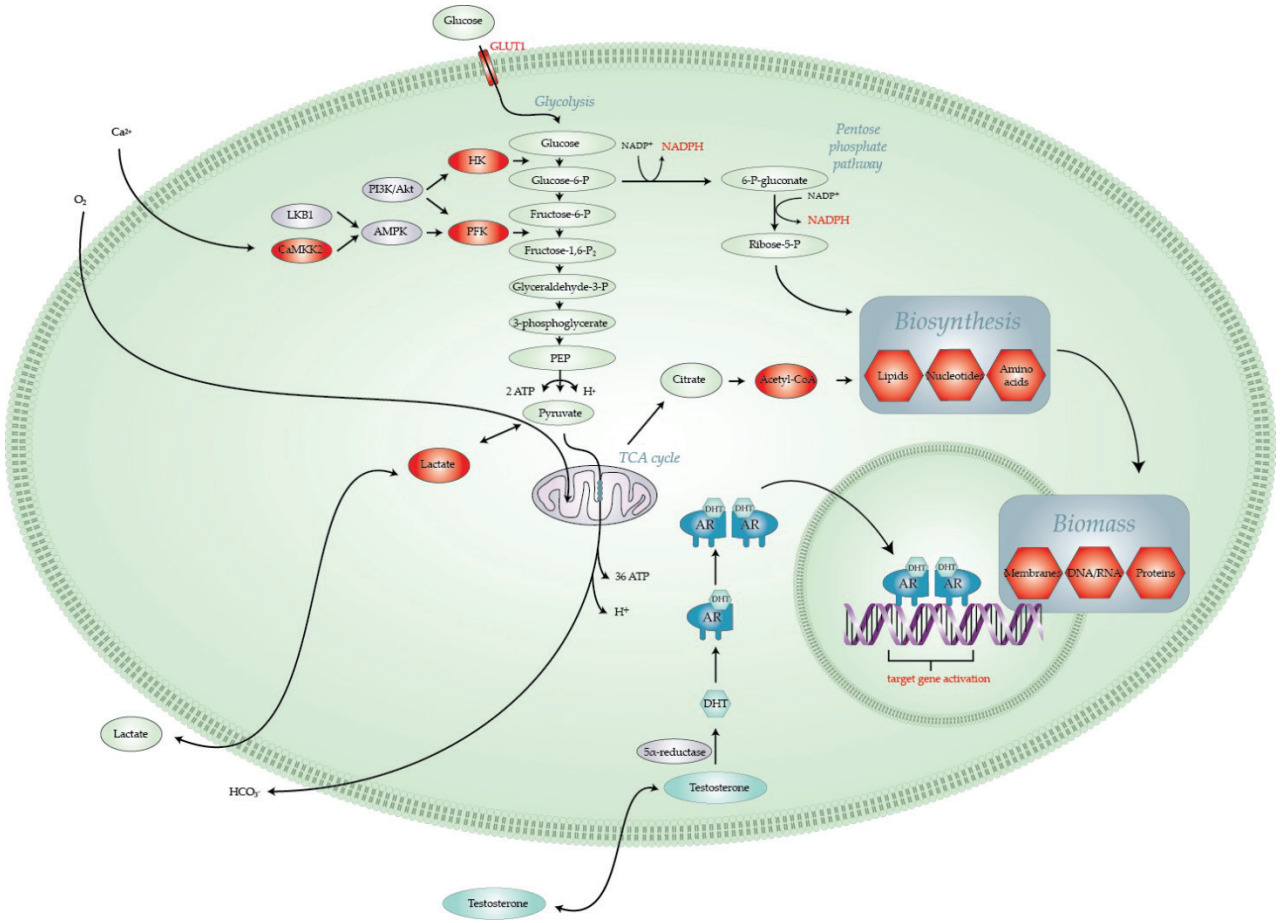

Figure 6. Cell metabolism in cancer. In normal cells, energy production is usually obtained via glycolysis and oxidative phosphorylation in the mitochondria through the TCA cycle, yielding 36 ATP per molecule glucose. Under oxygen starvation, cells switch to anaerobic glycolysis. Cancer cells however, undergo even in the presence of oxygen aerobic glycolysis, thereby downregulating ATP production but drastically enhancing biosynthesis which is so important for the increased proliferation rate of cancer cells. The AR regulates this process by upregulation of various genes important in these pathways (direct AR target genes in red) and thereby fuels PrCa cell growth by regulating metabolism (see also **1.3.4**).

1.3.2 Metabolic reprogramming in prostate cancer cells

In PrCa, metabolic reprogramming is fairly distinct compared to most cancers. Nonmalignant prostatic epithelial cells accumulate high levels of intramitochondrial zinc. The high zinc levels lead to a truncated TCA cycle and citrate accumulates. The surplus citrate is then secreted into the prostatic fluid where it plays an important role in maintaining sperm viability through calcium chelation and as an energy source [76]. The impaired efficiency of the TCA cycle in untransformed cells is predicted to mean that basal energy metabolism in the prostate gland is more glycolytic, or Warburg-like, than in other tissues [77]. Upon malignant transformation of prostate cells, intracellular zinc levels drop by ~ 66 %, partially due to downregulated zinc transporters [71]. This leads to the recovery of the functional TCA cycle and increased ATP production, important to provide energy for accelerated proliferation [78]. Presumably, this 'TCA cycle release' also helps to restore downstream intermediates that can again be conveyed for more biosynthetic reactions. Accordingly, this altered metabolism appears to be a key step in the tumour transformation, since the intracellular concentration of zinc is inversely correlated with the aggressiveness of PrCa [79]. Consequently, there are increased citrate oxidation and oxidative phosphorylation in PrCa cells, whereas aerobic glycolysis may have a less significant role [71].

1.3.3 Oncogenic regulators of metabolism

Several oncogenic pathways are associated with the metabolic dysregulation in cancer and PrCa in particular. The phosphoinositide 3-kinase (PI3K) pathway is one of the most commonly altered pathways in human cancer cells. Growth factors and mutations in the

tumour suppressor gene Phosphatase and TENsin homolog (PTEN), which loss of expression is observed in up to 40 % of PrCa patients and associated with a shorter survival of patients [80], can induce the activation of the PI3K pathway and its main effector Akt, also known as protein kinase B (PKB), that regulates glycolysis and plays a major role in the regulation of the cell's energy balance [81, 82]. Akt increases the expression and translocation of glucose transporters, and thereby stimulates glycolysis. Furthermore, Akt indirectly activates phosphofructokinase-1 (PFK1) by phosphorylating phosphofructokinase-2 (PFK2) which leads to fructose-2,6-biphosphate production which in turn is a powerful activator of PFK1 [83, 84]. Akt is in addition also a strong activator of the mechanistic target of Rapamycin (mTOR) by phosphorylating and inhibiting tuberous sclerosis 2 (TSC2), a negative regulator of mTOR. Activated Akt strongly stimulates the protein complex mTOR complex 1 (mTORC1) which positively regulates protein, lipid and nucleotide synthesis under sufficient nutrient and energy conditions. mTORC1 is known to be a strong antiapoptotic and prosurvival signal.

However, under poor cellular nutrient supply there is an increase in the AMP/ATP ratio which induces activation of AMP-activated protein kinase (AMPK) [85]. AMPK is the master regulator of energy homeostasis. As the main cellular energy sensor, AMPK works via phosphorylation of TSC2, the negative regulator of mTOR, which inhibits mTORC1. This inhibition slows down cellular metabolism in order to avoid anabolic reactions and cell death. AMPK can be activated via liver kinase B1 (LKB1, also known as serine/threonine kinase 11 (STK11)) in response to energy stress or independently via the calcium/calmodulin-dependent kinase kinase 2 (CaMKK2) in response to calcium flux [86-90]. This pathway is however often compromised in cancer cells, by either LKB1 mutations and/or TSC2

truncations (AR-induced isoform switch towards TSC2A) and cancer can progress with anabolic metabolism regardless of energy stress levels [91, 92].

Other oncogenic regulators in metabolism include ETS transcription factors [93], forkhead transcription factors [94-96], homeobox-containing proteins [97], and c-Myc [98], all cofactors or interactors which influence the AR-mediated transcriptional control in PrCa and CRPC [99].

1.3.4 The AR as the driving force of prostate cancer

The AR is the main therapeutical target in PrCa. However, androgens and the AR are both required for the normal development of the prostate gland and maintenance of its secretory functions [100]. Androgen binding is thought to promote dimerization of the transcription factor AR and its translocation to the nucleus where it then regulates AR-target genes (**Fig. 5**). The AR is known to be expressed in many other cell types in the body, but its expression is particularly high in the luminal epithelial cells of the prostate gland [101]. These are the specialised secretory cells that release prostate-specific proteins, including PSA. The AR regulates gene expression of metabolic enzymes and secreted proteins associated with glucose consumption, lipid turnover, and biomass production in untransformed prostate cells and at least in localised PrCa; although in the healthy prostate rather for secretion than tumorigenesis [74, 102-104].

Progression of PrCa to CRPC comes from an imposition of selection pressures on PrCa cells to maintain AR activity [99]. In order to preserve AR activity, PrCa cells adapt in various ways, for example AR copy number amplification, AR overexpression, expression of splice variants of AR lacking an intact ligand-binding domain for hormone binding, and selection

for somatic mutations that sustain AR activity by converting antagonistic drug responses to agonist responses [9, 99, 105, 106].

Overall, the AR displays a conserved and important role at all stages of the prostate development and throughout cancer progression by influencing the metabolism and biosynthesis at key regulatory steps. This has been especially highlighted by a study in 2011 in which Massie *et al.* have analysed combined direct AR targets and androgen-regulated genes and revealed significant enrichment of metabolic targets. In comprehensive metabolomics profiling, they assessed the effects of AR signalling and found increased glucose uptake, increased lactate production in normoxia, increased citrate levels while unchanged levels of TCA cycle metabolites and increased anabolic flux, all highlighting the AR as a stimulator of energy production by upregulating these rate-limiting steps in glycolysis [103].

1.4 CaMKK2 AND ITS ASSOCIATION WITH PROSTATE CANCER

In the previous described study by Massie *et al.*, CaMKK2 has been identified as a direct AR target gene in both androgen-dependent and CRPC [103] and to be overexpressed at the RNA and protein level [103, 107-109]. Massie *et al.* have also shown that inhibiting CaMKK2 using a small molecule inhibitor, STO-609, which targets the ATP binding pocket [110] reduces cell proliferation in AR-positive cell-lines, reduces tumour burden in a xenograft model of PrCa and sensitises cells to the apoptotic effects of another metabolic inhibitor that is used to treat diabetes called metformin. It has been shown that androgen stimulates CaMKK2 expression and nuclear translocation and that CaMKK2 is significantly increased in high Gleason grade PrCa relative to normal prostate [111]. It has been indicated that CaMKK2 works in a feedback circuit to maintain AR activity which promotes prostate growth and PrCa progression. Inhibition of the kinase significantly impairs tumorigenesis [103, 109]. It is also indicated that the induction of androgen-hypersensitivity after androgen-deprivation therapy (ADT) may be involved in down-regulation of CaMKK2 [112].

The AR stimulates CaMKK2 expression, as described previously. As a novel regulator, microRNA (miR)-224 has been identified to target CaMKK2 [113]. microRNAs have been associated with the development of various human cancers [114]. These RNAs regulate expression levels of their target genes via completely or partially complementarily binding to the 3'-untranslated region (3-UTR) of mRNA, leading to mRNA degradation or blocking translation [115]. miR-224 is involved in the regulation of cell migration and invasion in human hepatocellular carcinoma (HCC) [116]. In PrCa, miR-224 has been found to be down-regulated [117], and the miR-224-low / CaMKK2-high expression profile has now been linked

to frequently shorter overall survival of PrCa patients, demonstrating the significant association between this combined expression pattern and the aggressive clinicopathological characteristics of PrCa patients [113]. Another recently identified interacting protein in the CRPC cell line C4-2, nucleoporin 62 (Nup62), links CaMKK2 with nuclear pore complexes (NPCs). This study also finds highly similar patterns of CaMKK2/Nup62 up-regulation and similar perinuclear and nuclear subcellular localizations in clinical PrCa specimens [118]. Karacosta *et al.* have further shown that CaMKK2, Nup62, and the AR are recruited to androgen response elements of the AR target genes, PSA, and transmembrane protease, serine 2. Therefore, they indicate Nup62 as a potential transcriptional co-regulator [118]. However, evidence of direct phosphorylation of Nup62 by CaMKK2 remains pending which implies that further co-regulators might be involved in the interaction.

1.5 CaMKK2 – PHYSIOLOGY, PATHWAYS, AND PATHOLOGIC ASSOCIATIONS

CaMKK2 is functionally connected to obesity, insulin resistance, glucose intolerance, and autophagy [119-121]. Besides PrCa, CaMKK2 is also linked to various other diseases. **Fig. 7A** illustrates 57 diseases that have been associated with CaMKK2 (The Target Validation Platform, www.targetvalidation.org).

In normal tissue, CaMKK2 is ubiquitously expressed with higher levels in the brain, intermediate expression in spleen, prostate, thyroid, and leukocytes and lowest levels in the lung [122-127]. CaMKK2 is mainly present in the cytoplasm, but has also been reported to be located in the nucleus and perinuclear region [111, 122, 128]. Most work undertaken to identify functions of CaMKK2 has been undertaken in the brain, especially the hypothalamus, which are the tissues that show the highest normal levels of expression. The expression pattern of CaMKK2 in cancer tissue looks differently. Compared to normal expression, CaMKK2 is highly overexpressed in PrCa (**Fig. 7B**). The Oncomine database [107], which collects clinically annotated PrCa data sets presents CaMKK2 to be highly overregulated in 8 individual studies (**Fig. 7C**).

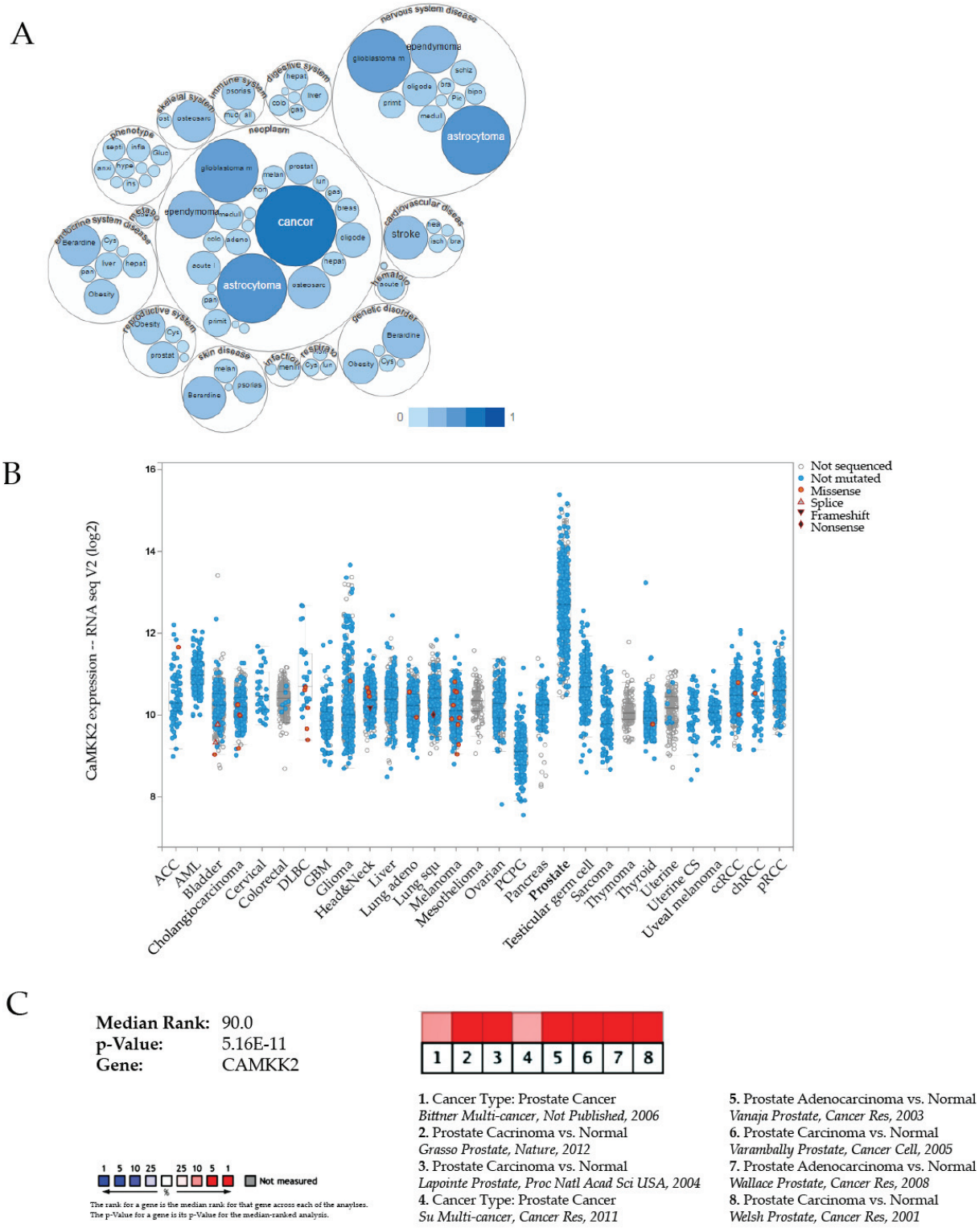

Figure 7. CaMKK2 in databases. (A) CaMKK2 is associated with 57 diseases. www.targetvalidation.org **(B)** CaMKK2 expression in human cancer tissues. cBioPortal [131, 132]. **(C)** CaMKK2 overexpression across 8 individual PrCa studies. Oncomine [107].

1.5.1 Ca^{2+}/CaM-dependent kinase cascade

CaMKK2 is one of over 120 enzymes and proteins that are controlled by the calcium/calmodulin (Ca^{2+}/CaM) complex [129]. Ca^{2+} is one of the most ubiquitous and critical second messengers in the cell and among its many binding partners calmodulin (CaM) serves as the most prevalent calcium sensor. CaM is a ubiquitously expressed 17 kDa protein with four Ca^{2+} binding motifs (EF hands). Upon Ca^{2+} binding, CaM assumes an active conformation that allows it to bind target proteins, including the CaMKKs and the CaMKs [130]. Binding of Ca^{2+}/CaM to CaMKs controls the conformation of the kinase's activation loop and allows its phosphorylation by CaMKKs, which themselves are activated by Ca^{2+}/CaM [131, 132]. Thus, CaMKK/CaMK signalling cascades are controlled at multiple levels by Ca^{2+}/CaM, thereby actively regulating downstream protein phosphorylation.

CaMKK2 was originally discovered to act by phosphorylating the downstream calcium/calmodulin-dependent kinases 1 and 4 (CaMKI and CaMKIV) [125]. CaMKI activation is involved in cell growth regulation, as observed in neurite elongation and branching [133], as well as during cell cycle control [134]. CaMKIV activity is involved in the regulation of protein synthesis and gene expression programmes responsive to nutrients [135] and hormones [136]. Moreover, CaMKK2 is a key driver of hepatic cancer via CaMKIV signalling and CaMKK2 overexpression correlates negatively with HCC patient survival [137]. This study also suggests the association between CaMKK2 and the mTOR/S6K/S6 pathway, a protein synthesis pathway that is well-known to be an oncogenic signalling cascade, routinely hijacked by cancer cells and critical for cancer cell growth [138]. More recently, CaMKK2 has also been identified to be a physiologically relevant upstream

activator of AMPK [86, 139-141]. CaMKK2-dependent activation of AMPK is involved in the regulation of energy balance, particularly in the brain [119], liver [142], and adipose [143]. AMPK acts as a crucial cellular energy sensor which can promote ATP production by activating the catabolic pathways, while conserving ATP by switching off biosynthetic pathways [86, 144]. Activated CaMKK2 phosphorylates the α subunit of AMPK and forms a multimeric protein complex comprising Ca^{2+}/CaM, CaMKK2, and the AMPKα and β subunits [145, 146]. Importantly, the regulatory γ subunit of AMPK that senses changes in the AMP:ATP ratio in the cell is not required for this interaction. Thus, the activation of AMPK by CaMKK2 is unique to fluctuations in intracellular Ca^{2+} [146]. Moreover, the interaction between CaMKK2 and AMPK occurs via their kinase domains and CaMKK2 must be present in its active conformation to interact with AMPKα, but not with CaMKI or CaMKIV [147]. The AMPK activation through CaMKK2 has recently been linked to gastric cancer. Like in hepatic cancer, CaMKK2 was found to be significantly overexpressed in tumour tissues and silencing via small interfering RNA (siRNA) or STO-609 reduced cell proliferation and tumorigenicity *in vivo* [137, 148]. Moreover, the CaMKK2-AMPK pathway is also linked to autophagy. CaMKK2 is thought to activate autophagy via AMPK-activation and mTOR-inhibition and to mediate autophagy in response to a wide variety of stimuli and cell types [120, 121].

In this context it is noteworthy to mention that CaMKK2 can be expressed as seven isoforms through alternative splicing and polyadenylation [124, 149]. Isoform 1, 2, and 3 are able to phosphorylate CaMKI and CaMKIV. Isoform 3 can phosphorylate CaMKID. Isoform 4, 5 and 6 lack part of the CaM-binding site and are inactive, whereas isoform 3 displays the CaM-binding site but is reported to be highly Ca^{2+}/CaM-independent [149] (see also **1.6.2**).

Interestingly, both normal prostate and PrCa cells predominantly express the 541 aa long CaMKK2 isoform 3, a splice variant lacking exon 16 [150].

1.6 CaMKK2 – STRUCTURAL PROPERTIES AND MOLECULAR FEATURES

The eukaryotic protein kinases comprise one of the largest superfamilies of homologous proteins [151] with 518 identified human protein kinases known to date [152]. Protein kinases are key regulators of diverse cellular functions by activating or deactivating substrate proteins and thus often found in signalling pathways. They catalyse the transfer of the γ-phosphate group from ATP to the hydroxyl group of serines, threonines, or tyrosines of substrate proteins. Phosphorylation of these target proteins may have profound effects on the target protein conformation and function, directing the activity, localization and overall function of many proteins, and serve to orchestrate the activity of almost all cellular processes.

The human kinome contains 478 kinases with the characteristic eukaryotic protein kinase (ePK) domain, encompassing the ATP and substrate-binding sites. The remaining 40 kinases lack sequence similarity to the ePK domain, but are known to have kinase activity. For this reason they are called atypical protein kinases (aPKs). The ePKs are further classified into eight groups based on sequence similarity within this domain [152] (**Fig. 8**). The majority of ePKs are serine/threonine protein kinases (STK) to which also the CaMKK family belongs to. CaMKK1 and CaMKK2 are however placed into the 'other' kinase subgroup, collecting 83 ePKs that lack sufficient sequence similarity to any of the eight ePK groups [153]. However, functionally CaMKK2 is supposed to work similar to the so-called CAMK group, comprising kinases regulated by Ca^{2+}/CaM-binding.

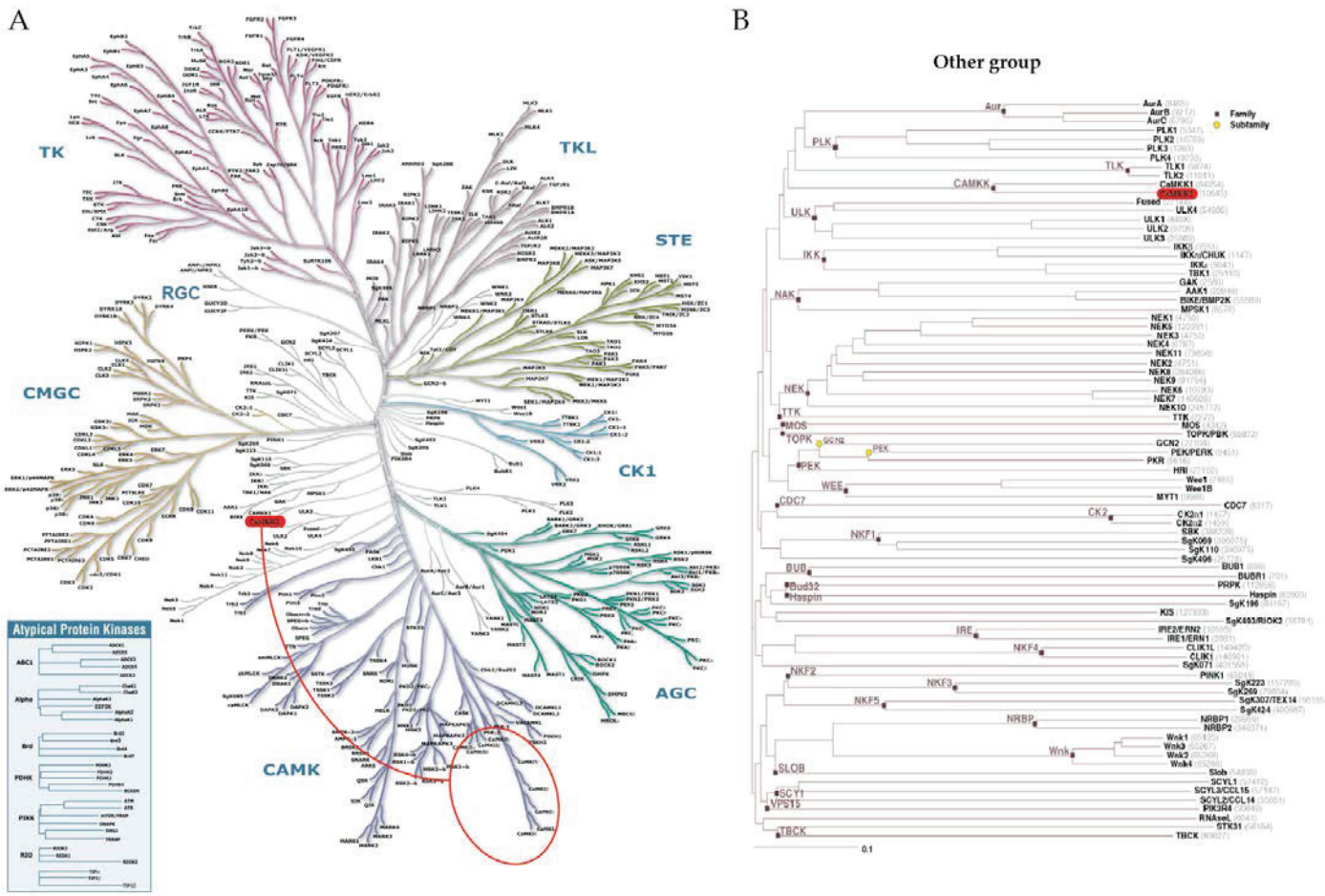

Figure 8. The human kinome with CaMKK2 highlighted in red. (A) The human kinase dendrogram. Human ePKs are classified into eight groups (i) TK (tyrosine kinase), (ii) TKL (tyrosine kinase-like), (iii) STE (STE20, STE11, and STE7 related), (iv) CK1 (casein kinase 1), (v) AGC (protein kinase A, protein kinase G, and protein kinase C related), (vi) CAMK (Ca^{2+}/CaM-dependent kinases), (vii) CMGC (Cdk, MAPK, GSK, Cdk-like related), and (viii) RGC (receptor guanylyl cyclase). Highlighted in red is CaMKK2 among the 'other' kinases group (grey). Functionally, CaMKK2 is similar to the CaMK family, encircled in red. **(B)** The kinome diagram showing the 'other' group kinases including CaMKK2 in red. Illustration is reproduced by courtesy of Cell Signaling Technology, Inc. (www.cellsignal.com), based on Manning *et al.*, 2002 [152].

The following paragraphs will describe structural properties of STKs, mainly the commonly found kinase domain (KD), and the characteristic features of CaMKK2 in more detail, like the exceptional autoregulatory domain, comprising the overlapping autoinhibitory domain (AID) and calmodulin-binding domain (CBD). CaMKK2 isoform 3 (Uniprot: Q96RR4-3) will be used as a reference model with regard to residue numberings in the following section. All currently available structural data for CaMKK2 comes from the kinase domain of CaMKK2 co-crystallised with the inhibitor STO-609 (PDB ID: 2ZV2) [154].

1.6.1 The kinase domain

All protein kinases contain a structurally conserved catalytic domain which is ~250 amino acids in length (in CaMKK2 aa 165-446). This kinase domain shows a conserved fold with highly conserved motifs (**Fig. 9A**). It consists of two lobes, referred to as the N-terminal lobe (N-lobe) and the C-terminal lobe (C-lobe) with a catalytic cleft in between, important for ATP binding and phosphate transfer [151, 155] (**Fig. 9C**). The N-lobe is the smaller one and consists of five β-sheets with one α-helix termed the αC-helix; the C-lobe forms the larger part of the kinase domain with mostly α-helical motifs and a small amount of β-sheets. The two lobes are connected by a linker region, a single polypeptide strand, in a flexible manner. That region acts as a hinge about which the two lobes can change orientation with respect to each other by domain movements during the catalytic cycle.

ATP is the phosphate donor for all catalytic kinase reactions, and the kinase domain is catalysing the transfer of the γ-phosphate group from ATP to the substrate. The ATP-binding pocket is located at the lobe interface and upon ATP binding the N-lobe approaches the C-lobe. The bound ATP is covered in the catalytic cleft by a highly conserved phosphate-

binding loop, the P-loop, connecting strands β1 and β2 of the N-lobe (**Fig. 9C**). The P-loop is also called glycine-rich loop according to its conserved glycine-rich sequence motif GXGXϕGXV, in which X stands for an arbitrary amino acid and ϕ for a hydrophobic residue. This could typically be tyrosine or phenylalanine, in CaMKK2 this is Tyr176. The flexibility allowed by the glycine residues in the loop enables the conformational changes needed to approach the β- and γ-phosphates of ATP and to coordinate them via backbone interactions [156]. The last residue of the consensus sequence, a valine residue, makes a hydrophobic contact to the nucleobase of ATP.

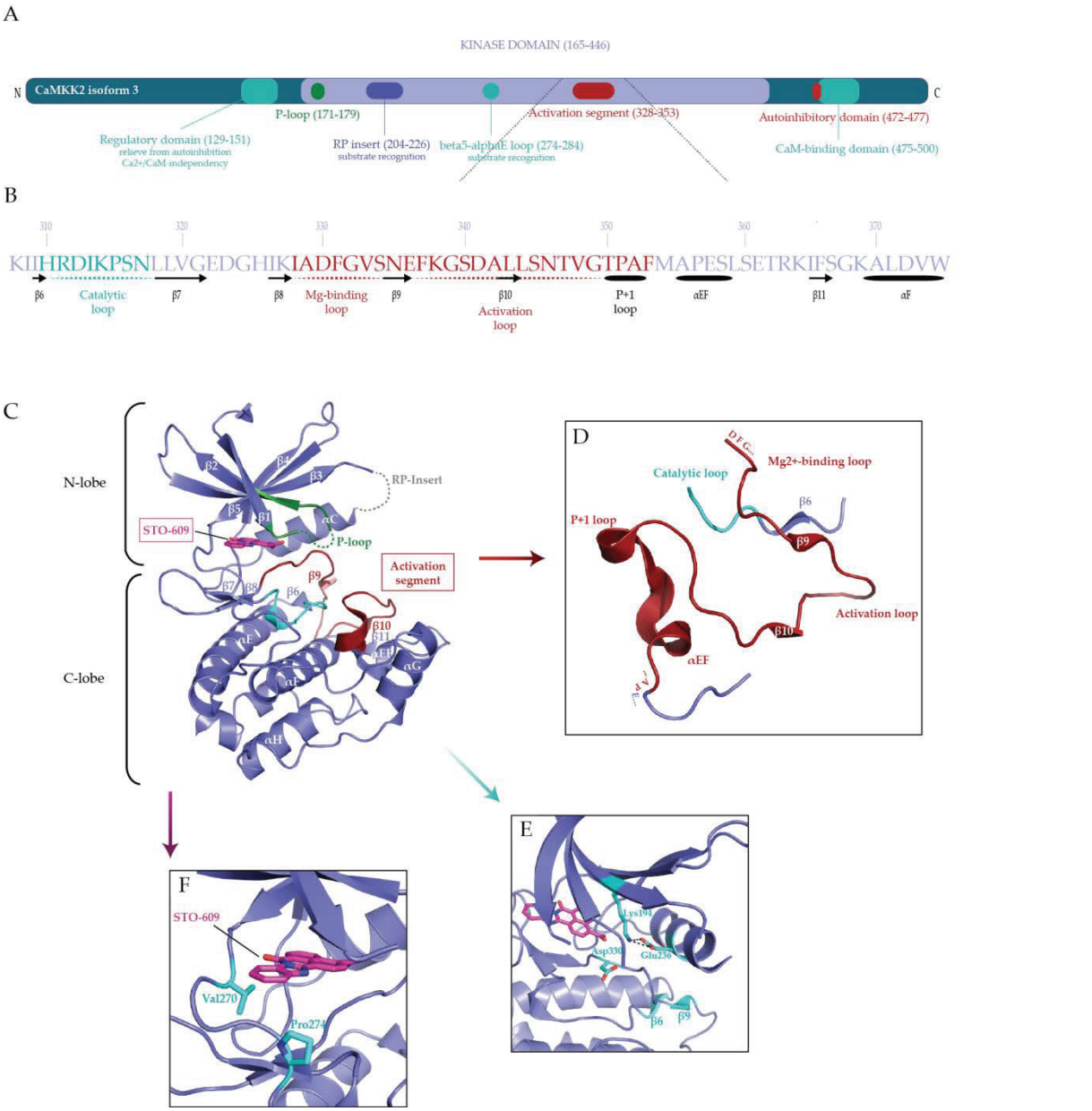

Figure 9. (A) Domain overview of full-length CaMKK2 isoform 3 protein (541 aa). Kinase domain (KD) (aa 165-446), arginine-proline-rich site (RP) (aa 204-226), autoinhibitory domain (AID) (aa 472-477), CaM binding domain (CBD) (aa 475-500). **(B)** Primary and secondary structural elements of the CaMKK2 activation segment and surrounding motifs. **(C)** The tertiary structure of the kinase domain of CaMKK2 (aa 165-446) in complex with STO-609 [154]. The N-terminal lobe (N-lobe) consists of β-sheets and one conserved α-helix (αC-helix). The C-terminal lobe (C-lobe) is largely helical and contains the activation segment which includes residue(s) that in many kinases are phosphorylated for activity. STO-609 is shown bound to the ATP-binding site (purple sticks**). (D)** Enlargement of the activation segment domain structure (redrawn and adapted for CaMKK2 from [157]). **(E)** Highlighted in cyan are Asp330 of the DFG motif, and the salt bridge between Lys194 and Glu236, implicating the active conformational state of STO-609 bound CaMKK2. Hydrogen bonds between beta sheets 6 and 9 (highlighted in cyan) are another characteristic of active kinase conformation. **(F)** Enlargement of the STO-609 binding site with two residues highlighted in cyan, causing the increased sensitivity of STO-609 towards CaMKK2. Among related kinases, Pro274 is only conserved in CaMKK1 and CaMKK2. Due to a single amino acid replacement in CaMKK1, Val270 to Leu270, STO-609 is more sensitive to CaMKK2.

1.6.2 Kinase activation

Whereas a kinase domain is defined by fold conservation, its activity is a complex product of kinase-specific phosphorylation events and protein-protein interactions. However, the timing and order of these events are crucial but difficult to assess as kinase activation states are usually temporary events.

Newly translated kinases are by default locked in an autoinhibited state to prevent untimely kinase activation. There are several mechanisms known for kinase autoinhibition. Some kinases, e.g. CaMKII and CaMKIV, undergo autophosphorylation on specific residues that prevent an active kinase conformation [158, 159]. Others, like all CaM-dependent kinases are held in an inactive state by their AID [160]. Members of the CaMK cascade share a common domain organization with the previously described catalytic kinase domain, terminating with the αI-helix [151], and the directly following C-terminal AID, which partially overlaps with the CBD (**see Fig. 9A**) [161, 162]. The interaction of the KD with AID prevents both substrate and ATP binding which maintains the kinase in an inactive state, based on the crystal structure of CaMKI KD-AID fragment [162]. The Ca^{2+}/CaM-binding to the CBD is believed to relief this intrasteric autoinhibition of the kinase and therefore leads to activation. CaMKK2 presents a different organisation of the KD-AID interaction compared to the CaMK family. One difference is that the autoregulatory AID and CBD are not immediately affiliated with the KD as there is a 26 aa separation between the KD and the N-terminal located AID/CBD domain. Moreover, the CaMKK2 KD lacks the αD-helix, involved in hydrophobic interactions with the AID, in CaMKI and CaMKII [154]. This suggests that there must be a different mechanism in the KD-AID interaction in CaMKK2, compared to CaMKI. Even more

interestingly, CaMKK2 is proposed to bind CaM in a unique way compared to other CaM-target complexes [163]. Structural data coming from nuclear magnetic resonance (NMR) spectroscopy of the rat CaMKK CaM-binding site (a 26 aa peptide) in complex with CaM revealed that the peptide forms an α-helix which binds to both the N- and C-terminal domains of CaM but in the opposite orientation to other helix-mediated interactions between CaM-binding kinases and CaM (PDB ID: 1CKK) (**Fig. 10**). The 26 aa CaM-binding sequence is conserved in the human CaMKK2 isoforms 1, 2, 3 and 7, whereas isoform 4, 5 and 6 lack parts of the CaM-binding site and are expected to be inactive [124]; CaMKK2 isoform 3 displays the CaM-binding site but is reported to be highly Ca^{2+}/CaM-independent [149].

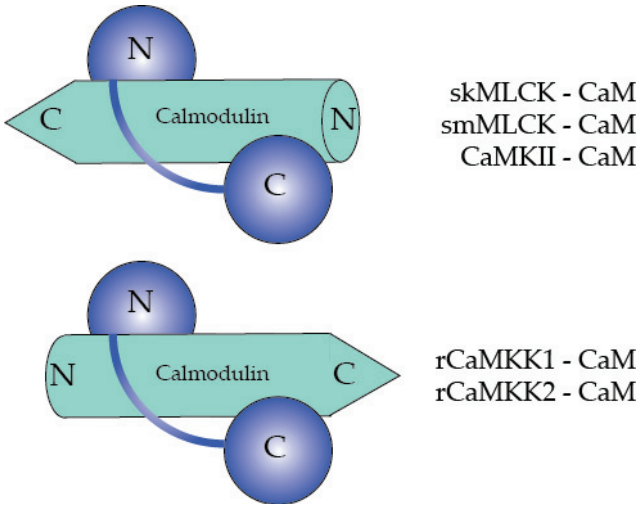

Figure 10. Orientation of interaction between CaM-binding kinases and CaM. CaMKKs are supposed to bind in an opposite orientation to the N- and C-terminal domains of CaM compared to all previously identified cases (CaM–skeletal muscle MLCK peptide, CaM–smooth muscle MLCK, CaM–CaMKII) in which the peptide helix is positioned such that the N-terminal portion mainly binds to C-domain of CaM, while the C-terminal portion binds to CaM N-domain. The peptide orientation in the CaM–CaMKK complex is reversed; the N-terminal portion of the CaMKK peptide interacts with N-domain, while the C-terminal portion of the peptide binds to C-domain. Figure redrawn and adapted from [163].

External stimuli needed for full kinase activation can either be interactions with other proteins, like the Ca^{2+}/CaM-binding in the case of CaMKK2. Most STKs however require phosphorylation events to adopt the active conformation. This can be achieved by upstream kinases, but some kinases are also capable of autophosphorylation, either by another molecule of the same kinase, a process known as trans-phosphorylation, or intramolecularly via cis-phosphorylation [164, 165]. In several of the eukaryotic systems the activating response cannot be achieved by phosphorylation on a single site; double or triple phosphorylation is needed [166]. Only when activated, the kinase comprises the catalytically competent conformation to bind to substrate peptides and catalyse the transfer of a phosphoryl group from the γ-phosphate of ATP to the hydroxyl group of serine or threonine residues in the protein substrate.

In most STKs, the activation through phosphorylation takes place on a serine or threonine residue located within the activation segment [166]. For this reason, this region of the kinase has become a major focus for understanding the relationship between the structure and function in protein kinases. The activation segment is defined by its primary structure as the region flanked by the conserved DFG- and APE-motifs within the C-lobe and includes the magnesium binding loop, β9, the activation loop, and the P+1 loop [157, 167] (**Fig. 9B-D**). The activation loop is for many kinases the site of regulatory phosphorylation or interaction with activity modulators [157]. Even though the N- and C-terminal neighbouring motifs of the activation loop are conformational preserved between kinases, the activation loop itself shows in both conformation and sequence a great range of diversity [157]. N-terminally, the activation loop is anchored by the magnesium binding loop with the conserved DFG-motif (in CaMKK2 330DFG332) and β9 (**Fig. 9D**). In an active state conformation, the aspartate of

the DFG motif (Asp330) points into the ATP-binding pocket (**Fig. 9E**) and coordinates a magnesium ion, which positions the phosphate groups of ATP for catalysis [168]. Since the asparagine side chain points toward the ATP binding pocket this is called the active 'DFG-in' conformation [155, 169]. This conformation is not a sufficient criterion for the active state of a kinase though. Another hallmark feature of an active state conformation is the orientation of the αC-helix which in an active state is rotated inwards towards the active site, together with a characteristic ion pair interaction between two strictly conserved residues; the lysine (Lys194) of the β3 strand in the N-lobe forms an important salt bridge to the glutamate (Glu236) (**Fig. 9E**), located in the αC-helix, and in addition makes contact to the α- and β-phosphate groups of ATP [169]. The αC-helix is connecting both lobes and believed to be important for the intramolecular communication between the two lobes [170]. In an active kinase state, the αC-helix is close to the activation loop and only in such way the lysine-glutamate salt bridge can be formed. C-terminal to the DFG motif is β9 which forms a short antiparallel sheet with β6, stabilised by three hydrogen bonds, which is another characteristic of the active state (**Fig. 9E**). The β6 sheet precedes the catalytic loop (conserved sequence: HRDLKxxN), containing the conserved HRD-motif (310HRD312). The arginine of the HRD motif (Arg311) can form a salt-bridge to a phosphorylated residue of the activation loop and thus stabilising it. The aspartate of HRD (Asp312) orientates the hydroxyl acceptor group of the peptide substrate. In inactive kinases on the other hand, the activation loop is disordered, thus untying the sheet, disrupting the N-terminal anchor, and distorting the magnesium binding loop. This prohibits correct ATP phosphate positioning, alters positioning of the αC-helix, in turn altering the interface between the N- and C-lobe of the kinase domain [171]. The P+1 loop, critical for the interaction between substrate and kinase, extends into the αEF-

helix, a short helix consistently present in most kinases. The αEF/αF loop is not part of what is commonly referred to as the activation segment. However, its location is critical and the intensive interactions of this loop with the activation loop indicate a key role in activation loop function [171]. In phosphorylation-dependent kinases, the αEF/αF loop may stabilize the active conformation of the phosphorylated loop while destabilizing the unphosphorylated loop, preventing it from folding into an active state [171].

As described, most STKs require phosphorylation on a serine or threonine residue within the activation segment [166]. However, even though CaMKK2 lacks the activation loop phosphorylation site (CaMKIV Thr200, CaMKI Thr177, and PKA Thr197 corresponding to CaMKK2 Asn346), the activation segment is folded in the active 'DFG-in' conformation as observed in the structural data of the CaMKK2/STO-609 complex formation [154]. The conserved Phe331 in the DFG motif interacts with the αC-helix, stabilizing the catalytically necessary ion pair (**Fig. 9E).** This indicates that CaMKK2 does not require activation loop phosphorylation to adopt the closed active conformation. The activation segment is furthermore stabilised by various interactions between amino acids conserved among the CaMKK isoforms, and shows intrinsic activity in *in vitro* assays. Therefore, CaMMK2 has been proposed to be an intrinsically active kinase, independent of activation via Ca^{2+}/CaM or phosphorylation of the activation loop [154]. Even though CaMKK2 does not comprise any reported phosphorylation events in the activation segment, phosphorylation has been detected throughout the kinase sequence, including one in the kinase domain, Thr216 [172]. Different extents of phosphorylation throughout the kinase sequence can affect the activity of the kinase and lead to variable binding characteristics [173-175]. CaMKK2 is expected to autophosphorylate *in vivo* and *in vitro* [123, 124, 172, 176] and the reported phosphorylation

sites are Ser22, Ser129, Ser133, and Ser137, Thr316, Thr483 and Thr517 [172, 177, 178]. There are also reported phosphorylation events by other kinases, the cyclin-dependent kinase 5 (CDK5) and glycogen synthase kinase 3 (GSK3) [179]. The N-terminal sequential CDK5/GSK3 phosphorylation of CaMKK2 (Ser129, Ser133, and Ser137) *in vitro* has been reported to decrease the Ca^{2+}/CaM-independent, autonomous activity of the enzyme and regulates its turnover *in vivo*. Moreover, the death-associated protein kinase 1 (DAPK1) has been shown to phosphorylate CaMKK2 on Ser511 [180]. In which cellular and functional context these activation pathways take place remains to be studied.

In an earlier study, it has been demonstrated that both CaMKK1 and CaMKK2 require Ca^{2+}/CaM-binding for maximal activation, whereas the latter exhibits substantial constitutive activity (60-70% of the total activity) in the absence of Ca^{2+}/CaM [123, 172]. In rat CaMKK2, a 23 amino acid N-terminal regulatory domain has been identified to participate in the release of its AID from the catalytic core, resulting in autonomous activity [181]. This conserved regulatory domain (aa 129-151 in human CaMKK2) (**Fig. 9A**) is required for threonine autophosphorylation of CaMKK2, including that at Thr483, which maintains the kinase in a partially active conformation in the absence of its activator, Ca^{2+}/CaM [172]. Whereas the presence of the N-terminal regulatory domain in CaMKK2 can explain the differences in Ca^{2+}/CaM-binding dependency between CaMKK1 and CaMKK2 for full activity, the reason for the substantial Ca^{2+}/CaM-independency of CaMKK2 isoform 3 remains unknown, but varying phosphorylation events might be pivotal.

1.6.3 Substrate-binding regions

Active STKs can interact with the specific substrates and catalyse the transfer of a phosphoryl group from the γ-phosphate of ATP to the hydroxyl group of serine or threonine residues in the substrate. In contrast to the well-defined ATP-binding site, the substrate-binding regions of kinases are shallow surface depressions, whose structures are still poorly understood [182]. The critical sites for substrate recognition that are known are the P+1 loop, described above, and the N-terminal activation loop (**Fig. 9A**). Substrate recognition and high-affinity binding rely partly on local residues, but in many cases it has become apparent that short peptide sequences do not exploit the complete binding capacity offered by the protein kinases. This suggests that kinases might use distal recognition elements that are likely to be important for efficient phosphorylation [166, 167].

CaMKK2 presents a unique feature for substrate recognition. In addition to the commonly conserved motifs of STKs, CaMKK2 displays an Arg-Pro-rich 22 residue insert (RP-insert) in the N-lobe (**Fig. 9A and C**), which plays an important role for the selective recognition of downstream substrates. The RP-insert is critical for activation of the downstream targets CaMKI and CaMKIV [183]. It is thought that the three conserved arginines in the RP insert (corresponding to Arg209, Arg210, and Arg214 in CaMKK2) are essential to recruit and stabilize the downstream substrates to maintain proper orientation towards the catalytic cleft which provides high affinity and specific interaction between CaMKK2 and these substrates. The RP-insert is not associated with autophosphorylation or activation of substrates [183]. In addition, CaMKK2 lacks the αD-helix with conserved acidic residues that in many other kinases recognizes basic residues in kinase substrates. The equivalent region, called β5-αE

loop (**Fig. 9A**), displays a hydrophobic molecular surface containing a conserved Pro274. Pro274 is in addition to Ser316 and Cys382 one of the replaced amino acids that leads to a hydrophobic pocket that is suitable to accommodate hydrophobic substrate residues. It has been proposed that CaMKK2 therefore prefers hydrophobic or non-polar rather than basic residues at the P-3 and P-2 positions of the substrate [154].

1.6.4 CaMKK2 in complex with STO-609

The only currently available structural data of CaMKK2 is the one of the KD in complex with STO-609 [154] and most of the above described structural features which are specific for CaMKK2 derive from that publication.

STO-609, is an ATP competitive inhibitor [110], and is the current standardly used small molecule CaMKK2 inhibitor in pre-clinical studies. The structural data of the co-crystallised STO-609 with the KD of CaMKK2 revealed that it binds within a narrow pocket of the KD adopting a closed conformation [154] (**Fig. 9C**). The interaction between CaMKK2 and STO-609 is mainly hydrophobic, involving Ile171, Val179, Ala192, Val249, and Phe267 from the N-lobe and Gly273, Pro274, Leu319, and Asp330 from the C-lobe of KD. Additionally, STO-609 binds to the backbones of Val270, Asp330 and Glu236 in a water-dependent manner. STO-609 is more sensitive to CaMKK2 than to CaMKK1 which is due to a single amino acid replacement in CaMKK1, Val270 to Leu270 [154] (**Fig. 9F**).

Even though STO-609 is a relatively selective inhibitor, it is not specific and can also inhibit at higher concentrations CaMKK1 and other protein kinases like CaMKI, CaMKII, CaMKIV, PKA, AMPK, and LKB1 (ranked in this order after *in vitro* filter-binding assay) [86, 110]. The conserved Pro274 in CaMKK2 and CaMKK1, which is replaced by other amino acids in all

the other kinases, is the most important determinant for the selective inhibition by STO-609. This proline residue is located at the so-called 'gate' of the STO-609-binding pocket (**Fig. 9F**), forms hydrophobic contacts with STO-609 which is likely to cause the stronger binding compared to other kinases.

1.7 DRUG DISCOVERY

Even though there has been a tremendous increase in the understanding of cancer biology in the recent decades, the ability to translate this knowledge into novel therapies is poor. The so-called 'Valley of death' in anticancer drug development describes the mismatch in progress between basic research and approved medicines [184]. This also applies for kinase inhibitor development and the long time span from the discovery of kinases as oncogenes and key players of tumorigenesis to the clinical approval of the first kinase small molecule inhibitor imatinib (STI-571, Gleevec) in 2001 provides evidence of the difficult path to success in this area [185]. Typically, the drug development process is divided into three major steps: (i) discovery, (ii) preclinical development, (iii) and clinical trials, whereas the transition from the discovery phase to preclinical development is often an interplay and continuum.

The drug discovery phase usually describes all basic research activities that lead to the identification of a drug target and candidate compounds. Early pharmacology studies, *in vivo* efficacy, and experimental toxicology can contribute to the selection of a lead candidate for preclinical development. Once a lead candidate/compound is identified, a preclinical development programme generally involves efficacy, pharmacology, and experimental toxicology studies to define the dose, route, and frequency required for subsequent studies. Bioavailability studies typically address the absorption, distribution, metabolism, and excretion (ADME) profile of the drug, whereas pharmacokinetics (PK) studies provide information on the drug exposure and pharmacodynamics (PD) on target engagement and downstream pathway inhibition [186, 187]. The active pharmaceutical ingredient (API) must ultimately be well characterised in terms of structure identity, counter ions (salts) and co-

crystals, impurities, stability, chirality and enantiomer(s), appearance, solubility, and other chemical and physical properties. Only when all these data is provided and documented, a drug can be filed as an Investigational New Drug (IND) application which is required prior to initiation of a clinical trial by the US Food and Drug Administration (FDA) [186].

In a typical drug discovery and development process, target selection and validation usually comes first. Evidence needs to be provided for the target to be sufficiently overactive or part of an overactive pathway that drives the defined cancer and contributes to cancer growth. The target might also support the oncogenic process or represents a vulnerability that can be exploited through synthetic lethality. Moreover, it is from importance to assess early on whether there is evidence that inhibition of the target will lead to the desired effect in patients, like tumour regression and increased survival at well tolerated doses [188]. A key question now is to evaluate the druggability of the proposed target by small molecules. Therefore, the target needs to contain a pocket or cavity to which the drug can bind with strong potency. Available 3-dimensional structural data for the target provides the possibility to assess whether there are such pockets available, either qualitatively by inspecting the structure or more quantitatively using computational tools to assess the druggability. It is however increasingly recognised that not all druggable pockets are fully present in static crystal structures, as they might only appear in particular conformational transition states which do not crystallise [187]. Once a target is chosen, chemical hit compounds need to be identified. A chemical hit describes the key compound which acts as an early prototype drug against the target. Conceptually, two approaches for hit identification can be distinguished: knowledge-based design and random screening. Knowledge-based design takes prior knowledge, such as a crystal structure of the target

protein or chemical structure of known inhibitors, into consideration whereas random screening involves the unbiased screening of large compound collections. In reality, most hit discovery campaigns combine both approaches [188]. Assessing the quality of a hit determines to a large extent how fast and efficiently the optimisation towards a preclinical candidate proceeds. As a rule of thumb when evaluating a chemical hit, oral drug candidates with high molecular weight (>550 Da) and excessive lipophilicity have a higher chance of failing during development, as initially described by Lipinski's 'rule of 5' [188, 189]. The lead optimisation then involves multi-parameter testing of potency, selectivity, tolerability, bioavailability, and metabolic stability in order to generate a safe and efficacious drug which can be used for subsequent biology-led clinical trials.

1.7.1 Considerations for the druggability of protein kinases

Although the catalytic characteristics of active protein kinases are alike and evolutionary conserved, the mechanisms by which individual kinases are physiologically inhibited and therefore inactive might vary considerably. Different strategies are utilized by nature to lock kinases into inactive conformations, including pseudosubstrate inhibition, adenine mimetic mechanisms as well as other mechanisms that involve locking the enzyme into an inactive conformation by using surfaces other than the active site [190].

Synthetically designed kinase inhibitors target to a great majority the ATP-binding site and act as ATP competitors / adenine mimetics. An important issue in the development of kinase inhibitors by targeting the ATP-binding site is specificity. As already described, protein kinase domains are evolutionary conserved as the catalytic mechanism requires the exact positioning of highly conserved active site residues, thus making the active state kinase rigid

and the ATP binding site highly conserved. This property suggests that targeting the active kinase conformation by conventional ATP mimetic inhibitors is innately challenging. However, recent expansion of structural data has provided a clear description of the ATP-binding pocket and proved that certain regions within the ATP-binding cleft are not in direct molecular contact with ATP and therefore potential target sites to increase selectivity of drugs designed against the ATP-binding pocket [191]. These hydrophobic regions are poorly conserved and show therefore structural diversity between kinases which has been used to increase selectivity of ATP-competitors [192-195]. In contrast, the inactive forms of kinases are structurally highly diverse and dynamic, suggesting the potential of drugs with a higher selectivity. The most explored inactive state is the 'DFG-out' conformation (see also **1.6.2**), leading to a rearrangement and thus creating an allosteric binding pocket adjacent to the ATP-binding pocket [185]. Another strategy of targeting kinases is allosteric inhibition or activation in which less conserved noncatalytic pockets are used for targeting. Such inhibitors can also bind to the kinase outside of the catalytic domain, either by interfering with the binding site of an interacting protein or the protein substrate-binding site (see also **1.6.3**). However, these regions are often shallow surface depressions, structurally poorly understood compared to the well-defined ATP-binding site, and thus have rarely been used as targets for inhibitor binding [182]. Still, this route potentially enables higher specificity as these less conserved pockets might distinguish a kinase from its peers [196].

2 AIMS OF THIS STUDY

1. **To improve the expression and purification of CaMKK2 in complex with calmodulin and develop new insights into its association with STO-609 to support drug development efforts.**

   No structural data existed for the CaMKK2:CaM protein complex and thus we set out to express and purify the full-length protein complex to use for crystallisation studies. We hoped to gain insight into the CaMKK2/CaM-binding interface to gain further understanding on kinase regulation. The relationship between inhibitor binding and calmodulin and other factors had not been well defined, and the recombinant protein complex shall support such studies.

2. **To identify interacting partners in prostate cancer cells.**

   At the outset of this PhD, no interacting proteins had been reported for CaMKK2 beside CaM. Thus we aimed to identify interacting proteins in prostate cancer cells using co-immunoprecipitation with subsequent mass spectrometry. Defining the CaMKK2 interactome will suggest additional pathways that may explain the biological impact of CaMKK2 on PrCa.

3. **To evaluate the contribution of CaMKK2 to autophagy using functional autophagic assays.**

   A limited number of studies had implicated CaMKK2 in autophagy but none had been undertaken in PrCa cells and all had relied on the use of microtubule-associated

protein 1A/1B-light chain 3 (LC3) as a marker reflecting autophagic activity. Using more functional assays of autophagy will help to determine whether CaMKK2 is likely to be associated with an autophagic phenotype during PrCa progression and it is vital to know whether the functional relationship is positive/driving autophagy or inhibiting it because autophagy can be both pro- and anti-tumorigenic depending on the molecular context.

3 SYNOPSIS OF THE INCLUDED PUBLICATIONS

3.1 PAPER I

Data for the co-expression and purification of human recombinant CaMKK2 in complex with calmodulin in *Escherichia coli*

Here, we describe the expression in *E. coli* and the purification protocol for the following constructs: full-length CaMKK2 in complex with CaM, CaMKK2 'apo', CaMKK2 (165-501) in complex with CaM, and the single mutation construct CaMKK2 F267G. The protocols described have been optimized for maximum yield and purity with minimal purification steps required. Protein purified according to this protocol has been used subsequently for crystallisation attempts, the development of a fluorescence-based assay for drug binding to the kinase (**Paper II**), Gemin4 interaction studies (**Paper III**), and further CaMKK2 characterisation.

3.2 PAPER II

The purified human CaMKK2:calmodulin complex shows phosphorylation-dependent binding properties with the fluorescent inhibitor STO-609

We have developed a novel fluorescence-based assay by exploiting the intrinsic fluorescence properties of STO-609. Here, we report an in vitro binding constant of K_D ~17 nM between STO-609 and purified CaMKK2 or CaMKK2:CaM complex (**Paper I**). Whereas high concentrations of ATP were able to displace STO-609 from the kinase, GTP was unable to

achieve this confirming the specificity of this association. Recent structural studies on the kinase domain of CaMKK2 had implicated a number of amino acids involved in the binding of STO-609. Our fluorescent assay enabled us to confirm that Phe267 is critically important for this association since mutation of this residue to a glycine abolished the binding of STO-609. An ATP replacement assay, as well as the mutation of the 'gatekeeper' amino acid (F267G), confirmed the specificity of the assay and once more confirmed the strong binding of STO-609 to the kinase. In further characterising the purified kinase and kinase-calmodulin complex we identified a number of phosphorylation sites some of which corroborated previously reported CaMKK2 phosphorylation and some of which, particularly in the activation segment, were novel phosphorylation events. In conclusion, the intrinsic fluorescent properties of STO-609 provide a great opportunity to utilise this drug to label the ATP-binding pocket and probe the impact of mutations and other regulatory modifications and interactions on the pocket. It is however clear that the number of phosphorylation sites on CaMKK2 will pose a challenge in studying the impact of phosphorylation on the pocket unless the field can develop approaches to control the spectrum of modifications that occur during recombinant protein expression in *E. coli*.

3.3 PAPER III

CaMKK2 is a novel interacting partner of Gemin 4

We set out to identify novel interaction partners for CaMKK2 in prostate cancer by using co-immunoprecipitation studies with subsequent mass spectrometry analyses. We identified a small number of proteins that reproducibly bound to CaMKK2 and were functionally relevant to prostate cancer. Over the course of several reciprocal co-immunoprecipitations of

our initial target set, we pulled repetitively down parts of a multiprotein complex which role lies within the assembly of spliceosomal small nuclear ribonucleoproteins (snRNPs). Of these interacting proteins, only Gemin4 validated through reciprocal co-immunoprecipitation and further peptide arrays validated the interaction between CaMKK2 and Gemin4. Recently, Gemin4 was reported to be an AR corepressor and CaMKK2 is known to be a coactivator. Consequently, future studies will need to address how their differential expression and protein-protein interaction affects AR activity in prostate cancer.

3.4 PAPER IV

CaMKK2 does not function as a central regulator of autophagy in prostate cancer cells

CaMKK2 is thought to activate autophagy via AMPK-activation and mTOR-inhibition and to mediate autophagy in response to a wide variety of stimuli in a wide range of cell types. CaMKK2 is overexpressed in PrCa cells and is a mediator of androgen-induced stimulation of catabolic metabolism and cell proliferation. In concordance with this, the CaMKK2-inhibitor STO-609 has been shown to inhibit tumour growth in a PrCa xenograft model. Applying functional autophagic assays to assess the impact of CaMKK2 as an autophagy mediator in PrCa cell lines, we provide evidence that CaMKK2 does not regulate autophagy. The used long-lived protein degradation (LLPD) assay and the lactate dehydrogenase (LDH)-sequestration assay are both well suited to quantitatively measure the dynamic process of autophagy flux. Here we report that pharmacological or siRNA-mediated inhibition of CaMKK2 affects neither basal autophagy nor autophagy inhibited or induced by various stimuli. However, we observed an activation of autophagy with STO-609 in VCaP cells.

4 DISCUSSION

We set out to study the structure and function of CaMKK2 in order to gain a better understanding of its influence on PrCa. Unique molecular characteristics of the CaMKK2 and CaM complex binding mode may provide a site for exclusive drug targeting opportunities. The physiological role of CaMKK2 is still not fully understood, however is likely involved with several interaction partners that regulate the kinase activity. Thus identifying new interaction partners was, and remains a high priority. Finally, the last project of this PhD was to investigate the role of CaMKK2 in autophagy in PrCa as it was reported to be a positive regulator of autophagy via AMPK. Autophagy is regarded as a mediator of drug resistance by acting as a pro-survival response; therefore, targeting CaMKK2 in PrCa therapy might inhibit the drug-induced autophagy resistance response.

In this chapter, the work conducted in this PhD, partially summarised in Papers I-IV will be discussed and put into context. For a more detailed discussion of the individual results, the reader is advised to consult the discussion sections of the respective papers.

4.1 MOTIVATION FOR THE STUDY

Over the course of the last decade CaMKK2 has been implicated in a range of conditions and pathologies from obesity [119] through to prostate [103, 150] and hepatic cancer [135] and the risk of developing schizophrenia [197]. Previous work has focussed, in particular, on the contribution of CaMKK2 as an AR target gene to the development and progression of PrCa [103]. Despite the very significant volume of research on the functional contribution of CaMKK2 to disease, the rate of progress in elucidating the structure of the kinase, defining

its biophysical properties and improvements on the existing small molecule inhibitor targeting CaMKK2, a drug called STO-609 first reported in 2002, has been much slower [110, 154]. Developing drugs against CaMKK2 with the aim to treat PrCa might have significant advantageous over other up-regulated targets in the disease. As described previously (see **1.5**), CaMKK2 is highly expressed in the brain and has low expression patterns in most other organs [122-127]. As such, novel drugs targeting CaMKK2 might have considerable little side effects for the patients if unable to pass the blood brain barrier (BBB). However, care must be taken as CaMKK2 (via CaMKIV) has been reported to be involved in preserving the integrity of the BBB [146, 198].

The currently most selective and potent pharmacological inhibitor against CaMKK2 is the small molecular inhibitor STO-609 [110]. In previous studies on PrCa xenografted mice, there has been shown enhanced efficacy for STO-609 in combination with a clinically improved drug for the treatment of type 2 diabetes, Metformin [103]. Metformin is currently being investigated in clinical trials in combinations with drugs targeting androgen biosynthesis (Abiraterone acetate) or the AR (Enzalutamide); registered clinical trials with the identification numbers NCT02339168, NCT01650194, NCT02640534, and NCT01677897. The combinatorial therapeutic approach is promising [199], however, both drugs, Abiraterone acetate and Enzalutamide, are expensive new therapeutics and in the case of Abiraterone acetate this was only made available to Norwegian patients through extensive price negotiations with the manufacturer and through political campaigning by patients and their families [200]. We foresee the need of novel drugs which could be used to fuel combination therapy approaches in order to allow the best possible treatment options for PrCa patients. In order though to consider STO-609 a candidate therapeutic agent, issues like the currently

limited availability of information regarding pharmacokinetic properties, toxicity, packaging and administration of the drug must be addressed first [146]. STO-609, being a selective inhibitor of CaMKK2, lacks however specificity and is known to inhibit a range of related kinases at higher concentrations [86, 110] (see also **1.6.4**). Even though this appears to be initially detrimental in terms of targeting a specific upregulated protein, the inhibition of several targets by a single compound is more recently discussed as a promising therapeutic approach [155]. Especially in cancer multiple parallel kinase-mediated pathways can contribute to increased cell proliferation and inhibition of related targets might synergistically promote the desired therapeutic response of the drug. This however, needs to be shown in the case of CaMKK2 and this study shall support both fields of unknowns: (i) generate information to promote current drug development against CaMKK2, as well as (ii) identify the cellular function of CaMKK2 in PrCa and dissect differences in CaMKK2 knockdowns vs STO-609 effects.

4.2 CRYSTALLISATION ATTEMPTS TOWARDS FULL-LENGTH CAMKK2 IN COMPLEX WITH CALMODULIN

The initial focus of this PhD was to crystallise full-length CaMKK2 in complex with CaM to generate novel information regarding the binding site of these two proteins. The binding is reported to be unique related to other CaMKs [163] (see also **1.6.2**). However, the current model of CaMKK2:CaM is only based on a 26 aa peptide sequence deriving from rat CaMKK2 in complex with CaM [163], whereas the only other structural data for CaMKK2 does not include the CBD and only spans the kinase domain [154]. Our main aim was to

generate the first structure of full-length CaMKK2 in complex with CaM. This could then inform the modelling of changes in CaMKK2 conformation upon CaM-binding, affecting kinase activity.

4.2.1 Expression and purification of CaMKK2:CaM

For protein expression, we have used CaMKK2 isoform 3, the dominant isoform in PrCa [150], in order to gain information on the most relevant model in terms of drug design towards PrCa treatment. We co-expressed and co-purified CaMKK2 in complex with CaM as described in **Paper I**. The successful purified CaMKK2:CaM complex has been used to set up crystallisation screens.

4.2.2 The crystallisation bottleneck

X-ray crystallography is one of the most important and powerful tools for determining the atomic structure of proteins. However, the major obstacle is often quality and amounts of diffracting crystals. The discrepancy between the number of soluble purified proteins and diffracting crystals is also called the crystallisation bottleneck [201] with getting proteins to crystallise still being the major rate-limiting step in the overall process from target selection to data collection [202]. Protein crystallisation describes the slow precipitation from an aqueous solution which is forced by the supersaturation of the protein. This process cannot be predicted and is normally a "trial and error" based process to identify conditions that will trigger nucleation and subsequent crystal growth (**Fig. 11D**). Initial screening is often carried out in a sparse matrix setup with a reduced number of experiments to test a larger space of combinations of chemical and physical parameters, such as concentration of both protein and

precipitant, type of precipitant (salt, polyethylene glycols (PEGs), alcohols or organic solvents), temperature, pH, or additives. By varying the individual chemical and physical parameters of the initial crystal condition, the crystal quality is further optimized.

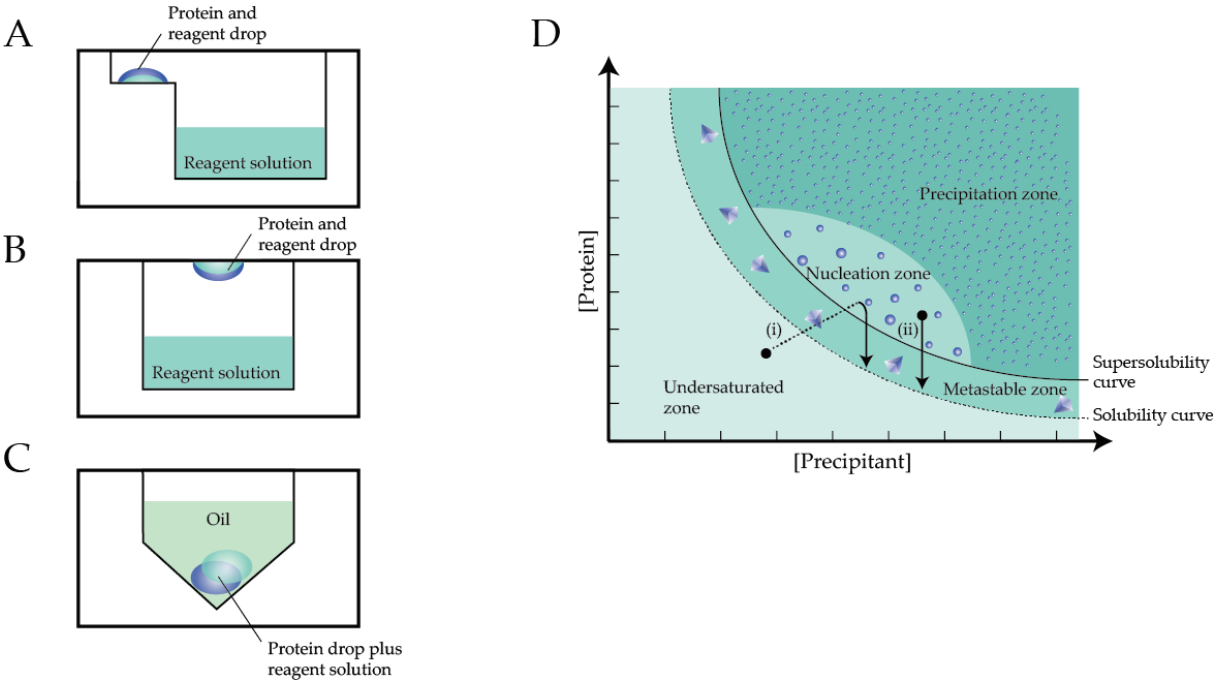

Figure 11. Crystallisation methods. (A) Vapour diffusion, sitting drop. **(B)** Vapour diffusion, hanging drop. **(C)** Microbatch under oil **(D)** Schematic illustration of a protein crystallisation phase diagram. Two major crystallisation methods are represented, showing their different routes to the nucleation and metastable zones. The black circles represent the starting conditions. **(i)** Batch. **(ii)** Vapour diffusion. The solubility is defined as the concentration of protein in the solute that is in equilibrium with crystals. The supersolubility curve is defined as the line separating conditions where spontaneous nucleation (or phase separation, precipitation) occurs from conditions where the crystallisation solution remains clear if left undisturbed. Figure redrawn and adapted from [203].

4.2.3 Crystallisation efforts towards CaMKK2:CaM were not successful

Extensive efforts have been put in the crystallisation of the CaMKK2:CaM complex, but not a single protein crystal hit has been identified. Crystal screening in-house, performed mostly by commercial sparse matrix chemical libraries, was complemented with the use of external crystallisation facilities at the automated high-throughput crystallisation (HT-X) facility at the EMBL Hamburg outstation, Germany, possible by received P-CUBE funding in June 2011, and the high-throughput screening (HTS) laboratory at the Hauptmann-Woodward Medical Research Institute (HWI) in Buffalo, US [204, 205]. Both vapour diffusion and batch crystallisation have been attempted **(Fig. 11A-C**). Additionally, we performed crystallisation screens with the protein complex soaked with its inhibitor STO-609, or in the presence of a non-hydrolysable ATP analogue (AMP-PCP) in order to stabilise a specific conformation [206]. The screening has been performed at both 19°C and 4°C. All obtained crystals appeared to be salt crystals as identified by poking or collecting diffraction images by x-rays (**Fig. 12**). Generally, a salt crystal is harder than a protein crystal and will be harder to destroy by poking it. This has to do with the higher solvent content generally present in protein crystals. The crystallographers at the crystallisation sites evaluated the diffraction images, however it is typically easy to distinguish salt diffraction from protein crystal diffraction, as the unit cell of a protein crystal is larger than the salt crystals. The protein crystal will often show diffraction in the low resolution region, while salt crystals will show single well separated spots that often collected at higher resolution. Due to the absence of protein crystals during crystallisation trials, interesting conditions with light, granular and

shiny precipitate were chosen for further testing based on the assumption that they would be close to the nucleation point and grid screens were set up, without progress. The screens performed at HT-X Hamburg and HTS Buffalo alone included more than 5500 conditions tested. None of the approaches were successful.

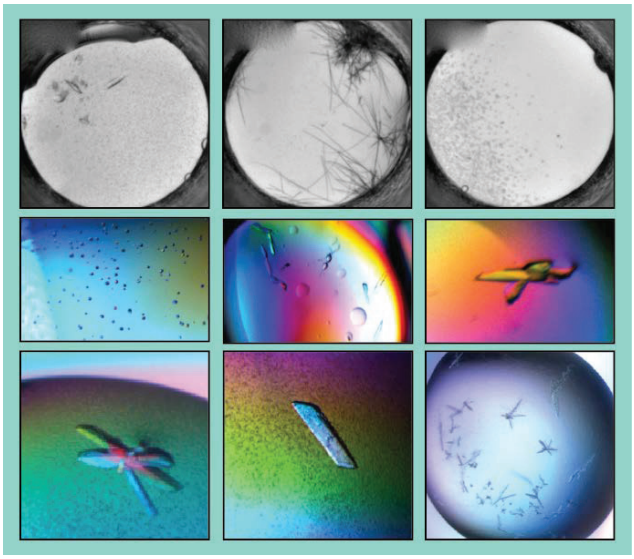

Figure 12. Collection of obtained non-protein crystals.

4.2.4 Phosphorylation of CaMKK2 prevents crystallisation

Having not succeeded in crystallising the CaMKK2:CaM complex, we went on to further validate the quality of the protein complex. With the help of MS analyses we detected high levels of phosphorylation on various residues throughout the sequence of CaMKK2 (with almost ~ 50 % of total spectral counts being phosphorylated) (**Paper II**). This could explain the crystallisation obstacles we faced. One of the key factors for successful crystallisation of a protein is a homogenous protein sample. The heterogeneity of our protein sample with its multiple species of protein in various states of phosphorylation was likely one of the major

contributors that prevented crystal formation. Phosphorylation changes the surface charge of a protein and consequently also the solubility and thus the behaviour of the sample potentially preventing crucial crystal contacts from being formed [207].

4.2.5 Future considerations

We did not succeed in solving the structure of the CaMKK2:CaM complex. In order to do so, there are several possibilities to enhance the chances. First, construct design is critical and the most obvious choice would be to chop the full-length kinase on both N- and C-terminus to reduce it to the functional domains, including KD, AID, and CBD. By eliminating the unconserved and most likely unstructured ends, we could increase the likelihood of crystallisation. A second option, and especially with regard to our phosphorylation issues, it would be useful to adapt the expression system. Even though it is not uncommon that recombinant expressed proteins, and especially kinases, are phosphorylated to different extents when overexpressed in *E.coli*, by either autocatalysis or by endogenous kinases [208], the amount of phosphorylation in our samples was surprisingly high (**Paper II**). Therefore, we could make use of other large-scale expression systems, such as yeast or mammalian cells. The only available structural data for CaMKK2 derives from protein expressed in a cell-free expression system [154]. In such, post-translational modifications are not of concern, and limited phosphorylation of the expressed kinase can be attributed to autocatalysis, however this system is expensive and not applicable to most medium sized laboratories.

4.3 DEVELOPMENT OF NOVEL *IN VITRO* BINDING ASSAY FOR CAMKK2

We have used the recombinant produced proteins 'apo' CaMKK2, CaMKK2:CaM, and CaMKK2 F267G (**Paper I**) for further binding studies towards the small inhibitor STO-609 (**Paper II**). Here, we speculated that STO-609 might display intrinsic fluorescence based on its structure and reported background fluorescence interfering with imaging experiments that was reported during the course of this study [209]. We established that the optimal excitation wavelength was 400 nm with a maximum emission wavelength at 533 nm for the free drug. Interestingly, we could observe a ~30 nm blue-shift towards a maximum emission wavelength of ~500 nm accompanied by a 10-fold enhancement of the signal upon saturation when recombinant protein was added.

The graphical abstract, only available in the online version of **Paper II** (http://dx.doi.org/10.1016/j.bbrc.2016.05.045) illustrates this discovery nicely (**Fig. 13**). Our results confirmed the reported binding of STO-609 within a narrow pocket of the KD that adopts a closed conformation and therefore decreases the solvent accessibility of STO-609 upon binding [154].

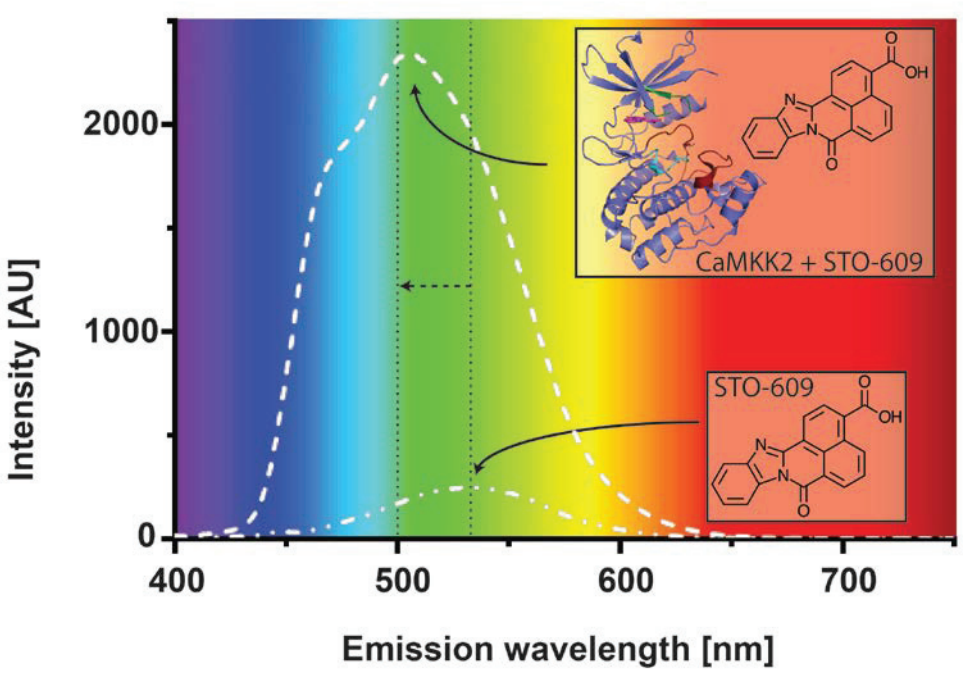

Figure 13. Graphical abstract of Paper II. Available only online: http://dx.doi.org/10.1016/j.bbrc.2016.05.045.

We could further verify the accuracy of our assay by displacing STO-609 with high concentrations of ATP, but not with GTP. The base of ATP and GTP is in both cases a purine conjugated π–system that could interfere with our measurement. To make sure that the drop in fluorescence yield, observed as a function of ATP titration, was due to replacement of STO-609 and not merely additional absorption of photons in the purine ring, we decided that GTP was the optimal control. It should be mentioned that a recent study in which CaMKK2 has been reported to be one of the few known kinases capable of using GTP as a phosphate donor for downstream target phosphorylation but with K_m values ~ 30 times higher than for ATP [210]. However as we observe no change of fluorescence yield when we titrate GTP into the CaMKK2:STO-609 complex we are not able to confirm or refute this observation.

4.3.1 Ca^{2+}/CaM-independent activity of CaMKK2

Firstly, we set out to assess the difference in the STO-609 binding properties of the purified 'apo' CaMKK2 and the CaM-bound CaMKK2 (**Paper I**). In this comparison we observed a similar K_D of STO-609 binding, 17 ± 6 nM and 17 ± 7 nM with and without CaM, respectively (**Paper II**). The ability of CaMKK2 to bind STO-609 is thus not dependent on Ca^{2+}/CaM-binding. This finding fits with the reported substantial constitutive activity of CaMKK2 in general [123, 172], but especially of the highly Ca^{2+}/CaM-independent isoform 3 [149] we work with as the predominantly expressed CaMKK2 isoform in both normal prostate and PrCa cells [150]. CaMKK2 displays, unlike CaMKK1, ~70 % activity in the absence of Ca^{2+}/CaM as a result of cis-autophosphorylation of Thr482 by the CaMKK2 specific N-terminal regulatory domain [123, 172, 181] (see also **1.6.2**). The phosphorylation of Thr482 is supposed to partially disrupt the autoinhibitory mechanism of the AID/KD interplay [172], and we found this residue also phosphorylated in our studies (**Paper II**). The causes for the high Ca^{2+}/CaM-independency of CaMKK2-3 are currently unknown, but in terms of validating this isoform towards a PrCa dug target it might be important to elucidate this further. We have also performed an ATP-displacement assay in which we could show that ATP is able to outcompete STO-609, and thus capable to bind CaMKK2 in the absence of Ca^{2+}/CaM (**Paper II**). However, a question remains whether the ability of CaMKK2 to bind ATP independently of Ca^{2+}/CaM-binding implies that the kinase is fully active, meaning to be able to hydrolyse ATP and bind and phosphorylate substrate proteins. In the literature reviewed, there is no clear definition about the term activity in regard to kinases. What defines an active kinase? Is it the kinase being able to bind ATP, is it the one folding in the

previously described active conformation, is a kinase only considered to be active when successfully phosphorylating substrates? As kinases are dynamic proteins, changing conformation upon several influences like activators (Ca^{2+}/CaM-binding in the case of CaMKK2), autophosphorylation, and endogenous phosphorylation but also upon ATP-binding and substrate phosphorylation, it is difficult to define the exact 'active' state. It is a dynamic process, and different for each single kinase, and as such only structural data comparing various conformational states of a kinase upon binding to its influencers and in different phosphorylation states can give the full overview needed to define its full activity potential.

4.3.2 Phosphorylation of CaMKK2 prevents binding to STO-609, especially in the absence of Ca^{2+}/CaM

Early in this binding study we noticed a discrepancy in the binding stoichiometry between CaMKK2 and STO-609 between the reported 1 : 1 binding stoichiometry in the literature (STO-609 : CaMKK2 KD) [154] and our data (**Paper II**). We observed saturation of the fluorescence signal with an STO-609 concentration equivalent to a stoichiometry of 0.35 : 1 (210 nM CaMKK2 or CaMKK2:CaM vs. 60 nM STO-609). This suggested that for some reason only a sub-fraction of the kinase in our preparation was able to bind to STO-609 since CaMKK2 is known to exist in a monomeric form. We confirmed that the kinase was a monomer using size exclusion chromatography and this did not show any signs of higher oligomeric states (**Paper I**). In the course of purifying the kinase for crystallization we had observed that it was highly phosphorylated when expressed in bacteria (see also **4.2.4**). This led us to hypothesize that phosphorylation was the cause for the unexpected binding

stoichiometry. We tested this hypothesis by dephosphorylating our protein samples, and indeed phosphatase treatment led to a clear shift in the fluorescence saturation such that we now achieved the expected 1 : 1 stoichiometry between the drug and the kinase, but only in complex with calmodulin. In the 'apo' situation the stoichiometry shifted to 1 : 2 (STO-609 : CaMKK2). Phosphatase treatment also altered the K_D for STO-609 binding to both the CaMKK2:CaM complex and to 'apo' CaMKK2 but in opposite directions. For the complex the K_D shifted from 17 nM to 7 nM whereas the K_D for the 'apo' form increased to 49 nM. An additional implication of this experiment is that calmodulin may indeed have an impact on STO-609 binding and the ATP binding pocket but that expression in bacteria and concomitant phosphorylation could mask this. This is the first time that the stoichiometry of binding has been addressed using full-length kinase and therefore also underscores the importance of addressing these interactions with multiple domains present rather than just the kinase domain alone. Since this initial phosphatase treatment did not completely remove phospho-groups from the kinase, we extended the incubation time to see if this would further impact on the binding properties of the kinase. Unfortunately, the extended incubation time led to protein degradation and the complete loss of binding (data not shown). This may reflect a trace contamination of bacterial proteases in the purified sample as well as suggesting that phosphorylation can help to maintain folding. Our results support the findings that CaMKK2 can bind STO-609 even when not phosphorylated [154], however with much lower binding affinity. Reported phosphorylation sites connected with regulation of CaMKK2 are, beside Thr482 as described above (**4.3.1**), Ser129, Ser133, and Ser137, sequentially phosphorylated *in vitro* by the endogenous kinases CDK5 and GSK3 and meant to decrease the Ca^{2+}/CaM-independent, autonomous activity of the enzyme and regulating its

turnover *in vivo* [179] (**1.6.2**). The DAPK1 phosphorylation on CaMKK2 Ser511 is reported to attenuate subsequent CaM-dependent autophosphorylation of CaMKK [180] (**1.6.2**). In our protein samples, we found upon many other sites also Thr482, Ser137, and Ser511 to be phosphorylated (**Paper II**). Future studies must show the impact of our identified phosphorylation sites in the presence and absence of Ca^{2+}/CaM. The fluorescent assay we have developed is an ideal way to easily examine the impact on STO-609 binding of specific phosphorylation site mutants, ideally in a combination of both phospho-null mutants expressed in our current *E.coli* system, and phospho-mimetic mutants expressed in a cell-free expression system.

Our findings also highlight the importance of careful consideration of recombinant protein samples in use when studying kinase activity. Most often, phosphorylation levels are not taken into considerations and the total sample quantity is anticipated to be active or inactive. This is, as we have shown, a premature conclusion because a kinase sample is most likely never completely homogenous in terms of dynamic conformational states.

4.3.3 CaMKK2 Phe267 is the gatekeeper residue

The so-called 'gatekeeper' residue in kinases is typically a bulky amino acid at the ATP binding pocket (see also **1.6.4**). This single residue separates the adenine binding site from an adjacent hydrophobic pocket and regulates access to the hydrophobic pocket [211]. Mutational replacement of the gatekeeper residue to a larger hydrophobic residue has been shown to confer drug resistance [212, 213]. Gatekeeper mutants in which the gatekeeper residue has been replaced with smaller ones, like glycine or alanine, have been used to support chemical genetic screens for novel kinase substrates in combination with bulky

nucleotide analogues [214, 215]. With this method, developed by Shokat and colleagues, a radioisotope-labelled bulky ATP analogue, which is a poor substrate of wild-type protein kinases, is efficiently accepted by any kinase of interest by virtue of a mutation that enlarges the ATP-binding site to accommodate the bulky ATP analogue. The bulky nucleotide analogues can access the deeper hydrophobic pocket, and in that way kinase specific phosphorylation can identify novel substrates [214].

Due to an initially planned chemical genetic screen to identify novel substrates for CaMKK2, we set out to identify the gatekeeper residue of CaMKK2 and aligned the kinase sequence against CaMKKI and cyclin-dependent kinase 2 (CDK2), a recently used kinase in a chemical genetics approach [215]. In CaMKK2, Phe267 is the conserved gatekeeper residue, corresponding to CaMKK1 Phe230 and CDK2 Phe80, and we used this information to produce a recombinant CaMKK2 mutant in which Phe267 is replaced with glycine (CaMKK2 F267G) (**Paper I and II**).

The relevance of the CaMKK2 Phe267 gatekeeper residue was supported by the fact that the mutant CaMKK2 F267G was unable to bind the ATP-competitor STO-609, as described in **Paper II**. It is also reported that Phe267 is one of the residues STO-609 interacts with via hydrophobic binding [154]. Even though we identified the gatekeeper residue of CaMKK2, two neighbouring amino acid residues, Val270 and Pro274, are from more importance for the increased sensitivity of STO-609 towards CaMKK2 in comparison to CaMKK1 and other kinases (see also **1.6.4**). However, whereas a CaMKK2 P274E mutant was still affected by STO-609 treatment in activity (24.2 % inhibition) [154], the F267G mutation in CaMKK2 abolished any STO-609 binding to the recombinant kinase (**Paper II**).

4.3.4 Novel phosphorylation events in the CaMKK2 activation segment

We found several novel phosphorylation sites in the activation segment of CaMKK2 and more specifically in the activation loop; the region of the kinase whose phosphorylation often determines the activity of the kinase (see also **1.6.2**). Both, CaMKK2:CaM and 'apo' CaMKK2, showed phosphorylation sites at Ser334, Ser340, Ser345, Thr347 and Thr350 of the activation segment (**Paper II**). The conserved activation loop phosphorylation site in CaMKIV and protein kinase A (PKA) is lacking in CaMKK2 [154], and no other phosphorylated residue have yet been reported in the activation segment. Still, the structural model of CaMKK2 bound to STO-609, supports CaMKK2 to be a kinase with intrinsic activity, active without phosphorylation of the activation loop. The kinase presents the DFG-in conformation (see **1.6.2**), which is on its own not a sufficient criterion for an active kinase, but in addition has been shown to be able to phosphorylate the AMPK peptide [154]. However, this study used protein expressed in a cell-free system prior to structural characterizations and activity assays, allowing minimal posttranslational modifications to the expressed proteins, and as such does not give evidence that phosphorylation in the activation segment might not alter activity levels. It remains to be proven whether our novel phosphorylation sites, three of which within the activation loop, are physiological present and relevant, and if so whether they have an additional effect on STO-609 binding, or the ability to phosphorylate substrates.

4.3.5 Future implications

In conclusion, it remains to be elucidated in what context and to which extent Ca^{2+}/CaM-binding, reported phosphorylation sites, so far undescribed phosphorylation events, or even other activating factors contribute to CaMKK2 activity. There is a great deal of inaccuracy in the fairly young field describing the kinase, often describing CaMKK without making a clear distinction between CaMKK1 and CaMKK2, not taking CaMKK2 isoform-varieties into account, or using different substrate peptides to predict activity, and thus making it tedious to draw conclusions. However, this information is crucial for drug design and only full knowledge about the activation cascade in the specific disease context might enable drugs with superior specificity and minimal side effects at the same time.

Our new fluorescent assay which we describe in **Paper II** is the first example of a kinetic binding study that utilises the intrinsic fluorescent properties of STO-609 to assess and describe the binding properties with its target kinase in a pure *in vitro* system. As such, this assay has potential to be utilized to uncover some of the above mentioned issues. First, it could be used to successfully screen for novel inhibitors of CaMKK2 in a competitive or displacement assay on the basis that more effective ATP competitors should out compete STO-609, observed as a drop in fluorescence at 500 nm. This could directly benefit the drug development efforts towards CaMKK2 if the ATP-binding pocket is used as the target site. This assay would be applicable both for CaMKK2 and other kinases inhibited by STO-609 [86]. Secondly, STO-609 could be used as a fluorescent chemical probe, as the emission wavelength overlaps with the excitation wavelength for yellow fluorescent protein YFP (maximum excitation at 515 nm) [23]. This could potentially be used for describing binding

modes and dynamics in fluorescent resonance energy transfer (FRET) assays, equivalent to the classical FRET assay using cyan fluorescent protein (CFP) and YFP [24]. Scenarios such as (i) STO-609 binding to the various CaMKK2 (YFP-tagged) isoforms, (ii) STO-609-bound CaMKK2 incubated with CaM (YFP-tagged), or (iii) the impact of mutations and other regulatory modifications and interactions on the ATP-binding pocket, would all increase structure/function-related knowledge on the important cancer target CaMKK2. Information accumulated in such *in vitro* settings could form the basis for analysing CaMKK2 and CaM binding directly in a cellular setting. The CaMKK2 and CaM interaction studies would, in addition, serve as the benchmark assay for exploring novel complex partners. The above described assay development could also be used to test whether our novel identified CaMKK2 interaction partner, Gemin4 (**Paper III**), interacts with the STO-609 bound CaMKK2 both *in vitro* and possibly in cells.

4.4 CaMKK2 IS ASSOCIATED WITH COMPONENTS OF THE SMN COMPLEX

At the time we initiated the project, there were no further interacting proteins for CaMKK2 known beside CaM. Searching for interaction partners is always important to evaluate the physiological significance of a protein. With CaMKK2 playing a role in metabolism and being AR-regulated and overexpressed in PrCa (see also **1.4**), we set out to identify novel interactors of CaMKK2 in general (human embryonic kidney cells, HEK293) and in PrCa in particular (LNCaP model). The LNCaP cell line is one of two PrCa cell lines we have used in the studies included in this thesis (**Paper III and IV**). Both, LNCaP and VCaP lines are widely used androgen-responsive and AR-expressing PrCa cell lines. The LNCaP cell line has derived from a metastatic lesion to the supraclavicular lymph node of a Caucasian CRPC patient [216, 217], whereas VCaP cells derived from a metastatic lesion to a lumbar vertebral body of a patient with CRPC [218]. The LNCaP cell line is one of the most used in vitro model systems for PrCa research due to its extensive characterization. After preparing CaMKK2-enriched cell lysates for HEK293 and LNCaP cells, we have co-immunoprecipitated proteins bound to CaMKK2 and identified them with subsequent mass spectrometry. Interestingly, we found consistently proteins belonging to a multiprotein complex, the survival of motor neurons (SMN) complex, with SMN and the DEAD box protein 20 (DDX20), also called Gemin3, present in all samples. Further reciprocal co-immunoprecipitation (co-IP) experiments validated the Gemin4 interaction. Via peptide arrays, we are currently working on mapping the specific binding site (**Paper III**).

4.4.1 Protein-protein interaction analysis

Both, co-IPs and peptide arrays are useful methods to explore and validate novel interaction partners of proteins. However, they do have their limitations. Co-IPs, in which the specific antibody precipitates the target antigen that again co-precipitates a binding partner/protein complex from a lysate, can be affected by various factors including protein folding, antibody specificity, the strength of the interaction between 'bait' and 'prey' protein and the stringency of the wash steps. To validate our initial hits, we used cells transfected with either a CaMKK2 or an SMN expression vector and then performed co-IPs. We both detected and co-immunoprecipitated endogenous Gemin3 and Gemin4 from these overexpression cell lysates (**Paper III**). In contrast to co-IPs, peptide arrays, which we used to validate further the interaction and map the exact binding sites between CaMKK2 and Gemin4 only assess interactions that are based on the primary sequence of the arrayed protein. Consequently bona fide interactions that are not dependent on short unstructured peptide motifs may give negative results however the protein-protein interaction can still be real. Whilst there are many examples of motif-based protein-protein interactions they are by no means the only determinants of protein-protein interactions. Often however, protein-protein interactions are dependent on the three-dimensional fold of the proteins which brings single binding motifs in close neighbourhood and only the sum of various binding sites enables the binding. The peptides, linked to a membrane, are simply too short to resemble physiologically folded proteins. Despite these caveats short peptide motifs are known to mediate protein-protein interactions and thus peptide arrays can be a powerful tool to identify interaction sites [219, 220]. We have identified a short 5 aa sequence (FSQNA) in Gemin4 which we consistently

detected in overlapping peptides (**Paper III**). Gemin4 is already known to bind to other proteins through short peptide motifs and in the same region there are multiple LxxLL motifs that mediate its binding to the mineralocorticoid receptor (MR) and other Nuclear Hormone Receptor (NHR) superfamily members including the AR [221]. For short peptide motifs to be accessible to mediate protein-protein interactions they often occur in relatively disordered regions of proteins. The fact that there are already validated peptide motifs in this region for other interactions implies that Gemin4 may be disordered [222].

Other methods to further validate and prove protein-protein interactions include (i) *in vitro* pull-down assays [223], label transfer [224], NMR, X-ray crystallography or (ii) *in vivo* yeast-two hybrid system, crosslinking reagents, or immunofluorescence or FRET assays. A modified FRET assay, based on our fluorescent assay described in **Paper II**, has been proposed earlier (**section 4.3.5**) as a means of further testing the interaction between CaMKK2 and Gemin4.

4.4.2 CaMKK2 interacts with Gemin4, a subunit of the SMN complex and a corepressor for NHRs

Gemin4 is, as described in more detail in **Paper III**, part of the multiprotein SMN complex. We propose a CaMKK2-Gemin4 interaction by co-immunoprecipitation, and we used lysis conditions that provide whole cell lysate. Both the SMN complex and CaMKK2 are reported to be present throughout the cell. The SMN complex is found throughout the cytoplasm and in lower levels in the nucleus where it is concentrated in discrete bodies called Gems [225, 226]. Gemin4 is the only component of the SMN complex that also localizes to the nucleolus [227]. In progressing PrCa, CaMKK2 has been reported to increasingly shift its localisation

pattern from predominantly cytoplasmic to a mixed cytoplasmic/perinuclear/nuclear pattern in high grade clinical specimens, with the nuclear translocation induced by DHT [111] (see also **1.4**). The SMN complex is functionally closely associated with the small nuclear ribonucleoproteins (snRNP) biogenesis in the cytoplasm and its import into the nucleus and this in turn is required for efficient RNA splicing [228-235]. Complex assembly is sensitive to metabolic stress and has been shown to be disrupted by the treatment of cells with metabolic inhibitors that are also anti-cancer drugs like Rapamycin (mTOR inhibitor) [236]. However, Gemin3 and Gemin4 can also form an SMN-independent complex with AGO1 (Argonuate) and in this setting they regulate microRNA biogenesis [237]. Gemin4 was in addition recently reported to interact with NHRs and to act as a transcriptional corepressor for the mineralocorticoid receptor but also at higher concentrations to function as an AR corepressor [221].

4.4.3 Future implications

The impact on the AR and NHR signalling is the most clinically relevant of the described functions Gemin4 is associated with. CaMKK2 is androgen regulated [103], nuclear in high-grade disease [111], and an AR coactivator [111]. We could show that Gemin4 is downregulated by androgen (**Paper III**), using the same array data than in Massie *et al.* [103]. Furthermore in clinical prostate cancer datasets there are small numbers of patient samples in which Gemin4 is downregulated and CaMKK2 is overexpressed [7, 238]. It would be interesting to determine whether these patients have a poorer prognosis than simply CaMKK2 overexpressing cases in total and whether there is competition between the AR and

CaMKK2 for Gemin4 binding given that Gemin4 has peptide motifs that can potentially allow both to bind to the same region.

4.5 CaMKK2 AND AUTOPHAGY

4.5.1 Autophagy and prostate cancer

Macroautophagy, hereafter referred to as autophagy, is the highly conserved homeostatic process coordinating the cellular use of available nutrients dependent on the cellular environment. Whereas basal autophagy maintains the cellular homeostasis by metabolizing cytoplasmic protein waste through the lysosomal system, starvation initiates autophagy by stimulation of AMPK which in turn blocks mTOR and thus activates autophagy [239] (see also **1.5.1**). Even though autophagy is generally thought of as a beneficial survival mechanism and can indeed suppress tumour development [240, 241], it is not always protective; in established tumours it can enhance nutrient utilization and therefore improve growth characteristics of cancer cells facilitating carcinogenesis [241-243]. Moreover, it can contribute to greater cellular robustness towards therapeutic intervention[244], making adjuvant autophagy inhibition a promising area of research.

In advanced PrCa, autophagy facilitates both disease progression and therapeutic resistance [239]. Knowing that AR is the key driver for progression of PrCa we and others hypothesized that AR-mediated CaMKK2-AMPK signalling could promote PrCa through increasing cellular rates of autophagy [245, 246]. Moreover, CaMKK2 has already been directly linked to autophagy as a mediator [120, 121, 247-250], thus potentially making CaMKK2 an ideal therapeutic target where inhibition of both cell proliferation and autophagy could be achieved simultaneously.

4.5.2 Challenges in the assessment of autophagy

A major challenge in the field of autophagy research is the controversy regarding appropriate assays. Even though there has been an exponential growth in the number of publications in the recent years, the consensus on how to assess properly a dynamic process such as autophagy is lacking behind [251]. The main challenge is how to capture a 'dynamic process' like autophagic flux with 'static measurements' [252]. Still, one of the most popular ways to assess autophagy is to measure LC3 lipidation levels by either fluorescence microscopy or biochemical assays. In these assays, LC3-I represents cytoplasmic LC3, whereas LC3-II is associated with inner and outer autophagosome membranes [253]. However, the often made conclusion that increased LC3-II levels correspond to higher cellular autophagic activity must be considered with care. Several reasons for increased number of autophagosomes can e.g. be increased autophagy induction or suppression of steps in the autophagy pathway downstream of autophagosome formation, perhaps due to delayed trafficking to the lysosomes, reduced fusion between compartments or impaired lysosomal proteolytic activity [252]. To measure autophagic flux as a dynamic process, researchers are prompted these days to use state-of-the-art assays that monitor autophagic flux such as the long-lived protein degradation (LLPD) or lactate dehydrogenase (LDH)-sequestration assays [251, 254-256], and that are the assays we have used to obtain our data (**Paper IV**).

4.5.3 CaMKK2 does not regulate autophagy in prostate cancer

To test the importance of CaMKK2, we have used direct CaMKK2 knockdowns via siRNA and STO-609 in both LNCaP and VCaP cells, two widely used androgen-responsive and AR-expressing PrCa cell lines [216-218] (see also **4.4**). No changes in basal, inhibited, or induced autophagy levels could be detected, indicating that CaMKK2 is not a mediator of autophagy in PrCa (**Paper IV**). This finding contradicts the reported role of Ca^{2+}-regulated autophagy via CaMKK2-AMPK signalling [120]. However, in this study Høyer-Hansen and colleagues have not only used different cell lines (the human breast cancer cell line MCF7), but also verified autophagy via LC3-lipidation with the implications described in the previous paragraph. Our group has previously shown that even though calcium ion perturbations in prostate cancer cell lines upregulate LC3 lipidation, functional autophagy is inhibited [257]. This further highlights the challenge in interpreting results from static autophagy markers such as LC3-II but also the importance of assessing carefully cell-type specific effects.

Our finding that CaMKK2 is not a central mediator of autophagy in prostate cancer cells obviates the need for further attempts to use the target CaMKK2 as a means of altering autophagy. Whether AMPK itself is not required for autophagy activation, as proposed by several groups [258, 259], or activation of AMPK in prostate cancer is achieved by another upstream kinase, like LKB1 or TGF-β activated kinase-1 (TAK1) [260], remains to be examined.

4.5.4 STO-609 activates autophagy in prostate cancer cells

Moreover, we found in our study that long-term treatment with STO-609 can actually activate autophagy (**Paper IV**). Our results indicated that STO-609 activates bulk autophagic activity in VCaP cells. Interestingly, the inducing effect of STO-609 on autophagy was accompanied by increases in LC3-II lipidation levels upon STO-609 treatment, at the same time AMPK phosphorylation was sustainably hampered by STO-609.

As described in earlier sections of this thesis, STO-609 is a selective but not highly specific inhibitor of CaMKK2 [86, 110] (see also **1.6.4** and **4.1**). Even though kinase inhibitors targeting several related kinases might be advantageous in terms of cancer treatment [155], kinase inhibitors employed as research tools should ideally only target one selective target. It makes it otherwise difficult to attribute certain results solely on the inhibited kinase, and uncharacterised off-target effects potentially falsify the results. Often, STO-609 is used at 10-20 μM when applied to cell lines, but inhibits a number of other kinases including one of its primary substrates, AMPK, at a 1 μM concentration in the same assays [86]. The 10-20 μM concentration used in cell lines is because that is the minimal STO-609 concentration needed to show an effect, based on a reduction in AMPK phosphorylation [140]. At these concentrations reduction in AMPK phosphorylation could be due to effects on CaMKK2 and /or on a number of inhibited kinases including AMPK and AMPK regulators, like LKB1. The lack of STO-609 specificity is also an explanation for the difference between our data from CaMKK2 inhibition with siRNA and STO-609 treatment (**Paper IV**). siCaMKK2 does not influence autophagy, whereas STO-609 leads to an increase of autophagy in VCaP cells. We have access to so far unpublished data of a STO-609 kinase screen in which the MRC Protein

Phosphorylation Unit (University of Dundee) repeated the panel published in 2007 [261] with more kinases (**Appendix 1**). In the original study from 2007, AMPK and other kinases were inhibited significantly *in vitro* with STO-609 concentrations of 1 μM or more [261]. In the new dataset, they detected the mammalian homologue of yeast ATG1, Ulk1, as a potential STO-609 activated target protein. Ulk1 is a downstream kinase of AMPK and mTOR and the only known STK in the autophagy pathway. Ulk1 phosphorylation can lead to activation, p-Ser555 via AMPK [262], or inhibition, p-Ser757 via mTOR [263]. We have detected an increase of S555 phosphorylation and a decrease of S757 phosphorylation in Ulk1 upon STO-609 treatment in VCaP cells (data not shown). These insights come from the basis of anew follow-up study.

Combining all our results, (i) CaMKK2 specific knockdown via siRNA does not influence autophagy (**Paper IV**), (ii) STO-609 enhances autophagy (**Paper IV**), (iii) STO-609 is a potential Ulk1 activator (**Appendix 1**), and (iv) STO-609 treatment might enhance Ulk1 p-Ser555 levels (our unpublished data), we propose that the increase in autophagy we see upon STO-609 might be via direct STO-609 activation of Ulk1, independently from CaMKK2 and/or AMPK activation. Moreover, DAPK1 which is another important regulator of cell death and autophagy is in the same level inhibited by STO-609 than CaMKK2, whereas the AMPK upstream kinase LKB1 has been found to be potentially activated by STO-609 in the same kinase screen (**Appendix 1**). In summary, STO-609 is known to inhibit CaMKK2, DAPK1, and AMPK, while potentially activating Ulk1 and LKB1, all five proteins involved to some extent in the early steps of autophagy initiation. This even more supports our endeavours to develop novel, more specific CaMKK2 inhibitors, and until then we

recommend using siCaMKK2 and more targeted genome editing approaches in order to specifically assess the role of the specific kinase in any cellular context.

4.5.5 Future implications

Even though STO-609 is not a specific CaMKK2 inhibitor, STO-609 might still be useful in treating metabolic diseases or cancer. In Massie *et al*. [103], they found that STO-609 treatment alone was cytostatic not cytotoxic – in other words it reduced proliferation but did not significantly kill the cells over time. They also found that STO-609 reduced glucose metabolism and downstream anabolic metabolism and so might be expected to impose metabolic stress on cells simulating nutrient deprivation. Our findings presented in **Paper IV** using VCaP cells corroborate with a recent study using the human gastric adenocarcinoma cell lines (SNU-1 and N87) that showed that STO-609 induced autophagy independently of CaMKK2 [264]. It is now of interest to determine whether autophagy inhibition sensitises VCaPs for STO-609. Dr. Engedal has got preliminary data indicating that STO-609 is more cytotoxic to LNCaP cells when co-treated with the well-studied autophagy inhibitor chloroquine (CQ; [265]) (data not shown). In future studies, CaMKK2 inhibition will need to be combined with other drugs and autophagy inhibitors are worthy of further study in this setting.

5 REFERENCES

1. *2016 Annual Report on Prostate Diseases*, G.M. B., Editor. 2016, Harvard Medical School.
2. Ferlay, J., et al., *Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012.* Int J Cancer, 2015. **136**(5): p. E359-86.
3. M. Ervik, F.L., J. Ferlay, L. Mery, I. Soerjomataram, F. Bray, *International Agency for Research on Cancer*, F. Cancer Today. Lyon, Editor. 2016: Available from: http://gco.iarc.fr/today.
4. Moller, M.H., et al., *Trends in stage-specific incidence of prostate cancer in Norway, 1980-2010: A population-based study.* BJU Int, 2015.
5. Stegeman, S., et al., *A Large-Scale Analysis of Genetic Variants within Putative miRNA Binding Sites in Prostate Cancer.* Cancer Discov, 2015. **5**(4): p. 368-79.
6. Al Olama, A.A., et al., *A meta-analysis of 87,040 individuals identifies 23 new susceptibility loci for prostate cancer.* Nat Genet, 2014. **46**(10): p. 1103-9.
7. Robinson, D., et al., *Integrative clinical genomics of advanced prostate cancer.* Cell, 2015. **161**(5): p. 1215-28.
8. Bancroft, E.K., et al., *Targeted prostate cancer screening in BRCA1 and BRCA2 mutation carriers: results from the initial screening round of the IMPACT study.* Eur Urol, 2014. **66**(3): p. 489-99.
9. Visakorpi, T., et al., *In vivo amplification of the androgen receptor gene and progression of human prostate cancer.* Nat Genet, 1995. **9**(4): p. 401-6.
10. Koivisto, P., et al., *Androgen receptor gene amplification: a possible molecular mechanism for androgen deprivation therapy failure in prostate cancer.* Cancer Res, 1997. **57**(2): p. 314-9.
11. Albright, F., et al., *Prostate cancer risk prediction based on complete prostate cancer family history.* Prostate, 2015. **75**(4): p. 390-8.
12. Carter, B.S., et al., *Mendelian inheritance of familial prostate cancer.* Proc Natl Acad Sci U S A, 1992. **89**(8): p. 3367-71.
13. Steinberg, G.D., et al., *Family history and the risk of prostate cancer.* Prostate, 1990. **17**(4): p. 337-47.
14. Crawford, E.D., *Epidemiology of prostate cancer.* Urology, 2003. **62**(6a): p. 3-12.
15. Center, M.M., et al., *International variation in prostate cancer incidence and mortality rates.* Eur Urol, 2012. **61**(6): p. 1079-92.
16. Yang, M., et al., *Dietary patterns after prostate cancer diagnosis in relation to disease-specific and total mortality.* Cancer Prev Res (Phila), 2015. **8**(6): p. 545-51.
17. Rider J, W.K., Kelly R, et al. *Ejaculation frequency and risk of prostate cancer: updated results from the health professionals follow-up study*. in *2015 Annual Meeting of the American Urological Association*. 2015. New Orleans, LA.
18. Dennis, L.K., C.F. Lynch, and J.C. Torner, *Epidemiologic association between prostatitis and prostate cancer.* Urology, 2002. **60**(1): p. 78-83.
19. Siddiqui, M.M., et al., *Vasectomy and risk of aggressive prostate cancer: a 24-year follow-up study.* J Clin Oncol, 2014. **32**(27): p. 3033-8.

20. Sfanos, K.S. and A.M. De Marzo, *Prostate cancer and inflammation: the evidence.* Histopathology, 2012. **60**(1): p. 199-215.

21. Siegel, R.L., K.D. Miller, and A. Jemal, *Cancer statistics, 2015.* CA Cancer J Clin, 2015. **65**(1): p. 5-29.

22. Draisma, G., et al., *Lead time and overdiagnosis in prostate-specific antigen screening: importance of methods and context.* J Natl Cancer Inst, 2009. **101**(6): p. 374-83.

23. Gaylis, F., et al., *Prostate Biopsy Complications: A Dual Analysis.* Urology, 2016. **93**: p. 135-40.

24. Whitson, J.M., K.S. Murray, and J.B. Thrasher, *Prostate Biopsy is Associated with an Increased Risk of Erectile Dysfunction.* J Urol, 2016. **196**(1): p. 21-3.

25. Cheng, L., et al., *Staging of prostate cancer.* Histopathology, 2012. **60**(1): p. 87-117.

26. Halabi, S., et al., *Meta-Analysis Evaluating the Impact of Site of Metastasis on Overall Survival in Men With Castration-Resistant Prostate Cancer.* Journal of Clinical Oncology, 2016.

27. Epstein, J.I., et al., *A Contemporary Prostate Cancer Grading System: A Validated Alternative to the Gleason Score.* Eur Urol, 2016. **69**(3): p. 428-35.

28. Bangma, C.H., M. Bul, and M. Roobol, *The Prostate cancer Research International: Active Surveillance study.* Curr Opin Urol, 2012. **22**(3): p. 216-21.

29. Bangma, C.H., et al., *Active surveillance for low-risk prostate cancer.* Crit Rev Oncol Hematol, 2013. **85**(3): p. 295-302.

30. Ganz, P.A., et al., *National Institutes of Health State-of-the-Science Conference: role of active surveillance in the management of men with localized prostate cancer.* Ann Intern Med, 2012. **156**(8): p. 591-5.

31. Tosoian, J.J., et al., *Intermediate and Longer-Term Outcomes From a Prospective Active-Surveillance Program for Favorable-Risk Prostate Cancer.* J Clin Oncol, 2015. **33**(30): p. 3379-85.

32. Lloyd, T., et al., *Lifetime risk of being diagnosed with, or dying from, prostate cancer by major ethnic group in England 2008-2010.* BMC Med, 2015. **13**: p. 171.

33. Martins, R.N. and S. Gandy, *Prostate cancer: Increased dementia risk following androgen deprivation therapy?* Nat Rev Urol, 2016.

34. Gandaglia, G., et al., *Comparative effectiveness of robot-assisted and open radical prostatectomy in the postdissemination era.* J Clin Oncol, 2014. **32**(14): p. 1419-26.

35. Healy, K.A. and L.G. Gomella, *Retropubic, laparoscopic, or robotic radical prostatectomy: is there any real difference?* Semin Oncol, 2013. **40**(3): p. 286-96.

36. Barqawi, A.B., et al., *Targeted focal therapy for the management of organ confined prostate cancer.* J Urol, 2014. **192**(3): p. 749-53.

37. Valerio, M., et al., *The role of focal therapy in the management of localised prostate cancer: a systematic review.* Eur Urol, 2014. **66**(4): p. 732-51.

38. Eisemann, N., et al., *The ProCaSP study: quality of life outcomes of prostate cancer patients after radiotherapy or radical prostatectomy in a cohort study.* BMC Urol, 2015. **15**: p. 28.

39. Resnick, M.J., et al., *Long-term functional outcomes after treatment for localized prostate cancer.* N Engl J Med, 2013. **368**(5): p. 436-45.

40. Sooriakumaran, P., et al., *Comparative effectiveness of radical prostatectomy and radiotherapy in prostate cancer: observational study of mortality outcomes.* BMJ, 2014. **348**: p. g1502.

41. *American Cancer Society*. 2016.

42. Feldman, B.J. and D. Feldman, *The development of androgen-independent prostate cancer.* Nat Rev Cancer, 2001. **1**(1): p. 34-45.

43. Yuan, X., et al., *Androgen receptor functions in castration-resistant prostate cancer and mechanisms of resistance to new agents targeting the androgen axis.* Oncogene, 2014. **33**(22): p. 2815-25.

44. Narayanan, S., S. Srinivas, and D. Feldman, *Androgen-glucocorticoid interactions in the era of novel prostate cancer therapy.* Nat Rev Urol, 2016. **13**(1): p. 47-60.

45. Gregory, C.W., et al., *Androgen receptor stabilization in recurrent prostate cancer is associated with hypersensitivity to low androgen.* Cancer Research, 2001. **61**(7): p. 2892-2898.

46. Madan, R.A. and P.M. Arlen, *Abiraterone. Cougar Biotechnology.* IDrugs, 2006. **9**(1): p. 49-55.

47. Tran, C., et al., *Development of a Second-Generation Antiandrogen for Treatment of Advanced Prostate Cancer.* Science, 2009. **324**(5928): p. 787-790.

48. James ND, S.M., Mason MD, et al. , *Docetaxel and/or zoledronic acid for hormone-naïve prostate cancer: first overall survival results from STAMPEDE (NCT00268476)*, in *American Society of Clinical Oncology Annual Meeting 2015*. 2015: Chicago, IL.

49. Coleman, R.E., *Clinical features of metastatic bone disease and risk of skeletal morbidity.* Clin Cancer Res, 2006. **12**(20 Pt 2): p. 6243s-6249s.

50. Parker, C., et al., *Alpha emitter radium-223 and survival in metastatic prostate cancer.* N Engl J Med, 2013. **369**(3): p. 213-23.

51. Strauss, J., R.A. Madan, and W.D. Figg, *Evaluating immune responses after sipuleucel-T therapy.* Cancer Biol Ther, 2015. **16**(8): p. 1119-21.

52. Delwar, Z., et al., *Oncolytic virotherapy for urological cancers.* Nat Rev Urol, 2016. **13**(6): p. 334-52.

53. DeWeese, T.L., et al., *A phase I trial of CV706, a replication-competent, PSA selective oncolytic adenovirus, for the treatment of locally recurrent prostate cancer following radiation therapy.* Cancer Res, 2001. **61**(20): p. 7464-72.

54. Freytag, S.O., et al., *Phase I study of replication-competent adenovirus-mediated double suicide gene therapy for the treatment of locally recurrent prostate cancer.* Cancer Res, 2002. **62**(17): p. 4968-76.

55. Freytag, S.O., et al., *Prospective randomized phase 2 trial of intensity modulated radiation therapy with or without oncolytic adenovirus-mediated cytotoxic gene therapy in intermediate-risk prostate cancer.* Int J Radiat Oncol Biol Phys, 2014. **89**(2): p. 268-76.

56. Small, E.J., et al., *A phase I trial of intravenous CG7870, a replication-selective, prostate-specific antigen-targeted oncolytic adenovirus, for the treatment of hormone-refractory, metastatic prostate cancer.* Mol Ther, 2006. **14**(1): p. 107-17.

57. Comins, C., et al., *REO-10: a phase I study of intravenous reovirus and docetaxel in patients with advanced cancer.* Clin Cancer Res, 2010. **16**(22): p. 5564-72.

58. Vidal, L., et al., *A phase I study of intravenous oncolytic reovirus type 3 Dearing in patients with advanced cancer.* Clin Cancer Res, 2008. **14**(21): p. 7127-37.

59. Cookson, M.S., et al., *Castration-resistant prostate cancer: AUA guideline amendment.* J Urol, 2015. **193**(2): p. 491-9.

60. Lund Haheim, L., et al., *Metabolic syndrome predicts prostate cancer in a cohort of middle-aged Norwegian men followed for 27 years.* Am J Epidemiol, 2006. **164**(8): p. 769-74.

61. Esposito, K., et al., *Effect of metabolic syndrome and its components on prostate cancer risk: meta-analysis.* J Endocrinol Invest, 2013. **36**(2): p. 132-9.

62. Hammarsten, J. and R. Peeker, *Urological aspects of the metabolic syndrome.* Nat Rev Urol, 2011. **8**(9): p. 483-94.

63. Lawrence, Y.R., et al., *Association between metabolic syndrome, diabetes mellitus and prostate cancer risk.* Prostate Cancer Prostatic Dis, 2013. **16**(2): p. 181-6.

64. Morote, J., et al., *Metabolic syndrome increases the risk of aggressive prostate cancer detection.* BJU Int, 2013. **111**(7): p. 1031-6.

65. Xiang, Y.Z., et al., *The association between metabolic syndrome and the risk of prostate cancer, high-grade prostate cancer, advanced prostate cancer, prostate cancer-specific mortality and biochemical recurrence.* J Exp Clin Cancer Res, 2013. **32**: p. 9.

66. Hitron, A., et al., *The influence of antidiabetic medications on the development and progression of prostate cancer.* Cancer Epidemiol, 2012. **36**(4): p. e243-50.

67. Lehman, D.M., et al., *Statin use as a moderator of metformin effect on risk for prostate cancer among type 2 diabetic patients.* Diabetes Care, 2012. **35**(5): p. 1002-7.

68. Zhang, Y. and T. Zang, *Association between statin usage and prostate cancer prevention: a refined meta-analysis based on literature from the years 2005-2010.* Urol Int, 2013. **90**(3): p. 259-62.

69. Andreoiu, M. and L. Cheng, *Multifocal prostate cancer: biologic, prognostic, and therapeutic implications.* Hum Pathol, 2010. **41**(6): p. 781-93.

70. Warburg, O., *On the origin of cancer cells.* Science, 1956. **123**(3191): p. 309-14.

71. Popovics, P., et al., *Targeting the 5'-AMP-activated protein kinase and related metabolic pathways for the treatment of prostate cancer.* Expert Opin Ther Targets, 2015. **19**(5): p. 617-32.

72. Dang, C.V., *Links between metabolism and cancer.* Genes Dev, 2012. **26**(9): p. 877-90.

73. Reitzer, L.J., B.M. Wice, and D. Kennell, *Evidence that glutamine, not sugar, is the major energy source for cultured HeLa cells.* J Biol Chem, 1979. **254**(8): p. 2669-76.

74. Gorlov, I.P., et al., *Candidate pathways and genes for prostate cancer: a meta-analysis of gene expression data.* BMC Med Genomics, 2009. **2**: p. 48.

75. Gorlov, I.P., et al., *GWAS meets microarray: are the results of genome-wide association studies and gene-expression profiling consistent? Prostate cancer as an example.* PLoS One, 2009. **4**(8): p. e6511.

76. Ford, W.C. and A. Harrison, *The role of citrate in determining the activity of calcium ions in human semen.* Int J Androl, 1984. **7**(3): p. 198-202.

77. Barfeld, S.J., et al., *Androgen-regulated metabolism and biosynthesis in prostate cancer.* Endocr Relat Cancer, 2014. **21**(4): p. T57-66.

78. Costello, L.C., et al., *Zinc inhibition of mitochondrial aconitase and its importance in citrate metabolism of prostate epithelial cells.* J Biol Chem, 1997. **272**(46): p. 28875-81.

79. Cortesi, M., et al., *New prospective for non-invasive detection, grading, size evaluation, and tumor location of prostate cancer.* Prostate, 2010. **70**(15): p. 1701-8.

80. Ferraldeschi, R., et al., *PTEN protein loss and clinical outcome from castration-resistant prostate cancer treated with abiraterone acetate.* Eur Urol, 2015. **67**(4): p. 795-802.

81. Lee, S.H., et al., *Crosstalking between androgen and PI3K/AKT signaling pathways in prostate cancer cells.* J Biol Chem, 2015. **290**(5): p. 2759-68.

82. Edlind, M.P. and A.C. Hsieh, *PI3K-AKT-mTOR signaling in prostate cancer progression and androgen deprivation therapy resistance.* Asian J Androl, 2014. **16**(3): p. 378-86.

83. Deprez, J., et al., *Phosphorylation and activation of heart 6-phosphofructo-2-kinase by protein kinase B and other protein kinases of the insulin signaling cascades.* J Biol Chem, 1997. **272**(28): p. 17269-75.

84. Elstrom, R.L., et al., *Akt stimulates aerobic glycolysis in cancer cells.* Cancer Res, 2004. **64**(11): p. 3892-9.

85. Hardie, D.G., F.A. Ross, and S.A. Hawley, *AMPK: a nutrient and energy sensor that maintains energy homeostasis.* Nat Rev Mol Cell Biol, 2012. **13**(4): p. 251-62.

86. Hawley, S.A., et al., *Calmodulin-dependent protein kinase kinase-beta is an alternative upstream kinase for AMP-activated protein kinase.* Cell Metab, 2005. **2**(1): p. 9-19.

87. Hawley, S.A., et al., *Complexes between the LKB1 tumor suppressor, STRAD alpha/beta and MO25 alpha/beta are upstream kinases in the AMP-activated protein kinase cascade.* J Biol, 2003. **2**(4): p. 28.

88. Hong, S.P., et al., *Activation of yeast Snf1 and mammalian AMP-activated protein kinase by upstream kinases.* Proc Natl Acad Sci U S A, 2003. **100**(15): p. 8839-43.

89. Shaw, R.J., et al., *The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress.* Proc Natl Acad Sci U S A, 2004. **101**(10): p. 3329-35.

90. Woods, A., et al., *LKB1 is the upstream kinase in the AMP-activated protein kinase cascade.* Curr Biol, 2003. **13**(22): p. 2004-8.

91. Munkley, J., et al., *A novel androgen-regulated isoform of the TSC2 tumour suppressor gene increases cell proliferation.* Oncotarget, 2014. **5**(1): p. 131-9.

92. Momcilovic, M. and D.B. Shackelford, *Targeting LKB1 in cancer - exposing and exploiting vulnerabilities.* British Journal of Cancer, 2015. **113**(4): p. 574-584.

93. Feldman, R.J., V.I. Sementchenko, and D.K. Watson, *The epithelial-specific Ets factors occupy a unique position in defining epithelial proliferation, differentiation and carcinogenesis.* Anticancer Res, 2003. **23**(3A): p. 2125-31.

94. Carlsson, P. and M. Mahlapuu, *Forkhead transcription factors: key players in development and metabolism.* Dev Biol, 2002. **250**(1): p. 1-23.

95. Robinson, J.L., et al., *Elevated levels of FOXA1 facilitate androgen receptor chromatin binding resulting in a CRPC-like phenotype.* Oncogene, 2014. **33**(50): p. 5666-74.

96. Sahu, B., et al., *Dual role of FoxA1 in androgen receptor binding to chromatin, androgen signalling and prostate cancer.* EMBO J, 2011. **30**(19): p. 3962-76.

97. Cillo, C., et al., *Homeobox genes in normal and malignant cells.* J Cell Physiol, 2001. **188**(2): p. 161-9.

98. Miller, D.M., et al., *c-Myc and cancer metabolism.* Clin Cancer Res, 2012. **18**(20): p. 5546-53.

99. Mills, I.G., *Maintaining and reprogramming genomic androgen receptor activity in prostate cancer.* Nat Rev Cancer, 2014. **14**(3): p. 187-98.

100. Pritchard, C.C. and P.S. Nelson, *Gene expression profiling in the developing prostate.* Differentiation, 2008. **76**(6): p. 624-40.

101. Bonkhoff, H. and K. Remberger, *Widespread distribution of nuclear androgen receptors in the basal cell layer of the normal and hyperplastic human prostate.* Virchows Arch A Pathol Anat Histopathol, 1993. **422**(1): p. 35-8.

102. Heemers, H., et al., *Androgens stimulate coordinated lipogenic gene expression in normal target tissues in vivo.* Mol Cell Endocrinol, 2003. **205**(1-2): p. 21-31.

103. Massie, C.E., et al., *The androgen receptor fuels prostate cancer by regulating central metabolism and biosynthesis.* EMBO J, 2011. **30**(13): p. 2719-33.

104. Sharma, N.L., et al., *The androgen receptor induces a distinct transcriptional program in castration-resistant prostate cancer in man.* Cancer Cell, 2013. **23**(1): p. 35-47.

105. Li, Y., et al., *Androgen receptor splice variants mediate enzalutamide resistance in castration-resistant prostate cancer cell lines.* Cancer Res, 2013. **73**(2): p. 483-9.

106. Balbas, M.D., et al., *Overcoming mutation-based resistance to antiandrogens with rational drug design.* Elife, 2013. **2**: p. e00499.

107. Rhodes, D.R., et al., *ONCOMINE: A cancer microarray database and integrated data-mining platform.* Neoplasia, 2004. **6**(1): p. 1-6.

108. Varambally, S., et al., *Integrative genomic and proteomic analysis of prostate cancer reveals signatures of metastatic progression.* Cancer Cell, 2005. **8**(5): p. 393-406.

109. Ashida, S., et al., *Molecular features of the transition from prostatic intraepithelial neoplasia (PIN) to prostate cancer: genome-wide gene-expression profiles of prostate cancers and PINs.* Cancer Res, 2004. **64**(17): p. 5963-72.

110. Tokumitsu, H., et al., *STO-609, a specific inhibitor of the Ca(2+)/calmodulin-dependent protein kinase kinase.* J Biol Chem, 2002. **277**(18): p. 15813-8.

111. Karacosta, L.G., et al., *A regulatory feedback loop between Ca2+/calmodulin-dependent protein kinase kinase 2 (CaMKK2) and the androgen receptor in prostate cancer progression.* J Biol Chem, 2012. **287**(29): p. 24832-43.

112. Shima, T., et al., *Down-regulation of calcium/calmodulin-dependent protein kinase kinase 2 by androgen deprivation induces castration-resistant prostate cancer.* Prostate, 2012. **72**(16): p. 1789-801.

113. Fu, H., et al., *MicroRNA-224 and its target CAMKK2 synergistically influence tumor progression and patient prognosis in prostate cancer.* Tumour Biol, 2015. **36**(3): p. 1983-91.

114. Bartel, D.P., *MicroRNAs: Genomics, biogenesis, mechanism, and function.* Cell, 2004. **116**(2): p. 281-297.

115. Bentwich, I., et al., *Identification of hundreds of conserved and nonconserved human microRNAs.* Nat Genet, 2005. **37**(7): p. 766-70.

116. Li, Q., et al., *miR-224 promotion of cell migration and invasion by targeting Homeobox D 10 gene in human hepatocellular carcinoma.* J Gastroenterol Hepatol, 2014. **29**(4): p. 835-42.

117. Lin, Z.Y., et al., *MicroRNA-224 inhibits progression of human prostate cancer by downregulating TRIB1.* International Journal of Cancer, 2014. **135**(3): p. 541-550.

118. Karacosta, L.G., et al., *Nucleoporin 62 and Ca(2+) /calmodulin dependent kinase kinase 2 regulate androgen receptor activity in castrate resistant prostate cancer cells.* Prostate, 2016. **76**(3): p. 294-306.

119. Anderson, K.A., et al., *Hypothalamic CaMKK2 contributes to the regulation of energy balance.* Cell Metab, 2008. **7**(5): p. 377-88.

120. Hoyer-Hansen, M., et al., *Control of macroautophagy by calcium, calmodulin-dependent kinase kinase-beta, and Bcl-2.* Mol Cell, 2007. **25**(2): p. 193-205.

121. Hoyer-Hansen, M. and M. Jaattela, *AMP-activated protein kinase: a universal regulator of autophagy?* Autophagy, 2007. **3**(4): p. 381-3.

122. Sakagami, H., et al., *Distinct immunohistochemical localization of two isoforms of Ca2+/calmodulin-dependent protein kinase kinases in the adult rat brain.* Eur J Neurosci, 2000. **12**(1): p. 89-99.

123. Anderson, K.A., et al., *Components of a calmodulin-dependent protein kinase cascade. Molecular cloning, functional characterization and cellular localization of Ca2+/calmodulin-dependent protein kinase kinase beta.* J Biol Chem, 1998. **273**(48): p. 31880-9.

124. Hsu, L.S., et al., *Human Ca2+/calmodulin-dependent protein kinase kinase beta gene encodes multiple isoforms that display distinct kinase activity.* J Biol Chem, 2001. **276**(33): p. 31113-23.

125. Okuno, S., T. Kitani, and H. Fujisawa, *Purification and characterization of Ca2+/calmodulin-dependent protein kinase kinase beta from rat cerebellum.* J Biochem, 1997. **121**(1): p. 155-60.

126. Tokumitsu, H., H. Enslen, and T.R. Soderling, *Characterization of a Ca2+/calmodulin-dependent protein kinase cascade. Molecular cloning and expression of calcium/calmodulin-dependent protein kinase kinase.* J Biol Chem, 1995. **270**(33): p. 19320-4.

127. Vinet, J., et al., *Cloning of mouse Ca2+/calmodulin-dependent protein kinase kinase beta (CaMKKbeta) and characterization of CaMKKbeta and CaMKKalpha distribution in the adult mouse brain.* Brain Res Mol Brain Res, 2003. **111**(1-2): p. 216-21.

128. Nakamura, Y., et al., *Immunohistochemical localization of Ca(2+)/calmodulin-dependent protein kinase kinase beta in the rat central nervous system.* Neurosci Res, 2001. **39**(2): p. 175-88.

129. Carafoli, E. and C.B. Klee, *Calcium as a cellular regulator*. 1999, New York: Oxford University Press. xiii, 642 p.

130. Means, A.R., *Regulatory cascades involving calmodulin-dependent protein kinases.* Mol Endocrinol, 2000. **14**(1): p. 4-13.

131. Chatila, T., et al., *A unique phosphorylation-dependent mechanism for the activation of Ca2+/calmodulin-dependent protein kinase type IV/GR.* J Biol Chem, 1996. **271**(35): p. 21542-8.

132. Tokumitsu, H. and T.R. Soderling, *Requirements for calcium and calmodulin in the calmodulin kinase activation cascade.* J Biol Chem, 1996. **271**(10): p. 5617-22.

133. Wayman, G.A., et al., *Regulation of axonal extension and growth cone motility by calmodulin-dependent protein kinase I.* J Neurosci, 2004. **24**(15): p. 3786-94.

134. Kahl, C.R. and A.R. Means, *Regulation of cyclin D1/Cdk4 complexes by calcium/calmodulin-dependent protein kinase I.* J Biol Chem, 2004. **279**(15): p. 15411-9.

135. Lin, F., et al., *The camKK2/camKIV relay is an essential regulator of hepatic cancer.* Hepatology, 2015. **62**(2): p. 505-20.

136. Oury, F., et al., *CREB mediates brain serotonin regulation of bone mass through its expression in ventromedial hypothalamic neurons.* Genes Dev, 2010. **24**(20): p. 2330-42.

137. Lin, F.M., et al., *The camKK2/camKIV relay is an essential regulator of hepatic cancer.* Hepatology, 2015. **62**(2): p. 505-520.

138. Zoncu, R., A. Efeyan, and D.M. Sabatini, *mTOR: from growth signal integration to cancer, diabetes and ageing.* Nat Rev Mol Cell Biol, 2011. **12**(1): p. 21-35.

139. Woods, A., et al., *Ca2+/calmodulin-dependent protein kinase kinase-beta acts upstream of AMP-activated protein kinase in mammalian cells.* Cell Metab, 2005. **2**(1): p. 21-33.

140. Hurley, R.L., et al., *The Ca2+/calmodulin-dependent protein kinase kinases are AMP-activated protein kinase kinases.* J Biol Chem, 2005. **280**(32): p. 29060-6.

141. Hamilton, S.R., et al., *AMP-activated protein kinase kinase: detection with recombinant AMPK alpha1 subunit.* Biochem Biophys Res Commun, 2002. **293**(3): p. 892-8.

142. Anderson, K.A., et al., *Deletion of CaMKK2 from the liver lowers blood glucose and improves whole-body glucose tolerance in the mouse.* Mol Endocrinol, 2012. **26**(2): p. 281-91.

143. Lin, F., T.J. Ribar, and A.R. Means, *The Ca2+/calmodulin-dependent protein kinase kinase, CaMKK2, inhibits preadipocyte differentiation.* Endocrinology, 2011. **152**(10): p. 3668-79.

144. Racioppi, L. and A.R. Means, *Calcium/calmodulin-dependent protein kinase kinase 2: roles in signaling and pathophysiology.* J Biol Chem, 2012. **287**(38): p. 31658-65.

145. Chin, D. and A.R. Means, *Calmodulin: a prototypical calcium sensor.* Trends Cell Biol, 2000. **10**(8): p. 322-8.

146. Marcelo, K.L., A.R. Means, and B. York, *The Ca2+/Calmodulin/CaMKK2 Axis: Nature's Metabolic CaMshaft.* Trends Endocrinol Metab, 2016.

147. Green, M.F., K.A. Anderson, and A.R. Means, *Characterization of the CaMKKbeta-AMPK signaling complex.* Cell Signal, 2011. **23**(12): p. 2005-12.

148. Subbannayya, Y., et al., *Calcium calmodulin dependent kinase kinase 2-a novel therapeutic target for gastric adenocarcinoma.* Cancer Biology & Therapy, 2015. **16**(2): p. 336-345.

149. Ishikawa, Y., et al., *Identification and characterization of novel components of a Ca2+/calmodulin-dependent protein kinase cascade in HeLa cells.* FEBS Lett, 2003. **550**(1-3): p. 57-63.

150. Frigo, D.E., et al., *CaM kinase kinase beta-mediated activation of the growth regulatory kinase AMPK is required for androgen-dependent migration of prostate cancer cells.* Cancer Res, 2011. **71**(2): p. 528-37.

151. Hanks, S.K. and T. Hunter, *Protein Kinases .6. The Eukaryotic Protein-Kinase Superfamily - Kinase (Catalytic) Domain-Structure and Classification.* Faseb Journal, 1995. **9**(8): p. 576-596.

152. Manning, G., et al., *The protein kinase complement of the human genome.* Science, 2002. **298**(5600): p. 1912-34.

153. Hanks, S.K., *Genomic analysis of the eukaryotic protein kinase superfamily: a perspective.* Genome Biol, 2003. **4**(5): p. 111.

154. Kukimoto-Niino, M., et al., *Crystal structure of the Ca(2)(+)/calmodulin-dependent protein kinase kinase in complex with the inhibitor STO-609.* J Biol Chem, 2011. **286**(25): p. 22570-9.

155. Duong-Ly, K.C. and J.R. Peterson, *The human kinome and kinase inhibition.* Curr Protoc Pharmacol, 2013. **Chapter 2**: p. Unit2 9.

156. Endicott, J.A., M.E. Noble, and L.N. Johnson, *The structural basis for control of eukaryotic protein kinases.* Annu Rev Biochem, 2012. **81**: p. 587-613.

157. Nolen, B., S. Taylor, and G. Ghosh, *Regulation of protein kinases; controlling activity through activation segment conformation.* Mol Cell, 2004. **15**(5): p. 661-75.

158. Colbran, R.J., *Inactivation of Ca2+/calmodulin-dependent protein kinase II by basal autophosphorylation.* J Biol Chem, 1993. **268**(10): p. 7163-70.

159. Watanabe, S., et al., *Inactivation of calmodulin-dependent protein kinase IV by autophosphorylation of serine 332 within the putative calmodulin-binding domain.* J Biol Chem, 1996. **271**(12): p. 6903-10.

160. Swulius, M.T. and M.N. Waxham, *Ca(2+)/calmodulin-dependent protein kinases.* Cell Mol Life Sci, 2008. **65**(17): p. 2637-57.

161. Tokumitsu, H., et al., *Calcium/calmodulin-dependent protein kinase kinase: identification of regulatory domains.* Biochemistry, 1997. **36**(42): p. 12823-7.

162. Goldberg, J., A.C. Nairn, and J. Kuriyan, *Structural basis for the autoinhibition of calcium/calmodulin-dependent protein kinase I.* Cell, 1996. **84**(6): p. 875-87.

163. Osawa, M., et al., *A novel target recognition revealed by calmodulin in complex with Ca2+-calmodulin-dependent kinase kinase.* Nat Struct Biol, 1999. **6**(9): p. 819-24.

164. Lochhead, P.A., et al., *A chaperone-dependent GSK3beta transitional intermediate mediates activation-loop autophosphorylation.* Mol Cell, 2006. **24**(4): p. 627-33.

165. Oliver, A.W., S. Knapp, and L.H. Pearl, *Activation segment exchange: a common mechanism of kinase autophosphorylation?* Trends Biochem Sci, 2007. **32**(8): p. 351-6.

166. Johnson, L.N. and R.J. Lewis, *Structural basis for control by phosphorylation.* Chem Rev, 2001. **101**(8): p. 2209-42.

167. Johnson, L.N., M.E. Noble, and D.J. Owen, *Active and inactive protein kinases: structural basis for regulation.* Cell, 1996. **85**(2): p. 149-58.

168. Adams, J.A., *Kinetic and catalytic mechanisms of protein kinases.* Chem Rev, 2001. **101**(8): p. 2271-90.

169. Vijayan, R.S.K., et al., *Conformational Analysis of the DFG-Out Kinase Motif and Biochemical Profiling of Structurally Validated Type II Inhibitors.* Journal of Medicinal Chemistry, 2015. **58**(1): p. 466-479.

170. Taylor, S.S., et al., *Integration of signaling in the kinome: Architecture and regulation of the alphaC Helix.* Biochim Biophys Acta, 2015. **1854**(10 Pt B): p. 1567-74.

171. Nolen, B., S. Taylor, and G. Ghosh, *Regulation of protein kinases: Controlling activity through activation segment conformation.* Molecular Cell, 2004. **15**(5): p. 661-675.

172. Tokumitsu, H., et al., *Generation of autonomous activity of Ca(2+)/calmodulin-dependent protein kinase kinase beta by autophosphorylation.* Biochemistry, 2011. **50**(38): p. 8193-201.

173. Salazar, C. and T. Hofer, *Multisite protein phosphorylation--from molecular mechanisms to kinetic models.* FEBS J, 2009. **276**(12): p. 3177-98.

174. Pitzschke, A., *Modes of MAPK substrate recognition and control.* Trends Plant Sci, 2015. **20**(1): p. 49-55.

175. Nishi, H., et al., *Regulation of protein-protein binding by coupling between phosphorylation and intrinsic disorder: analysis of human protein complexes.* Mol Biosyst, 2013. **9**(7): p. 1620-6.

176. Scott, J.W., et al., *Autophosphorylation of CaMKK2 generates autonomous activity that is disrupted by a T85S mutation linked to anxiety and bipolar disorder.* Sci Rep, 2015. **5**: p. 14436.

177. Tokumitsu, H., et al., *Regulatory mechanism of Ca2+/calmodulin-dependent protein kinase kinase.* J Biol Chem, 2000. **275**(26): p. 20090-5.

178. Green, M.F., et al., *Ca2+/Calmodulin-dependent Protein Kinase Kinase beta Is Regulated by Multisite Phosphorylation.* Journal of Biological Chemistry, 2011. **286**(32): p. 28066-28079.

179. Green, M.F., et al., *Ca2+/Calmodulin-dependent protein kinase kinase beta is regulated by multisite phosphorylation.* J Biol Chem, 2011. **286**(32): p. 28066-79.

180. Schumacher, A.M., et al., *A calmodulin-regulated protein kinase linked to neuron survival is a substrate for the calmodulin-regulated death-associated protein kinase.* Biochemistry, 2004. **43**(25): p. 8116-24.

181. Tokumitsu, H., et al., *Differential regulatory mechanism of Ca2+/calmodulin-dependent protein kinase kinase isoforms.* Biochemistry, 2001. **40**(46): p. 13925-32.

182. Breen, M.E. and M.B. Soellner, *Small molecule substrate phosphorylation site inhibitors of protein kinases: approaches and challenges.* ACS Chem Biol, 2015. **10**(1): p. 175-89.

183. Tokumitsu, H., et al., *Substrate recognition by Ca2+/Calmodulin-dependent protein kinase kinase. Role of the arg-pro-rich insert domain.* J Biol Chem, 1999. **274**(22): p. 15803-10.

184. Adams, D.J., *The Valley of Death in anticancer drug development: a reassessment.* Trends Pharmacol Sci, 2012. **33**(4): p. 173-80.

185. Muller, S., et al., *The ins and outs of selective kinase inhibitor development.* Nat Chem Biol, 2015. **11**(11): p. 818-21.

186. Steinmetz, K.L. and E.G. Spack, *The basics of preclinical drug development for neurodegenerative disease indications.* BMC Neurol, 2009. **9 Suppl 1**: p. S2.

187. Surade, S. and T.L. Blundell, *Structural biology and drug discovery of difficult targets: the limits of ligandability.* Chem Biol, 2012. **19**(1): p. 42-50.

188. Hoelder, S., P.A. Clarke, and P. Workman, *Discovery of small molecule cancer drugs: successes, challenges and opportunities.* Mol Oncol, 2012. **6**(2): p. 155-76.

189. Lipinski, C.A., et al., *Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings.* Advanced Drug Delivery Reviews, 1997. **23**(1-3): p. 3-25.

190. Taylor, S.S. and E. Radzio-Andzelm, *Protein kinase inhibition: natural and synthetic variations on a theme.* Curr Opin Chem Biol, 1997. **1**(2): p. 219-26.

191. Toledo, L.M., N.B. Lydon, and D. Elbaum, *The structure-based design of ATP-site directed protein kinase inhibitors.* Current Medicinal Chemistry, 1999. **6**(9): p. 775-805.

192. Liu, Y., et al., *Structural basis for selective inhibition of Src family kinases by PP1.* Chemistry & Biology, 1999. **6**(9): p. 671-678.

193. Schindler, T., et al., *Crystal structure of Hck in complex with a Src family-selective tyrosine kinase inhibitor.* Molecular Cell, 1999. **3**(5): p. 639-648.

194. Wang, Z.L., et al., *Structural basis of inhibitor selectivity in MAP kinases.* Structure with Folding & Design, 1998. **6**(9): p. 1117-1128.

195. Zhu, X.T., et al., *Structural analysis of the lymphocyte-specific kinase Lck in complex with non-selective and Src family selective kinase inhibitors.* Structure with Folding & Design, 1999. **7**(6): p. 651-661.

196. Kontopidis, G., et al., *Insights into cyclin groove recognition: complex crystal structures and inhibitor design through ligand exchange.* Structure, 2003. **11**(12): p. 1537-46.

197. Luo, X.J., et al., *Convergent lines of evidence support CAMKK2 as a schizophrenia susceptibility gene.* Mol Psychiatry, 2014. **19**(7): p. 774-83.

198. McCullough, L.D., et al., *Inhibition of calcium/calmodulin-dependent protein kinase kinase beta and calcium/calmodulin-dependent protein kinase IV is detrimental in cerebral ischemia.* Stroke, 2013. **44**(9): p. 2559-66.

199. Xie Y, W.L., Hussain A., *Metformin enhances the anti-prostate cancer activity of abiraterone and enzalutamide*, in *Poster presentation at: AACR Annual Meeting 2016*. 2016: New Orleans, LA.

200. Hansen, A., *Norway backs downs over plan to exclude anticancer drugs from coverage.* BMJ, 2013. **346**: p. f1707.

201. Chayen, N.E., *Tackling the bottleneck of protein crystallization in the post-genomic era.* Trends Biotechnol, 2002. **20**(3): p. 98.

202. Doerr, A., *Widening the protein crystallization bottleneck.* Nat Methods, 2006. **3**(12): p. 961.

203. Chayen, N.E., *Turning protein crystallisation from an art into a science.* Curr Opin Struct Biol, 2004. **14**(5): p. 577-83.

204. Luft, J.R., et al., *A deliberate approach to screening for initial crystallization conditions of biological macromolecules.* J Struct Biol, 2003. **142**(1): p. 170-9.

205. Mueller-Dieckmann, J., *The open-access high-throughput crystallization facility at EMBL Hamburg.* Acta Crystallogr D Biol Crystallogr, 2006. **62**(Pt 12): p. 1446-52.

206. Vedadi, M., et al., *Chemical screening methods to identify ligands that promote protein stability, protein crystallization, and structure determination.* Proc Natl Acad Sci U S A, 2006. **103**(43): p. 15835-40.

207. Ramage P., M.B., Fendrich G., Benoit R., *Purification of phosphorylated protein kinases using MonoBeads ion exchange media*, B. Novartis Institutes for Biomedical Research, Switzerland, Editor.

208. Du, P., et al., *Phosphorylation of serine residues in histidine-tag sequences attached to recombinant protein kinases: A cause of heterogeneity in mass and complications in function.* Protein Expression and Purification, 2005. **44**(2): p. 121-129.

209. Jurcsisn, J.G., et al., *The CamKKbeta Inhibitor STO609 Causes Artefacts in Calcium Imaging and Selectively Inhibits BKCa in Mouse Carotid Body Type I Cells.* Adv Exp Med Biol, 2015. **860**: p. 17-24.

210. Yurimoto, S., et al., *In vitro substrate phosphorylation by Ca(2)(+)/calmodulin-dependent protein kinase kinase using guanosine-5'-triphosphate as a phosphate donor.* BMC Biochem, 2012. **13**: p. 27.

211. Roskoski, R., Jr., *RAF protein-serine/threonine kinases: structure and regulation.* Biochem Biophys Res Commun, 2010. **399**(3): p. 313-7.

212. Azam, M., et al., *Activation of tyrosine kinases by mutation of the gatekeeper threonine.* Nat Struct Mol Biol, 2008. **15**(10): p. 1109-18.

213. Gibbons, D.L., et al., *The rise and fall of gatekeeper mutations? The BCR-ABL1 T315I paradigm.* Cancer, 2012. **118**(2): p. 293-9.

214. Shah, K. and K.M. Shokat, *A chemical genetic approach for the identification of direct substrates of protein kinases.* Methods Mol Biol, 2003. **233**: p. 253-71.

215. Chi, Y., et al., *Identification of CDK2 substrates in human cell lysates.* Genome Biol, 2008. **9**(10): p. R149.

216. Horoszewicz, J.S., et al., *The LNCaP cell line--a new model for studies on human prostatic carcinoma.* Prog Clin Biol Res, 1980. **37**: p. 115-32.

217. Horoszewicz, J.S., et al., *LNCaP model of human prostatic carcinoma.* Cancer Res, 1983. **43**(4): p. 1809-18.

218. Korenchuk, S., et al., *VCaP, a cell-based model system of human prostate cancer.* In Vivo, 2001. **15**(2): p. 163-8.

219. Frank, R., *The SPOT-synthesis technique. Synthetic peptide arrays on membrane supports--principles and applications.* J Immunol Methods, 2002. **267**(1): p. 13-26.

220. Kramer, A. and J. Schneider-Mergener, *Synthesis and screening of peptide libraries on continuous cellulose membrane supports.* Methods Mol Biol, 1998. **87**: p. 25-39.

221. Yang, J., et al., *GEMIN4 functions as a coregulator of the mineralocorticoid receptor.* J Mol Endocrinol, 2015. **54**(2): p. 149-60.

222. Blikstad, C. and Y. Ivarsson, *High-throughput methods for identification of protein-protein interactions involving short linear motifs.* Cell Commun Signal, 2015. **13**: p. 38.

223. Einarson, M.B., E.N. Pugacheva, and J.R. Orlinick, *Identification of Protein-Protein Interactions with Glutathione-S-Transferase (GST) Fusion Proteins.* CSH Protoc, 2007. **2007**: p. pdb top11.

224. Liu, B., et al., *Label transfer chemistry for the characterization of protein-protein interactions.* J Am Chem Soc, 2007. **129**(41): p. 12348-9.

225. Feng, W., et al., *Gemins modulate the expression and activity of the SMN complex.* Hum Mol Genet, 2005. **14**(12): p. 1605-11.

226. Liu, Q. and G. Dreyfuss, *A novel nuclear structure containing the survival of motor neurons protein.* EMBO J, 1996. **15**(14): p. 3555-65.

227. Charroux, B., et al., *Gemin4. A novel component of the SMN complex that is found in both gems and nucleoli.* J Cell Biol, 2000. **148**(6): p. 1177-86.

228. Battle, D.J., et al., *The SMN complex: an assembly machine for RNPs.* Cold Spring Harb Symp Quant Biol, 2006. **71**: p. 313-20.

229. Piazzon, N., et al., *Implication of the SMN complex in the biogenesis and steady state level of the signal recognition particle.* Nucleic Acids Res, 2013. **41**(2): p. 1255-72.

230. Liu, Q., et al., *The spinal muscular atrophy disease gene product, SMN, and its associated protein SIP1 are in a complex with spliceosomal snRNP proteins.* Cell, 1997. **90**(6): p. 1013-21.

231. Pellizzoni, L., et al., *A novel function for SMN, the spinal muscular atrophy disease gene product, in pre-mRNA splicing.* Cell, 1998. **95**(5): p. 615-24.

232. Pellizzoni, L., J. Yong, and G. Dreyfuss, *Essential role for the SMN complex in the specificity of snRNP assembly.* Science, 2002. **298**(5599): p. 1775-9.

233. Fischer, U., Q. Liu, and G. Dreyfuss, *The SMN-SIP1 complex has an essential role in spliceosomal snRNP biogenesis.* Cell, 1997. **90**(6): p. 1023-9.

234. Narayanan, U., et al., *SMN, the spinal muscular atrophy protein, forms a pre-import snRNP complex with snurportin1 and importin beta.* Hum Mol Genet, 2002. **11**(15): p. 1785-95.

235. Yong, J., et al., *Gemin5 delivers snRNA precursors to the SMN complex for snRNP biogenesis.* Mol Cell, 2010. **38**(4): p. 551-62.

236. Tsalikis, J., et al., *Intracellular Bacterial Pathogens Trigger the Formation of U Small Nuclear RNA Bodies (U Bodies) through Metabolic Stress Induction.* J Biol Chem, 2015. **290**(34): p. 20904-18.

237. Zhu, W., et al., *Genetic variants in the MicroRNA biosynthetic pathway Gemin3 and Gemin4 are associated with a risk of cancer: a meta-analysis.* PeerJ, 2016. **4**: p. e1724.

238. Taylor, B.S., et al., *Integrative genomic profiling of human prostate cancer.* Cancer Cell, 2010. **18**(1): p. 11-22.

239. Farrow, J.M., J.C. Yang, and C.P. Evans, *Autophagy as a modulator and target in prostate cancer.* Nat Rev Urol, 2014. **11**(9): p. 508-16.

240. Degenhardt, K., et al., *Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis.* Cancer Cell, 2006. **10**(1): p. 51-64.

241. Leone, R.D. and R.K. Amaravadi, *Autophagy: a targetable linchpin of cancer cell metabolism.* Trends Endocrinol Metab, 2013. **24**(4): p. 209-17.

242. Mathew, R., V. Karantza-Wadsworth, and E. White, *Role of autophagy in cancer.* Nat Rev Cancer, 2007. **7**(12): p. 961-7.

243. White, E., *Deconvoluting the context-dependent role for autophagy in cancer.* Nat Rev Cancer, 2012. **12**(6): p. 401-10.

244. Chen, N. and V. Karantza, *Autophagy as a therapeutic target in cancer.* Cancer Biol Ther, 2011. **11**(2): p. 157-68.

245. Shi, Y.T., Efrosini; Blessing, Alicia M.; Tennakoon, Jayantha; Han, Jenny J.; Ittmann, Michael M.; Frigo, Daniel E. . *Regulation of AMPK by androgen receptor signaling and its role in promoting prostate cancer through the use of autophagy* in *AACR 106th Annual Meeting 2015*. 2015. Philadelphia, PA.

246. Shi, Y., et al., *Androgens promote prostate cancer cell growth through induction of autophagy.* Mol Endocrinol, 2013. **27**(2): p. 280-95.

247. Gu, Y., et al., *Porcine Circovirus Type 2 Activates CaMMKbeta to Initiate Autophagy in PK-15 Cells by Increasing Cytosolic Calcium.* Viruses, 2016. **8**(5).

248. Xi, H., et al., *Endoplasmic reticulum stress induced by 2-deoxyglucose but not glucose starvation activates AMPK through CaMKKbeta leading to autophagy.* Biochem Pharmacol, 2013. **85**(10): p. 1463-77.

249. Son, S.M., et al., *Abeta-induced formation of autophagosomes is mediated by RAGE-CaMKKbeta-AMPK signaling.* Neurobiol Aging, 2012. **33**(5): p. 1006 e11-23.

250. Ghislat, G., et al., *Withdrawal of essential amino acids increases autophagy by a pathway involving Ca2+/calmodulin-dependent kinase kinase-beta (CaMKK-beta).* J Biol Chem, 2012. **287**(46): p. 38625-36.

251. Klionsky, D.J., et al., *Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition).* Autophagy, 2016. **12**(1): p. 1-222.

252. Kimmelman, A.C., *The dynamic nature of autophagy in cancer.* Genes Dev, 2011. **25**(19): p. 1999-2010.

253. Tanida, I., T. Ueno, and E. Kominami, *LC3 and Autophagy.* Methods Mol Biol, 2008. **445**: p. 77-88.

254. Seglen, P.O., et al., *Macroautophagic cargo sequestration assays.* Methods, 2015. **75**: p. 25-36.

255. Luhr, M., et al., *A simple cargo sequestration assay for quantitative measurement of non-selective autophagy in cultured cells*, in *Methods in Enzymology* 2016.

256. Bauvy, C., A.J. Meijer, and P. Codogno, *Assaying of autophagic protein degradation.* Methods Enzymol, 2009. **452**: p. 47-61.

257. Engedal, N., et al., *Modulation of intracellular calcium homeostasis blocks autophagosome formation.* Autophagy, 2013. **9**(10): p. 1475-90.

258. Pfisterer, S.G., et al., *Ca2+/calmodulin-dependent kinase (CaMK) signaling via CaMKI and AMP-activated protein kinase contributes to the regulation of WIPI-1 at the onset of autophagy.* Mol Pharmacol, 2011. **80**(6): p. 1066-75.

259. Samari, H.R., et al., *Stimulation of hepatocytic AMP-activated protein kinase by okadaic acid and other autophagy-suppressive toxins.* Biochem J, 2005. **386**(Pt 2): p. 237-44.

260. Mihaylova, M.M. and R.J. Shaw, *The AMPK signalling pathway coordinates cell growth, autophagy and metabolism.* Nat Cell Biol, 2011. **13**(9): p. 1016-23.

261. Bain, J., et al., *The selectivity of protein kinase inhibitors: a further update.* Biochem J, 2007. **408**(3): p. 297-315.

262. Bach, M., et al., *The serine/threonine kinase ULK1 is a target of multiple phosphorylation events.* Biochem J, 2011. **440**(2): p. 283-91.

263. Kim, J., et al., *AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1.* Nat Cell Biol, 2011. **13**(2): p. 132-41.

264. Ma, Z., et al., *Growth inhibition of human gastric adenocarcinoma cells in vitro by STO-609 is independent of calcium/calmodulin-dependent protein kinase kinase-beta and adenosine monophosphate-activated protein kinase.* Am J Transl Res, 2016. **8**(2): p. 1164-71.

265. Kimura, T., et al., *Chloroquine in cancer therapy: a double-edged sword of autophagy.* Cancer Res, 2013. **73**(1): p. 3-7.

6 APPENDIX

Appendix 1. Extended *in vitro* kinase screen of STO-609. The table ranks the extended panel of protein kinases according to their percentage activity remaining in the presence of STO-609 (averages of duplicate determinations). Further details of the assay are described in Bain *et al.,* 2007 [261].

	STO-609 [1μM]	
	average % activity [n=2]	SD
DAPK1	1	1
PIM3	1	0
CAMKK2	6	0
ERK8	7	0
IRAK1	8	1
MNK1	12	3
PIM2	14	1
MLK1	14	1
MLK3	15	4
DYRK1A	16	3
AMPK (hum)	16	1
GCK	19	2
TBK1	24	5
IKKe	25	2
CK2	33	3
TAK1	35	3
HIPK2	40	1
MAP4K5	43	4
PIM1	45	17
DYRK2	51	6
CLK2	51	3
MINK1	54	2
TTK	56	1
JAK2	57	13
GSK3b	58	8
NUAK1	59	9
SmMLCK	60	5
STK33	62	18
Aurora B	64	11
MNK2	65	15
ULK2	66	0
VEG-FR	68	3
HIPK3	68	5
MKK6	68	9
RPK1	69	2
CDK2-Cyclin A	69	1
DYRK3	69	0
CHK2	70	9
BRSK2	72	2
MARK3	73	5
CK1δ	76	1
JNK2	77	4
BRSK1	80	10
IGF-1R	81	12
MST2	81	3
PLK1	84	5
CDK9-Cyclin T1	84	9
MSK1	85	1
MARK1	86	5
PKD1	87	10
ASK1	88	2
EIF2AK3	89	3
S6K1	89	7
ERK5	90	3
MAPKAP-K3	91	1
EPH-B3	91	6
IRR	92	13
MARK2	92	4
MPSK1	92	1
EPH-B1	93	10
BRK	93	13
MARK4	95	11
TESK1	95	11
MELK	96	5
MAP4K3	97	0
HIPK1	98	17
ZAP70	99	13
YES1	99	1
JNK1	99	9
IRAK4	99	7
TAO1	99	19
EPH-A4	99	1
PKBb	99	7
MKK2	100	10
PAK2	100	5
p38d MAPK	100	10
Aurora A	101	3
EPH-A2	101	14
CK1γ2	101	2
MKK1	102	3
RSK2	102	3
CAMK1	102	9
NEK6	102	4
ROCK 2	102	2
SIK2	102	3
EPH-B4	103	18
PAK5	103	7
PDGFRA	103	5
JNK3	103	21
PRK2	104	0
PAK6	104	14
EF2K	105	17
NEK2a	105	16
PDK1	105	6
ABL	105	15
RIPK2	105	3
SIK3	106	5
PINK	106	0
PHK	106	8
SYK	106	11
PKCγ	106	14
TIE2	107	14
WNK1	107	4
PAK4	107	7
MAPKAP-K2	108	3
TrkA	108	3
PKA	108	5
Src	108	2
TGFBR1	109	4
MEKK1	109	0
BTK	109	8
p38g MAPK	109	19
p38b MAPK	110	10
ERK2	110	10
TLK1	111	17
RSK1	111	7
DDR2	111	8
p38a MAPK	112	12
EPH-B2	112	3
PKCz	113	8
SGK1	113	6
PKCa	113	3
Lck	113	8
PKBa	114	7
HER4	114	5
TTBK1	114	6
TSSK1	114	22
PRAK	117	1
MST4	118	20
TTBK2	119	3
CSK	122	3
IR	123	11
ULK1	123	5
ERK1	123	14
LKB1	124	1
MST3	125	25
OSR1	126	10
FGF-R1	128	2
CHK1	129	6
IKKb	129	24