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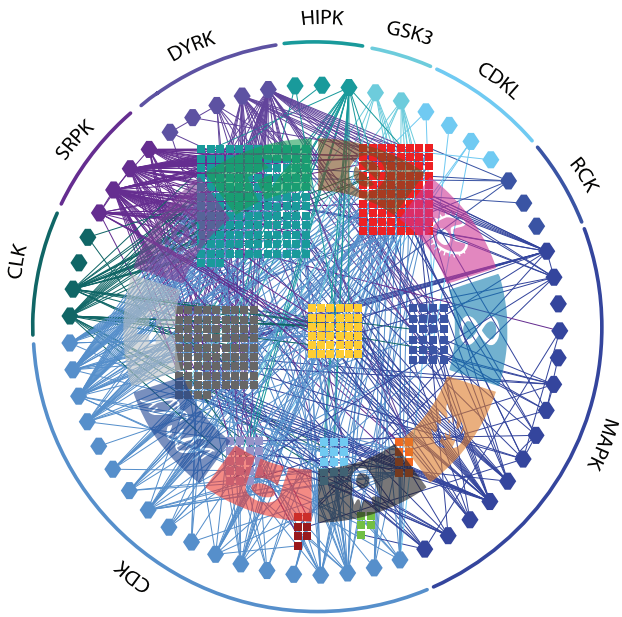
SALLA KESKITALO

CMGC Kinases and Cancer



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FACULTY OF BIOLOGICAL AND ENVIRONMENTAL SCIENCES
DOCTORAL PROGRAMME IN INTEGRATIVE LIFE SCIENCE
UNIVERSITY OF HELSINKI

Institute of Biotechnology and Department of Biosciences
Faculty of Biological and Environmental Sciences
University of Helsinki
Helsinki, Finland

Doctoral School in Health Sciences
Integrative Life Sciences (ILS) Doctoral Program

CMGC KINASES AND CANCER

Salla Keskitalo

A C A D E M I C D I S S E R T A T I O N

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their roman numerals I-IV:

I. Varjosalo M, **Keskitalo S**, Van Drogen A, Nurkkala H, Vichalkovski A, Aebersold R, Gstaiger M. The protein interaction landscape of the human CMGC kinase group. Cell Rep. 25;3(4):1306-20, 2013.
- SK performed AP-MS analyses, cloned the BiFC-constructs and optimized the method, designed and performed microscopy and co-IP western blotting analyses, analyzed data and participated in writing the paper

II. Turunen M*****, Spaeth JM*****, **Keskitalo S**, Park MJ, Kivioja T, Clark AD, Mäkinen N, Gao F, Palin K, Nurkkala H, Vähärautio A, Aavikko M, Kämpjärvi K, Vahteristo P, Kim CA, Aaltonen LA, Varjosalo M, Taipale J, Boyer TG. Uterine leiomyoma-linked MED12 mutations disrupt mediator-associated CDK activity. Cell Rep. 8;7(3):654-60, 2014.
- SK designed, cloned protein expression constructs and performed affinity-purification coupled to mass spectrometry, participated in data filtering and interpretation and writing the manuscript

III. Kämpjärvi K, Kim NH, **Keskitalo S**, Clark AD, von Nandelstadh P, Turunen M, Heikkinen T, Park MJ, Mäkinen N, Kivinummi K, Lintula S, Hotakainen K, Nevanlinna H, Hokland P, Böhling T, Bützow R, Böhm J, Mecklin JP, Järvinen H, Kontro M, Visakorpi T, Taipale J, Varjosalo M, Boyer TG, Vahteristo P. Somatic MED12 mutations in prostate cancer and uterine leiomyomas promote tumorigenesis through distinct mechanisms. Prostate. 76(1):22-31, 2016.
- SK designed and performed affinity-purification coupled to mass spectrometry, performed quantitative proteomics analyses, participated in data filtering and interpretation and writing the manuscript

IV. Heikkinen T*, Kämpjärvi K*, **Keskitalo S***, von Nandelstahl P, Rantanen V, Pitkänen E, Kuusanmäki H, Kontro M, Turunen M, Liu X, Mäkinen N, Hautaniemi S, Taipale J, Aaltonen LA, Heckman C, Lehti K, Mustjoki S, Varjosalo M, Vahteristo P. Somatic MED12 Nonsense Mutation Escapes mRNA Decay and Reveals a Motif Required for Nuclear Entry. Human Mutation, 2017.
- SK designed and performed AP-MS and BioID mass spectrometry analysis, data filtering and interpretation, participated in cloning the constructs, performed microscopy and participated in the analysis and interpretation of the data and writing the manuscript

*Equal contribution

ABBREVIATIONS

aa	amino acid
AP	affinity purification
BirA*	modified biotin ligase
C-terminus	carboxyterminus (of protein)
CDK	cyclin dependent kinase
CDKL	cyclin dependent kinase -like kinase
CLK	CDC2-like kinases
CMGC	kinase family consisting of CDK, MAPK, GSK3, and CDC-like kinases
COSMIC	Catalogue Of Somatic Mutations In Cancer
CTD	carboxyterminal domain (C-terminal domain)
DAP	disease-associated protein
DYRK	dual-specificity tyrosine (Y)-phosphorylation-regulated kinase
ER	estrogen receptor
ERK	extracellular signal-regulated kinase
GSK3	glycogen synthase kinase-3
HA	hemagglutinin
HCI	high confidence interacting protein
HER2	human epidermal growth factor receptor-2
HIPK	homeodomain-interacting protein kinase
JNK	c-Jun N-terminal kinase
LC-MS	liquid chromatography coupled to mass spectrometry
MAPK	mitogen-activated protein kinase
MED12	mediator complex subunit 12
MED13	mediator complex subunit 13
MS	mass spectrometry
NLS	nuclear localization signal
NPC	nuclear pore complex
N-terminus	aminoterminus (of protein)
OMIM	Online Mendelian Inheritance in Man
PRP4	pre-mRNA processing protein 4 kinase
RCK	tyrosine kinase gene v-ros cross-hybridizing kinase
RNA pol II	RNA polymerase II
SH-tag	Twin-Strep and hemagglutinin -tag
SRPK	SR-specific protein kinase
T-ALL	T-cell acute lymphoblastic leukemia
TF	transcription factor
WB	western blotting
WT	wild type

The standard single- and three-letter codes for amino acids

A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
Y	Tyr	Tyrosine
V	Val	Valine
W	Trp	Tryptophan

ABSTRACT

A decade after identification of the human kinome, only a fraction of the kinome has been systematically studied. This clearly illustrates the lack of knowledge regarding the functions and cellular effects that these proteins have in the establishment of cellular phenotypes. Surprisingly, recent evidence suggests that approximately 23% of all kinase genes were estimated to function as cancer genes.

The overall aim of this thesis project was to shed light on the mechanisms controlling protein kinase activity and function, particularly in human cancers. A global and comprehensive interactomics analysis of the signaling networks of the evolutionarily conserved CMGC-protein kinase family, consisting of cyclin-dependent kinases (**C**DK), mitogen-activated protein kinases (**M**APK), glycogen synthase kinase-3 (**G**SK3) and CDC-like kinases (**C**LK) was performed. From this physical and functional interaction analysis, hundreds of novel interactions, kinase-substrate relationships and previously unknown links to human diseases, such as cancer were identified (**I**). The unprecedented sensitivity and specificity of this approach was also illustrated, and highlighted, for example, by the purification and identification of the complete translational co-activator complex (the Mediator complex) with two of the CMGC kinases (CDK8 and CDK19). At the same time, the Mediator complex was identified mutated in benign uterine human tumors (uterine leiomyomas). To further deepen the understanding of the role of CDK8 and CDK19 kinases in controlling human disease pathways, we studied the functional role of two MED12 mutations in uterine leiomyoma and prostate cancer (**II-III**). Based on these studies, MED12 was found essential for the kinase activity of CDK8 in the context of the Mediator kinase module. Furthermore, disruption of the Mediator kinase module subunit interactions was shown as a common mechanism contributing to the formation of uterine leiomyoma. The MED12 mutants in leiomyoma and prostate cancer were shown functionally different. Finally, a novel MED12 nuclear localization signal was identified, and its importance in the correct nuclear localization of MED12 and subsequent proper assembly and function of the Mediator kinase module was experimentally proven (**IV**). The described results from the multidisciplinary studies (**I-IV**) clarify the cellular roles of CMGC kinases and Mediator subunits, facilitating the design of targeted future therapies/therapeutics against human diseases.

I REVIEW OF THE LITERATURE

1. PROTEIN KINASES

At any given time in any given eukaryote cell, multiple types of molecular networks are concurrently active. An important feature of these networks is the multiple reversible reactions of protein phosphorylation (catalyzed by protein kinases) and dephosphorylation (catalyzed by protein phosphatases) (**Figure 1**). Protein kinases form an essential part of signal transduction in eukaryotic cells by phosphorylating specific protein substrates. For plethora of proteins, changes in phosphorylation status are tightly linked to their activity, cellular location and/or association with other proteins. Thus, the change in phosphorylation status affects a multitude of cellular processes like metabolism, transcription, cell cycle progression, cytoskeletal rearrangement and cell motility, apoptosis and, differentiation. Alterations in protein phosphorylation have been functionally linked to a variety of human diseases.

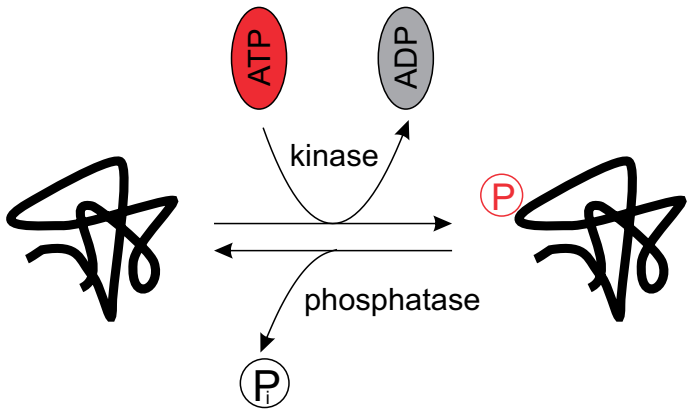

Figure 1: Phosphorylation and dephosphorylation are mediated by kinases and phosphatases. Protein kinases transfer the high-energy phosphate group from ATP to serine, threonine or tyrosine residues of substrate proteins. The work of protein kinases is undone by protein phosphatases that remove the phosphate by hydrolysis.

Simply put, phosphorylation is the transfer of a phosphate group of an ATP molecule to a target protein's serine (S), threonine (T) or tyrosine (Y) residues by protein kinases. Based on their selectivity of the target amino acid, protein kinases are divided into serine/threonine (Ser-/Thr-specific), tyrosine (tyrosine-specific) and dual-specificity protein kinases that are serine/threonine kinases, but are also able to phosphorylate on tyrosine. Most of cellular processes are controlled by serine/threonine protein kinases. The rates of cellular phosphorylation events differ dramatically, giving ratios of 1800(Ser):200(Thr):1(Tyr) (Mann et al., 2002).

1.1 Structure of protein kinases

A common feature for all eukaryotic protein kinases is the existence of a catalytic core. This highly conserved twin-lobed kinase core consists of N- and C-terminal lobes with an ATP binding

cleft between the lobes (**Figure 2**). The N-lobe has five β-strands and an α-helix, whereas C-lobe contains mostly helices (Taylor and Kornev, 2011). The N-lobe β-strands serve as binding surfaces for ATP, and the α-helix is a regulatory unit important for kinase activation also connecting N- and C-lobes. The C-lobe contains the substrate binding groove and the activation segment consisting of catalytic loop, DFG motif, activation loop, P+1 loop, and APE region (Kannan and Neuwald, 2004) (**Figure 2**). The catalytic and regulatory functions of the activation segment are needed for the correct transfer of the phosphate residue to the substrate.

Mammalian protein kinases, themselves, are under tight regulation, and DFG motif regulated phosphorylation of the activation loop acts as the switch for activation and deactivation (Taylor and Kornev, 2011). Indeed, activation loop phosphorylation is the most common mechanism to regulate kinase activity (Nolen et al., 2004). Upon phosphorylation most protein kinases are in their active 'on' state, and dephosphorylated in the basal 'off' state. It is, therefore, not surprising that the three-dimensional structure of activation segment in different active protein kinases is highly similar, but in contrast inactive conformations vary greatly allowing kinase specific activation mechanisms and, thus, high specificity (Taylor and Kornev, 2011). Activation of protein kinases can also depend on the priming phosphorylation of the substrate (Kannan and Neuwald, 2004).

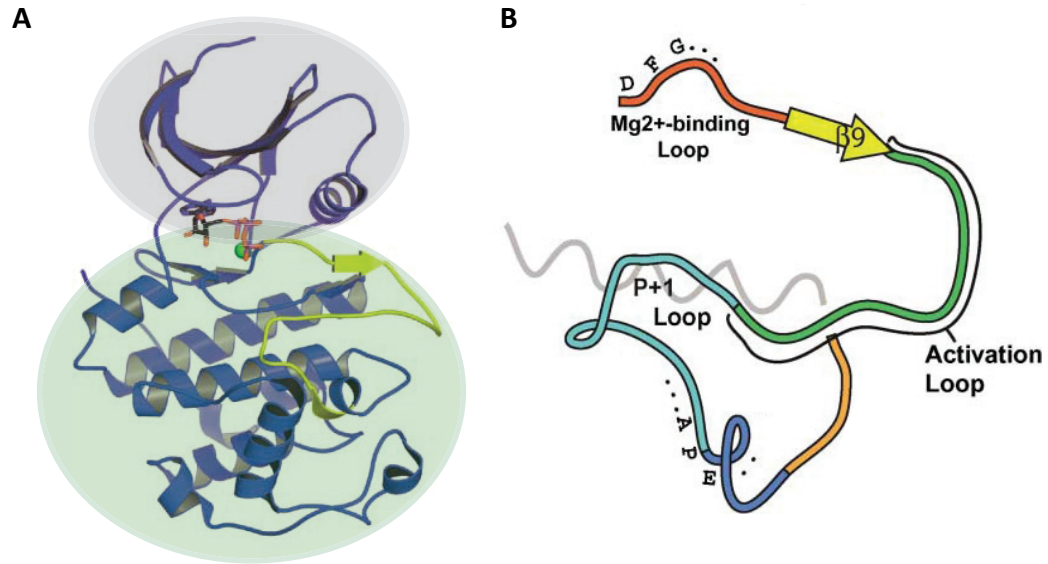

Figure 2: Structure of kinase core and schematic illustration of the activation segment. A) Structure of CDK2 kinase core. N- and C-lobes are indicated with grey and green background, respectively. Activation segment is visualized with yellow. B) Nomenclature of the activation segment extending from the conserved DFG motif till the conserved APE in the P+1 loop. Aspartate (D) in the DFG motif is a catalytic residue responsible of chelating magnesium (Mg^{2+}), which further positions ATP phosphates during phosphotransfer. Activation loop contains the primary phosphorylation site determining the conformation of the loop and the activity of the kinase, and which is the site of regulatory phosphorylation or interaction with kinase activity modulators. P+1 loop provides a docking site to the neighboring residues to the residues to be phosphorylated in the peptide substrate. APE region stabilizes the whole segment. (Modified from Nolen et al., 2004)

1.2 CMGC kinases - an evolutionarily conserved kinase family consisting of CDKs, MAPKs, GSK3, and CDKL kinases

In 2002, Manning and co-workers identified 518 protein kinases in the human genome, forming one of the largest families of genes in eukaryotes constituting approximately 2% of

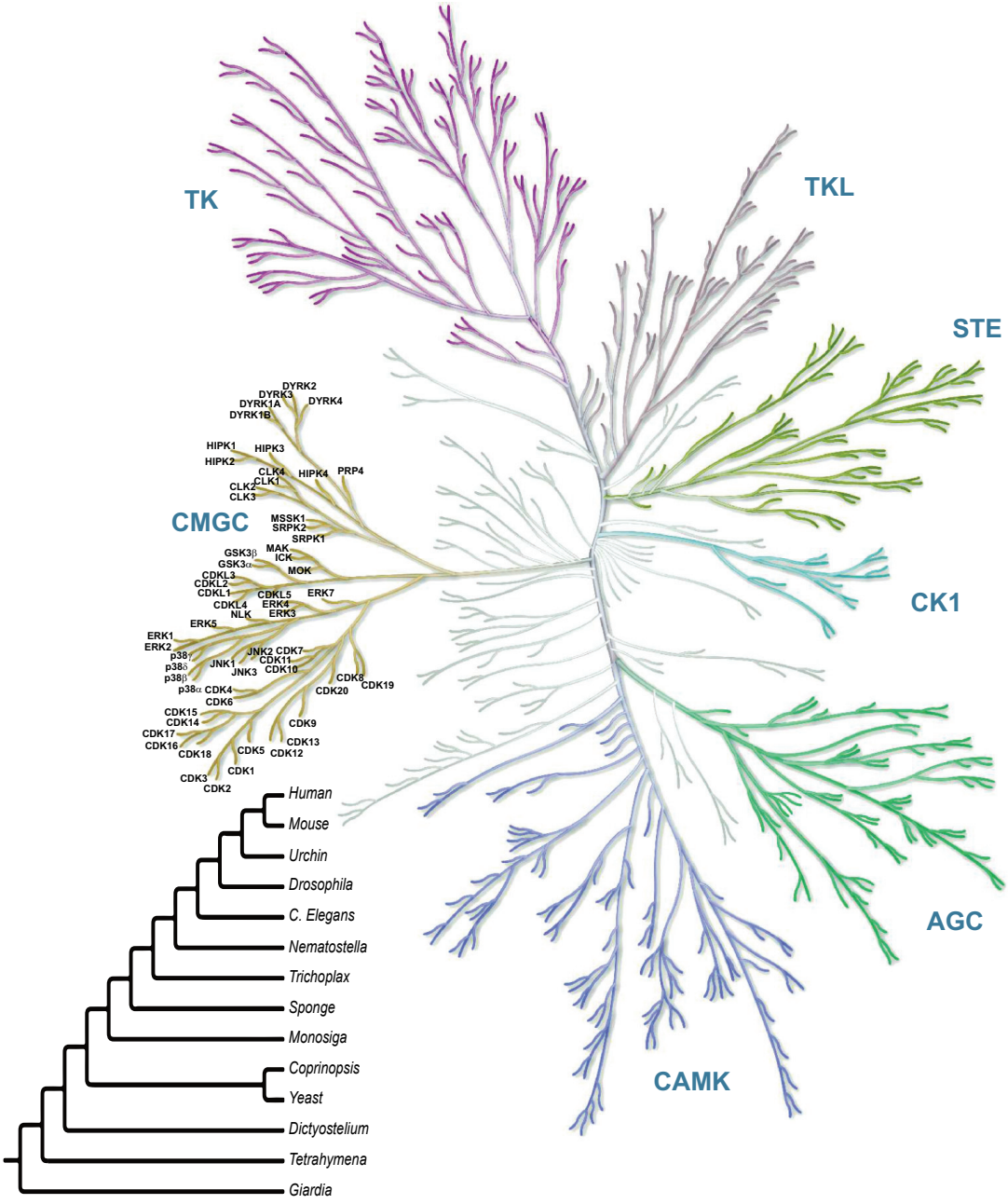

Figure 3: Human protein kinase family tree, and CMGC family conservation among species. The kinase tree shows the sequence similarity between protein kinases based on their kinase domain. ACG, containing PKA, PKG, PKC families; CAMK, calcium/calmodulin-dependent protein kinase; CK1, casein kinase-1; STE, homologs of yeast Sterile 7/11/20 kinases; TK, tyrosine kinase; TKL, tyrosine kinase-like. Illustration reproduced courtesy of Cell Signaling Technology, Inc. (www.cellsignal.com).

all human genes (Manning et al., 2002). This collection of over five hundred protein kinases, forming the human 'kinome', were further grouped into seven major families based on the structural similarities in their catalytic domain (**Figure 3**). According to the similarities in their kinase domains cyclin-dependent kinases (**C**DKs), mitogen-activated protein kinases (**M**APKs), glycogen synthase kinase-3 (**G**SK3s), CDC-like kinases (**C**LKs) and their related kinase families, form a kinase family termed CMGC (Manning et al., 2002). The CMGC family is highly conserved, implying that their functions are highly important for the existence of various species ranging from nematode to human (**Figure 3**).

CMGC kinases, as with other protein kinases have a conserved kinase core, but they also share a distinctive CMGC-insert segment, which is lacking in other protein kinases (Kannan and Neuwald, 2004). The CMGC-insert, located in the C-lobe, is associated with binding to co-proteins that participate in kinase function (Pawson and Nash, 2003) (**Figure 4**). This domain does not present high homology among the family members, thus enabling substrate specificity. CMGC kinases are mainly regulated either through tyrosine phosphorylation in the activation loop (Johnson et al., 1996), or through the pre-phosphorylation of the substrate, preparing it for sequential recognition and phosphorylation by these kinases (ter Haar et al., 2001). Another common functional feature is the preference to phosphorylate substrates having proline in P+1 position.

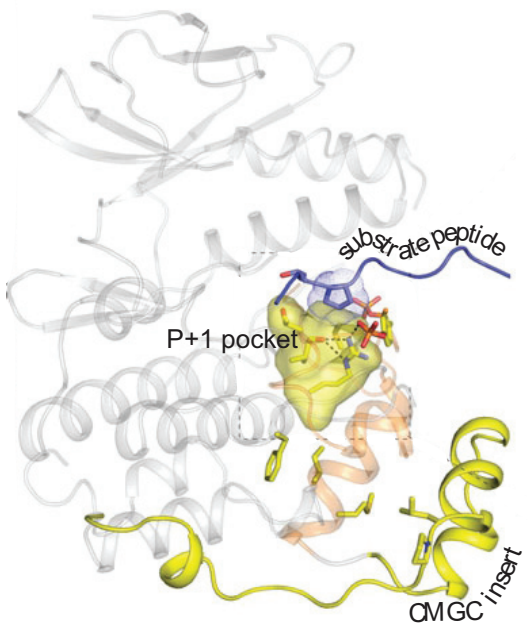

Figure 4. The CMGC-specific structural features P+1 and CMGC insert. (Modified from Oruganty and Kannan, 2012).

In total the CMGC family has 62 (uniprot.org, Varjosalo et al., 2008) members, which can be further classified into eight (Manning et al., 2002) subfamilies, where CDKs and MAPKs form the two most studied and largest families with 21 and 14 members, respectively (Malumbres et al., 2009). Other families are DYRKs (dual-specificity tyrosine (Y)-phosphorylation-regulated kinases; 10), CLKs (4), RCKs (tyrosine kinase gene v-ros cross-hybridizing kinase; 3), CDKLs (cyclin dependent kinase like; 5), GSK3s (2), and SRPKs (SR-specific protein kinase; 3) (**Figure 3**).

Most of the CMGC kinases can be assigned to particular biological functions including control of the cell cycle (CDKs), cell fate decisions (MAPKs), regulation of multiple signaling pathways (GSKs), and RNA splicing (CLKs). The individual subfamilies and their specific features will be described in detail below.

1.3 CMGC kinase subfamilies

1.3.1 ***CDK*** (cyclin dependent kinase)

CDKs or **c**yclin **d**ependent **k**inases form a family of 21 constitutively expressed serine/threonine kinases that regulate cell cycle, transcription, mRNA processing, and differentiation of cells (Malumbres, 2014). CDKs, as their name indicates, are dependent on cyclins - proteins whose levels constantly fluctuate in a tightly coordinated manner during the cell cycle (**Figure 5**). In the CDK/cyclin complexes, CDKs function as the catalytic subunits, whereas cyclins are the regulatory subunits that are needed for CDKs to present enzymatic activity. Indeed, the main mechanism to regulate the enzymatic activity of CDKs is the production and degradation rate of cyclins. The CDK activation loop contains the residues for both cyclin and subsequent ATP binding and as with most eukaryotic protein kinases, CDKs are also controlled by phosphorylation of the activation segment. Phosphorylation has either inhibitory effects via interference on ATP binding at the catalytic cleft or activating effects when the phosphorylation site acts as a substrate of CDK-activating kinases including other CDKs. Activating phosphorylation improves substrate binding by promoting full kinase activity (Jeffrey et al., 1995; Russo et al., 1996).

CDK activity is downregulated by two families of small proteins that function as CDK inhibitors during the cell cycle. A family of cyclin-dependent kinase inhibitors, INK4 proteins, acts during G1, specifically inhibiting the binding of CDK4/CDK6 with cyclin D (Harper and Brooks, 2005; Malumbres, 2014), whereas the broader spectrum inhibitors of the Cip/Kip family inhibit the activity of the CDK/cyclin -complexes throughout the cell cycle (Harper and Brooks, 2005; Malumbres, 2014).

Based on evolutionary clustering CDKs form kinase subfamilies that regulate separate entities within the cell. CDKs 1-4, and 6 are mainly involved in cell cycle regulation, whereas CDKs 7-9, 11-13, and 19 have established roles in transcription (Malumbres, 2014). Other CDKs have variable roles that will be discussed later.

Cell cycle regulation by CDKs (CDK1, -2, -3, -4 and -6)

Most living organism are unicellular, but others like humans consist of millions of cells. During development of these multicellular organisms multiple rounds of cell growth and division have to take place, and this process continues throughout the life of an individual. This elegant, tightly regulated universal process of cell growth and division, where a cell duplicates its contents and divides into two is known as the cell cycle.

In a normal situation, the cell cycle is under tight regulation at each state through the activation or deactivation of a multitude of proteins. The mammalian cell cycle has five phases: G0 (resting state), G1 and G2 (RNA and protein synthesis), S (DNA replication) and M (mitosis and completion of cell division), where multiple checkpoints ensure normal progression from one phase to another. Each checkpoint has its own specific cyclin/CDK complexes controlling the cell cycle progression (**Figure 5**). Briefly, cyclin C/CDK3 complex assists cells in G0-G1 transition (Ren & Rollins, 2004; uniprot.org). Cyclin D/CDK4(CDK6) initiate the transfer through G1 through phosphorylation of the retinoblastoma protein that leads to transcription of genes needed for DNA synthesis and further progression of the cell cycle. Cyclin E/CDK2 is a gatekeeper in the G1/S transition and is responsible for the hyper-phosphorylation of the retinoblastoma protein. During S phase, cyclin A accumulates, and the activity of cyclin A/CDK2 is essential for S phase termination via phosphorylation of transcription factor E2F1. Cyclin A/CDK1 and cyclin B/CDK1 are important for progression through G2 and M phase (Harper and Brooks, 2005; Malumbres, 2014). CDK7 can also influence cell cycle by acting as CDK-activating kinase, phosphorylating for example CDK1 and CDK2 (Fisher, 2012).

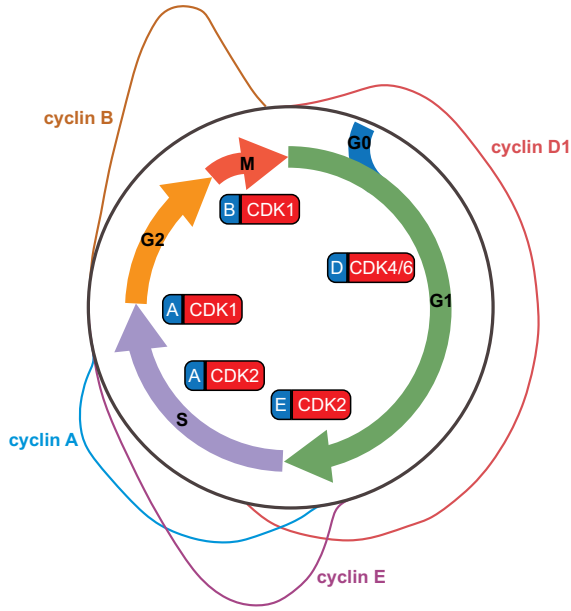

Figure 5: The cell cycle and fluctuation of cyclin levels during the cell cycle progression. The cell cycle is divided into five phases: G0 (senescence), G1 (cell decides to grow or to enter G0), S (DNA replication), G2 (cells prepare for entry into mitosis), and M (mitosis, cell division). The CDK/cyclin complexes important for different phases are drawn on the inner rim of the cell cycle, and the fluctuating levels of cyclins on the outer rim. The fluctuations in cyclin levels are tightly linked to the different phases of cell cycle. However, the levels of cyclin D are strongly influenced by extracellular signals from growth factors.

To study the functional roles of individual cell cycle related CDKs *in vivo* multiple knockout mice have been made (**Figure 6**). The analyses of knockout animals ablated of one or more of the cell cycle related CDKs, CDK1 (Santamaria et al., 2007), CDK2 (Ortega et al., 2003; Berthet et al., 2003), CDK4 (Rane et al., 1999; Tsutsui et al., 1999), and CDK6 (Malumbres et al., 2004)

have shown that only CDK1 is essential for embryonic cell division leading to early embryonic lethality. CDK2, 4, and 6 are mainly needed for the development of distinct cell types and the knockout animals develop normally into adulthood (Barrière et al., 2007). CDK4/CDK6 double knockout mutant embryos die in utero (Malumbres et al., 2004).

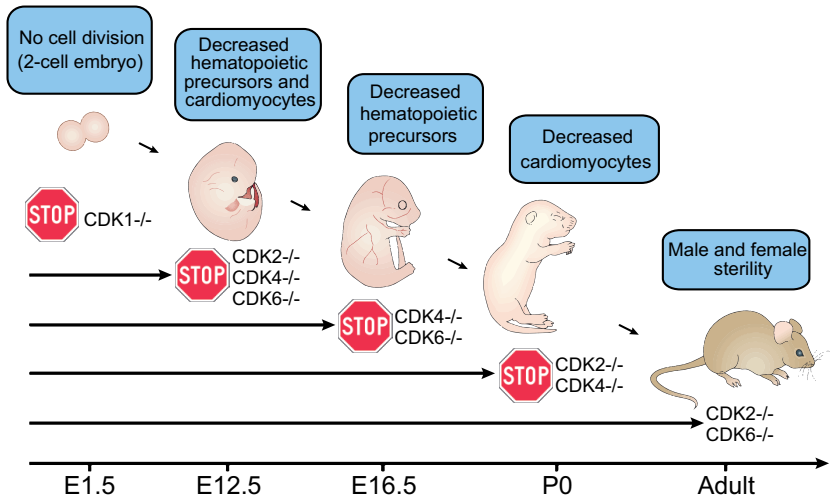

Figure 6: **Transgenic CDK-mice.** Genotypes of the various knockout mice used to study the role of cell cycle -linked CDKs are indicated. The arrows and stop signs indicate the embryonic development stage to which the corresponding CDK knockout animal develops. (Modified from Malumbres and Barbacid, 2009)

Transcription initiation, elongation and termination regulation by CDKs ('transcriptional CDKs': CDK7, -8, -9 -11, -12, -13 and -19)

To utilize the information that is stored in the cellular DNA, cells need to copy this genetic information to an RNA nucleotide sequence in a tightly regulated process termed transcription. Transcription on all protein-coding genes is dependent on RNA polymerase II (RNA pol II). RNA pol II has a C-terminal domain (CTD) composed of polypeptide repeats that are phosphorylated in a gene specific manner to regulate the activity of RNA pol II. The first step in transcription is the recruitment of RNA pol II to the promoter of the transcribed gene. This task, among others, is performed by transcription preinitiation complex (PIC), composed of several general transcription factors (TFIIA, TFIIB, TFIID, TFIIE, IFIIF, TFIIH), the Mediator complex, and RNA pol II itself (**Figure 7**).

The TFIIH has multiple subunits, one of which is the subcomplex composed of CDK7, cyclin H and MAT1. The TFIIH complex is important at the transcription start site as it unwinds the DNA, positions it correctly in the RNA pol II active site and phosphorylates a serine (primarily Ser-5, but can also be Ser-7) at the CTD of RNA pol II to initiate RNA production. When the transcript has gained a certain threshold length, RNA pol II sheds most of the first transcription factors, and new factors are recruited on site. During elongation one of these newly recruited factors is the CDK9/cyclin T containing positive transcription elongation factor, pTEFb. The CDK9 subunit phosphorylates another serine (Ser-2) in the CTD of RNA pol II. CDK12 interacts

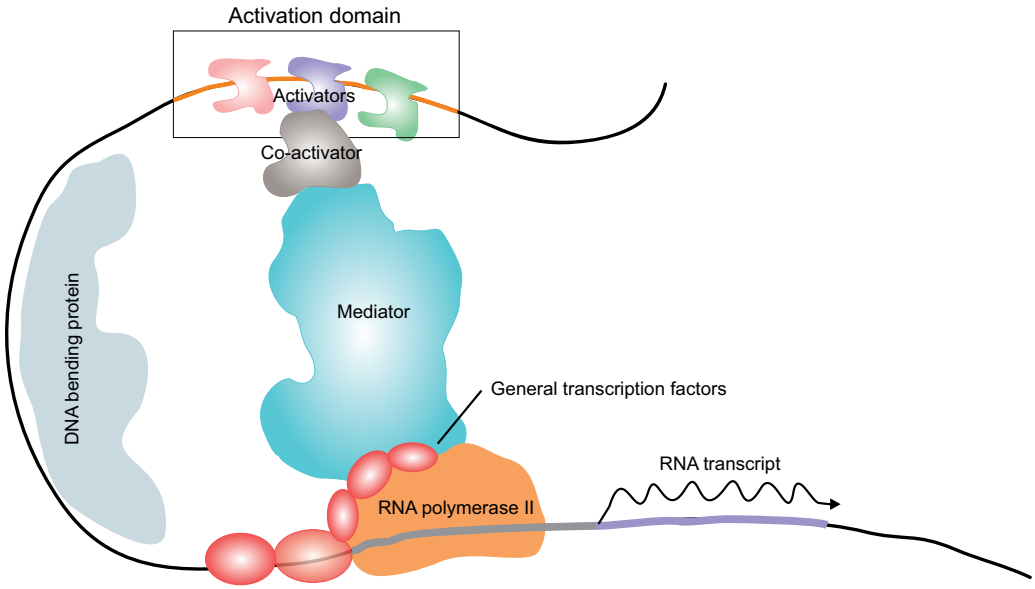

Figure 7: Illustration of the transcription preinitiation complex. Transcription activators bind to the activation domains (enhancers) and, thus, turn 'on' the gene. As indicated in the illustration, these enhancer regions (indicated with orange) might reside relatively distant to the start of the gene (lilac). Transcription factors assemble at the promoter region of the DNA. The Mediator complex conveys the signals from TFs to RNA pol II, and transcription begins at the initiation site (grey). (Modified from Vojnic et al., 2011)

with cyclin K and has been shown to phosphorylate the same serine-2 in the CTD. CDK12, CDK11 and CDK13 also act in alternative RNA splicing (uniprot.org; Even et al., 2006; Loyer et al., 2008). The last step of transcription is termination releasing both the RNA pol II from the template DNA and mRNA from the transcriptional complex.

The presence of CDK8 and CDK19 containing Mediator complex is also necessary for RNA pol II phosphorylation, although the main task of Mediator is to transfer gene-specific regulatory signals from multiple transcription factors to RNA pol II (Allen, Taatjes, 2015).

To date, knockout animal models of transcriptional CDKs are available only for CDK7 (Ganuza et al., 2012), CDK8 (Westerling et al., 2007), CDK11 (Li et al., 2004), and CDK12 (Juan et al., 2016). All of these knockouts are embryonic lethal during early development.

Other CDKs (CDK5, -10, -14, -15, -16, -17, -18, and -20)

CDK5 is expressed in post-mitotic cells like neurons (Liu, W. et al., 2017). The presence of CDK5 is essential for developing brain (Ohshima et al., 1996), and later in adult brain it is involved in multiple neuronal processes including learning and memory, survival, synaptic plasticity, and pain signaling (Shah and Lahiri, 2014). It is considered as a potential target in the treatment of neurodegenerative diseases such as Alzheimer's and Parkinson's disease (Shah and Lahiri, 2014). In brain, CDK5 is activated by interaction with the non-cyclins CDK5R1 (p35) or CDK5R2 (p39). Cyclins D1, D3, and E have been reported to form a complex with CDK5 (Shah and Lahiri, 2014), and in 2009 (Brinkkoetter et al., 2009), cyclin I was identified as a new CDK5 activator. Recently cyclin I-like (CCNI2), a homolog of cyclin I, was shown to activate CDK5, affect its

cellular localization and have a role in cell cycle progression and cell proliferation (Liu, C. et al., 2017).

Only recently the functional role and activating cyclin of CDK10 was detected (Guen et al., 2013). Previously, silencing of CDK10 was linked to ETS2-driven increased expression of c-RAF leading to MAPK pathway activation, and loss of estrogen responsiveness of breast cancer cells causing tamoxifen resistance in certain breast tumors (Iorns et al., 2008). The study of Guen et al. (2013) indeed showed that cyclin M interacts with CDK10 and this complex phosphorylates ETS2 transcription factor and positively controls ETS2 degradation by the proteasome.

CDK14 to CDK18 belong to the PFTAIRE and PCTAIRE kinase subfamilies (HUGO Gene Nomenculature Committee, genenames.org). CDK14 and CDK16 are activated at the plasma membrane by cyclin Y and participate in multitude of signaling cascades such as the Wnt pathway (Jiang et al., 2009; Shehata et al., 2012). This pathway is known to control transcription and its regulators are also important during mitosis. Noteworthy, cyclin Y expression has also been shown to peak during the G2-M phase of the cell cycle. Both of these observations suggest these enzymes playing a role in cell division (Malumbres, 2014). CDK15, CDK17 and CDK18 are the least studied CDKs. CDK15 has been shown to phosphorylate survivin and lead to reduction in tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induced apoptosis (Park et al., 2014). Elevated phosphorylation of CDK17 and CDK18 was recently identified in neuroblastoma cell line expressing amyloid precursor protein (APP) and also validated in brain samples from Alzheimer's disease patients (Chaput et al., 2016). This finding identifies novel targets for APP and raises further questions about the role of CDKs in neurodegenerative diseases.

CDK20 has a role in ciliary length control and knockdown of this protein promoted cilia formation and cilia stability in the absence of kinase activity (Yang, Y. et al., 2013).

1.3.2 ***MAPK*** (mitogen-activated protein kinase)

This family of serine/threonine kinases has 14 members that can be divided into atypical and conventional kinases. The latter can be further subdivided into four subfamilies: 1) extracellular signal-regulated kinases (ERKs), 2) c-Jun amino-terminal kinases (JNKs), 3) p38 MAPKs and 4) ERK5. All of these enzymes are characterized by a system where three kinases act in a row to phosphorylate the downstream kinase (**Figure 8**). For ERKs the activators are MEK1 and MEK2, for JNKs- MKK4 and MKK7, for p38- MKK3 and MKK6, and for ERK5- MEK5. MAPK are highly conserved, respond to extracellular signaling clues and regulate many important cellular processes like proliferation, differentiation, and stress responses together with transcriptional control of CDKs in organisms ranging from yeast to human. Enormous amount of research has focused on MAPKs during the last decades, from their substrates and functions, to their role in health and disease.

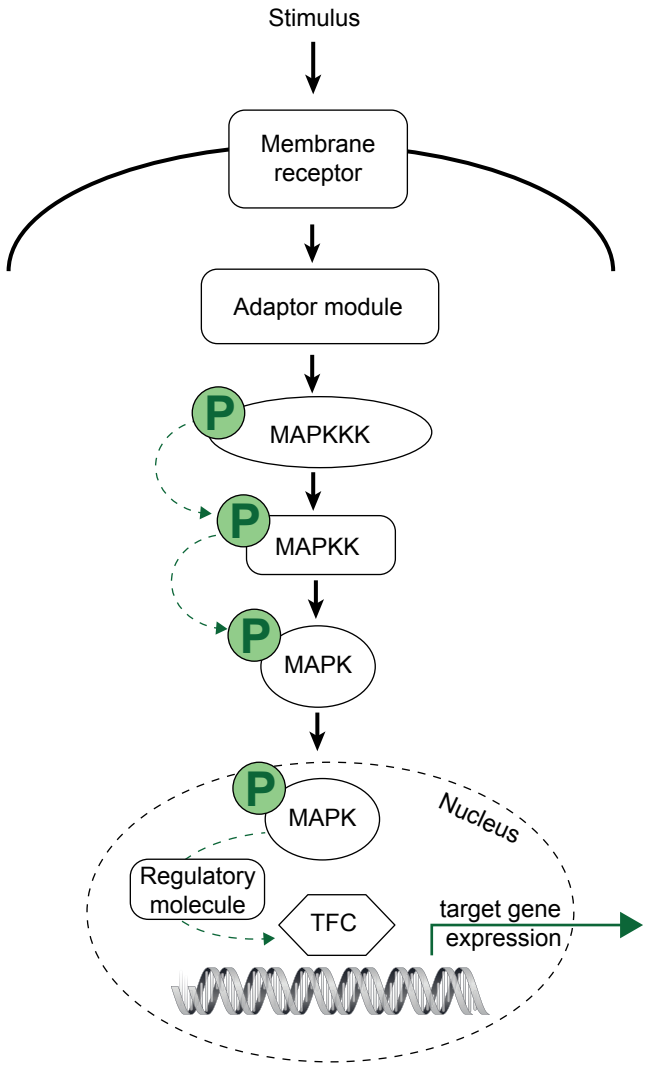

Figure 8: MAPK, MAPKK, MAPKKK. Each of the MAP kinases (MAPK) is activated by an upstream MAPK kinase (MAPKK), which is activated by MAPKK kinase (MAPKKK). The stimulus is specific to each MAPK subfamily. For ERK1/2, mitogenic stimulus leads to phosphorylation of Raf (MAPKKK) that further phosphorylates MEK1/2 (MAPKK) that finally phosphorylates ERK1/2. After activation by phosphorylation the MAPK translocates into the nucleus, and activates TCF family transcription factors. Most target genes for ERK1/2 are genes affecting proliferation, cell division and differentiation. For JNKs MKK4 and MKK7 act as MAPKK responding to stress signals, similar to p38 and MAPKKs MKK3 and MKK6. ERK5 responds to morphogenic clues via MEK5 promoted phosphorylation.

ERK (extracellular signal-regulated kinase)

ERKs respond to different stimuli ranging from growth factors to insulin to cytokines and carcinogens (Johnson and Lapadat, 2002; Cargnello and Roux, 2011). They are components of the cell surface receptor (receptor tyrosine kinase)/RasGTP/Raf/MEK/ERK -signaling cascade. Components of this signaling cascade have been shown to be essential for proper

cell proliferation. ERKs function both in the cytoplasm to promote cell proliferation (G1 to S phase transition), and in the nucleus to phosphorylate and activate multitude of transcription factors (TFs) (Cargnello and Roux, 2011; Burotto et al., 2014). Upon TF activation c-Jun is expressed, and stabilized by direct phosphorylation by ERK1/2 (Murphy et al., 2002) allowing association with c-Fos and subsequent formation of AP-1 complex. AP-1 is essential for cyclin D1 expression, which interacts with CDKs to drive G1 to S transition.

JNK (c-Jun N-terminal kinase)

All three JNKs are highly homologous (>85%), but have distinct tissue distribution. JNK1 and 2 are ubiquitously expressed, while the expression of JNK3 is mainly limited to the brain. In contrast to ERKs, JNKs are mostly activated by stress signals including oxidative stress, radiation, and DNA-damaging agents. JNKs are mainly localized to the cytoplasm, but their identified substrates are mostly TFs including c-Jun, p53, STAT3 and c-Myc. The phosphorylation of c-Jun leads to AP-1 complex formation and thus transcription of cyclin D1 promoting cell cycle progression, akin to ERK1/2 (Sabapathy et al., 2004). To date, only a few cytoplasmic interaction partners of JNKs have been identified (Bogoyevitch et al., 2010; Cargnello and Roux, 2011).

p38

The four members of p38 subfamily (α, β, γ, δ) respond to various environmental stress stimuli and cytokines like interleukin-1 and tumor necrosis factor α (TNF). Interestingly, p38 both regulates the production of cytokines and responds to them. Other targets of p38 regulation are TFs and other protein kinases. Based on the observations of p38 activation, it has a role in inflammation, cell cycle regulation and apoptosis (Cuadrado and Nebreda, 2010; Cargnello and Roux, 2011).

ERK5 (BMK1 or big MAP kinase 1)

ERK5 has a kinase domain that is similar to ERK1/2, having 51% similarity with ERK2. ERK5 is essential during normal embryogenesis (Regan et al., 2002). An upstream activator of ERK5 is MEK5, whose expression is elevated in metastatic prostate cancer (Mehta et al., 2003). Similar to ERK1/2 and JNK, ERK5 also promotes cyclin D1 expression and cell cycle progression (Mulloy et al., 2003).

1.3.3 GSK3 (glycogen synthase kinase-3)

Glycogen synthase kinase-3 is a conserved, widely expressed serine/threonine kinase. A unique feature of GSK3 is its high activity in resting cells. Humans have two forms of GSK3s: GSK3α and -β, which have 85% overall sequence homology (Woodgett, 1990). The kinase domain of these kinases is highly homologous (98%), whereas the N- and C-terminal domains differ (Woodgett, 1990). GSK3α and -β are functionally different, as homozygous GSK3β mice are embryonic lethal but GSK3α mice are viable (Hoeflich et al., 2000; MacAulay et al., 2007). Different from CDK and MAPK families described above, the activity of GSK's is regulated on multiple levels and ways ranging from phosphorylation by other kinases to autophosphorylation, and to need for priming phosphorylation of the target substrate by another kinase (Frame and Cohen, 2001; Cole et al., 2004; McCubrey et al., 2016). In most cases, phosphorylation of serine residues has been shown to inhibit GSK3's kinase activity, while tyrosine phosphorylation leads to increased

kinase activity (Medina and Wandosell, 2011). GSK3 (mainly GSK3α) has been extensive studied and have a known function in variety of signaling pathways ranging from Wnt, Notch and Hedgehog (proliferation) to growth factors affecting differentiation/survival. It is implicated to have multiple roles in many human pathological conditions ranging from neurodegenerative diseases to diabetes and several types of cancers (Cormier & Woodgett, 2017; McCubrey et al., 2016).

As GSK3 is a crucial component of the canonical Wnt signaling pathway, which is important during normal development and often gone awry in cancers. The following is a short summary of the main events during Wnt-β-catenin signaling. A central component of this pathway is the destruction complex that regulates the cytoplasmic β-catenin levels. The destruction complex is composed of tumor suppressors Axin and adenomatosis polyposis coli (APC) together with casein kinase-1α (CK1α) and GSK3 (Niehrs, 2012). In the absence of Wnt-ligand, β-catenin is assembled into this complex, phosphorylated by CK1 and GSK3 labelling it for proteasomal degradation, thus preventing the transcription of β-catenin dependent genes. Upon Wnt binding to its receptor Frizzled, the destruction complex is not able to phosphorylate and ubiquitinate β-catenin, which then translocates to the nucleus where it forms complexes with transcription factors and promotes transcription (**Figure 9**). Phosphorylation by GSK3 is essential also for targeting multiple other proteins for proteasomal degradation. (Kaidanovich-Beilin and Woodgett, 2011; Cormier and Woodgett, 2017)

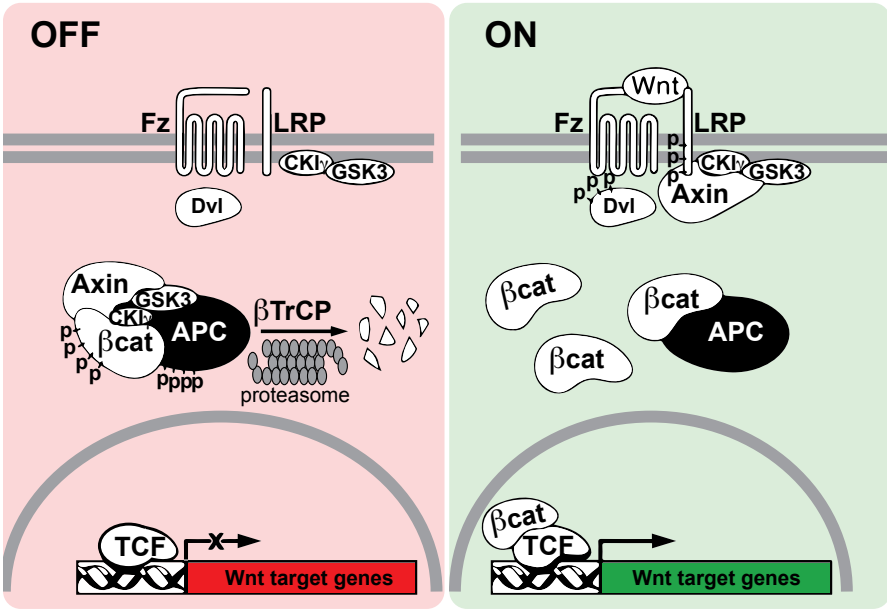

Figure 9: Simplified pathway of Wnt-β-catenin GSK3. In the absence of Wnt ligand, the cellular β-catenin levels are kept low. The phosphorylation of β-catenin by the destruction complex enables recognition and ubiquitination by E3 ubiquitin ligase β-TrCP (β-transducin repeats containing protein) further targeting β-catenin for proteasomal degradation (Wnt OFF state, left). Upon ligand binding to Frizzled and its co-receptor LRP (lipoprotein receptor-related protein), the destruction complex translocates to the plasma membrane. The binding of Axin to LRP is catalyzed by LRP phosphorylation by other destruction complex components (GSK3 or CK1). Cytoplasmic levels of β-catenin accumulate leading to its translocalization into the nucleus and subsequent induction of TCF family transcription factors.

*1.3.4 **CLK*** (cdc2-like kinase) ***and other less-studied kinases***

The four Cdc2-like kinases or CLKs are dual specificity kinases that autophosphorylate at tyrosines, but specifically phosphorylate serine/threonine residues on their substrates (Nayler et al., 1997). CLKs localize to cytoplasm and nucleus, where they phosphorylate the arginine and serine (RS)-rich domains on serine/arginine (SR) splicing factor proteins thus controlling their nuclear distribution (Bullock et al., 2009). SR splicing factor proteins are extensively phosphorylated by multiple kinases, and the correct phosphorylation status has been shown essential for the activity of SR proteins (Prasad et al., 1999). These protein are indispensable for the alternative mRNA splicing - a fundamental process responsible of the production of the complex human proteome during normal development.

In a recent study, CLKs were identified upstream of Aurora B acting as novel components in the final steps of cytokinesis, the division of cytoplasm into two cells (Petsalaki and Zachos, 2016). But as Petsalaki and Zachos concluded, our understanding, to date, of the cellular functions of CLKs is rather limited (Petsalaki and Zachos, 2016).

SRPK (SR-specific protein kinase)

Serine-arginine protein kinases specifically phosphorylate serine residues in serine/arginine/arginine-serine dipeptides. They are mainly localized in the cytoplasm, where these constitutively active kinases regiospecifically phosphorylate SR splicing factors on multiple serines inducing the translocation of these factors into nucleus, where they are further phosphorylated by CLKs (Giannakouros et al., 2011; Ghosh and Adams, 2011). The change in the cellular localization of the SR proteins is essential to phosphorylation by CLKs (Bullock et al., 2009). As mentioned above, SR protein phosphorylation is necessary for correct mRNA splicing and maturation.

Over 100 hundred SR-domain containing proteins have been identified in the human genome (Calarco et al., 2009), indicating multiple open questions of the functional roles of SRPKs in mammalian cells. SRPK1 expression is elevated in breast, colonic, and pancreatic cancers (Hayes et al., 2007) as well as acute T-cell leukemia (Hishizawa et al., 2005). SPRK2 has a specific role in phosphorylating apoptosis promoting protein ACIN1 and increasing cyclin A1 expression on leukemia cells (Jang et al., 2008).

RCK (tyrosine kinase gene v-ros cross-hybridizing kinase)

The RCK serine/threonine-protein kinase family consists of three kinases MAK, ICK and MOK, whose functions are poorly understood. Structurally RCK family resembles both MAPKs and CDKs (Manning et al., 2002). MAK and ICK autophosphorylate on a tyrosine residue, but need a second phosphorylation on their MAPK-like motif in their activation loop by an upstream kinase for full enzymatic activity (Fu, Z. et al., 2005; Wang and Kung, 2011; Miyata et al., 1999). Different from MAPK, the activating kinase for MAK and ICK is cell cycle related kinase (CCRK), but for MOK it remains unknown (Wang and Kung, 2011; Fu, Z. et al., 2006).

MAK (male germ cell-associated kinase) is mainly expressed in the testicular germs cells during spermatogenesis and in the retina in the back of the eye (Omori et al., 2010; Tucker et al., 2011). In the retina MAK localizes to connecting cilium in photoreceptor cells, negatively regulates the length of their cilia and is essential for the survival of these cells (Omori et al., 2010). Not surprisingly, MAK mutations are associated with retinitis pigmentosa, an illness of photoreceptor degeneration in the retina (Ozgul et al., 2011; Tucker et al., 2011).

ICK (intestinal cell kinase) is highly conserved, constitutively and widely expressed. Similarly to MAK, it negatively regulates ciliary length and, in addition, is identified as an important component of sonic hedgehog signaling (Moon et al., 2014). These two factors seem to be the underlying cause of human ECO syndrome, a multi-organ illness affecting endocrine, cerebral and skeletal systems, caused by a missense mutation in ICK gene (Lahiry et al., 2009).

The cellular function of the third RCK, MOK (MAPK/MAK/MRK-overlapping kinase), is largely unknown. To date, only the expression of MOK in mouse wildtype and cancerous intestinal cells has been analyzed by western blotting, showing downregulation of MOK in adenomas (Chen, T. et al., 2013). This interesting observation suggests that MOK might have a role in intestinal cancer development, but further studies are needed.

CDKL (cyclin dependent kinase like)

This family of five members of serine/threonine kinases with homology to MAPK and CDK families is hitherto rather uncharacterized. Studies focused on CDKL5, a ubiquitous protein mainly expressed in the brain, testes and thymus (Lin et al., 2005). CDKL5 mutations have been identified in a severe neurodevelopmental disorder, the Rett syndrome (Weaving et al., 2004; Tao et al., 2004). In postnatal brain, CDKL5 has similar expression pattern as metyl-CpG-binding protein (MECP2), whom CDKL5 directly interacts with and mediates its phosphorylation (Mari et al., 2005). MECP2 is also frequently found mutated in the Rett syndrome (Van den Veyver and Zoghbi, 2001), whether CDKL5 and MECP2 are components of a common cellular pathway remains an open question.

1.3.5 DYRK (Dual-specificity tyrosine (Y)-phosphorylation-regulated kinase)

DYRK is a relatively large mammalian dual-specificity protein kinase family, that based on homology analysis consists of three subfamilies having in total 10 members: DYRK (DYRK1A-B, 2-4), HIPK (**h**omeodomain-**i**nteracting **p**rotein **k**inase 1-4), and PRP4 (**p**re-m**R**NA **p**rocessing **p**rotein **4** kinase).

DYRK 1-4

The DYRK subfamily members are autophosphorylated on tyrosine within the activation loop during their translation resulting in a constitutively active mature protein (Lochhead et al., 2005). Autophosphorylation of this conserved tyrosine is essential for achieving full kinase activity (Himpel et al., 2001). As DYRKs are not subject to the classical MAPK-like regulation by an upstream protein kinase, other regulatory mechanisms have been proposed. All of these subfamily members experience changes in their cytoplasmic versus nuclear localization, which are speculated to limit substrate accessibility and, thus, regulate DYRK1-4 (Aranda et al., 2011). Other regulatory mechanisms for DYRKs might be alterations in gene expression and protein abundance or interactions with regulatory proteins. Thus far DYRKs are not shown to undergo regulation by reversible phosphorylation or dephosphorylation (Aranda et al., 2011).

The major function of mammalian DYRKs is the regulation of the cell cycle. Both DYRK1A and DYRK1B can be classified as survival and differentiation promoting factors. DYRK1A has been shown to control the length of G1 phase via regulating cyclin D1 levels and to promote the transition between quiescence and differentiation (Chen, J. Y. et al., 2013). DYRK1B is active in quiescent cells preventing entry into G1 by stabilizing CDK inhibitors and destabilizing cyclin D (Zou et al., 2004; Deng et al., 2004). In apoptosis DYRK1A, DYRK3, and DYRK2 have opposite

roles, as DYRK1A and DYRK3 phosphorylate either directly pro-apoptotic proteins or their inhibitors leading to inhibition of apoptotic activity (Seifert et al., 2008; Guo et al., 2010), while the phosphorylation of p53 by DYRK2 (or HIPK2) promotes apoptosis (Taira et al., 2007).

Another common character of the DYRKs is their action as priming serine/threonine kinases for consequent phosphorylation by GSK3 thus targeting proteins for proteasomal degradation (Aranda et al., 2011). To date DYRK family members have been identified as priming kinases for oncoproteins c-Jun and c-Myc in addition to transcription factor GLI2 (Taira et al., 2007; Varjosalo et al., 2008).

Although the functions of the fourth member of this subfamily, DYRK4, are ambiguous, based on the molecular roles of other DYRKs, one can conclude that the members of this subfamily are involved in cell survival, cell differentiation, gene transcription and translation (Aranda et al., 2011).

Clinical relevance of DYRK subfamily

The most studied member is DYRK1A – mostly due to the gene localization in chromosome 21, which is triplicated in Down syndrome. Individuals affected with Down syndrome have an elevated risk of developing Alzheimer's disease. Due to chromosomal trisomy leading to elevated DYRK1A expression on transcriptional and protein level, DYRK1A is thought to function in the pathogenesis of neurodegeneration in this patient population (Park et al., 2009). The hyperphosphorylation of tau, a hallmark protein of Alzheimer's disease, by DYRK1A observed in Down syndrome patients, potentially also contributes to neurodegeneration in normal population.

HIPK (homeodomain-interacting protein kinase)

HIPKs, like DYRKs, are autophosphorylated to reach full kinase activity (van der Laden et al., 2015). HIPK1 to 3 are highly homologous, whereas the later identified fourth member, HIPK4, is only related to others in its catalytic domain (Arai et al., 2007). Most of the knowledge of HIPKs comes from HIPK2, which is known to act in cell cycle, apoptosis and responses to DNA damage mostly via interactions with TFs. The activity of HIPK2 itself is orchestrated by multiple post-translational modifications and caspase-mediated cleavage (van der Laden et al., 2015). Due to structural similarity, HIPK1 is thought to have a similar role in cells as HIPK2 (Aikawa et al., 2006). A recent study identified HIPK2 as a facilitator of Dishevelled phosphorylation, maintaining cells responsive to Wnt ligand stimulation (Shimizu et al., 2014). The molecular functions of both HIPK3 and, especially, HIPK4 are still rather uncharacterized.

PRP4 (pre-mRNA processing protein 4 kinase)

A subfamily of ubiquitously expressed kinases that regulate pre-mRNA splicing and acts as a spindle assembly checkpoint protein (Kojima et al., 2001; Montembault et al., 2007). Recently the kinase domain of PRP4 was identified essential for pancreatic and colorectal tumor cell viability (Gao et al., 2013).

1.3.6 Protein kinase therapeutics – kinase inhibitors

The protein kinase domain is among the most commonly encountered domain in known cancer genes (Futreal et al., 2004). As described earlier, most of the kinase pathways are involved

in critical cellular processes like proliferation, apoptosis, or survival, and often, abnormal phosphorylation is either the cause or consequence in cancer (Cohen, 2002). Therefore, protein kinases are among the most studied druggable targets in pharmacological research to date (Knight et al., 2010; Abbassi et al., 2015). In human cancers, kinases are often found overexpressed or overactive, due to vast array of genetic and epigenetic events including point mutations, chromosomal gene amplifications (copy number alterations), and chromosomal translocations giving rise to gene fusions (Gross et al., 2015).

Oncogenic relations of CMGC family members

Combined data from six recent studies identified a total of 1100 genes that drive cancer development. From this list, an enrichment of protein kinases was observed (91 kinases were present) (Fleuren et al., 2016). Six of them (6/91; ~7%) belonged to CMGC kinase family (Fleuren et al., 2016) involving family members of CDK, MAPK and DYRK; namely CDK4, CDK6, CDK12, MAPK1 (Erk2), MAPK8 (JNK1)), and DYRK1A. Expectedly, the alterations observed in these genes were either point mutations or copy number changes (Fleuren et al., 2016). Other studies have also identified amplified CDK12 expression in breast cancers and inactivated in ovarian cancers (Kauraniemi et al., 2001 and 2003; Joshi et al., 2014). In addition, over-expression of CDK7 together with MAT1 and cyclin H was detected in >900 estrogen receptor positive (ER+) breast cancer samples (Patel et al., 2016). DYRK1B overexpression has been observed in pancreatic and ovarian cancer (Friedman, 2007), and in osteosarcomas and rhabdomyosarcomas (Yang et al., 2010; Mercer et al., 2006). Interestingly, the depletion of DYRK1B drove pancreatic and ovarian cancer cells to apoptosis (Deng, X. et al., 2006; Hu, et al., 2013).

CMGC kinase inhibitors

Based on the common structural features of protein kinases, presented in detail earlier in this work, the majority of protein kinase inhibitors target the ATP-binding site in the activation segment, thus competing with ATP. The second-generation inhibitors target protein kinase in a specific conformation and are thought to be more specific. However, there are highly selective and broad range inhibitors within both generations of kinase inhibitors (Treiber and Shah, 2013).

Several pan-CDK inhibitors have been studied in different phases in clinical trials, and currently multiple ongoing trials are announced to assess the effects of multitarget CDK inhibitors, seliciclic, dinaciclib, and AT7519, in different cancers either as single therapeutic agent or in combination with other agents (clinicaltrials.gov). Unfortunately, a common shortcoming of kinase inhibitors is the relatively low percentage of patients presenting satisfactory response (Knight et al., 2010). The best clinical outcome is usually reached with a polypharmacological approach, using drug combinations either within a single kinase pathway or targeting parallel kinase pathways in preselected patient populations (Knight et al., 2010; Gross et al., 2015).

The first US Food and Drug Administration (FDA) approved CMGC family kinase inhibitor was Palbociclib. It is a selective CDK4 and CDK6 inhibitor, approved for the treatment of ER positive and human epidermal growth factor receptor-2 negative (ER+/HER2-) advanced breast cancer (Beaver et al., 2015). Currently there are over 50 clinical trials listed for Palbociclib (clinicaltrials.gov), studying, among others, the effects in acute leukemias, neuroendocrine tumors, and metastatic breast cancer. Some of these studies combine Palbociclib with other

kinase inhibitors or already established pharmaceuticals (for example tamoxifen), or have targeted patient populations with certain predictive biomarkers.

Abemaciclib, another specific CDK4 and CDK6 inhibitor, received a breakthrough therapy status from FDA in October 2015, and a successful phase III combination trial in patients with hormone receptor positive (HR+), HER2- advanced breast cancer was published in March 2017 (https://investor.lilly.com/releasedetail.cfm?releaseid=1017952).

The third CDK4 and CDK6 specific inhibitor, Kisqali® (ribociclib), received FDA breakthrough therapy designation in 2016, and has been approved by FDA in March 2017 as a first-line treatment for HR+/HER2- advanced or metastatic breast cancer in combination with aromatase inhibitor (https://www.novartis.com/news/media-releases/novartis-kisqalir-ribociclib-lee011-receives-fda-approval-first-line-treatment).

Currently, there are many ongoing clinical trials assessing p38 MAPK inhibitors in inflammatory diseases, and ERK1/2 (MAPK1) inhibitor in advanced cancers, administered either alone or in combination with other pharmaceutical agents (clinicaltrials.gov).

A plethora of GSK3 inhibitors have been published and studied in clinical trials for the treatment of cancer or Alzheimer's disease (Pandey and DeGrado, 2016). The first GSK3 inhibitor was lithium chloride (LiCl), which has been used in the treatment of bipolar disorder since 1950s. The main focus of the clinical studies has been in the treatment of Alzheimer's disease. The neuroprotective mechanism of GSK3 inhibitors is the reduction of β-amyloid production and tau hyperphosphorylation (Kramer et al., 2011). However, the results of the studies have been controversial, and none of the inhibitors have made it into clinical use (Pandey and DeGrado, 2016).

A polyphenolic green tea flavonol, epigallocatechin-3-gallate (EGCG), is DYRK1A inhibitor that was shown to normalize DYRK1A activity in Down syndrome patients in a pilot-study reversing cognitive deficits (De la Torre et al., 2014). Inhibition of DYRK1A is an enchanting therapeutic approach for the treatment of cognitive impairment in Down syndrome together with inhibiting tau hyperphosphorylation in Alzheimer's disease (Abbassi et al., 2015). DYRK-family inhibitors have also been shown effective *in vitro* in reducing proliferation and inducing apoptosis either as single agents or as dual-inhibitors targeting CKL1 (Abbassi et al., 2015). At the moment several clinical studies are listed to evaluate the effect of EGCG in cancer patients (clinicaltrials.gov).

The oncogenic potential and inhibitors of CDK8 are discussed later in the context of the Mediator kinase module.

2. THE MEDIATOR COMPLEX

The mammalian Mediator is a large protein complex (~2 MDa) that has been described as the central hub and master regulator of transcription acting on multiple levels ranging from epigenetic regulation, transcription elongation and termination to mRNA processing and enhancer formation (Yin and Wang, 2014; Clark et al., 2015). As briefly mentioned earlier, the main function of the Mediator is to transmit the information of general transcription factors to RNA pol II. In mammals, the Mediator complex has 30 subunits that can exist in multiple compositions. The core Mediator complex consists of 26 subunits forming the head, middle, and tail, and additional subunits forming the kinase module (**Figure 10**). The Mediator kinase module is composed of 4 subunits: CDK8/CDK19, MED13 (or MED13L), MED12 (or MED12L) and cyclin C (Clark et al., 2015; Allen and Taatjes, 2015; Yin and Wang, 2014). MED13 serves as

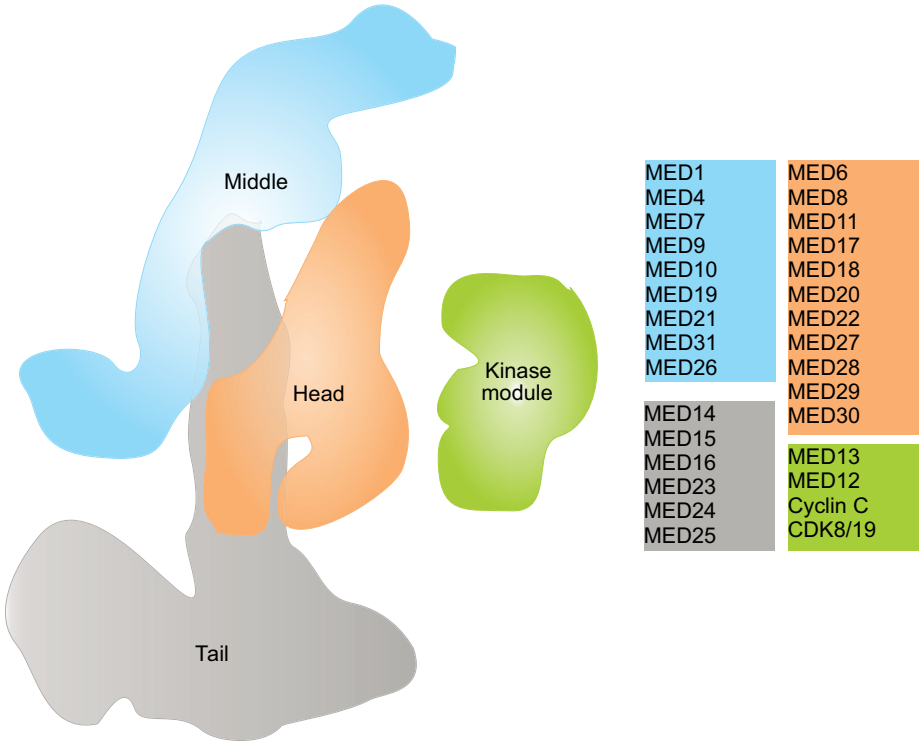

Figure 10: Illustration of the Mediator complex components. The Mediator subunits forming the middle, head, tail and kinase module are listed on the right. (Modified from Tsai et al., 2014.)

a direct link between CDK8 and other Mediator components (Knuesel et al., 2009a; Tsai et al., 2013).

Transcription factors bind to their specific subunits within the Mediator, thus permitting the simultaneous binding of multiple TFs. On the other hand, the absence of certain Mediator subunit only disrupts the downstream effects of certain TFs, and not all, as the binding partners for other TFs are still present (Allen and Taatjes, 2015).

The gigantic size of the Mediator complex offers an enormous platform for protein-protein interactions, at the same time causing problems in functional analysis (Allen and Taatjes, 2015). The Mediator was first discovered in yeast *Saccharomyces cerevisiae* (Kelleher 3rd et al., 1990; Flanagan et al., 1991), and has since been found across eukaryotes with conserved, basic functions in TF and RNA pol II binding. Interesting observation is, however, that the subunit composition and sequences of the Mediator subunits are not highly conserved from yeast to man, allowing fine-tuning of the processes they control (Allen and Taatjes, 2015).

2.1 Mediator kinase module: CDK8/CDK19, MED12, MED13, and cyclin C

CDK8 and CDK19 were previously classified in this work as transcriptional CDKs because of their association with the Mediator complex. More specifically, CDK8 and CDK19 form the kinase domain together with MED12, MED13, and cyclin C. CDK8 binds to cyclin C, but reaches full kinase activity only in CDK8-cyclinC-MED12 complex (Knuesel et al., 2009b). MED12 also serves

as a central hub within the kinase module, connecting CDK8/cyclin C and MED13. MED13 further connects the kinase module with the Mediator core (Tsai et al., 2013). Information from TF binding Mediator tail and kinase domains is conveyed through the Mediator middle and head to RNA pol II (Clark et al., 2015).

During transcription, the Mediator tail and kinase modules serve as binding sites for gene specific transcription factors working in context-dependent transcriptional repression or activation. Activation of signal transduction after TF phosphorylation by CDK8 has been observed, for example, in the interferon pathway, where STAT1 is a target for CDK8 (Bancerek et al., 2013). Another target of CDK8, E2F1, usually promotes β-catenin degradation. Upon phosphorylation by CDK8, the activity of E2F1 is repressed leading to increased β-catenin levels, often observed in colon cancer (Morris et al., 2008). Intrestingly, CDK8 has been identified as a colorectal cancer oncoprotein (Firestein et al., 2008), and as a coactivator of p53 tumor suppressor driven protein expression (Donner et al., 2007).

Only small fraction of cellular CDK8 is associated with the Mediator kinase module, and most likely there are other yet uncharacterized roles for CDK8 (Knuesel et al., 2009b). Although the kinase domain is now known to be essential for transcription, the full details of the physical and functional interactions with core Mediator and RNA pol II remain unclear.

2.1.1 Kinase module and cancer

In addition to having a plethora of functional roles during transcription, as described above, the Mediator complex, and especially its kinase module, have been linked to development of multiple different cancers (Clark et al., 2015). **Figure 11** presents a summary of cancer types, where known mutations or other alterations in the kinase module components have been reported.

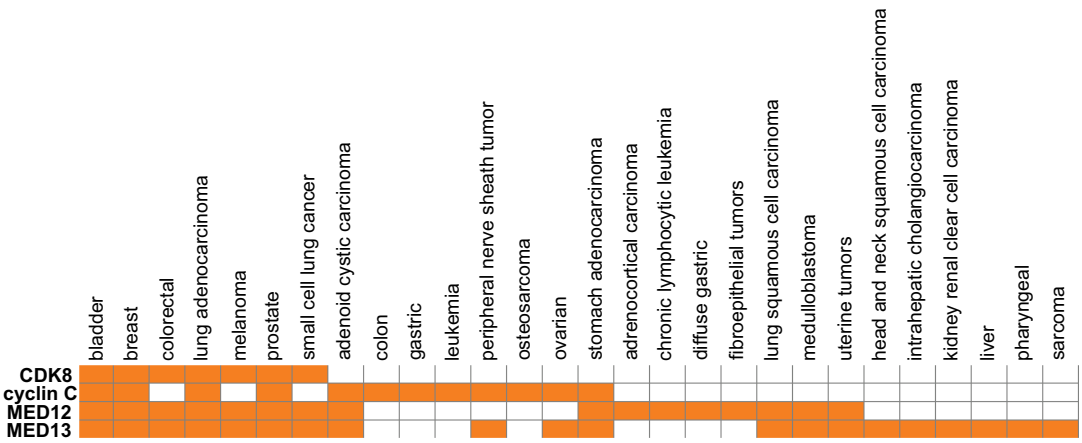

Figure 11: Oncogenic alterations in kinase module subunits (table based on the data on Clark et al., 2015).

An underlying common factor for tumorigenesis seems to be altered CDK8 kinase activity (Clark et al., 2015). The dysfunction of the kinase module can result either from the gain of CDK8 kinase activity, as observed in colorectal cancer, melanoma, pancreatic, breast and

gastric cancer, or from CDK8 loss of function, identified in uterine leiomyomas, T-cell acute lymphoblastic leukemias (T-ALL) and endometrial cancers (Clark et al., 2015). The changes in kinase activity can be due to mutations in CDK8 itself or happen through mutations in the other kinase module components, especially cyclin C or MED12. A phase I clinical study is currently recruiting patients to assess BCD115, a CDK8/CDK19 inhibitor, in combination therapy in ER+/HER- advanced breast cancer (clinicaltrials.gov).

MED12 in uterine leiomyoma and prostate cancer

In 2011, a study was conducted to find out high-frequency genetic alterations in benign, common uterine tumors, uterine leiomyomas (Makinen et al., 2011) (**Figure 12**). 70% of these tumors carried a mutation in exon 2 of MED12 gene. Almost 50% of studied leiomyomas displayed a missense mutation in codon 44, with all possible nucleotide substitutions. All of the mutations were predicted to result in an in-frame transcript with the most frequent mutation reported as G44D (20.9%; Makinen et al., 2011). To further characterize the role of the Mediator kinase module in uterine leiomyomas, 70 MED12 mutation-negative uterine leiomyomas were screened for mutations in other kinase module components CDK8/CDK19, Cyclin C or MED13. Interestingly, none of these tumors displayed mutations in the other kinase module components, further validating the tumorigenic role of MED12 mutations in uterine leiomyomas (Makinen et al., 2014).

After the identification of G44 as a mutational hotspot in uterine leiomyoma (Makinen et al., 2011), another MED12 gene mutation L1224F affecting exon 26 in 5 out of 152 patients was identified in prostate cancer (Barbieri et al., 2012) (**Figure 12**). The prostate cancer specific area of MED12 mutation is rather distant from the leiomyoma hotspot, thus presenting an open question of whether the tumorigenic impact of these two mutations is similar or different. The functional roles of these mutations need further studies.

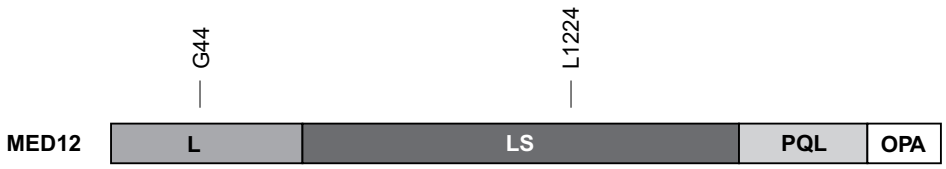

Figure 12. Schematic illustration of the human MED12 showing the location of the observed G44D, and L1224F mutations. Different domains of MED12 are also indicated.

3. 'OMICS' METHODS TO STUDY KINASE BIOLOGY

Most of the studies regarding kinase interactions have focused on a single kinase and its function or dysfunction in a disease. Additionally, the names of the CDK kinases were unified in 2009 (Malumbres et al., 2009), and that might have contributed to the slowness of studies of some these kinases. It is, after all, rather difficult to assign a kinase to this interesting family when the name is very different, such as PFTK1 or CRKRS.

3.1 Protein-protein interactions

The first high-throughput screenings of protein-protein interactions were performed using the yeast two-hybrid technique (Uetz et al., 2000). This method allows the identification of binary interactions, thus assigning proteins to a biological context. Also human protein-protein interactions were first studied in the yeast two-hybrid system as an important intermediate step towards a more systematic and comprehensive analysis of human interactome (Rual et al., 2005). The next development in interactomics was the detailed characterization of yeast protein complexes utilizing affinity purification coupled to mass spectrometry (AP-MS) (Krogan et al., 2006; Gavin et al., 2006). In 2009, Glatter and co-workers (Glatter et al., 2009) presented a sensitive, reproducible, high-throughput AP-MS workflow for human cells. The general workflow of an AP-MS experiment has three steps: 1) expression of the epitope-tagged bait protein, 2) single or double-step affinity purification of the protein complexes, and 3) analysis of the complex components with liquid chromatography coupled to a mass spectrometer (**Figure 13**). Several great advantages of this system are the use of Gateway®-compatible human Orfeome (Rual et al., 2004) collections, generation of isogenic cell lines, inducible expression of bait proteins at near physiological levels, high yield and reproducibility affinity purification together with direct, "gel free" analysis with LC-MS (Glatter et al., 2009). Later, this approach was also shown to be robust and highly reproducible between laboratories (Varjosalo et al., 2013a).

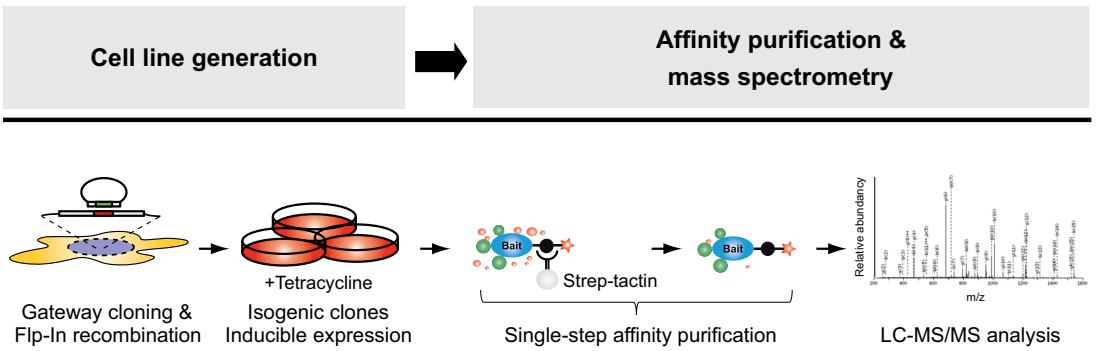

Figure 13: An overview of the AP-MS workflow. The isogenic, inducible HEK293 cell lines expressing Twin-Strep and hemagglutinin -tagged baits are generated. The tagged protein with its binding partners is single-step affinity purified from cell lysates utilizing Strep-tactin beads. The eluted proteins are trypsin digested and the resulting peptides analyzed by LC-MS/MS.

3.1.1 BioID proximity labeling

Proteins are not static components of cells forming only binary interactions, but can also form indirect interactions within a protein complex or with nearby proteins (Roux et al., 2012). The BioID method for proximity labeling of interacting proteins is based on the ability of a modified biotin ligase (BirA*) to covalently attach a biotin on the neighboring proteins lysine residues upon biotin supplementation in the growth media (Roux et al., 2012). Workflow for BioID sample analysis resembles AP-MS: the protein-of-interest (bait) is tagged with the BirA*, affinity purified with streptavidin beads and analyzed with LC-MS (**Figure 14**). Endogenous biotinylation

in mammalian cells is mainly limited to carboxylases, thus providing a rather specific way to label close proximity interactors in living cells and to detect transient and weak interactions with reasonable background (Roux et al., 2012; Varnaité and MacNeill, 2016). Noteworthy, the labeling radius is about 10 nm (Kim et al., 2014) indicating that spatiotemporally similar proteins will be labeled. Biotin will remain attached to the protein even though the BirA*-containing protein has moved further away, providing a molecular picture of the proteomic landscape in the cell.

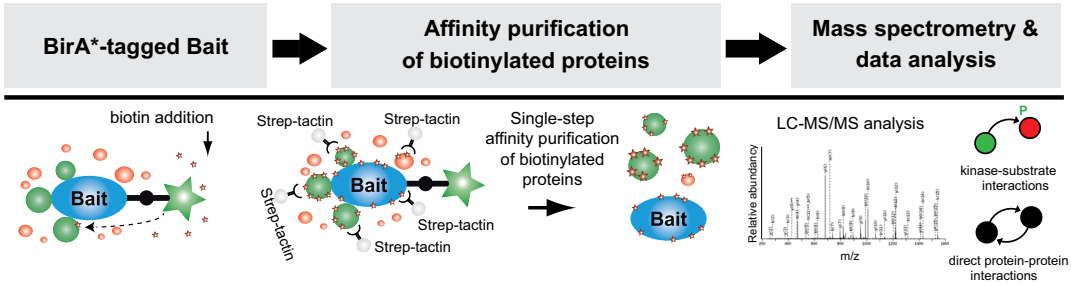

Figure 14: An overview of the BioID workflow. Addition of biotin to the cell culture media activates the bait protein tagged with modified biotin ligase, BirA* (indicated with a green star). Once active, the BirA* biotinylates the lysine residues in close proximity. The proteins from cell lysate are affinity purified using Strep-tactin beads, trypsin digested and analyzed by LC-MS/MS. The high-confidence interactors are identified using statistical filtering.

3.2 Assessment of kinase activity

As the function of protein kinases is to transfer a phosphate group, western blotting with antibodies against a substrate's phosphoserine, -threonine or -tyrosine is the most common and widely used method to indirectly identify kinase activity. This approach is very labor intensive, highly dependent on the specificity and sensitivity of the available antibodies, and not directly comparable with high-throughput approaches.

A more sensitive, direct approach to quantitatively measure kinase activity *in vitro* is to measure the incorporation of radiolabeled phosphate from γ-^{33}P ATP into the substrate (Hastie et al., 2006). A prerequisite to the solution-based *in vitro* kinase assays is a purified kinase, which in most cases is compared to the kinase dead mutant that abolishes ATP binding. An extensive study of 84 pairs of wild-type kinases and their kinase-dead counterparts (Varjosalo et al., 2008) confirmed, using *in vitro* kinase assays, that indeed the mutated "VAIK" or "HRD" motif is essential for kinase activity. The lysine in the "VAIK" motif is among the most conserved amino acids in all kinases as it is a central catalyzer of the phosphate transfer (Manning et al., 2002).

Functional protein microarrays are an efficient 'omics'-scale tool to detect kinase-substrate interactions (Zhu et al., 2001; Mok et al., 2009). The commercially available protein microarrays have thousands of purified proteins spotted onto a glass slide, providing an efficient means to identify phosphorylation of candidate substrates for the kinases of interest when incubated together with radiolabeled ATP (Meng et al. 2008) (**Figure 15**). To date, the only large-scale study utilizing protein microarrays in kinase substrate identification has been performed

with yeast protein kinases (Fasolo et al., 2011), although the human protein microarrays are nowadays commercially available.

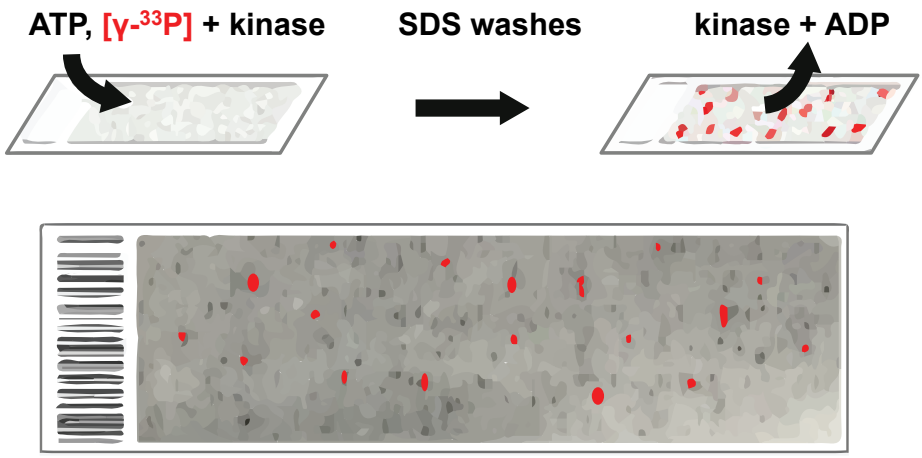

Figure 15: Kinase substrate identification on ProtoArray. After blocking the microarray, purified kinase is added in the presence of ^{33}P-ATP and incubated on slide. The unbound ^{33}P-ATP is removed by rigorous washing, and an image of the microarray is exposed to an X-ray film. The acquired image is further analyzed with ProtoArray Prospector software (Invitrogen).

II AIMS OF THE STUDY

The overall aim of this thesis project was to shed light on the cellular signaling networks of human CMGC-protein kinases. Of special interest were the physical and functional interactions of these kinases in human diseases. To validate our findings and further deepen the understanding of specific CMGC-kinase roles in human cancer, we focused on the functional roles of CDK8 and CDK19 within the Mediator complex in the newly identified MED12 mutation-linked diseases.

The specific aims in studies (**I-IV**) were as follows:

- To perform a global and comprehensive proteomics analysis of the signaling networks of the cancer-linked CMGC protein kinases (**I**).
- To study the impact of MED12 cancer-associated mutations to the Mediator kinase module assembly and function (**II-IV**).

III MATERIALS AND METHODS

The methods used in this thesis are summarized in this chapter. A more detailed description is found in the original publications (**I-IV**).

1. DNA CONSTRUCTS (I-IV)

Cloning of the DNA constructs was performed by using the Gateway® recombination technology (Thermo Fisher Scientific). The human CMGC kinase entry clones were obtained from the human Orfeome clone collection (Varjosalo et al., 2008) or from commercial sources (ThermoScientific or Dharmacon). The MED12 mutant plasmids were created using the QuikChange Site-Directed Mutagenesis kit (Agilent Technologies) either to a destination vector (Varjosalo et al., 2013b) or to a gateway pDONR221 entry clone containing wild type MED12 cDNA (Turunen et al., 2014). Finally, the entry clones were transferred by Gateway® recombination into destination vectors, that contained either N- or C-terminal tetracycline-inducible double affinity SH-tag (Twin-Strep-tag and hemagglutinin; pcDNA5_TO_StrepIII_HA_GW_FRT; Varjosalo et al., 2013b) or tetracycline-inducible BirA*/Myc-tag (biotin protein ligase and human c-myc; pcDNA5_TO_MYC_BirA; Heikkinen et al., 2017) (Table 1).

Table 1: Constructs and tags used in studies. The StrepIII or BirA are always proximal to protein of interest, other tags (Myc or HA) distal.

	Study I	**Study II**	**Study III**	**Study IV**
SH-tag				
HA_StrepIII-POI (N-terminal tag)	X			
POI-StrepIII_HA (C-terminal tag)	X	X	X	X
BirA*/Myc-tag				
POI-BirA*_Myc (C-terminal tag)				X

POI= Protein Of Interest

2. GENERATION OF ISOGENIC, TETRACYCLINE-INDUCIBLE, STABLE CELL LINES (I-IV)

Flp-In™ 293 T-REx cells (Invitrogen), containing an Flp Recombination target (FRT)-site at a transcriptionally active genomic locus and expressing the Tet repressor, were utilized in all of the original publications (**I-IV**) to create a stable, inducible cell lines. Upon cotransfection of pOG44 (Invitrogen) and destination vector of interest, the FLP recombinase is expressed from the pOG44 mediating a homologous recombination event between the FRT sites integrated into the genome of the cell line and on the destination vector. Integration confers hygromycin resistance and expression of the protein under investigation can be induced by the addition of tetracycline. This allows near intrinsic expression levels.

The pOG44 plasmid and corresponding kinase, MED12 WT or MED12 mutant destination vector were cotransfected into the Flp-In™ 293 T-REx cells using FuGENE 6 Transfection Reagent

(Promega) according to manufacturer's instructions. Selection with Hygromycin B (Thermo Fisher Scientific) was started two days after the transfections to create stable cell line. Cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM 1.5 g/l glucose) supplemented with 10% FBS, 2 mM L-glutamine, 50 mg/ml penicillin and 50 mg/ml streptomycin.

3. AFFINITY PURIFICATION OF PROTEIN COMPLEXES (I-IV)

For each pull-down experiment approximately 5×10^7 cells (5 x 15 cm confluent dishes) in two to three biological replicates were induced with 1 µg/ml tetracycline (Sigma) for 24 hours (for BioID additional 50 µM biotin was added). After induction, cells were washed with 0.1 mM $MgCl_2$, 0.1 mM $CaCl_2$ in PBS and harvested in 1 mM EDTA-PBS. Cells were pelleted by centrifugation 400 g for 5 min at 4 °C, then snap-frozen.

Cell pellets were affinity purified either with SH-tag or BirA*/Myc-tag purification method (**Table 1**). Strep-tag affinity purification was performed as described in (Turunen et al., 2014), and BioID purification with harsher conditions is explained in (Heikkinen et al., 2017). Briefly, cells were lysed in HNN-buffer, proteins were bound with Strep-Tactin sepharose beads (IBA GmbH) in Bio-Spin chromatography columns (Bio-Rad), and bound proteins were eluted with D-biotin in HNN-buffer (**I-IV**).

Obtained proteins were reduced and alkylated followed by trypsin digestion overnight at 37 °C. Peptides were quenched with trifluoroacetic acid (TFA), purified by reverse-phase chromatography C-18 Micro SpinColumns (Nest Group) and eluted with 0.1% TFA in 50% acetonitrile (ACN). Samples were vacuum dried and reconstituted in 30 µl buffer A (0.1% TFA and 1% ACN in LC-MS grade water) and vortexed thoroughly.

4. PROTEOMICS ANALYSIS (MASS SPECTROMETRY) (I-IV)

LC-MS/MS analysis was performed on an LTQ Orbitrap XL (**I**) and an Orbitrap Elite ETD Hybrid Ion Trap (**II-IV**) mass spectrometer coupled to an EASY-nLCII-system (all from Thermo Fisher Scientific) via a nanoelectrospray ion source. The detailed mass spectrometer settings are described in Varjosalo et al., 2013b (**I**) and Turunen et al., 2014 (**II-IV**).

Peak extraction and subsequent protein identification against the human reference proteome of UniProtKB/SwissProt database (www.uniprot.org) were performed with XTandem search algorithm with the k-score plug-in (**I**) and Proteome Discoverer software using SEQUEST search engine (Thermo Scientific) (**II-IV**).

The high confidence protein-protein interactions were identified using stringent filtering against CRAPome contaminant database (Mellacheruvu et al., 2013). The bait normalized relative protein abundances (% to the bait) were calculated from the spectral counts. Each average and standard deviation was calculated from 3 biological replicates. Statistical difference of the nuclear pore protein abundance of individual mutant versus WT were calculated with Student's t-test.

5. IMMUNOPRECIPITATION AND WESTERN BLOTTING (I-IV)

To analyze the protein-protein interactions with co-immunoprecipitation (**I**) transfected HEK293T cells were lysed 24 hours after transfection in HNN buffer supplemented with 0.5% NP-40, 1.5 mM $NaVO_3$, 0.5 mM PMSF and protease inhibitor cocktail for mammalian cells (Sigma). Cleared lysate was incubated with anti-HA agarose beads (Sigma) for 1 hour on a rotatory wheel at 4 °C. After incubation beads were washed five times with lysis buffer, and complexes were eluted in Laemmli Sample Buffer. Denatured samples were electrophorized on 7.5% SDS-PAGE gels and transferred to nitrocellulose membranes (Whatman GmbH). Immunoprecipitated proteins were detected wither with anti-HA (HA-11, Covance) or anti-V5 (Invitrogen) primary and horseradish peroxidase (HRP) -conjugated secondary antibodies (anti-mouse HRP 31450, Pierce). Signals were visualized by chemiluminescence using Amersham ECL Western Blotting analysis system (GE Healthcare).

The expression of constructs upon tetracycline induction was also confirmed by western blotting (**I-IV**). Proteins were separated on 10% SDS-PAGE gel, transferred to nitrocellulose membranes and detected with antibodies listed in **Table 2** as described above.

6. BIMOLECULAR FLUORESCENCE COMPLEMENTATION (BiFC) ASSAYS (I)

The bimolecular fluorescence complementation (BiFC) can be utilized to study protein-protein interactions in living cells. The technique is based on the observation, that two non-fluorescent fractions of a fluorescent protein form a fluorescent complex upon association (Kerppola, 2006). To validate the bait-prey interactions with the BiFC assay, the bait protein (a kinase) was tagged with N-terminal fragment of GFP, and the interacting prey protein with C-terminal fragment of GFP. When the bait and prey protein are in close proximity, the GFP molecule is reconstituted and GFP signal can be visualized. pBiFC BAIT and -ORF vectors were a kind gift from Dr. Darren Saunders (Vancouver, Canada). For analysis HeLa cells were seeded onto glass coverslips and transfected with Bait-V1_mCherry and Prey-V2_Cerulean. 24 hours after the transfection cells were fixed with 4% paraformaldehyde, permeabilized and their nuclei were stained with TOTO-3 (Invitrogen). After washing coverslips were mounted with Vectashield® hardening mounting media (Vector Laboratories) and staining was visualized with confocal microscopy.

7. IMMUNOFLUORESCENCE (IV)

The identified MED12 nuclear localization signal (NLS) was validated by cloning the MED12-NLS sequence (amino acids 11-20; HRPLKRPRLG) into pcDNA5_TO_StrepIII_HA_GW_FRT vector (Varjosalo et al., 2013b) containing green fluorescent protein (GFP). This construct was transfected using HelaFect (OZbiosciences) into HeLa cells plated on coverslips. The following day, the cells were fixed with 4% PFA, permeabilized with 0.1% Triton-X and stained with Alexa Fluor® 568 Phalloidin (Thermo Fisher Scientific). After rinsing, coverslips were mounted with Vectashield® mounting media (Vector Laboratories) containing DAPI (4', 6-diamidino-2-phenylindole) to visualize the nuclei. Cells were visualized with confocal microscopy.

Table 2: Antibodies and dyes used in studies.

Antibody / Dye	Method used in	Manufacturer	Catalog number	Study
Dyes				
TOTO-3	BiFC	Invitrogen	T3604	I
DAPI (4', 6-diamidino-2-phenylindole)	IF	Sigma	D9542	IV
AlexaFluor® 568 Phalloidin	IF	Thermo Fisher Scientific	A12380	III, IV
Primary antibodies				
anti-HA (HA-11)	WB, co-IP, IF	Covance	MMS-101R	I, III, IV
anti-V5	WB, co-IP	Invitrogen	46-0705	I
anti-GAPDH	WB	Abcam	Ab37168	III
Secondary antibodies				
anti-mouse HRP	WB	GE Healthcare	NA931	IV
anti-mouse HRP	WB	Pierce	31430	I
Anti-rabbit HRP	WB	Sigma-Aldrich	A0545	III
anti-mouse Alexa488	IF	Thermo Fisher Scientific	A11001	III, IV

IF= immunofluorescence; WB= Western Blotting; co-IP= co-immunoprecipitation

8. *IN VITRO* KINASE ASSAYS (I)

Kinase substrates were identified using ProtoArray® human protein microarrays (Thermo Fischer Scientific). After blocking the microarray was incubated with 50 nM kinase in the presence of ^{33}P-ATP (PerkinElmer). Unbound radiolabeled ATP was removed by washing, and after drying the microarray was exposed to Hyperfilm MP (GE Healthcare). Data was analyzed with ProtoArray® Prospector software (Thermo Fischer Scientific).

IV RESULTS AND DISCUSSION

1. THE PROTEIN INTERACTION LANDSCAPE OF THE HUMAN CMGC KINASE GROUP (I)

The recently developed AP-MS method (Glatter et al., 2009) was utilized in the study of CMGC kinase complexes to systematically determine the physical (protein-protein) interactions for 57 CMGC kinases. These selected kinases included members from all CMGC kinase subfamilies (**Figure 16**). After generation of the isogenic cell lines, expression of the tagged kinases was induced, cell pellets harvested, and affinity-purified. After trypsin digestion and subsequent C18-purification the samples were subjected to LC-MS/MS analysis. The resulting data-files were searched against the human reference proteome, and after statistical filtering using SAINT algorithm (Choi et al., 2011) high confidence interacting proteins (HCI) were identified. In total 481 interacting proteins were identified forming 652 interactions. When our data was compared with publicly available interaction data, 531 novel interactions within the CMGC kinases were identified. Not surprisingly, most of the novel interactions identified in this study clustered in the less-studied kinase subfamilies such as DYRK, SRPK or CLK (**Figure 16**). The enriched GO classifications (GO Biological Processes, GO-BP) among the HCIs were cell communication, cell growth, and cell cycle as many of the CMGC kinases have an established role in cell-cycle regulation and cellular signaling (**Figure 16**). Also the poorly studied kinases such as DYRKs could be assigned a distinct, previously unknown cellular function.

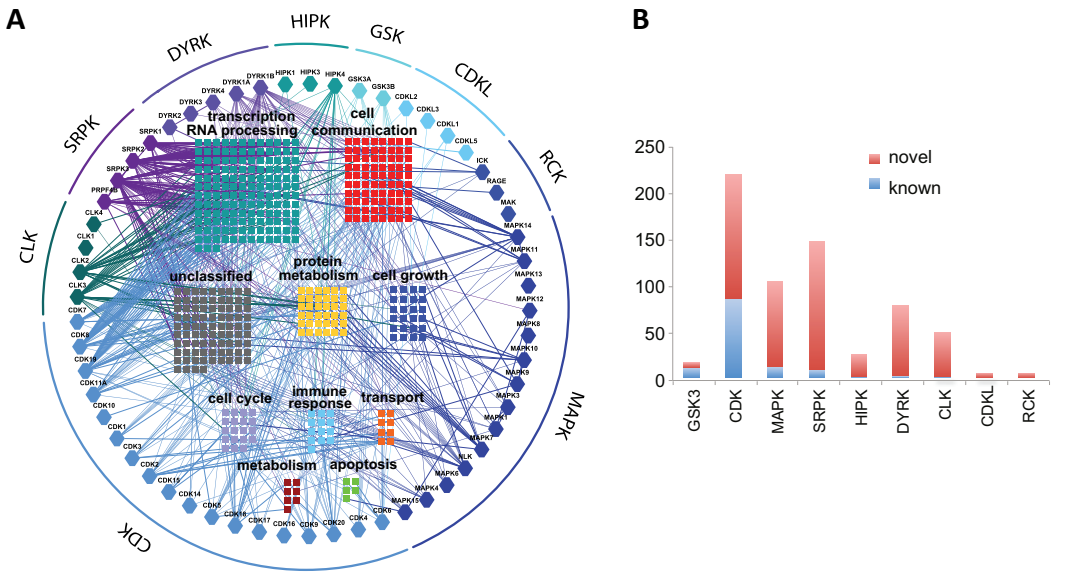

Figure 16: CMGC family members and their protein-protein interactions. A) Network model of CMGC kinase interactions. The CMGC families are shown on the outer rim of the network, and organized based on their sequence similarity. B) Novel (identified in this study) and known (from databases) interactions of the CMGC kinases. (Adapted from Varjosalo et al., 2013b)

Selection of the obtained interactions was validated via bimolecular fluorescence complementation (BiFC) assays in HeLa cells and by co-immunoprecipitation of HEK293T cells. High number of tested interactions were validated with either of the methods (23/23) or with both methods (19/23). On average, kinases form interactions with multiple partners, but the number of interactions per bait varies greatly between the CMGC kinase families (CDKL and RCK had only a few interactors, whereas SRPKs showed over three times higher bait-to-prey ratio compared to other CMGC kinases). Upon closer inspection it was discovered that related kinases often have multiple common HCIs, but some kinases can form unique, highly specific complexes. An excellent example of a kinase subfamily presenting both shared and specific interactions is GSK3α and GSK3β, where only GSK3β was observed in complex with β-catenin and APC further supporting the functional role of GSK3β in Wnt signaling.

As an example of the high specificity and sensitivity of the utilized workflow, we noticed that three members of the CDK family (CDK7, CDK8, CDK19) form complexes with HCIs involved in transcription initiation – an observation well in-line with the current literature. General transcription factor TFIIHH complex subunits were exclusively revealed in the CDK7 interactome. On the other hand, CDK8 and CDK19 were shown to interact with multiple Mediator complex components, providing, at the time, the most complete picture of interactions between CDK8, CDK19 and different MED subunits (**Figure 17**).

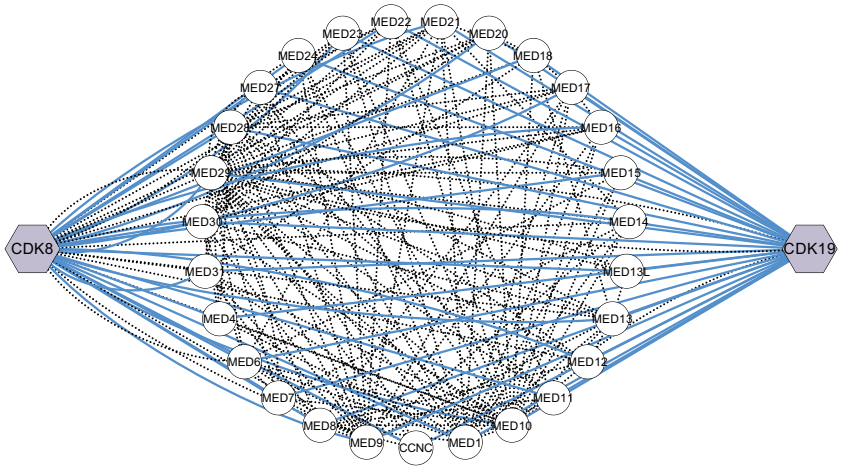

Figure 17: CDK8 and CDK19 form interactions with multiple Mediator subunits. The Mediator complex associates either with CDK8 or CDK19. Blue edges indicate interactions identified in this study, while known interactions are visualized with black dotted lines. (Adapted from Varjosalo et al., 2013b)

In addition, a previously unknown kinase-kinase interaction subnetwork in the CMGC interactome was found, as almost half of the CMGC kinases showed a preference to interact with protein kinases. Interesting observation was the enrichment of kinase-kinase interactions within the CMGC kinase family (44 out of over 100 identified kinase-kinase interactions), followed by interactions formed with Ca^{2+}/calmodulin-dependent protein kinase (CAMK; 24 interactions) and protein kinase A, G, and C (AGC; 13 interactions) groups.

To further characterize the functional, kinase-substrate, signaling networks, phosphopeptides were identified from the AP-MS data. Based on our phosphopeptide data, and data available from public databases a hypothetical network of substrates and their upstream kinases, utilizing known kinase-substrate data, was created. This network was further merged with the HCI AP-MS data to identify potential high confidence kinase-substrate interactions. This approach identified numerous kinases and their candidate substrates for kinase subfamilies such as GSK, CDK or MAPK. For SRPKs a different approach was selected, as substrate prediction tools, due to the low number of known substrates, did not cover these kinases. The *in vitro* kinases assays (IVK) were used to demonstrate the kinase-substrate association for SRPKs. After combining the data from IVK and AP-MS, a network of RNA splicing or processing proteins was observed.

Searching this proteomic CMGC kinase dataset against genetic human disease databases (OMIM and COSMIC) revealed 91 disease-associated proteins (DAPs) forming 143 interactions (108 novel) with CMGC kinases. The most pronounced disease class was cancer. The clustering of disease phenotypes with specific kinase families and complexes identified five kinase complex clusters, where the preys were linked to a specific disease phenotype. In total 43 kinase-associated proteins could be linked to various forms of human cancer, showing roughly three times higher frequency than expected from a random network of similar features (**Figure 18**), thus, several CMGC kinases could be classified as 'cancer-hubs'. Remarkably, some 'hubs' were associated with proteins linked to a specific type of cancer. Good examples of specific cancer-linked proteins are proteins identified in complexes with CDK9, and mostly linked to acute lymphoplastic leukemia (ALL) and acute myeloid leukemia (AML) (**Figure 18**).

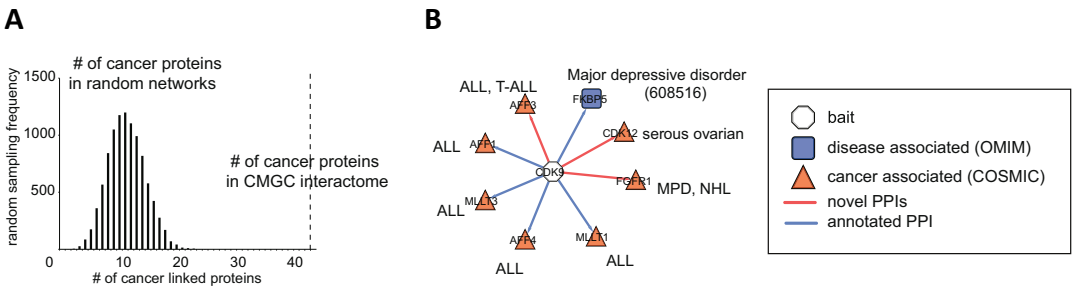

Figure 18: Complexes of CMGC kinases and disease associated proteins. A) Enrichment of cancer associated proteins in CMGC network. The number of cancer proteins in CMGC interactome was 43 (dashed line). B) CDK9 showed an enrichment of interactions with proteins mutated in ALL. Known interactions are visualized with blue edges, novel interactions are indicated with red edges. (Adapted from Varjosalo et al., 2013b)

To conclude, known interactions were confirmed and several novel interactions identified of the CMGC kinases leading to better understanding of their functional roles in mammalian cells. The phosphopeptide analysis identified multiple candidate substrates also for the poorly studied CMGC kinases, providing a good starting point for future experiments. The interactions of CDK8/19 and Mediator components were also comprehensively mapped, proving evidence that the approach is robust and sensitive.

Overall, the poorly studied subfamilies were overrepresented in this kinase interactome indicating that the database information is biased towards the well-known families. This is not surprising, but points out the need for systematic proteomics studies to better characterize these proteins and their cellular functions. This study showed more than 100 physical interactions of CMGC kinases and human DAPs not listed in public databases. Several diseases clustered around specific kinase families, hinting that these kinases might serve as future targets for the development of new therapeutic agents.

2. UTERINE LEIOMYOMA-LINKED MED12 MUTATIONS DISRUPT MEDIATOR-ASSOCIATED CDK ACTIVITY (II)

Although benign, leiomyomas are among the most common tumors in women of reproductive age, and the leading cause of hysterectomies worldwide (Ordulu 2016). Information about the molecular mechanisms underlying the tumor formation has remained sparse, but genetic factors have been implicated to have a role in tumorigenesis (Ordulu, 2016). Recently, whole-exome sequencing revealed that mutations in exon 2 of MED12 occur at high frequency (~70%) in uterine leiomyomas (Makinen et al., 2011). The impact of the most common uterine leiomyoma -linked MED12 mutation, G44D, to the functions of CDKs and the Mediator kinase module in tumorigenesis was studied using the AP-MS method. In AP-MS analysis, the G44D mutation showed reduced binding to the Mediator kinase module components cyclin C, CDK8, and CDK19, when compared to wild type (WT) MED12 (**Figure 21**). Interactions with other Mediator subunits remained unaffected. The reduced association of G44D with CDK8 and CDK19 was confirmed by co-immunoprecipitation and western blotting analysis. Other leiomyoma-linked mutations (L36R, Q34P, G44S) were also shown to lose their interactions with the Mediator kinase module.

To determine the mechanism behind the disrupted interaction between MED12 and cyclin C-CDK8, six MED12 constructs of variable lengths were designed. Recombinant proteins of these constructs and the full-length WT MED12 were produced and immunoprecipitated in the presence of CDK8 and cyclin C. Kinase activity of CDK8 was also monitored. These experiments mapped the cyclin C-CDK8 binding and activation domain of MED12 within its first 100 amino acids. Next, MED12 was shown to bind to cyclin C, which further binds to CDK8. MED12 also presented binding to MED13 that bound neither to cyclin C or CDK8. Distinct binding surfaces in cyclin C for MED12 and CDK8 were revealed, and the cyclin C surface groove was further identified as the main MED12 binding interface leading to CDK8 activation. Overall, these results show that MED12 first binds to cyclin C, which in turn binds to CDK8, revealing the molecular basis for the loss of interactions.

Taken together, this study shows that MED12 mutations in uterine leiomyoma severely compromise the interactions with Mediator kinase module components CDK8 and cyclin C. Importantly, the first 100 amino acids in MED12 were identified crucial for binding and activation of CDK8/cyclin C, suggesting a leiomyoma-linked functional Mediator kinase module defect contributing to the formation of these tumors. As uterine leiomyomas globally affect hundreds of millions of women, our finding is an important starting point for the design of targeted therapies.

3. SOMATIC MED12 MUTATIONS IN PROSTATE CANCER AND UTERINE LEIOMYOMAS PROMOTE TUMORIGENESIS THROUGH DISTINCT MECHANISMS (III)

Recently, inspection of MED12 mutations in exon 26 that lead to discovery of L1224F substitution were described in prostate cancer in 5 out of 152 patients (Barbieri et al., 2012). To analyze the presence of this MED12 L1224F mutation in Finnish patient samples, a sample set of nearly 800 tumors was analyzed. Within this set L1224F mutation was identified in one patient.

To elucidate the functional role of this prostate cancer-specific mutation in the context of Mediator kinase module, isogenic HEK293 cell lines expressing MED12 L1224F and MED12 WT were generated, and their protein-protein interactions analyzed with AP-MS. The L1224F mutant was able to interact with cyclin C, CDK8 and CDK19, and presented similar CDK8 kinase activity as MED12 WT. Instead, L1224F had reduced interactions with MED13, MED13L, MED17, MED1, MED14, MED15, and MED24 (**Figure 19**). Changes in interactions outside the Mediator complex were not observed. Cellular localization of both L1224F and G44D mutant MED12 remained mainly nuclear similar to WT MED12.

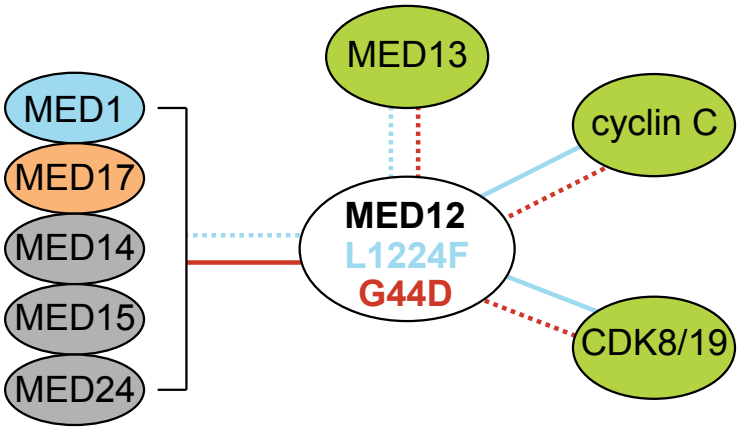

Figure 19. Interactions of MED12 WT, L1224F, and G44D. The prostate cancer-specific MED12 L1224F mutation presented reduced binding to MED13. However, interactions with other components of the Mediator kinase module remained at a level comparable to MED12 WT. The reduced interactions are indicated with dashed lines, whereas solid lines indicate interactions that remained similar to WT (average fold change at least 0.4). Color-coding of the Mediator head, tail, middle and kinase domain subunits according to Figure 10. (Adapted from Kampjarvi et al., 2016)

In summary, our results highlight the functional differences of MED12 mutants in uterine leiomyoma and prostate cancer by revealing the different interaction profiles of the prostate-specific MED12 L1224F mutation and the uterine leiomyoma MED12 hotspot mutation G44D. The interaction profile of L1224F was also biologically relevant, as interactions with MED1 and MED15 were decreased. MED1 has been reported to promote growth and survival of cancer cells (Jin et al., 2012; Vijayvargia et al., 2007; Jin et al., 2013), and overexpression of MED15 has been detected in castration-resistant prostate cancer (Shaikhibrahim et al., 2014a;

Shaikhibrahim et al., 2014b). At the end of the day, to be able to establish a causal relationship between an observed mutation and a disease, three criteria must be met: 1) genotype is not occurring just by change in population, 2) the genetic variant must impair or destroy gene expression or the function of the protein product, and 3) the causal relationship must be confirmed in a relevant model *in vitro* or *in vivo* (Casanova et al., 2014). Thus, to better understand the role in tumorigenesis and to confirm the causal role of MED12 L1224F mutation in prostate cancer, further studies with for example MED12 mutation-positive cells would be needed.

4. SOMATIC MED12 NONSENSE MUTATION ESCAPES mRNA DECAY AND REVEALS A MOTIF REQUIRED FOR NUCLEAR ENTRY (IV)

The first MED12 exon 1 nonsense mutation (c.97G>T, p.E33X) was identified in a T-ALL patient through exome sequencing (Kontro et al., 2014). Previously, MED12 missense mutations affecting site E33 were observed in chronic lymphocytic leukemia (CLL) samples (Kampjarvi et al., 2015). In study IV, this T-ALL nonsense mutation, which was predicted to result in a truncated protein, was validated in the genomic DNA of the patient.

The functional effects of the MED12 E33X mutation on translated protein products, its localization, and protein-protein interactions were studied utilizing the stable, isogenic, inducible HEK293 cell lines. In western blot analysis using an anti-HA antibody, the molecular weight of the MED12 E33X was clearly smaller compared to the WT MED12 or missense mutants G44D (uterine leiomyoma hotspot) or E33K (CLL hotspot). An interesting observation was, that an N-terminal MED12 antibody could not recognize the E33X mutant in western blot. Mass spectrometry analysis of the E33X MED12 peptides further validated this observation as the first peptide identified from the E33X AP-MS samples was ranging from amino acid 163 to amino acid 174 (**Figure 20**). MED12 alanine mutants M154A, M162A and M154A&M162A validated M154 as the translation initiation site in the E33X mutant. Thus, the E33X MED12 mutant was shown to escape nonsense mediated mRNA decay and lead to an N-terminally truncated protein.

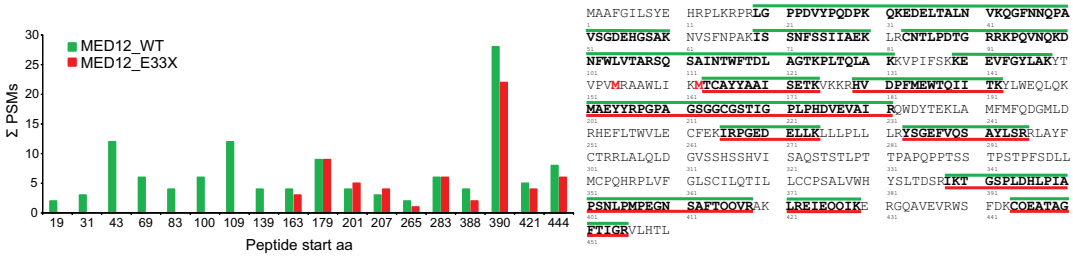

Figure 20: The identified peptides of MED12 E33X and MED12 WT. The peptides identified with mass spectrometry from MED12 WT are indicated with green, and red is used for MED12 E33X peptides. The alternative translation start sites, M154 and M162 are written in red. (Adapted and modified from Heikkinen et al., 2017)

The AP-MS analysis revealed that the MED12 E33X mutant fails to interact with all Mediator complex components (**Figure 21**). On the contrary, the missense mutation E33K resembled the uterine leiomyoma specific G44D mutant (Turunen et al., 2014) presenting diminished interactions between MED12 and the Mediator kinase module subunits CDK8, CDK19, and cyclin C. Other Mediator complex interactions were unimpaired (**Figure 21**).

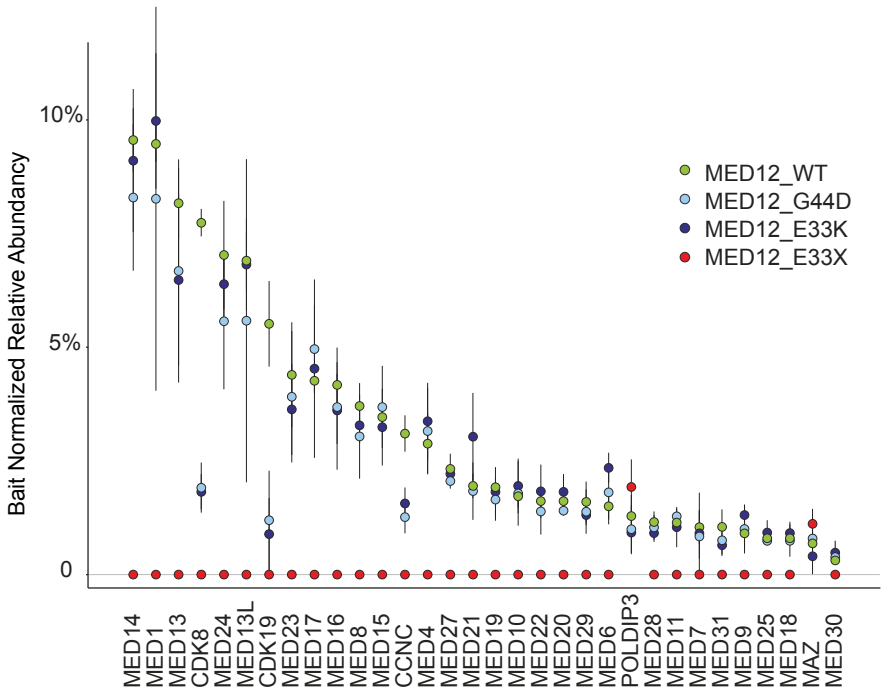

Figure 21: AP-MS results from MED12 WT, G44D, E33K, and E33X. The N-terminally truncated MED12 mutant E33X loses interactions with Mediator complex components, whereas the uterine leiomyoma hotspot, G44D, shows reduced interactions with the Mediator kinase module components CDK8, CDK19, and cyclin C (CCNC). Missense mutant E33K phenocopies the G44D mutant. (Adapted from Heikkinen et al., 2017)

Immunofluorescent staining revealed mislocalization of the N-terminally truncated E33X to the cytoplasm, while MED12 WT and E33K were nuclear. This observation suggested that MED12 might have an N-terminal sequence important for nuclear localization; thus, four *in silico* prediction tools were utilized to analyze the presence of a nuclear localization signal (NLS) in MED12. All of these tools suggested an NLS in the N-terminus of MED12 between amino acids 13-19. Two alanine mutant constructs of different lengths were generated (NLS1, amino acids 13-16; NLS2, amino acids 13-19) to disrupt the predicted NLS. Both constructs produced a protein localizing to the cytoplasm and had severely disrupted protein-protein interactions with the Mediator complex similar to E33X. Also, a fusion construct of MED12 amino acids 11-20 to GFP showed nuclear localization.

To reveal the functional mechanism of MED12 nuclear entry, we utilized the BioID proximity labelling. In the BioID analysis MED12 interactions with importins and nuclear pore complex

(NPC) components were detected. The E33X mutant presented either lost or diminished interactions with nuclear basket components, and importin-α-proteins similar to NLS1 and NLS2 mutants (**Figure 22**). Intriguingly, E33X and NLS mutants retained or even increased their interaction with importin-β compared to the MED12 WT. This suggests that MED12 mutants fail to bind to importin-α and enter the NPC central channel, thus remaining in the cytoplasm.

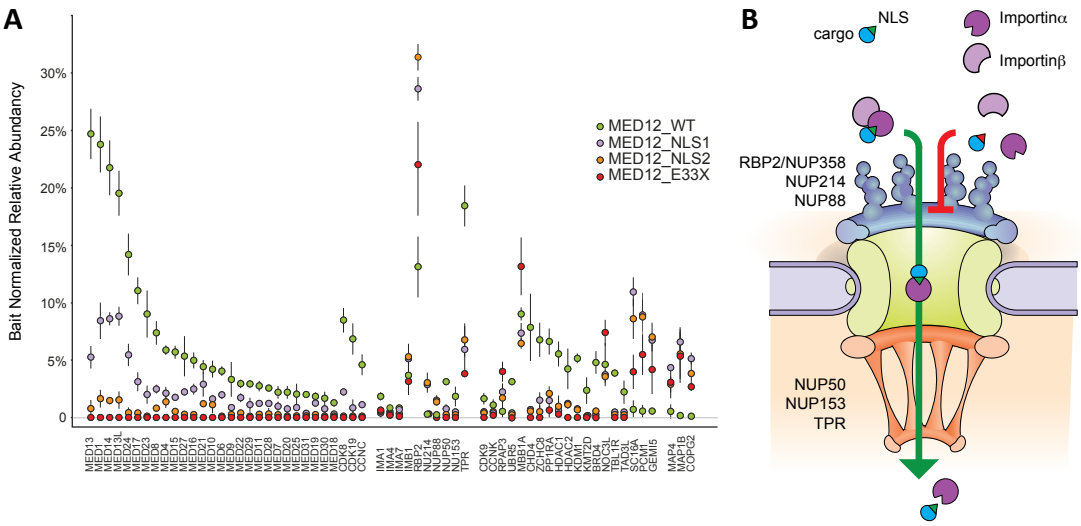

Figure 22: BioID analysis of MED12 WT, NLS1, NLS2, and E33X. A) Interactions of MED12 and proteins of the nuclear import machinery were identified in BioID analysis. E33X has reduced interactions with importin α proteins (IMA1, IMA4, and IMA7), and dimished interactions with the nuclear ring proteins (NUP50, NUP153, and TPR). B) Based on the BioID results a schematic presentation of the nuclear entry through the NPC could be built for MED12 WT (green arrow) and MED12 E33X (red arrow). (Adapted from Heikkinen et al., 2017)

In our previous work, the binding site for cyclin C in the N-terminus of MED12 was identified (Turunen et al., 2014), but hitherto the MED12 functional domains have remained rather uncharacterized outside the SOX9, β-catenin and Gli3 binding regions in the PQL domain (Zhou et al., 2002; Kim et al., 2006; Zhou et al., 2006). Now a nuclear localization signal was identified in the MED12 E33X T-ALL mutant producing a protein lacking the N-terminus of MED12. This truncated protein had severely impaired interactions with other Mediator complex components due to cellular mislocalization in the cytoplasm. To sum up, these results indicate that the nuclear entry of MED12 is essential for the interactions with other kinase module components and, further, for kinase activity. MED12 was recently identified as necessary for normal hematopoiesis (Aranda-Orgilles et al., 2016), giving room for speculation of the role of MED12 in hematological malignancies.

V CONCLUSIONS AND FUTURE PROSPECTS

The overall aim of this thesis was to provide a systematic characterization of a protein kinase family called CMGC. This family consists of 62 members of 8 subfamilies presenting both well-studied kinases as well as kinases without a known cellular function. Most of the kinases in this family are known to be important regulators of cell cycle progression and responders to autocrine or paracrine signals for determining functions such as cell entry into senescence or apoptosis. All of these processes are of crucial importance to normal cells. Cancer, simply put, is uncontrolled growth (division) of abnormal cells, where protein kinases are often found overexpressed or overactive. Cancer incidence rate is related to age, which is at least partly explained by the slow accumulation of mutations in the genes controlling cellular processes. Most cellular processes rely on the correct phosphorylation of substrates (or their dephoshorylation), and genetic and epigenetic events leading to aberrant regulation of protein kinases, not surprisingly, are implicated in multiple cancers (Cohen, 2002; Knight et al., 2010; Abbassi et al., 2015; Gross et al., 2015).

It is, therefore, not surprising that the systems wide identification of the protein-protein interactions of the CMGC kinases revealed physical interactions mostly with cancer-associated proteins (**I**). Our studies also suggest a common mechanism for MED12 mutations contributing to leiomyoma formation, and highlight the functional differences of MED12 mutants in uterine fibroids and prostate cancer (**II-III**). We also identified the novel MED12 NLS, and showed that the correct localization and subunit assembly is essential for proper function of the Mediator kinase module (**IV**).

The Mediator kinase module components and multiple other members of the CMGC kinase family have been linked to multiple human malignancies, and they can be considered as an important target for future therapeutic interventions. Based on the high number of kinase inhibitors in different stages of pre-clinical and clinical trials, either as single or combination therapies, they possess vast potential in the treatment of a range of human pathologies. To date, Mediator subunits have not been targeted, but a recent study (Al-Hendy et al., 2017) showed that the knockdown of MED12 in uterine leiomyoma cells slowed down cell proliferation and decreased the expression levels of CDK1, CDK2, and cyclin D1.

As mentioned earlier, data in public collections are biased towards the most-studied kinases, and only recently the HUGO Gene Nomenclature Committee (HGNC) (http://www.genenames.org/) renamed and unified the names of CDK subfamily members. The systematic analysis of the functions of these kinases in the cell has been challenging and only the recent advances of the 'omics' techniques has made it possible to study kinases as large entities in their physiological environment. AP-MS and the application of BioID-method in the study of kinase functions in pathological conditions will, without a doubt, shed more light on the biology of these cellular components. The global and complete analysis of CMGC kinase functional interactions, substrate-specificities, phosphorylation events and dynamics of these networks presents an important future goal for better understanding of the mechanisms of action in health and disease.

Finally, it may be concluded, that understanding the regulatory diversity and complexity of the human kinome is of utmost importance to develop new well-tolerated, specific, potent pharmacological interventions for the treatment of neurodegenerative diseases and multiple cancers.

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