Invited Lectures

L1

The PIF-pocket regulatory site in AGC kinases: relevance for cellular physiology and innovative therapies

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The group of AGC protein kinases includes more than 60 protein kinases in the human genome (e.g. PDK1 and isoforms of PKA, PKC, PRK, PKB/Akt, S6K, SGK, RSK, MSK, ROCK, GRK, amongst others). This group is also widely represented in other eukaryotes, including organisms that cause human infectious diseases. Human AGC kinases are involved in diverse cellular functions and are potential targets for the treatment of cancer, diabetes, obesity, neurological disorders, inflammation, viral infections, etc. Small molecule inhibitors of AGC kinases can also be considered as potential candidates for the development of targeted therapies against infectious diseases. AGC kinases are typically regulated by phosphorylation at three sites, the activation loop, the Zipper/turn-motif and the hydrophobic motif sites, and by other diverse mechanisms including intramolecular interactions with other regions of the protein, oligomerization, interaction with regulatory subunits, and second messengers. Interestingly, we were able to identify that a particular pocket on the kinase domain, termed the PIF-pocket, is a key mediator of the allosteric regulation of many AGC kinases. We first described the regulatory properties of the PIF-pocket on PDK1 [1]. Follow-up work verified that the PIF-pocket is also the key site that mediates the activation by Zipper/turn-motif and hydrophobic motif phosphorylation of PKB/Akt, S6K, RSK, SGK, PKC, PRK, and MSK [2–4]. Interaction with the PIF-pock*et al*so regulates the intramolecular inhibition of atypical PKCs by their N-terminal domains [5] and the oligomerization that inhibits PRK2 [6]. Moreover, we have developed reversible low-molecular-weight compounds that, by targeting the PIF-pocket site, allosterically activate PDK1 [7, 8], or allosterically inhibit atypical PKCs [5]. Our studies have shed light on the molecular details of the mechanism of allosteric regulation by small compounds and support the future development of drugs directed to the PIF-pocket of AGC kinases. The PIF-pocket regulatory site shares similar features with a dimerization regulatory site recently discovered on receptor tyrosine kinases, suggesting that the deep knowledge of the molecular mechanisms that operate in AGC kinases may support the rational development of allosteric compounds to protein kinases from other groups.

References:

1. Biondi RM, Cheung PC, Casamayor A, Deak M, Currie RA, Alessi DR (2000) Identification of a pocket in the PDK1 kinase domain that interacts with PIF and the C-terminal residues of PKA. *EMBO J* **19**: 979–988.
2. Frodin M, Antal TL, Dummler BA, Jensen CJ, Deak M, Gammeltoft S, Biondi RM (2002) A phosphoserine/threonine-binding pocket in AGC kinases and PDK1 mediates activation by hydrophobic motif phosphorylation. *EMBO J* **21**: 5396–5407.
3. Yang J, Cron P, Thompson V, Good VM, Hess D, Hemmings BA, Barford D (2002) Molecular mechanism for the regulation of protein kinase B/Akt by hydrophobic motif phosphorylation. *Mol Cell* **9**: 1227–1240.
4. Hauge C, Antal TL, Hirschberg D, Doehn U, Thorup K, Idrissova L, Hansen K, Jensen ON, Jorgensen TJ, Biondi RM, Frodin M (2007) Mechanism for activation of the growth factor-activated AGC kinases by turn motif phosphorylation. *EMBO J* **26**: 2251–2261.
5. Lopez-Garcia LA, Schulze JO, Frohner W, Zhang H, Suss E, Weber N, Navratil J, Amon S, Hindie V, Zeuzem S, Jorgensen TJ, Alzari PM, Neimanis S, Engel M, Biondi RM (2011) Allosteric regulation of protein kinase PKC ξ by the N-terminal C1 domain and small compounds to the PIF-pocket. *Chem Biol* **18**: 1463–1473.
6. Bauer AF, Sonzogni S, Meyer L, Zeuzem S, Piiper A, Biondi RM, Neimanis S (2012) Regulation of protein kinase C-related protein kinase 2 (PRK2) by an intermolecular PRK2-PRK2 interaction mediated by its N-terminal domain. *J Biol Chem* **287**: 20590–20602.
7. Engel M, Hindie V, Lopez-Garcia LA, Stroba A, Schaeffer F, Adrian I, Imig J, Idrissova L, Nastainczyk W, Zeuzem S, Alzari PM, Hartmann RW, Piiper A, Biondi RM (2006) Allosteric activation of the protein kinase PDK1 with low molecular weight compounds. *EMBO J* **25**: 5469–5480.
8. Hindie V, Stroba A, Zhang H, Lopez-Garcia LA, Idrissova L, Zeuzem S, Hirschberg D, Schaeffer F, Jorgensen TJD, Engel M, Alzari PM, Biondi RM (2009) Structure and allosteric effects of low molecular weight activators on the protein kinase PDK1. *Nat Chem Biol* **5**: 758–764.

L2

The curious case of the inhibition of the p90 ribosomal S6 kinase by a plant flavonol glycoside, SL0101

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The phosphorylation of either Ser/Thr or Tyr residues on target proteins is catalyzed by 518 protein kinases, the human kinome [1]. Intense interest in the structure and function of protein kinases is driven by their potential as drug targets, particularly in cancer therapy. Eleven drugs acting on protein kinases have been approved by the FDA, and approximately 150 other inhibitors are at various stages of clinical studies [2].

One of the families of Ser/Thr kinases recognized for their potential as drug targets are the p90 ribosomal S6 (RSK) kinases [3]. The four RSK isoforms (RSK1–4), as well as the two related mitogen- and stress-activated kinases MSK1 and MSK2, display a unique molecular architecture, with two distinct kinase domains in tandem, i.e. the regulatory C-terminal kinase domain (CTKD), and the physiologically active N-terminal kinase domain (NTKD) [3–5]. The activation of RSK kinases involves several phosphorylation steps, initiated by the docking of extracellular signal-regulated kinases (ERK1/2) at the C-terminus with subsequent phosphorylation and activation of CTKD, intramolecular phosphorylation of the interdomain linker by CTKD, the recruitment of phosphoinositide-dependent kinase 1 (PDK1) to this newly phosphorylated site, and finally PDK1-dependent phosphorylation and activation of the NTKD [3]. The two most ubiquitously expressed isoforms, i.e. RSK1 and RSK2, are increasingly attracting attention because of their involvement in various cancers [5–8].

Only two inhibitors with relative specificity for the RSK family have been described to date: BI-D1870 [9], and a naturally occurring compound, SL0101, identified in a tropical plant *Forsteronia refracta* [10]. It is a flavonol glycoside, kaempferol-3-O-(3",4"-di-O-acetyl-a-L-rhamnopyranoside. Free flavonols are known to be inhibitors of kinases [11]. The inhibitory activity is due to the fact that the planar benzopyran moiety of the flavonol is able to compete with ATP, by mimicking the purine heterocycle. However, low affinities of such interactions and poor selectivity have traditionally rendered the flavonol scaffold as unattractive for further drug development. Importantly, flavonols are synthesized in plants and ingested as either 7-O or 3-O glycosides. At least some glycosides do show kinase inhibitory activity [12], although no structural details regarding the mechanism are known. We were therefore intrigued by the molecular basis of the specificity of SL0101 towards the RSK family, and to address this we solved a crystal structure of the complex of the N-terminal domain of the mouse RSK2 kinase (residues 47–342) with SL0101, at 1.5 Å resolution. Most of the polypeptide chain is well ordered, with only two loops lacking interpretable electron density, i.e. residues 116–119 and 219–223, the latter being a part of the activation (or T) loop. The SL0101 molecule is very well resolved in the electron density maps, and is located, as expected, in the cleft between the N- and C-lobes. A comparison with the complex with AMP-PNP [13] shows that the helical cores of the C-lobes in the two structures are highly similar, with an rms difference of 0.56 Å for main chain atoms. In contrast, the N-lobe undergoes a dramatic rearrangement in the SL0101 complex compared to the AMP-PNP bound structure, including changes in both the topology and architecture of the novel three-stranded β-sheet. A closer structural comparison reveals additional differences between the two complexes within the C-lobe. The DFG-motif, located upstream of the T-loop undergoes a structural reorganization, while the C-terminal portion of the T-loop, beginning with residue 223, becomes ordered and clearly visible in the electron density map. Finally, the αD-helix, which normally remains inert and not affected by the binding of ATP or inhibitors, significantly alters its conformation.

The overall effect of the structural differences observed within the protein moiety of the two complexes is an unprecedented rearrangement of the nucleotide binding site. Although SL0101 binds in the cleft between the N- and C-lobes, as expected for most kinase inhibitors, the nature of this cleft and the identities of residues that make it up are significantly different from the canonical ATP-binding site. It is not clear if SL0101 binds through conformational selection, or if it forces a conformational change through an induced fit effect. The knowledge of the crystal structure reported in this paper will be of significant help in drug design efforts. In particular, the unique flexibility of the N-lobe and the P-loop in RSK2 revealed by the present structure may be exploited for the design of highly selective inhibitors that target the RSK-specific conformations.

Notes:

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References:

1. Manning G, *et al.* (2002) The protein kinase complement of the human genome. *Science* **298**: 1912–1934.
2. Zhang J, Yang PL, Gray NS (2009) Targeting cancer with small molecule kinase inhibitors. *Nat Rev Cancer* **9**: 28–39.
3. Anjum R, Blenis J (2008) The RSK family of kinases: emerging roles in cellular signalling. *Nat Rev Mol Cell Biol* **9**: 747–758.
4. Jones SW, *et al.* (1988) A *Xenopus* ribosomal protein S6 kinase has two apparent kinase domains that are each simi-

lar to distinct protein kinases. *Proc Natl Acad Sci USA* **85**: 3377–3381.
5. Pearce LR, Komander D, Alessi DR (2010) The nuts and bolts of AGC protein kinases. *Nat Rev Mol Cell Biol* **11**: 9–22.
6. Stratford AL, Dunn SE (2011) The promise and challenges of targeting RSK for the treatment of cancer. *Expert Opin Ther Targets* **15**: 1–4.
7. Romeo Y, Roux PP (2011) Paving the way for targeting RSK in cancer. *Expert Opin Ther Targets* **15**: 5–9.
8. Lu W, *et al.* (2011) SHAFTS: a hybrid approach for 3D molecular similarity calculation. 2. Prospective case study in the discovery of diverse p90 ribosomal S6 protein kinase 2 inhibitors to suppress cell migration. *J Med Chem* **54**: 3564–3574.
9. Alessi DR, *et al.* (2007) BI-D1870 is a specific inhibitor of the p90 RSK (ribosomal S6 kinase) isoforms *in vitro* and *in vivo*. *Biochem J* **401**: 29–38.
10. Smith JA, *et al.* (2005) Identification of the first specific inhibitor of p90 ribosomal S6 kinase (RSK) reveals an unexpected role for RSK in cancer cell proliferation. *Cancer Res* **65**: 1027–1034.
11. Hou DX, Kumamoto T (2010) Flavonoids as protein kinase inhibitors for cancer chemoprevention: direct binding and molecular modeling. *Antioxid Redox Signal* **13**: 691–719.
12. Goettert M, *et al.* (2010) Biological evaluation and structural determinants of p38α mitogen-activated-protein kinase and c-Jun-N-terminal kinase 3 inhibition by flavonoids. *Chembiochem* **11**: 2579–2588.
13. Malakhova M, *et al.* (2009) Structural diversity of the active N-terminal kinase domain of p90 ribosomal S6 kinase 2. *PLoS One* **4**: e8044.

L3

Regulation of bacterial transcription activators by phosphoenolpyruvate-dependent protein phosphorylation

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The phosphoenolpyruvate (PEP):carbohydrate phosphotransferase system (PTS) catalyzes the transport and phosphorylation of many sugars in bacteria [1]. The PTS is usually composed of four proteins forming a PEP-dependent phosphorylation cascade. In the last step, the phosphoryl group is transferred to a sugar molecule bound to the membrane-spanning PTS component EIIC. The phosphorylated sugar is subsequently released into the cytoplasm. Phosphorylation of the PTS proteins occurs at His or Cys residues.

In addition, PTS components function as the central processing unit in the regulation of carbon metabolism [2]. Among others they interact with or phosphorylate numerous transcription activators containing usually two PTS regulation domains (PRDs) as well as an EIIB and an EIIA domain [3]. A well-studied example is MtlR from *Bacillus subtilis*, which is required for the expression of the mannitol operon in this organism. MtlR becomes phosphorylated by the general PTS components EI and HPr at His-342 in PRD-2. This phosphorylation, which renders MtlR active, is prevented when a rapidly metabolizable carbon source, such as glucose, is present. It therefore serves as a carbon catabolite repression mechanism. Phosphorylation of MtlR at Cys-419 in the EIIBGat domain requires in addition to EI and HPr the sugar-specific PTS component $EIIA^{Mtl}$. This phosphorylation, which inhibits MtlR activity, is prevented when mannitol is present in the growth medium. Under these conditions $P\sim EIIA^{Mtl}$ transfers its phosphoryl group mainly to mannitol bound to the permease and MtlR is barely phosphorylated at Cys-419 and therefore active. This regulatory mechanism is used for induction of the *mtl* operon [4]. Deletion of $EIIA^{Mtl}$ therefore leads to constitutive expression of the *mtl* operon [4, 5]. Tests aimed at identifying inhibitors of the phosphoryl group transfer from $P\sim HPr$ to $EIIA^{Mtl}$ or from $P\sim EIIA^{Mtl}$ to Cys-419 in MtlR, which should also lead to constitutive expression of the lacZ reporter gene fused to the *mtl* promoter, are under way. The constitutive expression from the *mtl* promoter was prevented when both, $EIIA^{Mtl}$ and the $EIIB^{Mtl}$ domain, were deleted. Unphosphorylated $EIIB^{Mtl}$ therefore seems to be necessary for MtlR activation. An interaction between $EIIB^{Mtl}$ and MtlR could indeed be established. This interaction was prevented when $EIIB^{Mtl}$ was phosphorylated. $EIIB^{Mtl}$ is fused to the membrane-spanning $EIIC^{Mtl}$ and only $EIICB^{Mtl}$ was able to activate MtlR. Synthesis of un-fused cytoplasmic $EIIB^{Mtl}$ did not restore MtlR activity. However, when fused to other membrane proteins (YwqC) $EIIB^{Mtl}$ also activated MtlR, suggesting that it is the membrane localisation that stimulates MtlR function. This phosphorylation-dependent protein/protein interaction also serves as a second induction mechanim of the *B. subtilis mtl* operon. Unphosphorylated $EIIB^{Mtl}$ prevails when the cells transport mannitol *via* the PTS.

References:

1. Postma PW, Lengeler JW, Jacobson GR (1993) Phosphoenolpyruvate : carbohydrate phosphotransferase system of bacteria. *Microbial Rev* **57**: 543–594.
2. Deutscher J, Francke C, Postma PW (2006) How phosphotransferase system-related protein phosphorylation regulates carbohydrate metabolism in bacteria. *Microbiol Mol Biol Rev* **70**: 939–1031.
3. Greenberg DB, Stülke J, Saier MH Jr (2002) Domain analysis of transcriptional regulators bearing PTS regulatory domains. *Res Microbiol* **153**: 519–526.
4. Joyet P, Derkaoui M, Poncet S, Deutscher J (2010) Control of *Bacillus subtilis mtl* operon expression by complex phosphorylation-dependent regulation of the transcription activator MtlR. *Mol Microbiol* **76**: 1279–1294.
5. Heravi KM, Wenzel M, Altenbuchner J (2011) Regulation of *mtl* operon promoter of *Bacillus subtilis*: requirements of its use in expression vectors. *Microb Cell Fact* **10**: 83.

L4

A chemical approach to controlling cell fate

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Recent advances in stem cell biology may make possible new approaches for treatment of a number of diseases. A better understanding of molecular mechanisms that control stem cell fate/function as well as an improved ability to manipulate them are required. Toward these goals, we have developed and implemented high throughput cell-based screens of arrayed chemical libraries to identify and further characterize small molecules that can control stem cell fate in various systems. This talk will present latest discovery efforts in my lab that have advanced our ability and understanding toward controlling stem cell fate, including self-renewal, survival, differentiation and reprogramming of cells. Particular examples will be focused on the identification and characterizations of both kinase inhibitors and activators that affect cell fate and function [1–5].

References:

1. Lin T, Ambasudhan R, Yuan X, Li W, Hilcove S, Abujarour R, Lin X, Hahm HS, Hao E, Hayek A, Ding S (2009) A chemical platform for improved induction of human iPS cells. *Nat Methods* **6**: 805–808.
2. Xu Y, Zhu X, Hahm HS, Hao E, Li W, Hayek A, Ding S (2010) Revealing a core signaling regulatory mechanism for pluripotent stem cell survival and self-renewal by small molecules. *Proc Natl Acad Sci USA* **107**: 8129–8134.
3. Zhu S, Li W, Zhou H, Wei W, Ambasudhan R, Lin T, Kim J, Zhang K, Ding S (2010) Reprogramming of human primary somatic cells by OCT4 and chemical compounds. *Cell Stem Cell* **7**: 651–655.
4. Li W, *et al.* (2011) Rapid induction and long-term self-renewal of primitive neural precursors from human embryonic stem cells by small molecule inhibitors. *Proc Natl Acad Sci USA* **108**: 8299–8304.
5. Efe JA, Hilcove S, Kim J, Zhou H, Ouyang K, Wang G, Chen J, Ding S (2011) Conversion of mouse fibroblasts into cardiomyocytes using a direct reprogramming strategy. *Nat Cell Biol* **13**: 215–222.

L5

Plasmodium functional kinomics: opportunities for antimalarial drug discovery

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Malaria remains a major public health problem in large parts of the world, causing about one million deaths per year, and representing a significant hindrance to socio-economic development of affected countries. The spread of drug resistance in *Plasmodium falciparum*, the protozoan parasite responsible for the vast majority of mortality cases caused by malaria, renders the development of novel control tools an urgent task, especially with respect to as yet unexploited classes of targets to prevent cross-resistance. Based on (i) the success of protein kinase-selective inhibitors to treat various cancers and their prospects in other diseases, and (ii) the divergences that have been documented between mammalian and *Plasmodium* protein kinases (PKs) in many instances, the kinome of the parasite has been proposed as an attractive target for novel antimalarials [1].

The entire complement of *P. falciparum* genes encoding PKs was characterised shortly after publication of the *P. falciparum* genome [2]. This revealed that although some *Plasmodium* PKs are clear orthologues of mammalian PKs (e.g. CK1), the vast majority are either "orphan" enzymes (i.e. they do not cluster within any of the established eukaryotic PK families) or "semi-orphan" (i.e. they belong to an established PK family but are sufficiently divergent as to prevent detection of orthology to specific mammalian enzymes). A particularly dramatic example of "orphan" kinases is given by the so-called FIKK kinases, named after a conserved Phe-Ile-Lys-Lys motif in subdomain II of the kinase domain. Kinases of this family are strictly restricted to Apicomplexa (the taxon that includes malaria parasites) and expanded to 20 members in *P. falciparum* [3]. Interestingly, the kinome of the related Apicomplexan parasite *Toxoplasma gondii* also includes a vastly expanded kinase family, the ROP kinases. These are unrelated to the FIKKs, but some of them have also been shown to be exported to the host cell, where they regulate gene expression to tailor the host cell behavior [4]. Another divergence between the kinomes of Apicomplexan parasites and that of their host cells is the presence of a family of calcium-dependent kinases (CDPKs) characterized by a calmodulin-like domain fused to the kinase domain, an architecture found only in plants and Alveolates, the phylum that comprises Apicomplexa and Ciliates [5].

Thus, most *Plasmodium* PKs are divergent from mammalian enzymes at the level of primary structure, suggesting that species-selective inhibition is achievable (even for those kinases that possess a mammlian orthologue). Structural data published for kinases of apicomplexan parasites confirm that the catalytic pockets of these divergent enzymes offer unique opportunities selective inhibition. This is illustrated notably by the unusually small gatekeeper residues of these kinases, which renders them sensitive to the so-called "bumped" inhibitors [6]. A summary of antimalarial drug discovery based on interference with the parasite and host kinomes will be presented.

An initial assessment of essentiality of the *P. falciparum* kinome based on a reverse genetics approach identified 36 parasite kinases as likely to be essential for asexual proliferation of blood stage parasites [7]. We are now addressing the cellular function of essential kinases. We will present a general overview of the status of our current kinome-wide investigations, focusing on an interactomics approach based on immunoprecipitation of complexes from parasite lines expressing epitope-tagged kinases, which allowed us to identify chromatin dynamics [8] and mRNA splicing (unpublished) as processes likely to implicate PfCK2 and PfCK1, respectively, and that we intend to apply to the entire kinome. This will be followed by more detailed discussions of unpublished results on selected kinases that we found to be involved in mitosis (the Aurora-related kinases PfARK1, PfARK2 and PfARK3), control of proliferation rate (the atypical kinase PfPK7 and the cyclin-dependent kinase Pfcrk-5), sexual development (the NIMA-related kinase Pfnek-4), or to be secreted to the extracellular medium (PfCK1 and PfTKL2).

Notes:

This work was performed at Inserm U609, Wellcome Trust Centre for Molecular Parasitology, University of Glasgow, and at the Inserm-EPFL Joint Laboratory, Lausanne, Switzerland (Doerig lab), with major contributions by Luc Reininger, Dominique Dorin-Semblat, Jean-Philippe Semblat, Jean Halbert, Audrey Sicard, Marie-Paule Nivez, Yvan Bouza, Claudia Demarta, Guillem Dayer.

The project is ongoing at Monash with Teresa Carvalho and Megan Bird, in cooperation with Isabelle Lucet, Andrew Wilks and Brian Cooke (Monash) and Andrew Tobin (Leicester).

References:

1. Doerig C, Meijer L (2007) Antimalarial drug discovery: targeting protein kinases. *Expert Opin Ther Targets* **11**: 279–290.

2. Ward P, *et al.* (2004) Protein kinases of the human malaria parasite *Plasmodium falciparum*: the kinome of a divergent eukaryote. *BMC Genomics* **5**: 79.

3. Schneider AG, Mercereau-Puijalon O (2005) A new Apicomplexa-specific protein kinase family: multiple members in *Plasmodium falciparum*, all with an export signature. *BMC Genomics* **6**: 30.

4. Peixoto L, *et al.* (2010) Integrative genomic approaches highlight a family of parasite-specific kinases that regulate host responses. *Cell Host Microbe* **8**: 208–218.

5. Billker O, Lourido S, Sibley LD (2009) Calcium-dependent signaling and kinases in apicomplexan parasites. *Cell Host Microbe* **5**: 612–622.

6. Ojo KK, *et al*. (2012) Transmission of malaria to mosquitoes blocked by bumped kinase inhibitors. *J Clin Invest* **122**: 2301–2305.
7. Solyakov L, *et al*. (2011) Global kinomic and phosphoproteomic analyses of the human malaria parasite *Plasmodium falciparum*. *Nat Commun* **2**: 565.
8. Dastidar EG, *et al*. (2012) Involvement of *Plasmodium falciparum* protein kinase CK2 in the chromatin assembly pathway. *BMC Biol* **10**: 5.

L6

Switch helix: a novel site of interchain allosteric communication in cGMP-dependent protein kinase

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The cGMP-dependent protein kinase, PKG, is widely expressed and serves as an integral component of second messenger signaling in a number of biological contexts including vasodilation, motility and memory [1]. Despite extensive biochemical characterization, the molecular mechanisms of PKG activation remain obscure. Insights into the forces that govern kinase activation have recently been provided by the 2.5 Å crystal structure of a regulatory domain construct (aa 78–355) of type Iα PKG [2]. This 31 kDa PKG fragment contains both cGMP binding sites as well as a previously uncharacterized helical domain (switch helix) that promotes the formation of a hydrophobic interface between protomers.

Mutational disruption of this interface in full-length PKG implicates the switch helix as a critical site of dimer communication in PKG biology as demonstrated by kinase activation at lower cGMP concentrations [2]. Consequently, the switch helix interface may serve as a structural platform for targeted drug screening of cGMP-independent kinase activators. Following iterative and positional-scanning peptide library screens, a series of switch helix peptide derivatives demonstrated specific binding and dose-dependent activation of the kinase. Interestingly, switch helix specific activation constants was further augmented by the presence of cGMP suggesting a stereo-chemically contiguous and cooperative communication between both types of binding sites. Lastly, the functional significance of the switch helix interaction was demonstrated by probing the PKG-dependent activity of the calcium-sensitive potassium (BK) channel in excised patches from vascular smooth muscle from rat brain [3]. The switch helix peptide (D329–D358) significantly increased the open probability (NP_o) of the BK channel to approximately 85% of maximum.

The switch helix as part of a novel domain in PKG offers valuable additional structural insights into the mechanism of allosteric kinase activation. However, the pharmacological implications of our structural work are much broader. It is well understood that PKG serves as a critical intracellular mediator in the control of vasomotor reactivity and consequently, blood vessel relaxation. The blood pressure lowering effects associated with PKG activation are equally well documented. In fact, all existing pharmacological therapies aimed at PKG as a target, such as NO-donors (isosorbide dinitrate/ischemic heart disease) or phosphodiesterase inhibitors (sildenafil / erectile dysfunction), are geared towards the control of intracellular levels of endogenous cGMP. This work presents the first direct and cGMP-independent modulation of PKG activity. The discovery of the switch helix interaction site has the potential to elevate the search for more effective treatments of cardiovascular disorders and other cGMP-dependent pathologies to new levels.

References:

1. Hofmann F, Bernhard D, Lukowski R, Weinmeister P (2009) cGMP regulated protein kinases (cGK). *Handb Exp Pharmacol* **191**: 137–162.
2. Osborne BW, Wu J, McFarland CJ, Nickl CK, Sankaran B, Casteel DE, Woods VL Jr, Kornev AP, Taylor SS, Dostmann WR (2011) Crystal structure of cGMP-dependent protein kinase reveals novel site of interchain communication. *Structure* **19**: 1317–1327.
3. Robertson BE, Schubert R, Hescheler J, Nelson MT (1993) cGMP-dependent protein kinase activates Ca-activated K channels in cerebral artery smooth muscle cells. *Am J Physiol* **265**: C299–C303.

L7

Exploiting substrate recognition for selective inhibition of protein kinases. GSK-3 as an example

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Protein kinases are potential targets of drugs to treat many human diseases [1]. Intensive efforts have been made to develop protein kinase inhibitors, but a major challenge is achieving specificity [2]. Most protein kinase inhibitors described to date are small molecules that compete with ATP for its binding site. These inhibitors, although often very effective, generally show limited specificity. Selecting regulatory elements outside the ATP binding pocket may thus provide an alternative approach. Although has not been extensively exploited, substrate competitive inhibitors offer substantial selectivity and may be safer for clinical use with fewer off target side effects. Yet, discovery and development of substrate competitive inhibitors is a complicated task. In-depth understanding of substrate recognition by the protein kinase, under investigation, is essential for design and refinement of competitive inhibitors, and ideally combines experimental and computational analyses. These should take into consideration the dynamics of substrate's binding with the kinase making the design of the inhibitor even more sophisticated [3].

GSK-3, plays essential roles in diverse biological processes. Its elevated activity has been linked with various human diseases including diabetes, inflammation, neurodegenerative and psychiatric disorders [4]. Recently GSK-3 has been also implicated in self renewal and pluripotency in embryonic stem cells [5]. Hence, use of selective GSK-3 inhibitors is considered a promising therapy. We developed a novel cell-permeable peptide inhibitor termed, L803-mts, based on the unique recognition motif of GSK-3 that requires pre-phosphorylation. We focused on understanding the molecular basis of GSK-3 interaction with its substrates and with L803-mts. Accordingly, we identified common and non-overlapping elements: whereas substrates bind to the cavity delimited by Gln 89 and Asn 95, located within GSK-3 catalytic domain, L803-mts binds to only one residue, Phe 93, within this segment and in addition interacts with a hydrophobic patch surface located away from the ATP binding site [6, 7]. Based on this understanding, we synthesized new L803-mts variants aimed at enhancing the binding affinity to the Gln 89 and Asn 95 cavity, and strengthening the hydrophobic interactions with the kinase. These new peptides showed 3–10 fold improvement in kinase inhibition as compared to the 'original' L803-mts [7]. In summary, we concluded that knowledge of the molecular recognition of substrates is essential but not sufficient for design of an ideal inhibitor. The experimental approach is critically required in optimization and refinement of the inhibitors. The therapeutic benefits of substrate competitive inhibitors in in vivo models will be also discussed.

References:

1. Manning G, Plowman GD, Hunter T, Sudarsanam S (2002) Evolution of protein kinase signaling from yeast to man. *Trends Biochem Sci* **27**: 514–520.
2. Parang K, Till JH, Ablooglu AJ, Kohanski RA, Hubbard SR, Cole PA (2001) Mechanism-based design of a protein kinase inhibitor. *Nat Struct Biol* **8**: 37–41.
3. Taylor SS, Kornev AP (2011) Protein kinases: evolution of dynamic regulatory proteins. *Trends Biochem Sci* **36**: 65–77.
4. Eldar-Finkelman H, Martinez A (2011) GSK-3 nhibitors: preclinical and clinical focus on CNS. *Front Mol Neurosci* **4**: 32.
5. Kim WY, Wang X, Wu Y, Doble BW, Patel S, Woodgett JR, Snider WD (2009) GSK-3 is a master regulator of neural progenitor homeostasis. *Nat Neurosci* **12**: 1390–1397.
6. Ilouz R, Kowelsman N, Eisenstein M, Eldar-Finkelman H (2006) Identification of novel glycogen synthase kinase-3β substrate-interacting residues suggests a common mechanism for substrate recognition. *J Biol Chem* **281**: 30621–30630.
7. Licht-Murava A, Plotkin B, Eisenstein M, Eldar-Finkelman H (2011) Elucidating substrate and inhibitor binding sites on the surface of GSK-3β and the refinement of a competitive inhibitor. *J Mol Biol* **408**: 366–378.

L8

Non-ATP competitive kinase inhibitors: potentials and limitations

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Conformation provides a conceptual framework for understanding many aspects of protein kinase which act as molecular switches with remarkable plasticity and dynamics upon interaction with specific regulatory domains as well as modulators.

Conformational bias, i.e. a shift in the equilibrium between active and inactive conformations is a key determinant in kinase regulation and can be brought about by many factors including post-translational modifications, regulatory proteins, ligand binding etc. Pathologic kinase deregulation often shifts the conformation to an active "on" state, leading to constitutive signaling [1, 2]. Protein kinases with gain of function mutations appear to be more sensitive (and sometimes resistant) to kinase inhibitors compared to their wild type variants. Protein kinases escape resistance by either mutating key residues in their kinase domain or by pathway reactivation leading to a bypass of the targeted kinase. Thus, one of the major challenges in kinase drug discovery today, among others, is to understand the mechanisms of resistance to kinase inhibition [3, 4].

Kinase inhibitors can be viewed as particular ligands to protein kinases. As the mode of action is linked to the binding mode, the selectivity as well as the kinetics of kinase inhibitors can often be rationalized based on the target conformation. Our knowledge on the structural determinants of kinase inhibition by small molecules binding to the ATP pocket has advanced steadily in the past years [5, 6]. Selectivity of ATP directed kinase inhibitors and the limited set of chemotypes targeting the ATP binding site — a highly crowded area — are issues in kinase drug discovery. In contrast less is known about targeting the protein kinases outside of the "classical ATP pocket". However, a few well documented examples like the inhibitors for MEK, ABL, mTOR, AKT and IGF-1R have shown that protein kinase can be inhibited by mechanisms that are outside of the beaten tracks [6, 7].

In this lecture we will review the field of non-ATP site directed inhibitors and we will discuss the potentials as well as the limitation of these approaches that should lead to an improved target selectivity as well as the use of these types of inhibitors in preventing resistance protein kinase inhibition.

References:

1. Taylor SS, Kornev AP (2011) Protein kinases: evolution of dynamic regulatory proteins. *Trends Biochem Sci* **36**: 65–77.
2. Lahiry P, Torkamani A, Schork NJ, Hegele RA (2010) Kinase mutations in human disease: interpreting genotype-phenotype relationships. *Nat Rev Genet* **11**: 60–74.
3. Sellers WR (2011) A blueprint for advancing genetics-based cancer therapy. *Cell* **147**: 26–31.
4. Sawyers C (2004) Targeted cancer therapy. *Nature* **432**: 294–297.
5. Traxler P, Bold G, Buchdunger E, Caravatti G, Furet P, Manley P, O'Reilly T, Wood J, Zimmermann J (2001) Tyrosine kinase inhibitors: from rational design to clinical trials. *Med Res Rev* **21**: 499–512.
6. Zhang J, Yang PL, Gray NS (2009) Targeting cancer with small molecule kinase inhibitors. *Nat Rev Cancer* **9**: 28–39.
7. Möbitz H, Fabbro D (2012) The role of kinase conformation for kinase inhibition: from bench to bedside. *Eur Pharmaceutical* **17**: 41–51.

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Dynamical effects in the allosteric control of kinases

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Protein kinases (PK) show a remarkable conformational dynamics that is tightly regulated in physiological conditions, often by allosteric signals. A shift of the conformational ensemble towards the active state, with the consequent hyper-activation of the PK, leads to a number of human diseases, including cancer. Thus, understanding how allosteric signals regulate the inactive to active equilibrium in PK could lead to the rational design of a new class of allosteric drugs. Historically, drug discovery programs have been dominated by efforts to develop antagonists that compete for binding with endogenous ligands at orthosteric sites. However, allosteric drugs might offer several therapeutic advantages over traditional orthosteric ligands, including greater safety and/or selectivity.

Here, by combining state-of-the-art computer simulations with spectroscopy, chemical and molecular biology approaches we study in great details the role of conformational changes in the allosteric control of two pharmaceutically relevant kinases: Abl and FGFr.

In Abl a shift of the SH2 domain from the C- to the N-terminus of the catalytic domain has been found to be involved in activation [1]. The allosteric mechanism, by which the SH2 domain induces conformational changes at the active site, is still debated. We have used elastic network models, normal mode analysis, molecular dynamics simulation and mutagenesis to gain insight into the interplay between the SH2 domain and the relevant motifs at the catalytic site. We propose a mechanism, by which the SH2 domain influences the dynamics of crucial regulatory elements. We also find that the flexibility of the same structural elements regulate the stability of the DFG-out state in other kinases [2]. In FgFr we use free energy calculations, crystallography and NMR approaches to shed light on the mode of action of a novel allosteric inhibitor [3].

References:

1. Nagar B, Hantschel O, Seeliger M, Davies JM, Weis WI, Superti-Furga G, Kuriyan J (2006) *Mol Cell* **21**: 787–798.
2. Lovera S, Sutto L, Boubeva R, Scapozza L, Doelker N, Gervasio FL (2012) The different flexibility of c-Src and c-Abl kinases regulates the stability of the DFG-out inactive state. *J Am Chem Soc* **134**: 2496–2499.
3. Herbert C, *et al.* Elucidation of the molecular mode of action of SSR128129E, the first small molecule allosteric inhibitor of FGF receptor signaling. *Cancer Cell* (under revision).

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Regulation of protein synthesis *via* deregulated signaling networks in human gliomas

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Among human gliomas, glioblastoma multiforme (GBM) is the most aggressive and lethal form of brain cancer, with a mean survival of approximately one year. Currently there is a desperate need to fully understand mechanisms that drive gliomagenesis and therapy resistance. In a search for novel targets that could be therapeutically developed, molecular profiling of malignant brain tumors identified and characterized mechanisms leading to brain cancer development, progression and therapy resistance. Various genetic or epigenetic alterations can lead to hyperactivation of signal transduction pathways that become oncogenic and induce cancer growth associated with an increase in overall protein synthesis [1, 2]. Hyperactivated AKT-signaling pathway inactivates the tuberous sclerosis tumor-suppressor TSC1/2 complex that negatively regulates the mammalian target of rapamycin complex 1 (mTORC1), a major regulator of protein synthesis. In its active form, mTORC1 phosphorylates scaffold protein eIF4G, S6 kinase (S6K) and eIF4E-binding proteins (4E-BPs) leading to the release and activation of the cap binding protein eIF4E, that is a limiting factor for cap-dependent translation. MAP kinase-interacting protein kinases (MNKs) are closely associated with translation initiation complexes and can also influence protein synthesis. MNKs function downstream of p38 and ERK MAP kinases. They bind to initiation factor eIF4G and phosphorylate eIF4E. Role of eIF4E phosphorylation in translation regulation is not clear. Importantly, eIF4E phosphorylation at Ser209 by MNK kinases was required for eIF4E action in opposing apoptosis and promoting tumorigenesis *in vivo* [3] and an increase in phospho-eIF4E has been observed in various cancers [4]. Previously, a balance between mTORC1 and MNK signaling pathways was reported in human cancer cells where inhibition of one pathway was correlated with the activation of another, resulting in a defined level of translation that supported cancer cell survival [5, 7]. In our previous study targeting MNK1 activity suppressed eIF4E phosphorylation and reduced proliferation whereas, concomitant treatment with MNK inhibitor CGP57380 and mTORC1 inhibitor RAD001 resulted in an additive effect on growth inhibition in human GBM cells as well as in our recently established orthotopic GBM mouse model. Analysis of polysomal profiles indicated inhibition of global translation in CGP57380 and rapamycin-treated GBM cells. Furthermore, incorporation of stable isotope labeled amino acids (SILAC) showed the strongest inhibition in the global rate of protein synthesis in CGP57380 and RAD001 incubated cells as compared to single compound treatment. In order to analyze further how these pathways regulate assembly of translation initiation complexes we used cap analogue m7GTP for efficient precipitation of translation initiation factors followed by mass spectrometry-based quantitative proteomics. Association of eIF4E-BPs with analyzed complexes was regulated by mTORC1 pathway, whereas double blocking of MNK and mTORC1 increased the binding of eIF4BPs to eIF4E indicating an important point of signaling pathway convergence during protein synthesis. Our model for regulation of protein synthesis together with clinical implications will be presented and discussed.

References:

1. Grzmil M, Hemmings BA (2010) Deregulated signalling networks in human brain tumours. *Biochim Biophys Acta* **1804**: 476–483.
2. Grzmil M, Hemmings BA (2012) Translation regulation as a therapeutic target in cancer. *Cancer Res* **72**: 1–10. doi: 10.1158/0008–5472.
3. Wendel HG, Silva RL, Malina A, *et al.* (2007) Dissecting eIF4E action in tumorigenesis. *Genes Dev* **21**: 3232–3237.
4. Fan S, Ramalingam SS, Kauh J, *et al.* (2009) Phosphorylated eukaryotic translation initiation factor 4 (eIF4E) is elevated in human cancer tissues. *Cancer Biol Ther* **8**: 1463–1469.
5. Wang X, Yue P, Chan CB, *et al.* (2007) Inhibition of mammalian target of rapamycin induces phosphatidylinositol 3-kinase-dependent and Mnk-mediated eukaryotic translation initiation factor 4E phosphorylation. *Mol Cell Biol* **27**: 7405–7413.
6. Bianchini A, Loiarro M, Bielli P, *et al.* (2008) Phosphorylation of eIF4E by MNKs supports protein synthesis, cell cycle progression and proliferation in prostate cancer cells. *Carcinogenesis* **29**: 2279–2288.
7. Grzmil M, Morin P Jr, Lino MM, *et al.* (2011) MAP kinase-interacting kinase 1 regulates SMAD2-dependent TGF-beta signaling pathway in human glioblastoma. *Cancer Res* **71**: 2392–2402.

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Allosteric and ATP-competitive inhibitors of the Bcr-Abl tyrosine kinase

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Imatinib (Gleevec) was the first FDA-approved small-molecule tyrosine kinase inhibitor (TKI) and became the frontline therapy for chronic myeloid leukemia (CML) that is caused by the constitutively active tyrosine kinase BCR-ABL. This unprecedented success story was however diminished by the rapid emergence of drug resistance in a subset of patients that became one of the major obstacles preventing cure. This has triggered the development and rapid approval of the second-generation BCR-ABL inhibitors nilotinib and dasatinib, as well as several third generation inhibitors that are currently in clinical trials. All these TKIs inhibit the majority of imatinib resistance mutations. Using an unbiased chemical proteomics approach combined with detailed enzymatic, structural and functional studies in CML cells, we have analyzed the proteins that are targeted by the three generations of BCR-ABL TKIs. Differences in the target spectrum, possible second medical uses and impact of the Bcr-Abl TKIs on the transcriptome, proteome and interactome of CML cells will be discussed.

In addition to the targeting of BCR-ABL using ATP-competitive compounds, recent advances in our understanding of the structure, regulation and signaling of BCR-ABL have identified allosteric sites that are distant from the ATP binding pocket and critical for BCR-ABL dependent oncogenic transformation. I will discuss how targeting of allosteric sites could open new opportunities to inhibit resistant BCR-ABL mutants, either alone or in combination with conventional ATP-competitive inhibitors.

References:

1. Hantschel O (2012) Allosteric BCR-ABL inhibitors in Philadelphia chromosome-positive acute lymphoblastic leukemia: novel opportunities for drug combinations to overcome resistance. *Haematologica* **97**: 157–159.
2. Grebien F, Hantschel O, Wojcik J, *et al.* (2011) Targeting the SH2-kinase interface in Bcr-Abl inhibits leukemogenesis. *Cell* **147**: 306–319.
3. Hantschel O, Rix U, Superti-Furga G (2008) Target spectrum of the BCR-ABL inhibitors imatinib, nilotinib and dasatinib. *Leuk Lymphoma* **49**: 615–619.
4. Hantschel O, Rix U, Schmidt U, *et al.* (2007) The Btk tyrosine kinase is a major target of the Bcr-Abl inhibitor dasatinib. *Proc Natl Acad Sci USA* **104**: 13283–13288.
5. Rix U, Hantschel O, Durnberger G, *et al.* (2007) Chemical proteomic profiles of the BCR-ABL inhibitors imatinib, nilotinib and dasatinib reveal novel kinase and non-kinase targets. *Blood* **110**: 4055–4063.
6. Hantschel O, Superti-Furga G (2004) Regulation of the c-Abl and Bcr-Abl tyrosine kinases. *Nat Rev Mol Cell Biol* **5**: 33–44.

L12

Identification of novel non-canonical mTOR kinase targets

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Target of rapamycin (TOR) is a protein kinase, broadly expressed in all eukaryotic cells. TOR is involved in sensing nutrients availability, trophic factors support, cellular energy levels, as well as cellular stress and adjusting cellular metabolism accordingly [1]. TOR proteins, including mammalian homolog mTOR, were thought to primarily regulate protein translation *via* their best known downstream targets such as S6 kinase and 4E-BP1 protein (canonical function). However, two important findings challenged this view of mTOR kinase being solely involved in translational control. First, it has been shown that mTOR forms two different protein complexes, mTORC1 and mTORC2, with non-overlapping cellular functions and different resistance to mTOR-inhibitor, rapamycin. mTORC1 that contains protein called Raptor was shown sensitive to rapamycin and involved in phosphorylation of S6 kinase and 4E-BP1 [2]. On the other hand, mTORC2 containing Rictor and mSin proteins turned out to be rapamycin resistant and involved in control of AGC kinase activity and actin cytoskeleton dynamics [3, 4]. Yet, "chemical genomics", performed on yeast with use of rapamycin challenged further the view of TORC1 exclusive involvement in translational control. In course of this screen hundreds of mutants were identified to be functionally linked to TOR and their analysis suggested that TOR1 is involved in cellular functions other than translation [5], including transcription, membrane trafficking, lipid metabolism, different ways of protein degradation and cytoskeleton dynamics. This finding done in yeast was further supported by an observation of very broad cellular distribution of TOR proteins in different species through different cell compartments including endosomes, lysosomes, ER, cytoplasm and nucleus. Consequently, non-canonical functions of TOR in different types of organisms and different cell types started to be elucidated. For example, we showed that mTOR is needed for control of cytoskeleton dynamics [6]. In neurons mTOR phosphorylates protein called cytoplasmic linker 170 protein (CLIP-170). This in turn changes interaction of CLIP-170 with actin-binding protein IQGAP1 and with filamentous actin. Such interaction bridges dynamic microtubules with microfilaments and is needed for proper neuronal development. On the other hand, Hennig *et al.* [7] showed that *Drosophila* dTOR controls different modes of endocytosis. Also our work strongly suggests that mTOR is needed for protein endo- and exocytosis. mTOR was also linked to control of autophagy in several organisms [8]. Finally, mTORC1 as well as mTORC2 were found both, present in the nucleus as well as involved in several cellular processes occurring in this particular compartment. Solid body of evidence suggests participation of nuclear mTOR in control of transcription. For example, mTOR associates with the promoter regions of RNA polymerase I (PolI)- and Pol III-transcribed genes, which are regulated by growth factors and nutrients [9]. In case of PolIII it was postulated that promoter-bound, active mTOR phosphorylates transcription inhibitor Maf1, that upon phosphorylation releases PolIII [9]. Role of mTOR-dependent, PolII-driven transcription has been also postulated in control of mitochondrial proliferation and oxidative metabolism [10]. More elusive data regards role of mTOR in nuclear RNA processing or in mRNA nuclear export, but jointly such findings raise intriguing possibility that mTOR in nucleus can recruit mRNAs ready to be exported, assist them during nuclear export and facilitate their translation once the complex reaches the cytoplasm.

During, IPK2012 I will discuss several of the findings regarding "non-canonical" functions of mTOR mentioned above as well as by comparison of our mass spectrometry analysis of proteins bound to mTOR with recent results of Sabatini and Blenis labs regarding mTOR-phosphoproteom, I will attempt to provide a comprehensive picture of non-canonical activities of mTOR in neurons. I will also try to show the logical connections between canonical and novel mTOR functions.

Notes:

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References:

1. Sengupta S, Peterson TR, Sabatini DM (2010) Regulation of the mTOR complex 1 pathway by nutrients, growth factors, and stress. *Mol Cell* **40**: 310–322.
2. Kim DH, Sarbassov DD, Ali SM, King JE, Latek RR, Erdjument-Bromage H, Tempst P, Sabatini DM (2002) mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. *Cell* **110**: 163–175.
3. Sarbassov DD, Guertin DA, Ali SM, Sabatini DM (2005) Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* **307**: 1098–1101.
4. Jacinto E, Loewith R, Schmidt A, Lin S, Ruegg MA, Hall A, Hall MN (2004) Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. *Nat Cell Biol* **6**: 1122–1128.
5. Xie MW, Jin F, Hwang H, Hwang S, Anand V, Duncan MC, Huang J (2005) Insights into TOR function and rapamycin response: chemical genomic profiling by using a high-density cell array method. *Proc Natl Acad Sci USA* **102**: 7215–7220.
6. Swiech L, Blazejczyk M, Urbanska M, Pietruszka P, Dortland BR, Malik AR, Wulf PS, Hoogenraad CC, Jaworski J (2011) CLIP-170 and IQGAP1 cooperatively regulate dendrite morphology. *J Neurosci* **31**: 4555–4568.
7. Hennig KM, Colombani J, Neufeld TP (2006) TOR coordinates bulk and targeted endocytosis in the *Drosophila*

melanogaster fat body to regulate cell growth. *J Cell Biol* **173**: 963–974.
8. Jung CH, Jun CB, Ro SH, Kim YM, Otto NM, Cao J, Kundu M, Kim DH (2009) ULK-Atg13-FIP200 complexes mediate mTOR signaling to the autophagy machinery. *Mol Biol Cell* **20**: 1992–2003.
9. Tsang CK, Liu H, Zheng XF (2010) mTOR binds to the promoters of RNA polymerase I- and III-transcribed genes. *Cell Cycle* **9**: 953–957.
10. Cunningham JT, Rodgers JT, Arlow DH, Vazquez F, Mootha VK, Puigserver P (2007) mTOR controls mitochondrial oxidative function through a YY1-PGC-1alpha transcriptional complex. *Nature* **450**: 736–740.

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Delineating the design principles of proteins kinases using genomic data: Implications for inhibitor design

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Protein kinases display remarkable diversity and complexity in their modes of allosteric regulation. The complex modes of regulation have evolved as a consequence of natural selection operating on protein kinase sequences for billions of years. In this talk, I will describe how quantitative comparisons of protein kinase sequences from diverse organisms, in particular prokaryotes, have contributed to our understanding of the structural organization and evolution of allosteric regulation in the protein kinase domain [1–4]. An emerging view from these studies is that regulatory diversity and complexity in the protein kinase domain evolved in a "modular" fashion through elaboration of an ancient core component, which existed before the emergence of eukaryotes [4–7]. The core component provided the conformational flexibility required for ATP binding and phosphoryl-transfer in prokaryotic kinases, but evolved into a highly regulatable domain in eukaryotes through the addition of exaggerated structural features that facilitated tight allosteric control [6, 7]. Family-specific features are built upon the core component to provide additional layers of control [8]. In this talk, I will describe how identifying and characterizing family-specific features has allowed us to obtain new insights into the evolution of allosteric regulation in the protein kinase domain [9], and predict the impact of cancer mutations [10]. Finally, I will describe our recent efforts to classify the kinomes of apicomplexan parasites [11], which are implicated in numerous infectious diseases. I will discuss our preliminary findings on the evolutionary divergence of parasitic kinases, and the implications of our findings on protein kinase inhibitor design.

References:

1. Manning G, Plowman GD, Hunter T, Sudarsanam S (2002) Evolution of protein kinase signaling from yeast to man. *Trends Biochem Sci* **27**: 514–520.
2. Manning G, Young SL, Miller WT, Zhai Y (2008) The protist, *Monosiga brevicollis*, has a tyrosine kinase signaling network more elaborate and diverse than found in any known metazoan. *Proc Natl Acad Sci USA* **105**: 9674–9679.
3. Nidhi Tyagi, Krishanpal Anamika, Narayanaswamy Srinivasan (2010) A framework for classification of prokaryotic protein kinases. *PLoS One* **5**: e10608.
4. Kannan N, Neuwald AF (2005) Did protein kinase regulatory mechanisms evolve through elaboration of a simple structural component? *J Mol Biol* **351**: 956–972.
5. Lim WA (2002) The modular logic of signaling proteins: building allosteric switches from simple binding domains. *Curr Opin Struct Biol* **12**: 61–68.
6. Kannan N, Taylor SS, Yufeng Z, Venter C, Manning G (2007) Structural and functional diversity of the microbial kinome. *PLoS Biol* **5**: e17.
7. Taylor SS, Kornev AP (2011) Protein kinases: evolution of dynamic regulatory proteins. *Trends Biochem Sci* **36**: 65–77.
8. Kannan N, Haste N, Taylor SS, Taylor SS, Neuwald AF (2007) The hallmark of AGC kinase functional divergence is its C-terminal tail, a cis acting regulatory module. *Proc Natl Acad Sci USA* **104**: 1272–1277.
9. Mirza M, Mustafa M, Talevich E, Kannan N (2010) Co-conserved features associated with *cis* regulation of ErbB receptor tyrosine kinases. *PLoS ONE* **5**: e1431.
10. Gosal G, Kochut K, Kannan N (2011) ProKinO: An ontology for integrative analysis of protein kinases in cancer. *PLoS ONE* **6**: e28782.
11. Talevich E, Mirza A, Kannan N (2011) Structural and evolutionary divergence of eukaryotic protein kinases in Apicomplexa. *BMC Evol Biol* **11**: 321.

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Structure, mechanism and inhibition of the RIO kinases

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Ribosome biogenesis is an emerging therapeutic target due to increased ribosome production during cellular proliferation, and ribosomopathies caused by ribosome processing defects [1, 2]. Since two of the three known RIO kinases, Rio1 and Rio2, are essential for 40S ribosome biogenesis [3, 4], they are logical targets for inhibitor design. RIO kinases are classified as atypical protein kinases due to limited sequence homology to canonical eukaryotic protein kinases, and structures reveal unique features, including the lack of an activation loop and altered ATP binding conformation. Using the previously determined structure of *Archaeoglobus fulgidus* Rio1 kinase as a template [5], structure-based inhibitor design and screening were employed to identify high affinity small molecule inhibitors of Rio1 kinase. The X-ray crystal structures of complexes between Rio1 kinase and two of these inhibitors: toyocamycin, an analog of adenosine, and a synthesized caffeic acid benzyl ester (CABE), were determined to high resolution. These structures, which show that the inhibitor molecules to bind as predicted in the ATP-binding pocket of the RIO1 kinase domain, allowed determination of functionalization that may result in more specific inhibitors. In addition, we were able to determine the steady state inhibition parameters for these molecules, and, for toyocamycin, identified a novel mechanism of inhibitor-stabilized oligomerization that appears to be conserved for the human homolog [6].

In order to refine the target to eukaryotic RIO homologs, the crystal structures of two eukaryotic RIO kinases, Rio2 from *Chaetomium thermophilum* and human RioK1, were also determined. In these we were able to observe eukaryote specific C-termianl extensions to both Rio1 and Rio2. The placement of these extensions, which is different in each of the two molecules, is in agreement with a potential role in regulation of functional activity. Also, these structures reveal the presence of an unexpected phosphoaspartate intermediate. Acyl-phosphate intermediates, which are observed in P-type ATPases, are unusual for the serine protein kinases, and their presence provides novel insight into the catalytic mechanism of the RIO kinases. Additionally, the results of docking of the eukaryotic Rio2 kinase into a recently reported cryo-electron microscopy map of the yeast pre-40S particles [7] suggests an exciting new model for the functional mechanisms of the RIO kinases in pre-40S maturation that provides basis for a role for a transient intermediate. In this model, supported by yeast studies, Rio2 is positioned at the interface between the two major domains of the pre-40S, the head and body, poised to detect conformational changes that alter their relative positions. In addition, further functional analysis shows that dissociation of the RIO kinase from the pre-40S requires the ability to hydrolyze ATP, and ATP hydrolysis may occur in the absence of a protein substrate. Taken together, these data suggest that the strategy for the design of RIO kinase inhibitors must take into accounr the potential for oligomerization of these enzyme, the presence of a stable but transient intermediate, and the role of ATP hydrolysis in the pre-40S maturation.

Notes:

The reported work is a result of ongoing collaboration with the research groups of Bogdan Lesyng (University of Warsaw, Department of Biophysics, Warsaw, Poland) and Waldemar Priebe (University of Texas, MD Anderson Cancer Center, Houston, TX, USA) on the discovery of RIO kinase inhibitors, and Ed Hurt (Heidelberg University, Biochemistry Center, Heidelberg, Germany) on the functional mechanism of the Rio2 kinase. The described research contains major contributions from Irene Kiburu, Vatsala Sagar, Momar Diop and Eileen Chai from the University of Maryland, and Sebastian Ferreira-Cerca from Heidelberg University.

References:

1. Moss T (2004) At the crossroads of growth control making ribosomal RNA. *Curr Opin Genet Develop* **14**: 210–217.
2. Narla A, Ebert BL (2010) Ribosomopathies: human disorders of ribosome dysfunction. *Blood* **115**: 3196–3205.
3. Vanrobays E, Gelugne JP, Gleizes PE, Caizergues-Ferrer M (2003) Late cytoplasmic maturation of the small ribosomal subunit requires RIO proteins in *Saccharomyces cerevisiae*. *Mol Cell Biol* **23**: 2083–2095.
4. Schafer T, Strauss D, Petfalski E, Tollervey D, Hurt E (2003) The path from nuclear 90S to cytoplasmic 40S pre--ribosomes. *EMBO J* **22**: 1370–1380.
5. LaRonde-LeBlanc N, Guszczynski T, Copeland T, Wlodawer A (2005) Structure and activity of the atypical serine kinase Rio1. *FEBS J* **272**: 3698–3713.
6. Kiburu IN, LaRonde-LeBlanc N (2012) Interaction of rio1 kinase with toyocamycin reveals a conformational switch that controls oligomeric state and catalytic activity. *PLoS One* **7**: e37371.
7. Strunk BS, *et al.* (2011) Ribosome assembly factors prevent premature translation initiation by 40S assembly intermediates. *Science* **333**: 1449–1453.

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Probes of the mitochondrial cAMP-dependent protein kinase

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Members of the protein kinase family have been implicated in a myriad of processes, from ATP generation to unrestrained growth and division [1]. Although the cAMP-dependent protein kinase (PKA) is commonly held as the prototypical protein kinase, its mechanism of activation is atypical relative to nearly all other protein kinase family members. PKA is inactive in its holoenzyme form, a tetrameric species consisting of two regulatory (R) and two catalytic (C) subunits. Upon binding of cAMP to the R subunits, the C subunits are released, and are thus free to catalyze the phosphorylation of an array of proteins [2, 3]. However, it is now recognized that PKA can be activated in a cAMP-independent fashion as well [4–8].

There have been several reports describing the concerted action of PKA and calpain, including the regulation of platelet procoagulant activity [9], D1 receptor-mediated phosphorylation of tau [10], and the PKA-catalyzed phosphorylation of the calpain inhibitor calpastatin [11, 12], to name but a few. Calpains are a class of ubiquitously expressed Ca^{2+}-activated cysteine proteases. Recent studies have shown that calpain-1 (μ-calpain), calpain-2 (m-calpain), and calpain-10 are present in the mitochondria [13–15], an organelle with significant PKA activity [16–19]. Both calpain-1, which requires micromolar levels of Ca^{2+}, and calpain-2, which requires millimolar levels of Ca^{2+}, are located in the mitochondrial intermembrane space, while calpain-10 is embedded within the matrix. PKA, like the calpains, is interspersed throughout the major mitochondrial compartments [19]. Finally, the calpains [20, 21] and PKA [22] are known play key roles in mediating (and blocking) apoptosis.

We have examined the relationship between mitochondrial calpain and PKA activity, and have discovered that calpain activates the R_2C_2 holoenzyme in a cAMP-independent fashion. A variety of agents, including inhibitors of the electron transport chain, activate calpain in a Ca^{2+}-dependent manner, which in turn, catalyzes the proteolysis of the R subunit, thereby releasing the C subunit in its active form.

References:

1. Manning G, *et al.* (2002) The protein kinase complement of the human genome. *Science* **298**: 1912–1934.
2. Gullingsrud J, *et al.* (2006) Dynamic binding of PKA regulatory subunit RI alpha. *Structure* **14**: 141–149.
3. Kim C, Xuong NH, Taylor SS (2005) Crystal structure of a complex between the catalytic and regulatory (RIalpha) subunits of PKA. *Science* **307**: 690–696.
4. Zhong H, *et al.* (1997) The transcriptional activity of NF-kappaB is regulated by the IkappaB-associated PKAc subunit through a cyclic AMP-independent mechanism. *Cell* **89**: 413–424.
5. Dulin NO, *et al.* (2001) Cyclic AMP-independent activation of protein kinase A by vasoactive peptides. *J Biol Chem* **276**: 20827–20830.
6. Gambaryan S, *et al.* (2010) Thrombin and collagen induce a feedback inhibitory signaling pathway in platelets involving dissociation of the catalytic subunit of protein kinase A from an NFkappaB-IkappaB complex. *J Biol Chem* **285**: 18352–18363.
7. Nguyen PV, Woo NH (2003) Regulation of hippocampal synaptic plasticity by cyclic AMP-dependent protein kinases. *Prog Neurobiol* **71**: 401–437.
8. Chain DG, *et al.* (1999) Mechanisms for generating the autonomous cAMP-dependent protein kinase required for long-term facilitation in Aplysia. *Neuron* **22**: 147–156.
9. Yan R, *et al.* (2009) Role of cAMP-dependent protein kinase in the regulation of platelet procoagulant activity. *Arch Biochem Biophys* **485**: 41–48.
10. Lebel M, *et al.* (2009) Dopamine D1 receptor activation induces tau phosphorylation via cdk5 and GSK3 signaling pathways. *Neuropharmacology* **57**: 392–402.
11. Cong M, *et al.* (1998) The bovine calpastatin gene promoter and a new N-terminal region of the protein are targets for cAMP-dependent protein kinase activity. *J Biol Chem* **273**: 660–666.
12. De Tullio R, *et al.* (2009) Involvement of exon 6-mediated calpastatin intracellular movements in the modulation of calpain activation. *Biochim Biophys Acta* **1790**: 182–187.
13. Arrington DD, Van Vleet Schnellmann RG (2006) Calpain 10: a mitochondrial calpain and its role in calcium-induced mitochondrial dysfunction. *Am J Physiol Cell Physiol* **291**: C1159–C1171.
14. Kar P, *et al.* (2010) Mitochondrial calpain system: an overview. *Arch Biochem Biophys* **495**: 1–7.
15. Ozaki T, Yamashita T, Ishiguro S (2009) Mitochondrial m-calpain plays a role in the release of truncated apoptosis-inducing factor from the mitochondria. *Biochim Biophys Acta* **1793**: 1848–1859.
16. Sardanelli AM, *et al.* (2006) Occurrence of A-kinase anchor protein and associated cAMP-dependent protein kinase in the inner compartment of mammalian mitochondria. *FEBS Lett* **580**: 5690–5696.
17. Chen Q, Lin RY, Rubin CS (1997) Organelle-specific targeting of protein kinase AII (PKAII). Molecular and in situ characterization of murine A kinase anchor proteins that recruit regulatory subunits of PKAII to the cytoplasmic surface of mitochondria. *J Biol Chem* **272**: 15247–15257.
18. Schwoch G, Trinczek B, Bode C (1990) Localization of catalytic and regulatory subunits of cyclic AMP-dependent protein kinases in mitochondria from various rat tissues. *Biochem J* **270**: 181–188.
19. Agnes RS, *et al.* (2010) Suborganelle sensing of mitochondrial cAMP-dependent protein kinase activity. *J Am Chem Soc* **132**: 6075–6080.
20. Cao G, *et al.* (2007) Critical role of calpain I in mitochondrial release of apoptosis-inducing factor in ischemic neuronal injury. *J Neurosci* **27**: 9278–9293.
21. Norberg E, *et al.* (2008) An increase in intracellular Ca^{2+} is required for the activation of mitochondrial calpain to release AIF during cell death. *Cell Death Differ* **15**: 1857–1864.
22. Insel PA, *et al.* (2012) Cyclic AMP is both a pro-apoptotic and anti-apoptotic second messenger. *Acta Physiol (Oxf)* **204**: 277–287.

L16

Chemical proteomics and functional proteomics strategies for protein kinase inhibitor validation and protein kinase substrate identification: applications to protein kinase CK2

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Based on the universal role of protein kinases in the regulation of biological processes, protein kinase inhibitors have become indispensible research tools for interrogating specific functions of individual protein kinases in countless experimental systems. Furthermore, since numerous protein kinases have been implicated in a host of human diseases, kinase inhibitors have emerged as promising therapeutic agents [1–2]. Despite this promise, there has been a relative lag in the development of unbiased strategies to validate both inhibitor specificity and the ability to inhibit target activity within living cells. Consequently, for many protein kinase inhibitors, it remains challenging, if not impossible, to assess their effectiveness and mechanisms of action in complex biological systems [3]. To overcome these limitations, our efforts have been focused on the development of systematic strategies that employ chemical proteomics and functional proteomics. We have employed these strategies to evaluate small molecule inhibitors of protein kinase CK2, a constitutively active protein kinase that has recently emerged as a target for anti-cancer therapy in clinical trials [4–6]. Our chemical proteomics strategies have utilized ATP or CK2 inhibitors immobilized on sepharose beads to capture binding partners from cell extracts, followed by identification by mass spectrometry [7, 8]. These studies have verified that interactions between CK2 and its inhibitors occur in complex mixtures. However, in the case of CK2 inhibitors related to 4,5,6,7-tetrabromo-1*H*-benzotriazole (TBB), a number of off-target interactions were also identified [7]. To complement our strategies for interrogating TBB binding proteins in an unbiased manner, we have also used functional proteomics approaches to identify proteins that exhibit decreases in phosphorylation when cells are treated with CK2 inhibitors [9]. Mutants of CK2 that are engineered to have decreased inhibitor sensitivity [9, 10] are used to discriminate between CK2-dependent and CK2-independent effects of the inhibitors. Using this strategy, CK2 substrates that could be biomarkers for perturbations in CK2 activity are identified as those proteins exhibiting CK2 inhibitor-dependent decreases in phosphorylation that are directly phosphorylated by CK2 *in vitro* and where phosphorylation is rescued with inhibitor-resistant CK2. Overall, our studies have yielded systematic platforms for studying CK2 inhibitors that we believe will foster efforts to define the biological functions of CK2 and to rigorously investigate its potential as a candidate for molecular-targeted therapy.

References:

1. Lahiry P, Torkamani A, Schork NJ, Hegele RA (2010) Kinase mutations in human disease: interpreting genotype-phenotype relationships. *Nat Rev Genet* **11**: 60–74.
2. Zhang J, Yang PL, Gray NS (2009) Targeting cancer with small molecule kinase inhibitors. *Nat Rev Cancer* **9**: 28–39.
3. Graves LM, Litchfield DW (2011) "Going KiNativ" — Probing the native kinome. *Chem Biol* **18**: 683–684.
4. Ruzzene M, Pinna LA (2010) Addiction to protein kinase CK2: a common denominator of diverse cancer cells? *Biochim Biophys Acta* **1804**: 499–504.
5. Duncan JS, Litchfield DW (2008) Too much of a good thing: the role of protein kinase CK2 in tumorigenesis and prospects for therapeutic inhibition of CK2. *Biochim Biophys Acta Proteins Proteomics* **1784**: 33–47.
6. Duncan JS, Turowec JP, Duncan KE, Wu C, Luscher B, Li SSC, Gloor GB, Litchfield DW (2011) A peptide-based target screen of overlapping protein kinase/caspase substrates: global regulation of caspase pathways by phosphorylation and identification of pro-caspase 3 as a target for CK2. *Sci Signal* **4**: ra30.
7. Duncan JS, Gyenis L, Lenehan J, Bretner M, Graves LM, Haystead TA, Litchfield DW (2008) An unbiased evaluation of CK2 inhibitors by chemo-proteomics: characterization of inhibitor effects on CK2 and identification of novel inhibitor targets. *Mol Cell Proteomics* **7**: 1077–1088.
8. Duncan JS, Haystead TAJ, Litchfield DW (2012) Chemoproteomic characterization of protein kinase inhibitors using immobilized ATP. *Methods Mol Biol* **795**: 119–134.
9. Gyenis L, Duncan JS, Turowec JP, Bretner M, Litchfield DW (2011) Unbiased functional proteomics strategy for protein kinase inhibitor validation and identification of bona fide protein kinase substrates: application to identification of EEF1D as a substrate for CK2. *J Proteome Res* **10**: 4887–4901.
10. Battistutta R, Cozza G, Pierre F, Papinutto E, Lolli G, Sarno S, O'Brien SE, Siddiqui-Jain A, Haddach M, Anderes K, Ryckman DM, Meggio F, Pinna LA (2011) Unprecedented selectivity and structural determinants of a new class of protein kinase CK2 inhibitors in clinical trials for the treatment of cancer. *Biochemistry* **50**: 8478–8488.

L17

Fluorescent biosensors — probing protein kinase function in cancer and drug discovery

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One of the challenges of modern biology and medicine is to visualize biomolecules in their natural environment, in real-time and in a non-invasive fashion, so as to gain insight into their behaviour in both physiological and pathological settings, to monitor changes in their spatio-temporal dynamics, relative abundance and biological activity in response to specific stimuli, and thereby devise novel therapeutic strategies to target disease-related deregulations. Amongst the chemical biology tools which have been developed over the past recent years, molecular imaging approaches based on fluorescent biosensors have provided some of the most exciting advances in our understanding of protein activity, function and spatiotemporal regulation [1–3]. The discovery of the green fluorescent protein (GFP) lead to the development of GFP-based reporters and genetically-encoded FRET biosensors, which have been successfully applied to study protein kinase behaviour in living cells with high spatial and temporal resolution [3, 4]. In parallel, combined efforts in fluorescence chemistry and in chemical biology have lead to the design of an entirely different family of biosensors, based on peptide, polypeptide or polymeric scaffolds, onto which small synthetic probes with particular photophysical properties, such as environmental sensitivity, are incorporated [5, 6]. These nongenetic biosensors have been developed to monitor protein kinase activities *in vitro* and in more complex biological samples, including cell extracts and living cells, with an equally successful outcome. Fluorescent biosensor technology offers promising perspectives for development of fluorescence-based cancer diagnostics, for monitoring cancer progression and for evaluating the response to therapeutics [7]. Moreover it constitutes an attractive technology for drug discovery programmes, for high content, high throughput screening assays, to assess the potency of new hits, to optimize lead compounds, and to evaluate drugs developed through rational design [8, 9].

In this lecture, we will provide an overview of the fluorescent biosensors that have been developed to probe protein kinases for fundamental research, and which have been applied for biomedical applications and drug discovery programmes. We will present some of our own recent developments of biosensors to probe cyclin-dependent kinases, which are applicable to both diagnostic and drug discovery approaches. Finally we will discuss perspectives and challenges for the future.

References:

1. Tarrant MK, Cole AP (2009) The chemical biology of protein phosphorylation. *Annu Rev Biochem* **78**: 797–825.
2. Wang H, Nakata E, Hamachi I (2009) Recent progress in strategies for the creation of protein-based fluorescent biosensors. *Chembiochem* **10**: 2560–2577.
3. Morris MC (2010) Fluorescent biosensors of intracellular targets from genetically encoded reporters to modular polypeptide probes. *Cell Biochem Biophys* **56**: 19–37.
4. Zhang J, Allen MD (2007) FRET-based biosensors for protein kinases: Illuminating the kinome. *Mol Biosyst* **3**: 759–765.
5. Pazos E, Vázquez O, Mascareñas JL, Vázquez ME (2009) Peptide-based fluorescent biosensors. *Chem Soc Rev* **38**: 3348.
6. González-Vera JA (2012) Probing the kinome in real time with fluorescent peptides. *Chem Soc Rev* **41**: 1652–1664.
7. Morris MC (2012) In *Fluorescent biosensors for cancer cell imaging and diagnostics. Biosensors and Cancer*. Preedy V, Hunter J, eds. CRC Press, ISBN 978-1-57808-734-1. (in press)
8. Wolff M, Wiedenmann J, Nienhaus GU, Valler M, Heilker R (2006) Novel fluorescent proteins for high-content screening. *Drug Discov Today* **11**: 1054–1060.
9. Allen MD, DiPilato LM, Rahdar M, Ren YR, Chong C, Liu JO, Zhang J (2006) Reading dynamic kinase activity in living cells for high-throughput screening. ACS *Chem Biol* **1**: 371–376.

L18

Targeting tumour cell metabolism: fragment based drug discovery with PKM2

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In the 1920s Otto Warburg proposed that cancer cells preferentially utilise glycolysis over oxidative phosphorylation for energy production, even in the presence of oxygen. This unique metabolic phenotype, termed the Warburg effect, is characterised by high levels of glucose consumption and lactate production. There has been a recent resurgence of interest in the field of tumour cell metabolism and de-regulation of cellular energetics is now considered an emerging hallmark of cancer etiology [1, 2]. Thus 'normalisation' of cancer cell metabolism may offer therapeutic opportunities for the treatment of cancer.

Pyruvate kinase (PK) catalyses the final step of glycolysis, converting phosphoenolpyruvate (PEP) into ATP and pyruvate. There are four mammalian PK isoforms PK(L/R) and PKM(1/2), products of alternative splicing of the PKLR and PKM genes respectively. PKM2 is highly expressed in cancer cells and has been implicated in regulating multiple metabolic processes including aerobic glycolysis and anabolism (reviewed in [3] and [4]). PKM2 has also been shown to function as a protein kinase, phosphorylating and activating the transcription factor STAT3 [5]. PKM2 is a tightly regulated enzyme and PKM2 activity is modulated by multiple factors including oligomerisation, phosphorylation, acetylation, oxidation, nuclear localisation and binding of the natural allosteric activator fructose-1,6-bisphosphate (FBP), (reviewed in [3]). As such PKM2 appears to constitute a key metabolic regulatory node in cancer cells and is an attractive target for anti-cancer drug discovery.

Although PKM2 is overexpressed in tumour cells, it is believed, counterintuitively, that the enzyme resides in a predominantly dimeric form that has low basal activity. It has been argued that, while permitting some flux of pyruvate into the tricarboxcylic acid (TCA) cycle *via* conversion of pyruvate into acetyl-CoA, maintaining low PKM2 activity promotes an accumulation of upstream metabolites within the glycolytic pathway. These metabolites can then be shuttled to support cellular anabolic processes (reviewed in [6] and [7]). This observation suggests two potential therapeutic strategies for targeting PKM2. The first is to develop PKM2 activators [8] which abolish the accumulation of upstream metabolites, thereby compromising anabolic processes. The second is to develop PKM2 inhibitors which seek to metabolically stress cancer cells by perturbing intracellular ATP production, through reducing PKM2-mediated pyruvate production.

I will present data obtained during the preliminary phase of a PKM2 drug discovery programme. I will describe the ligands and ligand binding sites identified from a crystallographic fragment-based screen *versus* PKM2. I will discuss how low affinity fragments can be evolved into PKM2 activators and inhibitors as well as presenting data on previously uncharacterised PKM2 ligand binding sites. I will show how the biological relevance and function of one of these sites, an amino acid binding pocket, was elucidated and discuss the potential opportunities for future drug discovery.

References:

1. Hanahan D, Weinberg RA (2011) Hallmarks of cancer: The next generation. *Cell* **144**: 647–674.
2. Frezza C, Pollard PJ, Gottlieb E (2011) Inborn and acquired metabolic defects in cancer. *J Mol Med* **89**: 213–220.
3. Chaneton B, Gottlieb E (2012) Rocking cell metabolism: revised functions of the key glycolytic regulator PKM2 in cancer. *Trends Biochem Sci* **37**: 309–316.
4. Mazurek S (2011) Pyruvate kinase type M2: A key regulator of the metabolic budget system in tumour cells. *Int J Biochem Cell Biol* **43**: 969–980.
5. Gao X, Wang H, Yang JJ, Liu X, Liu Z-R (2012) Pyruvate kinase M2 regulates gene transcription by acting as a protein kinase. *Mol Cell* **45**: 598–609.
6. Ward PS, Thompson CB (2012) Metabolic reprogramming: a cancer hallmark even Warburg did not anticipate. *Cancer Cell* **21**: 297–308.
7. DeBerardinis RJ, Lum JJ, Hatzivassiliou G, Thompson C (2008) The biology of cancer: metabolic reprogramming fuels cell growth and proliferation. *Cell Metab* **7**: 11–20.
8. Walsh MJ, Brimacombe KR, Veith H, Bougie JM, Daniel T, Leister W, Cantley LC, Israelsen WJ, Vander Heiden MG, Shen M, Auld DS, Thomas CJ, Boxer MB (2011) 2-Oxo-N-aryl-1,2,2,4-tetrahydroquinoline-6-sulfonamides as activators of the tumour cell specific M2 isoform of pyruvate kinase. *Bioorg Med Chem Lett* **21**: 6322–6327.

L19

Global profiling of native kinases in cells and tissues: Bridging the gap between enzymatic assays and biological outcomes

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Protein kinases have been the subject of intense pharmaceutical development efforts over the last 15 years. Since the approval and clinical success of the first small molecule kinase inhibitor, imatinib, 16 small molecule kinase inhibitors have been approved for clinical use in the US. With more than 500 enzymes positioned in key growth, metabolic, and inflammatory pathways, the high expectations for protein kinase-based therapeutics have been tempered only by the unique challenges posed by this diverse enzyme group. In particular, the large number of protein kinases sharing a similar ATP site architecture presents considerable challenge for assessing and achieving selectivity. In addition, protein kinases are conformationally flexible and subject to numerous modes of regulation *in vivo*, complicating the setup and interpretation of *in vitro* assays.

In response to increased drug development activity in the protein kinase space, the biochemical tools for studying kinases have been dramatically improved [1, 2]. Biochemical binding/activity assays are now readily available for the vast majority of kinases, and hundreds of antibodies for profiling kinase phosphorylation sites in cellular systems have been validated. These advances have largely addressed one of the most critical early concerns in protein kinase-based therapeutics, namely the ability to achieve selectivity for a particular kinase where all kinases share a similar ATP-binding pocket. It has now clearly been shown for a number of protein kinase targets, that exquisite selectivity across the entire protein kinase family can be achieved with ATP-competitive inhibitors [1–3].

Even with the comprehensive tools available, translating data from *in vitro* assays into conclusive *in vivo* target and mechanism of action validation remains a major challenge. To this end, new methodologies have been developed to enable global quantitative profiling of drug interactions with protein kinases directly in cells and tissues. Two main approaches have been taken to achieve this goal, both relying on modern advances in mass spectrometry-based proteomics [4–6]. One strategy uses combinations of relatively non-selective kinase inhibitors immobilized on agarose beads to isolate kinases from cell and tissue lysates. The other method relies on soluble ATP-based covalent probes that transfer a desthiobiotin group to conserved lysine residues in the kinase ATP site. Both of these methods are sensitive to competition by ATP-site directed kinase inhibitors, and thus precise target binding potency can be determined. These *in situ* kinase profiling methodologies have been shown to provide more relevant inhibitor potency information than recombinant kinase binding/activity assays, and enable determination of selectivity across the specific subset of kinases present in cells or tissues where biological outcomes can be measured. Further, the ability of these methods to precisely measure target and off-target engagement levels from compound-treated cells or tissues effectively bridges the gap between *in vitro* profiles and *in vivo* outcomes, greatly improving critical development decisions.

This lecture will focus on the application of these proteomics-based kinase profiling methodologies to key steps in the kinase inhibitor development process.

References:

1. Anastassiadis T, Deacon SW, Devarajan K, Ma H, Peterson JR (2011) Comprehensive assay of kinase catalytic activity reveals features of kinase inhibitor selectivity. *Nat Biotechnol* **29**: 1039–1045.
2. Davis MI, Hunt JP, Herrgard S, Ciceri P, Wodicka LM, Pallares G, Hocker M, Treiber DK, Zarrinkar PP (2011) Comprehensive analysis of kinase inhibitor selectivity. *Nat Biotechnol* **29**: 1046–1051.
3. Zarrinkar PP, Gunawardane RN, Cramer MD, Gardner MF, Brigham D, Belli B, Karaman MW, Pratz KW, Pallares G, Chao Q, Sprankle KG, Patel HK, Levis M, Armstrong RC, James J, Bhagwat SS (2009) AC220 is a uniquely potent and selective inhibitor of FLT3 for the treatment of acute myeloid leukemia (AML). *Blood* **114**: 2984–2992.
4. Bantscheff M, Eberhard D, Abraham Y, Bastuck S, Boesche M, Hobson S, Mathieson T, Perrin J, Raida M, Rau C, Reader V, Sweetman G, Bauer A, Bouwmeester T, Hopf C, Kruse U, Neubauer G, Ramsden N, Rick J, Kuster B, Drewes G (2007) Quantitative chemical proteomics reveals mechanisms of action of clinical ABL kinase inhibitors. *Nat Biotechnol* **25**:1035–1044.
5. Sharma K, Weber C, Bairlein M, Greff Z, Kéri G, Cox J, Olsen JV, Daub H (2009) Proteomics strategy for quantitative protein interaction profiling in cell extracts. *Nat Methods* **6**: 741–744.
6. Patricelli MP, Nomanbhoy TK, Wu J, Brown H, Zhou D, Zhang J, Jagannathan S, Aban A, Okerberg E, Herring C, Nordin B, Weissig H, Yang Q, Lee JD, Gray NS, Kozarich JW (2011) *In situ* kinase profiling reveals functionally relevant properties of native kinases. *Chem Biol* **18**: 699–710.

L20

Intersection of selenoproteins and kinase signalling: a novel protein kinase-like domain in a selenoprotein, widespread in the tree of life

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Selenoproteins are an intriguing evolutionary creation, characterised by the presence of a rare atypical amino acid residue, selenocysteine. Apparently, the benefits of selenocysteine, instead of cysteine, in some enzymatic active sites can outweigh the costs of maintaining cellular infrastructure for selenocysteine synthesis and incorporation. Selenoproteins serve important functions in the cells of many organisms, usually providing essential oxidoreductase enzymatic activity, often for defense against toxic xenobiotic substances. Most eukaryotic genomes possess a small number of these proteins. Human selenoproteins are encoded by 25 genes, and most of those with known functions are oxidoreductases with a selenocysteine located in the active site. A few human selenogenes are functionally uncharacterized [1].

Selenoprotein O, SELO, has been listed among the top ten most-wanted "unknown unknowns", i.e. proteins of unknown structure and function posing exciting conceptual challenges for structure predictors, and functionally interesting, as judged by phyletic spread [2]. We have recently proven, using bioinformatics tools, that the SELO protein adopts a three-dimensional fold similar to protein kinases [3]. Furthermore, we argue that despite the lack of conservation of the "classic" catalytic aspartate residue of the archetypical H/Y-R-D motif, SELO kinases might have retained catalytic phosphotransferase activity, albeit with an atypical active site. The selenocysteine residue, located at the C-terminus, outside the predicted kinase-like domain provokes speculation of an oxidoreductase-regulated kinase function for SELO proteins. We also found a very broad phylogenetic spread of the SELO homologues, including almost all eukaryotic phyla, at least half of the bacterial phyla, and a few archaeal ones. The lifestyles of SELO hosts and functional data for SELO homologues suggest a role in reaction to oxidative stress.

Does the kinase prediction for SELO close the case for novel kinase discovery? Are all the kinases known now? The question cannot be convincingly answered, however, recent experience teaches us that there may still be unknown novel kinases remaining. Only recently, several novel kinase families with members in humans and possible medical relevance have been reported: PKDCC [4], FAM20 [5] and FAM69 [6].

Recently, Edwards convincingly presented the case for exploring the "unpopular" members of otherwise popular protein families [7]. The authors pointed out that even for the established and attractive drug targets, kinases, majority of scientific activity, focuses on a small minority of proteins that have been historically popular. The "uncharacterized" proteins are even less fortunate, often getting little attention even if the experimental data point at their involvement in interesting biological or disease processes [8]. Importantly, many kinase inhibitors targeted at well-known kinases may have off-target effects onto the less-studied ones [9]. It has been reported that some kinases affected by off-target effects of a compound have less than 20% sequence identity in their active sites as compared to the original target kinase. Thus, complete understanding of the kinome is important for proper assessment of the potential off-target effects of kinase-modulating compounds.

In this lecture, we will review the functional landscape of selenoproteins, including disease links, and present a surprising prediction of a novel protein kinase-like domain in a ubiquitous selenoprotein. I will discuss also other cases of recently discovered novel kinase families. In the end, we will attempt to summarize the reasons for failure to discover the novel kinase families earlier and will ask the question as to the completeness of the currently established human kinome.

References:

1. Lobanov AV, Hatfield DL, Gladyshev VN (2009) Eukaryotic selenoproteins and selenoproteomes. *Biochim Biophys Acta* **1790**: 1424–1428.
2. Galperin MY, Koonin EV (2004) ‚Conserved hypothetical' proteins: prioritization of targets for experimental study. *Nucleic Acids Res* **32**: 5452–5463.
3. Dudkiewicz M, Szczepinska T, Grynberg M, Pawlowski K (2012) A novel protein kinase-like domain in a selenoprotein, widespread in the tree of life. *PLoS One* **7**: e32138.
4. Imuta Y, Nishioka N, Kiyonari H, Sasaki H (2009) Short limbs, cleft palate, and delayed formation of flat proliferative chondrocytes in mice with targeted disruption of a putative protein kinase gene, Pkdcc (AW548124). *Dev Dyn* **238**: 210–222.
5. Tagliabracci VS, Engel JL, Wen J, Wiley SE, Worby CA, Kinch LN, Xiao J, Grishin NV, Dixon JE (2012) Secreted kinase phosphorylates extracellular proteins that regulate biomineralization. *Science* **336**: 1150–1153.
6. Dudkiewicz M, Lenart A, Pawlowski K (2012) A novel predicted calcium-regulated kinase family implicated in neurological disorders (submitted).
7. Edwards AM, Isserlin R, Bader GD, Frye SV, Willson TM, Yu FH (2011) Too many roads not taken. *Nature* **470**: 163–165.
8. Pawlowski K (2008) Uncharacterized/hypothetical proteins in biomedical ‚omics' experiments: is novelty being swept under the carpet? *Briefings in Functional Genomics & Proteomics* **7**: 283–290.
9. Fedorov O, Muller S, Knapp S (2010) The (un)targeted cancer kinome. *Nat Chem Biol* **6**: 166–169.

L21

Exploiting the repertoire of CK2 inhibitors to target other druggable kinases

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CK2 (an acronym derived from the misnomer "casein kinase-2") is probably the most pleiotropic member of the kinome, with hundreds *bona fide* substrates already identified and *in silico* analysis suggesting that it may be responsible alone for the generation of a substantial proportion of the human phospho-proteome [1]. Such an extraordinary pleiotropy is likely to account for the observation that, at variance with the great majority of protein kinases, CK2 is constitutively active.
Although no genetic alteration is known conferring to it an oncogenic potential, CK2 level is invariably elevated in malignant cells where it generates an environment favourable to the enhancement of the tumour phenotype through a mechanism known as "non oncogene addiction" [2].

Given this scenario it is not surprising that considerable efforts have been done to develop specific and potent cell permeable CK2 inhibitors. The first compound with these properties, DRB, was described by David Shugar in 1986 [3] and it is still employed as CK2 inhibitor. Subsequently however a large repertoire of DRB derivatives was generated by modifications of its scaffold, many of which, notably TBI, TBB and DMAT [4–6], inhibit CK2 more potently than DRB, with IC_{50} values in the sub-micromolar range. These compounds as well as others derived from different scaffolds were originally believed to be quite selective until, by profiling them on large panels of 50–125 protein kinases it turned out that they tend to inhibit a limited number of other kinases, notably DYRKs, PIMs and HIPKs, as drastically as CK2 [7]. This outcome fostered investigation aimed at improving the potency and selectivity of CK2 inhibitors, ending up with a new generation of compounds endowed with unprecedented selectivity [8], one of which, CX-4945, is now in clinical trials for the treatment of cancer.

On the other hand, the relative promiscuity of the commonest CK2 inhibitors provided an opportunity for developing from their scaffolds derivatives able to specifically inhibit those kinases that share with CK2 susceptibility to the parent compounds. This strategy has been successfully exploited to obtain dNBC, a selective inhibitor of DYRK1A [9]. By a similar approach, grounded on the rational modification of CK2 inhibitors we have recently obtained compounds capable to down-regulate *in vitro* and in cells HIPK2, a kinase controlling survival in response to external stimuli, for which specific inhibitors were not available to date. Likewise a potent (IC_{50} < 0.1 μM) and cell permeable dual inhibitor of CK2 and PIM1, capable to induce apoptosis of tumour cells more effectively than CX-4945 itself, has been recently obtained by altering the original scaffold of DRB.

The presentation will describe the properties of these compounds and discuss their potential to pave the road toward the development of new therapeutic tools.

Notes:

This work was performed at the University of Padua, Department of Biomedical Sciences, Padova, Italy (Pinna lab) with major contributions by Giorgio Cozza, Maria Ruzzene, Stefania Sarno, and with the external collaboration of Zygmunt Kazimierczuk (Institute of Chemistry, Warsaw University of Life Sciences, Poland), Conrad Kunick (Institut für Pharmazeutische Chemie, Technische Universitat Braunshweig, Germany), Roberto Battistutta (Venetian Institute of Molecular Medicine, Padova, Italy) and the teams of the Protein Production of the Division of Signal Transduction Therapy and the International Centre for Kinase Profiling, MRC Protein Phosphorylation Unit, University of Dundee, Scotland, UK.

References:

1. Salvi M, Sarno S, Cesaro L, Nakamura H, Pinna LA (2009) Extraordinary pleiotropy of protein kinase CK2 revealed by weblogo phosphoproteome analysis. *Biochim Biophys Acta* **1793**: 847–859.
2. Ruzzene M, Pinna LA (2010) Addiction to protein kinase CK2: a common denominator of diverse cancer cells? *Biochim Biophys Acta* **1804**: 499–504.
3. Zandomeni R, Zandomeni MC, Shugar D, Weinmann R (1986) Casein kinase type II is involved in the inhibition by 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole of specific RNA polymerase II transcription. *J Biol Chem* **261**: 3414–3419.
4. Szyszka R, Grankowski N, Felczak K, Shugar D (1995) Halogenated benzimidazoles and benzotriazoles as selective inhibitors of protein kinases CK I and CK II from *Saccharomyces cerevisiae* and other sources. *Biochem Biophys Res Commun* **208**: 418–424.
5. Sarno S, Reddy H, Meggio F, Ruzzene M, Davies SP, Donella-Deana A, Shugar D, Pinna LA (2001) Selectivity of 4,5,6,7-tetrabromobenzotriazole, an ATP site-directed inhibitor of protein kinase CK2 (‚casein kinase-2'). *FEBS Lett* **496**: 44–48.
6. Pagano MA, Meggio F, Ruzzene M, Andrzejewska M, Kazimierczuk Z, Pinna LA (2004) 2-Dimethylamino--4,5,6,7-tetrabromo-1*H*-benzimidazole: a novel powerful and selective inhibitor of protein kinase CK2. *Biochem Biophys Res Commun* **321**: 1040–1044.
7. Pagano MA, Bain J, Kazimierczuk Z, Sarno S, Ruzzene M, Di Maira G, Elliott M, Orzeszko A, Cozza G, Meggio F, Pinna LA (2008) The selectivity of inhibitors of protein kinase CK2: an update. *Biochem J* **415**: 353–365.
8. Battistutta R, Cozza G, Pierre F, Papinutto E, Lolli G, Sarno S, O'Brien SE, Siddiqui-Jain A, Haddach M, Anderes K, Ryckman DM, Meggio F, Pinna LA (2011) Unprecedented selectivity and structural determinants of a new class of protein kinase CK2 inhibitors in clinical trials for the treatment of cancer. *Biochemistry* **50**: 8478–8488.
9. Sarno S, Mazzorana M, Traynor R, Ruzzene M, Cozza G, Pagano MA, Meggio F, Zagotto G, Battistutta R, Pinna LA (2012) Structural features underlying the selectivity of the kinase inhibitors NBC and dNBC: role of a nitro group that discriminates between CK2 and DYRK1A. *Cell Mol Life Sci* **69**: 449–460.

L22

Halogen bonding in protein kinase/ligand complexes vs a balance of hydrophobic and electrostatic interactions drives binding of majority of ligands

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Approximately 20% of low-mass protein ligands listed in the PDB are halogenated, and their possible involvement in halogen bonding extensively reviewed [1, 2], but the thermodynamics of these interactions remains debatable [3, 4]. Inspection of over 1500 structures of protein kinase/ligand complexes, deposited in the PDB, revealed that almost 500 display at least one short contact (i.e. < 4 Å) between the halogen atom of a ligand and a potential halogen bond acceptor. According to the Anderson-Darling test, the distribution of distances between a halogen atom and the nearest potential acceptor of a halogen bond is Gaussian, when determined separately for chlorine, bromine and iodine of a ligand. For Cl and Br these distributions are perturbed, displaying visible shoulders centered below 3.0 Å. The distribution for iodine (μ = 3.21 Å, σ = 0.13 Å, ~ 70% of 32 structures) and additional distributions estimated for Cl (μ = 2.88 Å, σ = 0.16 Å, 10% of 389 contacts) and Br (μ = 2.94 Å, σ = 0.09 Å, 20% of 144 contacts), are valuable descriptors of halogen bonding in protein systems. An isolated water molecule and a π-electron system are also efficient halogen bond acceptors. The majority of halogen atoms are protected from accessibility to solvent, underlining the contribution of hydrophobic interactions to binding of halogenated ligands.

The first reported low-mass inhibitors of protein kinase CK2 are multihalogenated compounds, 4,5,6,7-tetrabromobenzotriazole (TBBt) and 4,5,6,7-tetrabromobenzimidazole (TBBz), with K_i values in the low μM and sub-μM range, with a reasonable degree of selectivity when tested against 60 other kinases. This selectivity may result from different modes of ligand binding, as shown for TBBt in complexes with CdK2 and CK2α [5].

Replacement of one of the halogen atom in TBBt, that at C(5)/C(6), by six neutral substituents differing in size, electronegativity and polarity [6] did not significantly affect inhibitory activity.

To further delineate the role of each Br atom of TBBt on inhibitory activity, we have synthesized, and determined the physico-chemical properties, of all possible two mono-, four di-, and two tri-bromobenzotriazoles [7]. Their solution properties varied substantially, the solubility decreasing with an increase in the number of bromines on the benzene ring, and modulated by the pattern of substitution.

Inhibition (IC_{50}) of human CK2α by the foregoing showed that halogenation of the vicinal C(5)/C(6) atoms is a key factor in inhibitory activity, e.g. 5,6-Br_2Bt and 4,5,6-Br_3Bt were almost as effective as TBBt, notwithstanding marked differences in solubility and pK_a for dissociation of the triazole proton.

Overall, inhibitory activity of halogenated benzotriazoles, in terms of ligand physicochemical properties, and modeled structures of complexes, results from a balance of electrostatic and hydrophobic interactions. The role of halogen bonding remains debatable, as noted for the crystal structure of TBBt with CK2α [8].

A general conclusion, based on the observed dominant effect of permutation of bromination sites, is that partial dehalogenation of known multi-halogenated inhibitors may result in significant enhancement of inhibitory activity. Finally, attention is directed to the promising applicability of our series of well-defined halogenated benzotriazoles to studies on inhibition of kinases other than CK2.

References:

1. Voth AR, Ho PS (2007) The role of halogen bonding in inhibitor recognition and binding by protein kinases. *Curr Top Med Chem* **7**: 1336–1348.
2. Parisini E, Metrangolo P, Pilati T, Resnati G, Terraneo G (2011) Halogen bonding in halocarbon-protein complexes: a structural survey. *Chem Soc Rev* **40**: 2267–2278.
3. Kraut DA, Sigala PA, Fenn TD, Herschlag D (2010) Dissecting the paradoxical effects of hydrogen bond mutations in the ketosteroid isomerase oxyanion hole. *Proc Natl Acad Sci USA* **107**: 1960–1965.
4. Hardegger LA, Kuhn B, Spinnler B, Anselm L, Ecabert R, Stihle M, Gsell B, Thoma R, Diez J, Benz J, Plancher JM, Hartmann G, Isshiki Y, Morikami K, Shimma N, Haap W, Banner DW, Diederich F (2011) Halogen bonding at the active sites of human cathepsin L and MEK1 kinase: efficient interactions in different environments. *Chem Med Chem* **6**: 2048–2054.
5. De Moliner E, Brown NR, Johnson LN (2003) Alternative binding modes of an inhibitor to two different kinases. *Eur J Biochem* **270**: 3174–3181.
6. Wąsik R, Łebska M, Felczak K, Poznański J, Shugar D (2010) Relative role of halogen bonds and hydrophobic interactions in inhibition of human protein kinase CK2α by tetrabromobenzotriazole and some C(5)-substituted analogues. *J Phys Chem B* **114**: 10601–10611.
7. Wąsik R, Wińska P, Poznański J, Shugar D (2012) Synthesis and physico-chemical properties in aqueous medium of all possible isomeric bromo analogues of benzo-1*H*-triazole, potential inhibitors of protein kinases. *J Phys Chem B* **116**: 7259–7268.
8. Battistutta R, De Moliner E, Sarno S, Zanotti G, Pinna LA (2001) Structural features underlying selective inhibition of protein kinase CK2 by ATP site-directed tetrabromo-2-benzotriazole. *Protein Sci* **10**: 2200–2206.

L23

Kinase inhibitor profiling in complex environments: the virtues and vices of quantitative readouts from cell-based extracts

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Protein kinases orchestrate virtually ever aspect of cellular life and death [1]. From cell–cell communication, cell growth and differentiation, to sensing of metabolic states, mediating intracellular transport and initiating apoptosis. Conversely, aberrant kinase activites and pathological alterations in the establishments of signaling mediated phosphorylation marks, whether due to hereditary changes or environmental cues [2], have been implicated in a great number human diseases, most prominently in many forms of cancer [3]. Thus, the study of cellular kinase activities has profound medical implications, both in the preventive and curative sense [4].

Despite the fact that protein kinases constitute the second largest group of drug targets to date [5], the overall efficacies of most kinase inhibitors are setback by poor discriminative behaviors and low kinase specificities. While kinase cross-inhibition can easily be tested under defined experimental conditions *in vitro*, it is more difficult to assess in complex cellular environments that contain large numbers of active, but mostly unknown kinases, as well as numerous regulatory networks of counteracting phosphatase activities. At the same time, important pharmacological parameters of kinase inhibitors, such as IC_{50} values for example, are often determined in cell extracts, or tissue homogenates that contain at least one known kinase activity, typically the one against which the kinase inhibitor has been raised. Quantitative profiling, by different methodological means, is then employed to 'measure' individual degrees of kinase inhibition.

In this lecture, we will focus on one particular aspect of kinase inhibitor profiling using cell-based assays: the apparent range of 'measured' modulations in cellular kinase activities and inhibition efficiencies that different cell-extract preparation protocols artificially introduce. To this end, we introduce a novel tool for direct kinase activity profiling in complex cellular environments: quantitative, time-resolved *in situ* NMR spectroscopy.

References:

1. Taylor SS, Kornev AP (2011) Protein kinases: evolution of dynamic regulatory proteins. *Trends Biochem Sci* **36**: 65–67.
2. Lahiry P, Torkamani A, Schork NJ, Hegele RA (2010) Kinase mutations in human disease: interpreting genotype-phenotype relationships. *Nat Rev Genet* **11**: 60–74.
3. Sellers WR (2011) A blueprint for advancing genetics-based cancer therapy. *Cell* **147**: 26–31.
4. Sawyers C (2004) Targeted cancer therapy *Nature* **432**: 294–297.
5. Zhang J, Yang PL, Gray NS (2009) Targeting cancer with small molecule kinase inhibitors. *Nat Rev Cancer* **9**: 28–39.

L24

Adding a new dimension to kinase activity and inhibitor profiling in translational medicine

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Traditional measurements of kinase activities in cell or tumor lysates are performed in most cases by Western-blotting and immuno-precipitation. These classical methods are focused and biased, because they are restricted by the choice of antibodies for detection of phosphorylation endpoints. Moreover they are time consuming, and lack throughput the ability to analyze large numbers of phosphorylation events.

This talk presents a novel multiplex kinase assay on a 3-dimensional flow-through microarray platform, where kinases in complex samples, as well as potential inhibitors, are tested against a panel of peptides, representing known kinase substrates. This multiplex assay is performed on a fully automated high-throughput 96-well microarray system, which generates thousands of kinetic read-out curves in less than 1 hour, allowing simultaneous measurements of kinase inhibitor potency/IC_{50} selectivity and mechanism of action assessments [1–4].

Drug effect profiles are obtained *via* pharmacology-on-the-chip, i.e. combined analysis of the kinase inhibitor drug and the lysate from the patient-derived tumor tissue. This generates drug-specific information from naive patient material. Discovery of drug-response prediction biomarkers at these pharmacological and enzymatic levels, are very different from other strategies where DNA mutations/amplification, RNA or protein levels/modifications are the source of investigation.

This new biomarker discovery platform is presented using examples including clinical response prediction in Tarceva-treated lung cancer patients [5], and in another trial, the prediction of response of rectal cancer patients to neoadjuvant chemoradiation therapy [6].

References:

1. Ter Elst A, Diks SH, Kampen KR, Hoogerbrugge P, Ruijtenbeek R, Boender PJ, Sikkema AH, Scherpen FJ, Kamps WA, Peppelenbosch MP, De Bont ES (2011) Identification of new possible targets for leukemia treatment by kinase activity profiling. *Leuk Lymphoma* **52**: 122–130.

2. Sanz AS, Ungureanu D, Hilhorst R, Touw I, Ruijtenbeek R, Silvennoinen O (2011) Analysis of Jak2 catalytic function by peptide microarrays: the role of the JH2 domain and V617F mutation. *PLoS One* **6**: e18522.

3. Tournoij E, Koekman CA, Du VX, Roest M, Ruijtenbeek R, Moll FL, Akkerman JW (2012) The platelet P2Y(12) receptor contributes to granule secretion through Ephrin A4 receptor. *Platelets* Jan 24 [Epub ahead of print].

4. Jarboe JS, Jaboin JJ, Anderson JC, Nowsheen S, Stanley JA, Naji F, Ruijtenbeek R, Tu T, Hallahan DE, Yang ES, Bonner JA, Willey CD (2012) Kinomic profiling approach identifies Trk as a novel radiation modulator. *Radiother Oncol* **103**: 380–387.

5. Ruijtenbeek H, *et al.* (2011) Blind prediction of response to erlotinib in early-stage non-small cell lung cancer (NSCLC) in a neoadjuvant setting based on kinase activity profiles. American Society for Clinical Oncology (ASCO) Annual Meeting, Chicago, USA, 2011. *J Clin Oncol* **29**: (Suppl. Abstract 10521).

6. Folkvord S, Flatmark K, Dueland S, de Wijn R, Kotanska Grøholt K, Nesland JM, Hole KH, Boender PJ, Ruijtenbeek R, Johansen M, Giercksky K-E, Hansen Ree A (2010) Prediction of response to preoperative chemoradiotherapy in rectal cancer by multiplex kinase activity profiling. *Int J Radiat Oncol Biol Phys* **78**: 555–562.

L25

Cell signalling in space and time

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Intracellular signal transduction events are precisely regulated in space and time. This is achieved in part by A-Kinase Anchoring Proteins (AKAPs) that tether signaling enzymes such as protein kinases and phosphatases in proximity to select substrates. AKAP targeting provides an efficient means to reversibly control the phosphorylation status of key substrates and contributes to the dynamic regulation of sophisticated cellular events. Using a variety of genetic, electrophysiological and live-cell imaging techniques we show that AKAPs, which enhance the precision of signaling events, are up-regulated under certain pathophysiological states. This leads to aberrant regulation of certain physiological processes and disorders such as diabetes and heart disease.

L26

Identification of a family of secreted protein kinases

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In 1883, Olof Hammarsten discovered that casein, an abundant milk protein secreted by the lactating mammary gland, contains stoichiometric amounts of phosphate [1]. In hindsight, this was the first indication for the existence of protein kinases, discovered and named almost one-century later using casein as a substrate [2]. By inspecting the sequences of casein fractions, it was predicted that the kinase responsible for this phosphorylation specifically recognized the consensus S-x-E/pS [3]. Furthermore, this motif is phosphorylated in some 75% of human plasma and cerebrospinal fluid phosphoproteins, suggesting that the kinase was not specific for casein but rather a ubiquitous and pleiotropic enzyme dedicated to the phosphorylation of several secreted proteins [4–7]. Historically, casein has been used as a model substrate for the detection of several protein kinases, including casein kinase-1 and casein kinase-2. However, these enzymes are physiologically unrelated to casein as they are mainly cytosolic and nuclear proteins that would be unlikely to encounter casein in the secretory pathway and have therefore been renamed protein kinase CK1 and protein kinase CK2 [8]. Given the history of casein in protein phosphorylation research, it is somewhat of a paradox that the *bona fide* casein kinase has yet to be molecularly identified. Nevertheless, a casein kinase activity from highly enriched Golgi fractions of the lactating mammary gland has been thoroughly characterized at the biochemical level, and termed Golgi casein kinase (GCK). GCK specifically recognizes the consensus S-x-E/pS, prefers Mn^{2+} over Mg^{2+}, and is extremely insensitive to staurosporine [9, 10]. Additionally, GCK activity has been detected in milk, suggesting that the kinase is a secreted protein [11].

In this lecture, we describe a family of atypical protein kinases that localize within the Golgi apparatus and are secreted. Fam20C appears to be the GCK responsible for phosphorylating secretory pathway proteins within S-x-E/pS motifs. Fam20C phosphorylates the caseins and several secreted proteins implicated in biomineralization, including the small integrin-binding ligand, N-linked glycoproteins (SIBLINGs). Consequently, mutations in Fam20C cause a devastating osteosclerotic bone dysplasia in humans known as Raine syndrome [12]. Our results establish the molecular identity of the first mammalian kinase dedicated to the phosphorylation of extracellular proteins.

References:

1. Hammarsten O (1883) Zür Frage ob Caseín ein einheitlicher Stoff sei. *Z Physiol Chem* **7**: 227–273.
2. Burnett G, Kennedy EP (1954) The enzymatic phosphorylation of proteins. *J Biol Chem* **211**: 969–980.
3. Mercier JC (1981) Phosphorylation of caseins, present evidence for an amino acid triplet code posttranslationally recognized by specific kinases. *Biochimie* **63**: 1–17.
4. Carrascal M, Gay M, Ovelleiro D, Casas V, Gelpi E, Abian J (2010) Characterization of the human plasma phosphoproteome using linear ion trap mass spectrometry and multiple search engines. *J Proteome Res* **9**: 876–884.
5. Zhou W, Ross MM, Tessitore A, Ornstein D, Vanmeter A, Liotta LA, Petricoin EF (2009) An initial characterization of the serum phosphoproteome. *J Proteome Res* **8**: 5523–5531.
6. Bahl JM, Jensen SS, Larsen MR, Heegaard NH (2008) Characterization of the human cerebrospinal fluid phosphoproteome by titanium dioxide affinity chromatography and mass spectrometry. *Anal Chem* **80**: 6308–6316.
7. Salvi M, Cesaro L, Tibaldi E, Pinna LA (2010) Motif analysis of phosphosites discloses a potential prominent role of the Golgi casein kinase (GCK) in the generation of human plasma phospho-proteome. *J Proteome Res* **9**: 3335–3338.
8. Allende JE, Allende CC (1995) Protein kinases 4. Protein kinase CK2: an enzyme with multiple substrates and a puzzling regulation. *FASEB J* **9**: 313–323.
9. Lasa-Benito M, Marin O, Meggio F, Pinna LA (1996) Golgi apparatus mammary gland casein kinase: monitoring by a specific peptide substrate and definition of specificity determinants. *FEBS Lett* **382**: 149–152.
10. Lasa M, Marin O, Pinna LA (1997) Rat liver Golgi apparatus contains a protein kinase similar to the casein kinase of lactating mammary gland. *Eur J Biochem* **243**: 719–725.
11. Duncan JS, Wilkinson MC, Burgoyne RD (2000) Purification of Golgi casein kinase from bovine milk. *Biochem J* **350**: 463–468.
12. Simpson MA, Hsu R, Keir LS, Hao J, Sivapalan G, Ernst LM, Zackai EH, Al-Gazali LI, Hulskamp G, Kingston HM, Prescott TE, Ion A, Patton MA, Murday V, George A, Crosby AH (2007) Mutations in FAM20C are associated with lethal osteosclerotic bone dysplasia (Raine syndrome), highlighting a crucial molecule in bone development. *Am J Hum Genet* **81**: 906–912.

L27

PKA: assembly of dynamic and isoform-specific macromolecular assemblies

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cAMP-dependent protein kinase (PKA), ubiquitous in every mammalian cell, regulates a plethora of biological processes. While the PKA catalytic (C) subunit is the best understood protein kinase and serves in many ways as a prototype for the entire superfamily, the C-subunit in cells is packaged as holoenzyme with regulatory (R) subunits so that the activity of PKA is fully dependent on the second messenger, cAMP. There are four functionally non-redundant R-subunits (RIa, RIb, RIIa, and RIIb) and all have a conserved domain organization with a dimerization/docking (D/D) domain at the N-terminus followed by a flexible linker that contains an inhibitor site and finally at the C-terminus two tandem cyclic nucleotide binding (CNB) domains. Specificity in PKA signaling is determined by the isoform diversity of the R-subunits and by targeting, which is typically mediated by A Kinase Anchoring Proteins (AKAPs), which bind through an amphipathic helix to the D/D domains of the R-subunits. In this way specific PKA holoenzymes are localized in close proximity to dedicated substrates. The functional non-redundancy of the R-subunits was not understood in molecular terms until structures of tetrameric holoenzymes were solved. Although the 1°, 2°, and 3° structures of all four isoforms are very similar, the quaternary structures of each R_2C_2 tetramer are remarkably different and define distinct mechanisms for allosteric regulation.

In contrast to metabolic enzymes, which have evolved to be efficient catalysts, protein kinases have evolved to be molecular switches, similar to the G proteins. The protein kinases are thus highly dynamic and use a variety of mechanisms to regulate the active enzyme. In each case it is the linkers, loops, and inserts that mediate this dynamic regulation. Phosphorylation of the activation loop, for example, is a common mechanism for activation, and we show how addition of a single phosphate can significantly enhance both stability and kinetics of the PKA C-subunit. In the absence of activation loop phosphorylation, the activation loop is highly disordered and the rapid pre-steady state burst of activity is completely abolished. The C-terminal and N-terminal tails of the C-subunit also contribute to the dynamic regulation of the C-subunit and of each holoenzyme. In the R-subunits it is the highly dynamic linker region that orchestrates the novel assembly of each holoenzyme complex. This diversity of the R-subunit isoforms and the novel mechanisms for allosteric regulation of each kinase are only defined in the structures of the full length holoenzymes.

References:

1. Taylor SS, Kornev AP (2011) Protein kinases: evolution of dynamic regulatory proteins. *Trends Biochem Sci Rev* **36**: 65–77.
2. Darshi M, Macke MR, Murphy AN, Koller A, Perkins GA, Ellisman MH, Taylor SS (2010) ChChD3, an inner mitochondrial membrane protein, is essential for maintaining cristae integrity and mitochondrial function. *J Biol Chem* **28**: 2918–2932. PMCID: PMC3024787.
3. Zhang P, Smith-Nguyen EV, Keshwani MM, Deal MS, Kornev AP, Taylor SS (2012) Structure and allostery of the PKA RIIβ tetrameric holoenzyme. *Science* **335**:712–716. PMID: 22323819.
4. Ilouz R, *et al.* (2012) Localization and quaternary structure of PKA RIb holoenzyme. *Proc Natl Acad Sci USA* **109**: 12443–12448.

L28

Navigating the Kinome — past, present and future

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The importance of selectivity for kinase-targeted drugs continues to be debated in the medicinal chemistry community. Some marketed drugs inhibit several kinases (e.g. sunitinib), while others are highly selective (e.g. gefitinib). The understanding and practical application of design principles which lead to selective kinase inhibitors are of high interest. Recently Davis [1], Anastassiadis [2] and Metz [3] published data sets of 70–3800 kinase drugs and inhibitors across 172 to 379 kinase targets, providing insights on pharmacological relationships between kinases and factors that confer promiscuity. In addition, we have characterized *via* dose-response curves about 4100 inhibitors against 105 kinases. We have investigated the concordance between these four different data sets from several perspectives and found modest agreement. For three sets (Davis, Metz and Lilly) where IC_{50}s or K_i are measured, more than 50% of activity results for the same compound and kinase differed by ≥3 fold between sources; Pearson R^2 values comparing non-qualified IC_{50}s or K_is (i.e. no qualifier '<' or '>') across sources range from 0.22 to 0.43. We also found kinase promiscuity (i.e. the frequency at which a kinase is inhibited) and pharmacological relationships between kinases (i.e. the propensity for two kinases to be similarly inhibited by compounds) derived from these different sources have relatively low level of concordance as judged by multiple statistical comparisons. When quantifying pharmacological similarity of two kinases *via* the Pearson correlation coefficient of pIC_{50}s for compounds tested in common, only 30%–51% of the variation from one source is explained by other sources, which is further reduced to 23%–39% when different compound sets are used. This indicates that pharmacological similarity is both assay format- and compound-dependent; the latter implies that even when using the same assays, trends observed in one compound collection have limited utility for others. What we found to be consistent across data sources is the overall promiscuity assessment of compounds (e.g. the percentage of kinases with IC_{50} < 100 nM). Unlike Davis [1], we find increased promiscuity for type II *vs.* type I inhibitors based on our data. We have also used the Lilly data set to access our computational technology's [4] in ability to predict selectivity of compounds and kinase space relationship. Using multiple approaches, we are able to correctly predict selectivity profiles (within 3 fold from experimental results) for 20% of compounds. Initial attempts to include compound docking based descriptors did not improve the results.

References:

1. Davis MI, Hunt JP, Herrgard S, Ciceri P, Wodicka LM, Pallares G, Hocker M, Treiber DK, Zarrinkar PP (2011) Comprehensive analysis of kinase inhibitor selectivity. *Nat Biotechnol* **29**: 1046–1051.
2. Anastassiadis T, Deacon SW, Devarajan K, Ma H, Peterson JR (2011) Comprehensive assay of kinase catalytic activity reveals features of kinase inhibitor selectivity. *Nat Biotechnol* **29**: 1039–1046.
3. Metz JT, Johnson EF, Soni NB, Merta PJ, Kifle L, Hajduk PJ (2011) Navigating the kinome. *Nat Chem Biol* **7**: 200–202.
4. Vieth M, Erickson J, Wang J, Webster Y, Mader M, Higgs R, Watson I (2009) Kinase inhibitor data modeling and de novo inhibitor design with fragment approaches. *J Med Chem* **52**: 6456–6466.

L29

A kinase-phosphatase battle in the control of meiotic chromosome segregation

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Chromosome segregation during cell division depends on the attachment of microtubules to centromeric DNA through large protein complexes called kinetochores. Kinetochore-microtubule interactions are stabilised by tension, which arises when microtubules from opposite spindle poles attach to a pair of physically connected kinetochores. During mitosis, kinetochores on sister chromatids attach to microtubules from opposite spindle poles (bipolar attachment). Sister kinetochores become linked and can develop tension because ring-shaped cohesin complexes entrap both sister chromatids during DNA replication [1]. At entry into anaphase, a protease called separase destroys cohesin along the entire length of chromosomes by cleaving its Scc1/Rad21 subunit. This enables the spindle to pull sister chromatids to opposite spindle poles, resulting in the generation of two genetically identical daughter nuclei. Separase is activated upon degradation of its inhibitor Pds1/securin, which results from ubiquitinylation by the anaphase-promoting complex (APC/C).

Meiosis generates haploid gametes from diploid germ cells. Thus, sister chromatid cohesion established during premeiotic DNA replication mediates first the segregation of homologous chromosomes (homologs) in meiosis I and subsequently the disjunction of sister chromatids in meiosis II. This depends on four meiosis-specific processes [2]: first, after premeiotic *S* phase, homologs undergo reciprocal recombination between non-sister chromatids, which generates crossovers that link homologs through sister chromatid cohesion on chromosome arms. This requires not only recombination enzymes but also cohesin containing the meiosis-specific Rec8 subunit instead of its mitotic counterpart Scc1/Rad21 [3]. Second, sister kinetochores adopt a conformation that enforces their attachment to microtubules from the same spindle pole (mono-polar attachment) [4, 5]. Since kinetochores on maternal and paternal centromeres are connected *via* crossovers and arm cohesion, microtubules pulling them in opposite directions now generate tension. Third, disjunction of homologs is triggered when separase resolves crossovers by cleaving cohesin's Rec8 subunit on chromosome arms [6]. Centromeric cohesin, however, is protected from separase during meiosis I by a mechanism that requires Rec8 and the kinetochore-associated shugoshin protein [7]. Fourth, cells do not re-replicate their DNA after meiosis I but progress into meiosis II where centromeric cohesion serves to bi-orient sister chromatids [4, 5]. A second wave of separase activity then disjoins sister centromeres leading to the formation of haploid gametes.

Although the stepwise loss of cohesion has long been recognized as a fundamental aspect of meiosis, the underlying mechanisms are only beginning to emerge. The finding that shugoshin protects centromeric Rec8 by recruiting the PP2A phosphatase to kinetochores implied that phosphorylation of some protein is necessary of Rec8 cleavage [8, 9]. Candidates for the PP2A substrate included separase and Rec8 itself. Equally important, the kinases counteracting PP2A in cohesin cleavage remained to be identified. To address these issues, we have identified and subsequently mutated phosphorylation sites in Rec8 from budding yeast. We have shown that multiple phosphorylation sites as well as two evolutionarily conserved kinases, casein kinase 1δ/ε (CK1δ/ε, Hrr25 in yeast) and Dbf4-dependent Cdc7 kinase (DDK), are required for Rec8 cleavage at meiosis I [10]. Rec8 with phospho-mimetic mutations is no longer protected from separase at centromeres and is cleaved even when the two kinases are inhibited. This suggests that PP2A protects centromeric cohesion by opposing CK1δε- and DDK-dependent phosphorylation of Rec8. Interestingly, the very same kinases have been found to trigger other processes essential for reductional chromosome segregation in meiosis I: DDK initiates DNA replication as well as recombination and promotes, together with CK1δ/ε, monopolar attachment of sister kinetochores [11, 12]. A better understanding of the regulation of meiotic cohesin is of medical relevance since deterioration of cohesion is thought to be a major factor in the age-dependent increase of aneuploidies in the human oocyte [13].

References:

1. Nasmyth K, Haering CH (2005) The structure and function of SMC and kleisin complexes. *Annu Rev Biochem* **74**: 595–648.
2. Petronczki M, Siomos MF, Nasmyth K (2003) Un menage a quatre: the molecular biology of chromosome segregation in meiosis. *Cell* **112**: 423–440.
3. Klein F, Mahr P, Galova M, Buonomo SB, Michaelis C, Nairz K, Nasmyth K (1999) A central role for cohesins in sister chromatid cohesion, formation of axial elements, and recombination during yeast meiosis. *Cell* **98**: 91–103.
4. Toth A, Rabitsch KP, Galova M, Schleiffer A, Buonomo SB, Nasmyth K (2000) Functional genomics identifies monopolin: a kinetochore protein required for segregation of homologs during meiosis I. *Cell* **103**: 1155–1168.
5. Watanabe Y, Nurse P (1999) Cohesin Rec8 is required for reductional chromosome segregation at meiosis. *Nature* **400**: 461–464.
6. Buonomo SB, Clyne RK, Fuchs J, Loidl J, Uhlmann F, Nasmyth K (2000) Disjunction of homologous chromosomes in meiosis I depends on proteolytic cleavage of the meiotic cohesin Rec8 by separin. *Cell* **103**: 387–398.
7. Kitajima TS, Kawashima SA, Watanabe Y (2004) The conserved kinetochore protein shugoshin protects centromeric cohesion during meiosis. *Nature* **427**: 510–517.
8. Riedel CG, Katis VL, Katou Y, Mori S, Itoh T, Helmhart W, Galova M, Petronczki M, Gregan J, Cetin B, Mudrak I,

Ogris E, Mechtler K, Pelletier L, Buchholz F, Shirahige K, Nasmyth K (2006) Protein phosphatase 2A protects centromeric sister chromatid cohesion during meiosis I. *Nature* **441**: 53–61.
9. Kitajima TS, Sakuno T, Ishiguro K, Iemura S, Natsume T, Kawashima SA, Watanabe Y (2006) Shugoshin collaborates with protein phosphatase 2A to protect cohesin. *Nature* **441**: 46–52.
10. Katis VL, Lipp JJ, Imre R, Bogdanova A, Okaz E, Habermann B, Mechtler K, Nasmyth K, Zachariae W (2010) Rec8 phosphorylation by casein kinase 1 and Cdc7-Dbf4 kinase regulates cohesin cleavage by separase during meiosis. *Dev Cell* **18**: 397–409.
11. Petronczki M, Matos J, Mori S, Gregan J, Bogdanova A, Schwickart M, Mechtler K, Shirahige K, Zachariae W, Nasmyth K (2006) Monopolar attachment of sister kinetochores at meiosis I requires casein kinase 1. *Cell* **126**: 1049–1064.
12. Matos J, Lipp JJ, Bogdanova A, Guillot S, Okaz E, Junqueira M, Shevchenko A, Zachariae W (2008) Dbf4-dependent CDC7 kinase links DNA replication to the segregation of homologous chromosomes in meiosis I. *Cell* **135**: 662–678.
13. Lister LM, Kouznetsova A, Hyslop LA, Kalleas D, Pace SL, Barel JC, Nathan A, Floros V, Adelfalk C, Watanabe Y, Jessberger R, Kirkwood TB, Hoog C, Herbert M (2010) Age-related meiotic segregation errors in mammalian oocytes are preceded by depletion of cohesin and Sgo2. *Curr Biol* **20**: 1511–1521.