

Regulations and Functions of ICK/MAK/MOK – A Novel MAPK-Related Kinase Family Linked to Human Diseases

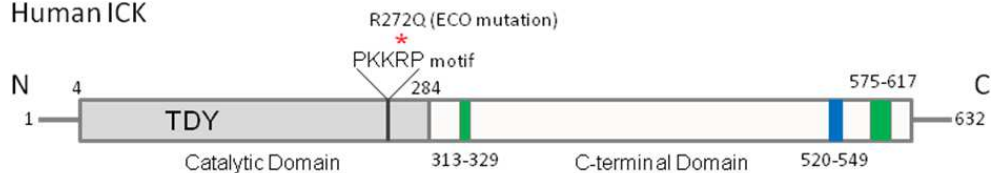
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1. Introduction

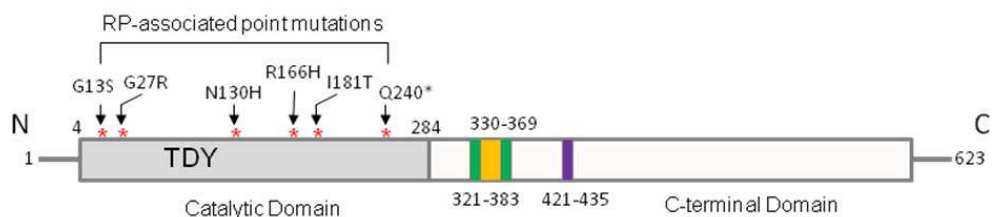
The RCK (the tyrosine kinase gene *v-ros* cross-hybridizing kinase) family within the CMGC (CDK/MAPK/GSK3/CLK) group of the human kinome consists of ICK/MRK (Intestinal cell kinase/MAK-related kinase) (Abe, Yagi et al. 1995; Togawa, Yan et al. 2000), MAK (male germ cell-associated kinase) (Matsushime, Jinno et al. 1990), and MOK (MAPK/MAK/MRK-overlapping kinase) (Miyata, Akashi et al. 1999) (Fig. 1). In the N-terminal catalytic domain, they all share significant sequence homology with MAPK (mitogen-activated protein kinase) and contain a MAPK-like TXY motif in the activation T-loop. However, they display significant divergence in the composition of their C-terminal non-catalytic domains which may determine their functional specificity and confer distinct regulatory mechanisms.

The biological functions of the ICK/MAK/MOK family have been elusive until recently. MAK is highly expressed in testis, however the MAK null mouse is viable and fertile, suggesting the existence of functional redundancy or compensation for the lack of MAK in testis (Shinkai, Satoh et al. 2002). In the MAK-null retina, photoreceptors exhibit elongated cilia and progressive degeneration, suggesting that MAK is required for regulation of ciliary length and retinal photoreceptor survival (Omori, Chaya et al. 2010). Exome sequencing has identified multiple point mutations in the kinase domain (Fig. 1) or an Alu-insertion in exon 9 of MAK as potential causes of retinitis pigmentosa (RP), a genetically heritable and autosomal recessive disease (Ozgul, Siemiatkowska et al. 2011; Tucker, Scheetz et al. 2011). MAK is a co-activator for androgen receptor (AR) in prostate cancer cells and is required for AR-mediated signaling and cell proliferation (Xia, Robinson et al. 2002; Ma, Xia et al. 2006). A loss-of-function point mutation R272Q of ICK (Fig. 1) has been recently identified as the causative mutation in a neonatal lethal multiplex human syndrome ECO (endocrine-cerebro-osteodysplasia), implicating a key role for ICK in development of multiple organ systems (Lahiry, Wang et al. 2009). Using shRNA knockdown, we have shown that suppression of ICK expression in intestinal epithelial cells markedly impaired cell proliferation and G1 cell cycle progression (Fu, Kim et al. 2009). Furthermore, ICK deficiency led to a significant decrease in the mTORC1 (mammalian target of rapamycin complex 1) activity, concomitant with reduced expression of specific mTORC1 downstream targets cyclinD1 and c-Myc (Fu, Kim et al. 2009). These results suggest that ICK may target

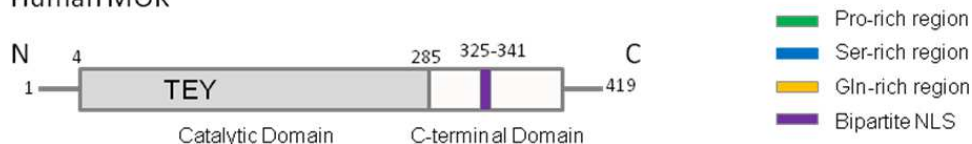
Human ICK



Human MAK



Human MOK



ICK	MNRYTTIRQLGDG TY GSVLLGRSIESGELIAIKKMKRFYSWEECMNLREVKSLKKLN-H	59
MAK	MNRYTTMRQLGDG TY GSVLMGKS NESGELVAIKRMKRFYSWDECMNLREVKSLKKLN-H	59
MOK	MKNYKAIGKIGEG TF SEVMKMQSLRDGNYYACKQMKQRFESIEQVNNLREIQALRRLNPH	60
	:	
ICK	ANVVKLKEVIRE--NDHLYFIFEYMKENLYQLIKERNKLPESAIRNIMYQILQGLAFIH	117
MAK	ANVIKLKEVIRE--NDHLYFIFEYMKENLYQLMKDRNKLFPESVIRNIMYQILQGLAFIH	117
MOK	PNILMLHEVVFDKSGSLALICELMDMNIYELIRGRYPLSEKKIMHYMYQLCKSLDHIH	120
	.*:*	
ICK	KHGFFHRDLKPENLLCMGPELVKIDFGLAREIRSKPPY TDY VSTRWYRAPEVLLRSTNY	177
MAK	KHGFFHRDMKPENLLCMGPELVKIDFGLARELRSQPPY TDY VSTRWYRAPEVLLRSSVY	177
MOK	RNGIFHRDVKPENILIK-QDVLKLGDFGSCR SVYSKQPY TEY I STRWYRAPECLLDGFY	179
	:::*	
ICK	SSPIDVWAVGCIMA EVYTLRPLFP GASEIDTI FKICQVLGTPKKT DWPEGYQLSSAMNFR	237
MAK	SSPIDVWAVG SIMAELYMLRPLFPGT SEVDEI FKICQVLGTPKSDWPEGYQLASSMNFR	237
MOK	TYKMDLWSAGCVFYBIASLQPLFPGVNELDQI SKIHDVIGTPAQKILTK-FKQSRAMNFD	238
	:*	
ICK	WPQCVPNLKTLPNASSEAVQLLRDMLQWDPKPKRPTASQALRYPYF	284
MAK	FPQCVPINLKTLPNASNEAIQLMTEMLNWDPKPKRPTASQALKHPYF	284
MOK	FPFKKGSGLPLLTNLSPOCLSLHAMVAYDPDERIAAHQALQHPYF	285
	:*	

Fig. 1. Schematic illustration of structural organization and features of human ICK, MAK and MOK; Sequence alignment of their catalytic domains. ECO, endocrine-cerebro-osteodysplasia syndrome; RP, retinitis pigmentosa

the mTORC1 signaling pathway to regulate cell proliferation and cell cycle progression. The biological functions of MOK are the least understood in the ICK/MAK/MOK family, except that a previous study indicated that MOK may be involved in growth arrest and differentiation in the intestinal epithelium (Uesaka and Kageyama 2004).

In this chapter, the current knowledge about the regulations and functions of this novel group of serine/threonine protein kinases will be reviewed. Furthermore, and more importantly, the many “unknowns” about the biology of ICK/MAK/MOK will be identified and discussed, the answers to which should provide new insights into their unique regulatory mechanisms, diverse biological substrates and physiological functions.

2. ICK signaling cascade

ICK was separately cloned from a rat heart cDNA library (Abe, Yagi et al. 1995) and a mouse small intestinal crypt cDNA library (Togawa, Yan et al. 2000) by using degenerate oligonucleotide primers recognizing sequences from highly conserved subdomains of serine-threonine kinases. ICK, named after its cloning origin the intestine, is actually a ubiquitously expressed Ser/Thr protein kinase. Northern analysis with specific ICK probes detected ICK mRNAs in most mouse, rat and human tissues examined (Abe, Yagi et al. 1995; Togawa, Yan et al. 2000). ICK and MAK contain nearly identical N-terminal catalytic domains (87% identity) but more divergent C-terminal noncatalytic domains. The ICK/MAK catalytic domain is related to both mitogen-activated protein kinases (MAPKs) and cyclin-dependent protein kinases (CDKs), with a MAPK-like TDY motif in the activation loop and the CDK-like regulatory sites T¹⁴Y¹⁵, but lacking the PSTAIRE cyclin-binding motif found in most CDKs. ICK and MAK are also conserved from yeast to humans. *Saccharomyces cerevisiae* has one closely related kinase, Ime2p (inducer of meiosis) that is a meiosis-specific homolog of human CDK2 and required for timing meiotic S phase (Foiani, Nadjar-Boger et al. 1996; Clifford, Stark et al. 2005). *Caenorhabditis elegans* has one homolog DYF5, a dye-filling defective mutant identified from a forward genetic screen that plays an important role in the control of cilia length and the docking and undocking of kinesin-2 motors from IFT (intraflagellar transport) particles (Burghoorn, Dekkers et al. 2007). *Danio rerio* (zebrafish) also has one homologous gene whose in situ expression at the basal level was detected in the retinal photoreceptor cell layer (<http://zfin.org>).

2.1 Regulation of activity by the TDY motif phosphorylation through a pair of yin-yang regulators

So far, we have established ICK as the prototype for a new group of kinases with MAPK-like regulation at TDY motifs (Fu, Schroeder et al. 2005). By mass spectrometry, we have shown that ICK can be specifically phosphorylated in the TDY motif *in vivo*. ICK requires an intact and doubly phosphorylated TDY motif for maximum activity. Autophosphorylation on Tyr-159 in the TDY motif only confers basal kinase activity. Full activation of ICK requires additional phosphorylation of Thr-157 in the TDY motif. Furthermore, we have identified PP5 (protein phosphatase 5) and CCRK (cell cycle related kinase) as a pair of *yin-yang* regulators for Thr-157 phosphorylation (Fu, Larson et al. 2006).

CCRK (Cell Cycle-Related Kinase): Since the catalytic domain of ICK is similar to those of both ERK2 (extracellular signal-regulated kinase) and CDK2 (cyclin-dependent protein kinase 2), we tested whether the ERK2 activator MEK1/2 (MAPK/ERK kinase 1/2) and CDK2

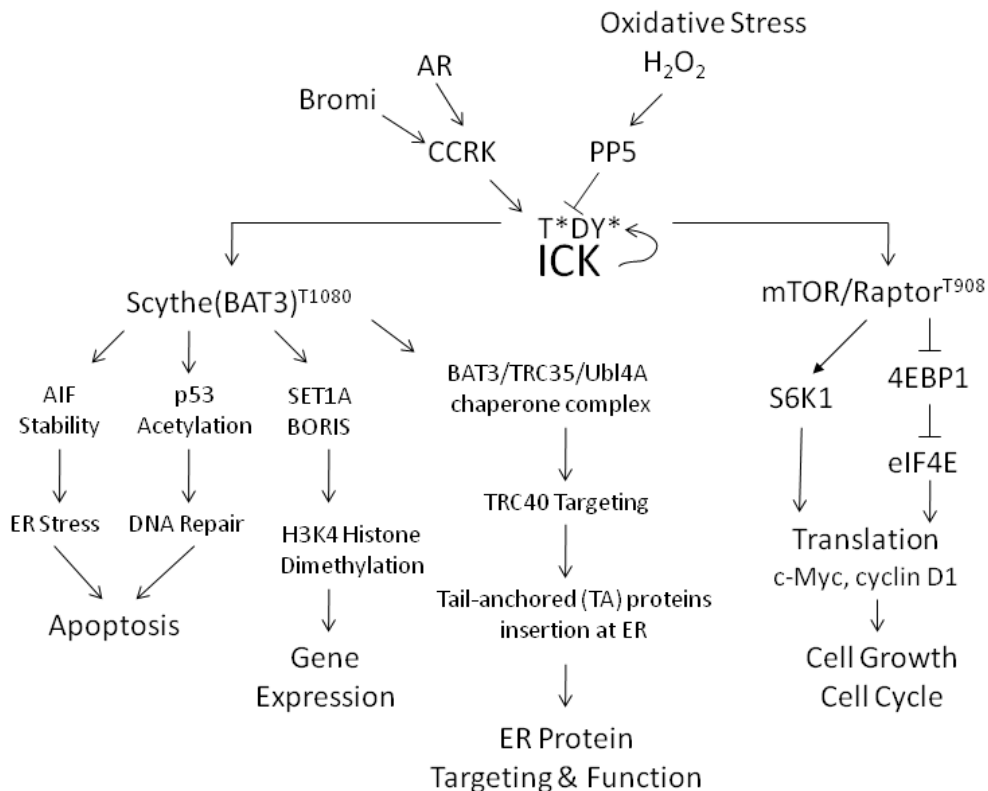


Fig. 2. Working model of the ICK signaling cascade. CCRK, cell cycle-related kinase; PP5, protein phosphatase 5; Bromi, broad-minded; AR, androgen receptor; mTOR, mammalian target of rapamycin; Raptor, regulatory associated protein of mTOR; Scythe/BAT3, HLA-b-associated transcript 3.

activator CAK (Cdk activating kinase) activates ICK. Surprisingly, neither MEK1/2 nor the Cdk7 complex (CDK7/cyclin H/MAT1) phosphorylates ICK in the T-loop, instead our data implicated ICK as a physiologic downstream target of CCRK (Fu, Schroeder et al. 2005; Fu, Larson et al. 2006). CCRK is most closely related to yeast CAK based on sequence homology, however it is a point of controversy as to whether CCRK has the intrinsic CAK activity (Liu, Wu et al. 2004; Wohlbold, Larochelle et al. 2006). CCRK may support a role for ICK in the regulation of proliferation and/or apoptosis. CCRK was identified in a large scale siRNA screen for suppressors of apoptosis (MacKeigan, Murphy et al. 2005). CCRK was also shown to be important for cell growth in HeLa, HCT116 and U2OS cells (Liu, Wu et al. 2004; Wohlbold, Larochelle et al. 2006). CCRK is a novel candidate oncogene in human glioblastoma (Ng, Cheung et al. 2007), colon cancer (An, Ng et al. 2010) and hepatocellular carcinoma (Feng, Cheng et al. 2011). The heart expresses a splice variant of CCRK, which promotes cardiac cell growth and survival; differs from the generic isoform in terms of protein-protein interactions, substrate specificity and regulation of the cell cycle; and is down-regulated significantly in heart failure (Qiu, Dai et al. 2008). Recently, CCRK was

shown to interact with Broad-minded (Bromi) to control cilia assembly and mammalian Sonic hedgehog (Shh) signaling transduction (Ko, Norman et al. 2010). The endogenous CCRK protein level was significantly reduced in Bromi mutant embryos and fibroblasts, while the CCRK mRNA level was unaffected, suggesting that Bromi promotes CCRK stability. CCRK was also implicated as a downstream mediator of AR (androgen receptor) signaling that drives hepatocarcinogenesis through a β -catenin and TCF (T cell receptor)-dependent pathway (Feng, Cheng et al. 2011). Ligand-bound AR is able to up-regulate CCRK transcription and protein expression through direct binding to the AR-responsive element of its promoter. What remains unknown is how the activity of CCRK is regulated independent of its expression level. Given CCRK is localized in both nuclear and cytoplasmic compartments, it will be important to know whether CCRK is differentially regulated and performs distinct biological functions in two different locations. It is also worth pointing out that a previous gel filtration study demonstrated the presence of at least two different CAK activities in human cells, with the second CAK activity detected at 30-40 KDa resembling the biochemical properties of Cak1p (Kaldis and Solomon 2000). This observation raises the possibility that a “small” CAK other than CCRK may be an upstream activator of ICK and MAK.

PP5 (Protein Phosphatase 5): PP5 plays important roles in cell cycle checkpoints, DNA damage response and proliferation (Golden, Swingle et al. 2008; Hinds and Sanchez 2008). Inhibition of PP5 expression results in a marked antiproliferative effect through the activation of the p53-dependent G1 checkpoint (Zuo, Dean et al. 1998). PP5 is required for both ATM (Ataxia telangiectasia mutated) and ATR (Ataxia telangiectasia and Rad3-related protein) checkpoint signaling, operant in S and G2/M (Ali, Zhang et al. 2004; Zhang, Bao et al. 2005). Hydrogen peroxide treatment induces activation of PP5 leading to the negative regulation of ASK1 (apoptosis signal-regulating kinase 1) and inhibition of apoptosis (Morita, Saitoh et al. 2001). PP5 dephosphorylates two functional sites in DNA-PKc (DNA-dependent protein kinase, catalytic subunit) that are required for functions in the DNA repair of double strand breaks (Wechsler, Chen et al. 2004). PP5 also dephosphorylates Raf1 (proto-oncogene c-Raf) at Ser-338 to inhibit Raf1 activity and its downstream signaling to MEK and ERK (von Kriegsheim, Pitt et al. 2006). Similarly, PP5 can inactivate ICK by dephosphorylating the essential phospho-threonine residue within the T-loop (Fu, Larson et al. 2006). We also showed that hydrogen peroxide treatment induces activation of the endogenous PP5 to negatively regulate ICK phosphorylation in the T-loop (Fu, Larson et al. 2006). Identifying ICK as a new downstream target of PP5 leads to our hypothesis that PP5 may modulate some branch of checkpoint signaling in response to stress and DNA-damage through the inactivation of ICK.

2.2 Regulation of activity by nuclear targeting

Prior studies from us and others have shown that GFP-tagged ICK is predominantly nuclear (Yang, Jiang et al. 2002; Fu, Schroeder et al. 2005). Our studies further established that the catalytic domain, but not the C-terminal domain, of ICK is required for nuclear localization. Neither the kinase activity nor the TDY phosphorylation appears to be necessary. Instead, an intact subdomain XI is required as well as the conserved arginine, R272, in the PKKRP motif and its interacting networks including W184 and E169. Loss of nuclear localization was associated with a significant reduction in its catalytic activity, suggesting that nuclear

targeting is important for the maximal activation of ICK, consistent with the predominant nuclear localization of its upstream activator CCRK.

It still remains elusive how the endogenous ICK is distributed in cells. Our unpublished data implicate the presence of endogenous ICK signals in the cytoplasm. Given that ICK has multiple splicing variants, it is possible that different isoforms of ICK may exhibit differential subcellular localization. Although CCRK is mainly localized to the nucleus, it is also present in the cytoplasm (Liu, Wu et al. 2004; An, Ng et al. 2010; Ko, Norman et al. 2010), thus capable of phosphorylating and activating cytoplasmic ICK as well. It remains to be determined whether cytoplasmic ICK and nuclear ICK have distinct biological activities.

2.3 The ICK substrate phosphorylation consensus

In order to identify putative substrates for ICK, a positional scanning peptide array method was used to determine the sequence specificity surrounding the ICK phosphorylation site (Fu, Larson et al. 2006). The phosphorylation consensus for ICK is [R]-[P]-[X]-[S/T]-[P/A/T/S], with the strongest selection for arginine at P-3 and proline at P-2. A preference for proline at P+1 was observed and was expected, given the similarity of ICK to ERK2 in the catalytic domain. However, the selection for proline at P+1 position is not absolutely stringent because alanine, threonine, and serine were also selected albeit less well. Despite some similarities, the ICK phosphorylation consensus is distinct from that of ERK2 due to the lack of absolute stringency for proline at P+1 or that of CDK2 due to the lack of a strong preference for basic residues (K/R) at P+3.

Due to the difficulty in obtaining a large quantity of highly purified and active full-length ICK protein, we were only able to use the catalytic domain of ICK as the kinase source in the peptide library scan (Fu, Larson et al. 2006). Therefore, despite our recent successes in using this consensus motif to identify several candidate physiological substrates for ICK (see Fig. 2), there still is the possibility that the full-length ICK including the long C-terminal domain may add additional features or modifications to the current substrate consensus sequence for ICK.

Given that ICK and MAK are essentially identical in the catalytic domain, we anticipate that this consensus sequence for ICK may also be useful for selecting putative substrates and/or phosphorylation sites for MAK. For example, we have identified Scythe/BAT3 as a candidate physiological substrate for ICK (Fu, Larson et al. 2006). Scythe/BAT3 is especially enriched in testis and abundant in male germ cells (Wang and Liew 1994), an expression pattern very similar to that of MAK (Matsushime, Jinno et al. 1990). In addition, both MAK and Scythe/BAT3 mRNAs increase dramatically in the mouse testis at around 14 to 20 days after birth. These correlations suggest an interesting hypothesis that Scythe may be a direct substrate downstream of MAK to function in some aspects of spermatogenesis.

2.4 Candidate physiological substrates

Scythe/BAT3 (HLA-b-associated transcript 3): We have established, *in vitro*, that ICK can phosphorylate Scythe (Fu, Larson et al. 2006), an important mediator of apoptosis and proliferation during mammalian development (Desmots, Russell et al. 2005). Scythe was originally identified as a novel reaper-binding apoptotic regulator in *Drosophila melanogaster* (Thress, Henzel et al. 1998; Thress, Evans et al. 1999). Recently, Scythe was identified as the

key interacting partner of the human small glutamine-rich TPR-containing protein (hSGT) that is required for progression through cell division (Winnefeld, Grewenig et al. 2006). Findings from studies of Scythe-deficient mice indicated that Scythe is a novel and essential regulator of p53-mediated responses to genotoxic stress by controlling DNA-damage induced acetylation of p53 (Sasaki, Gan et al. 2007). As a possible link of ICK to colon cancer, Scythe was a newly identified candidate tumor suppressor gene in colon cancer cells (Ivanov, Lo et al. 2007). Scythe is also essential for selective elimination of defective proteasomal substrate as a ubiquitin-like protein (Minami, Hayakawa et al. 2010) and acts as a transmembrane domain (TMD)-selective chaperon that effectively channels tail-anchored (TA) proteins into the ER membrane (Mariappan, Li et al. 2010). Using the ICK phosphorylation consensus sequence R-P-X-S/T, we reported that an *in vivo* phosphorylation site, Thr-1080, in Scythe is a major ICK phosphorylation site *in vitro* (Fu, Larson et al. 2006). Functions are yet to be defined for this ICK phosphorylation site in Scythe-regulated biological events such as proliferation, apoptosis and DNA damage control in response to genotoxic stress.

Raptor (Regulatory associated protein of mTOR): The serine/threonine protein kinase mammalian target of rapamycin (mTOR) is the core catalytic component of two structurally and functionally distinct protein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), which collectively integrates nutrient, hormonal, and energy signal inputs to control cell growth, proliferation and survival (Bhaskar and Hay 2007; Hall 2008; Laplante and Sabatini 2009). mTORC1, when activated by growth factors and nutrients, stimulate cell growth and proliferation by phosphorylating two key regulators of mRNA translation and ribosome biogenesis, S6K1 (ribosomal protein S6 kinase) and 4EBP1 (eukaryotic initiation factor 4E-binding protein 1) (Hara, Yonezawa et al. 1997; Fingar, Richardson et al. 2004; Proud 2004; Ma and Blenis 2009). In addition to the catalytic subunit mTOR, mTORC1 also contains four associated components, Raptor, mLST8/GβL, PRAS40, and Deptor (Kim, Sarbassov et al. 2002; Loewith, Jacinto et al. 2002; Kim, Sarbassov et al. 2003; Kim and Sabatini 2004; Vander Haar, Lee et al. 2007; Wang, Harris et al. 2008; Peterson, Laplante et al. 2009). Raptor plays an important role as a scaffolding protein to recruit substrates S6K1 and 4EBP1 to mTOR (Nojima, Tokunaga et al. 2003). Upon growth factor stimulation, Raptor binding to substrates can be enhanced by the dissociation of the competitive inhibitor PRAS40 (the proline-rich Akt substrate of 40KDa) from mTORC1 (Fonseca, Smith et al. 2007; Sancak, Thoreen et al. 2007; Wang, Harris et al. 2008; Nascimento and Ouwens 2009). Raptor can also positively regulate mTOR activity in response to nutrient sufficiency by directly interacting with Rag family GTPases to induce mTORC1 re-localization to an intracellular vesicular compartment containing RheB, a Ras-like GTP-binding protein that activates mTOR via an unknown mechanism (Hanrahan and Blenis 2006; Kim, Goraksha-Hicks et al. 2008; Sancak, Peterson et al. 2008).

Recently, multiple phosphorylation sites of Raptor have been identified, several of which are critical for the regulation of mTORC1 activity in response to insulin, nutrients or energy stress. Phosphorylation of Raptor Ser-722 and Ser-792 by AMPK is required for the inhibition of mTORC1 and cell cycle arrest induced by energy stress (Gwinn, Shackelford et al. 2008). RSK mediated phosphorylation of Ser-719/721/722 enhances mTORC1 activity stimulated by Ras/MAPK pathway (Carriere, Cargnello et al. 2008). Phosphorylation of Ser-863 by either mTOR or ERK1/2 promotes mTORC1 activation in response to various stimuli

including growth factors, nutrients and cellular energy (Wang, Lawrence et al. 2009; Foster, Acosta-Jaquez et al. 2010; Carriere, Romeo et al. 2011). Taken together, a plethora of emerging evidence indicates that the complex phosphorylation status of Raptor is tightly associated with the activity of mTORC1. Our data indicated that ICK associates with Raptor and phosphorylates Raptor at Thr-908 (Fu, Kim et al. 2009). More importantly, knockdown the ICK expression significantly reduced the phosphorylation of S6K1 at Thr-389 targeted by the mTORC1, suggesting that ICK is an upstream regulator of the mTORC1 activity in the regulation of cell growth and proliferation (Fu, Kim et al. 2009).

2.5 Role of ICK in ECO and multiple organ development during embryogenesis

Protein kinases comprise one of the largest and most abundant gene families in humans. Both inherited germ-line and somatic mutations in kinase genes have been associated with many human diseases including developmental and metabolic disorders and neoplastic malignancies (Lahiry, Torkamani et al. 2010). The human endocrine-cerebro-osteodysplasia (ECO) syndrome is a newly identified congenital neonatal-lethal disorder whose clinical manifestations include osteodysplasia, cerebral anomalies, and endocrine gland hypoplasia. A homozygous missense mutation R272Q in ICK was identified as the causative mutation for ECO. ICK, named after its cloning origin the intestine, is a misnomer because it is highly conserved and ubiquitously expressed in human tissues, which may explain why the R272Q mutation in ICK causes developmental defects in multiple organs. Previously we have established that the R272A mutation in ICK impairs the nuclear targeting and the catalytic activity of ICK; this result was confirmed with the R272Q mutation as well. Many of the malformations observed in ECO involve a defect in apoptosis, especially the cleft lip and palate, syndactyly, prolonged persistence of fusion of the eyelids, and unfused urogenital folds. ECO-affected infants also develop phenotypes observed with Scythe deficiency, including hydrocephalus, dilated and hypoplastic kidneys. Given the role of scythe in apoptosis, these observations support the hypothesis that ICK targets scythe to regulate apoptosis during mammalian organ development.

2.6 Role of ICK in the intestinal epithelium

A delicate balance of cell renewal, differentiation and cell death is crucial to maintain the gastrointestinal tissue architecture that forms the basis for the normal function of the gut. An early study using *in situ* hybridization showed that the expression of ICK mRNA was localized specifically to the crypt compartment of the small intestine (Togawa, Yan et al. 2000). The crypt is the compartment of the intestinal epithelium where stem cells, progenitor cells and rapidly replicating transit-amplifying cells reside raising the hypothesis that ICK may play a role in epithelial replication, lineage specification and cell fate determination in crypt epithelium. Using a conditional ICK knockout mouse model, we are currently addressing whether intestine-specific ablation of the ICK gene affects cell proliferation, differentiation, migration and lineage allocation in the intestinal epithelium during normal development and homeostasis, and whether the ICK expression is important for the expansion and proliferation of the intestinal stem cell population and their progenitors in the restoration of the normal epithelial architecture after mucosal injury.

Recently, we reported that suppression of ICK expression in cultured colorectal carcinoma and intestinal epithelial cell lines by short hairpin RNA (shRNA) interference significantly

impaired cellular proliferation and induced features of gene expression characteristic of colonic or enterocytic differentiation (Fu, Kim et al. 2009). Downregulation of ICK altered expression of cell cycle regulators (cyclin D1, c-Myc, and p21^{Cip1/WAF1}) of G1-S transition, consistent with the G1 cell cycle delay induced by ICK shRNA. ICK deficiency also led to a significant decrease in the expression and/or activity of S6K1, indicating that disrupting ICK function downregulates the mTORC1 signaling pathway. Our prior studies also provided biochemical evidence that ICK interacts with the mTOR/Raptor complex in cells and Raptor is an *in vitro* substrate for ICK (Fu, Kim et al. 2009). Recently, we investigated whether and how ICK targets Raptor to regulate the activity of mTORC1. Our results indicate that ICK is able to promote mTORC1 activation through phosphorylation of Raptor Thr-908 (Wu D et al., J Biol Chem, in press).

2.7 Role of ICK in cardiac development and hypertrophy

Abe, S and colleagues (Abe, Yagi et al. 1995) observed the ICK/MRK protein signals in the cytosol of cardiomyocytes at day 11 rat embryos, and the ICK/MRK immunohistological staining appeared to be weaker and in a patchy, speckled pattern in adult rat hearts, suggesting downregulation of the ICK/MRK signals during cardiac development. Furthermore, the intensity of the ICK/MRK staining and the number of ICK positive cardiomyocytes were both increased in hypertrophic hearts with experimentally induced stenosis of the abdominal aorta, implicating that the ICK/MRK expression is inducible by external stress such as pressure overload.

3. MAK signaling cascade

In 1990, MAK was first isolated from a human genomic DNA library by using weak cross-hybridization with a tyrosine kinase gene (*v-ros*) in Professor Masabumi Shibuya's laboratory. This gene was designated as MAK (male germ cell-associated kinase) because it is highly expressed in testicular germ cells. In contrast to the ubiquitous expression pattern of ICK, MAK expression is more restricted. MAK mRNAs are enriched in testis and expressed in male germ cells during and after meiosis (Matsushime, Jinno et al. 1990). MAK expression was also detected in prostate and retina. MAK was identified as an androgen-inducible gene in LNCaP prostate epithelial cells and as a co-activator of androgen receptor signaling in prostate cancer (Ma, Xia et al. 2006). Recently, MAK expression was detected in cilia of the retina where it was suggested to be involved in photoreceptor cell survival (Omori, Chaya et al. 2010).

3.1 cell cycle-dependent localization and regulation in the TDY motif

The subcellular localization of MAK is dynamic during cell cycle (Wang and Kung 2011). MAK displays uniform localization in the nucleus during interphase, and associates with mitotic spindles and centrosomes at metaphase and anaphase. This dynamic nuclear localization of MAK is associated with its cell cycle-related role (see 3.4). Similar to ICK, MAK also requires an intact and dually phosphorylated TDY motif for full activation. CCRK, but not MEK, is the upstream activating kinase for MAK in the TDY motif (Wang and Kung 2011). More interestingly, although the expression level of MAK remained constant, the TDY-dual phosphorylation level oscillated during cell cycle (Wang and Kung

2011). It increased at S phase, peaked at G2 to early M phase, and decreased at late M phase. It is not clear, however, whether this oscillation of the TDY-dual phosphorylation of MAK during cell cycle is associated with the expression and/or activity levels of CCRK. The high level TDY-phosphorylation of MAK at G2/M does provide the molecular basis for an important role of MAK during the metaphase-anaphase transition (see 3.4).

3.2 MAK in testis and spermatogenesis

Northern blot analysis revealed two discrete transcripts (2.6 and 3.8 kb) of the *mak* gene that are mainly expressed in germ cells at and/or after the pachytene stage (Matsushime, Jinno et al. 1990). Since these two *mak* transcripts display differential temporal expression patterns during spermatogenesis, it was speculated that they may have distinct physiological functions in germ cells differentiation. Subsequent studies from Professor Shibuya's lab identified two MAK protein products that are mainly localized in the cytoplasm and a phosphorylated 210-KDa protein as a candidate physiological substrate for MAK. The true identify of this MAK-associated 210-KDa protein still remains a mystery.

In 2002, phenotypic analysis of the MAK knockout mouse was reported by Yoichi Shinkai and colleagues. Overall, MAK-deficient mice developed normally with no gross abnormalities (Shinkai, Satoh et al. 2002). Surprisingly, most of the MAK null mice were fertile, suggesting no major defects in spermatogenesis in the absence of MAK gene in mice. The only mild phenotype in MAK-deficient male mice is reduced sperm motility. These data suggest that MAK is not essential for spermatogenesis and male fertility, raising the possibility that ICK may compensate for the role of MAK in spermatogenesis. Our studies indicate that both ICK and MAK, proteins are abundantly expressed in mouse testis, and in primary spermatocytes and sertoli cell lines (Fu Z et al., unpublished data), providing further molecular basis for the speculation that ICK and MAK may have redundant biological functions in testis. Knockout of both MAK and ICK genes in mice will be required to test this hypothesis.

3.3 MAK in retina, ciliogenesis and retinitis pigmentosa

A potential role of MAK and ICK in regulating cilia structure and functions has long been speculated based on the observations that loss of functions of their homologs in *Chlamydomonas reinhardtii* (*LF4p*) (Berman, Wilson et al. 2003), in *Caenorhabditis elegans* (*Dyf-5*) (Burghoorn, Dekkers et al. 2007), and in *Leishmania Mexicana* (*LmxMPK9*) (Bengs, Scholz et al. 2005) causes elongated cilia or flagella to various degrees. During a microarray screening to identify photoreceptor cell-specific genes involved in the conversion of photoreceptors to amacrine-like cells, Takahisa Furukawa and colleagues found a retina-specific isoform of MAK cDNA containing a 75-bp in-frame insertion to the originally reported form of MAK in testis. Using the same MAK-null mice that display no major phenotype in spermatogenesis, Furukawa's lab investigated whether MAK has any important functions in retina where MAK is predominantly expressed in photoreceptor cells and localized in the photoreceptor connecting cilia and outer segment axonemes. The MAK-null retina appears to be normal until the completion of retinogenesis at postnatal day 14, suggesting that MAK gene is not essential for cell fate determination in retina. However, photoreceptor cells in the MAK-null retina do exhibit progressive degeneration associated with two major hallmarks: elongated

cilia and aberrant outer-segment disk formation. The role of MAK in regulating cilia length was also confirmed in serum starved NIH 3T3 cells where MAK is mainly localized in the nuclei and in the cilia as well. Both the kinase activity and the C-terminal region of MAK are essential for the regulation of the cilia length. However, only the C-terminal noncatalytic domain, but not the kinase activity, of MAK is required for its ciliary localization.

Retinitis pigmentosa 1 (RP1) was implicated as a candidate physiological substrate for MAK in regulating ciliary structure and organization in that RP1 induces ciliary elongation and reduces the effect of MAK overexpression, and furthermore MAK physically interacts with RP1 and directly phosphorylates RP1 *in vitro* (Omori, Chaya et al. 2010). Scythe may be another candidate substrate for MAK related to its role in RP for the following reasons. Scythe was identified from a yeast two-hybrid screen using ICK kinase domain as the bait and was confirmed to be an *in vitro* and *in vivo* substrate for ICK (Fu, Larson et al. 2006). ICK and MAK are essentially identical in the kinase domain, therefore Scythe maybe a common substrate for both. RP is characterized by apoptotic death of photoreceptor cells and scythe is known to be important for regulating apoptosis and is abundantly expressed in retina.

MAK was found in two cell types involved in sensory transduction, photoreceptors and olfactory receptors as well as epithelial of the respiratory tract and choroid plexus (Bladt and Birchmeier 1993). Interestingly, Furukawa and his colleagues also noted the reduced MAK expression in respiratory epithelia of the nasal cavity and in epididymal sperm cells of the testis, yet the ciliary length of neither cell types differ in wild-type and MAK-KO mice (Omori, Chaya et al. 2010). One possible explanation for this retina-specific phenotype in MAK-KO mice is the tissue-specific functional redundancy/compensation. Our studies indicate that both ICK and MAK are expressed in respiratory systems and testis, but very little of ICK or MOK protein is detected in retina where MAK is highly expressed (Fu Z et al., unpublished data), consistent with the notion that functional redundancy may exist between MAK, ICK and MOK in testis and lung but not in retina.

The essential role of MAK in supporting the biological functions of retina was further substantiated recently by similar findings from two independent studies (Ozgul, Siemiatkowska et al. 2011; Tucker, Scheetz et al. 2011). Exome sequencing and cis-regulatory mapping identified six missense point mutations of highly conserved residues within the catalytic domain of MAK as a cause of retinitis pigmentosa (RP) (Fig. 1). Also by exome sequencing, a 353-bp Alu repeat insertion was found to disrupt the correct splicing of exon 9 of *Mak* gene, thereby preventing mature retinal cells from expressing the correct MAK isoform in retina. In either case, a lack of active form of MAK gene product in retina was implicated as a cause of RP. Interestingly, as pointed out in (Ozgul, Siemiatkowska et al. 2011), all of the MAK mutations identified in the retinal isoform should also be present in testis isoform, yet there were no reports of infertility in the identified male objects, consistent with the phenotype of the *Mak* knockout mice. This finding again raises the possibility that ICK may be able to compensate for the lack of MAK in testis where both genes are abundantly expressed.

3.4 MAK in prostate cancer, AR signaling and mitosis

In contrast to ICK, expression of its closely related kinase MAK is more restricted. However, in addition to testis and retina, MAK expression was also detected in prostate (Xia,

Robinson et al. 2002). MAK was identified as an androgen-inducible co-activator of androgen receptor (AR) in prostate cancer cells (Ma, Xia et al. 2006). Similar to the role of ICK in intestinal epithelial cells, MAK is also required for prostate epithelial cell replication (Ma, Xia et al. 2006). A recent study from Kung's lab indicated that over-expression of MAK in prostate cancer cells caused mitotic defects that are independent of AR signaling but are associated with deregulation of the APC/C(CDH1) (Wang and Kung 2011).

Unpublished data from Kung's lab and our own data have suggested that AR is not the substrate for MAK *in vitro* or *in vivo*. Given the tight physical association of MAK with AR complex in cells and the significant biological effect of MAK knockdown on AR signaling events, it is quite possible that an AR-associated protein within the AR complex serves as the direct target of MAK in regulating the AR signaling.

Recently, Kung's lab demonstrated that MAK is over-expressed in prostate cancer cells and causes mitotic defects such as centrosome amplification and lagging chromosomes via deregulation of APC/C^{CDH1}, thus providing an AR-independent mechanism to promote prostate cancer development (Wang and Kung 2011). This report also indicated that CDH1 is an *in vitro* substrate for MAK and MAK can negatively regulate APC/C^{CDH1} through phosphorylation of CDH1 at the same CDK-dependent sites. It requires further studies to determine whether CDH1 is a true physiologic substrate for MAK and how MAK and CDK coordinate to target the same sites to regulate APC/C^{CDH1} activities.

4. MOK signaling cascade

MOK was identified through *in silico* computer screening of the GENBANK EST database using MAP kinase consensus sequences as probes (Miyata, Akashi et al. 1999). MOK encodes a protein of 419 (human) and 420 (mouse) amino acids, containing the conserved kinase subdomains I-XI and the TEY motif in the activation loop. Structurally, MOK belongs to the MAP kinase superfamily, and is closely related to MAK and ICK/MRK, thus termed as MOK (MAPK/MAK/MRK overlapping kinase). Although MOK shares highest homologies to MAK and ICK, especially in the catalytic domains (41-43% identity), it also displays certain structural features that are distinct from MAK and ICK. MAK and ICK have the TDY motif in the activation loop, as compared with the TEY motif for MOK. Similar to Cdk2, MAK and ICK possess T¹⁴Y¹⁵ motif in the N-terminal end of their catalytic domains, while MOK exhibits T¹⁴F¹⁵ motif instead in the same position. More significantly, the C-terminal noncatalytic domain of MOK is much shorter than that of either MAK or ICK (Fig. 1) and show very little sequence homology to MAK and ICK or any other known protein kinases.

Interestingly, MOK was also isolated from a blast search to analyze homologies to the RAGE (renal cell carcinoma antigen)-gene family that encodes antigens of human renal carcinoma cells recognized by autologous cytolytic T lymphocytes (Eichmuller, Usener et al. 2002). Sequence alignment indicates MOK is identical to RAGE-1, -2, -3 at the 3'-region, but is completely different at the 5'-region, suggesting that MOK and RAGE genes may be the splicing products from the same gene or MOK may be aberrantly inserted into RAGE genes by translocation. What is the molecular genetic basis for this observation and whether MOK is involved in RAGE-gene family associated tumorigenesis are interesting questions that will motivate further studies.

4.1 Regulation of MOK activity in the TEY motif and by TPA

MOK possesses protein kinase activity towards exogenous substrates for MAPK such as c-Jun, MBP, cyclin B1 and c-Myc and undergoes autophosphorylation (Miyata, Akashi et al. 1999). Similar to ICK and MAK, an intact TEY motif in the activation loop of MOK is essential for its kinase activity (Miyata, Akashi et al. 1999). The autokinase activity of MOK was almost completely abolished when the TEY motif was mutated to AEF, although it is not clear whether the Thr and/or the Tyr in the TEY motif are the phosphor-acceptor sites by autophosphorylation.

Similar to ICK and MAK, MOK could not be significantly activated by many extracellular stimuli (serum, anisomycin, and hyperosmotic shock) that stimulate MAP kinases (Miyata, Akashi et al. 1999). TPA (phorbol 12-myristate 13-acetate), however, at a concentration of 100 ng/ml, was able to stimulate the MOK activity up to about threefold, albeit at a much longer time point after treatment (15-20 min) than that required to maximally activate ERK1/2 and p38 MAPK (5 min) (Miyata, Akashi et al. 1999). These results suggest that the activation mechanism of MOK by TPA is different from that of MAP kinases. The identity of the upstream activating kinase for MOK remains unknown.

4.2 Regulation of MOK activity and/or functional specificity by subcellular localization

Recombinant MOK is predominantly cytoplasmic (Miyata, Akashi et al. 1999). In our subcellular fractionation studies, we observed both cytoplasmic and nuclear localization of endogenous MOK in human cell lines, suggesting that MOK may shuttle between these two compartments (Fu, Z et al., unpublished data). The molecular size (48 KDa) of MOK allows its nuclear entry by diffusion. In addition, there is a consensus bipartite NLS located in its C-terminal domain (Fig. 1), possibly permitting selective nuclear targeting as well. Further studies are required to elucidate the molecular mechanisms underlying its subcellular distribution and to address whether MOK targets different subsets of substrates in different locations and thus performs distinct biological functions.

4.3 Regulation of MOK stability by chaperone proteins

Work from Professor Eisuke Nishida's lab suggested specific association of MOK with a set of molecular chaperones including HSP90, HSP70 and Cdc37 (Miyata, Ikawa et al. 2001). Inhibition of HSP90 chaperone activity caused rapid degradation of MOK through proteasome-dependent pathways, suggesting that chaperone association is required to stabilize MOK. Interestingly, in the same study both MAK and ICK/MRK were also reported to associate with HSP90, although the biological effects of HSP90 association with MAK and ICK/MRK were not examined. In our unpublished studies, we also observed robust association of chaperones (HSP70 and Cdc37) with ICK and its upstream activating kinase CCRK. These results taken together suggest that ICK/MAK/MOK may require the presence of chaperone proteins in the same protein complexes for assistance in folding and stabilization, a biochemical property that is strikingly different from classic MAP kinases that do not specifically associate with chaperones (Miyata, Ikawa et al. 2001).

4.4 MOK in testis and germ cell development

MOK (T/STK30, testis-derived serine/threonine kinase 30) was isolated from adult testis using a PCR-based strategy to identify novel protein kinases expressed in germ cells

(Gopalan, Centanni et al. 1999). T/STK30 transcripts are most abundantly expressed in testis and ovary, and were not detected in a sterile mutant testis that lacks germ cells, further demonstrating that T/STK30 expression in testis is restricted to germ cells. By in situ hybridization, Donovan P.J. and colleagues further demonstrated that T/STK30 transcripts were detected in pachytene spermatocytes and round spermatids, but not in spermatogonia or testicular somatic cells. Similarly, T/STK30 is highly expressed in female germ cells, but not in the surrounding somatic cells that are mostly proliferating. These results, taken together, suggest T/STK30 is involved in some aspects of germ cell differentiation and maturation. Functional studies (targeted gene disruption and/or over-expression) will be required to address the role of MOK (T/STK30) in mammalian gametogenesis.

4.5 MOK in intestinal cell differentiation

Although MOK is closely related to ICK in the N-terminal catalytic domain, they differ significantly in the structural organization of the C-terminal non-catalytic domain. While MOK mRNA appears to be restricted to the crypt compartment of the small intestine, MOK protein was detected in the upper crypt and lower villus epithelial cells (Uesaka and Kageyama 2004). In HT-29 cells, MOK activity was reported to be elevated by sodium butyrate, which is known to inhibit growth and induce differentiation of HT-29 cells (Uesaka and Kageyama 2004). This observation suggests a possible role for MOK in the regulation of intestinal epithelial differentiation. Does MOK play an important role in the induction of growth arrest and differentiation in the intestinal epithelium, which could be directly opposite to the role of ICK? What are the upstream modulators and the downstream physiological substrates of MOK? Does MOK crosstalk with ICK during the regulation of gastrointestinal proliferation and differentiation? If so, what is the molecular basis for this interaction? These questions about MOK and its relationship to ICK remain to be clarified.

5. Conclusion and significant questions

The ICK/MAK/MOK family shares significant sequence and structural homology to MAPKs and CDKs. They all contain a TXY motif in the activation loop that is required for full activity. ICK and MAK also possess a CDK-like TY motif, but so far it is unknown whether these sites are phosphorylated *in vivo* and be able to regulate the catalytic activity. The regulatory mechanisms of ICK/MAK/MOK appear to be very different from that of MAPKs. Unlike classic MAPKs, they are not acutely activated by growth factors or stress. The upstream activating kinase for ICK and MAK is CCRK, not MEK or CDK7 complex. Both ICK and MAK have a long C-terminal non-catalytic domain with postulated functions in protein-protein and protein-DNA interactions, enabling them to operate through signaling pathways distinct from that of classic MAPKs.

Emerging evidence strongly suggest that this group of kinases have important biological functions in mammalian development and human diseases. They are involved in regulating many fundamental biological processes including cell proliferation, differentiation, apoptosis and cell cycle. Yet the molecular basis underlying these regulations is still largely elusive.

Even though tremendous progress has been made to elucidate the regulations and functions of ICK/MAK/MOK since their discoveries some 20 years ago, many significant questions,

such as a few named below, are yet to be answered in order to fully understand the biology of this kinase family in mammalian development and human diseases.

Q1: What is the molecular basis to determine the substrate and/or signaling specificity for ICK/MAK/MOK? This information may be stored in their C-terminal non-catalytic domains since they share extensive homology in their N-terminal catalytic domains. The recent data showing that the C-terminal domain of MAK is essential for its ciliary localization and function in regulating ciliary length provided further support to this notion.

Q2: What are the upstream stimuli or environmental cues that activate ICK/MAK/MOK? Are the expression and activity levels of ICK/MAK/MOK regulated during development or stress?

Q3: Do ICK/MAK/MOK have different subsets of physiologic substrates in different subcellular compartments and thus regulate distinct biological processes?

Q4: Is there a functional redundancy between ICK and MAK in testis? More specifically, does ICK compensate for the lack of MAK in spermatogenesis in MAK KO mice? Do MAK and ICK signal through scythe or different substrates to regulate germ cell development during spermatogenesis?

Q5: Does MOK have an opposite function to that of ICK or MAK in the intestine and testis given the existing evidence seem to indicate that ICK and MAK are pro-proliferation and MOK is pro-differentiation?

Q6: How the ICK, MAK and MOK genes are regulated at the transcription level in a specific biological context during development and diseases? Currently very little is known on this topic. ICK and FBX9 are divergently transcribed from a bi-directional promoter that contains functional sites for β -catenin/TCF7L2 and FOXA (Sturgill, Stoddard et al. 2010). MAK transcripts can be down-regulated by retinol during spermatogonial proliferation phase of spermatogenesis (Wang and Kim 1993). Cdx2, a caudal-related homeobox transcription factor, interacts with the MOK promoter and induces expression of MOK transcripts (Uesaka and Kageyama 2004).

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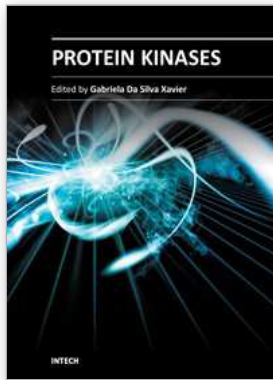
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Proteins are the work horses of the cell. As regulators of protein function, protein kinases are involved in the control of cellular functions via intricate signalling pathways, allowing for fine tuning of physiological functions. This book is a collaborative effort, with contribution from experts in their respective fields, reflecting the spirit of collaboration - across disciplines and borders - that exists in modern science. Here, we review the existing literature and, on occasions, provide novel data on the function of protein kinases in various systems. We also discuss the implications of these findings in the context of disease, treatment, and drug development.

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