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Global Approaches to Understanding Protein Kinase Functions

Jennifer L. Gorman and James R. Woodgett

1.1
A Brief History of the Structure of the Human Kinome

Initial guesstimates of the complexity of the human kinome suggested that our genomes would code for around 1000 protein kinases [1]. Subsequent sequencing of the human genome identified 518 protein kinases [2] (subsequently revised to add some atypical kinases and to delete pseudokinases), which were classified into subgroups based on sequence similarities of their catalytic domain. These subclasses are the AGC kinases, CMGC kinases, CAMK (calcium/calmodulin-dependent kinase), STE kinases, tyrosine kinases, casein kinases, tyrosine kinase-like protein kinases as well as the receptor guanylyl cyclase (RGC) kinases, and other/atypical protein kinases [2]. The degree of similarity of their kinase domains that enabled such classifications was also thought to preclude meaningful specificity of small molecule inhibitors, especially as most kinase inhibitors tend to interfere with ATP binding as a mechanism of action. The family, as a whole, was initially deemed undruggable. It is testament to the capabilities of synthetic chemists that kinase inhibitors with remarkable selectivity, potency, and specificity have become available. Add to this library the collection of gene knockouts and RNA interference (RNAi) reagents and global analysis of the 500+ gene family become feasible.

The remarkable scope of the kinase inhibitor libraries reflects the high level pharmaceutical interest in this class of proteins due to repeated examples of the dysregulation of these enzymes in human diseases. Here, we initially describe the subclasses of protein kinases, provide examples of their roles in disease, and discuss methods for their characterization and functional assignments.

1.1.1
AGC Kinases

This family of serine/threonine kinases [3] is named after three representative families PKA, PKG, and PKC [4] and contains 60 members with a propensity for phosphorylating Ser/Thr residues preceded by basic amino acids [5]. The master

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regulator of the AGC kinases is phosphoinositide-dependent kinase-1 (PDK1), which lies directly upstream of at least 23 other AGC kinase family members including Akt/PKB, protein kinase C (PKC), and serum- and glucocorticoid-induced protein kinase (SGK) and phosphorylates these protein kinases within their catalytic domain at the so-called "T-loop" [3]. Within the cell, PDK1 is constitutively active due to its intrinsic ability to autophosphorylate within its own active site and also contains a C-terminal pleckstrin homology (PH) domain that has high affinity for specific phosphoinositide lipids [3]. Given its wide range of substrates, PDK1 has very diverse cellular functions in regulating metabolism, cell proliferation, and cell survival [6]. PDK1 is inhibited by staurosporine (among other targets) which, given the proclivity of PDK1 to regulate many other kinases, explains why this drug was thought to be relatively nonselective, albeit a potent inducer of cellular apoptosis [7].

PDK1 was discovered by Alessi and colleagues while searching for a protein kinase activity that could phosphorylate and activate Akt/PKB. Akt/PKB is activated by agonists of phosphatidylinositol 3′kinase (PI3K) and, like PDK1, has a PH domain. Stimuli that induce PI3K activity result in generation of 3′-phosphorylate phosphoinositides that act to relocate Akt/PKB and PDK1 to colocalize at the plasma membrane, resulting in Akt/PKB phosphorylation and activation [3]. Full activation also requires phosphorylation of residues in the C-terminal domain of Akt/PKB, known as the hydrophobic motif. The kinase responsible for this modification is mTORC2, the rapamycin-insensitive complex of mammalian target of rapamycin (mTOR), Rictor, and other proteins. Isoform-specific knockouts of the three known isoforms (Akt1, 2, 3) have revealed nonredundant actions of each [8], as Akt1 knockout mice display increased rates of apoptosis [9], while Akt2 knockouts display a phenotype similar to type 2 diabetes with impaired glucose utilization [10]. Akt3 is primarily expressed in neuronal tissue, with Akt3 knockout mice displaying abnormalities in brain development [11]. Akt activation promotes cell proliferation via phosphorylation of substrates involved in regulating apoptosis and the cell cycle. For example, one group of targets, members of the FOXO family, induces transcription of genes that promote apoptosis and cell cycle arrest. Akt phosphorylation promotes exclusion of FOXO from the nucleus, turning down expression of these pro-apoptotic genes [12].

The PKC family consists of at least 12 isoforms, which are divided in four subcategories: conventional (α, β, and γ); novel (δ, ε, η, and θ); atypical (ζ and ι); as well as PKN- and PKC-related PKN1, PKN2, and PKN3 [3]. While PKC activation is regulated via phosphorylation at a C-terminal hydrophobic motif by mTORC2 and within the "T-loop" by PDK1, PKC activation can also be regulated through lipid second messengers. These vary based on the isoform, with conventional PKCs regulated by both diacylglycerol (DAG) and calcium, novel PKCs regulated solely by DAG, with neither required for atypical PKC activation [3, 13, 14]. PKC activation has two primary consequences; activation of the mitogen-activated protein kinase–extracellular signal regulated kinase (MAPK/ERK) pathway and activation of the PI3K/Akt pathway [15], leading to critical roles in cell survival, growth, and motility.

The SGKs are serine/threonine kinases that were initially found to act, as the name implies, in response to serum, glucocorticoids, cytokines, as well as other growth factors, through phosphorylation by both PDK1 and mTORC2 [13]. SGKs also have additional roles in the regulation of both ion channel and enzyme activity, including the ubiquitin ligase NEDD4, and with the transcription factors beta-catenin and p53. Through its downstream targets, the SGKs play a role in regulation of transport, hormone release, neuroexcitability, inflammation, cell proliferation, and apoptosis [16]. Of note, SGK3 harbors a PX domain that, while related to PH domains, has a distinct binding affinity for 3′-phosphoinositides found in the endosomal compartment [17].

1.1.2 The CaMK Family

The CaMKs also are basophilic amino acid directed with the majority of kinases in this group initially activated by calcium/calmodulin binding [5], followed by subsequent autophosphorylation. Binding of Ca^{2+}/calmodulin induces a conformational change that exposes the activation loop site for phosphorylation by other CaMK kinases [18]. As CaMKs are activated through Ca^{2+}, their ultimate functions lie in processes intimately associated with intracellular calcium levels, including excitation – contraction coupling in muscle [19], neuronal development, and synaptic plasticity [18].

Two other well-characterized members of this structurally related family are Myosin Light Chain Kinase (MLCK) and AMP-activated Protein Kinase (AMPK). MLCK is activated following release of calcium from sarcomeres in skeletal muscle [20]. Once activated, MLCK phosphorylates myosin, ensuring cross-bridge formation and subsequent muscle contraction [20]. Activation of AMPK occurs through binding of AMP, resulting in allosteric activation, and phosphorylation on Thr172 by the upstream kinase LKB1 [21]. AMPK acts to promote metabolic pathways that generate ATP: increasing glucose uptake, inactivating acetyl-coA carboxylase (ACC) to inhibit fatty acid synthesis, and enhancing fatty acid oxidation, as well as stimulating mitochondrial biogenesis through either phosphorylation of PGC1-α or activation of SIRT1, leading to inhibition of both protein and rRNA syntheses [22].

1.1.3 CMGC Family Kinases

The CMGC subfamily of protein kinases is composed of four groups; the cyclin-dependent kinases (Cdks), the mitogen-activated protein kinases (MAPKs), glycogen synthase kinase-3 (GSK-3), and Cdk-like kinases [23]. Cdks are a family of proline-directed serine/threonine protein kinases comprising 21 genes that encode a Cdk, with 5 additional genes encoding Cdk-related kinases. While nomenclature differs, approximately 20 different Cdk have been identified [24]. Cdk activation is typically achieved through binding of cyclin subunits, as in the

classic example of Cdk2 activation via cyclin A association. Other Cdks, such as Cdk4, require additional partners for full activation, as cyclin D binding alone does not confer the active conformation [25–27]. Cdk4 and Cdk6 play roles in promoting entry into the cell cycle and lead to inhibition of the retinoblastoma (Rb) protein. After DNA duplication in S phase, Cdk1 becomes activated promoting chromosome condensation and nuclear envelope breakdown [27]. Cdk5 is an atypical Cdk highly expressed in neural tissues. Its function is less associated with cell cycle regulation [28, 29] and is activated by the non-Cyclin proteins p35 and p39 [28]. Cdk5 functions in cytoskeletal organization, membrane trafficking, and neuronal differentiation [28].

MAPKs are also proline-directed and regulate proliferation, differentiation, apoptosis, survival, inflammation, and innate immunity [30]. The signaling cascades that lead to their activation consist of a hierarchy of kinases, each phosphorylating and activating the next kinase in the pathway. Following a stimulus, the activation of Extracellular signal-Regulated Kinase (ERK) cascade is triggered by the mitogen-activated protein kinase kinase kinases (MAP3Ks), which includes Raf [31, 32]. The MAP3K in turn activates the mitogen-activated protein kinase kinases (MAP2Ks), MEK1/2, leading to dual tyrosine and threonine phosphorylation of ERK [32–34]. Two other pathways that primarily respond to cellular stress stimuli are similarly structured. In these cases, the p38 MAPK and Stress-Activated Protein Kinase/Jun N-terminal Kinase (SAPK/JNK) proteins are activated by the MAP2Ks MKK3 and 6 or MKK4 and 7, respectively. These MKKs are respectively activated by MAP3Ks such as ASK1, MEKK, and MLK3 (mixed-lineage kinase) [32–34]. Each MAPK tends to comprise several isoforms with varying tissue distribution and function, although these typically overlap.

The ERK1/2 MAPK cascade is triggered by extracellular stimuli including growth factors and hormones and regulates cell proliferation and differentiation through control of certain transcription factors including myc, Ets, CREB, Jun, and Fos [32]. ERK5 is usually described separately from the rest of the ERK1/2 pathway as its activation pathway (MAPKKK and MAPKK) differs from that of ERK, p38MAPK, and SAPK/JNK. ERK5 activation is triggered by both cellular osmotic stress and mitogens [35, 36], and downstream substrates include SGK and the transcription factors c-myc, MEF2, and c-fos [37]. p38 MAPK has four isoforms termed α to δ. The α and β isoforms can be triggered in response to cellular stress, as well as immune responses and inflammation [37]. Downstream targets of p38 MAPK include the MAPKAPKs MNK1/2 and MSK1/2, heat shock proteins, and the transcription factors ATF2 and MEF2C [37]. Similar to p38 MAPK, SAPK/JNK is triggered in response to cellular stresses. Although the three known genes of SAPK/JNK are expressed in most tissues, SAPKβ/JNK3 expression is primarily found in neuronal cells [30, 38]. Following activation by MKK4/7, SAPK/JNK transmits the signal to a variety of mediators including the MAPKAPK3 and the transcription factors ATF2, c-Jun, and Elk1 [39].

Two genes encoding GSK-3 exist in mammals, α and β, which share high sequence homology of up to 98% in their catalytic domains [40]. GSK-3 is active

in the basal state and serves to regulate activation of numerous cell signaling pathways including the Wnt, Notch, Hedgehog, and growth factor signaling pathways [42]. Mitogenic pathways activated through stimulation of PI3K induce phosphorylation and inhibition of GSK-3 at serines 21 and 9 in the α and β isoforms, respectively. This mechanism is irrelevant to regulation of GSK-3 by other signals such as Wnt due to insulation of a small (<10%) of the protein kinase within specific complexes [41]. By this means, the same protein kinase can act downstream of several systems yet be controlled in a highly specific manner. A common theme to many of the substrates of GSK-3 is that their phosphorylation sites fall within "phospho-degrons" that when phosphorylated lead to targeting for ubiquitination and degradation by the 26S proteosome [42]. Other substrates are suppressed in their function (such as glycogen synthase, the rate-limiting enzyme of glycogen synthesis) [42]. GSK-3 might therefore be thought of as a constitutive cellular brake that acts to dampen functions, and its negative effects are selectively switched off by specific signals, permitting induction of a variety of cellular processes. Genetic studies have revealed that despite high homology, GSK-3α and β have nonredundant functions. GSK-3α knockout mice are viable, while mice lacking GSK-3β either die late in embryogenesis due to significant liver apoptosis or just after birth due to heart-patterning defects [43–45].

1.1.4
STE Family Kinases

The STE (for "sterile") family of kinases contains a variety of atypical kinases including WNK (With No Lysine) and the MAPK cascade kinases [5]. WNK kinases differ from all other kinases as they lack a lysine in β sheet 3, which is instead located in β sheet 2 [46]. These kinases are involved in a variety of signaling pathways where, for example, WNK1 acts as an upstream MAP4K for ERK5. The WNKs themselves can be regulated by Akt/PKB- and SGK-mediated phosphorylation [47]. Recognized functions of WNK include cell proliferation, differentiation, exocytosis, and critical roles in chlorine, sodium, and potassium transport [47].

The STE family also includes the MAPK cascade kinases (MEK1/2, MKK3/4/6/7), which transmit the extracellular stimulus signal to the MAPK, leading to alterations in cell proliferation and survival. The STE20 subfamily consists of MST, PAKA, and PAKB and serve as MAP4Ks, which transmit signals to the STE11 (MAP3K) family of which ASK1 and MEKK2 are two examples [48].

1.1.5
Tyrosine Kinases

This family consists of two distinct classes: membrane-bound receptor tyrosine kinases (RTKs) and non-RTKs. RTKs are transmembrane proteins harboring an extracellular domain that contains high-affinity binding sites for ligands and a

C-terminal, intracellular domain which includes a protein kinase domain with specificity for tyrosine. Examples include many growth factor receptors including those for platelet-derived growth factor, epidermal growth factor, colony stimulating factor 1, insulin, insulin-like growth factor, heregulins, vascular endothelial growth factor, angiopoeitins, nerve growth factor, and so on [49]. This family is most targeted pharmaceutically due to the identified roles of these enzymes in many forms of cancer (such as breast, lung, stomach) as well as angiogenesis (VEGF). Therapeutic agents targeting RTKs have flourished in the past decade and are either monoclonal antibodies to the extracellular domain (e.g., Herceptin) or small molecule inhibitors to the intracellular tyrosine kinase domain (e.g., Tarceva, Iressa) [50]. The substrates of tyrosine kinases tend to be primarily their dimeric partners, which are phosphorylated at multiple tyrosines lying within a C-terminal tail domain. The phosphorylated residues, within local amino acid sequence contexts, recruit proteins harboring Src Homology 2 (SH2) or PhosphoTyrosine Binding (PTB) domains that include the regulatory subunits of PI3K, certain protein tyrosine phosphatases, phospholipase Cγ, and adaptor proteins such as Grb2, SHC, Gab1, and the insulin receptor substrate (IRS) proteins, which, in turn, recruit other signaling proteins [51].

In comparison, non-RTKs are typically intracellular proteins and can also associate with membranes to serve as catalytic domain donors to RTK that lack such a domain [52]. This family includes the three Src-like kinases (Src, Fyn, and Yes) as well as Lck, which lies downstream of the T-cell receptor and c-Abl, the infamous kinase that as a result of the 9 : 22 translocation associated with the Philadelphia chromosome in chronic myeloid leukemia (CML) is oncogenically activated (Bcr-Abl) [53, 54]. This latter kinase is also the target for the poster-child of molecularly targeted anticancer agents, Gleevec/imatinib, which has had remarkable impact in the clinical treatment of CML. Indeed, Gleevec is so effective in blocking the activity of Bcr-Abl that patients in whom the disease returns virtually exhibit mutations in the tyrosine kinase that interfere with Gleevec binding. This has given rise to second-line tyrosine kinase inhibitors designed to target common resistance mutations such as nilotinib [55]. In spite of their name, many of the non-RTKs act in close association with RTKs.

1.1.6
Casein Kinases

There are two primary subclasses of casein kinases. Casein kinase 1 (CK1) consists of eight genes in mice, which function as monomeric enzymes: α, γ1, γ2, γ3, δ, ε1, ε2, and ε3. CK1 is basally active and serves as a priming kinase for other protein kinases such as GSK-3 (in the case of β-catenin and LRP5/6) and an upstream regulator of Cdk5 [56–58]. These are under-appreciated kinases that touch upon many cellular processes through selective association with macromolecular complexes. Like its distally related cousins, the casein kinase II subfamily is implicated in a wide panoply of cellular events, largely via basal activity although this kinase has also been implicated in animal tumors. Notably, neither subclass is a

physiological casein kinase. That role is owned by Fam20C, a protein without obvious structural similarity to protein kinases [59].

1.1.7 Tyrosine Kinase-Like Family

This is a diverse group containing both serine and tyrosine protein kinases that includes MLK, LISK, IRAK, Raf, RIPK, and STRK [2]. Despite its name, this family is strictly comprised of ser/thr specific kinases that act as intermediaries in a number of signaling cascades. Their structure suggests that they may have been evolutionary stepping stones toward tyrosine kinases (the yeast genomes are effectively barren of "true" tyrosine kinases, although Wee1 targets Cdc2 at Tyrosine 15 [60].

1.1.8 RGC Kinases

The RGCs is an atypical family with a domain sequence similar to tyrosine kinases that possess enzymatic capabilities [2]. Members of this family are activated by hormones, peptides, and low calcium-induced guanylyl cyclase-activating proteins [61]. They possess an active guanylate cyclase domain, which generates cGMP, and a catalytically inactive kinase domain [62]. RGC have a wide array of substrates and have roles in hypertension, cardiac hypertrophy, and vision [63–65].

1.1.9 Atypical/Other Protein Kinases

This subgroup includes kinases that lack primary sequence similarity to the canonical protein kinase profile, but have experimentally demonstrated protein kinase activity. The subgroups include Alpha Kinases, ABC1, pyruvate dehydrogenase kinases, RIO, Bromo Domain kinases (BRDs), TATA binding factor associated factors (TAFs), BCR, FASTK, G11, Transcriptional Intermediary Factor 1 family (TIF1), H11, Fam20C, and PIKK, a member of the PI3K-related kinases [2]. Of this diverse group, one PIKK in particular has been well studied, FRAP, better known as mTOR.

mTOR is an atypical serine/threonine kinase [13] involved in the regulation of cell growth, proliferation, and metabolism and is associated with two distinct complexes termed mTORC1 and mTORC2. In mTORC1, mTOR interacts with Raptor, Deptor, and GβL to regulate protein translation and metabolism through phosphorylation of downstream targets including 4E-BP1, S6K, SREBP [13]. In the mTORC2 complex, mTOR interacts with Rictor, GβL, Sin1, Deptor, and PRR5 and is activated in response to extracellular cues. It remains to be elucidated via what molecular mechanisms these cues are "sensed," though mTORC2 carries out its role via phosphorylation of substrate kinases in the AGC family [13, 66].

1.2 Why Study Protein Kinases – Their Roles in Disease

After the sequencing of the human kinome was completed, the kinase chromosomal map was compared to disease loci, revealing 164 kinases that map to sites frequently observed in tumors [2, 67], with 80 kinases mapping to other (non-cancer) disease loci [2]. The remarkably high proportion of kinases associated with such loci highlights the important role of protein kinases in disease initiation and progression. Below we exemplify the role of key protein kinases in the pathophysiology of just two of the chronic diseases that affect tens of millions of people worldwide: Alzheimer's disease (AD) and cancer.

1.2.1 Neurodegenerative Disease

AD is a progressive neurodegenerative disorder characterized by both the loss of acetylcholine and neuronal overstimulation, leading to neuronal decay and impairments in both cognition and behavior [68]. The pathological hallmarks of AD progression include protein deposits found inside (neurofibrillary tangles – NFTs) and outside (amyloid beta – Aβ plaques) of the neuron [68]. In healthy individuals, the processing of amyloid precursor protein (APP) is regulated by α-secretase, which cleaves a 695 amino acid fragment, which is further processed by a second enzyme, γ-secretase. This is described as the nonamyloidogenic pathway, as it does not produce the toxic Aβ peptides. The production of amyloid plaques in Alzheimer's patients is regulated by the amyloidogenic pathway in which Aβ peptides are generated through increased APP cleavage by β-secretase. The cleavage of APP by β-secretase releases a longer fragment that is subsequently cleaved by γ-secretase. APP cleavage initiated by β-secretase results in the formation of peptides of varying length including Aβ40 and Aβ42. These two peptides differ in their effects on neighboring neurons as Aβ42 peptides have neurotoxic properties, at least *in vitro* [69]. Recent studies have suggested that it is the relative ratio of these two peptides, and not their absolute quantities, which is key in the induction of neurotoxicity [70, 71].

The microtubule-associated protein Tau is important for the assembly and stabilization of neurofilaments, which play key roles in axonal growth and transport [72]. In healthy individuals, Tau phosphorylation typically occurs to target the protein to various compartments and is essential for its mobility within the neuron [72]. In AD, hyperphosphorylation of Tau leads to its dissociation from the microtubule and the formation of an insoluble complex of paired helical fragments. These fragments then aggregate together inside the neuron, leading to the formation of NFT [73, 74], resulting in decreased transport of essential components within the neuron and the eventual loss of the neuron.

Protein kinases have well-documented roles in the progression of AD and in the two pathological processes that are characteristic of the disease. The CMGC group of protein kinases have been of particular focus due to the significant

consequences of alterations in GSK-3, neuronal-enriched Cdks, and MAPKs in the brain. Increased GSK-3 activity has been documented in postmortem analysis of the brain of suspected Alzheimer's patients through increased protein expression and phosphorylation at tyrosine sites linked to increased activity [75–77]. Studies have connected GSK-3 expression to both amyloid- and tau-associated pathologies in AD [78–80]. Treatment of rat hippocampal neurons in culture with Aβ peptides increases GSK-3 activity [81], and GSK-3β was specifically shown to regulate processing of APP by β-secretase through NF-κB-induced BACE1 promoter activity and gene expression both in culture and in mouse models [82]. Tyrosine-phosphorylated GSK-3 also has been shown to colocalize with NFT [77], and GSK-3 acts as a physiological tau protein kinase, playing a significant role in the hyperphosphorylated state of tau observed in brain slices from AD-afflicted individuals [83].

The roles of GSK-3 in neurobehavior and neuronal health have been assessed in mice with altered GSK-3 expression in various brain regions. Transgenic overexpression of GSK-3β in neurons of the cortex and hippocampus resulted in tau hyperphosphorylation, neuronal death, and reduced spatial learning and memory, as assessed by the Morris water maze test [78, 79, 84]. The tau pathology was linked to the effects on spatial learning and memory, as performance on the Morris water maze test improved through tau deletion in these mice [85]. A conditional mouse model was later used to demonstrate the effect of both increased GSK-3 activity on forebrain neurons and the consequence of lowering activity back to normal levels. Increased GSK-3 activity leads to hyperphosphorylation of tau, neuronal loss, and deficits in spatial learning. Following reduction of GSK-3 activity, phosphorylation of tau decreased, neuronal loss was diminished, and deficits in spatial learning were prevented [86]. Also, inhibition of GSK-3 activity, either through pharmacological or genetic methods, in mouse models of AD had neuroprotective effects, with decreased Aβ production and reduced hyperphosphorylation of tau observed [87]. Treatment of rats with lithium, a relatively nonspecific inhibitor of GSK-3 [88, 89], before and after infusion of Aβ fibrils was shown to prevent spatial memory deficits, as well as tau hyperphosphorylation [90, 91]. In the TgCRND8 AD mouse model, lithium treatment also rescued deficits in spatial learning and memory [92, 93]; however, studies on the effects of lithium on AD and cognition have not all shown benefit. Chronic lithium treatment begun after amyloid plaque development in TgCRND8 mice showed no improvement in cognition, even though the average size of the Aβ plaques was reduced [93]. Also, treatment of early stage AD patients with lithium showed no improvement in cognition [94]. Currently, a series of small molecule inhibitors are being developed and tested to target GSK-3, and recent studies have shown cognitive improvements and reduced tau phosphorylation and neuron degeneration in mouse models of AD [92, 95, 96].

Cdk5 is a unique Cdk, as it is highly expressed in postmitotic neurons [97], and in the adult brain it regulates neuronal survival, synaptic plasticity, and in the formation of learning and memory [97]. In AD, truncation of the Cdk5-regulating protein p35 occurs in response to Aβ-induced activation of calcium-dependent proteases, the calpains, chopping down p35 to p25 [97]. As the p25 form of the

activating subunit is resistant to ubiquitin-mediated proteolysis, it extends the activation time of Cdk5, leading to hyperphosphorylation of its substrates, including tau [68, 74, 98]. The phosphorylation of tau by Cdk5 also serves to prime it for further phosphorylation by GSK-3, heightening the level of tau hyperphosphorylation [99]. Mice with regulatable expression of p25 displayed brain atrophy, synaptic dysfunction, and NFT formation [100]. Knockdown of Cdk5 expression in a mouse model of AD reduced tau phosphorylation as well as the accumulation of NFT and rescued hippocampal neuronal loss [101]. The development of Cdk5-specific inhibitors has been hampered by the high sequence homology between the various Cdk isoforms. A recently described Cdk5-specific inhibitor was shown to decrease Aβ production in both young and old APP mutant mice, but in young mice it also relieved inhibition of GSK-3, leading to increased hyperphosphorylation of tau [102].

All three MAPK family members have documented roles in AD pathophysiology, in part, through phosphorylation of tau at various sites [103, 104]. Both the ERK and SAPK/JNK pathways play a role in Aβ-induced neurotoxicity as exposure of hippocampal neuronal slices to Aβ peptides induced cell death via ERK-induced caspase-3 expression [105] and exposure of cortical neurons to Aβ peptides activated SAPK/JNK leading to Fas-dependent neuronal death [106]. Infusion of a MEK inhibitor into mice displaying Aβ- and tau-induced pathology and behavioral deficits reversed the decline in memory displayed by these animals [107]. In response to oxidative stress, SAPK/JNK activation also has been shown to increase phosphorylation of APP, which promotes generation of Aβ peptides [108]. The Aβ42 peptide has specifically been shown to induce SAPK/JNK-mediated BACE1 expression, further leading to increased Aβ peptide generation [109]. This effect of SAPK/JNK on Aβ generation has been confirmed *in vivo*, where SAPK/JNK inhibition decreased Aβ peptide generation and plaque deposition in a mouse model of AD [110]. SAPK/JNK activation also has been demonstrated in the synapse prior to the establishment of cognitive impairments and treatment of TgCRND8 mice, a model of AD, with a jun N-terminal kinase (JNK) inhibitor prevented both the loss of postsynaptic proteins and reduction in excitatory synapse size, preventing neural spine degeneration [30, 111].

Activation of the p38 MAPK pathway in AD has also been reported to play a significant role in pathophysiology of the disease [112–115]. Patients in the early stages of AD show evidence of p38 MAPK pathway activation in both their neurons and glia, which has been linked to glial proinflammatory cytokine production and synaptic dysfunction [112–114, 116]. p38 MAPK may also contribute to tau phosphorylation, leading to its dissociation from microtubules and aggregation into NFT [83, 117]. The role of p38 MAPK in AD-associated neuroinflammation was highlighted in recent studies, where p38 MAPK inhibitor treatment decreased both Aβ plaque accumulation and Aβ-induced production of inflammatory regulators IL-6 and TNF-α [30, 118]. A novel p38alpha MAPK inhibitor, MW150, has been tested for efficacy on memory-related deficits associated with AD in two mouse models of AD: one overexpressing APP, leading to rapid onset of symptoms, and one utilizing endogenous promoters with no overexpression and slow

progression. In both models, treatment with the p38α MAPK inhibitor halted AD-associated deficits in memory, as assessed by the radial arm water maze test and the contextual fear conditioning test [119].

Several members of the AGC kinase family also have described functions in the progression of AD. Under normal physiological conditions, mTOR plays a role in tau protein synthesis and phosphorylation via activation of p70S6K [120]. In contrast, it has recently been reported that Aβ accumulation inhibits the mTOR pathway and that upregulation of mTOR signaling prevents Aβ-induced deficits in long-term potentiation [121, 122]. In addition, treatment of rats with rapamycin, a potent and selective mTOR inhibitor, was shown to enhance the neurotoxicity of Aβ peptides [123]. Like mTOR, PKC isoforms appear to have contrasting roles with other kinases in AD. Activation of PKC isoforms stimulates APP cleavage by α-secretase, resulting in decreased Aβ peptide production, both in culture and in mouse models of AD [124–126]. Also, Aβ has been shown by various researchers to inhibit PKC activation via multiple mechanisms, including direct binding via a putative PKC pseudosubstrate domain [127, 128]. Recent studies have shown that human PKCε overexpression reduces Aβ expression and increases expression of endothelin-converting enzyme type 1, which plays a role in Aβ clearance [129, 130].

Overall, these studies have revealed the integral role for several protein kinases in the progression of AD, and future work needs to focus both on further increasing our understanding of the mechanisms of kinase action in the progression of this disease, as well as developing novel therapies to target these kinases to halt disease progression. A substantial challenge in the field is that onset of symptoms usually occurs long after neuronal loss has occurred due to the remarkable synaptic plasticity and neuronal rewiring. As a consequence, effective therapies are likely going to need to be used prior to presentation of symptoms, raising the bar for safety of any prophylactic intervention.

1.2.2 Hallmarks of Cancer

Cancer is the result of a complex series of events that lead to uncontrolled growth and spread of tumor cells. Hanahan and Weinberg described the six hallmarks of cancer, which include sustaining a proliferative signal, evading growth suppressors, activation of invasion and metastasis, resisting cell death, inducing angiogenesis, and enabling replicative immortality [131]. Protein kinases have documented roles in each of these cancer hallmarks, and therefore extensive study is ongoing to assess their role in cancer progression and to ascertain whether their inhibition would be a useful therapeutic approach.

The initiation of disruptions to normal growth restrictions within a cell typically occurs via mutation of critical signaling components. Mutations in protein kinase signaling pathways account for a high proportion of the total amount of driver mutations found in human cancers. Point mutations in the PIK3CA gene (hotspots include E542K, E545K, and H1047R), coding for the p110α subunit of

PI3K, are among the most frequent in human cancer and all result in enhanced catalytic activity of PI3K [132, 133]. Aside from gene mutation, activation of PI3K, and its downstream target Akt/PKB, are observed in breast, ovarian, pancreatic, esophageal, and thyroid cancer due to gene amplification [132]. In mouse models, expression of a single PIK3CA point mutation targeted to the mammary gland was sufficient for mammary tumor growth [134–137]. In contrast, PIK3CA point mutation expression in mouse models of colorectal, ovarian, endometrial, and lung cancer required a separate cooperating mutation in another pathway in order for tumor growth to occur [138–141]. Constitutively active Akt/PKB isoforms do not induce mammary gland tumorigenesis when expressed via the MMTV promoter [142, 143], indicating that overexpression of Akt/PKB alone is insufficient for mammary tumorigenesis. Following this result, the role of the Akt/PKB isoforms was examined in the context of mouse models of breast cancer driven by PyMT or ErbB-2 [8]. Constitutively active Akt1/PKBα was shown to accelerate tumor growth in the ErbB-2 mouse model of breast cancer, with tumors detectable at earlier time points but with less metastasis observed. In contrast, constitutively active Akt2/PKBβ had no effect on tumor latency but metastasis to the lung was increased [144, 145]. Akt1/PKBα and Akt2/PKBβ knockouts crossed to the PyMT and ErbB-2 mouse mammary tumorigenesis models also revealed opposing effects with loss of Akt1/PKBα resulting in delayed tumor onset, while loss of Akt2/PKBβ accelerated tumor growth [8, 146].

Due to its high mutation and amplification rate in human cancer, PI3K represents an important node for drug discovery research. Currently, numerous pan-PI3K and dual PI3K/mTOR inhibitors are at varying stages of clinical trials for treatment of breast cancer, nonsmall cell lung cancer, and renal cell carcinoma [147]. To date only one PI3K inhibitor has received regulatory approval. Idelalisib, a specific PI3Kδ inhibitor, was approved in mid-2014 for combination treatment of patients with B cell malignancies [148, 149].

mTOR lies downstream of both the Ras/MAPK and PI3K pathways, where mutations in oncogenes or tumor suppressors are found in a high percentage of human cancers, leading to constitutive activation of mTORC1 and resultant tumor growth [150, 151]. The mTORC1 inhibitors rapamycin and rapalogs Temsirolimus and Everolimus are approved for the treatment of renal cell carcinoma, hormone refractory breast cancer, and pancreatic neuroendocrine tumors [152–154]; however, effects in patients have been modest as while these inhibitors affect cell proliferation, they are not toxic to tumor cells [151]. Also, the mTORC1 plays a role in feedback loops to repress signaling through RTKs, and inhibition of the complex allows this signaling system to remain unchecked [151, 155].

Activating mutations in PKC isoforms in cancer are rare [15], while PKC isoforms show altered expression in various cancers including PKCε in stomach, lung, and breast cancer, PKCθ in GIST, and PKCη in nonsmall cell lung cancer [14]. Using selective isoform inhibitors in mouse models of breast cancer, it has been shown that augmenting PKCα expression abrogates metastasis without an effect on primary tumor growth [156]. The role of PKCβ in tumor progression is

somewhat controversial as inhibitor studies have shown growth suppression in mouse xenograft models, whereas overexpression in mouse mammary cell lines has revealed suppression of both tumor growth and metastasis [15, 157, 158]. The novel PKCε isoform is considered oncogenic as early studies revealed that overexpression of PKCε in NIH 3T3 cells injected into mice induced tumor growth, while untransfected and PKCδ overexpressing cells failed to grow [159, 160]. Knockdown of PKCε in MDA-MB-231 breast cancer cells has been shown to significantly reduce both primary mammary tumor growth and metastasis to the lung [161]. PKCι overexpression in cancer has been linked to the amplification of its gene PRKCI, which is located on chromosome 3q26, one of the most commonly amplified regions in human cancer [162], with copy number gains established in lung squamous cell carcinoma and serous epithelial ovarian cancer [162]. PKCι knockdown in esophageal cancer xenografts caused decreases in tumor growth and metastasis to the lung [163].

The CMGC group of protein kinases also have well-documented roles in cancer progression and serve as drug discovery targets for new therapeutics. Melanoma, thyroid cancer, and colon cancer all show evidence of alterations in the MAPK pathway, with this activation, in many cases, the result of activating point mutations in RAS family members (HRAS, KRAS, and NRAS) [164, 165]. Trametinib is the first MEK inhibitor approved for cancer treatment [164], and recent studies have shown that combination with B-Raf inhibitors results in enhanced antitumor activity in patients with melanoma [166]. The p38α MAPK isoform has also been shown in preclinical models to play a role in resistance to various chemotherapeutic drugs for the treatment of colon cancer, as well as to kinase inhibitor treatment of hepatocellular carcinoma [30, 167, 168]. Overactivation of SAPK/JNK has been observed in hepatocellular carcinoma and mouse models of this disease in which JNK1 has been inactivated show decreases in cell proliferation and tumor formation [169, 170]. JNK2 is also implicated in tumor progression, as ablation of this kinase blocks chemical-induced skin carcinogenesis in the mouse [171].

The transcriptional regulator Snail plays an important role in epithelial–mesenchymal transition (EMT) of various solid tumors including gastric, liver, and colorectal cancer [172–174]. This protein is rapidly turned over by ubiquitin-mediated degradation, triggered by phosphorylation by GSK-3. If GSK-3 activity is inhibited, Snail protein accumulates and represses transcription of the epithelial cell adhesion protein, E-Cadherin (as well as promoting mesenchymal gene expression). GSK-3 overexpression has been observed in colon, liver, ovarian, and pancreatic tumors [175], while inhibition of GSK-3β activity promoted development of hepatocellular carcinoma [176], as well as stabilization of β-catenin and mammary gland hyperplasia [177]. Knockdown of GSK-3 was shown to inhibit pancreatic cancer growth and angiogenesis [175, 178] associated with decreased Bcl-2 and VEGF expression. Studies have also highlighted GSK-3 as a tumor suppressor as the overexpression of a constitutively active form of GSK-3 lead to increased chemosensitivity, cell cycle arrest, and reduced tumorigenicity of breast cancers [175]. GSK-3β has also been identified in several "sleeping beauty" mediated screens for tumor suppressors [179, 180].

As observed in these studies, as well as many others we do not have space to include protein kinases play a complex role in the initiation and progression of cancer. Using our increased understanding of the role of kinases in these processes, coupled with remarkable advances in medicinal chemistry that is increasing selectivity and potency of kinase inhibitions, should allow for improved and precise therapeutics to help conquer kinase-inhibitor drug resistance typically observed in drugs currently approved for therapeutic use.

1.3 Methodology for Assessment of Protein Kinase Functions

While AD and the many forms of cancer clearly impose huge burdens on patients and healthcare, there are many other conditions for which modulation of protein kinase activities offer promising potential including diabetes, arthritis/autoimmunity, viral infections, and psychiatric disorders. That said, there are significant hurdles in targeting these cellular regulators. It is critical to remember that signaling pathways play many distinct roles in distinct tissues and organs in response to a wide array of stimuli. Protein kinases are the cells "mail delivery system" and most drugs are indiscriminate in terms of where they act. Hence, therapeutic options for targeting members of this class of signaling proteins must take into account the possible impact on other areas of the body where these signaling molecules are essential for providing normal cellular homeostasis. A first step in delineating physiological functions of these pleiotropic enzymes is measurement of their normal functions and how these are perturbed – a task assisted by new technologies that are relatively unbiased.

1.3.1 Mass Spectrometry

Protein mass spectrometry (MS) monitors the mass-to-charge ratio of ionized peptides followed by further fragmentation of the peptides to obtain the mass-to-charge ratio of the components producing a spectrum that identifies unequivocally not only the peptide but also any posttranslational modifications including phosphorylation [181]. As analysis of MS data accumulated, it became clear that previously identified phosphorylation sites represented a tiny portion of those actually present when assessed in an unbiased manner. Databases such as PhosphoELM (*http://phospho.elm.eu.org*) provide searchable interfaces for probing all phosphorylation, as well as other modifications, identified to date, by protein. This avalanche of new data uncovered a major gap in understanding and raised important questions. Firstly, because a phosphorylation site exists, does that mean it is physiologically relevant? Secondly, what are the kinases and phosphatases acting on these sites? Several approaches have been developed to try to answer these questions. One, termed NetworKIN (part of a suite of kinase bioinformatics tools from the University of Copenhagen: *http://kinomexplorer.info*), uses regular

expression analysis of PubMed abstracts along with databases such as PhosphoELM and known primary sequence preferences to predict the kinases responsible for modification of any given phosphorylated residue. This type of information can play a vital role in assessing the likely importance of a site and provides insight into experimental approaches for validation. Other approaches have mapped the changes observed in response to treatment of cells with protein kinase inhibitors. MS analysis of the impact of the broad-spectrum kinase inhibitor CTx-0294885 revealed 235 protein kinases that were targetable with this inhibitor in the human breast cancer cell line MDA-MB-231 [182]. Of note, this screen revealed the interaction of this broad-spectrum inhibitor with all Akt/PKB isoforms as well as previously unreported phosphosites on HIPK2, a CMGC protein kinase and MELK, a CaMK [182].

Affinity-purification mass spectrometry (AP-MS) combines the specificity of antibody-based affinity purification with the sensitivity of MS. A typical work flow involves immobilizing a ligand, usually a well-characterized antibody, on a solid support such as an agarose or magnetic bead, allowing for capture of a target protein or protein complex from the soluble phase [183, 184]. Advantages of AP-MS include scalability, as it can be performed on as many proteins as can be epitope-tagged and/or antibodies that are available for immunoprecipitation, the fact that it can be performed on proteins in their native cellular environment and that it can monitor dynamic changes in protein–protein interactions [181]. Following AP-MS, it is possible to integrate the information on disease loci with protein interaction data to gain a new understanding of how kinases may control disease pathways. In a study by Varjosalo *et al.*, AP-MS data was used to identify 91 disease-associated proteins that physically interacted with CMGC kinases, with cancer being the most common disease associated with the CMGC interaction proteome. As an example, CDK6 was found to interact with cyclin D2, cyclin D3, and the CDK inhibitor p18, which are linked to various tumor types including non-Hodgkin lymphomas and glioma. These results served to confirm previously obtained results as well as showcase new potential mediators as CLK2 was shown to form interactions with SETD2, a methyltransferase that is linked to renal carcinoma [23, 185].

Another study used AP-MS to identify interacting partners with three critical proteins in colorectal cancer, namely adenomatous polyposis coli (APC), a tumor suppressor protein that is mutated in over 80% of colon cancers, MRE11A, a component of the MRE11A-RAD50-NBN complex, involved in the response to DNA damage and DNMT1, a DNA methyltransferase involved in the regulation of DNA methylation. AP-MS confirmed previously identified binding partners, validating the system, and also combined subcellular fractionation to localize protein–protein interactions to specific cell compartments [184]. In particular, although levels of APC and Flightless-1 homolog (FLII) were higher in the cytosol compared with the nucleus, MS revealed that their interaction only occurred in the nucleus, which is in line with previously published reports of FLII associated with β-catenin in the nucleus [186, 187]. Although this study did not examine protein kinases in particular, the technique could be applied to kinase interactions

that are critical within a particular disease model and the subcellular compartment in which they take place – an important element of cellular compartmentalization and signaling specificity.

Liquid chromatography tandem mass spectrometry (LC-MS/MS) is a further MS technique useful in the study of protein kinases in disease as it allows protein identification within mixed solutions. Recent studies have employed this technique to search for novel liquid biopsy markers in AD. Analysis of plasma from Tau transgenic mice and control mice revealed that changes in protein levels could be detected in the plasma, even during early stages of disease progression. This study confirmed three potential biomarkers in Histone H2B type 1-F/J/L, Clusterin, and Transthyretin, which were increased in the plasma of tau mice. Adenosine kinase was also decreased in the plasma of Tau transgenic mice during the early stages of disease progression, which was linked to the neurotoxicity observed in AD, as decreased adenosine kinase activity can lead to adenosine accumulation within the cell [188].

A recent innovation variation of traditional MS is MIB/MS, in which beads containing multiple kinase inhibitors are used to capture any kinases capable of interacting with the inhibitor, with the identity of the captured kinases determined through normal MS workflow. This technique can provide a snapshot of the signaling occurring in a particular tumor or tissue and can also be used to assess changes in kinase activity following drug treatment, for example, revealing potential drug resistance within a given tumor [189]. In response to MEK inhibition, the kinase activation profile is altered within 24 h, leading to the reactivation of the MEK/MAPK pathway within a few days [190]. This technique has also been used to compare kinase activation profiles between leukemia cells lines that were sensitive or resistant to imatinib treatment. MIB/MS revealed 35 significant kinases that are altered in imatinib-resistant cells including increased activity of MEK and ERK, while activity of Kit and SAPK/JNK were decreased [191]. These data allowed for relevant drug combinations to be tested in human cancer cell lines, revealing that cotargeting with inhibitors of MEK or IKKα, resulted in decreased cell viability and increased apoptosis – offering potential new treatments following resistance to the original therapy [191].

1.3.2
Fluorescence Resonance Energy Transfer

Fluorescence resonance energy transfer (FRET) is a technique for assessing direct molecular interactions between two molecules in their natural state (i.e., within cells). FRET consists of energy transfer from an excited donor fluorophore to a proximal acceptor fluorophore due to overlapping emittance and absorbance spectra. The energy transfer alters the amount and wavelength of the emitted energy, which can be quantified. This technique allows determination of how specific molecules interact as well as how this interaction may be altered during drug treatment [192]. When measured by fluorescence lifetime imaging microscopy (FLIM), FRET can be used to track kinase activity in fixed cells as well

as tissue samples. Analysis of mean fluorescence lifetime images revealed that while there is a small degree of PKCα activation in unstimulated cells, treatment with TPA, a PKC activator, rapidly increased PKCα phosphorylation/activation, validating the technique in the assessment of kinase activity. This activation could then be tracked to differing regions of the cell with activated PKC relocating to the perinuclear compartment [193]. FRET has also been used to ascertain novel binding partners of mTOR and exactly which proteins directly interacted within the mTOR complex. Use of GFP-mTOR and DsRED-Rheb, a GTP-binding protein, revealed direct interaction between these two proteins, which had been postulated via immunoprecipitation experiments. Through the use of these biosensors, it was also determined that Raptor and mTOR directly interact but Raptor and Rheb do not [194].

FRET can also be performed on formalin-fixed breast tumor sections, and this analysis revealed that only about half of the examined tumors showed increased PKCα activity, while the rest showed increases in expression with no effect on activity [193]. Studies on primary breast cancer tissue assays also have examined the utility of FRET to assess activity of a given enzyme in relation to prognosis, as current methods rely on staining intensity measurements by independent observers. FRET sensors monitoring Akt/PKB phosphorylation at Thr308 revealed heterogeneous Akt/PKB activity in breast tumors although higher phospho-Thr308 levels were correlated with worse disease-free survival [195].

This technique can also be used to identify novel binding partners of a particular kinase under a certain set of conditions or confirm interaction results obtained via high-throughput screens. Previous results had revealed that the AGC kinase ROCK1 activated LIMK2, and this interaction was hypothesized to be critical in membrane blebbing-mediated cell motility. FRET-FLIM allowed the interaction between ROCK and LIMK to be assessed in cells with spread morphology and those displaying membrane blebs. No FRET was detected between GFP-ROCK1 and mRFP-LIMK2 in blebbing cells, while the interaction was confirmed in cells with a spread morphology [196]. Also, time-resolved–fluorescence resonance energy transfer (TR-FRET) was used to assess receptor heterodimerization in response to drug treatment. TR-FRET combines the standard FRET technique with time-resolved (TR) measurement of fluorescence, involving the delayed measurement of fluorescence following excitation, in order to reduce the inclusion of short-lived, nonspecific emissions, to only capture long-lived fluorescence engaged in a FRET process [197]. TR-FRET examining EGFR-HER2 heterodimer formation in response to combination therapy drug treatment revealed that treatment with two monoclonal antibodies, cetuximab (EGFR inhibitor), and trastuzumab (HER2 inhibitor) resulted in significantly reduced pancreatic xenograft tumor growth, in part, due to significantly reduced heterodimer formation [198].

FRET is also being used in investigations of the mechanisms of drug resistance and the effectiveness of second-generation therapies. Mizutani *et al.* tested two second-generation therapies on imatinib-resistant cell lines. The biosensor used enabled the evaluation of BCR–ABL kinase activity in living cells and utilized

the downstream substrate of BCR–ABL, CrkL. In this study, FRET efficiency increased up to 40% in the presence of the BCR–ABL fusion, which could be decreased with imatinib treatment. In cell lines expressing the BCR–ABL fusion, these new inhibitors showed faster kinetics compared with imatinib; however, in imatinib-resistant cell lines, some sensitivity was observed by decreased FRET efficiencies with one of the drugs, while no change was detected in FRET in the other cell line tested with both second-generation inhibitors [199].

FRET techniques allow for the study of protein kinase activity in a variety of disease-related contexts. A resource for further examples on the use of FRET/fluorescence biosensors in probing kinase function and drug discovery can be found in a recent compilation [200].

1.3.3 Assessment of Kinase Functions *in vitro*: Genetic and Chemical

Knockdown of kinase expression in cell lines has been instrumental in establishing the complex roles of protein kinases in cellular function in both the normal and disease states. Establishing kinase knockdown in cells has relied on two main technologies: RNAi and chemical inhibitors of kinase function. RNAi relies on endogenous cellular processes, which following introduction of double-stranded (ds) RNA, posttranscriptionally silence gene expression [201]. It requires the introduction of target-specific dsRNA (short hairpin RNA (shRNA)) into the cell of interest through viral or nonviral methods. Once in the cell, the dsRNA is processed into short RNA sequences of approximately 21–23 nucleotides in length by the endogenous DICER protein and integrated into the RNA-induced silencing complex (RISC). In this complex, the antisense strand binds to mRNA with the complementary sequence, leading to mRNA cleavage and degradation, resulting in protein loss [202]. Given the critical role protein kinases play in disease progression, intense focus has been on the development of chemical inhibitors for the treatment of disease. The first kinase inhibitor was approved for use in 2001 and to date well over 20 have received approval, primarily for the treatment of cancer [203]. Chemical suppression of kinase function is elicited via two broad types of inhibitors: those which compete with ATP for active site binding, and non-ATP-based small molecule inhibitors, which target the site of substrate binding [203].

Part of the power of these two methods of kinase knockdown lies in the fact that they can be used in large screens: for the assessment of all kinases in a particular signaling pathway, cellular function, or disease state. The two methods are often used in parallel in order to overcome specific limitations associated with each. The use of genome/kinase wide screens requires careful determination of what read out will provide the greatest amount of information on kinase function. These screens can assess both negative and positive functionality for a given readout, such as cell viability. Negative screens can identify kinases required for survival such that their suppression results in cell death, and therefore the causative shRNA, dropping out of the final cell population. Positive selection screens can

use cell viability to assess the development of therapeutic resistance with knockdown of kinases required for drug sensitivity resulting in their enrichment within the final cell population. Below we highlight the use of both RNAi- and inhibitor-based screens in the assessment of protein kinase function.

The readout for many screens is cell viability; however, altered cell state (differentiation) and pathway activation can also be used to assess kinase function in genome/kinase wide screens. In melanoma cancer cell lines, the role of various MAPK and AGC kinase isoforms was established *in vitro*, revealing that knockdown of ERK1/2, SAPK/JNK, and PKCβ resulted in cell death. Follow-up studies established that knockdown of PKCβ alone reduced cell proliferation, colony formation, and migratory capacity [204]. Genetic- and chemical inhibitor-based screens were combined to assess Notch pathway activation in breast cancer cells. In cells expressing a Hes1-firefly luciferase reporter, Notch activation was augmented in cells treated with an ERK inhibitor, while in a kinase-specific siRNA screen, knockdown of TRB3, a regulator of the MAPK–ERK pathway, significantly reduced cell proliferation and tumor growth [205]. Genetic and chemical inhibitor screens were also carried out in parallel to assess regulators of myeloid differentiation in cell lines of acute myeloid leukemia (AML). Two AML cell lines were treated with 84 kinases inhibitors, with pan GSK-3 inhibitors significantly affecting differentiation. Following this result, an independent genetic screen was carried out assessing the role of 1000 genes in myeloid differentiation with the α homolog of GSK-3 scoring highly. Follow-up work knocked down GSK-3α expression in AML cells, leading to decreased cell growth, increased apoptosis, and attenuated methylcellulose-induced colony formation [206]. Finally, Cronan *et al.* used siRNA to screen the metastatic human breast cancer cell line MDA-MD-231 to assess the role of seven MAP3Ks in tumor growth and metastasis. MDA-MB-231 cells with stable knockdown of one specific MAP3K were then injected into the fat pad of immune compromised mice. Results indicated roles for some MAP3Ks in both primary and metastatic tumor growth including MEKK2 and MLK3, while knockdown of MEKK1 had no effect on tumor growth but decreased metastatic tumor frequency. Tp12, MEKK3, and B-Raf inhibition was shown to decrease tumor growth with no effect on metastatic frequency, while TAK1 knockdown had no effect on primary or metastatic tumor growth [207].

Kinase screens can also be carried out to assess the effectiveness of drug combinations as well as synthetic lethal combinations. Liu-Sullivan *et al.* used human lung cancer cell lines with varying mutational profiles to screen for kinase knockdown that would influence the response to a polo-like kinase 1 (PLK1) inhibitor at both high and low doses. Results of this study revealed knockdown of 25 different kinases affected cellular response to PLK1 inhibition, with the majority increasing drug sensitivity including knockdown of the AGC kinases MAST4 and LATS2, while knockdown of another AGC kinase, SGK3, conferred resistance to the PLK1 inhibitor [208]. The development of drug resistance is a critical focus of research as it is an inevitable clinical occurrence for targeted therapies, and in many cases the mechanisms underlying resistance are unknown. The resistance

of ERα positive breast cancer to Tamoxifen has been well documented, thus an siRNA screen of known and putative kinases in MCF7 breast cancer cells was carried out to assess mediators of this acquired resistance. Results revealed a role for CDK10 in tumor cell sensitivity to Tamoxifen treatment, with follow-up studies attributing this resistance to CDK10 suppression-induced ETS2-driven transcription of C-Raf, leading to MAPK activation and loss of tumor growth reliance on estrogen signaling [209]. Another screen in human breast cancer cells sought to identify agents that modify resistance to PI3K inhibitor treatments. Over 40 agents affecting pathways involved in growth, metabolism, and apoptosis were applied to cell lines rendered insensitive to PI3K inhibitor treatment. The drug LEE011, a highly specific CDK4 and 6 inhibitor, emerged as the strongest sensitizer to PI3K inhibitor treatment, with sensitization also seen with two mTOR inhibitors and the Akt/PKB inhibitor MK2206. Following confirmation of results with siRNA-mediated knockdown, xenografts were treated with a PI3K inhibitor alone, or in combination with the CDK4 and CDK6 inhibitors. While PI3K inhibition alone slowed tumor growth, progression still occurred whereas combination treatment leads to a greater suppression of tumor growth [210].

An additional study examined the effect of suppressing GSK-3 activity on the functionality of oncology relevant drugs, with cell viability used as the readout. Results revealed that mTOR inhibitor function was perturbed to the greatest extent, with rapamycin and everolimus showing a complete loss of growth-inhibitory effects in combination with suppression of GSK-3 activity. In a second screen, a small molecule library of 367 kinase inhibitors was used to assess drug response rates and cell viability in combination with GSK-3 inhibition. The effectiveness of 89 separate compounds was altered upon GSK-3 inhibition, with mTOR inhibitor function again suppressed, while PLK1 inhibitor function was enhanced. Finally, the researchers performed a modifier RNAi screen of the entire human kinome in the presence/absence of GSK-3 inhibition. Their results revealed that approximately 35% of the kinome interacts with GSK-3 in the regulation of cell viability in human colonic epithelial cells, including previously described interactions with Akt, B-Raf, and CK1, as well as unidentified or underappreciated interactions with TNK2, LCK, ABL1, and PKCβ [211].

1.3.4
Functional Assessment of Kinase Function *in vivo*: Animal Models

While knockdown of kinase activity through RNAi and chemical inhibitors allows for the assessment of kinase function, ultimately these models do not fully recapitulate the system as a whole with its many interacting cell types. The development of mouse models in which a particular kinase has been deleted provides a more informative method to ascertain their complete biological function. Taking advantage of endogenous homologous recombination, it is possible to engineer a mutation at a given locus in mouse embryonic stem cells that can then be used to generate a knockout mouse. While efforts are underway to generate knockout mice for the over 25 000 murine genes, the critical nature of some of these genes

results in embryonic lethality, hampering our ability to isolate the roles they play in adulthood.

The introduction of Cre-LoxP technology allowed for control over both the spatial and temporal activation/deletion of a given gene in the mouse. Cre recombinase is derived from the P1 bacteriophage and excises DNA placed between two LoxP sites, which are 34 base-pair nucleotide sequences [212–215]. Following inclusion of these sites into the genomic DNA and mouse generation, these animals are crossed to transgenic Cre expressing mice, where a tissue-specific promoter is used to drive Cre expression and limit gene excision to a particular cell type. This system can also be further modified to restrict when the Cre-mediated excision occurs, through the ligation of the ligand-binding domain of the estrogen receptor that restricts transcription until the mice are administered Tamoxifen [216, 217]. Two other methods to control temporal gene deletion include using the reverse-tetracycline-controlled transactivator, in which doxycycline must be administered in order to induce Cre expression [218] or Cre can be delivered to tissues through injection of a viral vector [219]. Both global- and tissue-specific knockouts have had immense impact on our understanding of kinase function in disease, below we highlight the significant gains in understanding of one particular kinase, GSK-3, through the use of animal models.

As described earlier, the significant sequence homology between the two homologs of GSK-3, α and β, increased speculation that their functions would be highly similar with each being able to compensate for loss of the other. The use of knockout models revealed that the two homologs had critical nonredundant functions. Mice lacking GSK-3α are viable with no overt abnormalities but, depending on background strain, have improved glucose tolerance, with increased ability to clear glucose as well as decreased total body fat mass and increased glycogen deposition in the liver. These effects on glucose clearance were not found on the C57BL/6 strain under normal conditions [44, 220] but were revealed under metabolic stress [220]. Other groups also have examined the consequence of global knockout of GSK-3α expression in specific tissues, with global loss of this gene resulting in both decreased sperm motility and capacitation [221], increased cardiac hypertrophy and dysfunction following myocardial infarction [222, 223], and premature aging with the development of cardiac hypertrophy and dysfunction, impaired clearance of debris from skeletal muscle and loss of cartilage, and bone ossification from the knee joint [224]. In the brain, global loss of GSK-3α altered cerebellar structure and function, with mice displaying decreased aggression, exploratory activity, loss of social motivation, and deficits in fear conditioning [225]. Unlike previous studies on the global GSK-3α KO animals, a recent study described the compensatory upregulation in GSK-3β expression in the kidney medulla to which the lack of gross morphological changes was attributed. However, elevated water intake and urine output were seen in these mice, which was correlated with changes in aquaporin 2 expression [226].

The nonredundant functions in GSK-3α and β were initially highlighted by the embryonic lethality associated with the GSK-3β knockout mouse [45]. This

lethality can occur at two different time points during gestation with mice dying mid-gestation due to significant liver degeneration, as a result of TNFα-mediated toxicity or death occurs later in gestation due to heart-patterning defects including the aorta and pulmonary artery arising from the right ventricle and ventricular septal defects [43]. GSK-3β knockout mice are embryonic lethal, while global GSK-3β heterozygotes are viable and allow study of the effects of partial knockdown of GSK-3β expression. These mice display impairments in long-term memory formation [227], as well as behavioral changes including decreased exploratory behavior without deficits in locomotion. These mice also have varying responses to injury in the heart with GSK-3β inhibition exacerbating ischemic injury but have a protective role in injury from ischemia/reperfusion [228].

Despite the information gathered from global GSK-3α knockouts and GSK-3β heterozygotes, it was necessary to develop tissue-specific knockouts of both homologs to ascertain the effects of single cell type GSK-3α loss, as well as to understand the role of complete GSK-3β loss in specific tissues. In the heart, the role of both α and β has been investigated using Cre-targeted to cardiomyocytes. These studies revealed that GSK-3α inactivation limits remodeling and preserves function following myocardial infarction, with similar responses seen in cardiomyocyte-specific GSK-3β KO. The effect of GSK-3β knockout was also assessed in response to pressure overload; however, these mice exhibited a normal hypertrophic response [229, 230]. Isoform-specific effects have also been closely examined in the brain, with deletion targeted to highly specific regions. Neuron-specific knockdown of GSK-3α leads to alterations in basal synaptic transmission, as well as memory impairment, highlighting a role for the alpha homolog in synaptic plasticity [231]. Knockout of GSK-3β in D2-receptor-expressing neurons, but not D1-expressing neurons, was shown to mimic antipsychotic actions without affecting signaling pathways involved in mood-related behaviors [232]. Serotonin neuron-selective knockout of GSK-3β was also shown to decrease firing, cAMP production, and serotonin release affecting serotonin-regulated behaviors [233].

As GSK-3 has well-described roles in glucose handling, the knockout of either isoform has been studied in tissues linked to glucose metabolism. Liver-specific GSK-3β knockouts had normal glucose clearance and blood glucose levels, while skeletal muscle-specific knockout of GSK-3β resulted in improved glucose tolerance with no changes in glucose-stimulated insulin release [234]. Knockout of GSK-3β in skeletal muscle also enhanced recovery to muscle disuse with faster recovery of muscle mass following hind limb suspension and increased expression of myogenesis-associated genes [235]. Mice lacking GSK-3β in the β-islets of the pancreas showed mild improvement in glucose tolerance, as well as glucose-induced insulin secretion and increased β-cell mass. These mice were resistant to high-fat diet-induced diabetes as they showed improved glucose tolerance and expanded β-cell mass [236]. Breeding of β-cell-specific GSK-3β knockout mice with mice either lacking IRS1 expression (IRS1 −/−) or with reduced insulin receptor expression (IR +/− mice) prevented the development of insulin resistance and diabetes otherwise seen in these mice [237].

More recently, the consequence of tissue-specific loss of both GSK-3α and β has been examined. Loss of both GSK-3α and β was assessed in the brain through the use of nestin-Cre targeting neural progenitor cells. These mice displayed larger heads at every developmental stage until death at birth, with enlarged ventral brain areas and massive expansion of neuronal progenitor cell pools [238]. Targeted loss of both GSK-3α and β in mammary gland epithelial cells leads to transdifferentiation of the mammary gland, resulting in dams unable to support nourishment of pups. These mice also developed large mammary ductal adenocarcinomas by 6–7 months of age, with tumors displaying areas of atypical squamous differentiation. The loss of both GSK-3α and β leads to significant upregulation of β-catenin expression, and the development of these tumors closely resembled those seen in β-catenin mutant mice. To assess the importance of GSK-3α and β outside of their effects on β-catenin, triple transgenic mice lacking mammary gland expression of GSK-3α and β and β-catenin were established. While the lack of β-catenin corrected the gland transdifferentiation, these mice still developed tumors, though latency was increased by ~4 weeks and tumors displayed different histological characteristics [239].

These results highlight the complex, tissue- and isoform-specific functions of GSK-3 that could only be revealed in the context of a mouse model. Similar work has been undertaken to understand the role of other protein kinases in health and disease through the generation of global and conditional knockout animals. As a resource, several reviews are listed on the role of these other kinases [132, 240–242].

1.3.5 CRISPR/Cas9 Genomic Recombineering

Traditionally, as previously described, RNAi-mediated knockdown has been the technique most widely used to examine the importance of proteins to a given signaling pathway or disease model. However, given the reported issues with this technique including partial knockdown and off-target effects [241], improved approaches have been sought. Two promising techniques that allowed direct sequence-specific targeting of genes were Zinc Finger Nucleases (ZFNs) and Transcriptional Activator-Like Effector Nucleases (TALENs). Both of these techniques rely on DNA–protein interactions to guide the fused nuclease domain of the restriction enzyme Fok1, to specific sites within the genome to activate nuclease activity. However, both of these techniques also have limitations/complications, including design issues limiting the regions of the genome that could be targeted, as well as protein engineering challenges [243, 244]. In essence, each target must be built from scratch by serial addition of nucleotide-specific protein domains. While these techniques were being refined, a further tool for genomic editing emerged from studies of prokaryotic immune systems.

The CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas system in prokaryotes functions as a defense mechanism against bacteriophage infection. In bacteria, CRISPR exploits sequence-specific homology pairing

between nucleic acids to direct nuclease activity to phage-induced DNA. Early studies revealed that bacteria possess three separate CRISPR/Cas systems, type I, II, and III, but type II was most readily engineered for genomic editing as it only requires a single protein, Cas9, to locate the target sequence and cleave DNA [245–247]. The type II CRISPR assay has three critical components required to engineer the system including Cas9, a tracrRNA, that facilitates DNA cleavage by Cas9, and a crRNA, which contains the target genomic sequence [244]. The system can then be targeted to a gene of interest through modification of the 20 nucleotide spacer sequence of the crRNA [247]. One critical aspect of this sequence is that it must contain the Protospacer-Adjacent Motif (PAM) immediately proximal to the region of homology to the DNA target, as the PAM sequence functions in the DNA target search mechanism of Cas9 and also elicits the transition between the two conformations of Cas9, from binding to cleavage [244, 247]. Further studies revealed that the tracrRNA and crRNA could be fused to create a single guide RNA (sgRNA) [248], further simplifying the system.

Two papers were published in 2013 that engineered the CRISPR/Cas system for use in mammalian cells [249, 250]. In mammals, Cas9-induced double-stranded breaks (DSBs) can be repaired by one of two pathways; Non-Homologous End Joining (NHEJ) or Homology-Directed Repair (HDR). NHEJ is more common as it does not require a homologous template; however, it is error prone, often resulting in insertions or deletions leading to frame-shift mutations and the absence of functional protein [244, 247]. This reliance on the error-prone NHEJ pathway allows this tool to be used in the generation of knockouts in a specific gene directed by the sgRNA, while introduction of an sgRNA and a single-stranded DNA template with homology to the target gene allows for introduction of transgenes, tags of interest or single base changes into the genome [247]. Since 2013, CRISPR/Cas technology has become widely used in animal models and human cancer cell lines for rapid generation of mutations and assessment of protein expression with greater specificity and fewer limitations to those carried out with RNAi. Some issues do remain such as off-target effects and the need sequence each change given the unpredictable nature of the repair, but the advantages and power of the approach outweigh these problems and have not yet hindered application.

Due to the recent adoption of this technique, its usefulness in the study of protein kinases in health and disease is in its infancy; however, genome-wide screens using sgRNA libraries have already been published. Three such studies have highlighted the role of proteins in proliferation and drug resistance, including a role for protein kinases [251–253]. In a study by Shalem *et al.*, two screens were performed to assess essential genes for survival of A375 human melanoma cell line and to identify genes that confer resistance to the B-Raf inhibitor vemurafenib. While the screen on cell survival showed that most of the deleted sgRNAs targeted essential genes such as ribosomal structural components, the resistance screen revealed a small group of previously reported and unreported genes including NF1, NF2, and the E3 ligase Cullin 3 [253]. A similar experiment was carried out to assess genes whose loss conferred resistance to etoposide, a widely

employed chemotherapeutic agent, in human leukemia cell lines. Results revealed a previously unreported role for the protein kinase CDK6 in etoposide-induced cytotoxicity [251].

Matano *et al.* employed CRISPR/Cas9 to examine the progression of colon cancer from benign adenoma to advanced adenocarcinoma by introducing mutations in normal human intestinal epithelial cells cultured into organoids with media supplemented with niche factors. Progressive genomic editing of organoids with sgRNAs targeting APC, SMAD4, and TP53 for knockout, as well as sgRNAs and templates for HDR-repair induced point mutations in KRas and PIK3CA, allowed for growth of organoids without niche factors in those containing alterations in all five genes. This approach also allowed the study of the effect of mutating each gene alone or in combination in progression from benign adenoma to adenocarcinoma, through injection of organoids into the kidney subcapsule. Off-target effects of CRISPR technology were also assessed through exome sequencing with no off-target mutations detected [254].

CRISPR/Cas9 technology has also been used to assess the role of mutations in PKC isoforms in cancer progression. This is a controversial area as controlled trials examining chemotherapy alone or in combination with PKC inhibitors, revealed significantly decreased response and disease control rates in nonsmall cell lung cancers treated with chemotherapeutic agents and PKC inhibitors [255]. These results may not be all that surprising given that 46 cancer-associated mutations have been detected in PKC, with the majority reducing or abolishing PKC activity and none leading to activation of the kinase [256]. HCT116 colon cancer cells contain a loss-of-function mutation in PKCβ and CRISPR/Cas9 was used to correct this mutation to the wild-type version of the sequence. Restoration of the wild-type PKCβ resulted in increased PKC activity as well as a more tumor-suppressive phenotype, in that HCT116 cells showed decreased anchorage-independent growth [256].

Aside from generating mutations in human cancer cell lines, the CRISPR approach can also be used *in vivo* to generate mutations in animal models. Delivery of CRISPR/Cas9 components targeting Pten to the mouse liver via hydrodynamic tail vein injections resulted in just over 3% of hepatocytes becoming null for Pten, while an additional 0.4% showed intermediate staining, which may represent heterozygous deletion. These results were duplicated in Pten floxed mice transduced with Cre via hydrodynamic injection. Coinjection of sgRNA targeting both Pten and p53 using the same method resulted in tumor development with similar histology to combined Pten and p53 floxed conditional knockout mice. This method also was used to establish point mutations in β-catenin through the injection of sgRNA targeting β-catenin, along with oligos containing four point mutations in β-catenin. Mutations in β-catenin, resulting in nuclear expression, were detected in approximately 0.5% of hepatocytes, demonstrating the reduced efficiency of HDR, but still resulting in increased target gene expression 7 days post injection [257]. Another method of adopting CRISPR/Cas9 technology became possible through generation of a Cre-dependent Rosa26 Cas9 knockin mouse, followed by viral or nonviral sgRNA delivery to induce genome

editing. This method has been used to assess mutations in the serine/threonine kinase LKB1, as well as KRas and p53, in lung adenocarcinoma, requiring introduction of a single vector containing the sgRNAs for required mutations, as well as Cre recombinase, delivered, via the trachea, into Cre-dependent Cas9 mice. Results showed lung nodule development with progression to advanced adenocarcinoma within 2 months and that LKB1/p53 double knockout cells were highly enriched over the entire lung [258].

CRISPR/Cas9 technology is rapidly changing the way the contributions of various proteins can be assessed in disease models as it is highly specific and allows for rapid generation of mouse models with multiple allelic changes, compared with techniques requiring introduction of targeted mutations into ES cells, via homologous recombination, to generate chimeric animals, followed by many months of breeding to generate experimental animals. Given the rapid uptake of this technology and advances in usability and application, CRISPR-mediated recombineering is likely to become the method of choice for both *in vitro* and *in vivo* analysis. Several excellent reviews have recently been published on the development of this technique, the mechanisms that underlie its specificity, as well as its use in various models of disease [244, 247, 259, 260].

1.4 Final Thoughts

While there is little question that targeting members of the extensive kinome family has yielded a number of major and high profile successes, there is also a lesser appreciated but much greater volume of failures. Many highly selective and potent inhibitors have failed in preclinical and clinical trials for a variety of reasons. Should these targets be abandoned? The answer lies in understanding why these drugs failed and part of that derives from the ubiquity and dependencies of normal cellular regulation on this class of enzymes. Given the very finite number of potential targets, perhaps efforts should focus on precision targeting within tissues and even within cells. We know that signaling pathways are not freely diffusible and that a large fraction of signaling proteins are sequestered within macromolecular complexes, each tuned to specific functions, responses, and locations. This is how specificity and efficiency are maintained. Yet our clumsy techniques for modulating the functions of these proteins are indiscriminate in terms of subcellular location or context of complex. Whether it is a small molecule, siRNA or CRISPR recombination, all of the selected molecules in a given cell are inactivated. This may provide an opportunity for drug discovery whereby we target not the enzyme but its interaction with a specific set of complexes. This, coupled with more selective tissue targeting, may resuscitate targets that cause toxicity if universally inactivated. Clearly, an underappreciated subset of targeting proteins are the scaffolds that frame the macromolecular complexes, although, it should be pointed out that these scaffolds are often targeted by disease mutations, underscoring their importance.

Lastly, while the protein kinase family is large compared to most protein families, the research community has largely focused on a small subset. Of the 500+ members of this group, only 10–20% have been studied in any significant detail (*http://healthydebate.ca/opinions/canadian-biomedical-science-outstandingbut-also-redundant*). The emergence of genome-wide screens has started to change this picture, but the conservative, risk-averse nature of scientific funding acts as a disincentive for researchers to explore too far outside of the boundaries of current knowledge. For those who dare, there may be rich fruit to uncover.

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