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Identification of a multisubunit E3 ubiquitin ligase required for heterotrimeric G-protein beta-subunit ubiquitination and cAMP signaling

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Identification of a multisubunit E3 ubiquitin ligase required for heterotrimeric G-protein beta-subunit ubiquitination and cAMP signaling

A dissertation submitted in partial satisfaction of the

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in Molecular Biology

by

Brian Daniel Young

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ABSTRACT OF THE DISSERTATION

Identification of a multisubunit E3 ubiquitin ligase required for heterotrimeric G-protein beta-subunit ubiquitination and cAMP signaling

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Brian Daniel Young

Doctor of Philosophy in Molecular Biology

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Professor James Akira Wohlschlegel, Chair

GPCRs are stimulated by extracellular ligands and initiate a range of intracellular signaling events through heterotrimeric G-proteins. Upon activation, G-protein α-subunits (Gα) and the stable βγ-subunit dimer (Gβγ) bind and alter the activity of diverse effectors. These signaling events are fundamental and subject to multiple layers of regulation. In this study, we used an unbiased proteomic mass spectrometry approach to uncover novel regulators of Gβγ. We identified a subfamily of potassium channel tetramerization domain (KCTD) proteins that specifically bind Gβγ. Several KCTD proteins are substrate adaptor proteins for CUL3–RING E3 ubiquitin ligases. Our studies revealed that a KCTD2-KCTD5 hetero-oligomer associates with CUL3 through KCTD5 subunits and recruits Gβγ through both subunits. Using *in vitro* ubiquitination reactions, we demonstrated that these KCTD proteins promote monoubiquitination of lysine-23 within $G\beta_{1/2}$. This ubiquitin modification of $G\beta_{1/2}$ is also observed in human

cells and is dependent on these substrate adaptor proteins. Because these KCTD proteins bind Gβγ in response to G-protein activation, we investigated their role in GPCR signaling. Their deletion strongly impairs cAMP generation and downstream signaling pathways in response to signaling activation. Consistent with these results, depletion of CUL3, the component of the E3 ligase that binds KCTD proteins, causes similar defects. Together, our studies suggest that a KCTD2/KCTD5–CUL3–RING E3 ubiquitin ligase recruits Gβγ in response to signaling, monoubiquitinates lysine-23 within Gβ, and stimulates adenylyl cyclases—Gβγ effectors—to positively regulate cAMP signaling.

The dissertation of Brian Daniel Young is approved.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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A manuscript with the data from Chapter II is currently in preparation and data from Chapter III may be incorporated into a future manuscript.

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CHAPTER I

INTRODUCTION

FUNDAMENTALS OF GPCR SIGNALING

G-protein-coupled receptors (GPCRs) are a large class of cell-surface proteins that regulate a variety of intracellular signaling pathways in response to extracellular stimuli. Much of our understanding of GPCR signaling originates from studies of the adrenergic receptors that are activated by epinephrine and norepinephrine, two neurotransmitters that stimulate the sympathetic nervous system (1,2). Lefkowitz and colleagues initially purified the β-adrenergic receptor from frog erythrocytes using a known receptor antagonist covalently linked to an affinity matrix (3). His laboratory soon adopted this approach to isolate the β_2-adrenergic receptor from mammals (4), and was then able to clone the gene encoding this protein (5). The amino acid sequence of this receptor revealed seven distinct regions of high hydrophobicity that they predicted to be transmembrane domains (5). Other mammalian adrenergic receptors were soon cloned, and all shared this predicted organization (6-8). Over two decades later, a crystal structure of the β_2-adrenergic receptor was obtained (9). As anticipated, this structure revealed seven transmembrane helices connected by intracellular and extracellular loops—a hallmark of GPCRs.

Based on sequence homology, over 5000 unique GPCRs have now been identified across life from yeast to humans (10). Over 800 of these GPCRs are found in humans (10,11). A wide variety of ligands, including small molecules, lipids, peptides, and proteins, bind and activate this diverse set of receptors.

Each of these cell-surface receptors is tethered on their cytoplasmic face to heterotrimeric guanine nucleotide-binding proteins (G-proteins) (12,13). These

intracellular heterotrimeric G-proteins regulate different intracellular signaling pathways upon receptor stimulation with a ligand. The heterotrimer is composed of a G-protein α-subunit (Gα) bound to a stable G-protein βγ dimer (Gβγ). In humans, there are 21 Gα subunits, 6 Gβ subunits, and 12 Gγ subunits, ensuring great diversity in heterotrimers (13,14). These G-proteins are anchored to membranes with covalently attached lipids; Gα can be myristoylated or palmitoylated while Gγ is isoprenylated (15,16). A defining feature of Gα subunits is their GTPase domain capable of binding guanine nucleotides and hydrolyzing GTP to GDP. In the absence of receptor activation, Gα binds GDP. Gβ subunits form a β-propeller structure and have an N-terminal helical domain that facilitates dimerization by forming a coiled coil with Gγ (14,17,18). Gα and Gβγ form a heterotrimer through interactions between several flexible regions of Gα and several blades of the β-propeller within Gβγ (13). GPCRs bind this heterotrimer through interactions of its transmembrane domains and intracellular loops with several surfaces within Gα (12,19). FRET-based biochemical studies have demonstrated that this GPCR–heterotrimer complex forms in the absence of receptor activation (20-22).

Activation of a GPCR by ligand binding alters the structures of the receptor and heterotrimer. Crystallography studies of the β_2-adrenergic receptor have elucidated the effects of ligand binding to the receptor (2). Ligand binding is associated with modest (~2 Å) displacement of the transmembrane domains surrounding the ligand-binding site. These small changes near the extracellular portion of the GPCR are associated with a more pronounced (~14 Å) rearrangement of the transmembrane domains near the cytosolic portion (12). These changes to the cytoplasmic portion of the receptor create a deeper binding pocket for the C-terminus of Gα and induce structural changes within

Gα. This triggers the release of GDP from Gα, and recent crystallography and hydrogen-deuterium exchange mass spectrometry studies have revealed the structural basis for this release (12,23). This nucleotide-free heterotrimer–GPCR complex quickly binds GTP, which is more abundant than GDP (24). GTP binding further affects the structure of Gα by altering interaction domains that normally bind Gβγ (17,25,26). The disruption of the Gα–Gβγ interface causes dissociation of these subunits or induces them to adopt a more open conformation (13,21,27,28). Such changes to the Gα–Gβγ interface expose large protein-interaction surfaces that can then bind and regulate a wide variety of different signaling effectors ranging from enzymes to ion channels (13,29-32). This complex cascade of ligand-induced conformational changes in the receptor and heterotrimer facilitates GPCR signaling. Such signaling can be terminated by the Gα GTPase activity that hydrolyzes GTP to GDP to promote structural changes in Gα that favor formation of the inactive heterotrimer (24).

SIGNALING PATHWAYS REGULATED BY HETEROTRIMERIC G-PROTEINS

GPCR signaling is initiated by the GTP-dependent dissociation of Gα and Gβγ that unmasks large surfaces within the Gα–Gβγ binding interface. These signaling-exposed regions of Gα and Gβγ can then bind and regulate large classes of signaling effectors (13). Gα subunits can be clustered into families that each regulate distinct effectors.

GTP-bound $G\alpha_s$-family subunits bind and activate adenylyl cyclases while $G\alpha_i$-family subunits inhibit their activity (33-35). This nine-member family of integral membrane enzymes converts ATP to 3′,5′ cyclic AMP (cAMP). $G\alpha_s$-family subunits activate all adenylyl cyclases while a limited subset are negatively regulated by $G\alpha_i$-

family subunits (34). cAMP is a critical signaling molecule and can activate protein kinase A (PKA) to catalyze protein phosphorylation (36-38). One major PKA substrate is the cAMP response element-binding protein (CREB), a transcription factor activated by phosphorylation of serine-133 that regulates transcription of many genes (39). cAMP can also bind and regulate specific ion channels and proteins that activate Rap GTPases (36).

Other families of Gα subunits regulate additional signaling effectors. $G\alpha_q$-family subunits bind and activate phospholipase C-β (PLC-β) isoforms (40). These enzymes hydrolyze phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-triphosphate (IP_3) and diacylglycerol. IP_3 binds and activates a specific ion channel located on the endoplasmic reticulum that releases Ca^{2+} into the cytoplasm (41). Higher concentrations of cytoplasmic Ca^{2+} and membrane diacylglycerol localize protein kinase C (PKC) to membranes to activate its activity and trigger protein phosphorylation (42). The $G\alpha_{12/13}$ family binds and activates RhoGEF proteins, which promote nucleotide exchange on the RhoA GTPase (43,44). Active GTP-bound RhoA can then regulate a variety of cellular processes.

Selective pairing between receptors and different families of Gα subunits enables specificity in Gα signaling. GPCRs recognize specific residues within the C-terminus of Gα to couple primarily to a single family of Gα subunits (24,45). This ensures that GPCRs activate specific Gα-dependent signaling events. For example, agonist binding to a $G\alpha_s$-coupled receptor will stimulate cAMP signaling without affecting PKC or RhoA.

In contrast to Gα subunits, individual Gβγ dimers can regulate a multitude of signaling effectors (31). These effectors have overlapping binding sites on the signaling-exposed surface of Gβ that binds Gα in basal conditions (46). Some of these Gβγ effectors are also regulated by Gα. For example, Gβγ can positively or negatively regulate adenylyl cyclases (29,34). The activities of group II (isoforms 2/4/7) and group III (isoforms 5/6) adenylyl cyclases are all activated by Gβγ while those of group I enzymes (isoforms 1/3/8) are inhibited by this dimer. One group suggested that multiple adenylyl cyclases (isoforms 1/2/6) share the same binding surface on Gβ regardless of whether they are positively or negatively regulated by Gβγ (47). For each enzyme isoform, they measured its activity after adding purified $G\alpha_s$ with or without Gβγ. Addition of Gβγ increased activity of isoforms 2/6 and reduced that of isoform 1. This Gβγ-dependent stimulation or inhibition could be attenuated with a 15-residue peptide that binds the Gα-binding region of Gβ, suggesting that the peptide and the enzymes share the same binding site (48). Consistent with this observation, Gβ alanine-substitution mutations on that same Gα-binding interface perturb Gβγ-mediated activation of isoforms 2/5/6 (46,47).

Similar to $G\alpha_q$, Gβγ dimers can also positively regulate PLC-β isoforms (29). Gβγ dimers likely bind to multiple sites on these enzymes that are distinct from those interacting with $G\alpha_q$ (31). Indeed, mutation of several residues within the catalytic site impairs activation by Gβγ but not $G\alpha_q$ (49). The region of Gβ that promotes Gβγ-dependent activation of PLC-β isoforms likely overlaps with the region regulating different adenylyl cyclases (46). For example, mutation of a specific residue on the Gα-binding surface of Gβ blocks activation of both PLC-β and adenylyl cyclases (47,50).

While Gβγ shares two signaling effectors with Gα subunits, these dimers also have targets that are not regulated by other G-proteins. One of these effectors is the phosphoinositide 3-kinase (PI3K) (29). This enzyme phosphorylates phosphoinositides to generate phosphatidylinositol (3,4,5)-triphosphate, which promotes activation of AKT, a protein kinase regulating diverse pathways (51). PI3K-β and PI3K-γ are activated by Gβγ while the α and δ isoforms are not (52). One group demonstrated that Gβγ can bind the γ isoform of this kinase (53). As before, PI3K likely binds a region of Gβγ shared with other effectors because a peptide targeting the Gα-interacting surface of Gβ blocks Gβγ-dependent activation of PI3K and PLC-β (54).

Other classical Gβγ effectors include inwardly rectifying K^+ channels and voltage-dependent Ca^{2+} channels. These potassium channels function to hyperpolarize neurons (55). Biochemical studies revealed that Gβγ binds to these channels and patch-clamping experiments demonstrated that Gβγ promotes their activation (56-58). In contrast, Gβγ binds and inhibits Ca_v2 voltage-dependent Ca^{2+} channels (59,60). The Gβ residues regulating these channels are located on the signaling-exposed surface of Gβ that many other Gβγ effectors also bind (46,58,61).

All of these canonical Gβγ effectors share overlapping binding sites on the Gα-interacting region of Gβ. In contrast to a Gα subunit, a Gβγ dimer can regulate many different effectors. Specificity in Gβγ signaling might be achieved by controlling expression of different effectors in a given cell. Alternatively, specificity might be determined by differences between Gβγ isoforms. There are 6 Gβ subunits and 12 Gγ subunits—theoretically 72 distinct Gβγ dimers. It is becoming clear that there is

functional heterogeneity among these dimers. *In vitro* and *in vivo* dimerization studies have revealed that some pairs assemble more efficiently than others (14). In addition, specific Gβγ dimers regulate different effectors more strongly than others (13,29). Furthermore, certain GPCRs preferentially couple to heterotrimers containing specific dimers (62,63). This suggests that Gβγ signaling specificity may be achieved through differences in the Gβγ dimers—a given receptor might preferentially couple to a specific dimer that strongly activates one effector but not others. Larger-scale studies should bring the extent of such Gβγ isoform-dependent specificity into focus.

PHYSIOLOGIC ROLES OF GPCRS AND HETEROTRIMERIC G-PROTEINS

GPCR signaling is vitally important for both normal mammalian physiology and disease. Ligands of diverse nature—proteins, peptides, small molecules, and lipids—initiate signaling by binding to one or several of approximately 800 receptors present on plasma membranes of cells throughout the body (11). Receptor activation can then initiate a wide range of signaling events through Gα and Gβγ to regulate specific enzymes and ion channels (13,29,31,64). This process facilitates cell–cell communication within tissues and between different organs, and has an instrumental role in all organ systems (65).

Indeed, GPCR signaling is involved in many acquired and genetic diseases. For example, dysregulation of GPCR signaling is present in heart failure, hypertension, diabetes, and cancer—highly prevalent conditions associated with significant morbidity and mortality (66-69). Less-common genetic diseases are also linked to mutations

affecting GPCR signaling. For example, gain-of-function and loss-of-function mutations in hormone GPCRs cause a variety of endocrine syndromes (70-73).

In addition to GPCRs, heterotrimeric G-proteins have also been linked to disease. For example, somatic mutations in $G\alpha_s$ that disrupt the GTPase domain and render the subunit constitutively active were found in over one-third of growth hormone-secreting pituitary tumors (74). Identical somatic mutations during embryogenesis cause a complex endocrine syndrome (65). Similar activating somatic mutations of $G\alpha_{q/11}$ were reported in over 80% of uveal melanomas (75,76). In addition, Gβ somatic mutations have also been linked to myeloid and B-cell leukemias. One group demonstrated that these cancer-associated mutations on the Gβ signaling surface impaired interactions with multiple Gα subunits (77). They suggested that these mutations increased the amount of free Gβγ available to stimulate PI3K activity to increase AKT activation and promote malignant transformation.

Given this large role of GPCR signaling in human health, it is not surprising that approximately one-fourth of pharmaceuticals target specific GPCRs (78). Manipulating GPCRs with specific agonists and antagonists enables highly targeted therapies.

REGULATION OF GPCR SIGNALING

The importance of GPCR signaling necessitates multiple layers of regulation. Typically, these regulatory strategies rely on feedback inhibition to prevent excessive signaling during prolonged GPCR stimulation. Much is known about how GPCRs are regulated in this manner (1). Receptor activation dissociates Gα from Gβγ, allowing

Gβγ to bind and recruit GPCR kinases to the membrane (79,80). Receptor phosphorylation induces binding of arrestins, which prevents the receptors from coupling to G-proteins and promotes their clathrin-mediated endocytosis (81). In the case of the prototypical β_2-adrenergic receptor, β-arrestin-2 binding in response to signaling promotes receptor polyubiquitination and lysosomal degradation (82). Other GPCRs are also degraded by similar mechanisms. This inhibitory regulation, known as desensitization, depends on post-translational modifications catalyzed by GPCR kinases and E3 ubiquitin ligases.

Similar to receptors, G-protein subunits also undergo careful regulation to achieve similar goals. Control via the Regulator of G-protein Signaling (RGS) class of proteins that target GTP-bound Gα and enhance its GTPase activity is well-described (83,84). By promoting GTP hydrolysis, these RGS proteins facilitate formation of inactive heterotrimers. There is some evidence that different RGS proteins may regulate Gα subunits coupled to specific receptors (85). Much like receptors, Gα also appears to be regulated by post-translational modifications. One group reported that $G\alpha_{11}$ is phosphorylated after extended receptor stimulation (86). $G\alpha_{11}$ is a member of the $G\alpha_q$ subfamily and typically activates PKC. Indeed, the phosphorylated serine residue was a predicted PKC phosphorylation site. Mutation of this serine to an alanine reduces signaling desensitization after agonist treatment, suggesting that signaling-induced $G\alpha_{11}$ phosphorylation is part of a feedback inhibition mechanism.

Although Gβγ also has a large role in GPCR signaling, little is known about its regulation following receptor stimulation in higher eukaryotes (29,31). It remains a

mystery whether there are Gβγ-binding proteins that affect its ability to interact with specific effectors following GPCR stimulation. It is also unclear whether Gβγ contains any regulatory post-translational modifications that affect its ability to initiate signaling. Our understanding of how Gβγ is regulated in the context of signaling is limited.

UNBIASED STRATEGIES TO IDENTIFY PROTEIN-BINDING PARTNERS

One approach to identify novel regulators of proteins of interest is to characterize their protein-binding partners. Protein activities are frequently controlled by other proteins. For example, GPCRs are regulated by GPCR kinases and arrestins. In response to signaling, these kinases bind and phosphorylate the receptors to induce arrestin binding, which prevents receptor–G-protein coupling and promotes receptor trafficking. Such protein–protein interactions regulate receptor activity. Accordingly, new layers of regulation can be revealed by identifying protein-binding partners of proteins of interest.

Two strategies to define protein–protein interactions are co-immunoprecipitation (co-IP) and proximity labeling. The co-IP approach involves using a resin-conjugated antibody recognizing a protein of interest to purify protein complexes containing this protein and its binding partners (87). The negative control for such an experiment is typically a purification with a non-specific antibody. There are several challenges, however, associated with this approach, including low expression levels of the protein of interest and an absence of specific antibodies. Accordingly, many groups overexpress their protein of interest fused to an affinity tag. Overexpression ensures high yields of protein complexes containing proteins of interest, and affinity tags enable efficient

purifications with specific antibodies. Two frequently used short-peptide affinity tags are FLAG and HA tags (88). Negative controls for these experiments are most often affinity purifications from a sample not expressing the fusion protein.

Recently, several groups have developed proximity-labeling strategies as a co-IP alternative (89,90). With this approach, a protein of interest is fused to an enzyme that chemically labels protein residues in close physical proximity. One such enzyme is BioID, which is a promiscuous biotin ligase (91,92). BioID activates biotin in an ATP-dependent manner to form biotinoyl-5′-AMP, which can then label lysine residues with biotin. APEX is another proximity-labeling protein that is an engineered ascorbate peroxidase (93). In the presence of H_2O_2, this enzyme oxidizes biotin phenol to a biotin-phenoxyl radical that labels multiple amino acids, especially tyrosine residues (94). With both of these methods, proteins in proximity to the protein of interest are labeled with biotin. These biotin-labeled proteins can then be efficiently purified using resin-conjugated streptavidin, a protein that has high affinity for biotin. Negative controls for these proximity-labeling studies include purifications from samples expressing BioID or APEX fused to a protein with a localization that is similar to the protein of interest.

Proteomic mass spectrometry can be coupled to these co-IP and proximity-labeling approaches to identify potential protein interactors of a protein of interest in an unbiased manner (95). For co-IP experiments, protein complexes can be eluted from the antibody-conjugated resin by competitive elution or with denaturants, such as urea. The proteins present in these complexes can be enzymatically digested into peptides with multiple proteases (Lys-C and trypsin). For proximity-labeling studies, efficient elution

of biotinylated proteins is challenging, so streptavidin resin-bound biotinylated proteins are often directly digested with these proteases without elution (91).

These peptides can be separated by hydrophobicity using reversed-phase liquid chromatography before in-line analysis by high-resolution tandem mass spectrometry. Briefly, during the chromatographic separation, the mass spectrometer will complete rapid cycles of acquiring a full MS^1 precursor spectrum before selecting prominent peptide species for fragmentation by collision-induced dissociation to generate MS^2 product spectra (95). MS^1 precursor spectra measure mass-to-charge ratios of intact peptides while MS^2 product spectra measure mass-to-charge ratios of fragments of a specific peptide after its dissociation. Computational tools can be used to search these spectra against protein databases, allowing identification and quantification of peptides and ultimately proteins present in each digested sample (96-100). For both approaches, it is possible to quantify enrichment of a protein associated with or labeled by the protein of interest relative to the appropriate control samples, allowing identification of putative protein-binding partners.

CULLIN–RING E3 UBIQUITIN LIGASES

Ubiquitination is a post-translational modification that involves the covalent attachment of the small protein ubiquitin to a lysine in a target protein (101,102). This modification is facilitated by three classes of proteins: E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin ligases. E1 enzymes activate ubiquitin with ATP, and have a cysteine that forms a thioester with the C-terminus of ubiquitin. A transthiolation reaction transfers this ubiquitin molecule to a cysteine within

the E2 enzyme. RING-family E3 ligases then bind the ubiquitin-bound E2 enzyme, recruit a substrate protein, and promote transfer of the ubiquitin to a lysine residue within the substrate. Alternatively, HECT-domain E3 ligases transfer the ubiquitin to a cysteine within the E3 ligase before attaching it to a lysine within a substrate. For both types of E3 ligases, the ε-amino group of a lysine forms an amide bond with the C-terminus of ubiquitin.

Substrate specificity in this process is achieved by E3 ligases—these proteins recruit substrates to be modified by ubiquitin. Given this, it is not surprising that many E3 ligases exist to generate diversity in substrate specificity. One group identified 300 RING-family and 28 HECT-domain E3 ligases (103). Some RING-family E3 ligases further expand their diversity in substrate specificity by binding substrate adaptor proteins that can recruit discrete sets of target proteins.

Cullin proteins are a family of seven scaffolding proteins that bind a RING protein and large families of substrate adaptor proteins at their C-termini and N-termini, respectively (104). These resulting multisubunit complexes are known as Cullin–RING E3 ligases. Each type of cullin forms complexes with distinct families of substrate adaptor proteins (103,104). Cullin-1 and cullin-7 recruit F-box proteins through a SKP1 subunit. Cullin-2 and cullin-5 bind SOCS-box proteins through elongin B/C subunits while cullin-4 binds DCAF proteins through DDB1 (105). Finally, cullin-3 (CUL3) interacts with BTB domain-containing proteins. Based on sequence homology, multiple groups have identified several hundred putative substrate adaptor proteins for Cullin-RING E3 ligases in humans (103,104).

CUL3–RING E3 ligases were first recognized to interact with BTB domain-containing proteins in *C. elegans*. One group demonstrated that depletion of MEL-26 led to the stabilization of MEI-1, a protein known to be destabilized by CUL3 (ubiquitination frequently targets proteins for degradation) (106). They used co-IP studies to show interactions between MEL-26 and CUL3, and demonstrated that MEI-1 binds MEL-26 *in vitro*. With these observations, they proposed that MEL-26 was a substrate adaptor for CUL3. At the same time, another group used two-hybrid screening to identify 11 CUL3-interacting proteins, and noted that all of these proteins contained a BTB domain (107). They also suggested that MEL-26 was a CUL3 substrate adaptor for MEI-1. Within months, another group completed detailed biochemical studies to demonstrate the activity of a MEL-26–CUL3–RING E3 ligase on MEI-1 (108). Soon, other groups identified a human BTB domain-containing protein that functioned similarly as a substrate adaptor for a CUL3–RING E3 ligase (109,110). Recent proteomic mass spectrometry studies have indicated that CUL3 interacts with 53 BTB domain-containing proteins in human 293T cells (111).

KCTD PROTEINS: A FAMILY OF CUL3 SUBSTRATE ADAPTOR PROTEINS

One of the major classes of CUL3-specific substrate adaptor proteins is the potassium channel tetramerization domain (KCTD) protein family. There are 25 KCTD proteins in humans, and they share homology with the T1 cytoplasmic oligomerizing domain of voltage-gated potassium channels that contains a BTB domain (112,113). Consistent with BTB domains being a unifying feature of this protein family, several of these human KCTD proteins assemble into CUL3–RING E3 ligases. In various

biochemical studies, KCTD5, KCTD6, KCTD7, KCTD9, KCTD10, KCTD11, KCTD13, KCTD17, and KCTD21 have all been shown to interact with CUL3 (113-121). Crystal structures have revealed that KCTD5 and KCTD9 form pentamers, and electron microscopy studies have suggested that KCTD6 and KCTD11 also pentamerize (112,113,122). Electron microscopy of KCTD9–CUL3 and KCTD6–CUL3 complexes revealed 5:5 KCTD:CUL3 stoichiometry and showed that CUL3 subunits radiate outward from the KCTD pentamer (113,122). In contrast, other KCTD proteins, including KCTD1, KCTD12, KCTD15, and KCTD16, are unable to bind CUL3 (113,123). Some of these non-CUL3-binding KCTD proteins have ubiquitination-independent functions. For example, KCTD12 and KCTD16 are auxiliary subunits of $GABA_B$ receptors and regulate their activity (124).

Other CUL3-binding KCTD proteins have been demonstrated to target proteins for ubiquitination. For example, KCTD11, a known tumor suppressor involved in medulloblastoma, promotes polyubiquitination of HDAC1, a histone deacetylase, to facilitate its degradation (115,125). Two closely related KCTD proteins, KCTD6 and KCTD21, also target this enzyme for degradation (116). One group explored how regulation of this deacetylase by these functionally similar KCTD proteins might contribute to tumorigenesis (115,116). Another putative CUL3 substrate adaptor protein, KCTD17, binds and polyubiquitinates a positive regulator of Aurora-A kinase (118). Loss of KCTD17 causes an accumulation of this regulatory protein and increases Aurora-A kinase activity to impair ciliogenesis.

Additional KCTD proteins have yet to be deorphanized—the proteins that they target for ubiquitination and the pathways that they regulate remain unknown. Several of these have been linked to phenotypes in humans and model organisms alike. For example, homozygous KCTD7 missense, nonsense, and frameshift mutations are linked to a progressive myoclonic epilepsy syndrome (126-128). Despite these clinical phenotypes, the targets of this putative substrate adaptor protein are unidentified.

Another orphaned KCTD protein, Insomniac, was identified in two genetic screens for regulators of sleep homeostasis in *D. melanogaster* (129,130). In fly studies, depletion of Insomniac or Cul3 during development markedly reduces sleep in adults. Within neurons, Insomniac depletion is associated with alterations to synaptic architecture and transmission (131). This protein has close sequence homology with a subfamily of human KCTD proteins: KCTD2, KCTD5, and KCTD17. One group has suggested that KCTD2 and KCTD5 also share functional homology with Insomniac because they can reverse the sleep phenotypes in Insomniac-deficient flies (131). The physiologic functions of these putative substrate adaptor proteins and the molecular mechanisms underlying these striking neurological phenotypes are unclear because the proteins targeted for ubiquitination by Insomniac and its human homologs have yet to be identified.

FUNCTIONAL CONSEQUENCES OF UBIQUITINATION

E3 ubiquitin ligases attach ubiquitin to lysine residues within substrate proteins (102). Proteins can be modified by mono- or polyubiquitination. Monoubiquitination involves the attachment of a single ubiquitin to a lysine while polyubiquitination refers to

modification of a lysine with a chain of ubiquitin molecules. Polyubiquitin chains are formed when the ε-amino group of a lysine on an already-attached ubiquitin becomes covalently linked to the C-terminus of a free ubiquitin molecule (132). Alternative models suggest that ubiquitin chain formation may precede modification of the target protein (133). These polyubiquitin chains are classified by the position of the lysine residues that link the ubiquitin chains, and different chain topologies have distinct impacts on protein function. One group determined that lysine-48 and lysine-63-linked chains account for 90% of the polyubiquitin chains in human HEK-293 cells (134).

Lysine-48-linked polyubiquitin chains are the canonical form and primarily target proteins for degradation by the 26S proteasome (135,136). Proteins with at least 4 lysine-48-linked ubiquitin molecules are recognized by two ubiquitin-binding proteins, PSMD4 and ADRM1, within the 19S regulatory complex of the proteasome (137). Deubiquitinases within the regulatory complex cleave the attached ubiquitin molecules, and the substrates are unfolded and threaded into the barrel-shaped 20S catalytic complex. Within the catalytic complex, multiple active sites facilitate proteolysis to degrade the target protein (136).

Other types of polyubiquitin chains, however, have proteasome-independent functions (138). Lysine-63-linked chains can promote endocytosis and guide intracellular trafficking (139). Early work in yeast suggested that lysine-63-linked chains were important for endocytosis of a plasma membrane uracil permease (140). Subsequent studies identified three adaptor proteins required for clathrin-mediated endocytosis of ubiquitin-modified proteins: Epsin, Eps15, and Eps15r (139,141). All of

these proteins have multiple ubiquitin-binding motifs, and these domains within Epsin and Eps15 have relative specificity for lysine-63-linked chains compared to lysine-48-linked chains and monoubiquitin (142,143). Within endosomes, the ESCRT machinery directs these modified proteins into intraluminal vesicles. The ESCRT-0 complex has multiple ubiquitin-binding domains that also preferentially recognize lysine-63-linked chains compared to lysine-48-linked chains and monoubiquitin (144,145). Other ESCRT components also have ubiquitin-binding domains, and coordinate the deubiquitination of the trafficked protein and invagination of the endosomal membrane to create an intraluminal vesicle (139,141). Fusion of these endosomes with lysosomes results in the proteolysis of proteins within the intraluminal vesicles.

Monoubiquitination—the attachment of a single ubiquitin molecule to a lysine—also has diverse roles in regulating a target protein's protein–protein interactions, intracellular localization, and stability. One of the most-studied monoubiquitin-modified proteins is PCNA, a DNA replication processivity factor that forms a homotrimeric ring that encircles DNA and binds DNA polymerase δ, the primary replicative polymerase (146). In response to DNA damage, PCNA is monoubiquitinated at a specific lysine residue by the RAD18 E3 ligase (147). Disruption of this modification sensitizes cells to DNA damage because the attached ubiquitin recruits multiple permissive DNA repair polymerases to replication forks through their ubiquitin-binding domains in response to DNA damage (148). In this context, monoubiquitination induces an important protein–protein interaction to relocalize proteins containing ubiquitin-binding domains.

Monoubiquitination can also direct proteins for endocytosis in a similar manner to lysine-63-linked chains (149). Some models suggest that several endocytosis adaptors, such as Epsin and Eps15, and the ESCRT complex might also recognize monoubiquitinated proteins to promote their internalization and lysosomal degradation, respectively. In yeast, Gα is monoubiquitinated, and depletion of the requisite E3 ligase is associated with reduced localization of Gα to the vacuole, which is similar to a lysosome (150,151). Deletion of Eps15 and ESCRT complex homologs block this vacuolar localization, suggesting that these ubiquitin-binding proteins coordinate the internalization and trafficking of monoubiquitinated Gα (152). There are also examples of this monoubiquitination-dependent trafficking in mammals. For example, addition of a single ubiquitin to the EGF receptor tyrosine kinase induces its endocytosis and degradation (153). Interestingly, fusion of ubiquitin to membrane-localized GFP was also sufficient to induce endocytosis, suggesting that a single ubiquitin might be the sole signal needed to induce internalization (154).

CHAPTER II

IDENTIFICATION OF A NOVEL FAMILY OF HETEROTRIMERIC G-PROTEIN REGULATORS

INTRODUCTION

GPCR signaling is vitally important for both normal human physiology and disease. Ligands of diverse nature—proteins, small peptides, small molecules, and lipids—bind to one or several of approximately 800 receptors present on plasma membranes of cells throughout the body (11). Receptor stimulation induces Gα to exchange GDP for GTP. This nucleotide exchange promotes dissociation of Gα and Gβγ, exposing critical protein-interaction surfaces on Gα and Gβγ that facilitate signaling by binding and altering the activity of multiple effectors (13). $G\alpha_s$ and $G\alpha_i$ stimulate and inhibit adenylyl cyclases (ACs), respectively, while $G\alpha_q$ activates phospholipase-Cβ (PLCβ) (13). Gβγ can activate specific ACs (isoforms 2, 4, 5, 6, 7), PLCβ, phosphoinositide-3-kinase (PI3K), and Kir3 G-protein-coupled inwardly rectifying potassium channels (GIRKs). Gβγ can also reduce the activity of other ACs (isoforms 1, 3, 8) and Ca_v2 voltage-dependent Ca^{2+} channels (29,34). Subsequent hydrolysis of the Gα-bound GTP to GDP terminates signaling through these effectors by inducing structural changes that favor inactive heterotrimer formation (13).

The physiologic importance of GPCR signaling necessitates multiple layers of regulation. Typically, these regulatory strategies rely on feedback inhibition to prevent excessive signaling during prolonged GPCR stimulation. Signaling-induced receptor phosphorylation induces β-arrestin binding to impair receptor–G-protein coupling and promote receptor endocytosis (81). Stimulus-induced β-arrestin and receptor polyubiquitination also induce endocytosis and lysosomal degradation of receptors, respectively (82,155). This inhibitory regulation, known as desensitization, depends on

post-translational modifications catalyzed by G-protein-coupled receptor kinases and E3 ubiquitin ligases.

Similar to receptors, G-protein subunits also undergo careful regulation to achieve similar goals. Control via the Regulator of G-protein Signaling (RGS) class of proteins that bind GTP-bound Gα and enhance its GTPase activity is well described. By promoting GTP hydrolysis, RGS proteins accelerate formation of the inactive heterotrimer (84). Much like receptors, Gα is also regulated by post-translational modifications. Yeast studies have shown that Gα is mono- and polyubiquitinated, facilitating its trafficking to the vacuole and proteasomal degradation, respectively, although it is not yet clear if these modifications are conserved in higher eukaryotes (150,152). One group has also described a role for signaling-induced $G\alpha_{11}$ phosphorylation in negatively regulating signaling through 5-HT_{2A} receptors in mammalian cells (86).

Although Gβγ plays a large role in GPCR signaling and coordinates many of the signaling events following receptor stimulation, little is known about the mechanisms governing its regulation (29,31). It is unclear whether there are Gβγ-binding proteins that alter its ability to interact with specific effectors at the plasma membrane, or whether post-translational modifications affect its ability to transduce signals. In yeast, Gβ is phosphorylated and monoubiquitinated in response to activation of a GPCR with mating pheromone (156). Gβ phosphorylation is necessary for Gβ monoubiquitination, and this latter modification affects specific signaling events by negatively regulating polarized growth while having no effect on MAP kinase activation. The ubiquitin-

attachment site and adjacent residues within yeast Gβ, however, are not conserved in mammalian Gβ subunits.

In this study, we used proteomic mass spectrometry to identify novel regulators of heterotrimeric G-proteins. Our proteomic analysis revealed that Gβ interacts with a multisubunit E3 ubiquitin ligase. This result raises the possibility that a heterotrimeric G-protein might be modified by ubiquitin to regulate signaling. We have demonstrated that this E3 ubiquitin ligase recruits Gβγ in response to signaling and promotes monoubiquitination of a single lysine residue within Gβ. Depletion of subunits of this ubiquitin ligase impairs cAMP generation and downstream signaling pathways, suggesting that this complex and this novel Gβ monoubiquitin modification might function to positively regulate cAMP signaling.

RESULTS

Identification of novel regulators of heterotrimeric G-protein signaling

Heterotrimeric G-protein signaling has vast roles in both normal human physiology and disease. In response to GPCR stimulation, conformational changes in heterotrimeric G-proteins uncover surfaces on Gα and Gβγ that recruit and modulate a multitude of effectors, ranging from enzymes to ion channels. Given their physiologic importance, we sought to identify novel regulators of heterotrimeric G-proteins with an unbiased affinity purification-proteomic mass spectrometry approach. Briefly, we generated a HeLa cell line stably expressing 3×FLAG 3×HA-tagged $G\beta_1$, and analyzed anti-HA affinity purifications from this cell line and a control HeLa cell line with proteomic mass spectrometry. This analysis identified proteins significantly enriched in $G\beta_1$ complexes relative to control complexes to reveal the $G\beta_1$ interactome (**Figure 1**). As expected, $G\beta_1$ assembles into dimers with Gγ subunits and forms heterotrimers with a variety of Gα subunits. $G\beta_1$ also associates with CCT chaperonins and PhLP1, which guide Gβ folding and Gβγ assembly, respectively (14,157).

In addition to these known $G\beta_1$-binding partners, this analysis also revealed new $G\beta_1$ interactors. We identified two potassium channel tetramerization domain (KCTD) proteins that are highly enriched in $G\beta_1$ complexes: KCTD2 and KCTD5 (**Figure 1**). KCTD proteins share homology with the oligomerizing T1 cytosolic domain of voltage-gated potassium channels. Several of these proteins have been demonstrated to be substrate adaptor proteins for Cullin-3–RING E3 ubiquitin ligases (115,116,118). Other KCTD proteins, including KCTD2 and KCTD5, have been predicted to have this function

(111,114,158). Interestingly, we found that Cullin-3 (CUL3) is also enriched in $G\beta_1$ complexes (**Figure 1**). Together, these results from our analysis of the $G\beta_1$ interactome suggested that a KCTD2/KCTD5–CUL3–RING E3 ubiquitin ligase might interact with $G\beta_1$ and potentially regulate this central signaling molecule through ubiquitination.

A subfamily of ubiquitin ligase substrate adaptor proteins that recognize Gβγ

We were intrigued that our proteomic interactome analysis of $G\beta_1$ revealed that this G-protein subunit interacted with two putative ubiquitin ligase substrate adaptor proteins (KCTD2 and KCTD5) as well as the ubiquitin ligase scaffolding component (CUL3) predicted to utilize these substrate adaptor proteins. KCTD2 and KCTD5 are members of a subfamily of three KCTD proteins that share substantial sequence homology (**Figure 2A**). Unlike KCTD2 and KCTD5, KCTD17 has a C-terminal extension that is essential for its role in the degradation of a regulator of ciliogenesis (118). Some subfamilies of closely related KCTD proteins appear to be functionally redundant while others have distinct roles (115,116,124). The extent of functional overlap between the KCTD2/5/17 subfamily was unknown. It was also unclear whether these KCTD proteins recognized the Gβ monomer, Gβγ dimer, or Gαβγ heterotrimer.

To investigate the differences between this subfamily of KCTD proteins and identify the G-proteins that they target, we characterized the protein interactomes of these substrate adaptor proteins in HEK-293 cells using mass spectrometry (**Figure 2B**). As reported for other subfamilies of closely related KCTD proteins, KCTD2, KCTD5, and KCTD17 all interact with each other (116,159). Because crystallography studies of KCTD5 revealed a pentameric structure, our results suggest that this KCTD

subfamily might form hetero-pentamers (112). In addition, each member of this subfamily assembles into complexes with CUL3, indicating that they are all functionally active substrate adaptor proteins capable of delivering substrate proteins to a CUL3–RING ubiquitin ligase.

Interestingly, these interactome studies demonstrated that KCTD2 and KCTD5 interact with multiple Gβ and Gγ subunits while neither forms detectable complexes with Gα subunits (**Figure 2B**). This suggested that these substrate adaptor proteins specifically recognize Gβγ dimers. Co-immunoprecipitation experiments with overexpressed heterotrimeric G-proteins reinforced this observation that Gβ and Gγ, but not Gα, interact with KCTD2 and KCTD5 (**Figure 2C**).

In contrast to KCTD2 and KCTD5, KCTD17 interacts relatively weakly with Gβ and forms complexes with fewer Gβ isoforms (**Figure 2B and 3**). For example, $G\beta_1$ was enriched ~6500× relative to controls in KCTD5 complexes, but only ~15× in KCTD17 complexes. This suggests that there are functional differences in this subfamily of KCTD proteins despite their close sequence homology, hetero-oligomerization, and shared interactions with CUL3. Consistent with these findings, KCTD17 was not identified as a significant binding partner in our analysis of the $G\beta_1$ interactome (**Figure 1**). Together, these results show that KCTD2 and KCTD5 share a strong relative preference for multiple isoforms of Gβγ, and that KCTD17 might instead primarily recruit other proteins for ubiquitination through its C-terminal domain, such as a regulator of ciliogenesis that it was previously reported to polyubiquitinate (118).

Given that our interactome analyses of $G\beta_1$ and the KCTD2/5/17 subfamily suggested that a KCTD2/KCTD5–CUL3–RING ubiquitin ligase recruits Gβγ, we further explored the structural organization of this complex. Our earlier results demonstrated that both KCTD2 and KCTD5 could form complexes with Gβγ and CUL3 (**Figure 2B**). Their ability to hetero-oligomerize with each other, however, could potentially obscure differences in their recruitment of G-proteins and assembly into CUL3–RING ubiquitin ligases. Accordingly, we evaluated the interactions of KCTD2 and KCTD5 with CUL3 or Gβ in wild type HEK-293 cells or cells lacking either KCTD2 or KCTD5. Interestingly, deletion of *KCTD2* has no effect on the CUL3–KCTD5 interaction, but deletion of *KCTD5* results in the complete loss of the CUL3–KCTD2 interaction (**Figure 4A**). In contrast, these putative substrate adaptor proteins can recruit Gβ independently of each other (**Figure 4B**). These studies refined our understanding of these substrate adaptor proteins and contributed to our model of a KCTD2-KCTD5 hetero-pentamer that associates with CUL3 and Gβγ through KCTD5 subunits and KCTD2 or KCTD5 subunits, respectively (**Figure 4C**).

KCTD2 and KCTD5 facilitate Gβ monoubiquitination

To evaluate our hypothesis that KCTD2 and KCTD5 function as substrate adaptor proteins for a CUL3–RING ubiquitin ligase that binds and ubiquitinates Gβγ, we developed an *in vitro* ubiquitination reaction system to assess the activity of these KCTD proteins. Briefly, we isolated complexes from HEK-293 cells containing KCTD2 or KCTD5 bound to their endogenous substrates and incubated these complexes with CUL3/RBX1, ubiquitin, other enzymes required for ubiquitination, and ATP.

Immunoblots of the reaction products with antibodies recognizing $G\beta_1$ subunits demonstrate that these KCTD proteins monoubiquitinate Gβ subunits (**Figure 5A**). Importantly, this ATP-dependent enzymatic activity is strongly stimulated by the inclusion of CUL3/RBX1, suggesting that Gβ monoubiquitination is facilitated by a KCTD2/KCTD5–CUL3–RBX1 ubiquitin ligase and not a co-purifying ubiquitin ligase.

We also analyzed the products of these *in vitro* reactions with mass spectrometry to identify the ubiquitin-attachment sites within Gβ and to search for additional potential substrates related to G-protein signaling (**Figure 5B**). We identified ubiquitin-modified peptides by searching for peptides with lysine residues containing diglycine adducts, which are the remnants of a ubiquitin molecule attached to a lysine residue following digestion with trypsin (160). We examined proteins within the KCTD5 *in vitro* reactions that had diglycine-modified lysine residues and that were also enriched in the KCTD5 complexes. Four proteins met these criteria: $G\beta_1$, $G\beta_2$, DNA polymerase-κ, and KCTD5.

This approach identified a ubiquitin-attachment site at lysine-23 within two Gβ isoforms: $G\beta_1$ and $G\beta_2$. This lysine residue is conserved within other Gβ isoforms in humans and other model organisms, including *M. musculus*, *D. melanogaster*, and *C. elegans*. We also found multiple ubiquitin-attachment sites within DNA polymerase-κ, a low-fidelity translesion repair polymerase previously described to be ubiquitinated (161). In addition, we also observed a single modified lysine residue within KCTD5, which is consistent with other reports of autoubiquitination of CUL3-associated substrate adaptor proteins (162,163).

We augmented these *in vitro* studies with *in vivo* experiments by quantifying ubiquitination of lysine-23 within $G\beta_{1/2}$ in HEK-293 cells (**Figure 5C**). Interestingly, we could clearly detect these exact $G\beta_1$ and $G\beta_2$ modifications in membranes of wild type cells. In *KCTD2/KCTD5*-deletion cells, however, the levels of these modifications were sharply reduced. The abundance of lysine-23-modified $G\beta_1$ and $G\beta_2$ peptides were significantly decreased by 83% and 76%, respectively. Collectively, these *in vitro* and *in vivo* experiments suggest that KCTD2 and KCTD5 promote Gβ monoubiquitination of lysine-23—a novel post-translational modification of a central signaling protein.

KCTD2 and KCTD5 recruit Gβγ in response to G-protein signaling

Given that this KCTD2/KCTD5-containing ligase promotes Gβ ubiquitination *in vivo* and *in vitro*, we sought to understand whether its activity is regulated by G-protein activation as part of a regulatory mechanism to amplify or inhibit Gβγ signaling. We investigated whether these substrate adaptor proteins recruit Gβγ in a signaling-dependent manner. We activated G-protein signaling in HeLa cells with GDP and AlF_4^-, which bind within the Gα nucleotide-binding site and activate Gα subunits to promote their dissociation from Gβγ (164). Signaling activation markedly enhances the interactions between 3×FLAG 3×HA-tagged $G\beta_1$ and endogenous KCTD2 or KCTD5 (**Figure 6A**) as well as those between 3×FLAG 3×HA-tagged KCTD2 or KCTD5 and endogenous $G\beta_1$ (**Figure 6B and 7**). This strongly suggested that KCTD2 and KCTD5 recruit their substrate in response to signaling.

The molecular basis for this signaling-dependent adaptor–substrate interaction was clarified by experiments mapping the surface of Gβγ recognized by these substrate

adaptor proteins. Alanine-substitution mutations of multiple surface-exposed Gβ residues in its Gα-interacting surfaces severely impair its interaction with KCTD2 and KCTD5 in HEK-293 cells (**Figure 6C–D**). This demonstrates that KCTD2 and KCTD5 likely bind a region of Gβ that is only accessible during signaling activation when Gβγ is liberated from Gα. Together, these findings raise the possibility that Gβ ubiquitination might be induced by G-protein activation to fine-tune downstream signaling events.

A KCTD2/KCTD5–CUL3–RING E3 ligase positively regulates cAMP signaling

To augment our extensive biochemical studies of the KCTD2/KCTD5–CUL3–RING ubiquitin ligase and the novel Gβ modification that it facilitates, we next investigated the physiologic role of these substrate adaptor proteins in the context of GPCR signaling. One of the primary targets of Gα and Gβγ are adenylyl cyclases (ACs) (34). All ACs can be stimulated by $G\alpha_s$ and several are inhibited by $G\alpha_i$. Similarly, Gβγ positively regulates several isoforms (AC2, AC4, AC5, AC6, and AC7) and negatively regulates others (AC1, AC3, and AC8). When active, these enzymes produce cyclic AMP (cAMP), which can activate protein kinase A (PKA) and promote protein phosphorylation (36-38).

Given the significant role of Gβγ in regulating ACs, we measured cAMP generation in both control and *KCTD2/KCTD5*-deletion HEK-293 cells following treatment with two signaling activators: forskolin or PGE1 (**Figure 8A**). Forskolin directly binds all but one AC isoform and can sensitize these enzymes to activation by $G\alpha_s$ (165). Forskolin can also augment the activating effects of Gβγ on several AC isoforms (166). PGE1

activates multiple prostaglandin-stimulated GPCRs, including several which couple to $G\alpha_s$ heterotrimers.

Both signaling activators robustly increase cAMP levels in control HEK-293 cells. In the absence of KCTD2 and KCTD5, however, the cAMP response to these molecules is sharply attenuated (**Figure 8A**). For example, the forskolin-induced cAMP increase is 87% lower in *KCTD2/KCTD5*-knockout cells while the PGE1-induced cAMP increase is 51% lower in the cells lacking these substrate adaptor proteins. As expected, these large differences in cAMP generation are accompanied by corresponding changes in downstream signaling pathways. We examined phosphorylation of CREB—a canonical substrate of the cAMP-activated PKA—after treatment with different concentrations of PGE1 (**Figure 8B**). We observed a dose-dependent increase in phosphorylation of serine-133 within CREB. Compared to control cells, however, cells lacking KCTD2 and KCTD5 were less sensitive to PGE1. At moderate concentrations, we observed lower levels of CREB phosphorylation in *KCTD2/KCTD5*-knockout cells relative to control cells (**Figure 8B**). These decreases in CREB phosphorylation mirror the defects in cAMP generation.

Importantly, this signaling defect in the *KCTD2/KCTD5*-deletion cells is readily reversible. Transient overexpression of 6×MYC-KCTD2 and 6×MYC-KCTD5 can partially rescue the impaired signaling observed in *KCTD2/KCTD5*-knockout cells (**Figure 8C**). This indicates that these changes in cAMP generation are related to the deletion of these substrate adaptor proteins rather than off-target effects of the Cas9 crRNAs used to generate the *KCTD2/KCTD5*-deletion cells.

Although these results suggested a role for KCTD2 and KCTD5 in positively regulating cAMP signaling, it was not clear whether this signaling phenotype was related to the substrate adaptor function of these KCTD proteins. Accordingly, we examined how depletion of CUL3—the ubiquitin ligase scaffolding component that binds KCTD proteins—affects cAMP generation in response to signaling (**Figure 8D**). Much like deletion of *KCTD2* and *KCTD5*, depletion of CUL3 markedly impairs activation-dependent cAMP generation. CUL3 depletion reduces the forskolin and PGE1-induced increases in cAMP levels by 82% and 46%, respectively. The similarity of the changes in cAMP generation associated with loss of these substrate adaptor proteins and CUL3 suggests that ubiquitination and the KCTD2/KCTD5–CUL3–RING ubiquitin ligase positively regulate cAMP signaling.

To determine whether this ubiquitin ligase has a broad role in stimulating cAMP signaling, we also examined its importance in other human cancer cell lines. We quantified CREB phosphorylation in MCF-7 breast cancer-derived cells in the context of depletion of KCTD5 or CUL3 (**Figure 8E**). Depletion of these proteins reduces levels of CREB phosphorylation in cells treated with a phosphodiesterase inhibitor, which increases cAMP levels. This is consistent with our findings in HEK-293 cells and suggests that positive regulation of cAMP signaling by this ubiquitin ligase is shared among multiple cell types.

DISCUSSION

In an effort to identify novel regulators of $G\beta_1$, we used proteomic mass spectrometry to find $G\beta_1$ protein-binding partners. We identified a KCTD2/KCTD5–CUL3–RING E3 ubiquitin ligase that recognizes Gβγ in response to G-protein activation. We then developed an unbiased approach to identify the substrates of this E3 ligase. We showed that this complex likely facilitates monoubiquitination of lysine-23 within $G\beta_{1/2}$. We also demonstrated the functional role of this Gβ-ubiquitinating complex in positively regulating cAMP signaling.

Identification of a KCTD2/KCTD5–CUL3–RING E3 ligase that recognizes Gβγ

We identified a $G\beta_1$-binding multisubunit Cullin–RING E3 ligase composed of KCTD2, KCTD5, and CUL3. CUL3 is the scaffolding component of a multisubunit E3 ligase that binds both a RING protein, which is necessary for ubiquitination, and large families of substrate adaptor proteins at its C-terminus and N-terminus, respectively (104). CUL3 specifically recognizes substrate adaptor proteins containing BTB domains (106-108). Recent proteomic mass spectrometry studies have indicated that CUL3 interacts with 53 BTB domain-containing proteins in human 293T cells, including several proteins of the KCTD family (111). KCTD2 and KCTD5 have also been identified as putative substrate adaptor proteins because they form complexes with CUL3 in cells (114,158). This indicated that $G\beta_1$ interacts with a complete KCTD2/KCTD5–CUL3–RING E3 ubiquitin ligase.

Our studies revealed that this E3 ligase recognizes a multitude of Gβγ dimers. The Gβγ dimers recognized by these substrate adaptor proteins are functionally active because KCTD2 and KCTD5 can bind Gβγ released from heterotrimers following Gα activation. We have further demonstrated that a KCTD2–KCTD5 hetero-pentamer likely binds the signaling-exposed surface of Gβγ through either KCTD2 or KCTD5 while it recruits CUL3 through its KCTD5 subunits.

The structural basis for this apparent selectivity in CUL3 binding is unclear because KCTD5 residues predicted to coordinate its interaction with CUL3 are largely conserved in KCTD2 (113,167). The functional implications of this difference in CUL3 binding between KCTD2 and KCTD5 are also not understood. Electron microscopy of complexes containing the BTB domain of KCTD9 and the N-terminal domain of CUL3 revealed 5 CUL3 fragments radiating outward from a KCTD9 pentamer (113). It is possible that inclusion of KCTD2 in the substrate adaptor pentamer increases its activity by reducing steric hindrance caused by recruitment of multiple CUL3 molecules. This could increase the number of accessible binding sites for Gβγ. Alternatively, KCTD2 incorporation into the pentamers could impair activity by limiting CUL3 binding. Mapping the CUL3 and Gβγ-binding domains on these substrate adaptor proteins and precise measurement of complex activity will clarify the function of KCTD2 in these hetero-pentamers.

While we primarily focused on KCTD2 and KCTD5, we note these proteins share close sequence homology with KCTD17. Compared to KCTD2 and KCTD5, however, there were lower levels of Gβγ in KCTD17 complexes. In addition, in our initial $G\beta_1$

interactome analysis, KCTD17 was slightly enriched relative to controls but this did not reach statistical significance. This may indicate that KCTD17 binds Gβγ indirectly by hetero-oligomerizing with KCTD2 or KCTD5, or that KCTD17 binds Gβγ directly with low affinity. Regardless, its limited interactions with Gβγ suggest that it may not be involved in regulating Gβγ. These differences between KCTD2/KCTD5 and KCTD17 might be related to the C-terminal domain of KCTD17 that is involved in targeting a regulator of ciliogenesis for polyubiquitination and proteasomal degradation (118).

An approach to deorphanize a subfamily of E3 ligase substrate adaptor proteins

Our observation that a KCTD2/KCTD5–CUL3–RING E3 ligase recognized Gβγ prompted us to develop a unique method to identify substrates of this complex. One of the major challenges of deorphanizing substrate adaptor proteins is distinguishing protein-binding partners from actual substrates. Low-stringency affinity purifications of overexpressed substrate adaptor proteins frequently contain hundreds of significantly enriched binding partners. Others have developed substrate-trapping strategies to identify substrates of different substrate adaptor proteins. One method involves overexpression of 6×His-tagged ubiquitin and a substrate-adaptor protein of interest fused to a set of ubiquitin-binding domains (168). This facilitates purification of substrate adaptor complexes associated with their modified substrates, which can then be enriched with Ni^{2+}-NTA resin and detected by mass spectrometry. While this approach has been successful, it has limitations (169,170). For example, the ubiquitin-binding domains used in this method bind different types of ubiquitin modifications with varying affinities. The Dsk2 ubiquitin-binding domain binds monoubiquitin and lysine-

48-linked diubiquitin with a K_D of 1.7 and 0.056 μM, respectively (171). Accordingly, it may be challenging to capture proteins with certain types of ubiquitin modifications. In addition, overexpression of the substrate adaptor protein without the requisite cullin and RING protein may limit modification of substrate proteins.

Our substrate-identification strategy involves purification of complexes containing affinity-tagged substrate adaptor proteins associated with their endogenous substrates followed by incubation with components required for ubiquitination. These components include ubiquitin, a cullin, a RING protein, E1 and E2 enzymes, and ATP, and their inclusion should maximize the activity of the complexes. We then analyze the products of these *in vitro* ubiquitination reactions with proteomic mass spectrometry. We filter our results to identify proteins that are highly enriched in the substrate adaptor protein complexes and also modified by ubiquitin in the reactions containing these substrate adaptor proteins.

For KCTD5, this unbiased approach led to the identification of four potential substrates: $G\beta_1$, $G\beta_2$, DNA polymerase-κ, and KCTD5. This limited number of substrates suggests that KCTD5 is relatively specific in targeting proteins for ubiquitination, although it is possible that the actual number of substrates could be understated by limited sensitivity in detecting low-abundance modified peptides. Two of these substrates were $G\beta_1$ and $G\beta_2$. Both of these isoforms were modified at lysine-23—a modification previously reported in proteome-wide surveys of ubiquitination (172). We were also able to determine that KCTD2 and KCTD5 likely promote monoubiquitination of Gβ by analyzing these *in vitro* ubiquitination reactions by

immunoblotting with a $G\beta_1$-specific antibody. The substrate information gleaned from our unbiased *in vitro* ubiquitination reaction approach was validated by our observation that deletion of *KCTD2* and *KCTD5* sharply reduce ubiquitination of lysine-23 within $G\beta_{1/2}$ *in vivo*.

While we extensively characterized $G\beta_{1/2}$ as substrates of these KCTD proteins, our approach also identified multiple ubiquitin-attachment sites within another potential substrate: DNA polymerase-κ. This is noteworthy because this low-fidelity enzyme has been reported to be ubiquitinated, and its association with replication forks is predicted to be regulated by this modification (161).

We imagine that this overall approach may guide identification of substrates of other orphaned substrate adaptor proteins for cullin–RING ubiquitin ligases.

The role of the KCTD2/KCTD5–CUL3–RING E3 ligase as a signaling regulator

We anticipated that this ubiquitin ligase would regulate signaling because it promoted ubiquitination of a key signaling molecule and recruited its substrate in a signaling-dependent manner. Lysine-23 is located within the N-terminal helical domain of Gβ. Interactions between Gβγ and its multitude of signaling effectors are well described. Most of these canonical signaling effectors bind several blades of the Gβ β-propeller that are only exposed upon G-protein activation when Gβγ dissociates from Gα. Mutation of residues within this surface affects regulation of ACs, isoforms of PLCβ, GIRK channels, G-protein-coupled receptor kinases, and other classical effectors (46).

In contrast, the N-terminal helical domain of Gβ has not been as extensively studied. Early work demonstrated that it forms a coiled coil with Gγ to facilitate dimer formation (18). Recently, however, it has become apparent that its functional role extends beyond dimer assembly. Sequencing of human cancers has revealed multiple missense mutations within this domain, including mutation of lysine-23 to glutamic acid or glutamine within $G\beta_2$ (77). Intriguingly, this is the same residue modified by the KCTD2/KCTD5–CUL3–RING E3 ligase. In studies of lymphoid cells, this lysine–glutamic acid mutation in $G\beta_2$ enabled cytokine-independent growth, mirroring the effects of other missense mutations within the signaling surface of $G\beta_{1/2}$ (77). This suggested that lysine-23 and the N-terminal helical domain might have important signaling roles. Indeed, multiple groups have reported that combined mutations of lysine-23 to alanine (K23A), alanine-24 to asparagine (A24N), and aspartic acid-27 to alanine (D27A) within $G\beta_1$ affect Gβγ-mediated regulation of four signaling effectors: AC5, AC6, PLCβ2, and PLCβ3 (47,49).

In the presence of $G\alpha_s$, wild type Gβγ activates the *in vitro* activity of AC5 and AC6, but the triple-mutant Gβ largely loses this ability (47). In addition, this mutation impairs the interaction between Gβγ and AC5 in HEK-293 cells, suggesting that AC5 forms contacts with the N-terminal helical domain to stimulate its activity (47). Consistent with the importance of lysine-23 and the N-terminal domain in activating these two AC isoforms, we observed impaired cAMP generation in *KCTD2*/*KCTD5*-deletion cells or CUL3-depleted cells upon treatment with a GPCR agonist or forskolin. The GPCR agonist should activate $G\alpha_s$ and promote activation of ACs through $G\alpha_s$ and Gβγ. Forskolin activates all ACs except AC9, and can sensitize these enzymes to

activation by $G\alpha_s$ (34,165). In addition, Gβγ can activate AC5 or AC6 in the presence of forskolin *in vitro* (47,166). Our experiments suggest that this Gβ-ubiquitinating E3 ligase complex positively regulates cAMP signaling and ACs—a direct effector of Gα and Gβγ.

It is possible that the cAMP signaling defects associated with disruption of this ubiquitin ligase are related to the role of the N-terminal helical domain and lysine-23 of Gβ in stimulating these two AC isoforms (47). Additional studies should elucidate whether the effects of this ubiquitin ligase on cAMP signaling are directly related to its ability to modify lysine-23 of Gβ with ubiquitin. This modification may promote the association of Gβγ with AC5 or AC6 and enhance their enzymatic activities (47). Alternatively, this Gβ modification might promote activation of other Gβγ-stimulated ACs or block negative regulation of Gβγ-inhibited isoforms. It will be necessary to assess signaling-dependent interactions between Gβγ and all AC isoforms in the presence and absence of KCTD2 and KCTD5 using bioluminescence resonance energy transfer studies or proximity-labeling methods to address these possibilities.

An alternative mechanism for these cAMP defects may be related to the ability of the N-terminal helical domain of Gβ—including lysine-23—to regulate PLCβ activity (49). The combined K23A/A24N/D27A mutation within $G\beta_1$ increases Gβγ-mediated activation of PLCβ2 and PLCβ3. One group proposed a model where the classical signaling surface of Gβγ activates this enzyme while the Gβ helical domain attenuates this positive regulation. Higher levels of PLCβ activity can increase intracellular Ca^{2+} concentrations, which can affect the activity of multiple AC isoforms (34,41).

One group described how activation of $G\alpha_q$-coupled receptors could attenuate the effects of $G\alpha_s$-coupled receptor activation on cAMP signaling (173). Their experiments showed that this effect could be reduced by depletion of AC5 and AC6, which are inhibited by physiological concentrations of Ca^{2+}. In our studies, PGE1 should activate receptors coupled to $G\alpha_s$ and $G\alpha_q$, so it is possible that $G\alpha_q$-dependent increases in Ca^{2+} concentrations inhibit AC5 and AC6 to reduce their activation by $G\alpha_s$ and $G\beta\gamma$. Similar inhibition might also be observed with forskolin treatment if basal Ca^{2+} concentrations were also increased. Experiments with PLC inhibitors or Ca^{2+} chelators should clarify whether the defects in cAMP signaling observed in the absence of KCTD2/KCTD5 or CUL3 are related to increases in PLCβ activity and intracellular Ca^{2+} levels. In addition, other experiments could clarify whether the KCTD2/KCTD5–CUL3–RING E3 ligase can also regulate $G\alpha_q$ signaling by measuring cytoplasmic Ca^{2+} levels following treatment with agonists specific for $G\alpha_q$-coupled receptors.

While future studies will detail the mechanisms underlying the positive regulation of cAMP signaling by the Gβ-ubiquitinating KCTD2/KCTD5–CUL3–RING E3 ligase, it is apparent that this complex may also regulate other signaling events. Recently, in a genetic screen for regulators of AKT signaling in HAP1 pseudo-haploid cancer-derived cells, one group identified KCTD5 and CUL3 as possible negative regulators of AKT activation and found that the deletion of KCTD5 or CUL3 increased phosphorylation of serine-473 within AKT (174). They also performed a second screen in *KCTD5*-deletion cells to find suppressors of this AKT phenotype and identified Gβ and Gγ subunits as well as PI3K, which is stimulated by Gβγ to activate AKT. This group found that KCTD5 affected ubiquitination of lysine-23 within $G\beta_{1/2}$ *in vivo*, and hypothesized that this

modification might reduce activation of PI3K by Gβγ and decrease AKT phosphorylation. While we have not observed increased AKT phosphorylation in HEK-293 cells lacking KCTD5 or KCTD2/KCTD5 (*data not shown*), it is possible that these proteins also negatively regulate AKT in other cell types.

Importantly, this group also identified lysine-23 of Gβ as a potential target for a KCTD5–CUL3–RING E3 ligase (174). Our efforts to identify novel regulators of Gβ uncovered a hetero-oligomeric KCTD2/KCTD5–CUL3–RING E3 ligase that recruits Gβγ through KCTD2 or KCTD5 subunits in a signaling-dependent manner. Using *in vitro* ubiquitination reactions, we have demonstrated that purified complexes containing KCTD2 or KCTD5 can monoubiquitinate Gβ. We have localized this *in vitro* modification to lysine-23 of Gβ, and have confirmed that these substrate adaptor proteins facilitate this modification *in vivo*. While our studies suggest that this Gβ-modifying complex promotes cAMP signaling, others have reported that it impairs AKT signaling. Additional studies will clarify the breadth of signaling events regulated by this signaling-regulated E3 ligase, and illuminate how this novel Gβ modification affects Gβγ-mediated regulation of different signaling effectors.

MATERIALS AND METHODS

Plasmids and Cloning

cDNAs for KCTD5, KCTD17, GNAS, GNB1, GNG12, and CUL3 were obtained from Dharmacon (**Table 1**). ORFs were amplified by PCR using Phusion high-fidelity DNA polymerase (NEB) and forward and reverse primers fused to *attB1* or *attB2* sequences, respectively (**Table 2**). *attB*-flanked ORFs were purified with the PureLink Quick PCR Purification kit or the PureLink Quick Gel Extraction kit (both from Invitrogen). These amplified ORFs were inserted into pDONR221 using BP Clonase II (Thermo). A KCTD2 cDNA in pENTR223.1 was obtained from GeneCopoeia (**Table 1**). These entry clones were sequenced using the M13F(-21) primer to verify their identity and ensure that they were inserted in frame. Reaction of these entry clones with LR clonase II (Thermo) and a pcDNA5/FRT/TO destination vector yielded expression clones expressing N-terminally 3×HA 3×FLAG-tagged proteins under the control of a CMV/TetO2 promoter. Another destination vector was used to generate expression clones expressing N-terminally 6×MYC-tagged proteins.

Plasmids expressing 3×HA-tagged wild type and mutant GNB1 in the pCS2 backbone were generously provided by Ethan Lee (175). Mutations were verified by sequencing with the SP6 and T3 primers.

Cell Culture and Generation of Cell Lines

HEK-293 and MCF-7 cells were acquired from ATCC. HEK-293 and HeLa Flp-In T-REx cells containing the tetracycline repressor and a single integrated FRT site were

obtained from Invitrogen. Similar HEK-293 Flp-In cells lacking the tetracycline repressor were also acquired from Invitrogen. These cells were maintained in DMEM containing 25 mM glucose (Thermo; 11960) supplemented with 2 mM L-glutamine, 10% FBS (Gemini; Foundation B), and 1× Gibco Antibiotic-Antimycotic.

Stable cell lines expressing 3×HA 3×FLAG-tagged proteins were generated by transfecting HeLa or HEK-293 Flp-In T-REx cells with the appropriate expression vector and pOG44 in a 1:9 ratio using Lipofectamine 2000 (Thermo). HeLa and HEK-293 cells were selected with 500 or 250 µg/mL Hygromycin B Gold (Invivogen), respectively, and multiple colonies were combined to make stocks.

KCTD2 and *KCTD5*-knockout cells were generated using the Edit-R CRISPR-Cas9 system (Dharmacon). Briefly, HEK-293 Flp-In cells lacking T-REx were transfected with a crRNA (CR-032516-04 or CR-021199-03; Dharmcon), tracrRNA, and a Cas9 expression plasmid using Dharmafect Duo (Dharmacon). For double knockouts, cells were simultaneously transfected with crRNAs targeting both genes. Control cells were generated by transfecting cells with the Cas9 expression plasmid. Knockout and control cells were selected with 1.5 µg/mL puromycin (Invivogen) for several weeks before isolation of individual clones. Genotyping was performed by SDS-PAGE and immunoblotting with an antibody recognizing KCTD2 and KCTD5 (Proteintech; 15553-1-AP).

Protein Interactome Profiling

For protein interactome studies, 4×15-cm plates of HeLa or HEK-293 cells stably expressing 3×HA 3×FLAG-tagged proteins or control Flp-In cells were grown and

induced with 0.5 µg/mL doxycycline overnight before harvesting. Cell pellets were resuspended in 4 mL native lysis buffer (100 mM Tris (pH 8), 150 mM NaCl, 5 mM EDTA, 1 mM DTT, 5% glycerol, 0.1% NP-40) containing AEBSF, pepstatin, and leupeptin. After end-over-end rotation for 30 min at 4 ºC, lysates were clarified by centrifugation at 16,100×g for 15 min at 4 ºC. Lysates were normalized by absorbance at 280 nm and incubated with 40 µL of pre-equilibrated EZview Red Anti-HA affinity gel (Sigma). After 2 h of end-over-end rotation at 4 ºC, the beads were washed 5× with native lysis buffer and twice with native lysis buffer lacking NP-40. Protein complexes were eluted by incubating the beads with 55 µL 8 M urea in 100 mM Tris (pH 8.5) for 5 min. This elution was repeated twice and the eluates were combined.

Proteins were precipitated by addition of 4 volumes of -20 ºC acetone and incubation on ice for 2 h. After centrifugation at 16,100×g for 30 min at 4 ºC, the pellets were washed with -20 ºC acetone and centrifuged again. Dried pellets were resuspended in 50 µL 8 M urea in 100 mM Tris (pH 8.5), reduced with addition of 1.25 µL 200 mM TCEP for 20 min, and alkylated with 1.025 µL 0.5 M iodoacetamide for 20 min in darkness. Proteins were initially digested with 0.1 µg LysC for 4 h at 37 ºC in darkness before addition of 150 µL 100 mM Tris (pH 8.5), 2 µL 100 mM $CaCl_2$, and 0.8 µg Pierce trypsin. After overnight incubation at 37 ºC in darkness, digestions were quenched by addition of formic acid to 5%. Peptides were desalted with C18 tips, eluted with 40% acetonitrile and 5% formic acid, dried by vacuum centrifugation, and resuspended in 20 µL 5% formic acid.

Peptide samples were separated on C18 reversed phase (1.9 µm, 100-Å pores, Dr. Maisch GmbH) 75-µm internal diameter columns that were packed with 25 cm of resin. Peptides were eluted on a 140-min water–acetonitrile gradient with 3% DMSO in both mobile phases. After electrospray ionization at 2.2 kV, ionized peptides were analyzed by tandem mass spectrometry with a Thermo Orbitrap Fusion Lumos. Data-Dependent Acquisition (DDA) was used with MS^1 and MS^2 scan resolutions of 120,000 and 15,000, respectively, and a 3-second cycle time.

The resulting spectra were analyzed with the Galaxy-based MilkyWay analysis platform (http://github.com/wohllab/milkyway_compose). Acquired data files were converted from the RAW format to the mzML format using msconvert. The vendor peak-centroiding feature was used for the MS^1 and MS^2 scans. The MSGF+ search algorithm was used to search the spectra against the human Uniprot reference proteome database with a MS^1 search tolerance of 25 ppm (98,176). The search results were converted into Percolator inputs with the msgf2pin converter, and peptide and PSM score calibration was completed with the crux implementation of the Percolator support vector machine classifier (177-179). Confidence for protein identifications was calculated with the stand-alone implementation of FIDO and were calculated globally within each analysis (180). PSMs and proteins were filtered with a q-value threshold of ≤0.01. Skyline was used for label-free MS^1-based intensity-based quantification (181). Peak picking in Skyline was guided by training of mProphet peak-picking models using random mass shift decoy peptides, and filtered with a q-value threshold of ≤0.01 (182). Skyline-generated quantification values were exported to MSstats to make statistical comparisons between protein abundances in different

samples (100). Sample normalization was achieved with several abundant proteins present in all samples that bind non-specifically to the anti-HA beads.

Co-Immunoprecipitation Studies

Cells were transiently transfected with mammalian expression plasmids and BioT (Bioland Scientific). After two days, cells were harvested and resuspended in native lysis buffer containing AEBSF, pepstatin, and leupeptin. After end-over-end rotation for 30 min at 4 ºC, lysates were clarified by centrifugation at 16,100×g for 15 min at 4 ºC. Lysates were normalized by absorbance at 280 nm and rotated with pre-equilibrated EZview Red anti-HA affinity gel (Sigma) or anti-HA magnetic beads (Pierce) at 4 ºC for 2 h. After washing 4× with native lysis buffer, purified complexes were eluted with SDS-PAGE sample buffer. Whole-cell lysates and anti-HA immunopurifications were analyzed by SDS-PAGE and immunoblotting. PVDF membranes were probed with antibodies recognizing FLAG (Sigma; F1804), HA (Roche; 12CA5), KCTD2/KCTD5 (Proteintech; 15553-1-AP), and α-tubulin (Proteintech; HRP-66031). HRP-conjugated secondary antibodies, Pierce ECL Western Blotting Substrate, SuperSignal West Femto Maximum Sensitivity Substrate (Thermo), and X-ray film were used to visualize the membranes.

In vitro Ubiquitination Reactions

For immunoblotting analysis of *in vitro* ubiquitination reactions, 3×15-cm plates of HEK-293 cells stably expressing 3×HA 3×FLAG-tagged substrate adaptor proteins or control Flp-In cells were grown and induced with 0.5 μg/mL doxycycline overnight. Cells

were lysed in native lysis buffer containing 2 mM N-ethylmaleimide, AEBSF, pepstatin, and leupeptin. After clarification by centrifugation and normalization by absorbance at 280 nm, lysates were incubated with 40 µL EZview Red anti-HA affinity gel (Sigma) and rotated for 2 h at 4 ºC. After washing 5× with lysis buffer, the beads were washed 4× with ubiquitin ligase reaction buffer (50 mM Tris (pH 7.5), 5 mM $MgCl_2$, and 0.6 mM DTT) and separated into three tubes (183). The beads were then incubated with 12 µg ubiquitin (Boston Biochem; U-100H), 120 ng UBE1 (Boston Biochem; E-304), 300 ng UBCH5A (Boston Biochem; E2-616), ±150 ng CUL3/RBX1 (Ubiquigent; 63-1003-025), and ±60 nmol ATP in 30 µL ubiquitin ligase reaction buffer for 1 h at 37 ºC with shaking. The reactions were quenched with addition of SDS-PAGE sample buffer and boiling, and analyzed by SDS-PAGE and immunoblotting with antibodies recognizing FLAG (Sigma; F1804), ubiquitin (Abcam; ab19247), and GNB1 (Proteintech; 10247-2-AP). Membranes were visualized as described previously.

Mass spectrometry-based analysis of these reactions was conducted with 12×15-cm plates of HEK-293 control cells or cells stably expressing 3×HA 3×FLAG-tagged KCTD5. After overnight induction with doxycycline, lysis in 12 mL native lysis buffer containing AEBSF, pepstatin, and leupeptin, and a similar anti-HA immunopurification, the beads were incubated with 24 µg ubiquitin, 240 ng UBE1, 600 ng UBCH5A, 300 ng CUL3/RBX1, and 120 nmol ATP in 60 µL ubiquitin ligase reaction buffer for 1 h at 37 ºC with shaking. The reactions were centrifuged and the supernatant was saved. The beads were then incubated with 60 µL 8 M urea in 100 mM Tris (pH 8.5) for 5 min. After repeating this urea elution step, the three supernatants were combined. The reaction products were reduced with 5 mM TCEP for 20 min, alkylated with 10 mM

chloroacetamide for 20 min in darkness, and digested with 0.45 µg LysC for 4 h at 37 ºC in darkness. After dilution of the urea concentration to 2 M with 100 mM Tris (pH 8.5), $CaCl_2$ was added to 1 mM and the samples were digested with 1.8 µg Pierce trypsin overnight in darkness at 37 ºC. The urea concentration was then diluted to 0.8 M with water and the digests were quenched by adding trifluoroacetic acid to 0.5%. The peptides were fractionated with the Pierce High pH Reversed-Phase Peptide Fractionation kit and dried by vacuum centrifugation. Each of the 8 resulting fractions from each reaction were then resuspended in 20 µL 5% formic acid and analyzed separately by proteomic mass spectrometry as described for the interactome analyses.

Data from the 8 fractions for each reaction were analyzed using the IP2 platform (Integrated Proteomics Applications). The ProLUCID search parameters included a variable 114.042927-Da modification on lysine residues to identify diglycine-modified peptides (96). DTASelect parameters included a spectrum false-positive rate of 1% (97). Proteins identified in these reactions had at least two mapped peptides while modified peptides identified in these reactions lacked this constraint.

Quantification of Gβ Ubiquitination in HEK-293 Cells

HEK-293 *KCTD2*/*KCTD5*-deletion cells and control cells were grown and membrane fractions were isolated as described previously (184). Briefly, cell pellets from 15-cm plates were resuspended in 4 mL cytosol extraction buffer (50 mM HEPES (pH 7.4) 150 mM NaCl, 5 mM N-ethylmaleimde, and 25 µg/mL digitonin) containing AEBSF, pepstatin, and leupeptin. After end-over-end rotation for 10 min at 4 ºC and centrifugation at 2000×g for 5 min at 4 ºC, the pellets were washed with ice-cold PBS.

The washed pellets were then vortexed in membrane extraction buffer (50 mM HEPES (pH 7.4), 150 mM NaCl, 5 mM N-ethylmaleimde, and 0.1% NP-40) containing AEBSF, pepstatin, and leupeptin. After 30 min on ice, the extracts were centrifuged at 7000×g for 10 min at 4 ºC. The proteins within these supernatants (membrane fractions) were then precipitated with 20% trichloroacetic acid on ice for 1 h before two washes with ice-cold acetone.

Dried protein pellets were solubilized in 8 M urea 100 mM Tris (pH 8.5) and protein content was quantified with the Pierce BCA Protein Assay Kit. Equal amounts of protein were then reduced with 4.875 mM TCEP for 20 min and alkylated with 11.4 mM chloroacetamide for 20 min in darkness. The urea concentration was reduced to 2 M with addition of 100 mM Tris (pH 8.5), 1 mM $CaCl_2$ was added, and Worthington trypsin was added (1:20 enzyme:substrate ratio). After 2 h incubation at 37 ºC in darkness, additional trypsin was added (1:40 enzyme:substrate ratio) and the digestions continued overnight at 37 ºC in darkness. Digests were acidified with 5% formic acid and desalted using C18 cartridges (3M Empore).

Ubiquitinated peptides were then purified from the resuspended peptides using antibody-conjugated beads recognizing diglycine-modified lysine residues (PTMScan Ubiquitin Remnant Kit from Cell Signaling) (185). Briefly, peptides were resuspended in 200 µL IAP buffer (50 mM MOPS (pH 7.2), 10 mM Na_2HPO_4, and 50 mM NaCl), incubated with antibody-conjugated beads for 1 h at 4 ºC with rotation, washed 2× in IAP buffer, and washed 3× in PBS before elution in 0.15% trifluoroacetic acid. Peptides enriched from the membrane protein digests were then desalted again with C18 tips,

resuspended in 20 µL 5% formic acid, and analyzed by proteomic mass spectrometry with the Thermo Orbitrap Fusion Lumos and the MilkyWay data analysis platform as previously described. The acquisition methods for these samples were altered to allow targeted analysis of $G\beta_{1/2}$ peptides containing diglycine modifications at lysine-23. A Parallel Reaction Monitoring (PRM or t-MS^2) cycle was added to an abbreviated 2-sec DDA cycle. PRM scans were allowed a maximum injection time of 246 msec at a resolution of 120,000. The MSGF+ searches allowed for a 114.042927-Da variable modification on lysine residues to identify diglycine-modified peptides. Quantification of the modified $G\beta_{1/2}$ peptides was done manually with Skyline and peptide intensities were normalized to ubiquitin peptides modified at lysine-48 and lysine-63 (99).

G-Protein Signaling Activation Co-Immunoprecipitation Studies

HeLa Flp-In cells or cells expressing 3×HA 3×FLAG-tagged proteins were grown and induced with 0.5 µg/mL doxycycline overnight. After harvesting, cell pellets were lysed in a buffer containing 100 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1 mM DTT, 5% glycerol, 0.1% NP-40, AEBSF, pepstatin, and leupeptin. To activate G-proteins, the lysis buffer was supplemented with 60 µM AlF_3, 10 mM NaF, 10 mM $MgCl_2$, and 10 µM GDP. After rotation at 4 ºC for 30 min, lysates were clarified by centrifugation, normalized by absorbance at 280 nm, and incubated with EZview Red anti-FLAG affinity gel (Sigma) for 2 h at 4 ºC with end-over-end rotation. After washing 4× with the appropriate lysis buffer, the affinity gel was boiled in SDS-PAGE sample buffer. Whole-cell lysates and the anti-FLAG IPs were analyzed by SDS-PAGE and immunoblotting as described.

Quantification of cAMP

HEK-293 cells were seeded in 96-well poly-L-lysine-coated plates. The following day, the cells were rinsed with 200 µL PBS and grown in medium lacking FBS supplemented with 0.5 mM IBMX (Cayman). After 30 min at 37 ºC, the cells were treated with 10 µM forskolin (Cayman), 10 µM PGE1 (Cayman), or DMSO for 5 min in the presence of 0.5 mM IBMX. cAMP levels were quantified using with an ELISA-based kit (Cell Signaling; Cyclic AMP XP Assay Kit). Concentrations were normalized to relative protein content (Pierce BCA Protein Assay Kit).

For rescue experiments of our *KCTD2*/*KCTD5*-deletion HEK-293 cells, Lipofectamine 2000 or BioT was used to transiently transfect control and *KCTD2*/*KCTD5*-knockout HEK-293 cells with pcDNA3.1 control or 6×MYC-tagged expression plasmids. After 1 day of transfection, cells were seeded on coated 96-well plates and cAMP levels were measured the following day.

For CUL3-depletion experiments, HEK-293 cells were transfected with a pool of CUL3 siRNAs or a non-targeting control siRNA (both siGENOME from Dharmacon) using RNAiMAX (Thermo). After 2 days of transfection, the cells were seeded to coated 96-well plates and cAMP levels were measured the following day.

Quantification of CREB Phosphorylation

HEK-293 cells were seeded in 12-well coated plates. On the day of treatment, the cells were rinsed with PBS and grown in medium lacking FBS supplemented with 0.5

mM IBMX. After 30 min at 37 ºC, the cells were treated with varying concentrations of PGE1 for 5 min in the presence of 0.5 mM IBMX.

MCF-7 cells were transfected with a pool of siRNAs targeting KCTD5 or CUL3, or a non-targeting control siRNA (all siGENOME from Dharmacon) using RNAiMAX (Thermo). After 2 days of transfection, the cells were seeded to 12-well coated plates. On the next day, the cells were rinsed with PBS and grown in medium lacking FBS supplemented with 0.5 mM IBMX for 30 min.

The HEK-293 or MCF-7 cells were then rinsed twice with PBS before lysis in 25 mM Tris (pH 8), 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton-X100, protease inhibitors (AEBSF, pepstatin, and leupeptin), Simple Stop 2 phosphatase inhibitors (Gold Biotechnology), and Benzonase (Sigma) or Universal Nuclease (Pierce). Lysates were normalized (Pierce BCA Protein Assay Kit) before analysis by SDS-PAGE and immunoblotting with the indicated antibodies, including those recognizing CREB and phospho-Ser133-CREB (Cell Signaling; 8212). Quantification was performed using the LICOR Image Studio software.

FIGURES AND FIGURE LEGENDS

Figure 1—Proteomic interactome analysis identifies KCTD2, KCTD5, and CUL3 as potential regulators of Gβ

A proteomic approach was used to characterize the $G\beta_1$ interactome. Anti-HA immunopurifications (n=2) from control HeLa cells or cells stably expressing 3×HA 3×FLAG-tagged $G\beta_1$ (3H3F-$G\beta_1$) were analyzed by mass spectrometry. Intensity-based quantification data are shown in a volcano plot. For each quantified protein, the fold-change enrichment in $G\beta_1$ complexes relative to control complexes and adjusted p-values are shown. Significant $G\beta_1$ interactors were defined as being enriched >10× relative to controls with adjusted p-values <0.01. Proteins infinitely enriched in $G\beta_1$ or control complexes that were not detectable in control and $G\beta_1$ complexes, respectively, are marked by diamonds. Classes of $G\beta_1$-binding partners are also indicated.

Figure 1

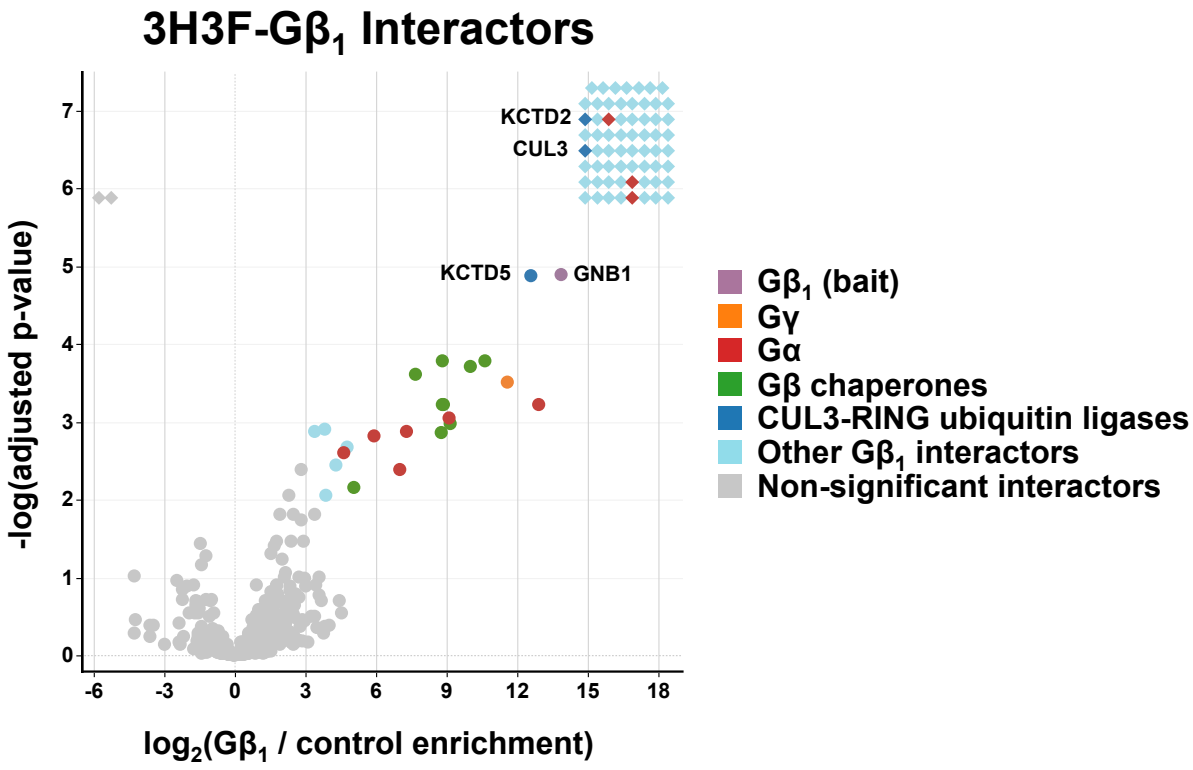

3H3F-Gβ1 Interactors
KCTD2
CUL3
KCTD5
GNB1
-log(adjusted p-value)
log2(Gβ1 / control enrichment)
Gβ1 (bait)
Gγ
Gα
Gβ chaperones
CUL3-RING ubiquitin ligases
Other Gβ1 interactors
Non-significant interactors

Figure 2—KCTD2 and KCTD5 are substrate adaptor proteins for a CUL3–RING E3 ubiquitin ligase that recognizes Gβγ

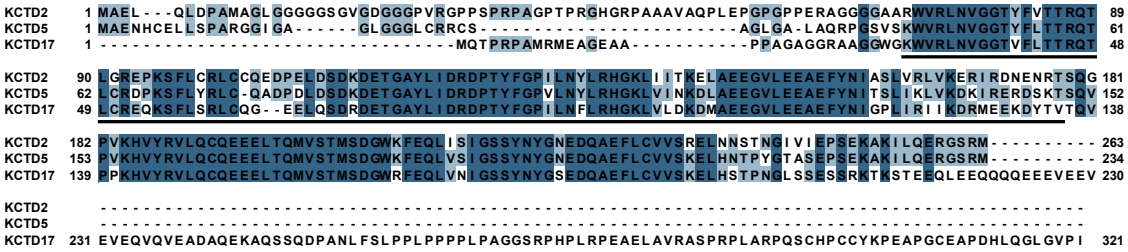
A: A sequence alignment of three closely related KCTD proteins, KCTD2, KCTD5, and KCTD17, was generated using Clustal Omega and Jalview. Residues sharing sequence identity in two or three KCTD subfamily members are marked in light and dark blue, respectively. The BTB domain, which is required for assembly of KCTD proteins into CUL3–RING ubiquitin ligases, is underlined.

B: Proteomic analysis of protein-binding partners of the KCTD2/5/17 subfamily. Anti-HA immunopurifications (n=2) from control HEK-293 cells or cells stably expressing 3×HA 3×FLAG-tagged KCTD proteins were analyzed by mass spectrometry. Columns and rows represent different bait and select prey, respectively. Colors and numerical values indicate the $\log_2$ of the fold-change enrichment of a given prey in bait complexes relative to control complexes. ∞ indicates infinite enrichment relative to controls while ND represents the absence of a signal for a given prey. All bait–prey interactions are significant (adjusted p-value <0.01) unless marked n.s..

C: HEK-293 cells were transiently transfected with 3×HA 3×FLAG-tagged heterotrimeric G-protein subunits or a control plasmid. Immunoblots of whole-cell lysates (WCL) and anti-HA immunopurifications (HA IP) with the specified antibodies are shown. The KCTD2/KCTD5 antibody recognizes both proteins; the upper and lower bands are KCTD2 and KCTD5, respectively. This is representative of three independent experiments.

Figure 2

A.



B.

Prey	Bait: 3H3F KCTD2	3H3F KCTD5	3H3F KCTD17
KCTD2	12.39	7.78	8.21
KCTD5	12.51	16.56	14.46
KCTD17	6.03	6.97	12.27
CUL3	8.60	10.68	5.81
GNB1	15.15	12.67	3.88
GNB2	13.63	11.47	1.93 *(ns)*
GNB4	∞	∞	∞
GNG7	∞	∞	ND
GNG12	13.61	10.92	1.95 *(ns)*

$\log_2$(KCTD / control enrichment)

0 ∞

C.

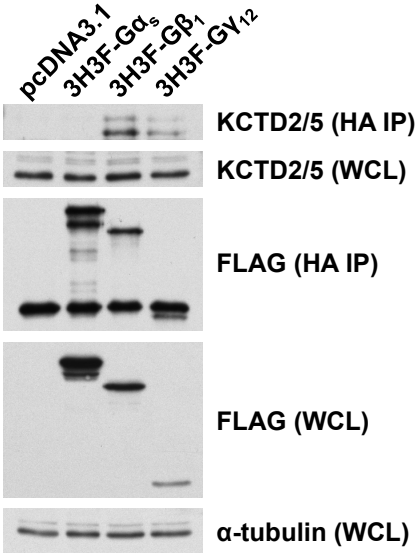


Figure 3—Spectral counting data for the protein interactomes of the KCTD2/5/17 subfamily

Anti-HA immunopurifications (n=2) from control HEK-293 cells or cells stably expressing 3×HA 3×FLAG-tagged KCTD proteins were analyzed by mass spectrometry. Columns and rows represent different bait and prey, respectively. Average spectral counts for select prey are shown.

Figure 3

Spectral Counts

	Control	KCTD2	KCTD5	KCTD17
KCTD2	0.0	335.8	21.8	44.0
KCTD5	0.0	27.0	181.0	86.5
KCTD17	0.0	49.8	62.0	526.0
CUL3	0.0	25.8	43.8	15.8
GNB1	0.0	153.8	42.5	4.3
GNB2	0.0	139.0	42.5	3.8
GNB4	0.0	83.5	28.0	2.5
GNG2	0.0	3.3	0.0	0.0
GNG4	0.0	2.8	0.3	0.0
GNG7	0.0	2.5	0.3	0.0
GNG12	0.0	16.0	5.5	0.0

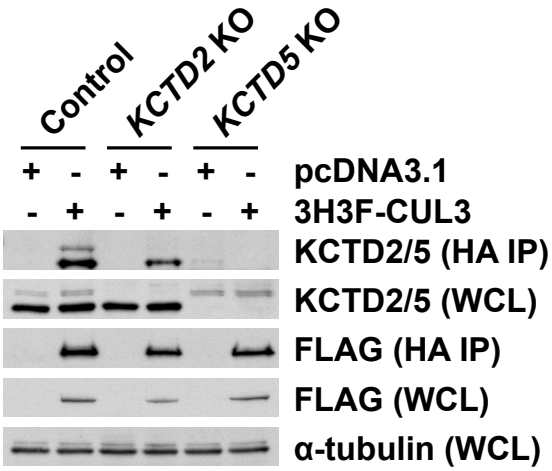
Figure 4—Architecture of a KCTD2/KCTD5–CUL3–RING E3 ubiquitin ligase

A–B: Control and *KCTD2* and *KCTD5*-deletion HEK-293 cells were transiently transfected with control plasmids or the specified 3×HA 3×FLAG-tagged constructs. Immunoblots of whole-cell lysates (WCL) and anti-HA immunopurifications (HA IP) with the indicated antibodies are shown. The KCTD2/KCTD5 antibody recognizes both proteins; the upper and lower bands are KCTD2 and KCTD5, respectively. This is representative of two independent experiments.

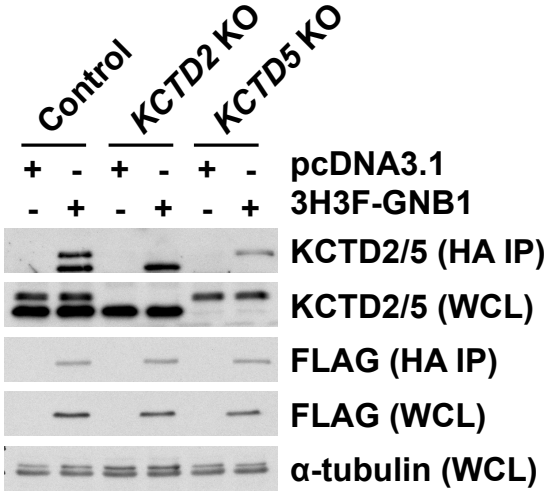
C: A model of a KCTD2/KCTD5–CUL3–RING E3 ubiquitin ligase complex and its putative substrate, Gβγ. CUL3 binding is dependent on KCTD5 subunits while KCTD2 and KCTD5 recruit Gβγ independently of each other.

Figure 4

A.


Control
KCTD2 KO
KCTD5 KO
+ - + - + -
- + - + - +
pcDNA3.1
3H3F-CUL3
KCTD2/5 (HA IP)
KCTD2/5 (WCL)
FLAG (HA IP)
FLAG (WCL)
α-tubulin (WCL)

B.


Control
KCTD2 KO
KCTD5 KO
+ - + - + -
- + - + - +
pcDNA3.1
3H3F-GNB1
KCTD2/5 (HA IP)
KCTD2/5 (WCL)
FLAG (HA IP)
FLAG (WCL)
α-tubulin (WCL)

C.

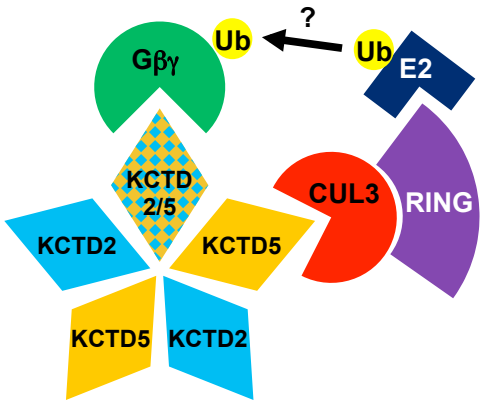

Gβγ
Ub
?
Ub
E2
KCTD 2/5
CUL3
RING
KCTD2
KCTD5
KCTD5
KCTD2

Figure 5—KCTD2 and KCTD5 promote Gβ monoubiquitination of lysine-23

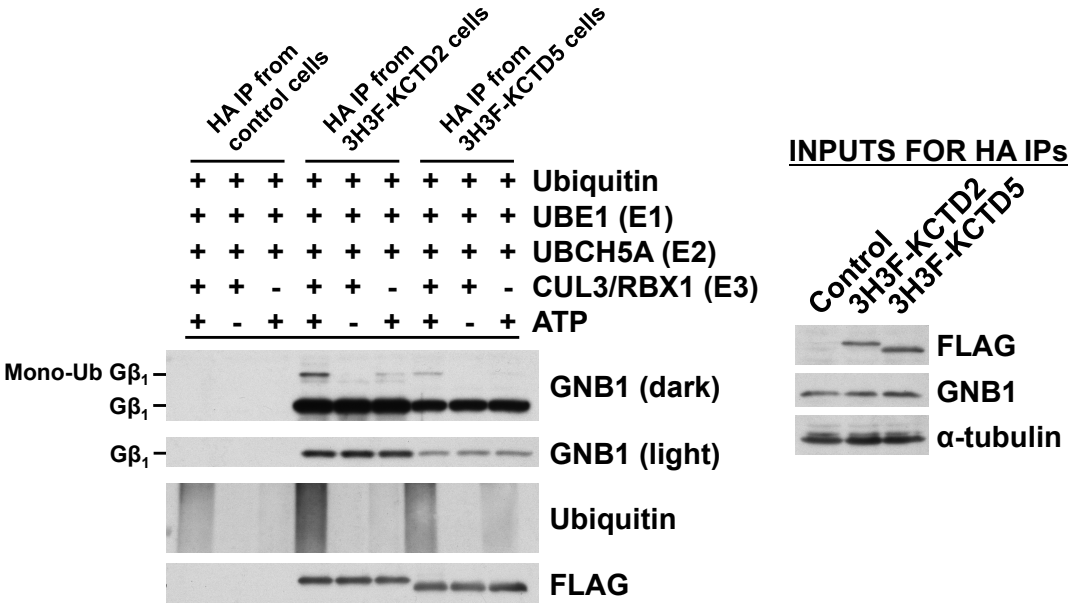
A: *In vitro* ubiquitination reactions were prepared with anti-HA immunopurifications from control HEK-293 cells or cells expressing the indicated 3×HA 3×FLAG-tagged KCTD protein. These purified complexes were incubated with ubiquitin, CUL3/RBX1, UBCH5A, UBE1, and ATP. Reactions lacking ATP or CUL3/RBX1 were also prepared as negative controls. Reaction products were analyzed by SDS-PAGE and immunoblotting with the indicated antibodies. Inputs for the initial anti-HA immunopurifications are shown. Representative of at least two experiments.

B: Larger-scale *in vitro* KCTD5 ubiquitination reactions containing all required components were prepared as described above and analyzed by mass spectrometry. A Venn diagram identifies proteins enriched in the KCTD5 *in vitro* reactions that also have diglycine-modified lysine residues. Numbers of proteins enriched in KCTD5 reactions ≥3× by spectral counting in both independent experiments and numbers of proteins containing identical diglycine-modified peptides in KCTD5 reactions in both independent experiments are shown. A table lists potential substrates of KCTD5 and experimentally observed ubiquitin-attachment sites.

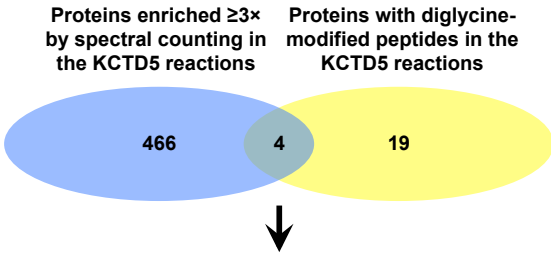
C: The abundances of $G\beta_{1/2}$ peptides containing +114-Da diglycine modifications at lysine-23 were quantified from peptide-level anti-diglycine purifications of membrane fractions isolated from control and *KCTD2*/*KCTD5*-deletion HEK-293 cells. Precursor peak areas for each modified Gβ peptide were normalized to the peak areas for several diglycine-modified ubiquitin peptides. Averages for samples from two independent experiments (n=6 for control and n=4 for *KCTD2/KCTD5*-deletion cells), standard deviation, and p-values generated from the Student's t-test are shown.

Figure 5

A.



B.



Protein	Ubiquitin-attachment sites
$G\beta_1$	K23
$G\beta_2$	K23
DNA Polymerase-κ	K7/K683/K691/K693/K722/K836
KCTD5	K223

C.

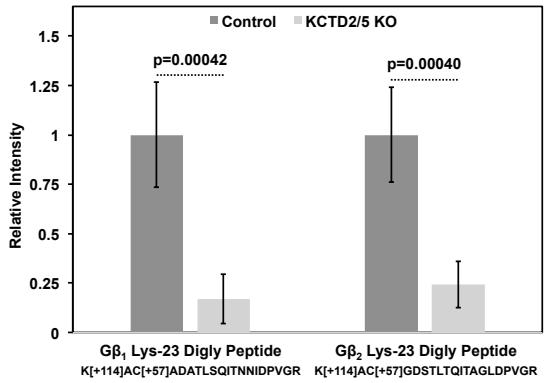


Figure 6—KCTD2 and KCTD5 bind Gβγ in response to G-protein activation

A–B: HeLa control cells and cells stably expressing 3×HA 3×FLAG-tagged $G\beta_1$ or KCTD5 were lysed in the presence and absence of G-protein signaling activators (AlF_4^-, GDP, and $MgCl_2$). Immunoblots of whole-cell lysates (WCL) and anti-FLAG immunopurifications (FLAG IP) with the indicated antibodies are shown. The KCTD2/KCTD5 antibody recognizes both proteins; the upper and lower bands are KCTD2 and KCTD5, respectively. This is representative of two independent experiments.

C: HEK-293 cells were transiently transfected with 3×HA-$G\beta_1$ constructs containing the indicated missense mutations or a control plasmid. Immunoblots of whole-cell lysates (WCL) and anti-HA immunopurifications (HA IP) with the indicated antibodies are shown. The KCTD2/KCTD5 antibody recognizes both proteins; the upper and lower bands are KCTD2 and KCTD5, respectively. This is representative of at least two independent experiments.

D: A structure of G-protein heterotrimer (PDB: 3SN6) highlighting residues in $G\beta_1$ coordinating its interactions with KCTD2 and KCTD5.

Figure 6

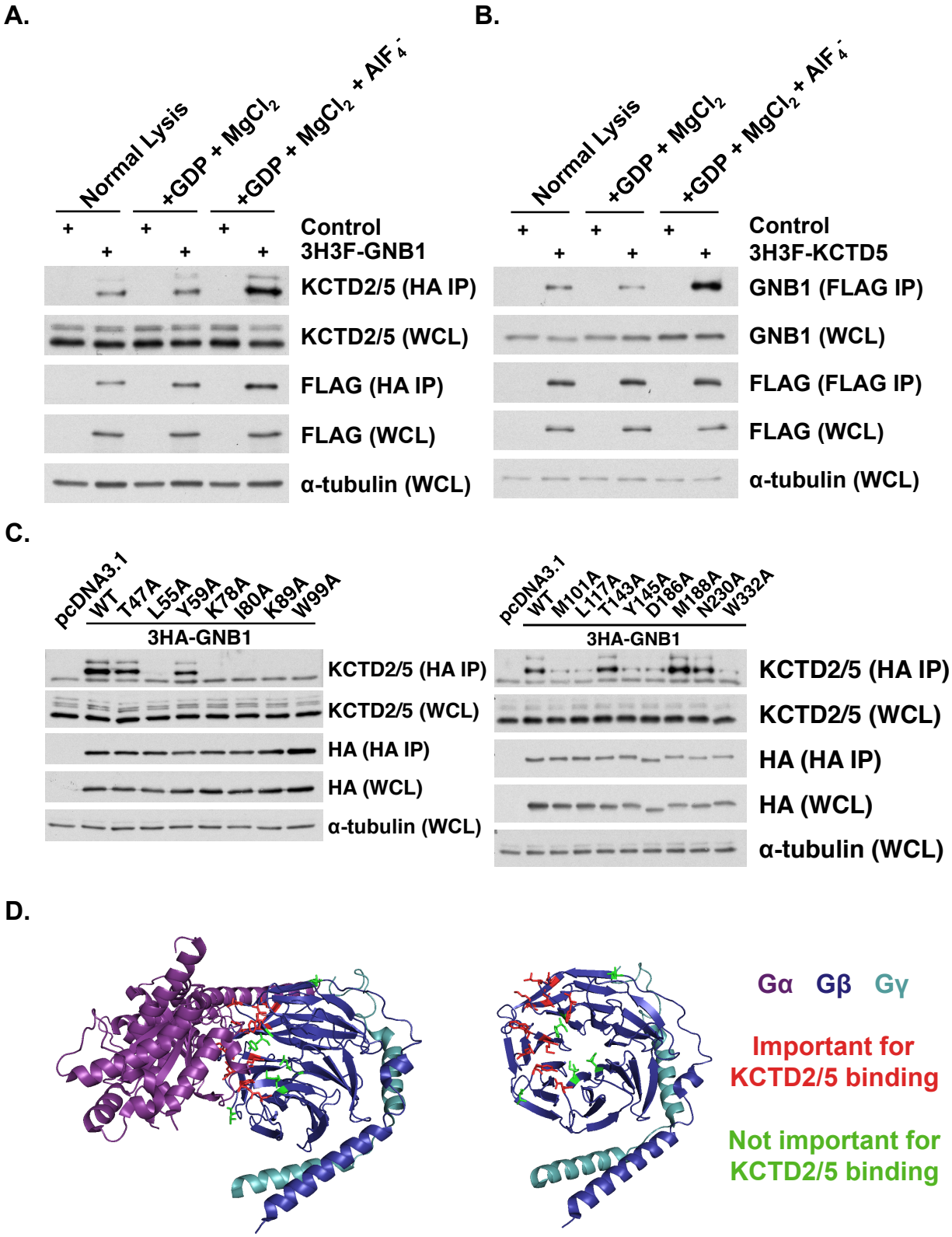

A.
Normal Lysis
+GDP + MgCl2
+GDP + MgCl2 + AlF4-
Control
3H3F-GNB1
KCTD2/5 (HA IP)
KCTD2/5 (WCL)
FLAG (HA IP)
FLAG (WCL)
α-tubulin (WCL)
B.
Normal Lysis
+GDP + MgCl2
+GDP + MgCl2 + AlF4-
Control
3H3F-KCTD5
GNB1 (FLAG IP)
GNB1 (WCL)
FLAG (FLAG IP)
FLAG (WCL)
α-tubulin (WCL)
C.
pcDNA3.1
WT
T47A
L55A
Y59A
K78A
I80A
K89A
W99A
3HA-GNB1
KCTD2/5 (HA IP)
KCTD2/5 (WCL)
HA (HA IP)
HA (WCL)
α-tubulin (WCL)
pcDNA3.1
WT
M101A
L117A
T143A
Y145A
D186A
M188A
N230A
W332A
3HA-GNB1
KCTD2/5 (HA IP)
KCTD2/5 (WCL)
HA (HA IP)
HA (WCL)
α-tubulin (WCL)
D.
Gα
Gβ
Gγ
Important for KCTD2/5 binding
Not important for KCTD2/5 binding

Figure 7—KCTD2 binds Gβ in response to G-protein activation

HeLa control cells and cells stably expressing 3×HA 3×FLAG-tagged KCTD2 were lysed in the presence and absence of G-protein signaling activators (AlF_4^-, GDP, and $MgCl_2$). Immunoblots of whole-cell lysates (WCL) and anti-FLAG immunopurifications (FLAG IP) with the indicated antibodies are shown. This is representative of two independent experiments.

Figure 7

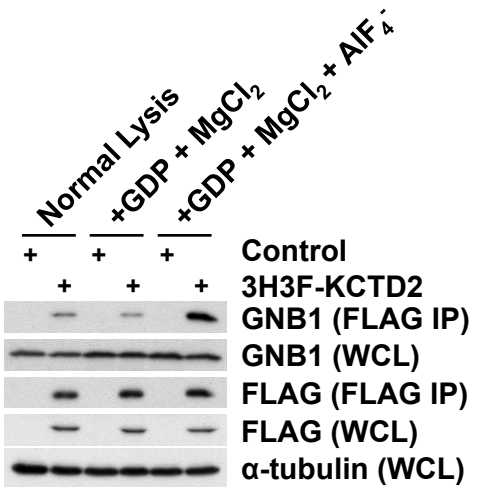

Normal Lysis
+GDP + MgCl2
+GDP + MgCl2 + AlF4-
+ + + Control
+ + + 3H3F-KCTD2
GNB1 (FLAG IP)
GNB1 (WCL)
FLAG (FLAG IP)
FLAG (WCL)
α-tubulin (WCL)

Figure 8—A KCTD2/KCTD5–CUL3–RING E3 ligase promotes cAMP signaling

A: Measurements of cAMP in control or *KCTD2/KCTD5*-deletion HEK-293 cells after treatment with 10 μM forskolin or 10 μM PGE1 (n=2). Averages and standard deviation are shown. P-values were generated with the Student's t-test. N.D. indicates that cAMP concentrations were less than limit of detection. This is representative of two independent experiments.

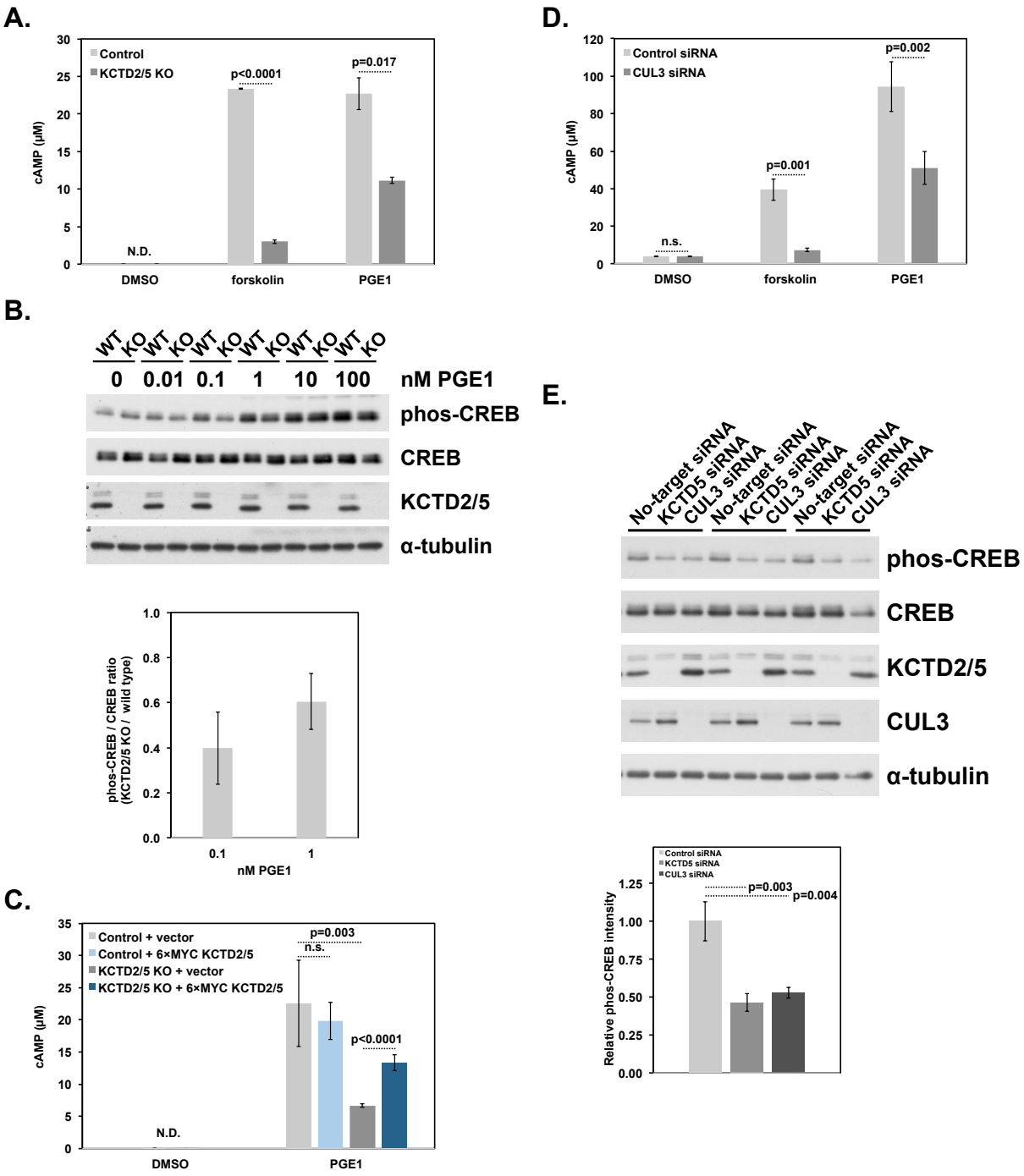
B: Measurement of CREB phosphorylation in control or *KCTD2/KCTD5*-deletion HEK-293 cells treated with 0.5 mM IBMX for 30 min before a 5-min treatment with varying concentrations of PGE1. Immunoblots with the indicated antibodies are shown. The KCTD2/KCTD5 antibody recognizes both proteins; the upper and lower bands are KCTD2 and KCTD5, respectively. Quantification of PGE1-induced phosphorylation of serine-133 within CREB is shown. For each sample from two independent experiments, the phos-CREB signal was normalized to the total CREB signal. Ratios for the normalized intensities between *KCTD2/KCTD5*-deletion cells and control cells are shown. Error bars represent 95% confidence intervals.

C: Measurements of cAMP in control or *KCTD2/KCTD5*-deletion HEK-293 cells transiently transfected with pcDNA3.1 or 6×MYC-tagged KCTD2/KCTD5 after 5 min treatment with 10 μM PGE1 (n=4) or DMSO (n=2). Averages and standard deviation are shown. P-values were generated with the Student's t-test. N.D. indicates that cAMP concentrations were less than limit of detection while n.s. indicates that a comparison was not statistically significant. This is representative of two independent experiments.

D: Measurements of cAMP in HEK-293 cells transfected with control or CUL3 siRNA after 5 min treatment with DMSO (n=2), 10 μM forskolin (n=4), or 10 μM PGE1 (n=4). Averages and standard deviation are shown. P-values were generated with the Student's t-test. n.s. indicates that a comparison was not statistically significant. This is representative of two independent experiments.

E: MCF-7 cells were transfected with the indicated siRNAs and treated for 30 min with 0.5 mM IBMX. Immunoblots of three biological replicates with the indicated antibodies are shown. The KCTD2/KCTD5 antibody recognizes both proteins; the upper and lower bands are KCTD2 and KCTD5, respectively. Phosphorylation of serine-133 within CREB was quantified in the biological replicates by normalizing the phos-CREB intensity to total CREB. Relative phos-CREB intensities for each siRNA are shown. Error bars mark standard deviation and p-values were generated with the Student's t-test.

Figure 8


A.
Control
KCTD2/5 KO
p<0.0001
p=0.017
N.D.
cAMP (μM)
DMSO
forskolin
PGE1
D.
Control siRNA
CUL3 siRNA
p=0.002
p=0.001
n.s.
cAMP (μM)
DMSO
forskolin
PGE1
B.
WT KO WT KO WT KO WT KO WT KO WT KO
0 0.01 0.1 1 10 100
nM PGE1
phos-CREB
CREB
KCTD2/5
α-tubulin
phos-CREB / CREB ratio (KCTD2/5 KO / wild type)
0.1
1
nM PGE1
E.
No-target siRNA
KCTD5 siRNA
CUL3 siRNA
phos-CREB
CREB
KCTD2/5
CUL3
α-tubulin
Control siRNA
KCTD5 siRNA
CUL3 siRNA
p=0.003
p=0.004
Relative phos-CREB intensity
C.
Control + vector
Control + 6×MYC KCTD2/5
KCTD2/5 KO + vector
KCTD2/5 KO + 6×MYC KCTD2/5
p=0.003
n.s.
p<0.0001
N.D.
cAMP (μM)
DMSO
PGE1

TABLES

Table 1: List of cDNA Plasmids

ORF	*GenBank*	*Vendor*	*Catalog Number*
KCTD2	BC160142.1	GeneCopoeia	Y3428
KCTD5	BC007314.2	Dharmacon	MHS6278-202826872
KCTD17	BC031038.1	Dharmacon	MHS6278-202808680
GNAS	BC002722.2	Dharmacon	MHS6278-202829474
GNB1	BC005888.2	Dharmacon	MHS6278-202829088
GNG12	BC005940.1	Dharmacon	MHS6278-202839798
CUL3	BC092409.1	Dharmacon	MHS6278-202759865

Table 2: List of PCR Primers

Primer	*Sequence*
KCTD5 Forward	5′-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC CGC GGA GAA TCA CTG CGA G-3′
KCTD5 Reverse	5′-*GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC TTA* TCA CAT CCT TGA GCC TCG TTC-3′
KCTD17 Forward	5′-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC CAG GAT GGA GGC CGG GGA G-3′
KCTD17 Reverse	5′-*GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC TTA* TCA GAT GGG AAC CCC AAG TCC-3′
GNAS Forward	5′-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC CGG CTG CCT CGG GAA CAG T-3′
GNAS Reverse	5′-*GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC TTA* TTA GAG CAG CTC GTA CTG ACG-3′
GNB1 Forward	5′-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC CAG TGA GCT TGA CCA GTT ACG G-3′
GNB1 Reverse	5′-*GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC TTA* TTA GTT CCA GAT CTT GAG GAA GCT-3′
GNG12 Forward	5′-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC CTC CAG CAA AAC AGC AAG CAC C-3′
GNG12 Reverse	5′-*GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC TTA* CTA TAA GAT GAT GCA AGT TTT TTT ATC CTT-3′
CUL3 Forward	5′-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC CTC GAA TCT GAG CAA AGG CAC G-3′
CUL3 Reverse	5′-*GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC TTA* TTA TGC TAC ATA TGT GTA TAC TTT GCG-3′

Underlined and *italicized* sequences mark the attB1 and attB2 sequences, respectively.

CHAPTER III

IDENTIFICATION OF POTENTIAL PROTEIN TARGETS OF A KCTD2 AND KCTD5 HOMOLOG LINKED TO SLEEP HOMEOSTASIS AND SYNAPTIC FUNCTION

INTRODUCTION

We have demonstrated that a KCTD2/KCTD5–CUL3–RING E3 ubiquitin ligase modifies Gβ with ubiquitin and positively regulates cAMP signaling in human cancer cell lines. The function of this multisubunit ubiquitin ligase has not yet been examined in the context of an organism, and mutations within the substrate adaptor proteins within this complex—KCTD2 and KCTD5—have not yet been linked to any human diseases. Interestingly, however, the sole homolog of KCTD2 and KCTD5 in *D. melanogaster* has been identified as a regulator of sleep homeostasis in two independent genetic screens (129,130).

This homolog, Insomniac, shares substantial sequence homology with human KCTD2 and KCTD5 (130). Loss of this protein in fruit flies is associated with marked changes in sleep homeostasis (129,130). Neuronal deficiency of Insomniac causes flies to sleep less than half as much as control flies. Insomniac-deficient flies have more periods of sleep than control flies, but each period of sleep has greatly reduced duration. These effects on sleep are related to the expression of Insomniac during development (129). Neuronal depletion of Cul3 during development has similar effects on sleep. Given that Insomniac can interact with Cul3, these results suggested that an Insomniac–Cul3–RING E3 ubiquitin ligase might function in neurons during development to promote sleep homeostasis in adult flies.

Subsequent efforts characterized cell-level phenotypes associated with loss of this sleep-regulating putative substrate adaptor protein during development (131). Insomniac is expressed in neurons and its deficiency reduces the amplitude of

excitatory postsynaptic potentials triggered by excitation of a presynaptic neuron at the larval neuromuscular junction. These changes were interpreted to indicate impaired synaptic transmission. Structurally, Insomniac deficiency also alters synapses by increasing the number of synaptic boutons at the larval neuromuscular junction. These changes to the synaptic architecture may be a consequence of impaired synaptic function during development in Insomniac-deficient larvae (131).

Although Insomniac deficiency during development is associated with striking changes in synaptic function in larvae and sleep homeostasis in adults, the proteins that it targets for ubiquitination remain unknown. Its human homologs, KCTD2 and KCTD5, can rescue the sleep defects associated with Insomniac deficiency, suggesting that there is functional overlap between the fly and human proteins (131). KCTD2 and KCTD5 assemble into a CUL3–RING E3 ubiquitin ligase to modify Gβ with ubiquitin. It is unclear whether Insomniac shares substrates or protein-binding partners with its human homologs. Identification of Insomniac's potential substrates should clarify the molecular basis for the behavioral and synaptic changes associated with its deficiency.

RESULTS

To investigate the extent of functional overlap between Insomniac and these human KCTD proteins, and better understand the behavioral and functional phenotypes associated with Insomniac deficiency, we characterized the Insomniac interactome. We created *D. melanogaster* Schneider 2 (S2) cells stably expressing 3×HA 3×FLAG-tagged Insomniac. We performed anti-HA affinity purifications from these S2 cells overexpressing Insomniac or control S2 cells. To obtain a more complete Insomniac interactome, we incubated these purified complexes with whole-fly extracts to be able to identify Insomniac-binding partners that might not be expressed in S2 cells. We then used proteomic mass spectrometry to identify proteins interacting with Insomniac. In our analysis of two independent experiments, we identified 36 proteins that were significantly enriched in the Insomniac complexes relative to control complexes (**Figure 9**). As expected, Insomniac was highly enriched in these complexes. In addition, Gbeta13F, the fly homolog of human $G\beta_1$, was also enriched in the Insomniac complexes. This suggested that Insomniac and its human homologs might both be able to target Gβ for modification by ubiquitin.

We augmented these studies using Insomniac-expressing S2 cells and whole-fly extracts by analyzing the Insomniac interactome within a more physiologically relevant context. CRISPR was used to insert a 3×FLAG tag upstream of *insomniac* at its endogenous genomic locus. This enabled us to examine the Insomniac interactome within an organism where affinity tagged-Insomniac is expressed at physiologic levels in tissues where it is normally expressed. Given that neural deficiency of Insomniac

causes striking alterations in sleep homeostasis, we examined the Insomniac interactome within fly heads using these transgenic flies. In one experiment, we performed anti-FLAG affinity purifications from heads of endogenously tagged flies and control flies. Analysis by mass spectrometry identified 166 proteins enriched in Insomniac complexes isolated from two distinct transgenic fly lines. As seen in our initial studies using S2 cells and whole-fly extracts, Insomniac and Gbeta13F were highly enriched in these complexes isolated from heads of these transgenic flies.

Given that others demonstrated that expression of human KCTD2 and KCTD5 could restore sleep in Insomniac-deficient flies, it is likely that these substrate adaptor proteins bind and target similar proteins for ubiquitination (131). Accordingly, we compared the interactomes of Insomniac generated in these studies with those of KCTD2 and KCTD5 that we previously characterized in HEK-293 cells. For each Insomniac-interacting protein, we identified its top-matching human homolog with FlyBase so we could identify shared interactors between these proteins. This comparison showed that there is relatively limited overlap between the interactomes of these substrate adaptor proteins across species (**Figure 10**). In addition to $G\beta_1$, there are 16 interactors shared by Insomniac and its human homologs.

DISCUSSION

Previous studies demonstrated that developmental expression of Insomniac regulates synaptic function in fly larvae and sleep homeostasis in adult flies (129-131). Given its sequence homology to KCTD proteins, it was predicted to be a substrate adaptor protein for a Cul3–RING E3 ubiquitin ligase. Indeed, neuronal depletion of Cul3 elicited similar sleep phenotypes in flies. The targets of this Insomniac–Cul3–RING ubiquitin ligase, however, were unknown and the molecular basis of these neurological phenotypes remained elusive.

In this study, we have performed multiple interactome analyses to reveal possible targets of Insomniac that might mediate these striking phenotypes. We purified Insomniac complexes from S2 cells, a macrophage-like fly cell line, and incubated them with whole-fly extracts to identify its binding partners. Inclusion of the whole-fly extract facilitated identification of binding partners that may be poorly expressed in S2 cells. We identified 36 possible substrates of Insomniac. We also purified Insomniac complexes from heads of a transgenic fly where an affinity tag had been inserted upstream of the open-reading frame of the endogenous *insomniac* gene. With this latter approach, we identified 166 possible binding partners from heads of an organism where Insomniac is expressed at normal levels in cells where it is normally expressed.

We were intrigued to find that Insomniac interacted with Gbeta13F—the fly homolog of mammalian $G\beta_1$—in both of these interactome analyses. We identified $G\beta_1$ as a target of Insomniac's human homologs: KCTD2 and KCTD5. The KCTD2/KCTD5–CUL3–RING ubiquitin ligase ubiquitinates multiple isoforms of this central signaling

protein at lysine-23. Our observation that Insomniac and these human KCTD proteins both recognize Gβ is consistent with a report demonstrating that these proteins have functional similarities and that KCTD2 and KCTD5 can replace Insomniac in flies without affecting sleep homeostasis (131).

Our studies of KCTD2 and KCTD5 in multiple human cancer cell lines suggested that these Gβ-modifying proteins positively regulate cAMP signaling. It is possible that loss of Gβ ubiquitination and reduced cAMP signaling in Insomniac-deficient flies may impair synaptic function during development to alter adult sleep homeostasis.

The role of cAMP signaling in sleep homeostasis has been investigated extensively in flies (186,187). Most of these studies, however, have focused on the role of cAMP signaling in regulating sleep during adulthood. When the catalytic subunit of PKA is overexpressed throughout all neurons in adult flies, sleep is decreased (187). This suggested that cAMP signaling during adulthood negatively regulates sleep. It is unclear, however, how cAMP signaling during development affects adult sleep homeostasis. Additional studies will clarify whether alteration of cAMP signaling in Insomniac-expressing neurons during development affects sleep homeostasis in adults.

It is possible that these studies will reveal functional conservation of KCTD2 and KCTD5, and show that Insomniac targets Gβ for ubiquitination and positively regulates cAMP signaling during development to alter synapses and adult sleep homeostasis. Alternatively, these interesting synaptic and sleep phenotypes associated with Insomniac deficiency might be related to other targets of Insomniac and its human homologs, such as those identified in our analysis of the Insomniac interactome.

MATERIALS AND METHODS

Plasmids and Cloning

A cDNA for Insomniac was obtained from the DGRC (clone LD43051) and amplified by PCR with Phusion high-fidelity DNA polymerase (NEB) and forward and reverse primers fused to *attB1* or *attB2* sequences, respectively (forward primer: 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTCCAGCACGGTGTTCATAAACTCGC and reverse primer: 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCTTATTAAATTCCAAGAATTCGCGATCCC). The *attB*-flanked ORF was purified with the PureLink Quick PCR Purification kit (Invitrogen), inserted into pDONR221 using BP Clonase II (Thermo), and sequenced using the M13F(-21) primer to verify its identity and ensure that it was inserted in frame. Reaction of this entry clone with LR clonase II (Thermo) and the pAFHW destination vector (DGRC) yielded an expression clone constitutively expressing N-terminally 3×HA 3×FLAG-tagged Insomniac.

Cell Culture and Generation of Cell Lines

Schneider 2 (S2) cells were cultured in Schneider's Drosophila Medium (Thermo; 21720-024) supplemented with 5% FBS (Gemini; Foundation B), and 1× Gibco Penicillin-Streptomycin or 1× Gibco Antibiotic-Antimycotic. For suspension culture, the growth medium was supplemented with 0.1% Pluronic F-68 (Gibco) and flasks were shaken at 120 rpm at 27 ºC. To generate S2 cells stably expressing 3×HA 3×FLAG-Insomniac, 3×10^6 S2 cells were transfected with 38 µg of the expression clone and 2 µg

of the pCoHygro selection vector using $CaCl_2$, and selected with 150 µg/mL Hygromycin B Gold (Invivogen).

Generation of Transgenic Flies

Transgenic flies containing a 3×FLAG tag directly upstream of the first *insomniac* exon were generated using CRISPR-Cas9 by the laboratory of Nicholas Stavropoulos at NYU. Briefly, embryos from the nos-Cas9(II-2A) fly strain expressing Cas9 (strain NIG-FLY#CAS-004; BestGene) were injected with two plasmids expressing guide RNAs targeting regions upstream and downstream of *insomniac*. A third plasmid contained a repair template containing a 3×FLAG tag upstream of the first *insomniac* exon. The repair template plasmid also contained a loxP-flanked dsRed fluorescent protein under the control of the 3xP3 eye-specific promoter. This enabled isolation of integrants and PCR was used to select strains with the correct integration event. The dsRed cassette was removed by crossing the strains with one expressing Cre recombinase. The transgenic flies were then backcrossed to the wild type Bloomington strain 5905 for 8 generations.

Interactome Analysis in S2 Cells with Whole-Fly Extracts

Control S2 cells or S2 cells stably expressing 3×HA 3×FLAG-Insomniac were grown in a 50-mL suspension culture before resuspension in native lysis buffer (100 mM Tris (pH 8), 150 mM NaCl, 5 mM EDTA, 1 mM DTT, 5% glycerol, and 0.1% NP-40) containing AEBSF, pepstatin, and leupeptin. After end-over-end rotation for 30 min at 4 ºC, lysates were sonicated for 5 min with a Bioruptor (30-sec on/off cycle; high-power

setting), and clarified by centrifugation at 16,100×g for 15 min at 4 ºC. Lysates were normalized by absorbance at 280 nm and incubated with 40 µL of pre-equilibrated EZview Red Anti-HA affinity gel (Sigma). After 2 h of end-over-end rotation at 4 ºC, the beads were washed 4× with native lysis buffer. The beads were then incubated with a whole-fly extract for 2 h with rotation at 4 ºC. This extract was generated by homogenizing 75 10-day-old female wild type white dahomey flies (a gift from Anil Rana at UCLA) in native lysis buffer containing protease inhibitors with a dounce before 30 min of rotation at 4 ºC, centrifugation at 16,100×g for 40 min at 4 ºC, and filtration through a 0.2-µm cellulose acetate membrane (Thermo). The beads were then washed again 4× in native lysis buffer before 3 washes with native lysis buffer lacking NP-40. Complexes were eluted in 100 µL 8 M urea in 100 mM Tris (pH 8.5) for 5 min. This elution was repeated twice and the eluates were combined.

Proteins were precipitated by addition of 4 volumes of -20 ºC acetone and incubation on ice for 2 h. After centrifugation at 16,100×g for 25 min at 4 ºC, the pellets were washed with -20 ºC acetone and centrifuged again. Dried pellets were resuspended in 50 µL 8 M urea in 100 mM Tris (pH 8.5), reduced with 1.25 µL 200 mM TCEP for 20 min, and alkylated with 1.2 µL 0.5 M iodoacetamide for 20 min in darkness. Proteins were initially digested with 0.2 µg LysC for 4 h at 37 ºC in darkness before addition of 150 µL 100 mM Tris (pH 8.5), 2 µL 100 mM $CaCl_2$, and 1.6 µg Pierce trypsin. After overnight incubation at 37 ºC in darkness, digestions were quenched by addition of formic acid to 5%. Peptides were desalted and analyzed by mass spectrometry with a Thermo Orbitrap Fusion Lumos and the MilkyWay data analysis platform as described for the earlier interactome studies.

Interactome Analysis in Transgenic Fly Heads

Transgenic flies containing a 3×FLAG tag upstream of *insomniac* at its endogenous genomic locus were grown and frozen in liquid nitrogen before vortexing and isolation of fly heads using several metal sieves (the fly heads were a gift from Nicholas Stavropoulos at NYU). Approximately 0.65 g of heads from two transgenic lines and a control line were homogenized in native lysis buffer using a dounce. After end-over-end rotation for 30 min at 4 ºC, lysates were clarified by centrifugation at 16,100×g for 30 min at 4 ºC and filtered through a 0.2-µm cellulose acetate membrane (Thermo). Lysates were normalized by protein content (Pierce BCA Protein Assay kit) and approximately 20.5 mg of extracted protein was incubated with 40 µL of pre-equilibrated EZview Red Anti-FLAG affinity gel (Sigma). After 2 h of end-over-end rotation at 4 ºC, the beads were washed 5× with native lysis buffer before 3 washes with native lysis buffer lacking NP-40. Complexes were then eluted in 80 µL 8 M urea in 100 mM Tris (pH 8.5) for 5 min. This elution was repeated twice and the eluates were combined.

Proteins were precipitated with acetone as described previously. Dried pellets were resuspended in 50 µL 8 M urea in 100 mM Tris (pH 8.5), reduced with addition of 1.25 µL 200 mM TCEP for 20 min, and alkylated with 1.025 µL 0.5 M iodoacetamide for 20 min in darkness. Proteins were initially digested with 0.1 µg LysC for 4 h at 37 ºC in darkness before addition of 150 µL 100 mM Tris (pH 8.5), 2 µL 100 mM $CaCl_2$, and 0.8 µg Pierce trypsin. After overnight incubation at 37 ºC in darkness, digestions were quenched and desalted before analysis as described for earlier interactome analyses.

FIGURES AND FIGURE LEGENDS

Figure 9—The Insomniac interactome in S2 cells and whole-fly extracts

A proteomic approach was used to characterize the Insomniac interactome. Anti-HA immunopurifications (n=2) from control S2 cells or S2 cells stably expressing 3×HA 3×FLAG-tagged Insomniac were incubated with whole-fly extracts before analysis by mass spectrometry. Significant Insomniac-binding partners, which were enriched ≥10× relative to controls with adjusted p-values ≤0.01, are listed. Top-matching human homologs of these proteins were identified using FlyBase and are also shown. Insomniac and Gbeta13F—a homolog of a protein targeted by Insomniac's human homologs—are marked.

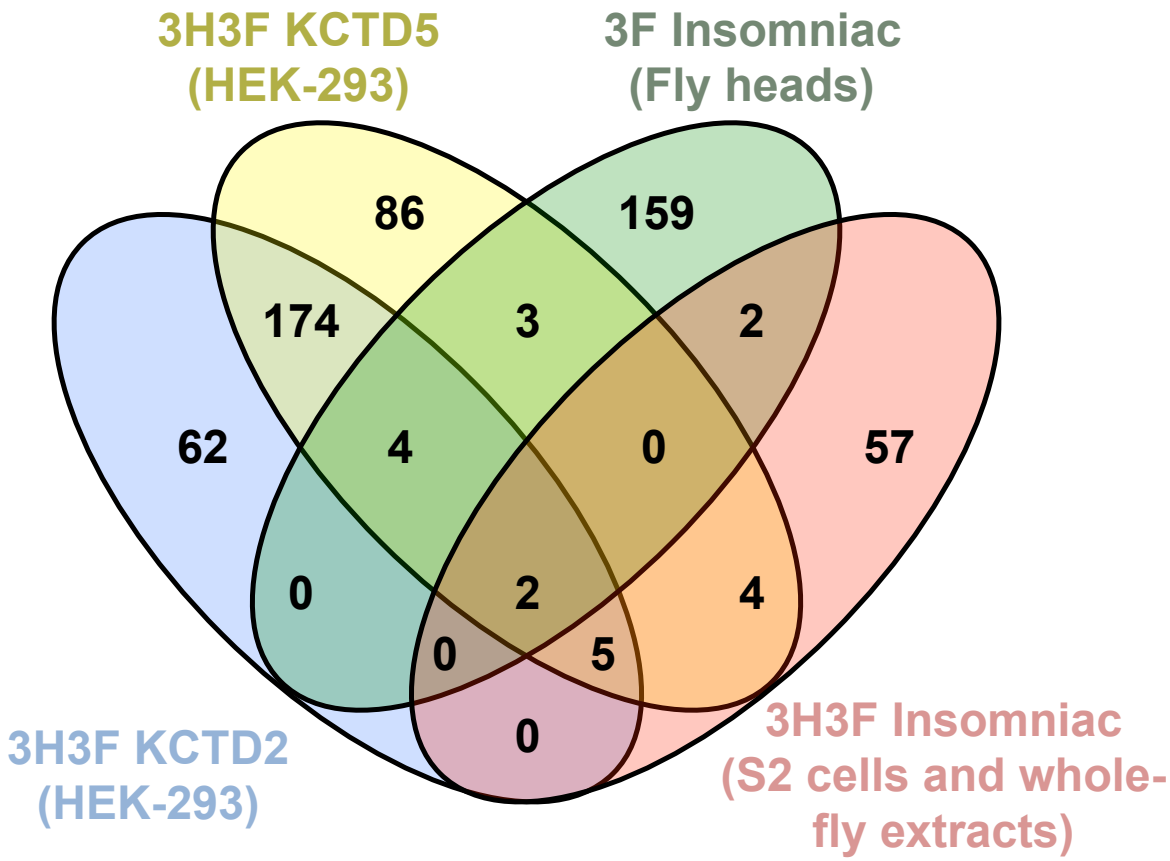
Figure 9

Fly Uniprot ID	Fly Gene Name	Top-matching human homolog gene
A0A0B4K897	CG5174	TPD52L2
A0A0B4KI34	cindr	SH3KBP1
A1Z8D3	CG18003	HAO1
A1Z9M5	CG30069	AHNAK / AHNAK2
A8DRW0	Cpr49Aa	-
A8DYP0	Unc-89	SPEG
E1JIH4	Dmel\CG8478	ZBTB1 / ZBTB34 / ZBTB47 / ZFP92 / ZNF107 / ZNF160 / ZNF184 / ZNF208 / ZNF264 / ZNF282 / ZNF324 / ZNF324B / ZNF347 / ZNF354B / ZNF473 / ZNF48 / ZNF497 / ZNF526 / ZNF594 / ZNF652 / ZNF665 / ZNF689 / ZNF729 / ZNF764 / ZNF771 / ZNF785 / ZNF805 / ZNF84 / ZNF845 / ZNF850 / ZNF99
O02649	Hsp60A	HSPD1
P05990	r	CAD
P07664	Sry-delta	GZF1 / ZNF200 / ZNF449
P11147	Hsc70-4	HSPA8
P26308	**Gbeta13F**	**GNB1**
P28166	zfh1	ZEB1 / ZEB2
P48598	eIF4E1	EIF4E
P48607	spz	-
P52168	pnr	GATA4
Q24133	DnaJ-1	DNAJB4
Q2PDT4	Pax	TGFB1I1
Q5BIC3	az2	ZNF133 / ZNF585A
Q6IDD9	Ect4	SARM1
Q8SX89	kuk	-
Q8SY33	gw	TNRC6C
Q8SZM2	Dmel\CG16885	GGN / TPRX1
Q8T4F7	ena	ENAH
Q95RB2	Cpr49Ae	-
Q9NFV7	Tep2	CD109
Q9V3P6	Rpn2	PSMD1
Q9V3Z3	meso18E	-
Q9VCH1	eIF4G2	EIF4G1 / EIF4G3
Q9VID5	anon-WO0118547.182	FAM102A
Q9VSA9	CT16169	HSPB2
Q9VXQ5	CCT6	CCT6A
Q9W3C4	Hexo2	HEXA / HEXB
Q9W4F9	Dmel\CG32772	OVOL2 / ZNF384
Q9W579	**inc**	**KCTD5**
X2JEI9	RhoGAP19D	ARHGAP23

Figure 10—Identification of proteins that bind Insomniac and KCTD2 or KCTD5

A Venn diagram comparing the interactomes of overexpressed Insomniac in 3×HA 3×FLAG-Insomniac S2 cells/whole-fly extracts, endogenously tagged 3×FLAG Insomniac in fly heads, and overexpressed 3×HA 3×FLAG-KCTD2 and KCTD5 in HEK-293 cells. For 3×HA 3×FLAG-tagged proteins, protein-binding partners were enriched ≥10× relative to controls with adjusted p-values ≤0.01. For 3×FLAG-Insomniac, protein-binding partners from two transgenic fly lines were enriched ≥10× relative to controls with adjusted p-values ≤0.05. Top-matching human homologs of fly proteins were identified using FlyBase. Proteins interacting with Insomniac and its human homologs are listed.

Figure 10



Human Gene	Description / Function	3H3F KCTD2 (HEK-293)	3H3F KCTD5 (HEK-293)	3F Insomniac (fly heads)	3H3F Insomniac (S2 cells and whole-fly extracts)
KCTD5	E3 ligase substrate adaptor				
GNB1	Heterotrimeric G-protein β-subunit				
KIF5B	Kinesin-1 heavy chain				
PSMC6	26S proteasome reg. subunit				
USP9X	Deubiquitinase				
PFDN2	Chaperone				
CAD	Pyrimidine metabolism enzyme				
EIF4G1	Translation initiation factor				
PSMD1	26S proteasome reg. subunit				
TGFB1I1	Signaling adaptor protein				
ZNF324	KRAB transcriptional repressor				
PPP5C	Serine/threonine phosphatase				
SEC13	Vesicular transport				
TAGLN2	Actin-binding protein				
HSPA8	Chaperone				
HSPD1	Mitochondrial chaperonin				
ZBTB34	Transcriptional repressor				
ZNF184	KRAB transcriptional repressor				

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