WNT Activates the AAK1 Kinase to Promote Clathrin-Mediated Endocytosis of LRP6 and Establish a Negative Feedback Loop

Graphical Abstract

Highlights

- Gain-of-function kinome screen identifies AAK1 as a repressor of WNT signaling
- AAK1 promotes clathrin-mediated endocytosis of LRP6
- Selective AAK1 inhibitor stabilizes β-catenin and activates WNT signaling
- WNT induces AAK1-dependent phosphorylation of AP2M1 and LRP6 endocytosis

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In Brief

WNT signal transduction is essential for normal development and contributes to many human diseases. Agajanian et al. used a kinase gain-of-function screen to show that WNT activates the AAK1 kinase to promote clathrin-mediated endocytosis of the WNT receptor. This work identifies an AAK-driven negative feedback loop that downregulates WNT signaling.

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WNT Activates the AAK1 Kinase to Promote Clathrin-Mediated Endocytosis of LRP6 and Establish a Negative Feedback Loop

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SUMMARY

β-Catenin-dependent WNT signal transduction governs development, tissue homeostasis, and a vast array of human diseases. Signal propagation through a WNT-Frizzled/LRP receptor complex requires proteins necessary for clathrin-mediated endocytosis (CME). Paradoxically, CME also negatively regulates WNT signaling through internalization and degradation of the receptor complex. Here, using a gain-of-function screen of the human kinome, we report that the AP2 associated kinase 1 (AAK1), a known CME enhancer, inhibits WNT signaling. Reciprocally, AAK1 genetic silencing or its pharmacological inhibition using a potent and selective inhibitor activates WNT signaling. Mechanistically, we show that AAK1 promotes clearance of LRP6 from the plasma membrane to suppress the WNT pathway. Time-course experiments support a transcription-uncoupled, WNT-driven negative feedback loop; prolonged WNT treatment drives AAK1-dependent phosphorylation of AP2M1, clathrin-coated pit maturation, and endocytosis of LRP6. We propose that, following WNT receptor activation, increased AAK1 function and CME limits WNT signaling longevity.

INTRODUCTION

β-Catenin-dependent WNT signal transduction is an evolutionarily conserved pathway that instructs cell and tissue-specific differentiation and proliferation programs. It is required for both normal development and maintenance of adult tissue homeostasis (Nusse and Clevers, 2017). Like many developmental signaling pathways, aberrant WNT signaling contributes fundamentally to human diseases, including cancer, bone density disorders, and neurodegeneration (Nusse and Clevers, 2017). The regulation of the WNT/β-catenin signaling axis is often described in three stages: proximal signaling, β-catenin destruction, and nuclear effectors of transcription (Angers and Moon, 2009). In the absence of WNT ligand, the LRPs/5/6 (low-density lipoprotein receptor related protein 5/6) and Frizzled (FZD) family of WNT receptors remain dissociated. Within the cytosolic compartment, a multi-protein β-catenin destruction complex comprises AXIN1/2, APC (adenomatous polyposis coli), CK1ε (casein kinase 1ε), and GSK3β (glycogen synthase kinase 3β). CK1ε and GSK3β sequentially phosphorylate β-catenin, resulting in its ubiquitylation by β-TRCP and ultimately proteasomal degradation (Hernández et al., 2012; Liu et al., 1999, 2002; Zeng et al., 2005). Extracellular WNT ligand physically couples LRPs/5/6 and FZD receptors, leading to phosphorylation-dependent recruitment of the DVL (Dishevelled1/2/3), GSK3β, AXIN1/2, and adenosomatous polyposis coli proteins (Bilić et al., 2007; Mao et al., 2001; Metcalfe et al., 2010; Tamai et al., 2000). This complex, referred to
as the WNT signalosome, transiently suppresses β-catenin phosphorylation by GSK3β (Bilic et al., 2007). Hence, WNT ligand transiently inactivates the destruction complex to promote cytoplasmic accumulation of β-catenin, resulting in its translocation to the nucleus, where it binds to members of the TCF/LEF family of transcription factors to modulate tissue-specific transcriptional programs and cell phenotypes (Behrens et al., 1996).

Through a gain-of-function screen of the kinome, we discovered that the AP2-associated kinase 1 (AAK1) kinase negatively regulates β-catenin-dependent WNT signaling by promoting clathrin-mediated endocytosis (CME) of LRP6. CME is a complex and multi-step process that removes lipids and transmembrane proteins from the plasma membrane. The assembly poly peptide 2 (adaptor-related protein 2 [AP2]) protein complex first recognizes and binds sorting signals on the intracellular domains of transmembrane cargo proteins (Höning et al., 2005; Ricotta et al., 2002). Activation of the AP2 machine occurs allosterically through binding cargo, clathrin, and PIP2 (phosphatidylinositol 4,5-bisphosphate) (Kadlecova et al., 2017). This activation coincides with a conformational shift in AP2 from “closed” to “open.” Open/active AP2 is required for continued clathrin polymerization, cargo recruitment, and ultimately the maturation of a nascent clathrin-coated pit to a stable, successful endocytic event (Conner and Schmid, 2002; Conner et al., 2003; Henderson and Conner, 2007; Kadlecova et al., 2017; Ricotta et al., 2002). In addition to PIP2, cargo, and clathrin, activation of the AP2 complex is promoted by phosphorylation of the AP2M1 subunit by AAK1 (Conner and Schmid, 2002; Conner et al., 2003; Henderson and Conner, 2007; Kadlecova et al., 2017; Ricotta et al., 2002). Importantly, AAK1-dependent phosphorylation of AP2M1 is promoted by clathrin assembly (Conner et al., 2003), which constitutes a positive feedforward loop to promote pit maturation. Once mature, cargo-loaded clathrin-coated pits evolve to endosomal vesicles through dynamin-mediated scission from the plasma membrane (Damke et al., 1994).

Numerous studies establish a regulatory role for CME in WNT signaling, although whether CME activates or inhibits signaling remains debated (Blitzer and Nusse, 2006; Hagemann et al., 2014; Jiang et al., 2012; Kim et al., 2013; Yamamoto et al., 2008). However, a specific role for AAK1 in WNT signaling has not previously been reported. First, clathrin, AP2, and PIP2 are required for WNT-induced LRP6 phosphorylation, signalosome formation, and subsequent WNT signaling (Bilic et al., 2007; Kim et al., 2013). AP2 and clathrin recruitment to the WNT signalosome is induced by WNT ligand, presumably via WNT-induced PIP2 production (Kim et al., 2013). Indeed, WNT ligand induces a gradual accumulation of PIP2 over a 4 hr window after treatment with WNT ligand (Pan et al., 2008). These studies suggest that clathrin-associated machinery is required for WNT-induced signalosome maturation and maintenance and, although not yet proved, possibly for its formation. Second, Gammons et al. (2016a) established that the WNT signalosome incorporates into clathrin-coated pits via DVL polymerization and that this is ultimately required for signalosome formation and WNT signal transduction. Third, De Robertis and colleagues described a mechanism by which the endocytosed signalosome is incorporated into multivesicular bodies (MVBs). Here, sequestration of GSK3β in MVBs suppresses β-catenin phosphorylation, thus potentiating signaling (Taelman et al., 2010). Finally, others have reported that CME of LRP6 and FZD downregulates WNT signaling (Hao et al., 2012; Jiang et al., 2012; Koo et al., 2012; Liu et al., 2014; Yamamoto et al., 2008). For example, disabled homolog 2 (DAB2) drives LRP6 toward CME and represses WNT signaling (Jiang et al., 2012). Additionally, phosphorylation of LRP6 on cytoplasmic tyrosine residues drives CME of LRP6, resulting in decreased WNT signaling (Liu et al., 2014). Furthermore, ubiquitylation of FZD by ZNRF3 and RNF43 drives receptor internalization and degradation, resulting in decreased WNT signaling (Hao et al., 2012; Koo et al., 2012). All together, these studies establish CME as a major regulatory feature in WNT signaling. Although still unresolved, it is likely contextual and temporal considerations play an important role in determining either a positive or negative role for CME in WNT signaling.

Cross-referencing our gain-of-function screen of the kinome with previously published loss-of-function screens, we identify AAK1 as a negative regulator of WNT-induced β-catenin transcription. Gain- and loss-of-function studies reveal that AAK1 negatively regulates WNT signaling by promoting CME of LRP6; FZD remains to be tested. We also report and use a potent and selective cell active AAK1 inhibitor, which potentiates WNT signaling. Full characterization of this small molecule establishes it as the best available tool compound to interrogate AAK1 biology. Unexpectedly, we discovered that WNT induces phosphorylation of AP2M1 by AAK1 in a temporally delayed fashion at 8–10 hr post-WNT3A treatment. Therefore, we propose a modified model whereby WNT-driven CME promotes a negative feedback mechanism to limit pathway longevity. Indeed, we show that WNT stimulation promoted CME of LRP6 in an AAK1-dependent fashion. More broadly, this work contributes to the ongoing discussion of endocytosis within WNT signaling by solidifying a role for the AAK1 kinase in removing LRP6 from the cell plasma membrane.

RESULTS

Identification of AAK1 as a Repressor of WNT Signaling

Thirty years of research on the WNT pathway has revealed its core components and basic mechanics. That said, recent improvements in genetic screening technologies and protein mass spectrometry continue to identify additional modifiers of WNT pathway activity. We, along with others, have reported genome-wide gain- and loss-of-function genetic screens of the WNT pathway, illuminating regulators (e.g., FOXP1, USP6, TFAP4) and their mechanisms of WNT control (Lebensohn et al., 2016; Madan et al., 2016; Walker et al., 2015). Here, we focused on the human kinome to determine which kinases when overexpressed activate or inhibit WNT3A-driven β-catenin transcription. We cloned 387 kinase open reading frames (ORFs) into a lentiviral expression vector before transient overexpression in HEK293T cells carrying the β-catenin-activated Firefly luciferase reporter (BAR) and a constitutively expressed Renilla (Ren) luciferase. Eighteen hours after kinase transfection, cells were treated with WNT3A conditioned media (CM) for an additional 16 hr before lysis and BAR/Ren reporter quantitation (Table S1). ANKRD6/Diversin and CTNNB1/β-catenin served...
as positive controls for repression and activation, respectively. Five kinases (AAK1, ADCK1, ADCK2, MAST1, and TGFBR3) were validated by low-throughput reporter assays in HEK293T-BAR/Ren cells (Figure 1A). Comparative analysis of this kinase gain-of-function screen in HEK293T cells with two previously published small interfering RNA (siRNA)-based loss-of-function screens in HT1080 sarcoma cells and A375 melanoma cells revealed a single common protein: AAK1 (Table S1) (Biechele et al., 2012; Madan et al., 2016). Because of the well-established functional connections between AAK1 and CME and the emerging data on CME in governing WNT pathway dynamics, we sought to understand how AAK1 negatively regulates the WNT pathway.

To validate and extend the discovery of AAK1 as a WNT inhibitor, we tested (1) whether siRNA-mediated silencing of AAK1 activated β-catenin-driven transcription, (2) the cell-type specificity of the AAK1-WNT phenotype, (3) whether AAK1 regulated the expression of endogenous β-catenin target genes, and (4) whether AAK1 affected the activity of non-WNT signaling pathways. First, in agreement with AAK1 overexpression blocking WNT signaling (Figure 1A), siRNA silencing of AAK1 using two non-overlapping siRNAs increased BAR expression in HT1080 fibrosarcoma cells and RKO colon cancer cells (Figures 1B and 1C). To visualize reporter expression in real time, we silenced AAK1 in HT1080 cells carrying a BAR-mCherry reporter. Quantitation of mCherry fluorescence confirmed that AAK1 knockdown activated the BAR reporter (Figure 1D), while AAK1 overexpression suppressed BAR activity (Figure 1E). Third, to rule out potential reporter-based artifacts, we quantified the expression of two endogenous WNT target genes after AAK1 perturbation. AAK1 knockdown increased RNA expression of AXIN2 and NDK1 in both HEK293T and HT1080 cells (Figures 1F and 1G). Conversely, AAK1 overexpression led to decreased RNA expression of AXIN2 and NDK1 in HEK293T cells (Figure 1H). Fourth, because of its established roles in CME, AAK1 might broadly regulate other signaling cascades. AAK1 overexpression did not affect TNFα-driven NFκB reporter activity or TGFβ-driven SMAD reporter activity (Figure 1I). Together, these data establish that AAK1 negatively regulates WNT signaling in cells derived from multiple tissue types. Importantly and consistently with its established role in CME, AAK1 did not affect β-catenin transcriptional activity in the absence of exogenous WNT3A stimulation.

**Discovery of a Potent and Selective Inhibitor of AAK1**

Indazole compound SB-742864 was previously identified as a semi-selective inhibitor of AAK1 (in vitro half maximal inhibitory concentration IC50 = 220 nM) (Elkins et al., 2016). Optimization of SB-742864 via iterative medicinal chemistry optimization led to compound 25A (Figure 2A), a potent and selective inhibitor of AAK1 and the closely related kinase BMP2K. Further optimization to improve the selectivity led to SGC-AAK1-1 (Figure 2A), a chemical probe for AAK1 and BMP2K. A closely related molecule, SGC-AAK1-1N (referred to as 34A), which is devoid of activity on AAK1 or BMP2K, was identified for use as a negative control compound. Full details of the chemistry program and analysis of the structure-activity relationships will be published elsewhere. Compounds 25A and SGC-AAK-1, but not 34A, both selectively inhibited AAK1 and BMP2K over the other members of the subfamily. Numb-associated kinases GAK and STK16, in a TR-FRET binding displacement assay (Figures 2B and S1A), with K_i values against AAK1 of 8 and 9 nM respectively for 25A and SGC-AAK1-1 and 2 μM for negative control 34A. Both 25A and SGC-AAK1-1 showed good selectivity over a panel of 406 wild-type human protein kinases at 1 μM concentration (Figures 2C and S1B; Table S2) with only a small number of other kinases inhibited with dissociation constant (BMP2K K_D bound to 25A) values within 30 times of those of AAK1. Kinases that exhibited >85% inhibition and/or are common kinase off-targets of acylaminoidazoles within this structural class were analyzed to determine whether the inhibition was confirmed in a dose-dependent K_D determination experiment. Kinases for which the compound of interest demonstrated a K_D < 100 nM are marked with a large red circle and 100 nM < K_D < 1 μM with a smaller red circle. One of these kinases, PIP5K1C, is required for PIP2 production, which plays a key role in regulating WNT signaling. To ensure that any observed phenotype is AAK1 dependent, we determined the PIP5K1C IC50 values for SGC-AAK1-1 and 34A as 6.6 and 3.6 μM, respectively, in a PIP5K1C enzyme assay at Reaction Biology (Anastasiadi et al., 2011) (Table S3). The in vitro IC50 values for inhibition of AAK1 enzymatic activity were determined for SGC-AAK1-1 and the negative control (34A) versus a positive control (staurosporine) using a coupled enzyme assay system. Inhibition of AAK1 enzymatic activity was confirmed for SGC-AAK1-1 (IC50 = 270 nM). The controls demonstrated the expected activity profiles: 34A was not active (IC50 > 1 μM), while staurosporine demonstrated potent AAK1 inhibition (IC50 = 120 nM) (Figure S1C). Isothermal titration calorimetry (ITC) measured K_D values for SGC-AAK1-1 of 120 and 490 nM against AAK1 and BMP2K, respectively, and showed that binding had favorable enthalpy (ΔH) and unfavorable entropy (ΔTS) (Figures 2D and S1D; Table S4). Attempts to co-crystallize either inhibitor with AAK1 were unsuccessful, but it was possible to obtain a co-crystal structure of 25A with BMP2K to 2.41 Å resolution (Figures 2E, S1E, and S1F; Table S5). The structure revealed 25A bound in the ATP site, making three hydrogen bonds to the kinase hinge region and two further hydrogen bonds via its sulfonamide oxygens to Gin137 and Asn185, while the central hydrophobic portion of the inhibitor packs against the gatekeeper residue Met130. Hydrogen bonds are indicated as dashed lines to the respective residues within the BMP2K binding pocket (Figure 2E). All pertinent statistics associated with data collection for the BMP2K co-crystal structure, the full-length structure with 25A bound, and the electron density map are included in the Supplemental Information (Table S5; Figures S1E and S1F).

To test that the inhibitors engage AAK1 and BMP2K in live cells, we used NanoBRET assays (Promega) (Vasta et al., 2018), in which human cells were transfected with a plasmid expressing the full-length kinase gene fused to Nanoluc luciferase. In the presence of a fluorescent-labeled ATP-competitive tracer compound, a bioluminescence resonance energy transfer (BRET) signal was measured. Displacement of the tracer by the inhibitor being tested, resulting in loss of BRET, demonstrated target engagement, and linear regression of the IC50 values at multiple tracer concentrations yielded the IC50 in the absence...
Figure 1. Gain-of-Function Kinome Screen Reveals AAK1 as a Repressor of WNT Signaling
(A) HEK293T-B/R cells were transfected with the indicated construct for 24 hr. Cells were then treated for 16 hr with WNT3A or Lcell CM. Bars represent average Firefly/Renilla fluorescence units (RFU) from three technical replicates.
(B and C) Luciferase assay of HT1080 (B) or RKO (C) stable B/R cells transfected with either control or AAK1 siRNA for 56 hr. Cells were then treated with either Lcell or WNT3A CM for 16 hr. Bars represent average Firefly/Renilla RFU from three technical replicates. Western blot analysis illustrates knockdown efficiency of two independent AAK1 siRNAs.
(D) IncuCyte imaging of HT1080 cells stably expressing a BAR-mCherry fluorescent reporter transiently transfected with indicated siRNA construct. WNT3A CM was added at 18 hr, then cells were imaged for 50 hr post-transfection. Graph represents data points averaged across four technical replicates.
(E) Live-cell imaging of HT1080 cells stably expressing a BAR-mCherry fluorescent reporter transiently transfected with the indicated expression construct, AAK1, or FLAG control. WNT3A CM was added at 18 hr, and cells were monitored for an additional 56 hr. Data represent the average of four technical replicates.
(F and G) qPCR analysis of AXIN2 and NKD1 in HEK293T (F) or HT1080 (G) cells 72 hr after transfection with the indicated siRNA. Cells were treated with WNT3A CM for 6 hr prior to harvest. Bars represent average GAPDH-normalized gene expression across three technical replicates.
(H) qPCR analysis of AXIN2 (left) and NKD1 (right) in HEK293T cells transfected with overexpression construct for 24 hr, then treated with WNT3A CM for 6 hr prior to harvest. Bars represent average GAPDH-normalized gene expression across three technical replicates.
(I) Luciferase assay of HEK293T cells transfected with indicated pathway-specific Firefly luciferase reporter constructs and expression constructs prior to a 16 hr treatment with recombinant human (rh) WNT3A (200ng/mL), rhTNFα (200ng/mL), or rhTGFβ1 (200ng/mL). Bars represent average Firefly/Renilla RFU from three technical replicates.

*p < 0.05, **p < 0.005, and ***p < 0.0005. All data are representative of biological triplicates, unless otherwise noted. Error bars represent SE. For complete statistics, see STAR Methods. See also Table S1.
Figure 2. SGC-AAK1-1 Is a Potent and Specific Inhibitor of AAK1 and BMP2K
(A) Chemical structures of the AAK1/BMP2K chemical probe SGC-AAK-1, the related AAK1/BMP2K inhibitor 25A, and the negative control compound 34A that has a similar chemical structure but does not inhibit AAK1 or BMP2K.
(B) SGC-AAK-1 selectively bound AAK1 and BMP2K with a more than 30-fold difference in $K_i$ in a TR-FRET binding displacement assay over the related kinases GAK and STK16. Sixteen-point dose-response curves were measured in duplicate.
(C) SGC-AAK1-1 has good selectivity over the human kinome. SGC-AAK1-1 was used at 1 μM concentration and tested against over 400 wild-type human kinases. $K_D$ determination was completed for all kinases that exhibited >85% inhibition and/or were observed as common off-targets for this structural class. Kinases for which SGC-AAK1-1 has a $K_D$ < 100 nM are marked with a large red circle and 100 nM < $K_D$ < 1.0 μM with a smaller red circle.
(D) ITC confirmed a $K_D$ of 120 nM for binding of SGC-AAK1-1 to AAK1.
(E) A co-crystal structure of BMP2K (2.41 Å) bound to 25A revealed the binding mode of the inhibitor in the ATP site. This structure revealed five key hydrogen bonds and revealed the orientation of the central hydrophobic portion of the inhibitor with respect to the binding pocket.
(F) NanoBRET cellular target engagement assays in HEK293T cells showed that 25A (F) and SGC-AAK1-1 (G) both entered cells and directly bound to AAK1 and displaced a fluorescent tracer molecule from the ATP site. Cells were treated with serially diluted inhibitors in the presence of four different concentrations of a NanoBRET tracer molecule, and IC50 values were calculated from a linear interpolation of the IC50 values at each tracer concentration to obtain the predicted IC50 in the absence of tracer. Measurements were made with three technical replicates.
(H) Binding of SGC-AAK1-1 to BMP2K in cells was weaker than to AAK1 with IC50 > 1 μM. Displacement of the same fluorescent tracer molecule required increased concentrations of SGC-AAK1-1, revealing an elevated IC50 value for binding to BMP2K versus AAK1 in the cellular target engagement assay.
(I) SGC-AAK1-1 inhibited the phosphorylation of the AP2M1 subunit at Thr156 in HEK293T cells in a concentration-dependent manner or with 10 μM 34A. HEK293T cells were treated with inhibitors for 2 hr before western blot analysis.
Error bars represent SE. See also Figure S1 and Tables S2, S3, S4, and S5.
of tracer. 25A and SGC-AAK-1 bound to AAK1 in HEK293T cells with IC50 values of about 240 nM, and more weakly to BMP2K, with IC50 values of 600 nM and 1.5 μM, respectively (Figures 2F–2H and S1G). To demonstrate that binding to AAK1 inhibited its enzymatic activity in cells, phospho-AP2M1 (Thr156) levels were observed by western blotting after 2 hr treatment of HEK293T cells with serially diluted SGC-AAK-1, resulting in a dose-dependent decrease of pAP2M1 starting below 1 μM, in agreement with the NanoBRET data (Figure 2I).

**AAK1 Inhibitor Activates WNT Signaling by Stabilizing β-Catenin Levels**

To confirm and extend these data, we next tested SGC-AAK-1 and 25A in a series of experiments to evaluate its potency of AAK1 inhibition in cells and regulation of WNT signaling. First, we treated HT1080 cells with SGC-AAK-1 and evaluated AAK1-dependent phosphorylation of AP2M1. SGC-AAK-1, along with 25A, both significantly reduced phosphorylation of AP2M1 (T156), compared with DMSO control and the negative control, 34A (Figure 3A). Second, treatment with SGC-AAK-1 or 25A activated WNT-driven BAR activity compared with controls (DMSO and 34A) in a dose-dependent manner (Figure 3B). Importantly, 25A also significantly upregulated WNT endogenous target genes AXIN2 and NKD1 in HT1080 cells, compared with DMSO control and 34A (Figure 3C).

Finally, we measured β-catenin protein abundance following a 15 min pulse of WNT3A ligand in the presence of control compound 34A or SGC-AAK1-1. In cells pulsed with WNT3A and cultured with 34A, β-catenin protein levels increased, reaching a maximum at about 7 hr before returning to baseline at 18 hr (Figures 3D and 3E). In the presence of SGC-AAK1-1, β-catenin protein accumulated more robustly at earlier time points and was sustained throughout the 24 hr time course (Figures 3D and 3E). Together, these results indicate that 25A and SGC-AAK1-1 block AAK1 kinase activity, resulting in increased β-catenin protein stability and β-catenin-dependent transcription.

**AAK1 Promotes CME of LRP6 to Inhibit WNT Signaling**

Several recent studies report that CME negatively regulates WNT signaling by reducing the presence of the receptor complex on the plasma membrane (Jiang et al., 2012; Liu et al., 2014; Yamamoto et al., 2008). Given the well-established role of AAK1 in phosphorylating AP2M1 to facilitate CME (Conner and Schmid, 2002; Conner et al., 2003; Henderson and Conner, 2007; Ricotta et al., 2002), our discovery of AAK1 as a negative regulator of
Figure 4. AAK1 Represses β-Catenin-Mediated WNT Signaling through Regulation of Endocytosis

(A) HT1080-B/R cells were transfected with indicated siRNA for 56 hr and then treated with WNT3A or CHIR99021 (1 μM) for 16 hr (left). HT1080-B/R cells were transfected with the indicated overexpression DNA construct and allowed to recover for 12 hr. Cells were then treated with WNT3A CM or CHIR99021 (1 μM) for 16 hr (right). Data are averaged over three technical replicates.

(B) BAR luciferase assay from HT1080 cells transiently transfected with BAR-firefly luciferase, TK-REN, and the indicated expression constructs. Twelve hours post-transfection, cells were treated for 16 hr with Lccll or WNT3A CM and then assayed for BAR activity. Western blot of FLAG-AAK1 and FLAG-GFP is shown to illustrate expression of AAK1 constructs. Presented data are averaged across three technical replicates.

(C) Luciferase assay in HT1080-B/R cells transfected with indicated siRNAs for 50 hr, then co-transfected with indicated expression constructs for 12 hr. Cells were then treated for 16 hr with either Lccll or WNT3A CM. Data are averaged over three technical replicates. Right: western blot of clathrin, indicating knockdown efficiency.

(D) Luciferase assay of HT1080-B/R cells transfected with indicated siRNA for 56 hr, then treated as indicated for 16 hr. Data are averaged over three technical replicates.

(E) Luciferase assay of HEK293T-B/R cells transfected with indicated constructs for 12 hr, then treated with either Lccll or WNT3A CM for 18 hr. (β-Catenin pm (point mutant) is a construct with the

GSK3β target residues (S33/S37/T41) on β-catenin mutated. Western blot analysis of FLAG and GFP shown to confirm expression of overexpression constructs. Data are averaged over three technical replicates.

WNT signaling is not entirely unexpected. That said, AAK1 has not previously been shown to regulate WNT signaling, and certainly, the molecular mechanisms governing CME within the WNT pathway and its homeostasis are not well defined, with several remaining questions and conflicting studies (Blitzer and Nusse, 2006; Gammons et al., 2016a; Hagemann et al., 2014; Jiang et al., 2012; Liu et al., 2014; Taelman et al., 2010; Yamamoto et al., 2008). To confirm that AAK1 functions within membrane proximal steps of WNT signaling, we stabilized β-catenin protein levels with a GSK3β inhibitor (CHIR99021) following either AAK1 overexpression or siRNA silencing. CHIR99021-mediated activation of the BAR reporter was not affected by AAK1 depletion or overexpression (Figure 4A). As expected, β-catenin knockdown blocked BAR activation by AAK1 silencing (Figure S2A). These results indicate that AAK1 functions upstream of β-catenin and the destruction complex. Next, the role of endocytosis in AAK1-mediated repression of WNT signaling was evaluated using two AAK1 mutants: (1) AAK1-74A, which lacks kinase activity, and (2) AAK1-AID, which codes for a truncated form of AAK1 that does not bind the AP2 complex (Conner and Schmid, 2002). Neither AAK1-74A nor AAK1-AID significantly repressed the BAR reporter (Figure 4B), suggesting that both AP2 interaction and kinase activity are required for AAK1-mediated repression of WNT signaling. A role for clathrin and CME in AAK1-driven WNT suppression was tested by (1) siRNA silencing of clathrin, (2) pharmacological inhibition of clathrin-coated vesicles, and (3) blocking dynamin1 function with an established dominant-negative K44A mutation. Two non-overlapping clathrin siRNAs effectively silenced clathrin expression and blocked AAK1-dependent suppression of the BAR reporter in HT1080 cells (Figure 4C). Similarly, treatment with chlorpromazine, an established, albeit promiscuous CME inhibitor (Blitzer and Nusse, 2006; Phompok and Rosenthal, 1991; Wang et al., 1993) suppressed WNT signaling induced by AAK1 silencing (Figure 4D). Furthermore, co-transfection of AAK1 and dominant-negative dynamin (DNM1) (K44A) rescued the AAK1-induced suppression of BAR activity in a dose-dependent manner (Figure 4E). These experiments suggest that AAK1 represses WNT signaling in a clathrin- and kinase-dependent manner.

We next examined the effect of AAK1 on LRP6 protein levels using cell surface biotinylation assays and flow cytometry. AAK1 overexpression decreased LRP6 protein levels at the plasma membrane (Figure 5A). Additionally, AAK1 knockdown
Together, these data indicate that SGC-AAK1-1, but not the negative control compound 34A decreased following WNT3A treatment; this decrease was observed in HEK293T cells. Here, AAK1 overexpression decreased LRP6 surface levels findings in an orthogonal assay, we quantified total LRP6 expression across six biological replicates and demonstrated that with AAK1 knockdown, total LRP6 levels increase (Figure 5C). To validate the changes in surface LRP6 expression findings in an orthogonal assay, we used flow cytometry to quantify cell surface LRP6 levels in HEK293T cells. Here, AAK1 overexpression decreased LRP6 surface levels while AAK1 knockdown increased LRP6 surface levels (Figures 5D and 5E). Importantly, LRP6 surface levels decreased following WNT3A treatment; this decrease was rescued by co-treatment with the active AAK1 inhibitor SGC-AAK1-1, but not the negative control compound 34A (Figure 5F; Table S6). Together, these data indicate that WNT3A treatment decreases LRP6 cell surface levels via an AAK1-dependent mechanism.

We next tested whether AAK1 and LRP6 co-complexed. Using HEK293T cells, immunoprecipitation, and western blot analysis of HA-LRP6 protein complexes revealed the presence of overexpressed FLAG-AAK1 (Figure S3A). Similarly, immunoprecipitation of HA-LRP6 co-purified endogenous AAK1, and reciprocally, immunopurification of overexpressed FLAG-AAK1 revealed co-complexed endogenous LRP6 (Figures S3B and S3C, respectively). Finally, immunoprecipitation of endogenous AAK1 showed co-complexed endogenously expressed LRP6 (Figure S3D). Interestingly, WNT3A stimulation suppressed the association of LRP6 and AAK1 in all experiments (Figures S3A–S3D). Because the AP2 complex is known to associate with LRP6 (Kim et al., 2013), we next tested if AP2M1 was co-immunoprecipitated with AAK1 and LRP6.

Figure 5. AAK1 Downregulates LRP6 Cell Surface Levels

(A and B) Western blot analysis of HEK293T cells transfected with an AAK1 overexpression construct for 24 hr (A) or AAK1 siRNA for 72 hr (B) prior to membrane fractionation using a surface biotinylation assay. Data represent average surface staining of four biological replicate experiments quantified by LiCOR software. (C) LiCOR quantification of western blot analysis of LRP6 levels with AAK1 knockdown. The box-and-whisker plot represents total LRP6 expression normalized to β-tubulin (loading control) from six biological replicates quantified by LiCOR software.

(D and E) LRP6 surface staining as detected by flow cytometry analysis in HEK293T cells transfected with an AAK1 overexpression construct for 24 hr (D) or an AAK1 siRNA construct for 72 hr (E). All experiments were quantified using FlowJo software. Experiment was performed in four biological replicates.

(F) LRP6 surface levels as detected by flow cytometry analysis in HEK293T cells treated as indicated for 8 hr. The box-and-whisker plot represents LRP6 surface levels normalized to BSA control for biological triplicates. Raw values for each experiment are shown in Table S6.

(G) HEK293T cells were transfected with FLAG-AAK1 or FLAG-GFP overexpression constructs. Twenty-four hours post-transfection, cells were treated for 6 hr with the autophagy inhibitor bafilomycin A1 (100 nm) or DMSO control prior to cell harvest, then analyzed using western blot for endogenous LRP6 and AAK1 levels. The box-and-whisker plot represents LRP6 expression normalized to β-tubulin from four biological replicates quantified by LiCOR software.

*p < 0.05 and **p < 0.005. All data are representative of biological triplicates, unless otherwise noted. For complete statistics, see STAR Methods. See also Figure S3 and Table S6.
required for the AAK1-LRP6 interaction. Co-overexpression of AAK1 constructs (WT, 74A, and dAID) and HA-LRP6 followed by FLAG affinity purification and western blot analysis demonstrated that the AAK1 AP2 binding domain is required for the AAK1-LRP6 interaction (Figure S3E).

AAK1 silencing potentiates WNT3A-driven β-catenin activation and increased LRP6 protein levels in whole cell lysate and on the membrane (Figures 5B, 5C, and 5E). Because cargo targeted by CME can be directed to lysosomes for degradation, we tested if the decreased LRP6 levels we observed following AAK1 overexpression were due to CME of LRP6 and subsequent lysosomal degradation. AAK1 was overexpressed in HEK293T cells before treatment with bafilomycin A1, an inhibitor of lysosomal acidification. Western blot analysis established that bafilomycin A1 treatment rescued AAK1-driven downregulation of LRP6 (Figure 5G). Together, these results suggest that AAK1 inhibits WNT signaling by inducing CME endocytosis and lysosomal degradation of LRP6.

**WNT3A Induces AAK1 Phosphorylation of AP2M1**

Within 15–30 min of WNT3A treatment, LRP6 is phosphorylated by GSK3β and CK1γ (casein kinase 1γ), resulting in transient suppression of β-catenin phosphorylation and degradation (Hernández et al., 2012; Liu et al., 1999, 2002; Zeng et al., 2005). Extended duration of WNT3A stimulation of 4–6 hr results in MVB formation and sequestration of GSK3β (Taelman et al., 2010). Here we asked if WNT3A treatment regulated AAK1-dependent phosphorylation of AP2M1. Time-course analysis in HT1080 or HEK293T cells revealed WNT3A-induced phosphorylation of AP2M1 at 8–10 hr post-treatment, with no change in total AP2M1 or AAK1 protein levels (Figure 6A). To rule out artifacts due to the use of WNT3A CM and to extend relevance to a second cell type, we treated HT1080 cells (Figure S4A) and HEK293T (Figure S4B) cells with recombinant human WNT3A (rhWNT3A) over a course of 4–16 hr. Western blot analysis revealed an increase in pAP2M1 protein levels 8–10 hr post-WNT3A stimulation. We also tested the importance of continued exposure to WNT ligand over the time course compared with transient pulse of WNT3A. Here, cells were treated with WNT3A for 15 min before replacement with fresh media (Figures S4C and S4D). These experiments demonstrated that a pulse of WNT3A induced a similar increase in AP2M1 phosphorylation compared with continuous exposure to WNT3A ligand.

The WNT3A-induced phosphorylation of AP2M1 required AAK1 expression and activity. First, HT1080 cells or HEK293T cells were treated with WNT3A CM and the AAK1 inhibitor 25A before western blot analysis of phosphorylated AP2M1; 25A blocked WNT3A-induced phosphorylation of AP2M1 (Figures S4C and S4D). Additionally, the AAK1 inhibitor blocked WNT3A-induced AP2M1 phosphorylation when added at any time point following WNT3A treatment (Figure S4E). Second, siRNA silencing of AAK1 also suppressed WNT3A-induced phosphorylation of AP2M1 (Figure 6B). These data suggest that at 8–10 hr post-WNT3A treatment, AAK1-dependent phosphorylation of AP2M1 increases to form a negative feedback loop of CME of LRP6 to decrease its abundance on the plasma membrane.

Intrigued with the delayed WNT3A-induced phosphorylation of AP2M1 by AAK1, we performed a series of functional assays to identify requisite signaling events. First, we found that AAK1-mediated pAP2M1 accumulation was induced by rhWNT3A (3A) and rhWNT5A (5A), the latter of which signals in a β-catenin-independent fashion (Figure 6C). Second, inhibition of GSK3β with CHIR99021 did not result in elevated pAP2M1 levels, suggesting that WNT3A-induced pAP2M1 did not require β-catenin-dependent transcription (Figure 6D). In agreement with these data, inhibiting β-catenin-dependent transcription with overexpressed dominant-negative TCF7L2/TCF4 (dnTCF7L2/dnTCF4) did not affect pAP2M1 increase (Figure 6E). Third, using HEK293T cells lacking all three DVL proteins via CRISPR-Cas9 triple-knockout (TKO) cells (clones 4 and 6) (Gammons et al., 2016b) and HEK293T LRP5/6 CRISPR-Cas9 double-knockout (DKO) cells, we tested whether WNT3A promoted phosphorylation of AP2M1 (Figure 6F). In the absence of LRP5/6 or DVL, AP2M1 phosphorylation did not increase following WNT treatment. Interestingly, background pAP2M1 levels are increased in both LRP5/6 DKO and DVL TKO cells compared with WT HEK293T cells (Figure 6F). Fourth, previous studies established that PIP2 levels increase following short-term (4 hr) WNT3A treatment and that PIP2 is required for WNT signalosome formation (Kim et al., 2013; Pan et al., 2008). Furthermore, PIP2 recruits AP2 to the signalosome, leading to CME of LRP6 (Kim et al., 2013). Thus, we hypothesized that PIP2 is required for WNT3A-induced phosphorylation of AP2M1 by AAK1. To test this hypothesis, we inhibited PIP2 by treating cells with neomycin or carbachol, both of which block PIP2 signaling (Baron et al., 1993; Huang et al., 1999). Treatment of HT1080 cells with neomycin or carbachol blocked WNT3A-induced phosphorylation of AP2M1 (Figure 6G). Furthermore, we blocked PIP2 production by silencing the PI4K2α kinase with two non-overlapping siRNAs. PI4K2α phosphorylates phosphatidylinositol (PI) to phosphatidylinositol 4-phosphate (PI4P) and is required for subsequent PIP2 production (Toker, 1998). In agreement with the neomycin or carbachol experiments, PI4K2α silencing in HT1080 cells blocked WNT3A-dependent increase in pAP2M1 (Figure 6H). We conclude that WNT ligands drive AAK1-dependent phosphorylation of AP2M1 in a manner that is (1) temporally delayed from signalosome formation, (2) independent of β-catenin-mediated transcription, (3) dependent on LRP5/6 and DVL protein expression, and (4) dependent on PIP2 production.

**DISCUSSION**

CME is a well-established regulator of WNT signaling, although whether CME promotes or inhibits the WNT pathway remains debated (Bilic et al., 2007; Blitzer and Nusse, 2006; Gammons et al., 2016a; Kim et al., 2013; Pan et al., 2008; Taelman et al., 2010; Yamamoto et al., 2008). Certainly, contextual and temporal features in CME-directed WNT studies are important. As a central and positive regulator of CME, our discovery of AAK1 as a WNT modulator is no surprise. Collectively our data show that AAK1 negatively regulates β-catenin-dependent WNT signaling through CME endocytosis of LRP6. Although the experiments have not yet been done, we speculate that FZD is similarly targeted by AAK1 and CME. In the course of our studies,
we discovered an unexpected WNT-induced phosphorylation event on T156 of AP2M1. This was mediated by AAK1 and reproducibly occurred at 8–10 hr post-WNT treatment. In Figure 7, we summarize our findings in a multi-step model that integrates WNT-driven signalosome formation, PI2 production, AAK1, AP2M1 phosphorylation, and CME of LR6.

In the absence of WNT ligand, Fzd and LR6 remain dissociated, allowing β-catenin phosphorylation, ubiquitylation, and degradation (Liu et al., 1999; Liu et al., 2002). Within minutes of WNT stimulation, the WNT signalosome forms, resulting in transient repression of β-catenin phosphorylation by GSK3β (Figure 7A) (Bilic et al., 2007). A key step in signalosome formation is recruitment of the signalosome to nascent clathrin-coated pits with high local concentrations of DVL, which allows the dimerization of DVL, thus potentiating WNT signaling (Gammons et al., 2016a). The newly formed WNT signalosome requires PI2 production and clathrin-coated pit stabilization and ultimately CME of LR6, thus constituting a WNT-driven and AAK1-dependent negative feedback loop.

Central to our model is the increase in phosphorylated AP2M1 levels at 8–10 hr post-WNT treatment. Phosphoinositide is converted to PI2 by PI4K2z and PI5K (Toker, 1998). Importantly, PI5K association with the DIX domain of DVL2/3 results in activation of PI5K1 (Hu et al., 2015; Pan et al., 2008). One
hypothesis is that through this mechanism, WNT-driven recruitment of DVL to the LRP/FZD receptor complex results in a time-dependent increase in local PIP2 production. PIP2 and cargo proteins allosterically activate the AP2 complex, thus driving clathrin polymerization and maturation of clathrin-coated pits (Honing et al., 2005; Kadlecova et al., 2017). Phosphorylation of AP2M1 by AAK1 stabilizes the AP2 complex in an open/active confirmation, promoting the binding of clathrin and cargo proteins and ultimately enhancing CME (Figure 7B) (Conner et al., 2003). Although we do not yet understand the mechanistic details, we speculate that PIP2 levels continue to rise hours after WNT stimulation, possibly triggering an increase in AAK1-dependent phosphorylation of AP2M1 and CME (Figure 7B). Further studies that quantify PIP2 levels at 8–10 hr post-WNT stimulation are needed.

Alternatively, the AP2 complex has been reported to cycle between phosphorylated and dephosphorylated states during CME and that this cycling is necessary for robust CME (Kadlecova et al., 2017). Therefore, it is possible that WNT stimulation shifts the AAK1/phosphatase balance to favor phosphorylated AP2M1. Another possibility is AAK1 itself is phosphorylated, either via AAK1 autophosphorylation or phosphorylation by another kinase, resulting in increased AAK1 activity, AP2M1 phosphorylation, and CME of LRP6. Interestingly, previous studies demonstrated that GSK3β inhibition results in increased dynamin-1 activity, leading to rapid and dysregulated CME (Liberali et al., 2014; Reis et al., 2015). It is possible that GSK3β sequestration within MVBs (Taelman et al., 2010) and increased AAK1 activity both contribute to WNT-driven CME of LRP6. Last, given the delayed nature of the AAK1-AP2M1 phospho-event, we were surprised that β-catenin-dependent transcription was not required. Indeed, we also show that the β-catenin-independent WNT ligand WNT5A similarly induces pT156-AP2M1. Interestingly, previous studies demonstrated that an interaction between DVL2 and AP2 is required for WNT5A-induced FZD4 endocytosis (Yu et al., 2007). Together, these data suggest a regulatory role for AAK1 in FZD endocytosis. Although not yet validated in vivo, we hypothesize that in certain developmental or disease contexts, WNT-induced pT156-AP2M1 may establish a negative feedback loop to remove LRP/FZD from the cell membrane. Collectively, the molecular events suggested in this time-delayed model are consistent with the timing reported in the GSK3-sequestration model proposed by De Robertis and colleagues (Taelman et al., 2010).

The AP2 complex binds Yxxφ or dileucine motifs within the intracellular domains of transmembrane cargo proteins (Chen et al., 1990; Collawn et al., 1990). Through phosphorylation of T156-AP2M1, AAK1 was previously shown to promote association of the AP2 complex with cargo (Ricotta et al., 2002). Interestingly, in co-immunoprecipitation experiments, we discovered AP2 regulates the association between AAK1 and LRP6. AP2 interacts with LRP6 through a conserved Yxxφ motif, which is required for WNT signalosome formation (Gammons et al., 2016a; Kim et al., 2013). We also report that the AAK1-LRP6 co-complex dissipates following WNT3A treatment. Although further experimentation is needed to define the dynamics of

Figure 7. Proposed Model for AAK1-Dependent and CME of LRP6

(A) Within minutes of WNT ligand exposure, FZD and LRP5/6 co-complex, allowing formation of the LRP6 signalosome in nascent clathrin-coated pits. Signalosome formation requires DVL domain swapping and polymerization, as well as PIP2 and AP2 recruitment. This promotes accumulation of β-catenin and activation of the WNT signaling pathway.

(B) Prolonged exposure to WNT ligand drives AAK1-dependent AP2M1 phosphorylation, CME, and ultimately removal of the WNT receptors from the plasma membrane.
protein-protein interactions during signalosome formation and CME, it is possible that following AAK1-dependent phosphorylation of AP2M1, AAK1 dissociates from the LRP6/AP2 complex.

AAK1 contributes to several neurological disorders, including neuropathic pain, Alzheimer’s disease, Parkinson’s disease, schizophrenia, and amyotrophic lateral sclerosis (Kostich et al., 2016; Shi et al., 2014). AAK1 has a described role in dendrite branching and spine development (Ultanir et al., 2012), and through the regulation of Neuregulin1/ErbB4, AAK1 has been linked to schizophrenia (Kuai et al., 2011). Just recently, the LX9211 AAK1 inhibitor passed phase 1 clinical trials for neuropathic pain. Here we report the development of a potent and selective AAK1 pharmacological inhibitor (SGC-AAK1-1). SGC-AAK1-1 demonstrates improved biochemical selectivity over the phase 1 clinical agent LX9211 and is confirmed to be cell active. Thus, we report the best available chemical tool to study AAK1/BMP2K pathways and related biology. Like the lead compound 25A, SGC-AAK1-1 inhibited AAK1-dependent phosphorylation of AP2M1 and activated WNT signaling. Looking forward, in vitro and in vivo neural-directed studies of SGC-AAK1-1 and its comparison with LX9211 are needed to evaluate the therapeutic potential of SGC-AAK1-1. Furthermore, while we describe a role for AAK1 in negatively regulating WNT signaling, AAK1 also inhibits Neuregulin1/ErbB4 and positively regulates the NOTCH pathway (Gupta-Rossi et al., 2011; Kuai et al., 2011). Therefore, AAK1 inhibitors may also regulate these signaling cascades and consequently prove therapeutically beneficial in diseases with misregulated NOTCH or Neuregulin1/ErbB4 signaling. In cancer, mutation and altered expression of several genes promote WNT receptor stability on the plasma membrane, resulting in hyperactive WNT signaling and tumorigenesis (e.g., ZNRF3/RNF43 and USP6; Hao et al., 2012; Koo et al., 2012; Madan et al., 2016; Ruffner et al., 2012; Schmid, 2017). Whether AAK1 expression or activity is suppressed in cancer and if this contributes to WNT activation remain to be determined.

STAR METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and seven tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.12.023.

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AUTHOR CONTRIBUTIONS

M.J.A. and M.P.W. co-led molecular characterization of AAK1, designed and carried out molecular biology experiments, and drafted and wrote the manuscript. A.D.A. synthesized SGC-AAK1-1, led the SGC probe declaration effort, and contributed to writing. R.R.R. carried out NanoBRET measurements and cellular IC_{50} measurements. D.S.S. carried out dose-response and pAP2M1 characterization of SGC-AAK1-1 and 25A and contributed to AAK1-LRP6 interaction studies. A.D.R. contributed to cloning and carrying out kinase gain-of-function screen and validation. D.M.G., M.B.R., T.T., and Y.N. aided in AAK1 signaling characterization. M.V.G. developed, generated, and validated LRP5/6 DKO HEK293T cells. J.M.B. carried out the NAK TR-FRET selectivity panel. R.M.C. made AAK1/BMP2K proteins and produced co-crystal structures. D.H.D. proposed and designed analogs. J.M.E. facilitated experimental design and contributed to writing. O.F. facilitated in vitro assays. C.G. made AAK1 proteins for crystallization/assays. O.G. facilitated protein synthesis and crystallization. P.H.G. carried out TR-FRET measurements and in vitro IC_{50} measurements. N.K. executed compound scale-up for validation studies. S.M. contributed ideas and managed the probe declaration effort. A.S.S. made AAK1/BMP2K proteins for crystallization/assays. F.J.S. made proteins for the NAK selectivity panel and ITC and carried out ITC experiments. C.I.W. synthesized 25A and SGC-AAK1-1N. T.M.W. managed chemical effort. A.S.S. made AAK1/BMP2K proteins for crystallization/assays. F.J.S. made proteins for the NAK selectivity panel and ITC and carried out ITC experiments. C.I.W. synthesized 25A and SGC-AAK1-1N. T.M.W. managed chemistry efforts and aided in the design of molecules. W.J.Z. designed molecules, facilitated SGC probe declaration, and contributed to writing. M.B.M. facilitated experimental design, data interpretation, project management, and contributed to writing.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Rabbit polyclonal anti-AAK1 | Bethyl Labs | Cat#A302-146A; RRID:AB_1720315 |
| Rabbit monoclonal anti-Clathrin Heavy Chain (D3C6) XP | Cell Signaling Technology | Cat#4796S; RRID:AB_10557412 |
| Rabbit monoclonal anti-LRP6 | Cell Signaling Technology | Cat#3395; RRID:AB_1950408 |
| Rabbit polyclonal anti-phospho-LRP6 (S1490) | Cell Signaling Technology | Cat#2568; RRID:AB_2139327 |
| Rabbit polyclonal Anti-Human AP2M1, phospho (Thr156) | Cell Signaling Technology | Cat#3843S; RRID:AB_2056358 |
| Rabbit monoclonal anti-PiP4K2A (D83C1) | Cell Signaling Technology | Cat#5527; RRID:AB_2722636 |
| Rabbit polyclonal anti-Phospho-beta-Catenin (Ser33/37/Thr41) | Cell Signaling Technology | Cat#95618; RRID:AB_331729 |
| Monoclonal mouse ANTI-FLAG M2 | Sigma-Aldrich | Cat#F3165; RRID:AB_259529 |
| Rat monoclonal Anti-HA High Affinity (clone 3F10) | Roche | Cat#11867423001; RRID:AB_390918 |
| Monoclonal mouse anti-beta-Tubulin | Sigma-Aldrich | Cat#T8328; RRID:AB_1844090 |
| Mouse monoclonal anti-LRP6 | Abcam | Cat#ab75358; RRID:AB_1950408 |
| Rabbit monoclonal anti-AP2M1 (phospho T156) | Abcam | Cat#ab109397; RRID:AB_10866362 |
| Rabbit monoclonal-AP2M1 [EP2695Y] | Abcam | Cat#ab75995; RRID:AB_1309955 |
| Mouse monoclonal anti-CTNNB1 | BD Biosciences | Cat#163510 |
| Mouse monoclonal anti-GSK-3 beta | BD Biosciences | Cat#610201; RRID:AB_397600 |
| Mouse monoclonal anti-Dvl-3 | Santa Cruz Biotechnology | Cat# sc-8027; RRID:AB_627434 |
| Mouse monoclonal beta-actin | Santa Cruz Biotechnology | Cat# sc-47778 HRP; RRID:AB_2714189 |
| Rabbit polyclonal AP2M1 | Thermo Fisher Scientific | Cat# PA5-21360; RRID:AB_11154082 |
|IRDye 800CW Goat anti Mouse IgG | LI-COR Biosciences | Cat# P/N 925-32210; RRID:AB_2687825 |
|IRDye 680LT Goat anti Mouse IgG | LI-COR Biosciences | Cat#P/N 925-68020; RRID:AB_2687826 |
|IRDye800CW Goat anti Rabbit IgG | LI-COR Biosciences | Cat#925-32211; RRID:AB_2651127 |
|IRDye 680LT Goat anti Rabbit IgG | LI-COR Biosciences | Cat#P/N 925-68021; RRID:AB_2713919 |
|Mouse monoclonal anti-LRP6 (Clone 25S302) | R and D Systems | Cat# MAB1505; RRID:AB_10889810 |
|Monoclonal mouse IgG2A Isotype Control (Clone 20102) | R and D Systems | Cat# MAB0003; RRID:AB_357345 |
|R-Phycocyanthr- AffiniPure F(ab')2 Fragment Goat Anti-Mouse IgG | Jackson Immunoresearch | Cat#115-116-146; RRID:AB_2338629 |
|Polyclonal rabbit anti-GFP | Abcam | Cat#ab290; RRID:AB_303395 |
| **Bacterial and Virus Strains** |        |            |
| BL21 (DE3) for recombinant protein expression | Laboratory of Dr. Opher Gileadi | N/A |
| **Chemicals, Peptides, and Recombinant Proteins** |        |            |
| CHIR99021 | Cayman Chemicals | Cat#13122 ; Cas#252917-06-9 |
| Chlorpromazine HCL | Sigma-Aldrich | Cat#C0982 ; Cas#69-09-0 |
| Bafilomycin A1 | Cayman Chemicals | Cat#11038 ; Cas#88899-55-2 |
| WNT3A | PeproTech | Cat#315-20 |
| WNT5A | R and D Systems | Cat#645-WN-010 |
| Neomycin trisulfate salt hydrate | Sigma-Aldrich | Cat#N63886 ; Cas#1405-10-3 |
| Carbachol | EMD Chemicals | Cat#212385 ; Cas#51-83-2 |
| Staurosporine | Sigma-Aldrich | Cat#S4400; Cas# 62996-74-1 |
| NanoBRET Tracer K-5 | Promega | Cat# N248A |
| AP-2 derived peptide | WatsonBio | Custom (biotin-GGSQITSQVTQGWRRAamide) |

(Continued on next page)
Continued

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Critical Commercial Assays** |        |            |
| Pierce Cell Surface Protein Isolation Kit | ThermoFisher | Cat#89881 |
| PureLink RNA Mini Kit | Invitrogen | Cat#12183018A |
| iScript cDNA Synthesis Kit | BioRad | Cat#1708891 |
| Dual-Luciferase Reporter Assay System | Promega | Cat#E1910 |
| **Deposited Data** |        |            |
| Gain-of-function kinome screen | This paper | Mendeley Data: https://doi.org/10.17632/352fxh8r6t.1 ; https://doi.org/10.17632/352fxh8r6t.1#file-94081320-ab20-4178-8be2-233c7ea23b62 |
| KINOMEscan | This paper | Mendeley Data: https://doi.org/10.17632/cz9bx7d52c.1 ; https://data.mendeley.com/datasets/cz9bx7d52c/draft/files/bd678698-6718-4979-8b9e-1b0f7645884e/KINOMEscan%20Results%20for%20AAK1.xlsx?dl=1 |
| BMP2K K0 bound to 25A | This paper | PDB ID: 5IKW ; https://www.rcsb.org/structure/5IKW |

| **Experimental Models: Cell Lines** |  |  |
| Human male: HT1080 | ATCC | CCL-121 |
| Human fetus: HEK293T/17 | ATCC | CRL-11268 |
| Human: RKO | ATCC | CRL-2577 |
| Mouse male: Lcells | ATCC | CRL-2648 |
| Mouse male: WNT3A expressing Lcells | ATCC | CRL-2647 |

| **Oligonucleotides** |  |  |
| See Table S7 for complete list |  | N/A |

| **Recombinant DNA** |  |  |
| Human kinase open reading frame library | Yang et al., 2011 ; Johannessen et al., 2010 | Addgene #1000000014 |
| Flag-AAK1-WT | Dr. Sean Conner | Conner et al., 2003 |
| Flag-AAK1-dAID | Dr. Sean Conner | Conner et al., 2003 |
| Flag-AAK1-74A | Dr. Sean Conner | Conner et al., 2003 |
| GFP-LRP6 | Dr. Randell Moon | This paper |
| HA-LRP6 | Dr. Randell Moon | This paper |
| ANKRd6 | Dr. Randell Moon | This paper |
| FLAG-dnTCF7L2 | Dr. Randell Moon | Walker et al., 2015, |
| TK-Renilla | Dr. Randell Moon | Walker et al., 2015, |
| BAR-reporter | Dr. Randell Moon | Walker et al., 2015, |
| NFkB-reporter | | Walker et al., 2015, |
| TGFβ-reporter | | Walker et al., 2015, |
| ADCK1 | Yang et al., 2011 ; Johannessen et al., 2010 | Addgene #1000000014 |
| ADCK2 | Yang et al., 2011 ; Johannessen et al., 2010 | Addgene #1000000014 |
| MAST1 | Yang et al., 2011 ; Johannessen et al., 2010 | Addgene #1000000014 |
| TGFRB3 | Yang et al., 2011 ; Johannessen et al., 2010 | Addgene #1000000014 |
| K44A Dynamin 1 pEGFP | Song et al., 2004 | Addgene #34681 |

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CONTACT AND REAGENT RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Michael B. Major (ben_major@email.unc.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell lines and tissue culture
All cells were cultured at 37°C and 5% CO2. HT1080 (human male), HEK293T/17 (human fetus), RKO (human, gender N/A), Lcells (mouse male), and WNT3A expressing Lcells (mouse male) were obtained from American Type Culture Collection (ATCC, Manassas, VA) and grown in DMEM with 10% FBS. Each cell line was tested for mycoplasma contamination and passaged no more than 20 passages from the original ATCC stock. Cells were treated at the indicated concentrations with the following compounds: CHIR99021 (Cayman Chemicals, Ann Arbor, MI), chlorpromazine HCL (Sigma-Aldrich, St. Louis, MO), bafilomycin A1 (Cayman Chemicals), rhWNT3A (PeproTech, Rocky Hill, NJ), rhWNT5A (R&D, Minneapolis, MN), neomycin trisulfate salt hydrate (Sigma-Aldrich) and carbachol (EMD Chemicals, Gibbstown, NJ).

METHOD DETAILS

WNT3A conditioned media
Conditioned media was collected as described by ATCC. Briefly, WNT3A and control Lcells were grown to 100% confluency before fresh media was added, conditioned for 48 hr, and then collected.

Immunoprecipitation and immunoblotting
Immunoprecipitation (IP) experiments were performed as previously described (Jiang et al., 2012). Briefly, cells were lysed in 1% Triton lysis buffer, passed through an 18 gauge needle, and cleared via centrifugation at max speed for 10 mins. For endogenous IP experiments, antibody was conjugated to activated Protein A/G beads (ThermoFisher) for 1 hr at 4°C with rotation. Conjugated beads were then added to cleared lysate and incubated for 4 hr at 4°C with rotation. For overexpression IP experiments, activated Flag (Sigma-Aldrich) or HA (Sigma-Aldrich) beads were added directly to the cleared lysate and incubated for 1 hr at 4°C with rotation. Following incubation, beads were washed 5 times with lysis buffer, then the sample was eluted at 95°C for 5 mins, and a W blot was performed. Standard W blotting techniques were utilized. Briefly, samples were run on a NuPAGE 4%–12% Bis-Tris Protein Gel (ThermoFisher), then transferred to a nitrocellulose membrane. The membrane was then blocked and incubated overnight with primary antibody at 4°C with rotation, washed with TBST, incubated with secondary antibody, washed with TBST and imaged.
All images were obtained using a LiCOR Odyssey imager and quantified with LiCOR software. For W.blot analysis, all antibodies were used at a concentration of 1:1,000, with the exception of loading controls, which were used at 1:10,000. Bethyl Labs (Montgomery, TX) – AAK1 (A302-148a); Cell Signaling (Danvers, MA) – Clathrin (4796), LRP6 (3395), phospho-LRP6 (S1490) (2568), phospho-AP2M1 (T156) (73995), PIP4K2A (S527), phospho-CTNNB1 (S33/37/T41) (9561S); Sigma-Aldrich – FLAG M2 (F3165), HA (11867423001), and beta-tubulin (T8328); Abcam (Cambridge, UK) – LRP6 (ab75358), AP2M1 (ab7995), p-AP2M1 (Thr156) (ab109397); BD Biosciences (San Jose, CA) – CTNNB1 (610153), GSK3β (610201); Santa Cruz (Dallas, TX) – DVL3 (sc-8027), beta-actin (sc-47778); Invitrogen (Carlsbad, CA) - AP2M1 (PA5-21360). Secondary antibodies were used at 1:5,000 dilution and purchased from LiCOR Biosciences. Specific antibodies used are as follows: IRDye® 800CW Goat anti Mouse IgG (925-32210), IRDye® 680LT Goat anti Mouse IgG (925-68020), IRDye® 800CW Goat anti Rabbit IgG (925-32211), and IRDye® 680LT Goat anti Rabbit IgG (925-68021).

**Surface staining of LRP6**

HEK293T cell were transfected for 48 hr prior to splitting into 60 cm plates with 400,000 cells/plate and allowed to attach overnight. Cells were dissociated with 0.5 mM EDTA in DPBS and washed with FACS Buffer (2% FBS in DPBS). Cells were stained in FACS buffer for 1 hour at ice using 5 μg/mL LRP6 antibody (R&D) or isotype control (R&D) and secondary staining with 1:100 anti-mouse PE (Jackson Immunoresearch, West Grove, PA) for 45 mins. After staining, cells were fixed with 2% paraformaldehyde in FACS buffer and analyzed by the UNC-Flow Cytometry Core.

**DNA constructs and siRNAs**

The human kinase open reading frame library was obtained from Addgene (cat# 1000000014) and cloned into a custom pHAGE-CMV-FLAG vector using Gateway cloning technology. All constructs were N-terminally sequence verified. Gain-of-function kinase data is presented in Table S3 and has been deposited on Mendeleu Data (https://doi.org/10.17837/049206r61.1#file-94081320-a2b2-4178-bbe2-233c7ea23b62). Wild-type AAK1 and mutants were a gift from Dr. Sean Conner (University of Minnesota). GFP-LRP6, HA-LRP6, ANKR6, and FLAG-dnTCF7L2 were kindly provided by Dr. Randell Moon (University of Washington). ADC1, ADC2, MAST1, and TGFBRII were obtained as follows: LRP6 antibody (R&D) or isotype control (R&D) and secondary staining with 1:100 anti-mouse PE (Jackson Immunoresearch, West Grove, PA) for 45 mins. After staining, cells were fixed with 2% paraformaldehyde in FACS buffer and analyzed by the UNC-Flow Cytometry Core.

**Transcriptional reporter assays**

All luciferase reporter assays and IncuCyte fluorescent reporter assays were performed as previously described (Walker et al., 2015). Briefly, for loss-of-function assays, cell lines stably expressing the BAR-Firefly luciferase reporter and TK-Ren luciferase were used and transfected with RNAiMAX (Life Technologies, Waltham, MA) using lipofectamine 2000 (ThermoFisher) for 24 hr. For gain of function studies, the BAR-reporter (20 ng), TK-Ren (10 ng), and indicated constructs (70 ng) were transfected with TansIT2020 (Mirus Bio) for 24 hr. For IncuCyte fluorescent reporter assays, stable BAR-mCherry cells were treated and imaged as indicated using the IncuCyte Live Cell Analysis System from Essen BioScience. Luciferase readouts were normalized using co-transfected TK-Ren (Luciferase assays) and IncuCyte Analysis was normalized to internal mCherry control. Conditions were plated in triplicate, and normalized values were averaged across triplicates to yield the data presented and standard error. Each assay was repeated in biological triplicate, unless otherwise stated. Firefly luciferase and the Renilla (Ren) control were detected using the Promega Dual-Luciferase Reporter Assay System per the manufacturer’s protocol (Promega, Madison, WI). Plates were read on the EnSpire plate reader from PerkinElmer.

**Real-Time Quantitative PCR**

Quantitative PCR (qPCR) was performed as described previously (Walker et al., 2015). Briefly, cells were treated as indicated and RNA was isolated using PureLink RNA Mini Kit (Invitrogen, Carlsbad, CA). cDNA was generated from 1ug of RNA using the iScript cDNA Synthesis Kit (BioRad, Hercules, CA) following kit specifications. qPCR was performed using Fast SYBER Green Master Mix (Applied Biosystems, Foster City, CA) on the Applied Biosystems 7400HT following manufacturer specifications. Samples were run in technical triplicate on a 384-well plate, with biological triplicates run on subsequent plates. Primers were previously published (Walker et al., 2015) and sequences are listed in Table S7.

**Generation of LRP5/6 DKO stable cell lines**

HEK293T cells were transfected with Cas9 and sgRNAs targeting LRP5/6. Sequences are listed in Table S7. Deletions were confirmed by immunoblotting and verified via sequencing.

**Surface biotinylation**

For surface biotinylation assays, the Pierce Cell Surface Protein Isolation Kit (ThermoFisher) was utilized and manufacturer specifications were followed. Briefly, cells were grown to 70% confluence, washed 3 times with cold PBS, and then biotinylated for 30 mins at 4 °C with NHS-SS-sulfo-linked biotin (0.25mg/mL). The free biotin was quenched, and then the samples were washed 3 times with cold TBS prior to lysis and sonication. Lysates were cleared and then incubated with streptavidin beads (GE Healthcare, Little Chalfont, UK) for 1 hour at 4°C with agitation. Beads were washed 4 times with cold TBS and then proteins were eluted with LDS protein loading buffer supplemented with DTT at 95°C for 10 mins.
Chemistry general procedures

Reagents were purchased from commercial suppliers and used without further purification. $^1$H and $^{13}$C NMR spectra were collected in methanol-$d_4$ and recorded on Varian Inova 400 or Inova 500 spectrometers. Peak positions are given in parts per million (ppm) downfield from tetramethylsilane as the internal standard; $^1$J values are expressed in hertz. Thin-layer chromatography (TLC) was performed on silica gel F254 to evaluate reaction courses and mixtures. Flash chromatography was performed on Merck 60 silica gel (0.063–0.200 mm). All non-aqueous reactions were performed under nitrogen atmosphere. Solutions containing the final products were dried over Na$_2$SO$_4$ before filtration and concentration under reduced pressure using a rotary evaporator.

Crystal structure determination

Diffraction data were integrated using XDS (Kabsch, 2010) and scaled using AIMLESS from the CCP4 software suite (Winn et al., 2011). Molecular replacement was performed with Phaser (McCoy et al., 2007) using BMP2K/AZD7762 (PDB: 4W9W) (Sorrell et al., 2016) as a search model. Automated model building was performed with Buccaneer (Cowtan, 2006). Automated refinement was performed in PHENIX (Adams et al., 2010). Coot (Emsley et al., 2010) was used for manual model building and real space refinement. Structure validation was performed using MolProbity (Chen et al., 2010). Structure factors and coordinates have been deposited in the PDB with code 5IKW.

Binding-displacement assays

The TR-FRET ligand binding-displacement assays for AAK1, BMP2K, GAK and STK16 were performed as previously described (Asquith et al., 2018). Inhibitor binding was determined using a binding-displacement assay, which measures the ability of inhibitors to displace a fluorescent tracer compound from the ATP binding site of the kinase domain. Inhibitors were dissolved in DMSO and dispensed as 16-point, 2x serial dilutions in duplicate into black multi-well plates (Greiner). Each well contained either 0.5 nM or 1 nM biotinylated kinase domain protein ligated to streptavidin-Tb-cryptate (Cisbio), 12.5 nM or 25 nM Kinase Tracer 236 (ThermoFisher Scientific), 10 mM HEPES pH 7.5, 150 mM NaCl, 2 mM DTT, 0.01% BSA, 0.01% Tween-20. Final assay volume for each data point was 5 µL, and final DMSO concentration was 1%. The kinase domain proteins were expressed in E. coli as a fusion with a C-terminal AVI tag (vector pNIC-Bio3, NCBI reference JN792439) which was biotinylated by co-expressed BirA, and purified using the same methods as used previously (Asquith et al., 2018). After setting up the assay plate it was incubated at room temperature for 1.5 hours and then read using a TR-FRET protocol. Residue ranges were AAK1: 31-396, BMP2K: 38-345, GAK: 12-347, STK16: 13-305, both on a PheraStarFS plate reader (BMG Labtech). The data was normalized to 0% and 100% inhibition control values and fitted to a four parameter dose-response binding curve in GraphPad Software (version 7, La Jolla, CA, USA). The determined IC$_{50}$ values were converted to Ki values using the Cheng-Prusoff equation and the concentration and $K_D$ values for the tracer (previously determined).

Kinome screening

The KinOMEscan assay panel was measured at DiscoverX Corporation as previously described (Davis et al., 2011). Data collection can be found in Table S2 and has been deposited on Mendeley Data (https://doi.org/10.17632/cz9bx7d52c.1#file-bd678698-6718-4979-8b9e-1b0f7645884e). Kinases were produced either as fusions to T7 phage or were expressed as fusions to NF-$\kappa$B in HEK293 cells and subsequently tagged with DNA for PCR detection. In general, full-length constructs were used for small, single-domain kinases, and catalytic domain constructs including appropriate flanking sequences were used for multidomain kinases. Briefly, for the binding assays, streptavidin-coated magnetic beads were treated with biotinylated affinity ligands to NF-$\kappa$B in HEK293 cells and subsequently tagged with DNA for PCR detection. In general, full-length constructs were used for small, single-domain kinases, and catalytic domain constructs including appropriate flanking sequences were used for multidomain kinases. Briefly, for the binding assays, streptavidin-coated magnetic beads were treated with biotinylated affinity ligands to generate affinity resins. The liganded beads were blocked to reduce nonspecific binding and washed to remove unbound protein. Bound kinase was eluted in the presence of non-native elution conditions. Binding reactions were assembled by combining kinase, liganded affinity beads and test compounds prepared as 100 µg/mL stock solutions in DMSO. DMSO was added to control assays lacking a test compound. Primary screen interactions were performed in 96-well plates. Assay plates were incubated at 25°C with shaking for 1 h, and the affinity beads were washed extensively to remove unbound protein. Bound kinase was eluted in the presence of non-native elution conditions for 30 min at 25°C with shaking. The kinase concentration in the eluates was measured by quantitative PCR. $K_D$ values were determined using 11 serial threefold dilutions of test compound and a DMSO control.

N-(6-[3-[(N,N-diethylsulfamoyl)amino]phenyl]-1H-indazol-3-yl)cyclopropanecarboxamide (SGC-AAK1-1)

3-Aminophenylboronic acid pinacol ester (1 eq., 50 mg, 0.228 mmol) in pyridine (1.2 mL) was cooled to 0 °C in an ice bath. Diethylsulfamoyl chloride (2.06 eq., 80.7 mg, 0.0754 mL, 0.47 mmol) was added drop wise, and the reaction mixture was stirred overnight. The reaction progress was checked by TLC, revealing complete consumption of starting material (SM). Solvent was removed and the residue was partitioned between dichloromethane and saturated aqueous sodium bicarbonate solution. The organic phase was dried (Na$_2$SO$_4$), filtered and concentrated. The residue was purified using an Isco CombiFlash companion automated purification system (4g (18 mL/min), SiO$_2$, 80%–20% to 25%–75% heptanes/EtOAc) to give diethyl[[3-[tetramethyl-1,3,2-dioxaborolan-2-yl]phenyl]sulfonyl]amine as an orange solid (44 mg, 54%), which was determined to be desired product by $^1$H NMR.

The following was added to a microwave vial: N-(6-bromo-1H-indazol-3-yl)cyclopropanecarboxamide (1 eq., 20 mg, 0.0677 mmol), diethyl[(3-[tetramethyl-1,3,2-dioxaborolan-2-yl]phenyl)sulfonyl]amine (1 eq., 24 mg, 0.0677 mmol), and Pd(dppf)Cl$_2$ (10%, 4.96 mg, 0.00677 mmol) in a mixture of dioxane (0.19 mL) and 1Maq sodium carbonate (0.16 mL). The vial was capped and heated in the microwave at 120°C for 30 mins. Once cool, the reaction progress was examined by TLC, which revealed complete
consumption of SM. The mixture was filtered through Celite and washed with EtOAc (ethyl acetate). The combined organic solution was washed with brine, dried (Na₂SO₄), filtered and concentrated. The residue was purified using an Isco CombiFlash companion automated purification system (4g (18 mL/min), SiO₂, 75%/25% to 20%/80% heptanes/EtOAc) to give SGC-AAK1-1 as a yellow solid (14.3 mg, 49%). ¹³H NMR (400 MHz, Methanol-d₄) δ 7.80 (d, J = 0.8 Hz, 1H), 7.58 (s, 1H), 7.48 (dt, J = 0.8, 2.1 Hz, 1H), 7.38 – 7.35 (m, 2H), 7.33 (d, J = 8.5 Hz, 1H), 7.17 – 7.12 (m, 1H), 3.29 (d, J = 1.6 Hz, 4H), 1.91 (tt, J = 4.5, 8.0 Hz, 1H), 1.05 (t, J = 7.1 Hz, 6H), 1.01 (dt, J = 3.0, 4.4 Hz, 2H), 0.95 – 0.88 (m, 2H); ¹³C NMR (101 MHz, Methanol-d₄) δ 174.1, 142.1, 141.9, 139.8, 139.0, 129.1, 122.2, 121.6, 119.7, 118.3, 118.1, 107.5, 48.2, 41.6, 13.5, 12.5, 6.8.

**General procedure for preparation of 3-acylamino-6-bromoindazoles**

To a solution of 6-bromo-1H-indazol-3-amine (48 mg, 0.23 mmol) in pyridine (0.75 mL) was added cyclopropanecarboxylic acid chloride (0.021 mL, 0.23 mmol, 1 eq.), Pd(dppf)Cl₂ (100.3 mg, 0.123 mmol, 0.1 eq). The reaction was run in the microwave at 160°C for 20 mins, at which time SM was consumed. The reaction mixture was poured into water and extracted 3 times with EtOAc. The combined organic layers were dried over Na₂SO₄ and concentrated to dryness. The compound was then purified by flash chromatography to afford 3-(3-acylamino)-1H-indazol-3-yl)cyclopropanecarboxamide (100 mg, 28% yield).

**N-(6-(3-cyclopropanesulfonamido)-3-phenyl)-1H-indazol-3-yl)cyclopropanecarboxamide (25A)**

To a solution of N-(6-(3-aminophenyl)-1H-indazol-3-yl)cyclopropanecarboxamide (67 mg, 0.2292 mmol) in pyridine (1mL) cooled to 0°C, cyclopropanesulfonyl chloride (1eq, 32.22mg, 0.2292 mmol) was added drop wise. The reaction mixture was stirred at 0°C for 4 hr and warmed to room temperature. After verification by LC/MS that SM had been consumed, solvent was removed under reduced pressure. The compound was then purified by high pressure liquid chromatography (HPLC) from 10% to 100% ACN/water + 0.05% TFA to yield 25A (44.5 mg, 49% yield). ¹H NMR (400 MHz, Methanol-d₄) δ 7.83 – 7.78 (d, J = 8.6 Hz, 1H), 7.61 – 7.54 (m, 2H), 7.46 - 7.30 (m, 3H), 7.29 – 7.23 (ddd, J = 7.7, 2.2, 1.3 Hz, 1H), 2.62 – 2.49 (tt, J = 8.0, 4.8 Hz, 1H), 1.95 – 1.84 (ddd, J = 12.5, 7.9, 4.2 Hz, 1H), 1.07 – 0.77 (m, 8H). ¹³C NMR (126 MHz, Methanol-d₄) δ 5.8 (2C), 8.4 (2C), 15.0, 30.5, 30.8, 109.1, 117.2, 121.2, 121.3, 121.4, 123.1, 124.7, 130.8, 140.1, 141.2, 143.4 (2C), 143.8, 175.6. MS+ (– ES API) - 397.1.

**N-(6-(3-cyclopropanesulfonamido)-3-phenyl)-1H-indazol-3-yl)isobutyramide (SGC-AAK1-1N):**

¹H NMR (400 MHz, Methanol-d₄) δ 7.82 – 7.75 (d, J = 8.6 Hz, 1H), 7.62 – 7.54 (dt, J = 10.9, 1.5 Hz, 2H), 7.47 – 7.31 (m, 3H), 7.31 – 7.22 (ddd, J = 7.8, 2.2, 1.3 Hz, 1H), 2.84 – 2.69 (hept, J = 6.8 Hz, 1H), 2.61 – 2.50 (tt, J = 8.0, 4.8 Hz, 1H), 1.29 – 1.15 (d, J = 6.8 Hz, 6H), 1.14 – 0.82 (m, 4H). ¹³C NMR (126 MHz, Methanol-d₄) δ 5.8 (2C), 20.0 (2C), 30.5, 36.3, 109.2, 117.4, 121.3, 121.4 (2C), 123.0, 124.7, 130.8, 140.1, 141.2, 143.4, 143.8, 179.3. MS+ (– ES API) - 399.1.

**Cloning, protein expression and purification**

For crystallization of BMP2K, a construct of BMP2K residues 38-345 (NCBI NP_942595) encompassing the kinase domain with surface mutations K320A and K321A in expression vector pNIC-Zb was used (Sorrell et al., 2016). The construct was transformed into BL21(DE3) cells that co-express λ-phosphatase and three rare tRNAs (plasmid pACYC-LIC+). The cells were cultured in TB medium containing 50 μg/mL kanamycin and 35 μg/mL chloramphenicol at 37°C with shaking until the OD600 reached ~3 and then cooled to 18°C for 1 hour. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM and the cultures were left overnight at 18°C. The cells were collected by centrifugation then resuspended in 2x lysis buffer (100 mM HEPES buffer, pH 7.5, 1.0 M NaCl, 20 mM imidazole, 1.0 mM tris(2-carboxyethyl)phosphine (TCEP), Protease Inhibitor Cocktail Set VII (Calbiochem, 1/500 dilution) and flash-frozen in liquid nitrogen. Cells were lysed by sonication on ice. The resulting proteins were purified using Ni-Sepharose resin (GE Healthcare) and eluted stepwise in binding buffer with 300 mM imidazole. Removal of the hexahistidine tag was performed at 4°C overnight using recombinant TEV protease. Protein was further purified using reverse affinity chromatography on Ni-Sepharose followed by gel filtration (Superdex 200 16/60, GE Healthcare). Protein in gel filtration buffer (25 mM HEPES, 500 mM NaCl, 0.5 mM TCEP, 5% [v/v] glycerol) was concentrated to 12 mg/mL (measured by UV absorbance, using the calculated molecular weight and estimated extinction coefficient, using a NanoDrop spectrophotometer (Thermo Scientific) using 30 kDa molecular weight cut-off centrifugal concentrators (Millipore) at 4°C.
NanoBRET measurements

Constructs for NanoBRET measurements of AAK1 and BMP2K were kindly provided by Promega. The AAK1 construct represents the short isoform of AAK1 with an N-terminal Nanoluc fusion, and the BMP2K construct represents residues the short isoform of BMP2K with an N-terminal Nanoluc fusion. HEK293T cells (ATCC) were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) (Life Technologies) with penicillin and streptomycin. NanoLuc-AAK1 or NanoLuc-BMP2K fusion constructs were complexed with Lipofectamine 2000 according to the manufacturer’s protocol (Invitrogen). DNA:Lipofectamine complexes were formed with 24 μg DNA and 60 μL Lipofectamine. The transfection complexes were then mixed with HEK293T cells in a 100 mm dish at 50%–70% confluence in serum-free DMEM (Lonza), followed by incubation in a humidified, 37°C, 5% CO2 incubator. After 4 hr of incubation, the medium was replaced by 10% FBS DMEM with antibiotics and the cells were incubated for an additional 20 hr. The NanoBRET assay was performed as previously described (Robers et al., 2015). Briefly, cells were trypsinized and resuspended in Opti-MEM without phenol red (Life Technologies). Cells (85 μL) were then seeded into white, nonbinding surface plates (Coming) at a density of 2 × 10⁴ cells/well. Diluted tracer was prepared from 200 μM stock in Tracer Dilution Buffer (32.25% PEG400 in 12.5 mM HEPES Buffer pH 7.5) at a 1 to 4 ratio, and 5 μL was added to the cells to have a final concentration of 2, 1, 0.5 or 0.25 μM. All chemical inhibitors were prepared as concentrated stock solutions in dimethylsulfoxide (DMSO) (Sigma-Aldrich). Serial dilutions of the inhibitors at 50x the final assay concentration were made by dilution with DMSO, before the inhibitors were added to the cells at 1:50 ratio (2% final DMSO concentration). Cells were then incubated for 2 hr before Bioluminescence resonance energy transfer (BRET) measurements. To measure BRET, NanoBRET NanoGlo Substrate (Promega) was added, and filtered luminescence was measure on a BMG LABTECH Clariostar luminometer equipped with 450 nm BP filter (donor) and 610 nm LP filter (acceptor), using 0.5 s integration time with gain settings of 3,600 for both filters. Background-corrected BRET ratios were determined by subtracting the BRET ratios of samples with no tracer added.

Measurement of in vitro IC₅₀ values for inhibition of AAK1 enzymatic activity

Enzymatic activity of AAK1 was monitored in 20 μL reaction volumes containing 25 nM AAK1 (27-365) in 15 mM MOPS pH 7.5, 2 mM MgCl₂ and 0.004% Triton X-100 buffer plus 46 μM ATP (at Kₐ) and 200 μM of an AP-2 derived peptide (biotin-GGSQITSQVTG QIGWRR-amide). Optimal activity was achieved with sample incubation at 37°C for 40 mins (steady-state conditions) in buffer conditions that were tailored by factorial design using Design-Expert software (Stat-Ease, version 10). To generate inhibition curves, end-point reactions were setup having compounds pre-incubated in a reaction mix without ATP for 10 mins at room temperature. Next, all samples (in PCR tubes) were transferred to a PCR instrument set at 37°C for another 5 mins. The reaction started with the addition of 46 μM ATP (at Kₐ), maintaining the incubation conditions for another 40 mins. The reactions were stopped and the amount of ADP produced was measured using a coupled enzyme assay system that converts Amplex Red to Resorufin (Charter et al., 2006). Fluorescence readings were made in a BMG Labtech Clariostar instrument with excitation peak set at 530 nm and emission peak reading at 590 nm.

Protein crystallization

The AAK1 inhibitor (dissolved in 100% DMSO) was added to the protein in a 3-fold molar excess and incubated on ice for approxi-mately 30 mins. The mixture was centrifuged at 14,000 rpm for 10 mins at 4°C prior to setting up 150 nL volume sitting drops at three ratios (2:1, 1:1, or 1:2 protein-inhibitor complex to reservoir solution). Crystallization experiments were performed at 20°C. Crystals were cryoprotected in mother liquor supplemented with 20%–25% glycerol before flash-freezing in liquid nitrogen for data collection. Diffraction data was collected at the Diamond Light Source. The best diffracting crystals grew in 10% (v/v) Broad MW PEG smear, 3.2 M MgCl₂, 100 mM HEPES pH 7.0 (Chaikuad et al., 2015). Data was collected at 100 K at the Diamond Synchrotron beamline I02. Data collection statistics can be found in Table S5.

Isothermal Titration Calorimetry

AAK1 and BMP2K proteins were produced as previously described (Sorrell et al., 2016). Isothermal titration calorimetry measurements were made on a Microcal VP-ITC instrument at 25°C. For the interaction of AAK1 with SGC-AAK1-1, the compound was diluted to 22 μM in ITC buffer from a stock at 10 mM in DMSO and loaded directly into the cell. AAK1 was dialyzed at 4°C overnight into ITC buffer (20 mM HEPES pH 7.5, 150 mM NaCl, 1 mM TCEP) and loaded into the ITC syringe at a final concentration of 218 μM. Following thermal equilibration, AAK1 was titrated into the cell using serial injections of 8 μL until saturation was observed in the thermogram. The same method was repeated for the BMP2K versus SGC-AAK1-1 interaction where protein was loaded into the syringe at a concentration of 286 μM and injected into a 32 μM solution of SGC-AAK1-1. The ITC data was analyzed with NITPIC (Keller et al., 2012) and SEDPHAT (Zhao et al., 2015). The final fitted data values are in Table S4.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistics

All error bars are ± standard error (SE). Statistical significance was evaluated by Mann-Whitney t test unless otherwise stated. N values are stated in figure legends. Each panel was performed in at least biological triplicate, with higher replicates noted in figure legend. *** p < 0.0005, ** p < 0.005, * p < 0.05.
DATA AND SOFTWARE AVAILABILITY

**Gain-of-function kinome screen**
The reference number for the gain-of-function kinome screen reported in this paper is Mendeley Data (https://doi.org/10.17632/352fxh8r6t.1).

**KINOMEsca**
The reference number for the KINOMEsca reported in this paper is Mendeley Data (https://doi.org/10.17632/cz9bx7d52c.1).

**BMP2K K_D bound to 25A**
The reference number for the BMP2K K_D bound to 25A is PDB: 5IKW.
Supplemental Information

WNT Activates the AAK1 Kinase to Promote Clathrin-Mediated Endocytosis of LRP6 and Establish a Negative Feedback Loop

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Figure S1 – SGC-AAK1-1 is a potent and specific inhibitor of AAK1 and BMP2K that is active in cells. Related to Figure 2. (A) Inhibitor 25A binds with high affinity to AAK1 and BMP2K but not to GAK and STK16 (related kinases from the same branch of the phylogenetic tree). Negative control compound 34A (illustrated as “neg”) does not bind to any of the kinases. The TR-FRET binding displacement assay was measured in duplicate. (B) Inhibitor 25A is most potent for AAK1 out of the 406 kinases tested and has good overall selectivity. (C) Inhibitor SGC-AAK1-1 inhibits AAK1 enzymatic activity in vitro with an IC50 of 233 nM at 46 µM ATP, similar to that of the potent but non-specific inhibitor staurosporine. Negative control compound 34A had an IC50 of 20 µM on AAK1. (D) SGC-AAK1-1 binds to BMP2K with weaker affinity than to AAK1, with a less favorable enthalpy of interaction. (E) The position of the inhibitor bound in the ATP binding site of BMP2K is shown. The Numb-associated-kinase-specific activation segment C-terminal helix (ASCH) is shown in red. (F) The inhibitor 25A is well resolved in the electron density. A 2Fo-Fc map is shown contoured at 1.0σ. (G) Inhibitor 25A binds to BMP2K in live HEK293T cells with an IC50 of 602 nM, derived from linear regression of the IC50 values at different concentrations of NanoBRET tracer. All experiments were performed in biological triplicates, unless otherwise noted.
Figure S2 – β-catenin knockdown impairs AAK1 regulation of WNT signaling. Related to Figure 4. Stable HT1080-B/R cells were transfected with the either β-catenin, AAK1 or control for 56 hrs. Cells were then treated for 16 hrs with WNT3A or Lcell CM. Bars represent average Firefly/Ren (RFU) from 3 technical replicates +/- standard error (S.E.). Data are representative of biological triplicates. * p<0.05.
Figure S3 – AAK1 interacts with LRP6. Related to Figure 5. (A) HEK293T cells were co-transfected with FLAG-AAK1 or control FLAG-hcRED and HA-LRP6 over-expression constructs. Twenty-four hrs post-transfection, cells were treated with WNT3A CM for 1 hour. W.blot analysis of immunoprecipitated HA-LRP6. (B) HEK293T cells were transfected with HA-LRP6 for 24 hrs and then treated with WNT3A CM for 1 hour. HA-LRP6 was immunoprecipitated, and the W.blot was probed for endogenous AAK1. (C) HEK293T cells were transfected with the FLAG-AAK1 or FLAG-GFP over-expression construct for 24 hrs and then treated with WNT3A for 1 hour. Affinity purification (AP) of FLAG-AAK1 was conducted to visualize endogenous LRP6 as detected by W.blot. (D) Endogenous AAK1 IP in HEK-293T cells treated as WNT3A CM for indicated times (untreated, 1, 9 hrs) then endogenous LRP6 levels were visualized via W.blot analysis. (E) HEK293T cells were transfected with HA-LRP6 and FLAG overexpression constructs (control, AAK1-WT, AAK1-74A, AAK1-dAID) for 36 hrs. AP of FLAG was used to visualize LRP6-HA levels via W.blot analysis. All panels are representative of biological triplicates, unless otherwise noted.
Figure S4 - WNT treatment induces AAK1 mediated phosphorylation of AP2M1. Related to Figure 6. (A,B) W.blot analysis of HT1080 (A) or HEK293T (B) cells treated with rhWNT3A (200 ng/mL) for indicated times. (C,D) W.blot analysis of HT1080 (C) or HEK293T (D) cells stimulated with WNT3A CM for 15 mins, then the media was replaced with complete DMEM. Cells were then treated with the AAK1 inhibitor, 25A, (1.25 µM) or small molecule analog, 34A, (1.25 µM) for the indicated times. (E) HEK293T cells treated with WNT3A for 8 hrs, with SGC-AAK1-1 or 34A added at indicated times into the WNT3A time course. All panels are representative of biological triplicates, unless otherwise noted.
Table S4 – Isothermal Titration Calorimetry data for binding of AAK1 or BMP2K proteins to SGC-AAK1-1. Related to Figure 2.

|                          | AAK1 | BMP2K |
|--------------------------|------|-------|
| $K_D$ (nM)               | 117.5| 487.5 |
| $\Delta H$ (kJ/mol)      | -70.8| -35.0 |
| $-T\Delta S$ (kJ/mol)    | 31.2 | -1.0  |
| Fraction of incompetent ligand | 0.28 | 0.11  |
Table S5 - Data collection and refinement statistics for the BMP2K co-crystal structure with inhibitor 25A. Related to Figure 2.

|                          |   |
|--------------------------|---|
| **PDB ID**               | 5IKW |
| **Space group**          | I222 |
| **Unit cell dimensions** |   |
| $a$, $b$, $c$ (Å), $\alpha$, $\beta$, $\gamma$ (°) | 42.0, 111.2, 164.1, 90, 90, 90 |
| **Data collection**      |   |
| Resolution range (Å)$^a$ | 28.6-2.4 |
|                          | (2.5-2.4) |
| Unique observations$^a$  | 81078 (7855) |
| Average multiplicity$^a$ | 5.3 (5.2) |
| Completeness (%)$^a$     | 99.1 (95.2) |
| $R_{merge}$              | 0.074 (0.49) |
| Mean $(I/\sigma(I))$     | 13.2 (3.1) |
| Mean CC(1/2)             | 0.991 (0.482) |
| **Refinement**           |   |
| Resolution range (Å)     | 21.75-2.41 |
| $R$-value, $R_{free}$    | 0.18, 0.22 |
| r.m.s. deviation from ideal bond length (Å) | 0.010 |
| r.m.s. deviation from ideal bond angle (°) | 1.04 |
| Ramachandran Outliers    | 0 |
| Favoured                 | 98.3% |

$^a$ Values within parentheses refer to the highest resolution shell.
Table S6 - AAK1 inhibition stabilizes LRP6 on the membrane. Related to Figure 5.

| Sample Name          | replicate 1 | replicate 2 | replicate 3 |
|----------------------|-------------|-------------|-------------|
| unstained            | 0           | 0           | 0.1         |
| isotype              | 1.2         | 0.7         | 0.1         |
| untreated            | 69.9        | 56.3        | 68.4        |
| BSA                  | 61.8        | 61.7        | 66.3        |
| 3A/1hr               | 28.8        | 38.5        | 50.8        |
| 3A/8hr               | 23.7        | 26.7        | 48.7        |
| 3A/8 +34A            | 21          | 30.9        | 47.8        |
| 3A/8 + SGC-AAK1-1    | 63.2        | 55.8        | 56.4        |

*Raw values from flow cytometry analysis of HEK293T cells treated as indicated.*
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Oligonucleotides** |        |            |
| siRNA targeting sequence: AAK1 #1 GGCUGAAGAUGAGUUUGACCUCUAUU | Life Technologies | Cat#AAK1/HSS176971 |
| siRNA targeting sequence: siAAK1 #2 CAAGAAAUUUGGGGUACAUUGAU | Life Technologies | Cat#AAK1/HSS176972 |
| siRNA targeting sequence: ANKRD6 CCAGAAGAACCUCUGCAUCUAUU | Life Technologies | Cat#NM_014942_stealth_1424 |
| siRNA targeting sequence: β-catenin – A CUAUCUGCUCUCUGUATT | Life Technologies | Madan B, et al. 2016 |
| siRNA targeting sequence: β-catenin – B CUGUUGGAUGUAUCGAATTT | Life Technologies | This paper |
| siRNA targeting sequence: CLTC – A CCGCCUUGCAGAUAGAAUAAUU | Life Technologies | Cat#HSS102017 |
| siRNA targeting sequence: CLTC – B GAGUGCUUUGGAGCUCUGUUUUA | Life Technologies | Cat#HSS174637 |
| siRNA targeting sequence: LRP6 GAUCCCAUGGGUUGGUACAUGUAUU | Life Technologies | Cat#LRP6/HSS180911 |
| siRNA targeting sequence: PI4K2α – 1 GGATCATTGCTGTCTTCAA | Sigma-Aldrich | Pan et al. 2008 |
| siRNA targeting sequence: PI4K2α – 2 GGAAGAGGACCTATATGAA | Sigma-Aldrich | Pan et al. 2008 |
| siRNA targeting sequence: Control – A CGUACGCGGAAAUACUCGATT | Life Technologies | Madan B, et al. 2016 |
| siRNA targeting sequence: Control – B UCGAAGUAUUCGCUGACGT | Life Technologies | Madan B, et al. 2016 |
| sgRNA targeting sequence: LRP5 GCGTCCACCGGCGGTACCGGCCG | This paper | N/A |
| sgRNA targeting sequence: LRP6 GCGATTGGTTGAGCTACAAATGG | This paper | N/A |
| **AXIN2** Forward Primer GCGATCCTGTTAATCCTTACAC | Thermo Scientific | N/A |
| **AXIN2** Reverse Primer AATTCCATCTACACTGCTGTC | Thermo Scientific | N/A |
| **NKD1** Forward Primer GGAGAAGGAGCCGGACCT | Thermo Scientific | N/A |
| **NKD1** Reverse Primer CTGCCGTGTTCTGCAAGT | Thermo Scientific | N/A |
| **GAPDH** Forward Primer ATGGGAAGTGAAGGTT | Thermo Scientific | N/A |
| **GAPDH** Reverse Primer AAGCTTCCCGTTTCTCAG | Thermo Scientific | N/A |