The E3 ubiquitin ligase component, Cereblon, is an evolutionarily conserved regulator of Wnt signaling

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Immunomodulatory drugs (IMiDs) are important for the treatment of multiple myeloma and myelodysplastic syndrome. Binding of IMiDs to Cereblon (CRBN), the substrate receptor of the CRL4CRBN E3 ubiquitin ligase, induces cancer cell death by targeting key neo-substrates for degradation. Despite this clinical significance, the physiological regulation of CRBN remains largely unknown. Herein we demonstrate that Wnt, the extracellular ligand of an essential signal transduction pathway, promotes the CRBN-dependent degradation of a subset of proteins. These substrates include Casein kinase 1α (CK1α), a negative regulator of Wnt signaling that functions as a key component of the β-Catenin destruction complex. Wnt stimulation induces the interaction of CRBN with CK1α and its resultant ubiquitination, and in contrast with previous reports does so in the absence of an IMiD. Mechanistically, the destruction complex is critical in maintaining CK1α stability in the absence of Wnt, and in recruiting CRBN to target CK1α for degradation in response to Wnt. CRBN is required for physiological Wnt signaling, as modulation of CRBN in zebrafish and Drosophila yields Wnt-driven phenotypes. These studies demonstrate an IMiD-independent, Wnt-driven mechanism of CRBN regulation and provide a means of controlling Wnt pathway activity by CRBN, with relevance for development and disease.
The Wnt signaling pathway is one of the major signal transduction cascades that regulate metazoan development and control tissue homeostasis. Consistent with these essential physiological roles, deregulated Wnt signaling is a driver of many human disease states. In the absence of Wnt ligands, the pivotal transcriptional coactivator β-Catenin is targeted for proteasomal degradation via its association with a macromolecular protein complex termed the destruction complex. This complex includes the Wnt regulators Adenomatous polyposis coli (APC), Axin2, 4, 5, Glycogen synthase kinase 3 (GSK3), and Casein kinase 1α (CK1α). Upon Wnt stimulation, the degradation of β-Catenin via the destruction complex is attenuated, allowing β-Catenin to accumulate in the nucleus and regulate the transcription of specific target genes. Consistent with its essential role in the destruction complex, CK1α has been identified as one of the crucial negative regulators of Wnt signaling. Although we and others have reported that CK1α is important to this process during metazoan development and in disease settings, little is known about how CK1α itself is regulated in cells.

A number of small molecules have been shown to control the activity of CK1α, amongst which the immunomodulatory drug (ImId) lenalidomide induces CK1α protein degradation. This process is mediated by a macromolecular E3 ubiquitin ligase complex consisting of the ligase scaffold Cullin-4 (CUL4), the RING-finger protein RING-box1 (RBX1), the adapter Damage-specific DNA binding protein 1 (DDB1), and the substrate receptor Cereblon (CRBN) (CRL4CRBN). This study, when bound to such ImIds, CRBN is able to target a number of neo-substrates for degradation, including Ikars and CK1α, thereby inhibiting cancer growth. Despite this established role of CRBN as an anticancer drug target, its physiological role remains poorly understood. Here, we demonstrate a small molecule-independent, evolutionarily conserved mechanism in which Wnt pathway activation stimulates CRBN to associate with CK1α and mediate ubiquitin-dependent CK1α degradation. This mechanism further modulates Wnt signal transduction, with importance for development and disease.

**Results**

**Wnt stimulation induces ubiquitin-dependent, proteasomal degradation of CK1α.** Based on our previous work, which showed reduced levels of CK1α in APC mutant cells, we postulated that Wnt signaling might regulate CK1α protein levels. To test this, we treated HEK293T (HEK) cells with recombinant Wnt3a for varying lengths of time or concentrations and analyzed CK1α levels in cell lysates. Wnt3a treatment reduced CK1α levels in both a time- and concentration-dependent manner, relative to the control protein α-Tubulin (Fig. 1a and Supplementary Fig. 1a). We also noted that recombinant Frizzled8 cysteine-rich domain (FZD8- CRD), a Wnt pathway antagonist, blocked the decrease of CK1α levels in response to Wnt3a (Supplementary Fig. 1b), consistent with Wnt3a acting in a specific manner. By contrast, CK1α gene expression was unaffected by Wnt exposure, relative to a Wnt target gene control (Fig. 1b), suggesting that Wnt downregulates CK1α protein levels via a posttranscriptional mechanism. Furthermore, we blocked new protein synthesis in HEK cells using cycloheximide and observed a substantially decreased CK1α half-life in response to Wnt3a (Fig. 1c and Supplementary Fig. 1c). Consistent with Wnt regulating the stability of only a subset of CK1α, the nuclear pool of CK1α was unaffected by Wnt treatment while its corresponding cytoplasmic pool was simultaneously degraded (Supplementary Fig. 1e). Further, unlike the general pool of cytoplasmic CK1α, CK1α associated with the Phosphatase and tensin homolog (PTEN) was not degraded in response to Wnt (Supplementary Fig. 1f). Taken together, these results show that Wnt signaling decreases the steady-state levels of a cytoplasmic pool of CK1α by promoting its turnover.

To determine if CK1α levels were regulated via a proteasome- or lysosome/autophagosome-mediated manner we stimulated HEK cells with Wnt3a in the presence of a proteasome (MG132) or lysosome/autophagosome (bafilomycin A1 (BA) inhibitor). MG132 treatment prevented Wnt3a-dependent CK1α degradation (Fig. 1d), while BA treatment did not (Fig. 1e), suggesting that Wnt-dependent CK1α degradation occurs via the proteasome. To determine if CK1α is ubiquitinated in response to Wnt exposure we treated HEK cells with Wnt3a and then immunoprecipitated CK1α from these cell lysates and immunoblotted for ubiquitin. Wnt3a treatment induced significant levels of CK1α ubiquitination (Fig. 1f). Similar results were obtained using a tandem ubiquitin-binding entity (TUBE) assay (Supplementary Fig. 1d). These results demonstrate that Wnt stimulation results in increased levels of CK1α ubiquitination and subsequent degradation via the proteasome.

**Wnt-induced degradation of CK1α requires CRBN, the substrate receptor of the CRL4CRBN E3 ubiquitin ligase complex.** We noted that a pan-inhibitor of the RING-finger family of E3 ubiquitin ligases, MLN4924, attenuated Wnt3a-induced CK1α degradation (Supplementary Fig. 2a). We, therefore, evaluated several candidate RING-finger E3 ubiquitin ligases previously implicated in CK1α or Wnt regulation, knocking down their expression in HEK cells and examining CK1α levels (Supplementary Fig. 2b). Interestingly, depletion of CRBN, the substrate receptor of the CRL4CRBN E3 ubiquitin ligase complex, attenuated Wnt-induced CK1α degradation (Fig. 2a and Supplementary Fig. 2b–d). Furthermore, knockdown of CRBN expression prevented the Wnt-induced decrease in CK1α half-life (Fig. 2b and Supplementary Fig. 2e–g), suggesting that the CRL4CRBN E3 ubiquitin ligase complex regulates Wnt-driven CK1α degradation.

To better understand how Wnt induces CRBN-dependent degradation of CK1α, we performed an in vitro binding assay between purified CRBN and recombinant CK1α (Fig. 2c). CRBN was only associated with recombinant CK1α when it was isolated from Wnt-treated cells (Fig. 2d). Notably, CK1α is known to interact with CRBN in a manner dependent on some ImIDs. Thus, the Wnt-dependent association of CRBN with CK1α is to a point consistent with this previous report, suggesting that the two proteins may require an additional factor/modification to associate upon Wnt stimulation. Next, we took advantage of these purified proteins to perform an in vitro CK1α ubiquitination assay, directly determining if Wnt signaling activates CRBN-dependent ubiquitination (Fig. 2c). We found that ubiquitination of recombinant CK1α was significantly enhanced when CRBN was isolated from Wnt-treated cells versus control cells (Fig. 2e). These results suggest that Wnt signaling induces CRBN to bind to and mediate the ubiquitination of CK1α.

**The β-Catenin destruction complex regulates CRBN-mediated CK1α degradation.** As CK1α is an essential component of the β-Catenin destruction complex, we next determined the kinetics of Wnt-driven CK1α degradation relative to that of other components in the complex. Wnt3a-induced CK1α degradation occurred prior to alterations in the levels of other destruction complex proteins (Fig. 3a and Supplementary Fig. 3a). Importantly, in contrast to CK1α, any Wnt-driven decreases in the levels of other destruction complex components occurred at a later time point (24 h) and were CRBN-independent (Fig. 3b).
Fig. 1 Wnt signaling regulates CK1α levels via ubiquitin-dependent proteasomal degradation. a Extracts of HEK cells treated with Wnt3a for various lengths of time were evaluated by immunoblotting. A representative immunoblot (left panel) or quantitation of immunoblots (mean ± SEM, \( n = 4 \) independent experiments; right panel) is shown. b RNA from HEK cells treated with Wnt3a for various lengths of time was used to determine the expression of the indicated genes, using quantitative RT-PCR analysis. Quantification of gene expression (mean ± SEM, \( n = 3 \) independent experiments) is shown. c HEK cells were co-treated with cycloheximide and PBS or Wnt3a for the indicated time and extracts of these cells were evaluated by immunoblotting for CK1α or HSP90. CK1α levels from immunoblots were quantitated, normalized to that of HSP90, and plotted to determine CK1α turnover in response to Wnt3a (mean ± SEM, \( n = 3 \) independent experiments). Asterisks indicate statistical significance (two-way ANOVA analysis, ****\( p \) value < 0.0001). d Extracts of HEK cells treated for 6 h with PBS or Wnt3a, together with DMSO or 10 \( \mu \)M MG132, were evaluated by immunoblotting. A representative immunoblot (left panel) and a quantification of immunoblots from (mean ± SEM, \( n = 3 \) independent experiments; right panel) are shown. e Extracts of HEK cells treated for 24 h with PBS or Wnt3a, together with DMSO or 20 nM bafilomycin A1 (BA), were evaluated by immunoblotting. A representative immunoblot (left panel) and a quantification of immunoblots (mean ± SEM, \( n = 3 \) independent experiments; right panel) are shown. P62 is a biomarker for lysosomal/autophagosomal inhibition. Asterisks in d and e indicate statistical significance (two-tailed Student’s t-test, *\( p \) value < 0.05, ***\( p \) value < 0.001). f CK1α was immunoprecipitated from the lysates of HEK cells treated with Wnt3a and MG132 for various time points, followed by analyses of the indicated proteins by immunoblotting. A representative immunoblot (\( n = 3 \) independent experiments) is shown.

Fig. 2 Wnt-induced degradation of CK1α requires CRBN, the substrate receptor of the CRL4CRBN E3 ubiquitin ligase complex. a HEK cells were transfected with the indicated smart-pool siRNA and subsequently treated with PBS or Wnt3a for 24 h. Extracts of these cells were evaluated by immunoblotting. A representative immunoblot (\( n = 5 \) independent experiments) is shown. b HEK cells were transfected with the indicated smart-pool siRNA and co-treated with Wnt3a and cycloheximide for the indicated time. Extracts of these cells were evaluated by immunoblotting. CK1α levels from immunoblots were quantitated and normalized to HSP90 levels (mean ± SEM, \( n = 3 \) independent experiments). Asterisks indicate statistical significance (two-way ANOVA analysis, ****\( p \) value < 0.0001). c A schematic outlining experimental details of the in vitro binding and ubiquitination assays shown respectively in panels d and e. d HEK cells were transfected with a plasmid encoding Flag-CRBN and subsequently treated with PBS or Wnt3a for 4 h. Flag-CRBN immunoprecipitated from extracts of these cells were incubated with the indicated amounts of recombinant GST-CK1α at 4 °C for 1 h. Flag-CRBN beads were re-isolated, washed, and CK1α bound was eluted using sample buffer. These immunoprecipitates were subjected to SDS-PAGE, followed by silver staining (top panel) or immunoblotting (bottom panel). IgG beads serve as a control for Flag-CRBN beads (left lane) and recombinant GST-CK1α a loading control (right lane). A representative gel image and immunoblot (\( n = 3 \) independent experiments) is shown. e HEK cells were transfected with a plasmid encoding Flag-CRBN and subsequently treated with PBS or Wnt3a for 4 h. Flag-CRBN immunoprecipitated from extracts of these cells were incubated with a mixture of recombinant GST-CK1α, ubiquitin, and a mixture of E1 and E2 ligases in the presence of vehicle or Mg-ATP, and incubated at 30 °C for 3 h. These reaction mixtures were subsequently analyzed by immunoblotting for the indicated proteins. A representative immunoblot (\( n = 3 \) independent experiments) is shown.
These results suggest that Wnt-driven, CRBN-dependent CK1α degradation occurs via an intact, functional destruction complex. Based on this finding, we speculated that CRBN is recruited to the destruction complex in response to Wnt stimulation in order to target CK1α. To test this hypothesis, we immunoprecipitated endogenous CRBN from lysates of HEK cells, treated with or without Wnt3a, and probed these immunoprecipitates for components of the destruction complex. We found that CRBN did indeed associate with CK1α and other components of the destruction complex in response to Wnt3a, including the two pivotal scaffolding proteins AXIN and APC (Fig. 3c). This result suggests that the destruction complex facilitates CRBN-dependent CK1α proteolysis upon Wnt stimulation.

Consistent with the destruction complex stabilizing CK1α in the absence of Wnt stimulation, knockdown of AXIN1 or APC expression induced CK1α degradation (Fig. 3d). Importantly, CK1α degradation following AXIN1 or APC depletion was also CRBN-dependent (Fig. 3d). In cells deficient for AXIN1 or APC, CK1α stabilization resulting from CRBN knockdown did not attenuate β-Catenin stabilization or downstream Wnt activity biomarkers (c-Myc). This observation is consistent with previous work demonstrating the importance of these proteins in efficiently targeting CK1α to β-Catenin3,37. Together, these results suggest that the integrity of the β-Catenin destruction complex is important for the regulation of CRBN-mediated CK1α proteolysis; the destruction complex both stabilizes CK1α in the absence of Wnt and promotes an activation step (via CRBN recruitment) upon Wnt induction.

Wnt stimulation modulates the interaction of CRBN with CK1α in a manner that requires the IMiD binding pocket. CRBN was reported previously to interact with and mediate CK1α ubiquitination only when the IMiD, lenalidomide, formed a molecular bridge between them18,21. This lenalidomide-induced, CRBN-dependent degradation of CK1α occurred via a β-hairpin loop anchored by Gly4021 (Fig. 4a, residues labeled magenta with white text). Similarly, using mutagenesis of key residues, we found that this β-hairpin loop of CK1α is also important for its lenalidomide-independent, Wnt-driven proteolysis (Fig. 4b). We therefore further investigated the role of the IMiD binding pocket of CRBN (Fig. 4a, binding pocket in blue and pivotal CK1α residues labeled green with white text) in Wnt-induced CK1α proteolysis. We mutated five pivotal CK1α residues within this pocket, all of which significantly attenuated their ability to degrade and bind to CK1α (Fig. 4c, d). Thus, Wnt signaling induces CRBN-dependent CK1α degradation in a manner that requires its previously described IMiD binding pocket21.

Due to primary sequence differences between human and mouse CRBN, lenalidomide is not able to induce CK1α degradation in mouse cells18. Thus, we determined whether Wnt-induced CK1α degradation also exhibits this specificity. We compared Wnt3a- and lenalidomide-induced degradation of CK1α in mouse fibroblasts (NIH3T3 cells) and found that Wnt3a-induced CK1α degradation and its binding to CRBN in these mouse cells, whereas lenalidomide did not (Supplementary Fig. 4a, b). This is consistent with a conserved function of Wnt signaling in CRBN-mediated CK1α proteolysis, unlike that of lenalidomide.

Wnt signaling induces CRBN-mediated degradation of a broad spectrum of endogenous substrates. We next determined the ability of Wnt3a to induce the degradation of a broader subset of known, endogenous CRBN substrates, including Glutamine synthetase (GS), c-Jun, and Meis homeobox 2 (MEIS2)40. We noted that in addition to causing decreased levels of CK1α, Wnt3a treatment also resulted in a decrease of the protein levels of these other CRBN substrates (Fig. 5a and Supplementary Fig. 5a), without affecting their levels of gene expression (Supplementary Fig. 5b–e). Similar to CK1α, the half-life of GS, c-Jun, and MEIS2 were also significantly decreased in response to Wnt3a, and this reduction was CRBN-dependent (Fig. 5b–g and Supplementary Fig. 5f–g). In contrast to CK1α, lenalidomide treatment had no significant effect on the degradation of these other CRBN substrates (Fig. 5h). Collectively, these results show that compared to lenalidomide, Wnt signaling activates the function of CRBN towards a wide spectrum of substrates.

**Fig. 3** The β-Catenin destruction complex regulates CRBN-mediated CK1α degradation. **a** Extracts of HEK cells treated with Wnt3a for various lengths of time were evaluated by immunoblotting. Quantitation of immunoblots (mean ± SEM, n = 3 independent experiments) is shown. Levels of phosphorylated β-Catenin were normalized to that of total β-Catenin. **b** HEK cells were transfected with control (CTRL) siRNA or one of three distinct CRBN siRNA, followed by PBS or Wnt3a treatment for 24 h. Extracts of these cells were evaluated by immunoblotting. **c** CRBN was immunoprecipitated from extracts of HEK cells treated with PBS or Wnt3a in the presence of MG132 for 4 h, followed by immunoblotting of the indicated proteins. **d** HEK cells were co-transfected with control (CTRL) siRNA or smart-pool AXIN1 or APC siRNA, along with one of two distinct CRBN siRNA. Extracts of these cells were evaluated by immunoblotting. A representative immunoblot (n = 3 independent experiments) is shown in **b–d**.

CRBN is a positive regulator of Wnt activity. We used a reporter cell line stably expressing a TOPFlash Wnt reporter gene (HEK293STF) in order to begin to probe the role CRBN may play in Wnt pathway activity. We found that CRBN knockdown significantly attenuated Wnt-stimulated reporter gene activity (Fig. 6a and Supplementary Fig. 6a). Additionally, overexpression of CRBN resulted in increased Wnt reporter activity in HEK293STF cells in the absence of exogenous Wnt stimulation (Fig. 6b and Supplementary Fig. 6b). Importantly, this increased Wnt activity was not observed upon ectopic expression of CK1α binding-deficient CRBN mutants (Figs. 4d, 6b and Supplementary Fig. 6b). Furthermore, both Wnt3a and overexpressed CRBN resulted in the degradation of CK1α in these cells (Supplementary Fig. 6c–e).
**Fig. 4** Wnt signaling induced CRBN-mediated degradation of CK1α requires its IMiD binding pocket. **a** A structural model of CRBN-lenalidomide-CK1α complex (PDB: 5fqd). The interacting residues at the interface of the CRBN (green with white text) and CK1α (magenta with white text) are shown as a ball and stick model. The IMiD binding pocket is depicted in blue. **b** HEK cells were transfected with a plasmid encoding wild-type (WT) HA-tagged CK1α or an HA-tagged CK1α mutant, followed by PBS or Wnt3a treatment for 24 h. Extracts of these cells were evaluated by immunoblotting. A representative immunoblot (left panel) and quantification of immunoblots (mean ± SEM, n = 3 independent experiments; right panel) are shown. HEK cells were transfected with a plasmid encoding WT Flag-tagged CRBN or the indicated Flag-tagged CRBN mutants. Extracts of these cells were evaluated by immunoblotting. A representative immunoblot (left panel) and quantification of immunoblots (mean ± SEM, n = 3 independent experiments; right panel) are shown. **c** HEK cells were transfected with a plasmid encoding WT Flag-tagged CRBN and subsequently treated with PBS or Wnt3a for 4 h. Flag-CRBN immunoprecipitated from extracts of these cells were incubated with the indicated amounts of recombinant GST-CK1α at 4 °C for 1 h. Flag-CRBN beads were re-isolated, washed, and CK1α bound was eluted using sample buffer. These immunoprecipitates were subjected to SDS-PAGE, followed by immunoblotting. IgG beads serve as a control for Flag-CRBN beads. A representative immunoblot (left panel) and a quantification of immunoblots (mean ± SEM, n = 3 independent experiments; right panel) are shown. Asterisks in **b**–**d** indicate statistical significance (two-tailed Student’s t-test, *p value < 0.05, ****p value < 0.0001).

**Fig. 5** Wnt signaling induces CRBN-mediated degradation of a subset of endogenous substrates. **a** HEK cells were transfected with the indicated smart-pool siRNA, followed by PBS or Wnt3a treatment for 24 h. Extracts of these cells were evaluated by immunoblotting. A representative immunoblot (n = 3 independent experiments) is shown. **b–d** HEK cells were co-treated with cycloheximide along with PBS or Wnt3a. Extracts of these cells were evaluated by immunoblotting. CK1α levels from immunoblots were quantitated, normalized to HSP90 levels, and plotted to determine the turnover of the indicated protein in response to Wnt3a (mean ± SEM, n = 3 independent experiments). **e–g** HEK cells were transfected with control (CTRL) or one of two distinct CRBN siRNA and co-treated with Wnt3a and cycloheximide. Extracts of these cells were evaluated by immunoblotting. CK1α levels from immunoblots were quantitated, normalized to HSP90 levels, and plotted to determine the turnover of the indicated protein in response to Wnt3a (mean ± SEM, n = 3 independent experiments). Asterisks in **b–g** indicate statistical significance (two-way ANOVA analysis, ***p value < 0.001, ****p value < 0.0001). **h** Extracts of HEK cells treated for 24 h with different doses of Wnt3a or lenalidomide were evaluated by immunoblotting. A representative immunoblot (n = 3 independent experiments) is shown.
Fig. 6 CRBN is a positive regulator of Wnt activity. a The Wnt reporter gene expressing cell line, HEK293STF, was transfected with control (CTRL) siRNA or one of two distinct CRBN siRNA and treated with PBS or Wnt3a (100 ng/mL) for 48 h. Luciferase activity was subsequently determined and normalized to total protein concentration. A quantification of Wnt reporter activity (mean ± SEM, n = 3 independent experiments) is shown. b HEK293STF cells were transfected with a control plasmid or a plasmid encoding wild-type (WT) Flag-tagged CRBN, or the indicated Flag-tagged CRBN mutants for 48 h. Luciferase activity was determined and normalized to total protein concentration. A quantification of Wnt reporter activity (mean ± SEM, n = 3 independent experiments) is shown. c A schematic of a mouse intestinal organoid showing the presumptive crypt (C) and villus (V) regions. d Mouse intestinal organoids were infected with control (Ctrl) or one of two distinct Crbn shRNA (marked by GFP expression) and then cultured in (i) basal media or (ii–iv) 25% exogenous Wnt conditioned media (L-WRN) for 5 days. Representative images are shown (n = 3 independent experiments). Scale bar = 100 μm. e Quantification of the percent of organoids that exhibit a more basal-like phenotype (mean ± SD, n = 3 technical replicates) in a representative experiment (n = 3 independent experiments) is shown. Asterisks indicate statistical significance (two-tailed Student’s t-test, **p-value < 0.01, ***p-value < 0.001).

Fig. 7 CRBN modulates Wnt function in Drosophila. a A schematic showing a Drosophila melanogaster wing imaginal disc. A anterior; P posterior; D dorsal; V ventral. b Representative confocal images showing the level of the Wg/Wnt biomarker Senseless (Sens, magenta) after RNAi-mediated knockdown of hh-Gal4 (Fig. 6c, ii–iv, e, Supplementary Fig. 6di). A quantification of wing disks with a defect in Sens levels is shown. Asterisks indicate statistical significance (Fisher’s exact test, **p-value < 0.0001).

CRBN modulates Wnt function in vivo. Wnt signaling is evolutionarily conserved across phyla, with many pivotal discoveries in the Wnt pathway originating from studies using Drosophila melanogaster. Wnt/Wingless (Wg) signaling directs cell fate specification during the development of the wing imaginal disc, the precursor of the adult wing. During this process, the Wg target gene senseless (sens) is transcriptionally activated on either side of a row of Wg-expressing cells at the dorsoventral boundary. We performed RNAi-mediated knockdown of the CRBN ortholog, ohgata (ohgt) in the posterior compartment of the wing imaginal disc and observed significantly disrupted Sens expression across the posterior region of wing disks (Fig. 7biv–vi, c). Of note, the expression of Wg was not disrupted by ohgt knockdown (Supplementary Fig. 7bii–iii, c, div–ix). Similar results were observed upon knockdown of the well-established regulator of Wg signaling disheveled (dsh), albeit in a manner that was more penetrant (Supplementary Fig. 7biv–vi, c). Canonical Wnt signaling plays a pivotal role in midline patterning during zebrafish development, with aberrant Wnt activation resulting in eye loss and Wnt/β-Catenin inhibition resulting in cyclopia. We injected either crbn mRNA, crbn morpholin (MO), or three distinct guide RNA (sgRNA) sequences targeting crbn along with dCas9 mRNA (CRISPRi), into zebrafish embryos and examined the phenotype of these head structures. Increased expression of crbn mRNA
Fig. 8 CRBN modulates Wnt function in zebrafish. a A schematic of transverse views of a zebrafish head indicating phenotypes regulated by Wnt signaling. White arrows indicate eyes and the balbis indicates the distance between eyes. b The transverse views of a (i) WT zebrafish head, a WT zebrafish head injected with (ii) crbn mRNA, or (iii–v) one of three distinct crbn guide RNAs (sgRNA) along with dCas9 mRNA, in the absence or presence of (iv) crbn mRNA or (v) β-catenin (ctnnb1) mRNA. White arrows indicate eyes, the gray arrow indicates merged eyes, and the balbis indicates the distance between eyes. Scale bar = 200 μm. c, e and g Quantifications of the indicated eye phenotypes are shown. Asterisks indicate statistical significance (Fisher’s exact test, *p < 0.05, ****p < 0.0001). d and f RNA extracts from the indicated zebrafish (20 hpf) were used to determine the expression of three Wnt target genes using quantitative RT-PCR. Quantification of expression indicated in zebrafish embryos (mean ± SEM, n = 3 independent pools of zebrafish embryo) is shown. Asterisks indicate statistical significance (two-tailed Student’s t-test, *p < 0.05, ***p < 0.001, ****p value < 0.0001). lef1 lymphoid enhancer-binding factor 1.
3.1 software. For Wnt reporter assays, following treatment HEK293STF cells were lysed in 1X passive lysis buffer (Promega) at room temperature for 10 min, followed by a luciferase assay (Promega). Luminescence was recorded by a Veritas 3.1 software. For Wnt reporter assays, following treatment HEK293STF cells were transfected with a nitrocellulose membrane and immunoblotted. The primary antibodies used were Ckinga (1:1000, ab108829) and SIAH1 (1:250, ab22377) from Abcam, Axin1 (1:1000, 2087), APC (1:1000, 2504), GSK3β (1:1000, 9315), phosphorylated β-Catenin S45 (1:1000, 9564), phosphorylated β-Catenin S33, 37, T41 (1:1000, 9561), β-Catenin (1:1000, 9562), c-Myc (1:5000, 3655), GAPDH (1:5000, 8884), c-Jun (1:5000, 9165), PTEN (1:5000, 9552), and α-Tubulin (1:1000, 9099) from Cell Signaling Technology, α-Tubulin (1:5000, T6199) and GS (1:1000, MAB2002) from Millipore, Ubiquitin (1:500, sc-8017) and Hsp90 (1:5000, sc-13119) from Santa Cruz Biotechnology, P62 (1:1000, 610832) from BD Biosciences, CRBN (1:1000, NBPI-91810) and MEIS2 (1:250, H80004122-M01) from Novus Biologicals, and Flag (1:5000, F7425) from Sigma (Supplementary Table 2). The secondary antibodies used were HRP-conjugated donkey anti-mouse or anti-rabbit (715-035-150 or 711-035-152), or rabbit anti-goat IgG (305-025-045) from Jackson ImmunoResearch (1:10000). ImageJ was used to quantify the signal of the indicated proteins in immunoblots.

Immunoprecipitation. Cells treated with PBS or Wnt3a for 4 h in the presence of 10 µM MG132 were lysed with Pierce IP lysis buffer (Thermo Fisher) containing a protease and phosphatase inhibitor cocktail (Thermo Fisher) and 10 µM MG132. For immunoprecipitation from HEK cells, the cell extracts were incubated with 1 µg of CRBN antibodies (Abcam ab18673) or PTEN antibodies (Santa Cruz Biotechnology sc-393186) overnight at 4 °C. Protein G agarose beads (Invitrogen) were then added for 1 h at 4 °C. For immunoprecipitation from NIH3T3 cells, CRBN antibodies (Cell Signaling Technology 71810) were first conjugated to agarose beads using an AminoLink Plus Immobilization Kit (Thermo Fisher) and these CRBN-conjugated beads were incubated in cell lysates overnight at 4 °C. Antibody-bound or -conjugated beads were then isolated by centrifugation and washed with lysis buffer for 10 min. For Flag immunoprecipitation, lysates were incubated with Flag antibody-conjugated beads (Sigma) overnight at 4°C, and the beads were subsequently isolated by centrifugation and washed with lysis buffer for 10 min three times. Antibody-bound or -conjugated beads were resuspended in SDS sample buffer, boiled for 5 min, and used for the subsequent analysis.

Ubiquitination assays. HEK cells were treated with recombinant Wnt3a (100 ng/mL) and MG132 for the indicated amount of time. Cells were lysed with Pierce IP lysis buffer (Thermo Fisher), a protease and phosphatase inhibitor cocktail (Thermo Fisher), 50 µM PR-619, and 10 µM MG132. Ckinga antibodies (Abcam ab200652) were conjugated to agarose beads using an AminoLink Plus Immobilization Kit (Thermo Fisher) and these CRBN-conjugated beads were incubated in cell lysates overnight at 4°C. Antibody-bound or -conjugated beads were then isolated by centrifugation and washed with lysis buffer for 10 min. For Flag immunoprecipitation, lysates were incubated with Flag antibody-conjugated beads (Sigma) overnight at 4°C, and the beads were subsequently isolated by centrifugation and washed with lysis buffer for 10 min three times. Antibody-bound or -conjugated beads were resuspended in SDS sample buffer, boiled for 5 min, and used for the subsequent analysis.

Model organisms. NHGRI-1 zebrafish embryos55 (1 cell) were injected with 15 ng crbn morpholinos (MO) in 3 nL into the yolk or 1 ng crbn mRNA in 1 nL into the single cell. The sequence of crbn mRNA and MO are as previously described55,56. For CRISPR/cas9 injections, embryos were injected with 250 pg dcr-2 mRNA and 300 pg crbn sgRNA. For rescue experiments, embryos were injected with 250 pg dcr-2 mRNA, 500 pg crbn sgRNA, and 250 pg mRNA encoding CRBN or β-Catenin. Sequences of crbn sgRNA (Alit-R CRISPR-Cas9 guide RNA, Integrated DNA Technologies) are as following: #A: CACCGGCAUUGGCUGCUGAGA GUUUUAGGCGAAGAAAAAGCGAAGCGCGUAA UCAACUGUGAAAGUGCCACAGAGCGGCGCUUUU; #B:GGAUGUUAAA CACACAGCUGUUGUUUGUGCUAAAAUGCAAGUGCGUUA GUCCGCUUCACUAUGCUAAAAGCGCCAGCAGGGCGUUUU; #E: CAGACGGGCAUUGUUAAAACACUGUAAUGAGCCAGAAGAAGGCA CAGCGGCUUCACUAUGCUAAAAGCGCCAGCAGGGCGUUUU; Embryos were raised, fixed, and phenotyped at 1 dpf or 2 dpf56.

Wnt pathway activation. For CRISPR/dCas9 injections, embryos were injected with 250 pg dcr-2 mRNA or/and 500 pg crbn sgRNA. For rescue experiments, embryos were injected with 250 pg dcr-2 mRNA, 500 pg crbn sgRNA, and 250 pg mRNA encoding CRBN or β-Catenin. Sequences of crbn sgRNA (Alit-R CRISPR-Cas9 guide RNA, Integrated DNA Technologies) are as following: #A: CACCGGCAUUGGCUGCUGAGA GUUUUAGGCGAAGAAAAAGCGAAGCGCGUAA UCAACUGUGAAAGUGCCACAGAGCGGCGCUUUU; #B:GGAUGUUAAA CACACAGCUGUUGUUUGUGCUAAAAUGCAAGUGCGUUA GUCCGCUUCACUAUGCUAAAAGCGCCAGCAGGGCGUUUU; #E: CAGACGGGCAUUGUUAAAACACUGUAAUGAGCCAGAAGAAGGCA CAGCGGCUUCACUAUGCUAAAAGCGCCAGCAGGGCGUUUU; Embryos were raised, fixed, and phenotyped at 1 dpf or 2 dpf56.

In vitro binding assay. Flag-CRBN was immunoprecipitated from HEK cells, treated with or without recombinant Wnt3a, using Flag antibody-conjugated beads (Sigma). IgG-conjugated beads (Sigma) served as a negative control for Flag-CRBN isolation. The antibody-conjugated beads were then blocked with 10% BSA in 1 M Tris-Cl (pH 7.5) for 6 h at 4°C as previously described55. The blocked beads were preincubated with 1 M NaCl for 1 h at 4°C, followed by the addition of recombinant GST-CRK1a in binding buffer (25 mM HEPES pH 7.25, 100 mM NaCl, 0.01% Triton X-100, 1 mM DTT, and 5% glycerol) for 1 h at 4°C. After one 5 min wash with binding buffer, the antibody-conjugated beads were resuspended in SDS sample buffer and boiled for 5 min, followed by silvestaining (Thermo Scientific) or immunoblotting.

Organoids. Wild-type mouse intestinal organoids were isolated and maintained as previously described57. Prior to shRNA lentiviral infection, organoids were collected and digested by Gentle Cell Dissociation Reagent (Stemcell Technologies) at room temperature for 10 min, followed by washing with basal culture medium. About 10,000 dissociated organoid cells were then resuspended in 25% conditioned L-WRN (Wnt3a, Rapaonind3, Noggin) media, produced from L-WRN cells according to ATCC’s protocol, which contained lentivirus particles (MOI = 10), 8 µM polybrene and 10 µM Y27632. Organoids were inoculated with the virus using a centrifugation-based protocol: 600 x g for 2 h at room temperature, followed by incubation for 1 h at 37°C. The cells were again pelleted, washed with culture media, resuspended in Matrigel, plated, and overlaid with basal culture media or 25% L-WRN conditioned media for 5 days. Images were obtained using an Olympus IX51 inverted fluorescence microscope and Olympus CellSens software.

+ Wnt:

Fig. 9 A model depicting Wnt-driven, CRBN-dependent regulation of Cki1α. Upon stimulation of Wnt signaling, CRBN is recruited to the β-Catenin destruction complex and targets Cki1α for degradation, further promoting Wnt pathway activation.
the posterior region was measured and normalized to the total length of the posterior compartment.

Animal care. Zebrafish studies were performed following the animal protocol of the University of Maryland's Institutional Animal Care and Use Committee.

Statistics. A minimum of three independent replicates were performed for each experiment. The error bars shown represent the standard error of the mean (SEM) of at least three independent experiments, except for organoid studies—which instead show the standard deviation (SD) of three technical replicates in one of three independent experiments. Statistical relevance was determined using a two-tailed Student's t-test, a two-way ANOVA (protein turnover comparisons), or a Fisher's exact test (Drosophila and zebrafish phenotype studies) using Prism Graphpad 9. Asterisks indicate statistical significance (*p values ≤ 0.05; **p values ≤ 0.01; ***p values ≤ 0.001; ****p values ≤ 0.0001).

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
All data supporting the findings of this study are available in the paper and the Supplementary information file. Raw data and original gel images are included in the Source Data file. All other relevant data were available from the authors upon reasonable request. Source data are provided with this paper.

Received: 25 June 2020; Accepted: 13 August 2021; Published online: 06 September 2021

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Acknowledgements

We thank all members of the Robbins, Capobianco, Lee, and Ahmed laboratories for their insightful advice and discussion regarding this work. We also thank GUMC Computational Chemistry Shared Resources (CCSR) for structural insights in this work. This work was supported by the National Institutes of Health (NIH) Grants: R01CA219189 to D.J.R., R35GM122516 to E.L., R01GM121421, R01GM122222, and R35GM136233 to Y.A., R01CA244188 to D.J.R., E.L., and Y.A., and R01GM118557 to C.C.H. This work was also supported by the Dwoskin Cancer Fund and the National Cancer Institute of the NIH under Award Number P30CA240139 to Sylvester Comprehensive Cancer Center.

Author contributions

C.S. and D.J.R. designed this project. E.L., Y.A., and N.G.A. provided insights with project design. C.S., A.N., L.R.N., A.A.A., M.S.-I., L.M.S., H.B., N.B., and S.D. obtained data and provided experimental support. C.S., A.N., L.R.N., A.A.A., L.M.S., H.B., N.B., S.D., and C.H.W. analyzed and interpreted the data. C.S. and D.J.R. wrote the manuscript. E.L., Y.A., N.G.A., B.L., H.B., N.B., C.C.H., and A.J.C. critically revised the manuscript. A.N., D.T.W., F.Y., and M.G.-C. proofread the manuscript.

Competing interests

D.J.R., E.L., and A.J.C. are founders of StemSynergy Therapeutics Inc., a company commercializing small-molecule cell signaling inhibitors, including Wnt inhibitors. The remaining authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-021-25634-z.

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Peer review information Nature Communications thanks the anonymous reviewers for their contributions to the peer review of this work. Peer review reports are available.

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