The polycystins are modulated by cellular oxygen-sensing pathways and regulate mitochondrial function

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ABSTRACT Autosomal dominant polycystic kidney disease is caused by mutations in the genes encoding polycystin-1 (PC1) and polycystin-2 (PC2), which form an ion channel complex that may mediate ciliary sensory processes and regulate endoplasmic reticulum (ER) Ca\(^{2+}\) release. Loss of PC1 expression profoundly alters cellular energy metabolism. The mechanisms that control the trafficking of PC1 and PC2, as well as their broader physiological roles, are poorly understood. We found that O\(_2\) levels regulate the subcellular localization and channel activity of the polycystin complex through its interaction with the O\(_2\)-sensing prolyl hydroxylase domain containing protein EGLN3 (or PHD3), which hydroxylates PC1. Moreover, cells lacking PC1 expression use less O\(_2\) and show less mitochondrial Ca\(^{2+}\) uptake in response to bradykinin-induced ER Ca\(^{2+}\) release, indicating that PC1 can modulate mitochondrial function. These data suggest a novel role for the polycystins in sensing and responding to cellular O\(_2\) levels.

INTRODUCTION Autosomal dominant polycystic kidney disease (ADPKD) affects ∼1 in 1000 individuals and is characterized by kidney cyst expansion that destroys surrounding normal tissue (Halvorson et al., 2010). Most cases (85%) result from mutations in Pkd1, which encodes the polycystin-1 protein (PC1), and the remainder result from mutations in Pkd2, which encodes the polycystin-2 protein (PC2; Harris and Torres, 2009). PC1 is a very large, multispanning transmembrane protein (450 kDa) that has been implicated in numerous signaling pathways (Chapin and Caplan, 2010; Harris and Torres, 2014; Ong and Harris, 2015), although its precise function is poorly understood. PC1 undergoes several cleavages, which include an autocatalytic N-terminal cleavage that generates a 150-kDa C-terminal fragment (CTF; Qian et al., 2002). PC2 is a Ca\(^{2+}\)-permeable nonselective cation channel and belongs to the transient receptor potential (TRP) channel family (Gonzalez-Perrett et al., 2001). The polycystins (PCs) form a complex that localizes to the plasma membrane (PM), as well as to intracellular compartments, where PC2 can modulate Ca\(^{2+}\) release from the endoplasmic reticulum (ER; Koulen et al., 2002). In addition, the PC proteins localize to the primary cilium, where they may participate in mechanosensory or chemosensory pathways (Pazour and Rosenbaum, 2002; Chapin and Caplan, 2010). The interaction between the PCs has been suggested to be important in defining the properties of the ion channel associated with the complex, whether through the modulation of PC2 channel activity or through new emergent channel properties of the complex (Hanaoka et al., 2000; Delmas et al., 2004). The factors that regulate the trafficking, localization, and channel activity of the PCs are unclear. Here we show that PC1 interacts with a component of the cellular O\(_2\)-sensing machinery, the prolyl hydroxylase domain-containing protein EGLN3 (or PHD3), and that O\(_2\) levels and the enzymatic activity of the EGLN proteins modulate the subcellular localization and the channel activity of the PC complex.
RESULTS AND DISCUSSION
The O$_2$ sensor EGLN3 and O$_2$ levels affect PC trafficking

To identify genes whose products modulate aspects of PC1 function, including its trafficking and the cleavage and nuclear translocation of its C-terminal tail (CTT; Chapin and Caplan, 2010), we designed a cell-based, high-content, genome-wide, small interfering RNA (siRNA)-based screen that employed HEK293 cells expressing tagged versions of PC1 and PC2. High-throughput confocal microscopy was used to quantify the fraction of PC1 present at the PM. EGLN3 was identified as a protein that profoundly influences PC1 trafficking to the cell surface. EGLN3 knockdown significantly increased the fraction of PC1 that localized to the PM (see the Supplemental Experimental Procedures for details concerning the design of the screen and the criteria for hit selection). The three paralogues of the EGLN proteins serve as cellular O$_2$ sensors, which regulate the hypoxia response pathway. In the presence of O$_2$, EGLN proteins hydroxylate proline residues on the hypoxia-induced factor HIF1α. This modification targets HIF1α for degradation (Jaakkola et al., 2001). Although it shares overlapping functions with the other two paralogues (EGLN1 and EGLN2), EGLN3 has the widest range of non-HIF targets (Jaakkola and Rantanen, 2013).

Because the screen was performed in nonpolarized HEK293 cells, we assessed whether EGLN3 knockdown influenced PC1 trafficking in polarized, ciliated LLC-PK$_1$ cells overexpressing both PC1 and PC2. An extracellular Flag epitope appended to the N-terminus of our PC1 construct allowed us to perform surface immunofluorescence (IF) labeling of only the pool of PC1 that is localized to the apical and ciliary membranes (Chapin et al., 2009). EGLN3 knockdown (Supplemental Figure S1A) increased the amount of PC1 at the apical surface (Figure 1A). Moreover, we found that the 150-kDa CTF that is generated by the autocatalytic N-terminal cleavage of PC1 (Qian et al., 2002) interacts with EGLN3 both in transfected cells and in mouse renal tissue in vivo (Figure 1B and Supplemental Figure S1B). Coimmunoprecipitation (coIP) experiments showed that PC1 also interacts to a lesser extent with EGLN1 and EGLN2 (Supplemental Figure S1C). To avoid any confounding effects from the potential compensatory activities of the EGLN1 and 2 proteins, we used pharmacological approaches that inhibit all three of the EGLN isoforms rather than an isoform-specific knockdown approach to examine the consequences for PC1 distribution and function of reduced EGLN activity.

To test whether the PC1-EGLN3 interaction was dependent on EGLN3 activity, we inhibited EGLN3 function by 1) incubating cells in hypoxic conditions (5% CO$_2$ and 95% N$_2$ for 2 h; 2) by incubating them for 24 h in the presence of the hypoxia-mimicking compound CoCl$_2$ to simulate prolonged hypoxia; or 3) by incubating them for 24 h with the pan EGLN inhibitor dimethylxallylglycine (DMOG). We also incubated cells in hypoxic conditions to increase O$_2$ availability and hence enhance EGLN3 activity. We found that the PC1-EGLN3 interaction is not dependent on EGLN3 activity because EGLN3 coimmunoprecipitates with the PC1 CTF regardless of the treatment (Figure 1B). Moreover, we found that PC2 also coimmunoprecipitates with EGLN3 (Supplemental Figure S2A). Because PC1 and PC2 interact with one another, these data do not reveal which of these proteins interacts with EGLN3. To determine whether EGLN3 can assemble with the cytoplasmic domains of PC1, we generated two constructs that embody distinct portions of the cytosolic regions of PC1. One of these is a concatenation of the five cytosolic loops of PC1 (PC1 C.L.1-5), in which each loop is connected to its neighbors by a flexible linker. The other corresponds to the PC1 CTT. Of interest, EGLN3 interacts with both PC1 C.L.1-5 and CTT (Supplemental Figure S2B) when it is coexpressed with either of these PC1 cytoplasmic domain constructs in HEK cells. These data suggest that EGLN3 participates in associations with multiple portions of the PC1 cytoplasmic domain. Although these data do not rule out the possibility that EGLN3 can also associate with PC2, they demonstrate that the cytoplasmic sequences of PC1 are sufficient to participate in interactions with EGLN3.

The effect of EGLN3 knockdown on PC1 localization suggested that O$_2$ levels might affect PC1 trafficking. To test this hypothesis, we incubated LLC-PK$_1$ cells overexpressing PC1 and PC2 in a hypoxia chamber for 2 h or in the presence of the hypoxia-mimicking compound CoCl$_2$ for 24 h and found that both treatments induced an increase in the amount of PC1 localized to the apical surface and to the primary cilium (Figure 1C). Pretreatment with brefeldin A (BFA) for 30 min prevented the increase in the size of the PC1 surface pool (Figure 1D), indicating that the increase in surface staining is likely due to an increase in the trafficking of newly synthesized PC1 from the ER rather than to a reduced rate of endocytosis or degradation of the apical pool of PC1. The PCs traffic together in a complex (Chapin et al., 2010; Kim et al., 2014), and so we assessed whether hypoxia had the same effect on PC2 trafficking. We performed surface biotinylation after 2 h of hypoxia or 24 h of CoCl$_2$ treatment and found that PC2 localization to the cell surface increased significantly after 2 h of hypoxia (80% increase in surface PC2) but not after the 24-h CoCl$_2$ treatment, suggesting that the effect of hypoxia on PC2 localization may be rapid and transient (Figure 1E). Quantification of ciliary PC2, performed by IF microscopy as described for PC1 (see Supplemental Figure S4), showed that hypoxic conditions did not increase the quantity of PC2 in the primary cilium (as shown in the graph in Figure 1E).

PC1 is modified by EGLN

To test whether PC1 is an EGLN substrate, we immunoprecipitated hemagglutinin (HA)-tagged PC1 from HEK cells overexpressing PC1 and PC2. Proline hydroxylation was assessed by Western blotting with an anti–hydroxylated proline (OH-Pro) antibody that has been used to assess the proline hydroxylation states of HIF and of several other proteins (Jung et al., 2012; Luo et al., 2014). We found that hydroxylated proline residues are present in the PC1 CTF (Figure 2A). No consistent hydroxylation signal was detected on PC2. The extent of PC1 proline hydroxylation is reduced after 48 h of treatment with DMOG (Figure 2A, graph). PC1 hydroxylation also occurs in renal tissue in vivo. We used a Pkd1-BAC model (Figure 2B, top) expressing three copies of Pkd1 (Fedeles et al., 2011) and a knock-in mouse model expressing physiological levels of Pkd1 (Figure 2B, bottom; Wodarczyk et al., 2009). In both cases, the PC1 protein is HA tagged at its C-terminus. The PC1 protein immunoprecipitated from both animal models was detected in Western blots probed with the antibody directed against OH-Pro. The presence of OH-Pro was also verified by mass spectrometry (MS) performed on the PC1 CTF immunoprecipitated from HEK293 cells overexpressing PC1 and PC2, which revealed that at least five peptides in the cytosolic regions of PC1 were hydroxylated at one or more residues (mapped onto a schematic representation of the PC1 CTF in Figure 2C; details in Supplemental Table S1). A representative tandem MS for one of the identified peptides, GELYRPAEPODYEMVELFLR, is shown in Figure 2D. This spectrum depicts the y and b ions that result from the progressive fragmentation of the specified peptide. The mass-to-charge (m/z) ratio of associated fragment ions is consistent with the presence of two OH-Pro residues in the positions indicated. The
O$_2$ levels regulate the channel activity of the PC complex

A number of proteins have been found to be EGLN substrates (Xie et al., 2009), including the TRP channel TRPA1 (Takahashi et al., 2011). EGLNs inhibit TRPA1 channel activity in normoxic conditions through hydroxylation of a specific proline residue, whereas reduced O$_2$ levels lead to activation of the channel. The O$_2$ levels also...
whether EGLN catalytic activity affects PC2 channel activity, we performed single-channel recordings using bilayer membranes into which were fused ER microsomes prepared from untransfected LLC-PK1 cells or from LLC-PK1 cells overexpressing PC1 and PC2. These cells had been treated under control conditions, exposed to a low-O2 environment for 2 h, or treated with DMOG overnight. The presence of both PCs in the microsome fraction was confirmed by Western blot analysis (Supplemental Figure S3A). Microsomes isolated

modulate the cell surface expression of TRPA1 (Takahashi et al., 2011). PC2 is a Ca2+-permeable cation TRP channel (Gonzalez-Perrett et al., 2001) that can mediate or modulate Ca2+ release from intracellular stores (Koulen et al., 2002). In addition, several membrane spans of PC1 share structural homology with the TRP family. Interaction between PC1 and PC2 may be important in modulating the localization or the intrinsic channel properties of PC2 (Hanaoka et al., 2000; Delmas et al., 2004; Casuscelli et al., 2009). To test whether EGLN catalytic activity affects PC2 channel activity, we performed single-channel recordings using bilayer membranes into which were fused ER microsomes prepared from untransfected LLC-PK1 cells or from LLC-PK1 cells overexpressing PC1 and PC2. These cells had been treated under control conditions, exposed to a low-O2 environment for 2 h, or treated with DMOG overnight. The presence of both PCs in the microsome fraction was confirmed by Western blot analysis (Supplemental Figure S3A). Microsomes isolated

FIGURE 2: PC1 proline hydroxylation. (A) Western blot analysis to detect the presence of OH-Pro on PC1 that was immunoprecipitated from HEK293 cells that overexpress PC1 and PC2 and were untreated (Control) or treated with 1 mM DMOG for 48 h; the graph shows the quantification of three independent experiments; *p < 0.05. (B) Western blot analysis of OH-Pro on PC1 that was immunoprecipitated from kidneys of mice expressing 3xHA-tagged Pkd1 (top; kidneys from two different transgenic animals, TG1 and TG2, and one wild-type mouse [WT]). The asterisk indicates a nonspecific band recognized by the anti-HA antibody in mouse tissue. Western blot analysis of OH-Pro on PC1 immunoprecipitated from kidneys of heterozygous (HA+/+) and homozygous (HA/HA) HA-tagged PC1 knock-in mice; material from a wild type mouse is also shown (bottom). (C) Schematic representation of the PC1 CTF, showing the localization of the peptides containing OH-Pro that were identified by MS (black dots). The position and sequence of the peptides corresponding to each black dot are presented in Supplementary Table S1. (D) Tandem MS of the peptide GELYRPAWEPPQDYEMVELFLR from PC1 confirming the presence of two OH-Pro residues (P). Characteristic b and y ions and internal ions were annotated for clarity.
We found that EGLN inhibition affected mitochondrial activity measured OCR in Pkd1-mediated effects on PC1 are implicated in OXPHOS regulation, we measured OXPHOS performed by these cells. To determine whether EGLN-1 found that Pkd1 renal proximal tubule cell lines, which completely lack or are heterozygous OXPHOS performed by these cells. To determine whether EGLN-1 found that Pkd1 renal proximal tubule cell lines, which completely lack or are heterozygous for Pkd1-affected cells and tissues (Rowe et al., 2013; Menezes et al., 2016). Some of these alterations are associated with high levels of aerobic glycolysis, reminiscent of the Warburg effect in tumor cells (Vander Heiden et al., 2009). The mechanisms responsible for this behavior have not been elucidated. Ca2+ release from the ER and its uptake by mitochondria regulates oxidative phosphorylation (OXPHOS) by activating critical enzymes, including pyruvate dehydrogenase (PDH), α-oxoglutarate dehydrogenase, and isocitrate dehydrogenase (McCormack and Denton, 1979; Cardenas et al., 2010; Rizzuto et al., 2012). Our data demonstrate that the PC channel activity is reduced during hypoxia. These observations together suggest the interesting possibility that the PCs may play a role in regulating mitochondrial OXPHOS. To test this hypothesis, we measured O2 consumption rate (OCR) in Pkd1-/- and Pkd1flox/flox mouse renal proximal tubule cell lines, which completely lack or are heterozygous for PC1 expression, respectively (Merrick et al., 2012). We found that Pkd1-/- cells manifest reduced OCR compared with Pkd1flox/flox cells (Figure 4A), consistent with a reduction in the quantity of OXPHOS performed by these cells. To determine whether EGLN-mediated effects on PC1 are implicated in OXPHOS regulation, we measured OCR in Pkd1-/- and Pkd1flox/flox cells treated with DMOG. We found that EGLN inhibition affected mitochondrial activity and induced a significant reduction in both the basal and maximal OCR observed in these cells. These data indicate that PC1 expression and EGLN activity play a physiologically important role in the regulation of baseline respiration. Moreover, treatment with dichloroacetate (DCA), an inhibitor of pyruvate dehydrogenase kinase, and therefore an OXPHOS activator (Cardenas et al., 2010), induced a moderate but significant increase in the OCR in cells lacking PC1 (Figure 4C). DCA had no effect on OCR in PC1-expressing cells, suggesting that pharmacological stimulation of mitochondrial respiration can partially rescue the metabolic perturbations observed in the absence of PC1.

Because PCs modulate ER Ca2+ release and mitochondrial Ca2+ uptake regulates OXPHOS, we tested whether mitochondrial uptake is perturbed in cells lacking PC1 expression. We performed confocal live imaging on Pkd1-/- and Pkd1flox/flox cells transfected with the MTS-RCaMP, a mitochondria-targeted version of the genetically encoded Ca2+ sensor RCaMP1h (Akerboom et al., 2013; Supplemental Figure S3B). Stimulation of ER Ca2+ release by treatment with bradykinin (performed in the absence of extracellular Ca2+) induced significantly higher mitochondrial Ca2+ uptake in cells expressing PC1 (Figure 4C). DCA had no effect on OCR in PC1-expressing cells, suggesting that pharmacological stimulation of mitochondrial respiration can partially rescue the metabolic perturbations observed in the absence of PC1.

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FIGURE 4: PC1 expression affects mitochondrial function and Ca\(^{2+}\) uptake. (A) OCR (picomoles/minute) in Pkd1\(^{-/-}\) and Pkd1\(^{flox/-}\) proximal tubule cells. (B) OCR in Pkd1\(^{-/-}\) and Pkd1\(^{flox/-}\) cells treated under control conditions or with 1 mM DMOG overnight. DMOG reduces OCR in Pkd1\(^{flox/-}\) cells but not in Pkd1\(^{-/-}\) cells. (C) OCR in Pkd1\(^{-/-}\) and Pkd1\(^{flox/-}\) cells under control conditions or after 5 mM DCA treatment for 1 h. DCA increases OCR in Pkd1\(^{-/-}\) cells but not in Pkd1\(^{flox/-}\) cells. OCR values from three independent experiments were normalized to total DNA content to account for variations in cell number. (D) Representative \(\Delta F/F_0\) graph showing bradykinin (BK)-induced mitochondrial Ca\(^{2+}\) uptake in Pkd1\(^{-/-}\) and Pkd1\(^{flox/-}\) cells in the absence of extracellular Ca\(^{2+}\) (18 cells from one experiment). (E) The maximum \(\Delta F/F_0\) fluorescence induced by BK (three experiments; 37 Pkd1\(^{flox/-}\) and 57 Pkd1\(^{-/-}\) cells analyzed). (F) Western blot analysis of total (60 μg of proteins), mitochondrial (12 μg), and MAM (12 μg) fractions blotted with antibodies directed against HA (to reveal PC1), PC2, the mitochondrial marker PDH, and the MAM marker FACL4. The asterisk indicates a nonspecific band generated by the anti-HA antibody in mouse tissue; the arrows represent the specific HA-PC1 band. Data are shown as mean ± SEM. *p < 0.05, **p < 0.01, and ***p < 0.001 determined by t test.
aposition between the ER and the mitochondrial membrane that represent hot spots for the regulation of Ca\(^{2+}\) transfer between these organelles (Patergnani et al., 2011). These results indicate that the PC complex can affect mitochondrial Ca\(^{2+}\) uptake and function.

Our findings reveal a novel mechanism that regulates the trafficking and channel activities of PCs and places the complex in the cellular O2-sensing pathway. The O2 levels affect the localization of the PC proteins and the Ca\(^{2+}\) sensitivity of their associated channel activity, suggesting that under normoxic conditions, the PC channel complex is triggered at relatively lower Ca\(^{2+}\) concentrations, permitting it to participate in ER Ca\(^{2+}\) release under normal levels of cytosolic Ca\(^{2+}\) (Figure 5A). This PC complex–mediated Ca\(^{2+}\) release can, in turn, affect mitochondrial Ca\(^{2+}\) uptake and therefore stimulate oxidative metabolism (Figure 5B). Conversely, under hypoxic conditions, the PC channel translocates to the cell surface and is less sensitive to activation by Ca\(^{2+}\), which results in less PC-mediated ER Ca\(^{2+}\) release in the presence of normal cytosolic Ca\(^{2+}\) concentrations and thus less Ca\(^{2+}\)-dependent OXPHOS. According to this model, lack of PC1 expression may mimic a low-O2 environment, leading to altered mitochondrial function, which results in a lower OCR. Consistent with this possibility, pharmacological activation of pyruvate dehydrogenase by treatment with DCA partially reverses the reduced OCR that is observed in cells lacking PC1 expression. Therefore, our results provide a possible explanation for at least some aspects of the previously observed metabolic perturbations in ADPKD–affected cells and tissues (Rowe et al., 2013).

**MATERIALS AND METHODS**

**Hypoxia/hyperoxia and brefeldin A treatment**

The hypoxia/hyperoxic environment was created using a hypoxia chamber (Billups-Rothenberg, San Diego, CA) by flowing different gas mixtures. For the hypoxia experiments, we used a 95% N\(_2\), 5% CO\(_2\) mixture, and for hyperoxia, we used an 85% O\(_2\), 5% CO\(_2\), and 10% N\(_2\) mixture. We ran the gas for 15 min at 2 psi and then clamped both the inlet and the outlet ports simultaneously to seal the chamber. The chamber containing the cells was incubated for 2 h at 37°C. The BFA-treated samples were preincubated in the presence of 20 μM BFA for 30 min before the hypoxia and during the 2-h incubation in the hypoxia chamber.

**Microsome preparation and single-channel recordings**

LLC-PK1 cells overexpressing PC1 and PC2 were grown to confluence in 100-mm dishes (Corning), treated as indicated, collected by scraping into phosphate-buffered saline, and centrifuged at 200 \(\times\) g for 5 min. Cell pellets were resuspended in 1 ml of microsome buffer (250 mM sucrose, 10 mM Tris, pH 7.6, 50 mM NaCl) plus protease inhibitor cocktail (Roche), probe sonicated three times for 10 s each (40% power, 4°C), and centrifuged for 10 min at 8000 \(\times\) g at 4°C. Supernatants were ultracentrifuged at 100,000 \(\times\) g for 45 min at 4°C. Pellets containing PC1- and PC2-enriched ER microsomes were resuspended in 100 μl of microsome buffer.

Microsomes from cells subjected to differing O2 environments or DMOG treatment (1 mM for 24 h) were fused to lipid bilayers as described (Koulen et al., 2002). Experiments were performed with 250 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)–Tris solution, pH 7.35, on the cis side and 250 mM HEPES,

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**FIGURE 5:** Models of the EGLN3-dependent regulation of PC trafficking and the influence of EGLN3 and the PCs on the regulation of OXPHOS. (A) Regulation of PC trafficking and channel activity. In the presence of O2, the fraction of the PC complex localized to the ER may facilitate ER Ca\(^{2+}\) leak and its uptake by the mitochondrion. Under hypoxic conditions, the PC complex translocates to the PM, and its Ca\(^{2+}\) sensitivity is reduced, possibly resulting in a reduction in ER Ca\(^{2+}\) leak. (B) Regulation of OXPHOS by the PC complex. ER-to-mitochondrion Ca\(^{2+}\) leak, resulting in decreased OXPHOS. Activation of PDH, through pharmacological treatment with the PDH kinase inhibitor DCA at least partially bypasses the need for PC function and restores OXPHOS.
55 mM Ba(OH)$_2$ solution, pH 7.35, on the trans side. Incorporation of channels was monitored by the addition of KCl on the cis side. Once incorporation of channels was established, the solution on the cis side was exchanged with 10x volume of 250 mM HEPES-Tris solution, pH 7.35. PC2 channels were then activated by the sequential addition of Ca$^{2+}$ to the cis side. Channel activity was recorded for 2 min at each Ca$^{2+}$ concentration under voltage-clamp conditions (~20 mV holding potential) using a Bilayer Clamp BC-525C (Warner Instruments, Hartford, CT), filtered at 1 kHz, and digitized at 5 kHz. Data were acquired and analyzed with pClamp9 (Axon Instruments, Burlingame, CA). Channel characteristics (open probability, Ca$^{2+}$ sensitivity, current amplitude) were compared with previously obtained traces of PC2.

**Oxygen consumption**

The OCR was measured using an XF24 extracellular analyzer (Seahorse Bioscience, North Billerica, MA). Cells were seeded at confluent density in XF24 cell culture microplates (Seahorse Bioscience) the day before the experiment. During the assay, the cells were sequentially exposed to oligomycin (10 μg/ml), 2,4-dinitrophenol (1 mM), and antimycin A (10 μM) plus rotenone (10 μM). After each injection, OCR was measured for 3 min, and the medium was mixed for 2 min and again measured for 3 min. Every point in each experiment represents an average of nine (three experiments in triplicate) different wells. Data were normalized for DNA content to account for variations in cell number by performing Hoechst 33342 (Molecular Probes) staining, followed by fluorescence reading in an Infinite M1000 PRO microplate reader (Tecan, San Jose, CA).

**Genome-wide siRNA screen**

The Genome Wide siRNA screen was performed in collaboration with the Yale Center for High Throughput Cell Biology. Additional details on the experimental procedures are given in the Supplemental Experimental Procedures.

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