Supporting Information

- A. Materials and Methods
- **B.** Experimental Procedures and Characterization Data
- C. References for supporting information
- D. Summary and captions for supporting information figures, tables and datasets:

A. Materials and Methods

LIM1215 sub cellular cytosolic extracts

Cytosolic extracts of LIM1215 colonic carcinoma cell lines(1) were used as protein sources in affinity experiments, which were prepared following a digitonin extraction process as previously described (2-4). Briefly, LIM1215 cells were grown to confluence in 10 dishes (10 cm diameter), the culture media was then removed, and the dishes were rinsed with cold PBS. Cells (2 x 108 cells) were scraped and extracted for 30 min at 4 °C by using cytosol extraction buffer (10 mL of 10 mM Pipes, 100 mM NaCl, 120 mM sucrose, 2.5 mM MgCl2, 2 mM EGTA, pH 6.8, containing 0.015% (w/v) digitonin) and protease (cOmplete, EDTA free, cocktail, Roche) and phosphatase inhibitor (PhosStop, Roche) was added. Cell permeation was assessed under a light microscope using 1% eosin. The cytosolic extracts were obtained by sequential centrifugation at 480g for 20 minutes followed by 436,000g (Beckman rotor TLA100.2, 100 000 rpm) for 15 minutes at 4°C.

Conjugation of amino IP₆ with Dynabeads M-270 Carboxylic acid

Procedure for the conjugation of amino IP₆ to Dynabeads M-270 was performed at the concentration of 38 µg amino IP₆ per 100 µL Dynabeads. Firstly, 100 µL Dynabeads were washed twice with 100 µL of 25 mM MES buffer (pH=5) and then amino IP₆ (38 µg, 0.051 µmol in 10 µL milliQ H₂O) in 50 µL of 25 mM MES was added to the beads and the suspension was incubated on rotation at room temperature for 30 min. A cold EDC solution (3 mg in 30 µL of 100 mM MES buffer) was then added to the Dynabeads and mix well, and another 10 µL of 25 mM MES buffer was added to a final volume of 100 µL for 100 µL beads. The mixture was incubated at 4 degree for 3 hours. Allow the suspension to settle on a magnet to separate the supernatant from the given beads, and the beads were washed three times with milli Q water and another three times with PBS. The supernatant and washings were kept for further analysis of unconjugated amino IP₆. The rest of the activated groups on the beads' surface were blocked by incubation with ethanolamine (1 M, pH 8.0, 250 µL) for 1 hour at room temperature. The resulting IP₆ conjugated Dynabeads were washed three times with PBS and finally stored in 100 µL PBS. Blank Dynabeads were always made in parallel with IP₆ beads by EDC coupling and ethanolamine blockage.

Fractionation of cytosolic lysates with Mono Q anion-exchange chromatography

LIM1215 cytosolic extracts (4 mL) were diluted to 15 mL with Buffer A (10 mM Tris-HCl (pH 7.5) containing 0.1% (v/v) Tween 20) and then injected three times using a superloop (5 mL) onto an anion

exchange column (MonoQ HR10/100). The proteins were eluted at 4 °C from the column using a linear gradient system with 0% to 100 % of Buffer B (10 mM Tris-HCl (pH 7.5) containing 0.1% (v/v) Tween 20, 1 M NaCl) in Buffer A, over 30 column volumes at a flow rate of 3 mL/min. Fractions (8 mL each) were collected automatically and proteins were detected by absorbance at 280 nm. According to the elution profile, every two to three adjacent fractions were combined and concentrated to 0.5 mL. The resulting fractions were then submitted to buffer exchange column (Sephadex G-25, GE Healthcare Life Sciences, MiniTrap) and proteins were eluted with 1 mL TBS (20 mM Tris-HCl, 150 mM NaCl, pH 7.35). The samples were then incubated with IP₆ beads or blank beads and processed as described below.

Affinity capture experiments

Each concentrated sample (1 mL in TBS) of the fractionated cytosolic extracts off the Mono Q column were pre-cleared by incubation with blank Dynabeads (15 μ L beads bed for 1 mL cell extracts) for 2 h at 4 °C to remove proteins which bind non-specifically to the beads. After removal of the blank beads by magnet, the pre-cleared supernatant was split into half (2 x 500 μ L) and incubated for 2 h at 4 °C with IP₆-Dynabeads or blank beads (15 μ L beads for 500 μ L of fraction). The beads were then washed twice with 200 μ L of 10 mM Tris-HCl buffer containing 0.1% (v/v) Chaps and twice with 200 μ L TBS (Tris buffer Saline, 0.15M NaCl). The bound proteins were then desorbed off the using 25 μ L of SDS-PAGE buffer (LDS NuPAGE sample buffer) at 95 °C for 5 min. Eluted proteins were separated using SDS-PAGE (NuPAGE 4-12% Bis-Tris gel) in MES buffer and detected by Coomassie staining (Imperial Stain, Pierce) overnight. The protein bands can be visualized by de-staining the protein gel in milliQ water.

LC/MS-MS analysis

<u>Tryptic digest:</u> Excised protein gel bands from the SDS-PAGE gel lane were subjected to manual in-gel tryptic digestion procedure. Briefly, the gel spots were de-stained and dehydrated with acetonitrile before submitted to reduction with 50 mM TCEP solution (50 μ L of 0.5M TCEP / 125 μ L of 200 mM TEAB / 325 μ L milliQ water) and alkylation with 100 mM Iodoacetamide solution in 50 mM TEAB buffer. Trypsin solution was prepared by adding 50 μ L of 50 mM TEAB to each Trypsin single vial (1 μ g trypsin), and rehydrate dried gel plug/bands with approximately 10 μ L trypsin working solution for 60 minutes in fridge. Excess trypsin solution was removed with pipette before adding 35 μ L of 50 mM TEAB to sample vial to ensure proper hydration during digestion at elevated temperatures. Digestion was performed at 37 °C for overnight. The second day, the digestion was stopped by adding 5 μ L of 10% formic acid before collecting the supernatant for peptide mass mapping on LC/MS/MS.

<u>Mass Spectrometry Analysis</u> Analyses were performed using an ABSCIEX 5600 qTOF LC/MS/MS coupled with an Eksigent μ LTRA nanoflow LC fitted with a nanoflex chip cube. The ion source was an ABsciex nano ionspray III source (2400V) fitted with a new objective 10 μ m Emitter. The sample was loaded onto a cHiPLC Trap, ChormXP, C18-CL, 3 μ m 120Å with a cHiPLC Column ChromXP, C18-CL, 3 μ m, 120Å, separating column (Eksigent USA). Peptides were eluted using a 5-50% Acetonitrile/ 0.1% Formic acid gradient over 25min. Data dependent acquisition was performed on ions between the mass range of 400 - 1600 Da with an intensity threshold of >60cps and product ion scans that covered the mass range of 100-1800 Da.

Database Searching and Peptide Identification:

The MGF files were analyzed using the Mascot search engine (Matrix Science, London, UK; version 2.4.02) and searched against the LudwigNR_Q314 sequence database (taxonomy filter *Homo sapiens*) including common contaminants (http://www.wehi.edu.au/faculty/advanced_research_technologies/ proteomics/wehi_systems_biology_mascot_server). Search parameters consisted of enzyme Trypsin/P, 2 missed cleavages, fragment ion mass tolerance of 0.040 Da and a parent ion tolerance of 20 PPM. Carbamidomethylation of cysteine was specified as a fixed modification and Gln->pyro-Glu of the peptide n-terminus, oxidation of methionine and acetylation of the protein n-terminus were specified as variable modifications.

Protein Inference and Validation:

Scaffold (version Scaffold_4.3.4, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide identifications and protein inferences. Peptide identifications were accepted if they could be established at greater than 95.0% probability based on Scaffold's Local FDR algorithm. Proteins were accepted if they could be established at greater than 95.0% probability and contained at least 2 identified peptides. Proteins inferred on the basis of single peptide identifications were manually inspected in accordance with previously established guidelines (5) and included if their probabilities were greater than 99% and all major fragment ions were annotated. Proteins that contained similar significant scoring peptides that could not be differentiated based on MS/MS analysis alone, were grouped into clusters to satisfy the principles of parsimony.

Bioinformatic analysis

Proteins identified on control beads were removed from protein lists of IP6 beads, along with known non-specific contaminant protein identified in previous studies, such as keratins, actins, histones, tubulins, translation initiation factors, tRNA ligases and ATP synthesase and subunits, heat-shock proteins, peroxiredoxins, enolases, vimentins, , glyceraldehyde-3-phosphate dehydrogenases, ribosomal proteins and nucleotide interacting proteins (2, 6, 7).

UniProt (Universal Protein Resource) (http://www.uniprot.org/) was used for protein annotation. PANTHER (http://www.pantherdb.org/) and GOSlim ontology were used for protein function classification GeneCodis database (http://genecodis.cnb.csic.es/) (8-10) was used for singular enrichment analysis of proteomics data by integrating information of Gene Ontology categories (Biological Process, and Molecular Function from http://www.geneontology) and KEGG pathways (from http://www.genome.jp/kegg/). p Values are obtained through hypergeometric analysis corrected by False Discovery Rate method.

Protein/protein and protein/IP6 interaction network were analysed using the STITCH is a database of protein-chemical interactions (http://stitch.embl.de).

Production and purification of recombinant His-tagged bovine beta-arrestin 2

beta-arrestin 2 (P32120: 1-420) was produced as a His-fusion protein in BL21 competent bacterial cells using a bovine beta-arrestin 2 cDNA (with a Histidine tag added at the N-terminal, made by DNA 2.0) cloned into the expression vector pJExpress404 (11, 12). Briefly, cells were grown in superbroth containing 100 ug/mL ampicillin and 1 mM IPTG for 18 h at 30 °C, harvested by centrifugation (12). The given pellets were lysed by sonication in lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM Immidazole, pH 7.5) and then purified using NiNTA beads eluted in elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM Imidazole, pH 7.5), followed by size exclusion chromatography with a SuperdexTM 200 column equilibrated in PBS and connected to an AKTA Purifier System. Protein identity was confirmed using LC/MS/MS analysis.

Biosensor Analysis

Experiments were performed using a Biacore 2000 biosensor (Biacore Life Sciences, GE Healthcare). Amino coupling of NH2-IP₆ derivatives is not feasible due to the negative charged of IP₆ molecule that inhibited surface enrichment. Therefore, NH2-IP₆ analogue (5 μ g/ μ L in PBS), was conjugated to biotin using Sulfo-NHS-biotin (Thermo Scientific) (1/2 ratio, 2 hours incubation at RT). Biotin conjugated IP₆ was separated from free NHS-Biotin using an anion exchange column (SAX Analytical HPLC Column, 4.6 x 150 mm, Agilent,) connected to an HP1100 system (Agilent). Biotinated IP₆ was eluted using a linear 0-2M (NH₄)HCO₃ generated over 30 minutes at a flow rate of 1 mL/min. Biotin derivatised IP₆ was then immobilised onto neutravidin derivatised sensor surface according to described protocol (4) Various concentrations of His-tagged beta-arrestin 2 (5 μ M, 2.5 μ M, 1.25 μ M, 0.625 μ M, 0.312 μ M) were injected over immobilised IP₆ (40RU immobilised). A neutravidin channel was used as control. Kinetic constants were derived from the resulting sensorgrams with BIAEVALUATION 4.1 software (Biacore Life Sciences, GE Healthcare) using Global analysis using a 1:1 model that includes terms for mass transfer of analyte to the surface. The fit between experimental data and fitted curves was estimated by chi-squared analysis using the equation given below:

$$\chi^{2} = \frac{\sum_{1}^{n} \left(\mathbf{r}_{\mathrm{f}} - \mathbf{r}_{\mathrm{x}}\right)^{2}}{n - p}$$

where rf is the fitted value at a given point, rx is the experimental value at the same point, n is the number of data points and p is the number of degrees of freedom.

Additional experiments with high concentration of Magnesium

In order to investigate the importance of magnesium, further pull-down experiments were carried out, using a modified extraction buffer containing no EGTA and 5 mM MgCl₂ (10 mL of 10 mM Pipes, 100 mM NaCl, 120 mM sucrose, 5 mM MgCl₂, pH 6.8, containing 0.015% (w/v) digitonin). The resulting material was used directly for the affinity step (without further purification on the MonoQ column or undergoing any buffer exchange procedure). The results from these experiments were comparable to our original experiment. See Data set 5 for the full protein and peptide lists (those highlighted in red were previsously identificied in our original experiments). In additions please see Table S3 for the biological process enrichment of the newly identified proteins with magnesium (excluding the proteins previously identified in the original study).

B. Experimental Procedures and Characterization Data

General Synthetic Methods and Apparatus

¹*H-NMR spectra* were recorded on a Varian Unity 500 (500 MHz) instrument, using deuterochloroform (or other indicated solvents) as reference or internal deuterium lock. The chemical shift data for each signal are given as δ in units of parts per million (ppm) relative to residual chloroform (δ 7.26 ppm) or other solvent. The number of protons (n) for a given resonance is indicated by n H; the multiplicity of each signal is indicated by: s (singlet), d (doublet), t (triplet), q (quartet), and m (multiplet). Broad peaks are prefixed with "br"; apparent multiplicity is prefixed with "app". Coupling constants (*J*) are quoted in Hz and are recorded to the nearest 0.1 Hz. ¹³*C NMR spectra* were recorded on Varian Inova 500 (125 MHz) instruments using the central resonance of the triplet of CDCl₃ at δ 77.0 ppm as an internal reference. The chemical shift data for each signal are given as δ in units of parts per million (ppm). ³¹*P NMR spectra* were recorded on a Varian Inova 500 (202 MHz) instrument with proton decoupling. The chemical shift data for each signal are given as δ in units (ppm), using the chemical shift of 85% H₃PO₄ at δ 0.00 ppm as an external reference. *Infrared spectra* were recorded on a Perkin Elmer Spectrum One FT-IR spectrometer in the region 4000-650 cm⁻¹. The samples were analysed as thin films from dichloromethane or as solutions in the indicated solvents.

Mass spectra were recorded using the LCMS system composed of an Agilent 1100 HPLC connected to an Agilent 6220 esiTOF mass spectrometer fitted with a standard Agilent electrospray ion source, in the Research Transfer Facility (RTF) at Bio 21 Institute, or a hybrid linear ion trap and Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer (Finnigan LTQ-FT San Jose, CA), which is equipped with ESI, by the Mass Spectrometry Service at the University of Melbourne chemistry department. *Flash chromatography* was carried out on silica gel [Merck Kieselgel 60 (230 – 400 mesh)] under a pressure of nitrogen. *Analytical thin layer chromatography (TLC)* was done on precoated 0.2 mm thick Merck Kieselgel 60 F_{254} silica gel plates and visualised by absorption of UV light and ethanolic phosphomolybdic acid (PMA) or aqueous potassium permanganate solution.

Solvents such as *n*-hexane and *N*,*N*-dimethylformamide were purchased from Aldrich and dried by sitting over freshly activated 4Å molecular sieves for 1 hour. Anhydrous THF, diethyl ether, and dichloromethane were dried by passage through a packed column of activated neutral alumina under a nitrogen atmosphere, and toluene being passed through a coloumn with additional R3-11 copper-based catalyst (BASF Australia) (14). Petroleum ether refers to the fraction of boiling point range 40-60 °C. Procedures using moisture or air sensitive reagents were undertaken in a nitrogen-filled dual manifold employing standard Schlenk line techniques. Brine refers to a saturated aqueous solution of sodium chloride. Where appropriate and if not stated otherwise, all reactions were performed in flame-dried apparatus under an atmosphere of dry nitrogen. *Melting points* were determined with an Electrothermal Engineering IA9100 or a Büchi 510 melting point apparatus and are uncorrected.

Experimental Procedures and Characterization Data

The following compounds were synthesised following literature procedures: **1** (15, 16), **2** (17), **10** (18), (benzyloxy)bis(N,N-diisopropylamino) phosphine (19, 20) and bis(benzyloxy)(N,N-diisopropylamino)phosphine (21).

1,3-O-Methylene-2,4,6-tri-O-p-methoxybenzyl-myo-inositol (3)



To a solution of **2** (10.0 g, 18.2 mmol, 1 equiv.) in dichloromethane (90 mL) under nitrogen at 0 $^{\circ}$ C was added diisobutyl aluminium hydride (45.5 mL, 1.5 M solution in toluene, 2.5 equiv.) dropwise. The colorless solution was stirred at 0 $^{\circ}$ C for 30 min and then was allowed to warm up to room temperature and stirred for 4 h. The resulting solution was transferred *via* cannula into a vigorously stirring solution of sodium potassium tartrate (1 M, 100 mL) and saturated ammonium chloride solution (100 mL) at 0 $^{\circ}$ C, and the mixture was kept stirring at 0 $^{\circ}$ C for 2 h followed by stirring at room temperature for a further 3 h to solubilize the aluminum salts. Dichloromethane was added and the organic phase was separated, the aqueous phase was extracted with dichloromethane. The combined organic fractions were washed with brine, dried over MgSO₄ and concentrated to afford viscous yellow clear oil. Flash chromatography (30 - 50% ethyl acetate/ petroleum spirit) afforded the acetal **3** as pale yellow oil and solidifies when left in freezer (9.75 g, 98%).

R_f 0.52 (30% ethyl acetate in petroleum spirits); v_{max} (neat)/cm⁻¹ 3500-3300 (br s), 2912, 1612, 1512, 1464, 1302, 1244, 1174, 1137, 1074, 1030, 818; $\delta_{\rm H}$ (500 MHz; CDCl₃) 7.27 (d, *J* = 8 Hz, 2H), 7.21 (d, *J* = 9 Hz, 4H), 6.86 (d, *J* = 8 Hz, 6H), 5.55 (d, *J* = 5 Hz, 1H), 4.68 (d, *J* = 5 Hz, 1H), 4.63-4.50 (m, 6H), 4.42-4.40 (m, 2H), 4.27 (s, 1H), 4.01 (t, *J* = 3 Hz, 2H), 3.95 (d, *J* = 10 Hz, 1H), 3.82 (s, 6H), 3.80 (s, 3H), 3.00 (d, *J* = 10, 1H); $\delta_{\rm C}$ (125 MHz; CDCl₃) 130.0, 129.5, 129.2, 113.9, 113.8, 85.6, 80.6, 72.7, 71.6, 70.3, 69.7, 69.6, 55.24, 55.22; HRMS (ESI+) m/z 575.2251 (575.2266 calc. for C₃₁H₃₆O₉Na (M+Na)⁺).

5-O-Allyl-1,3-O-methylene-2,4,6-tri-O-p-methoxybenzyl-myo-inositol (4)

To a stirred solution of the alcohol **3** (500 mg, 0.91 mmol) in dry DMF (15 mL) under nitrogen at 0 $^{\circ}$ C was added sodium hydride (80 mg, 60% dispersion in mineral oil, 2.0 mmol) and imidazole (catalytic, 15 mg). The suspension was stirred at 0 $^{\circ}$ C for 15 min followed by at room temperature for 30 min, and then cooled to 0 $^{\circ}$ C for the addition of allyl bromide (166 mg, 119 uL, 1.37 mmol). The reaction mixture was stirred at room temperature overnight and quenched by addition of ethanol (2.0 mL),

followed by water (2.0 mL). The solvent was removed *in vacuo*. The residue was partitioned between ethyl acetate (50 mL) and water (50 mL) and the organic layer was separated. The aqueous phase was extracted with ethyl acetate (4×50 mL) the combined organic extracts washed with brine and dried over MgSO₄. Evaporation and chromatography (30% ethyl acetate/petroleum spirit) afforded the acetal **4** as a clear yellow oil (480 mg, 0.81 mol, 89%).

R_f 0.30 (30% EtOAc in petroleum spirits); v_{max} (neat)/cm⁻¹ 2907, 1612, 1586, 1512, 1464, 1302, 1245, 1174, 1076, 1031, 1010, 818; $\delta_{\rm H}$ (500 MHz; CDCl₃) 7.29 (d, *J* = 9 Hz, 2H), 7.24 (d, *J* = 9 Hz, 4H), 6.88-6.84 (m, 6H), 5.89 (ddd, *J* = 23, 11, 6 Hz, 1H), 5.25 (dd, *J* = 3, 2 Hz, 1H), 5.22 (dd, *J* = 3, 2 Hz, 1H), 5.18-5.16 (m, 1H), 5.16-5.13 (m,1H), 4.81 (d, *J* = 6 Hz, 1H), 4.59 (d, *J* = 11 Hz, 2H), 4.58 (s, 2H), 4.52 (d, *J* = 11 Hz, 2H), 4.20 (br s, 2H), 4.13 (t, *J* = 1 Hz, 1H), 4.12 (t, *J* = 1Hz, 1H), 3.86 (d, *J* = 6 Hz, 2H), 3.81 (s, 6H), 3.80 (s, 3H), 3.77 (t, *J* = 2 Hz, 1H), 3.49 (t, *J* = 6 Hz, 1H); $\delta_{\rm C}$ (125 MHz; CDCl₃) 159.3, 135.0, 129.8, 129.7, 129.5, 129.4, 116.9, 113.9, 113.8, 85.4, 81.7, 80.3, 72.6, 72.1, 71.5, 70.6, 69.8, 55.3; HRMS (ESI+) m/z 615.2580 (615.2565 calc. for C₃₄H₄₀O₉Na (M+Na)⁺).

5-O-Allyl- myo-inositol (5)



To a solution of **4** (470mg, 0.79 mmol) in methanol (23.5 mL) was added concentrated hydrochloric acid (5.9 mL). The mixture was stirred at 80 °C for 4 h then cooled to rt and concentrated to complete dryness by high vacuum overnight. The residue was suspended in ethyl acetate (10 mL) and stirred for one hour. The suspension was submitted to centrifugation (3500 rpm, 9 min) and the supernatant was discarded. The white solid on the bottom was washed with more ethyl acetate (3500 rpm, 9 min x 2). The product **5** was collected as white solids after complete dryness (165 mg, 0.75 mmol, 95%). m.p. 239 – 241 °C; v_{max} (neat)/cm⁻¹ 3340-3000, 1138, 1032, 724; δ_{H} (500 MHz; DMSO) 5.94 (qd, J = 10, 5 Hz, 1H), 5.23 (dd, J = 17, 2 Hz, 1H), 5.06 (d, J = 10 Hz, 1H), 4.57-4.35 (m, 5H), 4.23 (d, J = 5 Hz, 2H), 3.69 (dd, J = 6, 3 Hz, 1H), 3.45 (dt, J = 9, 5 Hz, 3H), 3.13 (m, 2H), 2.84 (t, J = 9 Hz, 1H); δ_{C} (125 MHz; DMSO) 136.8, 115.1, 83.6, 72.7, 72.4, 71.8; HRMS (ESI+) m/z 243.0845 (243.0839 calc. for C₉H₁₆O₆Na (M+Na)⁺).

5-O-Allyl-myo-inositol-1,2,3,4,6- pentakis(dibenzyl)phosphate (6)



To the solution of 1*H*-tetrazole in acetonitrile (12.80 mL, 0.45M, 5.76 mmol, 25 equiv.) was added to phosphoramidite Bis(benzyloxy)(*N*,*N*-diisopropylamino)phosphine (BnO)₂PNⁱPr₂ (1 M in CH₂Cl₂, 2.88

mL, 2.88 mmol, 12.5 equiv.) under nitrogen. A solution of the penta-ol **11** (50 mg, 0.23 mmol, 1 equiv.) in dry DMF (5.0 mL) was added to the mixture dropwise. After stirring at room temperature overnight the reaction mixture was cooled to -78 °C followed by addition of *m*CPBA (1.06 g, 4.31 mmol, 18.75 equiv.). The resulting reaction mixture was then warmed to room temperature, stirred for 1 h, and then quenched with sat. NaHSO₃ (25 mL) and diluted with ethyl acetate (50 mL). The aqueous layer was separated and extracted with more ethyl acetate (3 x 50 mL). The combined organic extract was washed with brine and dried over MgSO₄. The solvent was removed *in vacuo* to give the crude product. Flash chromatography (50% -100 % ethyl acetate/petroleum spirit) afforded the product **6** (314 mg, 90%) as a colorless gum.

R_f 0.55 (1:1 ethyl acetate/petroleum spirit); v_{max} (neat)/cm⁻¹ 3040-3000, 1457, 1279, 1019, 887, 738, 696; $\delta_{\rm H}$ (600 MHz; CDCl₃) 7.33-7.10 (m, 50H), 5,85 (m, 1H), 5.59 (d, *J* = 9 Hz, 1H), 5.20-4.90 (m, 24H) 4.33 (t, *J* = 9 Hz, 2H), 4.26 (d, *J* = 6 Hz, 2H), 3.41 (t, *J* = 9 Hz, 1H); $\delta_{\rm C}$ (125 MHz; CDCl₃) 135.9, 135.8, 135.7, 135.6, 134.4, 128.4, 128.3, 128.2, 128.1, 128.0, 127.9, 117.5, 77.9, 76.1, 74.1, 73.8, 73.7, 70.1, 70.0, 69.8, 69.7, 69.6, 69.5, 69.4; $\delta_{\rm P}$ (202 MHz; CDCl₃) -0.85 (2 P), -1.50 (2 P), -2.86 (1 P); HRMS (ESI+) m/z 1543.3889 (1543.3851 calc. for C₇₉H₈₁O₂₁P₅Na (M+Na)⁺); 783.6905 (783.6911 calc. for C₇₉H₈₁O₂₁P₅Na (M+2Na)²⁺).

myo-inositol-1,2,3,4,6-pentakis(dibenzyl)phosphate (7)

To a solution of the penta-phosphate **6** (120mg, 0.079 mmol, 1 equiv.) in MeOH (5 mL) was added palladium dichloride (20 mg, 0.095 mmol, 1.5 equiv.) in a single portion. The resulting suspension was stirred vigorously for 3 h. The solvent was dried and the given dark residue was suspended in CH₂Cl₂ (20 mL) and H₂O₂ (20 mL, 15% v/v in H₂O) for 15 min. The reaction mixture was filtered though Celite to remove Pd catalyst, and washed with CH₂Cl₂. The organic phase was separated and the aquase phase was extracted with more CH₂Cl₂ (3 x 50 mL) and then the solvent was removed *in vacuo* before re-suspension in CH₂Cl₂ (20 mL) and H₂O₂ (6 mL, 30%) for 20 min. The organic phase was then separated from aqueous phase and then extracted with CH₂Cl₂ (20 mL x 4). The combined organic phase was removed *in vacuo* to give the crude product. Flash chromatography (50%-80 % ethyl acetate/petroleum spirit) afforded the product 7 (99 mg, 85%) as a colorless oil.

R_f 0.33 (1:1 ethyl acetate/petroleum spirit); v_{max} (neat)/cm⁻¹ 3040-2950, 1456, 1274, 1216, 1034, 1017, 896, 737, 696; $\delta_{\rm H}$ (500 MHz; CDCl₃) 7.31-7.07 (m, 50H), 5.52 (d, *J* = 9 Hz, 1H), 5.26 (d, *J* = 2 Hz, 1H), 5.13-4.91 (m, 20H), 4.83 (dd, *J* = 18, 9 Hz, 2H), 4.41 (t, *J* = 10 Hz, 2H), 3.78 (dt, *J* = 9, 2 Hz, 1H); $\delta_{\rm C}$ (125 MHz; CDCl₃) 135.6, 135.5, 128.5, 128.4, 128.3, 128.1, 127.9, 127.8, 78.2, 76.5, 73.6, 72.3, 70.0, 69.9, 69.7; $\delta_{\rm P}$ (202 MHz; CDCl₃) -0.16 (2 P), -1.58 (2 P), -2.51 (1 P); HRMS (ESI+) m/z 1503.3572 (1503.3538 calc. for C₇₆H₇₇O₂₁P₅Na (M+Na)⁺); 763.1736 (763.1715 calc. for C₇₆H₇₇O₂₁P₅Na₂ (M+2Na)²⁺).

Benzyloxy-N-diisopropylamino ethoxy ethyl-phosphine (11)

Firstly, Cbz protected 2-(2-aminoethoxy)ethanol was synthesised by treatment of 2-(2aminoethoxy)ethanol with benzyl chloroformate in aqueous NaOH and quenched with 1 M HCl. The compound was purified by flash chromatography (1:1 DCM:EA to 100% EA) as an oil as reported.(17) To synthesise the phosphoramidite, a solution of the (aminoethoxy)ethanol (66 mg, 0.28 mmol, 1 equiv.) in dry dichloromethane (2 mL) was added to a stirred solution of (benzyloxy)bis(N,Ndiisopropylamino)phosphine (0.75 mmol, 3 equiv.) and 1H-tetrazole (1.78 mL, 0.45 M, 0.80 mmol, 3.2 equiv.) in dry dichloromethane (1 mL) under nitrogen. After stirring at room temperature overnight, the reaction mixture was diluted with dichloromethane (10 mL) and quenched by addition of saturated sodium hydrogenearbonate solution (10 mL). The organic phase was separated and the aqueous phase was extracted with dichloromethane (3 x 10 mL). The combined organic phases were washed with brine (50 mL), dried over MgSO₄ and the solvent was removed in vacuo. Flash chromatography (5/15/80 triethylamine/ethyl acetate/petroleum spirit) afforded the phosphoramidite (130 mg, 0.28 mmol, quantit.) as a pale oil. $R_f 0.9$ (50% ethyl acetate in petroleum spirit); δ_H (500 MHz; CDCl₃) 7.40-7.25 (m, 10H), 5.32 (br s, 1H), 5.10 (s, 2H), 4.76 (dd, J = 12, 8 Hz, 1H), 4.67 (dd, J = 12, 8 Hz, 1H), 3.85-3.70 (m, 2H), 3.69-3.61 (m, 4H), 3.61-3.53 (m, 2H), 3.39 (dd, J = 10, 5 Hz, 2H), 1.19 (t, J = 7, 7 Hz, 12H); δ_C (125 MHz; CDCl₃) 156.4, 139.4, 136.6, 128.4, 128.2, 128.1, 128.0, 127.2, 127.012, 71.2, 71.1, 69.9, 66.6, 65.4, 65.2, 62.8, 62.6, 43.0, 42.9, 40.9, 24.6, 24.5; δ_P (202 MHz; CDCl₃) 147.7; HRMS (ESI+) m/z 477.2539 (477.2518 calc. for C₂₅H₃₈N₂O₅P (M+H)⁺).

5-O-(Benzyloxycarbonyl amino) ethoxy ethyl-benzylphosphoryl-*myo*-inositol 1,2,3,4,6-pentakis (dibenzyl) phosphate (8)



To a solution of 1*H*-tetrazole in acetonitrile (0.34 ml, 0.45M, 0.15 mmol, 7 equiv.) was added a solution of phosphoramidite **11** (64 mg, 0.134 mmol, 6 equiv.) in dry dichloromethane (2 mL) under nitrogen. After stirred at room temperature for 30 min, a solution of **7** (28 mg, 0.0189 mmol, 1 equiv.) in dry dichloromethane (1 mL) was added to the mixture dropwise. After stirring at room temperature overnight, *m*CPBA (50 mg, 70%, 0.201 mmol, 9 equiv.) was added in a single portion at -78 °C and stirred for 30 min. Then the mixture was warmed up to room temperature and after stirring for 1.5 h, the reaction mixture was worked up by dilution with NaHSO₃ (2 mL) and dichloromethane (5 mL). The organic phase was washed with NaHCO₃, brine and dried through MgSO₄. The organic extract was concentrated *in vacuo* to give the crude product. Flash chromatography eluting with 50-100% ethyl acetate/petroleum spirit afforded the protected lipid **8** (22 mg, 11.8 µmol, 62%) as a colorless oil.

 $R_f = 0.25$ (60% ethyl acetate/petroleum spirit); v_{max} (neat)/cm ⁻¹ 1627, 1277, 1018, 738, 698; δ_H (500

MHz, CDCl₃) 7.40-7.00 (m, 60H), 5.61 (d, J = 9 Hz, 1H), 5.57 (t, J = 5 Hz, 1H), 5.25-4.85 (m, 27H), 4.50-4.32 (m, 2H), 4.14-4.05 (m, 1H), 4.04-3.92 (m, 1H), 3.42-3.33 (m, 2H), 3.33-3.25 (m, 2H), 3.17 (dd, J = 10, 5 Hz, 2H); $\delta_{\rm C}$ (125 MHz, CDCl₃) 156.5, 136.7, 136.0, 135.9, 135.8, 135.7, 135.6, 128.4, 128.3, 128.20, 128.1, 128.0, 127.9, 127.8, 75.1, 74.6, 74.5, 73.3, 70.0, 69.9, 69.8, 69.7, 69.4, 67.2, 67.1, 66.4, 40.8; $\delta_{\rm P}$ (202 MHz, CDCl₃) -1.0 (1P), -1.2 (2P), -1.6 (2P), -2.5 (1P); HRMS (ESI+) m/z1894.4798 (1894.4722 calc. for C₉₅H₉₉NO₂₇P₆Na (M+Na)⁺); 958.7304 (958.7307 calc. for C₉₅H₉₉NO₂₇P₆Na₂ (M+2Na)²⁺).

5-O-Aminoethoxyethyl-phosphoryl-myo-inositol 1,2,3,4,6-pentakisphosphate (12)

$$(HO)_2OPO OPO(OH)_2 OPO(OH)_2 OPO(OH)_2 OPO OPO OPO(OH)_2 OPO OPO OPO(OH)_2 OPO(OH)_2 OPO(OH)_2 OPO(OH)_2 OPO(OH)_2 OPO OPO(OH$$

A solution of protected intermediate **8** (10 mg, 5.4 µmol, 1 equiv.) in 200 uL dry CDCl₃ was added (iodomethyl)trimethylsilane solution (0.162 mmol, 22 uL, 30 equiv.) at 0 °C, stirred for 10 min and then warmed up to room temperature for further 45 min. The reaction was monitored by TLC until the starting material **8** was consumed. The reaction mixture was concentrated at 0 °C *in vacuo* to give a brown residue. 2 mL of dry THF and 2 mL of DMF was added to re-dissolve the residue and then was dried again *in vacuo* at 0 °C. The given residue was dried under high vacuum for 3 days before exposed to 2 mL dry MeOH for 20 min at 0 °C followed by 50 min at room temperature. The solvent was evaporated off and given residue was dissolved up in 5 mL ethyl acetate and 3 mL milli Q water. The water was separated and was washed with more ethyl acetate (2 mL X 4) followed by CHCl₃ (2 mL X 2). The water phase was lyophilized to give **18** as a slight yellow solid (2.4 mg, 3.22 µmol, 60%). $\delta_{\rm H}$ (500 MHz, D₂O) 5.04-4.95 (m, 1H), 4.62 (dd, J = 11, 7 Hz, 2H), 4.47-4.40 (m, 3H), 4.28-4.19 (m, 2H), 3.87-3.67 (m, 4H), 3.26 (dd, J = 9, 4 Hz, 2H) ; $\delta_{\rm C}$ (125 MHz, D₂O/CD₃OD) 76.7, 76.2, 75.5, 73.0, 71.6, 69.9, 66.3, 66.0, 60.4, 39.1; HRMS (ESI+) *m/z* 747.9384 (747.9371 calc. for C₁₀H₂₈NO₂₅P₆ (M+H)⁺); 769.9208 (769.9190 calc. for C₁₀H₂₇NO₂₅P₆Na [(M+Na-H)+H]⁺); $\delta_{\rm P}$ (202 MHz; D₂O) 0.5 (6P); HRMS (ESI-) *m/z* 745.9244 (745.9225 calc. for C₁₀H₂₆NO₂₅P₆ (M-H)⁻); 767.9058 (767.9045 calc.

for $C_{10}H_{26}NO_{25}P_6Na[(M+Na-H)-H]^-)$; 372.4585 (372.4576 calc. for $C_{10}H_{25}NO_{25}P_6(M-2H)^{2-}$).

The phosphorus NMR spectrum recorded of the free acid form of **12** (~5 mM in D₂O) is broad, consistent with previous observation (22). The spectrum behavior of IP₆ has been extensively studied previously (22-25) and the broadening of the free acid form of IP₆ was reported. The complication of the phosphorus spectrum of IP₆ was observed and was stated to be highly sensitive to the specific nature of the solvating medium (cation types, pH, the ionic strength and concentration etc) (22, 25). Variation in any of the conditions can cause shifts in the spectrum. The broadening of resonances in the proton NMR spectrum of the free acid form was also observed due to the presence of an interconverting mixture of the axial/equatorial conformers.(22, 25) To maintain the purity of final compound and avoid possible contaminations, the phosphors NMR was not further tuned by changing pH or introducing ions/salts to the solvent system.

5-OH-tethered IP₆ conjugated-Dynabeads (13)



Firstly, Dynabeads M-270 Carboxylic Acid (Invitrogen, DYNAL) suspension 200 uL (0.90 µmol activate chemical functionality, 4.5 µmol/mL, 150 µmol/g activate chemical functionality at concentration of 30 mg/mL) was washed twice with 25 mM MES (pH = 5), using the equal volume of Dynabeads (200 µl) pipetted out of the vial, for ten minutes with good mixing each time. NH₂-IP₆ **12** (76 µg, 0.1012 µmol in 20 uL milliQ H₂O₂) in 100 µL of 25 mM MES (pH = 5) was added to the washed Dynabeads. Mix well and incubate with slow tilt rotation at room temperature for 30 minutes. Immediately before use dissolve 6 mg EDC in 60 uL of cold 100 mM MES (pH = 5) and add EDC solution to the Dynabeads/ligand suspension. Mix well. Add another 20 uL of 25 mM MES (pH = 5) to make the final volume of 200 µL. Incubate the given suspension for four hours or longer at 4 °C with slow tilt rotation. To remove any uncoupled NH₂-IP₆, place the tube containing given Dynabeads on the magnet for 1 min to remove the supernatant and the beads were washed three times with milliQ water (200 µL x 3), and then three times with PBS (200 µL x 3). The supernatant and all the washes were kept for further analysis.

The procedure was repeated three times with NH₂-IP₆ **12** (0.354 µmol) used for coupling to make 700 uL of IP₆ conjugated Dynabeads M-270. The supernatant and washes from three experiments were combined for lyophilization to obtain more accurate 1H-NMR analysis of any uncoupled NH₂-IP₆ left. To the combined supernatant and washes (containing unreacted amino-IP₆ **12**) was added a solution of *myo*-inositol orthoformate (0.526 µmol, 100 µL, C = 1.0 mg/mL in D₂O), and no NH₂-IP₆ was observed in the given ¹H NMR compared to its own ¹H NMR spectrum carefully, and with sharp signals from *myo*-inositol orthoformate as reference. Therefore, all of NH₂-IP₆ (0.1012 µmol) to beads (0.90 µmol), which is 11%.

C References for supporting information

- Whitehead RH, Macrae FA, St John DJ, & Ma j (1985) A colon cancer cell line (LIM1215) derived from a patient with inherited nonpolyposis colorectal cancer. J. Natl. Cancer Inst. 74(4):759-765.
- 2. Catimel B, et al. (2008) The PI(3,5)P2 and PI(4,5)P2 interactomes. J. Proteome Res. 7(12):5259-5313.
- 3. Catimel B, et al. (2009) PI(3,4,5)P3 Interactome. J. Proteome Res. 8(7):3721-3726.
- 4. Catimel B, *et al.* (2013) The PI(3)P interactome from a colon cancer cell. *Journal of Proteomics* 82(0):35-51.
- 5 Kapp, E.A et. al. (2003) Mining a tandem mass spectrometry database to determine the trends and global factors influencing peptide fragmentation. Anal. Chem. 75 (22):6251-6254)
- Nguyen TN & Goodrich JA (2006) Protein-protein interaction assays: eliminating false positive interactions. *Nat. Meth.* 3(2):135-139.
- 7. Petrak J, *et al.* (2008) Déjà vu in proteomics. A hit parade of repeatedly identified differentially expressed proteins. *Proteomics* 8(9):1744-1749.
- Carmona-Saez P, Chagoyen M, Tirado F, Carazo J, & Pascual-Montano A (2007) GENECODIS: a web-based tool for finding significant concurrent annotations in gene lists. *Genome Biol.* 8(1):R3.
- 9. Nogales-Cadenas R, *et al.* (2009) GeneCodis: interpreting gene lists through enrichment analysis and integration of diverse biological information. *Nucleic Acids Res.* 37(suppl 2):W317-W322.
- Tabas-Madrid D, Nogales-Cadenas R, & Pascual-Montano A (2012) GeneCodis3: a nonredundant and modular enrichment analysis tool for functional genomics. *Nucleic Acids Res.* 40(W1):W478-W483.
- Gaidarov I, Krupnick JG, Falck JR, Benovic JL, & Keen JH (1999) Arrestin function in G protein-coupled receptor endocytosis requires phosphoinositide binding. *EMBO J.* 18(4):871-881.
- Milano SK, Kim Y-M, Stefano FP, Benovic JL, & Brenner C (2006) Nonvisual Arrestin Oligomerization and Cellular Localization Are Regulated by Inositol Hexakisphosphate Binding. J. Biol. Chem. 281(14):9812-9823.
- Milano SK, Pace HC, Kim Y-M, Brenner C, & Benovic JL (2002) Scaffolding Functions of Arrestin-2 Revealed by Crystal Structure and Mutagenesis[†],[‡]. *Biochemistry (Mosc.)* 41(10):3321-3328.
- 14. Pangborn AB, Giardello MA, Grubbs RH, Rosen RK, & Timmers FJ (1996) Safe and Convenient Procedure for Solvent Purification. *Organometallics* 15:1518-1520.
- 15. Lee HW & Kishi Y (1985) Synthesis of mono- and unsymmetrical bis-orthoesters of scylloinositol. *The Journal of Organic Chemistry* 50(22):4402-4404.
- 16 Billington DC, *et al.* (1989) The total synthesis of myo-inositol phosphates via myo-inositol orthoformate. *Journal of the Chemical Society, Perkin Transactions 1* (8):1423-1429.
- 17 Lampe D, Liu CS, & Potter BVL (1994) Synthesis of selective non-Ca2+ Mobilizing Inhibitors of D-myo-Inositol 1,4,5-trisphosphate 5-phosphatase. J. Med. Chem. 37(7):907-912.
- 18. Boyce JP, et al. (2008) Identification of 14-3-3ζ by Chemical Affinity with Salicylanilide

Inhibitors of Interleukin-12p40 Production. Bioconjug. Chem. 19(9):1775-1784.

- Yu K-L & Fraser-Reid B (1988) A novel reagent for the synthesis of myo-inositol phosphates: N,N-diisopropyl dibenzyl phosphoramidite. *Tetrahedron Lett.* 29(9):979-982.
- 20. Bannwarth W & Trzeciak A (1987) A Simple and Effective Chemical Phosphorylation Procedure for Biomolecules. *Helv. Chim. Acta* 70(1):175-186.
- Perich JW & Johns RB (1987) A new, convenient and efficient general procedure for the conversion of alcohols into their dibenzyl phosphorotriesters using N,N-diethyl dibenzyl phosphoramidite. *Tetrahedron Lett.* 28(1):101-102.
- 22. Costello AJR, Glonek T, & Myers TC (1976) 31P Nuclear magnetic resonance □pH titrations of myo-inositol hexaphosphate. *Carbohydr. Res.* 46(2):159-171.
- Barrientos LG & Murthy PPN (1996) Conformational studies of myo-inositol phosphates. Carbohydr. Res. 296(1–4):39-54.
- Isbrandt LR & Oertel RP (1980) Conformational states of myo-inositol hexakis(phosphate) in aqueous solution. A carbon-13 NMR, phosphorus-31 NMR, and Raman spectroscopic investigation. J. Am. Chem. Soc. 102(9):3144-3148.
- 25. Emsley J & Niazi S (1981) THE STRUCTURE OF myo-INOSITOL HEXAPHOSPHATE IN SOLUTION: 31P N.M.R. INVESTIGATION. *Phosphorous and Sulfur and the Related Elements* 10(3):401-407.
- 26. Xiao K, *et al.* (2007) Functional specialization of β-arrestin interactions revealed by proteomic analysis. *Proceedings of the National Academy of Sciences* 104(29):12011-12016.
- Parisis N, Metodieva G, & Metodiev MV (2013) Pseudopodial and β-arrestin-interacting proteomes from migrating breast cancer cells upon PAR2 activation. *Journal of Proteomics* 80(0):91-106.

D Summary and captions for supporting information figures, tables and datasets:

Fig. S1. Interaction of IP6 beads with cytosolic fractions following Mono Q anion exchange chromatography referring to Experiment 2. (A) Chromatographic profile of LIM1215 cytosolic extracts on Mono Q (see Materials and Methods for chromatographic conditions). (B) SDSPAGE analysis of proteins purified using IP6 beads. Fractions (8 mL each) where indicated were pooled, combined and concentrated by centrifugation into 0.5 mL of volume and then buffer exchanged to 1 mL in TBS before incubation with blank beads (lanes on the left) or IP6 beads (lanes on the right). After incubation and extensive washings, retained proteins on beads were subjected to SDS-PAGE and excised bands (indicated by numbers) were taken for MS-MS analysis. (C) Specific IP6 interacting proteins identified by MS-MS analysis. Specific binding proteins were identified by comparison with blank derivatised beads used under the same experimental conditions. Non specific binding proteins were removed.

Fig. S2. Singular enrichment analysis in biological processes (Fig. S2.A) and KEGG pathways (Fig. S2.B) of IP6 binding proteins using GeneCodis3 database. The figure is categorized by the most number of proteins identified in the first annotations found using GeneCodis3.

Fig. S3. String analysis of protein-protein interactions of identified IP6 specific proteins generated by STRING 9.05 database (http://string-db.org). Disconnected proteins were not shown. The interactions are shown as various coloured lines between proteins and the required confidence (score) is above medium confidence 0.400.

Table S1-Specific IP₆ **binding proteins isolated in both experiments.** MudPIT scores and numbers of significant unique peptides identified are shown in brackets.

Table S2-Proteins identified as potential β **-arrestin interactors.** The IP₆ binding proteins we identified were compared with the previously reported β -arrestin 1 and β -arrestin 2 interacters (26 27). The overlapped proteins shown in the list are potentially pulled down complexed with β -arrestins.

Table S3 - Biological process enrichment of the newly identified proteins with magnesium (excluding the proteins previously identified in the original study).

Dataset 1- Summery of specific proteins identified with IP_6 beads for two experiments using the ABSCIEX 5600 qTOF mass spectrometer. Non-specific proteins were removed. Annotations and legends for MS results are identical to Datset 2 and 3.

Dataset 2-A.Data analysis of proteins identified on blank beads using the ABSCIEX 5600 qTOF mass spectrometer. Original results were searched using the MASCOT version 2.3.01 search algorithm against SWISSPROT database. The MS/MS results interpretation and mass spectrometric annotations can be found on Mascot website (http://www.matrixscience.com/). Each peptide match/hit is listed individually with all mass spectrometric parameters including score, rank, sequence etc (http://www.matrixscience.com/help/export_help.html). Protein list is ordered by numbers of peptide hits found for each protein. B. Data analysis of proteins identified on IP₆ beads using the ABSCIEX 5600 qTOF mass spectrometer.

Dataset 3- A. Singular Enrichment Analysis of Biological Process. The table shows the top single biological process annotations found by GeneCodis3. NGR = Number of annotated genes in the reference list, TNGR = Total number of genes in the reference list; NG = Number of annotated genes in the input list, TNG = Total number of genes in the input list, Hyp = Hypergeometric pValue, Hyp* = Corrected hypergeometric pValue. **Dataset 3- B. Singular Enrichment Analysis of KEGG Pathways.** The table shows the top single KEGG pathway annotations found by GeneCodis3.

Dataset 4-NMR Spectra

Dataset 5 – Proteins identified from the additional experiments with 5 mM Magnesium – proteins identified in the previous experiments with no magnesium present are highlighted in red. Data set includes both the protein report and the peptide report.