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Mechanisms of biased beta-arrestin mediated signaling downstream from the cannabinoid 1 receptor (CB1R)

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Non-Standard Abbreviations:

GRK, G protein-coupled receptor kinases; GPCRs, G protein-coupled receptors; 2-AG, 2-arachidonoylglycerol; AEA, anandamide; CB1R, cannabinoid 1 receptor; CP; CP55,940; PTX, Pertussis toxin; Δ 9-THC, Δ 9-tetrahydrocannabinol; WIN, WIN55,212-2; ERK1/2, Extracellular signal-regulated protein kinases 1 and 2; CREB, cAMP response element-binding protein; Src, SRC Proto-Oncogene, Non-Receptor Tyrosine Kinase; p38a, Mitogen-Activated Protein Kinase 14; RFP, monomeric Red Fluorescent Protein; SEP, Super-ecliptic phluorin.

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Abstract

Activation of G protein-coupled receptors (GPCR) result in multiple waves of signaling which are mediated by heterotrimeric G proteins and the scaffolding proteins beta-arrestin 1/2. Ligands can elicit full or subsets of cellular responses, a concept defined as ligand bias or functional selectivity. However, our current understanding of beta-arrestin mediated signaling is still very limited. Here we provide a comprehensive view of beta-arrestin mediated signaling from the cannabinoid 1 receptor (CB1R). Utilizing a signaling biased receptor, we define the cascades, specific receptor kinases and molecular mechanism underlying beta-arrestin mediated signaling: We identify the interaction kinetics of CB1R and beta-arrestin 1 during their endocytic trafficking as directly proportional to its efficacy. Finally, we demonstrate that signaling results in the control of genes clustered around prosurvival and proapoptotic functions among others. Together, these studies constitute a comprehensive description of beta-arrestin mediated signaling from CB1Rs and suggest modulation of receptor endocytic trafficking as a therapeutic approach to control beta-arrestin mediated signaling.

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Introduction

Ligand induced signaling from G protein-coupled receptors (GPCRs) was initially conceptualized as a linear series of sequential steps leading to specific biological outcomes. Research over the last 20 years has shown that many ligands can differentially stabilize receptors into multiple signaling conformations resulting in pluridimensional efficacies, a concept defined as functional selectivity or biased signaling (Kenakin, 2011; Shenoy and Lefkowitz, 2011; Urban et al., 2007). This complexity at the signaling level has significantly changed our understanding of GPCRs function and provides new challenges and opportunities for drug discovery (Atwood et al., 2012; Chang and Bruchas, 2014; Kenakin, 2007). Upon ligand activation, GPCRs undergo conformational changes leading to activation of heterotrimeric G proteins and their effectors such as adenylyl cyclase among others. These conformations are detected by G protein-coupled receptor kinases (GRKs) and they differentially phosphorylate GPCRs, generating specific patterns or barcodes depending on the ligand (Liggett, 2011; Nobles et al., 2011). These barcodes are subsequently recognized by beta-arrestins, which are recruited to the plasma membrane sterically hindering G-protein-receptor interactions and terminating the first wave, while initiating the second wave, of receptor signaling (Pierce et al., 2002; Shenoy and Lefkowitz, 2011). More recently a third wave has been described, where some GPCRs can re-engage in G protein signaling after internalization in specific intracellular compartments (Irannejad and von Zastrow, 2014).

Beta-arrestins have two major roles—as negative regulators of heterotrimeric G protein signaling during receptor desensitization and internalization, and as signaling scaffolds (Gainetdinov et al., 2004; Schmid and Bohn, 2009; Tzingounis et al., 2010). As negative regulators of receptor activity, beta-arrestins block G protein signaling and recruit components of the endocytic

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machinery to initiate receptor endocytosis (Claing et al., 2002; Goodman et al., 1996). As a signaling scaffold molecule, the focus has been placed on their role during the activation of selected downstream cascades such as MAPK (DeFea, 2011). At the mechanistic level, the kinetics of interaction between receptors and beta-arrestins during endocytosis have been suggested as a mechanism to control beta-arrestin mediated signaling efficacy (Flores-Otero et al., 2014; Shenoy et al., 2009). In this scenario, ligand activation results in specific phosphorylation barcodes, that control the recruitment and kinetics of receptor-arrestin interactions and beta-arrestin mediated signaling (Reiter et al., 2012; Shenoy and Lefkowitz, 2011). However, our understanding of beta-arrestin mediated signaling is still rudimentary and limited to selected well-studied signaling pathways without much information on their roles or the mechanisms controlling them; yet, beta-arrestin mediated pathways have been proposed as therapeutic targets in several disorders (Allen et al., 2011; Gurevich, 2014; Urs et al., 2015).

To delineate the complex mechanisms, physiological roles and therapeutic potential of beta-arrestin mediated signaling, a more comprehensive approach including cell networks analysis and transcriptomics is needed (Maudsley et al., 2013). To achieve this goal, we sought to investigate the dynamic mechanisms of beta-arrestin mediated signaling downstream from the cannabinoid 1 receptor (CB1R), one of the most abundant GPCRs in the CNS and the target of Δ^9 -THC, the main psychoactive ingredient in marijuana. Our results distinguish beta-arrestin mediated signaling from G protein signaling, illustrate the pharmacological complexity and mechanisms contributing to this pathway and propose modulation of the interaction between receptor and beta-arrestin as a mechanism to control signaling output. Furthermore, these results suggest that therapeutic drugs developed to control beta-arrestin mediated signaling for the CB1R could present a novel approach to target the long-term effects of CB1R activation.

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Finally, our work also provides a framework to investigate beta-arrestin signaling from other GPCRs and suggests endocytic dwell times as new biomarkers for identifying beta-arrestin biased compounds.

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Materials and Methods

Cell Culture and Transfections

Human embryonic kidney (HEK) 293 cells (ATCC, Manassas,VA) were maintained in Dubecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 3.5 mg/ml glucose at 37°C in 5% CO₂. SEP-CB1R cDNA in the pcDNA3.1 vector was a generous gift from Andrew Irving (University of Dundee, Scotland, UK) and the SEP-S426A/S430A construct was generated by site-directed mutagenesis (QuickChange, Agilent Technologies, Santa Clara, CA) using the SEP-CB1R construct as a template. The plasmids pcDNA3.1-3xHA-CB1Rs encoding the N-terminal 3xHA epitope-tagged CB1Rs were generated by PCR using the HA-CB1 plasmid (Daigle et al., 2008 J. Neurochem 106:70-82). For transfection, HEK 293 cells were seeded in a 6-well plate (300,000 cells per well) and transfected with a total of 2 µg of plasmids using lipofectamine (Life technologies, Grand Island, NY) according to the manufacturer's instructions. 24 hrs post-transfection, cells were incubated for an additional 16 hrs in serum-free growth media prior to agonist treatment. siRNA (Qiagen, Valencia, CA) transfection was carried out as previously described (Flores-Otero et al., 2014; Roman-Vendrell et al., 2012). GRK expression was targeted using the following siRNA sequences: GRK2, 5'-CCGGGAGATCTTCGACTCATA-3'; GRK3, 5'-AAGATGTTTCAGTGTTGGGTAA-3'; GRK4, 5'-CCGGGTGTTTCAAAGACATCA-3'; GRK5, 5'-AGCGTCATAACTAGAACTGAA-3', GRK6, 5'-AAGGATGTTCTGGACATTGAA-3'. Silencing of GRK expression was assessed by immunoblotting using anti-GRK2 antibody (1: 2000; Cell Signaling, Cell Signaling Technology, Danvers, MA), anti-GRK3, anti-GRK5, and anti-GRK6 antibodies (1:3000, 1: 6000, and 1:3000,

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respectively; Abcam, Cambridge, MA) and anti-GRK4 antibody (1: 3000; Sigma-Aldrich, St Louis, MO).

Immunoblotting Studies

Following serum starvation for 16 hrs cells expressing SEP-CB1R were washed twice and exposed to either 1 μ M WIN or 10 μ M 2-AG diluted in DMEM for 5, 10, 15 and 30 min at 37 °C. To observe the effect of pertussis toxin (PTX) on ERK1/2 phosphorylation, cells were pretreated with 10 ng/ml for 16 hours at 37 °C. For gene silencing experiments, siRNA transfection for beta-arrestin 1 and beta-arrestin 2 was carried out as previously described (Flores-Otero et al., 2014). Cells were then washed with ice-cold PBS and lysed in ice-cold lysis buffer consisting of 150 mM NaCl, 1.0% IGEPAL[®] CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 7.5, and a protease inhibitor cocktail 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), pepstatin A, E-64, bestatin, leupeptin, and aprotinin; Sigma-Aldrich, St Louis, MO). Cell lysates were cleared by centrifugation at 18,500 g for 15 min at 4 °C. 13 μ g of total proteins were resolved by SDS-PAGE gel electrophoresis in 10% gels, and transferred onto polyvinylidene fluoride (PVDF) membrane. After incubating with blocking reagent (Fisher Scientific, Pittsburgh, PA), the membrane was incubated for 1hr at room temperature with the primary antibody (1:4000 phospho-p44/42 and p44/42 antibodies; Cell Signaling Technology, Danvers, MA). After incubation with anti-rabbit peroxidase-conjugated secondary antibody (1:6000; Cell Signaling Technology, Danvers, MA) for 50 min at room temperature, the specific immunoreactivity was visualized using the SuperSignal West Femto Chemiluminescent Substrate System (Thermo Fisher Scientific, Rockford, IL). Immunoreactive bands of phospho-ERK1/2 were quantified by densitometric analysis using the ImageJ program

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(<http://rsb.info.nih.gov/ij/>) and normalized to the intensity of total-ERK1/2. Data are expressed as a fold increase above the basal level of phosphorylation.

Co-immunoprecipitation

HEK293 cells were co-transfected with HA-CB1R and either beta-arrestin 1-RFP or beta-arrestin 2-RFP and treated with WIN and 2-AG as described above. Cells were lysed in a buffer containing 1% n-dodecyl- β -D-maltoside, 10% glycerol, 250 mM NaCl, 50 mM Tris (pH 8), 0.5 mM EDTA, and protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO). The pre-cleared supernatant fraction (approximately 400 μ g) was incubated overnight at 4 °C with 2 μ g of anti-HA antibody (Roche, Indianapolis, IN) and Protein A/G Plus-Agarose (Santa Cruz Biotechnology, Dallas, TX). The beads were washed four times in lysis buffer, and elution was performed in 40 μ l of reducing 1X Laemmli buffer at 37 °C for 30 min. Samples were separated by SDS-PAGE gel electrophoresis in 10% gels and subjected to Western blotting using anti-RFP and anti-HA antibodies as described above.

Total Surface fluorescence

Receptor expression levels were compared between wild-type and mutant receptors by total surface fluorescence. HEK293 cells transiently expressing SEP-CB1R or SEP-CB1R S426A/S430A 72 hours after transfection were analyzed with a Cellometer Vision from Nexcelom Bioscience (Lawrence, MA) following manufacturer protocol (Chan et al., 2011). Briefly, cells were harvested and centrifuged for 5 minutes at 1,500 RPM. Cells were resuspended in PBS and transferred to imaging chambers. Captured bright-field and fluorescence images using the GFP filter set (optic module 1: VB535-402) were saved and analyzed utilizing the equipment software. Acquisition exposure was set to 7.5 seconds.

TIRF and confocal microscopy.

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TIRF microscopy was performed as previously described (Roman-Vendrell and Yudowski, 2015; Yudowski and von Zastrow, 2011). Briefly, HEK293 cells transiently expressing SEP-CB1R or SEP-CB1R S426A/S430A were imaged utilizing a Motorized Nikon Ti-E inverted microscope with a CFI-Apo X 100 1.49 oil TIRF objective lens and a motorized stage with perfect focus (Melville, NY, USA). Light sources were 488- and 561-nm Coherent sapphire lasers (Coherent Inc., Santa Clara, CA, USA) with 50 and 100mW, respectively. The microscope was coupled to an iXonEM + DU897 back illuminated EMCCD camera (Andor, Belfast, UK). Imaging settings were kept constant throughout our imaging: readout speed: 10 Hz, exposure time: 100 ms every 3 s, EM gain 300, binning: 1 X 1, image: 512 X 512, BitDepth = 14 bits, temperature: -75 and laser power: 10%. Cells were kept at 37°C with a Stable Z stage and objective warmer (Bioptechs, Butler, PA, USA). Cells were gently rinsed three times with OptiMem supplemented with 20mM HEPES (Life Technologies) and kept in the incubator for 10–30 min to acclimate before imaging. TIRF microscopy recording was conducted in the same imaging media for 1–3 min under basal condition (without any treatment) and was followed by bath application of selected ligand using a custom-built perfusion chamber as previously described (Flores-Otero et al., 2014; Roman-Vendrell and Yudowski, 2015). Total time of live-imaging visualization and recording was less than 30 min. Confocal microscopy was performed utilizing a Zeiss LSM 5 Pascal laser-scanning confocal microscope equipped with Ar 488 nm, and HeNe 543 nm laser-lines, using a 63x Plan-APOCHROMAT oil-immersion objective (NA = 1.4), with the following emission filters: BP 505-530, and BP 560-615, respectively. Images were collected at 1024 x 1024 pixel resolution using sequential scanning mode.

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Image processing and analysis

Analysis was performed using the public domain NIH Image program ImageJ/FIJI software, which is freely available at <http://fiji.sc/Fiji> as described before (Flores-Otero et al., 2014; Roman-Vendrell et al., 2014; Yudowski and von Zastrow, 2011). Briefly, raw images were first background-subtracted and flat field-corrected. Individual endocytic events were quantified by an observer blinded to experimental details, multiple times manually and using the particle tracking algorithm two-dimensional spot tracker. Event location, time and fluorescence profile were logged and recorded. Individual endocytic events were identified and scored according to the following criteria: (1) individual events appeared and disappeared within the time series; (2) endocytic events displayed limited movement in the x and y axes as described for clathrin endocytic pits during their maturation and (3) the events did not collide or merge with other structures. Dwell times were calculated as the time between the first frame where spot tracker detected an event above background fluorescence levels and the last. As the fluorescence from individual events can fluctuate and the algorithm from the tracking software can misinterpret endocytic events, we manually verify all individual events after automated analysis. To analyze statistical significance between groups, we counted the number of events in each independent experiment (i.e., each separate imaging session and different dish of cultured cells were treated as independent experiments), analyzed their normality by D'Agostino and Pearson test and used unpaired two-tailed Student's t -tests to test for statistical significance. All data are expressed as means \pm s.e.m unless stated. Statistical analyses between dwell times were calculated utilizing the GraphPad Prism Software (La Jolla, CA, USA). Box and whiskers plot represent minimum and maximum values, the box extends from 25 to 75% with the mean value.

Phospho-kinase Array Analysis

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The phosphor-kinase array was performed using the Proteome Profiler Human Phospho-Kinase Array Kit (R&D Systems, Minneapolis, MN). Briefly, HEK293 cells expressing the CB1R wild-type or S426/430A mutant receptor at similar expression levels (Supplemental figure 4) were treated with either 1 μ M WIN or 10 μ M 2-AG for 5 and 15 min. PTX treatment and siRNA transfection for beta-arrestins were carried out as described above. Cells were lysed with lysis buffer (R&D Systems) and agitated for 30 min at 4 °C. Cell lysates were subjected to protein assay. Pre-blocked nitrocellulose membranes spotted with antibodies for 43 kinases were incubated with 400 μ g of the lysates overnight at 4 °C on a rocking platform. The membranes were incubated with a biotinylated detection antibody cocktail and then streptavidin-HRP. Chemiluminescent detection reagents were applied to detect spot densities. Array images were analyzed and quantified by densitometric analysis using the ImageJ program (<http://rsb.info.nih.gov/ij/>). Every spot was subtracted by the averaged background level from negative control spots. The phospho-antibody array experiment was repeated three times. Data of duplicated spots from three array results were expressed with the relative fold change over the basal level (in the absence of agonist). Statistical analysis was performed using one-way ANOVA followed by Bonferroni's post hoc test.

RNA-seq Experiments and Transcriptome Analysis

HEK293 cells expressing the HA-tagged rat wild-type or S426/430A mutant receptors were treated with 1 μ M WIN for 2 hrs. After extensive washing, total RNA was isolated using TRIzol reagent (Life Technologies, Grand Island, NY). The RNA samples were further cleaned up using RNeasy Mini Kit (Qiagen, Valencia, CA), with purity and quantity assessed spectrophotometrically. RNA was polyA enriched and libraries constructed. Sequencing (50 nt, single strand) was performed using the Illumina HiSeq2000 sequencing system (CGB, Indiana

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University). Each sample gave ~10,000,000 reads/sample with >90% of reads uniquely mapped using Tophat2 ver 2.0.10 and using GRCh38 as the reference genome. Differential expression was examined using DESeq2 (Bioconductor) at a 5% false discovery rate.

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RESULTS

beta-arrestin mediated signaling is enhanced in the CB1R S426A/S430A receptor

To define the signaling cascades mediated by beta-arrestins downstream from the CB1R, we took advantage of the wild-type and S426A/S430A receptors. These serines have been shown to be phosphorylated in previous mass spec analysis and our previous work revealed that mutation of the putative GRK phosphorylation sites S426A/S430A to alanines resulted in lower levels of receptor internalization, reduced desensitization and persistent activation of ERK1/2 independently from Gi/Go, suggestive of a beta-arrestin mediated pathway (Daigle et al., 2008; Huttlin et al., 2010; Morgan et al., 2014; Trinidad et al., 2012; Wiśniewski et al., 2010). This persistent ERK1/2 activation was identical to the response we observed when prolonging receptor-arrestin interactions at the endocytic pit, a mechanism controlling beta-arrestin signaling (Flores-Otero et al., 2014). Sustained ERK1/2 activation by the S426A/S430A receptor led us to the hypothesis that signaling mediated by beta-arrestins could be enhanced in this receptor and used as a tool to investigate this type of signaling. To test this hypothesis, first we analyzed ERK1/2 phosphorylation in HEK293 cells expressing the wild-type CB1R or S426A/S430A receptor exposed to maximal concentrations of the synthetic CB1R agonist WIN 55,212-2 (WIN) (Figure 1A). Second, we assessed the role of beta-arrestins during the sustained phosphorylation of ERK1/2 by using small interfering RNA (siRNA) against beta-arrestin 1 and 2. Silencing beta-arrestin 1 resulted in a complete reduction of ERK1/2 phosphorylation for the S426A/S430A receptor while no significant effect was observed for the wild-type receptor. The effectiveness of siRNAs for beta-arrestins 1 and 2 was confirmed by western blots (Supplemental figure 1). In contrast, reduction of beta-arrestin 2 expression did not show a substantial effect at 5 min (Figure 1B and C). Interestingly however, both wild-type and

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S426A/S430A receptors show a smaller but sustained level of ERK1/2 phosphorylation at the later time points (10 and 15 min). Collectively, our data suggest that beta-arrestin 2 may not have a primary role in ERK1/2 signaling. Rather, as our previous studies indicate, β -arrestin 2 is critical for receptor internalization, and its removal may impair receptor internalization (Ahn et al., 2013; Flores-Otero et al., 2014). The effectiveness of siRNAs for beta-arrestins 1 and 2 was confirmed by western blots (Supplemental figure 1). Finally, consistent with previous findings PTX completely blocked ERK1/2 phosphorylation for the wild-type receptor (Daigle et al., 2008; Flores-Otero et al., 2014) while it had no effect on the mutant receptor (Figure 1D). Together these results suggest that WIN induced prolonged activation of ERK1/2 in the mutant receptor that is solely mediated by beta-arrestin 1.

To further characterize signaling from the S426A/S430A receptor, we evaluated the phosphorylation of ERK1/2 elicited by the endogenous CB1R agonist 2-AG. As we have previously reported for cells expressing the wild-type receptor (Flores-Otero et al., 2014), 10 μ M 2-AG induced a peak at 5 min followed by a slow decay in ERK1/2 phosphorylation. A similar response was observed with the S426A/S430A receptor (Figure 2A). However, only in the latter case, silencing of beta-arrestin isoform 1 and not 2 resulted in a complete reduction of ERK1/2 phosphorylation at 5 min (Figure 2B and C). On the other hand, preincubation with PTX fully abrogated the peak at 5 min in the wild-type receptor, but had no effect on the S426A/S430A receptor (Figure 2D). Interestingly, removal of beta-arrestin 2 also increased beta-arrestin 1 signaling as in figure 1. These results together with data from WIN (Figure 1) support the idea that activation of the S426A/S430A receptor leads to increased beta-arrestin 1 mediated signaling, probably by reducing beta-arrestin 2 induced internalization.

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The kinome downstream from CB1R/ beta-arrestins

beta-arrestins have been shown to coordinate multiple signaling networks downstream from many GPCRs (Maudsley et al., 2013). To identify additional pathways regulated by beta-arrestins downstream from CB1Rs we applied an unbiased screen to detect the phosphorylation of kinases upon CB1R activation. We utilized a human phospho-kinase antibody array to simultaneously identify the phosphorylation of 43 different kinases and two related proteins, including ERK and CREB whose activation were previously shown to be mediated by CB1Rs (Flores-Otero et al., 2014; Laprairie et al., 2014). Cells expressing the wild-type receptor were incubated with 10 μ M 2-AG for 5 and 15 min (Figure 3A). The effects of siRNA against beta-arrestin 1 and preincubation with PTX were also evaluated to resolve pathways from beta-arrestin 1 and Gi/Go proteins, respectively (Supplemental figure 2). Consistent with Figure 2 and our prior findings (Flores-Otero et al., 2014), ERK1/2 phosphorylation elicited by 2-AG was mediated by PTX sensitive G proteins at 5 min and exclusively by beta-arrestin 1 at 15 min (Figure 3A and B). Significant increases in phosphorylation levels upon 2-AG treatment were also observed in JNK1/2/3 and CREB while there is a decrease in the phosphorylation of HSP60. The levels of CREB and HSP60 phosphorylation were specifically affected by beta-arrestin 1 siRNA and PTX treatment, respectively (Figure 3C and supplemental figure 2). In contrast, JNK1/2/3 phosphorylation was abolished by PTX treatment and beta-arrestin 1 siRNA at 5 min and 15 min, respectively. Interestingly, these pathways have been previously described as regulated by CB1Rs further validating our approach, but the involvement of beta-arrestins was previously unknown (Derkinderen et al., 2001; Hart et al., 2004; Rueda et al., 2000). These results suggest that prolonged stimulation of CB1Rs with the endocannabinoid 2-AG leads to a beta-arrestin 1 dependent activation of multiple signaling pathways. We did not observe changes

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in p-AKT 1/2/3 as previously reported (Laprairie et al., 2014) although phosphorylation levels were significantly elevated under basal conditions, possibly masking any changes (Figure 3A and 4A). These differences could also be explained by quantitative relationship between receptors and signaling proteins or sensitivity issues in our detection methods. For the wild-type receptor upon WIN treatment, results were comparable to those of 2-AG treatment for 5 min (Figure 3B and C). In contrast, no further phosphorylation of these kinases were observed at 15 min.

Next, since beta-arrestin mediated signaling is enhanced in the S426A/S430A receptor, we utilized the phospho-kinase antibody array to investigate the pathways downstream from CB1R/beta-arrestin 1 (Figure 4A). While phosphorylation levels for kinases were similar to those described for the wild-type receptor exposed to WIN at 5 min (Figure 3B), key differences were identified. For example, unlike the wild-type receptor, ERK1/2 phosphorylation for the mutant receptor at 5 min is beta-arrestin 1 mediated, and the phosphorylation level at 15 min remained increased and all were dependent on beta-arrestin 1, not Gi/Go proteins (Figure 4A and B). Significant increases in phosphorylation upon WIN treatment were also observed for JNK1/2/3, CREB, and EGFR (Figure 4B and supplemental figure 2). These were specifically abolished by beta-arrestin 1 siRNA further suggesting that the mutation S426A/S430A resulted in enhanced beta-arrestin mediated signaling. Taken together these results indicate that this mutant receptor is an ideal tool to investigate beta-arrestin mediated signaling.

The differential interaction between receptor and beta-arrestin isoforms

Since activation of the S426A/S430A receptor resulted in enhanced beta-arrestin, we utilized this receptor to investigate the molecular mechanisms by which receptor activation translates into

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beta-arrestin mediated signaling. Previously, we have proposed ligand-specific endocytic dwell times, the time during which receptors and beta-arrestins are clustered at the cell surface during the endocytic process, as a possible mechanism modulating beta-arrestin mediated signaling (Flores-Otero et al., 2014). This work suggested that ligands inducing prolonged dwell times (>140 sec) were more efficacious at beta-arrestin mediated signaling than ligands inducing short dwell times (<120 sec). Here, we sought to compare the endocytic dwell times of the wild-type and the mutant S426A/S430A receptor in the presence of WIN and 2-AG using total internal reflection microscopy (TIRFM) (Figure 5A). Upon treatment, receptors clustered into individual endocytic events as previously described (Figure 5A top). By analyzing individual endocytic events, we found that dwell times of the S426A/S430A mutant receptor were prolonged when compared to wild-type receptors in the presence of 5 μ M WIN (Figure 5A, kymographs and individual traces). Analysis of multiple endocytic dwell times indicated that mutation of S426A/S430A significantly prolonged dwell times elicited by 1 and 5 μ M WIN (Figure 5B), supporting the enhanced beta-arrestin mediated signaling observed with the mutant receptor. However, no changes were observed in the wild-type versus the mutant in the presence of 10 μ M 2-AG (Figure 5B). These prolonged dwell times correlate and support their role as a predictor of beta-arrestin mediated signaling (Flores-Otero et al., 2014). Next, we investigated if the prolonged interaction between S426A/S430A receptors and beta-arrestins during the endocytic process at the cell surface extends to intracellular compartments. HEK293 cells expressing the wild-type or mutant receptors were transfected with beta-arrestin 1-RFP, incubated with 1 μ M WIN for 20 min and imaged by live-cell confocal microscopy. After 20 min, wild-type receptors were internalized and localized into intracellular vesicles while beta-arrestin 1 was homogeneously distributed in the cytoplasm (Figure 5C, middle panel). Interestingly,

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S426A/S430A receptors and beta-arrestin 1 were highly localized in intracellular clusters (Figure 5 C bottom panel). These clusters were frequently observed with the S426A/S430A receptor after WIN treatment but not with the wild-type receptor (Figure 5D). To test the idea that the interaction between beta-arrestin 1 and the mutant receptor is enhanced, we performed co-immunoprecipitations. beta-arrestins are notoriously difficult to immunoprecipitate, however, we observed a major band corresponding to beta-arrestin 1-RFP when the S426A/S430A receptor was immunoprecipitated after incubation with 1 μ M WIN (Figure 5E). In marked contrast, no beta-arrestin 1 band was observed upon WIN treatment of the wild-type receptor. We observed a weaker band for RFP-beta-arrestin 2 after 1 μ M WIN with immunoprecipitation of either the wild-type or the mutant receptors though the level of the latter was substantially reduced. (Figure 5F). Finally, we investigated the recruitment kinetics of beta-arrestins to the cell surface in cells expressing either CB1R or S426A/S430A by TIRFM (Supplemental figure 3). Interestingly, beta-arrestin 2 recruitment to the plasma membrane was severely impaired in the mutant receptor, strongly supporting the roles of S426/430 on beta-arrestin 2 recruitment (Supplemental figure 3 C). However, no significant difference was observed on the recruitment kinetics of beta-arrestin 1 (Supplemental figure 3 B)

Taken together these results indicate that strong physical interactions between the receptor and beta-arrestin 1 correlate with beta-arrestin mediated signaling. This interaction is initiated at the cell surface (i.e. prolonged dwell times) and continues after internalization in intracellular compartments suggesting prolonged interaction as a mechanism to engage sustained beta-arrestin mediated signaling.

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Specific GRK isoforms are responsible for beta-arrestin mediated signaling from the CB1R

GRKs have been proposed to recognize different ligand-induced receptor conformations and generate phosphorylation barcodes that lead to G protein or beta-arrestin mediated signaling (Liggett, 2011; Nobles et al., 2011, 2012; Zidar et al., 2009). To examine the mechanisms controlling beta-arrestin mediated signaling, we sought to investigate the roles of specific GRKs in the signaling of the wild type and mutant receptor. We utilized siRNA technology to specifically remove GRK isoforms 2-6 and investigated ligand induced ERK1/2 phosphorylation at 5 and 15 min by the two receptors (Figure 6). Consistent with Figure 1 and 3, incubation with WIN induced robust ERK1/2 phosphorylation at 5 min in the wild-type receptor. This phosphorylation was independent of GRKs as siRNAs had no effect, consistent with a G protein mediated mechanism (Bouaboula et al., 1995; Howlett, 2005) (Figure 6A and B). However, when GRK3 was knocked down, a small but significant increase in ERK1/2 phosphorylation was observed for the wild-type receptor upon WIN treatment for 15 min (Figure 6A and B bottom panels). These data are consistent with results suggesting that GRK3 may play a key role in CB1R internalization, and thus in its absence, the receptor remains at the cell surface signaling slightly longer via G protein (Jin et al., 1999). Interestingly, in cells expressing the S426A/S430A receptor, removal of GRK 4 or 5 significantly reduced ERK activation at 5 min and the knockdown of GRKs 5 and 6 reduced ERK1/2 phosphorylation further at 15 min (Figure 6C). These results indicate that GRKs are not involved in the phosphorylation of ERK1/2 at 5 min in the wild-type receptor but control phosphorylation at 5 and 15 min in the S426A/S430A receptor, strongly suggesting a beta-arrestin dependent mechanism at these time points. To control for the effectiveness of each siRNA, we analyzed the expression levels of endogenously expressed GRKs in HEK293 cells (Figure 6E). Taken together the reductions in phosphorylation

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in the mutant receptor strengthen the barcode hypothesis where specific GRKs mediate beta-arrestin mediated signaling (GRKs 4, 5, 6, but not 3) versus G protein activation, even when other GRKs are present in HEK293 cells (Atwood et al., 2011) and indicate that beta-arrestin mediated signaling is controlled by a specific subsets of GRKs.

Genes specifically controlled by beta-arrestin mediated signaling

beta-arrestin mediated signaling has been shown to regulate protein synthesis and gene transcription (DeWire et al., 2008; Maudsley et al., 2015). Interestingly, activation of the delta opioid receptor has been shown to translocate beta-arrestin 1 to the nucleus and affect histone modification and gene transcription (Kang et al., 2005). We took advantage of the enhanced beta-arrestin signaling of the S426A/S430A receptor to explore the transcriptional profile of beta-arrestin mediated signaling from the CB1R. We extracted mRNA and performed RNA sequencing from HEK293 cells expressing these receptors with and without 1 μ M WIN treatment for two hours. We examined differentially expressed genes utilizing a false discovery rate of 5% to compare the activation by WIN of the mutant S426A/S430A receptor versus activation of the wild type receptor (Figure 7A and supplemental figure 5 and 6). Several differentially regulated genes reflected activation of CB1R and induction of kinase stimulation as described in supplemental figure 5 and 6, further validating our results. The phosphatases *DUSP1*, 5, and 16 are all significantly upregulated in S426A/S430A cells following prolonged treatment with WIN (Supplemental figure 5). These phosphatases play roles as negative regulation of ERK, JNK and P38, and suggest crosstalk downstream from beta-arrestin mediated signaling and the dampening MAP kinase and cAMP dependent pathways.

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Interestingly, the E3 ubiquitin ligase *MDM2*, which regulates ubiquitination of beta-arrestin 2 and signaling from the beta2 adrenergic receptor (Shenoy et al., 2009) was also upregulated selectively in S426A/S430A expressing cells (Supplemental figure 5). Finally, we investigated the transcripts specifically regulated by WIN in the mutant receptor versus the wild-type receptor (Figure 7Aa and Supplemental figure 6). From this list, ~70% of the genes are involved in gene regulation, mRNA processing and protein translation and degradation (Figure 7 B), underlining the significant role of beta-arrestin mediated signaling in the long term effects of CB1R activation. Remarkably, some of the genes downstream of beta-arrestin include *VEGFA*, *GHI* and *ADAMTS1* which have been involved in cancer growth and neurodegeneration.

Discussion

Our understanding of GPCR signaling has dramatically changed over the last 15 years. Initially described as on and off switches, GPCRs are now likened to microprocessors where their activation can lead to multiple active states and cellular responses (Kenakin, 2006, 2011). This current understanding of GPCR pharmacology is integrated into the concepts of functional selectivity and biased signaling (Kenakin, 2011; Urban et al., 2007). In this paradigm, ligands can be biased to activate selected signaling pathways from the full signaling repertoire available to individual GPCRs. G protein-dependent pathways have been extensively described from the functional to the structural level (Pierce et al., 2002; Rosenbaum et al., 2009; Venkatakrisnan et al., 2013). However, despite its immense therapeutic potential, our general understanding of beta-arrestin mediated signaling is currently very limited (Maudsley et al., 2013; Shenoy and Lefkowitz, 2011; Srivastava et al., 2015; Venkatakrisnan et al., 2013).

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Here we sought to apply a comprehensive approach to investigate the beta-arrestin “signalosome”, the repertoire of cascades elicited downstream from the CB1R/ beta-arrestin. To achieve this goal, first we identified a CB1R with enhanced beta-arrestin signaling. Mutation of the putative GRK phosphorylation sites S426/S430 to alanines resulted in lower levels of receptor internalization (Supplemental figure 4), reduced desensitization and persistent activation of ERK1/2 independently from Gi/Go and reduced beta-arrestin 2 recruitment to receptors (Supplemental figure 3). Reduced internalization rates could give the receptors more opportunity to interact with beta-arrestin 1 and increase their signaling (Supplemental figure 4) (Daigle et al., 2008; Morgan et al., 2014). Interestingly, mice with these mutations have an increased response to agonist, supporting the idea of impaired desensitization. Next, we compared the kinases activated by G proteins and beta-arrestin using a human phospho-antibody array. This approach in combination with siRNA technology and toxin treatments, allowed us to simultaneously screen for signaling pathways specifically mediated by beta-arrestins. Consistent with our previous work and the work of others (Flores-Otero et al., 2014; Laprairie et al., 2014), signaling from the wild-type receptor elicited by the endogenous 2-AG was mediated by G proteins at 5 min and it was completely replaced with beta-arrestin mediated signaling at the later time point. In addition, the beta-arrestin mediated signaling from the S426A/S430A receptor showed significant switch from G protein to beta-arrestin pathways. Results from this receptor strengthens the idea that this pathway is involved in the regulation of long term cellular events such as protein translation, gene transcription and epigenetic regulation (Ma and Pei, 2007; Maudsley et al., 2013). Activation of ERK 1/2, JNK1/2/3, CREB, and EGFR RTK was dependent of beta-arrestin-1, strengthening the specific role of this molecule during CB1R signaling (Supplemental figure 2, 3 and 4)(Ahn et al., 2013; Srivastava et al., 2015). These

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cascades have been previously associated to CB1R signaling in different cellular backgrounds, but not all of the cascades were shown to be mediated by beta-arrestins (Dalton and Howlett, 2012; Derkinderen et al., 2001, 2003; Hoffman and Lupica, 2013). Interestingly, not all previously described cascades mediated by beta-arrestins such as AKT were observed in our model (Gómez et al., 2000; Ozaita et al., 2007; Trazzi et al., 2010). This lack in response could be explained by differences in the quantitative relationship between receptor and signaling proteins, detection issues, differential basal kinase activity levels in these cells or by the possibility that beta-arrestin mediated signaling activates only a subset of the complete signalosome available to CB1Rs in these particular cells. However, receptor expression levels and their cellular localization were not significantly different between wild type and mutants (Supplemental figure 4). Nevertheless, it is interesting to note that downstream cascades are in general very conserved among different cellular environments as previously observed for the PTH receptor (Maudsley et al., 2015).

How is GPCR activation translated into beta-arrestin mediated signaling? Our previous work (Flores-Otero et al., 2014) with TIRFM suggested that a prolonged interaction between receptors and beta-arrestins at endocytic pits could play a role in beta-arrestin mediated signaling. Supporting this, chemical or genetic prolongation of the dwell times of receptors/beta-arrestins at endocytic pits dramatically increased beta-arrestin mediated signaling (Flores-Otero et al., 2014). Ubiquitination has been also proposed as a molecular mechanism to control this interaction and signaling (Shenoy et al., 2009). We explored the interaction of CB1Rs with beta-arrestins at the cell surface and in intracellular compartments by microscopy and immunoprecipitations (Figure 5). WIN, which induced little or no beta-arrestin mediated signaling, elicited short endocytic dwell times in cells expressing the wild-type receptor (Flores-Otero et al., 2014). Remarkably,

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WIN elicited prolonged dwell times in the S426A/S430A receptor, similar to the dwell times obtained with 2-AG, which show a strong correlation with beta-arrestin signaling. Next, we explored the intracellular localization of CB1R and beta-arrestin 1. Surprisingly, we observed receptor-beta-arrestin 1 clusters in intracellular compartments after incubation with WIN only with the mutant receptor, suggesting a “stronger” interaction that is maintained after internalization. We tested this interaction biochemically by immunoprecipitation. Supporting the idea of a stronger (perhaps also a prolonged) interaction as a mechanism to control beta-arrestin mediated signaling, the S426A/S430A receptor successfully precipitated beta-arrestin 1 after WIN treatment. Little or no beta-arrestins were co-precipitated with the wild-type receptor. Finally, we looked into the hypothesis that specific GRKs are required for beta-arrestin mediated signaling. By using siRNA technology, we observed that GRKs were not necessary for the early activation of ERK (G protein dependent). However, GRKs 4-6 were necessary for ERK1/2 phosphorylation by the S426A/S430A receptor. Taken together this suggests that ligands eliciting beta-arrestin mediated signaling induce conformations that are recognized by specific GRKs leading to a stronger interaction between receptors and beta-arrestins during the endocytic trafficking. This prolonged interaction results in enhanced beta-arrestin mediated signaling. Interestingly, these results indicate that a high throughput approach to analyze this interaction could be utilized to screen for beta-arrestin biased compounds, providing new tools for drug discovery.

Because beta-arrestin mediated signaling activates kinases that control gene expression, we used transcriptomics to identify genes targeted by the CB1R/beta-arrestin pathways. Interestingly, ~70% of the genes specifically regulated by beta-arrestin mediated signaling control gene

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transcription and protein synthesis, suggesting a significant role of beta-arrestin mediated signaling on the long-term effects of CB1R activation.

MDM2, an E3 ubiquitin ligase previously implicated in beta-arrestin mediated signaling from the beta2 adrenergic receptor was upregulated selectively in S426A/S430A expressing cells suggesting a role for *MDM2* in the regulation of CB1R signaling (Shenoy et al., 2009). We additionally noted upregulation of several genes of the unfolded protein response pathway (*ATF4*, *ASNS*, *MTHFD2*, *HERPUD1*, *ATF3*, *TRIB3*, *EIF2AK3*, *GADD45B*, *HSPA5*, *XBPI*, *GADD45A* and *PPP1R15A*). Comparing activation of the ER-stress pathway genes in cells expressing the S426A/S430A receptor we noted important differences. While the components of ER-stress pathway were also activated in these cells, we noted the *ATF4* arm of the ER-stress pathway was induced to a lower extent. Two robust transcriptional targets of *ATF4*, *CHAC1* and *TRIB3* were among the highest differentially expressed genes noted comparing agonist stimulated wild-type versus beta-arrestin selective receptor expressing cells (Figure 7B and supplemental info). Previous work has shown that the pro-apoptotic and anti-tumor activity of cannabinoids is partially dependent on *TRIB3* and the Akt/mTORC1 pathway (Salazar et al., 2009). Taken together these data indicate that beta-arrestin could mediate the and antitumor action of CB1Rs (Velasco et al., 2012). Remarkably, the vascular endothelial growth factor A (*VEGFA*) gene and the growth hormone *GHI* were downregulated by β -arrestin mediated signaling, hinting at a molecular link between cannabinoids and cancer (Blázquez et al., 2004). The transcription factor *IRF2BPL* also known as *EAP1* was among the highest genes upregulated specifically by beta-arrestin mediated signaling. Although little information is available on this gene and its function, it has been proposed to modulate proenkephalin expression, suggesting a new link between the cannabinoid system and pain (Heger et al., 2007).

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The complexity and specificity of GPCR signaling networks controlled by beta-arrestins provides the opportunity to develop new therapeutic compounds with desired bias and reduced side effects (Kenakin and Christopoulos, 2012; Luttrell, 2014; Violin et al., 2010). Our work together with others, suggest identification and screening of beta-arrestin signatures as a rational approach to the development of biased drugs and propose that some of the positive effects associated with cannabis use may be dependent on beta-arrestin mediated signaling.

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FOOTNOTES

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Legends for Figures

Figure 1. WIN induced signaling from CB1R S426A/S430A is biased to beta-arrestins.

(A) HEK293 cells expressing SEP-CB1Rs or S426A/S430A were exposed to 1 μ M WIN 55212-2 for 5, 10, 15 and 30 min. Cell lysates were analyzed using western blots with phospho-ERK1/2 (p-ERK1/2, top panel) or total ERK1/2 (bottom panel). Representative western blot images and analysis of ERK1/2 phosphorylation are shown. Time course showing ERK1/2 phosphorylation levels in the wild-type (red) and S426A/S430A (blue) receptors. (B) HEK293 cells expressing SEP-CB1Rs or the S426A/S430A receptor were co-transfected with beta-arrestin 1 siRNA and exposed to WIN. The quantified time course shows complete abrogation of signal in the mutant receptor. (C) HEK293 cells expressing SEP-CB1Rs or S426A/S430A were co-transfected with beta-arrestin 2 siRNA and exposed to WIN. Graph provide quantified time course for the wild-type and mutant receptor. (D) HEK293 cells expressing SEP-CB1Rs or S426A/S430A were pretreated with PTX (16 hours). Quantified time course indicates complete inhibition of phosphorylation in the wild-type receptor. Data represent the mean \pm s.e.m. of at least three independent experiments. Statistically significant differences between the wild-type and mutant receptor were assessed using one-way analysis of variance and Bonferroni's post hoc test. *** $p < 0.005$.

Figure 2. 2-AG induced signaling from CB1R S426/430 is biased to beta-arrestins.

(A) HEK293 cells expressing SEP-CB1Rs or the S426A/S430A receptor were exposed to 10 μ M 2-AG for 5, 10, 15 and 30 min. Cell lysates were analyzed using western blots with phospho-ERK1/2 (p-ERK1/2, top panel) or total ERK1/2 (bottom panel). Representative western blot images and analysis to quantify ERK1/2 phosphorylation are shown. Time course showing

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ERK1/2 phosphorylation levels in the wild-type (red) and S426A/S430A (blue) receptor. (B) HEK293 cells expressing SEP-CB1Rs and S426A/S430A were co-transfected with beta-arrestin 1 siRNA and exposed to 2-AG as indicated. Quantified time course shows complete abrogation of signal in the mutant receptor. (C) HEK293 cells expressing SEP-CB1Rs or the S426A/S430A receptor were co-transfected with beta-arrestin 2 siRNA and exposed to WIN. Graphs provide quantified time courses for the wild-type and mutant receptor. (D) HEK293 cells expressing SEP-CB1Rs or the S426A/S430A receptor were pretreated with PTX (16 hours). Quantified time courses indicate inhibition of phosphorylation only at 5 min in the wild-type receptor. Data represent the mean \pm s.e.m. of at least three independent experiments. Statistically significant differences between the wild-type and mutant at each time point were assessed using one-way analysis of variance and Bonferroni's post hoc test. *** $p < 0.005$.

Figure 3. Signaling networks elicited from CB1R activation.

(A) Representative dot blots evaluated by profiling phosphorylation of 43 human kinases. HEK293 cells expressing SEP-CB1R were incubated with 1 μ M WIN and 10 μ M 2-AG for 5 and 15 min. Cell lysates from untreated and treated cells were applied to a nitrocellulose membrane spotted with the antibodies for 43 kinases. Three kinases (CREB, ERK1/2 and JNK1/2/3) with significant changes in phosphorylation level after agonist treatment were highlighted with boxes. AKT1/2/3 and reference spots (loading control) are shown for comparison. (B) Graphs provide the quantified time course showing ERK1/2 phosphorylation induced by 1 μ M WIN and 10 μ M 2-AG. Data are expressed as the fold change over the basal (no compound) level for each compound. The effects of beta-arrestin 1 knockdown (red) and PTX treatment (green) are shown. (C) Graphs provide the quantified time course showing JNK1/2/3 and CREB phosphorylation induced by 1 μ M WIN and 10 μ M 2-AG. Data are

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expressed as the fold change over the basal level for each compound. Statistical significance of agonist-induced phosphorylation compared with basal (0 min) was assessed using one-way analysis of variance and Bonferroni's post hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

Figure 4. CB1R S426A/S430A is a beta-arrestin 1 biased receptor.

(A) Representative dot blots evaluated by profiling phosphorylation of 43 human kinases. Lysates from untreated and agonist-treated (1 μM WIN for 5 and 15 min) cells expressing the S426A/S430A receptor were applied to a nitrocellulose membrane spotted with antibodies for 43 kinases along with control antibodies. ERK1/2 phosphorylation levels were highlighted with boxes. The effects of beta-arrestin 1 knockdown (red) and PTX treatment (green) are shown. (B) Graphs provide the quantified time course showing ERK1/2, JNK1/2/3 and CREB phosphorylation induced by 1 μM WIN. Data are expressed as the fold change over basal level for each compound. The effect of beta-arrestin 1 knockdown (red) and PTX treatments are included. Statistical significance of the differences compared with basal (0 min) was assessed using one-way analysis of variance and Bonferroni's post hoc test $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

Figure 5. CB1R S426A/S430A interacts with beta-arrestin1 more strongly than CB1R

(A) Total internal reflection images showing HEK293 cell expressing SEP-CB1Rs before and after 1 μM WIN (time indicated in seconds) showing individual endocytic events (arrow heads). Kymographs from HEK293 cell expressing either SEP-CB1Rs (top panel) or S426A/S430A (bottom panel) in the presence of 1 μM WIN. Individual endocytic events are indicated by the yellow rectangle. Intensity measurements from indicated events are represented to the right. (B) Box and whiskers plot (mean values with min/max range) from endocytic dwell times were

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analyzed for the indicated concentrations and receptors (n=10-19 cells). (C) Live HEK293 cells expressing either SEP-CB1Rs (top panel) or SEP-CB1R S426A/S430A (bottom panel) were imaged by confocal before and after 20 min incubation with 1 μ M WIN. (D) Intracellular beta-arrestin 1 particles were quantified after treatments (n=7-10 cells). (E) HEK293 cells were co-transfected with HA-CB1R and either beta-arrestin 1-RFP or beta-arrestin 2-RFP, and treated with 1 μ M WIN for 5 min. Lysates were immunoprecipitated with anti-HA antibody and subjected to immunoblot analysis using anti-RFP and anti-HA antibodies. Representative blot images show substantial beta-arrestin1 pull-down was observed with the HA-S426A/S430A mutant receptor, but not the HA-wild-type receptor after 5 min treatment. The lower panels show the input levels of HA-CB1Rs and beta-arrestins-RFP, respectively. (F) The bar graph provides quantification of beta-arrestin-RFP shown in (E). Data are the mean \pm s.e.m. from three independent experiments. The statistical significance of the differences compared with control (0 min) was assessed using one-way analysis of variance and Bonferroni's post hoc test. *** p <0.001.

Figure 6. GRK subtype specific regulation of beta-arrestin 1 signaling.

(A-D). Cells expressing either the wild-type (A) or S426A/S430A (C) receptors were co-transfected with control, GRK2, GRK3, GRK4, GRK5, or GRK6 siRNAs as described in Experimental Procedures. After exposure to 1 μ M WIN for 5 min and 15 min, cells were lysed and the lysates were separated by SDS-PAGE and blotted with anti-phospho-ERK1/2 and anti-total ERK1/2 antibodies. Representative western blot images are shown. Graphs provide the quantified levels of ERK1/2 phosphorylation for the wild-type (B) and S416/430A (D) receptors. The statistical significance of the differences compared with control was assessed using one-way

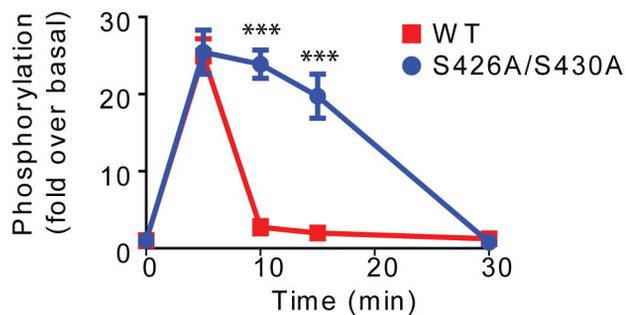
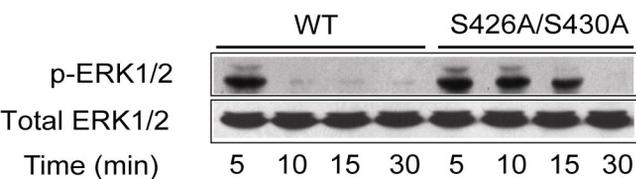
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analysis of variance and Bonferroni's post hoc test. *** $p < 0.001$. (E) Representative Western blot bands demonstrates specific knockdown of GRKs endogenously expressed in HEK293 cells.

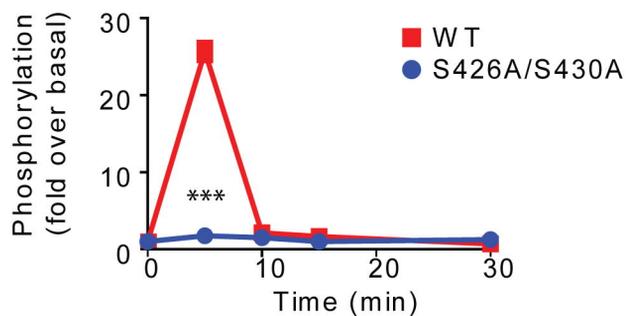
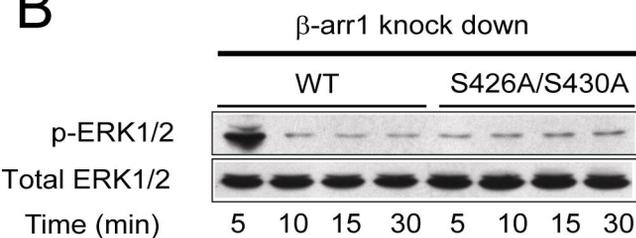
Figure 7. Transcripts selectively regulated by beta-arrestin 1 signaling

HEK293 cells expressing either the wild-type or S426A/S430A receptors were stimulated with 1 μ M WIN for 2 hours. RNA was isolated and sequenced and results analyzed as described in Methods. A. Transcripts that were differentially expressed in S426A/S430A expressing cells treated with WIN. Shown as \log_2 fold change. B. Varied function of the differentially regulated genes. The majority of differentially-regulated genes are involved in transcription and protein synthesis or degradation.

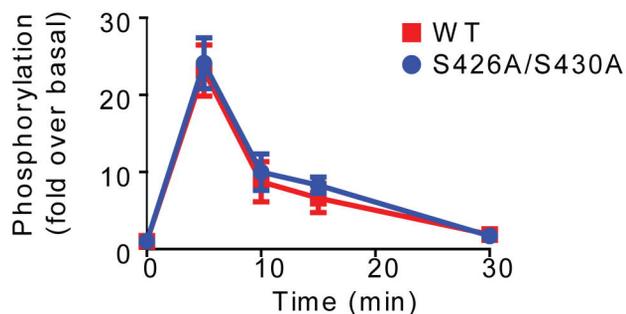
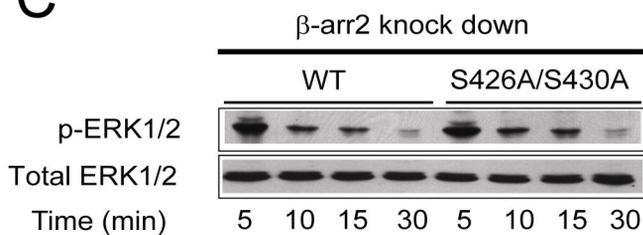
A



B



C



D

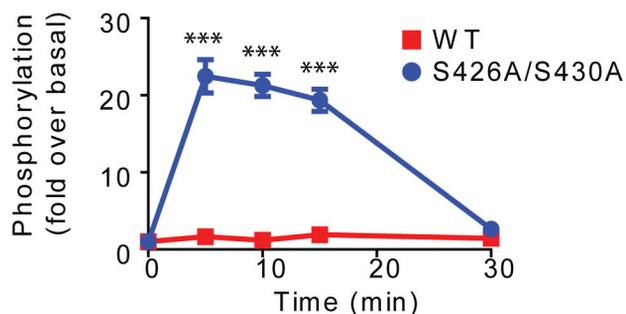
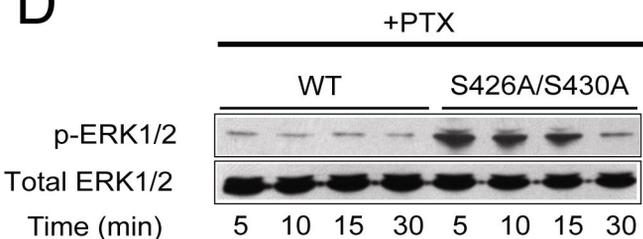
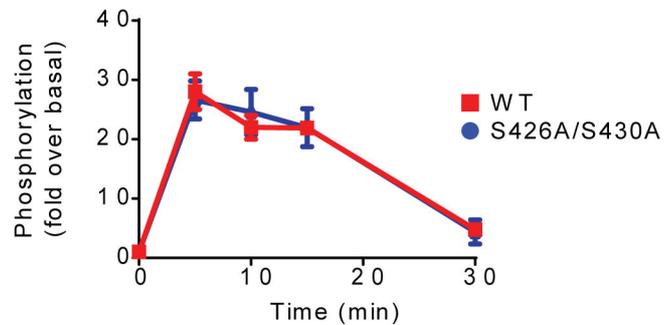
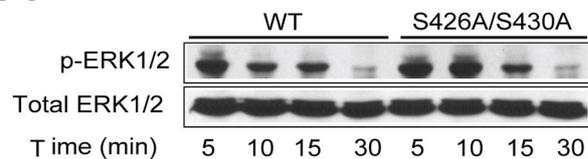
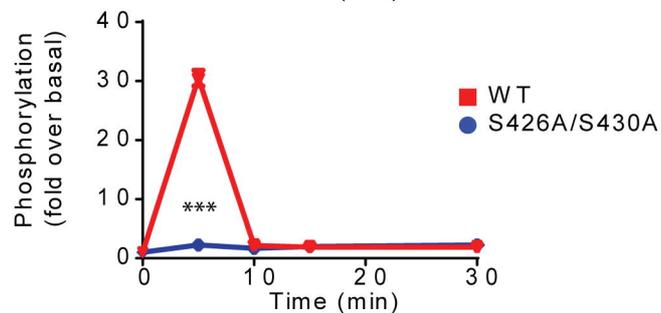
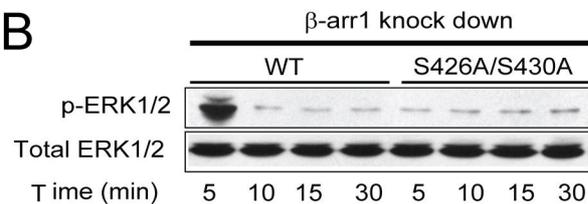


Figure 1

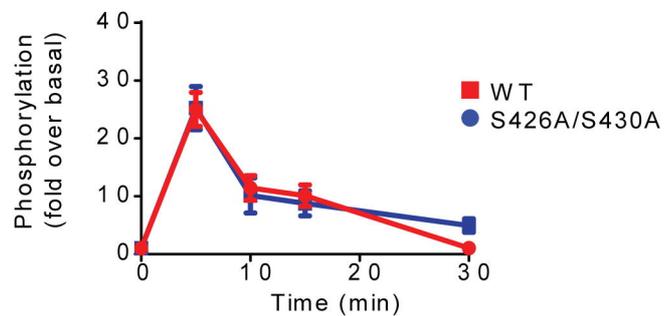
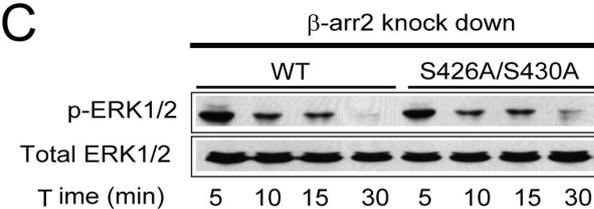
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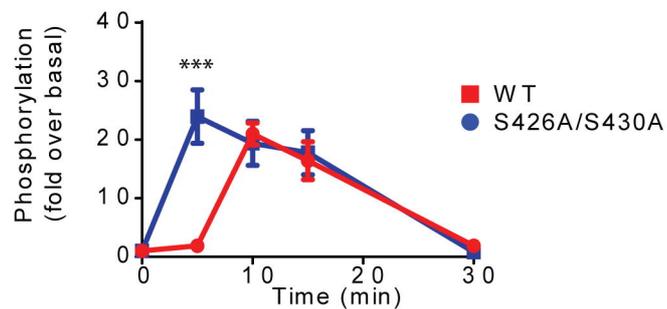
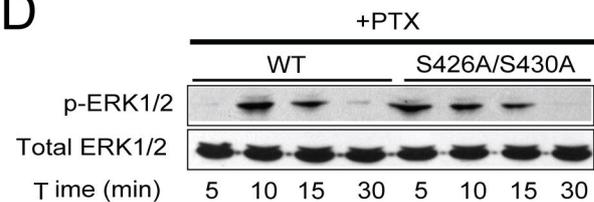
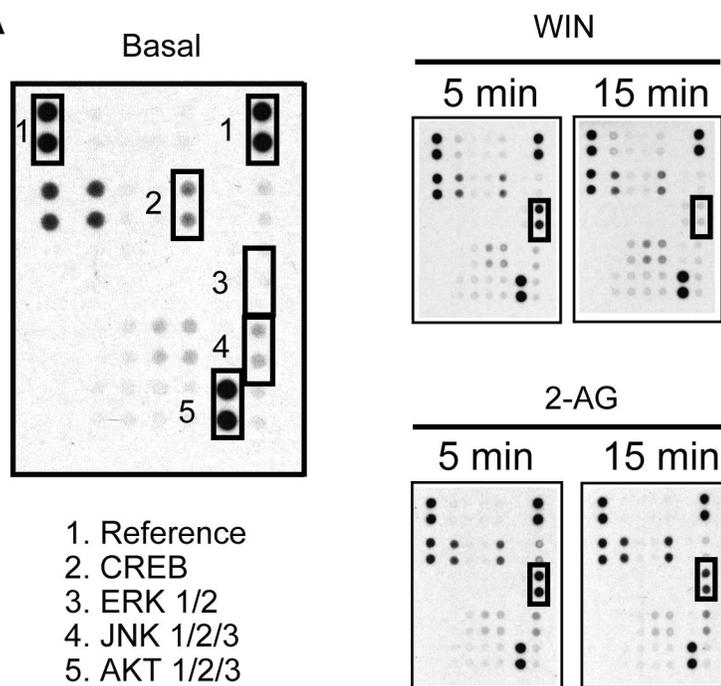
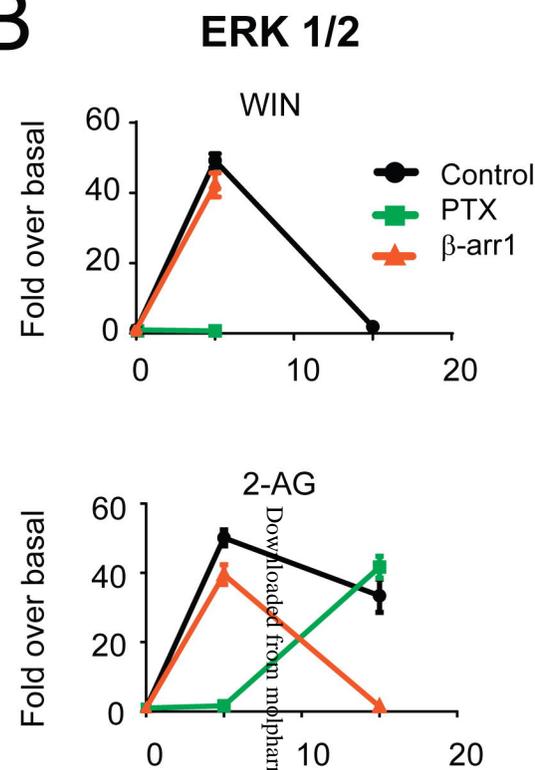
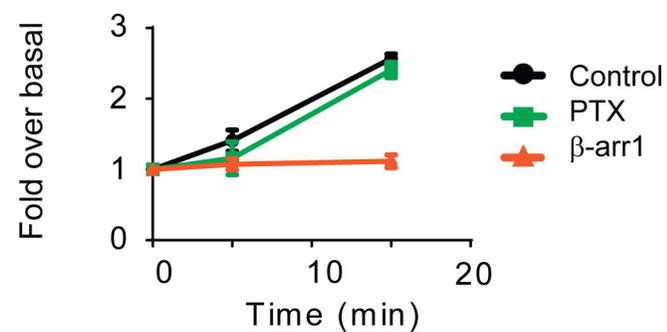
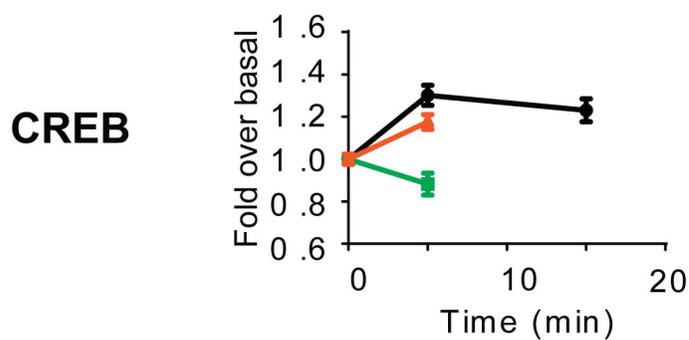
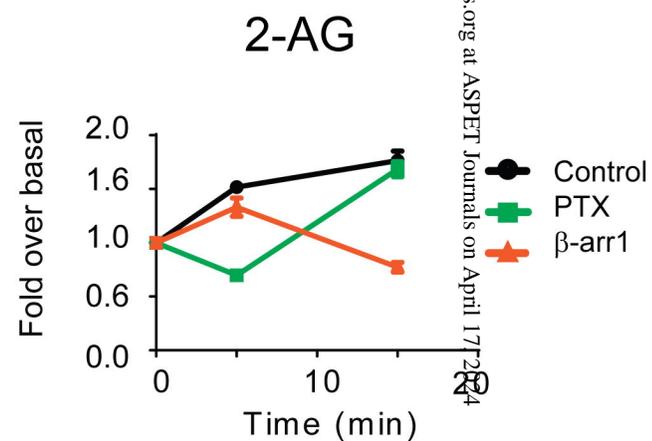
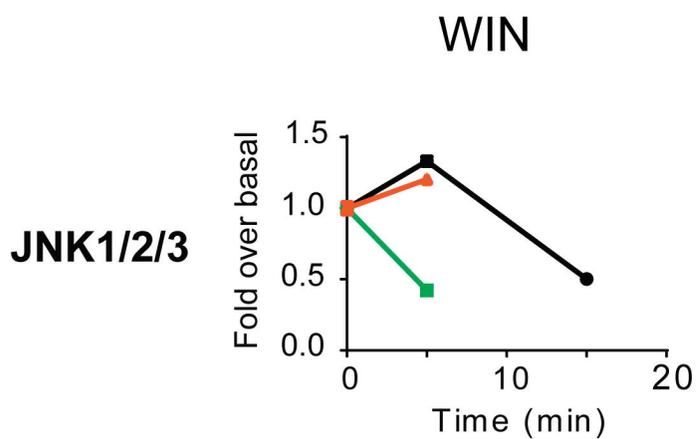


Figure 2

A**B****C****Figure 3**

A



B

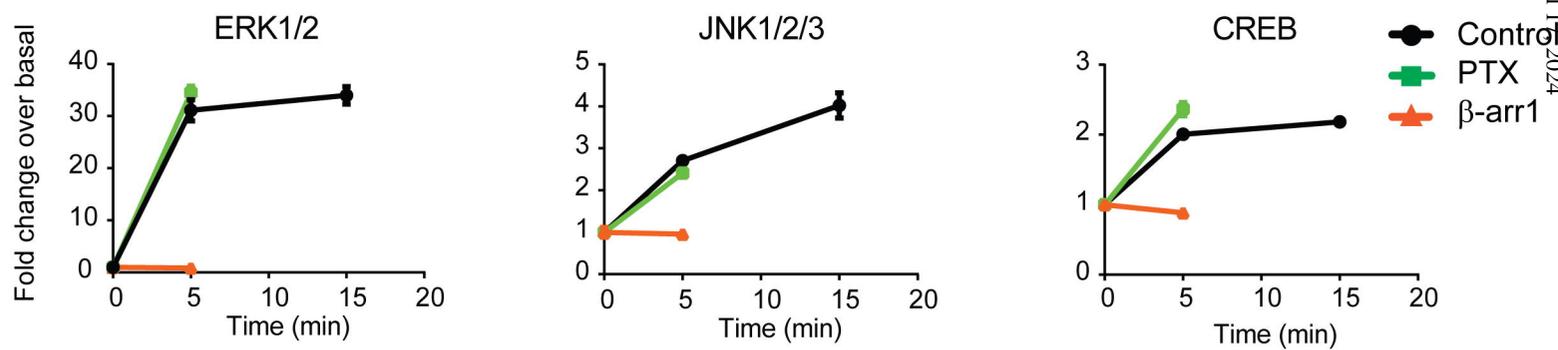


Figure 4

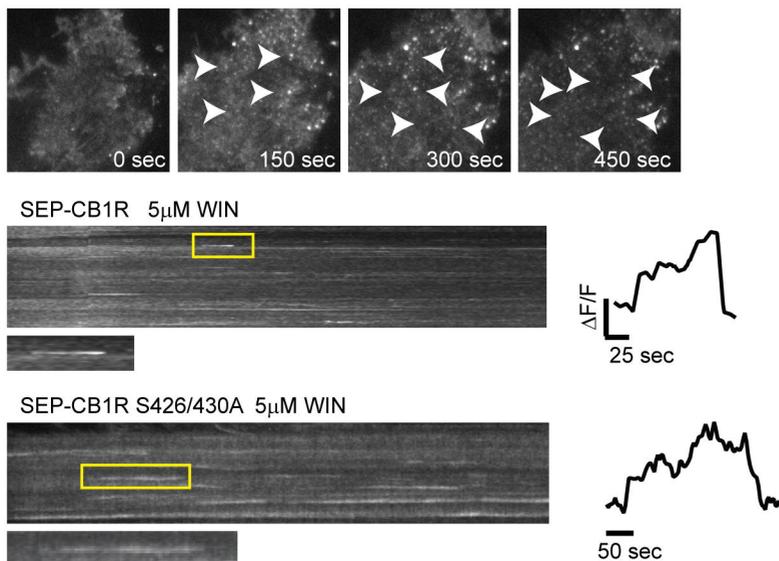
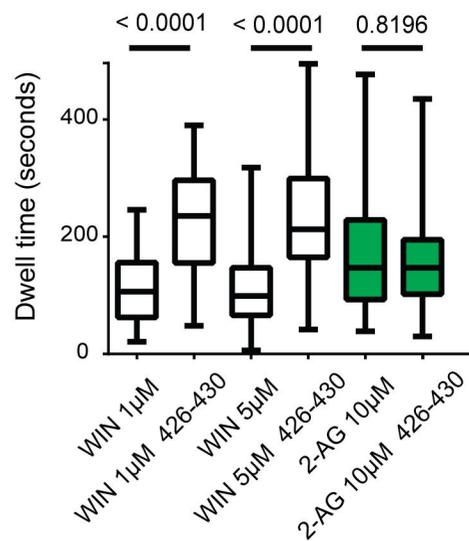
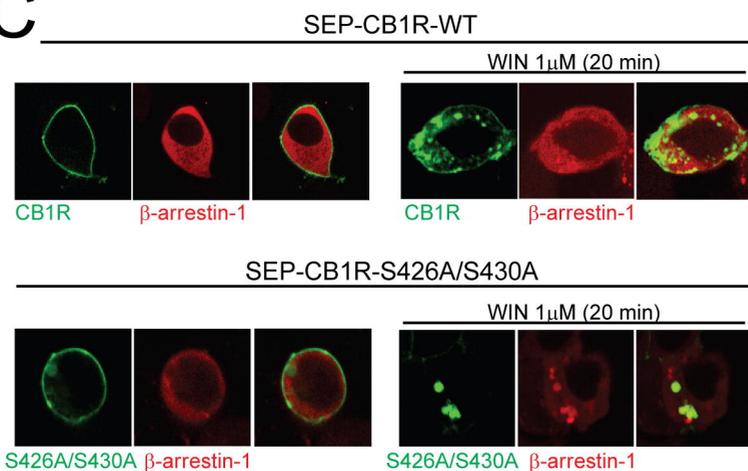
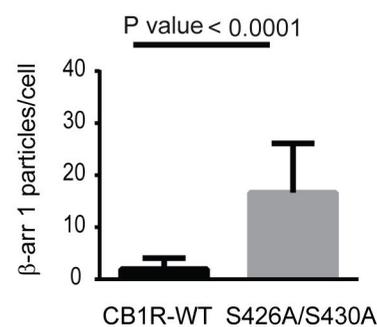
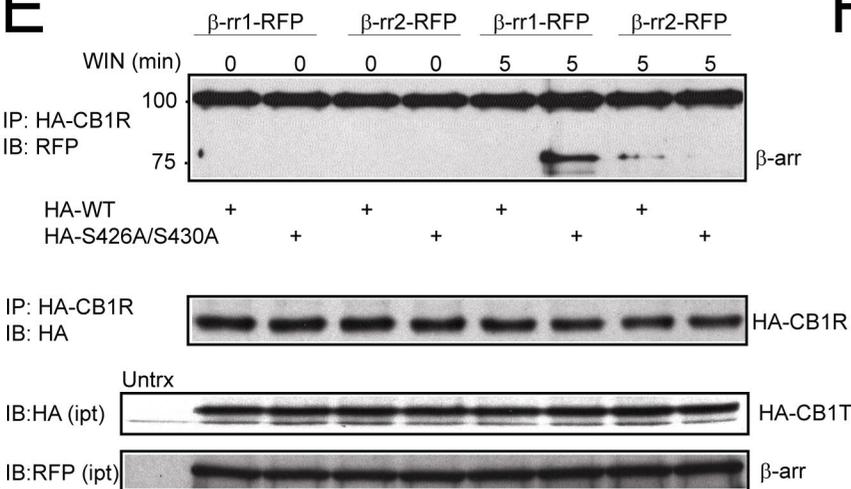
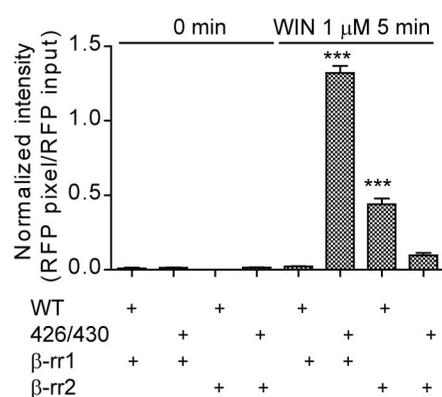
A**B****C****D****E****F**

Figure 5

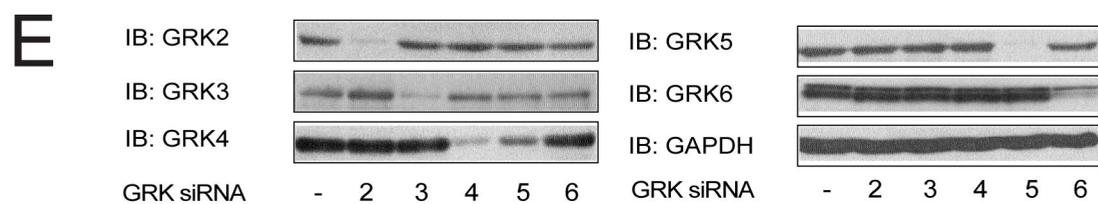
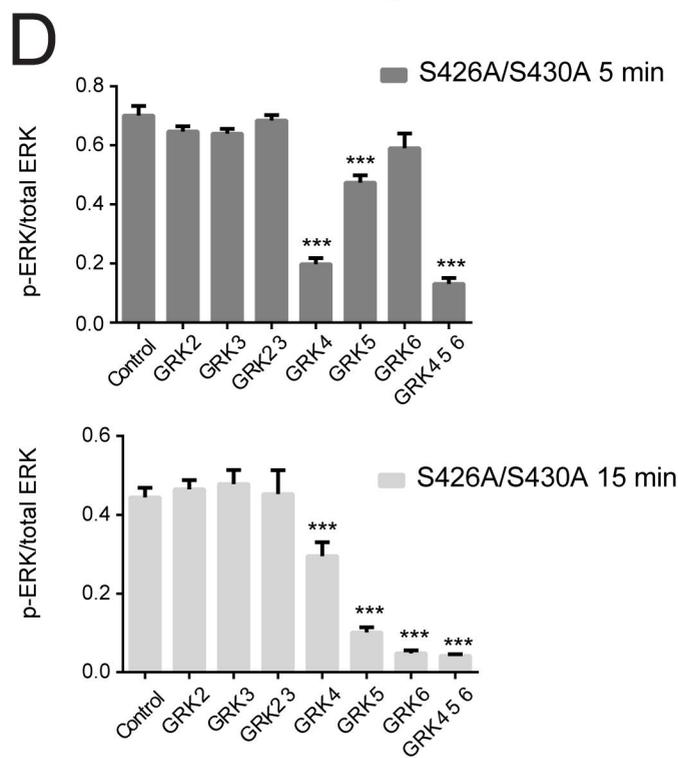
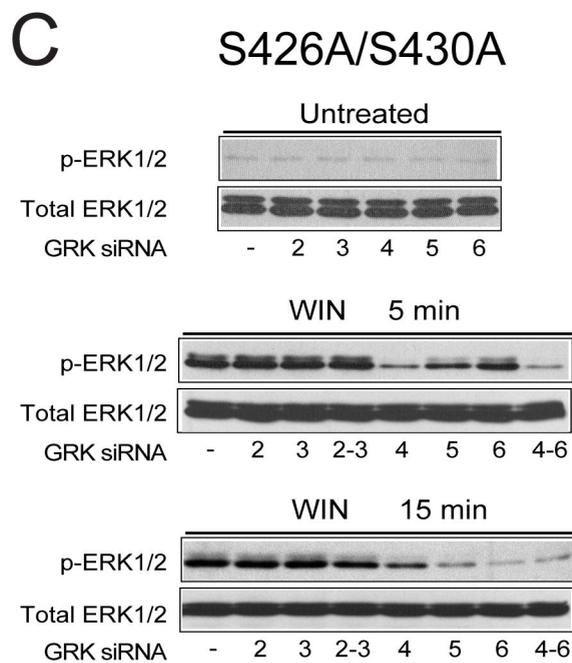
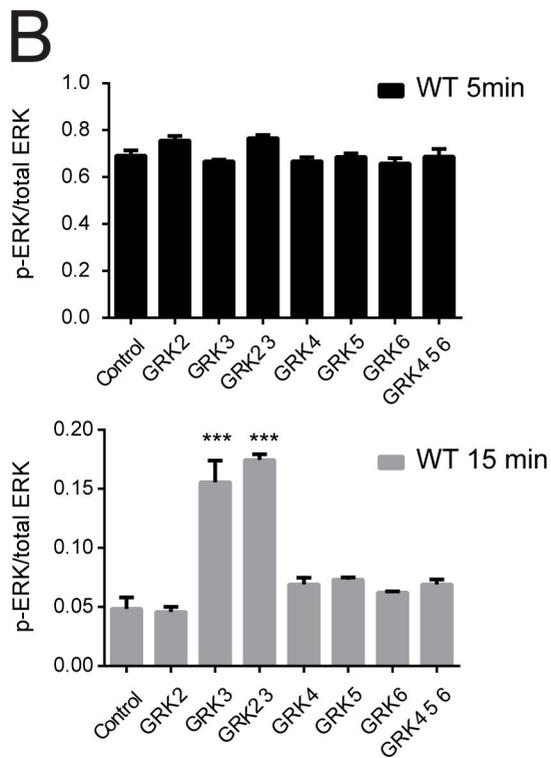
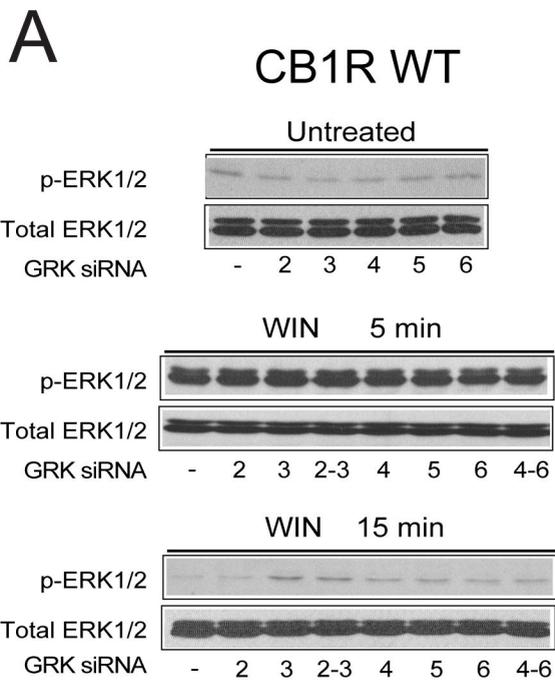


Figure 6

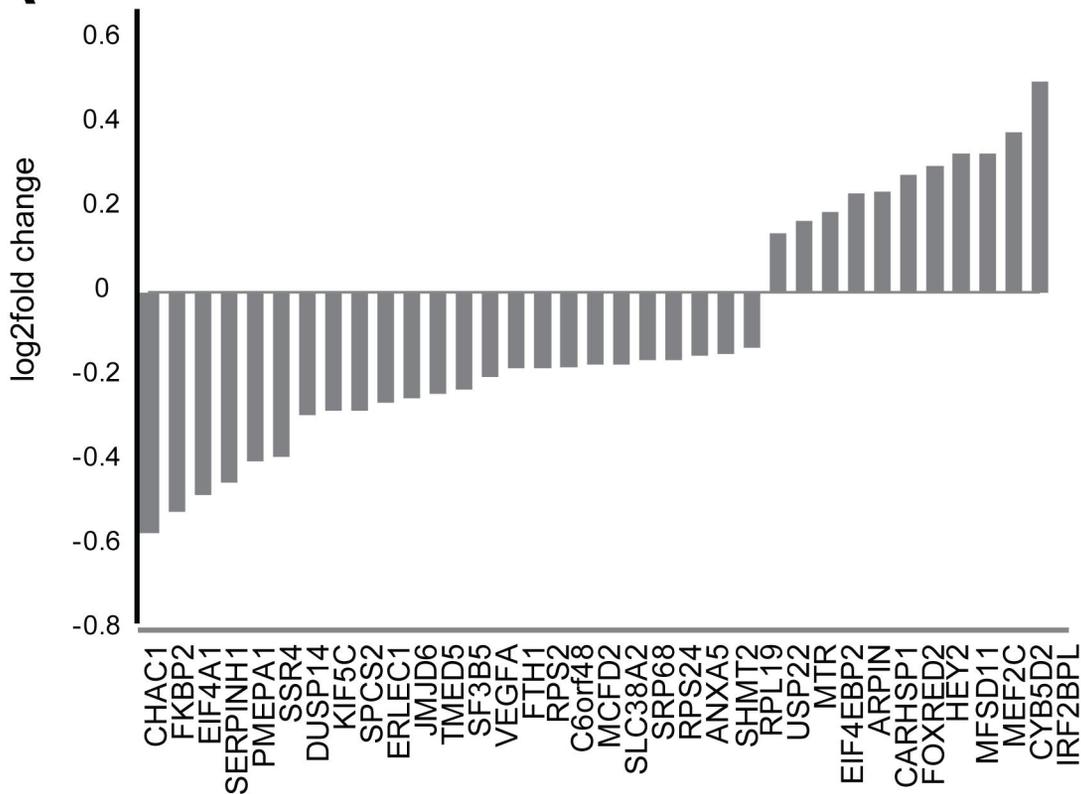
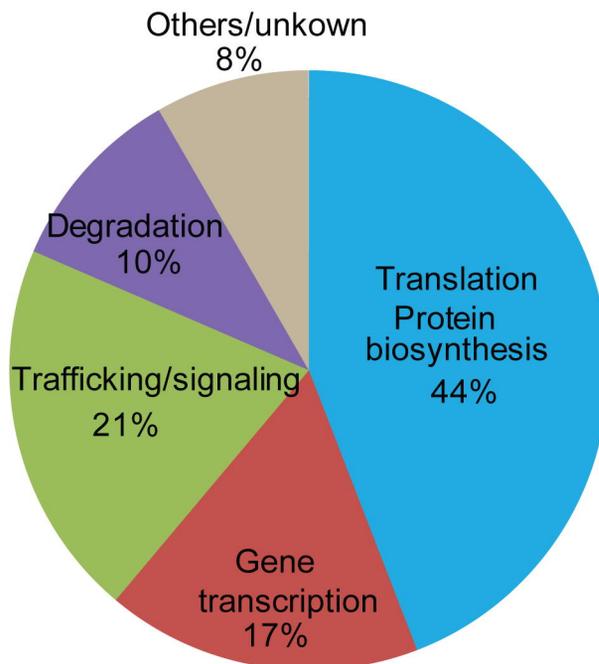
A**B**

Figure 7