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Jeffrey Chu April 8, 2020

Synaptic G Protein-Coupled Receptor BAI1 Regulation of Interacting Partners: Functional Interaction of Brain Angiogenesis Inhibitor 1 (BAI1) with Murine Double Minute 2 (MDM2) & β-Arrestin 2

By

Jeffrey Chu

Randy Hall
Adviser

Biology

Randy Hall

Adviser

John Hepler

Committee Member

Amanda Starnes

Committee Member

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Science with Honors

Biology

2020

Abstract

Synaptic G Protein-Coupled Receptor BAI1 Regulation of Interacting Partners: Functional Interaction of Brain Angiogenesis Inhibitor 1 (BAI1) with Murine Double Minute 2 (MDM2) & β-Arrestin 2

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The objective of this thesis is to determine the nature of Brain Angiogenesis Inhibitor 1 (BAI1) association with Murine Double Minute 2 (MDM2) and β-Arrestin 2 and how the co-expression of BAI1 with these proteins affects their respective post-translational modifications. BAI1 is an adhesion G protein-coupled receptor (aGPCR) that is predominantly expressed in the central nervous system and found mainly in neurons. Because BAI1 has been implicated in learning and memory and pathologies such as cancer and schizophrenia, it is essential to map out its crucial protein-protein interactions and post-translational modifications. The N-terminus of adhesion GPCRs are known to be cleaved via autoproteolysis. To shed light on how this might affect signaling, we generated 3 forms of the BAI1 receptor (BAI1 FL, ΔNT, and SL) and found that removal of the BAI1 N-terminus resulted in an increase in G protein-dependent signaling via the Rho-A pathway. Furthermore, we have found that MDM2, the E3 ubiquitin ligase that is responsible for the ubiquitination and subsequent degradation of proteins such as p53 and PSD-95, interacts to a greater degree with the truncated (and more active) forms of BAI1. We have also performed studies involving β-Arrestin 2, a protein responsible for the desensitization and internalization of overactive GPCRs and a known binding partner of both MDM2 and BAI1. We have found that MDM2 and β-Arrestin 2 mutually facilitate each other's interaction with BAI1. In regard to post-translational modifications, we have discovered that the presence of MDM2 enhances β-Arrestin 2-dependent ubiquitination of BAI1. We have also found that β-Arrestin 2 enhances MDM2 self-ubiquitination, while BAI1 inhibits this process, and that MDM2 co-expression increases ubiquitination of β-Arrestin 2. This thesis further elucidates the interaction of MDM2 with BAI1 and provides evidence that MDM2 and BAI1 form a triple complex with β-Arrestin 2. Ultimately, this thesis maps and characterizes the interactions between these key synaptic proteins, with the goal of enhancing the understanding of the fundamental biology of these proteins and providing insights that may ultimately lead to treatments of a wide range of pathologies.

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Acknowledgements

I would like to express my gratitude to my adviser, Dr. Randy Hall, for his mentorship and guidance for my research and studies. His enthusiasm was truly infectious, as my passion for research has grown every day since joining his lab. I could not have asked for a better mentor, and I'm so grateful that I was able to complete my honors thesis and undergraduate research experience in Dr. Hall's lab.

I would also like to express my sincere gratitude to my mentor, Trisha Lala, for teaching me fundamental lab techniques and being a constant source of guidance and ideas.

I would also like to express my deepest thanks to my committee members, Dr. John Hepler and Dr. Amanda Starnes, who have shared their vast knowledge and given me invaluable advice on the direction of my honors thesis.

Lastly, I want to sincerely thank my friends and family for their endless support and encouragement for my academic endeavors.

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Introduction

G Protein-Coupled Receptors

G protein-coupled receptors (GPCRs) are a superfamily of 7 transmembrane protein receptors that are found within a wide range of organisms, including mammals, plants, microorganisms and invertebrates. They constitute one of the largest gene families in the human genome with as many as 1,000 types encoded (28). GPCRs respond to a diverse range of substances, including light, hormones amines, neurotransmitters, and lipids and thus are heavily involved in many physiological processes. Because they are involved in so many physiological processes, they are popular targets for therapeutic drugs. As many as one-third to one-half of all marketed drugs act by binding to GPCRs (19). Prominent examples of GPCRs include the beta-adrenergic receptor which binds epinephrine, prostaglandin E2 receptors which bind inflammatory substances called prostaglandins, and rhodopsin which responds to light signals that helps us see (7). GPCRs recognize many difference extracellular stimuli, but a commonality is that they all signal through their heterotrimeric G proteins. Before agonist stimulation of the GPCR, the Gα and G βγ subunits associate together at the receptor's C-terminus with a GDP attached to the Gα subunit . However, upon agonist stimulation, the GDP is swapped for a GTP and the Gα subunit dissociates from the G βγ subunit. At this point, the activated Gα and G βγ subunit can interact with various effectors such as enzymes and ion channels and trigger downstream signaling cascades (7) Many GPCRs however, do not have identified ligands and these receptors are termed orphan receptors.

Adhesion G Protein-Coupled Receptors

The largest family of orphan GPCRs is the adhesion GPCR (aGPCR) family. The adhesion GPCR family is comprised of 33 heptahelical transmembrane proteins that constitute the second largest family of GPCRs in humans (15). They are involved in a host of physiological processes that included but not limited to neuronal development, angiogenesis, and immune system regulation. Adhesion GPCRs are characterized by a large extracellular N-terminus and intracellular C-terminus which are separated by the 7 transmembrane domains. A key feature of the aGPCR's N-terminus is the GPCR autoproteolysis-inducing domain (GAIN domain) which contains the GPCR proteolysis site (GPS motif). The GPS motif is where autoproteolytic cleavage of the N-terminus occurs which produces a cleaved fragment that can remain non-covalently associated with the receptor for a period of time (34). The N-terminus cleavage is thought to be a crucial activation step for many aGPCRs as many aGPCR truncated mutants that mimic post-cleavage receptors, show increased constitutive activity. Furthermore, the N terminus of aGPCRs are heavily glycosylated and contain various domains that aid in cellular adhesion and other biological functions (35).

Brain Angiogenesis Inhibitor 1

Brain Angiogenesis Inhibitor 1 (BAI1) is an aGPCR that is one of three protein receptors (4) that make up the BAI family. The BAI proteins are predominately expressed within the brain (26) and they were originally discovered and studied because of their ability to inhibit angiogenesis and tumor formation. They were initially identified in a screen for gene targets of the tumor suppressor, p53 (26). BAI1 was specifically found to be down regulated in

glioblastomas through epigenetic factors (44) and independent of p53 expression (11). Upon autoproteolytic cleavage at the GPS motif, BAI1 produces a 120 kDa N-terminus fragment called Vstat120 (vasculostatin 120) which has been shown to exhibit antiangiogenic and antitumorigenic properties (12). Restoration of BAI1 into tumors derived from gliomas and renal cell carcinomas has also been shown to limit the growth these tumors (17, 41, 42, 13).

BAI1's antitumorigenic properties traces back to well defined protein components in the N-terminus, including thrombospondin type 1 repeats which can regulate angiogenesis (44) and phagocytosis of apoptotic cells by macrophages (27). Other conserved domains across the BAI1 family include the hormone-binding domain (HBD), the GPS motif within the GAIN domain, the 7 transmembrane region, and the PDZ-binding motif which is a protein interaction module at the C-terminus. Unlike BAI2 and BAI3, BAI1's N terminus features an integrin-binding RGD) (Arg-Gly-Asp) motif as well as five thrombospondin type 1 repeats while BAI2 and BAI3 only have four thrombospondin type 1 repeats. The C-terminus of BAI1 also uniquely features a proline-rich region (PRR) that binds to Src homology 3 (SH3) domains that are present on proteins such as IRSp53 (14). Specifically, BAI1 is highly expressed in the cerebral cortex, the hippocampus, the olfactory bulb, thalamic nuclei, and basal ganglia (16, 33, 25).

As with other orphan GPCRs, BAI1 does not have an identified ligand at present but does exhibit constitutive coupling to the Gα12/13 signaling pathway that is enhanced by removal of the receptor's extracellular N-terminus (NT) (35). This was accomplished by constructing 3 receptor forms of BAI1: BAI1 FL ("full-length"), BAI1 ΔNT, and BAI1 SL ("stalk-less"). BAI1 FL contains the entire N-terminus and exhibits baseline G protein-dependent signaling via the

RhoA pathway. BAI1 ΔNT lacks the N-terminus at the site where autoproteolytic cleavage occurs and shows an increase in signaling compared to BAI1 FL. Lastly, BAI1 SL is a receptor form that even lacks the stalk region that usually remains after autoproteolysis and exhibits an even more drastic increase in signaling (15). However, it is important to note that only the BAI1 FL and ΔNT forms are physiologically relevant.

BAI1 is of special interest as it is enriched in the post-synaptic density (PSD) and is associated with spatial memory. Previous studies have shown that BAI1 associates with the postsynaptic density protein PSD-95, a key scaffold that helps anchor synaptic proteins and stabilizes synaptic changes during long-term potentiation (34). Also, BAI1 knockdown (KD) has been shown reduce spine density in hippocampal culture (36), while BAI1 knockout (KO) results in hippocampal learning and spatial memory deficits as evidenced by the Morris water maze test (45). All these studies suggest that BAI1 is a key regulator of synaptic plasticity and spine stability. However, little is currently known about how exactly BAI1 regulates synaptic function at the molecular level. Thus, it is crucial to determine the critical protein interactions of BAI1 as well as the functional consequences of these interactions.

BAI1 Interacting Partners

One protein that BAI1 is known to interact with is Murine double minute 2 (MDM2). Oncoprotein MDM2 is widely known as a key negative regulator of p53, a tumor suppressor protein that inhibits cell growth via growth arrest and apoptosis (20, 40, 37). Studies have shown that mice that incurred homozygous deletions of MDM2 died at approximately day 5 of embryogenesis while mice that possessed homozygous deletions of MDM2 and p53 were viable

and experienced normal development (10, 24). However, studies have also showed that overexpression of MDM2 leads to the reduction of p53 and subsequent tumorigenesis (23). This is partially due to MDM2's function as a RING finger-dependent E3 ubiquitin ligase which promotes the ubiquitination and degradation of p53 by the 26S proteasome (23, 9). Although p53 is the most famous substrate of MDM2 ubiquitination, it most certainly is not the only one. Other MDM2 ubiquitination targets include PSD-95 as well as MDM2 itself via self-ubiquitination (45, 38). Ubiquitination is a process that MDM2 helps facilitate by binding ubiquitin protein to lysine residues of other proteins. The attached ubiquitin chains can target the protein to the 26S proteasome for subsequent recycling and degradation (29). MDM2 also interacts with various cellular proteins in addition to p53 that regulate the MDM2/p53 feedback loop. This can occur via changes in the stability of MDM2, inhibition of the E3 ligase activity of MDM2, or through changes in the cellular distribution of MDM2 (23, 9, 3, 5). For example, ARF plays a role in MDM2 shuttling and distribution by sequestering MDM2 in the nucleoli and preventing its export to the cytoplasm (39, 43). Another prominent example of a protein that interacts with MDM2 to modulate is cellular distribution is BAI1. MDM2 has been shown to interact with BAI1 at its VSV motif to prevent PSD-95 and p53 polyubiquitination and degradation (45, 46). The VSV motif lies on the first intracellular loop of the receptor and may allow BAI1 to sequester MDM2 to prevent MDM2 from carrying out its ubiquitination function. This was elucidated within the context of medulloblastoma, one of the most common and aggressive malignant brain tumors in children, as BAI1 is a tumor suppressor that is down regulated in the pathology. It was shown that BAI1 knockout mice exhibit dramatically accelerate tumor growth in a mouse model of medulloblastoma, with BAI1 binding to the

ubiquitin E3 ligase, MDM2 being critical for regulation of medulloblastoma growth by BAI1 (46). This was attributed to BAI1 sequestering MDM2 in order to prevent MDM2 from ubiquitinating and degrading the tumor suppressor, p53. These findings suggest that activators of BAI1 could have potential as novel medulloblastoma therapies.

In addition to MDM2, BAI1 is also known to associate with the β-Arrestin 2 protein. β-Arrestin 2 was originally identified as a protein involved in the desensitization of β-adrenergic receptors (21, 1), but it is now known that recruitment is a prominent feature of highly active GPCRs in general (15). Agonist binding to GPCRs usually result in receptor phosphorylation by G protein-coupled receptor kinases (GRKs) which promotes β-Arrestin 2 association and receptor uncoupling from its respective G proteins (18). The exact location of β-Arrestin 2 binding to most GPCRs is not yet known but work on a handful of receptors has shown that determinants of β-Arrestin interaction are typically located in the third cytoplasmic loop as well as the C-terminus (18). β-Arrestin 2 is known to play a role in the endocytic process by interacting proteins such as clathrin (6), *N*-ethylmaleimide–sensitive factor (NSF) (22), and Mdm2 (30) and these interactions help facilitate GPCR desensitization and internalization into clathrin- coated pits for subsequent recycling or degradation. Ultimately, β-Arrestin 2 and β-Arrestins in general act as scaffolding proteins to physically link GPCRs to the cell's endocytic processes.

In our lab, we have shown that β -Arrestin 2 has a baseline interaction with BAI1 FL, but strongly interacts with the ΔNT version of BAI1. While our lab has also shown that BAI1 interacts with β-Arrestin 2 (15), the Lefkowitz lab at Duke and the Pei lab in China confirmed that MDM2 interacts with B-arrestin 2 (30, 38). A previous study conducted using the opioid

GPCR demonstrated the ternary complex formation of the opioid receptor, MDM2, and β-arrestin 2 (38). Because of this precedent, we logically hypothesized that BAI1, MDM2, and β-Arrestin 2 similarly, form a ternary complex.

In the work described here, we investigated the association of MDM2 and β -Arrestin 2 with BAI1. Because of MDM2's primary function as an E3 ubiquitin ligase and regulator of protein stability, we also wanted to investigate the ubiquitination state of MDM2, β -Arrestin 2, and BAI1. Previous studies have also shown that β_2AR stimulation results in accumulation of chromosomal DNA damage in both somatic and germ cells via β-arrestin-dependent mechanisms. These mechanisms include AKT mediated phosphorylation and activation of MDM2 which directs MDM2 to the nucleus where it can carry out MDM2-mediated ubiquitination and proteasomal degradation of p53 (8). Thus, in addition to ubiquitination state we also wanted to investigate the effect of BAI1 and β -Arrestin 2 on the phosphorylation state of MDM2 as these critical post-translational modifications play essential roles in protein stability and localization. These binding partners would be crucial for understanding BAI1 regulation and cellular pathways in a manner that might facilitate the future development of novel therapeutics for numerous pathologies.

Materials & Methods

Reagents

Complete Media: DMEM Incomplete Media (Gibco) + 10% FBS (Rockland) + 5% P/S filtered. 0.05% Trypsin EDTA (Millipore Sigma). DNA plasmids: BAI1 FL, BAI1 ΔNT, BAI1 SL, myc-MDM2, HA β-Arrestin 2, FLAG β-Arrestin 2, HA Ubiquitin, Empty Vector, Luciferase, Renilla. Mirus Transfection Reagent (Mirus TransIT-LT1). PEI reagent: Dispense 1g polyethylenimine (PEI) (MW: 25000) into beaker and suspend in 900ml of water; Add HCL dropwise until pH is <2.0; Stir until powder completely dissolve; Add NaOH until pH is 6.9-7.1; Add water until total volume is 1L; Filter solution and aliquot into 1.5mL tubes to store at -20C. PBS 1X with calcium (Fisher). RIPA 10X Lysis Buffer (Millipore Sigma). MYC-tagged agarose beads (Thermo). HA-tagged agarose beads (Thermo). Opti-MEM (Gibco). Pre-mixed firefly reagent aliquot; Renilla substrate; Renilla buffer (Promega). Protease Inhibitor (Thermo). Dimethyl sulfoxide (DMSO). 2X Laemmli Sample Buffer (BioRad). Intercept Blocking Buffer (Licor). Primary antibodies: BAI1 (ThermoFisher Scientific); HA (Cell Signaling Technology); MYC (Cell Signaling Technology); FLAG (Cell Signaling Technology); GAPDH (ThermoFisher Scientific). Tris-glycine 4-20% 10 & 15 well gradient gel (BioRad). 1X SDS Running Buffer (BioRad). Precision Plus Protein Dual Color Standard (BioRad). Secondary Antibodies: Rabbit 800 and Mouse 680 (Licor). MG-132 (Sigma Aldrich). Pierce N-ethylmaleimide (ThermoFisher Scientific).

Cell Culture/Splitting

The purpose of this technique is to split confluent cell plates for maintenance and for other lab uses such as transfections, etc. Warm Reagents in 37C water bath. The 10 cm plate should be confluent. Aspirate media off under the hood. Add 1 mL Trypsin and let the plate sit in the incubator for 5 min. Add 9 mL of Complete Media to trypsinized cells and make sure they lift off the plate. Triturate gently and plate cells into new dishes for (have 10 mL in each plate at the very end). Mix.

Polyethylenimine (PEI) Transfection

The purpose of this technique is to insert DNA plasmid into the genome of the cells. From a 10 cm confluent dish, aspirate media. Add 1ml trypsin and while cells are trypsinizing, prepare 6 well plates. Add 2 mL complete media/well. Add 9 mL complete media and triturate cells very gently to break them apart. Plate 800,000 cells/well. Gently tilt plate along length and side of 6 well plate to spread them gently. Transfect cells roughly 40 hours after plating. Warm Opti-MEM and thaw PEI aliquot. Thaw DNA and add DNA, then PEI into 1.5 mL tubes. Ratio of DNA to PEI is 1 ug DNA: 2 uL PEI. Add 100 uL Opti-MEM to each tube and vortex. Incubate at room temperature for 15 min. During 15 min incubation period, remove complete media from each well until the volume is 1.5 mL/well. Add transfection solution to the 6-well plate. Roughly 24 hr after transfection, add 1 mL of complete media back to cells. Harvest 48 hr after transfection.

Mirus Transfection

The purpose of this technique is to insert DNA plasmid into the genome of cells. On day 1, plate cells 1:10 day before transfection. On day 2, transfect cells by first thawing out DNA needed. Using 1.5 mL tubes, add reagents in this order: Add 750 uL Incomplete Media (DMEM). Add the DNA in the tube. Wait 5 min. Add 10 uL Mirus. Add 750 uL Incomplete Media (DMEM). Tap 8-10 times to mix. Let it sit for 15 min. Add dropwise and mix gently. Let it transfect in the incubator for 48 hr.

Dual-Glo Luciferase 96 Well Assay

The purpose is to insert DNA plasmid into the genome of cells and detect G protein-dependent signaling via Rho-A pathway activation. On day 1, from a confluent 10 cm dish, add 1 mL of Trypsin and 9 mL of complete media. Pre-load each well with 75 uL of complete media. Triturate cells to mechanically dissociate cells into solution. Pipette 50 uL of cell solution to each well. On day 2, transfect the cells. Thaw out DNA and label tubes. Pipette DNA to each tube. Create a master mix of DMEM, Firefly Luciferase, Renilla Luciferase. Pipette master mix to each tube ~250 uL. Set for 5 min. Add 5 uL of Mirus to each tube condition and flick 8-10 times. Let transfection solution sit at RT in hood for 30 min. Label wells on plate lid. Remove 80 uL of media from each well to be transfected. Pipette 50 uL of transfection solution to each well. Let transfection sit for 48 hours. On day 4, read the plate. Thaw firefly luciferase reagent RT (use only RT water, do not use warm water or incubator). Remove plate from incubator and allow to sit on bench for up to 5 min to cool to RT. Pipette 75 uL of firefly substrate to each well (do not

remove media). Let sit at RT for 10 min. Read plate and save data. Prepare Renilla reagent. Calculate amount needed: 75 uL/well + 15% for pipetting error. Dilute green substrate 1:100 in Stop and Glo buffer. For 10 conditions of 3 replicates, use 37.5 uL of green substrate and 3712.5 uL of buffer. Pipette 75 uL to each well (do not remove any liquid from wells). Let plate sit for 10 min at RT. Read plate and save data.

Co-Immunoprecipitation Protocol

The purpose is to use antibody incubated agarose beads to pull down a specific protein and the complex of proteins with which it associates. Make sure cells are confluent and aspirate off media. Remove excess media by adding 1 mL of PBS 1X to side of plate and aspirate off. Harvest cells in 500 uL RIPA 1X Buffer + Protease Inhibitor. Solubilize overnight in cold room end-over-end. Centrifuge samples: 15000 rpm for 20 min at 4C. 22.5 uL 4x + 67.5 uL lysate samples; 50 uL MYC beads or 15 uL HA beads (wash once with 1 mL of PBS 1X) + remaining lysate samples. PD O/N. 8x washes in RIPA 1X buffer w/ 0.2% DMSO. Elute O/N at RT in 100 uL 2X Laemmli Sample Buffer.

Western Blot

The purpose of this technique is to detect the presence of proteins through the use of antibodies. On day 1, run SDS-PAGE gel for 10 min at 120V, 50 min at 130V. Remove gel, rinse in water, soak blotting paper and membrane in transfer buffer, prepare sandwich and complete transfer. Ponceau stain if needed and use 0.1M NaOH to remove stain (rinse with water before

and after NaOH). Block in blocking buffer (20g milk, 10mL HEPES in fridge, 50mL NaCl in fridge, 1mL Tween20, fill to 1L with water) or Intercept Blocking Buffer for 1hr on shaker. Primary antibody overnight in cold room. On day 2, rinse 3x with 1X PBS-T. 2 PBS-T washes, 10 min. 1 hr RT on shaker secondary antibody in milk solution. Rinse 3x with 1x PBS. 3 PBS washes and image.

Results

MDM2 Exhibits Greater Association with Truncated Versions of BAI1

To assess whether MDM2's interaction with BAI1 was activity state dependent, we performed co-immunoprecipitation assays. The level of interaction between MDM2 and the 3 BAI1 receptor forms: BAI1 FL, BAI1 ΔNT, and BAI1 SL was assessed by coexpressing BAI1 FL, BAI1 ΔNT, and BAI1 SL, and myc-tagged MDM2 within HEK293T cells. As shown in Fig. 1, IP of myc-MDM2 revealed a baseline level of interaction between BAI1 FL and myc-MDM2. However, we witnessed a moderate increase in binding between BAI1 ΔNT and myc-MDM2 as well as a slight increase in binding between BAI1 SL and myc-MDM2. These data suggest that cleavage of BAI1's N-terminus, which increases receptor signaling activity, can modulate the interaction between BAI1 and MDM2 (15).

MDM2 & β -Arrestin 2 may Mutually Facilitate each other's Interaction with BAI1

From previous studies, we knew that MDM2 was able to bind β -Arrestin 2 and that BAI1 was able to bind to both MDM2 and β -Arrestin 2 (15, 30, 38, 46). We wanted to assess the relationship among these 3 proteins and determine whether these proteins might form a ternary complex. To accomplish this, we first performed a co-immunoprecipitation assay in which we IPed myc-MDM2. BAI1 FL and BAI1 ΔNT were coexpressed with myc-MDM2 in the presence or absence of coexpressed β -Arrestin. As shown in Fig. 2, in the presence of β -

Arrestin 2, myc-MDM2 was able to moderately co-immunoprecipitate BAI1 FL and more significantly co-immunoprecipitate BAI1 ΔNT. The increase in binding between BAI1 FL, ΔNT and myc-MDM2 suggests that β -Arrestin 2 can promote the interaction between BAI1 and MDM2.

We then took a different approach and performed another co-immunoprecipitation assay in which we IPed using HA- β -Arrestin 2. Similar to the previous experiment, BAI1 FL and BAI1 ΔNT were coexpressed with HA β -Arrestin 2 in the presence of absence of coexpressed MDM2. As depicted in Fig. 3, in the presence of MDM2 , HA β -Arrestin 2 was able to significantly co-immunoprecipitate BAI1 FL and BAI1 ΔNT. The increase in binding between BAI1 FL, ΔNT and β -Arrestin 2 suggests that MDM2 can facilitate the interaction between BAI1 and β -Arrestin 2. Ultimately, these findings suggest that both MDM2 and β -Arrestin 2 mutually promote each other's binding to BAI1.

BAI1 & β -Arrestin 2 Effect on MDM2 Ubiquitination Pattern

For all of our ubiquitination experiments we treated transfected HEK293T cells with 10 uM of MG-132 6 hours before harvest. We also harvested and washed the IP bead samples with 10 mM NEM. Because of MDM2 is an E3 ubiquitin ligase, we then wanted to assess how BAI1 and β -Arrestin 2 might affect MDM2's ubiquitination function. To accomplish this, we conducted a co-immunoprecipitation assay in which we IPed using transfected HA tagged ubiquitin. We assessed the level of protein ubiquitination by blotting for BAI1, myc-MDM2, and FLAG β -Arrestin 2.

BAI1 FL and ΔNT were coexpressed with myc-MDM2 , FLAG β -Arrestin 2, and with both myc-MDM2 and FLAG β -Arrestin 2. As depicted in Fig. 4, BAI1 ubiquitination for both FL and ΔNT did not increase with myc-MDM2 coexpression. However, BAI1 ubiquitination did increase when BAI1 was coexpressed with FLAG β -Arrestin 2 and increased even more drastically when BAI1 was coexpressed with both myc-MDM2 and FLAG β -Arrestin 2. These findings suggest that the presence of MDM2 can enhance the β -Arrestin 2-dependent ubiquitination of BAI1.

As shown in Fig. 5, we expressed myc-MDM2 in the presence and absence of FLAG β -Arrestin 2 and found that β -Arrestin 2 promotes MDM2 self-ubiquitination. We saw this trend even across the conditions in which myc-MDM2 was coexpressed with BAI1 FL and ΔNT. In addition, we also wanted to assess how BAI1 might affect MDM2 self-ubiquitination. Myc-MDM2 was expressed in the presence and absence of BAI1 FL and ΔNT and we observed a trend toward reduced MDM2 self-ubiquitination. As we saw that β -Arrestin 2 enhanced MDM2 self-ubiquitination, we also wanted to assess the effect of BAI1 FL and ΔNT on MDM2 self-ubiquitination when MDM2 was coexpressed with β -Arrestin 2. We found a similar trend in which the presence of BAI1 inhibited MDM2 self-ubiquitination, with the presence of BAI1 ΔNT exerting an even greater inhibitory effect.

In further studies, we wanted to determine the effect of MDM2 on β -Arrestin 2 ubiquitination levels. β -Arrestin 2 was expressed in the presence and absence of MDM2. As shown in Fig. 6, we observed an increase in β -Arrestin 2 ubiquitination levels in the presence of MDM2. This trend can also be seen in the presence of β -Arrestin 2 and BAI1 in the presence and absence of MDM2. Lastly, we were curious to see how BAI1 affected MDM2-mediated

ubiquitination of β -Arrestin 2. In these experiments, little difference was observed when β -Arrestin 2 and MDM2 were coexpressed in the presence and absence of BAI1 FL, but we did observe a clear reduction in β -Arrestin 2 ubiquitination levels when BAI1 ΔNT was co-transfected.

BAI1 & β -Arrestin 2 Effect on MDM2 Phosphorylation

Because previous studies have linked GPCR and β -Arrestin 2 signaling pathways via AKT mediated phosphorylation of MDM2, we proceeded to investigate how the presence of BAI1 and β -Arrestin 2 might affect the phosphorylation state of MDM2. HEK293T cells express a low baseline level of endogenous MDM2, and to increase MDM2 over this low endogenous level we transfected myc-MDM2 into every condition besides the first empty vector condition to assess how transfected myc-MDM2 might affect total MDM2 levels. We coexpressed BAI1 FL, β -Arrestin 2 with myc-MDM2 individually and together. As shown in Fig. 7, we found that the presence of β -Arrestin 2 drastically increased the phosphorylation level of MDM2. However, we also found that in the first empty vector condition in which we did not transfect in myc-MDM2, that we observed close to no phosphorylated MDM2.

Discussion

In our quantification of protein association in our co-IPs, we employed a semi-quantitative method. Quantifying IPs is challenging due to variability in IP efficiency as well as questions as to how best to normalize the data. However, in the IPs in our study, we quantified and averaged the proteins in the IP blots and normalized them to lysate levels of expression but not any loading controls. Thus, we derived semi-quantitative estimates of the degree of protein association within the context of the expression levels of the individual proteins. In addition, we attempted to perform statistical analysis on the degree of protein interaction via unpaired t tests. However, because we derived semi-quantitative estimates of protein interaction, we want to acknowledge that the p values likely do not represent the true significance. Instead, we want to draw attention and focus on the general trend of protein interactions. While we cannot say to what degree one protein facilitates the interaction of another pair of proteins, we can say that the trend appears to be an increase in protein association.

As the Van Meir lab demonstrated that MDM2 interacts with BAI1 at its VSV motif to prevent PSD-95 and p53 polyubiquitination and degradation (45, 46), we initially wanted to investigate whether the activity state of BAI1 (FL, ΔNT, SL) might have an effect upon interaction with MDM2. We found through our Co-IP that MDM2 interacted with the BAI1 N-terminus-truncated versions to a greater degree than BAI1 FL. The fact that MDM2 interacted with BAI1 ΔNT and SL to a greater degree than with BAI1 FL suggests that the large extra-cellular N-terminus as well as the stalk region from post-GPS cleave are not only unnecessary but might aid in MDM2's interaction with BAI1. This idea is further reinforced by the fact that BAI1 ΔNT and SL appear to express at lower levels that BAI1 FL. Thus, the perceived increase in

interaction between BAI1 ΔNT, SL with MDM2 may be even greater than meets the eye. Given the higher receptor activity levels of BAI1 ΔNT and SL relative to FL, we interpret these co-IP data to indicate that BAI1 interaction with MDM2 is dependent on receptor activity state.

Because other GPCRs have been known to form a ternary complex with MDM2 and β -Arrestin 2, and because BAI1 is known to interact with both of these proteins, we hypothesized that a ternary complex may form between BAI1, MDM2 and β -Arrestin 2. In our first experiment to investigate these protein relationships, we conducted a co-IP in which we IPed using myc-MDM2 to investigate the effect of β -Arrestin 2 on the BAI1-MDM2 interaction. We observed a slight increase in interaction between BAI1 FL and MDM2 in the presence of β -Arrestin 2. In these experiments, BAI1 FL expression appeared relatively consistent, so we can speculate that there was a real increase in the extent of interaction. In addition to BAI1 FL, we also observed an increase in interaction between BAI1 ΔNT and MDM2 in the presence of β -Arrestin 2. We noticed that in the absence of β -Arrestin 2, expression of BAI1 ΔNT appeared to be slightly lower than with of β -Arrestin 2. However, because the fold increase of BAI1 ΔNT co-IPed with β -Arrestin 2 compared to without β -Arrestin 2 was significantly greater than the difference in BAI1 ΔNT expression, we can logically conclude that β -Arrestin 2 facilitated the interaction between BAI1 ΔNT and MDM2.

We also conducted another co-IP in which we IPed using HA β -Arrestin 2 to investigate the effect of MDM2 upon the BAI1- β -Arrestin 2 interaction. We observed a moderate increase in the interaction between BAI1 FL and β -Arrestin 2 in the presence of MDM2. BAI1 FL expression for the conditions with and without β -Arrestin 2 appeared to be relatively

consistent throughout, reaffirming the apparent increase in interaction between BAI1 FL and β -Arrestin 2 observed in the IP blots. Furthermore, we investigated how the presence of MDM2 affected the interaction between BAI1 ΔNT and β -Arrestin 2. We observed that in the presence of MDM2, we saw a dramatic increase in the association between BAI1 ΔNT and β -Arrestin 2. Examination of BAI1 ΔNT expression in the lysates revealed that the condition with only BAI1 ΔNT and β -Arrestin 2 exhibited higher expression than the condition that included MDM2. However, because we observed even greater BAI1 ΔNT co-IP in the condition with lower BAI1 ΔNT expression , this further strengthens the notion that MDM2 facilitates the interaction between BAI1 ΔNT and β -Arrestin 2. Our two co-IPs suggest that MDM2 and β -Arrestin 2 mutually facilitate each other's interaction with BAI1.

We have proposed three possible models by which this mutual facilitation may happen. The first model as shown in Fig. 8a, we term as the "Stable Ternary Complex Model" in which all three proteins come together and fit like a jig-saw puzzle. In such a model, all three proteins would likely facilitate each other's stability, but we do acknowledge that the model might not be as simple as this. Thus, we also have proposed the "Conformation Change Model" as shown in Fig. 8b, in which β -Arrestin 2's binding to BAI1 at its C-terminus and MDM2's binding to BAI1 at its VSV domain on the first intracellular loop alters BAI1's conformation to make the other protein's respective binding locations more accessible. However, it is also possible that β -Arrestin 2 and MDM2 facilitate each other's interaction with BAI1 by shuttling each other to BAI1. This would be probable as β -Arrestin 2 is known to interact extensively with highly active GPCRs and other studies have also shown that as β -Arrestin 2 undergoes constitutive nucleocytoplasmic shuttling which enables it to distribute nuclear binding cargo such as MDM2

throughout the cytoplasm (2). We termed this model the “Recruitment and Hand-off Model” as shown in Fig. 8c.

In our ubiquitination co-IP experiment where we IPed using transfected HA ubiquitin and performed Western blotting to determine level of ubiquitination, we investigated a variety of questions, including: how do MDM2 and β -Arrestin 2 affect BAI1 ubiquitination? How do BAI1 and β -Arrestin 2 affect MDM2 self-ubiquitination? And how does BAI1 and MDM2 affect β -Arrestin 2 ubiquitination? In regard to the first question, we found that co-transfection with β -Arrestin 2 drastically increased the ubiquitination of both BAI1 FL as well as BAI1 ΔNT. We noticed that once myc-MDM2 was transfected alongside β -Arrestin 2, there was an even more drastic increase in BAI1 FL and BAI1 ΔNT. This suggested that MDM2 may increase β -Arrestin 2 dependent ubiquitination of BAI1. This observation has not previously been reported, although one study did show that β -Arrestin 2 can serve as an adaptor to recruit the E3 ligase, Nedd4, to ubiquitinate the β2-adrenergic receptor (32). We interpret our data to mean that only in the presence of β -Arrestin 2 is MDM2 able to be effectively recruited to ubiquitinate BAI1. The ubiquitination we see when MDM2 is not transfected could due to either endogenous MDM2 or other endogenous E3 ligases. Although the expression levels of BAI1 FL and ΔNT across conditions are not exactly equal, the dramatic differences in BAI1 ubiquitination likely render this point moot. Our lab has previously reported that BAI1 ΔNT exhibits far greater ubiquitination than BAI1 FL (15), and the present findings are consistent with that report given that the total expression levels of BAI1 ΔNT are much lower than BAI1 FL, but yet IP with HA-ubiquitin is greater for BAI1 ΔNT.

In our investigation of the effect of BAI1 and β -Arrestin 2 on MDM2 self-ubiquitination, we found that co-expression with β -Arrestin 2 increased while co-expression with BAI1 slightly decreased MDM2 self-ubiquitination. Our finding that β -Arrestin 2 promotes MDM2 self-ubiquitination is particularly interesting as a previous study had shown that the binding of β -Arrestin 2 to MDM2 suppressed the self-ubiquitination of MDM2 (38). However, this difference in β -Arrestin 2 function might be attributed to the difference in cell type as the previous study used Saos2 cells while we used HEK293T cells. Nevertheless, this modulation of MDM2 self-ubiquitination by β -Arrestin 2 is crucial as it can indirectly regulate the stability of p53 and other MDM2 substrates. Ultimately, this suggests that β -Arrestin 2 may serve as a point of crosstalk between GPCR and tumorigenic pathways. We also observed a slight decrease in MDM2 self-ubiquitination when BAI1 FL and ΔNT were coexpressed. Prior studies have suggested that BAI1 functions to sequester MDM2 at its VSV motif to prevent PSD-95 and p53 polyubiquitination and degradation (45, 46). This sequestering function of BAI1 could result in MDM2 no longer being physically able to ubiquitinate other MDM2 proteins. Another possibility could be that the interaction between BAI1 and MDM2 alters MDM2's conformation and thus inhibits its enzymatic activity as an E3 ubiquitin ligase.

We also found that MDM2 coexpression with β -Arrestin 2 increased β -Arrestin 2 ubiquitination while BAI1 coexpression (especially BAI1 ΔNT) reduced levels of β -Arrestin 2 ubiquitination. Our finding that co-expression with MDM2 increased β -Arrestin 2 ubiquitination was consistent with previous studies, which have repeatedly shown that MDM2 catalyzes arrestin ubiquitination (30). However, it is important to note that while MDM2 appeared to increase β -Arrestin 2 ubiquitination in the absence of BAI1 and in the BAI1 FL

conditions, there appeared to be minimal change in BAI1 ΔNT condition. One possible explanation as to why there might be less β -Arrestin 2 ubiquitination in the presence of BAI1 ΔNT is that the overactive version of BAI1 might require more β -Arrestin 2 to facilitate desensitization and internalization of the receptor. Thus, it would be counterproductive for the cell to ubiquitinate and degrade the much needed β -Arrestin 2. This ties into the last conclusion that we made regarding β -Arrestin 2 ubiquitination, which is that BAI1 ΔNT reduced β -Arrestin 2 ubiquitination when coexpressed with MDM2. The same explanation follows, in that β -Arrestin 2 is needed to desensitize and aid in internalization of the overactive BAI1 ΔNT receptor, and thus it is probable that its ubiquitination and subsequent degradation is inhibited.

In our study to investigate the effect of BAI1 and β -Arrestin 2 on the phosphorylation state of MDM2, we found that in the empty vector condition, in which we did not transfect any myc-MDM2, we saw little to no phosphorylated MDM2. This was in contrast to the rest of the conditions in which we transfected myc-MDM2 and saw substantial levels of phosphorylated MDM2. The data suggests that the myc tag drastically increases the level of phosphorylation of MDM2. It might be possible that the myc tag on the C-terminus of MDM2 prevents certain phosphatases from binding and dephosphorylating MDM2. This idea could be tested in the future by adding a phosphatase inhibitor to our cells and/or exploring the set of binding partners for MDM2 in the absence and presence of the myc tag. Furthermore, we were surprised to see that total MDM2 levels appeared to be relatively unchanged even after transfection of myc-MDM2. One potential explanation could be that the total MDM2 antibody might only recognizes endogenous MDM2, if for example the myc tag on the MDM2 C-terminus disrupts binding of the anti-MDM2 antibody. This could lead to a perceived unchanged level of

total MDM2 when in actuality, the increase is substantial. In regard to our experimental design of serum starving the HEK293T cells, we hoped that that this would broadly reduce the baseline of most signaling pathways. Thus, we hoped that if there was an effect of BAI1 and β -Arrestin 2 on Akt signaling, that it would appear more prominent. However, we did not notice any substantial differences between serum and serum starved conditions. We did observe a substantial increase in phosphorylation of MDM2 when β -Arrestin 2 was coexpressed. The increase in MDM2 phosphorylation did not seem to be attributed to BAI1 specifically, as there was minimal change in MDM2 phosphorylation in the presence of BAI1. Although the literature does not report any direct mechanism by which β -Arrestin 2 can promote the phosphorylation of MDM2, it is conceivable that β -Arrestin 2 acts through other endogenous GPCRs in HEK293T cells to promote the phosphorylation of MDM2 (8).

In terms of future directions, we would like to investigate what effect BAI1 and β-Arrestin 2 have on phosphorylated MDM2 distribution. Via other GPCRs and β-Arrestin 2 mediated mechanisms, Akt could phosphorylate MDM2 and redirect MDM2 back to the nucleus (8). Through immunocytochemistry, we could visualize to what extent this phenomenon is occurring. We also plan on repeating the MDM2 phosphorylation experiment with a phosphatase inhibitor to assess the level of endogenous MDM2 phosphorylation. In addition, we want to further clarify the BAI1, MDM2, & β-Arrestin 2 relationship. Experiments to date have IPed using myc-MDM2 and HA β-Arrestin 2, so the next step would be to IP with the receptor and assess how MDM2 and β-Arrestin 2's interaction with BAI1 affects BAI1's interaction with β-Arrestin 2 and MDM2, respectively. In addition to BAI1, MDM2, and β-Arrestin 2, we also want to assess the post-translational modifications of proteins more directly

involved in pathological conditions such as synaptic deficits and tumorigenic pathways. For example, we plan to investigate how BAI1, MDM2, and β-Arrestin 2 affect the ubiquitination state of PSD-95 and p53 as well as the phosphorylation state of p53.

In summary, our findings elucidate the nature of interaction between BAI1, MDM2, and β -Arrestin 2 and the subsequent effects on their respective post-translational modifications, specifically ubiquitination and phosphorylation. Our results identify BAI1 as a potential regulator and target of MDM2 ubiquitination function that has wide ranging implications from serving as a crosslink in p53 tumorigenic pathways to modulating synaptic stability and long-term potentiation. Ultimately, BAI1's diverse function within the brain marks it as an attractive target for the understanding and potential treatment of a wide range of neurological pathologies.

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Figures

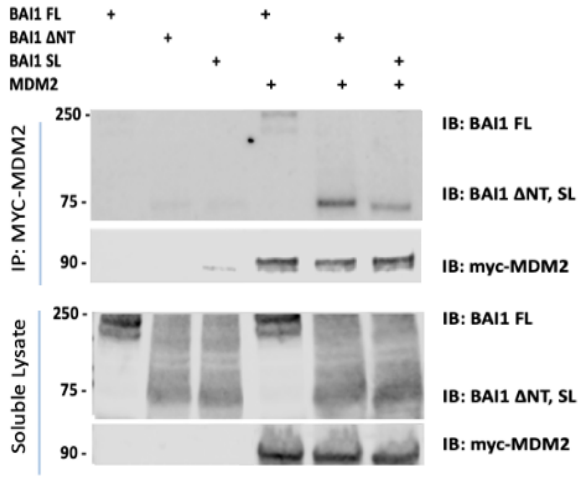

Figure 1a. MDM2 interacts to a greater degree with truncated forms of BAI1. Co-IP studies were performed to investigate the association between BAI1 FL, BAI1 ΔNT, BAI1 SL, and MDM2 in transfected HEK293T cells. Samples were analyzed via SDS-PAGE followed by Western blot. The lysate blot data demonstrate comparable protein expression levels across conditions.

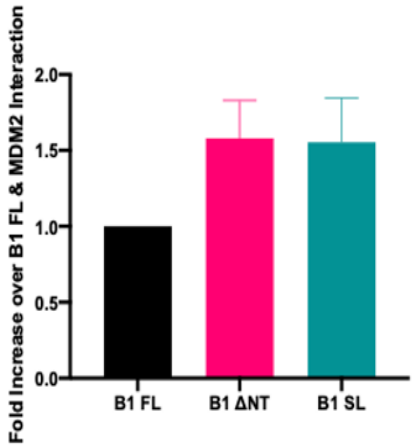

Figure 1b. MDM2 interacts to a greater degree with truncated forms of BAI1. Quantification of BAI1 from co-IP to investigate the association between BAI1 FL, BAI1 ΔNT, BAI1 SL, and MDM2. IP was normalized to lysates. Triplicates were averaged and level of BAI1-MDM2 interaction was displayed as fold over BAI1 FL-MDM2. (+/- S.E.M shown, BAI1 ΔNT p=0.08, BAI1 SL p=0.13 *versus* BAI1 FL, unpaired t test)

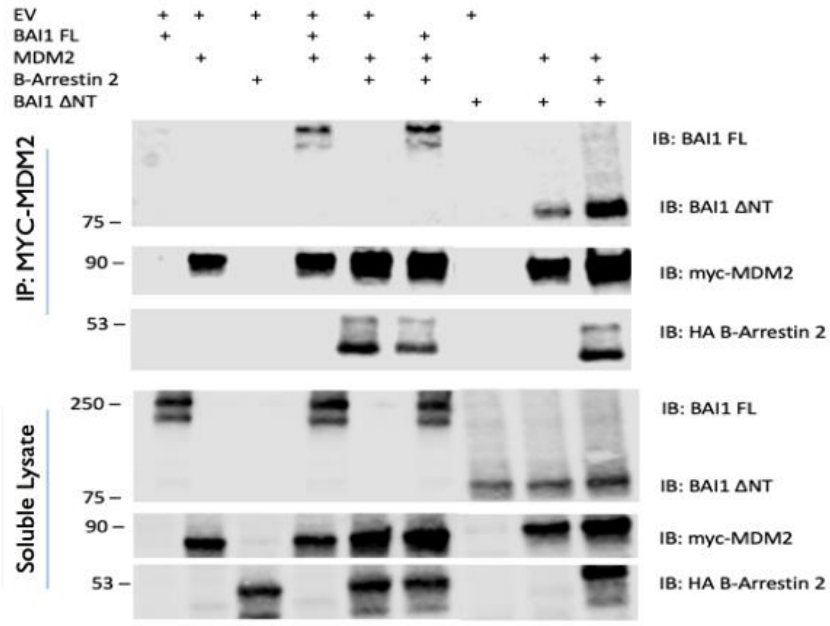

Figure 2a. β -Arrestin 2 increased association between MDM2 and BAI1. Co-IP studies were performed to investigate the association between BAI1 FL, BAI1 ΔNT and MDM2 in the presence and absence of β -Arrestin 2 in transfected HEK293T cells. Samples were analyzed via SDS-PAGE followed by Western blot. The input blot data demonstrate comparable protein expression levels across conditions.

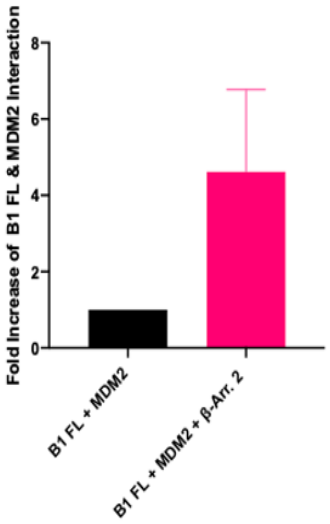

Figure 2b. β -Arrestin 2 facilitates BAI1 FL-MDM2 interaction. Quantification of BAI1 from co-IP to investigate the association between BAI1 FL and MDM2 in the presence and absence of β -Arrestin 2. IP was normalized to lysates. Triplicates were averaged and level of BAI1 FL-MDM2 interaction was displayed as fold over BAI1 FL-MDM2 baseline. (+/- S.E.M shown, Condition with β -Arrestin 2 p=0.168 *versus* baseline, unpaired t test)

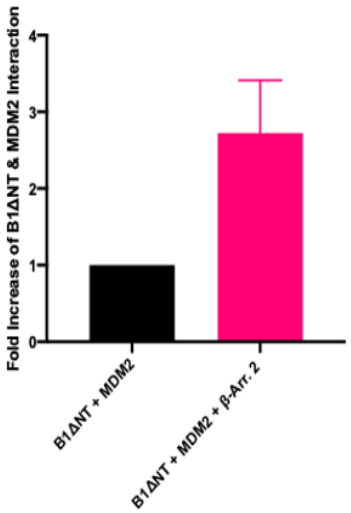

Figure 2c. β -Arrestin 2 facilitates BAI1 ΔNT-MDM2 interaction. Quantification of BAI1 from co-IP to investigate the association between BAI1 ΔNT and MDM2 in the presence and absence of β -Arrestin 2. IP was normalized to lysates. Triplicates were averaged and level of BAI1 ΔNT-MDM2 interaction was displayed as fold over BAI1 ΔNT -MDM2 baseline. (+/- S.E.M shown, Condition with β -Arrestin 2 p=0.0667 *versus* baseline, unpaired t test)

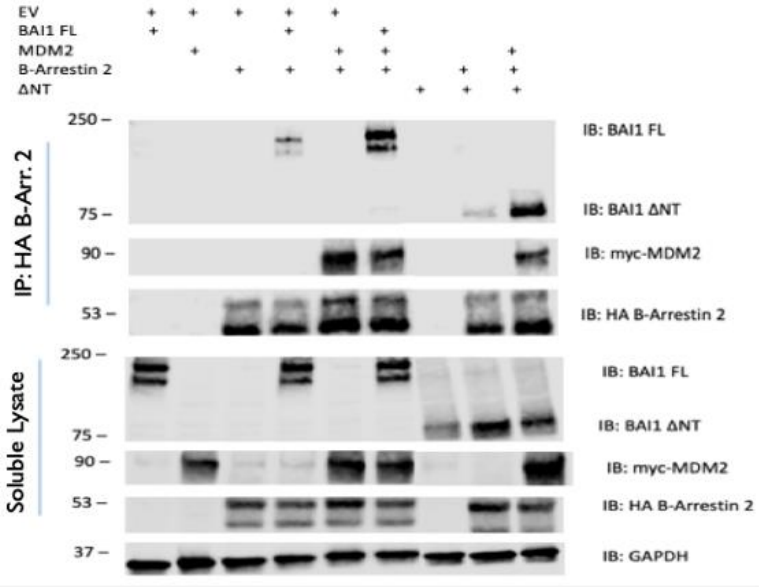

Figure 3a. MDM2 increases association between β -Arrestin 2 and BAI1. Co-IP studies were performed to investigate the association between BAI1 FL, BAI1 ΔNT and β -Arrestin 2 in the presence and absence of MDM2 in transfected HEK293T cells. Samples were analyzed via SDS-PAGE followed by Western blot. The input blot data demonstrate comparable protein expression levels across conditions.

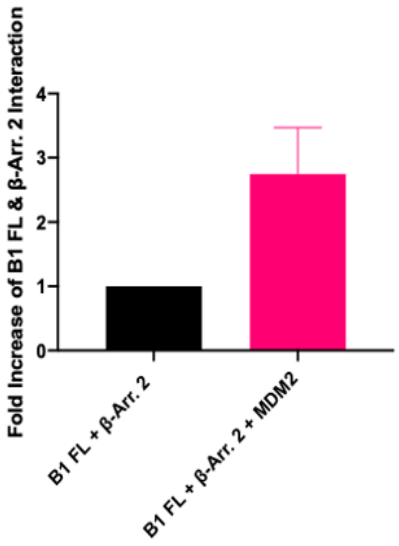

Figure 3b. MDM2 facilitates BAI1 FL- β -Arrestin 2 interaction. Quantification of BAI1 from co-IP to investigate the association between BAI1 FL and β -Arrestin 2 in the presence and absence of MDM2. IP was normalized to lysates. Triplicates were averaged and level of BAI1 FL- β -Arrestin 2 interaction was displayed as fold over BAI1 FL- β -Arrestin 2 baseline. (+/- S.E.M shown, Condition with MDM2 p=0.0731 *versus* baseline, unpaired t test)

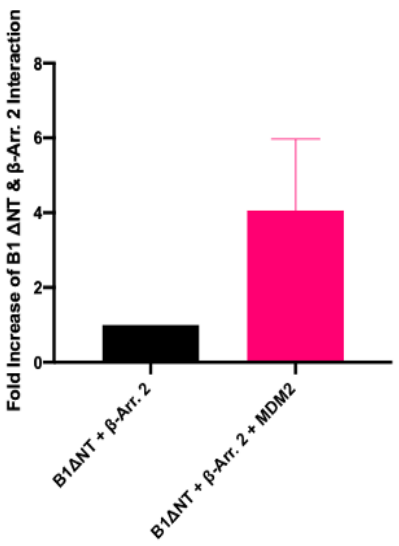

Figure 3b. MDM2 facilitates BAI1 ΔNT - β -Arrestin 2 interaction. Quantification of BAI1 from co-IP to investigate the association between BAI1 ΔNT and β -Arrestin 2 in the presence and absence of MDM2. IP was normalized to lysates. Triplicates were averaged and level of BAI1 ΔNT - β -Arrestin 2 interaction was displayed as fold over BAI1 ΔNT - β -Arrestin 2 baseline. (+/- S.E.M shown, Condition with MDM2 p=0.1852 *versus* baseline, unpaired t test)

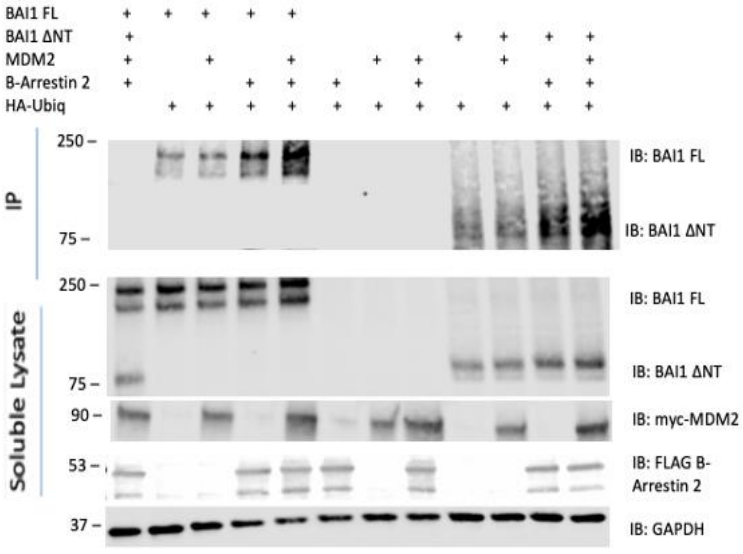

Figure 4a. MDM2 presence increased β -Arrestin 2 mediated ubiquitination of BAI1. Co-IP studies were performed to investigate the level of ubiquitination of BAI1 FL and BAI1 ΔNT in the presence and absence of MDM2 and β -Arrestin 2 in transfected HEK293T cells. IP was conducted using transfected HA tagged ubiquitin. BAI1 protein was subsequently blotted for. Samples were analyzed via SDS-PAGE followed by Western blot. The input blot data demonstrate comparable protein expression levels across conditions.

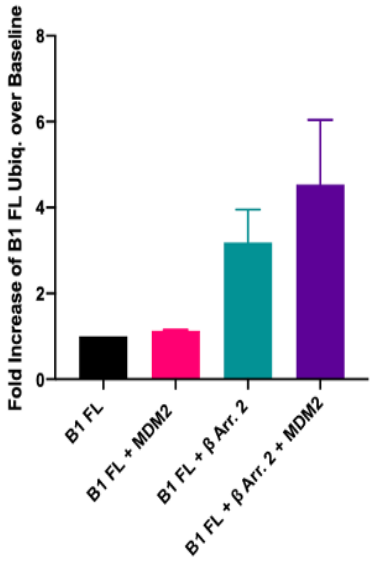

Figure 4b. MDM2 presence increases β -Arrestin 2 mediated ubiquitination of BAI1 FL. Quantification of BAI1 from co-IP to investigate the association between BAI1 FL and HA ubiquitin in the presence and absence of MDM2 and β -Arrestin 2. IP was normalized to lysates. Triplicates were averaged and level of BAI1 FL ubiquitination was displayed as fold over BAI1 FL ubiquitination baseline levels. (+/- S.E.M shown, B1 FL + β-Arr.: p=0.0472, B1 FL + β-Arr. + MDM2: p=0.0790 *versus* B1 FL baseline, unpaired t test)

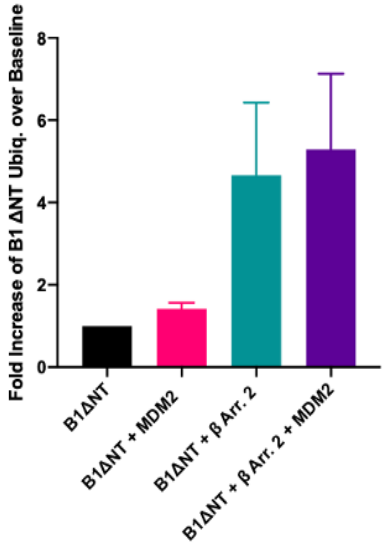

Figure 4c. MDM2 presence increases β -Arrestin 2 mediated ubiquitination of BAI1 ΔNT. Quantification of BAI1 from co-IP to investigate the association between BAI1 ΔNT and HA ubiquitin in the presence and absence of MDM2 and β -Arrestin 2. IP was normalized to lysates. Triplicates were averaged and level of BAI1 ΔNT ubiquitination was displayed as fold over BAI1 ΔNT ubiquitination baseline levels. (+/- S.E.M shown, B1 ΔNT + β-Arr.: p=0.1072, B1 ΔNT + β-Arr. + MDM2: p=0.0802 *versus* B1 ΔNT baseline, unpaired t test)

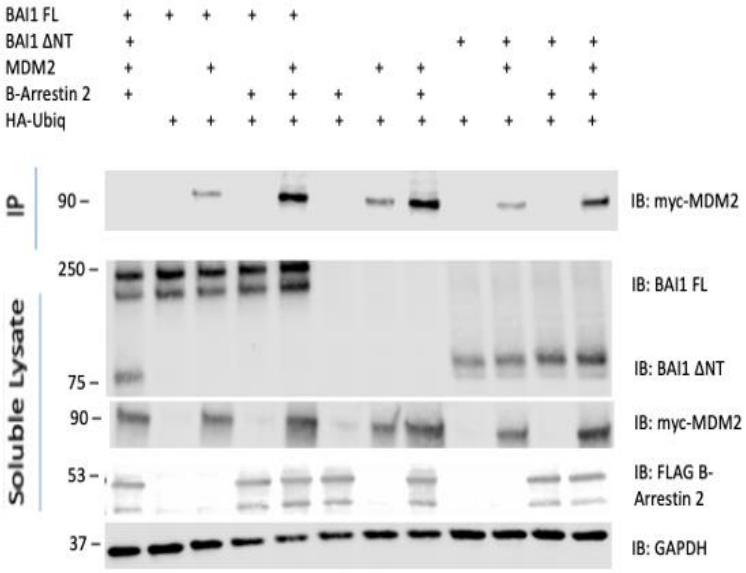

Figure 5a. MDM2 self-ubiquitination increases in the presence of β -Arrestin 2 and that it decreases in the presence of BAI1. Co-IP studies were performed to investigate the level of ubiquitination of MDM2 in the presence and absence of BAI1 and β -Arrestin 2 in transfected HEK293T cells. IP was conducted using transfected HA tagged ubiquitin. Myc tagged MDM2 was subsequently blotted for. Samples were analyzed via SDS-PAGE followed by Western blot. The input blot data demonstrate comparable protein expression levels across conditions.

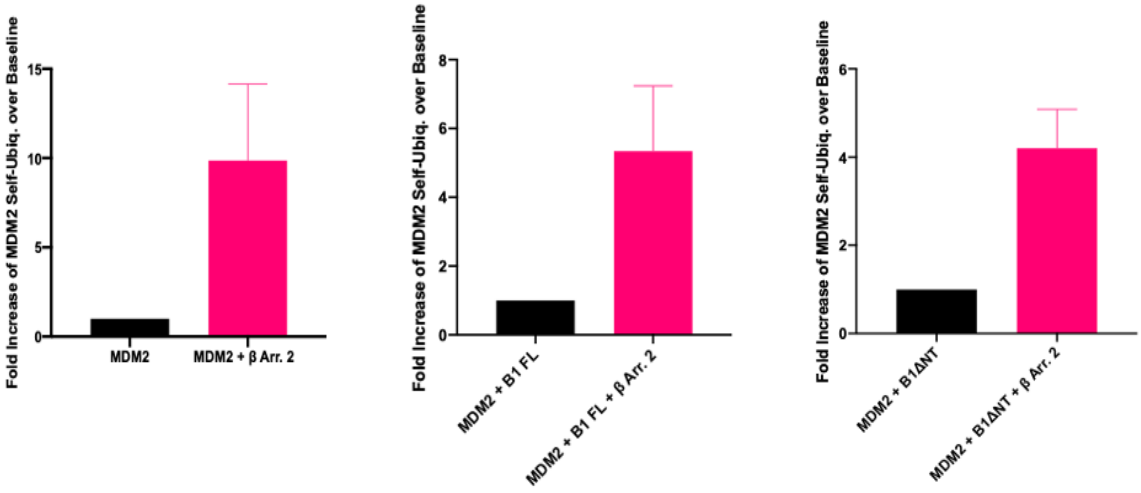

Figure 5b. β-Arrestin 2 presence increases MDM2 self-ubiquitination. Quantification of myc-MDM2 from co-IP to investigate the association between myc-MDM2 and HA ubiquitin in the presence and absence of β -Arrestin 2. IP was normalized to lysates. Triplicates were averaged and level of myc-MDM2 self-ubiquitination was displayed as fold over myc-MDM2 self-ubiquitination baseline levels. (+/- S.E.M shown, MDM2 + β -Arr. 2: p=0.1075, MDM2 + B1 FL + β -Arr. 2: p=0.085, MDM2 + B1 ΔNT + β -Arr. 2: p=0.0221 *versus* respective baseline, unpaired t test)

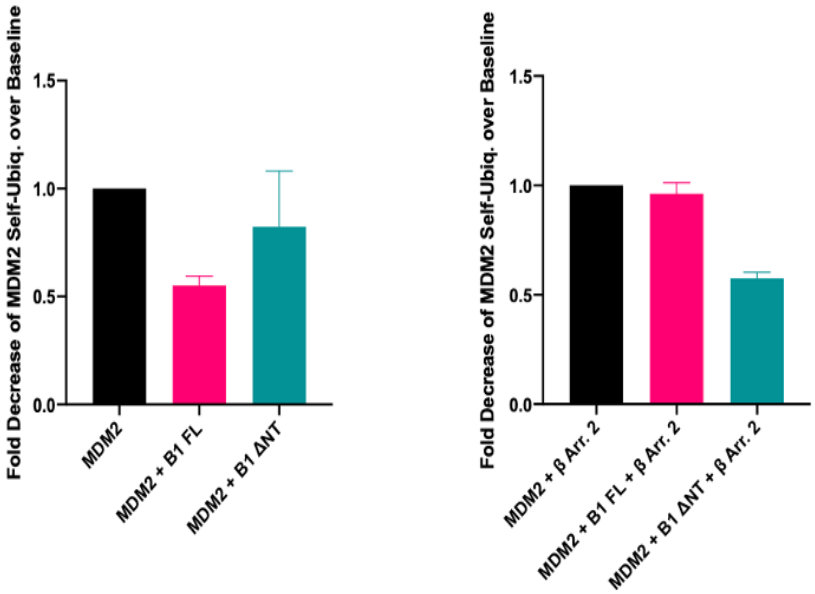

Figure 5c. BAI1 presence decreases MDM2 self-ubiquitination. Quantification of myc-MDM2 from co-IP to investigate the association between myc-MDM2 and HA ubiquitin in the presence and absence of BAI1. IP was normalized to lysates. Triplicates were averaged and level of myc-MDM2 self-ubiquitination was displayed as fold over myc-MDM2 self-ubiquitination baseline levels. (+/- S.E.M shown, MDM2 + B1 FL: p=0.0005, MDM2 + B1 ΔNT: p=0.5306, MDM2 + B1 FL + β -Arr. 2: p=0.4921, MDM2 + B1 ΔNT + β -Arr. 2: p=0.0001 *versus* respective baseline, unpaired t test)

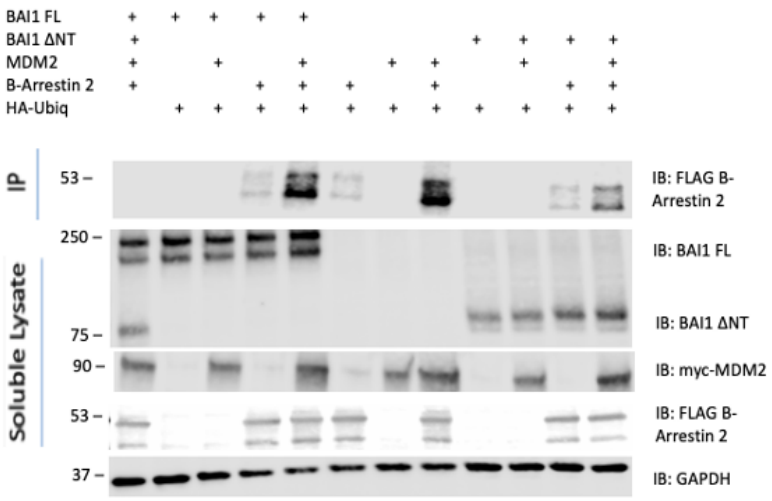

Figure 6a. β-Arrestin 2 ubiquitination increases in the presence of MDM2 and that it decreases in the presence of BAI1 ΔNT when MDM2 is coexpressed. Co-IP studies were performed to investigate the level of ubiquitination of β -Arrestin 2 in the presence and absence of BAI1 and MDM2 in transfected HEK293T cells. IP was conducted using transfected HA tagged ubiquitin. FLAG tagged β -Arrestin 2 was subsequently blotted for. Samples were analyzed via SDS-PAGE followed by Western blot. The input blot data demonstrate comparable protein expression levels across conditions.

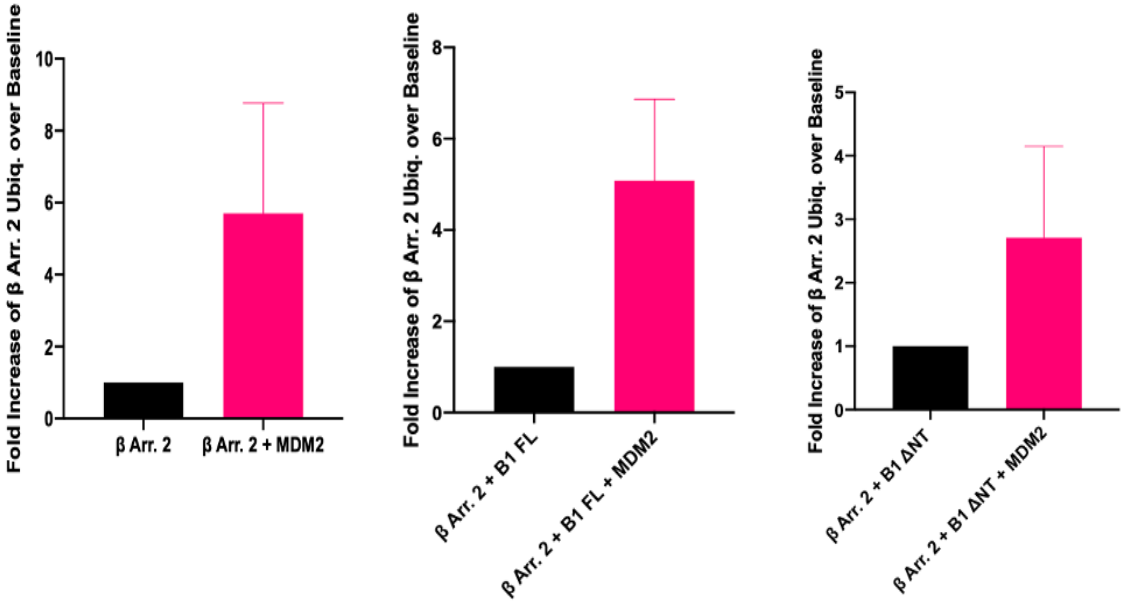

Figure 6b. MDM2 presence increases FLAG β -Arrestin 2 ubiquitination levels. Quantification of FLAG β -Arrestin 2 from co-IP to investigate the association between FLAG β-Arrestin 2 and HA ubiquitin in the presence and absence of MDM2. IP was normalized to lysates. Triplicates were averaged and level off FLAG β -Arrestin 2 ubiquitination was displayed as fold over FLAG β -Arrestin 2 ubiquitination baseline levels. (+/- S.E.M shown, β -Arr. 2 + MDM2: p=0.2005, β -Arr. 2 + B1 FL + MDM2: p=0.0841, β -Arr. 2 + B1 ΔNT + MDM2: p=0.3011 *versus* respective baseline, unpaired t test)

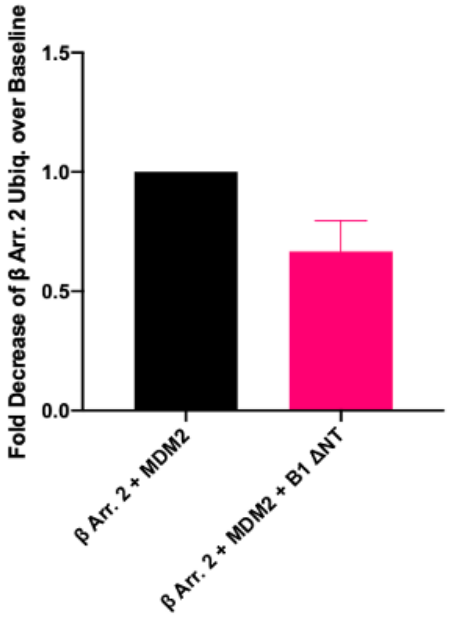

Figure 6c. BAI1 ΔNT presence decreases FLAG β -Arrestin 2 ubiquitination levels when coexpressed with MDM2. Quantification of FLAG β -Arrestin 2 from co-IP to investigate the association between FLAG β-Arrestin 2 and HA ubiquitin in the presence and absence of MDM2. IP was normalized to lysates. Triplicates were averaged and level off FLAG β -Arrestin 2 ubiquitination was displayed as fold over FLAG β -Arrestin 2 ubiquitination baseline levels. (+/- S.E.M shown, β -Arr. 2 + MDM2 + B1 ΔNT: p=0.0616, *versus* baseline, unpaired t test)

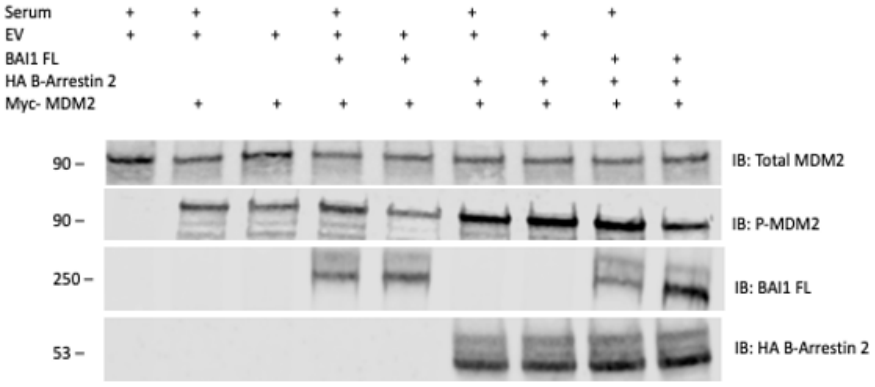

Figure 7a. Myc-MDM2 and β-Arrestin 2 increase phosphorylated MDM2 levels. Protein expression studies were performed to investigate the affect of BAI1 FL and β-Arrestin 2 on phosphorylated MDM2 levels. The condition that lacked transfected myc-MDM2 exhibited little to no phosphorylated MDM2.

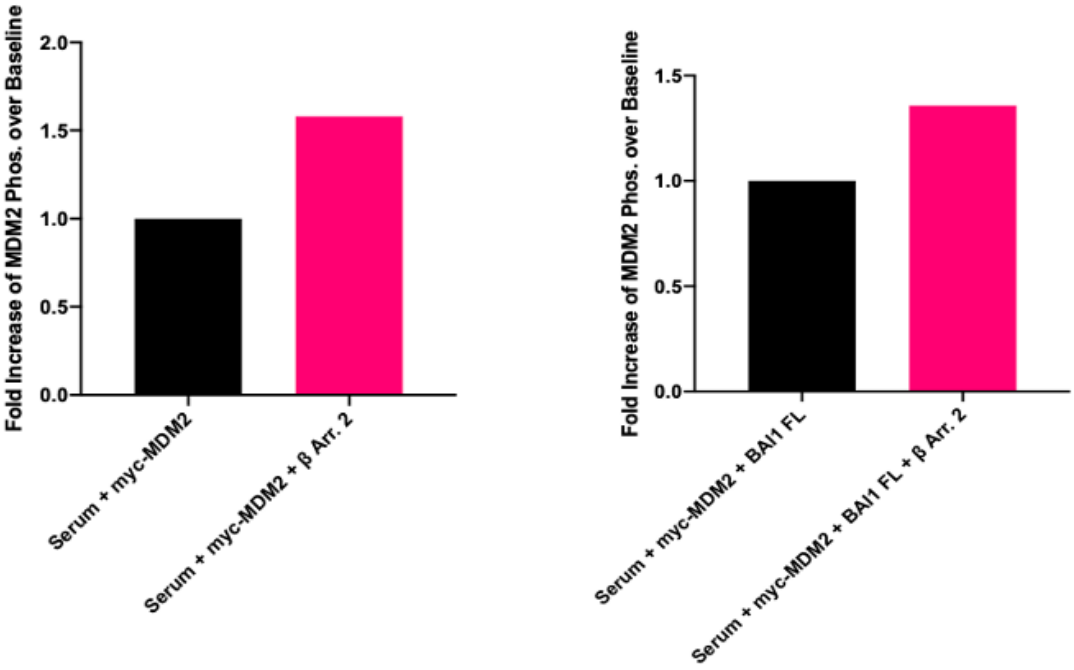

Figure 7b. β-Arrestin 2 presence increase phosphorylated MDM2 levels. Quantification of phosphorylated MDM2 in the presence and absence of β-Arrestin 2 . Single replicate displayed as fold over phosphorylated baseline levels.

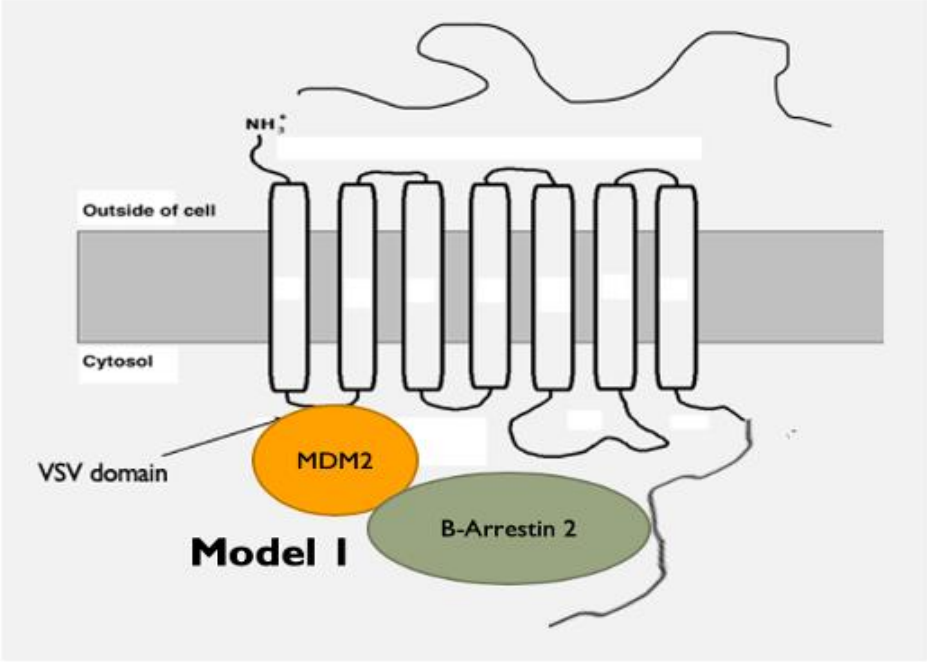

Figure 8a. "Stable Ternary Complex Mode." The first model by which MDM2 and β -Arrestin 2 mutually facilitate each other's interaction.

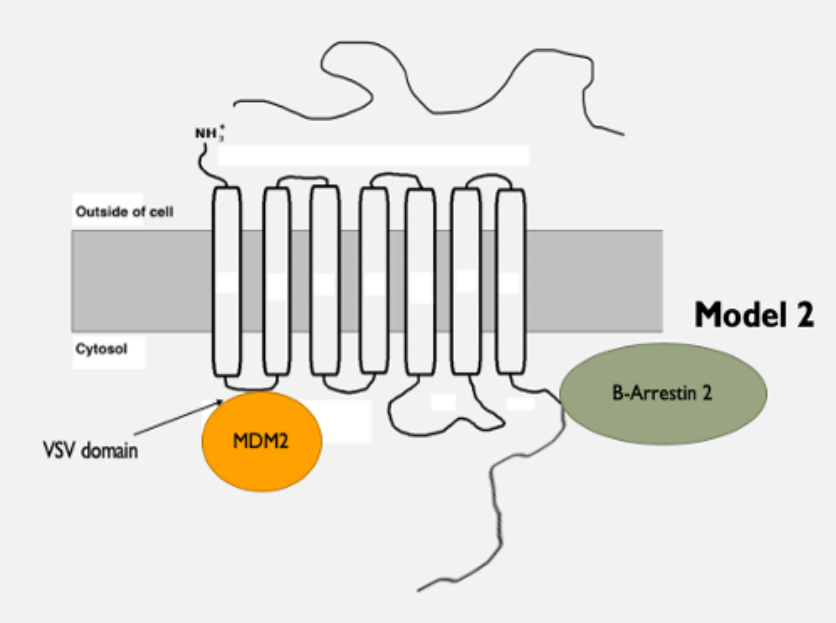

Figure 8b. "Conformation Change Model." The second model by which MDM2 and β -Arrestin 2 mutually facilitate each other's interaction.

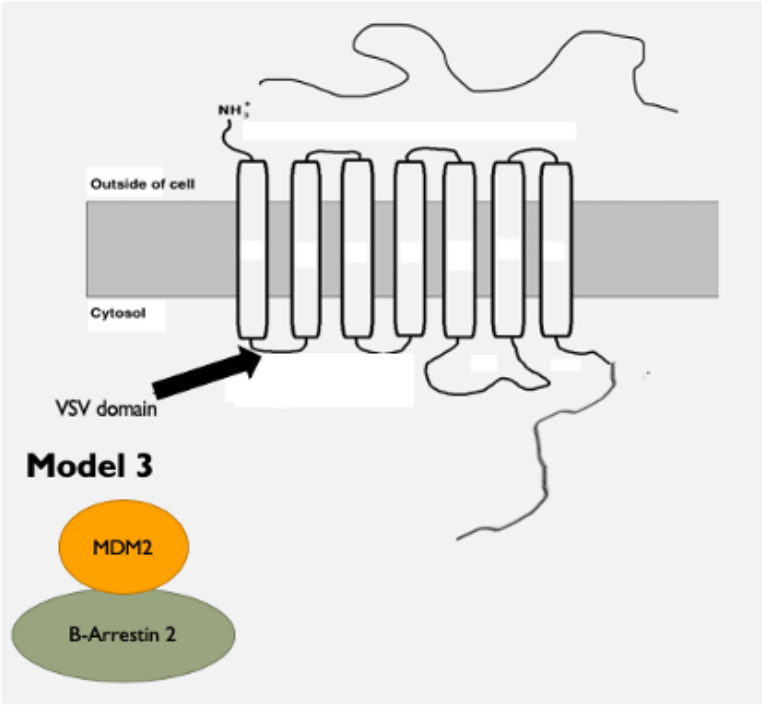

Figure 8c. "Recruitment and Hand-off Model." The third model by which MDM2 and β -Arrestin 2 mutually facilitate each other's interaction.

References

1. Attramadal H, Arriza JL, Aoki C, Dawson TM, Codina J, Kwatra MM, Snyder SH, Caron MG, Lefkowitz RJ. β -Arrestin2, a novel member of the arrestin/ β -arrestin gene family. *J Biol Chem*. 1992;267:17882–17890.

2. Blondel-Tepaz E., Guilbert T., Scott M.G.H. (2019) Methods to Investigate the Nucleocytoplasmic Shuttling Properties of β-Arrestins. In: Scott M., Laporte S. (eds) Beta-Arrestins. Methods in Molecular Biology, vol 1957. Humana Press, New York, N

3. Colman, M. S., Afshari, C. A., and Barrett, J. C. (2000) *Mut. Res.* 462, 179 –188

4. Cork, S.M. and Van Meir, E.G. (2011) Emerging roles for the BAI1 protein family in the regulation of phagocytosis, synaptogenesis, neurovasculature, and tumor development. J. Mol. Med. (Berl.) 89, 743–752

5. Daujat, S., Neel, H., and Piette, J. (2001) *Trends Genet.* 17, 459–464

6. Goodman OB Jr, Krupnick JG, Santini F, Gurevich VV, Penn RB, Gagnon AW, Keen JH, Benovic JL. β -Arrestin acts as a clathrin adaptor in endocytosis of the B2 -adrenergic receptor. *Nature*. 1996;383:447–450.

7. Hall RA, Lefkowitz RJ. Regulation of G Protein–Coupled Receptor Signaling by Scaffold Proteins. Circulation Research. 2002;91(8):672-80.

8. Hara MR, Kovacs JJ, Whalen EJ, Rajagopal S, Strachan RT, Grant W, Towers AJ, Williams B, Lam CM, Xiao K, Shenoy SK, Gregory SG, Ahn S, Duckett DR, Lefkowitz RJ. A stress response pathway regulates DNA damage through beta2-adrenoreceptors and beta-arrestin-1. Nature. 2011;477:349–353.

9. Honda, R., Tanaka, H., and Yasuda, H. (1997) *FEBS Lett.* 420, 25–27

10. Jones, S. N., Roe, A. E., Donehower, L. A., and Bradley, A. (1995) *Nature* 378, 206 –208

11. Kaur, B., Brat, D. J., Calkins, C. C., and Van Meir, E. G. (2003) Brain angiogenesis inhibitor 1 is differentially expressed in normal brain and glioblastoma independently of p53 expression. *Am. J. Pathol.* 162, 19 –27

12. Kaur, B., Brat, D.J., Devi, N.S., and Van Meir, E.G.(2005)Vasculostatin, a proteolytic fragment of brain angiogenesis inhibitor 1, is an antiangiogenic and antitumorigenic factor. *Oncogene* 24, 3632–3642

13. Kaur, B., Cork, S. M., Sandberg, E. M., Devi, N. S., Zhang, Z., Klenotic, P. A., Febbraio, M., Shim, H., Mao, H., Tucker-Burden, C., Silverstein, R. L., Brat, D. J., Olson, J. J., and Van Meir, E. G. (2009) Vasculostatin inhibits intracranial glioma growth and negatively regulates *in vivo* angio- genesis through a CD36-dependent mechanism. *Cancer Res.* 69, 1212–1220

14. Kay, B.K .et al.(2000) The importance of being proline: the interaction of proline-rich motifs in signaling proteins with their cognate domains. FASEB J. 14, 231–241

15. Kishore A, Purcell RH, Nassiri-Toosi Z, Hall RA. Stalk-dependent and Stalk independent Signaling by the Adhesion G Protein-coupled Receptors GPR56 (ADGRG1) and BAI1 (ADGRB1). J Biol Chem. 2016;291(7):3385-94.

16. Koh, J.T. et al. (2001) Characterization of mouse brain-specific angiogenesis inhibitor 1 (BAI1) and phytanoyl-CoA alpha- hydroxylase-associated protein 1, a novel BAI1-binding protein. Brain Res. Mol. Brain Res. 87, 223–237

17. Kudo, S., Konda, R., Obara, W., Kudo, D., Tani, K., Nakamura, Y., and Fujioka, T. (2007) Inhibition of tumor growth through suppression of angiogenesis by brain-specific angiogenesis inhibitor 1 gene transfer in murine renal cell carcinoma. *Oncol. Rep.* 18, 785–791

18. Krupnick JG, Benovic JL. The role of receptor kinases and arrestins in G protein–coupled receptor regulation. *Annu Rev Pharmacol Toxicol*. 1998; 38:289 –319.

19. Lee Y, Basith S, Choi S. Recent Advances in Structure-Based Drug Design Targeting Class A G Protein-Coupled Receptors Utilizing Crystal Structures and Computational Simulations. J Med Chem. 2018;61(1):1-46.

20. Levine, A. J. (1997) *Cell* 88, 323–331

21. Lohse MJ, Benovic JL, Codina J, Caron MG, Lefkowitz RJ. β -Arrestin: a protein that regulates B2-adrenergic receptor function. *Science*. 1990;248: 1547–1550.

22. McDonald PH, Cote NL, Lin FT, Premont RT, Pitcher JA, Lefkowitz RJ. Identification of NSF as a β -arrestin1-binding protein: implications for B2 -adrenergic receptor regulation. *J Biol Chem*. 1999;274:10677–10680.

23. Momand, J., Wu, H., and Dasgupta, G. (2000) *Gene* (*Amst*.) 242, 15–29

24. Montes de Oca Luna, R., Wagner, D. S., and Lozano, G. (1995) *Nature* 378, 203–206

25. Mori, K. et al. (2002) Brain-specific angiogenesis inhibitor 1 (BAI1) is expressed in human cerebral neuronal cells. Neurosci. Res. 43, 69–74

26. Nishimori, H., Shiratsuchi, T., Urano, T. *et al.* A novel brain-specific p53-target gene, BAI1, containing thrombospondin type 1 repeats inhibits experimental angiogenesis. *Oncogene* **15,** 2145–2150 (1997). https://doi.org/10.1038/sj.onc.1201542

27. Park, D. et al. (2007) BAI1 is an engulfment receptor for apoptotic cells upstream of the ELMO/Dock180/Rac module. Nature 450, 430–434

28. Pierce KL, Premont RT, Lefkowitz RJ. Seven-transmembrane receptors. Nat Rev Mol Cell Biol. 2002;3(9):639-50.

29. Ranaweera, Ruchira S, and Xiaolu Yang. "Auto-ubiquitination of Mdm2 enhances its substrate ubiquitin ligase activity." *The Journal of biological chemistry* vol. 288,26 (2013): 18939-46. doi:10.1074/jbc.M113.454470

30. Shenoy SK, Lefkowitz RJ. (2005) Receptor-specific ubiquitination of beta-arrestin directs assembly and targeting of seven-transmembrane receptor signalosomes. J Biol Chem 280:15315–15324

31. Shenoy SK, McDonald PH, Kohout TA, Lefkowitz RJ. Regulation of receptor fate by ubiquitination of activated B2 -adrenergic receptor and β -arrestin 2. *Science*. 2001;294:1307–1313

32. Shenoy S.K., Xiao K., Venkataramanan V., Snyder P.M., Freedman N.J., Weissman A.M. Nedd4 mediates agonist-dependent ubiquitination, lysosomal targeting, and degradation of the β2-adrenergic receptor. J. Biol. Chem. 2008;283:22166–22176. doi: 10.1074/jbc.M709668200.''

33. Sokolowski, J.D. et al. (2011) Brain-specific angiogenesis inhibitor-1 expression in astrocytes and neurons: implications for its dual function as an apoptotic engulfment receptor. Brain Behav. Immun. 25, 915–921

34. Stephenson JR, Paavola KJ, Schaefer SA, Kaur B, Van Meir EG, Hall RA. Brain-specific angiogenesis inhibitor-1 signaling, regulation, and enrichment in the postsynaptic density. J Biol Chem. 2013;288(31):22248-56.

35. Stephenson JR, Purcell RH, Hall RA. The BAI subfamily of adhesion GPCRs: synaptic regulation and beyond. Trends Pharmacol Sci. 2014;35(4):208-15.

36. Tu YK, Duman JG, Tolias KF. The Adhesion-GPCR BAI1 Promotes Excitatory Synaptogenesis by Coordinating Bidirectional Trans-synaptic Signaling. J Neurosci. 2018;38(39):8388-406.

37. Vogelstein, B., Lane, D., and Levine, A. J. (2000) *Nature* 408, 307–310

38. Wang P, Gao H, Ni Y, Wang B, Wu Y, Ji L, et al. Beta-arrestin 2 functions as a G-protein-coupled receptor-activated regulator of oncoprotein Mdm2. J Biol Chem. 2003;278(8):6363-70.

39. Weber, J. D., Taylor, L. J., Roussel, M. F., Sherr, C. J., and Bar-Sagi, D. (1999) *Nat. Cell Biol.* 1, 20–26

40. Woods, D. B., and Vousden, K. H. (2001) *Exp. Cell Res.* 264, 56–66

41. Xiao, X. R., Kang, X. X., and Zhao, J. Z. (2006) [Therapeutic effect of brain-specific angiogenesis inhibitor 1 on glioblastoma: an animal experiment. *Zhonghua Yi Xue Za Zhi* 86, 1342–1346

42. Yoon, K.C., Ahn, K.Y. ,Lee, J.H., Chun, B.J., Park, S.W., Seo, M.S., Park, Y. G., and Kim, K. K. (2005) Lipid-mediated delivery of brain-specific angiogenesis inhibitor 1 gene reduces corneal neovascularization in an *in vivo* rabbit model. *Gene Ther.* 12, 617– 624

43. Zhang, Y., and Xiong, Y. (1999) *Mol. Cell* 3, 579–591

44. Zhu, D., Hunter, S.B., Vertino, P.M., and Van Meir, E.G.(2011) Overexpression of MBD2 in glioblastoma maintains epigenetic silencing and inhibits the antiangiogenic function of the tumor suppressor gene BAI1. *Cancer Res.* 71, 5859 –5870

45. Zhu D, Li C, Swanson AM, Villalba RM, Guo J, Zhang Z, et al. BAI1 regulates spatial learning and synaptic plasticity in the hippocampus. J Clin Invest. 2015;125(4):1497-508.

46. Zhu D, Osuka S, Zhang Z, Reichert ZR, Yang L, Kanemura Y, et al. BAI1 Suppresses Medulloblastoma Formation by Protecting p53 from Mdm2-Mediated Degradation. Cancer Cell. 2018;33(6):1004-16 e5.