ABIN-1 Negatively Regulates μ -Opioid Receptor Function^[S]

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ABSTRACT

The μ -opioid receptor (MOR) is a $G_{i/o}$ protein-coupled receptor that mediates analgesic, euphoric, and reward effects. Using a bacterial two-hybrid screen, we reported that the carboxyl tail of the rat MOR associates with A20-binding inhibitor of nuclear factor κ B (ABIN-1). This interaction was confirmed by direct protein-protein binding and coimmunoprecipitation of MOR and ABIN-1 proteins in cell lysates. Saturation binding studies showed that ABIN-1 had no effect on MOR binding. However, the interaction of ABIN-1 and MOR inhibited the activation of G proteins induced by DAMGO ([p-Ala²,N-Me-Phe⁴,Gly⁵-ol]-Enkephalin). MOR phosphorylation, ubiquitination, and internalization induced by DAMGO were decreased in Chinese hamster ovary cells that coexpressed MOR and ABIN-1. The suppression of forskolin-stimulated adenylyl

cyclase by DAMGO was also inhibited by the interaction of ABIN-1 with MOR. In addition, extracellular signal-regulated kinase activation was also negatively regulated by overexpression of ABIN-1. These data suggest that ABIN-1 is a negative coregulator of MOR activation, phosphorylation, and internalization in vitro. ABIN-1 also inhibited morphine-induced hyperlocomotion in zebrafish larvae (AB strain). By utilization of an antisense morpholino oligonucleotide (MO) gene knockdown technology, the ABIN-1 MO-injected zebrafish larvae showed a significant increase (approximately 60%) in distance moved compared with control MO-injected larvae after acute morphine treatment ($P < 0.01$). Taken together, ABIN-1 negatively regulates MOR function in vitro and in vivo.

Introduction

Opioid receptors belong to the super-family of G protein-coupled receptors (GPCRs). The μ -opioid receptors (MOR) are one of the opioid receptors that mediate the characteristics associated with morphine, including analgesia, dependence, and tolerance. MOR generally undergoes rapid desensitization after being activated by their agonists and gets sequentially phosphorylated by G protein-coupled receptor kinases (GRKs). The phosphorylated MOR recruits β -arrestins and then uncouples G proteins from itself, thereby promoting rapid endocytosis of the receptor (Lefkowitz, 1998; Bohn et al., 1999, 2000; Lefkowitz and Shenoy, 2005; Williams et al., 2013). Agonist-induced MOR internalization is also dependent on opioid agonist properties as well as intracellular proteins that interact with MOR (Arden et al., 1995; Zhang et al., 1998; Schulz et al., 2004; Groer et al., 2007). These data indicate that intracellular proteins that interact with the

receptor might participate in MOR regulation and trafficking. However, the dynamic processes that underlie MOR phosphorylation, internalization, and desensitization remain to be elucidated.

Recent studies have focused on opioid receptor signaling, regulation, and trafficking. The C-terminal tail and the third intracellular loop of opioid receptors are critical for mediating signal transfer to G proteins and protein-protein interactions (Hawes et al., 1994; Morou and Georgoussi, 2005). Several proteins have been identified that physically interact with the carboxyl termini and third intracellular loop of MOR, which regulate the signal transduction of MOR (Georgoussi et al., 2012). Chronic opioid administration results in several changes to gene and protein expression in animals. For example, chronic morphine addition has been shown to induce the upregulation of heat shock protein families, silencer factor B, and Krox20 in the frontal cortex (Ammon et al., 2003), as well as tyrosine hydroxylase and galanin prodynorphin serum/glucocorticoid-regulated kinase in the locus ceruleus (McClung et al., 2005) of rats. These changes may be the key to understanding the mechanisms of morphine dependence and how the interaction of specific proteins with MOR regulates its function in morphine-dependent animals.

In our previous study, a morphine-dependent rat brain cDNA library was screened using C-terminal tail of MOR in a

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ABBREVIATIONS: ABIN-1, A20-binding inhibitor of nuclear factor κ B; ANOVA, analysis of variance; BSA, bovine serum albumin; CHO, Chinese hamster ovary; DAMGO, [p-Ala²,N-Me-Phe⁴,Gly⁵-ol]-enkephalin; ERK, extracellular signal-regulated kinase; GPCRs, G protein-coupled receptors; GRK, G protein-coupled receptor kinase; GST, glutathione S-transferase; [³⁵S]GTP γ S, guanosine 5'-O-(3-[³⁵S]thio)triphosphate; HRP, horseradish peroxidase; MO, morpholino oligonucleotide; MOR, μ -opioid receptors; PBS, phosphate-buffered saline; qPCR, quantitative polymerase chain reaction; RT, reverse transcription; TNF, tumor necrosis factor.

bacterial two-hybrid system. A20-binding inhibitor of nuclear factor (NF)- κ B 1 (ABIN-1, also known as TNIP1, Naf1, and VAN) was one of the 19 known proteins that were identified (Zhou et al., 2015). ABIN-1 is predominantly expressed in the human lymphatic system and blood (Verstrepen et al., 2009). Little is known about the function of ABIN-1 in the central nervous system and the ABIN-1-mediated regulation of MOR signaling. In our present study, we are the first to provide evidence that ABIN-1 interacts with MOR and this interaction negatively regulates MOR function in Chinese hamster ovary (CHO) cells and zebrafish (*Danio rerio*) larvae.

Materials and Methods

Column Overlay Assays. The C-terminus of rat MOR (aa 402–460) was subcloned into vector pGEX-4T-3 for expression in *Escherichia coli* strain DH5 α as a glutathione *S*-transferase (GST) fusion. Full-length ABIN-1 (BC008186, BCCM/LMBP) was subcloned into the pET-28a vector (Novagen, Madison, WI) to generate a fusion His-ABIN-1 protein. His-ABIN-1 was expressed in *E. coli* strain BL21 and affinity-purified using a Pro-Bond Nickel-chelating resin. Binding assay between GST-C and His-ABIN-1 fusion proteins was carried out as previously described (Zhou et al., 2015). GST-C and GST were immobilized on glutathione agarose beads and incubated with purified His-ABIN-1 at 4°C for 16 hours. Bound proteins were eluted with 10 mM glutathione in 50 mM Tris buffer (pH 8.0). Fractions of eluted proteins were separated by SDS/PAGE, and immunoblotting was visualized using the enhanced chemiluminescence method following overnight incubation at 4°C with monoclonal anti-polyhistidine (1:500 dilution; Santa Cruz Biotechnology, Dallas, TX) or polyclonal anti-GST antibodies (1:1000 dilution; Sigma-Aldrich, St. Louis, MO). Equal amounts of protein were added to each lane, based on the amounts of GST and His-ABIN-1 estimated by immunoblotting.

Immunofluorescence Staining. The colocalization of ABIN-1 with MOR using immunocytochemistry was carried out as previously described (Zhou et al., 2015). Chinese hamster ovary cells that were stably transfected with DDK-tagged MOR (MOR-CHO), ABIN-1 (ABIN-1-CHO), or cotransfected with DDK-tagged MOR and ABIN-1 (MOR-ABIN-1-CHO) were maintained in F12 media containing 10% fetal bovine serum (FBS) (plus 200 μ g/ml geneticin with or without 40 μ g/ml hygromycin). The C-terminal single flag-tagged MOR was subcloned into pCMV6 Entry (DDK-tagged MOR) (RC210383; OriGene, Rockville, MD). Cells were seeded on glass coverslips in 24-well plates at 70–80% confluence and then treated with or without 5 μ M DAMGO for 30 minutes. After washing in phosphate-buffered saline (PBS) and fixing in 3% freshly prepared paraformaldehyde for 20 minutes, the cells were once more washed in PBS and blocked in PBS containing 1% bovine serum albumin (BSA) and 3% normal donkey serum for 1 hour. Cells were then permeabilized with PBS/0.2% Triton X-100 for 5 minutes. After washing again in PBS, the cells were incubated with 20 μ g/ml M2 anti-FLAG antibody (Sigma-Aldrich) with or without 20 μ g/ml polyclonal anti-ABIN-1 antibody (Cell Signaling, Danvers, MA) at room temperature for 90 minutes. After washing in PBS, the cells were incubated in the dark with rhodamine-conjugated AffiniPure goat anti-mouse IgG (1:100; Santa Cruz Biotechnology), Alexa Fluor 488 goat anti-mouse or rabbit IgG (1:200 dilution; Invitrogen, Karlsruhe, Germany) to determine colocalization, or with Alexa Fluor 488 goat anti-mouse IgG (1:200 dilution; Invitrogen) for 1 hour at room temperature to determine MOR internalization. After washing in PBS with or without Hoechst (10 μ g/ml), the cells were mounted onto glass slides and examined using a laser-scanning Zeiss LSM510 confocal microscope at the following settings: 488 nm excitation and 527 nm emission for fluorescein isothiocyanate (FITC), 543 nm excitation and 560 nm emission for tetramethyl rhodamine (TRITC), 460 nm excitation and 535 nm emission for Hoechst staining.

Human neuroblastoma SH-SY5Y cells were also seeded on coverslips and cultured for 24 hours. The immunocytochemistry process was similar to MOR-CHO cells. To detect the expression of MOR and ABIN-1, 0.3 μ g/ml monoclonal rabbit anti-MOR (Abcam, Cambridge, UK), 20 μ g/ml polyclonal rabbit anti-ABIN-1 and Alexa Fluor488 (green)-coupled goat anti-rabbit IgG were used.

MOR-CHO and MOR-ABIN-1-CHO cells were seeded in 96-well plates at a density of 8×10^3 cells/well. After incubation for 24 hours at 37°C with 5% CO₂, the culture medium was replaced with F12 culture medium without FBS for 2 hours. Media was then replaced with F12 containing 0 or 10 μ M DAMGO for 30 minutes. Cell fixation and antibody incubation were carried out as described above. The cells were imaged using IN Cell Analyzer 2000 (20 $\times$ objective; GE Healthcare Life Sciences, Pittsburgh, PA) with the following settings: exposure time of 500 milliseconds/field, 475 nm excitation, and 535 nm emission for FITC. The images were analyzed using the Granularity Analysis Module.

Coimmunoprecipitation. MOR-CHO, ABIN-1-CHO, and MOR-ABIN-1-CHO cells in 60-mm plates at 90% confluence were washed twice with ice-cold PBS and lysed in 200 μ l RIPA⁺ buffer (1% Nonidet P-40, 0.05% deoxycholic acid, 0.1% SDS, 50 mM Tris-HCl (pH 8.0), 150 mM NaCl) supplemented with a standard protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany) for 30 minutes on ice. The lysate was centrifuged at 15,000g for 15 minutes at 4°C. Supernatant (50 μ l) was removed to determine protein concentration and permit equal protein loading and immunoblotting. An additional 350 μ l of extraction buffer was then added to give a final volume of 500 μ l, and the lysate was incubated overnight at 4°C with 2 μ g of M2 anti-Flag antibody (Sigma-Aldrich). Subsequently, 40 μ l of protein A/G agarose (Santa Cruz Biotechnology) was added for 2 hours at 4°C. Immunoprecipitates were collected by centrifugation at 3000g for 5 minutes at 4°C, and then washed three times with 1 ml of extraction buffer before being eluted with 50 μ l of 1 $\times$ SDS sample buffer at 95°C for 5 minutes. Eluted samples (20 μ l) were separated by SDS/PAGE, and immunoblot analysis was performed using a polyclonal anti-ABIN-1 antibody (1:800; Cell Signaling) or anti-FLAG M2-horseradish peroxidase (HRP) antibody (1:1000; Sigma-Aldrich) to determine protein levels.

SH-SY5Y cells were also used for coimmunoprecipitation experiments to verify endogenous ABIN-1 and MOR interaction. The process was similar to that used for CHO cells. SH-SY5Y cell lysates were incubated overnight at 4°C with 1 μ g of polyclonal anti-MOR antibody (Millipore Sigma, Burlington, MA). Eluted samples were separated by SDS/PAGE, and immunoblotting analysis was performed using polyclonal anti-ABIN-1 antibody (1:800; Cell Signaling) to detect ABIN-1 and monoclonal anti-MOR antibody (1:500; Abcam) to detect MOR.

Receptor Radioligand Binding. Cell membranes from MOR-CHO and MOR-ABIN-1-CHO cells were prepared as described previously (Li et al., 2002a). Membrane proteins (20 μ g/tube) were incubated with 0.06–1.2 nM [³H]-diprenorphine (50 Ci/mmol; PerkinElmer, Waltham, MA). Nonspecific binding was evaluated in the presence of 10 μ M naloxone. The binding reactions were carried out in duplicate in 50 mM Tris-HCl buffer (pH 7.4) at 37°C for 30 minutes in a total volume of 0.5 ml and terminated by inserting the assay tubes into ice-cold water. The membrane-bound [³H]-diprenorphine was rapidly separated by filtration through GF/C filters. The filters were washed three times with 5 ml of ice-cold Tris-HCl buffer, and filter-bound radioactivity was counted using a liquid scintillation counter (LS6500; Beckman Inc., Brea, CA).

[³⁵S]-GTP γ S Binding Assay. MOR-CHO and MOR-ABIN-1-CHO cells were harvested in Tris/EGTA/MgCl₂ (50 mM Tris-HCl, 10 mM EGTA, 5 mM MgCl₂, pH 7.4). The cells were subsequently dispersed by agitation. After centrifugation at 500g, the cell pellet was resuspended in ice-cold Tris/EGTA/MgCl₂ and passed through a 29G3/8 syringe needle at least 10 times. The pellet was then centrifuged at 20,000g for 30 minutes, resuspended in Tris/EGTA/MgCl₂, and passed again through the syringe needle and centrifuged in the same manner. Finally, the pellet was resuspended in membrane buffer (50 mM Tris-HCl,

100 mM NaCl, 10 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 0.1% BSA, pH 7.4). Membrane protein (20 µg) was incubated with DAMGO (10⁻¹¹ to 10⁻⁵ M) in buffer A (100 mM Tris, 100 mM NaCl, 5 mM MgCl₂, pH 7.4) containing [³⁵S]-GTPγS (200 pM) (1250 Ci/mmol; PerkinElmer) and 30 µM guanosine 5'-diphosphate (GDP; Sigma-Aldrich) in a total volume of 0.5 ml. Nonspecific binding was determined following incubation in the presence of 40 µM unlabeled GTPγS with 0.2 nM [³⁵S]-GTPγS. Basal nonspecific binding was determined following incubation in the presence of [³⁵S]-GTPγS (0.2 nM). Basal binding was defined as the level of [³⁵S]-GTPγS binding in the absence of agonist. After 1 hour of incubation at 25°C, binding was terminated in ice-cold water, and the mixture was then passed through GF/C filters under reduced pressure and rinsed three times with ice-cold buffer B (50 mM Tris-HCl, 50 mM NaCl, 5 mM MgCl₂, pH 7.4). The level of radioactivity on the filters was determined by liquid scintillation counting using a LS 6500 scintillation counter (Beckman Coulter, Fullerton, CA). Net-stimulated [³⁵S]-GTPγS binding was defined as stimulated binding minus nonspecific binding. Percentage stimulation was defined as (net stimulated binding/basal binding minus nonspecific binding) × 100%. EC₅₀ values were calculated using nonlinear regression.

Phosphorylation and Ubiquitination of MOR. MOR-CHO and MOR-ABIN-1-CHO cells were plated onto 60-mm dishes and grown to 80–90% confluence. Cells were exposed to 0 or 5 µM DAMGO for 30 minutes then cooled to 4°C by washing with ice-cold PBS. Proteins were lysed for 30 minutes at 4°C with 0.2 ml of RIPA⁺ buffer supplemented with protease inhibitors. The lysate was centrifuged at 15,000g for 15 minutes at 4°C. The supernatant was then removed and solubilized proteins were incubated with 1 µg of anti-FLAG antibody (Sigma-Aldrich) for 2 hours. A total of 40 µl of protein A/G (Santa Cruz Biotechnology) was then added for an overnight incubation at 4°C to immunoprecipitate DDK-tagged MOR. The immunoprecipitated proteins were washed three times with RIPA⁺ buffer before being eluted with 50 µl of 1× SDS sample buffer at 95°C for 5 minutes or 56°C for 20 minutes. Eluted samples (20 µl) were separated using SDS/PAGE and immunoblotting was performed using an anti-phospho-MOR (Ser375) antibody (1:1000; Cell Signaling) or anti-FLAG M2-HRP antibody to verify equal receptor levels. Anti-MOR antibody (1:1000; Millipore Sigma) was also used to verify receptor expression levels.

MOR-CHO and MOR-ABIN-1-CHO cells plated in 100-mm dishes were grown to 80–90% confluence and treated with 0 or 5 µM DAMGO for 30 minutes. The cells were then lysed with solubilization buffer (50 mM Tris HCl, 150 mM NaCl, 1 mM EDTA, 2% Triton X-100, 20 µM N-ethylmaleimide, pH 7.4) supplemented with protease inhibitors for 30 minutes at 4°C, and subsequently centrifuged at 15,000g for 30 minutes. Solubilized proteins were incubated overnight at 4°C with anti-FLAG affinity gel (Sigma-Aldrich) or anti-ubiquitin antibody to immunoprecipitate DDK tagged-MOR or ubiquitinated MOR. The immunoprecipitated proteins were washed three times with Tris-buffered saline before being eluted with 20 µl of Laemmli sample buffer without 2-mercaptoethanol or dithiothreitol at 95°C for 5 minutes or 56°C for 20 minutes. Eluted samples were separated using SDS/PAGE, and immunoblotting was performed using an anti-ubiquitin antibody (1:500; Santa Cruz Biotechnology), anti-Flag antibody (1:1000; Sigma-Aldrich) or anti-MOR antibody (1:1000; Millipore Sigma). Blots were then stripped (Restore Western Blot Stripping Buffer; Pierce, Thermo Fisher Scientific, Waltham, MA) and reprobed with an anti-FLAG M2-HRP antibody (Sigma-Aldrich) to verify equal receptor levels.

cAMP Assays by ELISA. Stably transfected MOR-CHO and MOR-ABIN-1-CHO cells were harvested using Versene dissociation solution (Gibco/Invitrogen, Thermo Fisher Scientific) and washed with Hanks' balanced salt solution buffer. The cells were then resuspended at a density of 2 × 10⁶/ml in stimulation buffer (Hanks' balanced salt solution containing 5 mM HEPES, 0.1% BSA, and 0.05 mM 3-isobutyl-1-methylxanthine). Alexa Fluor647-labeled antibodies (PerkinElmer) were added to the final cell suspension prior to the addition of forskolin, in the presence of DAMGO. Following incubation at 37°C for either, 1, 5, 15, or 20 minutes, the detection

mix was added and the signal was read using the Victor 2D Instrument (PerkinElmer). The LANCE signal was subsequently measured after a 1-hour incubation step. The LANCE signal obtained at 665 nM was used to directly analyze cAMP standard curves and cellular cAMP levels. The signal at 615 nM was used to identify dispensing or quenching issues. cAMP inhibition was defined as LANCE signal (DAMGO minus forskolin)/(basal minus forskolin) × 100%.

Extracellular Signal-Regulated Protein Kinase Activation. MOR-CHO and MOR-ABIN-1-CHO cells were plated onto 60-mm dishes and grown to 80–90% confluence. Cells were exposed to 0 or 5 µM DAMGO for 10 minutes then cooled to 4°C by washing with ice-cold PBS. Proteins were extracted for 30 minutes at 4°C with 0.2 ml of RIPA⁺ buffer supplemented with protease inhibitors. The lysate was centrifuged at 15,000g for 15 minutes at 4°C. The whole-cell protein was analyzed by Western blotting using a phospho-p44/42MAPK [extracellular signal-regulated protein kinase (ERK) 1/2] (Thr202/Tyr204) antibody (Cell Signaling). Blots were then stripped (Restore Western Blot Stripping Buffer; Pierce) and reprobed with p44/42 MAPK (ERK1/2) antibody (Cell Signaling).

Microinjection of Zebrafish Embryos and Morphine Challenge. Morpholino antisense oligos (MO) were designed and manufactured by Gene Tools (Philomath, OR). The ABIN-1 MO sequence was 5'-GGCCTTTTCCCTCCATCTGTCACCC-3'. The control MO, 5'-CCTCTTACCTCAGTTACAATTTATA-3', was used as a morpholino injection control. A second MO and control, e11 5'-GTCAGTTTCAGACCCCTCACCTAAA-3' and e11-mispair 5'-GTAAGTTTAAGACCCCTAACGTAAA-3', were also used to validate the locomotion of ABIN-1 knockdown larvae following morphine administration. To study the effects of ABIN-1 overexpression on larvae, mABIN-1 (LMBP5392) was subcloned into pEGFPn1 vector (Clontech, Mountain View, CA) to generate an ABIN-1-GFP-tagged fusion protein, and pEGFPn1 empty vector was used as the control. Morpholinos and cDNA were diluted to 250 µM injection stocks in RNase-free water.

Micropipettes were fabricated by heating and pulling borosilicate glass capillary tubes (Zheng tian yi Inc., Beijing, China) using a micropipette puller device (Flaming/Brown P-97; Sutter Instruments Inc., Novato, CA). Zebrafish randomly mate during the first few hours of each morning. The resultant embryos were subsequently collected at 1-cell stage and placed in 1.5% agarose in E3 medium plates molded with wedge-shaped troughs that were used to hold the embryos during injection (Hogan et al., 2008; Yuan and Sun, 2009).

Morpholino injection volumes ranged from 1 to 2 nl. Injection pressure was varied to compensate for variable needle bore sizes, thereby ensuring appropriate injection volumes. After injection, embryos were removed from the holding plates and transferred to Petri dishes with E3 media. Media was replaced on a daily basis in addition to removal of nonviable embryos. Only normally developing embryos were included in subsequent dosing experiments. Embryos hatched around 3 days postfertilization (dpf) and the larvae were nurtured to 7 dpf in a 28°C incubator. On the seventh day, media was changed to E3 medium with or without morphine (at a concentration of 10 mg/l) for 1 hour. To determine the specificity of morphine on the larvae, naloxone (at a concentration of 10 mg/l) was added into E3 medium for 30 minutes prior to morphine addition. The larvae were then transferred to individual wells (of 48-well plates) containing 100 µl of fresh E3 medium. The plate was placed on the bench of the Zebrafish (ViewPoint Behavior Technology, France) and a 10-minute trial for each larvae was recorded using a digital camera (up to 60 frames/second at 640 × 480; ViewPoint Behavior Technology). Each group (control MO, control MO + morphine, ABIN-1 knockdown, ABIN-1 knockdown + morphine, ABIN-1 knockin, ABIN-1 knockin + morphine) had 24 or 36 zebrafish larvae. Trials were analyzed using ZebraLab 3.3 software (ViewPoint Behavior Technology). Each experiment was performed at least twice. After the track recorded trial, the larvae were assessed for expression of ABIN-1 using quantitative polymerase chain reaction (qPCR). RNA was extracted from the larvae using the RNeasy Mini kit according to the manufacturer's instructions

(Qiagen, Hilden, Germany). First-strand cDNA was synthesized in a final reaction volume of 20 μ l. Each reaction mixture contained 1 μ g of RNA, 5 $\times$ first strand buffer (Invitrogen), 1 μ l of Oligo(dT)₁₈ primer (10 μ M), 1 μ l of deoxyribonucleoside 5'triphosphates (dNTPs 10 mM), 1 μ l of moloney murine leukemia virus (MML-V 200 IU/ μ l), 1 μ l of ribonuclease inhibitor (40 IU/ μ l), and nuclease-free water. Reverse transcription reactions were performed for 1 hour at 40°C, then 10 minutes at 70°C. SYBR green-based qPCR was performed next using an ABI PRISM 7300 Sequence Detection System (Applied Biosystems, Foster City, CA) with the following primers: mABIN-1: forward primer 5'-GACCGTGAACGCATGAATGA-3', reverse primer 5'-GACCTGGGCCTGCAGCTT-3'; DrABIN-1: forward primer 5'-GGAGGACGTCTGACCTACGT-3', reverse primer 5'-AACGGCCCGCAGTATGG-3'; β -Actin: forward primer 5'-TC-TGTGTGGATTGGTGGCTCTA-3', reverse primer 5'-CTGCTTGCTGATCCACATCTG-3'. All experiments were performed in duplicate and repeated at least three times. Three controls aimed at detecting genomic DNA contamination in the RNA samples during the reverse transcription (RT) or qPCR reactions were included in all experiments. An RT mixture that lacked reverse transcriptase and an RT mixture that contained reverse transcriptase but no RNA were used as negative controls (reaction mixture without cDNA template). The data were collected and analyzed using OneStep Software Applied Biosystems (ABI 7300; Waltham, MA). Relative quantification was performed using the comparative threshold (Ct) method ($\Delta\Delta$ Ct) after determining reference Ct values for β -Actin and the target gene (ABIN-1).

Statistical Analysis. Statistical and curve-fitting analyses were performed using PRISM 5.0 (GraphPad Software Inc., La Jolla, CA). The data were expressed as mean $\pm$ S.D. Two-way analysis of variance (ANOVA) followed by Bonferroni post-tests or one-way ANOVA were used for the analysis of multiple treatments. A value of $P < 0.05$ was considered statistically significant.

Results

ABIN-1 Interacts with the C-Terminus of MOR

Column overlay experiments were performed to confirm the direct interaction between MOR and ABIN-1. GST fusion proteins of the C-terminus of MOR (GST-C) and the hexahistidine fusion protein ABIN-1 (aa 1–647) were generated. Glutathione-agarose beads, bound to GST fusion proteins or GST, were incubated with purified His-ABIN-1. The resin-bound proteins were eluted with glutathione elution buffer and analyzed by immunoblotting with anti-polyhistidine antibodies. Positive bands corresponding to His-ABIN-1 were detected in the lanes with eluate containing GST-C, whereas no bands were detected in the lanes with GST. To ensure that approximately equal amounts of eluted GST and GST-C fusion proteins were loaded on the gel, nitrocellulose membranes were probed with anti-GST antibodies. His-ABIN-1 was also detected by Western blotting to ensure equal loading (Fig. 1A).

ABIN-1 Interaction with MOR by Coimmunoprecipitation and Immunofluorescence Colocalization

Coimmunoprecipitation experiments were performed to determine the direct interaction between MOR and overexpressed ABIN-1. DDK-tagged MOR was immunoprecipitated from lysates from MOR-CHO, ABIN-1-CHO, or MOR-ABIN-1-CHO cells, using a monoclonal anti-Flag antibody. The immunoprecipitates were immunoblotted with antibodies against ABIN-1. As shown in Fig. 1B, ABIN-1 was detected in immunoprecipitates from MOR-ABIN-1-CHO cells, whereas no bands were detected in MOR-CHO or ABIN-1-CHO cells, suggesting that MOR physically associates with ABIN-1 *in vitro*. Endogenous ABIN-1 interaction with MOR was detected in SH-SY5Y

cells by coimmunoprecipitation. ABIN-1 was also coimmunoprecipitated with MOR from SH-SY5Y cells (Fig. 1C). ABIN-1 did not coimmunoprecipitate with normal rat IgG.

Confocal microscopy revealed that DDK-tagged MOR was predominantly distributed in the plasma membrane in MOR-ABIN-1-CHO cells (Fig. 1D, a). ABIN-1 was mainly distributed in the cytoplasm and a distinct corona of staining was observed close to the plasma membrane (Fig. 1D, b). Following the coexpression of MOR and ABIN-1, there was clear overlapping of signals corresponding to the presence of the two proteins at the cell surface. The orange areas represent the colocalization of MOR and ABIN-1 (Fig. 1D, c). In MOR-CHO cells, MOR was predominantly distributed in the plasma membrane (Fig. 1D, d). In ABIN-1-CHO cells, ABIN-1 was mainly distributed in the cytoplasm (Fig. 1D, e). In SH-SY5Y cells, endogenous MOR was predominantly distributed in the plasma membrane (Fig. 1D, f), and ABIN-1 was mainly distributed in the cytoplasm (Fig. 1D, h).

ABIN-1 Has No Effect on Ligand Binding but Inhibited [³⁵S]-GTP γ S Binding with MOR

Saturation binding studies ($n = 6$) revealed no substantial difference between MOR-CHO and MOR-ABIN-1-CHO cells. The binding properties of MOR with [³H]-diprenorphine in MOR-CHO cells ($B_{\max}$, 2.53 ± 0.84 pmol/mg; K_d , 0.44 ± 0.14 nM) were similar to those in MOR-ABIN-1-CHO cells ($B_{\max}$, 2.05 ± 0.64 pmol/mg; K_d , 0.33 ± 0.12 nM). Thus, ABIN-1 does not appear to affect the binding affinity of diprenorphine for MOR.

To investigate whether ABIN-1 interferes with G protein activation by MOR, the stimulation of [³⁵S]-GTP γ S binding by DAMGO was examined. No difference was observed in [³⁵S]-GTP γ S basal binding, nonspecific binding, or basal minus nonspecific binding between MOR-CHO and MOR-ABIN-1-CHO cells (Fig. 2A). DAMGO potently stimulated [³⁵S]-GTP γ S binding in a concentration-dependent manner ($EC_{50} = 18.0 \pm 5.4$ nM) in MOR-CHO cells, with a maximal value of $574.8 \pm 99.1\%$ compared with basal levels. In contrast, DAMGO-mediated stimulation of [³⁵S]-GTP γ S binding in MOR-ABIN-1-CHO cells was attenuated ($EC_{50} = 45.9 \pm 10.3$ nM), with a maximal value of $274.4 \pm 93.1\%$ compared with basal levels (Fig. 2B).

ABIN-1 Inhibited MOR Phosphorylation, Ubiquitination, and Internalization

The basal level of MOR phosphorylation at Ser375 was similar between MOR-CHO and MOR-ABIN-1-CHO cells. After DAMGO (5 μ M) treatment of 30 minutes, MOR phosphorylation at Ser375 increased approximately 9-fold in MOR-CHO cells and 5-fold in MOR-ABIN-1-CHO cells compared with the phosphorylation of MOR without DAMGO exposure. MOR phosphorylation at Ser375 induced by DAMGO in MOR-ABIN-1-CHO cells was significantly lower (by 30–40%) compared with that of MOR-CHO cells (Fig. 3A, $P < 0.01$).

Since receptor ubiquitination could be influenced by receptor phosphorylation, we examined the effect of ABIN-1 on agonist-induced MOR ubiquitination in MOR-CHO and MOR-ABIN-1-CHO cells. MOR ubiquitination induced by DAMGO (5 μ M) was significantly increased in MOR-CHO cells ($P < 0.001$) but was impaired in MOR-ABIN-1-CHO cells ($P < 0.01$, Fig. 3B). Ubiquitinated MOR were also detected

using anti-Flag antibody or anti-MOR antibody in MOR-CHO and MOR-ABIN-1-CHO cells (Supplemental Fig. 1). It is probable that ABIN-1 associates with MOR and then inhibits the phosphorylation and ubiquitination of MOR induced by DAMGO.

To determine whether this postulated inhibition of MOR phosphorylation and ubiquitination affected agonist-induced MOR endocytosis, we examined MOR internalization. MOR was expressed primarily on the plasma membrane, as assessed by immunolabeling both in MOR-CHO and MOR-ABIN-1-CHO cells (Fig. 4A). MOR were not internalized following saline treatment in either MOR-CHO or MOR-ABIN-1-CHO cells (Fig. 4A). Exposure to DAMGO (5 μ M) resulted in rapid internalization of MOR into the cytoplasm of MOR-CHO cells, but this internalization was impaired in MOR-ABIN-1-CHO cells. It should be noted that the membrane receptor area of MOR-ABIN-1-CHO cells was similar to that of MOR-CHO cells after saline treatment. After DAMGO treatment, the membrane receptor area of MOR-CHO cells decreased significantly ($P < 0.001$). The membrane receptor area of MOR-ABIN-1-CHO also decreased ($P < 0.05$) compared with saline treatment (Fig. 4B). These results suggest that internalization of MOR is partially inhibited in the presence of ABIN-1.

The Effect of ABIN-1 on cAMP Accumulation and ERK Activity Following Acute Agonist Treatment

MOR is thought to inhibit adenylyl cyclase via G_i/G_o proteins. Hence, we aimed to determine whether the interaction of ABIN-1 and MOR interfered with G protein activation after agonist treatment. The effect of ABIN-1 on MOR signaling was measured by an assay that measured forskolin-stimulated cAMP accumulation by DAMGO in MOR-CHO or MOR-ABIN-1-CHO cells. In MOR-CHO cells, DAMGO (3.2×10^{-9} – 10^{-5} M) inhibited in a concentration-dependent manner forskolin-stimulated cAMP production after incubation for 15 minutes, with a maximum cAMP inhibition of $66.0 \pm 21.34\%$. DAMGO (3.2×10^{-9} – 10^{-5} M) also in a concentration-dependent manner inhibited forskolin-stimulated cAMP production in MOR-ABIN-1-CHO cells, but the maximum inhibition was significantly lower than that of MOR-CHO cells ($P < 0.05$, Fig. 5A1). The cAMP inhibition following DAMGO (5.0×10^{-6} M) incubation for 5 and 15 minutes were 39.95% and 62.95%, respectively, in MOR-CHO cells, which was higher than the cAMP inhibition of 18.35% and 33.23%, respectively, in MOR-ABIN-1-CHO cells ($P < 0.05$, $P < 0.01$, Fig. 5A2).

The agonist-induced activation of MOR in CHO cells has previously been shown to cause a rapid and transient increase in mitogen-activated protein kinase (MAPK) activity (Fukuda et al., 1996). ERK is the best-studied member of the MAPK family and plays a critical role in intracellular signal transduction (Ji and Woolf, 2001). To assess ERK activity in the current study, MOR-CHO and MOR-ABIN-1-CHO cells were exposed to DAMGO (0 or 5 μ M) for 10 minutes, and levels of phospho-ERK1/2/ERK1/2 were analyzed by Western blotting. ERK1/2 levels did not vary after DAMGO treatment either in MOR-CHO cells or MOR-ABIN-1-CHO cells. Moreover, basal levels of phospho-ERK1/2 were similar in the two cell types. However, following 5 μ M DAMGO treatment, phospho-ERK1/2 levels increased to eight times to that of untreated MOR-CHO cells ($P < 0.001$), whereas phospho-ERK1/2 levels were

upregulated by about three times after DAMGO treatment in MOR-ABIN-1-CHO cells. The levels of phospho-ERK1/2 in MOR-ABIN-1-CHO cells were significantly lower than that of MOR-CHO cells following 5 μ M DAMGO treatment ($P < 0.05$) (Fig. 5B).

Analysis of Zebrafish Larvae Mobility. The mobility parameter of the ZebraLab 3.3 software is able to detect distance traveled following stationary state to movement of specimens. In the control MO-injected larvae, morphine-exposed larvae showed a 20% increase in distance traveled compared with saline-treated larvae ($P < 0.05$) (Fig. 6, A1 and B1). Following morphine treatment in ABIN-1 MO-injected zebrafish larvae, there was a significant increase in distance traveled (about 60% compared with the control MO-injected zebrafish larvae) ($P < 0.001$) (Fig. 6, A1 and B1). There was no significant differences in mobility following comparison with noninjected, control MO-injected or ABIN-1 MO-injected larvae following saline treatment. The *ABIN-1* mRNA levels of ABIN-1 MO-injected larvae were about 50% of those in control MO-injected or noninjected larvae (Fig. 6C1) as determined by qPCR using forward primer 5-GGAGGACG-TCTGACCCCTACGT-3 and reverse primer 5-AACGGCCCCG-CAGTATGG-3. In the control pEGFPn1-injected cohorts, morphine-exposed larvae showed a 20% increase in distance traveled compared with saline-treated larvae ($P < 0.01$). Following morphine treatment, the ABIN-1 knockin larvae showed a 15% decrease in distance traveled compared with the control pEGFPn1-injected larvae ($P < 0.05$). In the ABIN-1 knockin larvae, morphine-exposed larvae showed no obvious variation in distance traveled compared with saline-treated larvae (Fig. 6, A2 and B2). The *ABIN-1* mRNA levels for ABIN-1 knockin larvae were about 40–50% higher than those for the control larvae (Fig. 6C2) determined by qPCR using forward primer 5-GACCGTGAACGCATGAATGA-3 and reverse primer 5-GACCCTGGGCCCTGCAGCTT-3. The locomotion of a second ABIN-1 MO-injected larvae also increased substantially following morphine administration. Furthermore, the locomotion of the larvae following acute morphine administration was antagonized by pretreatment with naloxone (Supplemental Fig. 2).

Discussion

The bacterial two-hybrid system that we used exhibited high screening efficiency. When employed in our previous study, this system facilitated the identification of a total of 19 proteins that interacted with rat MOR-C (Zhou et al., 2015). Among the MOR-C-interacting proteins we found, calmodulin 1 and heat shock protein 70 were also reported to interact with opioid receptors (Wang et al., 1999, 2000; Wannemacher et al., 2008). One of the 19 MOR-C interacting proteins, ABIN-1, was reported to be elevated in different brain regions following chronic morphine treatment (Zhou et al., 2015). In the present study, the activity of MOR was negatively regulated by ABIN-1 both *in vitro* and *in vivo*.

ABIN-1 is a protein that inhibits both transduction of transmembrane receptors such as tumor necrosis factor (TNF) α -receptor (TNF α -R), epidermal growth factor receptor (EGFR), and Toll-like receptor, and the activity of nuclear receptors peroxisome proliferator-activated receptor and retinoic acid receptor (Mauro et al., 2006; Gurevich and Aneskievich, 2009; Flores et al., 2011; Ramirez et al., 2012). These receptors play key roles in regulating inflammatory diseases.

Fig. 1. Interaction of MOR and ABIN-1. (A) In vitro binding of ABIN-1 and MOR C-terminus. Bacterial lysates containing GST-C and GST fusion proteins immobilized on glutathione agarose beads were tested for their ability to bind to purified His-ABIN-1. The immunoblot of the eluates was probed

Fig. 2. Stimulation of [35 S]-GTP γ S binding to membranes of MOR-CHO and MOR-ABIN-1-CHO cells. (A) [35 S]-GTP γ S basal binding, nonspecific binding, and basal minus nonspecific binding between MOR-CHO and MOR-ABIN-1-CHO cells. (B) [35 S]-GTP γ S binding was performed in MOR-CHO (circles) and MOR-ABIN-1-CHO cells (triangles). Results are expressed as DAMGO-stimulated [35 S]-GTP γ S binding over basal binding. The mean $\pm$ S.D. was obtained from five independent experiments performed in duplicate. * $P < 0.05$; ** $P < 0.01$, MOR-ABIN-1-CHO cells compared with MOR-CHO cells using two-way ANOVA followed by Bonferroni post-tests.

ABIN-1 was originally discovered to interact with the cytoplasmic protein A20 and to dampen subsequent NF- κ B signaling following TNF α -R activation (Heyninck et al., 1999). ABIN-1 also confers cellular protection by inhibiting apoptosis. ABIN-1 was found to inhibit caspase-8-mediated activation and apoptosis in response to TNF α (Oshima et al., 2009). Furthermore, ABIN-1 overexpression protected mice

from TNF α /galactosamine-induced acute liver failure. This protective effect was believed to result from its antiapoptotic properties in addition to its role in regulating inflammation (Wullaert et al., 2005). These in vivo and in vitro models strongly suggest that ABIN-1 plays a crucial role in regulating several cellular pathways involved in inflammation and immune-related disorders.

In the present study, the interaction of exogenously overexpressed ABIN-1 with MOR was confirmed in CHO cells. The interaction of endogenous ABIN-1 with MOR was replicated in SH-SY5Y cells. Specific [3 H]-diprenorphine binding in relation to MOR was not inhibited by ABIN-1 overexpression. ABIN-1 overexpression had no effect on basal [35 S]-GTP γ S binding without DAMGO treatment. However, the interaction of ABIN-1 and MOR inhibited the activation of G proteins induced by DAMGO. DAMGO-induced MOR phosphorylation, ubiquitination and internalization were decreased in CHO cells overexpressing MOR and ABIN-1. ERK activation was negatively regulated by the interaction of ABIN-1 and MOR.

Downregulation or desensitization of opioid receptors after agonist exposure can be one of the mechanisms for tolerance. Like most GPCRs, short-term exposure of opioid receptors to agonists leads to receptor desensitization that results in uncoupling of receptor from G proteins (Chaturvedi et al., 2001). The mechanism underlying homologous desensitization involves phosphorylation of agonist-activated GPCRs mediated by members of the GRK family, which promotes binding of GPCRs to arrestin proteins, which effectively uncouples productive interaction of activated GPCR to heterotrimeric G proteins (Lefkowitz, 1998). The C-terminal domain of MOR is an important site for post-translational modifications, such as phosphorylation, and is crucial for receptor desensitization and transportation. DAMGO-induced desensitization was completely abolished in C-terminal-truncated MOR (Deng et al., 2000). Agonist-induced phosphorylation of MOR occurred at a conserved 10-residue sequence 370TREHPSTANT379 in the receptor's carboxyl-terminal cytoplasmic tail (El Kouhen et al., 2001; Schulz et al., 2004; Doll et al., 2011, 2012; Lau et al., 2011; Just et al., 2013). Ser375 was the primary site of agonist-dependent phosphorylation (Doll et al., 2011). Mutation of Ser375 to Ala (S375A) completely abrogated detectable phospho-Ser375 immunoreactivity and strongly reduced agonist-driven receptor endocytosis (Schulz et al., 2004). The interaction of ABIN-1 with the C-terminal tail of MOR diminished Ser375 phosphorylation induced by DAMGO (Fig. 3). Zhang and colleagues (2002) found that ERK2 bound to ABIN-1, and ABIN-1 in turn blocked ERK2 nuclear signaling in Saos-2

with anti-His antibody. The bands corresponding to His-ABIN-1 (~85 kDa) were eluted from GST-C columns. (B) Proteins from MOR-CHO, ABIN-1-CHO, or MOR-ABIN-1-CHO cells were extracted and immunoprecipitated using anti-Flag antibodies. The blots were probed with an anti-ABIN-1 antibody. ABIN-1 was detected in the lysates from ABIN-1-CHO or MOR-ABIN-1-CHO cells (input) but not in the lysates from MOR-CHO cells. ABIN-1 coimmunoprecipitated with MOR from MOR-ABIN-1-CHO cell lysates but not from MOR-CHO or ABIN-1-CHO cell lysates. (C) Endogenous ABIN-1 interaction with MOR was detected in SH-SY5Y cells by coimmunoprecipitation. ABIN-1 coimmunoprecipitated with MOR from SH-SY5Y cells. Western blots are representative results from three independent experiments. The molecular mass marker is indicated on the left (in kilodaltons). (D) Subcellular distribution of MOR and ABIN-1 in CHO and SH-SY5Y cells. MOR-ABIN-1-CHO cells were fixed and subjected to dual immunofluorescent staining using mouse anti-Flag and rabbit anti-ABIN-1 antibodies; MOR-CHO, ABIN-1-CHO, or SH-SY5Y cells were fixed and subjected to immunofluorescent staining using mouse anti-Flag, rabbit anti-MOR, or rabbit anti-ABIN-1 antibodies. Rhodamine (red)-coupled donkey anti-mouse IgG (a) and Alexa Fluor 488 (green)-coupled goat anti-rabbit IgG (b) secondary antibodies were used to visualize the subcellular distribution of MOR and ABIN-1 in MOR-ABIN-1-CHO cells. The orange areas represent the colocalization of MOR and ABIN-1 (c). Alexa Fluor 488 (green)-coupled goat anti-mouse or rabbit IgG secondary antibodies were used to visualize the subcellular distribution of MOR (d) or ABIN-1 (e) in MOR-CHO or ABIN-1-CHO cells. Alexa Fluor 488 (green)-coupled goat anti-rabbit IgG secondary antibodies were used to visualize the subcellular distribution of MOR (f) or ABIN-1 (h) in SH-SY5Y cells. The confocal microscopic images are representative results from three independent experiments.

Fig. 3. ABIN-1 attenuates DAMGO-induced MOR phosphorylation and ubiquitination. (A) MOR phosphorylation was detected in MOR-CHO and MOR-ABIN-1-CHO cells by immunoprecipitation after incubation with DAMGO (0, 5 μ M) for 30 minutes. (A1) MOR was detected with anti-Flag antibody; (A2) MOR was detected with anti-MOR antibody. The protein bands were analyzed using ImageJ and normalized to MOR levels. Data are shown as the mean $\pm$ S.D. value of six independent experiments. *** P < 0.001; ** P < 0.01, MOR phosphorylation compared with control (DAMGO, 0 μ M); ## P < 0.01, MOR-ABIN-1-CHO compared with MOR-CHO cells. The analysis was conducted using a two-way ANOVA followed by Bonferroni post-tests. (B) MOR ubiquitination was detected from MOR-CHO and MOR-ABIN-1-CHO cells by immunoprecipitation after incubation with DAMGO (0, 5 μ M) for 30 minute. The immunoblotting result was representative of three and the quantification was representative of nine independent experiments. *** P < 0.001, MOR ubiquitination in MOR-CHO compared with control (DAMGO, 0 μ M); ## P < 0.01, MOR-ABIN-1-CHO compared with MOR-CHO cells. The analysis was conducted using two-way ANOVA followed by Bonferroni post-tests. The molecular mass marker is indicated in kilodaltons.

cells. In addition to the direct interaction with MOR Ser375, ABIN-1 might be an attenuator of GRK as well as ERK2. In a previous study, DAMGO promoted MOR ubiquitination in a time- and β -arrestin-1-dependent manner in mouse embryonic fibroblasts (Groer et al., 2011). hKOR ubiquitination was previously reported to be enhanced by receptor phosphorylation (Li et al., 2008), and MOR phosphorylation has long been known to influence receptor function and transportation. Ubiquitination is another form of post-translational modification that can be used to specify or regulate membrane receptor

trafficking in the vacuolar/lysosomal pathway (Hislop and von Zastrow, 2011). Ser375 phosphorylation is critical for DAMGO-induced MOR internalization. Indeed, the rate of agonist-induced endocytosis was altered by preventing MOR ubiquitination (Henry et al., 2012). In the present study, the interaction between ABIN-1 and MOR-C decreased DAMGO-induced phosphorylation and ubiquitination of MOR, resulting in the inhibition of agonist-induced MOR internalization (Fig. 4). ABIN-1 also physically linked A20 to NEMO, thereby facilitating A20-mediated de-ubiquitination

Fig. 4. ABIN-1 attenuates DAMGO-induced MOR endocytosis. (A) MOR-CHO or MOR-ABIN-1-CHO cells were treated with saline or 5 μ M DAMGO for 0 or 30 minutes then fixed and subjected to immunofluorescent staining using mouse anti-Flag antibodies and Alexa Fluor 488 (green)-coupled goat anti-mouse IgG antibodies, then examined by confocal microscopy. In untreated cells, MOR was confined to the plasma membrane. (B) After DAMGO treatment, the internalization of MOR was impaired in MOR-ABIN-1-CHO cells compared with that of MOR-CHO cells. The cells were imaged using IN Cell Analyzer 2000 and analyzed using the Granularity Analysis Module. The mean $\pm$ S.D. was obtained from four independent experiments performed in triplicate. * $P < 0.05$; *** $P < 0.001$, the membrane receptor area of MOR-ABIN-1-CHO or MOR-CHO cells after DAMGO treatment compared with saline treatment. The analysis was conducted using two-way ANOVA followed by Bonferroni post-tests.

of NEMO (Wagner et al., 2008). ABIN-1 has also been identified as a HIV-1 Nef-binding protein that increased the expression of cell surface CD4 (Fukushi et al., 1999). In addition to the direct interaction with MOR, ABIN-1 might facilitate de-ubiquitination of MOR, which needs to be clarified.

In the present study, ABIN-1 knockdown by antisense MO was used in zebrafish to determine its *in vivo* function. Control

MO-injected embryos, as well as wild-type embryos, showed an increase (approximately 20%) in locomotion following morphine exposure (Fig. 6, B1 and B2), and this result was in accordance with observations made in rodent studies (Carroll and Sharp, 1972; Elhabazi et al., 2012). Administration of a different MOR agonist, heroin, also produced an increase in adult zebrafish locomotion (Stewart and Kalueff, 2014). The motor-stimulatory and associated neurochemical

Fig. 5. ABIN-1 attenuates the inhibition of cAMP accumulation activity and down-regulates ERK phosphorylation after DAMGO treatment. (A1) DAMGO concentration-dependently inhibited forskolin-stimulated cAMP production in MOR-CHO and MOR-ABIN-1-CHO cells. The mean $\pm$ S.D. was obtained from five independent experiments performed in duplicate. (A2) The cAMP inhibition following DAMGO (5.0×10^{-6} M) incubation for 1, 5, 15, 20 minutes in MOR-CHO and MOR-ABIN-1-CHO cells. The mean $\pm$ S.D. was obtained from four independent experiments performed in duplicate. * $P < 0.05$; ** $P < 0.01$, compared with MOR-CHO cells with similar DAMGO treatment using a two-way ANOVA followed by Bonferroni post-tests. (B) Western blot analysis of ERK1/2 phosphorylation in MOR-CHO and MOR-ABIN-1-CHO cells after DAMGO (0.5 μ M) treatment. The protein bands were analyzed using ImageJ and normalized to ERK1/2 levels. Data are shown as mean $\pm$ S.D. of four independent experiments. * $P < 0.05$; *** $P < 0.001$; compared with DAMGO (0) treatment in the same cells; # $P < 0.05$, compared with MOR-CHO cells following DAMGO treatment (5 μ M). The analysis was conducted using two-way ANOVA followed by Bonferroni post-tests.

Fig. 6. Larvae travel activity in ABIN-1 MO- or ABIN-1-injected embryos in response to morphine exposure (10 mg/l). (A) EthoVision tracks (10-minute duration) recorded after morphine exposure in the ABIN-1 knockdown (A1, $n = 24$) or knockin (A2, $n = 36$) larvae. (B) Percentage of total distance traveled in the ABIN-1 knockdown (B1, $n = 24$) or knockin (B2, $n = 36$) larvae. Data are shown as mean $\pm$ S.D., * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. (C) ABIN-1 mRNA expression in the ABIN-1 knockdown (C1) or knockin (C2) larvae. qPCR was used to detect the expression of ABIN-1 mRNA. * $P < 0.05$; ** $P < 0.01$. The analysis was conducted using two-way ANOVA (B1, C1, and C2) and one-way ANOVA (B2) followed by Bonferroni post-tests.

effects of opioids are dependent on MOR (Tian et al., 1997; Contarino et al., 2002; Yoo et al., 2003). The increased locomotion of zebrafish and rodents following acute morphine administration is probably the result of monoamine neurotransmitter upregulation (Carroll and Sharp, 1972; Lau et al., 2006). The locomotion of ABIN-1 knockin larvae was reduced, whereas the locomotion of ABIN-1 knockdown larvae increased after acute morphine administration (Fig. 6). In CHO cells, ABIN-1 interacted with MOR and inhibited MOR

activation. ABIN-1 overexpression dampened morphine activity, and ABIN-1 knockdown increased morphine activity. It needs to be investigated whether the full ABIN-1 protein or its functional domain may prevent opioid addiction. However, the effects of ABIN-1 on brain neurotransmitters or neural pathways are unclear, especially under chronic opioid treatment.

As a potent analgesic, opioids have been widely used for decades. Long-term opioid administration leads to tolerance, which limits its clinical efficacy. Morphine tolerance is modulated

by proinflammatory cytokines such as TNF- α (Mao et al., 1995; Shen et al., 2012). Chronic morphine infusion has been demonstrated to induce a significant increase in TNF- α mRNA expressions of tolerant rats. Administration of the TNF- α inhibitor etanercept reduced proinflammatory cytokine production and microglial activation, thus preserving the antinociceptive effects of morphine (Li et al., 2002b). ABIN-1 increased significantly in the frontal cortex, striatum, and ventral tegmental area of rats after chronic morphine exposure (Zhou et al., 2015). The increase in NF- κ B signaling by TNF- α was inhibited by the upregulation of ABIN-1 in the cytoplasm after chronic morphine exposure, which may prevent excessive tolerance or addiction to morphine. In addition to its anti-inflammatory effects, ABIN-1 also protects cells by inhibiting apoptosis. ABIN-1 overexpression may protect morphine-induced apoptosis and preserve the antinociceptive effects of morphine. Morphine has been shown to induce apoptotic cell death in vitro (Goswami et al., 1998; Singhal et al., 2000, 2002; Yin et al., 2000; Tegeder et al., 2003). Morphine has been shown to induce apoptosis of human microglia and neurons (Hu et al., 2002), which is consistent with studies in rodent peritoneal macrophages and a macrophage cell line (Singhal et al., 2000). Caspase-3 is involved in the execution phase of morphine-induced apoptosis (Hu et al., 2002). In vivo, morphine also increases cortex and amygdala apoptosis with cleaved caspase-3-positive cells significantly increased after repeated administration to neonatal rats (Bajic et al., 2013). Apoptotic cell death was also increased following morphine administration associated with development of antinociceptive tolerance.

In summary, this study is the first to provide evidence that ABIN-1 protein interacts with MOR, leading to the suppression of MOR activation, phosphorylation, and internalization. ABIN-1 also inhibits morphine-induced hyperlocomotion in zebrafish larvae. Therefore, ABIN-1 appears to be an important inhibitor for agonist-bound MOR. Immunoinflammatory factors are involved in the formation of opioid tolerance. ABIN-1 has been reported to inhibit the transcription of inflammatory factors through the NF- κ B pathway. In addition to functioning as an inhibitor for agonist-bound MOR, ABIN-1 may be an important regulator for opioid tolerance. The exact mechanism by which ABIN-1 regulates chronic morphine tolerance needs to be investigated.

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Authorship Contributions

Participated in research design: Zhou, Su, Gong.
Conducted experiments: Zhou, Jiang, Yan, Li, Zhao, Wang.
Contributed new reagents or analytic tools: Yan, Li.
Performed data analysis: Zhou, Yan.
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