

Mitogen-activated Protein Kinase p38 Regulates the Wnt/Cyclic GMP/Ca²⁺ Non-canonical Pathway*

Received for publication, April 3, 2007, and in revised form, August 3, 2007 Published, JBC Papers in Press, August 7, 2007, DOI 10.1074/jbc.M702840200

Li Ma and Hsien-yu Wang¹

From the Departments of Physiology & Biophysics, Diabetes & Metabolic Diseases Research Center, School of Medicine, State University of New York/Stony Brook, Stony Brook, New York 11794-8661

The non-canonical Wnt/cyclic GMP/Ca²⁺/NF-AT pathway operates via Frizzled-2, a member of the superfamily of G protein-coupled receptors. In scanning for signaling events downstream of the Frizzled-2/Gat2/PDE6 triad activated in response to Wnt5a, we observed a strong activation of the mitogen-activated protein kinase p38 in mouse F9 teratocarcinoma embryonal cells. The activation of p38 is essential for NF-AT transcriptional activation mediated via Frizzled2. Wnt5a-stimulated p38 activation was rapid, sensitive to pertussis toxin, to siRNA against either Gat2 or p38 α , and to the p38 inhibitor SB203580. Real-time analysis of intracellular cyclic GMP using the Cygnet2 biosensor revealed p38 to act at the level of cyclic GMP, upstream of the mobilization of intracellular Ca²⁺. Fluorescence resonance energy transfer (FRET) imaging reveals the changes in cyclic GMP in response to Wnt5a predominate about the cell membrane, and likewise sensitive to either siRNA targeting p38 or to treatment with SB203580. Dishevelled is not required for Wnt5a activation of p38; siRNAs targeting Dishevelleds and expression of the Dishevelled antagonist Dapper-1 do not suppress the p38 response to Wnt5a stimulation. These novel results are the first to detail a Dishevelled-independent Wnt response, demonstrating a critical role of the mitogen-activated protein kinase p38 in regulating the Wnt non-canonical pathway.

Wnts are secreted, palmitoylated, and glycosylated ligands that play a central role in early development (1, 2). Heptahelical, G protein-coupled Frizzleds are the cellular receptors for Wnt ligands (3–8). The Wnt-sensitive “canonical” pathway was the first to be elucidated, is mediated by Frizzled-1, and regulates the cellular stability of β -catenin (*i.e.* the Wnt/ β -catenin pathway) (2). Wnts that regulate the canonical pathway increase the nuclear accumulation of β -catenin and thereby activate the transcription of developmentally essential genes that are sensitive to members of the lymphocyte enhancer factor/T cell factor (Lef/Tcf) transcription factors (2, 9, 10). An example of a “non-canonical” Wnt-sensitive pathway is that mediated by Frizzled-2, regulating cyclic GMP accumulation and Ca²⁺

mobilization (11–15). Activation of Frizzled-2 by Wnt5a leads to activation of the phosphatidylinositol pathway (11), activation of the cyclic GMP phosphodiesterase PDE6 (13, 16), and inhibition of protein kinase G (PKG)² (17), which leads to Ca²⁺ mobilization (17). Ca²⁺ imaging with Fura-2 dye in zebrafish embryos (13, 18), mouse F9 teratocarcinoma cells (17), and human embryonic stem cells in culture (17) has revealed Wnt5a-stimulated Ca²⁺ mobilization in this non-canonical pathway to be downstream of PKG. Wnt5a-stimulated mobilization of intracellular Ca²⁺ stimulates activation of Ca²⁺-sensitive enzymes, including the protein kinases (*e.g.* calcium/calmodulin-sensitive protein kinase II, CamKII, protein kinase C) (12, 14) as well as the phosphoprotein phosphatase calcineurin (19). The mobilization of intracellular Ca²⁺ is essential for Wnt5a activation of NF-AT (nuclear factor of activated T cells)-sensitive gene transcription (17).

We scanned signaling pathways that might intersect with the Wnt/cyclic GMP, Ca²⁺ pathway that ultimately regulates NF-AT-sensitive gene transcription. Of central interest in the analysis was the mitogen-activated protein kinase (MAPK) cascades and the possible role of p38 in the Wnt-sensitive non-canonical signaling pathway. Our results indicate a central role of p38 MAPK in the Wnt5a/Frizzled-2/Gat2/PDE6/cyclic GMP and Ca²⁺ mobilization pathway that regulates NF-AT. Notably, the Wnt5a-stimulated activation of MAPK signaling cascade to the level of p38 is the first Wnt pathway demonstrated to operate independent of Dishevelleds.

EXPERIMENTAL PROCEDURES

Cell Culture—Mouse F9 teratocarcinoma cells were obtained from the ATCC collection (Manassas, VA). The cells were propagated and maintained in Dulbecco's modified Eagle's medium supplemented with 15% heat-inactivated fetal bovine serum (Hyclone, South Logan, UT) at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. Clones stably co-transfected with pcDNA3.1 harboring rat Frizzled2 (Fz2) and pNFAT-Luc (Stratagene, La Jolla, CA) were selected in medium containing 0.4 mg/ml neomycin analogue, G418 (Invitrogen, Carlsbad, CA). At least three independent clones were selected for each transfection. Clones were propagated in Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine

* This work was supported by Grant GM 069375 from the NIGMS, National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ To whom correspondence should be addressed: Dept. of Physiology & Biophysics, Diabetes & Metabolic Diseases Research Center, School of Medicine, SUNY/Stony Brook, Stony Brook, NY 11794-8661. Tel.: 631-444-3489; Fax: 631-444-3432; E-mail: wangh@pharm.stonybrook.edu.

² The abbreviations used are: PKG, protein kinase G; FRET, fluorescence resonance energy transfer; MAPK, mitogen-activated protein kinase; NF-AT, nuclear factor of activated T cells; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; GST, glutathione S-transferase; DN, dominant negative.

serum, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 0.1 mg/ml G418.

Immunoblotting—F9 clones stably expressing either rat Frizzled1 (Fz1) or rat Frizzled2 (Fz2) were grown in 6-well plates. Cells were lysed in 250 μ l of lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β -glycerolphosphate, 1 mM Na_3VO_4 , 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, and 200 μ M phenylmethylsulfonyl fluoride) and the mixture was subjected to centrifugation ($20,000 \times g$ for 10 min at 4 °C). The supernatant, designated as whole cell lysates, was collected and protein concentration was determined by Lowry's method (20). Protein aliquots (40 μ g/lane) were subjected to SDS-polyacrylamide gel electrophoresis and the resolved proteins were transferred onto nitrocellulose membranes. The blots were probed with antibodies specifically against p38 MAPK, phosphorylated p38 MAPK, MKK3, phosphorylated MKK3/6, phosphorylated ATF2 (Cell Signaling Technology, Danvers, MA), or against the indicated G protein subunits (anti-G α 1, and anti-G α 2 from Santa Cruz Biotechnology, Santa Cruz, CA; anti-G α o from Chemicon, Temecula, CA) and followed by incubation with a corresponding peroxidase-conjugated secondary antibody (anti-mouse from Santa Cruz Biotechnology; anti-rabbit from Kirkegaard and Perry Laboratories, Gaithersburg, MD). Immune complexes were determined by enhanced chemiluminescence method.

p38 MAP Kinase Assay—Confluent F9 clones stably expressing Fz2 were cultured in 6-well plates in media without serum for 16 h, and thereafter cells were pretreated without or with pertussis toxin (100 ng/ml), 8-Br-PET-cyclic GMP (10 μ M), or Zaprinast (1 μ M) for 30 min to 1 h prior to Wnt5a (50 ng/ml, cat. 645-WN, R&D Systems, Minneapolis, MN) stimulation for the indicated periods. The activity of p38 MAP kinase was measured by using p38 MAP kinase assay kit (Cell Signaling Technology, Danvers, MA) according to the manufacturer's instruction. Briefly, cells were lysed in 250 μ l of lysis buffer (20 mM Tris, pH 7.5; 150 mM NaCl, 1 mM EDTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β -glycerolphosphate, 1 mM Na_3VO_4 , 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, and 200 μ M phenylmethylsulfonyl fluoride) and the lysates were centrifuged at $20,000 \times g$ for 10 min at 4 °C. Aliquots (500 μ g of protein) of the supernatant were incubated with 20 μ l of immobilized phospho-p38 MAPK monoclonal antibody for 4 h at 4 °C. The immune complexes were washed twice with lysis buffer and twice with kinase buffer (25 mM Tris, pH 7.5; 5 mM β -glycerolphosphate, 2 mM dithiothreitol, 0.1 mM Na_3VO_4 , and 10 mM CaCl_2). The immobilized phospho-p38 was incubated in 30 μ l of kinase buffer containing 1 μ g of p38 substrate peptide GST-ATF2-(19–96) and 20 μ M ATP at 30 °C for 30 min. The reaction mixtures were subjected to the 10% SDS-PAGE and separated proteins were transferred electrophoretically onto nitrocellulose membrane. The activity of p38 kinase was assessed by detection of GST-ATF2 phosphorylation via an anti-phospho-ATF2 antibody.

NF-AT-sensitive Luciferase Gene Reporter Assay—Mouse F9 clones stably co-transfected with NF-AT reporter gene (pNFAT-Luc) and an expression vector harboring Fz2 were cultured on 12-well plates. Confluent cells were serum-starved

for 12 h. Thereafter cells were pretreated for 30 min with vehicle (as control), or PD98059 (20 μ M; Calbiochem, San Diego, CA) or SB203580 (2 μ M; Calbiochem, San Diego, CA), followed by incubation without or with Wnt5a (50 ng/ml) for 6 h. Cells were lysed with $1 \times$ luciferase cell culture lysis reagent (Promega, Madison, WI) and supernatants from cell lysates were subjected to luciferase assay according to the manufacturer's instruction (Stratagene, La Jolla, CA). Briefly, 20 μ l of supernatant were mixed with 100 μ l of luciferase assay buffer (40 mM Tricine, pH 7.8; 0.5 mM ATP, 10 mM MgSO_4 , 0.5 mM EDTA, 10 mM 1,4-dithiothreitol, 0.5 mM coenzyme A, and 0.5 mM luciferin), and the intensity of luminescence was measured 10 s after by using a luminometer (Lumat LB 9507; Berthold Technologies, Oak Ridge, TN). Samples were assayed in triplicate, and the luciferase activity was normalized based on protein concentrations. Results are presented as ratios of relative light units of treatment groups to those of control groups.

Cytoplasmic Calcium Measurements—Cells were plated on collagen-coated, glass-bottomed 35-mm dishes (MatTek Corporation, Ashland, MA) and cultured overnight. The intracellular Ca^{2+} was measured as described previously (17). Briefly, cells were loaded with 2 μ M Fura-2 acetoxymethyl ester (Molecular Probes, Inc., Eugene, OR) in Krebs-Ringer buffer composed of 128 mM NaCl, 5 mM KCl, 77.5 mM NaH_2PO_4 , 1.3 mM MgSO_4 , and 1.3 mM CaCl_2 for 40 min at 37 °C. Cells were then washed twice with Krebs-Ringer buffer and treated with Wnt5a (50 ng/ml). In the experiment that p38 inhibitor SB203580 was used, it was incubated together with Fura-2 for the last 30 min of the total 45 min loading time. Cells were immediately monitored via a Hamamatsu OREA ER-AG digital CCD camera coupled to a Nikon inverted microscope. Measurement of fluorescence intensity was performed and the ratio of absorbance at 340/380 nm (A_{340}/A_{380}) was computed using Dynamic Intensity Analysis (Compix Inc., Cranberry Township, PA).

Assay of PKG Activity—PKG activity was determined as described previously (21) with modifications. Briefly, supernatants (20 μ l) from the whole cell lysates were employed with 200 μ g/ml PKG-specific heptapeptide substrate (RKRSRAE; Bachem, San Diego, CA), 50 μ M ATP, and 2.5 μ Ci of [γ - ^{32}P]ATP in a buffer (TMG) containing 20 mM Tris-HCl, pH 7.4, 20 mM magnesium acetate, 10 mM glycerophosphate, 100 mM okadaic acid, and a mixture of protease inhibitors (10 μ g/ml leupeptin, 10 μ g/ml aprotinin, and 200 μ M phenylmethylsulfonyl fluoride). The reaction mixture was incubated at 37 °C for 20 min, and the reaction was terminated by addition of 4 μ l of 2 N HCl. Preboiled or HCl-treated supernatants were used as blank samples. Twenty microliters of the resultant mixture were spotted onto P-81 phosphocellulose filters. Air-dried P-81 filters were washed with H_3PO_4 (75 mM) three times. Incorporated ^{32}P in substrates on filters were measured by liquid scintillation spectrometry. The samples were assayed in triplicate. Kinase activity is normalized with protein concentration and presented in percentage of PKG activity of treatment group to that of the control group.

Measurement of Intracellular Cyclic GMP in Live Cells—The change of intracellular cyclic GMP in response to Wnt5a in the absence or presence of SB203580 was measured by using a cyclic GMP indicator Cygnet-2.1 (a kind gift from Dr. Wolfgang

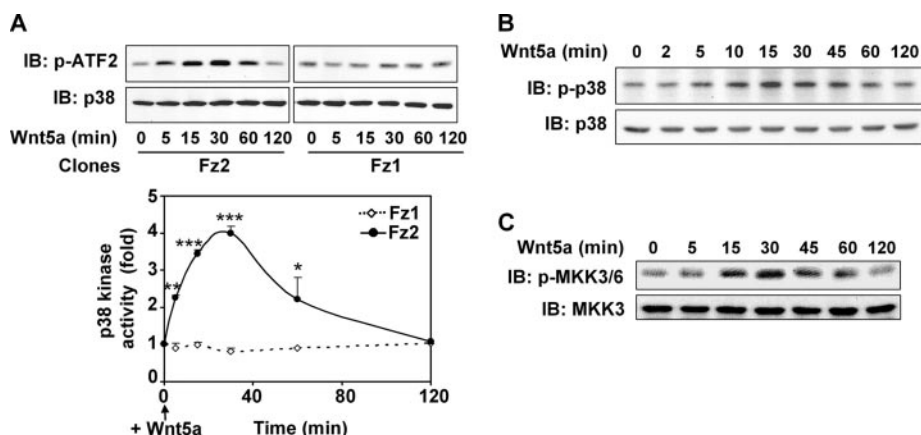


FIGURE 1. Stimulation of Frizzled-2 by Wnt5a activates p38 MAPK. A, mouse F9 clones stably expressing either rat Frizzled-1 (Fz1) or rat Frizzled-2 (Fz2) were treated with Wnt5a (50 ng/ml) for periods indicated. The activity of MAP kinase p38 was measured as described under "Experimental Procedures." Representative immunoblots are shown (upper panel). A quantitative analysis based upon results from scans of blots is presented (lower panel). The results displayed as mean $\pm$ S.E. were derived from at least three separate experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ versus Fz2 clone at 0 min. B, a representative blot of p38 MAPK activation in response to Wnt5a stimulation is displayed. Mouse F9 clones stably expressing Fz2 were treated with Wnt5a (50 ng/ml) for periods indicated. Total cell lysates were collected and subjected to SDS-gel electrophoresis followed by immunoblotting by using antibody specifically recognizing activated p38 which was characterized by pT-G-pY (pT and pY represent phosphorylated threonine and phosphorylated tyrosine, respectively) within the catalytic core (IB: p-p38). The same blot was stripped and probed by antibody against p38 (IB: p38) to establish the total p38 in the same fraction of cell lysates. C, a determination of MKK3/6 activation in response to Wnt5a stimulation by using anti-phospho-MKK3/6 (S189/207) antibody is shown (IB: p-MKK3/6). The same blot was stripped and probed by antibody against MKK3 (IB: MKK3). The data presented are representative of at least three separate determinations performed with separate cell lysates.

Dostmann, University of Vermont, Burlington, VT). Cygnet-2.1 was designed utilizing catalytically inactive mutant $\Delta 1-77$ /T516A bovine cGMP-dependent protein kinase I α (PKG I α) as the central cGMP sensor flanked by enhanced cyan fluorescence protein (eCFP) and citrine, a pH-insensitive version of yellow fluorescence protein (YFP). This biosensor binds cyclic GMP with a high affinity and undergoes a conformational change, interrupting the native FRET observed in the absence of cyclic GMP (22, 23). F9 Fz2 expressing clones were transfected with pcDNA3 harboring Cygnet-2.1 on a 35-mm glass-bottomed dish by using Lipofectamine 2000 as per the manufacturer's instruction (Invitrogen, Carlsbad, CA). Twenty-four hours later, cells were bathed in phenol red-free Dulbecco's modified Eagle's medium in the absence or presence of SB203580 (2 μ M) and imaged at 37 $^{\circ}$ C with a thermostatted chamber supplied with 5% CO₂ on a Zeiss LSM 510 META confocal microscope with a 100/1.45 Alpha Plan-Fluor objective and an Argon 458 nm laser line. Dual-emission ratio (525/475 nm) imaging of the indicator was controlled by Zeiss LSM Image software. Emission intensities were monitored from 460 to 560 nm at an interval of 10 nm.

Intracellular Cyclic GMP Accumulation—The second method for determining intracellular cyclic GMP concentrations was using a commercial cyclic GMP ELISA kit (Cayman, Chemical, Ann Arbor, MI) as previously described (13, 24). Briefly, mouse F9 cells stably expressing Fz2 were seeded onto 12-well plates. Control siRNA or siRNA specifically against p38 MAP kinase (Santa Cruz Biotechnology) were introduced into cells by using Lipofectamine 2000 according to the manufacturer's instruction. After siRNA treatment for 48 h and serum starvation for 12 h, cells were stimulated with Wnt5a (50 ng/ml) for 45 min and lysed in 0.2 ml of 0.1 M HCl. Supernatants from lysates were

collected by centrifugation and cyclic GMP concentrations were determined. The coefficients of variation within and among assays were 7.5 and 9.8%, respectively. The results were expressed as percentage of cyclic GMP measured in the "control" cells.

Treatment of Cells with Antisense Morpholinos—Morpholino phosphorodiamidate antisense oligonucleotides (morpholinos) targeting the translational initiation sites of Gat2, Gat1, and Gao were purchased from Gene Tools (Corvallis, OR). The sequences of morpholinos for Gat2, Gat1, and Gao are as follows: CACTCCCCATTCTGCTGTCTCCTC is for Gat2, CTCCCCGGCCTCCTCAGACGACCTT is for Gat1, and CCTCTGCGCTCAGCGTACATCCCAT is for Gao. Mouse F9 clones expressing Fz2 were treated with morpholino antisense oligo according to the manufacturer's protocol as described

previously (17). Seventy-two hours after morpholino antisense treatment, cells were stimulated without or with Wnt5a (50 ng/ml) for 15 min and p38 kinase activity was assessed. The expression levels of target proteins were evaluated by immunoblotting.

Treatment of Cells with siRNA—F9 cells expressing Fz2 were grown to $\sim$ 60% confluence. siRNA (final concentration = 100 nM) was introduced into cells by using Lipofectamine 2000 (Invitrogen). Cells were cultured in the presence of siRNA for additional 48 h according to the manufacturer's recommendation. siRNAs targeting either mouse p38 α (cat. SC-44217) or human p38 α (cat. SC-29433) were purchased from Santa Cruz Biotechnology.

PDE Activity Assay—F9 cells stably expressing Fz2 were seeded onto 12-well plates. siRNA treatment was performed 48 h prior to the Wnt5a (50 ng/ml) stimulation, as described previously. After incubation with Wnt5a for 15 min, cells were collected and homogenized in the buffer containing 20 mM Tris-HCl, pH 8.0; 2 mM MgCl₂, 1 mM EDTA, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, and 100 μ M phenylmethylsulfonyl fluoride. Twelve milligrams of cytosol fraction of cell lysates ($\sim$ 1 ml) were applied to a Resource Q chromatography (Amersham Biosciences). The starting buffer was 10 mM Tris-HCl, pH 8.0 with 50 mM NaCl; the washing buffer was 10 mM Tris-HCl, pH 8.0 with 300 mM NaCl. Samples were eluted with a linear gradient of NaCl (50–300 mM) at a flow rate of 1 ml/min. PDE6 in the eluted fractions was identified by immunoblotting. PDE6 activity was measured from the three fractions containing immunostained PDE6. Briefly, 50 μ l of samples were employed in a total volume of a 100- μ l reaction mixture containing 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 8 mM MgSO₄, 100 μ M cGMP, and 0.02 μ Ci of purified [³H]cGMP. The reaction mixture was incu-

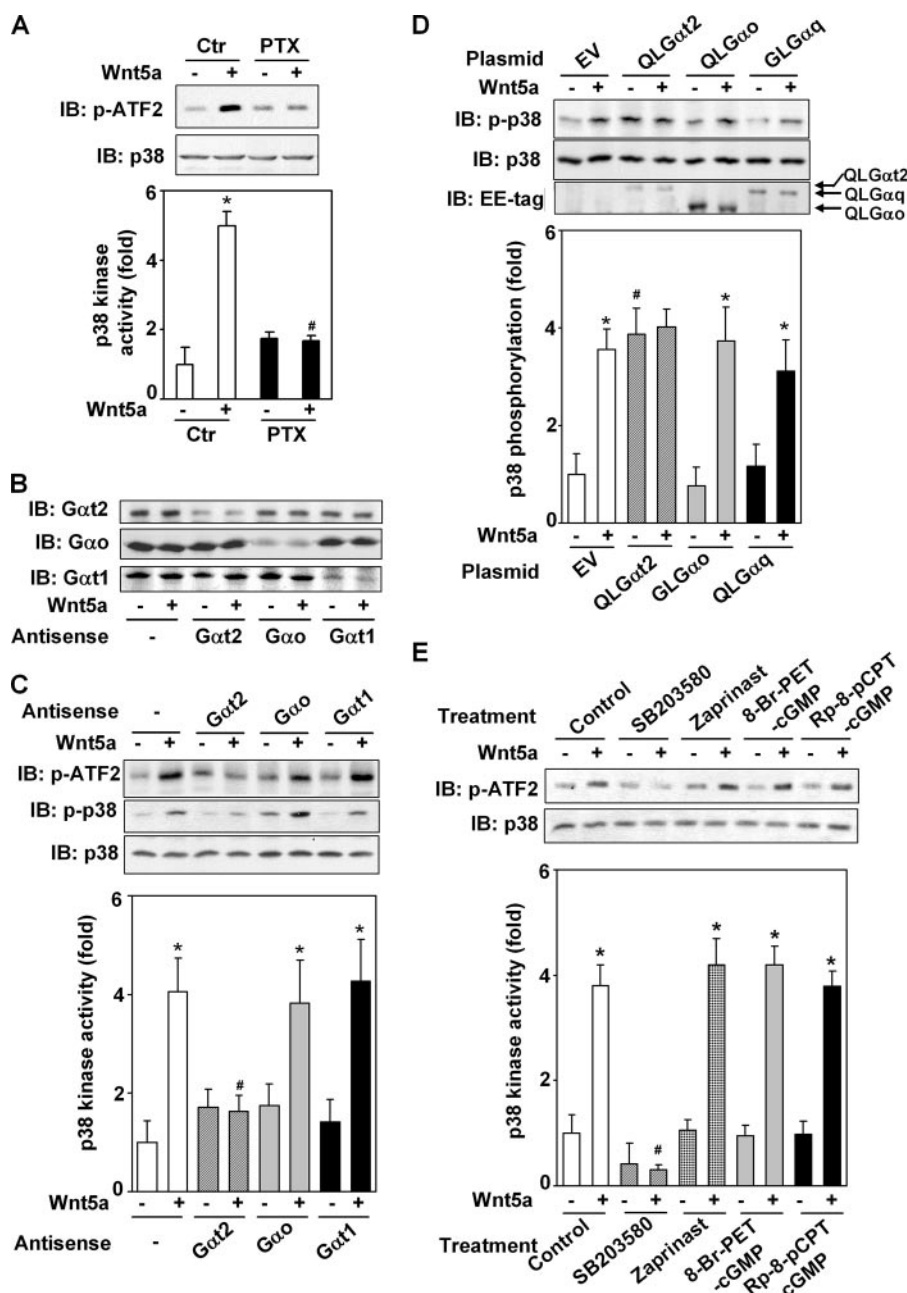


FIGURE 2. Wnt5a stimulation of MAPK p38 is blocked by pertussis toxin and by suppression of $G\alpha t2$, while mimicked by expression of constitutively active QL $G\alpha t2$. *A*, mouse F9 cells stably expressing Fz2 were pretreated without (as control, Ctr) or with pertussis toxin (PTX, 50 ng/ml) for 2 h prior to stimulation of the cells with Wnt5a. After incubation with Wnt5a for 15 min, cells were lysed, and the lysates were subjected to the p38 kinase activity assay by using ATF2 peptide as substrate as described under "Experimental Procedures." Total p38 in the same lysate was monitored by immunoblotting by using anti-p38 antibody. Representative blots were shown (upper panel). A quantitative analysis of p38 activity is presented (lower panel). The results displayed as mean $\pm$ S.E. were derived from at least three separate experiments. *, $p < 0.01$ versus the control without Wnt5a group; #, $p < 0.01$ versus the control with Wnt5a group. *B* and *C*, F9 cells stably expressing Fz2 were treated with morpholino antisense for 72 h to knockdown $G\alpha t2$, $G\alpha o$, or $G\alpha t1$ individually. *B*, protein levels of the designated G protein α -subunits were assessed by immunoblotting (IB). *C*, morpholino-treated cells were stimulated with Wnt5a (50 ng/ml) for 15 min. Cells were lysed, and p38 kinase activity was assessed as described under "Experimental Procedures." Representative immunoblots probed by antibodies against phospho-ATF2 (IB: p-ATF2), or phospho-p38 (IB: p-p38), or p38 are shown (upper panel). A quantitative analysis based upon results from scans of p-ATF2 blots is presented. *, $p < 0.05$ versus the control without Wnt5a group; #, $p < 0.05$ versus the control with Wnt5a group. *D*, F9 cells expressing Fz2 were transiently transfected with empty vector (EV) or plasmids encoding EE-tagged constitutively active mutants of $G\alpha t2$ (QL $G\alpha t2$), $G\alpha o$ (QL $G\alpha o$), or $G\alpha q$ (QL $G\alpha q$) for 24 h prior to the treatment with or without Wnt5a (50 ng/ml) for 15 min. Activated p38 and total p38 in the lysates were determined by immunoblotting by using anti-phospho-p38 antibody (IB: p-p38) or anti-p38 antibody (IB: p38), respectively, as described in the legend of Fig. 1. Expression of EE-tagged QL mutants of $G\alpha t2$, $G\alpha o$, and $G\alpha q$ were determined by immunoblotting by using anti-EE antibody. *, $p < 0.05$ versus the corresponding "-Wnt5a" groups; #, $p < 0.05$ versus the control without Wnt5a group. *E*, F9 cells stably expressing Fz2 were treated without (Control) or with SB203580 (2 μ M), 8-Br-PET-cGMP (10 μ M), zaprinast (1 μ M), or Rp-8-pCPT-cGMP (5 μ M) for 30 min prior to the stimulation of Wnt5a (50 ng/ml). p38 kinase activity was measured in cells treated with or without Wnt5a for 15 min. SB203580 (2 μ M) was present in the kinase reaction mixture during the incubation of immunoprecipitated p38 and its substrates. Representative immunoblots probed by antibodies against phospho-ATF2, or p38 are shown (upper panel). A quantitative analysis based upon results from scans of p-ATF2 blots is presented. *, $p < 0.01$ versus the corresponding "-Wnt5a" groups; #, $p < 0.001$ versus the control group treated with Wnt5a. The data presented are from at least three separate determinations performed with separate cell lysates.

bated at 30 °C for 20 min. Reactions were stopped by heat denaturation, 2 min at 100 °C. To convert GMP to guanosine, 0.1 unit/reaction of alkaline phosphatase (P-5931, Sigma) were added to each sample, and the reaction was conducted for 20 min at 25 °C. The labeled guanosine was separated by AG 1-X8 resin (Bio-Rad) and quantified by scintillation counting. PDE6 activity is expressed as the mean value (cpm) of three separate samplings.

Expression of Constitutively Active G Protein α -Subunits—Gln to Leu substitution mutants of G protein α -subunits: *Gat2* (Q204L), *GaoA* (G205L), and *Gaq* (Q209L) were purchased from UMR cDNA Resource Center (University of Missouri-Rolla, Rolla, MO). F9 cells stably expressing Fz2 were plated onto 12-well plates and transiently transfected with an expression vector harboring either *Gat2* (Q204L), *GaoA* (G205L), or *Gaq* (Q209L) by using Lipofectamine 2000 according to the manufacturer's protocol. Twenty-four hours later, cells were treated with Wnt5a for 15 min, and cell lysates were used for immunoblotting.

Statistical Analysis—The experiments were conducted at least in triplicate. All of the data are expressed as the means $\pm$ S.E. from at least three separate experiments. Comparisons of data among groups were performed with one-way analysis of variance followed by the Newman-Keuls test. Statistical significance (p value of less than 0.05) is denoted with asterisks or pound symbols.

RESULTS

Wnt5a Activates p38 Mitogen-activated Protein Kinase—Mouse F9 clones expressing rat Frizzled-2 (Fz2) were treated with purified Wnt5a for up to 2 h and the activity of p38 MAPK assayed. In response to stimulation with Wnt5a, p38 activity increases, as measured by the phosphorylation of the substrate ATF2 (Fig. 1A). ATF2 is a transcription factor that is a member of the leucine zipper family of DNA-binding proteins. Phosphorylated ATF2 (p-ATF2) was detected within 5 min of stimulation of the cells with Wnt5a. The amount of p-ATF2 increases progressively to 30 min, declining to basal levels from 60–120-min post-stimulation with Wnt5a. The amount of p38, established by immunoblotting, was unchanged by the treatment with Wnt5a. Clones expressing Fz1, rather than Fz2, display no significant accumulation of p-ATF2. To further test the hypothesis that Wnt5a treatment activates p38, we assayed the phosphorylation state of p38 MAPK itself, by immunoblotting (Fig. 1B). Wnt5a stimulates accumulation of the phosphorylated/activated form of p38 (p-p38), first detected at 5 min and peaking at 15 min following stimulation by Wnt5a (Fig. 1B). Analysis of known upstream regulators of p38 MAPK reveals that the phosphorylated/activated form of MAPK kinases (MKK) MKK3/6 (p-MKK3/6) is detected within 5 min of treatment with Wnt5a (Fig. 1C). The accumulation of p-MKK3/6 in response to stimulation with Wnt5a peaks at 30 min (Fig. 1C). These data clearly demonstrate that Wnt5a stimulates the p38 MAPK signaling pathway, culminating in the activation/phosphorylation of ATF2.

Activation of p38 MAPK by Wnt5a Is G Protein (*Gat2*)-mediated—Because *Gao* and *Gat2* mediate Wnt5a signaling to PDE6 and Ca^{2+} mobilization in F9 cells (13, 17), we examined

TABLE 1

Efficiency of knockdown of G protein subunits targeted by antisense morpholinos

The cellular abundance of each G protein subunit measured by immunoblotting in samples from cells untreated with antisense morpholinos is set to a value of 1.00. The results are present as means $\pm$ S.E. from three or more separate experiments.

Protein antisense	Control	<i>Gat2</i>	<i>Gao</i>	<i>Gat1</i>
<i>Gat2</i>	1.00 $\pm$ 0.04	0.23 $\pm$ 0.04 ^a	1.06 $\pm$ 0.04	0.99 $\pm$ 0.03
<i>Gao</i>	1.00 $\pm$ 0.04	0.95 $\pm$ 0.08	0.19 $\pm$ 0.04 ^a	0.96 $\pm$ 0.08
<i>Gat1</i>	1.00 $\pm$ 0.09	0.99 $\pm$ 0.11	1.01 $\pm$ 0.04	0.23 $\pm$ 0.02 ^a

^a $p < 0.001$ for the difference from the value observed for cells that were not treated with antisense morpholinos.

their possible involvement in the Wnt5a activation of p38 MAPK. Treating these cells with pertussis toxin (50 ng/ml, for 2 h), which inactivates both *Gao* and *Gat2*, abolishes the ability of Wnt5a to activate p38, as measured by accumulation of p-ATF2 (Fig. 2A). We made use of antisense morpholinos to suppress *Gat2*, *Gao*, or *Gat1* (as a control, Fig. 2B). The morpholinos effectively target their cognate G protein α -subunits (13) and effectively suppressed the expression of their targets by >75% (Fig. 2B, Table 1). Fz2 expressing F9 clones treated with antisense morpholinos targeting G protein subunits were assayed for the ability of Wnt5a to stimulate p38 MAPK phosphorylation as well as the phosphorylation of ATF2 (Fig. 2C). Suppression of *Gat2* effectively abolishes the ability of Wnt5a to stimulate p38 phosphorylation and activation (Fig. 2C). Suppression of either *Gao* or *Gat1*, in contrast, has no effect on the Wnt5a-stimulated signaling to p38 MAPK.

If *Gat2* mediates Wnt5a-stimulated activation of p38 MAPK, one would predict that expression of a constitutively activated (CA-) mutant form of this G protein α -subunit might mimic the effects of Wnt5a, in the absence of the ligand. F9 clones were transiently transfected with an empty expression vector (EV) or an expression vector harboring a CA mutant form of various G protein α -subunits. Expression of Q204L*Gat2* mimics the ability of Wnt5a to stimulate activation of p38 MAPK, as measured by immunoblotting with phosphospecific antibodies for active p38 (Fig. 2D). Treating the clones expressing Q204L*Gat2* with Wnt5a does not further increase the activation of p38 MAPK observed by expression of the Q204L*Gat2* alone. Expression of either Q205L*Gao* or Q209L*Gaq*, in contrast, does not activate p38 MAPK signaling (Fig. 2D). Thus, on the basis of knock-down studies (Fig. 2C) and expression of CA mutant forms of G α -subunits, *Gat2* appears to be obligate for Wnt5a to stimulate p38 MAPK.

The best-known effector of *Gat2* is the cyclic GMP phosphodiesterase PDE6, which has been shown to be the effector for the *Gat2*-mediated, Wnt5a-stimulated decline in intracellular cyclic GMP (13, 25). Based upon this knowledge, we explored if a cyclic GMP analogue (*i.e.* β -phenyl-1, N^2 -etheno-8-bromo-cGMP or 8-Br-PET-cGMP), a PDE6-selective inhibitor (zaprinast), or a protein kinase G inhibitor (*i.e.* 8-(4-chlorophenylthio) guanosine-3',5'-cyclic monophosphate Rp-isomer or Rp-8-pCPT-cyclic GMP) would impact the ability of Wnt5a to stimulate the activity of p38 MAPK. Inhibition of PDE6 with zaprinast and introduction of a cyclic GMP analogue have no significant effect on Wnt5a regulation of p38 (Fig. 2E). Likewise, treatment with the PKG inhibitor does not effect Wnt5a

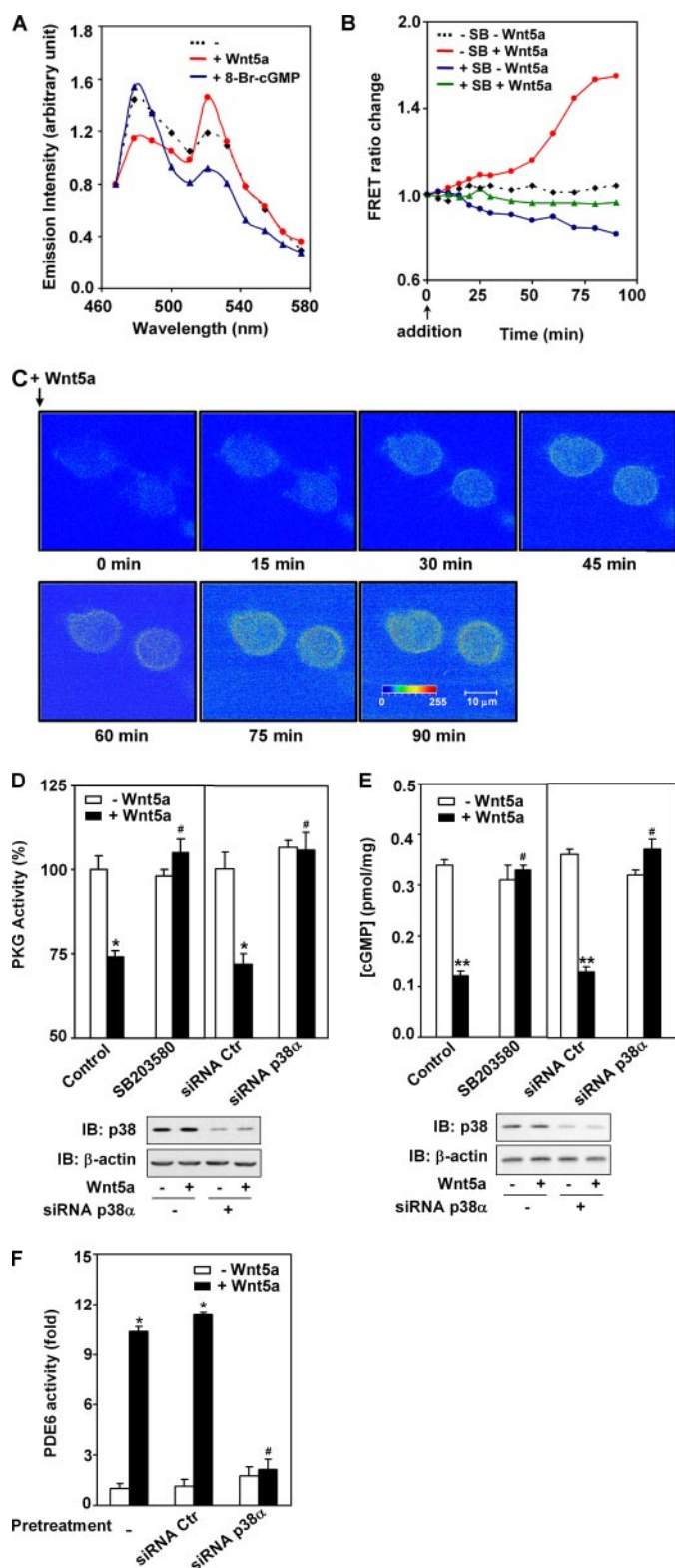


FIGURE 3. Wnt5a stimulation of p38 MAPK is essential for activation of the Wnt5a/PDE6/cyclic GMP response. A, F9 cells stably expressing Fz2 were transiently transfected with the cyclic GMP-sensing biosensor Cygnet-2.1 for 24 h. Cells were treated without or with either Wnt5a (50 ng/ml) or 8-Br-cGMP (100 µM), and the emission intensities from 465 to 580 nm were monitored. The results displayed are scans of untreated cells and cells treated for 90 min with either Wnt5a (+Wnt5a) or 8-Br-cGMP (+8-Br-cyclic GMP). B, F9 cells stably expressing Fz2 were transiently transfected with Cygnet-2.1 for 24 h. The cells then were treated with either vehicle or SB203580 (2 µM) and dual emission images at 525 nm and 475 nm were monitored for 90 min. Cells

signaling to the level of p38 MAPK (Fig. 2E). As a positive control, the p38-selective inhibitor SB203580 was tested and found to effectively abolish the ability of Wnt5a to stimulate p38 activity. Thus, Wnt5a signaling to p38 was G protein (Gαt2)-dependent, but not dependent upon the ability of Wnt5a to regulate either cyclic GMP PDE or protein kinase G.

p38 Regulates Wnt5a/NF-AT Signaling at the Level of Cyclic GMP—The inability of changes in intracellular cyclic GMP, PDE activity, or PKG activity to impact Wnt5a-stimulated p38 MAPK, clearly indicates that the Wnt5a effects on p38 activity are not because of changes in cyclic GMP, we wondered if the p38 MAPK itself may participate in the signaling from Wnt5a to the level of cyclic GMP. To test this possibility we made use of the Cygnet2.1 biosensor, specifically designed to report on intracellular cyclic GMP levels using a FRET-based read-out (22, 23). FRET signals from Cygnet2.1 decline as cyclic GMP levels increase, as the binding of the cyclic GMP to the biosensor disrupts the interaction of the fluorescence donor and acceptor moieties in the protein. By transient transfection, we analyzed the expression of Cygnet2.1 in the Fz2-expressing F9 clones and measured the emission intensity of the FRET signal in response to treatment of the cells with 8-bromo-cyclic GMP (Fig. 3A). Elevation of intracellular cyclic GMP yields a sharp decline in the 525 nm emission from Cygnet2.1, as predicted by earlier studies (23). Treating the cells with Wnt5a, which activates PDE6 and catalyzes a sharp decline in intracellular con-

were then untreated or treated with Wnt5a (50 ng/ml), and the dual emission images sampled for another 90 min. The intensity of emission ratio (525 nm/475 nm) was computed and the FRET ratio change is presented. The initial emission ratio is set as 1. C, a set of representative pseudo-colored images of the FRET ratio data (525 nm/475 nm in arbitrary unit) in live cells expressing the Cygnet2.1 biosensor and treated without or with Wnt5a. D, F9 cells stably expressing Fz2 were treated with either vehicle or SB203580 (2 µM) for 30 min prior to stimulation with Wnt5a. Additional sets of F9 cells expressing Fz2 were treated for 2 days with either a commercially designed control siRNA (siRNA Ctr) or siRNA targeting p38α MAP kinase (siRNA p38α). The cells were stimulated without or with purified Wnt5a (50 ng/ml) for 60 min, and cell lysates were collected for the measurement of activity of PKG as described under "Experimental Procedures." *, $p < 0.01$ versus the control -Wnt5a group; #, $p < 0.01$ versus the control +Wnt5a group. The data presented are from at least three separate determinations performed with separate cell lysates. The effect of siRNA treatment on the protein levels of p38 was assessed by immunoblotting. Representative immunoblots probed by antibodies against p38 (IB: p38) or β-actin (as a loading control) are shown (lower panel). E, F9 cells stably expressing Fz2 were treated with either vehicle or SB203580 (2 µM) for 30 min prior to stimulation with Wnt5a. Additional sets of F9 cells expressing Fz2 were treated for 2 days with either a control siRNA (Ctr) or siRNA targeting p38α MAP kinase (p38α). The cells were stimulated without or with purified Wnt5a (50 ng/ml) for 15 min and the accumulation of intracellular cGMP was assayed as described under "Experimental Procedures." The amount of cGMP was determined from standard dilutions and is presented as pmol of cyclic GMP/mg protein. *, $p < 0.01$ versus the corresponding -Wnt5a groups; #, $p < 0.001$ versus the control group treated with Wnt5a. The data presented are mean ± S.E. values from at least three separate determinations performed with separate cell lysates. The effect of siRNA treatment on the protein levels of p38 was assessed by immunoblotting. Representative immunoblots of p38 (IB: p38) or β-actin (as a loading control) are shown (lower panel). F, F9 cells stably expressing Fz2 without or with pretreatment of either control siRNA or siRNA targeting p38α were incubated with or without Wnt5a (50 ng/ml) for 15 min. Cell lysates were then applied to a source Q chromatography. The fractions containing PDE6 were collected, and the activity was measured as described under "Experimental Procedures." PDE6 activity presented as fold, setting the basal activity of control cells (-Pretreatment and -Wnt5a) as 1. *, $p < 0.001$ versus the corresponding -Wnt5a group; #, $p < 0.001$ versus the siRNA control +wnt5a group. The data are shown as mean ± S.E. Values from three separate determinations performed with separate cell lysates.

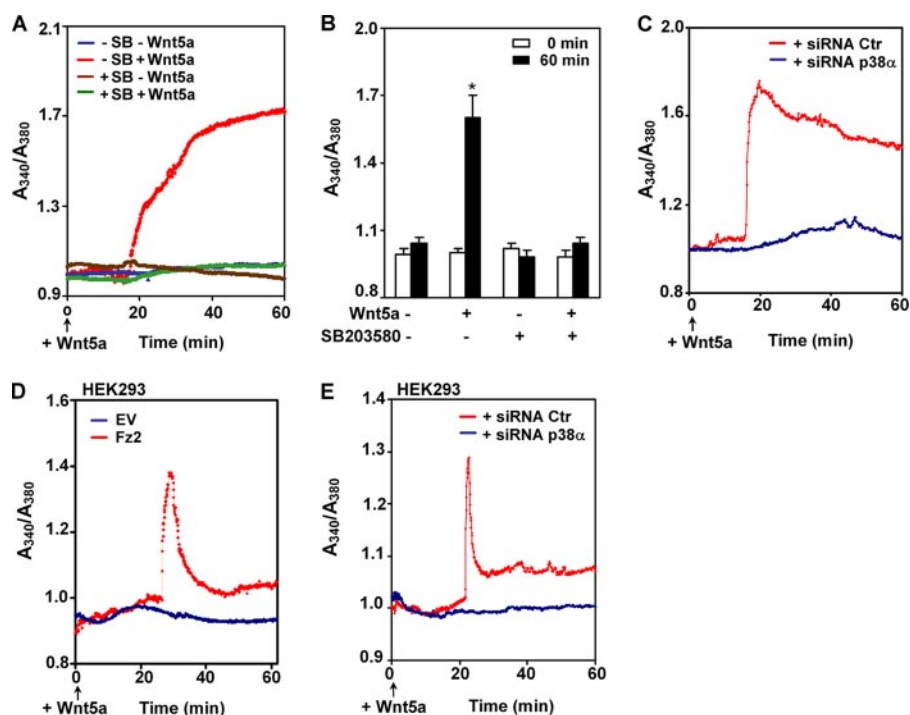


FIGURE 4. p38 MAPK activation by Wnt5a mediates the mobilization of intracellular Ca^{2+} . A and B, F9 cells stably expressing Fz2 were preloaded with Fura-2 for 45 min and then treated with either vehicle or SB203580 (2 μM) for the last 30 min of incubation. Cells then were treated without or with Wnt5a (50 ng/ml) and the intracellular free Ca^{2+} was monitored as described under "Experimental Procedures." Datum lines are summations of multiple imaging experiments, each performed on separate clones. These data are expressed as the ratio of absorbance at 340/380 nm (A_{340}/A_{380}). *, $p < 0.001$, versus the corresponding 0 min group. The data are presented as mean $\pm$ S.E. from at least three separate determinations, each performed with separate cell preparations. C, F9 cells expressing Fz2 were treated for 2 days with control siRNA (siRNA Ctr) or with siRNA targeting p38 α MAP kinase (siRNA p38 α). Calcium mobilization in response to Wnt5a (50 ng/ml) was then monitored as described under "Experimental Procedures." D, HEK-293 cells were transfected with either an empty vector (EV) or expressing vector pcDNA3.1 harboring rat Frizzled 2 (Fz2). Intracellular Ca^{2+} change in response to Wnt5a (50 ng/ml) stimulation was monitored as described under "Experimental Procedures." E, HEK-293 cells expressing Fz2 were treated for 2 days with either control siRNA or siRNA targeting p38 α . Intracellular Ca^{2+} change in response to Wnt5a (50 ng/ml) stimulation was monitored. The results are presented as ratios of intensity of absorbed fluorescence at excitation 340 over 380 nm monitored from one single experiment, representative of more than three separate experiments with similar results.

centrations of cyclic GMP (13, 15, 16), stimulated a decline in the 475 nm donor emission and a corresponding increase in the acceptor emission at 525 nm (Fig. 3A). Thus, Cygnet2.1 provides a sensitive read-out with which to measure intracellular levels of cyclic GMP in live cells, demonstrating by FRET that activation of Frizzled-2 by Wnt5a stimulates an increased acceptor emission characteristic of a decline in intracellular cyclic GMP levels. The Cygnet2.1 biosensor read-out was employed to test the effects of the chemical inhibition of p38 MAPK on the ability of Wnt5a to provoke a sharp decline in cyclic GMP accumulation (Fig. 3B). As displayed in a time course of the change in FRET ratio (525 nm/475 nm), Cygnet2.1 displays a sharp progressive increase in signal over 100 min in response to stimulation with Wnt5a. Treating the cells with the p38 inhibitor SB203580 alone has slightly negative effect on the Cygnet2.1 FRET ratio (Fig. 3B). Treating the cells with Wnt5a in the presence of SB203580, in contrast, abolishes the Cygnet2.1 response, demonstrating an obligate role of p38 activity in the downstream signaling from Wnt5a/Frizzled-2 to at the level of cyclic GMP.

The successful application of Cygnet2.1 biosensor to our studies enabled us to image the FRET signal in the live cells

under conditions in which the Fz2-expressing F9 clones are stimulated with Wnt5a (Fig. 3C). Within 15 min of stimulation of the cells with Wnt5a, FRET signal presented as dual-emission ratio (acceptor emission at 525 nm/donor emission at 475 nm) was obvious. Temporally, the FRET signal increases progressively during the time course following the treatment with Wnt5a. Spatially, the signal can be observed throughout the cells with increased localization of FRET signal about the cell membrane of the treated cells.

We tested the linkage between p38 MAPK activity and cyclic GMP levels by several additional, independent approaches, each with a different read-out. PKG activity was measured in cells treated with or without Wnt5a (Fig. 3D), displaying a decline in activity attributed to the decline in intracellular cyclic GMP stimulated by activation of the Wnt5a/Frizzled-2/Gat2/PDE6 pathway (17). Treating the cells with either the p38 inhibitor SB203580 or siRNA targeting p38 α abolishes the ability of Wnt5a to attenuate PKG activity (Fig. 3D). We also measured by direct means cyclic GMP accumulation in F9 cells treated with control versus p38-targeted siRNA or SB203580, and then

stimulated with Wnt5a (Fig. 3E). Cyclic GMP accumulation, as measured by enzyme-linked immunosorbent assay, declines in control siRNA-treated cells by >50% in response to stimulation with Wnt5a. Treating cells with siRNA targeting p38 α MAPK, in contrast, suppresses expression of p38 by more than 80% and abolishes the ability of Wnt5a to stimulate the sharp decline in intracellular cyclic GMP levels. Both the siRNA targeting p38 α MAPK as well as the p38 inhibitor SB203580 suppress the ability of Wnt5a to decrease intracellular cGMP accumulation equally well.

How then does p38 MAPK modulate cellular cGMP? We demonstrated previously that Wnt5a stimulation activates PDE6 (16). We tested the linkage between p38 MAPK and PDE6 by measuring the effect of siRNA targeting p38 α on PDE6 activation in response to Wnt5a stimulation. PDE6 activity was increased up to >10 fold in Fz2-expressing F9 clones stimulated by Wnt5a (Fig. 3F). Treating cells with control siRNA did not affect Wnt5a-stimulated PDE6 activation. Suppression of p38 α expression by siRNA, in contrast, abolished the ability of Wnt5a to stimulate activation of PDE6 (Fig. 3F). Treating cells with the p38MAPK-specific inhibitor SB203580 also blocked Wnt5a-stimulated activation of PDE6 (data not shown).

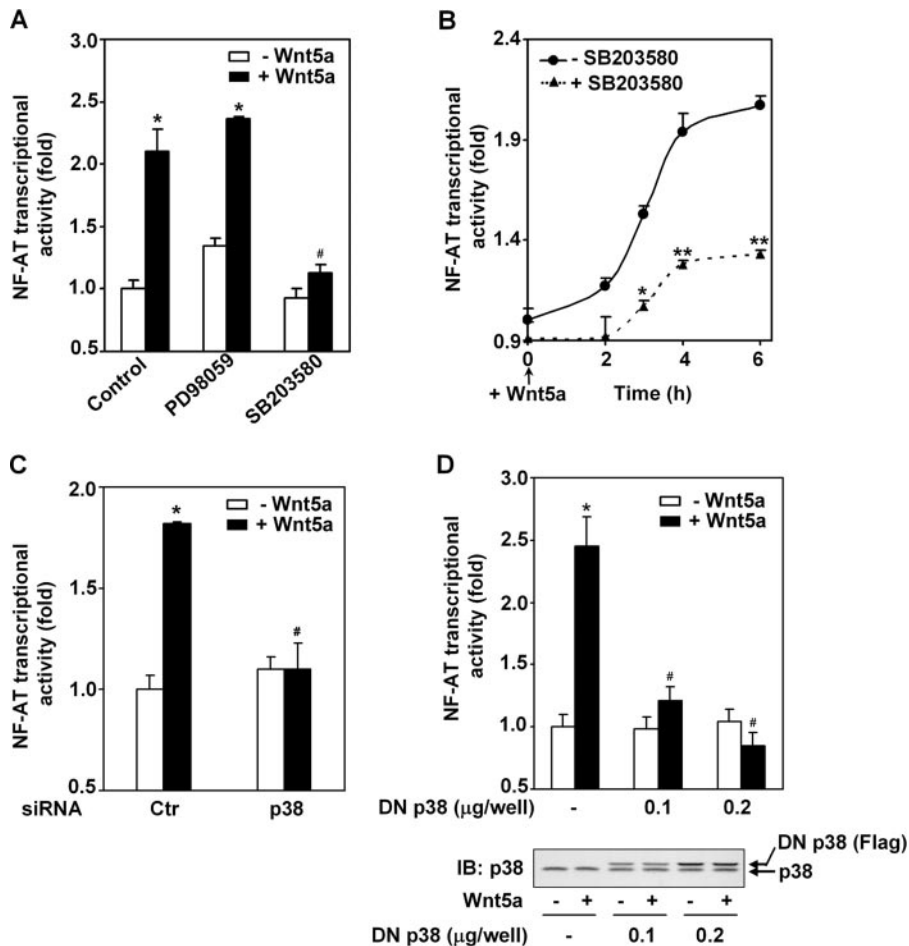


FIGURE 5. Chemical inhibition, knockdown of p38 α MAP kinase, or expression of dominant negative mutant of p38 α attenuates NF-AT transcriptional activation in response to Wnt5a stimulation. A, mouse F9 cells were stably co-transfected with Fz2 and NF-AT luciferase reporter gene. Stable clones were treated without (Control) or with either PD98059 (20 μ M) or SB203580 (2 μ M) for 30 min prior to treatment with Wnt5a (50 ng/ml). The activity of NF-AT-dependent luciferase reporter was measured after 6 h of incubation with or without Wnt5a. *, $p < 0.001$ versus the control without Wnt5a (-Wnt5a); #, $p < 0.001$ versus the control with Wnt5a (+Wnt5a). B, F9 cells stably co-transfected with an expressing vector harboring Fz2 and a plasmid containing NF-AT luciferase reporter gene were treated with SB203580 (2 μ M) for 30 min and then treated without or with Wnt5a (50 ng/ml). Cell lysates were collected at indicated time point and the activity of NF-AT-dependent luciferase reporter was measured. *, $p < 0.01$; **, $p < 0.001$ versus the corresponding -SB203580 groups. C, F9 cells stably co-transfected with an expressing vector harboring Fz2 and a plasmid containing NF-AT luciferase reporter gene were treated for 48 h with either a commercially designed control siRNA (Ctr) or siRNA targeting p38 MAP kinase (p38). The activity of NF-AT-dependent luciferase reporter was measured following a 6 h treatment without or with Wnt5a (50 ng/ml). *, $p < 0.001$ versus the control siRNA -Wnt5a group; #, $p < 0.001$ versus the control siRNA +Wnt5a group. D, F9 cells stably transfected with Fz2 and NF-AT luciferase reporter gene were cultured on a 12-well plate. Stable clones were transfected with empty vector (-) or pCMV harboring FLAG-tagged dominant negative p38 MAP kinase (p38 α (AGF)) at a concentration of 0.1 or 0.2 μ g/well. Twenty-four hours after transfection, the cells were incubated with or without Wnt5a (50 ng/ml) for 6 h, and the activity of NF-AT-dependent luciferase reporter was measured. *, $p < 0.001$ versus the corresponding -Wnt5a group; #, $p < 0.001$ versus empty vector +Wnt5a group. The expression of DN p38 was assayed by immunoblotting. Representative immunoblots of endogenous p38 (p38) as well as FLAG-tagged DN p38 are shown (lower panel).

p38 MAPK Is Obligate for Wnt5a-stimulated Mobilization of Intracellular Ca^{2+} —Recently it was shown that the ability of Wnt5a to mobilize intracellular Ca^{2+} is downstream of changes in cyclic GMP accumulation and PKG activity in zebrafish embryos as well as mouse F9 cells in culture (13, 17). Intracellular Ca^{2+} levels were measured by use of the Fura-2 calcium indicator. Treating the Fz2-expressing F9 clones with Wnt5a stimulates mobilization of intracellular Ca^{2+} (Fig. 4A). Treating the clones with the p38 MAPK inhibitor SB203580 abolishes the ability of Wnt5a to stimulate Ca^{2+} mobilization, as shown

in a Ca^{2+} level tracing (Fig. 4A) or in a summary obtained from independent trials of Fura-2 based quantification of a number of separate trials (Fig. 4B). Suppression of p38 α MAPK expression by siRNA in these clones abolished the ability of Wnt5a to stimulate Ca^{2+} mobilization (Fig. 4C). The essential role of p38 MAPK on Wnt5a-stimulated mobilization of intracellular Ca^{2+} was further tested in human embryonic kidney (HEK) 293 cells. In contrast to HEK293 cells transiently transfected with empty vector (EV), those cells expressing rat Frizzled-2 displayed Ca^{2+} transients in response to Wnt5a stimulation (Fig. 4D). Treatment of HEK293 cells with siRNA targeting p38 α blocked the Wnt5a-stimulated Ca^{2+} transients in these cells (Fig. 4E). Clearly the influence p38 MAPK on Wnt5a signaling is exerted at the levels of cyclic GMP accumulation, PKG activity, as well as Ca^{2+} mobilization.

p38 MAPK Is Obligate for the Activation of NF-AT-sensitive Gene Transcription by Wnt5a via the Non-canonical Pathway—Wnt regulation of NF-AT-sensitive gene expression is essential to ventral fate in *Xenopus* embryos (19). Wnt5a treatment alone stimulated NF-AT transcriptional activity in Fz2-expressing F9 clones, measured by an NF-AT sensitive luciferase-based gene reporter (17). We tested the effects of the p38 inhibitor SB203580 and of the MEK inhibitor PD98059 on the ability of Wnt5a to activate NF-AT-sensitive gene reporter. The MEK inhibitor displays no effect on the Wnt5a-stimulated gene response. Treating the cells with p38 inhibitor, in contrast,

effectively abolishes the ability of Wnt5a to stimulate NF-AT-sensitive gene transcription (Fig. 5A). A time course of NF-AT transcriptional activation by Wnt5 was performed in untreated cells (-SB203580) as well as cells treated with the p38 inhibitor (+SB203580). Activation of NF-AT-sensitive transcription was detected within 2 h of Wnt5a stimulation in cells untreated with the p38 inhibitor. The Wnt5a-stimulated response increases from 2–4 h and reaches a plateau of a 2-fold increase in NF-AT transcriptional activity from 4–6 h (Fig. 5B). Pretreating the cells with SB203580 for 30 min

sharply attenuates the ability of Wnt5a to stimulate the gene reporter. In cells pretreated with the p38 inhibitor, activation of NF-AT by Wnt5a was less than 40% of the wild-type levels and could only be detected at 3-h post-stimulation with Wnt.

On the basis of the ability of SB203580 to block Wnt5a-stimulation of NF-AT transcriptional activation, we explored the role of p38 in the Wnt5a regulation of this transcriptional activator by repression of p38 by using siRNA treatment (Fig. 5C). Cells were either treated with a control siRNA or a mixture of siRNA targeting p38 α MAPK. The siRNA targeting p38 α suppressed expression of the MAPK by >80% (Fig. 3, D and E). The ability of Wnt5a to stimulate NF-AT-sensitive transcriptional activity was probed in the control cells and the cells made deficient of p38 α . The results show that suppression of p38 MAPK abolishes the ability of Wnt5a to stimulate the NF-AT response in these cells. Clearly, the knock-down of p38 α was superior to chemical inhibition of p38 with SB203580, which yields a substantial, but not full, suppression of Wnt5a-stimulated NF-AT transcriptional activation. To further test the role of p38 MAPK in the Wnt5a regulation of transcriptional activity of NF-AT, we employed an expressing vector harboring a dominant negative (DN) mutant of p38 α MAPK (FLAG-tagged p38(AGF), a kind gift from Dr. Roger Davis, University of Massachusetts Medical Center, and examined the effect of its expression on NF-AT transcriptional activation. Expression of this DN mutant of p38 α MAPK clearly blocked Wnt5a-stimulated NF-AT-dependent luciferase reporter activation (Fig. 5D). Thus, p38 MAPK is obligate for the activation of NF-AT-sensitive gene transcription by Wnt5a via the non-canonical pathway.

The phosphoprotein Dishevelled (Dsh/Dvl) has been shown to be an essential downstream signaling element in Wnt canonical and non-canonical pathways as well as in planar cell polarity (27, 28). Immunoblotting of mouse F9 cells identified all three mammalian Dvls, Dvl1, Dvl2, and Dvl3 (Fig. 6A). If Dvls are mediating the ability of Wnt5a to activate p38, one would predict that suppression of their expression would impact negatively on the ability of Wnt5a to act. By use of siRNA targeting each of the mammalian Dvls, we were able to suppress expression by more than 75% (Fig 6A and Table 2). In F9 cells expressing Fz2 and targeted for suppression of Dvl1, Dvl2, or Dvl3 individually, no change in the ability of Wnt5a to activate p38 was observed (Fig. 6B). Similarly, suppression of three Dvl, the activation of p38 by Wnt5a was not affected (Fig 6B). Dapper1 has been reported as a general Dvl inhibitor (29). Expression of Dapper-1 blocks Wnt3a activation of the Lef/Tcf-sensitive transcriptional response, Wnt5a activation of the non-canonical cyclic GMP pathway, as well as Wnt3a stimulation of JNK activation (data not shown). Expression of Dapper-1 in the F9 cells expressing Fz2 did not diminish, however, the ability of Wnt5a to activate p38 MAPK (Fig. 6B). Taken together these data demonstrate the Wnt5a signals to the level of NF-AT-sensitive transcription via G α t2, PDE6, PKG, and Ca²⁺ mobilization, a response dependent upon the early activation of p38 MAPK for this Wnt non-canonical signaling pathway. The p38 activation in response to Wnt5a is the first example of a Wnt-sensitive pathway that operates independent of Dishevelled.

DISCUSSION

We reveal a novel role of the p38 MAPK in the Wnt/cyclic GMP/Ca²⁺/NF-AT transcriptional activation pathway mediated by Frizzled-2 (Fig. 7). Activation of the non-canonical Wnt/Ca²⁺ pathway promotes ventral cell fate in the *Xenopus* embryo. Wnt5a stimulates phosphatidylinositol signaling and Ca²⁺ transients that are essential to normal development in the zebrafish embryo (11, 18). Mouse embryonic F9 cells were employed to probe the role of p38 MAPK in the signal linkage map from a proximal step (*i.e.* activation of Frizzled-2) downstream to the activation of the developmentally regulated, luciferase reporter gene sensitive to NF-AT. The results from these studies provided sev-

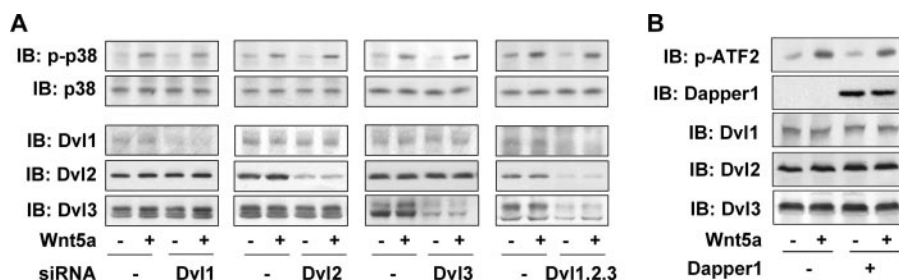


FIGURE 6. Suppression of Dvl1, Dvl2, or Dvl3 as well as expression of the Dvl antagonist Dapper-1 do not inhibit the ability of Wnt5a to activate p38 MAPK. A, F9 cells expressing Fz2 were treated for 48 h with commercially designed control siRNA or siRNA targeting Dvl1, Dvl2, or Dvl3. The cells were then incubated without or with Wnt5a (50 ng/ml) for 15 min and both activated phospho-p38 and total p38 were quantified in whole cell lysates by immunoblotting. Quantification of the data is presented as mean values $\pm$ S.E. from at least three separate experiments. B, F9 cells expressing Fz2 were transiently transfected with an expression vector harboring the Dvl antagonist Dapper-1 for 48 h. The cells were then treated without or with Wnt5a (50 ng/ml) for 15 min, and p38 activity was analyzed as described. Expression of Dapper1 as well as Dvl1, 2, and 3 was quantified in whole cell lysates by immunoblotting. Quantification of the data is presented as mean values $\pm$ S.E. from at least three separate experiments.

TABLE 2

Efficiency of knockdown of Dishevelleds targeted by siRNA

The cellular abundance of each Dvl measured by immunoblotting in samples from cells untreated with the targeted siRNA is set to a value of 1.00. The results are present as means $\pm$ S.E. from three or more separate experiments.

Protein siRNA	Control	Dvl1	Dvl2	Dvl3	Dvl123
Dvl1	1.00 $\pm$ 0.10	0.20 $\pm$ 0.11 ^a	0.96 $\pm$ 0.05	0.96 $\pm$ 0.05	0.23 $\pm$ 0.04 ^a
Dvl2	1.00 $\pm$ 0.03	1.15 $\pm$ 0.07	0.19 $\pm$ 0.04 ^a	0.97 $\pm$ 0.06	0.14 $\pm$ 0.03 ^a
Dvl3	1.00 $\pm$ 0.09	1.09 $\pm$ 0.13	0.96 $\pm$ 0.03	0.16 $\pm$ 0.03 ^a	0.26 $\pm$ 0.02 ^a

^a $p < 0.001$ for the difference from the values observed for untreated and control siRNA-treated cells.

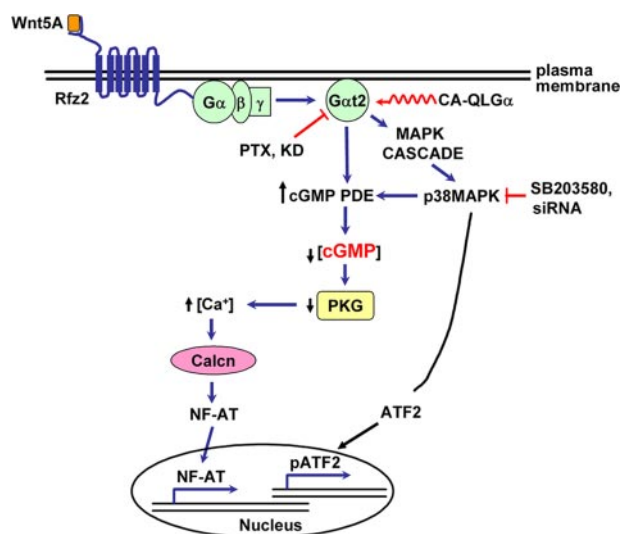


FIGURE 7. A schematic diagram of Wnt/cGMP/PKG/Ca²⁺/NF-AT transcriptional pathway: obligate role of p38 MAPK activation for Wnt5a response. See text for details.

eral key and novel insights about Wnt signaling in the non-canonical pathway.

First, although MAPK family members have been implicated in Wnt signaling, the current study is the first report to identify p38 MAPK as downstream in a Wnt-sensitive pathway. Earlier studies of the planar cell polarity pathway in *Drosophila* and Wnt pathways regulating convergent extension in vertebrate demonstrate the activation of N-terminal c-Jun protein kinase, JNK (30, 31). Erk1/2 MAPK have not yet been implicated in Wnt signaling, but it is likely that cross-talk must exist between Wnt-sensitive pathways and the MAPK cascade of downstream signaling (26). For the Wnt5a/cyclic GMP/Ca²⁺/NF-AT-sensitive transcription pathway, p38 not only regulates the signaling, but is essential for the overall function of the pathway from Wnt5a to the activation of NF-AT (Fig. 7).

Second, the activation of p38 by Wnt5a feeds into the Wnt5a/cyclic GMP/Ca²⁺/NF-AT pathway at the level of cyclic GMP, upstream of Ca²⁺ mobilization. The ability of Wnt5a to activate p38 MAPK itself is not sensitive to the elevation of intracellular cyclic GMP by addition of 8-bromo-cyclic GMP or by inhibition of PDE6 with zaprinast. Furthermore, inhibiting PKG activity does not alter the ability of Wnt5a to activate p38. What is clear is that inhibition of p38 MAPK interrupts the signaling of this pathway at the level of cyclic GMP. This important information was deduced both by read-outs of direct cyclic GMP measurement, as a reflection of PKG activity, and in live cells, making use of the Cygnet2.1 biosensor for cyclic GMP. Our understanding of how p38 MAPK modulates cGMP levels is not complete. Experimental results (Fig. 3F) provide a line of evidence indicating that p38 MAPK is necessary for the PDE6 activation in response to Wnt5a. Although Wnt5a stimulation leads to the activation of *Gat* and PDE6, mimicking the pathway in the visual system, the mechanism by which p38 MAPK regulates the PDE6 is not clear.

Third, the activation of p38 appears to operate via two interacting signaling paradigms, a GPCR cascade and a traditional MAPK cascade. The Fz2/*Gat*2/PDE6 triad operates down to

the level of NF-AT-sensitive transcriptional activation, while the MEKK/MKK/MAPK cascade culminates in activation of p38, which is also required for the activation of NF-AT. This configuration has marked similarities to the Fz1-mediated regulation of planar cell polarity, operating in mammals and in flies (25, 32) through Fz1/*Gαo*/*Dvl* and downstream to a MEKK/MKK/JNK cascade. Thus GPCRs relay information from Wnt ligands to G proteins and their cognate effectors downstream to MEKKs that control MAPKs and the activity of transcription factors (Fig. 7).

Finally, we reveal for the first time the operation of a Wnt-sensitive signaling pathway that to the level of the effector, p38, operates independent of the phosphoprotein *Dvl*. Knock-down of *Dvl1*, *Dvl2*, *Dvl3*, or the expression of the *Dvl* inhibitor *Dapper-1* has no effect on the ability of Wnt5a to activate p38, although the signaling to the level of NF-AT does.³ Taken together, these novel observations reveal an essential role of p38 MAPK in Wnt-sensitive signaling via the non-canonical pathway.

Acknowledgments—We thank Dr. Ken-Ichi Takemaru for *Dapper1* plasmid. We thank Dr. Roger Davis for pCMV-FLAG-tagged p38α and pCMV-FLAG-tagged p38α(AGF). We thank Dr. Noriko Yokoyama for help in PDE activity assay.

REFERENCES

- Mikels, A. J., and Nusse, R. (2006) *Oncogene* **25**, 7461–7468
- Cadigan, K. M., and Nusse, R. (1997) *Genes Dev.* **11**, 3286–3305
- Bhanot, P., Fish, M., Jemison, J. A., Nusse, R., Nathans, J., and Cadigan, K. M. (1999) *Development* **126**, 4175–4186
- Bhanot, P., Brink, M., Samos, C. H., Hsieh, J. C., Wang, Y., Macke, J. P., Andrew, D., Nathans, J., and Nusse, R. (1996) *Nature* **382**, 225–230
- Chen, C. M., and Struhl, G. (1999) *Development* **126**, 5441–5452
- Wang, H. Y., Liu, T., and Malbon, C. C. (2006) *Cell. Signal.* **18**, 934–941
- Malbon, C. C. (2004) *Front. Biosci.* **9**, 1048–1058
- Wang, H. Y., and Malbon, C. C. (2004) *Cell Mol. Life Sci.* **61**, 69–75
- Korinek, V., Barker, N., Morin, P. J., van Wichen, D., de Weger, R., Kinzler, K. W., Vogelstein, B., and Clevers, H. (1997) *Science* **275**, 1784–1787
- Molenaar, M., van de Wetering, M., Oosterwegel, M., Peterson-Maduro, J., Godsave, S., Korinek, V., Roose, J., Destree, O., and Clevers, H. (1996) *Cell* **86**, 391–399
- Slusarski, D. C., Corces, V. G., and Moon, R. T. (1997) *Nature* **390**, 410–413
- Kuhl, M., Sheldahl, L. C., Malbon, C. C., and Moon, R. T. (2000) *J. Biol. Chem.* **275**, 12701–12711
- Ahumada, A., Slusarski, D. C., Liu, X., Moon, R. T., Malbon, C. C., and Wang, H. Y. (2002) *Science* **298**, 2006–2010
- Kuhl, M., Sheldahl, L. C., Park, M., Miller, J. R., and Moon, R. T. (2000) *Trends Genet.* **16**, 279–283
- Wang, H. Y., and Malbon, C. C. (2003) *Science* **300**, 1529–1530
- Wang, H., Lee, Y., and Malbon, C. C. (2004) *Biochem. Soc. Trans.* **32**, 792–796
- Ma, L., and Wang, H. Y. (2006) *J. Biol. Chem.* **281**, 30990–31001
- Slusarski, D. C., Yang-Snyder, J., Busa, W. B., and Moon, R. T. (1997) *Dev. Biol.* **182**, 114–120
- Saneyoshi, T., Kume, S., Amasaki, Y., and Mikoshiba, K. (2002) *Nature* **417**, 295–299
- Lowry, O. H., Rosebrough, H. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Wolfe, L., Francis, S. H., Landiss, L. R., and Corbin, J. D. (1987) *J. Biol.*

³ L. Ma and H. Wang, unpublished data.

- Chem.* **262**, 16906–16913
22. Honda, A., Sawyer, C. L., Cawley, S. M., and Dostmann, W. R. (2005) *Methods Mol. Biol.* **307**, 27–43
23. Honda, A., Adams, S. R., Sawyer, C. L., Lev-Ram, V., Tsien, R. Y., and Dostmann, W. R. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 2437–2442
24. Chen, L., Salafranca, M. N., and Mehta, J. L. (1997) *Am. J. Physiol.* **273**, H1854–H1859
25. Malbon, C. C. (2005) *Nat. Rev. Mol. Cell. Biol.* **6**, 689–701
26. Jeon, S. H., Yoon, J. Y., Park, Y. N., Jeong, W. J., Kim, S., Jho, E. H., Surh, Y. J., and Choi, K. Y. (2007) *J. Biol. Chem.* **282**, 14482–14492
27. Habas, R., and Dawid, I. B. (2005) *J. Biol.* **4**, 2
28. Sheldahl, L. C., Slusarski, D. C., Pandur, P., Miller, J. R., Kuhl, M., and Moon, R. T. (2003) *J. Cell Biol.* **161**, 769–777
29. Cheyette, B. N., Waxman, J. S., Miller, J. R., Takemaru, K., Sheldahl, L. C., Khlebtsova, N., Fox, E. P., Earnest, T., and Moon, R. T. (2002) *Dev. Cell* **2**, 449–461
30. Boutros, M., Paricio, N., Strutt, D. I., and Mlodzik, M. (1998) *Cell* **94**, 109–118
31. Yamanaka, H., Moriguchi, T., Masuyama, N., Kusakabe, M., Hanafusa, H., Takada, R., Takada, S., and Nishida, E. (2002) *EMBO Rep.* **3**, 69–75
32. Katanaev, V. L., and Tomlinson, A. (2006) *Proc. Natl. Acad. Sci. U. S. A.* **103**, 6524–6529

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Li Ma and Hsien-yu Wang

J. Biol. Chem. 2007, 282:28980-28990.

doi: 10.1074/jbc.M702840200 originally published online August 7, 2007

Access the most updated version of this article at doi: [10.1074/jbc.M702840200](https://doi.org/10.1074/jbc.M702840200)

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