Supporting Information for

Arginine Methylation is Required for Canonical Wnt Signaling and Endolysosomal Trafficking

Lauren V. Albrecht, Diego Ploper, Nydia Tejeda-Muñoz, Edward M. De Robertis

Corresponding author: Edward M. De Robertis

Email: ederobertis@mednet.ucla.edu

This PDF file includes:

Supplementary Materials and Methods

Figs. S1 and S2

Captions for movies S1 and S2

References for SI reference citations

Other supplementary materials for this manuscript include the following:

Movies S1 and S2

www.pnas.org/cgi/doi/10.1073/pnas.1804091115

Supplementary Materials and Methods

Transfections. For the transfection of cDNA expression constructs into HEK-293T cells, 2 million cells were seeded in 10 cm dishes. Cells were transfected 24 hours after seeding with the indicated pCS2 based expression vectors using BioT transfection reagent (Bioland Scientific). Experiments were performed 24-48 hours after transfection. The total amount of DNA transfected was always normalized with empty pCS2 vector, with a total of 2 μg DNA per well of a 12-well plate.

Cell Lysis, Immunoprecipitation and Western Blots. Smad4 immunoprecipitation assays were performed as described previously (1). In brief, cells were rinsed with cold PBS and lysed in RIPA lysis buffer (0.1% NP40, 20 mM Tris/HCl pH 8, 10% Glycerol, with fresh protease inhibitors (Roche #04693132001) and phosphatase inhibitors (Calbiochem #524629). Cell lysates were cleared by centrifugation in a microcentrifuge (15,000 rpm for 10 minutes at 4°C). Cell lysates were prepared by addition of 2x Laemmli sample buffer (4% SDS, 20% Glycerol, 120 mM Tris-Cl pH 6.8, 0.02% bromophenol blue, and 5% β-Mercaptoethanol, resolved by 4%-20% SDS-PAGE), and analyzed by immunoblotting.

For anti-Flag immunoprecipitations, cleared Smad4-transfected cell extracts were incubated with the anti-Flag antibody (2 μg) for 4 hours at 4°C, incubated with slurry of protein A/G agarose beads (Santa Cruz, Biotechnology sc-2003), and washed 5 times with lysate buffer. Final immunoprecipitated products were denatured and eluted from beads by addition of 2x Laemmli sample buffer and boiling. Samples were resolved by 4%-20% SDS-PAGE, and

analyzed by immunoblotting.

Western blots were performed using standard protocols. Odyssey™ Blocking Buffer (LI-COR) diluted in PBS (1:1 ratio) was used to block nitrocellulose membranes for one hour at room temperature. All antibodies were diluted in PBS:Odyssey™ Blocking Buffer supplemented with 0.1% Tween 20. Primary antibodies were incubated overnight at 4°C, washed with Tris-buffered saline Tween 20 (TBST buffer) and incubated with secondary antibodies for one hour at room temperature. Images were acquired using a LI-COR Odyssey 9120 infrared imaging system.

Wnt Activity Reporter Assays and siRNA Rescue. The β-Catenin-activated (BAR) reporter Luciferase/Renilla system (2) was used to measure Wnt signaling activity. On day one, 2 million HEK-293T cells were plated in 6-well culture dishes. On day two, knockdown was performed with either siRNA targeting PRMT1 or Scrambled sequences using BioT transfection reagent, in triplicate. On day three, cells were re-plated onto 12-well plates. On day four, cells were transfected with BAR-Luc Wnt reporter DNA and either *Xenopus* PRMT1 or pCS2 carrier DNA. *Xenopus* PRMT1 is not targeted by human PRMT1 siRNA and rescued Wnt signaling. To normalize transfection efficiency pCS2+ Renilla was co-transfected, with each well receiving a total of 2 μg of DNA. The following DNA amounts were used: 1.2 μg BAR-Luc reporter, 0.4 μg pCS2+ Renilla, and either 0.4 μg of xPRMT1 or pCS2+ empty vector. 24 hours following DNA transfection, cells were incubated with Wnt3a or control medium for 16 hours, and Wnt activation analyzed by Luciferase assays. For all Luciferase assays, growth factors were added to cells at 60% confluency since confluency affects growth factor signaling (1). After treatment, cells were lysed in 70 μL of Passive Lysis Buffer (Promega) and Luciferase assays were

performed with the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions, using a Glomax Luminometer (Promega). To validate knockdown of PRMT1, cell lysates were collected and analyzed by immunoblotting of PRMT1.

Movies. Time-lapse movies were acquired with a Zeiss Observer.Z1 microscope equipped with Apotome.2. The microscope has a fully automated stage and a Temperature/CO2 Module S for cell culture. Images were collected using a Colibri LED using red filters with Apotome and DIC visible light optics with a 20x LD (Long working Distance) Apochromat objective. Cells were grown on 4-well Nunc Lab-Tek II Chamber Slide™ treated with Fibronectin. Each chamber was incubated with 0.1 ml of 100 μg/ml of sterile Fibronectin (Sigma) for 30 min at room temperature, and washed 3 times with PBS before seeding cells in DMEM medium containing 10% Fetal Bovine Serum. Image acquisition was 40 frames in 20 min. Image acquisition started 1-2 min after Wnt3a addition. Fluorescence filters were controlled by Axiovision 4.8 software and saved in this program. Movies were then processed using Windows Media Player. Similar results were obtained in 3 independent experiments.

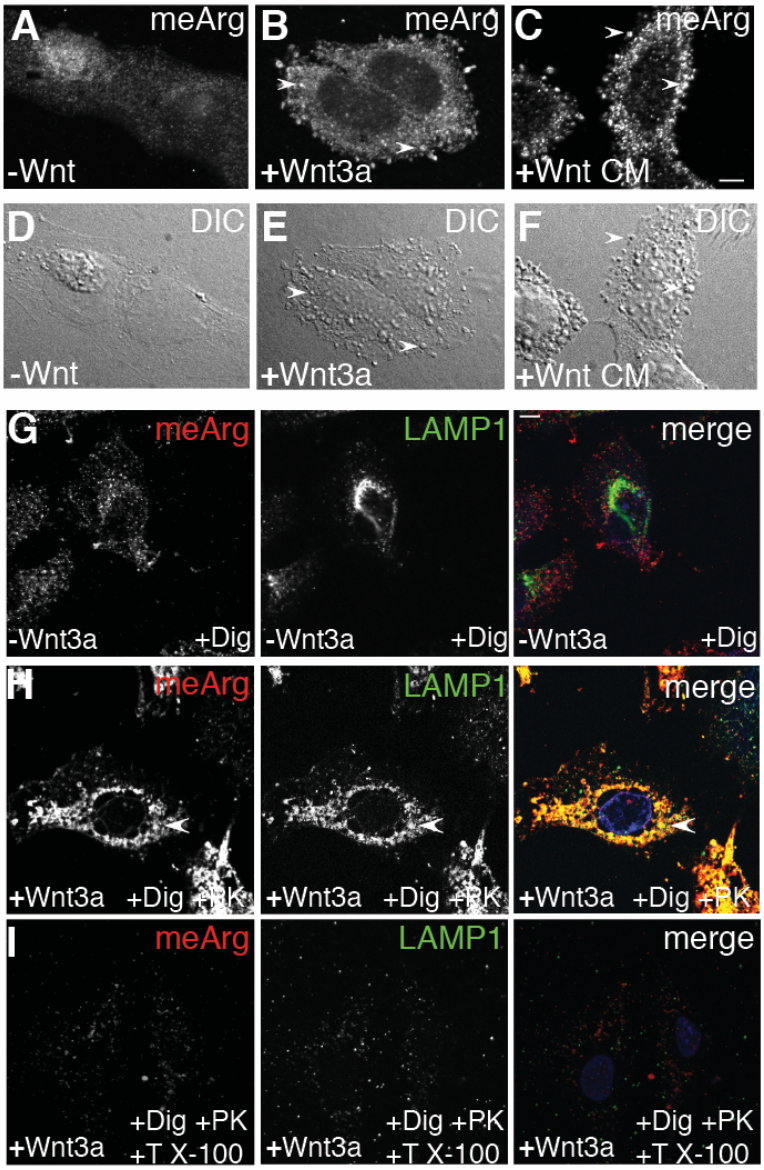

Fig. S1. Wnt-induced Arginine methylated proteins translocate into endolysosomes. (*A-F*) meArg was recruited into vesicles visible by DIC in HeLa cells (arrowheads), with either purified Wnt3a protein (PeproTech) or conditioned medium from Wnt3a secreting L-cells (3). (*G-I*) Arginine methylation was protected inside LAMP1-positive vesicles following Wnt signaling in protease protection analyses (arrowheads). HeLa cells were treated for 15 minutes with Wnt3a, placed on ice, treated with Digitonin for 15 minutes, washed in ice cold PBS, digested with Proteinase K (+/- Triton X-100) for 10 minutes at room temperature, followed by fixation with 4% paraformaldehyde for 10 minutes and immunostained for meArg and LAMP1. The LAMP1 antibody used (Cell Signaling C54H11) is directed against the intraluminal domain of LAMP1 and was therefore expected to be protected while the endolysosomal limiting membrane is intact. This is consistent with Fig. 1A showing that meArg becomes localized into LAMP1 vesicles in NIH 3T3 cells. Scale bar, 10 μm.

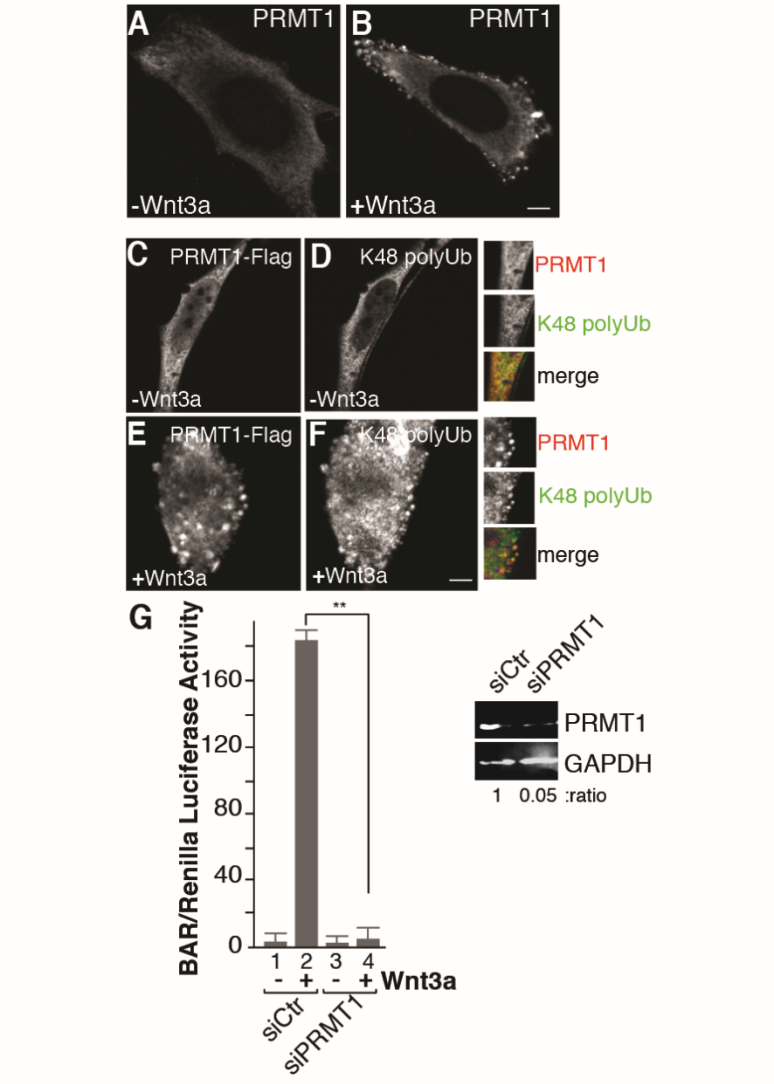

Fig. S2. PRMT1 is rapidly relocalized by Wnt signaling and is required for Wnt signaling in HeLa cells. (*A, B*) Endogenous Protein Arginine Methyltransferase 1 (PRMT1) relocalized to vesicles 15 minutes after adding Wnt3a. (*C-F*) PRMT1-Flag was sequestered in the same Wnt-induced MVBs as Lys48-polyUb (4) after 15 minutes of Wnt signaling. Note that PRMT1 and Lys48-polyUb overlap only during Wnt signaling. (*G*) PRMT1 was required for Wnt signal transduction Luciferase analyses in HeLa cells. The western blot shows the extent of depletion of PRMT1 by siRNA. Scale bars, 10 μm.

Supporting Information Movies

Movie S1. NIH 3T3 fibroblasts were incubated with 5 µg/ml BSA-DQ for 3 hours and then filmed for 20 min, with images collected in DIC brightfield and red fluorescence filter. BSA-DQ is normally self-quenched, as a result of heavy labeling by BODIPY dyes. When added to the culture medium BSA-DQ enters the liquid-phase cellular compartment by non-receptor mediated endocytosis. After digestion into smaller fragments in the lysosome, a fluorescent signal is generated when self-quenching is relieved. Note that fluorescence increased very little over the filming time. This movie serves as a control Movie 2 in which the same cells were treated with Wnt3a.

Movie S2. The same group of NIH3T3 cells as in Movie 1 filmed after the addition of 1 µl of Wnt3a (to a final concentration of 100 ng/ml, PreproTech) 1-2 min before the beginning of the 20 min filming. Note that Wnt3a causes a strong increase in BSA-DQ endocytosis and degradation in lysosomes, when compared to the control in Movie 1. This is remarkable, as the culture medium contained 10% Fetal Bovine Serum that contains high concentrations of unlabeled BSA (in humans serum contains 35-55 mg/ml Albumin). Despite the abundance of unlabeled carrier proteins, the degradation of the dilute (5 µg/ml) BSA-DQ was strongly increased. This indicates that Wnt3a strongly activated non-receptor mediated endocytosis and degradation of proteins from the extracellular milieu in lysosomes. This Wnt-mediated metabolic switch towards the lysosomal pathway will be addressed in future investigations.

References

1. Demagny H, Araki T, De Robertis EM (2014) The tumor suppressor Smad4/DPC4 is regulated by phosphorylations that integrate FGF, Wnt, and TGF-β signaling. *Cell Rep* 9(2): 688–700.
2. Biechele TL, Moon RT (2008) Assaying beta-catenin/TCF transcription with beta-catenin/TCF transcription-based reporter constructs. *Methods Mol Biol* 468:99–110.
3. Willert K et al. (2003) Wnt proteins are lipid-modified and can act as stem cell growth factors. *Nature* 423(6938):448–452.
4. Kim H, Vick P, Hedtke J, Ploper D, De Robertis, EM (2015) Wnt signaling translocates Lys48-Linked polyubiquitinated proteins to the lysosomal pathway. *Cell Rep* 11(8):1151–1159.