Supplementary Information for

Canonical Wnt is Inhibited by Targeting One Carbon Metabolism through Methotrexate or Methionine Deprivation

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Supplementary Materials and Methods

Fig. S1

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Supplementary SI Materials and Methods

Endocytosis and Immunofluorescence Experiments. To study endocytosis into lysosomes, cells were incubated with BSA-DQ (DQ Red BSA, Molecular Probes, #D-12051) diluted in pre-warmed culture medium at a concentration of 10 µg/ml for 30 min at 37°C (1). Endocytosis was monitored in cells that were incubated in Methionine-free medium, methotrexate, or control medium for 2 hours and subsequently with BSA-DQ and Wnt3a or control medium for 30 min at 37°C. Cells were then washed twice with PBS on ice (phosphate buffered saline, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) to prevent endocytosis and remove medium, fixed in 4% (wt/vol) paraformaldehyde (Sigma, P6148) in PBS for 20 min, and permeabilized with 0.2% (vol/vol) Triton X-100 in PBS for 10 min on ice. Next, coverslips were washed with PBS, blocked for 1 hr in Blocking buffer containing 3% (wt/vol) BSA in PBS at room temperature, and incubated with primary antibodies in blocking buffer overnight at 4°C. Primary antibodies were removed by three washes with PBS. Cells were mounted onto glass slides in ProLong Gold antifade reagent with DAPI (Life Technologies) to stain cell nuclei. Images were acquired on a Zeiss Imager Z.1 microscope with Apotome using a 63× oil immersion objective. ImageJ and Zeiss (Zen) imaging software was used for image analyses.

PRMT1 depletion. The procedure to examine whether PRMT1 was required for endocytosis was performed as described previously (1). On day one, HeLa cells were plated onto a 6-well dish at 30% confluency. On day two, knockdown was performed with either siRNA targeting PRMT1 or Scrambled sequences using BioTransfection reagent, in triplicate. On day three, cells were re-plated onto 12-well plates. On day four, cells were incubated with BSA-DQ and treated with Wnt3a for 30 min prior to fixation and analyses, as above.

Methionine Depletion Experiments. To assess Methionine depletion by immunofluorescence, HeLa cells were first grown on 12-well plates containing glass coverslips to a density of 60%. 24 hours after plating, the cell culture medium was replaced with RPMI 1640 or Methionine-free RPMI 1640 and incubated for 2 hours at 37°C. Cells were always washed briefly with pre-warmed Methionine-free medium before a 2 hour incubation period to normalize levels of Methionine in the background between experiments. Cells were then stimulated with Wnt3a protein for 30 min, washed with ice cold PBS, and fixed in 4% PFA. To study the kinetics of Methionine depletion on Wnt signaling, cells were incubated in Methionine-free medium for time points between 5 to 240 min, while Wnt3a (100 ng/mL) was always added to cells for 30 min before fixation. Cells were fixed with 4% PFA and primary antibodies used were mouse anti-PRMT1 (1:500 dilution) and rabbit anti-GSK3 (1:500 dilution) in blocking buffer. Cells were washed three times with PBS, incubated with secondary antibodies diluted in blocking buffer for 45 min at room temperature and mounted onto glass slides in ProLong Gold antifade reagent with DAPI (Life Technologies) to stain cell nuclei. Secondary antibodies used were: Donkey anti-mouse 488 and Donkey anti-rabbit Cy3 (both at 1:10,000 dilution, Thermo). Images were acquired on a Zeiss Imager Z.1 microscope with Apotome using a 63× oil immersion objective. ImageJ and Zeiss (Zen) imaging software were used for image analyses. For Luciferase assays, HEK293T BAR-Renilla cells (2) were plated onto 12-well dishes at 60% confluence. Cells were incubated in control or Methionine-free medium for 12 hr with or without Wnt3a, and subjected to Luciferase assays. Methionine depletion was performed using Methionine free-RPMI 1640 supplemented with 10% fetal calf serum (FCS) in both dialyzed and non-dialyzed forms (3). The small amount of Methionine present in FCS did not affect the results.

Luciferase Reporter Analyses. The β-Catenin-activated (BAR) reporter Luciferase/Renilla system (1) was used to measure Wnt signaling activity. On day one, 2 million HEK-293T cells permanently transfected with BAR/Renilla were plated in 12-well culture dishes. On day two, cells were incubated in

indicated treatment condition including Methionine-free RPMI, 20 μM methotrexate (2 hr preincubation), 8 mM nicotinamide (2 h preincubation), or normal control medium. Wnt3a stimulation was performed for 12 h and Wnt activation was assessed by Luciferase assays. For all Luciferase assays, growth factors were added to cells at 60% confluency. 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).

Nicotinamide experiments. Metabolites that undergo methylation modifications such as nicotinamide can serve as sinks for methyl-groups (4). The addition of nicotinamide in our experiments served as a mechanism to decrease cellular SAM levels without pharmacological agents. HEK293T cells permanently transfected with BAR-Renilla were incubated with nicotinamide solubilized in water at a concentration of 8 mM for 12 hr.

Methotrexate Experiments. Folate cycle inhibition with methotrexate (20 μM) was performed in HeLa cells for 1 hour, and assessed by *in situ* proteinase K protection analyses examining vesicle formation and PRMT1 and GSK3 localization during Wnt signaling. Cells displayed normal morphology. BAR-Renilla HEK293T cells were used to assess Wnt/β-catenin activity in the presence of 20 μM methotrexate diluted in folate-free medium with a preincubation for 2 hr and overnight treatment with Wnt3a for 12 hr (5). Cellular toxicity was assessed at methotrexate concentrations ranging from 5-60 μM. For these experiments, cells were preincubated with folate-free RPMI medium prior to methotrexate treatment.

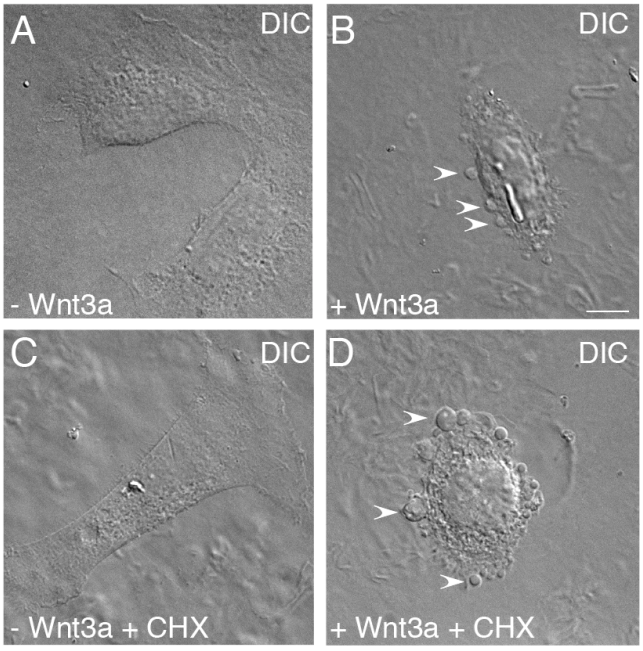

Fig. S1. Formation of Wnt-induced Endolysosomal Vesicles does not Require New Protein Synthesis. *(A-D)* HeLa cells treated with Wnt3a form large vesicles after 30 minutes by phase microscopy (arrowheads) regardless of the presence or absence of pretreatment with cycloheximide for 6 hours at a concentration (10 μg/ml) that blocks protein synthesis. (Scale bars, 10 μm; **P<0.01).

References

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