

# PDGFR $\alpha$ Signaling Is Regulated through the Primary Cilium in Fibroblasts

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## Supplemental Experimental Procedures

### Cell Cultures

Swiss NIH3T3 mouse fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal calf serum and 10 ml l<sup>-1</sup> penicillin-streptomycin, at 37°C, 5% CO<sub>2</sub>, 95% humidity. Mouse embryonic fibroblasts (MEF) were isolated after Freshney protocols 11.1 and 11.5 [S1]. Primary cultures of MEFs from wild-type and *Tg737<sup>orpk</sup>* mice were grown in 45% DMEM and 45% F12 + L-glutamine (Invitrogen, Taastrup, Denmark) supplemented with 10% heat-inactivated fetal calf serum and 10 ml l<sup>-1</sup> penicillin-streptomycin at 37°C, 5% CO<sub>2</sub>, and 95% humidity. Cells were examined at either 70%–80% confluency (interphase, nonarrested cells) or at 90%, followed by serum starvation for 0, 6, 12, 24, and 48 hr to induce growth arrest. In some experiments, cells were stimulated with 50 ng/ml PDGF-AA (Oncogene; VWR International Aps, Albertslund, Denmark) or PDGF-BB (Calbiochem; VWR International Aps). Cell cultures were passaged every 3–4 days by trypsination (0.5%) and only passages 10–30 for NIH3T3 cells and passages 10–20 for MEF cells were used for experiments.

### Antibodies and Blocking Peptides

Monoclonal mouse antibodies from SigmaAldrich, Saint Louis, MI were mouse anti-acetylated  $\alpha$ -tubulin (T6793) and mouse monoclonal anti- $\beta$ -actin (A5441). Polyclonal rabbit antibodies from Sig-

maAldrich were anti-phospho-PDGFR $\alpha$  Y<sup>742</sup> (P8246). Polyclonal goat antibodies from R&D Systems Europe, Ltd., Abingdon, UK were goat anti-PDGFR $\alpha$  (AF1062). Polyclonal rabbit antibodies from Santa Cruz Biotechnology, Inc., Santa Cruz, CA were anti- $\gamma$ -tubulin (sc-10732), anti-PDGFR $\alpha$  (sc-338), anti-phospho-PDGFR $\alpha$  Y<sup>720</sup> (sc-12910); and anti-PDGFR $\beta$  (sc-432), anti-PDGFR $\beta$  Y<sup>657</sup> (sc-12907-R), anti-IR $\alpha$  (sc-710) and anti-phosphotyrosine (sc-18182). Polyclonal rabbit antibodies from Cell Signalling Technology (Medinova Scientific A/S, Glostrup, Denmark) were anti-MEK1/2 (9122), anti-phospho-Mek1/2 (9121), anti-Erk1/2 (9102), anti-phospho-Erk1/2 (9101), anti-Akt (9272), and anti-phospho-Akt (587F11). Blocking peptide for rabbit anti-PDGFR $\alpha$  (Santa Cruz Biotechnology, Inc., sc-338 P). Mouse anti-pericentrin was a generous gift from Prof. J.L. Salisbury, Mayo Clinic College of Medicine.

### Immunofluorescence Microscopy Analysis

Cells grown on glass coverslips in six-well test plates (NUNC A/S, Roskilde, Denmark) were fixed in 4% paraformaldehyde, permeabilized in 0.2% Triton X-100, quenched in PBS with 2% BSA, and incubated with primary antibodies at room temperature for 2 hr (mouse anti-acetylated  $\alpha$ -tubulin [1:2,000–1:50,000], rabbit anti- $\gamma$ -tubulin [1:2000], rabbit anti-pericentrin [1:5000], goat anti-PDGFR $\alpha$ , rabbit anti-PDGFR $\alpha$ , rabbit anti-PDGFR $\beta$ , rabbit anti-IR $\alpha$ , rabbit anti-phosphotyrosine, and rabbit phospho-Mek1/2 [1:300]). Cells were washed in PBS (137 mM NaCl, 2.6 mM KCl, 6.5 mM Na<sub>2</sub>HPO<sub>4</sub>, and 1.5 mM KH<sub>2</sub>PO<sub>4</sub>) and incubated with DAPI and Alexa Fluor<sup>488/568</sup>-conjugated goat anti-mouse IgG or IgG F(ab)<sub>2</sub>, Alexa Fluor<sup>488/568</sup>-conjugated goat anti-rabbit IgG and Alexa Fluor<sup>488</sup>-conjugated chicken anti-mouse IgG (all 1:600, Molecular Probes, Eugene, OR) for 1 hr. Fluorescence was visualized on either a Microphot-FXA and Eclipse E600 microscopes (Nikon, Tokyo, Japan) with EPI-FL3 filters and MagnaFire cooled CCD camera (Optronics, Goleta, CA) or an IX70 confocal laser scanning microscope (Olympus, Tokyo, Japan) with a Kr/Ar laser by which special care was taken to avoid

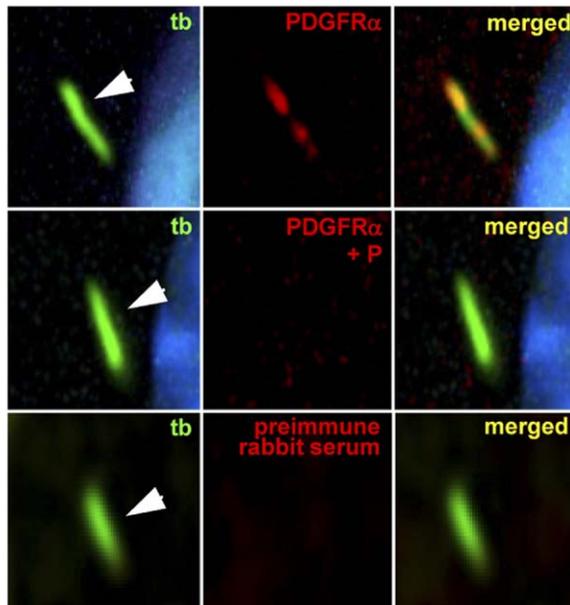


Figure S1. PDGFR $\alpha$  Specifically Localizes to the Primary Cilium of NIH3T3 Fibroblasts

Ciliary localization (Tb, green, arrows) of rabbit anti-PDGFR $\alpha$  in the absence (red, upper panel row) and in the presence of blocking peptide, P (red; middle panel row), in 24 hr serum-starved cells. Control, substitution of rabbit anti-PDGFR $\alpha$  with preimmune rabbit serum (red, lower panel row).

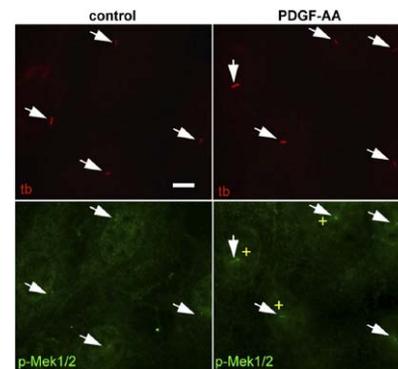


Figure S2. PDGF-AA Activates Mek1/2 in the Primary Cilium and at the Ciliary Basal Body of NIH3T3 Fibroblasts

Immunofluorescence microscopy analysis showing that PDGF-AA (10 min stimulation) increases the level of phospho-Mek1/2 (green; c-Raf-dependent Mek1/2 phosphorylation in the activation loop on serines in positions 217 and 221 that activates Erk1/2) in the mother centriole and along the primary cilium (red, tb, arrows, and marked by a plus sign). Scale bar: 10  $\mu$ m.

bleed through between channels using different filters. Differential interference contrast (DIC) images were obtained separately after fluorescence image acquisition, and digital images were processed by Adobe Photoshop 6.0.

#### **SDS-PAGE, Immunoprecipitation, and Western Blotting**

Cells grown in Petri dishes were quickly washed in ice-cold PBS, treated with 100  $\mu$ l boiling lysis buffer with 1% SDS, scraped off with a rubber policeman, and processed ten times through a 27 gauge needle. The lysates were centrifuged at  $16,000 \times g$  to precipitate nonsoluble material. The protein concentrations were estimated with a BCA protein kit (Pierce Biotechnology, Rockford, IL) so that equal concentrations of protein could be loaded on the gels. Rabbit anti-PDGFR $\alpha$  was added to lysates for immunoprecipitation in RIPA buffer with no SDS and incubated overnight at 4°C. Protein A- and G-conjugated sepharose (1:1) equilibrated in RIPA was then added and incubated for 2 hr at room temperature. The beads were washed with ice-cold RIPA and the precipitate was dissolved in sample buffer. Proteins from whole-cell lysates and immunoprecipitates were resolved by gel electrophoresis by SDS-PAGE under denaturing and reducing conditions and electrophoretically transferred to nitrocellulose membranes as previously described [S2] using the NOVEX XCell I system (Invitrogen A/S, Taastrup, Denmark). The membranes were incubated either with primary antibodies (1:300) overnight at 4°C or at room temperature for 2 hr. Crossreactivity of the antibodies was identified with species-specific alkaline phosphatase-coupled secondary antibodies (1:1200, Jackson Laboratory) followed by developing with BCIP/NBT (KPL, Gaithersburg, MD). Mark 12 (Invitrogen A/S, Taastrup, Denmark) was used as molecular mass standard in SDS-PAGE. Band intensities of proteins in Western blot analysis were measured using UN-SCAN-IT Version 5.1 (Silk Scientific, Inc., Orem, Utah, USA). Data are presented either as individual experiments representative of at least three independent experiments, or as mean values  $\pm$  SEM from a minimum of three individual experiments.

#### **Supplemental References**

- S1. Freshney, R.I. Culture of Animal Cells. (New York: Wiley-Liss, Inc.), pp. 150–163.
- S2. Christensen, S.T., Guerra, C.F., Wada, Y., Valentin, T., Angeletti, R.H., Satir, P., and Hamasaki, T. (2001). A regulatory light chain of outer arm dynein in *Tetrahymena thermophila*. J. Biol. Chem. 276, 20048–20054.