Supplement 3
Methods Supplement – Experimental Methods

1. Materials

Chemicals. EGFR inhibitor C225 was a gift from Imclone Systems. TGF-β1 receptor inhibitor, ALK4, was purchased from Sigma-Aldrich (St. Louis, MO). Dodecylmaltoside was from Anatrace, Inc (Maumee, OH). All enzymes were from New England Biolabs, USA. All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO), unless stated otherwise.

Plasmid constructs. The cytoplasmic domain (C-EGFR) and cytoplasmic plus juxtamembrane domain (C+J-EGFR) and extracellular domain (N-EGFR) of the EGFR were tagged for ease of purification. His-tagged N-EGFR, 1D4-tagged C-EGFR, 1D4-tagged C+J EGFR plasmids were constructed as follows. The genes were amplified by standard PCR techniques from the initial plasmid construct containing full-length human EGFR gene.

The construct corresponding to the amino acid residues 2-646 with N-terminal 7His-tag (HHHHHHH) of the human EGFR was amplified using the following primers: 5'-CGCCATGGCTCATCACCATCACCATCACCATGAGGAAAAGAAAGTTTGCCAAGG-3' as forward and 5'-CGAAGCTTCTACCTTCGCATGAAGAGGCCGAT-3' as reverse, respectively. The resulting amino acid sequence of the construct was (MHHHHHHHEEKKV…GLFMRR).

The following primers were designed for the construct corresponding to the amino acid residues 689-1186 of the human EGFR carrying C-terminal 1D4-tag (ETSQVAPA): 5'-CGCCATGGCTAAAAAGATCAAAGTGCTGGGCTC-3' as forward and 5'-CGAAGCTTCTATGCAGGTGCCACCTGAGAGGTTTCTGCTCCAATAAATTCACTGCTTTG-3' as reverse, respectively. The resulting amino acid sequence of the construct was MAKKIKV…EFIGAETSQVAPA.

For the construct corresponding to the amino acid residues 644-1186 of the human EGFR with C-terminal 1D4-tag (ETSQVAPA) 5'-CCATGGCTCGAAGGCGCCACATCGTTCGG-3' was used as forward primer, and the reverse primer was as above. The resulting amino acid sequence of the construct was MRRRH…EFIGAETSQVAPA.

Purified PCR products were ligated into pCR Blunt (Invitrogen) and subsequently sequenced. The clones with confirmed sequences and suitable insert orientation were then excised by NcoI, blunted and subsequently cut by NotI. The obtained DNA fragment was ligated into pMT vector based on the pMT4 vector [1] with the rhodopsin gene cut out by EcoRI and NotI. The EcoRI site was blunted to complete the ligation with the corresponding EGFR fragments.

The Green Fluorescent Protein (GFP)-tagged dynamin 2 (Dyn2) construct [2] was kindly provided by Mark A. McNiven, Mayo Clinic.

Baculovirus. Histidine-tagged Hck virus stock [3] was kindly provided by Tom Smithgall, University of Pittsburgh.

Purified Proteins. Pure TGF-β1 was purchased from Biosource (Carlsbad, CA), PHG9204. EGF was purchased from CalBioChem (La Jolla, CA). GST-EGFR was purchased from Cell Signal (Danvers, MA).

Antibodies. Anti-GFP Mouse IgG2b Clone 1 E4 was from MBL, Goat Anti-Mouse IgG (H + L)-HRP Conjugate was from Biorad, bovine rhodopsin C-terminal antibody 1D4 was from the University of British Columbia, Anti-His-HRP was from Qiagen. Anti-EGFR antibody SC-03 was from Santa Cruz Biotechnology, Santa Cruz, CA. Anti-TGF-β1 antibody MAB2401was from R & D Systems (Minneapolis, MN) and anti-GST was from Cell signaling Technology (Danver, MA). Anti- MAPK and Protein A-sepharose used for immunoprecipitation was from Sigma (St. Louis, MO). Anti-phospho-MAPK antibodies were from Cell Signaling (Beverly, MA).

Cells. PCI-37A cell line is part of a large collection of squamous cell carcinoma of the head and neck (SCCHN) cell lines in the Department of Otolaryngology at the University of Pittsburgh. Hep3B human hepatoma cell were from ATCC, Manassas, VA.

For all manipulations with DNA *E.coli* strain Top10 (Novagen) was used.

2. Transfection

Plasmid DNA was transfected into COS-1 cells by the DEAE-dextran method. COS-1 cells are grown in controlled environment with 5% CO_2 and 37^{o}C. All buffers and media described here were equilibrated to 37^{o}C. Buffers used were: PBS – phosphate buffered saline: 137mM NaCl, 2.7mM KCl, 1.8mM KH_2PO_4, 10mM Na_2HPO_4 pH 7.2. DMEM – Dulbecco's Modified Eagle Medium was from Gibco BRL. Buffer P: DMEM containing penicillin / streptomycin (Gibco BRL, 100x). DNA-Mix: Approximately 15μg of DNA was mixed into 15ml of solution containing 0.25 mg/ml DEAE-dextran (Pharmacia) and 0.1 M Tris-HCl pH 8.0 in Buffer P. Shock Buffer: PBS with 10% DMSO and 6mM Dextrose. Complete Media: DMEM with 10% Bovine serum, 1x penicillin/streptomycin. Chloroquine Buffer: Complete media containing 0.1mM chloroquine.

For transfection 2-3 days old confluent COS-1 cell culture dishes were used. The cells were washed twice gently with 25 ml DMEM. The cells were then incubated with 15ml of DNA-Mix for 6 hours. The DNA-Mix was removed and replaced with 2 ml of shock buffer and incubated exactly for 2 minutes. The shock buffer was removed and replaced with Chloroquine Buffer. The cells were then incubated for 2 hours. After this the cells were washed with DMEM twice and replaced with 25ml of complete media. The cells were then incubated for ~55 hours and then harvested. To collect the cells, they were washed with ice cold PBS twice and were scraped using a rubber police man into 2ml of ice cold PBS with 0.7mM PMSF and 0.005% Benzamidine. The harvested cells were either immediately solubilized or were snap frozen in liquid nitrogen and stored at -20 C for later use.

3. Protein gels and western blots

Harvested cell samples (5µl) were mixed with SDS-PAGE buffer (5µl) and separated by electrophoresis (200V for 1-1.5 hrs) on 7.5% SDS-PAGE gels. Proteins were transferred from the gels to polyvinylidene difluoride (PVDF, Biorad) membrane using a transfer apparatus, for 1 hour at 100V. Membranes were then blocked in blocking buffer: PBS with 0.1% Tween 20 and 5% dry milk powder at room temperature for 30-60min or at 4^{o}C over night. The blots were then incubated for 1 hr at room temperature in primary antibody diluted 1:25000 in PBS+0.1% Tween. The blots are then blots washed thrice for 15 minutes in wash buffer (PBS with 0.1% Tween 20). Later, the blots were incubated in Horseradish Peroxidase conjugated goat anti-mouse secondary antibodies (Biorad) diluted 1:50000 in wash buffer for 30 minutes at room temperature. The blots were washed three times for 15 minutes in wash buffer. The blots were then incubated with 6ml of West Pico Chemiluminescent Substrate (Pierce). The blots were then exposed to film in a dark room and were developed.

4. In-vivo interaction studies of EGFR-TGF-β1

PCI-37A cells were treated with 10ng/ml EGF and 5pM TGFβ1. The western blot of the cell lysate was probed with anti-MAPK antibody and anti-phospho-MAPK to detect the expression levels of MAPK and phospho-MAPK. The same cell line was preincubated with 6ug/ml C225, an EGFR inhibitor binding to its extracellular domain, and in a separate experiment with 5uM of TGFB1 receptor inhibitor or ALK4 inhibitor, for 2hrs before treatment with EGF and TGFb1. The western blot of this cell lysate was detected for Erk and phospho-Erk expression.

5. In-vitro interaction studies of EGFR-TGF-β1

20 ng of GST-EGFR was incubated with 20 ng, 100 ng, 500 ng, or 1 µg of TGF-β1 protein in 100 ml of PBS at 37 ^{o}C for 1 hr. GST-EGFR was immunoprecipitated with anti-GST antibody and protein A-sepharose. The co-immunoprecipitated TGF-β1 was detected by Western blots, which were probed with anti-TGF-β1 antibody.

6. Expression and purification of EGFR domains

The cytoplasmic domain (C-EGFR) and cytoplasmic plus juxtamembrane domain (C+J-EGFR) and extracellular domain (N-EGFR) of the EGFR were expressed in COS-1 cells as described above. Transfected cells were harvested at 48h, 55 h and 72 h post transfection to identify the time needed for optimal expression levels. For N-EGFR construct the optimal time of harvest was about 55 h after transfection (Fig. S3.4a). In the case of C-EGFR and C+J-EGFR the optimal time for harvesting was about 72 h (Fig. S3.4b).

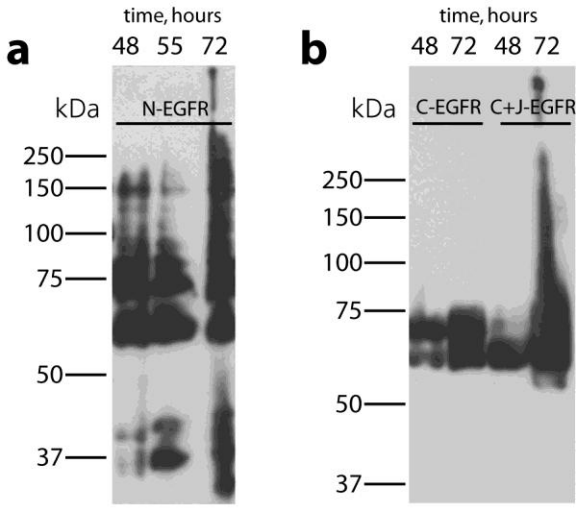

Figure S3.1 Expression of EGFR constructs. Western blots for detecting expression of N-EGFR (panel a), C-EGFR and C+J-EGFR (panel b).

7. Expression of GFP tagged dynamin-2

The dynamin-2 construct was expressed in COS-1 cells and the cells were harvested 55hrs post transfection. The GFP tagged dynamin-2 expression was confirmed by fluorescence imaging and western blots (Figure S3.2). GFP-Dyn was detected on the western blot as a band with an apparent molecular weight of 120Kda.

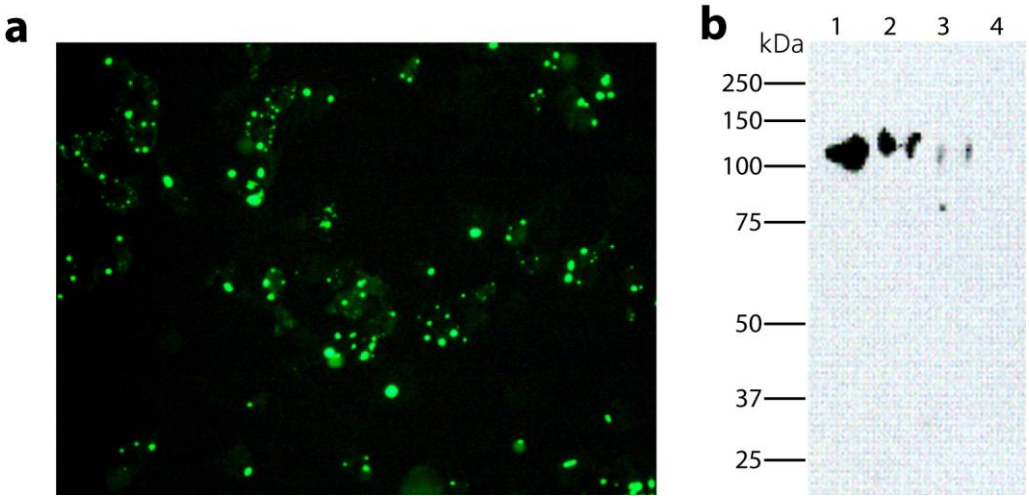

Figure S3.2 Expression of dynamin-2. (a). Fluorescence imaging. (b). Western blots. For detecting expression of GFP-tagged dynamin-2 using both techniques anti-GFP antibody was used. Lanes in panel b are identified as follows. Lanes 1 and 2: GFP-Dynamin-2, lane 3 CFP-GRIF-YFP and lane 4 YFP-Kv2.1. Lanes 3 and 4 are cyan fluorescent and yellow fluorescent proteins conjugated to GRIF and Kv.1 as controls for anti-GFP cross reactivity.

8. siRNA Downregulation of Hck

To investigate the putative role of Hck for cancer progression, we conducted cell proliferation and cell invasion assays in the presence and absence of Hck siRNA. UM-22A cells were transiently transfected with different concentrations of Hck siRNA for 72 hours. Lysates were collected and resolved by SDS-PAGE and probed for Hck and c-Src expression using specific antibodies. The effective down-regulation of Hck is shown in Figure S3.3.

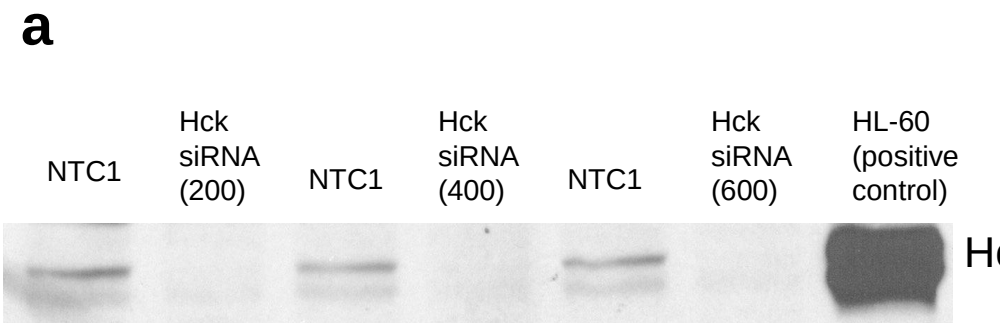

Figure S3.3. Hck siRNA specifically downmodulates Hck expression. HNSCC cells were transfected with non-targeting control siRNA (NTC 1) or different concentrations of Hck siRNA (200pmol, 400pmol and 600 pmol) for 72 hours. Lysates were resolved by SDS-PAGE for Hck expression. HL-60 was used as a positive control for Hck expression. Lysates were probed with c-src to determine specificity of Hck siRNA.

Bibliography

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