Figure 5. Tumor xenograft and biopsy specimens. U87 cells transfected with control empty vector (Vo) or heparanase plasmid (Hepa) were inoculated subcutaneously ($5x10^6$/ 0.1ml) and tumor volume was measured twice a week starting on day 21 (**A**). At the end of the experiment on day 35, tumors were resected, weighted (A, inset) and fixed in formalin. Paraffin-embedded 5 micron sections were stained with anti-phospho-EGFR (Tyr1173, **B**, lower panels), and anti-phospho-Src (Tyr416, **B**, upper panels) antibodies. **C**. Immunohistochemical staining of phospho-EGFR in human head and neck tumor specimens. Formalin-fixed, paraffin-embedded 5 micron sections of head and neck tumors were subjected to immunostaining of phosphorylated EGFR, applying anti-phospho-EGFR (Tyr1173) antibody, as described under 'Materials and Methods'. Shown are representative photomicrographs of phospho-EGFR negative (upper panel), and positively stained specimens scored as weak (+1; second panel) and strong (+2; third panel) intensity.

Supplementary figure legends

Suppl. Figure 1. Heparanase stimulates EGFR phosphorylation: densitometry analysis. **A**. Control (Vo) and heparanase transfected (Hepa) LNCaP cell lysates were subjected to immunoprecipitation (IP) with anti-phospho-tyrosine (PY) antibody, followed by immunoblotting with anti-EGFR antibody (upper panel), or the reciprocal experiment (IP: EGFR, blot: PY; middle panel), or immunoblotting with anti-phospho-EGFR (Tyr1173; lower panel). Shown are bar graphs of EGFR phosphorylation index calculated by densitometry analysis (Vo cells arbitrary set to a value of 1) of at least 5 independent experiments (average±SE). **B**. Shown is densitometry analysis (fold increase) of phospho-EGFR (Tyr1173; upper panel) and phospho-Akt (lower panel) levels in U87 cells following exogenous addition of wild type (left) and mutated (DM, right) heparanase proteins.

Suppl. Figure 2. **A**, **B**. Src inhibitors. **A**. U87 (upper panels) and A431 (lower panels) cells over expressing heparanase were incubated with the indicated kinase inhibitor (30 min, 37^{o}C) and cell lysates were subjected to immunoblotting with anti-phospho-EGFR (Tyr1173; upper and third panels) or anti-EGFR (second and fourth panels). **B**. A431 cells were incubated with the indicated PP2 concentration (μM) and total cell lysates were subjected to immunoblotting with anti-phospho-EGFR (Tyr1173; upper

panel), anti-EGFR (second panel), anti-phospho-Src (Tyr416, p-Src, third panel), phospho-Akt (p-Akt, fourth panel) and anti-Akt (lower panel) antibodies. **C-E**. Src regulation by heparanase. **C**. heparanase over expression. Control (Vo) and heparanase transfected (Hepa) A431 (left panels) and LNCaP (right panels) cell lysates were subjected to immunoblotting with anti-phospho-EGFR (Tyr1173; upper panels), anti-phospho-Src (Tyr416; middle panels), and anti-EGFR (lower panels) antibodies. Note enhanced Src phosphorylation following heparanase over expression**.** **D.** Densitometry analysis of phospho-EGFR (Tyr1173, upper panels) and phospho-Src (Tyr416; lower panels) of control Vo, and heparanase transfected (Hepa) A431 (left), U87 (middle), and LNCaP (right) cells. Data is presented as fold increase of EGFR phosphorylation compared with control, Vo cells, set arbitrary to a value of 1. **E**. Shown is densitometry analysis (±SE) of phospho-Src (Tyr416) levels following transfection of LNCaP cells with anti-GFP (si-GFP) or anti-heparanase (si-Hepa) siRNA oligonucleotides. Note decreased phospho-Src levels following heparanase gene silencing. **F**. p120 catenin phosphorylation. LNCaP cells were transfected with control vector (Vo) or heparanase plasmid. Cell lysates were subjected to IP with anti-phosphotyrosine antibody (PY), followed by immunoblotting with anti-$p120^{cat}$ antibody (arrow). Note that $p120^{cat}$ phosphorylation levels are enhanced by heparanase over expression.

Supp. Figure 3. Shown are densitometry analyses (average fold increase±SE) of phospho-EGFR (Tyr1173, upper panels) and phospho-Src (Tyr416; lower panels) levels following PP2 treatment of heparanase transfected LNCaP cells (left panels), or following addition of wild type (WT; middle panels) or double mutated (DM, right panels) heparanase together with DMSO or 5μM PP2.

Suppl. Figure 4. **A**. Kinetics study. MDA-MB-231 cells were left untreated (0) or stimulated with heparanase (1 μg/ml) for the time indicated. Cell lysates were subjected to immunoblotting with anti-phospho-EGFR (Tyr1173; upper panel), anti-phospho-EGFR (Tyr845, second panel), and anti-EGFR (lower panel) antibodies. Densitometry analyses (average fold increase±SE) of phospho-EGFR (Tyr1173; upper panel) and phospho-EGFR (Tyr845; lower panel) are shown in the second and third panels, respectively. EGFR phosphorylation levels in untreated cells (0) were set arbitrarily to 1. **B**. Densitometry analyses of phospho-EGFR (Tyr1173; upper panel),

phospho-EGFR (Tyr845; middle panel), and phospho-Src (Tyr416; third panel) (average fold increase±SE) of control (Vo) and heparanase (Hepa) transfected LNCaP cells untreated (0) or treated with the indicated concentration (μM) of the Src inhibitor PP2.

Suppl. Figure 5. **A**. Heparanase modulates cell proliferation. Heparanase transfected LNCaP cells were plated at $5x10^4$ cells per dish without (Hepa) or with Src inhibitor (Hepa+PP2; 2.5μM) and cell number was determined 3, 5, and 7 days following plating by a Coulter counter and hemacytometer. Mock transfected cells (Vo) incubated without or with the Src inhibitor (Vo+PP2; 2.5μM) were used as control. **B.** Colony formation in soft agar. Control (Vo) LNCaP cells ($5x10^3$ cells/dish) were mixed with soft agar and cultured for 3 weeks in the absence (Vo+DMSO) or presence of Src (Vo+PP2; 5μM) or EGFR (Vo+1478; 10μM) inhibitors. Note that treatment with the inhibitors does not affect colony formation by control, untransfected cell. **C**. Heparanase-stimulated cell migration is mediated by Src and EGFR. Control (Vo) and heparanase transfected U87 cells were plated on fibronectin-coated inserts (8 μm) and were allowed to migrate for 6 h without (Hepa, second panel) or with Src (Hepa+PP2, 2.5μM, third panel) and EGFR (Hepa+1478, 5μM; fourth panel) inhibitors. Cells migrating into the lower compartment were visualized by crystal violet staining (**C**) and quantified by counting of at least 8 random microscopic fields (**D**).

Supplementary "Materials and Methods"

Immunocytochemistry. Cells were grown on glass cover slips for 18 h and fixed with cold methanol for 10 min. Cells were then washed with PBS and subsequently incubated in PBS containing 10% normal goat serum for 1 h at room temperature, followed by 2 h incubation with the indicated primary antibody. Cells were then extensively washed with PBS and incubated with the relevant Cy2/Cy3-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA) for 1 h, washed and mounted (Vectashield, Vector, Burlingame, CA). Staining was observed under a fluorescent microscope and photographs were taken with a digital camera, both from Nikon. Cell migration on fibronectin-coated insert filters was carried out essentially as described (8).

Cell lysates, immunoprecipitation and protein blotting. Preparation of cell lysates, immunoprecipitation, and protein blotting was performed essentially as described (8, 11, 16, 17). Cell cultures were pretreated with 1 mM orththovanadate for 10 min at 37°C, washed twice with ice cold PBS containing 1 mM orththovanadate and scraped into lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton-X100, 1 mM orththovanadate, 1 mM PMSF) containing a cocktail of proteinase inhibitors (Roche). Total cellular protein concentration was determined by the BCA assay, according to the manufacturer's instructions (Pierce, Rockford, IL). Thirty μg of cellular protein were resolved on SDS polyacrylamid gel (SDS-PAGE) and immunoblotting was performed, as described (8, 11, 16, 17). Immunoprecipitation was carried out essentially as described (18). Briefly, 100 μg of cellular protein were brought to a volume of 1 ml in buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA and 0.5% NP-40, incubated with the appropriate antibody for 2 h on ice followed by incubation with protein A/G-Sepharose (30 min on ice). Beads were washed twice with the same buffer supplemented with 5% sucrose. Sample buffer was added and samples were boiled and subjected to gel electrophoresis and immunoblotting, as described above.

Immunostaining. Staining of formalin-fixed, paraffin-embedded 5 micron sections was performed essentially as described (20, 21). Briefly, slides were deparaffinized, rehydrated and endogenous peroxidase activity was quenched (30 min) by 3% hydrogen peroxide in methanol. Slides were then subjected to antigen retrieval by boiling (20 min) in 10 mM citrate buffer, pH 6. Slides were incubated with 10% normal goat serum (NGS) in PBS for 60 min to block non specific binding and incubated (20 h, 4^{o}C) with anti phospho-EGFR (Tyr1173) antibody diluted 1:50 in blocking solution. Slides were extensively washed with PBS containing 0.01% Triton X-100 and incubated with a secondary reagent (Envision kit) according to the manufacturer's (Dako, Glostrup, Denmark) instructions. Following additional washes, color was developed with the AEC reagent (Dako), sections were counterstained with hematoxylin and mounted, as described (20, 21). Immunostained specimens were examined by senior pathologist (IN) who was blind to clinical data of the patients, and were scored according to the intensity (0: none, 1: weak- moderate; 2: strong) and extent (percent of tumor cells that were stained; 0: <10%; 1: 10-50%; 2>50%) of

staining, as described (21). Specimens that were similarly stained with pre-immune serum, or applying the above procedure but lacking the primary antibody, yielded no detectable staining.

Heparanase activity assay. Preparation of ECM-coated 35 mm dishes and determination of heparanase activity were performed as described in detail elsewhere (22, 23). To evaluate heparanase activity in cell extracts, control Vo and heparanase-transfected cells ($1x10^6$) expressing the wild type (WT) or deletion constructs of the enzyme, were lysed by three freeze/thaw cycles and the resulting cell extracts were incubated (18 h, 37^0C, pH 6.0) with ^{35}S-labeled ECM. The incubation medium (1 ml) containing sulfate labeled degradation fragments was subjected to gel filtration on a Sepharose CL-6B column. Fractions (0.2 ml) were eluted with PBS and their radioactivity counted in a β-scintillation counter. Degradation fragments of HS side chains generated by heparanase are eluted at $0.5< K_{av}<0.8$ (fractions 15-30).

Table 1. Enhanced EGFR phosphorylation correlates with cytoplasmic, rather than nuclear localization of heparanase.

p-EGFR Intensity	**Cytoplasmic n (%)**	**Nuclear n (%)**	**Total**
0	6 (27)	16 (73)	22
1	12 (41)	17 (59)	29
2	11 (69)	5 (31)	16
	29	38	67

P=0.03

Supplementary Table 1. Phopsho-EGFR (Tyr1173) staining intensity correlates with head and neck carcinoma T stage.

p-EGFR Intensity	**T0-2 n (%)**	**T3-4 n (%)**	Total
0	11 (50)	11 (50)	22
1	6 (21)	23 (79)	29
2	1 (6)	15 (94)	16
	18	49	67

P=0.0067

Supplementary Table 2. Phopsho-EGFR (Tyr1173) staining extent correlates with head and neck carcinoma T stage.

p-EGFR Extent	**T0-2 n (%)**	**T3-4 n (%)**	Total
0	11 (50)	11 (50)	22
1	4 (17)	19 (83)	23
2	3 (14)	19 (86)	22
	18	49	67

P=0.01

Supplementary Table 3. Multi variant logistic regression for patient status at the end of the study (dead or alive), including phospho EGFR levels and heparanase localization [nuclear (N) vs. cytoplasmic (Cy)].

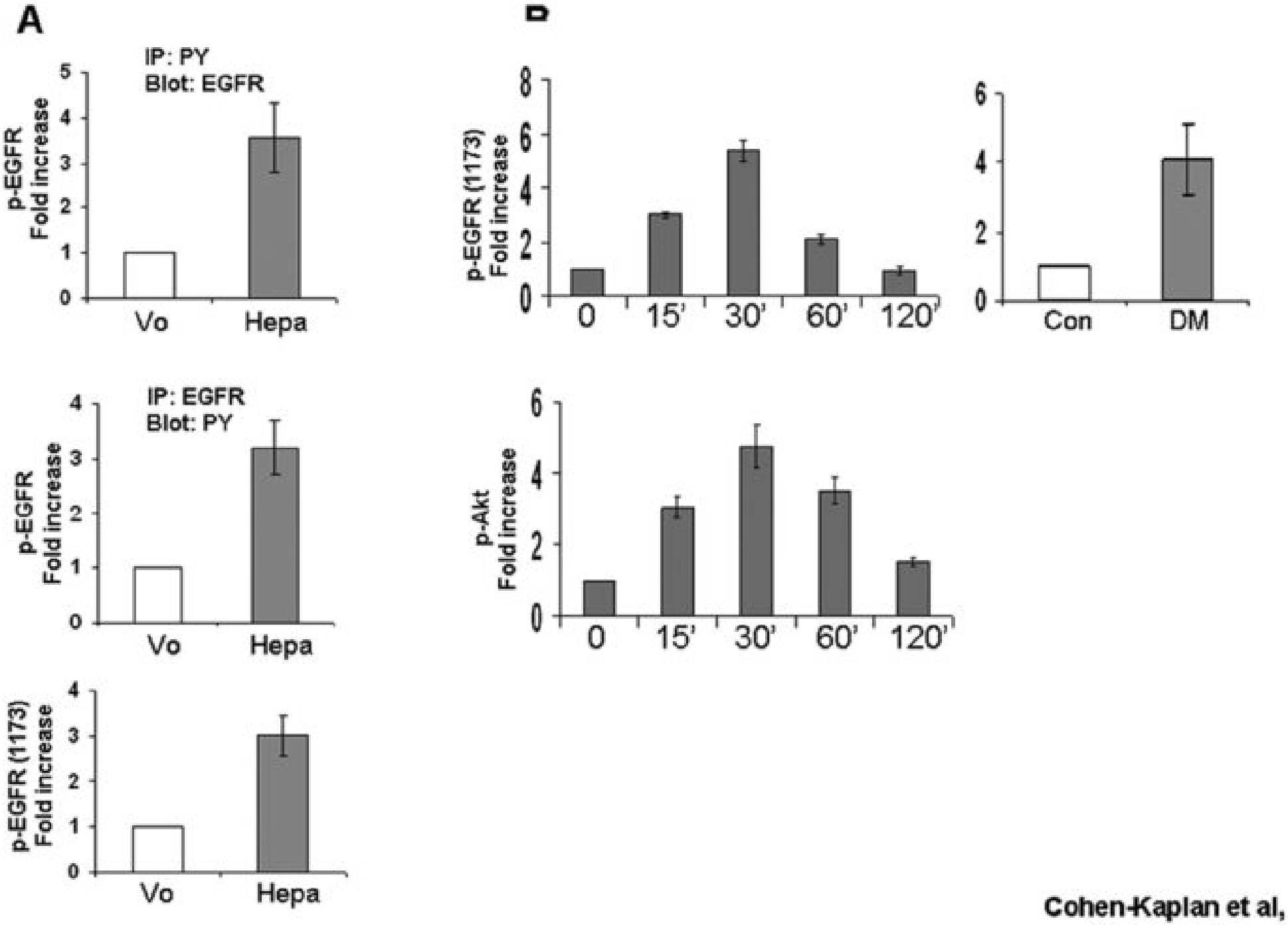
Parameter	OR	p
phospho-EGFR	1.42	0.2336
Heparanase localization (N/Cy)	8.33	0.0039

OR-odds ratio; N-nuclear; Cy-cytoplasmic

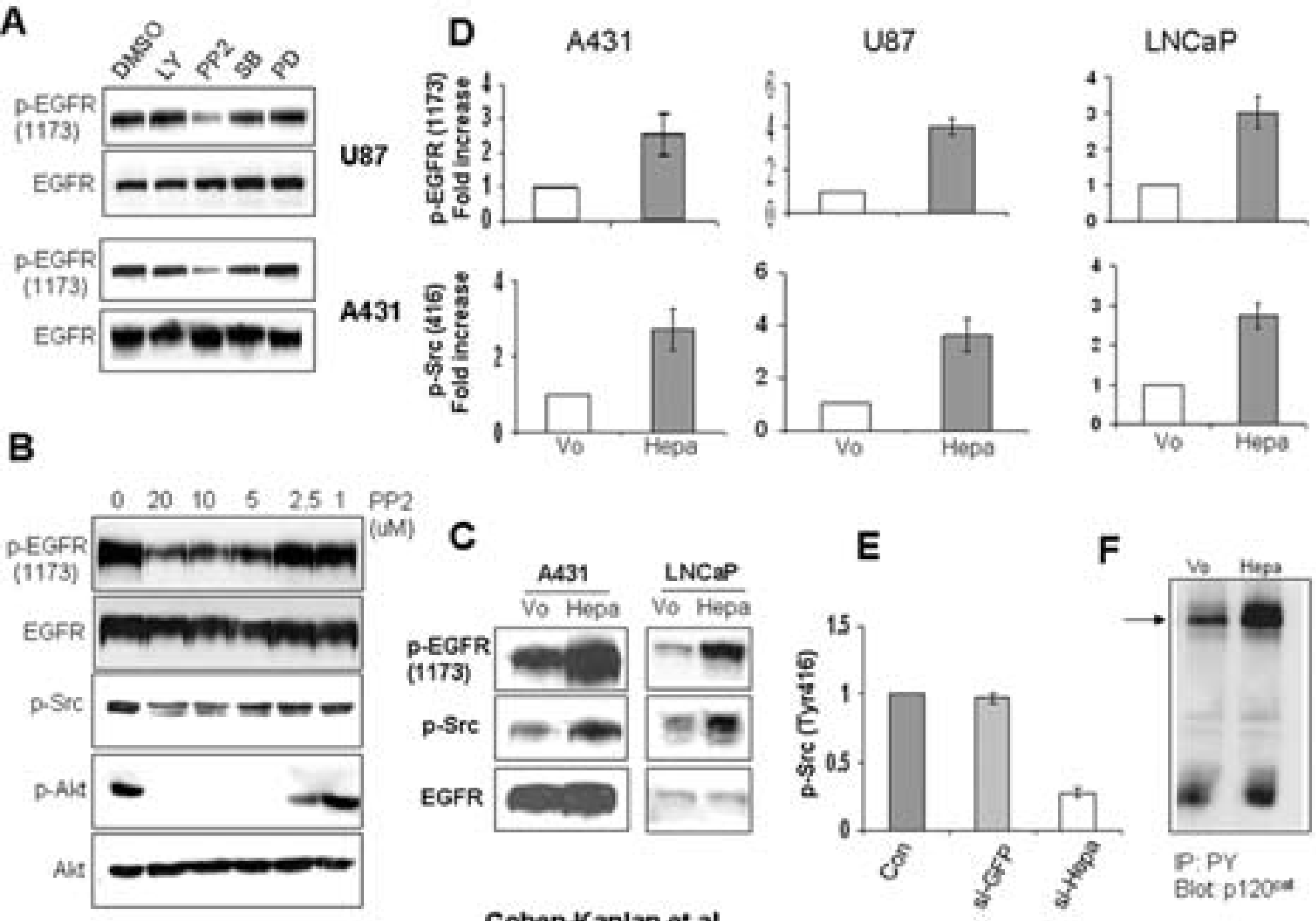
Supplementary Table 4. Multi variant logistic regression for patient status at the end of the study (dead or alive), including the T stage (T0-2 vs. T3-4), heparanase localization [nuclear (N) vs. cytoplasmic (Cy)], and phospho EGFR levels.

Parameter	OR	p
T Stage (0-2 vs. 3-4)	4.6478253	0.0311
Heparanase localization (N/Cy)	7.47164369	0.0063
p-EGFR (1173)	0.15602644	0.6928

OR-odds ratio; N-nuclear; Cy-cytoplasmic



Cohen-Kaplan et al,
Suppl. Fig. 1



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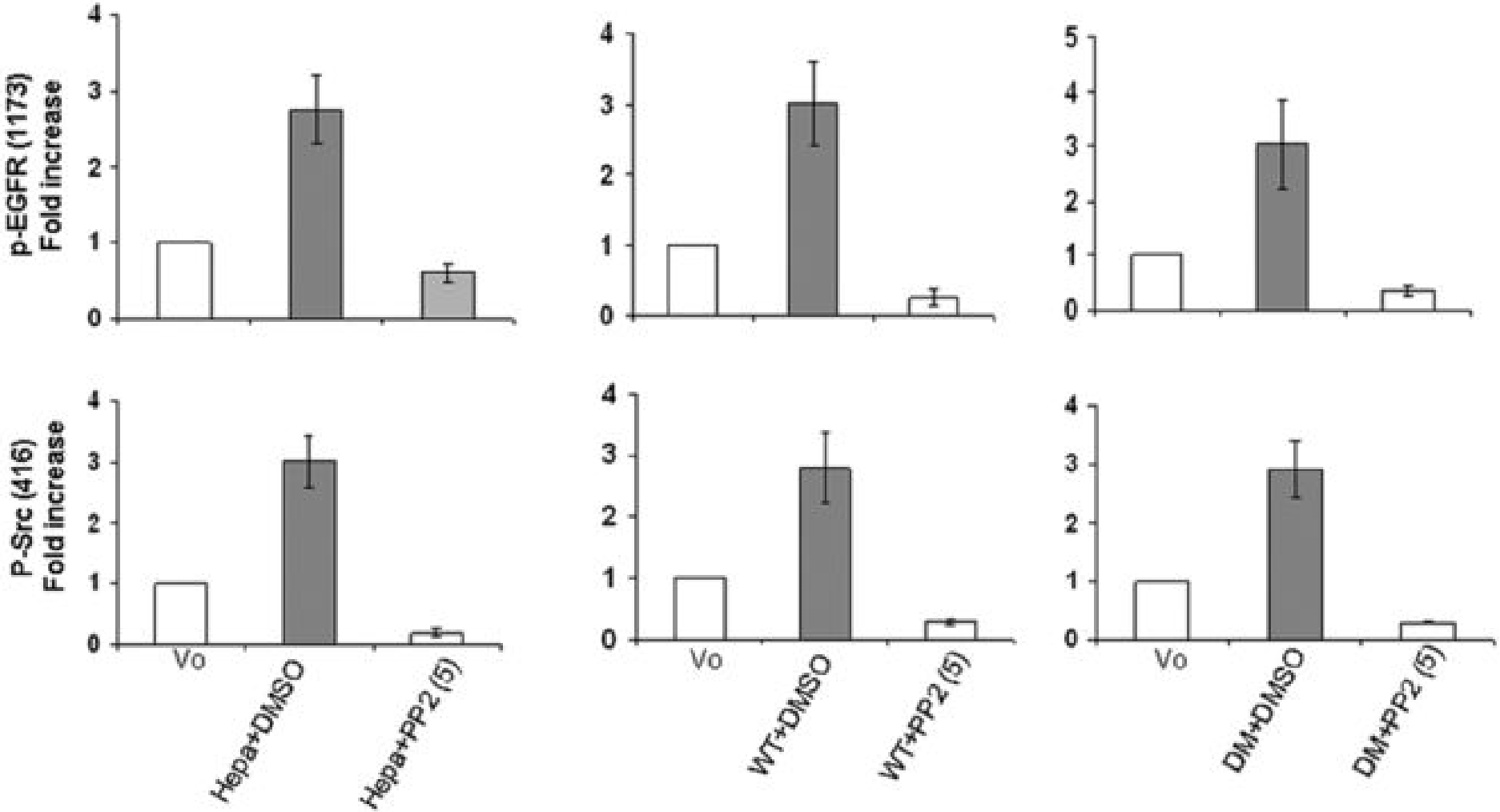
Suppl. Fig. 2



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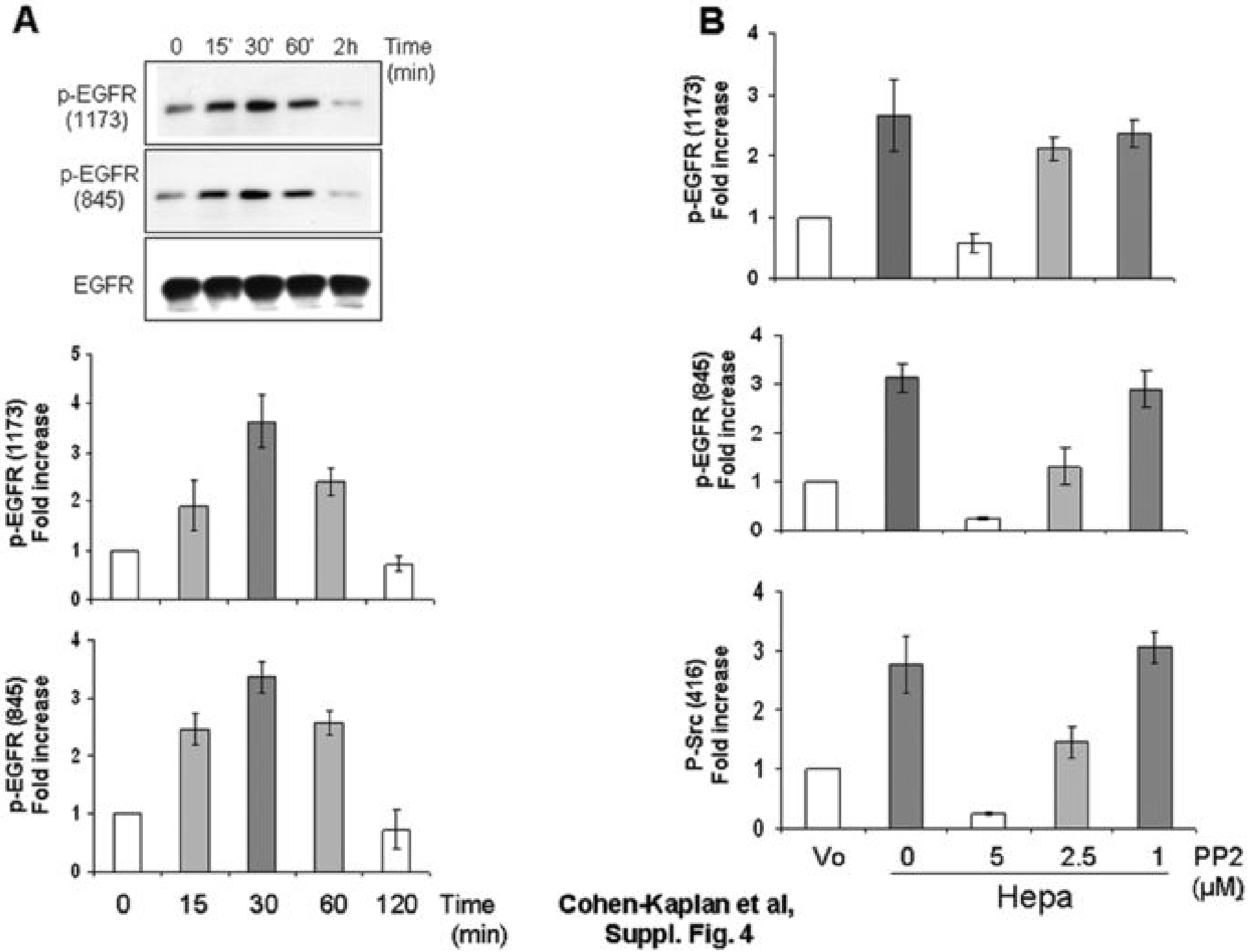

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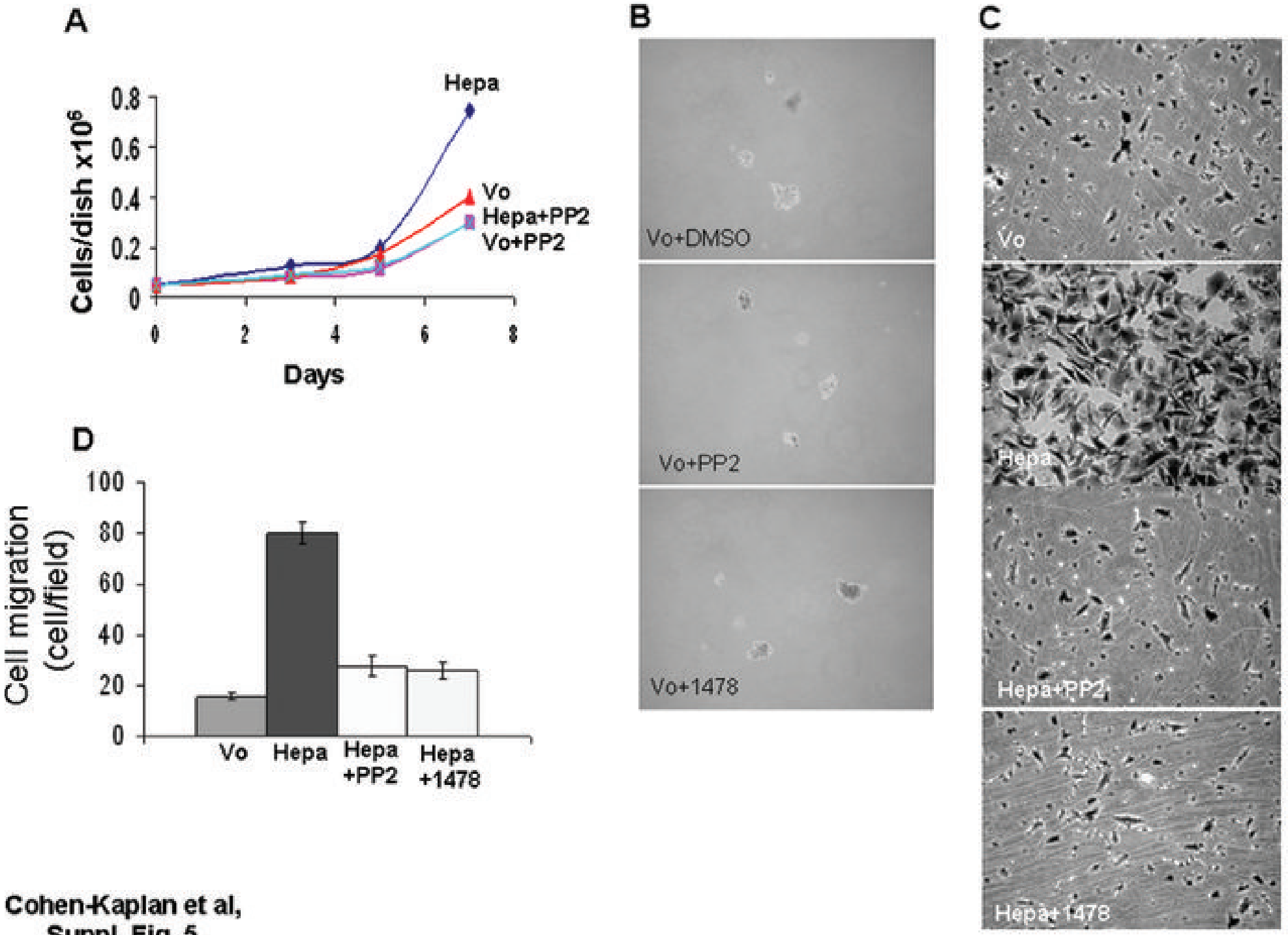

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