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Supplementary Materials for

The neuropilin 2 isoform NRP2b uniquely supports TGFβ-mediated progression in lung cancer

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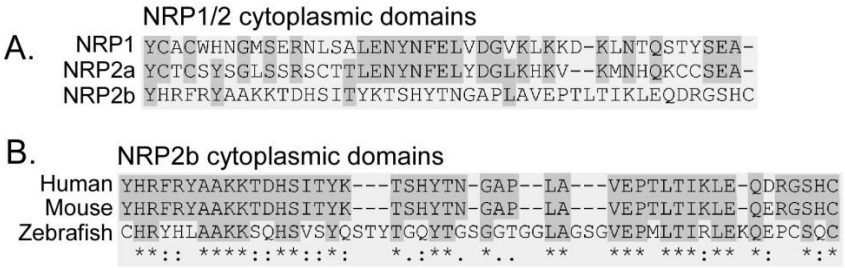

Fig. S1. Sequence alignments of NRP2b with NRP2a and NRP1. (**A**) Clustal W alignment of CYT sequences from NRP1, NRP2a and NRP2b, as indicated; identities are shaded. This region of NRP2a is 54% identical to NRP1 but only 9% identical to NRP2b. Among all three, only 1 amino acid is completely conserved. (**B**) Multiple sequence alignment of the NRP2b cytoplasmic domain from *H. sapiens, M. musculus* and *D. rario* (56% identical; 32% similar).

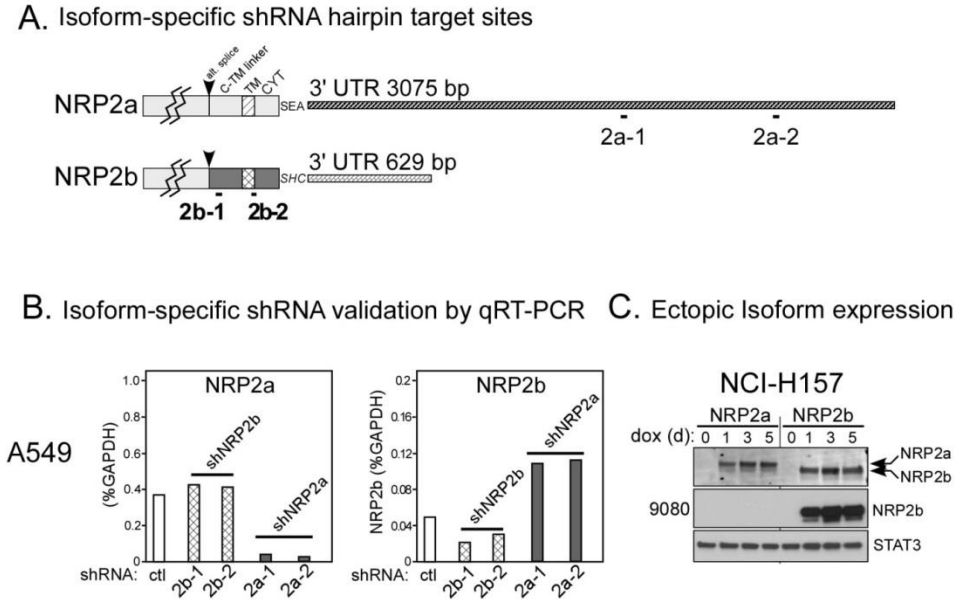

Fig. S2. Development and validation of NRP2 isoform-specific shRNAs. (**A**) Short hairpin sequences (table S3) targeting unique regions of NRP2a or NRP2b were designed using the BLOCK-It designer web site from Ambion (Life Technologies). Two independent NRP2a hairpins target sites within the 3'UTR while the sites recognizing NRP2b are present within the C-terminal alternative exon 16b. Double stranded oligonucleotides were annealed and cloned into AgeI/EcoRI cleaved pLKO.1 lentiviral vector. (**B**) Sequence verified clones were packaged and infected into A549 cells. RNA was isolated from puromycin-resistant cultures and analyzed by realtime RT-PCR using isoform-specific primers (table S3). The NRP2a-1 and NRP2a-2 shRNAs reduced the corresponding message by 87 and 92%, respectively, while NRP2b-1 and NRP2b-2 made reductions of NRP2b mRNA by 51 and 28%, respectively. We note that knockdown of NRP2a consistently increased NRP2b mRNA levels but not *vice versa*, consistent with additional levels of regulation. (**C**) Isoform re-expression in NCI-H157 FlpIn TRex cells. shRNA-resistant wild-type NRP2a and NRP2b were stably transfected into NCI-H157 cells genetically modified with the FlpIn TRex system to allow stable integration and doxycycline-inducible transgene expression. Following selection for hygromycin and blasticidin resistance, cells were treated with dox for the indicated times (days). Lysates were analyzed on Western blots with the indicated antibodies. The NRP2b-specific antiserum, 9080, detected only dox-induced NRP2b (see fig. S3). Data are representative of 3experiments.

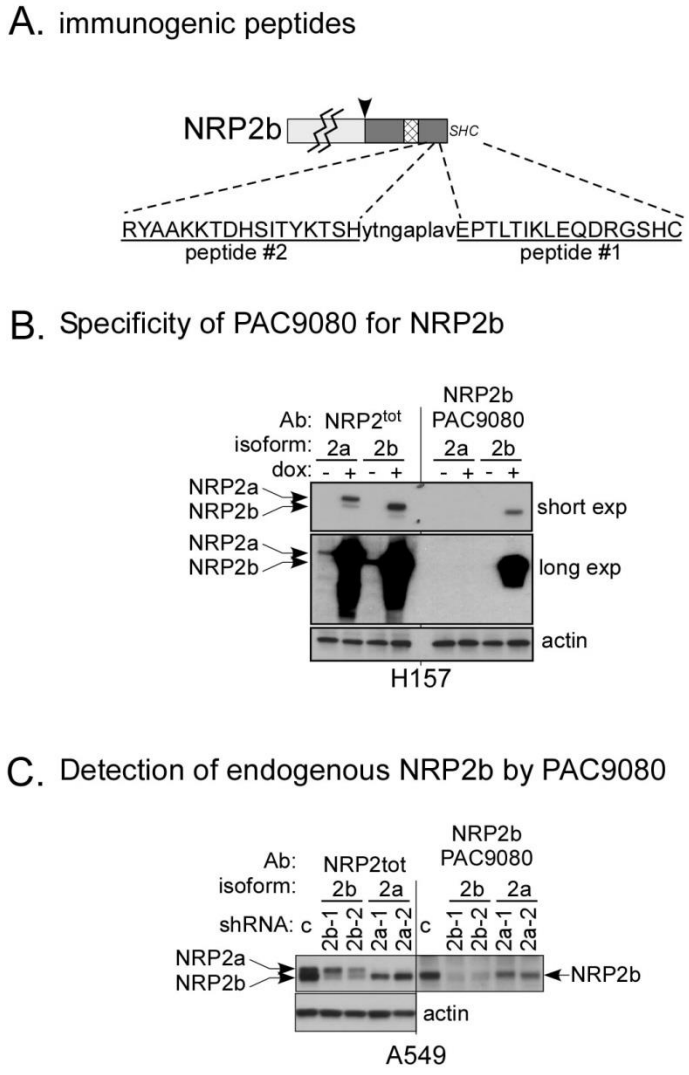

Fig. S3. Development and validation of NRP2b-specific antibody 9080. (**A**) Diagram of the NRP2b C-terminus with location and sequence of antigenic peptides used for immunization. Two rabbits were immunized with the peptide mixture. Animal 9080 produced antisera with high titre and specificity; NRP2b-reactive antibodies were purified via affinity chromatography. (**B**) Western blots prepared from NCI-H157 cells expressing dox-inducible NRP2a or NRP2b were probed with total NRP2 antibody (R&D Systems, AF2215) and with affinity purified NRP2b-9080 antibody. The NRP2b antibody reacted only with NRP2b with no detectable reactivity against NRP2a. Short exposures demonstrated that the sensitivity of 9080 is reasonably close to that of commercial AF2215 anti-NRP2 antibody. (**C**) Western blots of TGFβ-treated A549 cells bearing NRP2 isoform-specific knockdowns were probed with total NRP2 antibody and with NRP2b-9080 antibody to confirm detection of endogenous NRP2b. Blots in panels B and C are representative of 3 experiments.

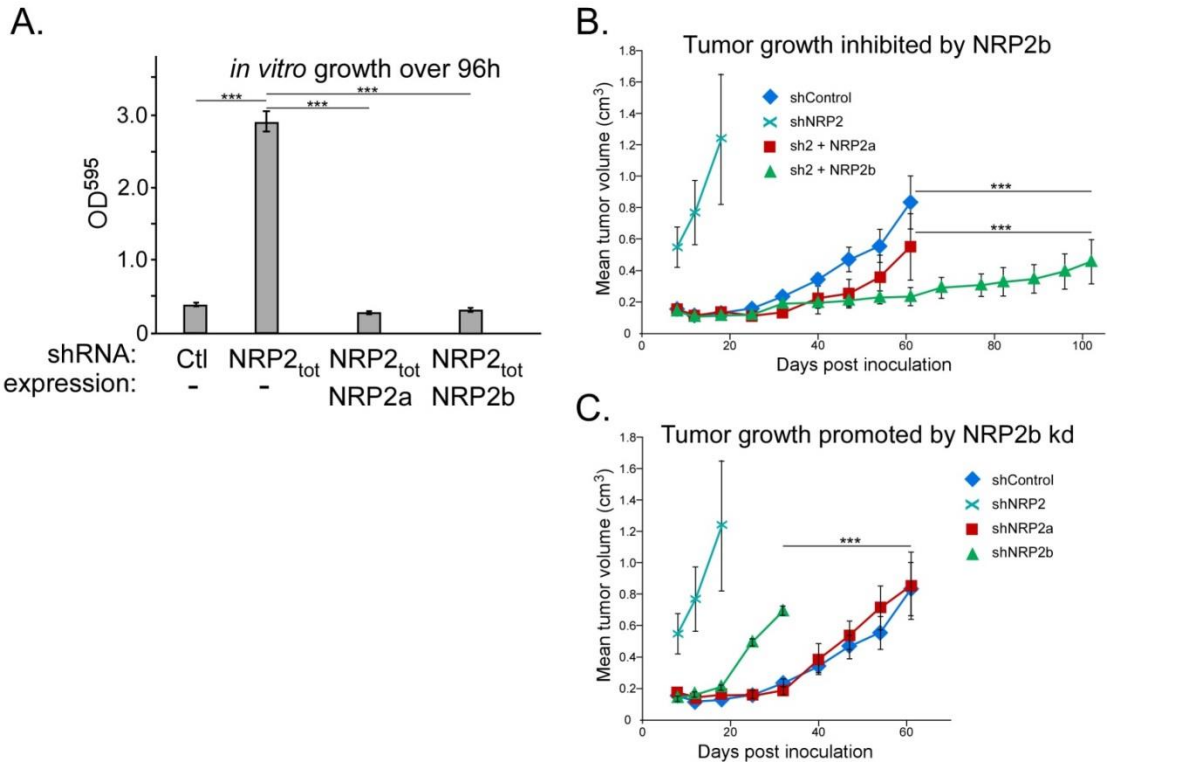

Fig. S4. NRP2b inhibits proliferation in vitro and in vivo. (**A**) H358 cells knocked down for total NRP2 and re-expressing either NRP2a or NRP2b were plated and allowed to grow for 96 hours. Crystal violet staining was used to determine relative growth after solubilization and spectrophotometric detection. Knockdown of NRP2 substantially increased growth over shControl cells while re-expression of either NRP2a or NRP2b suppressed growth below that of controls. N = 3 experiments; linear combinations of ANOVA parameters analyzed on a log scale. (**B**) Subcutaneous tumor growth of H358-shNRP2 knockdown cells re-expressing either NRP2a or NRP2b. NRP2a expressing tumors were not significantly different from shControls while NRP2b expressing tumors grew significantly more slowly. Data are means ± S.E.M. from 10 animals. Tumor growth over time was modeled using mixed effects linear regression on the log scale. Slopes (growth rates) for each group were estimated and compared using linear combinations of the resulting model coefficients (Wald test). (**C**) Subcutaneous tumor growth of H358 cells knocked down for either NRP2a or NRP2b, the reciprocal to the experiment shown in (B). shControl and shNRP2a cells were not significantly different. However, shNRP2b tumors grew significantly faster. Data were analyzed as in (B).

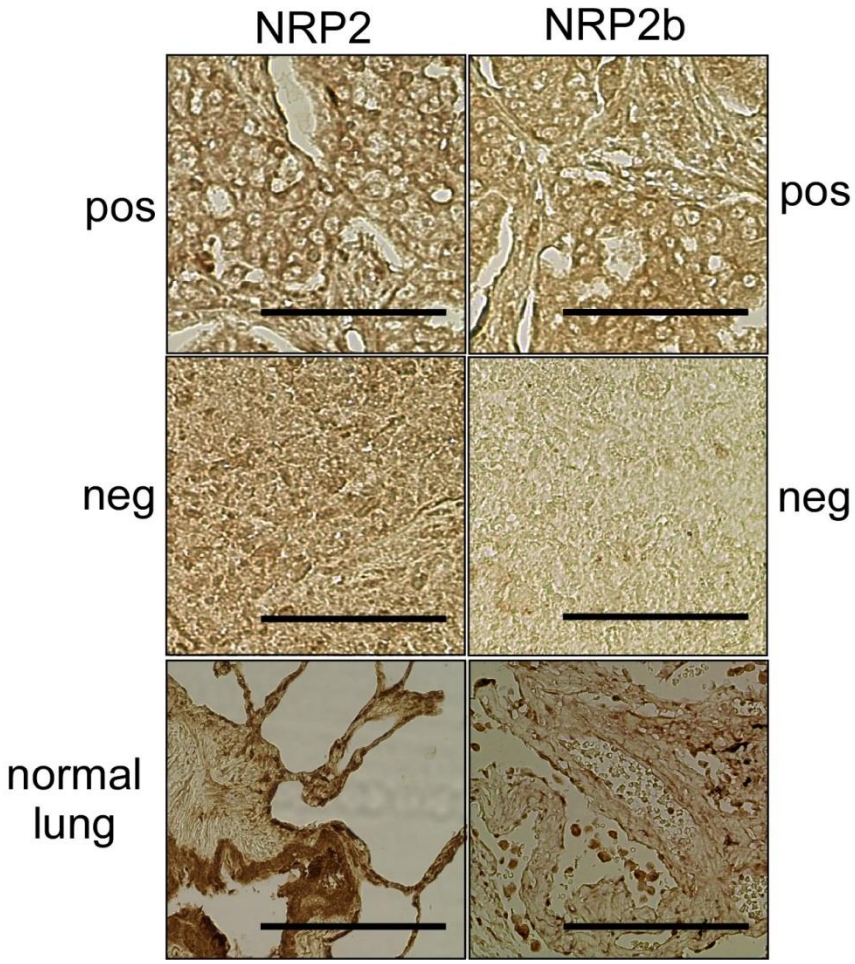

Fig. S5. Immunohistochemical staining of human lung tumors with NRP2 antibodies. Examples of membrane-specific staining for total NRP2 and NRP2b in lung tumors from TMAs. Because both antibodies show some background staining, only positivity at the membrane was scored in these analyses. The bottom two panels show uninvolved lung staining positive for NRP2 but negative for NRP2b. Scale bar, 200 μm.

Table S1. Patient characteristics and histology of the 436-case TMA. Tumor and patient characteristics among the 436-case tissue microarray.

	#	%
TOTAL CASES:	**436**	**100**
Median age, (range)	**66 (32-85)**	
M/F	**355/81**	**81/19**
Smoking history		
Never smoker	**42**	**10**
Former smoker	**231**	**53**
Current Smoker	**163**	**37**
Histology		
Adenocarcinoma	**231**	**53**
Squamous cell carcinoma	**148**	**34**
Large cell	**5**	**1**
Neuroendocrine	**23**	**5**
Undifferentiated	**9**	**2**
Adenosquamous	**20**	**5**
Stage		
I	**160**	**37**
II	**98**	**22**
III	**144**	**33**
IV	**34**	**8**

Table S2. Patient characteristics and histology of the 110-case BioMax Inc. TMA. Tumor and patient characteristics among 110-case tissue microarray sourced from BioMax, Inc.

	#	%
TOTAL CASES:	**110**	**100**
Median age, (range)	**57 (32-76)**	
M/F	**74/36**	**67/33**
Histology		
Adenocarcinoma	**52**	**47**
Squamous cell carcinoma	**41**	**37**
Large cell	**4**	**4**
Neuroendocrine	**5**	**5**
Undifferentiated	**6**	**5**
Adenosquamous	**2**	**2**
Stage		
I	**48**	**44**
II	**26**	**24**
III	**29**	**26**
IV	**1**	**1**

Table S3. Secretome analysis of TGFβ-exposed H358 cells. H358-TGFβ cells were induced to express the cytokine for 24 hours with doxycycline. Secreted proteins in serum-free medium were concentrated and analyzed on reverse-phase antibody arrays, according to the manufacturer's directions (RayBiotech, Inc.). Values represent the fold-change comparing H358 control cells to H358-TGFβ cells.

TGFβ-induced secretion

Rank	factor	fold change
1	IGFBP-1	5.12
2	**HGF**	**4.29**
3	TGF-b	4.14
4	bNGF	3.76
5	EGF	3.52
6	GDNF	3.42
7	HB-EGF	3.06
8	IGFBP-4	2.84
9	G-CSF	2.48
10	IGF-I	2.37
11	NT-4	2.29
12	TGF-a	2.12
13	FGF-4	2.11
14	VEGF D	1.85
15	SCF	1.80
16	IGF-II	1.74
17	PlGF	1.73
18	PDGF-AA	1.71
19	M-CSF	1.48
20	NT-3	1.42
21	TGF-b2	1.33
22	VEGF	1.25
23	IGFBP-2	1.19
24	GM-CSF	1.08
25	IGFBP-3	1.02
26	IGFBP-6	0.99
27	TGF-b3	0.98
28	PDGF-AB	0.29
29	PDGF-BB	0.21
	bFGF*	Pos
	FGF-6*	Pos
	FGF-7*	Pos

*not detected in control cells;
fold-change cannot be determined

Table S4. Oligonucleotide sequences used in this study. Oligonucleotide sequences to generate isoform-specific shRNA hairpin clones for RT-PCR, NRP2b cloning, and in vitro mutagenesis, including: 1) synonymous mutations in the shNRP2-1 target site to render clones resistant to this shRNA; 2) premature STOP codons positioned to delete the C-terminal cytoplasmic domains of both NRP2a and NRP2b; 3) creation of a NRP2b/a chimera (NRP2b+SEA) by conversion of the last 6 amino acids of NRP2b into the last 6 amino acids of NRP2a, thus creating an artificial GIPC1 binding site at the C-terminus of NRP2b; 4) insertion of an N-terminal FlagHA tandem affinity purification tag just 3' to the signal sequence.

Designation	Isoform-specific shRNA hairpins (top sequence only)
shNRP2a-1	CCGGGGAGGCATCAGGAATAGAATGCTCGAGCATTCTATTCCTGATGCCTCCTTTTTG
shNRP2a-2	CCGGGCACTTCACCTCTAAGTTATGCTCGAGCATAACTTAGAGGTGAAGTGCTTTTTG
shNRP2b-1	CCGGGCCTACTGGTATTACGTAATGCTCGAGCATTACGTAATACCAGTAGGCTTTTTG
shNRP2b-2	CCGGGGAGCCCACCCTAACCATTAACTCGAGTTAATGGTTAGGGTGGGCTCCTTTTTG
	Isoform-specific amplification primers
NRP2tot-648For	GGATGGCATTCCACATGTTG
NRP2tot-800Rev	ACCAGGTAGTAACGCGCAGAG
NRP2a 2590For	AAAAGAAGCAGGGTACGCAGG
NRP2a 2697Rev	CTGGCCAAGTCAAAATTGGG
NRP2b 46For	AACATGTTGCCTCGATTTTGC
NRP2b 168Rev	ACCCAAGGAGGATGGTTGG
NRP2-F-For	GAGAGCAAGTTGCTGTGGGT
NRP2a-G-Rev	AATTGCTCCAGTCCACCTCG
NRP2b-356Rev	CAGCGCGACGGAGACC
	Oligonucleotides for NRP2b cloning
Nrp2ab-2567 For	TGACTGGACAGACTCCAAGCC
NotI-Nrp2b-3512 Rev	GACTCGAGCGGCCGCTCAGCAGTGCGAGCCACG
	Mutagenic oligonucleotides
shNRP2-1 resist For	TTCCGGAGAGATTGCCATTGACGATATTAGGATAAGCACTGATGTCCC
NRP2a-delCYT For	TACTGCACCTGTTCCTAATCGGGCCTGAGC
NRP2b-delCYT For	CCACTACCACCGGTAACGCTATGCGGCCAAG
NRP2b-6SEA For	CTAACCATTAAGCTAGAGCAAAAATGTTGCTCGGAGGCCTGAGCGGCCGCTCGAGTCTAG
FlagHA-NRP2 For	GTGAGAGGCCAAGACTACAAAGACGATGACGACAAGTACCCATACGATGTTCCAGATTACGCTCCAGACCCACC