

Supplemental Table 1: Summary of crystallographic analysis

Data collection	FGFR3-IIIb: R3Mab Fab
Space group	P2 ₁ 2 ₁ 2 ₁
Cell parameters	a=58.5, b=99.3, c=143.7
Resolution (Å)	25-2.1
R _{sym} ^a	0.098 (0.663) ^b
Number of observations	288498
Unique reflections	49851
Completeness (%)	99.9 (100.0) ^b

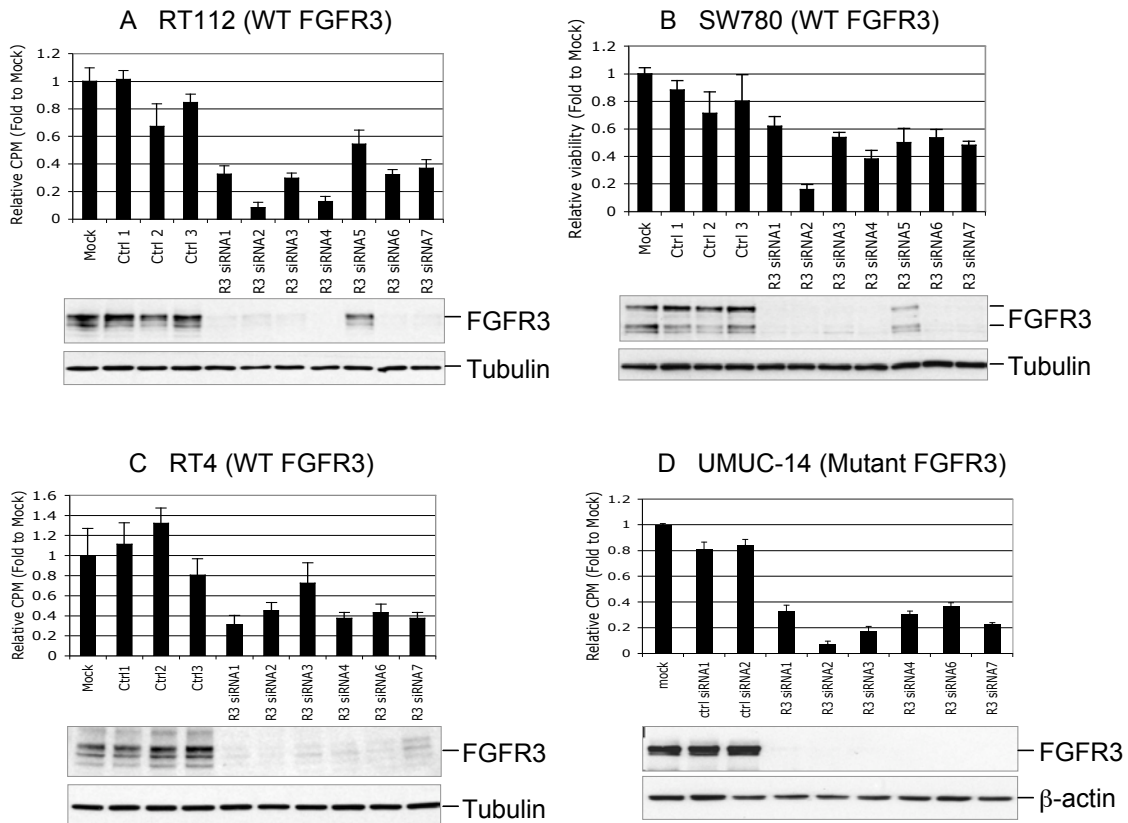
Refinement

Resolution (Å)	20-2.1
Number of reflections	46714
Final R ^c , R _{free} (F>0)	0.187, 0.224
Number of non-H atoms	5270
Number of Amino Acids	650
Sulfate	1
Sugar	1
Solvent atoms	274
Rmsd bonds (Å)	0.011
Rmsd angles (°)	1.4

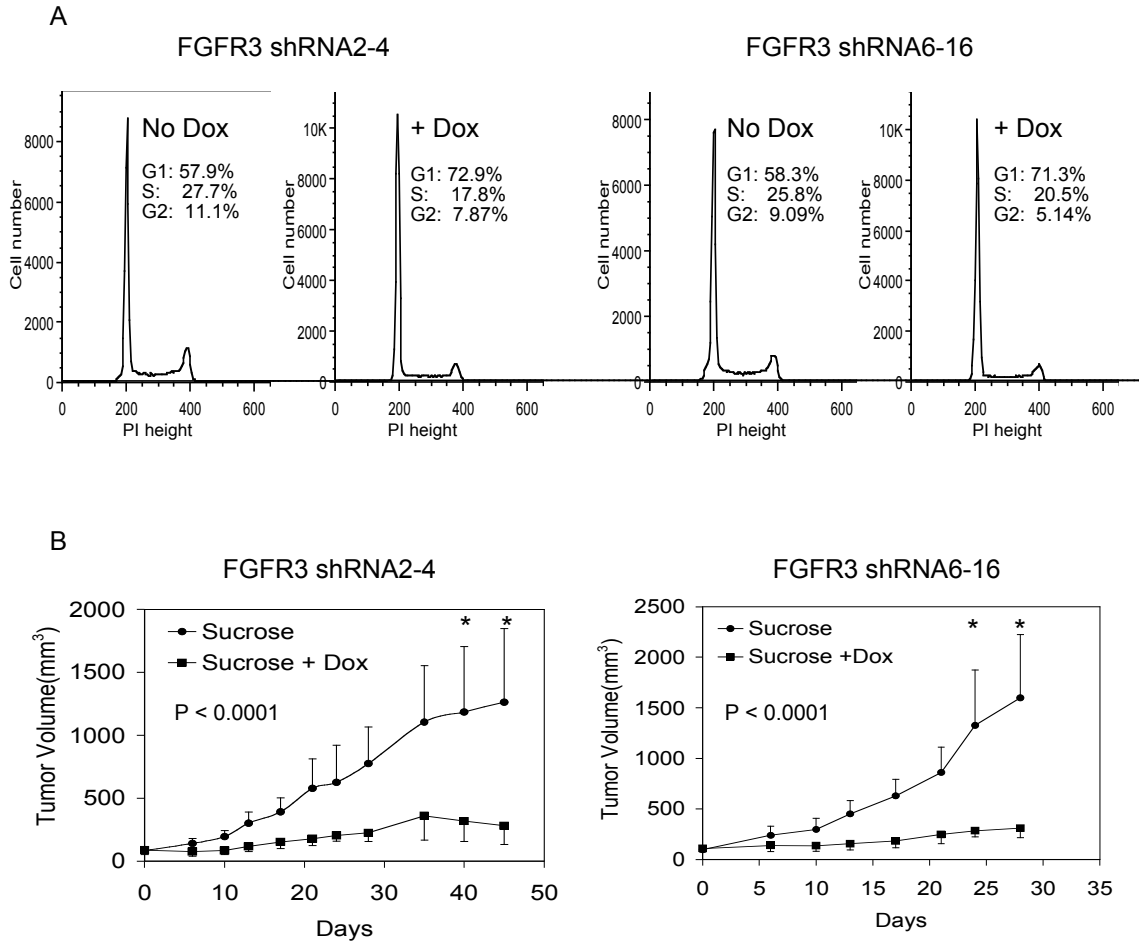
^a $R_{sym} = \sum |I - \langle I \rangle| / \sum I$. $\langle I \rangle$ is the average intensity of symmetry related observations of a unique reflection.

^b Numbers in parentheses refer to the highest resolution shell.

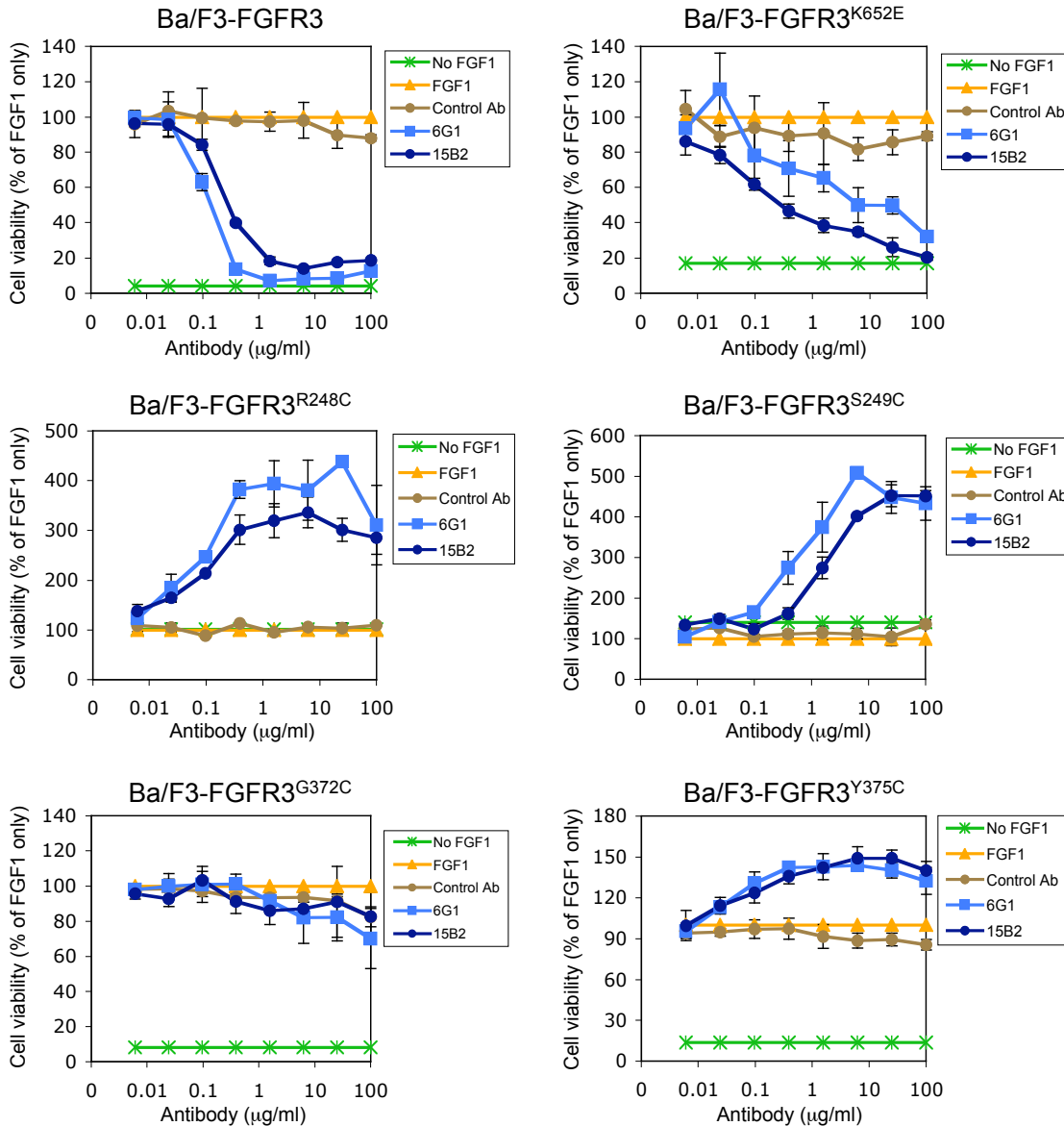
^c $R = \sum |F_o - F_c| / \sum F_o$. R_{free} is calculated as R, but for 5 % of the reflections excluded from all refinement.



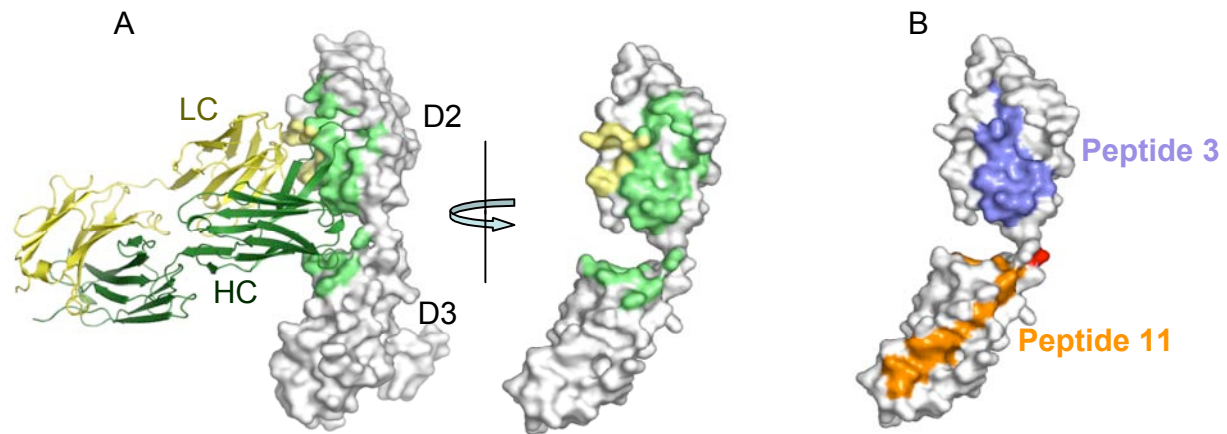
Supplemental Figure 1 Knockdown of FGFR3 with siRNA inhibits cell proliferation of bladder cancer cell lines. Six to seven different FGFR3 siRNAs and three non-specific control siRNAs were designed and synthesized in Genentech. Bladder cancer cell lines RT112 (A), SW780 (B), RT4 (C) and UMUC-14 (D) were plated into 96-well plate (3000 cells per well) and allowed to attach overnight, and transiently transfected with 25 nM siRNA in complex with RNAiMax (Invitrogen). 72 hr post-transfection, [³H]-thymidine (1 μCi per well) was added to the culture (A, C, and D) for another 16 hr incubation. Incorporated [³H]-thymidine was quantitated with TopCount. Data were normalized to that from cells transfected with RNAiMax alone (Mock). Error bars represent SEM. Lower panel: Representative blots showing FGFR3 expression in siRNA transfected cells. (B) Cell viability was measured with CellTiter-Glo (Promega) 96 hr after transfection. Error bars represent SEM



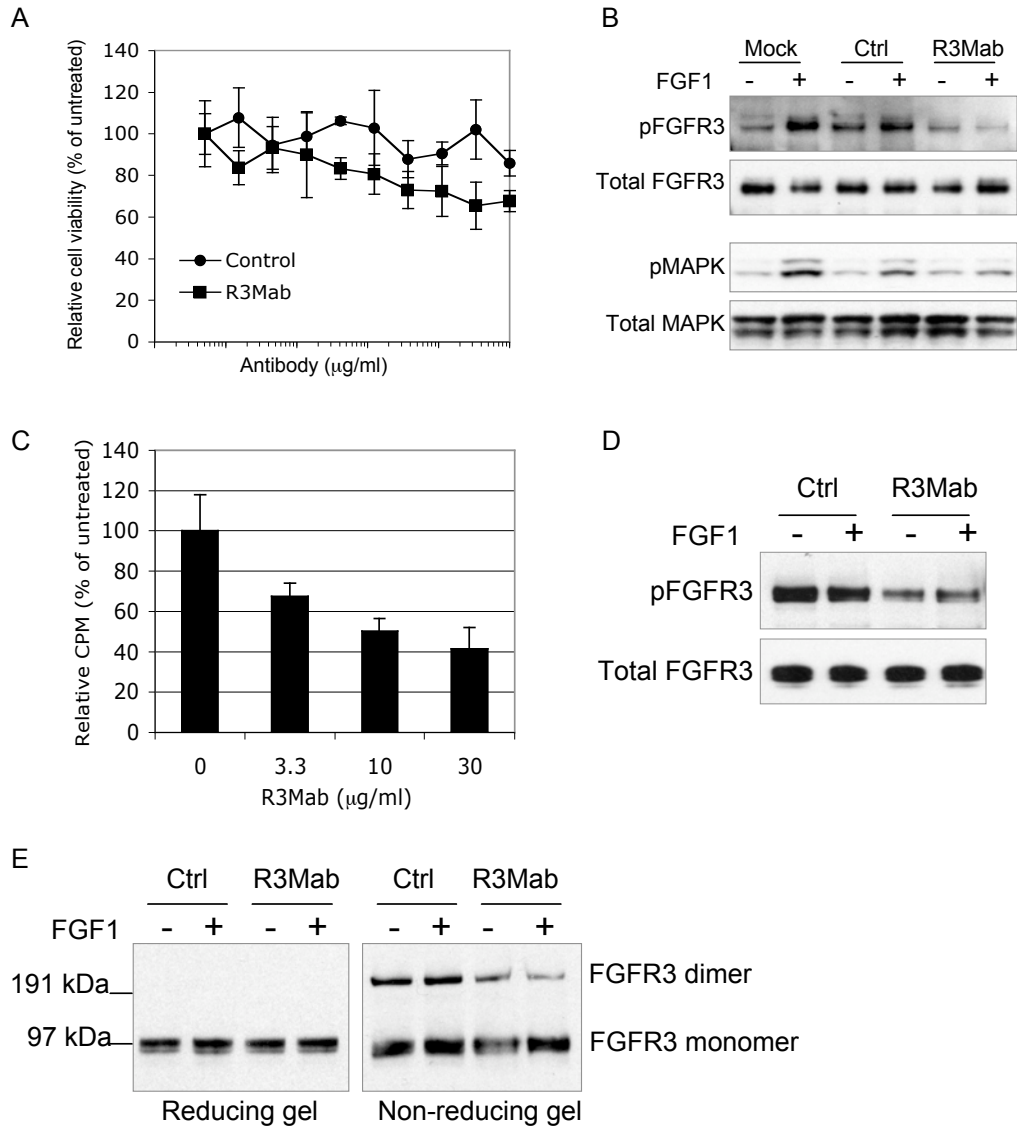
Supplemental Figure 2 FGFR3 knockdown in bladder cancer cell line RT112 induces G1 cell cycle arrest in vitro, and suppresses tumor growth in vivo. Three different FGFR3 shRNAs were designed and cloned into a Tet-inducible shRNA expression retroviral vector. RT112 stable clones expressing FGFR3 shRNAs or control shRNA were established with puromycin selection. (A) DNA fluorescence flow cytometry histograms of propidium iodide (PI)-stained nuclei obtained from RT112 stable cells expressing FGFR3 shRNA4 or shRNA6 following treatment with or without 1 μ g/ml doxycycline for 72 hr. (B) The growth of RT112 stable cells expressing FGFR3 shRNA2-4 (n=11 per treatment group) or FGFR3 shRNA6-16 (n=10 per treatment group) in nu/nu mice. Tumor bearing mice received 5% sucrose only (solid circle) or 5% sucrose plus 1 mg/ml doxycycline (solid square), and tumors were measured with calipers twice a week. Error bars represent SEM.



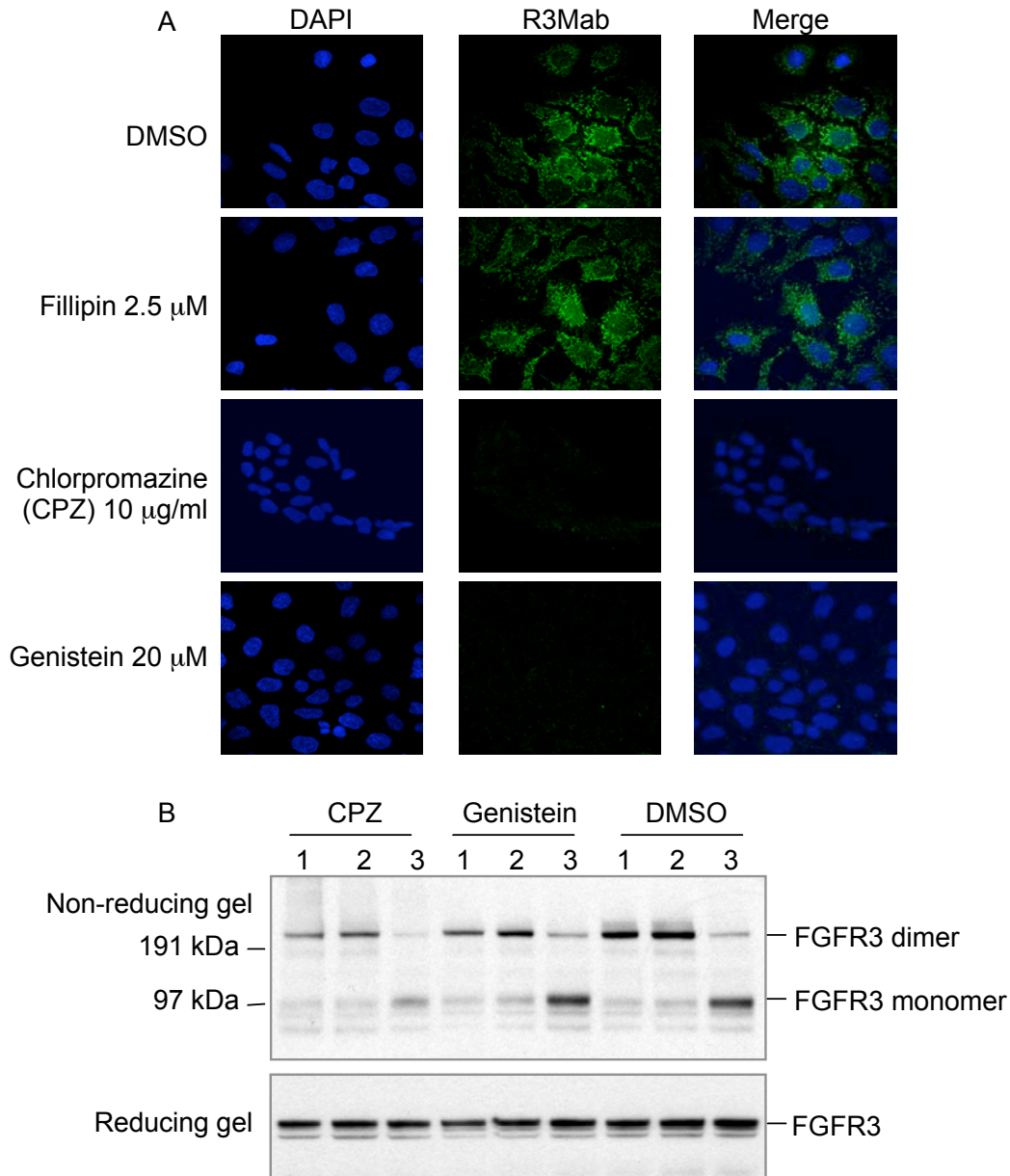
Supplemental Figure 3 Effect of anti-FGFR3 hybridoma antibodies 6G1 and 15B2 on Ba/F3 cell proliferation driven by wild type and mutated FGFR3. Anti-FGFR3 hybridoma antibodies were generated by immunizing BALB/c mice with human FGFR3-IIIb /Fc or human FGFR3-IIIc /Fc chimera. Fused hybridoma cells were selected using hypoxanthin-aminopterin-thymidine selection in Medium D from the ClonaCell® hybridoma selection kit (StemCell Technologies, Inc., Vancouver, BC, Canada). Hybridoma antibodies were sequentially screened for their ability to bind to FGFR3-IIIb and FGFR3-IIIc by ELISA and to recognize cell surface FGFR3 by FACS. Selected hybridomas were then cloned by limiting dilution. 6G1 and 15B2 are two clones used in this study to assess their effect on the proliferation of Ba/F3 cells expressing wild type or mutated FGFR3 similarly as described in Figure 3A. Error bars represent SEM.



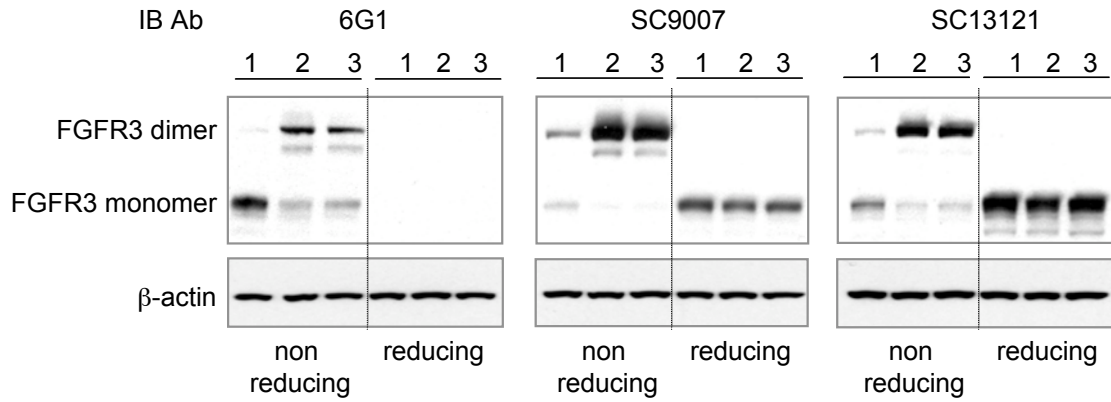
Supplemental Figure 4 Comparison of R3Mab epitopes determined by peptide mapping and crystal structure analysis. (A) Epitope revealed by the structure of the R3Mab Fab fragment in complex with the extracellular IgD2-D3 segment of human FGFR3. FGFR3 residues contacted by Fab heavy chain and light chain are colored in green and yellow, respectively. (B) Location of peptides 3 (colored in purple) and 11 (colored in orange) on FGFR3.



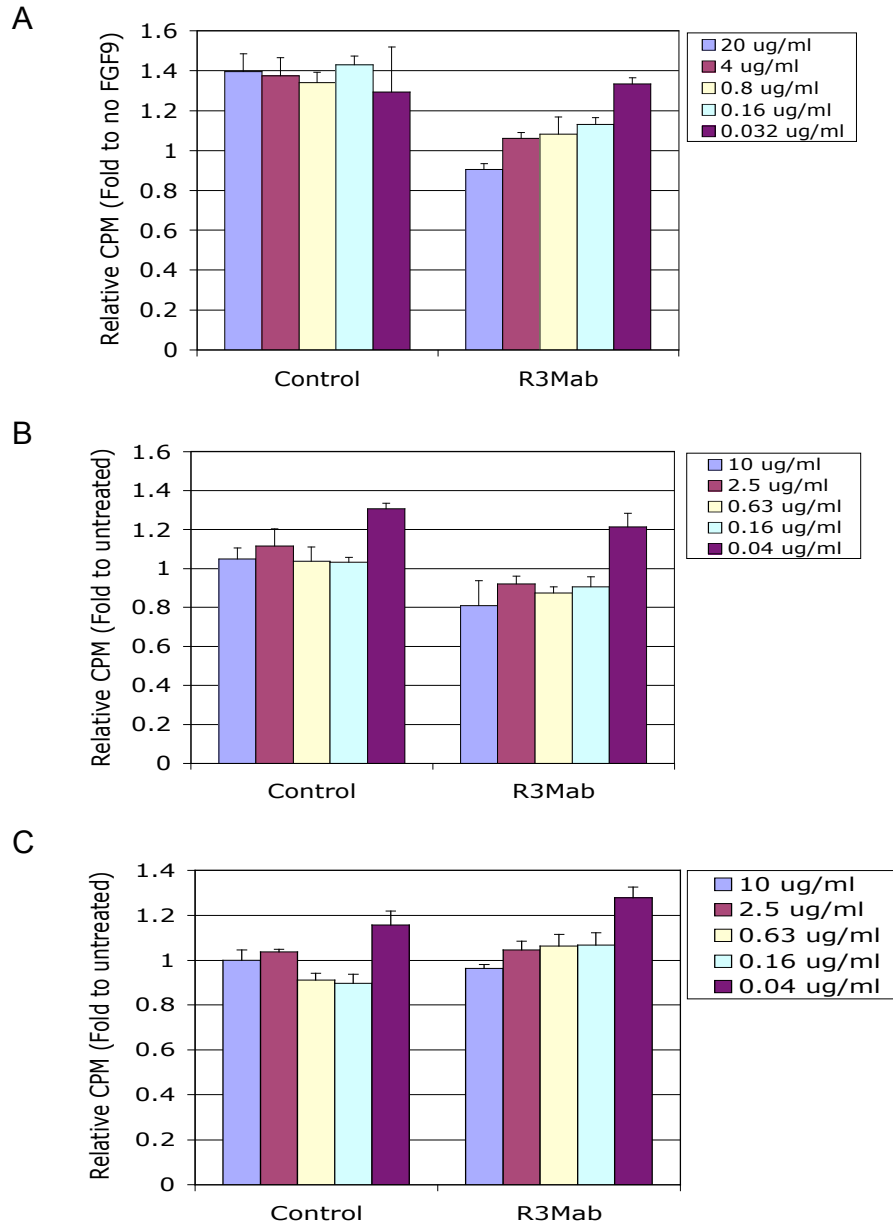
Supplemental Figure 5 R3Mab inhibits proliferation and FGFR3 signaling in bladder cancer cells containing wild type or mutated FGFR3^{S249C}. (A) Inhibition of cell viability by R3Mab in bladder cancer cell line RT4. Cell viability was assessed with CellTiter-Glo (Promega) after 96 hr incubation with the antibody. Error bars represent SEM. (B) Blocking of FGF1-activated FGFR3 signaling by R3Mab (15 µg/ml) in bladder cancer cell line RT4. (C) Inhibition of [³H]-thymidine incorporation by R3Mab in bladder cancer cell line TCC-97-7 (containing FGFR3^{S249C}). Error bars represent SEM. (D) Inhibition of FGFR3 phosphorylation in TCC-97-7 cells by R3Mab (15 µg/ml). (E) Decrease of FGFR3^{S249C} dimer in TCC-97-7 cells after 3 hr incubation with R3Mab (15 µg/ml) compared with a control antibody (Ctrl).



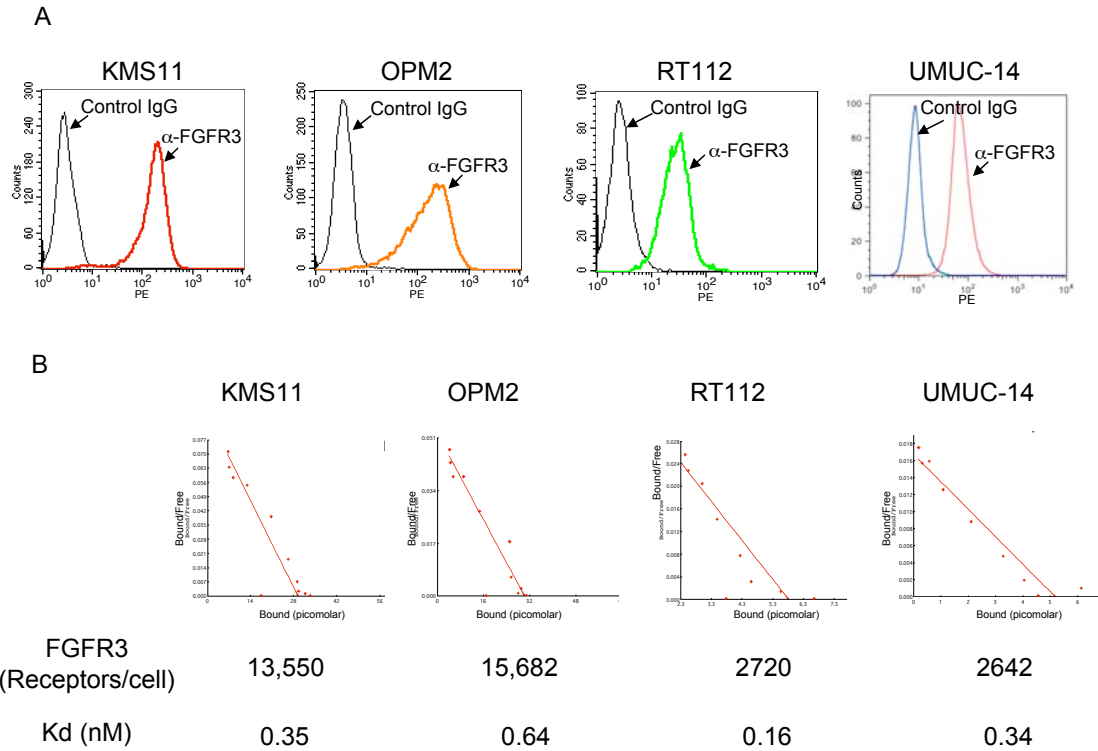
Supplemental Figure 6 Effect of endocytosis inhibitors on the internalization of R3Mab and FGFR3^{S249C} dimer in UMUC-14 cells. (A) Effect of endocytosis inhibitors on the internalization of R3Mab. UMUC-14 cells, pre-treated with various endocytosis inhibitor or DMSO for 1 hr at 37°C, were incubated with R3Mab (15 μ g/ml) for 3 hr at 37°C to allow internalization. A low pH wash was used to remove cell surface R3Mab to visualize internalized antibody. Cells were fixed and stained with Alexa 488-labeled anti-human IgG. Image was taken using confocal microscopy. (B) Effect of endocytosis inhibitors on FGFR3^{S249C} dimer in UMUC-14 cells treated with R3Mab. UMUC-14 cells, pre-treated with various endocytosis inhibitor or DMSO for 1 hr at 37°C, were incubated with mock (Lane 1), a control antibody (Lane 2), or R3Mab (15 μ g/ml, Lane 3) for 3 hr at 37°C. Cell lysates were analyzed for FGFR3 protein under non-reducing or reducing conditions by immunoblot. Note that chlorpromazine (inhibitor of clathrin-mediated endocytosis) blocked R3Mab internalization, but had no effect on R3Mab-induced decrease of FGFR3^{S249C} dimer.



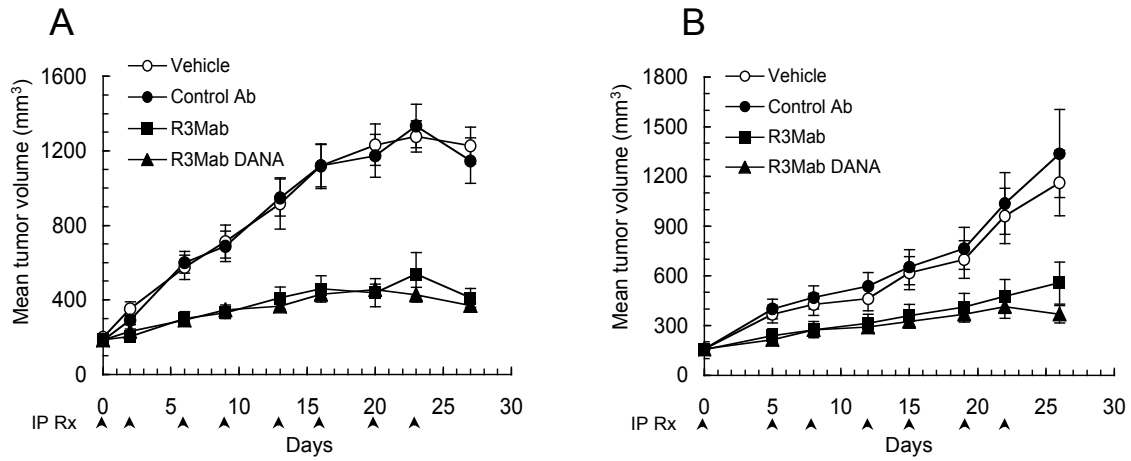
Supplemental Figure 7 Detection sensitivity of different anti-FGFR3 antibodies toward monomeric and dimeric FGFR3^{S249C} under non-reducing conditions. UMUC-14 cells were lysed after treatment with R3Mab (Lane 1), a control IgG1 (Lane 2), or PBS (Lane 3) for 3 hr, and cell lysates were subject to immunoblot analyses under reducing or non-reducing conditions. Note that 6G1 (murine hybridoma antibody generated at Genentech) detected both FGFR3^{S249C} dimer and monomer, whereas sc9007 (rabbit polyclonal antibody, Santa Cruz Biotechnology) or sc13121 (murine hybridoma antibody, Santa Cruz Biotechnology) preferentially detected the dimeric FGFR3^{S249C}.



Supplemental Figure 8 Effect of R3Mab on the proliferation of t(4;14)+ multiple myeloma cells. (A) Inhibitory effect of R3Mab on [³H]-thymidine incorporation by UTMC-2 cells. UTMC-2 cells were grown in medium containing R3Mab or a control antibody in the presence of 25 ng/ml FGF9 and 5 μg/ml heparin or heparin alone (No FGF9). After 6 days incubation, [³H]-thymidine was added for 16 hr incubation. Data were normalized to that from cells grown in the absence of FGF9 and antibody. (B-C) Effect of R3Mab on [³H]-thymidine incorporation by OPM2 (B) and KMS11 (C) cells. Cells grown in 1.5% FBS medium were treated with R3Mab or a control antibody for 6 days. Data were normalized to that from untreated cells. Error bars represent SEM.



Supplemental Figure 9 Cell surface expression levels of FGFR3 in myeloma and bladder cancer cells. (A) Cell surface FGFR3 expression in myeloma cells and bladder cancer cells assessed by FACS analysis. Cells were stained with phycoerythrin-conjugated mouse mAb against human FGFR3 (FAB766P, R&D Systems) or phycoerythrin-conjugated isotype control mouse IgG1(BD Pharmingen). (B) Scatchard analysis of FGFR3 density in myeloma cells and bladder cancer cells. R3Mab was radioiodinated, and incubated with cells in suspension with excess unlabeled antibody. After incubation at RT for 2 hr, cells were pelleted by centrifugation and washed twice. Specifically bound ^{125}I was determined. Receptor density and binding affinity (Kd) represent the mean from two binding experiments.



Supplemental Figure 10 Effect of R3Mab or its DANA mutant on xenograft growth of bladder carcinoma cells. (A) Effect of R3Mab and its DANA mutant (50 mg/kg each) on the growth of pre-established RT112 tumors. (B) Effect of R3Mab and its DANA mutant (50 mg/kg each) on the growth of pre-established UMUC-14 tumors. Error bars represent SEM.

Supplemental Methods

Generation of FGFR3 shRNA stable cells

Three independent FGFR3 shRNA were cloned into pHUSH vector as described (1). The sequence for FGFR3 shRNAs used in the studies is as follows: shRNA2:

5'GATCCCCGCATCAAGCTGCGGCATCATTCAAGAGATGATGCCGCAGCTTGA

TGCTTTTTTGGAAA; shRNA4: 5'GATCCCCTGCACAACCTCGACTACTA

TTCAAGAGATAGTAGTCGAGGTTGTGCATTTTTTGGAAA; shRNA6: 5'-

GATCCCCAACCTCGACTACTACAAGATTCAAGAGATCTTGTAGTAGTCGAGG

TTTTTTTTGGAAA. All constructs were confirmed by sequencing. EGFP control

shRNA was described in our previous study (1). The shRNA containing retrovirus was

produced by co-transfecting GP2-293 packaging cells (Clontech Laboratories, Mountain

View, CA) with VSV-G (Clontech Laboratories) and pHUSH-FGFR3 shRNA constructs,

and viral supernatants were harvested 72 hr after transfection, and cleared of cell debris

by centrifugation for transduction experiment.

RT112 cells were maintained in RPMI 1640 medium containing tetracycline-free FBS (Clontech Laboratories), and transduced with retroviral supernatant in the presence of 4 µg/ml polybrene. 72 hr after infection, 2 µg/ml puromycin (Clontech Laboratories) was added to the medium to select stable clones expressing shRNA. Stable cells were isolated, treated with 0.1 or 1 µg/ml doxycycline (Clontech Laboratories) for 4 days, and inducible knockdown of FGFR3 protein expression was assessed by Western blotting analysis. Cell cycle analyses were performed as described (2).

Selecting phage antibodies specific for FGFR3

Human phage antibody libraries with synthetic diversities in the selected complementary determining regions (H1, H2, H3, L3), mimicking the natural diversity of human IgG repertoire were used for panning. The Fab fragments were displayed bivalently on the surface of M13 bacteriophage particles (3). His-tagged IgD2-D3 of human FGFR3-IIIb and IIIc were used as antigens. 96-well MaxiSorp immnoplates (Nunc) were coated overnight at 4°C with FGFR3-IIIb-His protein or FGFR3-IIIC-His protein (10 µg/ml) and blocked for 1 hr with PBST buffer (PBS with 0.05% Tween 20) supplemented with 1% BSA. The antibody phage libraries were added and incubated overnight at room temperature (RT). The plates were washed with PBST buffer and bound phage were eluted with 50mM HCl and 500 mM NaCl for 30 min and neutralized with equal volume of 1M Tris base. Recovered phages were amplified in E.coli XL-1 blue cells. During subsequent selection rounds, the incubation time of the phage antibodies was decreased to 2 hr and the stringency of plate washing was gradually increased (4). Unique and specific phage antibodies that bind to both IIIb and IIIc isoforms of FGFR3 were identified by phage ELISA and DNA sequencing. Out of 400 clones screened, four were selected to reformat to full length IgGs by cloning VL and VH regions of individual clones into LPG3 and LPG4 vectors, respectively, transiently expressed in mammalian cells, and purified with protein A columns (5).

For affinity maturation, phagemid displaying monovalent Fab on the surface of M13 bacteriophage (3) served as the library template for grafting light chain (V_L) and heavy chain (V_H) variable domains of the phage Ab. Stop codons was incorporated in

CDR-L3. A soft randomization strategy was adopted for affinity maturation as described (4). Two different combinations of CDR loops, H1/H2/L3, H3/L3, or L1/L2/L3 were selected for randomization. For selecting affinity-matured clones, phage libraries were sorted against FGFR3 IIIb or IIIc–His protein, subjected to plate sorting for the first round and followed by four rounds of solution phase sorting as described (3). After five rounds of panning, a high-throughput single-point competitive phage ELISA was used to rapidly screen for high-affinity clones as described (6). Clones with low ratio of the absorbance at 450 nm in the presence of 10 nM FGFR3-His to that in the absence of FGFR3-His were chosen for further characterization.

ELISA Binding studies

cDNAs encoding the extracellular domains (ECD) of human FGFR1-IIIb, IIIc, FGFR2-IIIb and IIIc, FGFR3-IIIb and IIIc, and FGFR4 were cloned into pRK-based vector to generate human FGFR-human Fc chimeric proteins. The recombinant proteins were produced by transiently transfecting Chinese hamster ovary (CHO) cells and purified via protein A affinity chromatography. To test R3Mab binding to human FGFRs, Maxisorp 96-well plates (Nunc) were coated overnight at 4°C with 50 µl of 2 µg/ml of FGFR ECD-human Fc chimeric proteins. After blocking with phosphate-buffered saline (PBS)/3% BSA, R3Mab was added and incubated at RT for 2 hr. Specifically bound R3Mab was detected using an HRP-conjugated anti-human Fab and the TMB peroxidase colorigenic substrate (KPL, Gaithersburg, MD).

To test the effect of R3Mab on FGF/FGFR3 interaction, FGFR3-Fc chimeric proteins were captured on Maxisorp plate coated with anti-human immunoglobulin Fcγ

fragment-specific antibody (Jackson ImmunoResearch, West Grove, PA). After wash, increasing amount of R3Mab was added to the plate and incubated for 30 min. Then, FGF1 or FGF9 and heparin were added for incubation at RT for 2 hr. The plates were washed and incubated for 1 hr with biotinylated FGF1-specific polyclonal antibody (BAF232) or biotinylated FGF9 antibody (BAF273, R&D Systems), followed by detection with streptavidin-HRP and TMB.

Generation of Ba/F3-FGFR3 stable cells

cDNA encoding full-length human FGFR3 IIIb or IIIc was cloned into pQCXIP vector (Clontech Laboratories, Mountain View, CA) to generate pQCXIP-FGFR3-IIIb or IIIc. Specific mutations, i.e., R248C, S249C, G372C, Y375C and K652E, were introduced into the cDNA via QuickChange (Stratagene, La Jolla, CA). To generate Ba/F3 stable cells expressing wild type or mutant FGFR3, various pQCXIP-FGFR3 constructs were co-transfected into packaging cells GP2-293 with VSV-G plasmid (Clontech Laboratories). After selection with 2 μ g/ml puromycin for two weeks, cells expressing wild type or mutant FGFR3 were stained with Phycoerythrin-conjugated anti-human FGFR3 mAb (FAB766P, R&D Systems), and selected through fluorescence-activated cell sorting (FACS) for functional assays. For cell proliferation assay in 96-well micro-titer plate, the following cell density was used: For cells expressing wild type FGFR3-IIIb and FGFR3-K652E: 5,000 cells/well; for the rest: 10,000 cells/well. Cells were seeded in RPMI 1640 medium supplemented with 10% fetal bovine serum, 10 ng/ml FGF1 plus 10 μ g/ml heparin (Sigma-Aldrich, St. Louis, MO). R3Mab was added at

indicated concentration. After incubation for 72 hr, cell viability was assessed with CellTiter-Glo (Promega, Madison, WI).

Cell proliferation assay

For proliferation assays for RT112, RT4 and TCC-97-7 cells, 3000 cells /well were seeded into 96-well micro-titer plate and were allowed to adhere overnight. The medium was then replaced with low serum medium (0.5% FBS) with control or R3Mab at concentrations indicated. Following 4 days incubation, 1 μ Ci of [Methyl-³H] thymidine (PerkinElmer, Waltham, MA) was added to each well, and incubated for additional 16 hr. Cells were transferred to UniFilters using Packard Filtermate Harvester, and [³H]-thymidine incorporated into the genomic DNA of growing cells was measured using TopCount (PerkinElmer). In some cases, cell viability was assessed with CellTiter-Glo (Promega) following incubation with antibodies for 4 days. Values are presented as mean +/- SEM of quadruplets.

Clonal growth assay

The effect of R3Mab on cell clonogenicity was assessed following a previously described protocol (1). In brief, 400 UMUC-14 cells were seeded into 6-well plate in DMEM medium supplemented with 10% fetal bovine serum to allow adhesion overnight. Then R3Mab or control antibody diluted in 0.1% BSA medium was added to a final concentration of 10 μ g/ml. Equal volume of 0.1% BSA medium alone (Mock) was used as another control. The cells were incubated for about 12 days till cells in control groups formed sufficiently large colonies. Colonies were stained with 0.5% crystal violet, and

the number and size of colonies were quantitated using GelCount (Oxford, UK). The number of colonies larger than 120 μm in diameter was presented as mean \pm SEM (n=12).

Immunoprecipitation and immunoblotting analyses

To study the effect of antibodies on FGFR3 signaling, cells were starved in serum-free medium overnight prior to the beginning of treatment. Cells were incubated with either antibodies diluted in 0.1% BSA (w/v), RPMI 1640 medium, or with 0.1% BSA medium alone (Mock). After 3 hr at 37°C, FGF1 (final concentration of 15 ng/ml) and heparin (final concentration of 5-10 $\mu\text{g/ml}$) were added to half of the samples. As controls, a similar volume of heparin alone was added to the other half of samples. The incubation was continued for 10 min. Supernatants were removed by aspiration, and cells were washed with ice-cold PBS, then lysed in RIPA buffer (Upstate, Charlottesville, VA) supplemented with 1 mM sodium orthovanadate and Complete protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). The lysates were cleared of insoluble materials by centrifugation.

FGFR3 was immunoprecipitated using a rabbit polyclonal antibody (sc-123, Santa Cruz Biotechnology, Santa Cruz, CA) and analyzed by sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot. Phosphorylated FGFR3 was assessed with a monoclonal antibody against phospho-tyrosine (4G10, Upstate). Total FGFR3 was probed with a monoclonal antibody against FGFR3 (sc-13121, Santa Cruz Biotechnology). Phosphorylation and activation of FGFR3 signaling pathway were probed using the following antibodies: anti-FGFR^{Y653/654}, anti-FRS2 α ^{Y196}, anti-phospho-

p44/42 MAPK^{T202/Y204}, anti-total p44/42 MAPK and anti-AKT^{S473} were obtained from Cell Signaling Technology (Danvers, MA); anti-total FRS2 α (sc-8318) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The blots were visualized using a chemiluminescent substrate (ECL Plus, Amersham Pharmacia Biotech, Piscataway, NJ).

Antibody epitope mapping

To determine the epitope of R3Mab, 13 overlapping peptides, each of 15 amino acids in length, were synthesized to cover the extracellular domain of human FGFR3 from residues 138 to 310. The peptides were biotinylated at the C-terminus, and captured on streptavidin plates (Pierce, Rockford, IL) overnight. After blocking with PBS/3% BSA, the plates were incubated with R3Mab and detected using an HRP-conjugated anti-human IgG (Jackson ImmunoResearch) and the TMB peroxidase colorigenic substrate (KPL, Gaithersburg, MD).

Crystallization, structure determination and refinement

The human FGFR3-IIIb ECD (residues 143-374) was cloned into pAcGP67A vector (BD Bioscience, San Jose, CA), produced in T. ni Pro cells and purified using Ni-NTA column followed by size exclusion chromatography. The R3Mab Fab was expressed in *E.coli* and purified sequentially over a protein G affinity column, an SP sepharose column and a Superdex 75 column. Fab-FGFR3 complex was generated by incubating the Fab with an excess of FGFR3 ECD, and the complex was then deglycosylated and purified over a Superdex-200 sizing column in 20 mM TrisCl pH 7.5 and 200 mM NaCl buffer. The complex-containing fractions were pooled and concentrated to 20 mg/ml and

used in crystallization trials. Crystals used in the structure determination were grown at 4°C from the following condition: 0.1 M sodium cacodylate pH 6.5, 40% MPD and 5% PEG8000 using vapor diffusion method. Data was processed using HKL2000 and Scalepack (7). The structure was solved with molecular replacement using program Phaser (8) and the coordinates of 1RY3 (FGFR3) and 1N8Z (Fab-fragment). The model was completed using program Coot (9) and the structure refined to R/R_{free} of 20.4%/24.3% with program Refmac (10). Coordinates and structure factors were deposited in the PDB with accession code 3GRW.

ADCC assay

Human PBMCs were isolated by Ficoll gradient centrifugation of heparinized blood, and ADCC was measured using the multiple myeloma cell lines OPM2 or KMS11 or bladder cancer cell lines RT112 or UMUC-14 as target and PBMCs as effector cells at a 1:100 target : effector ratio. The target cells (10,000 cells/well) were treated with R3Mab or with control human IgG1 for 4 hr at 37°C. Cytotoxicity was determined by measuring LDH release using the CytoTox-ONE Homogeneous Membrane Integrity Assay following manufacturer's instructions (Promega, Madison, WI). The results are expressed as percentage of specific cytolysis using the formula: Cytotoxicity (%) = [(Experimental lysis - Experimental spontaneous lysis)/ (Target maximum lysis - target spontaneous lysis)] x 100, where spontaneous lysis is the nonspecific cytolysis in the absence of antibody, and target maximum lysis is induced by 1% Triton X-100.

Reference

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