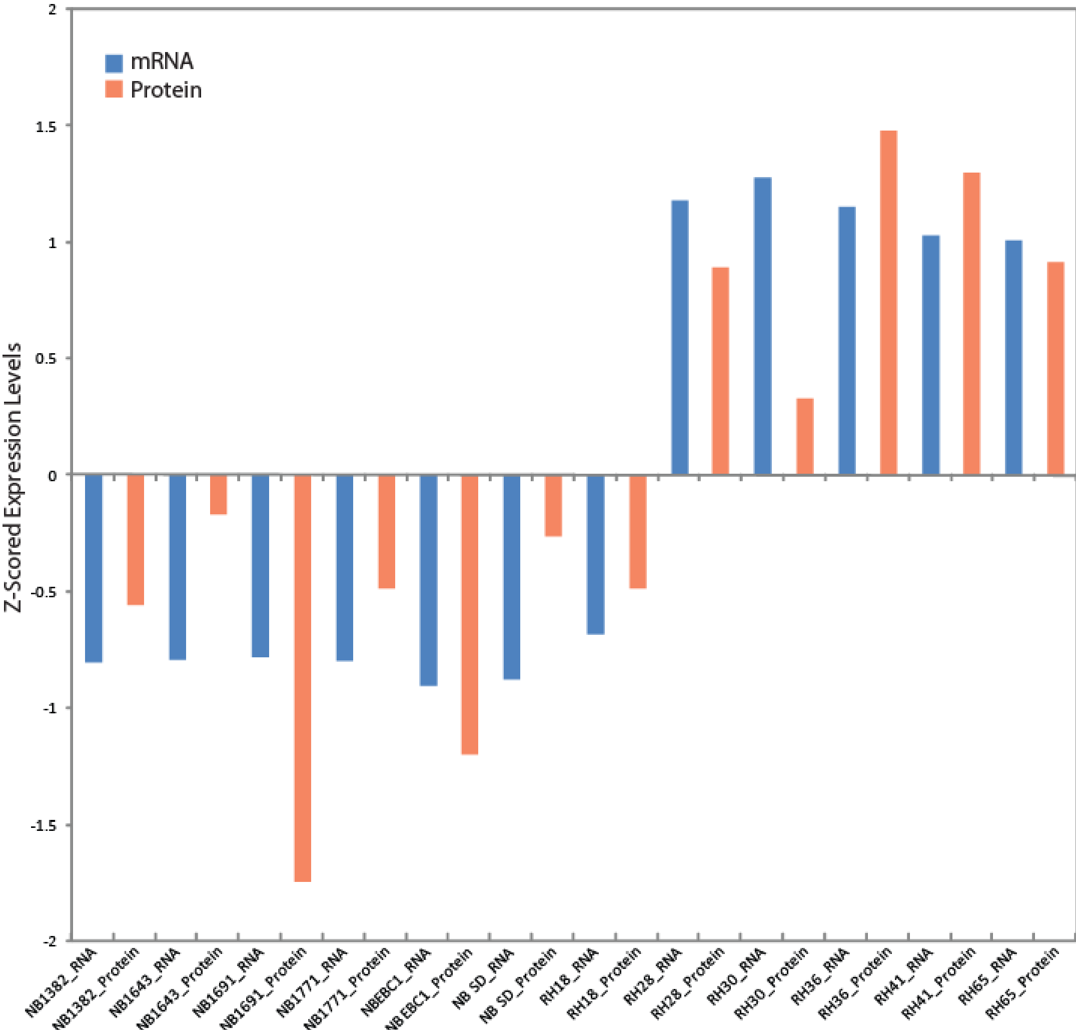
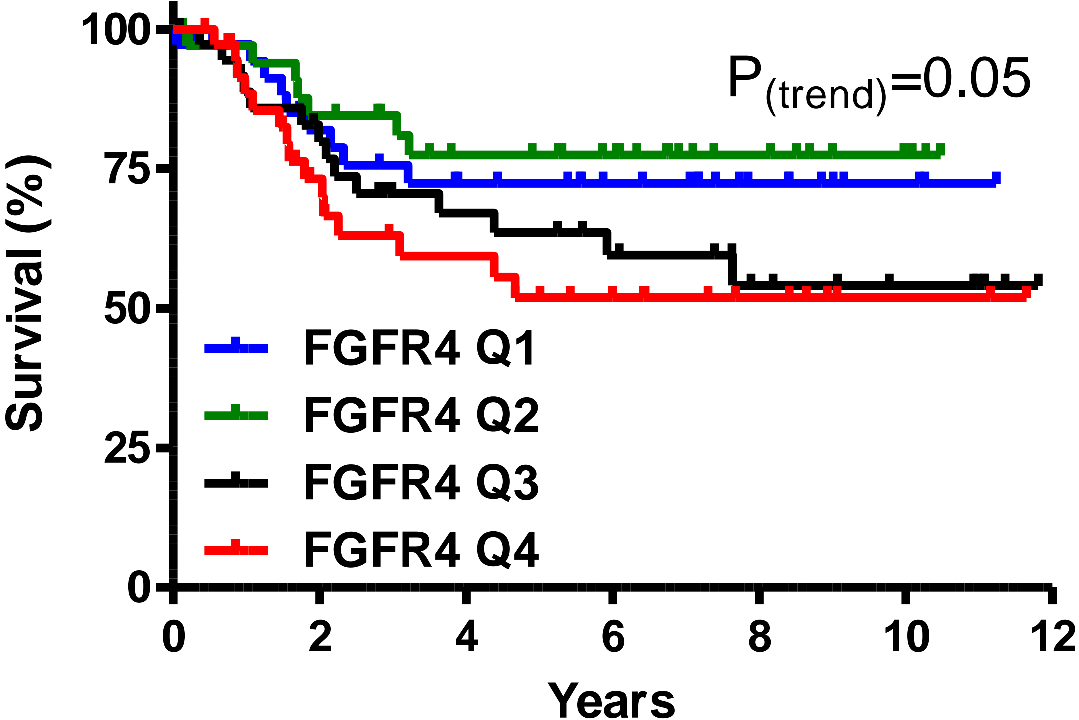
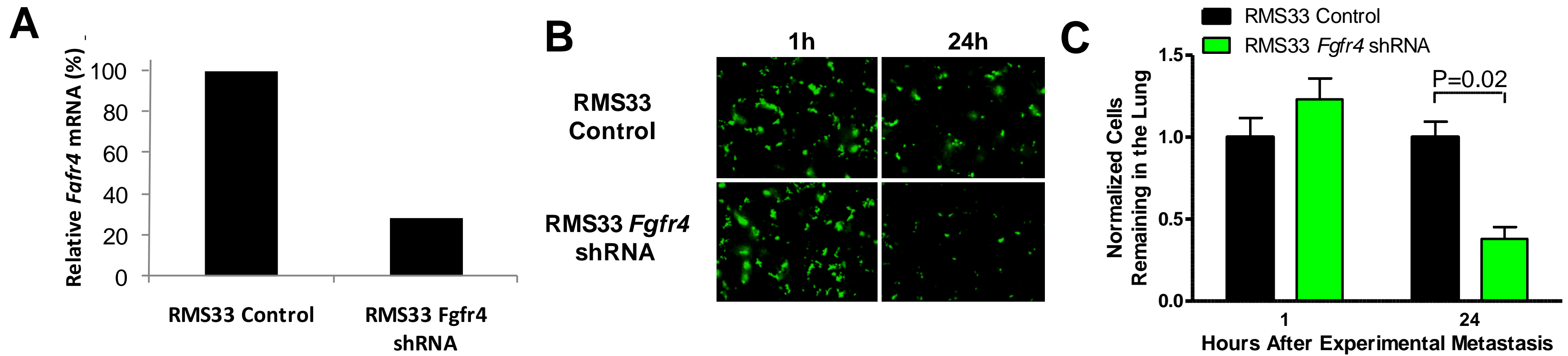
Supplemental Figure 1: Comparison of FGFR4 mRNA and protein levels in a panel of rhabdomyosarcoma (RMS; labeled with RH prefix) and neuroblastoma (NB; labeled with NB prefix) xenografts (2). FGFR4 protein was quantified with the Meso Scale Discovery platform from 6 RMS and 6 NB xenograft lines.



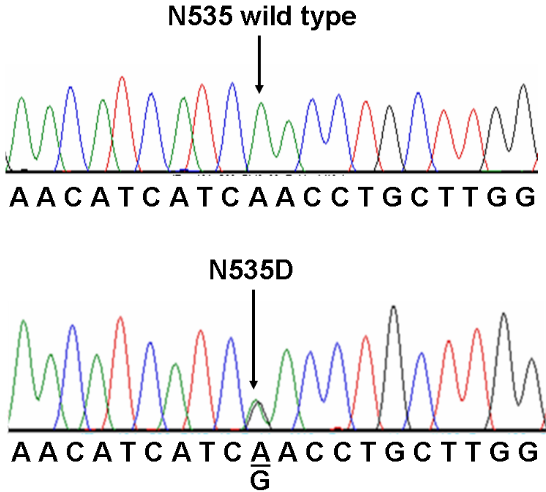
Supplemental Figure 2: *FGFR4* expression quartiles demonstrate a significant trend towards earlier mortality with higher expression by Kaplan Meier analysis ($P_{(trend)}$=0.05 by Logrank test).

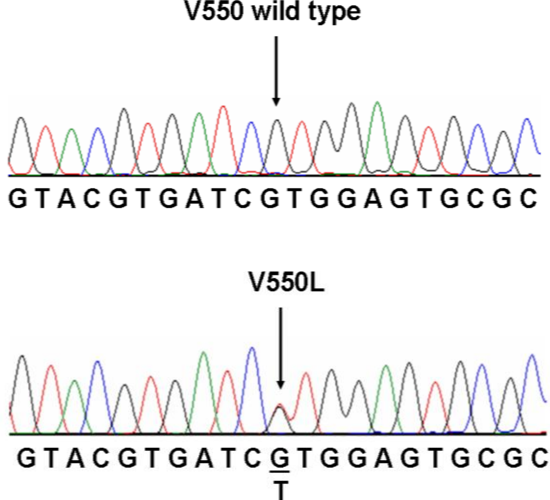


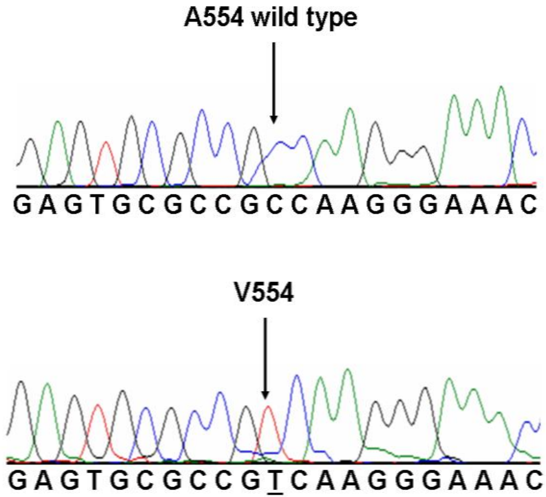
Supplemental Figure 3: (A) Quantitative RT-PCR shows a 70% *Fgfr4* mRNA reduction in RMS33 cells stably transduced with an shRNA targeting *Fgfr4*. **(B and C)** Quantification of IVVM early pulmonary metastases at 1 and 24 hours shows significantly fewer malignant cells remaining in the lungs with *Fgfr4* suppression (normalized values with SEM, n=5 mice per group, Mann Whitney test).

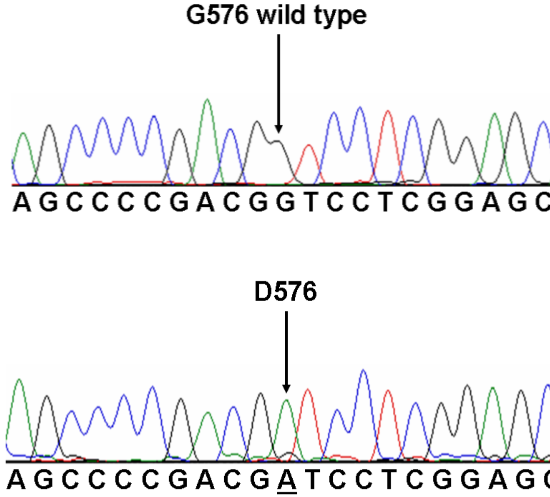


Supplemental Figure 4: Sequence chromatograms of the remaining *FGFR4* TK domain mutations observed in RMS tumors.

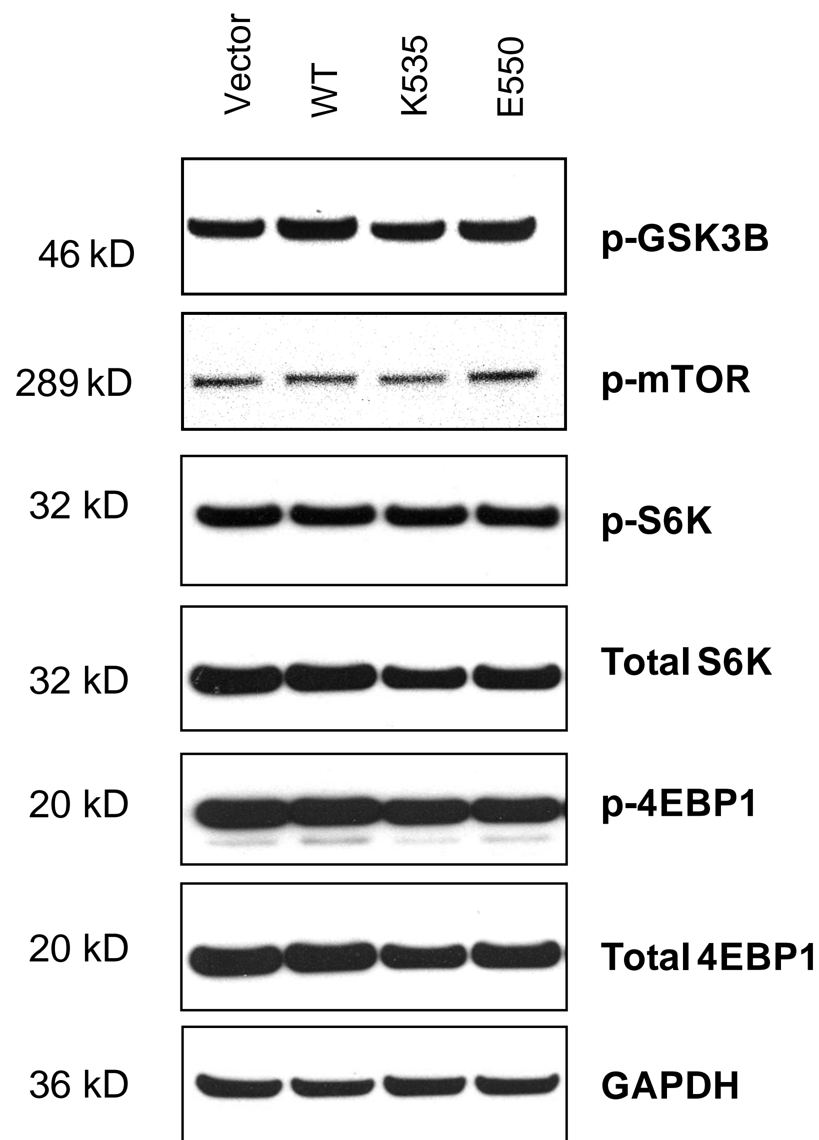








Supplemental Figure 5. Western blot analysis for total and phosphoprotein levels for the indicated members of the mTOR and GSK pathways.



Supplemental Table 1. *FGFR4* expression level in RMS samples with CNV (Copy Number Variation) call number greater than 2.5

Case ID	Copy number	Relative expression level (-ΔCT)[A]
1	4.02	-1.12
6	4.40	-0.13
8	3.81	-2.44
10	8.77	-3.37
200	4.13	-0.44
201	5.17	-1.51
202	4.41	-0.35
204	2.55	-1.54
208	2.62	-2.40
210	3.03	-1.68
217	2.87	-1.8
226	2.98	-0.09
228	3.47	-2.05
236	2.99	-2.52
240	6.61	-1.28

[A] Relative expression level was determined by qRT-PCR. -ΔCT was calculated by $-(CT_{(FGFR4)}-CT_{(GAPDH)})$. Correlation between CNV and mRNA expression r2=0.0127, P=NS.

Supplemental Table 2. *FGFR4* DNA Sequence Variants in Primary Tumors and Healthy Controls.

Site	Chromosome 5 Position	Amino Acid Change	Unpaired Tumor Variant Alleles (88 chrom)	Paired Tumor Variant Alleles (100 chrom)[A]	Healthy Control Variant Alleles (n=2060 chrom)[C]
rs3135918 (C/G)	176449143	-	3	3	ND
rs3135919 (C/T)	176449146	-	0	1	ND
rs1122528 (C/T)	176449148	-	1	0	ND
none (T/C)	176449169	-	0	1	ND
rs1966265 (G/A)	176449237	Ile10Val	19	24	ND
rs422421 (T/C)	176449932	-	67	62	ND
rs446382 (T/G)	176450067	Arg54Arg	52	58	ND
none (G/C)	176450072	Cys56Ser	0	2	ND
none (G/T)	176450120	Arg72Leu	0	1	ND
none (A/G)	176450360	Thr122Ala	1	0	ND
rs376618 (C/T)	176450403	Leu136Pro	70	76[A]	ND
none (G/A)	176450407	Ser137Ser	1	1	ND
none (G/A)	176450631	Ala175Thr	1	0	ND
rs45581232 (G/A)	176451271		0	2	ND
rs3135923 (C/T)	176451372	Asn228Asn	1	0	ND
none (G/A)	176451389	Arg234His	0	1	ND
rs452885 (C/T)	176451390	Arg234Arg	70	77	ND
rs393923 (G/A)	176451893	-	74	83	ND
rs45460599 (C/T)	176451983	Ala261Ala	1	0	ND
none (C/T)	176452061	Ile287Ile	0	1	ND
rs3135925 (A/G)	176452122	-	1	0	ND
none (C/G)	176452330	Thr332Thr	0	1	ND
none (C/T)	176452336	Leu334Leu	2	1	ND
rs351855 (G/A)	176452849	Gly388Arg	35	43	ND
rs34284947 (G/A)	176455003	Arg529Gln	0	0	1
none (A/G)	176455020	Asn535Asp	2	0	0
none (C/A)	176455022	Asn535Lys	2	0	0
none (G/C)	176455157	Val550Leu	1	1[B]	0
none (T/A)	176455158	Val550Glu	1	0	0
rs351854 (C/T)	176455168	Ala553Ala	2	5	0
none (C/T)	176455170	Ala554Val	0	2[B]	0
none (G/A)	176455236	Gly576Asp	0	2[B]	0
none (A/T)	176455303	Arg598Arg	0	1	0
rs42409 (C/T)	176455334	-	59	70	ND
none (A/T)	176455361	-	1	0	ND
rs45523032 (G/A)	176455792	-	1	2[A]	ND
rs31777 (C/A)	176456168	-	74	80	ND
rs31776 (A/G)	176456203	-	63	75[A]	ND
rs168446 (T/G)	176456404	-	0	8	ND
none (C/T)	176457071	-	3	2	ND
rs873652 (A/T)	176457559	-	0	2[A]	ND
none (C/T)	176457146	-	0	1	ND

ND=Not determined.

FGFR4 tyrosine kinase (TK) catalytic domain exons and intron/exon borders corresponding to codons 507 to 607 were sequenced in all tumor samples and all controls.

[A] One tumor sample (RMS205) had loss of heterozygosity at markers rs376618, rs45523032, rs31776, and rs873652.

[B] Somatic mutations absent in germline genomic DNA.

[C] P value = 2.0 x 10-7 for TK domain mutations in tumors vs. controls: 7 individuals / 94 tumors = 7.4% versus 1 individual / 1030 healthy controls = 0.1%.

Supplemental Table 3. Computational Predictive Analysis for FGFR4 Missense Mutations.

Functional Domain	**Missense Mutation**	**SIFT**	**Polyphen**	**SNPs3D SVM profile**	**MAPP P value**
Signal peptide	I10V[B]	T (1.00)	Unknown	-	0.77
Extra-cellular	C56S[B]	T (0.46)	Benign	1.91	0.93
Extra-cellular	R72L[B]	T (0.32)	**Possibly**	0.72	0.06
Extra-cellular	T122A[B]	T (0.41)	Benign	0.91	**0.005**
Extra-cellular	L136P[B]	T (0.32)	Benign	1.28	0.33
Ig like domain 2[A]	A175T[B]	T (0.70)	Benign	2.98	**0.003**
Extra-cellular	R234H[B]	**AFP (0.04)**	Benign	1.08	0.83
Transmembrane domain	G388R[B]	T (0.15)	**Possibly**	**-0.22**	**0.00002**
Tyrosine kinase domain[A]	N535D[B]	**APF (0.00)**	**Probably**	**-2.67**	**0.00003**
Tyrosine kinase domain[A]	N535K[B]	**APF (0.00)**	**Probably**	**-0.61**	**0.00006**
Tyrosine kinase domain[A]	V550E[B]	**APF (0.00)**	**Probably**	**-3.02**	**0.00001**
Tyrosine kinase domain[A]	V550L[B]	**APF (0.00)**	**Possibly**	**-0.95**	**0.0002**
Tyrosine kinase domain[A]	A554V[B]	**APF (0.03)**	Benign	**-0.84**	**0.001**
Tyrosine kinase domain[A]	G576D[B]	T (0.62)	Benign	2.06	**0.00002**

Abbreviations: SIFT=Sorting Intolerant From Tolerant; PolyPhen=Polymorphism Phenotyping; SNPs3D; MAPP=Multivariate Analysis of Protein Polymorphism; SIFT: T=tolerated, AFP=Affect protein function, and the value in parentheses is the SIFT probability score. Results for each method predicted to alter protein function are in bold.

[A] Domains as defined by the results of a search of the NCBI Conserved Domain database (NCBI CD-Search).

[B] Missense mutations detected in genomic DNA extracted from tumor samples

Supplemental Table 4. Rhabdomyosarcoma Clinical and Disease Demographics.

	Unpaired Tumors N=44 No. (%)	Paired Tumors N=50 No. (%)
Age, years	8.0 (5.7)[A]	8.0 (5.7)
Sex, male[B]	13 (52%)	23 (46%)
Race		
Caucasian	-	29 (58%)
African American	-	5 (10%)
Other or unknown	-	16 (32%)
Histology		
Alveolar	17 (39%)	19 (38%)
Embryonal	19 (43%)	27 (54%)
Botryoid	0 (0%)	4 (8%)
Unknown	8 (18%)	0 (0%)
Stage[C]		
1	7 (16%)	22 (44%)
2	6 (14%)	6 (12%)
3	11 (25%)	11 (22%)
4	5 (11%)	6 (12%)
unknown	15 (34%)	5 (10%)
Site		
Parameningeal	1 (2%)	1 (2%)
Orbit	0 (%)	3 (6%)
Other head and neck	8 (18%)	5 (10%)
Extremity	10 (23%)	5 (10%)
Genitourinary	7 (16%)	16 (32%)
Other	6 (14%)	20 (40%)
Unknown	12 (27%)	0 (0%)

Tumor demographics are presented in 2 groups: n=44 without paired germline DNA samples and n=50 that had paired germline DNA samples. Total tumors evaluated in the study was n=94.

[A] Mean and standard deviation. Number = 23 for Unpaired Tumors and n=50 in Paired Tumors.

[B] Number =25 for Unpaired Tumors and n=50 in Paired Tumors.

[C] Intergroup Rhabdomyosarcoma Study Group pretreatment staging classification (1).

Supplemental Table 5. Primers Used for Sequencing PCR Amplified Fragments of *FGFR4*.

Amplicon Name	Primer Name	Primer Sequence[A]
Exon 2	Exon2 Forward	GGCCACTTCCTGTCTCAGTTTCC
Exon 2	Exon2 Reverse	CTGGGCAAGGATCCTTTCCAGC
Exon 3	Exon3 Forward	GGTCAAGGAGTCTACATCAGGG
Exon 3	Exon3 Reverse	CCTTCAGCATGCGTTGCAAAG
Exon 4	Exon4 Forward	CTCACCTTGATTACAGGTGG
Exon 4	Exon4 Reverse	GTTTCTTCTCCATGCGCTG
Exon 5	Exon5 Forward	CAGTAGGTCTCCAAGGAC
Exon 5	Exon5 Reverse	CCGCAATCGCTTCACTCATTCG
Exon 6	Exon6 Forward	GTTCTCAGGGCCTAGAGAG
Exon 6	Exon6 Reverse	CTCACCAAGCTGCCTGACTC
Exon 7	Exon7 Forward	GAGACAGACAAGAAGCTGCAG
Exon 7	Exon7 Reverse	CCACCTCTGAGCTATTGATGTC
Exon 8	Exon8 Forward	CATTCTTCTCCCACCTTGGG
Exon 8	Exon8 Reverse	CCCACAAATCCACACACTG
Exon 9 / Exon 10	Exon9_10 Forward	GCTGGGAGGGACTGAGTTAG
Exon 9 / Exon 10	Exon9_10 Reverse	TGGAGAAAGTCCAGCCTCAG
Exon 11	Exon11 Forward	CTACCTCTCGACCCACTATG
Exon 11	Exon11 Reverse	GTCTTGCCATGTTGCCCAGG
Exon 12	Exon12 Forward	GATTCAGCCCTAGACCTACG
Exon 12	Exon12 Reverse	CACTCCACGATCACGTAC
Exon 13	Exon13 Forward	CAACCTGCTTGGTGTCTG
Exon 13	Exon13 Reverse	GGAAAGCGTGAATGCCTG
Exon 14	Exon14 Forward	CTAACCCTTGACCTCCTCCTCTG
Exon 14	Exon14 Reverse	CATCCACTTCACAGGCAG
Exon 15	Exon15 Forward	CCAGCAACGTGAGGGAGATG
Exon 15	Exon15 Reverse	CCAAATCTGAAGGAGCCCTCG
Exon 16	Exon16 Forward	GGCTCCTTCAGATTTGGTCTG
Exon 16	Exon16 Reverse	GTTAGTGTTGTCCTTCTGGCC
Exon 17	Exon17 Forward	CTACTGATGACCCTCCTATC
Exon 17	Exon17 Reverse	GAATAGGGTCCGAAGGTCAG
Exon 18	Exon18 Forward	GTCTCTGAGGAGGTACAGC
Exon 18	Exon18 Reverse	GACACGGCACAGCAACTCTG

[A] Each primer pair listed was first PCR amplified and then sequenced utilizing M13 universal sequencing tags added to the 5 prime end of each primer listed above (forward primers have M13 forward added to their 5 prime end: TGTAAAACGACGGCCAGT; reverse primers have M13 reverse added to their 5 prime end: CAGGAAACAGCTATGACC).

Supplemental Methods

Well-based protein array. Proteomic expressional signal of FGFR4 was detected using Meso Scale Discovery (MSD) Multi-Spot™ plates (MA2400 96 HB Plate) and an MSD Sector Imager 2400 reader (MSD). RMS and neuroblastoma (NB) xenografts used have been previously described, including RMS cell xenograft lines RH18, RH28, RH30, RH36, RH41, and RH65 (2). Frozen RMS and NB (for negative control) tissues were lysed in RIPA buffer containing 50 mM Tris–HCl, 150 mM NaCl, 1% Nonidet P-40 (NP-40), 0.5% sodium deoxycholate, 2 mM sodium fluoride, 2 mM EDTA, 0.1% SDS and protease inhibitor cocktail (Roche Diagnostics GmBH). The lysates were centrifuged for 15 minutes at 12,000 ×g at 4°C and the supernatant stored at -80°C until use. Five hundred nanograms of protein were added to the 96 well plates, the plate was allowed to dry at room temperature for 90 minutes, and the plates were subsequently further incubated at 37°C for 30 minutes. The antigen-coated plates were blocked for 1 h with 5% skim milk (Bio-Rad), and were then incubated overnight with FGFR4 (1:1,000) and GAPDH (1:5,000) antibodies at 4°C. After washing with PBST, the plates were incubated for 1 hour with goat anti-mouse SULFO-TAG™ antibodies at a dilution of 1:2,000 (1 mg/mL). The plates were then aspirated and washed three times with PBST. Finally, MSD-T read buffer was added to the plates and they were read on the Sector Imager 2400. In addition, BSA-coated wells were included on each plate as a control for nonspecific binding effects. The values from nonspecific wells were subtracted from all standard samples to calculate the actual value.

Copy number variation. FGFR4 copy number was determined by quantitative real-time PCR using a Gene Copy Number Assay on an Applied Biosystems 7900HT (ABI). Taqman RNasP Control reagent kits (ABI), where the RNasP gene copy number is known to be 2 in most populations, were used as an internal control. A PCR primer pair targeting amplification of a 97 bp region adjacent to *FGFR4* exon 9 and an *FGFR4* FAM labeled probe were designed using Primer Express (ABI-Perkin Elmer). Twenty nanograms of DNA were amplified using a multiplex reaction mixture of *FGFR4* primers (forward TTGTCTGTCTGTGTGTGTCCATGT, reverse CGTACAGGATGATGTCCGTATACC and FAM probe CAGAGGAGGACCCCACAT), 3 RNaseP gene primers (including VIC labeled probe), dNTPs, $MgCl_2$, DNA polymerase, and 10X PCR reaction buffer in a 96-well optical reaction plate. Relative quantification (ΔC_t) was performed in triplicate, where mean $\Delta C_t = C_{tFGFR4} - C_{tRNaseP}$, and these values were determined with SDS 2.2 software. Anti-$\log_2$ (meanΔC_t) represented the absolute *FGFR4* copy number. Reactions were performed in triplicate in tumor DNA and duplicate on available genomic DNA.

Intravital Videomicroscopy. The highly metastatic murine RMS cell line, RMS33, was transduced with an shRNA (oligo target sequence: GTCCACCACATTGACTACTAT) targeting *Fgfr4* (3). A clone of this stable

transductant was used for early metastasis assays by IVVM after injection of 1.0 x 10^6 labeled cells as described in the methods section and in previous reports (4). SCID Beige mice were used for these experiments.

Supplemental References

1. Raney, R.B., Anderson, J.R., Barr, F.G., Donaldson, S.S., Pappo, A.S., Qualman, S.J., Wiener, E.S., Maurer, H.M., and Crist, W.M. 2001. Rhabdomyosarcoma and undifferentiated sarcoma in the first two decades of life: a selective review of intergroup rhabdomyosarcoma study group experience and rationale for Intergroup Rhabdomyosarcoma Study V. *J Pediatr Hematol Oncol* 23:215-220.
2. Whiteford, C.C., Bilke, S., Greer, B.T., Chen, Q., Braunschweig, T.A., Cenacchi, N., Wei, J.S., Smith, M.A., Houghton, P., Morton, C., et al. 2007. Credentialing preclinical pediatric xenograft models using gene expression and tissue microarray analysis. *Cancer Res* 67:32-40.
3. Yu, Y., Khan, J., Khanna, C., Helman, L., Meltzer, P.S., and Merlino, G. 2004. Expression profiling identifies the cytoskeletal organizer ezrin and the developmental homeoprotein Six-1 as key metastatic regulators. *Nat Med* 10:175-181.
4. Khanna, C., Wan, X., Bose, S., Cassaday, R., Olomu, O., Mendoza, A., Yeung, C., Gorlick, R., Hewitt, S.M., and Helman, L.J. 2004. The membrane-cytoskeleton linker ezrin is necessary for osteosarcoma metastasis. *Nat Med* 10:182-186.