Supplementary Materials: Novel FGFR4-Targeting Single Domain Antibodies for Multiple Targeted Therapies Against Rhabdomyosarcoma

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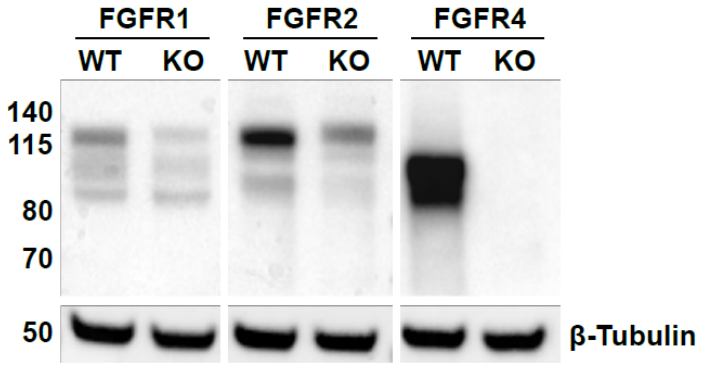

Figure S1. FGFR protein levels in Rh4 cells. Cells were transduced with a lentiviral vector containing Cas9 and sgRNA against FGFR4. Western blot analysis of Rh4-FR4wt (WT) and Rh4-FR4ko clone 8 (KO) were performed with antibodies against FGFR1, 2 and 4.

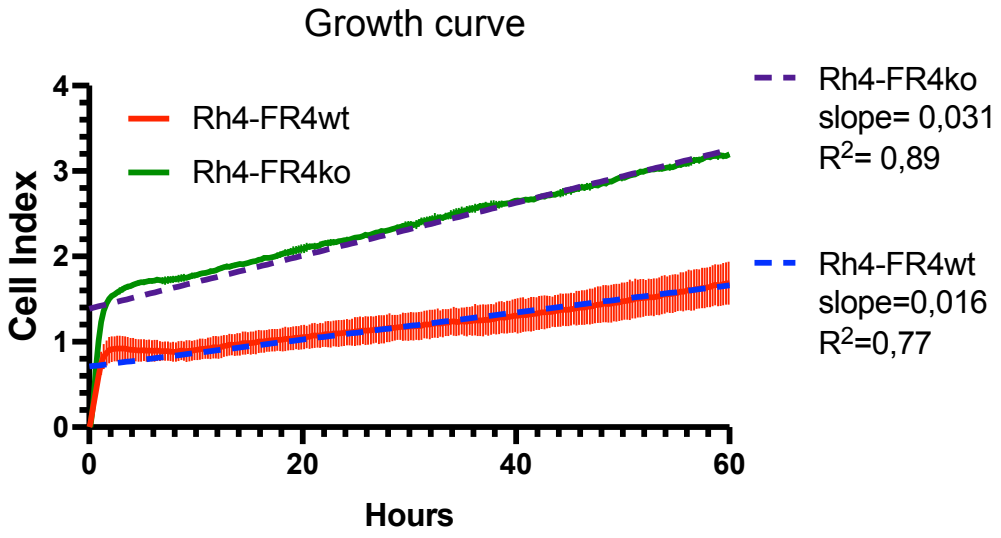

Figure S2. Real-time cell analysis of Rh4-FR4wt and Rh4-FR4ko cells growth. Rh4-FR4wt and Rh4-FR4ko cells were plated at 10,000 cell per well and let grow for 60 h. In this condition, the Rh4-FR4wt cells grown approximately twice shower than the KO cells, as indicated by the slope. The slope of the curve was determined using GraphPad Prism.

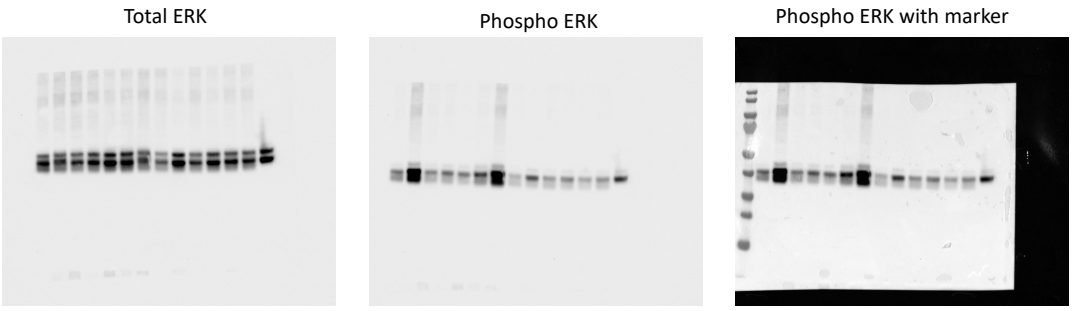

Figure S3. Original unmodified pictures of western blot shown in Figure 2C. Note that the first half of the samples (from the left) corresponds to Rh4-FR4wt and the other half to Rh4-FR4ko cells, which do not show any activation as expected.

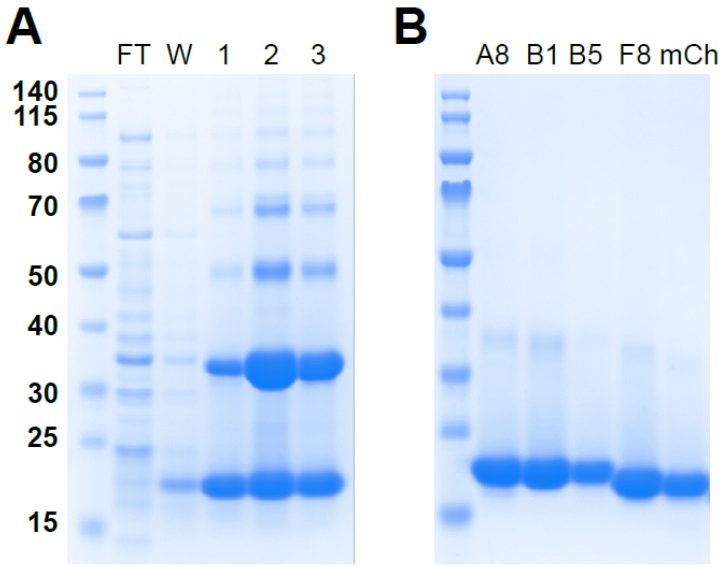

Figure S4. Purification of recombinantly expressed sdAb. (**A**) Flow-through and wash fraction of sdAb Co^{2+} beads purification and three elution steps with 300 mM imidazole (elution 1–3). (**B**)**.** Size exclusion purified sdAb candidates and mCh negative control. Molecular weights are indicated on the left side in kDa.

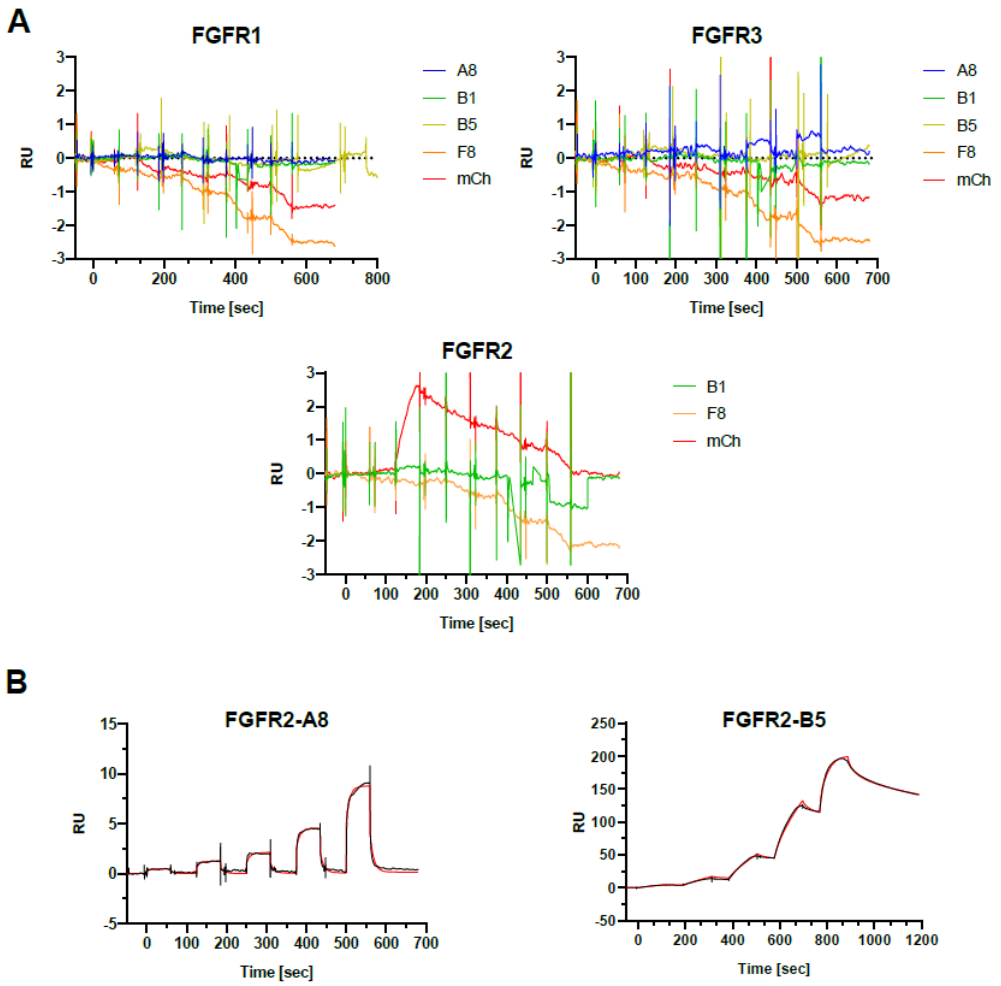

Figure S5. Surface plasmon resonance spectroscopy binding analysis of sdAb to FGFR1, 2 and 3. Single cycle kinetics measurements were performed with identical concentrations of sdAb used for affinity determination to FGFR4 by immobilizing recombinant FGFR1, 2 and 3 on a dextran based sensor chip. The analytes A8, B1, B5, F8 and mCh were injected in 5 different concentrations for the measurement of k_{on} and k_{off} rates. (**A**) Sensograms of non-binding sdAb to FGFR1, 2 and 3. (**B**). Candidates A8 and B5 bind to FGFR2. The black curves represent the measured data and red curves show the fit analysis (heterogeneous ligand model) performed with the BIAevaluation software.

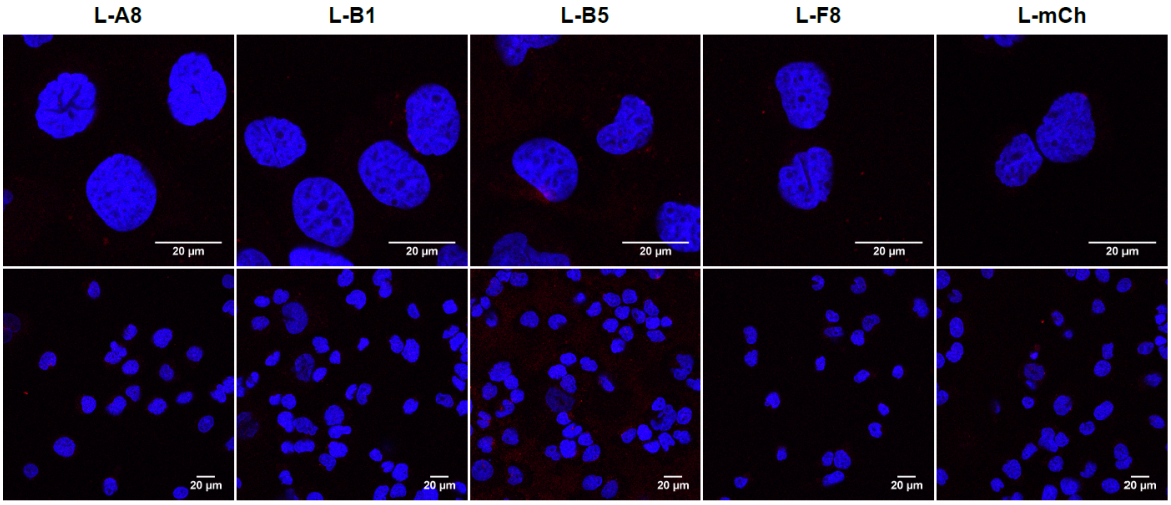

Figure S6. SdAb-coupled liposomes on Rh4-FR4ko cells. The cells were incubated for 2 h at 37 °C and 5% CO_2 with FGFR4-targeting and mCh control liposomes. The total lipid concentration was 3 mM. Cells were washed, fixed and mounted with DAPI containing medium.

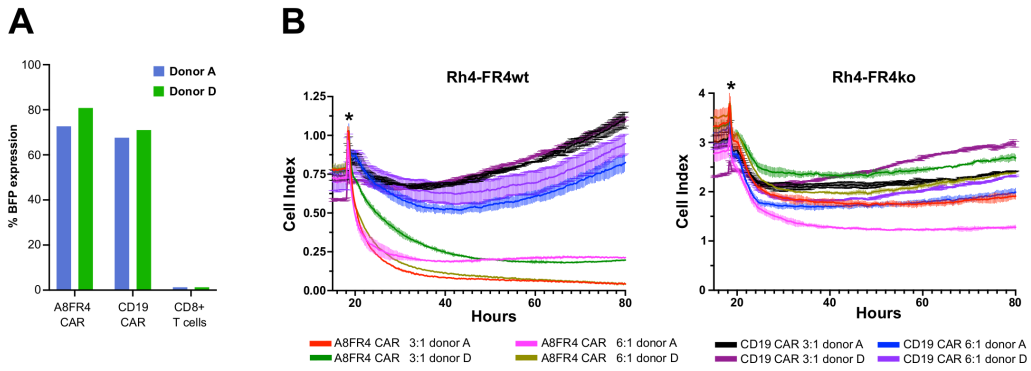

Figure S7. Cytotoxicity of FGFR4 CAR T cells towards RMS cells. (**A**) CD8+ T cell transduction efficiencies of donor A and D were determined by flow cytometry analysis of BFP signal. (**B**) Real-time cell death analysis of Rh4 cells co-cultured with effector T cells from donor A and D using xCELLigence RTCA DP. FGFR4 CAR T cells showed higher killing activities at the indicated E:T cell ratios in Rh4-FR4wt compared to non-specific CD19-CAR T cells. In Rh4-FR4ko cells no or reduced cytotoxicity was observed. The asterisks indicate the time of addition of the effector T cells.

Table S1. Affinity measurements of sdAb performed via surface plasmon resonance spectroscopy.

SdAb	Asso (s)	Disso (s)	Final diss (s)	C_1 (M)*	C_2 (M)	C_3 (M)	C_4 (M)	C_5 (M)
A8	120	70	500	2.80×10^{-8}	5.60×10^{-8}	1.13×10^{-7}	2.25×10^{-7}	4.50×10^{-7}
B1	240	70	650	6.25×10^{-9}	1.25×10^{-8}	2.50×10^{-8}	5.00×10^{-8}	1.00×10^{-7}
B5	180	70	300	6.30×10^{-10}	1.90×10^{-9}	5.60×10^{-9}	1.68×10^{-8}	5.00×10^{-8}
F8	60	60	120	3.40×10^{-8}	6.75×10^{-8}	1.35×10^{-7}	2.70×10^{-7}	5.40×10^{-7}
mCh	60	60	120	3.40×10^{-8}	6.75×10^{-8}	1.35×10^{-7}	2.70×10^{-7}	5.40×10^{-7}

*The analytes were injected in five different concentrations (C_1-C_5) for the indicated association time followed by a short dissociation phase. A final dissociation step was performed after the last and highest concentration injection.

Table S2. Affinity determination of sdAb A8 and B5 to FGFR2.

SdAb	$k_{on}1$ (1/M*s)	$k_{off}1$ (1/s)	K_D1 (M)	$k_{on}2$ (1/M*s)	$k_{off}2$ (1/s)	K_D2 (M)	$R_{max}1$ (RU)	$R_{max}2$ (RU)
A8	5.75×10^{4}	1.10×10^{-1}	1.91×10^{-6}	7.06×10^{2}	3.25×10^{-5}	4.60×10^{-8}	36.3	8.1
B5	7.88×10^{4}	5.94×10^{-4}	7.53×10^{-10}	1.04×10^{10}	5.74×10^{1}	5.54×10^{-9}	159.8	47.8

Surface plasmon resonance spectroscopy measurements and heterogeneous ligand binding fit revealed association- and dissociation constants (kon and koff) of the analytes for the immobilized recombinant protein. Binding affinities are expressed by the dissociation equilibrium constants KD (koff/kon) with respective maximal binding signals Rmax.