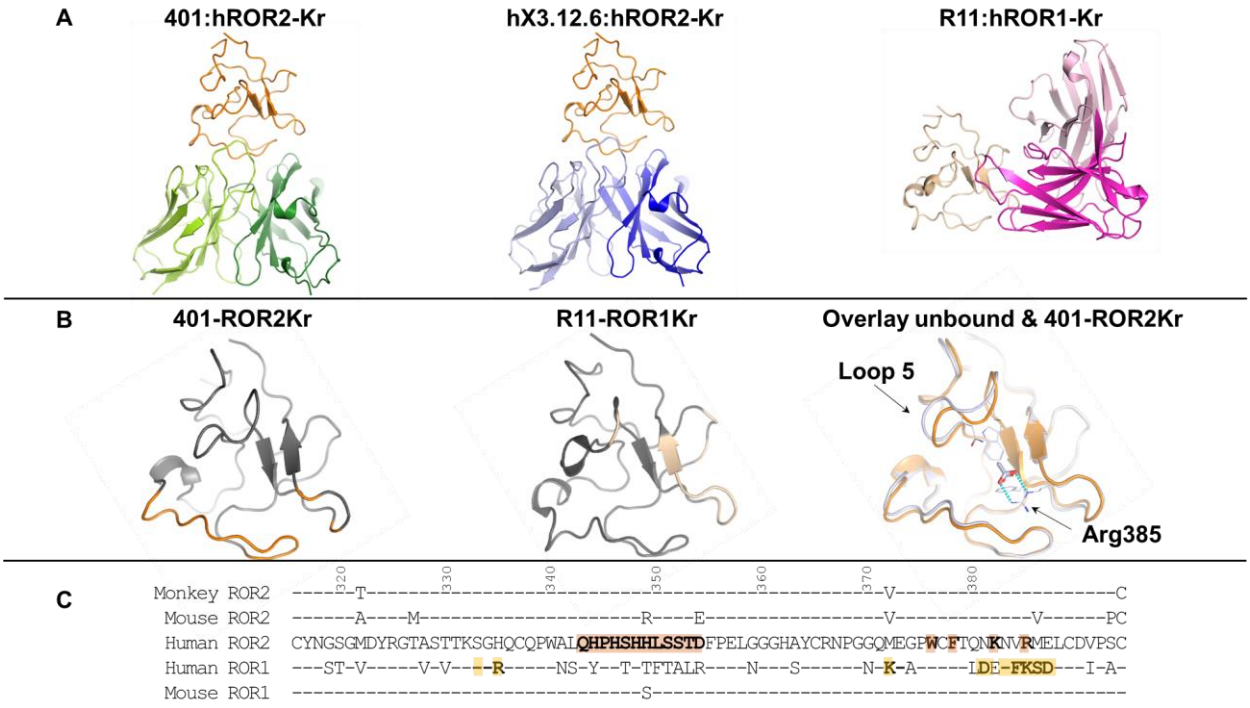
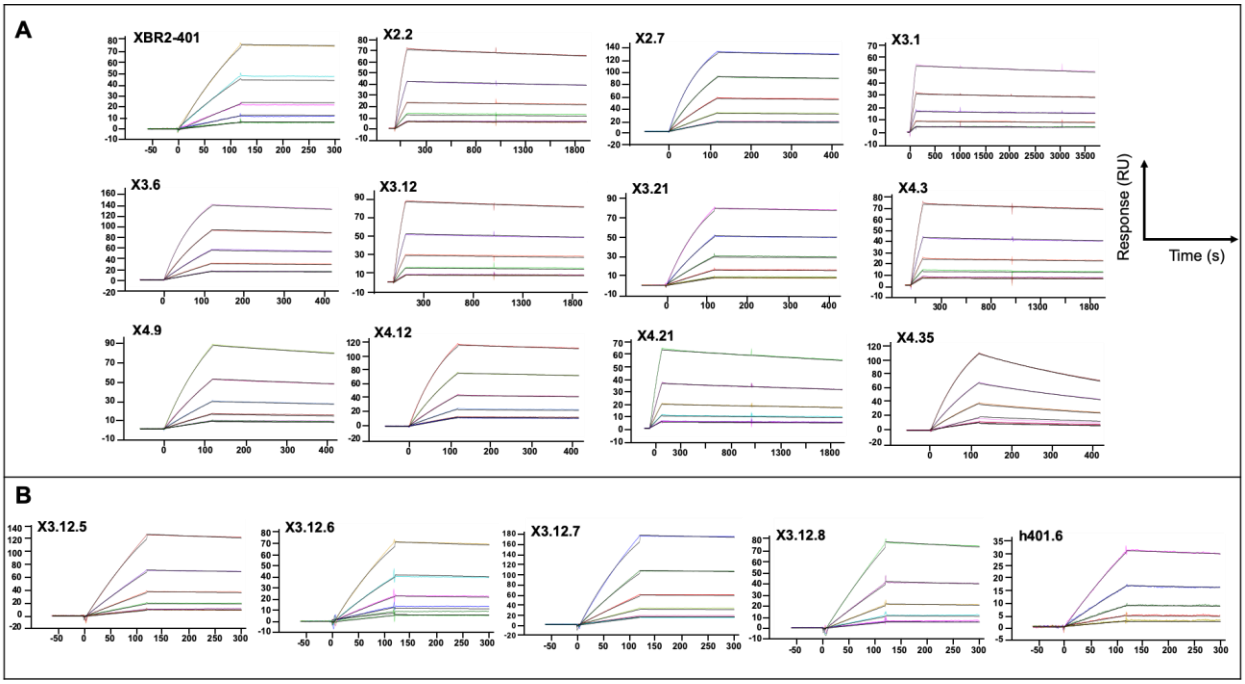
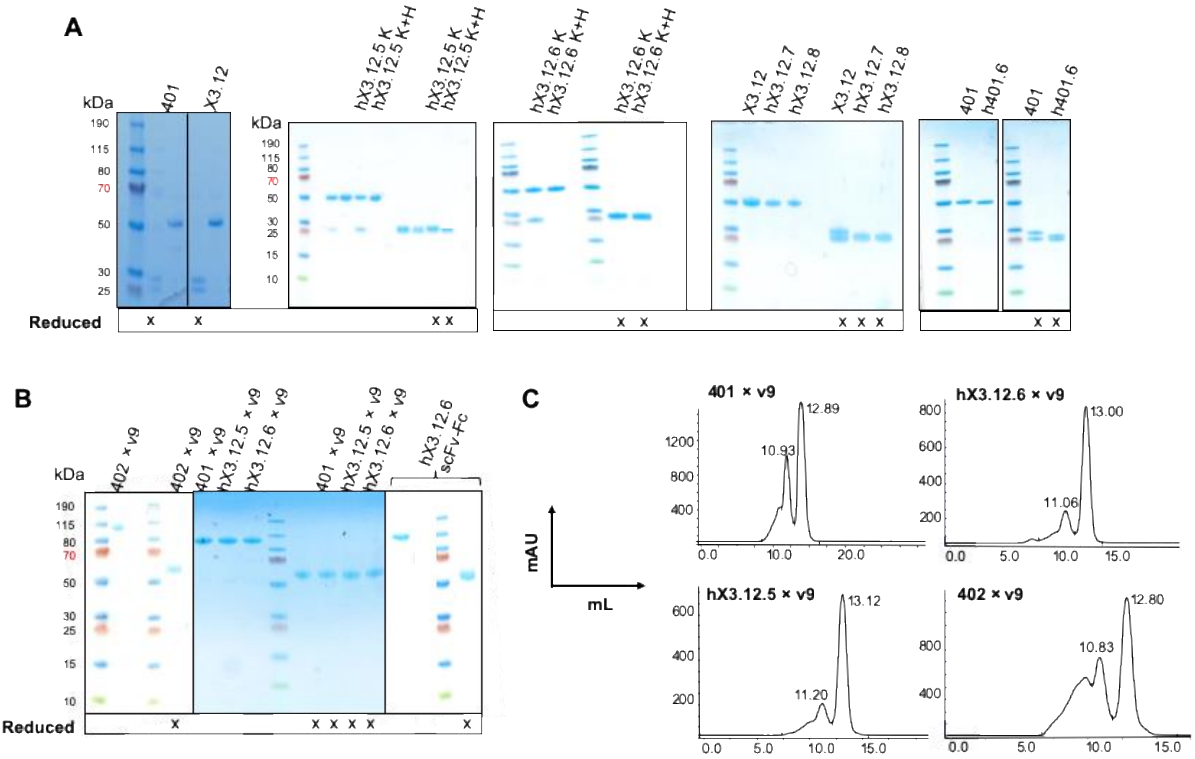
Supplementary Figures



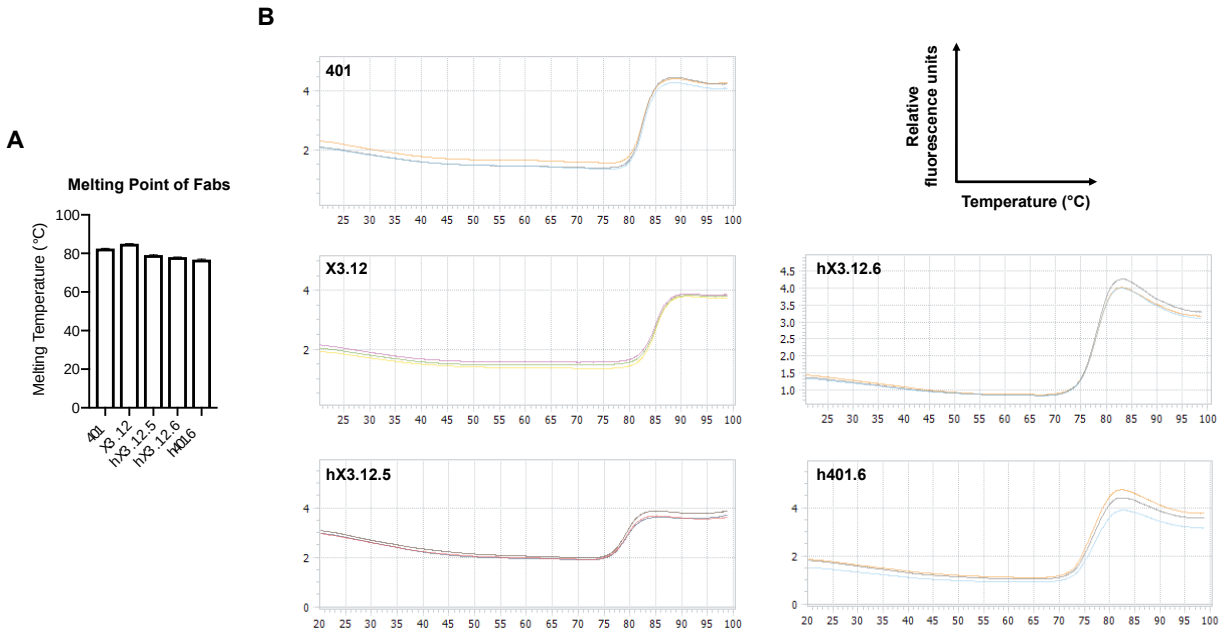
Suppl. Fig. 1. Comparison of 401:hROR2-Kr, hX3.12.6:hROR2-Kr, and R11:hROR1-Kr crystal structures, epitopes, and kringle domains. (**A**) Visual depiction where epitopes can be compared between the anti-ROR2 and anti-ROR1 mAbs. Darker and lighter shades denote V_H and V_L domains, respectively. The ROR2 and ROR1 kringle domains are shown in darker and lighter shades of orange, respectively. The overall rmsd of Cα positions between the 401:hROR2-Kr and hX3.12.6:hROR2-Kr complexes was found to be 0.474 Å. (**B**) Comparison of co-crystallized kringle domains of ROR2 and ROR1 from left to right: Crystal structure of hROR2-Kr based on the crystallized 401:hROR2-Kr complex. The epitope of 401 is marked in dark orange. Crystal structure of hROR1-Kr based on the crystallized R11:hROR1-Kr complex. The epitope of R11 is marked in light shaded orange. Overlay of unbound hROR2-Kr (gray) and hROR2-Kr from the 401:hROR2-Kr complex (orange). The rmsd of Cα positions was 0.383 Å. An acetate ion is bound to Arg385 of unbound hROR2-Kr through mixed salt bridge/hydrogen bond interactions. (**C**) Alignment of mouse, human, and monkey ROR2 and ROR1 kringle domain amino acid sequences (numbering from uniprot.org for hROR2). Residues that comprise the epitopes of 401 and R11 are marked in dark and light shaded orange, respectively. hROR2-Kr epitope residues which are not listed in Table I interact with 401 and hX3.12.6 through van der Waals interactions.



Suppl. Fig. 2. Analysis of affinity matured and humanized mAbs by SPR. (**A**) Shown are Biacore X100 sensorgrams of the top 12 chimeric rabbit/human anti-human ROR2 Fabs selected from the focused mutagenesis library by phage display. (**B**) Shown are Biacore X100 sensorgrams of the humanized anti-human ROR2 Fabs. A CM5 chip immobilized with a mouse anti-human Fcγ mAb was used to capture Fc-hROR2. Fabs were injected at five different concentrations (200, 100, 50, 25, and 12.5 nM for chimeric Fabs and starting at 100 nM for humanized Fabs). Kinetic (k_{on} and k_{off}) and thermodynamic ($K_D = k_{off}/k_{on}$) parameters of 1:1 binding were calculated and compiled in **Table III** and **IV**.



Suppl. Fig. 3. Purification of affinity matured and humanized Fabs and purification of ROR2 × CD3 biAbs. (**A**) Following tandem purification on KappaSelect and IMAC columns, the indicated Fabs were analyzed by SDS-PAGE and Coomassie blue staining**.** Expected bands were seen under nonreducing (~50 kDa) and reducing (~25 kDa) conditions. (**B**) Protein A-purified biAbs in heterodimeric scFv-Fc format (and a monospecific scFv-Fc negative control) were confirmed by SDS-PAGE and Coomassie blue staining showing the expected nonreduced bands at ~100 kDa and reduced bands ~50 kDa. (**C**) SEC elution profile of Protein A-purified biAbs. The major peak at ~13 mL is the monodisperse biAb while the minor peak at ~11 mL contains higher molecular weight aggregates. The activity assays shown in **Fig. 5** are based on tandem Protein A and SEC purification of biAbs.



Suppl. Fig. 4. Melting temperature and curves of parental and humanized Fabs. (**A**) Melting temperature of the indicated Fabs. Error bars represent standard deviations of the average of triplicates (mean ± SD) (**B**) Melting temperatures were determined using a LightCycler 480 protein melting protocol. Curves of triplicates of each Fab are shown. Melting of Fabs was measured up to 99°C.

Suppl. Table I: Crystal structure data collection

Protein (PDB ID)	**401:hROR2-Kr (6OSH)**	**hX3.12.6:hROR2-Kr (6OSV)**	**hROR2-Kr (6OSN)**
Beam line	LS-CAT 21-ID-F	ALS 5.0.2	ALS 5.0.2
Wavelength (Å)	0.9787	1.00	0.9787
Space group	$P\ 2_1 2_1 2_1$	$P\ 2_1 2_1 2_1$	$P\ 2_1 2_1 2_1$
Cell dimensions a, b, c (Å)	45.2, 67.2, 89.6	46.1, 60.1, 102.8	33.6, 41.9, 52.2
Asymmetric unit	1 complex	1 complex	1 molecule
Resolution (Å) [1]	53.76 - 1.2 (1.236 - 1.201)	51.4 - 1.4 (1.44 - 1.4)	22.1 - 1.1 (1.12 - 1.08)
Total reflections [1]	389303 (9481)	321703 (9887)	89198 (3544)
Unique reflections [1]	77000 (3850)	53044 (2652)	27454 (1821)
Mean I/σI [1]	18.5 (1.9)	23.6 (1.5)	37.6 (10.8)
Completeness (%) [1]	92.4 (72.5)	94.6 (65.3)	85.8 (58.1)
Multiplicity [1]	5.1 (2.5)	6.1 (3.7)	3.2 (1.9)
R_{merge} [1]	0.044 (0.518)	0.032 (0.575)	0.021 (0.059)
R_{pim} [1]	0.021 (0.372)	0.014 (0.317)	0.013 (0.510)
CC1/2 [1]	0.99 (0.70)	1.00 (0.73)	1.00 (0.99)
Wilson B-factor	10.0	19.2	5.8
Refinement			
Resolution (Å) [1]	40.4 - 1.2 (1.25 - 1.2)	51.4 - 1.4 (1.46 - 1.4)	22.1 - 1.1 (1.12 - 1.08)
No. of reflections [1]	76670 (6172)	53563 (4565)	27453 (1821)
No. of reflections in R_{free} [1, 2]	1150 (94)	1072 (94)	1367 (81)
R_{work} [1]	0.160 (0.234)	0.162 (0.231)	0.154 (0.174)
R_{free} [1, 2]	0.169 (0.234)	0.188 (0.242)	0.162 (0.179)
R.m.s. Bond length (Å)	0.008	0.009	0.009
R.m.s. Bond angle (º)	0.98	1.05	1.26
B-factor, average ($Å^2$)	17.1	30.7	11.5
B-factor, ScFv ($Å^2$)	16.05	28.50	-
B-factor, ROR2-Kr ($Å^2$)	19.20	35.6	7.99
B-factor, Water ($Å^2$)	29.31	37.52	25.57
Number of atoms			
Protein	2386	2383	666
Water	426	340	165
Model quality (%)			
Ramachandran favored	96.3	97.7	96.4
Ramachandran allowed	3.4	2.3	3.6
Ramachandran outliers	0.3	0.0	0.0
Rotamer outliers	0.0	0.0	0.0

[1] Parentheses refer to statistics for the highest resolution shell.

[2] R_{free} is calculated with removal of 2 % of the reflections as the test set before the refinement.