SUMOylation regulates nuclear accumulation and signaling activity of the soluble intracellular domain of the ErbB4 receptor tyrosine kinase

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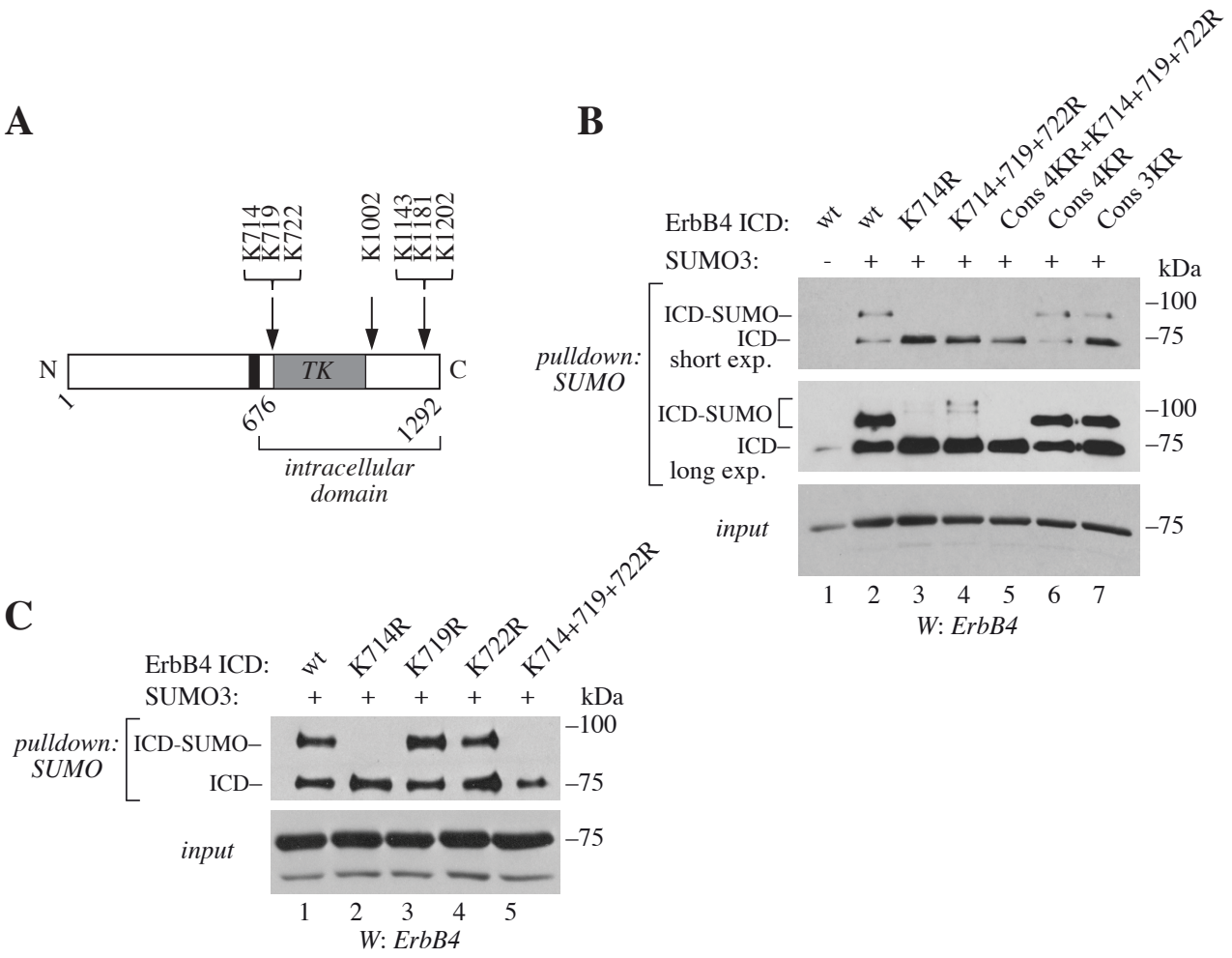

FIGURE S1. Identification of the ErbB4 SUMOylation site.

A) Schematic structure of ErbB4. Arrows indicate the mutated amino acids analyzed in B and C. Black color indicates the transmembrane domain and TK indicates the tyrosine kinase domain.

B) and C) MCF-7 cells were transfected with wild-type or mutant HA-tagged ErbB4 ICD constructs and His-tagged SUMO3. Cells were lysed in a denaturing buffer, and lysates were incubated with Ni^{2+}-NTA agarose to pull down His-SUMO conjugates. Whole cell extracts or pull-down samples were analyzed by Western with anti-ErbB4. Cons 4KR includes K1002R+K1143R+K1181R+K1202R; Cons 3KR includes K1143R+K1181R+K1202R. ErbB4 lysine-to-arginine mutant constructs were analyzed for SUMOylation in 4-11 independent experiments.

Fig. S1

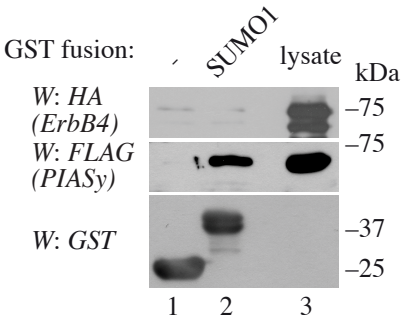

FIGURE S2. ErbB4 ICD does not interact with SUMO1 non-covalently.
A plasmid encoding GST-SUMO1 fusion protein (1) was expressed in BL-21 DE3 strain of *Escherichia coli* (Invitrogen), affinity-purified using Glutathione Sepharose 4B (GE Healthcare), and eluted with a buffer containing 20 mM glutathione, 100 mM NaCl, 0.5% Triton X-100, and 1 mM dithiothreitol. The non-covalent interaction of GST-SUMO1 with ErbB4 was analyzed using GST pull-down assay. COS-7 cells were transiently transfected with plasmids encoding HA-tagged ErbB4 ICD or FLAG-tagged PIASy (2). Aliquots of lysates corresponding to 1000 µg of total protein were incubated with 20 µg of GST-SUMO1 or GST and 25 µl of Glutathione Sepharose 4B at 4°C for 2 hours. Beads were washed five times with 1 ml of lysis buffer to remove non-specific binding, and heated at 95 °C for 5 minutes in Laemmli loading buffer to elute and denature precipitated proteins. Pull-downs were analyzed by Western blotting with anti-HA (Roche), anti-FLAG, and anti-GST (GE Healthcare). PIASy contains a SIM and interacts with SUMO1 non-covalently, and was used as a positive control (3).

Fig. S2

Lysine #	Exposed/Buried	
678	E	
681	E	
682	E	
683	E	
714	E	
719	E	kinase domain
722	E	kinase domain
734	B	kinase domain
745	E	kinase domain
751	B	kinase domain
760	E	kinase domain
812	E	kinase domain
829	E	kinase domain
852	E	kinase domain
858	B	kinase domain
873	E	kinase domain
881	E	kinase domain
885	B	kinase domain
896	E	kinase domain
919	E	kinase domain
935	E	kinase domain
955	E	kinase domain
966	E	kinase domain
968	E	kinase domain
994	E	
1002	E	
1097	E	
1141	E	
1143	E	
1160	E	
1181	E	
1202	E	
1207	E	
1216	E	
1218	E	
1219	E	
1249	E	
1253	E	
1275	E	

FIGURE S3. Surface accessibility of ErbB4 lysine residues.
The prediction of surface accessibility of lysine residues in the ErbB4 intracellular domain using NetSurfP Protein Surface Accessibility and Secondary Structure Predictions (http://www.cbs.dtu.dk/services/NetSurfP/) (4). The numbers refer to the amino acid sequence of ErbB4 JM-a CYT-2 isoform.

Fig. S3

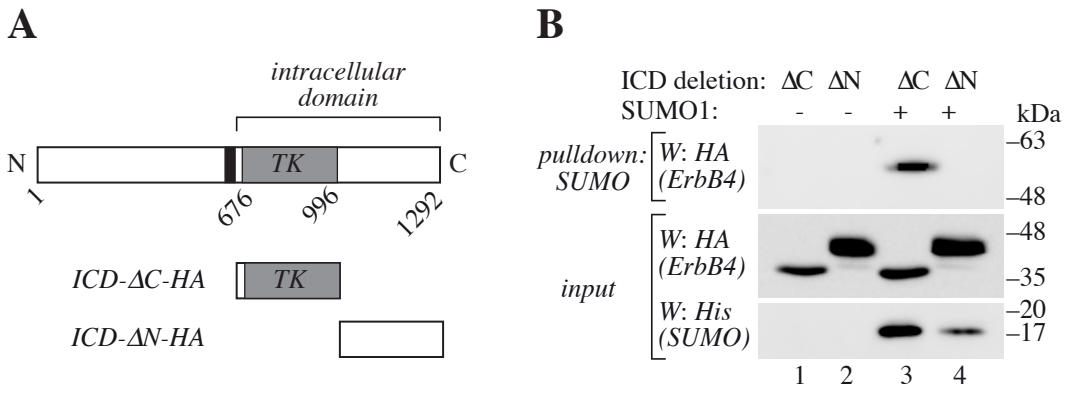

FIGURE S4. SUMOylation of the ErbB4 kinase domain.

A) Schematic structure of ErbB4 and deletion constructs ICD-ΔC (containing amino acid residues 676-996) and ICD-ΔN (containing amino acid residues 997-1292) (5). Black color indicates the transmembrane domain and TK indicates the tyrosine kinase domain.

B) COS-7 cells were transfected with HA-tagged ErbB4 ICD deletion constructs and His-tagged SUMO1 as indicated. Cells were lysed in a denaturing buffer, and lysates were incubated with Ni^{2+}-NTA agarose to pull down His-SUMO conjugates. Whole cell extracts or pull-down samples were analyzed by Western with anti-HA (H3663) or anti-His (H1029; Sigma-Aldrich). SUMOylation of the deletion constructs was examined in two independent experiments.

Fig. S4

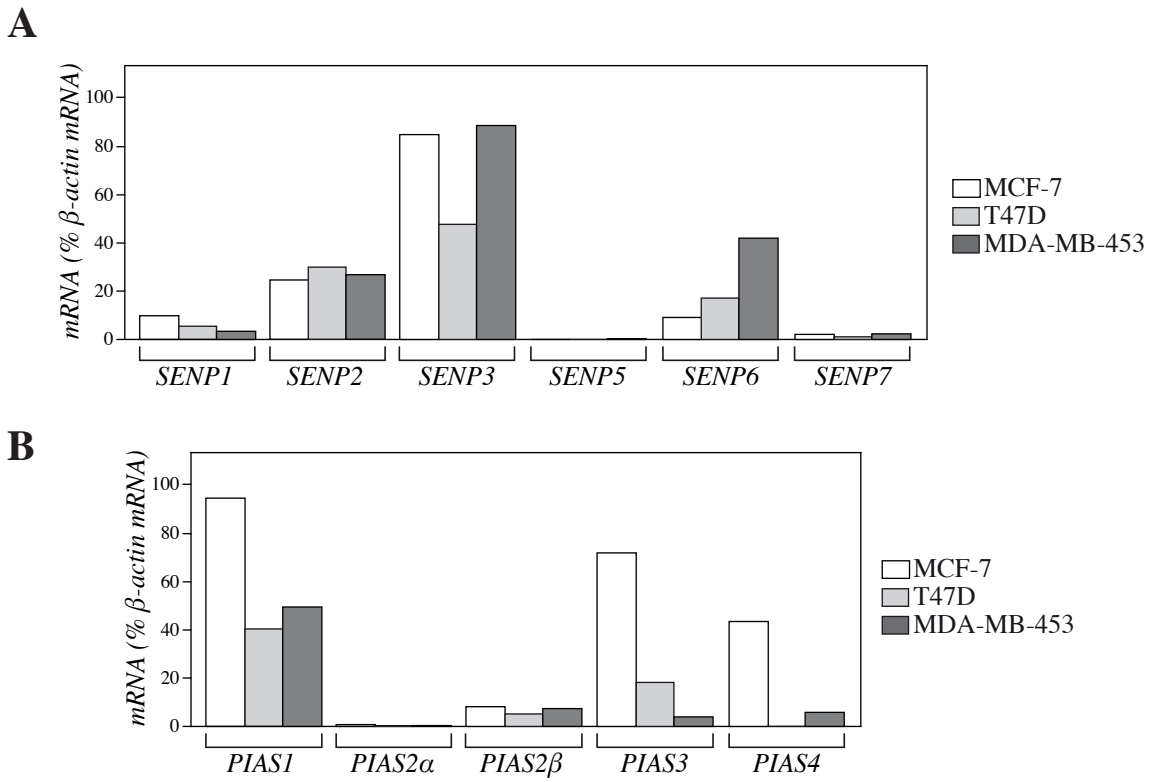

FIGURE S5. Expression of SUMO isopeptidases and E3 ligases in breast cancer cells.
MCF-7, T47D and MDA-MB-453 breast cancer cells were analyzed for the expression of the SENP family of SUMO isopeptidases (A) and the PIAS family of SUMO E3 ligases (B) by qRT-PCR. Expression relative to *β-actin* mRNA was determined from triplicate qRT-PCR reactions of each cDNA. MCF-7 and T47D are estrogen receptor-positive cell lines, and MDA-MB-453 is estrogen receptor-negative (6).

Primers and Universal Probe Library probes for SUMO isopeptidases and ligases were: *SENP1* (left 5'-GCCACCATCAGAAAGAAACG-3', right 5'-TCTTTTCACATCACTGACTTTAGCA-3', probe #15); *SENP2* (left 5'-TCACTGGCTCAATGATGAAGTC-3', right 5'-AGTGCTGGATAGCCTTGCTT-3', probe #31); *SENP3* (left 5'-GGACTCAGGTGGACTCCAAA-3', right 5'-AACCGTTGGGCAGAGTACAT-3', probe #82); *SENP5* (left 5'-CCAGGAACGAGGGTAGCAG-3', right 5'-TGGATTCCTTTCCTCCATAGAA-3', probe #6); *SENP6* (left 5'-TTCCTCGCTGATGACAACTG-3', right 5'-AGTGAGTCCATAAGTAGGATACAAGGT-3', probe #59); *SENP7* (left 5'-AGCGCCTGCAACTGAAAT-3', right 5'-TTTCCTTTTTCCTTCTGTGATGA-3', probe #7); *PIAS1* (left 5'-GACCTGTCCTTCCCTATCTCC-3', right 5'-CTGGAGATGCTTGATGTGGA-3', probe #9); *PIAS2α* (left 5'-CCAATATCAAGCATGTCATCAGA-3', right 5'-TGTTCCAAGCTTCAGTTCATTATT-3', probe #2); *PIAS2β* (left 5'-ACAAAGCAGCCCAACCAA-3', right 5'-AGTCTGTTAATGAAGGCGGAAT-3', probe #18); *PIAS3* (left 5'-GGAGCTGGGCGAATTAAAG-3', right 5'-AGCACCTGGAGCTCAGACAC-3', probe #9); *PIAS4* (left 5'-GGTGAAGCTGCCGTTCTTTA-3', right 5'-TCTCGTTGTTCTGTGGGACTAA-3', probe #42).
β-actin primers and probe were: left 5'-ATCTGGCACCACACCTTCTACAAT-3', right 5'-CCGTCACCGGAGTCCATCA-3', probe 5'-TGACCCAGATCATGTTTGAGACCTTCAACAC-3').

Fig. S5

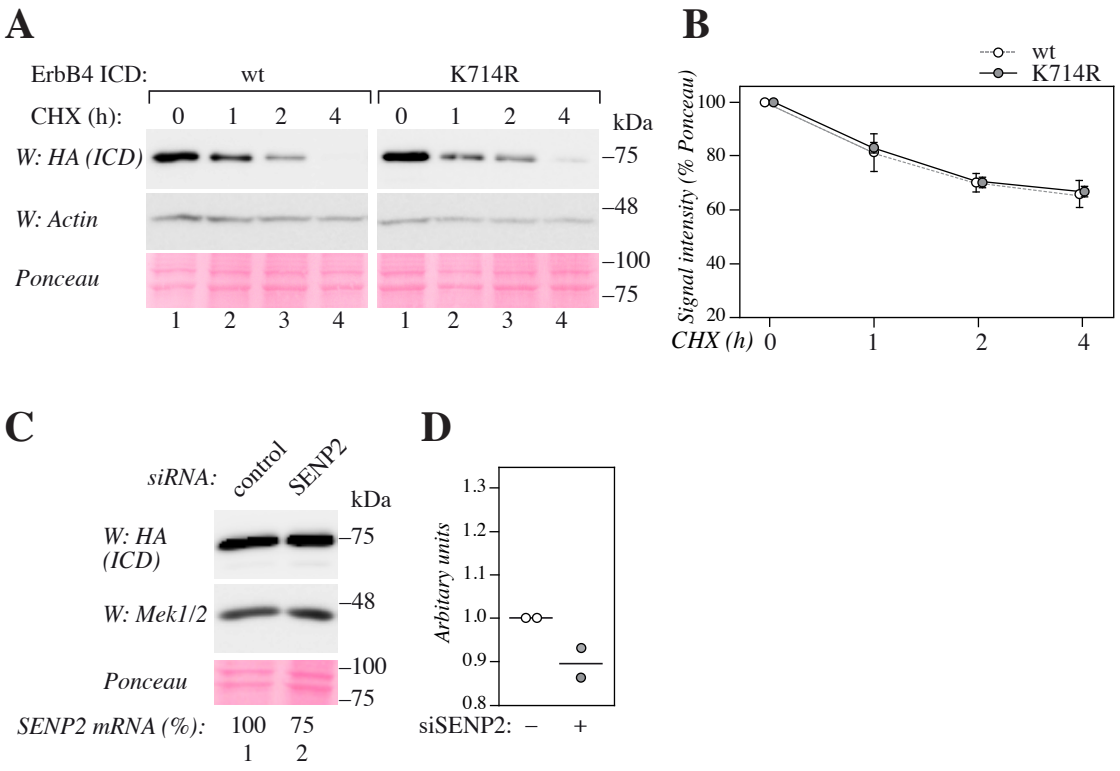

FIGURE S6. Analysis of the stability and abundance of ErbB4 ICD in total cell lysates.
A) MCF-7 cells were transfected with HA-tagged wild-type or K714R ErbB4 ICD, treated with 100 µg/ml cycloheximide (CHX) for the indicated periods of time and lysed. Total cell lysates were analyzed by Western with anti-HA (H3663) and anti-actin. Total protein loading was controlled with Ponceau staining. Representative data of two independent experiments are shown.
B) Quantification of HA (ErbB4 ICD) signal intensities (shown in A) normalized to Ponceau. Data are represented as mean ± range from two independent experiments.
C) MCF-7 cells were transfected with HA-tagged ErbB4 ICD together with control or SENP2 siRNA and lysates. Total cell lysates were analyzed by Western with anti-HA (H3663) and anti-Mek1/2. Total protein loading was controlled with Ponceau staining. Efficiency of RNA interference analyzed by qRT-PCR is indicated as relative SENP2 mRNA levels in cells treated with SENP2-targeting siRNA compared to cells treated with control siRNA (SENP2 mRNA %). Representative data of two independent experiments are shown.
D) Quantification of HA (ErbB4 ICD) signal intensities (shown in C) normalized to Ponceau. The circles indicate individual data points from two independent experiments and the horizontal line indicates the mean.

Fig. S6

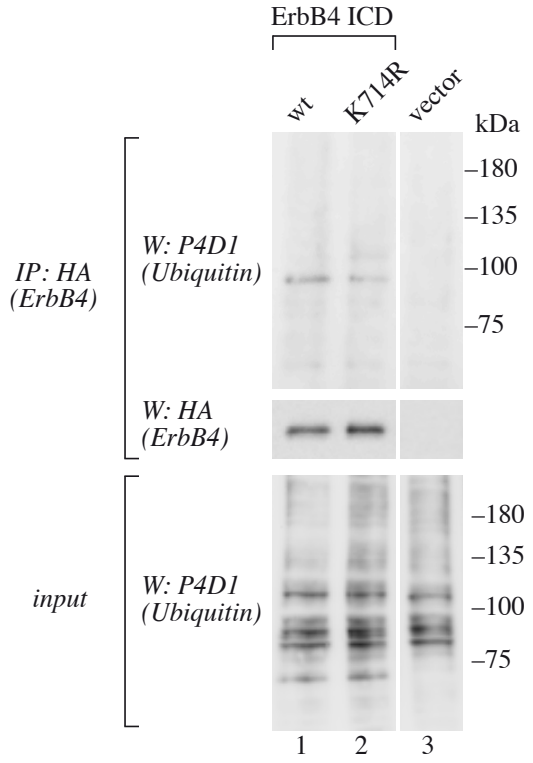

FIGURE S7. Analysis of the ubiquitination of ErbB4 ICD.
MCF-7 cells were transfected with HA-tagged wild-type of K714R ErbB4 ICD and subjected to fractionation to isolate nuclear proteins. Nuclear lysates were immunoprecipitated with anti-HA (H3663) and analyzed by Western using anti-Ubiquitin (P4D1; Santa Cruz Biotechnology) and anti-HA (H3663). Representative data of two independent experimets are shown.

Fig. S7

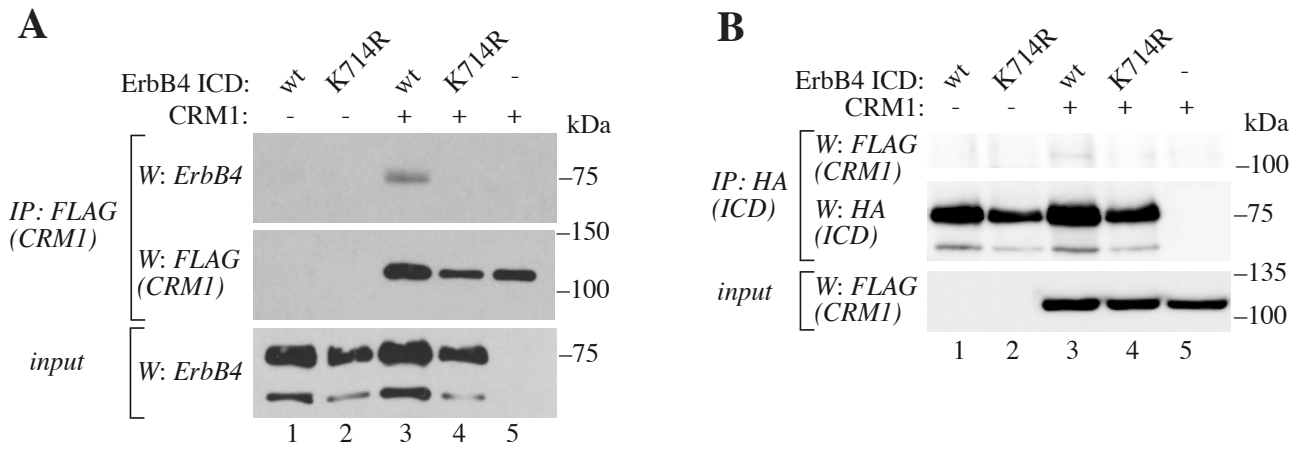

FIGURE S8. Coprecipitation of wild-type and K714R ErbB4 ICD with CRM1.
A) COS-7 cells were transfected with wild-type or K714R ErbB4 ICD with or without FLAG-tagged CRM1. Lysates were immunoprecipitated with anti-FLAG followed by Western with anti-ErbB4. The membrane was reprobed with anti-FLAG.
B) MCF-7 cells were transfected with wild-type or K714R mutant HA-tagged ErbB4 ICD with or without FLAG-tagged CRM1. Lysates were immunoprecipitated with anti-HA (H3663) followed by Western with anti-FLAG. The membrane was reprobed with anti-FLAG.
The experiment comparing the interaction of wild-type and K714R ErbB4 ICD was carried out three times.

Fig. S8

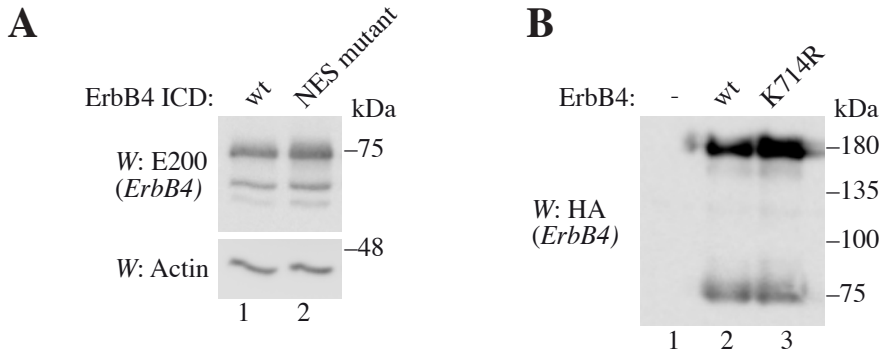

FIGURE S9. Expression levels of ErbB4 constructs.
A) COS-7 cells were transfected with ErbB4 ICD wild-type or a NES mutant construct used in Fig 5D and E (that encodes the V721A, V723A, and L724A amino acid substitutions). The expression was analyzed with anti-ErbB4 (E200). Equal loading was controlled with anti-actin.
B) MCF-7 cells were transfected with HA-tagged wild-type or K714R ErbB4, and the expression levels of ErbB4 constructs were analyzed with anti-HA (H3663). The Western analysis was conducted simultaneously with the experimentation described in Fig 5F and G.

Fig. S9

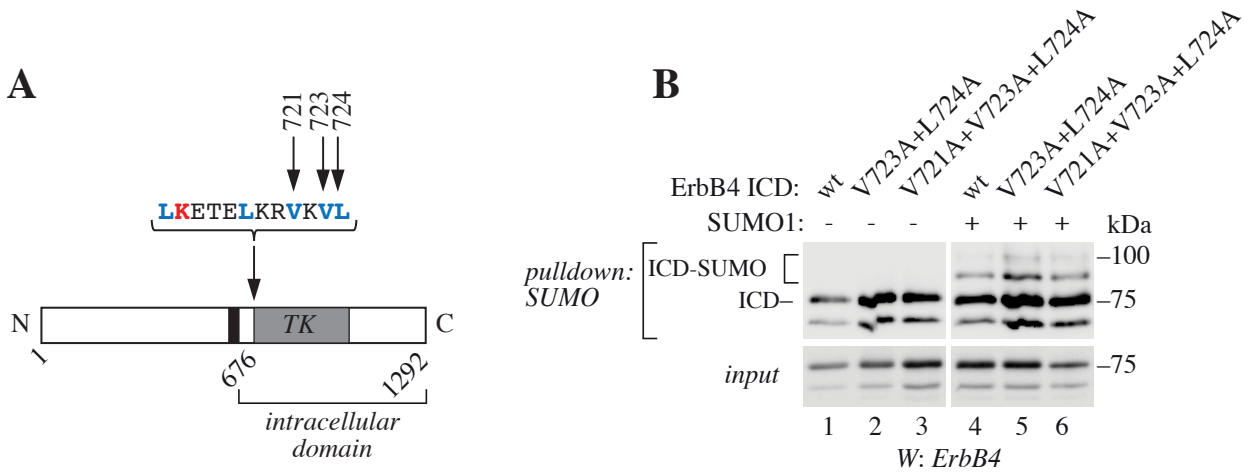

FIGURE S10. SUMOylation of ErbB4 ICD with V721A, V723A, L724A mutations.
A) Schematic structure of ErbB4. Arrows indicate the mutated amino acids analyzed in B. Black color indicates the transmembrane domain and TK indicates the tyrosine kinase domain.
B) COS-7 cells were transfected with wild-type or mutant HA-tagged ErbB4 ICD constructs and His-tagged SUMO1. Cells were lysed in a denaturing buffer, and lysates were incubated with Ni^{2+}-NTA agarose to pull down His-SUMO conjugates. Whole cell extracts or pull-down samples were analyzed by Western with anti-ErbB4. Representative data of two independent experiments are shown.

Fig. S10

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