Supplementary Material

A novel prodrug-like fusion toxin with protease-sensitive bioorthogonal PEGylation for tumor targeting

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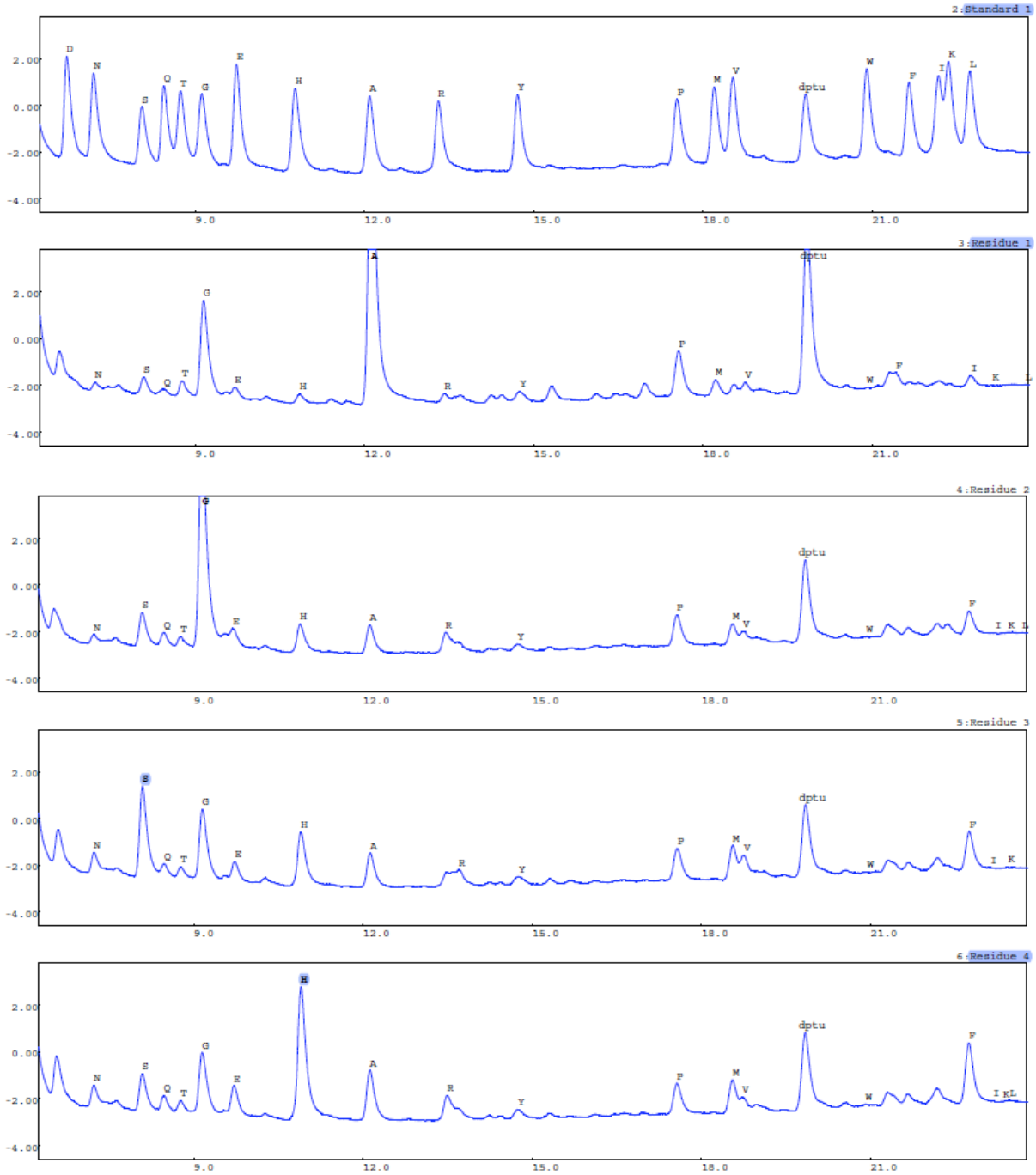


Figure S1 N-terminal sequence of Aha-Ala-Gly-Ser-His$_6$-Ec1-ETA" expressed in M9 medium containing Aha, as determined by Edman degradation. The top graph represents the standards, the following four graphs the first four Edman cycles. The major sequence of the first four residues was Ala-Gly-Ser-His, with a minor fraction of Gly-Ser-His-His, confirming complete processing of the initiator Aha (elution time of Aha: 16 min).

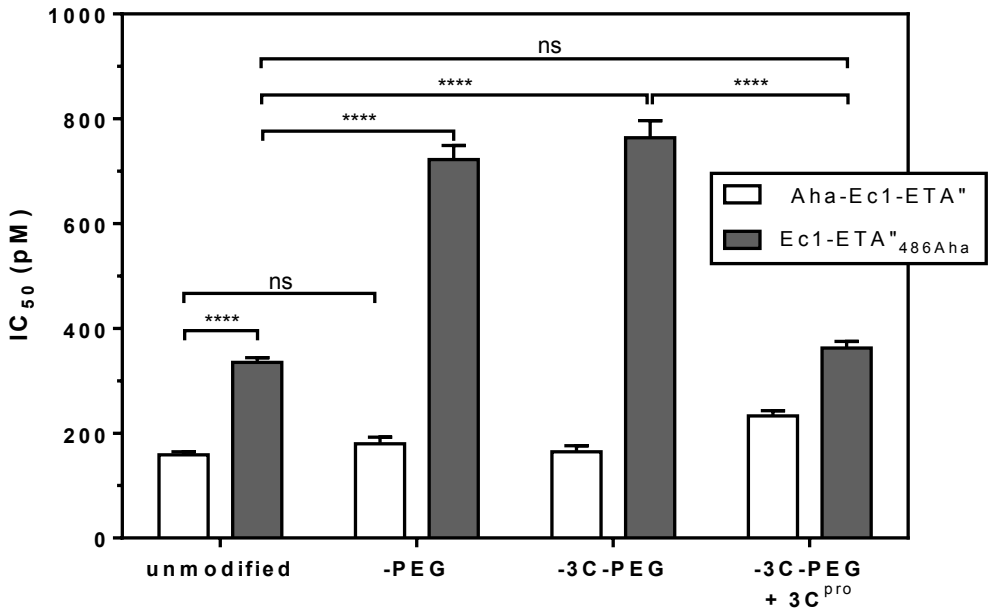
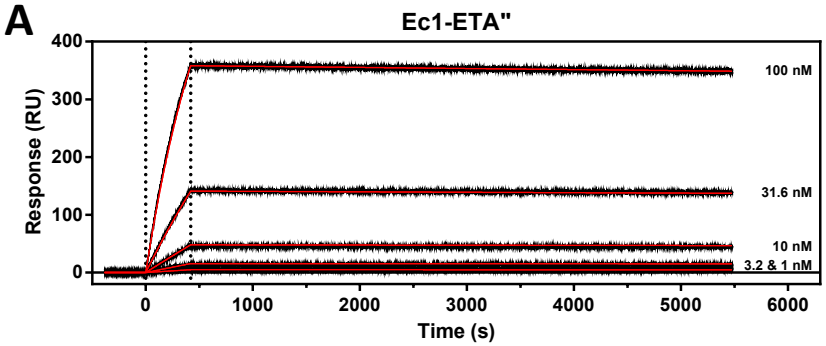
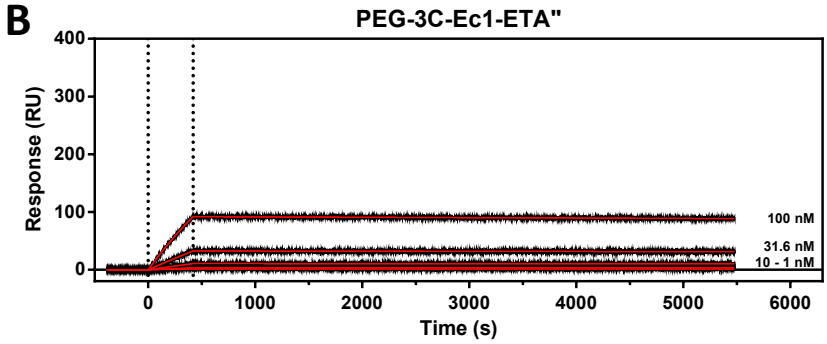
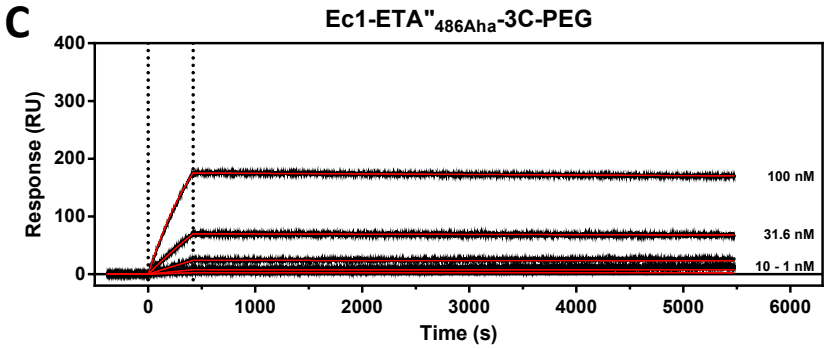
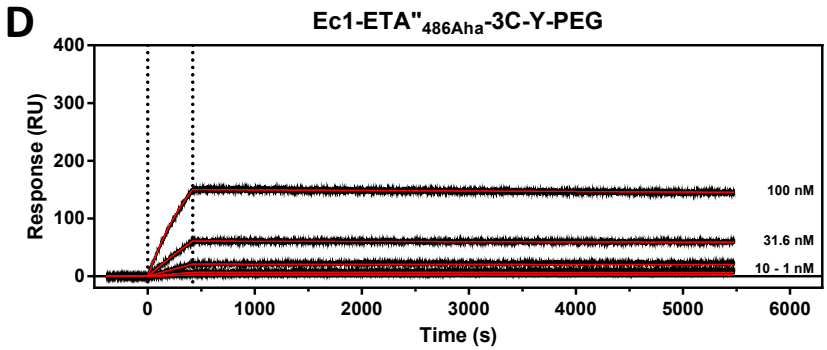


Figure S2 Catalytic activity (ADP-ribosylation activity) of wild-type Ec1-ETA" and Ec1-ETA"$_{486Aha}$ in the unPEGylated, PEGylated and proteolytically dePEGylated form as calculated from inhibition of protein translation *in vitro*. Data are presented as mean IC_{50} values ± SD. Statistical analysis was done using two-way ANOVA with Tukey post-test; ns: not significant ($p \geq 0.05$), **** $p < 0.0001$


A
Ec1-ETA"
Response (RU)
Time (s)
100 nM
31.6 nM
10 nM
3.2 & 1 nM


B
PEG-3C-Ec1-ETA"
Response (RU)
Time (s)
100 nM
31.6 nM
10 - 1 nM


C
Ec1-ETA"486Aha-3C-PEG
Response (RU)
Time (s)
100 nM
31.6 nM
10 - 1 nM


D
Ec1-ETA"486Aha-3C-Y-PEG
Response (RU)
Time (s)
100 nM
31.6 nM
10 - 1 nM

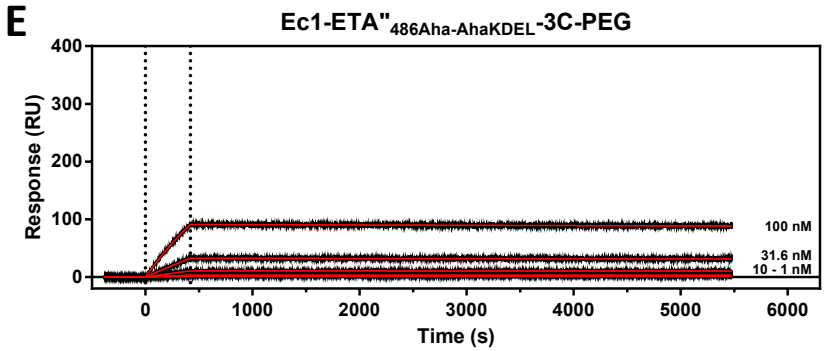

E
Ec1-ETA"486Aha-AhaKDEL-3C-PEG
Response (RU)
Time (s)
100 nM
31.6 nM
10 - 1 nM

Figure S3 Affinity of fusion toxins determined by surface plasmon resonance measurements. EpCAM binding was measured in duplicates using a ProteOn XPR36 (Bio-Rad Laboratories). Data were fitted to a 1:1 model (Langmuir) to obtain kinetic parameters using the ProteOn Manager software (Bio-Rad) and exported Prism (GraphPad) for illustration. Calculated values are shown in Table 2.

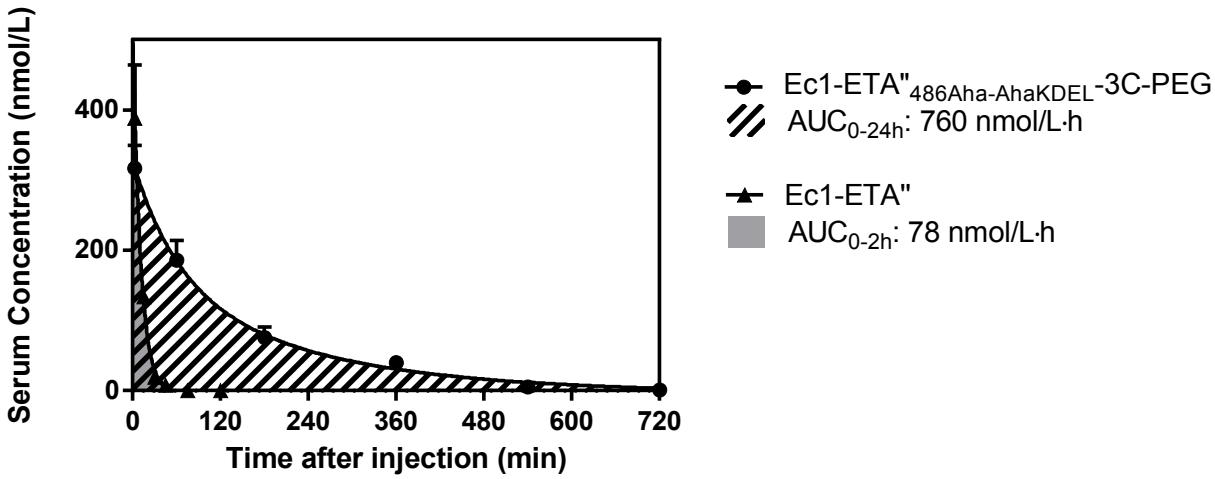


Figure S4 Blood clearance of Ec1-ETA" (n = 4) and Ec1-ETA" 486Aha-AhaKDEL-3C-PEG (n = 3) following intravenous injection. C57BL/6 mice received a single dose of 20 nmol/kg bodyweight and blood samples were drawn at the indicated time points (for clarity, the 24 h time point was omitted in the graph). Serum concentration of the ETA" constructs was analyzed by ELISA.

Table S1 Cytotoxicity of single Aha mutants of Ec1-ETA”

Protein	IC_{50} (M)[a]	Relative activity (%)[b]
Ec1-ETA“	1.9×10^{-13}	100
Aha-Ec1-ETA“	1.6×10^{-13}	120
Ec1-ETA“$_{420Aha}$	3.9×10^{-13}	49
Ec1-ETA“$_{459Aha}$	2.0×10^{-13}	96
Ec1-ETA“$_{461Aha}$	3.3×10^{-13}	56
Ec1-ETA“$_{463Aha}$	4.3×10^{-13}	44
Ec1-ETA“$_{476Aha}$	2.9×10^{-13}	65
Ec1-ETA“$_{486Aha}$	2.5×10^{-13}	75
Ec1-ETA“$_{489Aha}$	3.3×10^{-13}	56
Ec1-ETA“$_{519Aha}$	3.2×10^{-13}	59
Ec1-ETA“$_{547Aha}$	2.6×10^{-13}	73
Ec1-ETA“$_{548Aha}$	1.7×10^{-13}	108
Ec1-ETA“$_{553Aha}$	4.5×10^{-12}	4.2
Ec1-ETA“$_{574Aha}$	1.1×10^{-12}	16
Ec1-ETA“$_{583Aha}$	2.7×10^{-13}	71
Ec1-ETA“$_{585Aha}$	4.2×10^{-13}	45
Ec1-ETA“$_{586Aha}$	3.5×10^{-13}	54
Ec1-ETA“$_{AhaKDEL}$	2.2×10^{-13}	86

[a]IC_{50} values calculated in XTT assays with HT29 cells. Data were derived from the curves shown in Fig. 2.

[b]Relative activity is defined as 100 multiplied by the IC_{50} of Ec1-ETA divided by the IC_{50} of the mutant.

Table S2 Cytotoxicity of reversibly PEGylated Ec1-ETA" mutants

IC_{50} (M)[a]				Relative activity (% of Aha-Ec1-ETA")[b]			
no PEG	3C-PEG	3C-PEG + $3C^{pro}$	Protein	no PEG	3C-PEG	3C-PEG + $3C^{pro}$	Reactivation index[c]
1.8×10^{-13}	2.3×10^{-12}	1.9×10^{-13}	Aha-Ec1-ETA"	100.0	7.8	94.5	12.1
2.7×10^{-13}	5.9×10^{-12}	2.8×10^{-13}	Ec1-ETA"$_{459Aha}$	68.7	3.1	64.4	20.8
2.9×10^{-13}	2.8×10^{-12}	3.1×10^{-13}	Ec1-ETA"$_{461Aha}$	63.2	6.5	59.9	9.3
1.5×10^{-13}	4.2×10^{-12}	1.3×10^{-13}	Ec1-ETA"$_{486Aha}$	119.8	4.4	139.2	31.6
3.3×10^{-13}	6.8×10^{-12}	2.9×10^{-13}	Ec1-ETA"$_{489Aha}$	56.0	2.7	63.6	23.6
3.7×10^{-12}	8.6×10^{-11}	1.0×10^{-11}	Ec1-ETA"$_{553Aha}$	5.0	0.2	1.8	8.3
9.0×10^{-13}	4.2×10^{-11}	4.8×10^{-12}	Ec1-ETA"$_{574Aha}$	20.4	0.4	3.8	8.8
2.8×10^{-13}	4.3×10^{-12}	5.6×10^{-13}	Ec1-ETA"$_{586Aha}$	64.4	4.3	32.9	7.7
3.0×10^{-13}	9.8×10^{-12}	6.8×10^{-13}	Ec1-ETA"$_{AhaKDEL}$	61.0	1.9	27.0	14.4

[a]IC_{50} values and reactivation indices calculated in XTT assays with HT29 cells. The first column is the unPEGylated mutant, the second the PEGylated variant, and the third the PEGylated variant after cleavage with 3C protease.

[b]Relative activity is defined as 100 multiplied by the IC_{50} of unPEGylated Aha-Ec1-ETA divided by the IC_{50} of the respective variant.

[c]The reactivation index is defined in eq. 1.

Table S3 Cytotoxicity of PEGylated fusion toxins against various tumor cell lines

	IC_{50} (M)[a]					
	no PEG	3C-PEG	3C-PEG + $3C^{pro}$	PEG	Protein	Reactivation index[b]
HT29	5.3×10^{-14}	9.0×10^{-13}	7.1×10^{-14}	7.8×10^{-13}	Aha-Ec1-ETA“	12.6
	9.9×10^{-11}	1.7×10^{-9}	1.6×10^{-10}	1.8×10^{-9}	Ec1 + Aha-Ec1-ETA“	10.9
	5.2×10^{-14}	9.5×10^{-13}	4.6×10^{-14}	1.3×10^{-12}	Ec1-ETA“$_{486Aha}$	20.6
	1.0×10^{-10}	3.0×10^{-9}	1.1×10^{-10}	5.3×10^{-9}	Ec1 + Ec1-ETA“$_{486Aha}$	28.4
MCF7	3.2×10^{-14}	2.0×10^{-13}	4.3×10^{-14}	1.6×10^{-13}	Aha-Ec1-ETA“	4.8
	1.3×10^{-11}	1.1×10^{-10}	1.5×10^{-11}	1.7×10^{-10}	Ec1 + Aha-Ec1-ETA“	7.1
	2.7×10^{-14}	3.7×10^{-13}	4.6×10^{-14}	4.7×10^{-13}	Ec1-ETA“$_{486Aha}$	8.1
	1.1×10^{-11}	2.2×10^{-10}	1.2×10^{-11}	2.6×10^{-10}	Ec1 + Ec1-ETA“$_{486Aha}$	18.8
A431	1.5×10^{-11}	1.7×10^{-10}	2.3×10^{-11}	*n.d.*	Aha-Ec1-ETA“	7.6
	2.1×10^{-9}	1.1×10^{-10}	2.2×10^{-9}	*n.d.*	Ec1 + Aha-Ec1-ETA“	4.8
	1.5×10^{-11}	7.1×10^{-10}	2.3×10^{-11}	*n.d.*	Ec1-ETA“$_{486Aha}$	30.2
	3.1×10^{-9}	9.1×10^{-8}	3.6×10^{-9}	*n.d.*	Ec1 + Ec1-ETA“$_{486Aha}$	25.6
MKN-45	6.2×10^{-13}	8.8×10^{-12}	8.7×10^{-13}	*n.d.*	Aha-Ec1-ETA“	10.1
	1.1×10^{-9}	1.6×10^{-8}	1.3×10^{-9}	*n.d.*	Ec1 + Aha-Ec1-ETA“	11.7
	1.3×10^{-12}	1.7×10^{-11}	8.9×10^{-13}	*n.d.*	Ec1-ETA“$_{486Aha}$	18.9
	1.5×10^{-9}	4.1×10^{-8}	1.4×10^{-9}	*n.d.*	Ec1 + Ec1-ETA“$_{486Aha}$	29.6
SKOV3	2.9×10^{-12}	2.2×10^{-11}	4.1×10^{-12}	*n.d.*	Aha-Ec1-ETA“	5.4
	1.5×10^{-8}	7.2×10^{-8}	2.3×10^{-8}	*n.d.*	Ec1 + Aha-Ec1-ETA“	3.2
	2.6×10^{-12}	3.2×10^{-11}	3.4×10^{-12}	*n.d.*	Ec1-ETA“$_{486Aha}$	9.5
	1.9×10^{-8}	2.3×10^{-7}	1.7×10^{-8}	*n.d.*	Ec1 + Ec1-ETA“$_{486Aha}$	13.6
SKBR3	1.2×10^{-11}	5.9×10^{-11}	1.3×10^{-11}	*n.d.*	Aha-Ec1-ETA“	4.4
	1.9×10^{-8}	7.4×10^{-8}	2.2×10^{-8}	*n.d.*	Ec1 + Aha-Ec1-ETA“	3.4
	1.9×10^{-11}	5.8×10^{-11}	2.0×10^{-11}	*n.d.*	Ec1-ETA“$_{486Aha}$	3
	2.9×10^{-8}	2.4×10^{-7}	2.3×10^{-8}	*n.d.*	Ec1 + Ec1-ETA“$_{486Aha}$	10.7

[a]IC_{50} values and reactivation indices calculated in XTT assays.

[b]The reactivation index is defined in eq. 1.

Table S4 Cytotoxicity of differentially PEGylated Ec1-ETA" and Off7ΔM-ETA" fusion toxins against various tumor cell lines

	IC_{50} (M)[a]			
	HT29	MCF-7	MDA-MB-468	MDA-MB-231
Ec1-ETA"$_{486Aha}$	4.8×10^{-14}	2.5×10^{-14}	3.6×10^{-13}	3.0×10^{-11}
Ec1-ETA"$_{486Aha}$-3C-PEG	9.2×10^{-13}	2.2×10^{-13}	4.7×10^{-12}	1.0×10^{-09}
$3C^{pro}$ + Ec1-ETA"$_{486Aha}$-3C-PEG	3.6×10^{-14}	3.2×10^{-14}	4.0×10^{-13}	2.1×10^{-11}
Reactivation index[b]	***26.0***	***6.9***	***12.0***	***49.5***
Off7-ETA"$_{486Aha}$	2.0×10^{-9}	4.0×10^{-11}	1.1×10^{-9}	9.7×10^{-9}
Off7-ETA"$_{486Aha}$-3C-PEG	5.4×10^{-8}	1.0×10^{-9}	3.2×10^{-8}	1.7×10^{-7}
$3C^{pro}$ + Off7-ETA"$_{486Aha}$-3C-PEG	1.4×10^{-9}	2.8×10^{-11}	5.7×10^{-10}	4.5×10^{-9}
Reactivation index[b]	***39.0***	***35.6***	***55.6***	***37.4***
Ec1-ETA"$_{486Aha\text{-}AhaKDEL}$	5.5×10^{-14}	3.4×10^{-14}	4.4×10^{-13}	2.8×10^{-11}
Ec1-ETA"$_{486Aha\text{-}AhaKDEL}$-3C-PEG	3.7×10^{-11}	4.1×10^{-12}	1.1×10^{-10}	5.7×10^{-8}
$3C^{pro}$ + Ec1-ETA"$_{486Aha\text{-}AhaKDEL}$-3C-PEG	5.9×10^{-14}	3.7×10^{-14}	5.9×10^{-13}	4.6×10^{-11}
Reactivation index[b]	***637.0***	***110.8***	***185.6***	***1221.4***
Off7-ETA"$_{486Aha\text{-}AhaKDEL}$	2.2×10^{-9}	4.5×10^{-11}	1.1×10^{-9}	1.0×10^{-8}
Off7-ETA"$_{486Aha\text{-}AhaKDEL}$-3C-PEG	1.5×10^{-6}	3.9×10^{-8}	1.3×10^{-6}	1.6×10^{-6}
$3C^{pro}$ + Off7-ETA"$_{486Aha\text{-}AhaKDEL}$-3C-PEG	3.7×10^{-9}	6.0×10^{-11}	1.7×10^{-9}	1.1×10^{-8}
Reactivation index[b]	***412.6***	***650.3***	***750.9***	***143.1***
Ec1-ETA"$_{486Aha}$	5.0×10^{-14}	*n.d.*	*n.d.*	*n.d.*
Ec1-ETA"$_{486Aha}$-3C-Y-PEG	3.7×10^{-12}	*n.d.*	*n.d.*	*n.d.*
$3C^{pro}$ + Ec1-ETA"$_{486Aha}$-3C-Y-PEG	4.6×10^{-14}	*n.d.*	*n.d.*	*n.d.*
Reactivation index[b]	***80.5***			
Off7-ETA"$_{486Aha}$	2.5×10^{-9}	*n.d.*	*n.d.*	*n.d.*
Off7-ETA"$_{486Aha}$-3C-Y-PEG	8.7×10^{-8}	*n.d.*	*n.d.*	*n.d.*
$3C^{pro}$ + Off7-ETA"$_{486Aha}$-3C-Y-PEG	1.4×10^{-9}	*n.d.*	*n.d.*	*n.d.*
Reactivation index[b]	***62.1***			

[a]IC_{50} values as calculated in XTT assays.

[b]The reactivation index is defined in eq. 1.

Table S5 Primers used for site-directed mutagenesis, altered positions are indicated by small caps.

Name	Sequence
RBS_f	TCATCACCATAAAGACGAGC
RBS_r	ATCCCGCCATATTTTGCTCCTTACTGC
MAGS_f	ATGGCGGGATCGCATCACCATCACC
MAGS_r	ATATGGATCCGTGATGGTGATGGTG
MKDEL_f	TCATCACCATatgAAAGACGAGCTGTGATGATAG
MKDEL_r	GCTCGTCTTTcatATGGTGATGATGATGGTGAG
E420M_f	TTGGACAGTCatgCGCCTGCTGCAAGCCCATCG
E420M_r	GCAGCAGGCGcatGACTGTCCAATTCTGTGTCC
S459M_f	CCGTGCTCGTatgCAAGACCTGGACGCCATTTG
S459M_r	CCAGGTCTTGcatACGAGCACGGACCCCGCCAA
D461M_f	TCGTAGTCAAatgCTGGACGCCATTTGGCGTGG
D461M_r	TGGCGTCCAGcatTTGACTACGAGCACGGACCC
D463M_f	TCAAGACCTGatgGCCATTTGGCGTGGGTTTTATATC
D463M_r	GCCAAATGGCcatCAGGTCTTGACTACGAGCAC
A476M_f	CGGTGATCCAatgCTGGCATATGGTTATGCTCAG
A476M_r	CATATGCCAGcatTGGATCACCGGCGATATAAAACC
E486M_f	TCAGGATCAGatgCCTGACGCTCGTGGTCGTAT
E486M_r	GAGCGTCAGGcatCTGATCCTGAGCATAACCAT
A489M_f	GGAGCCTGACatgCGTGGTCGTATTCGTAACGG
A489M_r	TACGACCACGcatGTCAGGCTCCTGATCCTGAG
A519M_f	TCTGACACTGatgGCCCCGGAAGCAGCTGGGGA
A519M_r	CTTCCGGGGCcatCAGTGTCAGACCAGTACGAT
E547M_f	AGGCCCGGAAatgGAAGGAGGCCGTCTGGAGAC
E547M_r	GGCCTCCTTCcatTTCCGGGCCTGTAATAGCATCC
E548M_f	CCCGGAAGAAatgGGAGGCCGTCTGGAGACTAT
E548M_r	GACGGCCTCCcatTTCTTCCGGGCCTGTAATAG
E553M_f	AGGCCGTCTGatgACTATTCTGGGTTGGCCAC
E553M_r	CCAGAATAGTcatCAGACGGCCTCCTTCTTCTTCC
D574M_f	TATCCCGACCatgCCTCGTAATGTTGGTGGTGATC
D574M_r	CATTACGAGGcatGGTCGGGATAGCGCTCGGAA
D583M_f	TGGTGATCTGatgCCTAGCAGTATCCCTGATAAAG
D583M_r	TACTGCTAGGcatCAGATCACCACCAACATTAC
S585M_f	TCTGGACCCTatgAGTATCCCTGATAAAGAACAGGC
S585M_r	CAGGGATACTcatAGGGTCCAGATCACCACCAAC
S586M_f	GACCCTAGCatgATCCCTGATAAAGAACAGGCAATC
S586M_r	TTATCAGGGATcatGCTAGGGTCCAGATCACCAC