Supporting Information

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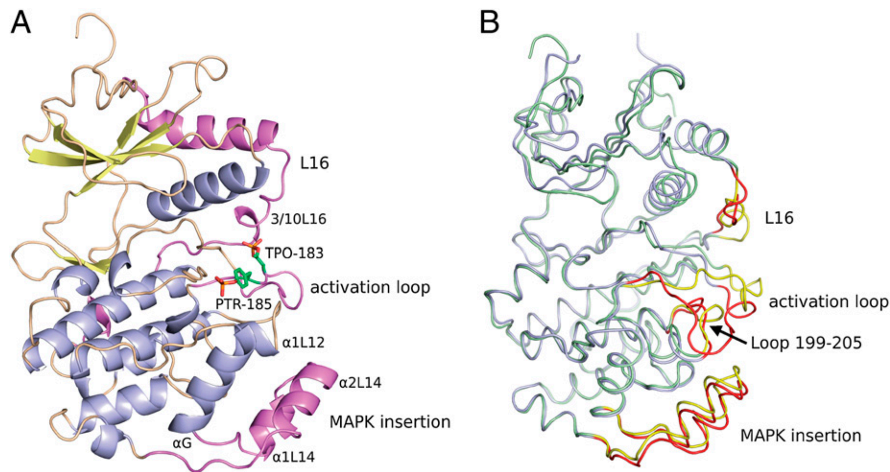

Fig. S1. Topology and structural comparison of extracellular signal-regulated kinase 2 (ERK2) and doubly phosphorylated ERK2. (*A*) Ribbon diagram of phosphorylated ERK2 (pERK2) [Protein Data Bank (PDB) ID 2ERK]. Structural segments of ERK2/pERK2 that are implicated in binding of designed ankyrin repeat proteins (DARPins) E40 to ERK2 and pE59 to pERK2 contain the activation loop, the mitogen-activated protein kinase (MAPK) insertion, α1L12, and αG. Regions unique to MAP kinases are indicated in magenta. The side chains of pThr-183 (TPO-183) and pTyr-185 (PTR-185) are indicated. (*B*) A superposition of ERK2 (PDB ID 1ERK, blue) and pERK2 (green) is shown. Segments of structural divergence are highlighted in red (ERK2) and gold (pERK2).

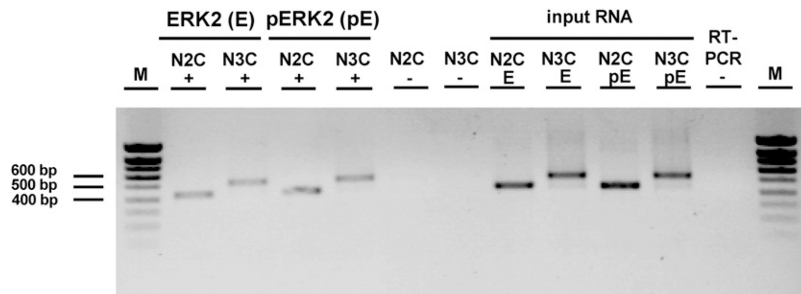

Fig. S2. Enrichment and specificity of ribosome display (RD) selections. The outcome of RD was monitored at the level of RT-PCR product yield by agarose gel electrophoresis. The RT-PCR yields of the fourth selection round were compared for the N2C and the N3C library selected against ERK2 and phospho-ERK2 (pERK2) (+). The specificities of selected N2C and N3C pools were checked by panning against neutravidin + BSA (–). The amount and quality of the selection input RNA was verified separately for each selection by RT-PCR amplification. A RT-PCR without RNA added (–) served as a negative control. All RT-PCR reactions yielded products of the expected size.

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        N-cap                          1. repeat
N2C     DLGKKLLEAARAGQDDEVRILMANGADVNA XDXXGXTPLHLAAXXGHLEIVEVLLKXGADVNA
E8      ------------------------------ M-NT-F-------QI-----------Y------
E38     -----------V--------P--------- R-DI-F-L---S-SM-----------N------
pE55    ---------T-------------------T R-QI-L-------QI-----------H------
pE57    ---------------------V-------- L-EE-L-------QM-----------N------
pE59    ------------------------------ L-ED-L-------QL-----------Y------
pE63    ------------------------------ S-WADS-------KK-----------H------
EpE82   ------------------------------ F-QI-L------SFE-----------Y------
EpE89   ------------------------------ F-NI-L-------QW-----------H------

        2. repeat                         C-cap
N2C     XDXXGXTPLHLAAXXGHLEIVEVLLKXGADVNA QDKFGKTAFDISIDNGNEDLAEILQKL
E8      K-AY-V-------DF-----------H------ ---------------------------
E38     T-FF-V-------DM-----------Y------ ---------------------------
pE55    Y-QW-I-------QK-----------Y------ ---------------------------
pE57    N-SY-I-------TR-----------Y------ ---------------------------
pE59    E-NF-I-------IR-----------H------ ---------------------------
pE63    K-DY-H-------FV-----------Y------ ---------------------------
EpE82   I-SY-I-------LH-----------Y------ ---------------------------
EpE89   K-IY-I-------AK-----------H------ ---------------------------

        N-cap                          1. repeat
N3C     DLGKKLLEAARAGQDDEVRILMANGADVNA XDXXGXTPLHLAAXXGHLEIVEVLLKXGADVNA
E39     ------------------------------ I-HD-S-------TY--------V--N------
E40     ------------------------------ H-DQ-S-------WI--P--------H------

        2. repeat                         3. repeat
N3C     XDXXGXTPLHLAAXXGHLEIVEVLLKXGADVNA XDXXGXTPLHLAAXXGHLEIVEVLLKXGADVNA
E39     T-DF-F-------LN-----------N------ N-SF-I-------IY-----------Y------
E40     R-TD-W-------DN-----------Y------ Q-AY-L-------DR-----------H------

        C-cap
N3C     QDKFGKTAFDISIDNGNEDLAEILQKL
E39     ---------------------------
E40     ---------------------------
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Fig. S3. Sequences of selected DARPins. Sequences of selected N2C DARPins and N3C DARPins were aligned using ClustalW and are shown in single-letter code. Only residues that differ from the consensus sequence are indicated. Positions randomized in the original design are framed and marked with X in the N2C and N3C consensus. Framework positions that mutated during selections are shown in boldface type. The positions of the designed cap regions and internal repeat modules are presented schematically.

Fig. S4. Competition ELISA of selected DARPins with ERK2 and pERK2. Selected DARPins (10 nM) were incubated with varying concentrations of free ERK2 or pERK2 before binding to the corresponding immobilized ERK2 form. The blue bars indicate the signal in the absence of competitor. The binding of selected DARPins was specifically inhibited by increasing concentrations of ERK2 or pERK2 (bars in shades of magenta). (*A*) DARPins selected for ERK (named E) and ERK/pERK (named EpE) binding were examined for specific inhibition by increasing concentrations of free ERK2. (*B*) DARPins selected for pERK (named pE) and ERK/pERK were analyzed for specific inhibition by free pERK2.

Fig. S5. Epitope mapping of selected DARPins by ELISA. Selected DARPins were tested in a sandwich ELISA format for binding to ERK2 or phospho-ERK2 (pERK2) in the presence and absence of ERK2-binding DARPin E40 or pERK2-binding DARPin pE59. (*A*) Immobilization of DARPins E40 and pE59 was confirmed by ELISA-based detection of the DARPin His_6-tag. (*B*) In a second step, biotinylated ERK2 (bERK2) and biotinylated pERK2 (bpERK2) were captured by the immobilized DARPins E40 (bERK2 capture) and pE59 (bpERK2 capture) shown in *A* and detected by an antibiotin antibody. (*C*) Selected ERK2-binding DARPins were tested for their binding capability (solid bars) to ERK2 previously captured by E40 (*B*). The background ELISA signal was determined in wells to which no DARPin was added to immobilized E40 and captured ERK2 (open bars). The nonbinding DARPin E3_5 was used as a negative control. DARPins were detected by their MRGS-His_6-tag. The detection of the MRGS-His6-tag is specific, because the His_6-tag of the capture DARPins is not detected by the anti–RGS-His antibody. (*D*) For selected pERK2-binding DARPins, the assay was perfomed in the same way as described in *C*.

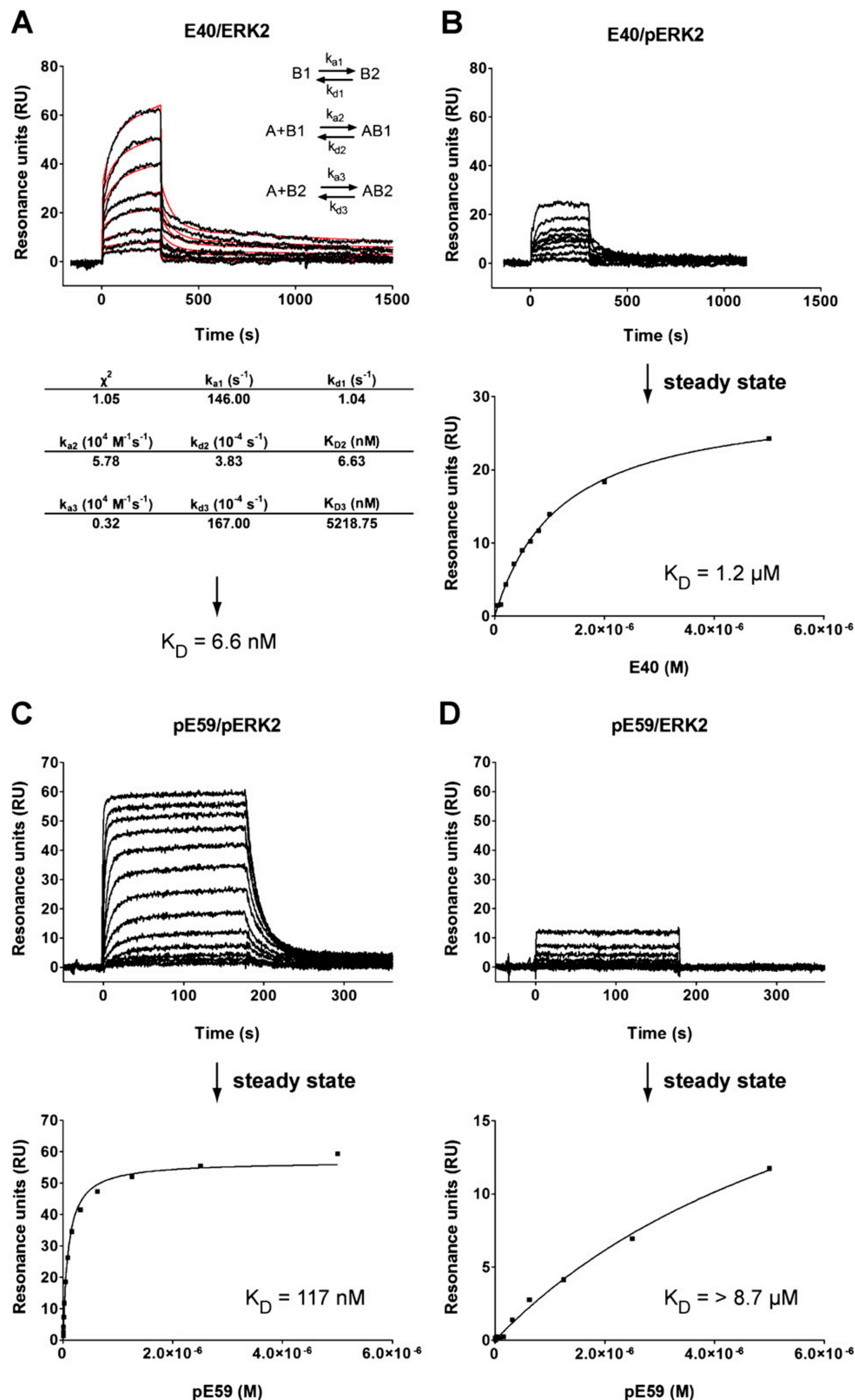

Fig. S6. Affinity determination of DARPins E40 and pE59 using surface plasmon resonance (SPR). The binding kinetics of DARPins E40 and pE59 to ERK2 and pERK2 were monitored using Biacore. ERK2 and pERK2 were immobilized at low concentrations and the response of varied amounts of DARPins was compared with an empty flow cell. Three independent experiments were carried out for each DARPin/kinase combination. Representative results are depicted. (*A*) Different concentrations of E40 (25, 50, 100, 200, 300, 400, 500, 650, 1,000, and 2,000 nM) were applied to a flow cell with immobilized ERK2. The global fit using the indicated model is shown in red. Extracted kinetic data are shown accordingly. (*B*) Binding of E40 (50, 100, 200, 350, 500, 650, 800, 1,000, 2,000, and 5,000 nM) to immobilized pERK2 was analyzed. The K_D was derived from the equilibrium binding responses. (*C* and *D*) Increasing concentrations of pE59 (1.2, 2.4, 4.9, 9.8, 19.5, 39.1, 78.1, 156.3, 312.5, 625, 1,250, 2,500, and 5,000 nM) were applied to flow cells with immobilized pERK2 (*C*) or ERK2 (*D*). The data were evaluated by fitting the equilibrium binding responses to obtain affinity values.

Table S1. Statistics for data collection and refinement of the E40/ERK2 and pE59/pERK2 complexes

Complex	Statistics
E40/ERK2	
Data collection	
Space group	P 2_1 2_1 2_1
Cell dimensions, Å	a = 68.04, b = 89.37, c = 99.54 α = β = γ = 90°
Resolution limits, Å	19.60–1.87
Observed reflections	Total, 255,810; unique, 48,301
Completeness, %	93.9 (44.8)*
Redundancy	5.3
Refinement	
Resolution range, Å	19.43–1.97
R factor/R_{free}, %	22.1/26.9
Ordered water molecules	236
Rms deviation	
Bond lengths, Å	0.023
Bond angles, °	1.957
Average B factor, $Å^2$	23.9
pE59/pERK2	
Data collection	
Space group	P 2_1 2_1 2_1
Cell dimensions, Å	a = 113.67, b = 150.45, c = 104.97 α = β = γ = 90
Resolution limits, Å	90.69–2.72
Observed reflections	Total, 201,276; unique, 90,072
Completeness, %	100 (74.3)*
Redundancy	2.2
Refinement	
Resolution range, Å	77.12–2.72
R factor/R_{free}, %	17.8/23.0
Ordered water molecules	203
Rms deviation	
Bond lengths, Å	0.02
Bond angles, °	1.939
Average B factor, $Å^2$	40.5

*Values in parentheses refer to the highest-resolution shell.

Table S2. List of the major interaction contacts in the E40/ERK2 complex

E40 interaction residue (repeat module)*			H-bond (Å)	ERK2 interaction residue (structural element)*		
GLN	46[†]	(1)		LYS	229	
ASP	79[†]	(2)	OD2-NZ (2.03)	LYS	229	
TRP	81[†]	(2)		LYS	229	
TRP	81[†]	(2)		HIS	230	
ASP	89[†]	(2)		LYS	229	
ASP	110	(3)		HIS	230	
TYR	112[†]	(3)		HIS	230	
TYR	112[†]	(3)		LEU	232	(αG)
TYR	112[†]	(3)	OH-OD1 (2.74)	ASP	233	(αG)
TYR	112[†]	(3)		ASN	236	(αG)
LEU	114[†]	(3)		HIS	230	
LEU	114[†]	(3)		LEU	232	(αG)
LEU	119	(3)		HIS	230	
ASP	122[†]	(3)		TYR	185	(activation loop)
ASP	122[†]	(3)	OD2-NH2 (2.75)	ARG	189	(activation loop)
ASP	122[†]	(3)	O-NH1 (3.19)	ARG	189	(activation loop)
ARG	123[†]	(3)	NH1-O (2.71)	TYR	185	(activation loop)
ARG	123[†]	(3)		VAL	186	(activation loop)
ARG	123[†]	(3)	NH1-O (3.33)	ALA	187	(activation loop)
ARG	123[†]	(3)		ARG	189	(activation loop)
HIS	125	(3)		VAL	186	(activation loop)
ASP	143	(C-cap)	OD2-OH (2.53)	TYR	231	(αG)
LYS	144	(C-cap)		LEU	232	(αG)
LYS	144	(C-cap)	NZ-OH (3.23)	TYR	261	(α2L14)
PHE	145	(C-cap)		TYR	231	(αG)
PHE	145	(C-cap)		LEU	232	(αG)
PHE	145	(C-cap)		LYS	257	(α2L14)
PHE	145	(C-cap)		TYR	261	(α2L14)
LYS	147	(C-cap)		ASN	199	
LYS	147	(C-cap)		TYR	231	(αG)
ASP	155	(C-cap)		PHE	181	(activation loop)
ASP	155	(C-cap)		ASN	199	
ASN	156	(C-cap)		PHE	181	(activation loop)
ASN	156	(C-cap)		THR	183	(activation loop)
ASN	156	(C-cap)	O-OH (2.95)	TYR	185	(activation loop)
GLY	157	(C-cap)		PHE	181	(activation loop)
GLY	157	(C-cap)		TYR	185	(activation loop)
ASN	158	(C-cap)		TYR	185	(activation loop)

*A cutoff of 4 Å was applied for interactions.
[†]Amino acids are located in a randomized library position of E40.

Table S3. List of the major interaction contacts in the pE59/pERK2 complex

pE59 interaction residue (repeat module)*			Chain	H-bond (Å)	ERK2 interaction residue (structural element)*			Chain
ASP	44	(1)	B	OD2-NZ (3.02)	LYS	229		A
ASP	46[†]	(1)	B	OD2-NZ (2.34)	LYS	229		A
GLN	56[†]	(1)	B		ARG	189	(activation loop)	A
GLN	56[†]	(1)	B	NE2-O (2.98)	LYS	229		A
GLN	56[†]	(1)	B		HIS	230		A
ASP	77	(2)	B	OD2-NE2 (2.76)	HIS	230		A
PHE	79[†]	(2)	B		HIS	230		A
PHE	79[†]	(2)	B		LEU	232	(αG)	A
PHE	79[†]	(2)	B		ASP	233	(αG)	A
ILE	81[†]	(2)	B		TYR	231	(αG)	A
LEU	86	(2)	B		HIS	230		A
ILE	89[†]	(2)	B		PTR	185	(activation loop)	A
ILE	89[†]	(2)	B		TYR	231	(αG)	A
ARG	90[†]	(2)	B	NH1-O2P (2.54)	PTR	185	(activation loop)	A
ARG	90[†]	(2)	B		ARG	189	(activation loop)	A
ASP	110	(C-cap)	B	OD2-OH (2.25)	TYR	231	(αG)	A
ASP	110	(C-cap)	B		LEU	232	(αG)	A
LYS	111	(C-cap)	B		TYR	261	(α2L14)	A
PHE	112	(C-cap)	B		ALA	258		A
PHE	112	(C-cap)	B		TYR	261	(α2L14)	A
ILE	121	(C-cap)	B		LYS	201		A
ASP	122	(C-cap)	B		GLU	184	(activation loop)	A
ASP	122	(C-cap)	B	O-NZ (2.89)	LYS	201		A
ASP	44	(1)	D		LYS	229		C
GLU	45[†]	(1)	D	OE2-NZ (3.02)	LYS	229		C
ASP	46[†]	(1)	D	OD2-NZ (2.44)	LYS	229		C
LEU	48[†]	(1)	D		LYS	229		C
GLN	56[†]	(1)	D		ARG	189	(activation loop)	C
GLN	56[†]	(1)	D	NE2-O (3.08)	LYS	229		C
ASP	77	(2)	D	OD2-NE2 (2.78)	HIS	230		C
PHE	79[†]	(2)	D		HIS	230		C
PHE	79[†]	(2)	D		LEU	232	(αG)	C
PHE	79[†]	(2)	D		ASP	233	(αG)	C
ILE	81[†]	(2)	D		HIS	230		C
ILE	81[†]	(2)	D		TYR	231	(αG)	C
ILE	81[†]	(2)	D		LEU	232	(αG)	C
ILE	89[†]	(2)	D		PTR	185	(activation loop)	C
ILE	89[†]	(2)	D		TYR	231	(αG)	C
ARG	90[†]	(2)	D	NH2-O2P (2.64)	PTR	185	(activation loop)	C
ARG	90[†]	(2)	D		ARG	189	(activation loop)	C
ASP	110	(C-cap)	D	OD2-OH (2.54)	TYR	231	(αG)	C
ASP	110	(C-cap)	D		LEU	232	(αG)	C
LYS	111	(C-cap)	D		TYR	261	(α2L14)	C
PHE	112	(C-cap)	D		LEU	198	(α1L12)	C
PHE	112	(C-cap)	D		TYR	231	(αG)	C
PHE	112	(C-cap)	D		LEU	232	(αG)	C
PHE	112	(C-cap)	D		LYS	257	(α2L14)	C
PHE	112	(C-cap)	D		ALA	258		C
PHE	112	(C-cap)	D		TYR	261	(α2L14)	C
GLY	113	(C-cap)	D		LYS	257	(α2L14)	C
LYS	114	(C-cap)	D		TYR	231	(αG)	C
LYS	114	(C-cap)	D		LYS	257	(α2L14)	C
ASP	118	(C-cap)	D	OD2-NZ (3.52)	LYS	257	(α2L14)	C
ASP	122	(C-cap)	D		GLU	184	(activation loop)	C
ASP	122	(C-cap)	D		LYS	201		C

*A cutoff of 4 Å was applied for interactions.
[†]Amino acids are located in a randomized library position of pE59.

Table S4. Overview of oligonucleotides

Oligonucleotide	Oligonucleotide sequence (5′→3′)
darpin1f-XhoI	GGACTCGAGATGGACCTGGGTAAGAAACTG
darpin1rstop-HindIII	TCCAAGCTTCTACTATTGCAGGATTTCAGCCAGGT
darpin2f-BglII	GGAAGATCTATGGACCTGGGTAAGAAACTGCTG
darpin2r-HindIII	TCCAAGCTTTTGCAGGATTTCAGCCAGGT
ERK1f	CTATCCATGGGCGCGGCGGCGGCGGCTCAG
ERK1r	GCGCCCAAGCTTGGGGGGCCTCCAGCAC
ERK2f	CTATCCATGGGCGCGGCGGCGGCGGCGGCGGGCC
erk2f-XhoI	GGACTCGAGCCATGGGCGCGG
ERK2r	GCGCCCAAGCTTGAGATCTGTATCCTGGCTGGAATCGAGC
erk2rstop-HindIII	TCCAAGCTTCTAAGATCTGTATCCTGGCTG
jnk1f-XhoI	GGACTCGAGATGTCCCGTAGCAAGCGT
jnk1rstop-HindIII	TCCAAGCTTCTACTATTGCTGCACCTGTGCTAA
JNK1α1f	CTATCCATGGGCTCCCGTAGCAAGCGTGAC
JNK1α1r	GCGCCCAAGCTTGCTGCACCTGTGCTAAAG
jnk2f-XhoI	GCACTCGAGATGTCCGACTCTAAATGTGA
jnk2rstop-HindIII	TCCAAGCTTCTATCGACAGCCTTCAAGG-3
JNK2α1f	CTATCCATGGGCTCCGACTCTAAATGTGACAG
JNK2α1r	GCGCCCAAGCTTGCTGCATCTGTGCTGAAG
MEK1R4Ff	TAGCGCTGAGCACGACTAAGGAGGTTTGACCTATGCCCAAGAAGAAGC
MEK1R4Fr	TAGCGCTCAGCTCATTAGACGCCAGCAGCATG
MEK4f	TAGCGCTGAGCACGACTAAGGAGGTTTGACCTATGGCGGCTCCGAGC
MEK4r	TAGCGCTCAGCTTATCAATCGACATACATGGGAG
p38αf	CTATCCATGGGCTCTCAGGAGCGTCCGAC
p38αr	GCGCCCAAGCTTGGGACTCCATCTCTTCTTGGTC