SUPPLEMENTAL FIGURE LEGENDS

FIGURE S1. Initial full models for MEK6 and p38α phosphorylation. (A) ASK1 phosphorylation of MEK6 and (B) MEK6 phosphorylation of p38α are depicted as detailed kinetic models. Each step of the cascade was represented by a series of reversible and irreversible steps. Initial reversible protein complexes leading to a catalytically competent interaction are followed by an irreversible phosphorylation step, then reversible dissociation and rebinding of the monophosphorylated substrate. The second phosphorylation step is similarly modeled leading to a final, doubly phosphorylated product.

FIGURE S2. LC-MS/MS identification of p38α/K53M phosphorylation intermediates. p38α/K53M was phosphorylated by MEK6/S*T*, proteolyzed with chymotrypsin and separated on a RP-C18 column (see Methods). Total ion trace shows separation of individual peptides (bottom). Extracted ion chromatographs of masses corresponding to doubly charged activation loop peptides (middle). MASCOT analysis of collision-induced dissociation generated peptide fragments for peptide identification (top).

FIGURE S3. Sequence Alignment of MAPKs with ERK3 Activation loops of p38α, ERK2 and JNK are aligned with phosphorylation sites shown in red. S189, the activating phosphorylation site of ERK3, aligns with T180 of p38α and T183 of ERK2.

SUPPLEMENTAL TABLES

Table S1 Proteolytically-derived phosphorylation site peptides observed by LC-MS for indicated kinases

Kinase	Protease	Peptide	Mass	Charge
MEK6	Chymotrypsin	LVDSVAKTIDAGCRPY	840.6	2
		LVDSVAKT*IDAGCRPY	880.6	2
		LVDS*VAKTIDAGCRP	880.6	2
		LVDS*VAKT*IDAGCRP	920.3	2
p38α	Trypsin	HTDDEMTGYVATR	748.6	2
		HTDDEMTGY*VATR	788.6	2
		HTDDEMT*GYVATR	788.6	2
		HTDDEMT*GY*VATR	828.6	2
ATF2	Trypsin	NDSVIVADQTPTPTR	807.6	2
		NDSVIVADQT*PTPTR	847.6	2
		NDSVIVADQTPT*PTR	847.6	2
		NDSVIVADQT*PT*PTR	887.6	2
		FGPAR NDSVIVADQTPTPTR	1072.8	2
		FGPAR NDSVIVADQT*PTPTR	1112.8	2
		FGPAR NDSVIVADQTPT*PTR	1112.8	2
		FGPAR NDSVIVADQT*PT*PTR	1152.8	2

Table S2 Equations used in final models.

(A) Differential equations used in modeling ASK1 phosphorylation of MEK6.

$$\frac{d[ASK1]}{dt} = -k_1[ASK1][MEK6ST] - k_2[ASK1][MEK6ST] + k_3[ASK1\_MEK6STbindT] + k_4[ASK1\_MEK6STbindS] - k_5[ASK1][MEK6ST^*] - k_6[ASK1][MEK6S^*T] + k_7[ASK1\_MEK6ST^*] + k_8[ASK1\_MEK6S^*T] + k_9[ASK1\_MEK6STbindT]$$

$$\frac{d[MEK6ST]}{dt} = -k_1[ASK1][MEK6ST] - k_2[ASK1][MEK6ST]$$

$$\frac{d[ASK1\_MEK6STbindT]}{dt} = k_1[ASK1][MEK6ST] - k_3[ASK1\_MEK6STbindT] - k_9[ASK1\_MEK6STbindT]$$

$$\frac{d[ASK1\_MEK6STbindS]}{dt} = k_2[ASK1][MEK6ST] - k_4[ASK1\_MEK6STbindS]$$

$$\frac{d[MEK6ST^*]}{dt} = k_3[ASK1\_MEK6STbindT] - k_5[ASK1][MEK6ST^*]$$

$$\frac{d[MEK6S^*T]}{dt} = k_4[ASK1\_MEK6STbindS] - k_6[ASK1][MEK6S^*T]$$

$$\frac{d[ASK1\_MEK6ST^*]}{dt} = k_5[ASK1][MEK6ST^*] - k_7[ASK1\_MEK6ST^*]$$

$$\frac{d[ASK1\_MEK6S^*T]}{dt} = k_6[ASK1][MEK6S^*T] - k_8[ASK1\_MEK6S^*T]$$

$$\frac{d[MEK6S^*T^*]}{dt} = k_7[ASK1\_MEK6ST^*] + k_8[ASK1\_MEK6S^*T] + k_9[ASK1\_MEK6STbindT]$$

(B) Differential equations used in modeling MEK6 phosphorylation of p38α. k_7 was introduced to account for a small fraction of each unphosphorylated or partially phosphorylated p38α species becoming inactive to account for the persistence of these species through the time course.

$$\frac{d[ASK1]}{dt} = -k_1[ASK1][MEK6ST] - k_2[ASK1][MEK6ST] + k_3[ASK1\_MEK6STbindT] + k_4[ASK1\_MEK6STbindS] - k_5[ASK1][MEK6ST^*] - k_6[ASK1][MEK6S^*T] + k_7[ASK1\_MEK6ST^*] + k_8[ASK1\_MEK6S^*T] + k_9[ASK1\_MEK6STbindT]$$

$$\frac{d[MEK6ST]}{dt} = -k_1[ASK1][MEK6ST] - k_2[ASK1][MEK6ST]$$

$$\frac{d[ASK1\_MEK6STbindT]}{dt} = k_1[ASK1][MEK6ST] - k_3[ASK1\_MEK6STbindT] - k_9[ASK1\_MEK6STbindT]$$

$$\frac{d[ASK1\_MEK6STbindS]}{dt} = k_2[ASK1][MEK6ST] - k_4[ASK1\_MEK6STbindS]$$

$$\frac{d[MEK6ST^*]}{dt} = k_3[ASK1\_MEK6STbindT] - k_5[ASK1][MEK6ST^*]$$

$$\frac{d[MEK6S^*T]}{dt} = k_4[ASK1\_MEK6STbindS] - k_6[ASK1][MEK6S^*T]$$

$$\frac{d[ASK1\_MEK6ST^*]}{dt} = k_5[ASK1][MEK6ST^*] - k_7[ASK1\_MEK6ST^*]$$

$$\frac{d[ASK1\_MEK6S^*T]}{dt} = k_6[ASK1][MEK6S^*T] - k_8[ASK1\_MEK6S^*T]$$

$$\frac{d[MEK6S^*T^*]}{dt} = k_7[ASK1\_MEK6ST^*] + k_8[ASK1\_MEK6S^*T] + k_9[ASK1\_MEK6STbindT]$$

Table S3 Derived kinetic constants based on experimentally observed progress curves of ASK1 phosphorylation of MEK6/K82M

Parameter	Value	Std. Error	90% confidence interval
k_1‡	2.0 μM^{-1} min^{-1}	NA	NA
k_2	0.044 μM^{-1} min^{-1}	0.0092	0.003 – 0.057
k_3	11 min^{-1}	0.75	10 – 12
k_4	0.72 min^{-1}	0.24	0.47 – 1.7
k_5	2.5 μM^{-1} min^{-1}	0.11	2.3 – 2.7
k_6	4.7 μM^{-1} min^{-1}	1.3	3.5 – 6.6
k_7	5.3 min^{-1}	1.2	3.7 – 8.5
k_8	0.011 min^{-1}	0.0086	0.00002 – 0.024
k_9	4.6 min^{-1}	0.35	4.1 – 5.2

‡ k_1 was determined by trial and error, and is similar to the approximate turnover for loss of MEK6/ST.

Table S4 Derived kinetic constants based on experimentally observed progress curves of MEK6 phosphorylation of p38α/K53M

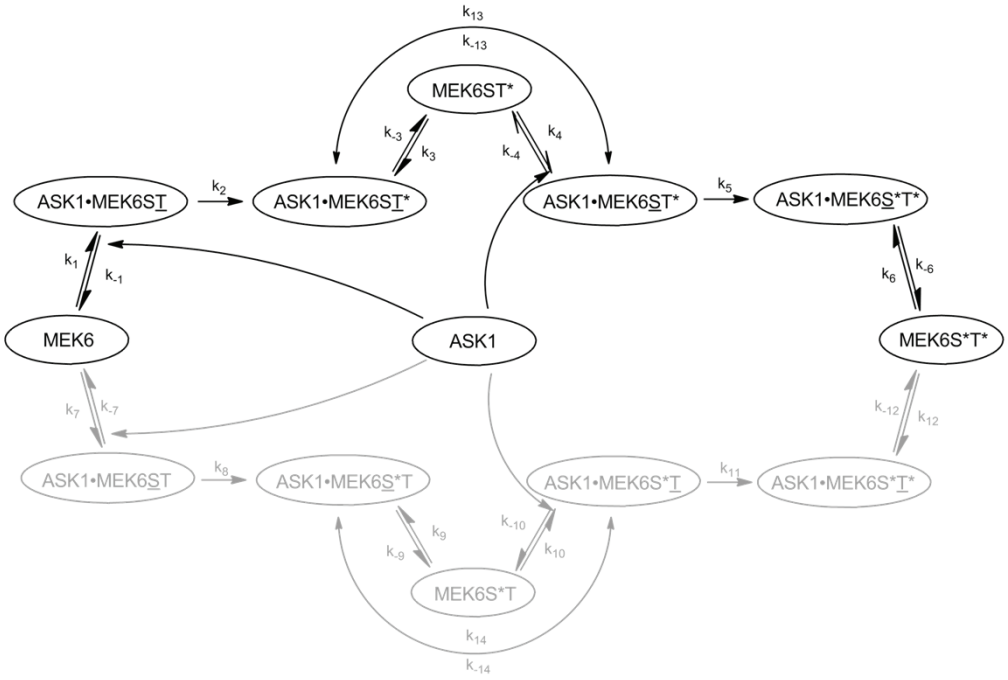
Parameter	Value	Std. Error	90% confidence interval
k_1[§]	154 μM^{-1} min^{-1}	NA	NA
k_2	31 μM^{-1} min^{-1}	1.7	28 – 34
k_3	101 μM^{-1} min^{-1}	8.9	87 - 116
k_4	15 min^{-1}	4.1	9.8 - 23
k_5	6.6 min^{-1}	0.43	6.0 – 7.3
k_6	44 μM^{-1} min^{-1}	5.6	36 - 54
k_7[‡]	0.03 μM^{-1} min^{-1}	NA	NA

[§] A range of values for k_1 fit the model; we picked the lowest, which put k_1 midrange of values reported in the literature (1). [‡] Rate of p38α species inactivation.

SUPPLEMENTAL REFERENCES

1. Fujioka, A., Terai, K., Itoh, R. E., Aoki, K., Nakamura, T., Kuroda, S., Nishida, E., and Matsuda, M. (2006) Dynamics of the Ras/ERK MAPK cascade as monitored by fluorescent probes. *J Biol Chem* **281**, 8917-8926

A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k-13
MEK6ST*
k-3
k3
k4
k-4
ASK1•MEK6ST
k2
ASK1•MEK6ST*
ASK1•MEK6ST*
k5
ASK1•MEK6S*T*
k1
k-1
k6
k-6
MEK6
ASK1
MEK6S*T*
k7
k-7
k-12
k12
ASK1•MEK6ST
k8
ASK1•MEK6S*T
ASK1•MEK6S*T
k11
ASK1•MEK6S*T*
k-10
k9
k-9
k10
MEK6S*T
k14
k-14

B

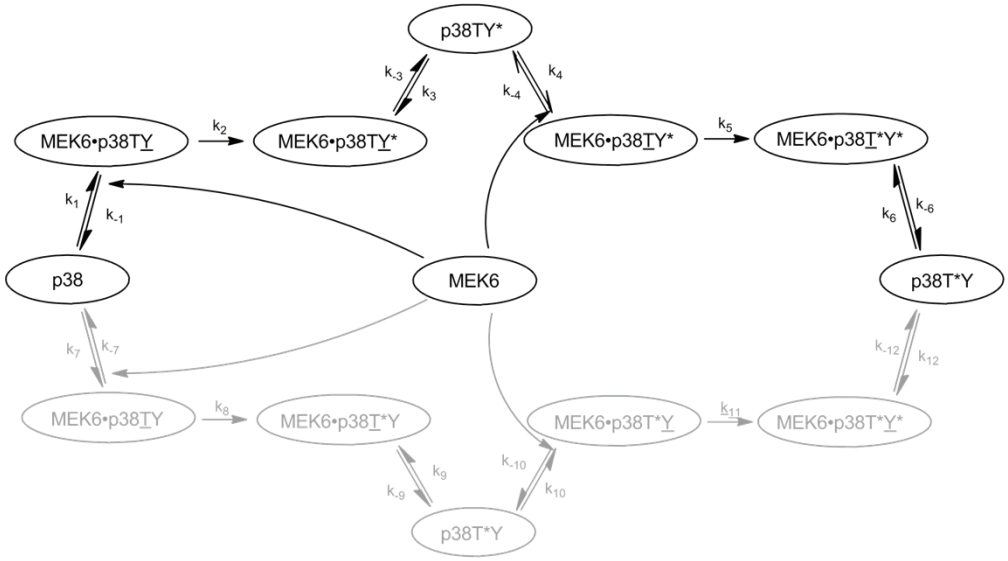
p38TY*
k-3
k3
k4
k-4
MEK6•p38TY
k2
MEK6•p38TY*
MEK6•p38TY*
k5
MEK6•p38T*Y*
k1
k-1
k6
k-6
p38
MEK6
p38T*Y
k7
k-7
k-12
k12
MEK6•p38TY
k8
MEK6•p38T*Y
MEK6•p38T*Y
k11
MEK6•p38T*Y*
k-10
k9
k-9
k10
p38T*Y

Figure S1

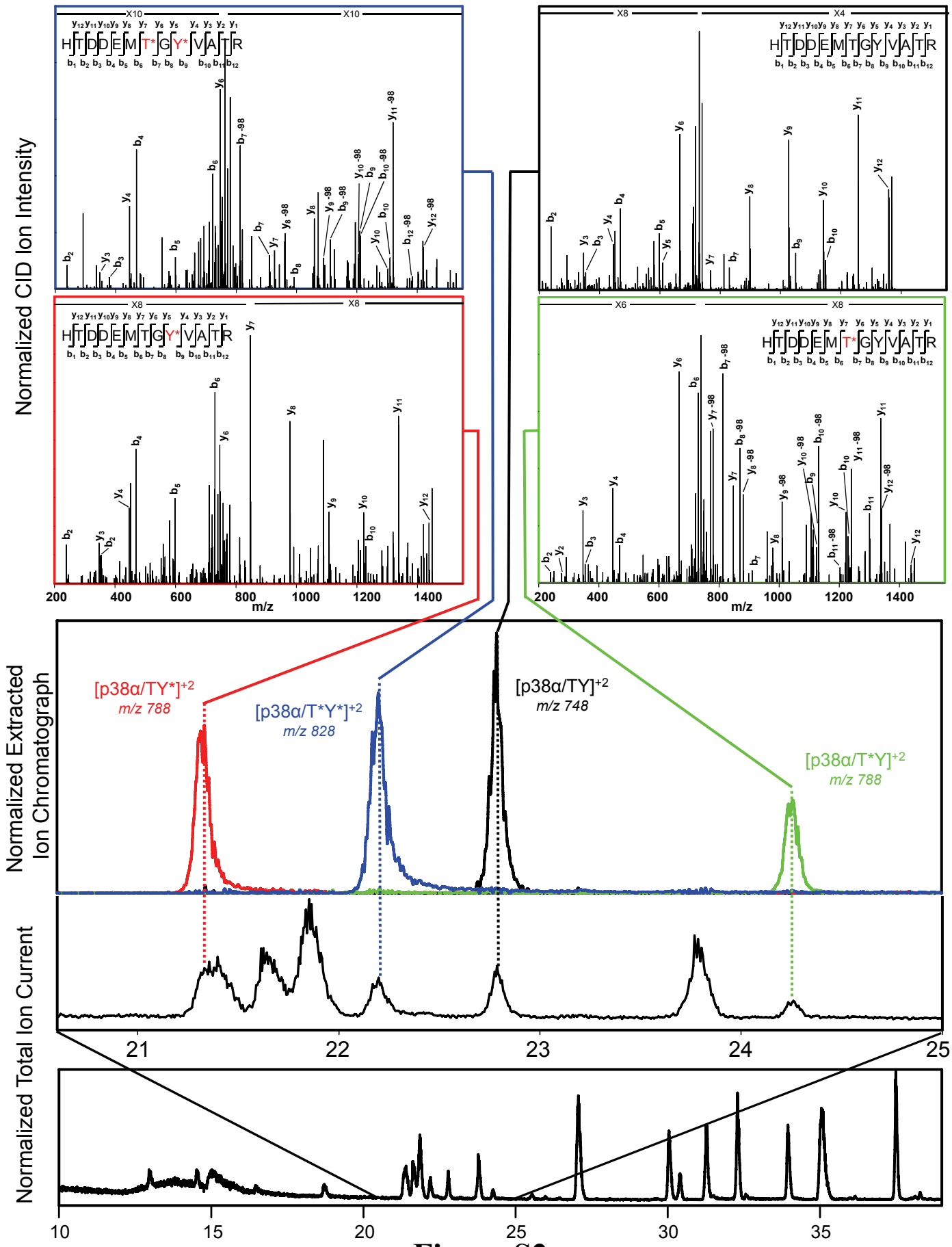
Normalized CID Ion Intensity
X10
X10
X8
X4
X8
X8
X6
X8
HTDDEMT*GY*VATR
HTDDEMTGY*VATR
HTDDEMTGY*VATR
HTDDEMT*GY*VATR
m/z
200
400
600
800
1000
1200
1400
Normalized Extracted Ion Chromatograph
[p38α/TY*]+2
m/z 788
[p38α/T*Y*]+2
m/z 828
[p38α/TY]+2
m/z 748
[p38α/T*Y]+2
m/z 788
Normalized Total Ion Current
21
22
23
24
25
10
15
20
25
30
35

Figure S2

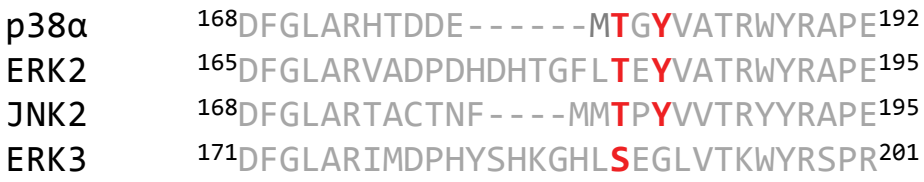
p38α 168DFGLARHTDDE------MTGYVATRWYRAPE192
ERK2 165DFGLARVADPDHDHTGFLTEYVATRWYRAPE195
JNK2 168DFGLARTACTNF----MMTPYVVTRYYRAPE195
ERK3 171DFGLARIMDPHYSHKGHLSEGLVTKWYRSPR201

Figure S3