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oxidation of ERK2 within its D-recruitment

site alters its substrate selection

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Figure S1.

Α.	ERK2	MAAAAAGPEMVRGQVFDVGPRYTNLSYIGEGAYGMVCSA 40	в.	Unphosphorylated ERK1 (PDB ID: 4QTB)				C.	Doubly-phosphorylated ERK1 (PDB ID: 6GES)				
	ERK1	MAAAAAAPGGGGGEPRGTAGVVPVVPGEVEVVKGQPFDVGPRYTQLQYIGEGAYGMVSSA 60		Prediction	Position	E-score	S-score		Prediction	Position	E-score	S-score	
					C82	\sim	$\mathbf{\circ}$			C82	\sim		
	ERK2	YDNLNKVRVAIKKISPFEHQTYCQRTLREIKILLRFRHENIIGINDIIRAPTIEQMKDVY 100			C1 4 4					C144		^	
	ERKI	YDHVRKTRVAIKKISPFEHQTYCQRTLREIQILLRFRHENVIGIRDILRAPTLEAMRDVY I20 **::.*.*******************************			C144	C144 ·\ •	• I •			0144			
				\checkmark	C178		$\mathbf{\circ}$		\checkmark	C178	~	Ç	
	ERK2	IVQDLMETDLYKLLKTQHLSNDHICYFLYQILRGLKYIHSANVLHRDLKPSNLLLNTTCD 160			C102					C183	^	•	
	ERKI	1VQDLMETDLYKLLKSQQLSNDHICYFLYQ1LKGLKYIHSANVLHKDLKPSNLLINTT <u>C</u> D 180			C183	-				0105			
					C233	^	$\mathbf{\circ}$			C233	~	C	
	ERK2	LKICDFGLARVADPDHDHTGFLTEYVATRWYRAPEIMLNSKGYTKSIDIWSVGCILAEML 220			0074	-	Ì			C271		•	
	FULT	**************************************		\checkmark	C2/1		C			C271	1		
). Unphosphorylated ERK2 (PDB ID: 1ERK					Doubly-phosphorylated ERK2 (PDB ID:				
			D.	Unphosp	horylated	ERK2 (PDB	ID: 1ERK)	E.	Doubly-pho	osphorylat	ed ERK2 (F	PDB ID: 2ERK)	
	ERK2 FRK1	SNRPIFPGKHYLDQLNHILGILGSPSQEDLNCIINLKARNYLLSLPHKNKVPWNRLFPNA 280 SNRPIFPGKHYLDOLNHILGILGSPSOFDLNCIINMKARNYLOSLPSKTKVAWAKLFPKS 300	D.	Unphosp Prediction	horylated Position	ERK2 (PDB E-score	ID: 1ERK) S-score	E.	Doubly-pho Prediction	osphorylat Position	ed ERK2 (F E-score	PDB ID: 2ERK) S-score	
	ERK2 ERK1	SNRPIFPGKHYLDQLNHILGILGSPSQEDLNCIINLKARNYLLSLPHKNKVPWNRLFPNA 280 SNRPIFPGKHYLDQLNHILGILGSPSQEDLNCIINMKARNYLQSLPSKTKVAWAKLFPKS 300 ***********************************	D.	Unphosp Prediction	horylated Position C38	ERK2 (PDB E-score	ID: 1ERK) S-score	E.	Doubly-pho Prediction	osphorylat Position C38	ed ERK2 (F E-score	PDB ID: 2ERK) S-score	
	ERK2 ERK1	SNRPIFPGKHYLDQLNHILGILGSPSQEDLNCIINLKARNYLLSLPHKNKVPWNRLFPNA 280 SNRPIFPGKHYLDQLNHILGILGSPSQEDLNCIINMKARNYLQSLPSKTKVAWAKLFPKS 300 ***********************************	D.	Unphosp Prediction	Position C38	ERK2 (PDB E-score	ID: 1ERK) S-score	E.	Doubly-ph Prediction	Position C38 C63	ed ERK2 (F E-score	PDB ID: 2ERK) S-score	
	ERK2 ERK1 ERK2 ERK1	SNRPIFPGKHYLDQLNHILGILGSPSQEDLNCIINLKARNYLLSLPHKNKVPWNRLFPNA 280 SNRPIFPGKHYLDQLNHILGILGSPSQEDLNCIINMKARNYLQSLPSKTKVAWAKLFPKS 300 ***********************************	D.	Unphosp Prediction	horylated Position C38 C63	ERK2 (PDB E-score	ID: 1ERK) S-score	E.	Doubly-pho Prediction	Position C38 C63	E-score	S-score	
	ERK2 ERK1 ERK2 ERK1	SNRPIFPGKHYLDQLNHILGILGSPSQEDLNCIINLKARNYLLSLPHKNKVPWNRLFPNA 280 SNRPIFPGKHYLDQLNHILGILGSPSQEDLNCIINMKARNYLQSLPSKTKVAWAKLFPKS 300 ***********************************	D.	Unphosp Prediction	horylated Position C38 C63 C125	ERK2 (PDB E-score	ID: 1ERK) S-score	E.	Doubly-pho Prediction	Position C38 C63 C125	ed ERK2 (F E-score	PDB ID: 2ERK) S-score	
	ERK2 ERK1 ERK2 ERK1	SNRPIFPGKHYLDQLNHILGILGSPSQEDLNCIINLKARNYLLSLPHKNKVPWNRLFPNA 280 SNRPIFPGKHYLDQLNHILGILGSPSQEDLNCIINMKARNYLQSLPSKTKVAWAKLFPKS 300 ***********************************	D.	Unphosp Prediction	horylated Position C38 C63 C125 C125	ERK2 (PDB E-score	ID: 1ERK) S-score	E.	Doubly-pho Prediction	Position C38 C63 C125 C159	ed ERK2 (I E-score	PDB ID: 2ERK) S-score	
	ERK2 ERK1 ERK2 ERK1 ERK2 ERK1	SNRPIFPGKHYLDQLNHILGILGSPSQEDLNCIINLKARNYLLSLPHKNKVPWNRLFPNA 280 SNRPIFPGKHYLDQLNHILGILGSPSQEDLNCIINMKARNYLQSLPSKTKVAWAKLFPKS 300 ***********************************	D.	Unphosp Prediction	horylated Position C38 C63 C125 C125 C159	ERK2 (PDB E-score	ID: 1ERK) S-score	E.	Doubly-pho Prediction	Position C38 C63 C125 C159 C164	ed ERK2 (I	S-score	
	ERK2 ERK1 ERK2 ERK1 ERK2 ERK1	SNRPIFPGKHYLDQLNHILGILGSPSQEDLNCIINLKARNYLLSLPHKNKVPWNRLFPNA 280 SNRPIFPGKHYLDQLNHILGILGSPSQEDLNCIINMKARNYLQSLPSKTKVAWAKLFPKS 300 ***********************************	D.	Unphosp Prediction	horylated Position C38 C63 C125 C125 C159 C164	E-score	ID: 1ERK) S-score	E.	Doubly-pho Prediction	Position C38 C63 C125 C159 C164	E-score	S-score	
	ERK2 ERK1 ERK2 ERK1 ERK2 ERK1	SNRPIFPGKHYLDQLNHILGILGSPSQEDLNCIINLKARNYLLSLPHKNKVPWNRLFPNA 280 SNRPIFPGKHYLDQLNHILGILGSPSQEDLNCIINMKARNYLQSLPSKTKVAWAKLFPKS 300 ***********************************	D.	Unphosp Prediction	horylated Position C38 C63 C125 C125 C159 C164 C214	E-score	ID: 1ERK) S-score	Ε.	Doubly-pho Prediction	Desphorylat Position C38 C63 C125 C159 C164 C214	E-score	S-score	

Figure S1. Computational prediction of redox-sensitive sites in ERK1 and ERK2, related to Fig. 1. A. Multiple sequence alignment of ERK2 and ERK1. Sequence alignments were done using Clustal Omega. **B-E***. Cy-Preds prediction of redox-sensitive Cys residues in unphosphorylated or doubly phosphorylated ERK1 and ERK2. The pdb ID used for the prediction is given in paratheses.*

Figure S2.



Figure S2. Bromobimane fluorescence labeling of recombinant ERK2, related to Fig. 1. ERK2 was incubated with (green triangles) or without (blue circles) the C159-specific inhibitor, BI-78D3, for 60 minutes at room temperature. After incubation, the solution was incubated in the dark for an additional 60 minutes with monobromobimane in phosphate buffered saline at the indicated pH. Error bars represent the standard error about the mean of 3 independent experiments.





Fig. S3. Non-reducing PAGE, related to Fig. 1. A) Purified ERK2 was treated with the indicated concentration of H^2O^2 for 10 minutes at room temperature. Excess H_2O_2 was then removed by catalase and the samples were resolved by SDS-PAGE under either reducing (left) or non-reducing (right) conditions. After electrophoresis, protein bands were visualized by western blotting using an anti-ERK1/2 antibody. B) Western blot analysis of endogenous ERK1/2 in cell lysates derived from three different cell lines following stimulation with lysophosphatidic acid (SK-OV3 cells), platelet-derived growth factor (NIH3T3 cells) or epidermal growth factor (HeLa cells) for the indicated time periods. Lysates were resolved under either reducing (left) or non-reducing (right) conditions and probed with an anti-ERK1/2 antibody.



Fig. S4. Dependence of RSK1 phosphorylation on MAPK signaling in HeLa cells, related to Fig. 6. A. Representative western blot of *HeLa cell lysates from cells pre-treated with the MEK1 inhibitor, UO126, or vehicle alone followed by incubation with or without EGF for 10 minutes before lysis.* **B.** Average normalized response of western blots. Error bars represent standard error about the mean of three independent experiments (n = 3). **: p < 0.01.



Fig. S5. Overexpression of ERK2(C159S) abolishes NAC-dependent changes in ERK2-mediated phosphorylation of RSK1, related to Fig. 6. A. Representative western blot of HeLa cell lysates from cells that were transfected with the mammalian expression vector, pcDNA3.1, encoding ERK2(C159S) under the control of the strong CMV promoter. Transfected cells were serum-starved before being treated with N-acetyl-cysteine (NAC) for 45 minutes followed by stimulation with epidermal growth factor (EGF) or vehicle alone for 10 minutes. Lysates were probed with the indicated antibodies. B. Average normalized response of western blots. Error bars represent standard error about the mean of three independent experiments (n = 3). n.s.: not significant.





Fig. S6. NAC treatment does not affect ERK1/2-mediated phosphorylation of the F-site substrate, ERF, related to Fig. 6. A. Representative western blot of HeLa cell lysates treated with N-acetyl-cysteine (NAC) for 45 minutes followed by stimulation with epidermal growth factor (EGF) or vehicle alone for 10 minutes. Lysates were probed with the indicated antibodies. **B.** Average normalized response of western blots. Error bars represent standard error about the mean of three independent experiments (*n* = 3). n.s.: not significant.





Fig. S7. Impact of oxidation of ERK2 on its interaction with pepHePTP, related to Figure 6. A. Fluorescence polarization competition assays using oxidized (red) and reduced (blue) ERK2. *B.* Average $K_{D,app}$ of oxidized (red) and reduced (blue) ERK2 for unlabeled pepHePTP. Error bars represent the standard error about the mean of three independent experiments done in duplicate. **p < 0.01.

Figure S8.

Α	ERK1	ND <mark>H</mark> IC <mark>Y</mark> FL	YQILRGI	LKYIHSA	N <mark>V</mark> L <mark>HRDI</mark>	LKPSNL	LI <mark>N</mark> -T	TCDLK	ICDFGI	LARIA-I	DPE-HD	197		
	ERK2	ND <mark>H</mark> IC <mark>Y</mark> FL	YQILRGI	LKYIHSA	N <mark>V</mark> L <mark>HRDI</mark>	LKPSNL	LL <mark>N</mark> -T	TCDLK	ICDFGI	LARVA-1	DPD-HD	177		
	ERK5	L <mark>EH</mark> VR <mark>Y</mark> FL	YQLLRGI	LKY <mark>MHSA</mark> (2 <mark>VIHRDI</mark>	LKPSNL	L <mark>VN-E</mark>	NCELK	IG <mark>DFG</mark> N	1 <mark>ar</mark> glc'	ISP-AE	213		
	ERK7	DI <mark>H</mark> KRCIF	'YQL <mark>LR</mark> A'	T <mark>KFIHS</mark> GI	RVIHRDÇ	2KP <mark>A</mark> NV	LLD-A	ACRVK	LC <mark>DFG</mark> I	L <mark>AR</mark> SLSI	OFPEGP	170		
	JNK1	H <mark>e</mark> rms <mark>yl</mark> l	YQMLC <mark>G</mark>	I <mark>K</mark> HL <mark>HSA</mark> (GI <mark>IHRD</mark> I	LKPSNI	V <mark>V</mark> K-S	DCTLK	ILDFGI	LART <mark>A</mark> G	Г	178		
	JNK2	H <mark>e</mark> rms <mark>yl</mark> l	YQMLCG	I <mark>K</mark> HL <mark>HSA</mark> (GI <mark>IHRD</mark> I	LKPSNI	V <mark>V</mark> K-S	DCTLK	ILDFGI	LARTAC:	Г	178		
	JNK3	H <mark>e</mark> rms <mark>yl</mark> l	YQMLCG	I <mark>K</mark> HL <mark>HSA</mark> (GI <mark>IHRD</mark> I	LKPSNI	V <mark>V</mark> K-S	DCTLK	ILDFGI	LARTAG	Г	216		
	p38α	DD <mark>H</mark> VQFLI	YQILRGI	LKYIHSAI	DI <mark>IHRDI</mark>	LKPSNL	A <mark>VN-E</mark>	DCELK	ILDFGI	LARHTDI)	177		
	p38 β	DEHVQFLV	YQLLRGI	LKYIHSA	GI <mark>IHRD</mark> I	LKPSNV	AVN-E	DCELR	ILDFGI	LARQADI	<u> </u>	184		
	p38γ	EDRIQFLV	YOMLKGI	LKYIH <mark>A</mark> A	GVIHRDI	LKP <mark>G</mark> NL	A <mark>VN</mark> -E	DCELK	ILDFGI		5	180		
	δ^{1}	EEKVO <mark>YL</mark> V	YOMLKG	LKYIHSA	GIV <mark>hrdi</mark>	LKPG <mark>NL</mark>	A <mark>VN-</mark> E	DCELK	ILDFGI	ARHTD	A	177		
	1	~												
В	Human		LMETDI	YKLLK-T	OHLSND	HICYFI	LYOILF	RGLKY	IHSANV	LHRDLK	PSNLLI	NTTC	DTKI	165
_	Chimpanz	ee	LMETDI	YKLLK-T	OHLSND	HICYFI	LYOILE	RGLKY	IHSANV	LHRDLK	PSNLLI	NTTC	DTKI	165
	Rat		T.METDI	'AKT'T'K-L	OHLSND	HTCYFI	LYOTT.F	RGLKY	THSANV		PSNLL	NTTC		163
	Mouse		T.METDI	.YKT.T.K-T	OHLSND	HTCYFI	2 [.YOTT.F	RGLKY	THSANV		PSNLLT	NTTC		163
	Zebrafish Drosophila		LMETDI	YKLLK-T	QHLSND	HICYFI	LYQILI	RGLKYI	IHSANV	LHRDLK	PSNLLI	LNTTC	DLKI	174
			LMETDI	YKLLK-T	'QRLSND	HICYFI	LYQILI	RGLKYI	IHSANV	LHRDLK	PSNLLI	NKTC	DLKI	178
	Yeast		LMQTDI	HRVIS-1	'OMLSDD	HIQYE	IYOTLE	RAV <mark>K</mark> VI	LHGSNV	IHRDLK	PSNLLI	INSNC	DLKV	153
	Arabidop	sis	LMDTDI	HQII <mark>K</mark> SS	QVLSND	HCQYFI	LFQLLE	RGLKYI	IHSANI	LHRDLK	PGNLL	/NANC	DLKI	174

Figure S8. Conservation of ERK2^{C159} *Among Mitogen-Activated Protein Kinase Family Members, related to Discussion. A) Multiple sequence alignment of* Rattus norvegicus *MAPK family members. Identical residues are highlighted in green while* ERK2^{C159} *is boxed in red.* **B)** *Multiple sequence alignment of* ERK2 *orthologs from human to Arabidopsis. Highlighting is as in A. All alignments were done using Clustal-Omega (EMBL-EBI).*