**Supplementary Table 1.** Phosphopeptides identified during data-dependent tandem MS analysis using either CID or ETD. Listed are the sequence, phosphorylation site identified, expected mass, mass difference, Mascot ion score, Mascot delta score, expectation value and charge state of all identified phosphorylated peptides.

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Protein** | **Peptide** | **Site** | **Ref.** | **Discovery studies** | ***in vitro*** | **Expected mass (Da)** | **Mass difference (Da)** | **Ion score** | **Delta score** | **Expectation value** | **Charge state** | **CID/ETD** |
| RelA/p65 | SAG**pS**IPGER | S45 | (1) | Y | Y | 952.14 | -0.26 | 53 | 23.8 | 2.40E-03 | 2 | CID |
| RelA/p65 | **pS**AG**pS**IPGER | S42/S45 | - | Y/Y |  | 1032.4 | -0.22 | 28 | 5.6 | 1.2E-1 | 2 | CID |
| RelA/p65 | CIH**pS**FQNLGIQCVK | S112 | - |  | Y | 1782.43 | -0.36 | 59 | 51.7 | 7.70E-04 | 3 | ETD |
| RelA/p65 | DLEQAI**pS**QR | S131 | - | Y | Y | 1138.22 | -0.31 | 48 | 19.8 | 8.30E-03 | 2 | CID |
| RelA/p65 | IQ**pT**NNNPFQVPIEEQR | T136 | - | Y | Y | 2005.54 | -0.38 | 78 | 62.7 | 1.60E-05 | 2 | ETD |
| RelA/p65 | N**pS**GSCLGGDEIFLLCDK | S203 | - |  | Y | 1963.44 | -0.36 | 106 | 12.3 | 2.10E-08 | 2 | CID |
| RelA/p65 | NSG**pS**CLGGDEIFLLCDK | S205 | (2, 3) |  | Y | 1963.44 | -0.36 | 101 | 0 | 7.90E-08 | 2 | ETD |
| RelA/p65 | G**pS**FSQADVHR | S238 | - |  | Y | 1182.16 | -0.32 | 55 | 25.9 | 1.10E-03 | 3 | ETD |
| RelA/p65 | GSF**pS**QADVHR | S240 | - |  | Y | 1182.16 | -0.32 | 39 | 1.1 | 5.80E-02 | 2 | CID |
| RelA/p65 | **pT**PPYADPSLQAPVR | T254 | (4) | Y |  | 1591.69 | -0.29 | 22 | 2.1 | 1.2E-01 | 2 | CID |
| RelA/p65 | TPPYADP**pS**LQAPVR | S261 | - | Y | Y | 1590.38 | -0.36 | 33 | 21.2 | 3.00E-01 | 2 | CID |
| RelA/p65 | TPPYADPSLQAPVRV**pS**MQLR | S269 | - | Y | Y | 2304.79 | -0.34 | 45 | 32.8 | 3.00E-02 | 3 | CID |
| RelA/p65 | EL**pS**EPMEFQYLPDTDDR | S281 | (2) |  | Y | 2163.48 | -0.39 | 29 | 13.7 | 7.3E-01 | 3 | CID |
| RelA/p65 | **pT**YETFK | T305 | - |  | Y | 867.12 | -0.22 | 18 | 9.3 | 1.2E-01 | 2 | CID |
| RelA/p65 | TYETFK**pS**IMK | S311 | (5) |  | Y | 1326.34 | -0.25 | 57 | 32.2 | 1.00E-03 | 3 | ETD |
| RelA/p65 | A**pS**VDD**pS**EFEQL | S468/S472 | (6, 7) | Y/Y |  | 1398.30 | -0.23 | 46 | 0 | 1.90E-03 | 2 | CID |
| p105 | LMFTAFLPD**pS**TGSFTR | S223 | - |  | Y | 1869.52 | -0.34 | 45 | 19.1 | 2.60E-02 | 2 | CID |
| p105 | LMFTAFLPDSTG**pS**FTR | S226 | - |  | Y | 1869.52 | -0.34 | 49 | 8.5 | 1.00E-02 | 2 | CID |
| p105 | RLEPVV**pS**DAIYDSK | S236 | - |  | Y | 1670.52 | -0.30 | 50 | 31.1 | 8.40E-03 | 2 | CID |
| p105 | TAGCV**pT**GGEEIYLLCDK | T263 | - |  | Y | 1964.48 | -0.34 | 74 | 25.2 | 3.00E-05 | 2 | CID |
| p105 | **pS**DLETSEPKPFLYYPEIK | S337 | (8) |  | Y | 2234.74 | -0.30 | 45 | 25.8 | 2.60E-03 | 3 | CID |
| IKKβ | HDSGLD**pS**MKDEEYEQMVK | S32 | - |  | Y | 2219.44 | -0.43 | 42 | 5.8 | 1.90E-03 | 3 | ETD |
| IKKβ | HD**pS**GLD**pS**MKDEEYEQMVK | S32/S36 | - |  | Y | 2299.39 | -0.45 | 44 | 26.2 | 2.10E-03 | 3 | CID |
| IKKβ | LEPQEVPRG**pS**EPWK | S63 | - |  |  | 1730.47 | -0.33 | 39 | 15.4 | 9.80E-02 | 3 | ETD |

**Supplementary Table 2.**  **Selected Reaction Monitoring (SRM) transitions of RelA peptides.** List of targeted peptides included in the SRM analysis. Sequences of peptides, including phosphorylation sites, are listed along with their respective precursor ions, precursor ion charge state, retention times (min) and product ions. C represents carbamidomethylated cysteine; -98 indicates loss of H3PO4 from the precursor ion; -116 indicates loss of (H3PO4+H2O) from precursor ion; -232 indicates loss of 2x(H3PO4+H2O) from precursor ion; n.d. - not detected; consistently observed product ions are highlighted in bold. Green reflects SRM assays for phosphopeptide quantification for which reproducible data was acquired; blue indicates the reference non-phosphorylated peptide.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Peptide** | **Site** | **Precursor ion m/z** | **Charge state** | **RT (min)** | **Product ions** |
| pSAGSIPGER | S42 | 477.20 | 2 | n.d. | -98; -116; y8; y7; y7; y5 |
| SAGpSIPGER | S45 | 477.20 | 2 | 22.3 | **-98; 116**; y7; **y7; y5**; y4 |
| pSAGpSIPGER | S42, S45 | 517.19 | 2 | 37.0 | **-**196;**-214**; y8; y8; **y5; y4** |
| CIHpSFQNLGIQCVK | S112 | 592.27 | 2 | 34.5 | **-98**; **y8**; **y7**; **y6**; y5; **y4** |
| DLEQAIpSQR | S131 | 570.26 | 2 | 28.1 | **-98; -116**; y7; y5; **y4; y3** |
| IQpTNNNPFQVPIEEQR | T136 | 669.65 | 3 | 38.7 | -98; y8; **y7; y6; y4; y102+** |
| VNRNSGpSCLGGDEIFLLCDK | S205 | 778.68 | 3 | 34.3 | **-98; y182+; y172+; y162+; y152+; y142+** |
| GpSFSQADVHR | S238 | 592.25 | 2 | 21.1 | **-98; -116; y9;** y8; **y7; y6** |
| GSFpSQADVHR | S240 | 592.25 | 2 | n.d. | -98; -116; y8; y8; y7; y7 |
| pTPPYADPSLQAPVR | T254 | 796.38 | 2 | n.d. | -98; -116; y13; y12; y11; y10 |
| QVAIVFRpTPPYADPSLQAPVR | T254 | 802.42 | 3 | 34.1 | **-98; y8**; y192+; y182+; y172+; y162+ |
| TPPYADPpSLQAPVR | S261 | 796.38 | 2 | 35.2 | **-98**;- 116; y12; y9; **y8**; y8 |
| QVAIVFRTPPYADPpSLQAPVR | S261 | 802.42 | 3 | n.d. | -98; y11; y10; y7; y162+; y152+ |
| pTPPYADPpSLQAPVR | T254, S261 | 836.36 | 2 | n.d. | -98; -196; y10; y9; y8; y7 |
| QVAIVFRpTPPYADpPSLQAPVR | T254, S261 | 829.07 | 3 | n.d. | -98; -196; y8; y7; y152+; y142+ |
| TPPYADPSLQAPVRVpSMQLR | S269 | 769.38 | 3 | 34.7 | **-98; -116**; y10; y182+; **y172+; y142+** |
| VSMQLRRPpSDR | S276 | 475.56 | 3 | 26.3 | **-98**; y7; y6; **y102+;** y92+; y82+ |
| RPpSDRELSEPMEFQYLPDTDDR | S276 | 926.07 | 3 | n.d. | -98; y8; y4; y202+; y192+; y182+ |
| RPSDRELpSEPMEFQYLPDTDDR | S281 | 926.07 | 3 | n.d. | -98; y8; y202+; y192+; y182+; y152+ |
| ELpSEPMEFQYLPDTDDR | S281 | 722.29 | 3 | 33.4 | **-98**; -116; **y132+;** y102+; **y122+;** y8; y7 |
| RPpSDRELpSEPMEFQYLPDTDDR | S276, S281 | 714.79 | 4 | n.d. | -98; -196; -232; y11; y10; y9 |
| pTIETFK | T305 | 434.68 | 2 | 32.0 | -**98**; -116; y5; **y4**; y3; **y2** |
| TYETFKpSIMK | S311 | 664.30 | 2 | 28.3 | **-98**; y8; -116; y7; y6; y5 |
| DGFYEAELCPDR | Reference | 736.31 | 2 | 36.2 | **y9; y8; y7; y6; y5; y4** |

**Supplementary Figure 1.** Product ion spectra generated either by CID or ETD of peptides from RelA/p65. The identified phosphorylation site is indicated and the sequence is detailed on the mass spectrum. (A) doubly charged ion at *m/z* 477.1 (CID): pSer45; (B) triply charged ion at *m/z* 595.1 (ETD): pSer112; (C) doubly charged ion at *m/z* 982.7 (CID): pSer203; (D) triply charged ion at *m/z* 655.5 (ETD): pSer205; (E) triply charged ion at *m/z* 395.1 (ETD): pSer238; (F) doubly charged ion at *m/z* 592.1 (CID): pSer240; (G) triply charged ion at *m/z* 557.9 (CID): pThr254 and pSer261; (H) doubly charged ion at *m/z* 722.3 (CID): pSer281; (I) doubly charged ion at *m/z* 434.6 (CID):pThr305; (J) triply charged ion at *m/z* 443.2 (ETD): pSer311; (K) doubly charged ion at *m/z* 467.1 (CID):pSer468 and pSer472 (CID).



***In vitro* phosphorylation of IκBα**

As part of these studies, we were also able to assess the phosphorylation status of IκBα following the *in vitro* kinase assays. Incubation of the p65:p50:IκBα trimer with IKKβ promoted phosphorylation of IκBα on Ser32 and Ser36 (Supp. Fig. 2), supporting the role of IKKβ in mediating the phosphorylation event on IκBα that leads to ubiquitination and degradation of the inhibitory proteins, as occurs in the canonical activation pathway. PKA was also able to induce phosphorylation of IκBα on Ser32 in addition to phosphorylation of a novel site at Ser63 (Supp. Fig. 2).

**Supplementary Figure 2. Tandem mass spectra of IκBα tryptic phosphopeptides.** (A) Triply charged ion at *m/z* 740.8 indicating phosphorylation of Ser32 (ETD); (B) Triply charged ion at *m/z* 767.5indicating phosphorylation of Ser32 and Ser36 (CID); (C) Triply charged ion at *m/z* 577.8 indicating phosphorylation of Ser63 (ETD).

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**Supplementary Figure 3. *In vitro* site-specific RelA phosphorylation is significantly increased in the presence of IκBα and/or p50.** The sites identified following *in vitro* phosphorylation of RelA with PKA or IKKβ, either alone or in the presence of stoichiometric amounts of p50, or p50 and IκBα are detailed. Selected reaction monitoring (SRM) determined relative change in phosphopeptide level compared to RelA alone with IKKβ. Statistical significance was assessed using a one-way ANOVA and a post-hoc Tukey test; \* represents *p*<0.05; \*\* *p*<0.01; \*\*\* *p*<0.001 with respect to RelA alone for each site and kinase. # represents *p*<0.05; ## *p*<0.01; ### *p*<0.001 with respect to RelA:p50 for each site and kinase. N.B. Relative phosphorylation cannot be directly compared between phosphopeptides and thus phosphorylation sites due to differences in peptide ionisation efficiency.

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**Supplementary Figure 4. Cross-species sequence alignment of RelA/p65.** Thirteen higher eukaryote RelA/p65 protein sequences: Zebrafish (NP\_001001839.2), Chicken (NP\_990460.1), Xenopus laevis (NP\_001081048.1), Opossum (XP\_001379658.2), Mouse (NP\_033071.1), Cow (NP\_001073711.1), Pig (NP\_001107753.1), Marmoset (XP\_002807426.1), Rhesus macaque (AFE79532.1), Dog (XP\_540850.2), Panda (XP\_002916737.1), Horse (XP\_001490867.3), Human (NP\_068810.3), were aligned with MUSCLE. Sites of phosphorylation are highlighted red (serine) or yellow (threonine). Non-conservative substitutions are highlighted cyan.

NP\_001001839.2|Zebrafish --MDGMFHQWGTSQ-----VPQGPPHVEIIEQPKSRGMRFRYKCEGRSAGSIPGEKSNDT

NP\_990460.1|Chicken MEPADLLPLYLQPEWGEQEPGGATPFVEILEQPKQRGMRFRYKCEGRSAGSIPGEHSTDS

NP\_001081048.1|Xenopus\_laevis --MDGFHWTDIVSS-----MPPSIPPVEIIEQPKQRGMRFRYKCEGRSAGSIPGERSTDT

XP\_001379658.2|Opossum --MEGLLPMIFASD----SPPARGPFVEIIEQPKQRGMRFRYKCEGRSAGSIPGERSTDT

NP\_033071.1|Mouse --MDDLFPLIFPSE----PAQASGPYVEIIEQPKQRGMRFRYKCEGRSAGSIPGERSTDT

NP\_001073711.1|Cow --MDDLFPLIFPAE----PAQASGPYVEIIEQPKQRGMRFRYKCEGRSAGSIPGERSTDT

NP\_001107753.1|Pig --MDDLFPLIFPSE----PAPASGPYVEIIEQPKQRGMRFRYKCEGRSAGSIPGERSTDT

XP\_002807426.1|Marmoset --MDELFPLIFPAE----PAQASGPYVEIIEQPKQRGMRFRYKCEGRSAGSIPGERSTDT

AFE79532.1|Rhesus\_macaque --MDELFPLIFPAE----PAQASGPYVEIIEQPKQRGMRFRYKCEGRSAGSIPGERSTDT

XP\_540850.2|Dog --MDDLFPLIFPAE----PAQASGPYVEIIEQPKQRGMRFRYKCEGRSAGSIPGERSTDT

XP\_002916737.1|Panda --MADLFPLIFPSE----PAQASGPYVEIIEQPKQRGMRFRYKCEGRSAGSIPGERSTDT

XP\_001490867.3|Horse --MEDLFPLIFPSE----PAQASGPYVEIIEQPKQRGMRFRYKCEGRSAGSIPGERSTDT

NP\_068810.3|Human 1 --MDELFPLIFPAE----PAQASGPYVEIIEQPKQRGMRFRYKCEGRSAGSIPGERSTDT

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NP\_001001839.2|Zebrafish TKTHPAIRVHNYSGPVRVRISLVTKNQPYKPHPHELVGKDCKHGYYEADL-QERRIHSFQ

NP\_990460.1|Chicken ARTHPTIRVNHYRGPGRVRVSLVTKDPPHGPHPHELVGRHCQHGYYEAELSPERCVHSFQ

NP\_001081048.1|Xenopus\_laevis SKTHPTIKINNYQGPARIRISLVTKDSPHKPHPHELVGKDCKDGYYEAELSPDRSIHSFQ

XP\_001379658.2|Opossum TKTHPTIKIHGYLGPGTVRISLVTKEPPHRPHPHELVGKDCRDGFYEAELCPDHSIHSFQ

NP\_033071.1|Mouse TKTHPTIKINGYTGPGTVRISLVTKDPPHRPHPHELVGKDCRDGYYEADLCPDRSIHSFQ

NP\_001073711.1|Cow TKTHPTIKINGYTGPGTVRISLVTKDPPHRPHPHELVGKDCRDGFYEAELCPDRCIHSFQ

NP\_001107753.1|Pig TKTHPTIKINGYTGPGTVRISLVTKDPPHRPHPHELVGKDCRDGFYEAELCPDRCIHSFQ

XP\_002807426.1|Marmoset TKTHPTIKINGYTGPGTVRISLVTKDPPHRPHPHELVGKDCRDGFYEAELCPDRCIHSFQ

AFE79532.1|Rhesus\_macaque TKTHPTIKINGYTGPGTVRISLVTKDPPHRPHPHELVGKDCRDGFYEAELCPDRCIHSFQ

XP\_540850.2|Dog TKTHPTIKINGYTGPGTVRISLVTKDPPHRPHPHELVGKDCRDGFYEAELCPDRCIHSFQ

XP\_002916737.1|Panda TKTHPTIKINGYTGPGTVRISLVTKDPPHRPHPHELVGKDCRDGFYEAELCPDRCIHSFQ

XP\_001490867.3|Horse TKTHPTIKINGYTGPGTVRISLVTKDPPHRPHPHELVGKDCRDGFYEAELCPDRCIHSFQ

NP\_068810.3|Human 55 TKTHPTIKINGYTGPGTVRISLVTKDPPHRPHPHELVGKDCRDGFYEAELCPDRCIHSFQ

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NP\_001001839.2|Zebrafish NLGIQCVKKKDVGEAVSCRLQTQNNPFKIPDAKIWEEEFDLNAVRLCFQVSITL-SSGDL

NP\_990460.1|Chicken NLGIQCVKKRELEAAVAERIRTNNNPFNVPMEER-GAEYDLSAVRLCFQVWVNG-PGG-L

NP\_001081048.1|Xenopus\_laevis NLGIQCVKKREVEDAVAHRIRTNNNPFNVSPEEL-KADYDLNTVCLCFQVFIPDQAAGRM

XP\_001379658.2|Opossum NLGIQCVKKRDLEQAIAHRMQTNNNPFNVPLEEQ-RGDYDLNAVRLCFQVTIQD-SAGRP

NP\_033071.1|Mouse NLGIQCVKKRDLEQAISQRIQTNNNPFHVPIEEQ-RGDYDLNAVRLCFQVTVRD-PAGRP

NP\_001073711.1|Cow NLGIQCVKKRDLEQAISQRIQTNNNPFQVPIEEQ-RGDYDLNAVRLCFQVTVRD-PAGRP

NP\_001107753.1|Pig NLGIQCVKKRDLEQAINQRIQTNNNPFQVPIEEQ-RGDYDLNAVRLCFQVTVRD-PAGRP

XP\_002807426.1|Marmoset NLGIQCVKKRDLEQAINQRIQTNNNPFQVPIEEQ-RGDYDLNAVRLCFQVTVRD-PSGRP

AFE79532.1|Rhesus\_macaque NLGIQCVKKRDLEQAITQRIQTNNNPFQVPIEEQ-RGDYDLNAVRLCFQVTVRD-PSGRP

XP\_540850.2|Dog NLGIQCVKKRDLEQAISQRIQTNNNPFQVPIEEQ-RGDYDLNAVRLCFQVTVRD-PAGRP

XP\_002916737.1|Panda NLGIQCVKKRDLEQAISQRIQTNNNPFQVPIEEQ-RGDYDLNAVRLCFQVTVRD-PAGRP

XP\_001490867.3|Horse NLGIQCVKKRDLEQAISQRIQTNNNPFQVPIEEQ-RGDYDLNAVRLCFQVTVRD-PAGRP

NP\_068810.3|Human 115 NLGIQCVKKRDLEQAISQRIQTNNNPFQVPIEEQ-RGDYDLNAVRLCFQVTVRD-PSGRP

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NP\_001001839.2|Zebrafish FPLEPVVSQPIYDNRAPNTAELKICRVNRNSGSCRGGDEIFLLCDKVQKEDIEVRFFLDS

NP\_990460.1|Chicken CPLPPVLSQPIYDNRAPSTAELRILPGDRNSGSCQGGDEIFLLCDKVQKEDIEVRFWAEG

NP\_001081048.1|Xenopus\_laevis LPLPFVVSQPIYDNRAPNTAELKICRVNKNSGSCLGGDEIFLLCDKVQKEDIEVIFGLGN

XP\_001379658.2|Opossum LVLPPVLSHPIYDNRAPNTAELKICRVNRNSGSCLGGDEIFLLCDKVQKEDIEVCFSGPG

NP\_033071.1|Mouse LLLTPVLSHPIFDNRAPNTAELKICRVNRNSGSCLGGDEIFLLCDKVQKEDIEVYFTGPG

NP\_001073711.1|Cow LRLAPVLSHPIFDNRAPNTAELKICRVNRNSGSCLGGDEIFLLCDKVQKEDIEVYFTGPG

NP\_001107753.1|Pig LRLPPVLSHPIFDNRAPNTAELKICRVNRNSGSCLGGDEIFLLCDKVQKEDIEVYFTGPG

XP\_002807426.1|Marmoset LRLLPVLSHPIFDNRAPNTAELKICRVNRNSGSCLGGDEIFLLCDKVQKEDIEVYFTGPG

AFE79532.1|Rhesus\_macaque LRLPPVLSHPIFDNRAPNTAELKICRVNRNSGSCLGGDEIFLLCDKVQKEDIEVYFTGPG

XP\_540850.2|Dog LRLSPVLSHPIFDNRAPNTAELKICRVNRNSGSCLGGDEIFLLCDKVQKEDIEVYFTGPG

XP\_002916737.1|Panda LRLSPVLSHPIFDNRAPNTAELKICRVNRNSGSCLGGDEIFLLCDKVQKEDIEVYFTGPG

XP\_001490867.3|Horse LRLSPVLSHPIFDNRAPNTAELKICRVNRNSGSCLGGDEIFLLCDKVQKEDIEVYFTGPG

NP\_068810.3|Human 173 LRLPPVLSHPIFDNRAPNTAELKICRVNRNSGSCLGGDEIFLLCDKVQKEDIEVYFTGPG

\* \*:\*:\*\*:\*\*\*\*\*.\*\*\*\*.\* :.\*\*\*\*\* \*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\* \* .

NP\_001001839.2|Zebrafish WESKGSFSQADVHRQVAIVFRTPPYCDTNLTEPLRVKMQLRRPSDREVSEPMDFQYLPSD

NP\_990460.1|Chicken WEAKGSFAAADVHRQVAIVFRTPPFRERSLRHPVTVRMELQRPSDRQRSPPLDFRYLPHQ

NP\_001081048.1|Xenopus\_laevis WEARGIFSQADVHRQVAIVFRTPAFQDTKIRQSVKVQMQLRRPSDKEVSEPMEFQYLPDE

XP\_001379658.2|Opossum WEARGSFSQADVHRQVAIVFRTPPYAEAALQAPVRVHMQLRRPSDRELSEPMEFQYLPDT

NP\_033071.1|Mouse WEARGSFSQADVHRQVAIVFRTPPYADPSLQAPVRVSMQLRRPSDRELSEPMEFQYLPDT

NP\_001073711.1|Cow WEARGSFSQADVHRQVAIVFRTPPYADPGLQAPVRVSMQLRRPSDRELSEPMEFQYLPDT

NP\_001107753.1|Pig WEARGSFSQADVHRQVAIVFRTPPYADPSLQAPVRVSMQLRRPSDRELSEPMEFQYLPDT

XP\_002807426.1|Marmoset WEARGSFSQADVHRQVAIVFRTPPYADPSLQAPVRVFMQLRRPSDRELSEPMEFQYLPDT

AFE79532.1|Rhesus\_macaque WEARGSFSQADVHRQVAIVFRTPPYADPSLQAPVRVSMQLRRPSDRELSEPMEFQYLPDT

XP\_540850.2|Dog WEARGSFSQADVHRQVAIVFRTPPYADPSLQAPVRVTMQLRRPSDRELSEPMEFQYLPDT

XP\_002916737.1|Panda WEARGSFSQADVHRQVAIVFRTPPYADPSLQAPVRVSMQLRRPSDRELSEPMEFQYLPDT

XP\_001490867.3|Horse WEARGSFSQADVHRQVAIVFRTPPYADPSLQAPVRVSMQLRRPSDRELSEPMEFQYLPDT

NP\_068810.3|Human 233 WEARGSFSQADVHRQVAIVFRTPPYADPSLQAPVRVSMQLRRPSDRELSEPMEFQYLPDT

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NP\_001001839.2|Zebrafish PDEHRLMEKRKRTEGMLHNLKLSSIITG----SSMSAE-RRPFPTAKRTLPVSKQPVAAS

NP\_990460.1|Chicken GDLQCIEEKRKRTRDTFRAFVQRAPLPGLEPNPEPRPP-RRIAVPSR-----PP-PAPQQ

NP\_001081048.1|Xenopus\_laevis GDPHHIDEKRKRTLDNFKHYVKNNPFAG----GETRPQ-RRIAVANRNVPTKSE-PIRPS

XP\_001379658.2|Opossum DDRHRIEEKRKRTLGTFKNIMKTSPFRG---NPDTWTSPRRIAVPSRTTGSTPKPPVPQP

NP\_033071.1|Mouse DDRHRIEEKRKRTYETFKSIMKKSPFNG---PTEPRPPTRRIAVPTRNSTSVPK-PAPQP

NP\_001073711.1|Cow DDRHRIEEKRKRTYETFKSIMKKSPFNG---PTDPRPPTRRIAVPNRGSASIPK-PAPQP

NP\_001107753.1|Pig DDRHRIEEKRKRTYETFKSIMKKSPFNG---PTDPRPATRRIAVPSRSSASVPK-PAPQP

XP\_002807426.1|Marmoset DDRHRIEEKRKRTYETFKSIMKKSPFNG---PTDPRPPPRRIAVPSRSSASIPK-PAPQP

AFE79532.1|Rhesus\_macaque DDRHRIEEKRKRTYETFKSIMKKSPFSG---PTDPRPPPRRIAVPSRSSV-VPK-PAPQP

XP\_540850.2|Dog DDRHRIEEKRKRTYETFKSIMKKSPFNG---PTDPRPPPRRIAVPSRSTTSVPK-PAPQS

XP\_002916737.1|Panda DDRHRIEEKRKRTYETFKSIMKKSPFNG---PTDPRPPPRRIAVPSRSTPSVPK-PAPQP

XP\_001490867.3|Horse DDRHRIEEKRKRTYETFKSIMKKSPFNG---PTDPRPPPRRIAVPARSSASVPK-PAPQP

NP\_068810.3|Human 293 DDRHRIEEKRKRTYETFKSIMKKSPFSG---PTDPRPPPRRIAVPSRSSASVPK-PAPQP

\* : : \*\*\*\*\*\* :. : \* . . \*\* . . . \*

NP\_001001839.2|Zebrafish AP--ASVPAV----------------SAAPPLKPPPTSFF-------------SPPPGQL

NP\_990460.1|Chicken PP---------------------------SMVGAPPAPLFPLGVPPASSP---TPEP-LA

NP\_001081048.1|Xenopus\_laevis IP----------------------VPNPVVSCLPFSMPVLKAENVTSPST---LLSTVNI

XP\_001379658.2|Opossum YTFPPPLSTINLEELSPIVFSASQV--QAPALASAPTPAP----SLVPAPTSTAPAPA--

NP\_033071.1|Mouse YTFPASLSTINFDEFSPMLLPSGQISNQALALAPSSAPVL------AQTM---VPSSAMV

NP\_001073711.1|Cow YSFTPSLSTINFEEFSPMVFPSGQIPSQTSALAPAPTPVLTQTQVLAPAP---APAPGMA

NP\_001107753.1|Pig YPFTPSLSTINFDEFTPMAFASGQIPGQTSALAPAPAPVL----VQAPAP---APAPAMA

XP\_002807426.1|Marmoset YPFSPSLNTINYDEFPTMVF----------------------------------------

AFE79532.1|Rhesus\_macaque YPFTSSLSTINYDEFPTMVFPSGQI-SQASALA--PPQVL----PQAPAP---APAPAMV

XP\_540850.2|Dog YPFTPSLSTINFEEFSPMVFSSGQISSQTSALASAPAPAP----ILAPAP----PAPA--

XP\_002916737.1|Panda YPFTPSLSTINFEEFSPMVFPSGQIPNQTSALAPAPAPIL----AQAPAP---APAP---

XP\_001490867.3|Horse YPFTPSLSTINFEEFSPMVFPSGTIPSQTSALAPAPAPVL------AQAP---APAPAMA

NP\_068810.3|Human 349 YPFTSSLSTINYDEFPTMVFPSGQI-SQASALAPAPPQVL----PQAPAP---APAPAMV

.

NP\_001001839.2|Zebrafish ----------------FTQQKMEPSPLPA-------------------------------

NP\_990460.1|Chicken EALLQLQFDDGVGGSGPPPSTTTTTTTTQCALGGGIPDP-----GGSPLDLGALLGDPP-

NP\_001081048.1|Xenopus\_laevis SDFSN-----------LGFSSQPPSQSDHDRLESMLNYP--SFPGDANLDLVEMLPHENE

XP\_001379658.2|Opossum ----------------------PALPPISQSGEGTLSEALLGLQFDTDGDLAEILADPD-

NP\_033071.1|Mouse -PLAQPPAPAPVLTPGPPQSLSAPVPKSTQAGEGTLSEALLHLQFDADEDLGALLGNSTD

NP\_001073711.1|Cow STLAQA------LAPGLAQAVTPPAPRTNQTGEGTLTEALLQLQFDTDEDLGALLGNNTD

NP\_001107753.1|Pig SALAQAPAPVPVLAPGLAQAVAPPAPKTNQAGEGTLTEALLQLQFDTDEDLGALLGNNTD

XP\_002807426.1|Marmoset -----------------------------------------------------LLGNSTD

AFE79532.1|Rhesus\_macaque SPLAQA----PVLAPGPPQAVAPPAPKPTQAGEGTLSEALLQLQFD-DEDLGALLGNSTD

XP\_540850.2|Dog --------PAPILAPGLAQAMAPPAPKTTQAGEGTLTEALLQLQFDADEDLGALLGNSAD

XP\_002916737.1|Panda ----------------------PPIPKTTQAGEGTLSEALLHLQFDADEDLGALLGNSTD

XP\_001490867.3|Horse SALAQAPAPVPVLAAGLAQAVAPPAPRTTQAGEGTLTEALLQLQFDADEDLGALLGNNTD

NP\_068810.3|Human 401 SALAQAPAPVPVLAPGPPQAVAPPAPKPTQAGEGTLSEALLQLQFD-DEDLGALLGNSTD

NP\_001001839.2|Zebrafish -------SSSDIWKYLQAMS--------------------------VDSQ----------

NP\_990460.1|Chicken ------FDTIDAAELQRLLGPPETPPGGIGAGGGFGELLSLPTNFGDPPSSTAATFGPSP

NP\_001081048.1|Xenopus\_laevis SRC-TSLSSIDNSDFSQLLSESQS--------------SGTLSAALQEPG-------TSQ

XP\_001379658.2|Opossum -STYTNLAAIDNSEFQQLLN------------------QGIPGTLEGPGP-------SGE

NP\_033071.1|Mouse PGVFTDLASVDNSEFQQLLN------------------QGVS----MSHS-------TAE

NP\_001073711.1|Cow PAVFTDLASVDNSEFQQLLN------------------QGVP----MGPH-------TAE

NP\_001107753.1|Pig PTVFTDLASVDNSEFQQLLN------------------QGVS----MPPH-------TAE

XP\_002807426.1|Marmoset PAVFTDLASVDNSEFQQLMN------------------QSLP----VAPH-------TAE

AFE79532.1|Rhesus\_macaque PTVFTDLASVDNSEFQQLLN------------------QGVP----VAPH-------TTE

XP\_540850.2|Dog PAVFTDLASVDNSEFQQLLN------------------QGVS----VAPH-------TAE

XP\_002916737.1|Panda PAVFTDLASVDNSEFQQLLN------------------QGVS----VAPH-------TAE

XP\_001490867.3|Horse PAVFTDLASVDNSEFQQLLN------------------QGVS----MAPH-------TAE

NP\_068810.3|Human 460 PAVFTDLASVDNSEFQQLLN------------------QGIP----VAPH-------TTE

: \* . . :.

NP\_001001839.2|Zebrafish ------PKAV----------------PVLPFPSGTVST----GRDARLITAARGENTVLH

NP\_990460.1|Chicken PMLLSYPEAITRLVQCQTPGGSGGGGPPVGPPQDLGGPLHPPGAPPQP-----TEDSLPS

NP\_001081048.1|Xenopus\_laevis GTFMAYPESIARLM-------T--NRPNEDEGGERIDS----GLINGMFDISREEIHLTS

XP\_001379658.2|Opossum PMLMEYPESITRLM-------TGSQRPPEPTPAPPGAS----GLANGLLG---ADEVFPS

NP\_033071.1|Mouse PMLMEYPEAITRLV-------TGSQRPPDPAPTPLGTS----GLPNG-LS---GDEDFSS

NP\_001073711.1|Cow PMLMEYPEAITRLV-------TGSQRPPDPAPTPLGPP----GLTNGLLS---GDEDFSS

NP\_001107753.1|Pig PMLMEYPEAITRLV-------TGSQRPPDPAPTPLGAS----GLTNGLLS---GDEDFSS

XP\_002807426.1|Marmoset PMLMEYPEAITRLV-------TGAQRPPDPAPAPLGAP----GLPNGLLS---GDEDFSS

AFE79532.1|Rhesus\_macaque PMLMEYPEAITRLV-------TGAQRPPDPAPAPLGAP----GLPNGLLS---GDEDFSS

XP\_540850.2|Dog PMLMEYPEAITRLV-------TGSQRPPDPVPAPVGAS----GLPNGLLS---GDEDFSS

XP\_002916737.1|Panda PMLMEYPEAITRLV-------TGSQRPPDPAPAPLGAS----GLPNGLLS---GDEDFSS

XP\_001490867.3|Horse PMLMEYPEAITRLM-------SGSQRPPDPAPAPLGPX----GLPNGLLS---GDEDFSS

NP\_068810.3|Human 491 PMLMEYPEAITRLV-------TGAQRPPDPAPAPLGAP----GLPNGLLS---GDEDFSS

\*::: \* \* : .

NP\_001001839.2|Zebrafish PYTLHYTHLTHVLLLV

NP\_990460.1|Chicken LGDLDFSAFLSQFPSS

NP\_001081048.1|Xenopus\_laevis LFELDFSSLLSNMK--

XP\_001379658.2|Opossum MGDLDISAFLSQISS-

NP\_033071.1|Mouse IADMDFSALLSQISS-

NP\_001073711.1|Cow IADVDFSALLSQISS-

NP\_001107753.1|Pig IADMDFSALLSQISS-

XP\_002807426.1|Marmoset IADMDFSALLSQISS-

AFE79532.1|Rhesus\_macaque IADMDFSALLSQISS-

XP\_540850.2|Dog IADMDFSALLSQISS-

XP\_002916737.1|Panda IADMDFSALLSQISS-

XP\_001490867.3|Horse ISDMDFSALLSQISS-

NP\_068810.3|Human 537 IADMDFSALLSQISS-

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**Supplementary Figure 4.** Example SRM chromatograms of the control peptide DGFTEAELCPDR (A) and the phosphopeptide SAGpSIPGER [pS45] (B). Prec-98 refers to neutral loss of H3PO4 from the precursor ion; Prec-116 refers to neutral loss of (H3PO4+H2O) from the precursor ion.

**A**

Fig3 SRM.tifFig3 SRM.tif

**B**

**Supplementary Figure 5.** Quality control of the SRM transitions for the RelA/p65 reference peptide DGFYEAELCPDR as a function of time post-TNFα stimulation: Depicted are the relative peak areas (top) and the measured retention time (min) (bottom) for each of the transitions. RSD of retention times are 0.14%.

**suppfigure5.TIF**

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