Supplementary material related to

Phosphoproteomic analysis of FLCN inactivation highlights differential kinase pathways and regulatory TFEB phosphoserines

Iris E. Glykofridis¹, Alex A. Henneman², Jesper A. Balk¹, Richard Goeij-de Haas², Denise Westland³, Sander R. Piersma², Jaco C. Knol², Thang V. Pham², Michiel Boekhout³, Fried J.T. Zwartkruis³, Rob M.F. Wolthuis¹* & Connie R. Jimenez²*

¹ Amsterdam UMC, location VUmc, Vrije Universiteit Amsterdam, Human Genetics, Cancer Center Amsterdam, De Boelelaan 1118, 1081 HV Amsterdam, The Netherlands
² Amsterdam UMC, location VUmc, Vrije Universiteit Amsterdam, Medical Oncology, Cancer Center Amsterdam, De Boelelaan 1118, 1081 HV Amsterdam, The Netherlands
³ University Medical Center Utrecht, Center for Molecular Medicine, Molecular Cancer Research, Universiteitsweg 100, 3584 CG Utrecht, The Netherlands

* corresponding authors: c.jimenez@amsterdamumc.nl & r.wolthuis@amsterdamumc.nl

Supplementary Figures 1- 7 including legends

Reference

Supplementary figure 1. Comparative phosphoproteomic analyses of RPTEC FLCN<sup>POS</sup> vs. FLCN<sup>NEG</sup> cell line pairs.

A) Western blot showing FLCN expression status in six RPTEC/TERT1 cell lines used for phosphoproteomic profiling. Tubulin was used as loading control. B) Coomassie staining of gel containing cell line lysates for phosphoproteomic analyses. C) Western blot of global tyrosine levels among samples used for phosphoproteomic analyses. D) Bar graphs of identified pY phosphosites per individual cell line, with the amount of singly, doubly or triply phosphorylated sites indicated. E) Bar graphs of identified pSTY phosphosites per individual cell line, with the amount of singly, doubly or triply phosphorylated sites indicated. F) Unsupervised hierarchical cluster analyses of FLCN differential pY peptide intensities. G) Unsupervised hierarchical cluster analyses of FLCN differential pSTY peptide intensities.
Supplementary figure 2. Volcano plot and top 20 of most significant pY phosphosites that are either higher or lower phosphorylated in FLCN<sup>NEG</sup> RPTEC.

A) Volcano plot of differential pY phosphosites detected in FLCN<sup>POS</sup> vs. FLCN<sup>NEG</sup> comparison. An interactive version of this volcano plot, which is created using the VolcaNoseR [1] web tool, is available via this link.

B) Bar graphs of the top 20 of most significant pY phosphosites that are more phosphorylated in FLCN<sup>NEG</sup> RPTEC.

C) Bar graphs of the top 20 of most significant pY phosphosites that are less phosphorylated in FLCN<sup>NEG</sup> RPTEC.
Supplementary figure 3. Volcano plot and top 20 of most significant pSTY phosphosites that are either higher or lower phosphorylated in FLCN\textsuperscript{NEG} RPTEC.

A) Volcano plot of differential pSTY phosphosites detected in FLCN\textsuperscript{POS} vs. FLCN\textsuperscript{NEG} comparison. An interactive version of this volcano plot, which is created using the VolcaNoseR [1] web tool, is available via this link.

B) Bar graphs of the top 20 of most significant pSTY phosphosites that are more phosphorylated in FLCN\textsuperscript{NEG} RPTEC.

C) Bar graphs of the top 20 of most significant pSTY phosphosites that are less phosphorylated in FLCN\textsuperscript{NEG} RPTEC.
Supplementary figure 4. Top 20 of active kinases identified by INKA per cell line.

A) INKA based ranking of top 20 most active kinases (pY) identified per individual cell line.

B) INKA based ranking of top 20 most active kinases (pSTY) identified per individual cell line.
Supplementary figure 5. Aggregated networks of kinases and substrates identified in FLCN\textsuperscript{POS} and FLCN\textsuperscript{NEG} RPTECs.

\textbf{A)} Aggregated network (pY + pSTY) of kinases and substrates identified in FLCN\textsuperscript{POS}, which are used to calculate aggregated INKA scores (shown in figure 2).

\textbf{B)} Aggregated network (pY + pSTY) of kinases and substrates identified in FLCN\textsuperscript{NEG}, which are used to calculate aggregated INKA scores (shown in figure 2).
Supplementary figure 6. FLCN-dependent kinase phosphorylation in UOK257 and UOK257-2.
A) Supervised hierarchical cluster analyses of FLCN differential pY peptide intensities. B) Supervised hierarchical cluster analyses of FLCN differential pSTY peptide intensities. C) INKA based ranking of top 20 most active kinases (pY and pSTY) identified in UOK257 and UOK257-2. Kinases strongly dependent on FLCN expression in RPTEC are highlighted in orange. D) Overlap of kinases with highest activity (INKA score >75) in FLCN<sup>NEG</sup> RPTEC and FLCN<sup>NEG</sup> UOK257 cells. E) Activities of kinases (pY and pSTY) in UOK257 and UOK257-2 that were top differential kinases dependent on FLCN in RPTEC. Activity of EPHA2, EPHB1, RPS6KA3, RPS6KA1, MAPK1, MAPK3, MAPK10 and PAK1 are affected similarly in both cell line backgrounds (boxed orange). EGFR and MET activities appear to be more active FLCN<sup>NEG</sup> RPTEC but were less active in the FLCN<sup>NEG</sup> UOK257 tumor cell line. F) Normalized intensities of individual FLCN-dependent phosphosites, of which phosphorylation was significantly affected upon FLCN loss in RPTEC, detected in UOK257 and UOK257-2 cell lines too.
Supplementary figure 7. FLCN dependent HIPK2, CDK1/2 and ROCK1/2 phosphorylation and TFEB/TFE3 western blots. 
A) Differential phosphosites of HIPK2 and its substrates HMGA1 and TRIM28. B) Silmitasertib dose-response curves of FLCN^{POS} versus FLCN^{NEG} RPTECs show no difference in sensitivity to HIPK2/CK2 inhibition. C) Western blot of total TFEB showing a decrease in its phosphorylation levels, indicated by a shift downwards upon amino acid starvation of FLCN^{POS} RPTECs. TFEB is also less phosphorylated in FLCN^{NEG} cells, independent of nutrient availability. TFE3 behaves similarly upon amino acid starvation or FLCN loss in RPTECs. RRAGD expression is upregulated upon FLCN loss and TFEB/TFE3 activation. D) siRNA mediated knockdown of TFEB in RPTEC confirms antibody specificity in western blots of TFEB E) siRNA mediated knockdown of TFEB in RPTEC FLCN^{-KO} confirms antibody specificity in immunofluorescent stainings of TFEB. F) Differential phosphosites of CDK1/2 and WEE1 kinases and substrates NPM, TMPO, RB1, TOP2B and LMNA. G) Differential phosphosites of ROCK1/2 kinases and CFL1/2