Supporting Information

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SI Materials and Methods

MS analysis was performed at the W. M. Keck Foundation Biotechnology Resource Laboratory, Yale University, using a Thermo Scientific LTQ Orbitrap mass spectrometer equipped with a Waters nanoAcquity UPLC system. Liquid chromatography separation was performed with a Waters Symmetry C18 180-μm × 20-mm trap column and a 1.7 μm, 75-μm × 250-mm nanoAcquity UPLC column (35 °C) for peptide separation. Trapping was done at 15 μL/min, 99% (vol/vol) Buffer A (100% water, 0.1% formic acid) for 1 min. Peptide separation was performed at 300 nL/min with Buffer A: 100% water, 0.1% formic acid, and Buffer B: 100% CH_3CN, 0.075% formic acid. A linear gradient (91 min) was run with 5% (vol/vol) Buffer B at initial conditions, 40% (vol/vol) Buffer B at 90 min, and 85% (vol/vol) Buffer B at 91 min. MS was acquired in the Orbitrap using 1 microscan, and a maximum inject time of 900 ms followed by six data-dependent MS/MS acquisitions in the ion trap. All MS/MS spectra were searched in-house using the Mascot (www.matrixscience.com) Distiller program to generate Mascot-compatible files before using the Mascot algorithm to search the National Center for Biotechnology Information, nr (non-redundant) database. Parameters used for searching were variable methionine oxidation and carbamidomethylated cysteine, a peptide tolerance of ± 20 ppm, MS/MS fragment tolerance of ± 0.6 Da, and peptide charges of +2 or +3. Normal and decoy database searches were run.

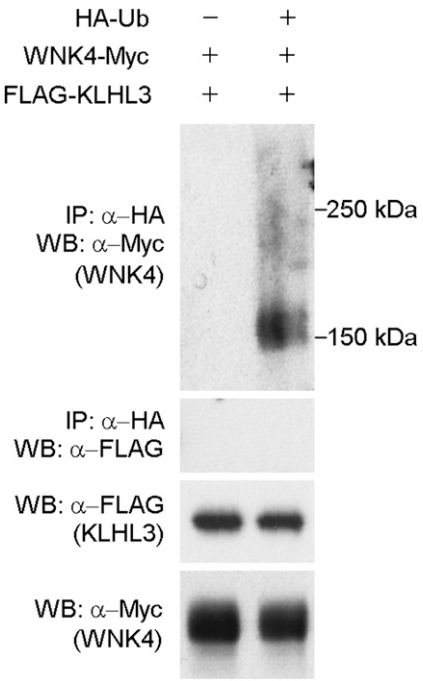

Fig. S1. Polyubiquitination of WNK4. Cell lysates expressing the indicated proteins were immunoprecipitated with an antibody to HA in denaturing condition, followed by Western blotting. Anti–Myc-reactive proteins in anti-HA immunoprecipitates correspond to polyubiquitinated WNK4. KLHL3, Kelch-like 3; IP, immunoprecipitation; Ub, ubiquitin; WB, Western blotting; WNK4, with no lysine kinase 4.

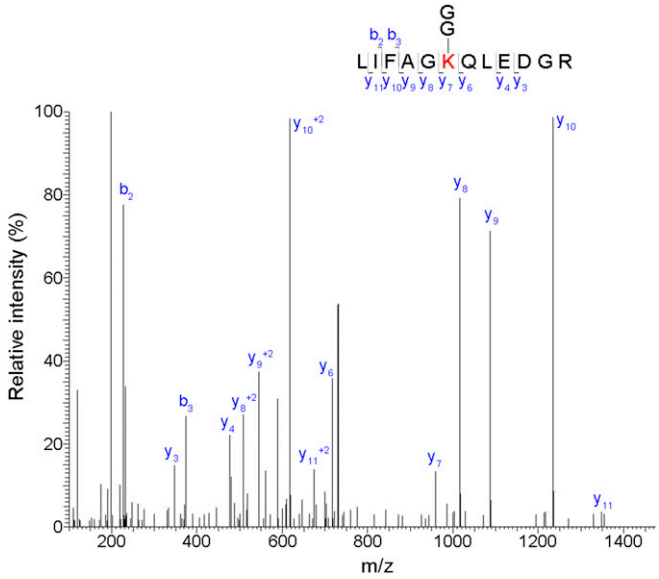

Fig. S2. A representative MS/MS spectrum showing assignment of the peptide containing ubiquitinated K48 in ubiquitin. Specific y and b fragment ions allowed the identification of lysine residue with di-glycine modification.

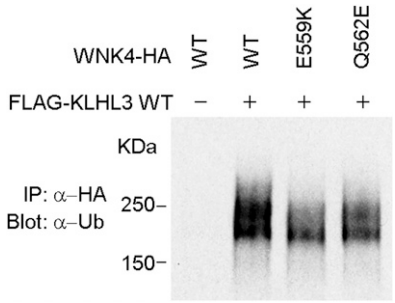

Fig. S3. Polyubiquitination of WNK4 through KLHL3 was reduced by E559K or Q562E substitution. COS-7 cell lysates expressing the indicated proteins were immunoprecipitated with an antibody to HA, followed by Western blotting with anti-ubiquitin antibody.

Table S1. List of CUL3 peptides identified by LC-MS/MS

Score	Peptide sequence	M/Z	Charge
84	MQHNVLVAEVTQQLK	869.47	2
66	GLTEQEVETILDK	737.88	2
65	EDGSEVGVGGAQVTGSNTR	910.42	2
60	YVNSIWDLLK	625.84	2
60	ALQSLAC*GKPTQR	715.38	2
57	VLTTGYWPTQSATPK	825.43	2
47	NAC*QMLMILGLEGR	803.40	2
41	NAYTMVLHK	538.78	2
40	KNNSGLSFEELYR	778.89	2
38	FLLESFNNDR	627.81	2
37	SVYEEDFEAPFLEMSAEFFQMESQK	1,006.77	3
35	HAFEIFR	460.24	2
31	VYTYVA	715.36	1
31	LKTEC*GC*QFTSK	486.89	3
31	NAIQEIQR	971.52	1
30	TEC*GC*QFTSK	609.25	2
19	MQHNVLVAEVTQQLK	579.98	3
16	TMC*EC*MSSYLR	719.28	2
16	ALVSEEGEGKNPVDYIQGLLDLK	829.77	3
9	FLPSPVVIKK	564.36	2

C*, carbamidomethylated cysteine; CUL3, cullin 3; LC, liquid chromatography.

Table S2. List of WNK1 peptides identified by LC-MS/MS

Score	Peptide sequence	M/Z	Charge
77	IGDLGLATLK	500.80	2
52	GPVLATSSGAGVFK	645.85	2
38	LGAAAADAGTGR	1,030.53	1
29	VPPAVIIPPAAPLSGR	777.97	2
24	GLQHPNIVR	517.30	2
15	VTSGVKPASFDK	618.33	2
11	EGPVASPPFMDLEQAVLPAVIPK	1,203.14	2

LC, liquid chromatography.