

Figure S1. Bound chloride ions in the KLHL3-WNK3 and KLHL3-WNK4 structures. (A and B) A bound chloride ion is observed in the KLHL3-WNK3 and KLHL3-WNK4 complexes and forms electrostatic interactions with positive charged Arg339 and Arg360 in KLHL3. KLHL3 residues are labelled in green, WNK3 peptide is shown in yellow, and WNK4 peptide is shown in pale pink. The chloride ions are shown as magenta spheres. The electrostatic interactions are demonstrated using dashed lines. The distances (Å) are indicated. (C-F) Stick representation and 2Fo-Fc electron density maps contoured at 0.5 σ (2.52 rmsd) for KLHL3 Arg339 and the interfacing Cl⁻ (C and E) or a replaced water molecule (D and F) in the indicated structures. Blue meshes represent the experimental electron density. The green mesh around the water molecule represents a positive discrepancy comparing experimental data with the structure model.

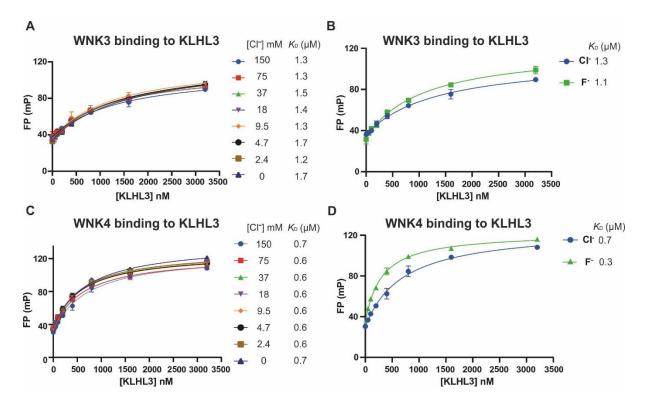


Figure S2. The effect of chloride on the interaction between KLHL3 and WNK peptides. (A-D) Analysis of the interaction between KLHL3 and WNK peptides by fluorescence polarisation in buffers containing various CI⁻ concentrations. Purified KLHL3 (a.a. 298-587) was diluted appropriately and mixed at a 1:1 volume ratio with 20 nM Rhodamine-110 fluorophore labelled WNK3 or WNK4 peptides to the concentration stated in the Figure, with the peptide concentration consistent at 10 nM. In panels **A** and **C**, buffers containing various CI⁻ concentrations were balanced with phosphate buffer to keep a constant ionic strength of 154 mM. In panels **B** and **D**, 150 mM CI⁻ or 150 mM F⁻ buffers were tested. Fluorescence polarisation measurements were recorded and corrected to the fluorescent probe alone. Each data point represents two technical replicates. For data analysis, one site-total with constant NS (slope of non-specific binding) equal to zero was assumed (model Y=Bmax*X/(K_D +X) + Background) and the disassociation constant was obtained. Binding curves were then generated with Prism6 using milli-polarization (mP) units.

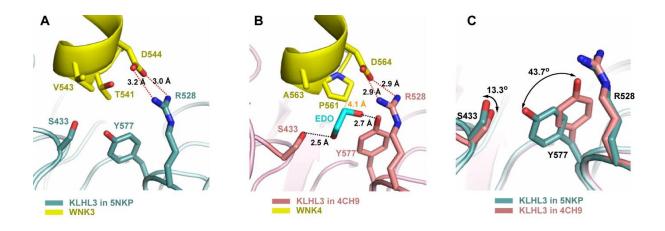


Figure S3. Side chain packing differences around the key salt bridge between WNKs and KLHL3 Arg528. (A) KLHL3 Arg528 forms a key salt bridge interaction with WNK3 Asp544. Selected side chains in the vicinity are displayed in stick representation, including KLHL3 Tyr577 as well as WNK3 Thr541 and Val543. KLHL3 is shown in green. WNK3 is shown in yellow. Salt bridge interactions are indicated with red dashed lines and labelled with distance measurements. (B) In the equivalent WNK4 complex, KLHL3 Arg528 forms a salt bridge with WNK4 Asp564. The diverged WNK4 degron residues Pro561 and Ala563 allowed an ethylene glycol (EDO) molecule from the cryo-protectant to pack in the crystal lattice adjacent to the salt bridge and to form hydrogen bonds with the side chains of KLHL3 Ser433 and Tyr577, as indicated with black dashed lines and distance measurements. The distance between WNK4 Pro561 and the EDO is also indicated in orange. KLHL3 is shown in red, WNK4 in yellow. (C) Superposition of the two KLHL3-WNK complexes highlighting the KLHL3 residues Ser433, Arg528 and Tyr577. Side chain packing in the vicinity of KLHL3 Arg528 is altered by the WNK3-specific substitutions of Thr541 and Val543 and the shift in the position of KLHL3 Tyr577. KLHL3 in PDB 5NKP (KLHL3-WNK3 co-structure) is coloured in green and KLHL3 in PDB 4CH9 (KLHL3-WNK4 co-structure) is coloured in red.

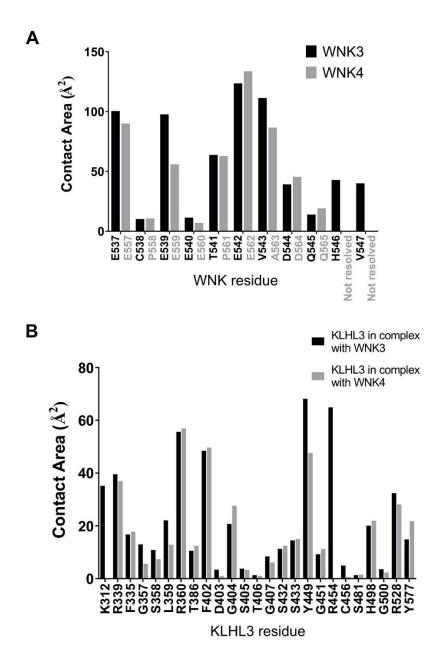


Figure S4. Comparison of contact residues in the KLHL3-WNK3 and KLHL3-WNK4 complex structures. (A) Buried interface surface areas for interacting residues in the WNK3 (black bars) and WNK4 (grey bars) peptide sequences, respectively. The side chain of Glu559 was only partially resolved in the KLHL3-WNK4 structure. (B) Buried interface surface areas for interacting residues in KLHL3 in the WNK3 (black bars) and WNK4 (grey bars) complex structures, respectively. For KLHL3 Lys312, the interacting residues in WNK4 were not fully resolved.

C Carbamidomethylation (+57.02) Deamidation (NQ) (+0.98) Oxidation (M) (+15.99) Phosphorylation (STY) (+79.97)

MATDSGDPAS TEDSEKPDGI SFENRVPQVA ATLTVEARLK EKNSTFSASG ETVERKRFFR KSVEMTEDDK VAESSPKDER
 IKAAMNIPRV DKLPSNVLRG GQEVKYEQCS KSTSEISKDC FKEKNEKEME EEAEMKAVAT SPSGRFLKFD IELGRGAFKT
 VYKGLDTETW VEVAWCELQD RKLTKAEQQR FKEEAEMLKG LQHPNIVRFY DSWESILKGK KCIVLTELM TSGTLKTYLK
 RFKVMKPKVL RSWCRQILKG LQFLHTRTPP IIHRDLKCDN IFITGPTGSV KIGDLGLATL MRTSFAKSVI GTPEFMAPEM

321 YEEHYDESVD VYAFGMCMLE MATSEYPYSE CONAAQIYRK VTSGIKPASF NKVTDPEVKE IIEGCIRONK SERLSIRDLL

	401 <mark>d</mark>		421 C			458 2		475 P
401	NHAFFAEDTG	LRVELAEEDD	502	511 514	KHKDNEALEF.	532 538	EVAYEMVKSG	557 560
481		IKKTREKKPA	C	CKSMGNVFPQ	PQNTTLPLAP	d c AQQTGAECEE	TEVDQHVRQQ	d c
561	SSVTGDNLSE	AGAASVIHSD	TSSQPSVAYS	SNQTMGSQMV	SNIPQAEVNV	PGQIYSSQQL	VGHYQQVSGL	QKHSKLTQPQ
641	ILPLVQGQST	VLPVHVLGPT	VVSQPQVSPL	TVQKVPQIKP	VSQPVGAEQQ	AALLKPDLVR	SLNQDVATTK	71415 718 720 ENVSSPDNPS
721	722 725 GNGKQDRIKQ	73436 RRASCPRPEK	GTKFQLTVLQ	VSTSGDNMVE	CQLETHNNKM	VTFKFDVDGD		EDNFVLESEK
801	EKFVEELRAI	VGQAQEILHV	HFATERATGV	DSITVDSNSS		851 853 856 57 859 D D D D NSTSTOTSNE	SAPQSSPVGR	WRFCINQTIR
		894	905		927	936 P	~~ 949 ℃	~
881	-	-	HPLPSPKNTS				VVDGKISECA	
961	962 970 d 0 YOVEDNROIM	APVTNSSSYS	TTSVRAVPAE	CEGLTKOASI	FIPVYPCHOT			P
	~ ~	1051	1065 36 1069 70		~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	1095	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	~
1041	PQTLSVQQPA	MDAEFISQEG		PKTVIPTQTP	~		PKLEFADNRI	KTLDEKLRNL
1121	LYOFHSTSST	VPESOKDTOS	TOSPESSE	DTLSCPVTEV	C	1171 117475 1180 P dP d SPVOSPNFOO	TGSKLLSNVA	ASOPANTSVE
1101	arganoroor	1215 1219	1224	1238	INIDIOJIND	1253	TODALLON VA	1278
1201	KRDLNVITSV	PSELCLHEMS	SDASLPGDPE	AYPAAVSSGG	AIHLQTGVET	EEMRSAIAPD	PIPLTRESTA	DTRALNRCKA
1001			10 800 100	1318 d			1346 1348	
1281	MSGSFØRGRF	1379	1385 1387		1402	SQLVEIEPAT	QNPKTSFSYE 1424 1426	KLQALQETCK
1361	ENKGVPKQGD	NFLSFSAACE	TDVSSVTPEK	EFEETSATGS	SMQSGSELLL	KEREILTAGK	QPSSDSEFSA	SLAGSGKSVA
	1447 1449 <mark>d</mark> C						1508 <mark>cl</mark>	
1441	KTGPESNQCL 1530		TQSSLFYSPS	SPMSSDDESE	IEDEDLKVEL 1561 1570		VVNLQTQQNK 1588	ELQELYERLR
1521	P	P	r pr sfksklr	SRPQSLTHVD	d		SPASKKGMFT	DDLHKLVDDW
1601		KPSLNQLKQS	QHKLETENWN	KVSENTPSTM		SQIRGAVPTS	LPQGLSLPSF	PGPLSSYGMP
1681	HVCQYNAVAG	A GYPV QWVGI	SGTTQQSVVI	PAQSGGPFQP	1722 1724 GMNMQAFPTS	SVQNPATIPP	GPK	

Figure S5. Overview of WNK3 peptide hits from hypotonic treatment. Residues recovered in elastase MS/MS are shaded in grey. Modifications are labelled above residues. Peptide hits in the kinase activation segment are illustrated.

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Carbamidomethylation (+57.02)
 Deamidation (NQ) (+0.98)
 Oxidation (M) (+15.99)
 Phosphorylation (STY) (+79.97)

321 YEEHYDESVD VYAFGMCMLE MATSEYPYSE CONAAQIYRK VTSGIKPASF NKVTDPEVKE IIEGCIRQNK SERLSIRDLL

	401 d		421			401 408 P P		
401	NHAFFAEDTG	LRVELAEEDD	CSNSSLALRL	WVEDPKKLKG	KHKDNEAIEF	SFNLETDTPE	EVAYEMVKSG	FFHESDSKAV
			502	511 514 C D		538	545	560
481	AKSI RDRVTP	IKKTREKKPA			PQNTTLPLAP		TEVDOHVRQQ	LLQRKPQQHC
	562			595 599			628	
561	SSVTGDNLSE	AGAASVIHSD	TSSQPSVAYS	SNQTMGSQMV	SNIPQAEVNV	PGQIYSSQQL	VGHYQQVSGL	QKHSKLTQPQ
	646 648		668	673	682 P		701 704	71415 718 720
641	ILPLVQGQST	VLPVHVLGPT	VVSQPQVSPL	TVQKVPQIKP	VSQPVGAEQQ	AALLKPDLVR	SLNQDVATTK	ENVSSPDNPS
	722 725 730	73435		758	761 52 770		789	793 C
721	GNGKQDRIKQ	RRASCPRPEK	GTKFQLTVLQ	VSTSGDNMVE	CQLETHNNKM	VTFKFDVDGD	APEDIADYMV	EDNFVLESEK
				83738 840 Pd P	841 844 45 d PP	851 853 856 57 58 59 d P d P P d	861 86536 P P P	874
801	EKFVEELRAI	VGQAQEILHV	HFATERATGV	DSITVDSNSS	QTGSSEQVQI	NSTSTQTSNE	SAPQSSPVGR	WRFCINQTIR
	886	894	905 909 P P	911 <mark>d</mark>	927 C	936	949 C	
881	NRETQSPPSL	QHSMS AVPGR	HPLPSPKNTS	NKEISRDTLL	TIENNPCHRA	LFTSKSEHKD	VVDGKISECA	SVETKQPAIL
	962 966 970 <mark>d d</mark> o	975 <mark>d</mark>		991	1007	1012 1018 1012 1018 1	1026 27 28 1030 d P P d	
961	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	APVTNSSSYS		~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	~~~~	PGESTQTSGN	SLTTLAFDQK
		1051 1057	1065 106970 d PP	P	1088 <mark>d</mark>	1095		
1041	PQTLSVQQPA	MDAEFISQEG					PKLEFADNRI	
		1134 1140 P P	1143	1155	1166	1171 117475 1177 1179 D D D D		1196 <mark>d</mark>
1121	LYQEHSISSI		IDSPFSSSAE		IAISHCGIKD	SPVQSPNFQQ	TGSKLLSNVA	ASQPANISVF
	1209	1212 1215 121920 P C D		1237 38 P P	1245 46 C P	1253		1278
1201		PSELCLHEMS	SDASLPGDPE		~			
	1281 32 0 P			1318 <mark>d</mark>	1321 P	1331 P	1345 46 1348 PPP	1359 C
1281	x				SEEAFIKTAK	SQLVEIEPAT	~	KLQALQETCK
	1368 <mark>C</mark>	1374 1379 P	1385 1387 P P	q q q			1424 P	
1361		NFLSFSAACE	TDVSSVTPEK	EFEETSATGS	SMQSGSELLL	KEREILTAGK		
	1446 47 48 49 P C C C						1503	1513 d
1441	KTGPESNQCL	PHHEEQAYAQ	TQSSLFYSPS	SPMSSDDESE		NN		ELQELYERLR
		1538		1555	1561 1570 d	d	1588	
1521		EIPLPPASPR	RPRSFKS KLR	SRPQSLTHVD	NGIVATDPLC	VESNAASCQQ	SPASKKGMFT	
	1607 d							1679
1601		KPSLNQLKQS	QHKLETENWN	KVSENTPSTM		SQIRGAVPTS	LPQGLSLPSF	PGPLSSYGMP
	1683 1686 C d				1722 23 24			

Figure S6. Overview of WNK3 peptide hits from isotonic treatment. Residues recovered in elastase MS/MS are shaded in grey. Modifications are labelled above residues. Peptide hits in the kinase activation segment are illustrated.

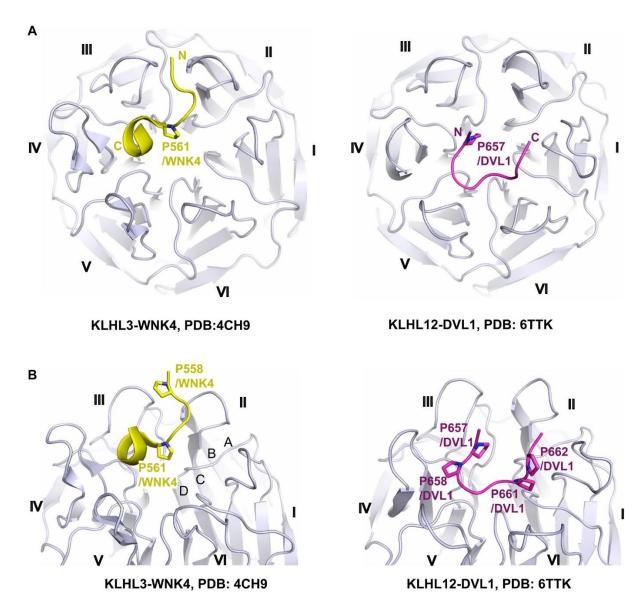


Figure S7. Diverse binding modes of Kelch-interacting PXXP motifs in substrate degrons. (A) Overviews of the KLHL3-WNK4 and KLHL12-DVL1 co-structures showing the substrate peptide conformations that contact multiple blades in the Kelch domains. WNK4 Pro561 and DVL1 Pro657 in stick representation both anchor the degron peptides in the centre of the Kelch domain binding pockets. Kelch domains are shown in grey, WNK4 in yellow and DVL1 in purple. N and C-termini of the degron peptides are labelled. Blades I to VI of the β -propeller are labelled for each Kelch domain. (B) The proline-rich motifs in the peptide degrons bind to distinct parts of the Kelch domains. Prolines in each degron motif are shown in stick representation. Blades I to VI and the β -strands A to D in Blade II are labelled for the Kelch domain.