Supplementary Table S1. Analysis of putative TNIK inhibitor-resistant mutants

Site	Region	Mutation	Active	Inhibitor-resistant
V31	Pocket Protector (G-loop -1)	V31R	Yes	No
		V31W	**Yes**	**Yes**
Y36	G-loop +5	Y36F	Reduced	No
F107	Gatekeeper + 2	F107G	Yes	No
C108	Gatekeeper + 3 (Hydrogen bond with NCB-0846)	C108M	Yes	No
G111	Gatekeeper + 6	G111Q	Yes	No
		G111R	Yes	No
S112	Gatekeeper + 7	S112Q	No	n.a.
V170	DGF -1	V170T	No	n.a.
		V170W	No	n.a.

n.a., not applicable.

Supplementary Table S2. Alignment of Merlin Human and Fly sequences around the Merlin residues that conform to the TNIK consensus phosphorylation motif (1).

Site	Species	Sequence								
S13	Human	M	S	F	S	S	L	K	R	K
	Fly	S	P	F	G	S	K	K	N	R
T23	Human	P	K	T	F	T	V	R	I	V
	Fly	N	R	S	L	S	V	R	V	S
T272	Human	D	K	E	F	T	I	K	P	L
	Fly	D	K	K	F	T	I	R	L	V
S315	Human	R	K	A	D	S	L	E	V	Q
	Fly	R	K	P	D	T	M	E	I	Q
T576	Human	S	K	H	N	T	I	K	K	L
	Fly	N	K	Y	S	T	L	K	K	L

Supplementary Table S3. Mass spectrometry phospho-peptide analysis (HEK 293T cells; Merlin and TNIK co-expression).

Merlin p-site (PSM)	**Condition**	**Relative Abundance ($\times 10^2$)**	**Fold**
pS13 (33)	Empty vector	n.d.	-
	Merlin	0.044	1.00
	Merlin + TNIK	0.501	11.35
	Merlin + TNIK + NCB-0846	0.170	3.86
	Merlin + TNIK K54R	0.015	0.33
pT272 (83)	Empty vector	n.d.	-
	Merlin	0.010	1.00
	Merlin + TNIK	1.170	116.56
	Merlin + TNIK + NCB-0846	0.841	84.11
	Merlin + TNIK K54R	0.002	0.19
pS315 (175)	Empty vector	0.89	-
	Merlin	0.179	1.00
	Merlin + TNIK	25.663	143.74
	Merlin + TNIK + NCB-0846	23.872	133.71
	Merlin + TNIK K54R	0.114	0.64
pS518 (139)	Empty vector	0.804	-
	Merlin	0.236	1.00
	Merlin + TNIK	3.876	0.81
	Merlin + TNIK + NCB-0846	2.462	0.52
	Merlin + TNIK K54R	4.794	1.00
pS566 (35)	Empty vector	n.d.	-
	Merlin	0.236	1.00
	Merlin + TNIK	0.536	2.27
	Merlin + TNIK + NCB-0846	0.366	0.67
	Merlin + TNIK K54R	0.157	0.16
pT576 (n.d.)	Empty vector	n.a.	-
	Merlin	n.a.	-
	Merlin + TNIK	n.a.	-
	Merlin + TNIK + NCB-0846	n.a.	-
	Merlin + TNIK K54R	n.a.	-
pT581 (5)	Empty vector	n.d.	-
	Merlin	0.009	1.00
	Merlin + TNIK	0.012	1.32
	Merlin + TNIK + NCB-0846	0.004	0.49
	Merlin + TNIK K54R	0.003	0.31

n.d., not detected; n.a., not applicable.

Supplementary Table S4. Molecular dynamics simulations of Merlin WT and following phosphorylation of different Merlin residues.

Site(s)	RMSF (Å)	ΔRMSF (Å)	RG (Å)	ΔRG(50%) (Å)
S13	2.4	0.5	35.6	0.81
T272	1.9	0.2	35.4	0.80
S315	2.3	0.4	35.0	0.79
S518	2.8	0.7	37.5	1.20
T576	2.2	0.4	37.4	1.02
S13, T272, S315, T576	2.0	0.3	35.1	1.11
WT	1.9	-	36.0	0.80

RMSF: root-mean square fluctuation; RG: radius of gyration.

Supplementary Table S5. List of oligonucleotides used in this study.

shRNA control
Forward:
5’ CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTTGTTTTT 3’
Reverse:
5’ AATTAAAAACAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTTG 3’

shRNA TNIK#1
Forward:
5' CCGGGCTCCTAAACCGTATCATAAACTCGAGTTTATGATACGGTTTAGGAGCTTTTT 3'
Reverse:
5' AATTAAAAAGCTCCTAAACCGTATCATAAACTCGAGTTTATGATACGGTTTAGGAGC 3

shRNA TNIK#2
Forward:
5' CCGGGTGGCCTACATTCATTCCAATCTCGAGATTGGAATGAATGTAGGCCACTTTTT 3'
Reverse:
5' AATTAAAAAGTGGCCTACATTCATTCCAATCTCGAGATTGGAATGAATGTAGGCCAC 3'

Cloning primers *attB*-TNIK
Forward: 5’ GGGGACAAGTTTGTACAAAAAAGCAGGCTTTATGGCGAGCGACTCCCCGGCTCGAA 3’
Reverse: 5’ GGGGACCACTTTGTACAAGAAAGCTGGGTATTACCAGTTCATCATGGAATTTCTG 3’

Mutagenesis TNIK K54R (a161g)
Forward: 5’ GCCAGCTTGCAGCCATCAGGGTTATGGATGTC 3’
Reverse: 5’ GACATCCATAACCCTGATGGCTGCAAGCTGGC 3’

Mutagenesis TNIK V31W (g91t_t92g_t93g_)
Forward: 5’ GGGATCTTTGAATTGGTGGAACTTTGGGGAAATGGAACATACGGGC 3’
Reverse: 5’ GCCCGTATGTTCCATTTCCCCAAAGTTCCACCAATTCAAAGATCCC 3’

Mutagenesis TNIK shRNA#1 resistant (t3858c, t3861c, a3864g, g3867t, t3870c, t3873c, a3876g)
Reaction 1
Forward: 5’ ATATATGCTTGGGCCCCTAAGCCGTACCATAAGTTCATGGCATTT 3’
Reverse: 5’ AAATGCCATGAACTTATGGTACGGCTTAGGGGCCCAAGCATATAT 3’

Reaction 2:
Forward: 5’ ATATATGCTTGGGCCCCCAAGCCTTACCACAAGTTCATGGCATTT 3’
Reverse: 5’ AAATGCCATGAACTTGTGGTAAGGCTTGGGGGCCCAAGCATATAT 3’

Mutagenesis pDONR221-TNIK ΔSTOP (for generation of pLenti6.3/TO/TNIK-V5)
Forward: 5’ AATTCCATGATGAACTGGGACCCAGCTTTCTTGTAC 3’
Reverse: 5’ GTACAAGAAAGCTGGGTCCCAGTTCATCATGGAATT 3’

Mutagenesis Merlin S13A (t37g)
Forward: 5’ CGCATGAGCTTCAGCGCTCTCAAGAGGAAGC 3’
Reverse: 5’ GCTTCCTCTTGAGAGCGCTGAAGCTCATGCG 3’

Mutagenesis Merlin S13D (t37g_c38a_)
Forward: 5’ CCCGCATGAGCTTCAGCGATCTCAAGAGGAAGCAAC 3’
Reverse: 5’ GTTGCTTCCTCTTGAGATCGCTGAAGCTCATGCGGG 3’

Mutagenesis Merlin T23A (a67g)
Forward: 5’ CAACCCAAGACGTTCGCCGTGAGGATCGTCA 3’
Reverse: 5’ TGACGATCCTCACGGCGAACGTCTTGGGTTG 3’

Mutagenesis Merlin T272A (a814g)
Forward: 5’ CGTACAGTGACAAGGAGTTTGCTATTAAACCACTGGATAAG 3’
Reverse: 5’ CTTATCCAGTGGTTTAATAGCAAACTCCTTGTCACTGTACG 3’

Mutagenesis Merlin T272D (a814g_c815a_) Forward: 5’ CATCTCGTACAGTGACAAGGAGTTTGATATTAAACCACTGGATAAGAAAATT 3’ Reverse: 5’ AATTTTCTTATCCAGTGGTTTAATATCAAACTCCTTGTCACTGTACGAGATG 3’
Mutagenesis Merlin S315A (t943g) Forward: 5’ GGAGAAGGAAAGCCGATGCTTTGGAAGTTCAGCAG 3’ Reverse: 5’ CTGCTGAACTTCCAAAGCATCGGCTTTCCTTCTCC 3’
Mutagenesis Merlin S315D (t943g_c944a_) Forward: 5’ GAGGAGAAGGAAAGCCGATGATTTGGAAGTTCAGCAGATG 3’ Reverse: 5’ CATCTGCTGAACTTCCAAATCATCGGCTTTCCTTCTCCTC 3’
Mutagenesis Merlin T576A (a1726g) Forward: 5’ GGTGGCAGCAGCAAGCACAATGCCATTAAAAAGC 3’ Reverse: 5’ GCTTTTTAATGGCATTGTGCTTGCTGCTGCCACC 3’
Mutagenesis Merlin T576D (a1726g_c1727a_) Forward: 5’ GTGGCAGCAGCAAGCACAATGACATTAAAAAGCTCACCTTGC 3’ Reverse: 5’ GCAAGGTGAGCTTTTTAATGTCATTGTGCTTGCTGCTGCCAC 3’
Mutagenesis Merlin T581A (a1741g) Forward: 5’CAATACCATTAAAAAGCTCGCCTTGCAGAGCGCCAA 3’ Reverse: 5’ TTGGCGCTCTGCAAGGCGAGCTTTTTAATGGTATTG 3’
***TNIK* qPCR** Forward: 5’ TCCACCAAAGGTGCCTCAAA 3’ Reverse: 5’ CCCAGAGCACTACCATTCCC 3’
***GAPDH* qPCR** Forward: 5’ AGCCACATCGCTCAGACAC 3’ Reverse: 5’ GCCCAATACGACCAAATCC 3’

Supplementary References

1. Miller CJ, Lou HJ, Simpson C, van de Kooij B, Ha BH, Fisher OS*, et al.* Comprehensive profiling of the STE20 kinase family defines features essential for selective substrate targeting and signaling output. PLoS Biol **2019**;17(3):e2006540 doi 10.1371/journal.pbio.2006540.