Supporting information

The interaction of the mitochondrial protein importer TOMM34 with HSP70 is regulated by TOMM34 phosphorylation and binding to 14-3-3 adaptors

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Supplementary table S1.

pET21-14-3-3β
MTMDKSELVQKAKLAEQAERYDDMAAAMKAVTEQGHELSNEERNLLSVAYKNVVGARRSSWRVISSIEQK
TERNEKKQQMGKEYREKIEAELQDICNDVLELLDKYLIPNATQPESKVFYLKMKGDYFRYLSEVASGDNK
QTTVSNSQQAYQEAFEISKKEMQPTHPIRLGLALNFSVFYYEILNSPEKACSLAKTAFDEAIAELDTLNE
ESYKDSTLIMQLLRDNLTLWTSENQGDEGENLYFQSLEHHHHHH

pET21-14-3-3γ
MVDREQLVQKARLAEQAERYDDMAAAMKNVTELNEPLSNEERNLLSVAYKNVVGARRSSWRVISSIEQKT
SADGNEKKIEMVRAYREKIEKELEAVCQDVLSLLDNYLIKNCSETQYESKVFYLKMKGDYYRYLAEVATG
EKRATVVESSEKAYSEAHEISKEHMQPTHPIRLGLALNYSVFYYEIQNAPEQACHLAKTAFDDAIAELDT
LNEDSYKDSTLIMQLLRDNLTLWTSDQQDDDGGEGNNENLYFQSLEHHHHHH

pDEST17-HIS-14-3-3ε
MSYYHHHHHHLESTSLYKKAGFEGDRTMDDREDLVYQAKLAEQAERYDEMVESMKKVAGMDVELTVEERN
LLSVAYKNVIGARRASWRIISSIEQKEENKGGEDKLKMIREYRQMVETELKLICCDILDVLDKHLIPAAN
TGESKVFYYKMKGDYHRYLAEFATGNDRKEAAENSLVAYKAASDIAMTELPPTHPIRLGLALNFSVFYYE
ILNSPDRACRLAKAAFDDAIAELDTLSEESYKDSTLIMQLLRDNLTLWTSDMQGDGEEQNKEALQDVEDE
NQ

pNIC28-Bsa4-14-3-3σ
MHHHHHHSSGRENLYFQGHMERASLIQKAKLAEQAERYEDMAAFMKGAVEKGEELSCEERNLLSVAYKNV
VGGQRAAWRVLSSIEQKSNEEGSEEKGPEVREYREKVETELQGVCDTVLGLLDSHLIKEAGDAESRVFYL
KMKGDYYRYLAEVATGDDKKRIIDSARSAYQEAMDISKKEMPPTNPIRLGLALNFSVFHYEIANSPEEAI
SLAKTTFDEAMADLHTLSEDSYKDSTLIMQLLRDNLTLWT

pDEST17-HIS-14-3-3τ
MSYYHHHHHHLESTSLYKKAGFEGDRTMEKTELIQKAKLAEQAERYDDMATCMKAVTEQGAELSNEERNL
LSVAYKNVVGGRRSAWRVISSIEQKTDTSDKKLQLIKDYREKVESELRSICTTVLELLDKYLIANATNPE
SKVFYLKMKGDYFRYLAEVACGDDRKQTIDNSQGAYQEAFDISKKEMQPTHPIRLGLALNFSVFYYEILN
NPELACTLAKTAFDEAIAELDTLNEDSYKDSTLIMQLLRDNLTLWTSDSAGEECDAAEGAEN

pDEST17-HIS-14-3-3ζ
MSYYHHHHHHLESTSLYKKAGFEGDRTMDKNELVQKAKLAEQAERYDDMAACMKSVTEQGAELSNEERNL
LSVAYKNVVGARRSSWRVVSSIEQKTEGAEKKQQMAREYREKIETELRDICNDVLSLLEKFLIPNASQAE
SKVFYLKMKGDYYRYLAEVAAGDDKKGIVDQSQQAYQEAFEISKKEMQPTHPIRLGLALNFSVFYYEILN
SPEKACSLAKTAFDEAIAELDTLSEESYKDSTLIMQLLRDNLTLWTSDTQGDEAEAGEGGEN

Supplementary table S1. Vectors for expression of 14-3-3 proteins and protein sequences of 14-3-3 isoforms used in this study. Sequences highlighted in blue represent tag sequences.

Supplementary table S2.

>sp_Q15785_TOM34_HUMA	309	amino	acids			
Sequence	**#**	**x**	**Context**	**Kinase**	**Answer**	**Score**
sp_Q15785_TOM34_HUMA	8	S	KFPDSVEEL	unsp	YES	0,998
sp_Q15785_TOM34_HUMA	93	S	LRRASAYEA	unsp	YES	0,997
sp_Q15785_TOM34_HUMA	186	S	NRVPSAGDV	unsp	YES	0,997
sp_Q15785_TOM34_HUMA	174	S	AKSKSKETT	unsp	YES	0,994
sp_Q15785_TOM34_HUMA	172	S	EMAKSKSKE	unsp	YES	0,939
sp_Q15785_TOM34_HUMA	84	S	LVPFSIKPL	unsp	YES	0,897
sp_Q15785_TOM34_HUMA	120	S	DNVTSAVEG	unsp	YES	0,883
sp_Q15785_TOM34_HUMA	25	Y	RNGQYAEAS	unsp	YES	0,878
sp_Q15785_TOM34_HUMA	230	Y	ESATYSNRA	unsp	YES	0,842
sp_Q15785_TOM34_HUMA	**93**	**S**	**LRRASAYEA**	**PKA**	**YES**	**0,827**
sp_Q15785_TOM34_HUMA	**160**	**S**	**KRWNSLPSE**	**PKA**	**YES**	**0,826**
sp_Q15785_TOM34_HUMA	180	T	ETTATKNRV	PKC	YES	0,82
sp_Q15785_TOM34_HUMA	54	Y	ESVLYSNRA	unsp	YES	0,819
sp_Q15785_TOM34_HUMA	55	S	SVLYSNRAA	PKC	YES	0,767
sp_Q15785_TOM34_HUMA	280	S	DYKSSFADI	unsp	YES	0,749
sp_Q15785_TOM34_HUMA	45	S	AQGSSDPEE	unsp	YES	0,744
sp_Q15785_TOM34_HUMA	45	S	AQGSSDPEE	CKII	YES	0,707
sp_Q15785_TOM34_HUMA	172	S	EMAKSKSKE	PKC	YES	0,678
sp_Q15785_TOM34_HUMA	129	T	INRMTRALM	PKA	YES	0,66
sp_Q15785_TOM34_HUMA	280	S	DYKSSFADI	PKC	YES	0,637
sp_Q15785_TOM34_HUMA	146	S	LKLPSIPLV	PKA	YES	0,619
sp_Q15785_TOM34_HUMA	160	S	KRWNSLPSE	DNAPK	YES	0,616
sp_Q15785_TOM34_HUMA	93	S	LRRASAYEA	PKG	YES	0,614
sp_Q15785_TOM34_HUMA	44	S	QAQGSSDPE	CKII	YES	0,611
sp_Q15785_TOM34_HUMA	231	S	SATYSNRAL	PKC	YES	0,592
sp_Q15785_TOM34_HUMA	44	S	QAQGSSDPE	cdc2	YES	0,587
sp_Q15785_TOM34_HUMA	45	S	AQGSSDPEE	cdc2	YES	0,584
sp_Q15785_TOM34_HUMA	120	S	DNVTSAVEG	CKII	YES	0,565
sp_Q15785_TOM34_HUMA	177	T	KSKETTATK	PKC	YES	0,564
sp_Q15785_TOM34_HUMA	29	S	YAEASALYG	cdc2	YES	0,561
sp_Q15785_TOM34_HUMA	105	Y	YPMAYVDYK	unsp	YES	0,558
sp_Q15785_TOM34_HUMA	19	S	AGNESFRNG	unsp	YES	0,556
sp_Q15785_TOM34_HUMA	223	S	SLLCSNLES	CKII	YES	0,556
sp_Q15785_TOM34_HUMA	119	T	DDNVTSAVE	CKII	YES	0,541
sp_Q15785_TOM34_HUMA	186	S	NRVPSAGDV	PKA	YES	0,541
sp_Q15785_TOM34_HUMA	95	Y	RASAYEALE	unsp	YES	0,534
sp_Q15785_TOM34_HUMA	265	Y	VKAFYRRAQ	EGFR	YES	0,534
sp_Q15785_TOM34_HUMA	25	Y	RNGQYAEAS	EGFR	YES	0,53

Supplementary table S2. NetPhos 3.1 prediction (1) of kinase target sites in TOMM34 protein sequence. PKA-targeted Ser^{93} and Ser^{160} residues are highlighted.

Supplementary table S3.

a

		TOMM34 protein form			
		WT	S93A	S160A	S93A/S160A
site	Ser^{-1}	3%	2%	3%	2%
	Ser^{93}	94%	*N/A*	97%	*N/A*
	Ser^{146}	4%	1%	3%	2%
	Ser^{160}	65%	58%	*N/A*	*N/A*
	Ser^{186}	8%	6%	7%	6%
	Ser^{280}	24%	17%	30%	24%

b

		TOMM34 protein form							
		WT		S93A		S160A		S93A/S160A	
	fraction	12.8 ml	14.3 ml	12.8 ml	14.3 ml	12.8 ml	14.3 ml	12.8 ml	14.3 ml
site	Ser^{93}	98%	93%	-	-	98%	96%	-	-
	Ser^{160}	99%	7%	*98%*	*9%*	-	-	-	-

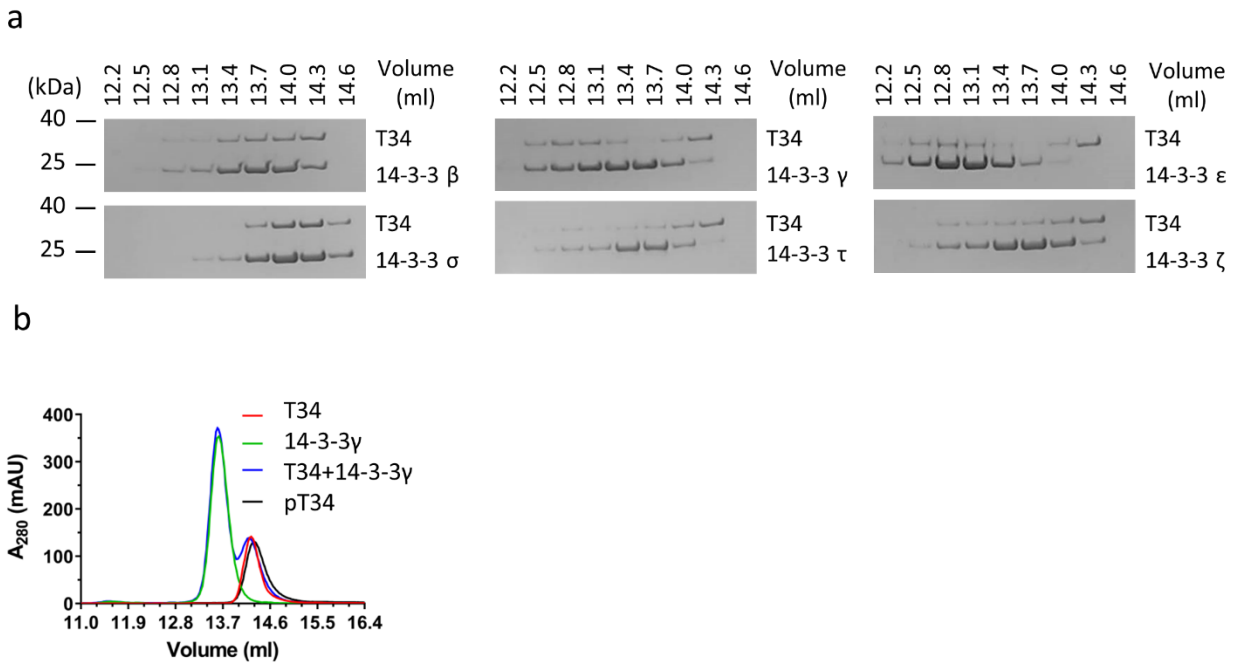
Supplementary table S3. Efficacy of PKA-mediated phosphorylation of TOMM34 residues. (a) The table summarizes phosphorylation levels at PKA-targeted TOMM34 residues inferred from extracted ion chromatograms corresponding to non- and phosphorylated peptide variants. (b) The same approach was used to estimate the levels of Ser^{93} and Ser^{160} residues phosphorylation in 14-3-3γ-bound (elution volume 12.8 ml) and unbound (elution volume 14.3 ml) SEC fractions (see Figure 3).

Supplementary table S4.

Site	Peptide_[-6:4]	ANN	PSSM	SVM	consensus,pSer/Thr
160	**AQKRWNsLPSE**	**0,93**	**1,44**	**1,49**	**1,28**
93	**PLLRRAsAYEA**	**0,91**	**1,20**	**1,05**	**1,05**
186	TKNRVPsAGDV	0,85	0,96	0,38	0,73
279	ALKDYKsSFAD	0,63	0,22	0,16	0,34
174	EMAKSKsKETT	0,40	0,30	0,15	0,29
146	WRLKLPsIPLV	0,59	0,58	0,01	0,39
45	LQAQGSsDPEE	0,37	0,32	-0,12	0,19
76	CIKDCTsALAL	0,50	0,28	-0,14	0,21
219	IEKYSEsLLCS	0,48	0,27	-0,14	0,20
172	HKEMAKsKSKE	0,39	0,05	-0,16	0,09
280	LKDYKSsFADI	0,33	0,05	-0,24	0,05
110	AYVDYKtVLQI	0,39	0,08	-0,38	0,03
135	TRALMDsLGPE	0,35	0,07	-0,41	0,00
129	EGINRMtRALM	0,19	0,10	-0,55	-0,09
244	LVLKQYtEAVK	0,18	0,08	-0,58	-0,10
19	RAAGNEsFRNG	0,30	-0,04	-0,65	-0,13
231	LESATYsNRAL	0,14	-0,01	-0,76	-0,21
153	IPLVPVsAQKR	0,19	-0,15	-0,79	-0,25
178	SKSKETtATKN	0,35	-0,01	-0,90	-0,19
180	SKETTAtKNRV	0,28	-0,04	-0,90	-0,22
251	EAVKDCtEALK	0,23	-0,08	-0,91	-0,25
8	APKFPDsVEEL	0,19	-0,15	-0,93	-0,29
119	QIDDNVtSAVE	0,20	-0,10	-0,96	-0,29
120	IDDNVTsAVEG	0,15	-0,12	-0,96	-0,31
75	DCIKDCtSALA	0,36	-0,04	-1,00	-0,23
84	LALVPFsIKPL	0,05	-0,24	-1,00	-0,40
163	RWNSLPsENHK	0,12	-0,15	-1,04	-0,35
229	SNLESAtYSNR	0,11	-0,02	-1,10	-0,33
44	VLQAQGsSDPE	0,13	-0,14	-1,13	-0,38
55	EESVLYsNRAA	0,08	-0,16	-1,15	-0,41
29	GQYAEAsALYG	0,16	-0,08	-1,18	-0,36
217	KAIEKYsESLL	0,06	-0,02	-1,23	-0,40
227	LCSNLEsATYS	0,10	-0,17	-1,49	-0,52
177	KSKSKEtTATK	0,07	-0,41	-1,49	-0,61
285	SSFADIsNLLQ	0,07	-0,32	-1,66	-0,64
223	SESLLCsNLES	0,07	-0,26	-2,02	-0,74
51	SDPEEEsVLYS	0,02	-0,44	-2,43	-0,95

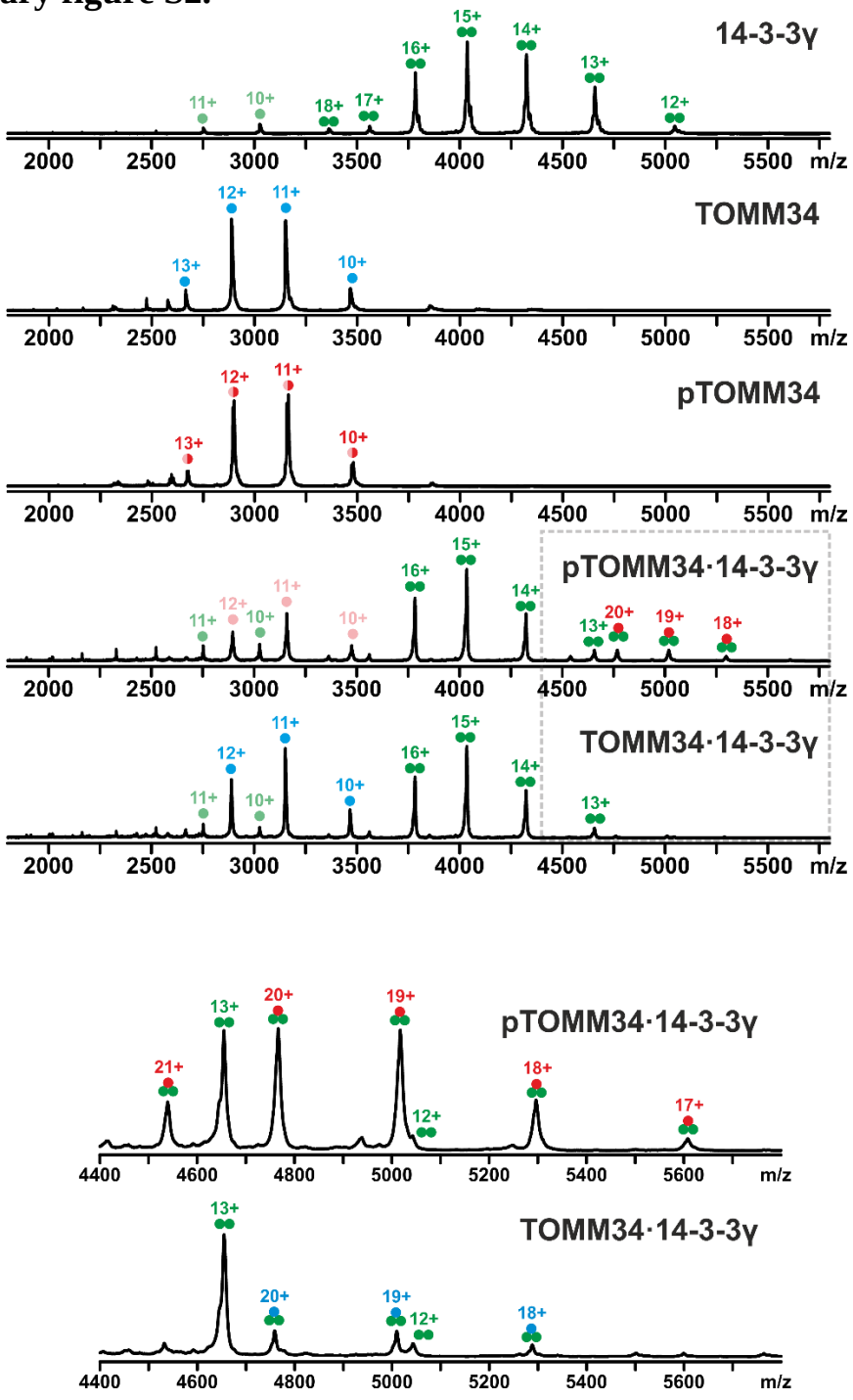
Supplementary table S4. 14-3-3 Pred (2) prediction of 14-3-3 binding sites in TOMM34 protein sequence. PKA-targeted Ser^{93} and Ser^{160} residues are highlighted.

Supplementary figure S1.



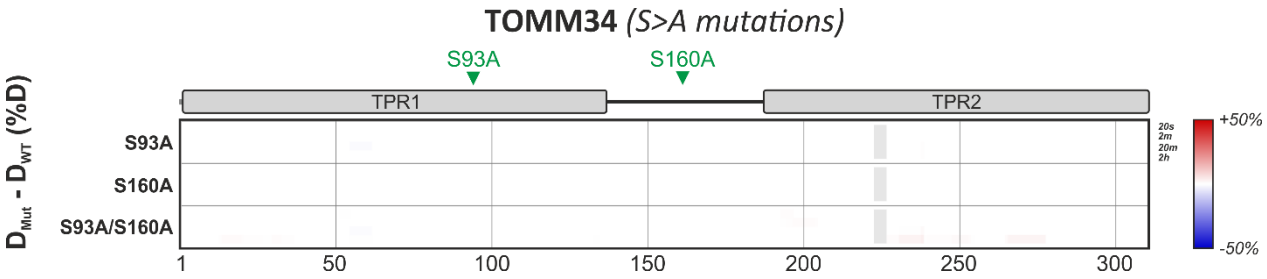
Supplementary figure S1. (a) Indicated fractions from Figure 2 were analyzed by gel electrophoresis and Coomassie staining. (b) SEC analysis of non-phosphorylated TOMM34 (35 μM) in a mixture with 14-3-3γ isoform (70 μM).

Supplementary figure S2.



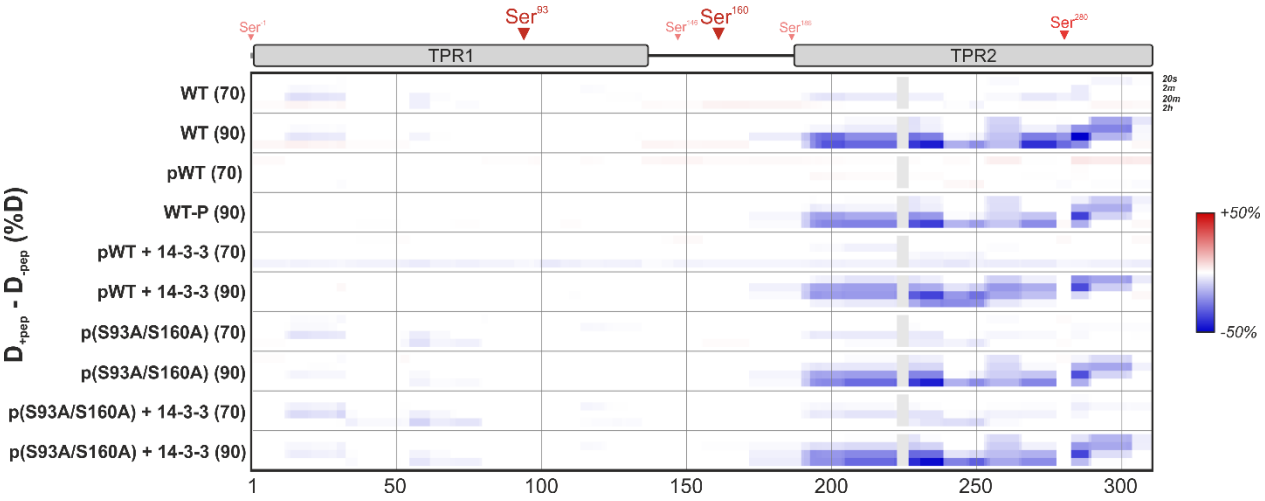
Supplementary figure S2. Native ESI-MS spectra of 14-3-3γ (green), unmodified TOMM34 (blue), PKA-phosphorylated pTOMM34 (red) and their mixtures. The charged states corresponding to monomers and dimers are labelled with single and double dots, respectively. Grey dashed box represents magnification of spectra belonging to a tripartite complex of 14-3-3γ dimer with phosphorylated TOMM34. Halved dots in native ESI-MS spectrum for pTOMM34 mirror the presence of single ($pSer^{93}$) and doubly phosphorylated protein ($pSer^{93}$/$pSer^{160}$, see Figure 1B).

Supplementary figure S3.



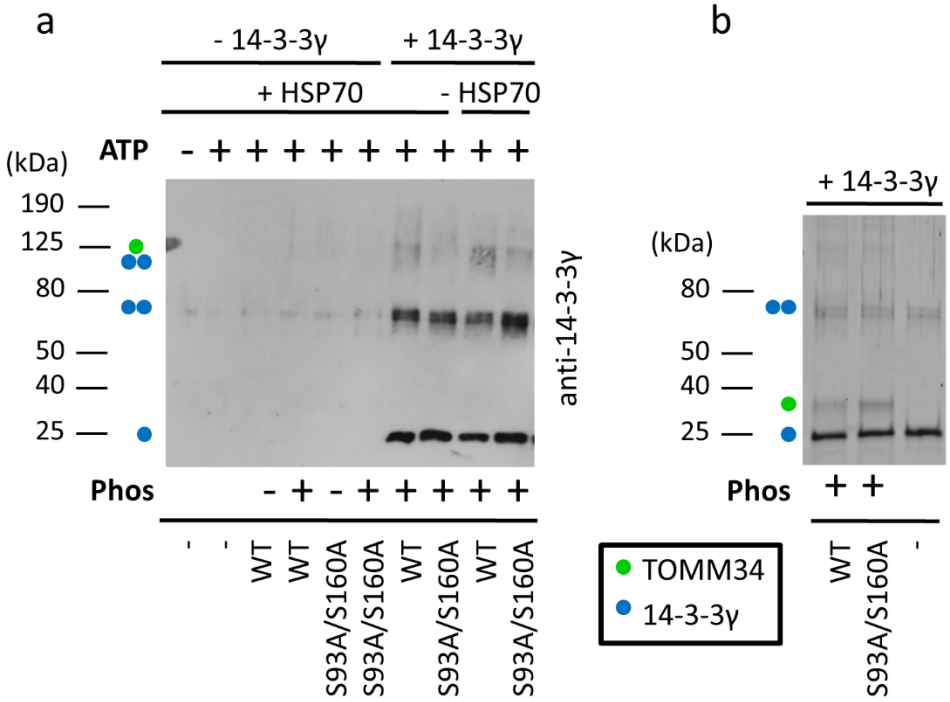
Supplementary figure S3. Time resolved deuteration level differences between TOMM34 WT and S93A, S160A and S93A/S160A mutant forms (indicated on the left side) presented in the form of heat maps. Horizontal grey lines separate individual experimental conditions. Within each experimental conditions, four lines are corresponding to exchange times (20 sec, 2 min, 20 min, 2 h, from top to bottom) – indicated on the right side. Vertical grey lines mark sequence positions (spaced by 50 amino acids). TOMM34 domain structure is depicted above the heat-map with mutations indicated by arrowheads. Regions in grey boxes were not covered during peptide mapping.

Supplementary figure S4.



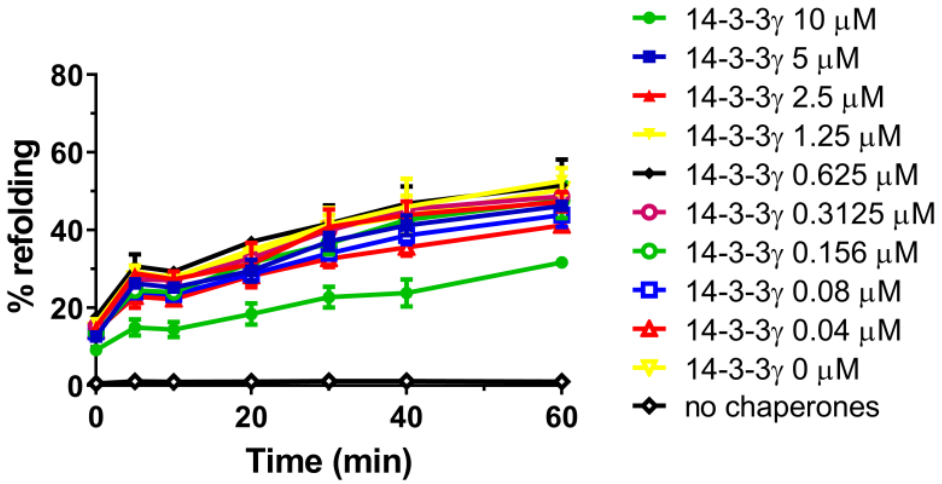
Supplementary figure S4. Time resolved deuteration level differences between non-phosphorylated/PKA-phosphorylated (p) TOMM34 WT and S93A/S160A proteins detected in the presence of HSP70 (70) and HSP90 (90) C-terminal peptides and in the peptides absence. The effect of 14-3-3γ presence on peptide-induced deuteration changes is also shown. Horizontal grey lines separate individual experimental conditions. Within each experimental conditions, four lines are corresponding to exchange times (20 sec, 2 min, 20 min, 2 h, from top to bottom) – indicated on the right side. Vertical grey lines mark sequence positions (spaced by 50 amino acids). TOMM34 domain structure is depicted above the heat-map with PKA-modified sites (see Figure 1 and Supplementary table S3) indicated by arrowheads. Regions in grey boxes were not covered during peptide mapping.

Supplementary figure S5.



Supplementary figure S5. (a) Unmodified or PKA-phosphorylated WT or S93A/S160A TOMM34 forms (final concentration 60 μM) proteins were mixed with 14-3-3γ (final concentration 120 μM) or buffer. HSP70 protein (60 μM) pre-incubated with or without ATP (0.4 mM) was added to the samples in 1:1 ratio and the mixture was chemically cross-linked by glutaraldehyde addition. The reactions were stopped after 20 min with Tris, pH 8 and separated by SDS-PAGE, blotted and probed with anti-14-3-3 antibody. Molecular weight markers and captured protein assemblies are indicated by numbers and dots, respectively. (b) Coomassie staining of pWT and p(S93A/S160A) forms of TOMM34 cross-linked to 14-3-3γ revealed clear separation of pWT, p(S93A/S160A) and 14-3-3γ monomers/dimers.

Supplementary figure S6.



Supplementary figure S6. Firefly luciferase incubated with HSP70 (1 μM), HSP40 (2 μM), and BAG1 (0.5 μM) proteins was thermally denatured at 42 °C for 30 min in the presence of increasing concentration of 14-3-3γ dimers. The kinetics of luciferase reactivation was measured after shifting the reaction temperature to 37 °C. The signal from samples with native luciferase was set as 100%. As negative controls we measured the luciferase activity of denatured luciferase only (no chaperones, black line). Error bars represent S.D.; n = 3 independent experiments.

References:

1. Blom, N., Gammeltoft, S., and Brunak, S. (1999) Sequence and structure-based prediction of eukaryotic protein phosphorylation sites. *J Mol Biol* **294**, 1351-1362
2. Madeira, F., Tinti, M., Murugesan, G., Berrett, E., Stafford, M., Toth, R., Cole, C., MacKintosh, C., and Barton, G. J. (2015) 14-3-3-Pred: improved methods to predict 14-3-3-binding phosphopeptides. *Bioinformatics* **31**, 2276-2283