

**Supplemental Table S1. Yeast strains used in this study.**

Strain	Genotype	Source
<i>tim23</i> <sub>Δ1-50-316</sub>	<i>MATa ade2 his3 ura3 leu2 trp1 can1 tim23Δ::CgHIS3</i> [pRS316- <i>tim23</i> <sub>Δ1-50</sub> ]	<sup>1</sup> Yasushi Tamura et al. 2009
<i>TIM23</i>	<i>MATa ade2 his3 ura3 leu2 trp1 can1 tim23Δ::CgHIS3</i> [pRS314-Tim23]	This study
<i>tim23</i> <sub>Y105N/G120D</sub>	<i>MATa ade2 his3 ura3 leu2 trp1 can1 tim23Δ::CgHIS3</i> [pRS314- <i>tim23</i> <sub>Y105N/G120D</sub> ]	This study
<i>tim23</i> <sub>L107R</sub>	<i>MATa ade2 his3 ura3 leu2 trp1 can1 tim23Δ::CgHIS3</i> [pRS314- <i>tim23</i> <sub>L107R</sub> ]	This study
<i>tim23</i> <sub>L109P</sub>	<i>MATa ade2 his3 ura3 leu2 trp1 can1 tim23Δ::CgHIS3</i> [pRS314- <i>tim23</i> <sub>L109P</sub> ]	This study
<i>tim23</i> <sub>I111N/M117K</sub>	<i>MATa ade2 his3 ura3 leu2 trp1 can1 tim23Δ::CgHIS3</i> [pRS314- <i>tim23</i> <sub>I111N/M117K</sub> ]	This study
<i>tim23</i> <sub>L138P</sub>	<i>MATa ade2 his3 ura3 leu2 trp1 can1 tim23Δ::CgHIS3</i> [pRS314- <i>tim23</i> <sub>L138P</sub> ]	This study
<i>tim23</i> <sub>N139E</sub>	<i>MATa ade2 his3 ura3 leu2 trp1 can1 tim23Δ::CgHIS3</i> [pRS314- <i>tim23</i> <sub>N139E</sub> ]	This study
<i>tim23</i> <sub>G149E</sub>	<i>MATa ade2 his3 ura3 leu2 trp1 can1 tim23Δ::CgHIS3</i> [pRS314- <i>tim23</i> <sub>G149E</sub> ]	This study
<i>tim23</i> <sub>G153V</sub>	<i>MATa ade2 his3 ura3 leu2 trp1 can1 tim23Δ::CgHIS3</i> [pRS314- <i>tim23</i> <sub>G153V</sub> ]	This study
<i>tim23</i> <sub>D174A</sub>	<i>MATa ade2 his3 ura3 leu2 trp1 can1 tim23Δ::CgHIS3</i> [pRS314- <i>tim23</i> <sub>D174A</sub> ]	This study
<i>tim23</i> <sub>R170</sub>	<i>MATa ade2 his3 ura3 leu2 trp1 can1 tim23Δ::CgHIS3</i> [pRS314- <i>tim23</i> <sub>R170</sub> ]	This study
<i>tim23</i> <sub>K172</sub>	<i>MATa ade2 his3 ura3 leu2 trp1 can1 tim23Δ::CgHIS3</i> [pRS314- <i>tim23</i> <sub>K172</sub> ]	This study
<i>tim23</i> <sub>L107R</sub> /pRS416 Tim17	<i>MATa ade2 his3 ura3 leu2 trp1 can1 tim23Δ::CgHIS3</i> [pRS314- <i>tim23</i> <sub>L107R</sub> / pRS416-Tim17]	This study
<i>tim23</i> <sub>I111N/M117K</sub> /pRS416 Tim17	<i>MATa ade2 his3 ura3 leu2 trp1 can1 tim23Δ::CgHIS3</i> [pRS314- <i>tim23</i> <sub>I111N/M117K</sub> / pRS416-Tim17]	This study
<i>tim23</i> <sub>G149E</sub> /pRS416 Tim17	<i>MATa ade2 his3 ura3 leu2 trp1 can1 tim23Δ::CgHIS3</i> [pRS314- <i>tim23</i> <sub>G149E</sub> / pRS416-Tim17]	This study
<i>TIM23</i> /pRS416 Tim21	<i>MATa ade2 his3 ura3 leu2 trp1 can1 tim23Δ::CgHIS3</i> [pRS314-Tim23/ pRS416-Tim21]	This study
<i>tim23</i> <sub>D174A</sub> /pRS416 Tim21	<i>MATa ade2 his3 ura3 leu2 trp1 can1 tim23Δ::CgHIS3</i> [pRS314- <i>tim23</i> <sub>D174A</sub> / pRS416-Tim21]	This study
<i>tim23</i> <sub>R170</sub> /pRS416 Tim21	<i>MATa ade2 his3 ura3 leu2 trp1 can1 tim23Δ::CgHIS3</i> [pRS314- <i>tim23</i> <sub>R170</sub> / pRS416-Tim21]	This study
<i>TIM23</i> -FLAG	<i>MATa ade2 his3 ura3 leu2 trp1 can1 tim23Δ::CgHIS3</i> [pRS316- <i>tim23</i> <sub>Δ1-50</sub> pRS314- Tim23 -FLAG]	This study

<i>tim23</i> <sub>Y105N/G120D</sub> -FLAG	<i>MATa ade2 his3 ura3 leu2 trp1 can1 tim23Δ::CgHIS3</i> [pRS316- <i>tim23</i> <sub>Δ1-50</sub> ] pRS414- <i>tim23</i> <sub>Y105N/G120D</sub> -FLAG	This study
<i>tim23</i> <sub>L107R</sub> -FLAG	<i>MATa ade2 his3 ura3 leu2 trp1 can1 tim23Δ::CgHIS3</i> [pRS316- <i>tim23</i> <sub>Δ1-50</sub> ] pRS414- <i>tim23</i> <sub>L107R</sub> -FLAG	This study
<i>tim23</i> <sub>L109P</sub> -FLAG	<i>MATa ade2 his3 ura3 leu2 trp1 can1 tim23Δ::CgHIS3</i> [pRS316- <i>tim23</i> <sub>Δ1-50</sub> ] pRS414- <i>tim23</i> <sub>L109P</sub> -FLAG	This study
<i>tim23</i> <sub>I111N/M117K</sub> -FLAG	<i>MATa ade2 his3 ura3 leu2 trp1 can1 tim23Δ::CgHIS3</i> [pRS316- <i>tim23</i> <sub>Δ1-50</sub> ] pRS414- <i>tim23</i> <sub>I111N/M117K</sub> -FLAG	This study
<i>tim23</i> <sub>G149E</sub> -FLAG	<i>MATa ade2 his3 ura3 leu2 trp1 can1 tim23Δ::CgHIS3</i> [pRS316- <i>tim23</i> <sub>Δ1-50</sub> ] pRS414- <i>tim23</i> <sub>G149E</sub> -FLAG	This study
<i>tim23</i> <sub>G153V</sub> -FLAG	<i>MATa ade2 his3 ura3 leu2 trp1 can1 tim23Δ::CgHIS3</i> [pRS316- <i>tim23</i> <sub>Δ1-50</sub> ] pRS414- <i>tim23</i> <sub>G153V</sub> -FLAG	This study
<i>tim23</i> <sub>Δ1-50</sub> -316/ <i>tim21Δ</i>	<i>MATa ade2 his3 ura3 leu2 trp1 can1 tim23Δ::CgHIS3</i> [pRS316- <i>tim23</i> <sub>Δ1-50</sub> ] <i>tim21Δ::kanMX4</i>	This study
<i>tim23</i> <sub>Δ1-50</sub> -316/ <i>pam17Δ</i>	<i>MATa ade2 his3 ura3 leu2 trp1 can1 tim23Δ::CgHIS3</i> [pRS316- <i>tim23</i> <sub>Δ1-50</sub> ] <i>pam17Δ::LEU2</i>	This study
<i>tim23</i> <sub>Δ1-50</sub> -316/ <i>tam41Δ</i>	<i>MATa ade2 his3 ura3 leu2 trp1 can1 tim23Δ::CgHIS3</i> [pRS316- <i>tim23</i> <sub>Δ1-50</sub> ] <i>tam41Δ::kanMX4</i>	This study
<i>tim23</i> <sub>Δ1-50</sub> -316/ <i>pam17Δ/GAL1-TAM41</i>	<i>MATa ade2 his3 ura3 leu2 trp1 can1 tim23Δ::CgHIS3</i> [pRS316- <i>tim23</i> <sub>Δ1-50</sub> ] <i>pam17Δ::LEU2</i> <i>tam41Δ::TRP1/GAL1-TAM41</i>	This study
<i>tim23</i> <sub>Δ1-50</sub> -316/ <i>tim21Δ/GAL1-TAM41</i>	<i>MATa ade2 his3 ura3 leu2 trp1 can1 tim23Δ::CgHIS3</i> [pRS316- <i>tim23</i> <sub>Δ1-50</sub> ] <i>tim21Δ::kanMX4</i> <i>tam41Δ::TRP1/GAL1-TAM41</i>	This study
<i>tim23</i> <sub>Y105N</sub>	<i>MATa ade2 his3 ura3 leu2 trp1 can1 tim23Δ::CgHIS3</i> [pRS314- <i>tim23</i> <sub>Y105N</sub> ]	This study
<i>tim23</i> <sub>G120D</sub>	<i>MATa ade2 his3 ura3 leu2 trp1 can1 tim23Δ::CgHIS3</i> [pRS314- <i>tim23</i> <sub>G120D</sub> ]	This study
<i>tim23</i> <sub>I111N</sub>	<i>MATa ade2 his3 ura3 leu2 trp1 can1 tim23Δ::CgHIS3</i> [pRS314- <i>tim23</i> <sub>I111N</sub> ]	This study
<i>tim23</i> <sub>M117K</sub>	<i>MATa ade2 his3 ura3 leu2 trp1 can1 tim23Δ::CgHIS3</i> [pRS314- <i>tim23</i> <sub>M117K</sub> ]	This study
<i>tim23</i> <sub>L138P</sub> /pRS316 Tim17	<i>MATa ade2 his3 ura3 leu2 trp1 can1 tim23Δ::CgHIS3</i> [pRS314- <i>tim23</i> <sub>L138P</sub> / pRS316-Tim17]	This study
<i>tim23</i> <sub>N139E</sub> /pRS316 Tim17	<i>MATa ade2 his3 ura3 leu2 trp1 can1 tim23Δ::CgHIS3</i> [pRS314- <i>tim23</i> <sub>N139E</sub> / pRS316-Tim17]	This study

<sup>1</sup>Yasushi Tamura , Yoshihiro Harada ,Takuya Shiota ,Koji Yamano ,Kazuaki Watanabe, Mihoko Yokota , Hayashi Yamamoto , Hiromi Sesaki , and Toshiya Endo. 2009. Tim23 – Tim50 pair coordinates functions of translocators and motor proteins in mitochondrial protein import. *J. Cell Biol.*184:129-141.

## Supplemental Table S2. Plasmids used in this study.

Plasmid	Expressed protein	Vector	Restriction Site	Source
pRS316-tim23 <sub>Δ1-50</sub>	Tim23 <sub>Δ(1-50)</sub>	pRS316	EcoRI/XhoI	<sup>1</sup> Yasushi Tamura et al., 2009
pRS314-Tim23	Tim23	pRS314	Spe1/Xho1	This study
pRS314-tim23 <sub>Y105N/G120D</sub>	tim23 <sub>Y105N/G120D</sub>	pRS314	Spe1/Xho1	This study
pRS314-tim23 <sub>L107R</sub>	tim23 <sub>L107R</sub>	pRS314	Spe1/Xho1	This study
pRS314-tim23 <sub>L109P</sub>	tim23 <sub>L109P</sub>	pRS314	Spe1/Xho1	This study
pRS314-tim23 <sub>I111N/M117K</sub>	tim23 <sub>I111N/M117K</sub>	pRS314	Spe1/Xho1	This study
pRS314-tim23 <sub>L138P</sub>	tim23 <sub>L138P</sub>	pRS314	Spe1/Xho1	This study
pRS314-tim23 <sub>N139E</sub>	tim23 <sub>N139E</sub>	pRS314	Spe1/Xho1	This study
pRS314-tim23 <sub>G149E</sub>	tim23 <sub>G149E</sub>	pRS314	Spe1/Xho1	This study
pRS314-tim23 <sub>G153V</sub>	tim23 <sub>G153V</sub>	pRS314	Spe1/Xho1	This study
pRS314-tim23 <sub>D174A</sub>	tim23 <sub>D174A</sub>	pRS314	Spe1/Xho1	This study
pRS314-tim23 <sub>R170</sub>	tim23 <sub>R170</sub>	pRS314	Spe1/Xho1	This study
pRS314-tim23 <sub>K172</sub>	tim23 <sub>K172</sub>	pRS314	Spe1/Xho1	This study
pRS416-Tim17	Tim17	pRS416	Spe1/Xho1	This study
pRS416-Tim21	Tim21	pRS416	Spe1/Xho1	This study
pRS314-Tim23 FLAG	TIM23-FLAG	pRS314	Spe1/Xho1	This study
pRS414-tim23 <sub>Y105N/G120D</sub> FLAG	tim23 <sub>Y105N/G120D</sub> -FLAG	pRS414	Spe1/Xho1	This study
pRS414-tim23 <sub>L107R</sub> FLAG	tim23 <sub>L107R</sub> -FLAG	pRS414	Spe1/Xho1	This study
pRS414-tim23 <sub>L109P</sub> FLAG	tim23 <sub>L109P</sub> -FLAG	pRS414	Spe1/Xho1	This study
pRS414-tim23 <sub>I111N/M117K</sub> FLAG	tim23 <sub>I111N/M117K</sub> -FLAG	pRS414	Spe1/Xho1	This study
pRS414-tim23 <sub>G149E</sub> FLAG	tim23 <sub>G149E</sub> -FLAG	pRS414	Spe1/Xho1	This study
pRS414-tim23 <sub>G153V</sub> FLAG	tim23 <sub>G153V</sub> -FLAG	pRS414	Spe1/Xho1	This study
pET 3a-Pam17His <sub>6</sub>	Pam17 His <sub>6</sub>	pET 3a	Nde1/BamHI	This study
pET 3a-Tim21His <sub>6</sub>	Tim21 (103-239) His <sub>6</sub>	pET 3a	Nde1/BamHI	This study
pET 3a-Tim23His <sub>6</sub>	Tim23 (1-100) His <sub>6</sub>	pET 3a	Nde1/BamHI	This study
pRSF-duet cytb <sub>2</sub> (167) <sub>Δ</sub> 19-DHFR His <sub>6</sub>	cytb <sub>2</sub> (167) <sub>Δ</sub> 19-DHFR His <sub>6</sub>	pRSF-duet	Nde1/Xho1	This study
pRSF-duet cytb <sub>2</sub> (167)-DHFR His <sub>6</sub>	cytb <sub>2</sub> (167)-DHFR His <sub>6</sub>	pRSF-duet	Nde1/Xho1	This study
pRSF-duet Tim23 His <sub>6</sub>	Tim23-His <sub>6</sub>	pRSF-duet	Nde1/Xho1	This study

pRSF-duet tim23 <sub>Y105N/G120D</sub> His <sub>6</sub>	tim23 <sub>Y105N/G120D</sub> His <sub>6</sub>	pRSF-duet	Nde1/Xho1	This study
pRSF-duet tim23 <sub>L107R</sub> His <sub>6</sub>	tim23 <sub>L107R</sub> His <sub>6</sub>	pRSF-duet	Nde1/Xho1	This study
pRSF-duet tim23 <sub>L109P</sub> His <sub>6</sub>	tim23 <sub>L109P</sub> His <sub>6</sub>	pRSF-duet	Nde1/Xho1	This study
pRSF-duet tim23 <sub>I111N/M117K</sub> His <sub>6</sub>	tim23 <sub>I111N/M117K</sub> His <sub>6</sub>	pRSF-duet	Nde1/Xho1	This study
pRSF-duet tim23 <sub>G149E</sub> His <sub>6</sub>	tim23 <sub>G149E</sub> His <sub>6</sub>	pRSF-duet	Nde1/Xho1	This study
pRSF-duet tim23 <sub>G153V</sub> His <sub>6</sub>	tim23 <sub>G153V</sub> His <sub>6</sub>	pRSF-duet	Nde1/Xho1	This study
pRS316-Tim17	Tim17	pRS316	Spe1/Xho1	This study

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<sup>1</sup>Yasushi Tamura ,Yoshihiro Harada ,Takuya Shiota ,Koji Yamano ,Kazuaki Watanabe, Mihoko Yokota , Hayashi Yamamoto , Hiromi Sesaki , and Toshiya Endo. 2009. Tim23 – Tim50 pair coordinates functions of translocators and motor proteins in mitochondrial protein import. *J. Cell Biol.*184:129-141.