Supplemental Information

Cross-priming of $CD8^+$ T cells is controlled by dipeptidyl peptidase 3 and thimet oligopeptidase 1 present in necrotic cells

Jaba Gamrekelashvili, Tamar Kapanadze, Miaojun Han, Josef Wissing, Chi Ma, Lothar Jaensch, Michael P. Manns, Todd Armstrong, Elizabeth Jaffee, Ayla O. White, Deborah E. Citrin, Firouzeh Korangy, Tim F. Greten

Figure S1

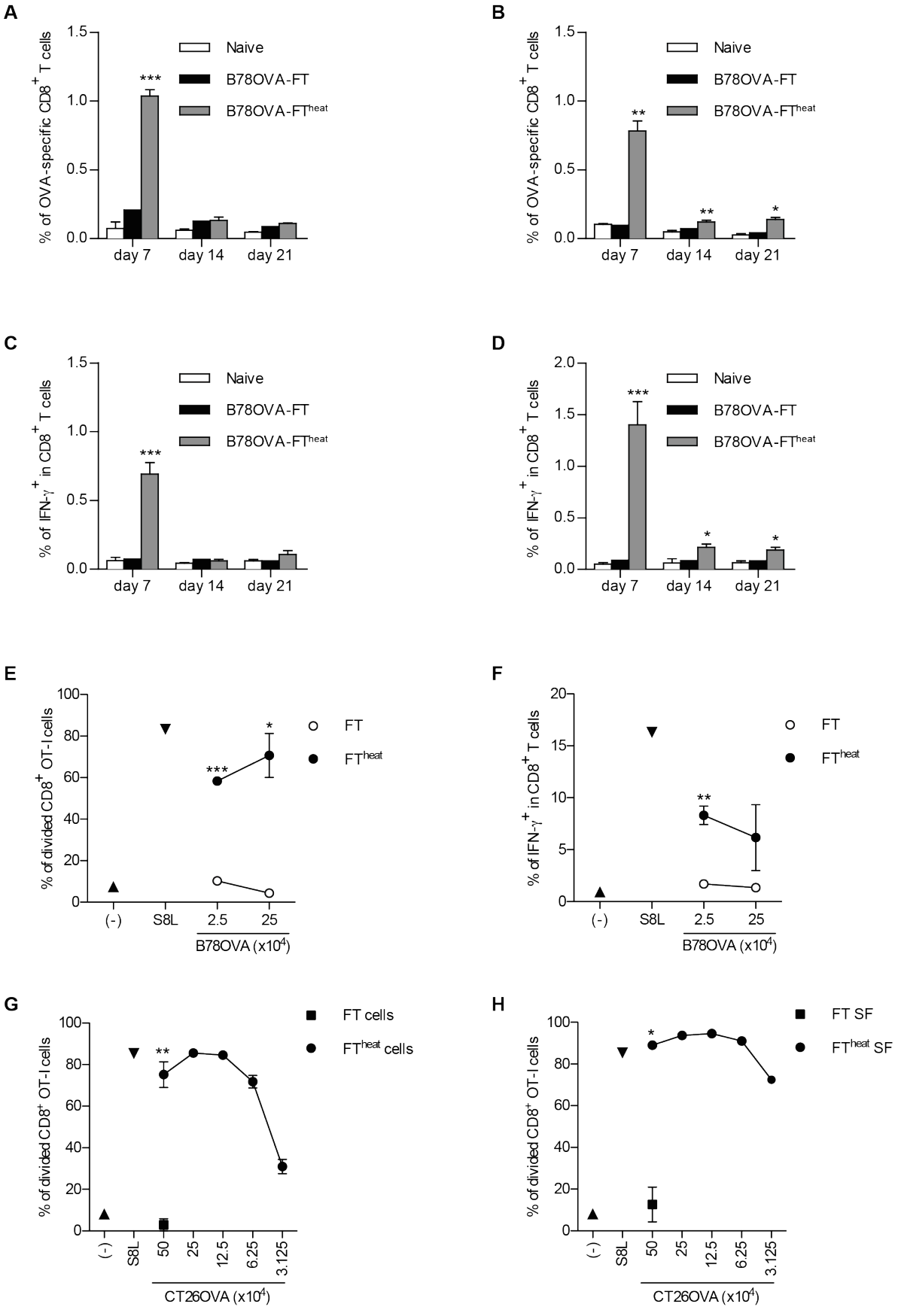
A
% of OVA-specific CD8+ T cells
Naive
B78OVA-FT
B78OVA-FTheat
day 7
day 14
day 21
B
% of OVA-specific CD8+ T cells
C
% of IFN-γ+ in CD8+ T cells
D
% of IFN-γ+ in CD8+ T cells
E
% of divided CD8+ OT-I cells
FT
FTheat
(-)
S8L
B78OVA (x10^4)
F
% of IFN-γ+ in CD8+ T cells
G
% of divided CD8+ OT-I cells
FT cells
FTheat cells
CT26OVA (x10^4)
H
% of divided CD8+ OT-I cells
FT SF
FTheat SF

Figure S1 Primary freeze-thawed necrotic cells do not induce $CD8^+$ T cell activation.
(A and B) Mice were vaccinated with $5x10^6$ B78OVA-FT or B78OVA-FT^{heat} cells on days 0, 2. On days 7, 14 and 21 number of OVA-specific $CD8^+$ T cells was determined in DLN (A) or spleen (B).
(C and D) DLN or splenic cells from vaccinated mice were cultured *in vitro* with S8L peptide for 16 hours in the presence of Brefedin A and IFN-γ expression in $CD8^+$ T cells was analyzed. (A-D) Data shown are from 2 independent experiments with 2 mice in each group.
(E and F) Serial dilutions of B78OVA-FT or B78OVA-FT^{heat} whole cells were cultured *in vitro* with $2.5x10^5$ CFSE labeled OT-I splenocytes for 48 hours. Brefeldin A was added during the last 5 hours and proliferation or IFN-γ expression of $CD8^+$ T cells was analyzed. 10pM S8L peptide was used as a control. Data shown is representative of minimum of 3 independent experiments.
(G and H)Serial dilution of CT26OVA-FT or CT26OVA-FT^{heat} cells (G) or SF (H) were cultured *in vitro* with $2.5x10^5$ CFSE labeled OT-I splenocytes for 48 hours and proliferation of $CD8^+$ T cells was analyzed. 10pM S8L peptide was used as a control. Data shown is representative of 2 independent experiments. (A-H) * $P<0.05$, ** $P<0.01$, *** $P<0.001$; Student's t test.

Figure S2

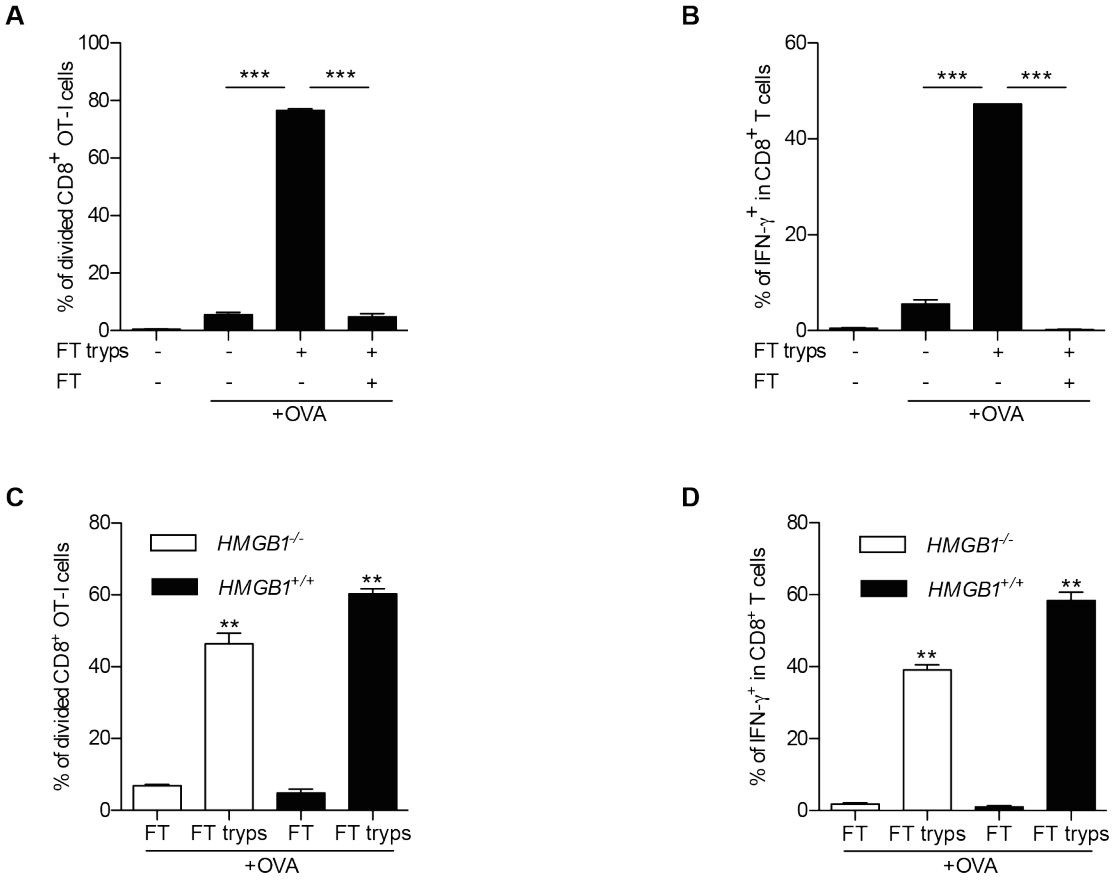

Figure S2 Primary freeze-thawed necrotic cells abort cross-priming of CD8⁺ T cells.
(A and B) Soluble fraction from human HLXPXT-FT cell line was subjected to trypsin digest. Digested or undigested SFs were cultured *in vitro* in the presence/absence of OVA protein with OT-I cells. Proliferation and IFN-γ expression of $CD8^+$ T cells were analyzed 48 hours after. Data shown is representative of 3 independent experiments. * $P<0.05$, ** $P<0.01$, *** $P<0.001$; Student's t test.
(C and D) Soluble fractions from FT *HMGB1*$^{-/-}$ or control *HMGB1*$^{+/+}$ MEFs were subjected to digest with sequencing grade modified trypsin or were incubated with buffer only. After buffer exchange and concentration SFs were added to the CFSE labeled OT-I splenocyte cultures in the presence of 20μg/ml OVA and antigen specific proliferation and IFN-γ expression were tested. Data are representative of at least 2 experiments. * $P<0.05$, ** $P<0.01$, *** $P<0.001$; Student's t test.

Figure S3

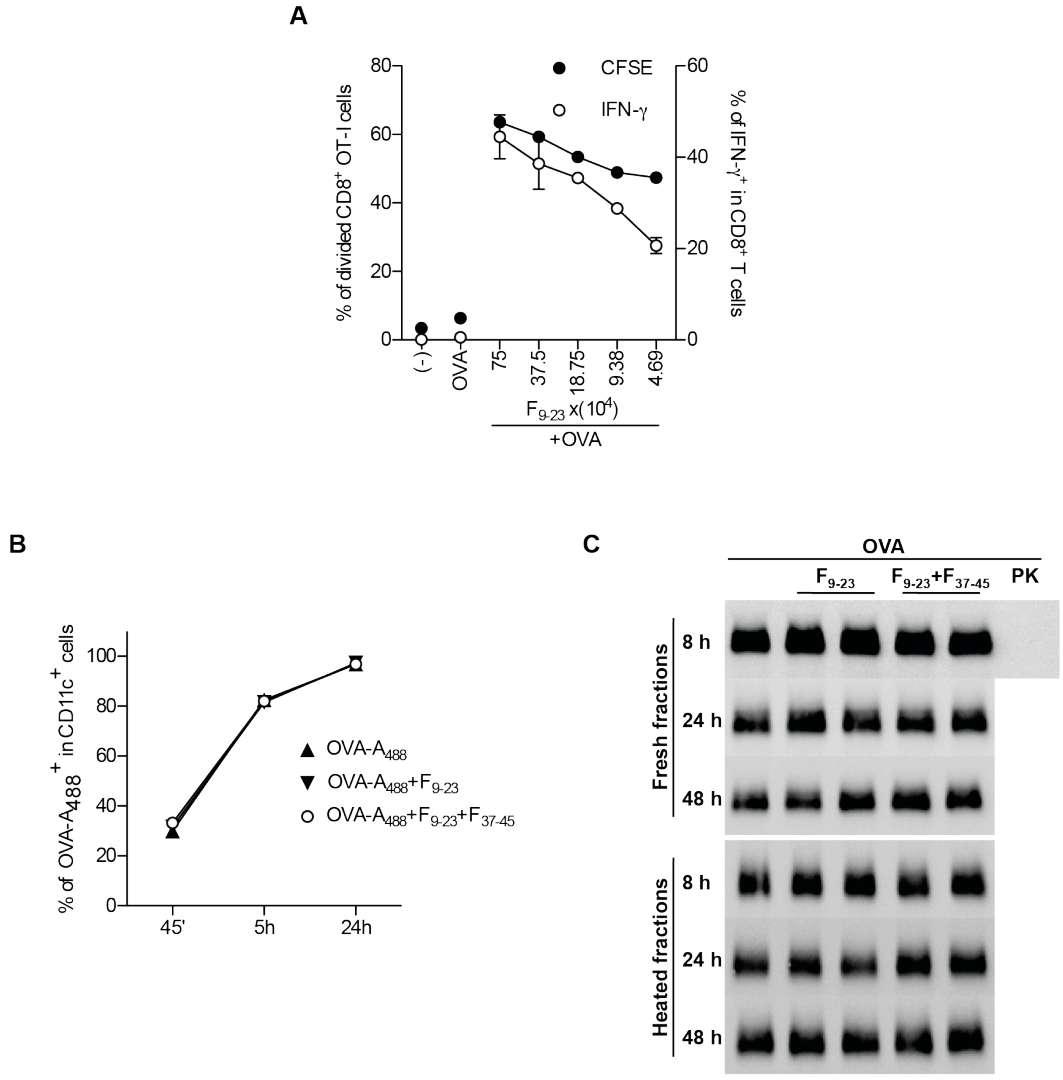

Figure S3 Necrotic cells contain high molecular weight factors capable of activating CD8⁺ T cells.

(A) Titrated amounts of F_{9-23} was added to the cultures of CFSE labeled OT-I splenocytes in the presence of 20μg/ml OVA. After 48 hours, proliferation and IFN-γ expression of $CD8^+$ T cells were analyzed. Shown are mean±s.e.m. representative of 2 independent experiments.

(B) $1x10^5$ splenocytes were cultured with 5μg/ml OVA-A_{488} in the presence of F_{9-23} and/or F_{37-45} at 37°C for the indicated time points. After culture cells were washed, stained and analyzed for the uptake of labeled OVA. Results shown are representative of 3 independent experiments.
(C) OVA (120ng) was incubated with 1μg Proteinase K or fresh/heat inactivated F_{9-23} and/or F_{37-45} at 37°C. Samples were collected at the indicated time points, resuspended in loading buffer and heat inactivated. Samples were loaded onto SDS gel and analyzed by Western Blot using anti-OVA specific antibody. Results from 1 out of 2 independent experiments are shown.

Figure S4

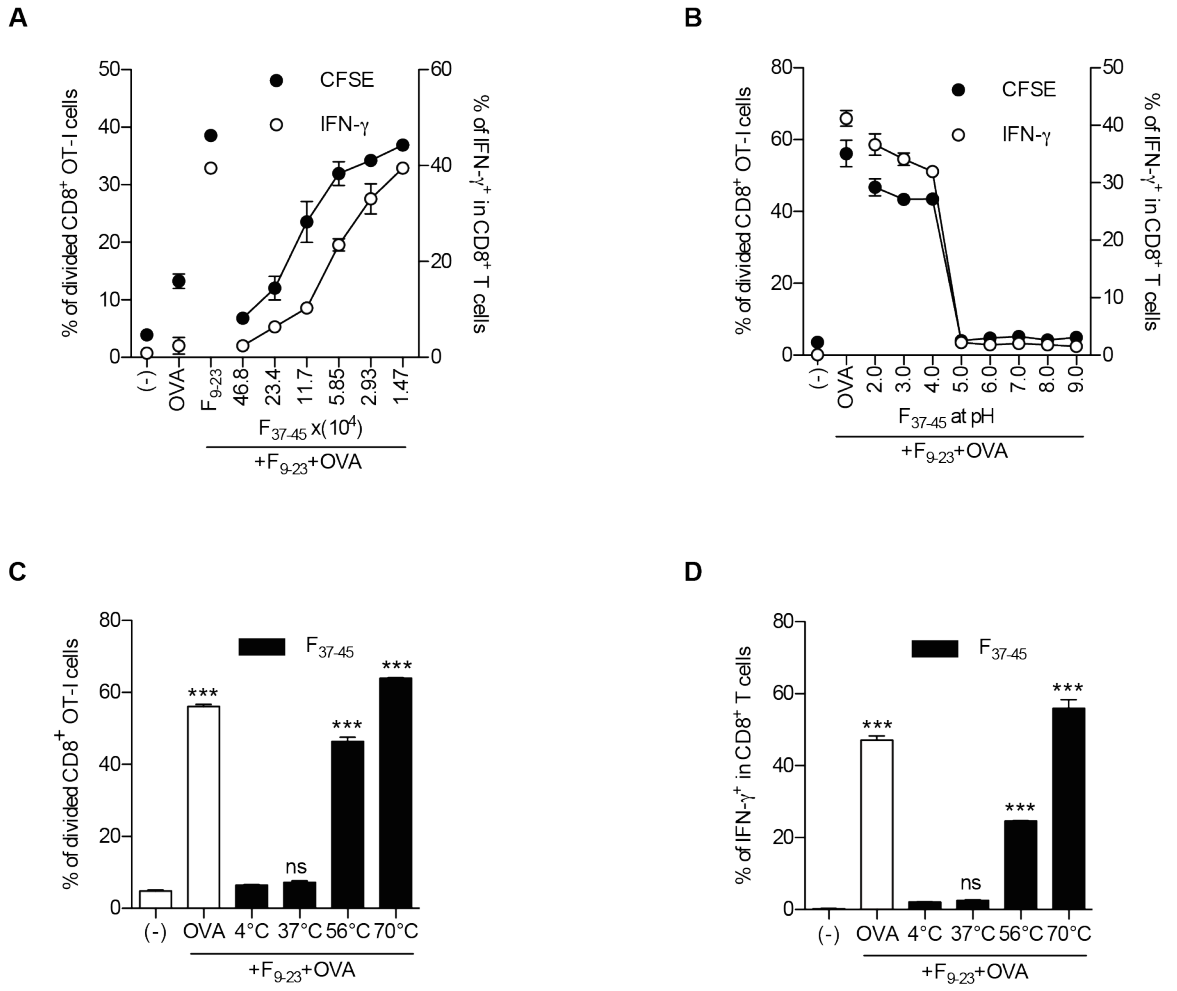

Figure S4 Characterization of F_{37-45} fraction.

(A) Titrated amount of F_{37-45} was added to the cultures of CFSE labeled OT-I splenocytes in the presence of 20μg/ml OVA and F_{9-23}. Proliferation and IFN-γ expression of $CD8^+$ T cells were analyzed after 48 hours. Shown are mean±s.e.m. representative of 2 independent experiments.

(B) F_{37-45} were incubated in PBS at different pH at room temperature for 1 hour and added to the OT-I cell culture in the presence of OVA+F_{9-23}. IFN-γ expression and proliferation of OVA specific $CD8^+$ T cells were determined. Data shown is representative of 2 independent experiments.

(C and D) F_{37-45} was incubated at different temperatures for 1 hour and added to OT-I cells in the presence of OVA+F_{9-23}. IFN-γ expression and proliferation of OVA specific $CD8^+$ T cells were determined. Data shown is mean±s.e.m. representative of 3 independent experiments. *** P<0.001; one-way ANOVA with Dunnett's multiple comparison test.

Figure S5

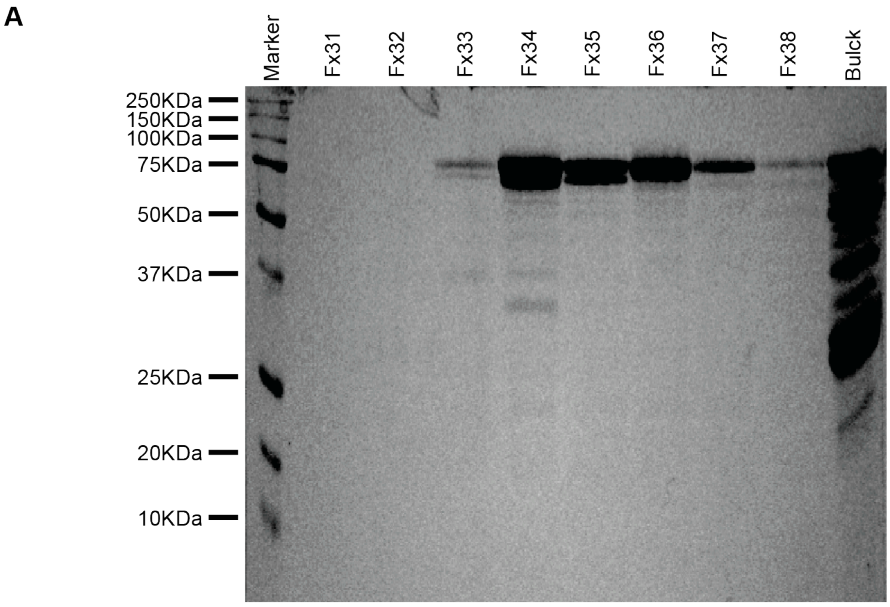

Figure S5 Purification and identification of peptidases controlling cross-priming of $CD8^+$ T cells.

(A) Fx fractions (10μl each) were run on the 12% polyacrylamide gel and proteins were stained using Coomassie-blue staining.

Supplemental Table 1 Relative reporter intensities of 3 representative Peptides from dipeptidyl peptidase 3 and thimet oligopeptidase 1.

ID	Name	Seq. Cov.	Uniq. Pept.	Peptide Sequence	Pept. Score	Spectr. ID	Max. Intens.	114	115	116	117
gil244791124	dipeptidyl peptidase 3	80%	48	STGDVVAGR	53,6	1557	6,60E+06	< 2,5	100	52	15
				NVSLGNVLAVAYAAK	112,3	6211	2,18E+05	< 2,5	100	50	15
				YEFQGNHFQVTR	58,2	4078	1,13E+05	< 2	100	56	15
gil239916005	thimet oligopeptidase 1	60%	41	TGSEAPQDLLEK	95,5	4145	1,10E+06	15	100	25	10
				ALTTQLIEQTK	75,9	4773	2,72E+04	23	100	20	12
				VGAQNFEDVSYESTLK	98,2	5091	8,71E+05	20	100	25	8

The reporters 114, 115, 116 and 117 correspond to the fractions Fx 33, 34, 35 and 36 respectively. The sequence coverage and the number of unique peptides for the proteins are given, as well as three representative sequences, the individual peptide scores, the spectrum ID and the maximum absolute reporter intensities of the individual peptides and the relative reporter intensities of the respective fractions setting fraction 34 = label 115 as 100%.

Supplemental Table 2 A six-step procedure for the purification and identification of peptidases.

	1st step [a]	**3rd step**	**4th step [b]**	**6th step**
	Gel-filtration	Affinity Chromatography	Negative Chromatography	Ion-Exchange Chromatography
Column Material	Sephacryl-S-200	Phenyl-Superose	Hi-Trap Heparin Sepharose	Resource Q Sepharose
Volume	1L	10ml	5ml	1ml each
Buffer A (BA)	0.5M NaCl/PBS pH 7.2	$(H_4N)_2SO_4$/PBS 35% (AS), pH7.2	50mM MES pH 6.3	50mM HEPES pH 7.6
Buffer B (BB)	-	20mM Na-Phosphate buffer pH 7.2	1.5M NaCl/50mM MES pH 6.3	1.5M NaCl/50mM HEPES pH 7.6
Flow rate	1.5ml/min	0.5ml/min	1ml/min	0.5ml/min
Fraction volume	5ml	10ml	3ml	1ml
Waiting time	200min	0min	0min	0min
Start gradient	-	3 Vol.	5 Vol.	3 Vol
Stop gradient	-	8 Vol.100%BB	25 Vol. 100% BB	100 Vol. 50%BB
Fractions for the next step	No 37-45	No 4-8	Flow through (No1-8)	No 33-38

[a] As a second step samples from Gel-Filtration (1st step) were precipitated using 35% AS ($(H_4N)_2SO_4$ in PBS pH7.2) and soluble, non-precipitated sample was used for the Affinity-Chromatography (3rd step).

[b] After the 4th step and buffer exchange from MES to HEPES and additional negative chromatography (5th step) using S-Sepharose and Blue-Sepharose final chromatography was performed onto Q-Sepharose (6th step).

HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), MES (2-(N-Morpholino)ethanesulphonic acid).

Supplemental Table 3 shRNA Sequences for gene silencing.

Gene	Clone ID	Sequence
TOP-1	NM_022653.2-345s1c1	5′-CCGGGCAGAGGAACATTCTCGACTTCTCGAGAAGTCGAGAATGTTCCTCTGCTTTTTG
TOP-1	NM_022653.2-1933s1c1	5′-CCGGGCTCAGTACTATGGCTACTTACTCGAGTAAGTAGCCATAGTACTGAGCTTTTTG
TOP-1	NM_022653.2-1959s1c1	5′-CCGGCGAAGTGTACTCGATGGACATCTCGAGATGTCCATCGAGTACACTTCGTTTTTG
DPP-3	NM_133803.1-2399s21c1	5′-CCGGTTACCGTATGTCCTCAGTTTCCTCGAGGAAACTGAGGACATACGGTAATTTTTG
DPP-3	NM_133803.1-1491s21c1	5′-CCGGAGAGACGTGGGATAGCAAATTCTCGAGAATTTGCTATCCCACGTCTCTTTTTTG
DPP-3	NM_133803.2-2070s21c1	5′-CCGGAGGAGTCCCGGAAGCTTATTGCTCGAGCAATAAGCTTCCGGGACTCCTTTTTTG