nature | methods

Universal sample preparation method for proteome analysis

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Note: Supplementary Data 1–7 ares available on the Nature Methods website.

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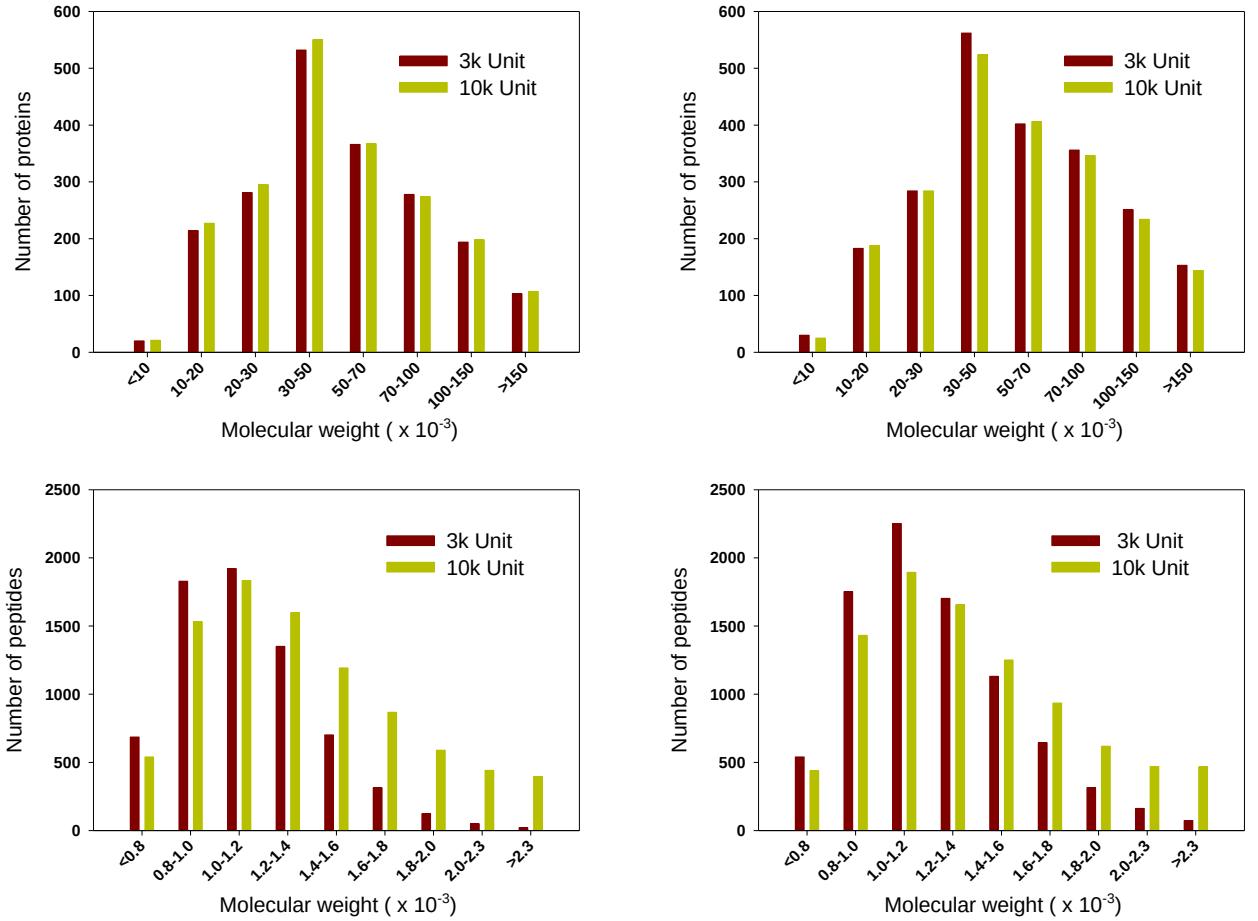
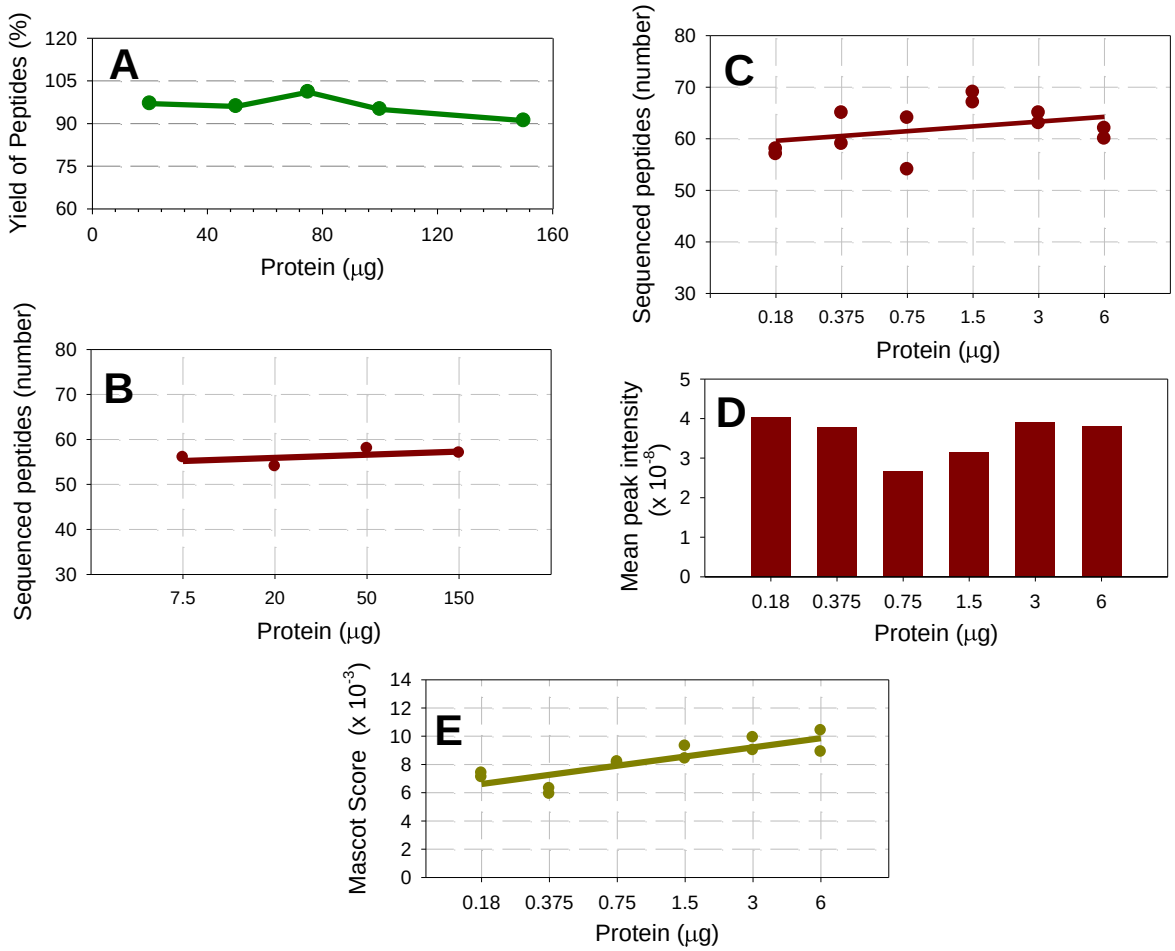
Supplementary Figures

Supplementary Figure 1. Retention and permeability of 3k and 10 k filtration devices for proteins and peptides. 100µg aliquots of SDS-lysates of total membrane (A and B) or cytosolic (C and D) fractions of HeLa cells were processed in 3k and 10k filtration units and analyzed by LC-MS/MS. Each sample was run in duplicate. A and C, Comparison of protein size. B and D, Comparison of peptide size.

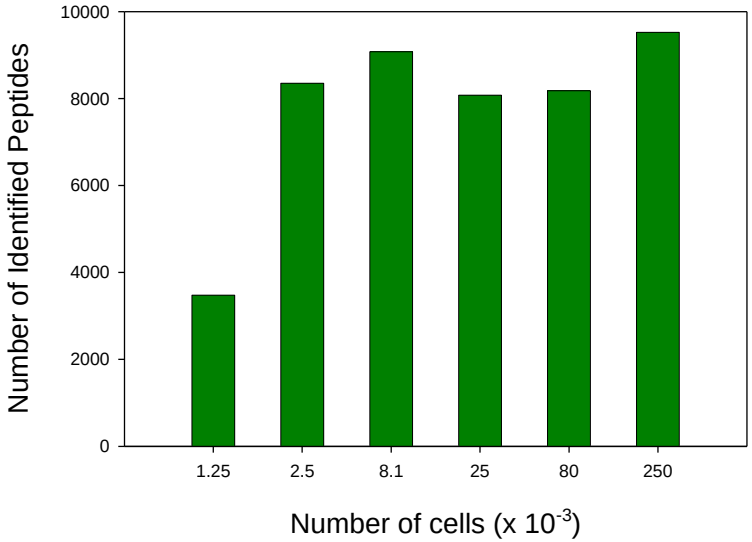


Supplementary Figure 2. Analysis of peptide recovery. Different amounts of BSA ranging 0.18-150 µg were processed in the 10k filter device and the obtained peptides were analyzed either by UV spectrometry (A) or LC- by MS/MS (B -D). A, Protein range 20-150 µg. B and C, Number of identified BSA peptides in two different protein ranges 7.5 - 150 µg (B) and 0.18 - 6 µg (C). D, Mean peak intensity of identified peptides E, Protein Mascot Score in 0.18 - 6 µg BSA range.

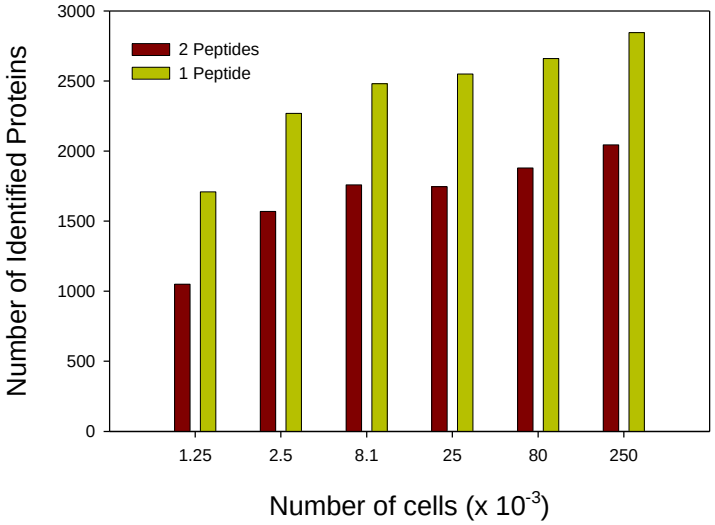


Supplementary Figure 3. Analysis sensitivity of the FASP. Total lysates corresponding to various amounts of HeLa cells ranging 1,250-250,000 were FASP processed and the obtained peptides were analyzed LC- by MS/MS. A, Number of identified peptides. B, Number of identified proteins with one or two peptides.

A.

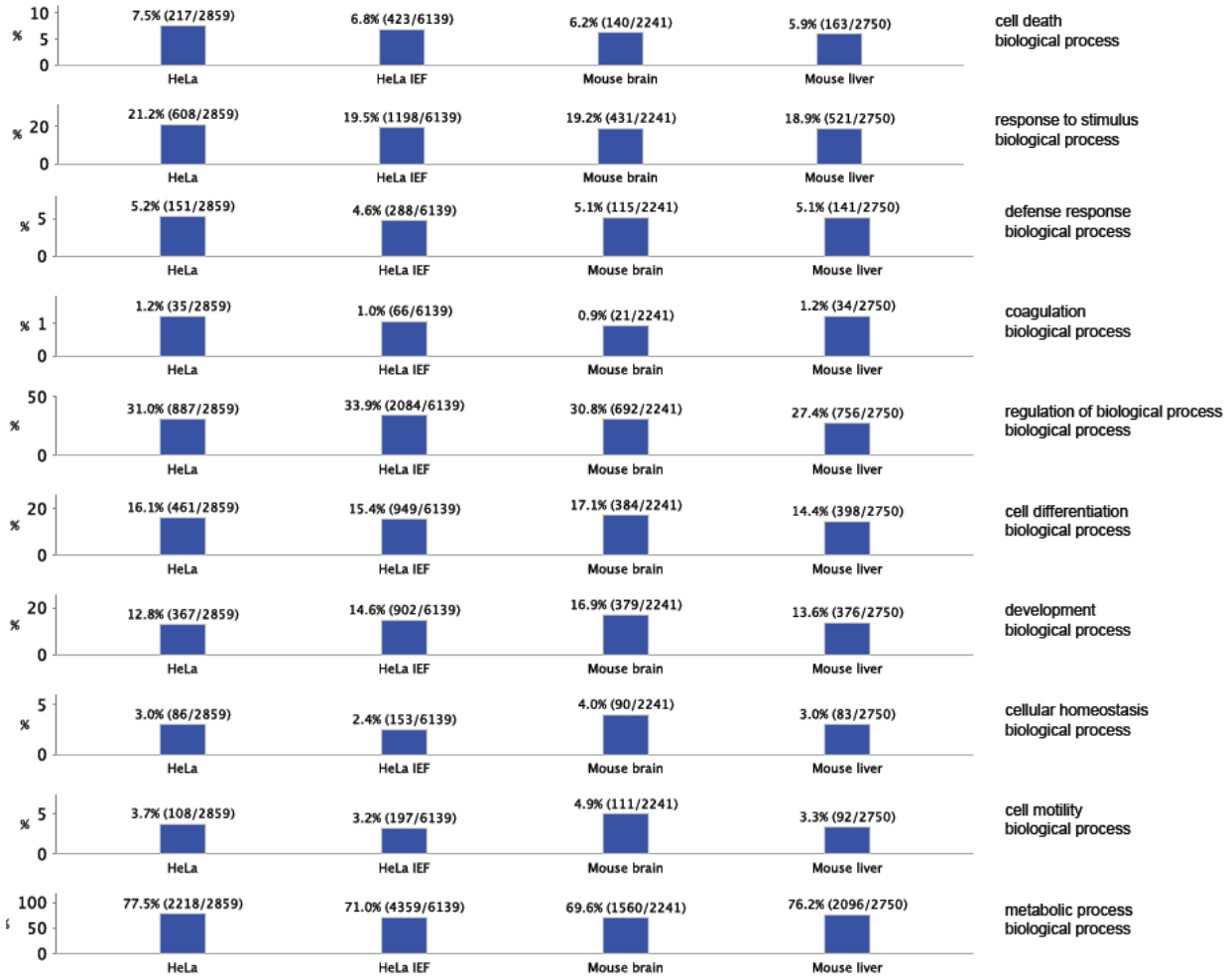


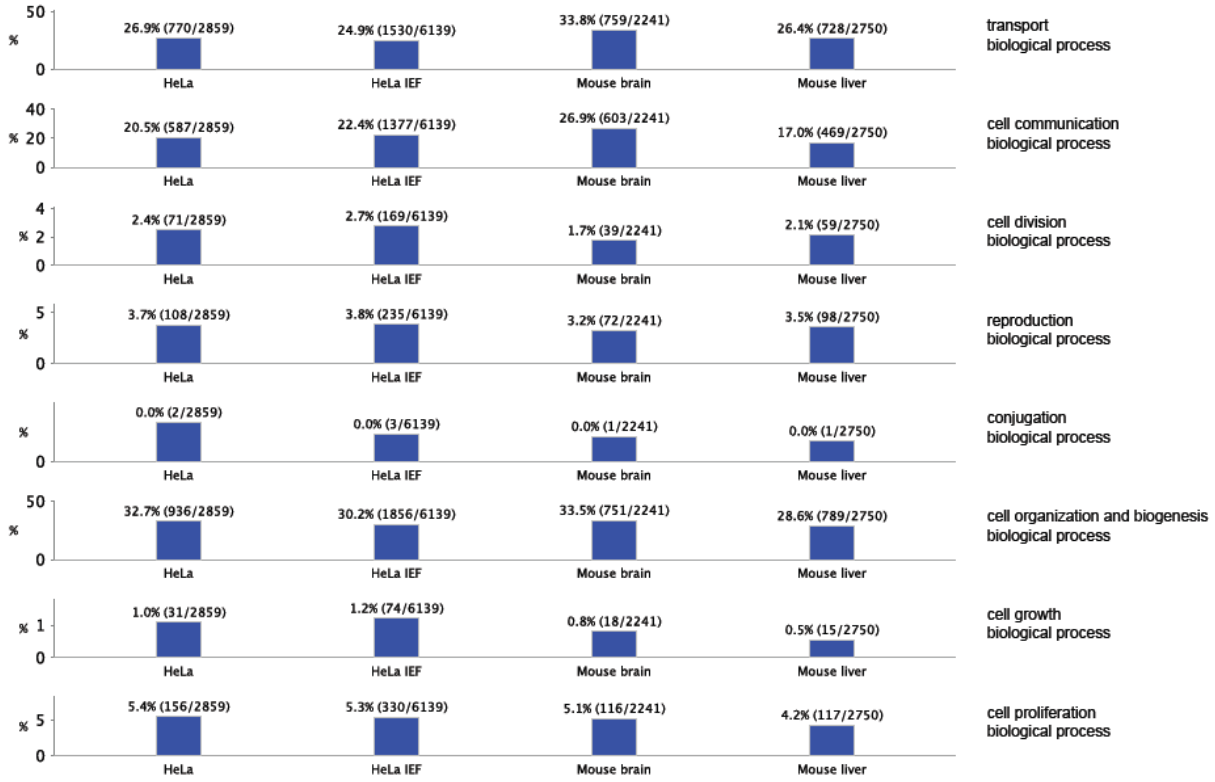
B.



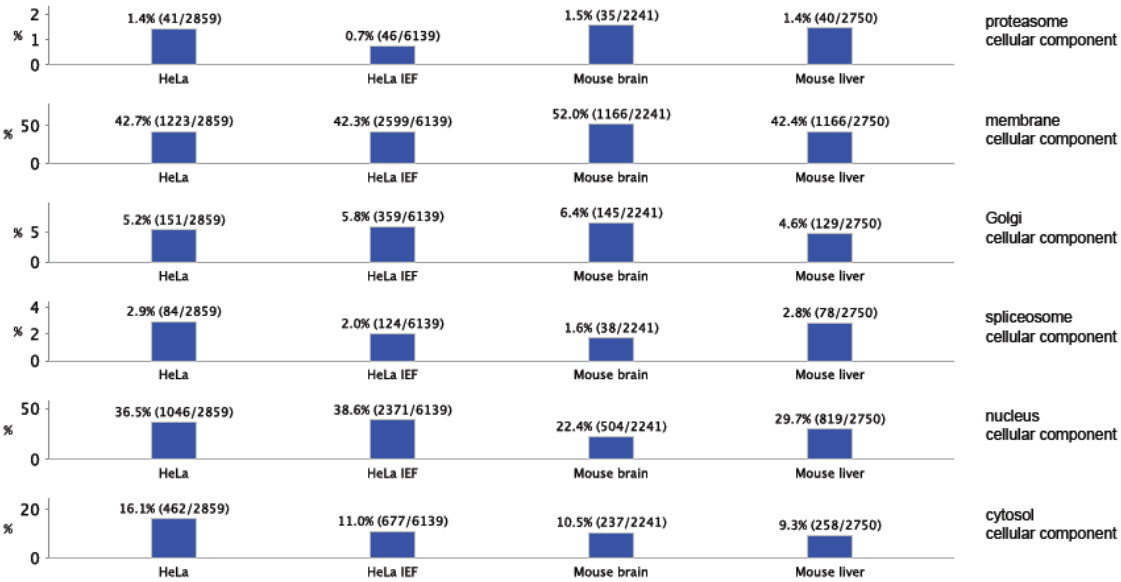
Supplementary Figure 4. GO-Annotations to proteins identified in whole cell lysates of HeLa cells (single run and 12 IEF fractions), moue brain and liver. (A) Biological Process, (B) Cellular component, and (C) molecular function.

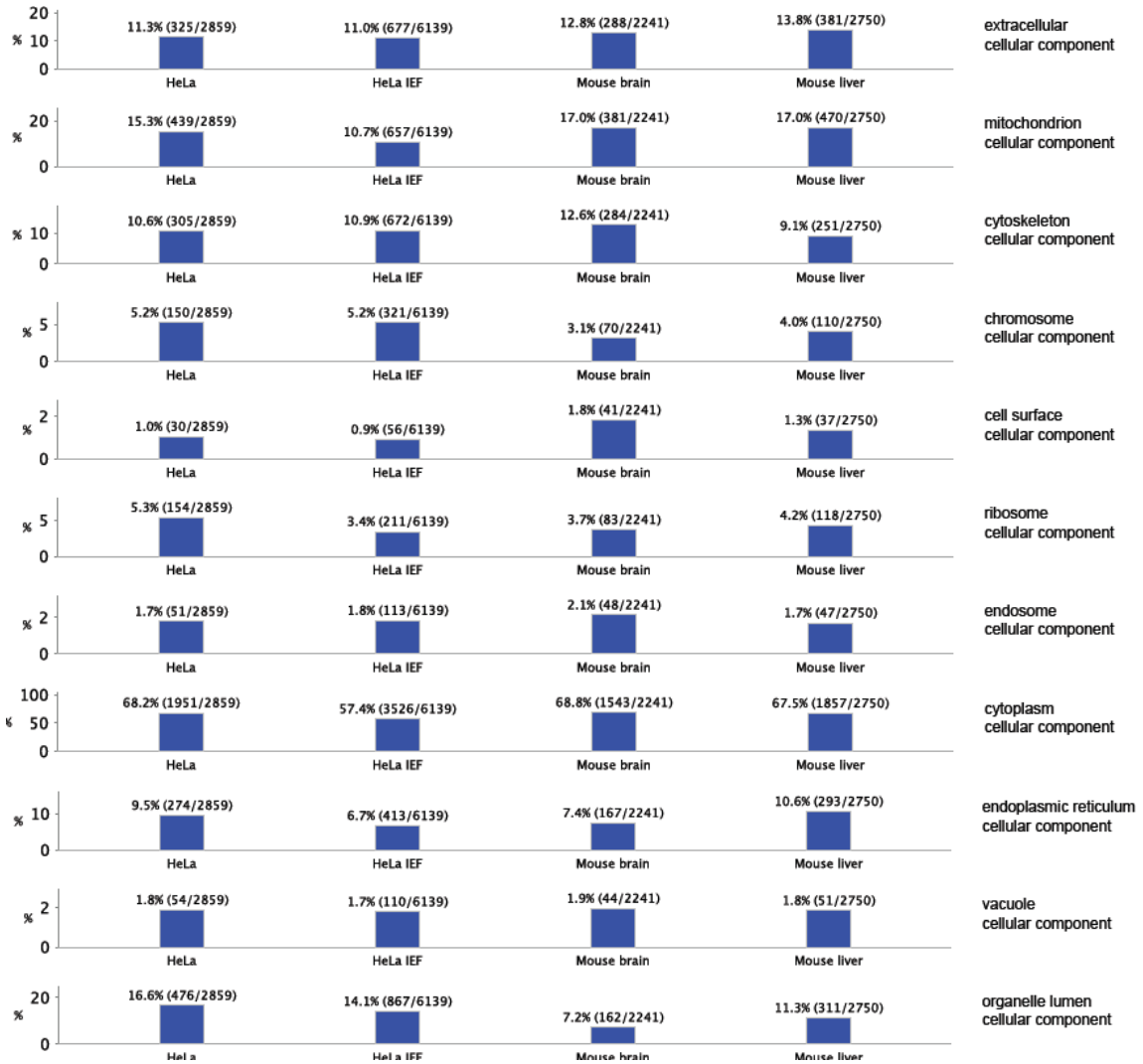
A. **Biological Process**



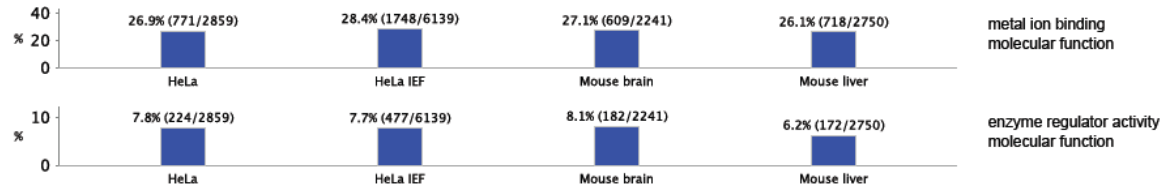


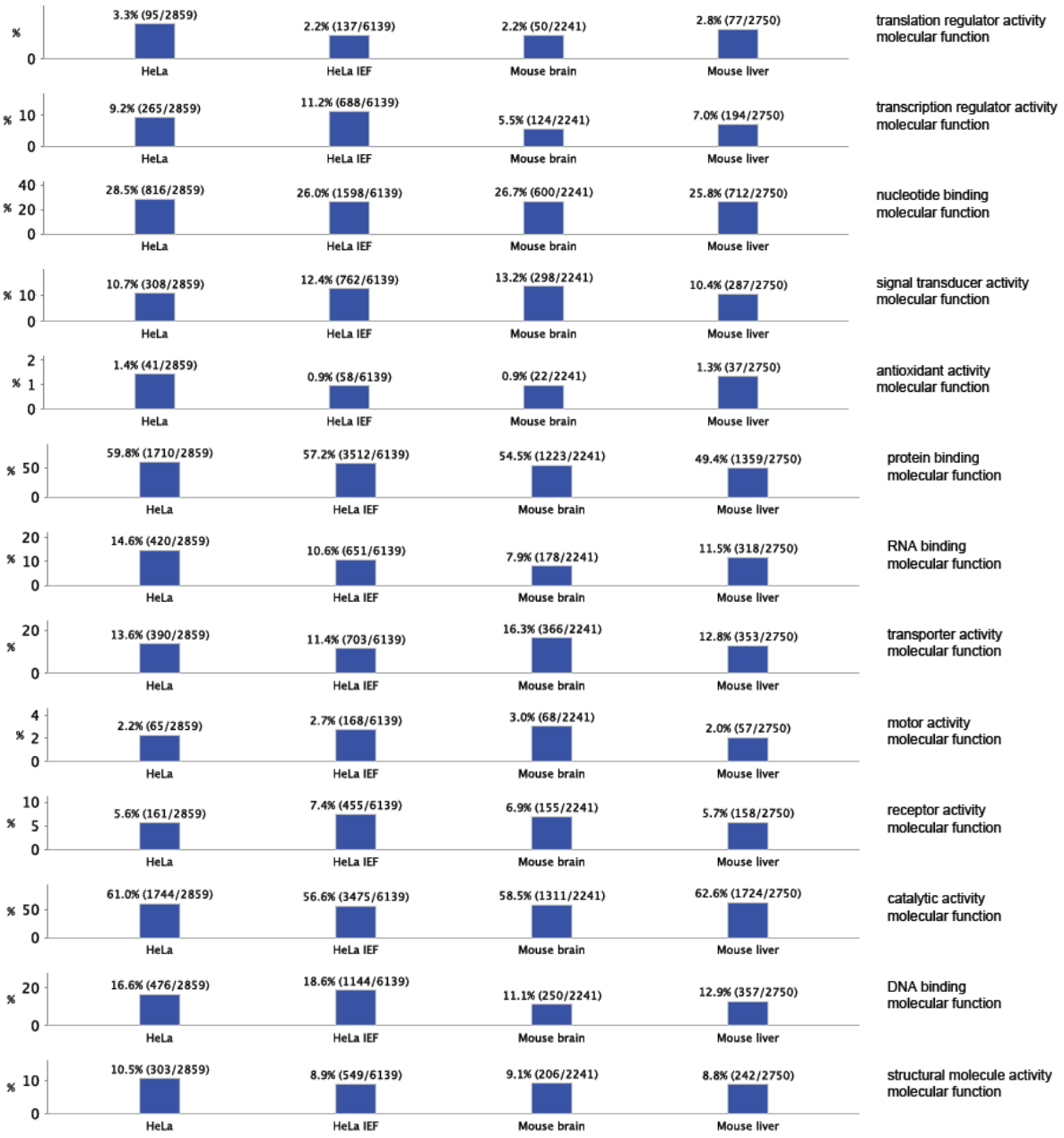
A. Cellular Component



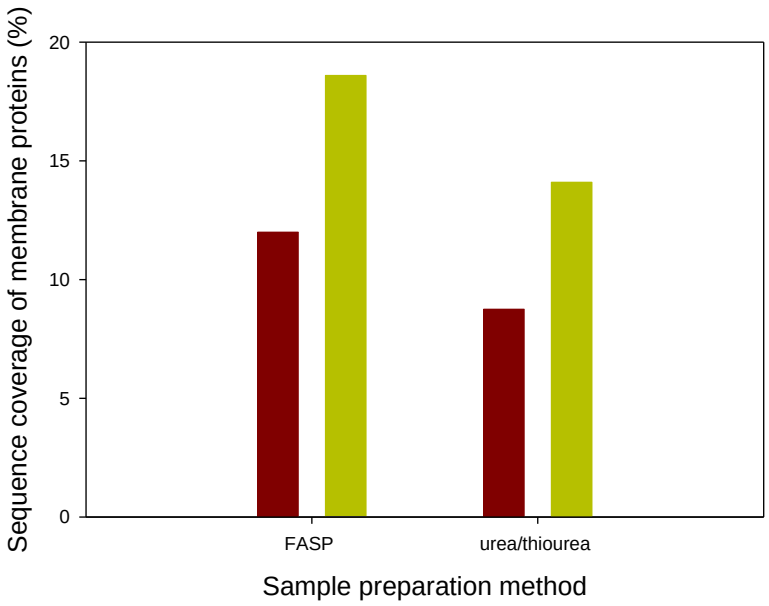


B. Molecular Function

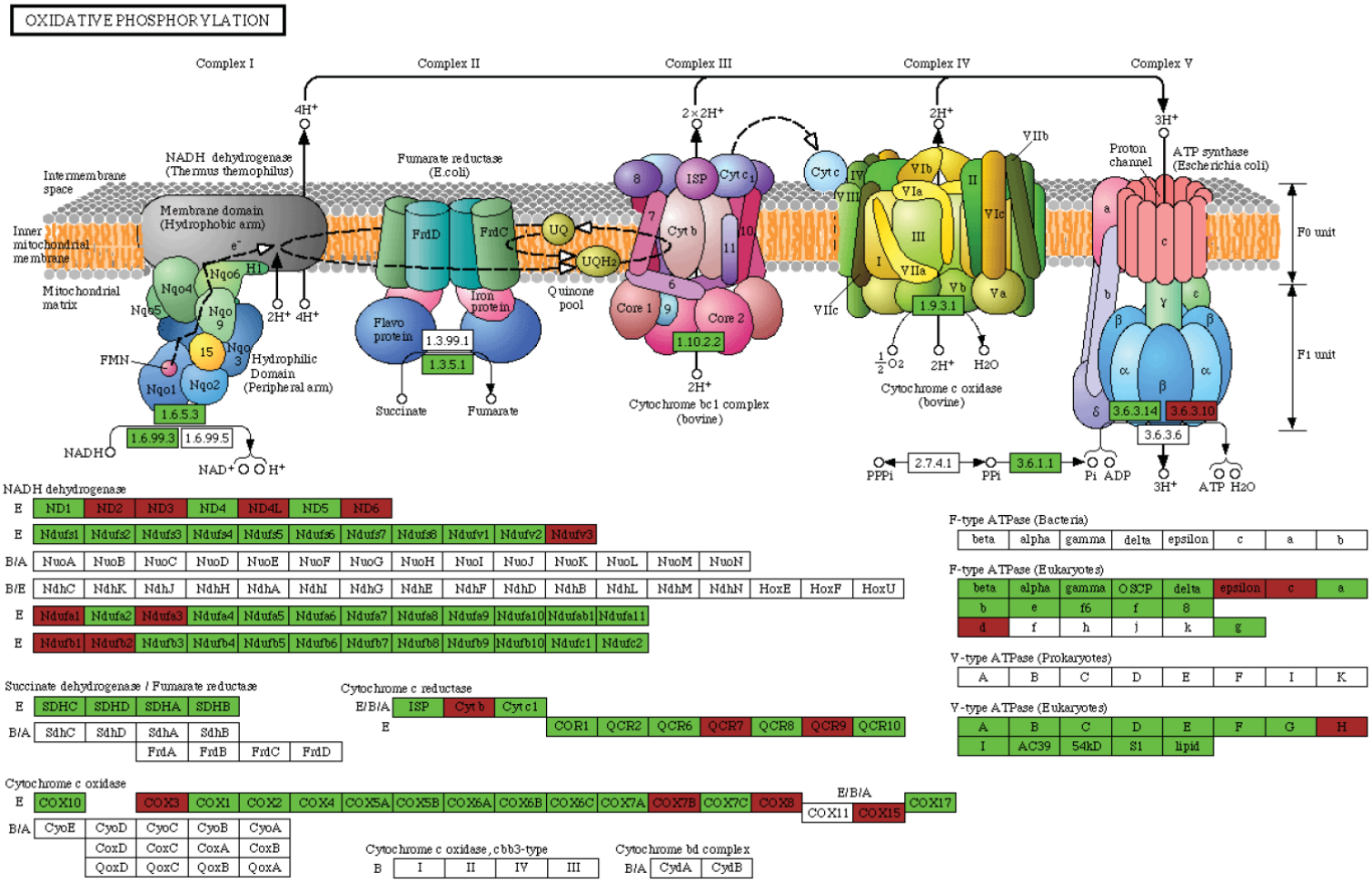



translation regulator activity molecular function
3.3% (95/2859)
2.2% (137/6139)
2.2% (50/2241)
2.8% (77/2750)
transcription regulator activity molecular function
9.2% (265/2859)
11.2% (688/6139)
5.5% (124/2241)
7.0% (194/2750)
nucleotide binding molecular function
28.5% (816/2859)
26.0% (1598/6139)
26.7% (600/2241)
25.8% (712/2750)
signal transducer activity molecular function
10.7% (308/2859)
12.4% (762/6139)
13.2% (298/2241)
10.4% (287/2750)
antioxidant activity molecular function
1.4% (41/2859)
0.9% (58/6139)
0.9% (22/2241)
1.3% (37/2750)
protein binding molecular function
59.8% (1710/2859)
57.2% (3512/6139)
54.5% (1223/2241)
49.4% (1359/2750)
RNA binding molecular function
14.6% (420/2859)
10.6% (651/6139)
7.9% (178/2241)
11.5% (318/2750)
transporter activity molecular function
13.6% (390/2859)
11.4% (703/6139)
16.3% (366/2241)
12.8% (353/2750)
motor activity molecular function
2.2% (65/2859)
2.7% (168/6139)
3.0% (68/2241)
2.0% (57/2750)
receptor activity molecular function
5.6% (161/2859)
7.4% (455/6139)
6.9% (155/2241)
5.7% (158/2750)
catalytic activity molecular function
61.0% (1744/2859)
56.6% (3475/6139)
58.5% (1311/2241)
62.6% (1724/2750)
DNA binding molecular function
16.6% (476/2859)
18.6% (1144/6139)
11.1% (250/2241)
12.9% (357/2750)
structural molecule activity molecular function
10.5% (303/2859)
8.9% (549/6139)
9.1% (206/2241)
8.8% (242/2750)
HeLa
HeLa IEF
Mouse brain
Mouse liver

Supplementary Figure 5. Sequence coverage of membrane proteins from liver prepared with FASP and by means of the standard urea/thiourea method. *Red*, median values, *yellow*, mean values.



Supplementary Figure 6. Pathway analysis of oxidative phosphorylation in human cells (http://www.genome.jp/kegg/tool/search_pathway.html). Proteins in green and red boxes indicate the presence or absence of proteins in the 7,093 HeLa data set, respectively. The genes for the proteins in grey boxes were not identified in human cells



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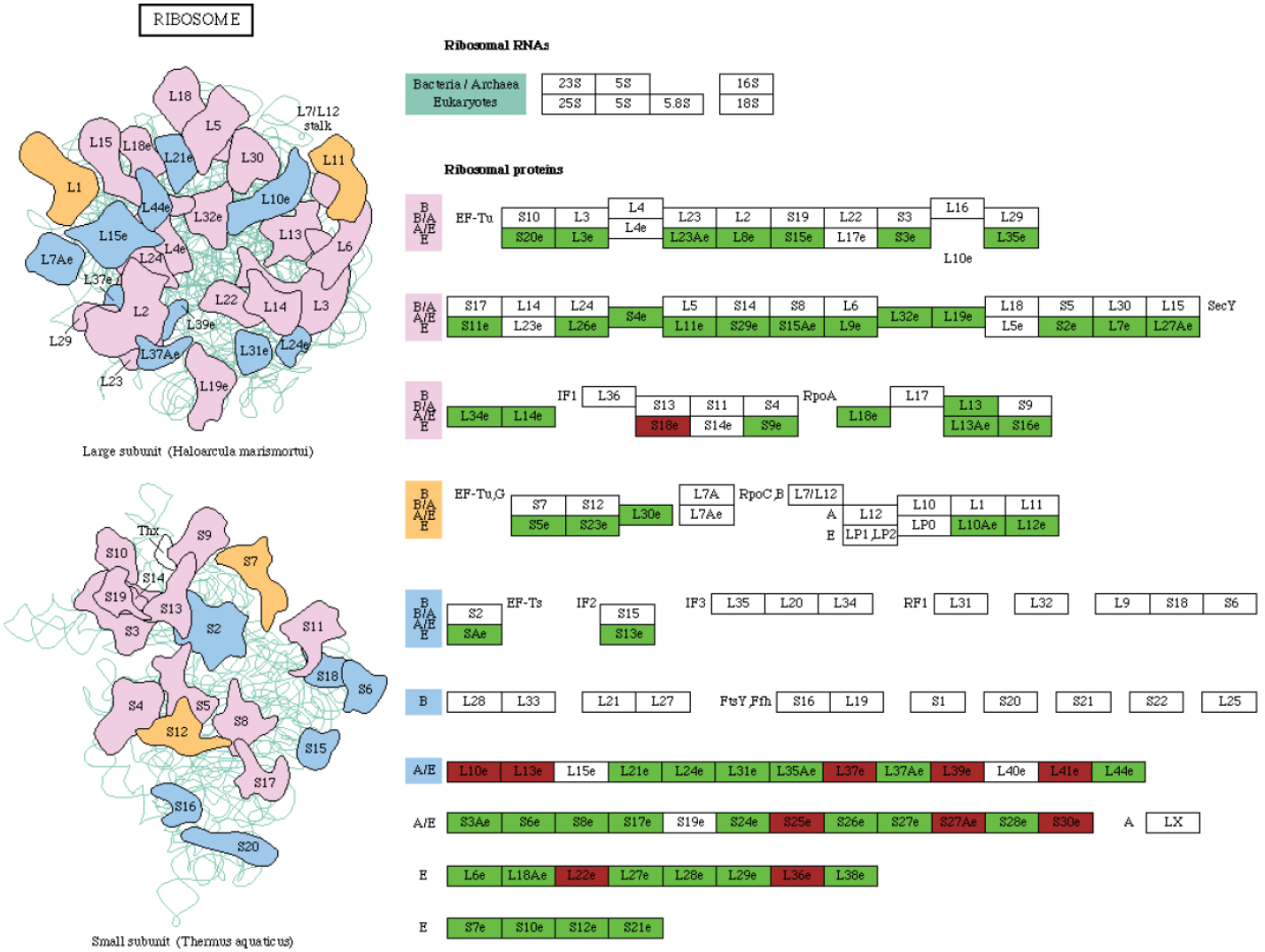
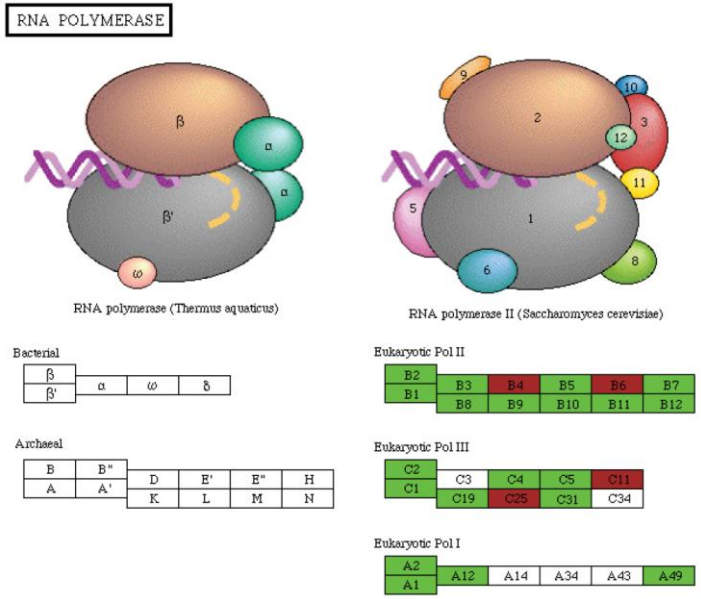
Supplementary Figure 7. KEGG-analysis of compositions of ribosomal subunits in human cells (http://www.genome.jp/kegg/tool/search_pathway.html**).** Proteins in green and red boxes indicate the presence or absence of proteins in the 7,093 HeLa data set, respectively. The genes for the proteins in grey boxes were not identified in human cells.

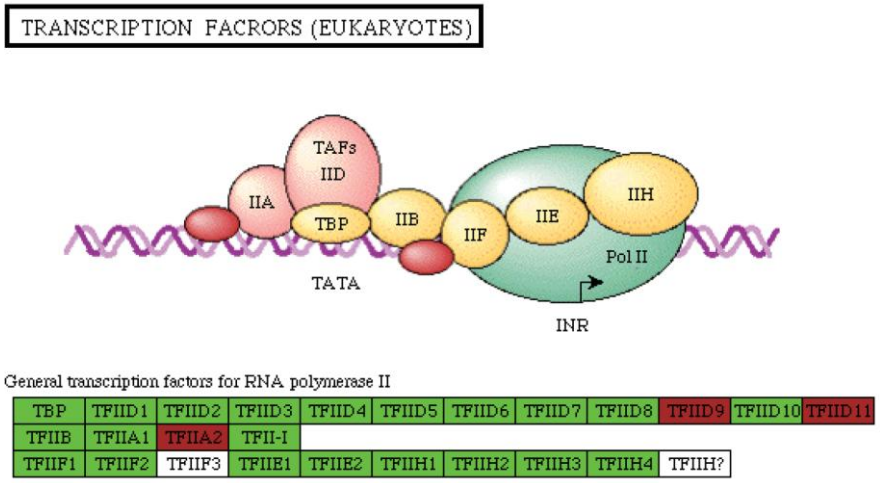


Supplementary Figure 8. KEGG-analysis of compositions of complexes of RNA Polymerase (A) and General Pol II-Transcription (B) in human cells (http://www.genome.jp/kegg/tool/search_pathway.html). Proteins in green and red boxes indicate the presence or absence of proteins in he 7,093 HeLa data set, respectively. The genes for the proteins in grey boxes were not identified in human cells.

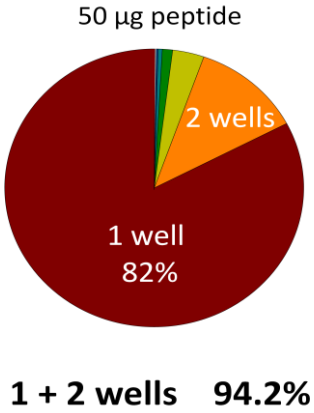
A.



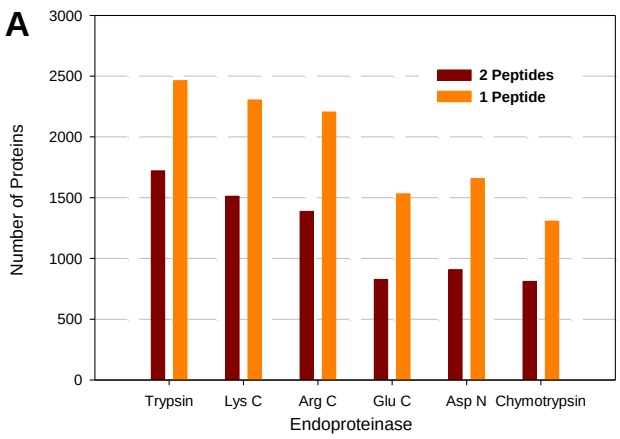
B.

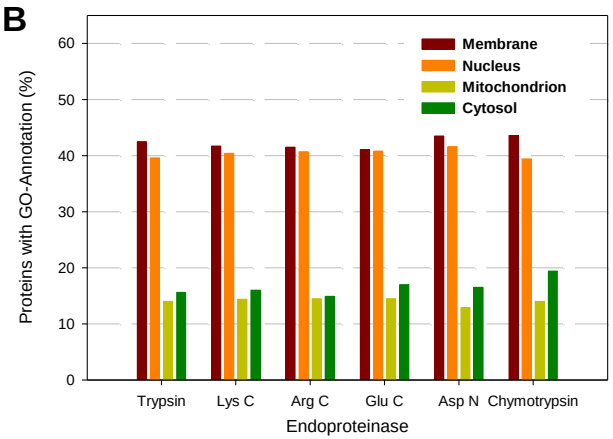


Supplementary Figure 9. Distribution of peptides after IEF.



Supplementary Figure 10. FASP based proteomic analysis of SDS -HeLa lysates using different digesting enzymes. A. Comparison of the number of proteins identified by LC-MS after digestion with trypsin, chymotrypsin, and endoproteases Lys C, Arg C, Glu C, and Asp N. B. Percentage of proteins annotated to membrane, nucleus, mitochondrion, and cytosol. C. Number of unique protein identifications achieved in two LC-MS runs of digests prepared with different enzymes. Data are average values of two independent experiments (**Supplementary Table 3, Supplementary Data 6)**





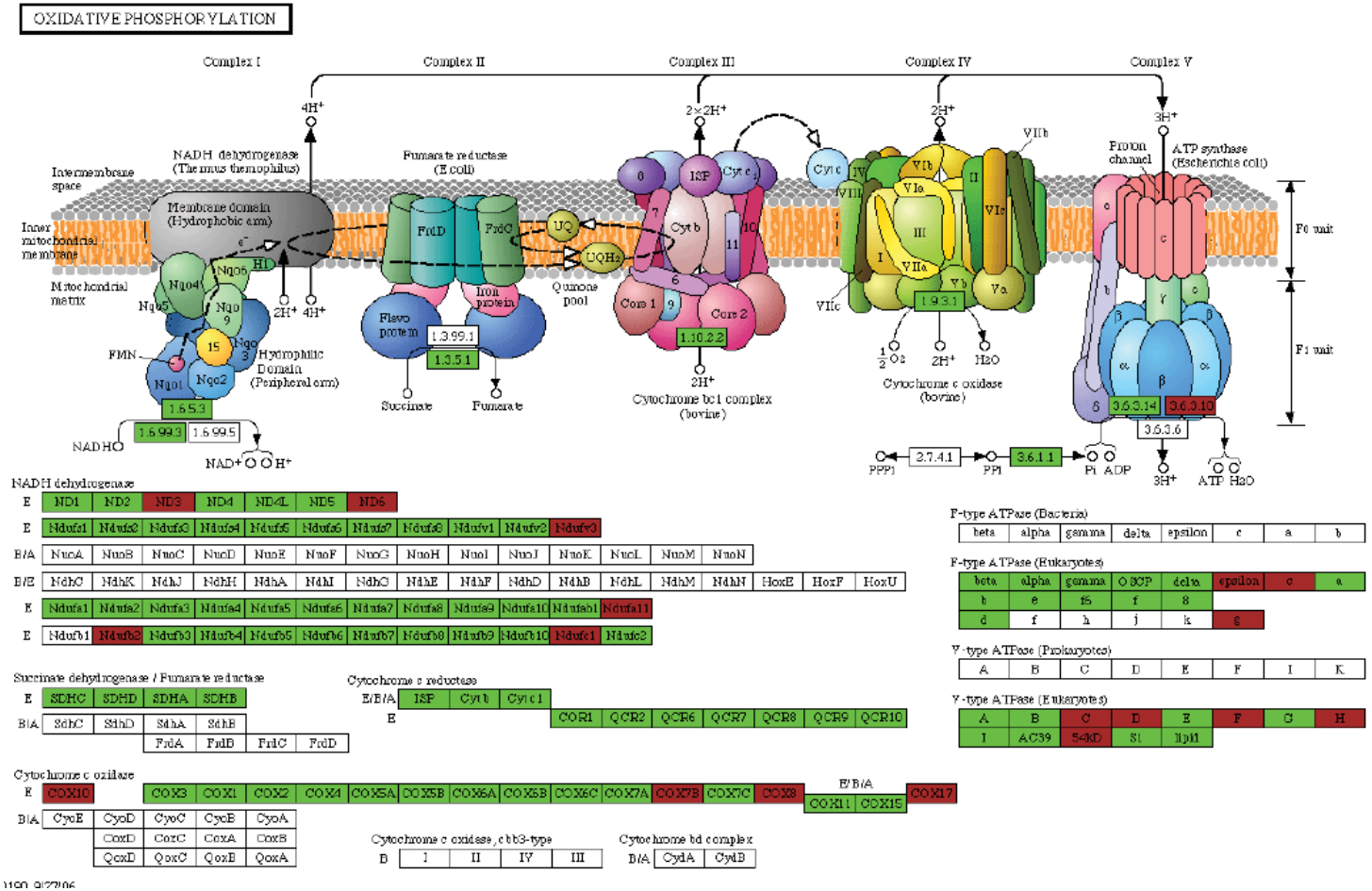
C

Number of Proteins

	Trypsin	Arg C	Lys C	Asp N	Glu C	Chymotrypsin
Trypsin	2,699	3,145	3,042	2,931	2,912	2,787
Arg C	2,499	3,054	2,759	2,717	2,503	
Lys C	2,304	2,704	2,677	2,588		
Asp N	1,907	2,324	2,140			
Glu C	1,787	2,066				
Chymotrypsin	1,403					

>15%	1>10%	>5%

Supplementary Figure 11. Pathway analysis of oxidative phosphorylation in mouse liver mitochondria.
(http://www.genome.jp/kegg/tool/search_pathway.html). Proteins in green and red boxes indicate the presence or absence of proteins in the 516 proteins mouse mitochondria data set, respectively. The genes for the proteins in grey boxes were not identified in mouse cells.



Supplementary Tables

Supplementary Table 1. FASP-based proteomic analysis of SDS-lysates.

Sample	MS/MS identified (%)[a]	Unique Peptides Identified	Proteins identified by		Protein Annotation			
			2 Peptides[b]	1 Peptide[c]	Membrane	Nucleus	Mitochondrion	Cytosol
HeLa Cells[d]	78.0 ± 2.0	9,728 ± 988	1,934 ± 52	2,745 ± 91	42.7	36.5	15.3	16.1
HeLa Cells 12 Fractions	41.5[d] ± 11.5	40,582	6,124	7,093	42.3	39.4	11.2	11.3
Mouse Liver	77.6	12,537	2,174	2,750	42.4	29.7	17.0	9.3
Mouse Brain	77.7	8,434	1,797	2,241	52.0	22.4	17.0	10.5

HeLa cells were analyzed in 3 independent experiments whereas tissue samples in single experiments. For single LC-MS run analyses 1 mg of tissues (wet tissue) or $2x10^5$ were processed with FASP using the standard two step digestion . The numbers shown are mean values ± standard deviations. a, percentage of sequencing events being matched to a peptide sequence; b, Identified by $\geq$2 peptides containing at least 1 unique peptides; c, identified by at least 1 unique peptide; d, mean ± standard deviation of 12 fractions. All unique peptides and proteins identified in these analyses are listed in Supplementary Data 1-4.

Supplementary Table 2. FASP based proteomic analysis of SDS -HeLa lysates using different digesting enzymes.

Enzyme[a]	Peptide[b] yield (µg)	MS/MS identified (%)[c]	Unique Peptides Identified	Proteins identified by		Protein Annotation			
				2 Peptides[d]	1 Peptide[e]	Membrane	Nucleus	Mitochondrion	Cytosol
Trypsin	95	77.7	9,617	1,685	2,457	42.5	39.6	14.0	15.6
	105	73.9	10,333	1,758	2,482				
Lys C	73	60.0	7,493	1,508	2,308	41,7	40.4	14.4	16.0
	84	53.4	7,418	1,517	2,307				
Arg C	45	47.6	6,304	1,402	2,173	41.5	40.7	14.5	14.9
	44	49.1	6,102	1,373	2,206				
Glu C	34	33.0	3,972	847	1,520	41.1	40.8	14.5	17.0
	34	31.9	4,175	809	1,555				
Asp N	66	23.3	3,855	946	1,686	43.5	41.6	12.9	16.5
	63	26.2	3,616	868	1,640				
Chymotrypsin	100	25.7	4,293	808	1,298	43.9	39.4	14.0	19.4
	99	24.7	4,462	813	1,325				

a, For each enzyme and condition two independent experiments were performed; b, SDS-lysate of $2x10^5$ HeLa cells was used as a starting material. The peptide concentration was determined by A280 nm measurement; c, percentage of sequencing events being matched to a peptide sequence; d, Identified by $\geq$2 peptides containing at least 1 unique peptides; e, identified by $\geq$ 1 unique peptide. All unique peptides and proteins identified in these analyses are listed in **Suppl. Data 5**.

Since digestion with proteases other than trypsin produces peptides carrying unique information we decided to test different proteases in FASP. To ensure that the enzymes remained stable we carried out digestions in 2M urea. All tested enzymes resulted in substantial yields as judged by UV spectrometry and LC MS analysis identified large number of peptides and proteins (**Suppl. Table 1**). As expected, the ubiquitously employed enzymes trypsin and LysC performed best (**Suppl. Fig. 9A**). Intriguingly, the much less used ArgC identified almost as many proteins, even though its efficiency of digestion was significantly lower. Conversely, chymotrypsin digested the sample very efficiently but identified a smaller number of proteins. This is probably due to the fact that ArgC peptides are longer and fragment better than those of chymotrypsin, contributing more to statistically significant protein identifications. All tested enzymes covered major cellular compartments in a similar way (**Suppl. Fig. 9B** and **Suppl. Table 1**). We next asked if application of different proteases would lead to deeper coverage of the proteome and tested all double combinations of the above proteolytic enzymes. Compared to the subsequent analysis of two trypsin digests the combination of one trypsin digest with either LysC or ArgC led to the largest increase in identifications (**Suppl. Fig. 9C**). Combination of trypsin with AspN and GluC likewise resulted in a larger identified proteome.

Supplementary Table 3. FASP based proteomic analysis of SDS -HeLa lysates using different digestion conditions.

Enzyme[a]	Peptide[b] yield (µg)	MS/MS identified (%)[c]	Unique Peptides Identified	Proteins identified by		Protein Annotation			
				2 Peptides[d]	1 Peptide[e]	Membrane	Nucleus	Mitochondrion	Cytosol
Standard two step digestion	77 75	77.0 80.5	11,666 11,180	2,008 1,917	2,773 2,682	44.1	40.4	15.1	14.5
Trypsin no urea 90 min digest	71 65	79.3 81.9	12,046 11,424	1,935 1,868	2,701 2,631	44.1	41.2	14.9	14.6
Trypsin no urea 18h digest	76 60	82.9 81.3	11,178 11,095	1,902 1,912	2,679 2,618	45.1	39.8	15.5	14.6
Trypsin 2M Urea 18 h digest RT	63 74	80.2 80.1	13,361 13,330	2,121 2,094	2,839 2,781	44.2	40.3	15.3	14.2

a, For each condition results of two independent experiments are shown; b, SDS-lysate of $2x10^5$ HeLa cells was used as a starting material. The peptide concentration was determined by A280 nm measurement; c, percentage of sequencing events being matched to a peptide sequence; d, Identified by $\geq$2 peptides containing at least 1 unique peptides; e, identified by $\geq$ 1 unique peptide. All unique peptides and proteins identified in these analyses are listed in Supplementary Data 6.

In-solution digests are frequently performed in a two step, Lys-C and trypsin procedure because the high stability of LysC allows initial digestion in the chaotropic environment of 8M urea. Intriguingly, we observed that FASP digestion with trypsin in 2 M urea alone led to equivalent results as the two enzyme standard method. This suggests that the backbone of proteins is readily accessible to the enzyme even with relatively mild denaturing conditions, because they remain unstructured after initial SDS and high urea solubilization. To directly test if FASP allows efficient digestion even without denaturing agent, we depleted urea by two additional washes of the filter with buffer and subjected the sample to tryptic digestion at 37°C for 90 min or 18h. Under both conditions single LC-MS runs allowed identification of about 12,000 peptides corresponding to about 2,600 proteins. No preferences for membrane or soluble proteins were observed between the two–step digestion, digestion in 2M urea, and without urea. Thus FASP enables digestion of membrane proteins under conditions previously applied only to soluble proteins.

Supplementary Table 4. Digestion conditions used.

Digest type	Enzyme (Supplier)	Protein to Enzyme ratio	Buffer	Digestion time	Digestion Temperature
Standard two step digestion	Step 1: LysC (Wako)	1:100	8M urea; 0.1M Tris/HCl pH7.9	18h	20°C
	Step 2: Trypsin (Promega)	1:100	2M urea; 0.1M Tris/HCl pH7.9	4h	20°C
Trypsin no urea 90 min digest	Trypsin (Promega)	1:100	50 mM $(NH)_4HCO_3$	1.5h	37°C
Trypsin no urea 18h digest	Trypsin (Promega)	1:100	50 mM $(NH)_4HCO_3$	18h	37°C
Trypsin 2M Urea 18 h digest RT	Trypsin (Promega)	1:100	2M urea; 0.1M Tris/HCl pH7.9	18h	20°C
Lys C	LysC (Wako)	1:100	2M urea; 0.1M Tris/HCl pH7.9	18h	20°C
Arg C	ArgC (Roche)	1:50	2M urea; 0.1M Tris/HCl pH7.6, 5 mM $CaCl_2$	18h	20°C
Glu C	Glu C (Roche)	1:50	2M urea; 50 mM $(NH)_4HCO_3$	18h	20°C
Asp N	Asp N (Roche)	1:50	0.025M Tris/HCl pH7.8,	18h	20°C
Chymotrypsin	Chymotrypsin (Roche)	1:100	0.025M Tris/HCl pH7.8, 10 mM $CaCl_2$	18h	20°C

Supplementary Protocols

Two FASP protocols are described below. The first one was used in all experiments presented in the manuscript except for analysis of mitochondria where protocol II was applied. Both protocols provide similar results; however, the protocol II is much faster, due to use of 30 k filtration unit, which enables more rapid sample concentration in comparison to the 10 k unit.

Filter Aided Sample Preparation (FASP) I.

[10k filter, two-step digestion, max. 250 µg total protein]

1. Materials

Solutions and Reagents

SDT-lysis buffer: 4%(w/v) SDS, 100mM Tris/HCl pH 7.6, 0.1M DTT

UA: 8 M urea (Sigma, U5128) in 0.1 M Tris/HCl pH 8.5

UB: 8 M urea (Sigma, U5128) in 0.1 M Tris/HCl pH 8.0

IAA solution: 0.05 M iodoacetamide in UA

Endoproteinase Lys-C from Wako Bioproducts (Richmond, VA) Stock 5 µg/µl.

Trypsin, Stock 0.4 µg/µl

0.5M NaCl in water

ABC: 0.05M NH_4HCO_3 in water.

Note:

UA, UB, and IAA solutions must be prepared freshly and used within a day.

Equipment

Microcon YM-10 (Millipore, Cat. No. number 42407)

3M Empore HP Extraction disk cartridge (C18-SD); 7mm/ 3 ml (Varian Cat. No. 12144002)

Refrigenerated Bench-top centrifuge (Eppendorf 5415R), temperature 20°C

Multifuge 3L-R (Heraeus), (for peptide desalting in cartridges), temperature 20°C

Wet chamber with a rack for Eppendorf tubes

Thermo-mixer set to 20°C

UV-Spectrophotometer, Quartz-cuvettes

2. Methods

2.1 Sample lysis

Lyse cells or tissues in SDT-lysis buffer using 1:10 sample to buffer ratio for at 95°C for 3-5 min. The DNA has to be sheared by sonication to reduce the viscosity of the sample. Before starting sample processing the lysate has to be clarified by centrifugation at 16,000 x g for 5 min.

Notes:

1. The tissues have to be homogenized with a blender in the lysis solution before heating.
2. Avoid temperatures below 15°C and potassium salts to avoid precipitation of concentrated SDS.

2.2 Sample processing

1. Mix up to 30µl of a protein extract with 200µl of UA in the filter unit and centrifuge at 14,000 x g for 40 min.
2. Add 200µlof UA to the filter unit and centrifuge at 14,000 x g for 40 min.
3. Discard the flow-through form the collection tube.
4. Add 100 µl IAA solution and mix at 600 rpm in thermo-mixer for 1 min and incubate without mixing for 5 min.
5. Centrifuge the filter units at 14,000 x g for 30 min.

6. Add 100 µl of UB to the filter unit and centrifuge at 14,000 x g for 40 min. Repeat this step twice.
7. Add 40 µl of UB with Lys-C (enzyme to protein ration 1:50) and mix at 600 rpm in thermo-mixer for 1 min.
8. Incubate the units in wet chamber overnight.
9. Transfer the filter units to new collection tubes.
10. Add 120 µl ABC with trypsin (enzyme to protein ration 1:100) and mix at 600 rpm in thermo-mixer for 1 min.
11. Incubate the units at RT for 4 h.
12. Centrifuge the filter units at 14,000 x g for 40 min.
13. Add 50 µl 0.5 M NaCl and centrifuge the filter units at 14,000 x g for 20 min.
14. Acidify with CF_3COOH and desalt the filtrate.

2.3 Desalting of peptides

Small amounts of digest for direct LC-MS analysis can be desalted on StageTips.

Large amounts of peptide mixtures used for OFFGEL separation have to be desalted on SPE cartridges according to the following protocol:

1. Place a 3 ml MILI-SPE Extraction disk cartridge (C18-SD) in and 15 ml conical tube.
2. Add 1 ml of CH_3OH and centrifuge at 1,500 x g for 1 min.
3. Add 0.5 ml of 0.1% CF_3COOH, 70% CH_3CN in water and centrifuge at 1,500 x g for 1 min.
4. Add 0.5 ml of 0.1% CF_3COOH in water and centrifuge at 1,500 x g for 1 min.
5. Load the filtrate (2.2 step 15) and centrifuge at 150 x g for 3 min.
6. Add 0.5 ml of 0.1% CF_3COOH in water and centrifuge at 150 x g for 3 min.
7. Transfer the cartridge to anew tube, add 0.5 ml 70% CH_3CN in water and centrifuge at 150 x g for 3 min.
8. The eluate contains desalted peptides.

2.4 Yield determination

Concentration of the peptides can be estimated by UV spectrometer assuming that 0.1% solution of vertebrate proteins has at 280 nm an extinction of 1.1 absorbance units. (1mg/ml solution has 1.1 au).

Filter Aided Sample Preparation (FASP) II.

[30 k filter, tryptic digestion 37°C; max. 250 µg total protein]

1. Materials

Solutions and Reagents

UA: 8 M urea (Sigma, U5128) in 0.1 M Tris/HCl pH 8.5. Prepare 1 ml per 1 sample.

IAA solution: 0.05 M iodoacetamide in UA. Prepare 0.1 ml per 1 sample.

Trypsin, Stock 0.4 µg/µl

0.5 M NaCl in water. Prepare 0.05 ml per 1 sample

ABC: 0.05M NH_4HCO_3 in water. Prepare 0.25 ml per 1 sample

Note:

UA and IAA solutions must be freshly prepared and used within a day.

Equipment

Microcon YM-30 (Millipore, Cat. No. 42410)

Refrigenerated Bench-top centrifuge (Eppendorf 5415R), temperature 20°C

Wet chamber with a rack for Eppendorf tubes

Thermo-mixer set to 20°C

See *FASP 10k filter two-step digestion protocol* for sample lysis and desalting of peptides protocol.

2. Protocol

1. Mix up to 30µl of a cell lysate with 200µl of UA in the filter unit and centrifuge at 14,000 x g for 15 min.
2. Add 200µlof UA to the filter unit and centrifuge at 14,000 x g for 15 min.
3. Discard the flow-through form the collection tube.
4. Add 100 µl IAA solution and mix at 600 rpm in a thermo-mixer for 1 min and incubate without mixing for 20 min.
5. Centrifuge the filter units at 14,000 x g for 10 min.
6. Add 100 µl of UA to the filter unit and centrifuge at 14,000 x g for 15 min. Repeat this step twice.
7. Add 100 µl of ABC to the filter unit and centrifuge at 14,000 x g for 10 min. Repeat this step twice.
8. Add 40 µl ABC with trypsin (enzyme to protein ratio 1:100) and mix at 600 rpm in thermo-mixer for 1 min.
9. Incubate the units in a wet chamber at 37°C for 4 -18 h.
10. Transfer the filter units to new collection tubes.
11. Centrifuge the filter units at 14,000 x g for 10 min.
12. Add 50 µl 0.5 M NaCl and centrifuge the filter units at 14,000 x g for 10 min.
13. Acidify with CF_3COOH and desalt the filtrate.