Proteomics of dying cells

(characterizing **protein cuts** on a proteome-wide level)

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3rd Training Course on Concepts and Methods in Programmed Cell Death

"Role of organelles in cell death", Cagliari, Sardinia, Italy

OUTLINE:

- 2D PAGE analysis of dying cells
- gel-free analysis of dying cells
- COFRADIC analysis of dying cells
- Example 1: Fas-induced apoptosis in human Jurkat T-cells
- Example 2: searching for granzyme B substrates

How to analyze the substrates of proteases?

“PEPTIDE/PROTEIN-BASED”

- (1D)/2D-PAGE
- “trial-and-error” immunoblotting
- peptide libraries
- gel-free (ICAT studies)

“GENETICS”

- yeast-2-hybrid and derivatives
- phage display
- CLIP-CHIP

2D-gel proteomics of dying cells

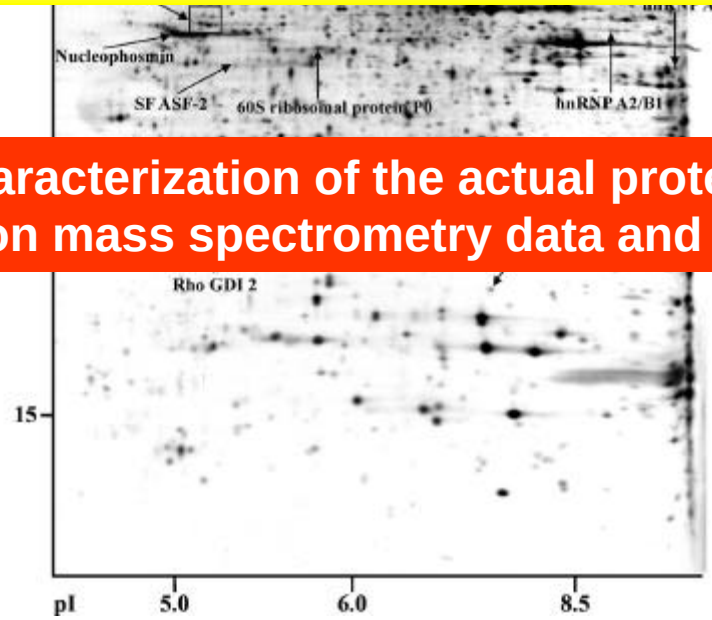
living Jurkat cells

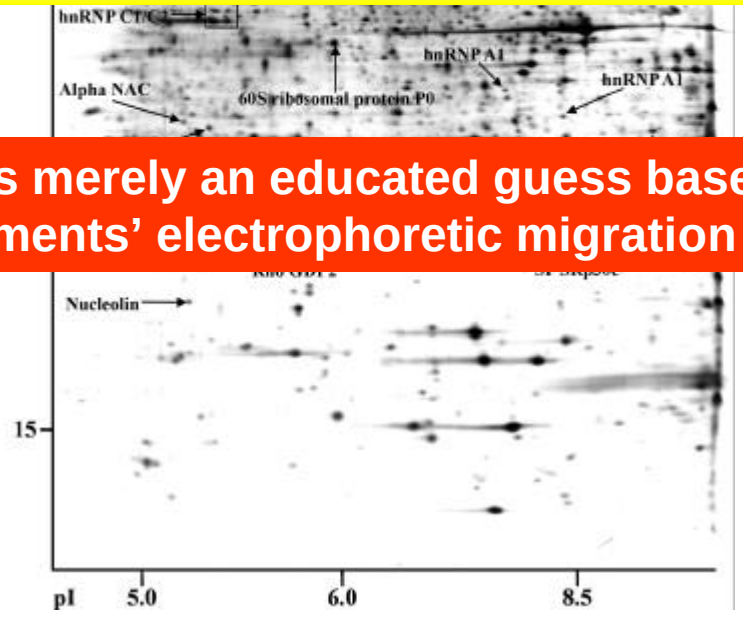
Fas-induced Jurkat cells

find protein spots that disappear in the living proteome (substrates)

&

protein spots that appear in the "dying proteome" (stable protein fragments)





characterization of the actual protein site is merely an educated guess based on mass spectrometry data and the fragments' electrophoretic migration

Thiede B *et al.* (2001) *J. Biol. Chem.* **276**, 26044-26050.

General disadvantages of 2D-gel proteomics

NOT ALL PROTEINS MAY BE CHARACTERIZED

Under-representation of

- **hydrophobic proteins (integral membrane proteins)**

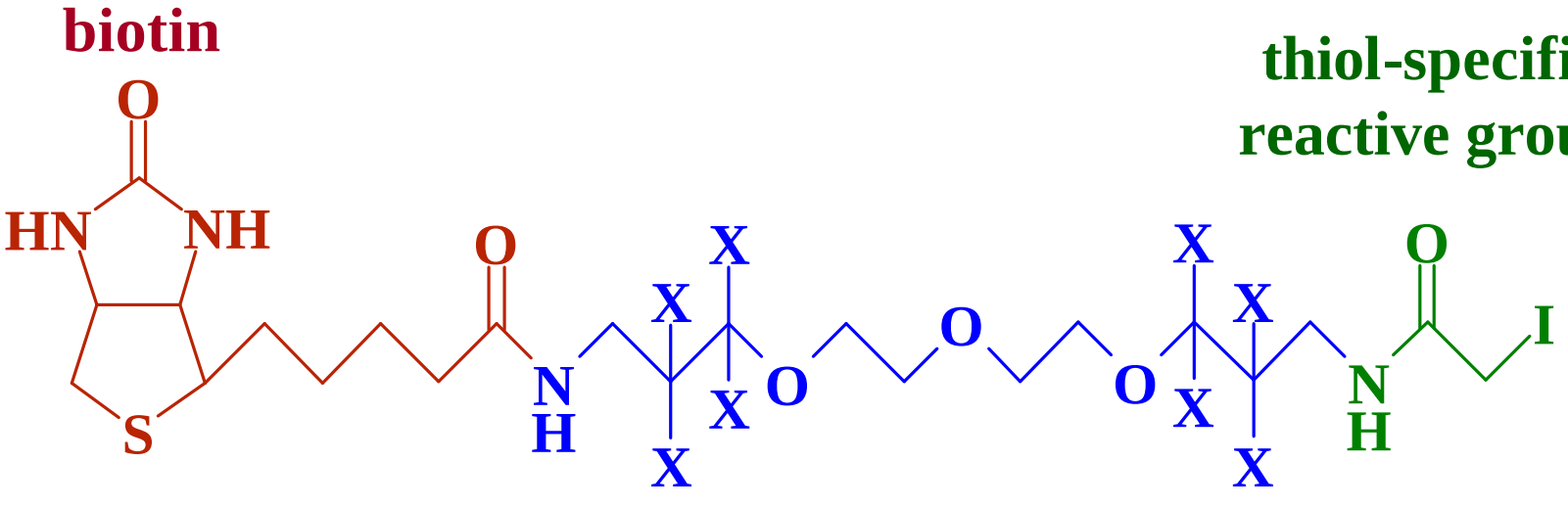
Solutions?
Use gel-free, peptide-centric, shotgun etc … proteomics

MASS SPECTROMETRY DRIVEN PROTEOME ANALYSIS

Due to both technical limitations of 2D-gel techniques and intrinsic complexity of eukaryotic proteomes.

Gel-free assessment of protease substrates (I)

The original ICAT molecule (isolation of cysteinyl peptides):



heavy reagent: X=deuterium
light reagent: X=hydrogen

The linker allows differential proteome analysis!

Evoked mass difference = 8 amu's.

Gygi SP *et al.* (1999) *Nat. Biotechnol.* **17**, 994-999

Gel-free assessment of protease substrates (II)

Example: work of Chris Overall on potential substrates of MT1-MMP

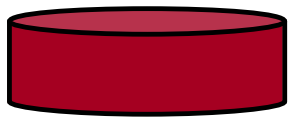
Proteomic comparison (AEX & ICAT) of MDA-MD-231 human breast carcinoma cell line expressing MT1-MMP versus its E240A inactive mutant.

General hypothesis:

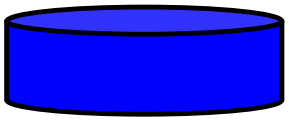
- **concentration of MT1 substrates can be altered because of:**
  - ✓ **secreted substrates are processed (concentration drops)**
  - ✓ **shedded protein fragments (concentration increases)**
- **concentration changes are picked up following MS & MS/MS analysis**

Tam EM *et al.* (2004) *PNAS* **101**, 6917-6922

Gel-free assessment of protease substrates (III)



Cell state 1
(light ICAT)



Cell state 2
(heavy ICAT)

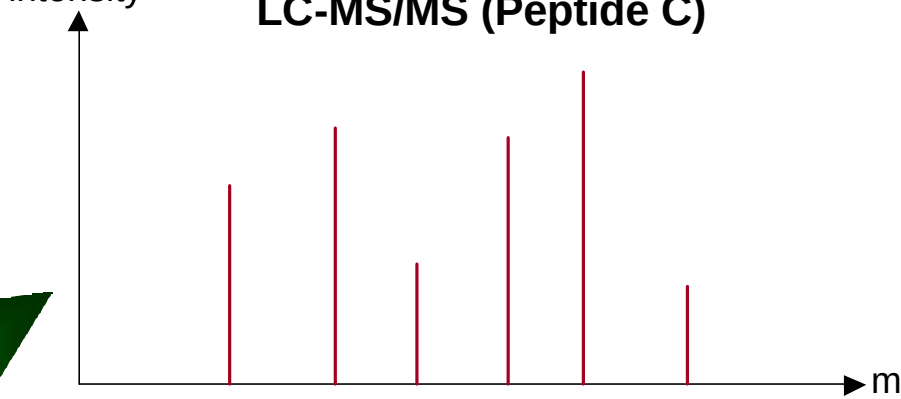
peptide sequenced
&
protein identified

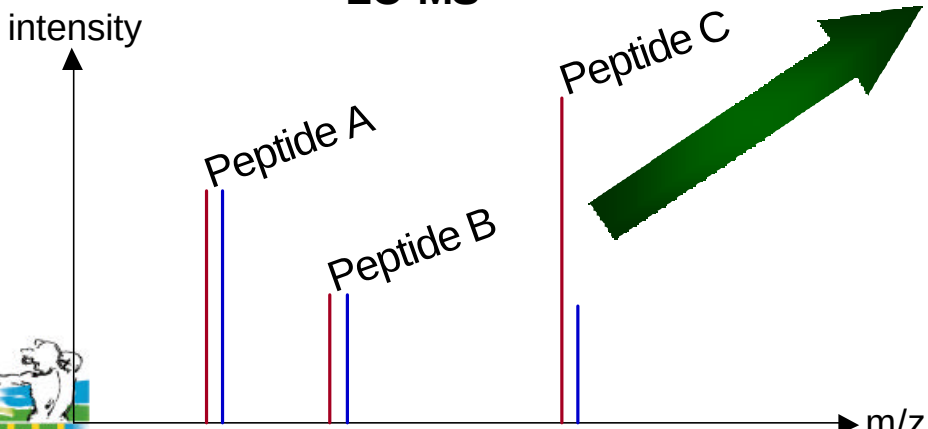
Combine and digest

Affinity isolate ICAT-peptides

LC-MS

LC-MS/MS (Peptide C)





Gel-free assessment of protease substrates (IV)

Proteins with increased concentration due to MT1 activity:

Protein	Ratio (active/mutant)	Peptide sequences
SLPI	4.95	CCMGMCGK
		YKKPECQSDWQCPGK
EFEMP-1	3.90	ADQVCINLR
		CVNHYGGYLCLPK
DR-6	3.79	VCSSCPVGTFTR
		EYLGAICSCTCFGGQR
		GEWTCIAYSQLR
ProMMP-1	2.05	ACDSKLYDAFHK
		CGVPDVAQFVLTEGNPR
Neuropilin-1	1.90	SPGFPEKYPNSLECTYIVFAPK
SKALP	1.85	CAMLNPPNR
		CLKDTDCPGIK
CTGF	1.74	DGAPCIFGGTVYR
LTBP-4S	1.73	AGPDLASCLDVDECRER
		DGGCSLPILR
		IQQCPGTETAEYQSLCPHGR
TNF	1.29	SWLPAGCETAILFPMR
TIMP-1	1.25	EPGLCTWQSLR
		FVYTPAMESVCGYFHR
		LQSGTHCLWTDQLLQGSEK

BUT:

a) Restricted to proteins with "identifiable" cysteinyl peptides

b) No clear assingment of protein cleavage sites

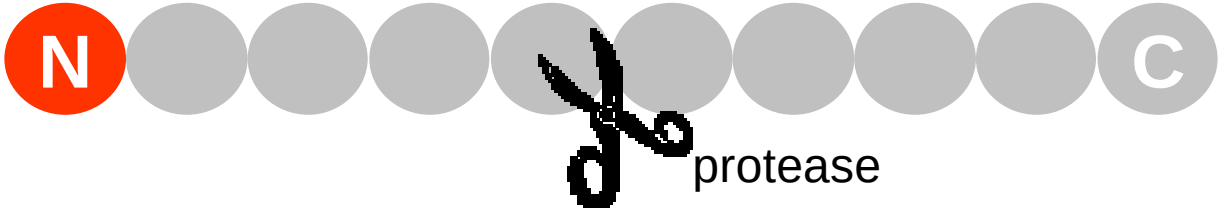
THUS: additional experiment/validation necessary

Adapted from Table 1 in Tam EM *et al.* (2004) *PNAS* **101**, 6917-6922

Our hypothesis?

Identify the processed site = unambiguous identification of the substrate of proteases in dying cells …

Which protein chemical techniques are available?



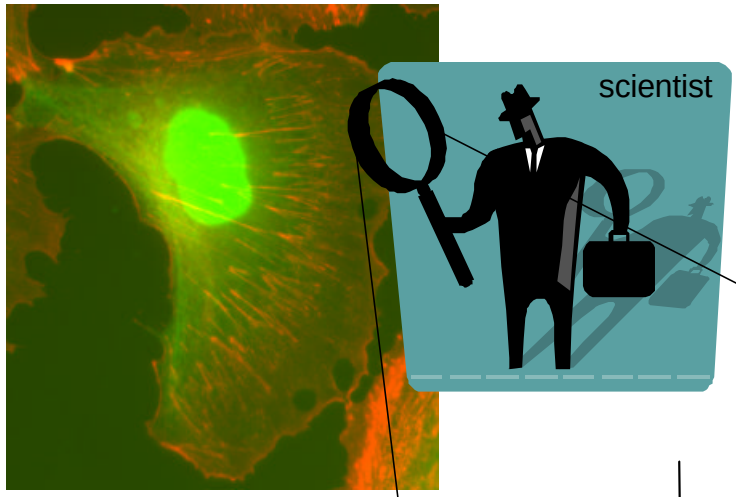
anhydrotrypsin
chemical sequencing
C-terminal Edman
C-terminal MS/MS

N-terminal COFRADIC
ion exchange chromatography
Protein Sequence Tags (PST)
positional proteomics

Gevaert K *et al*. *BBA*, in press (review).

COFRADIC (principle) COmbined FRActional Diagonal Chromatography

Cells contain thousands of proteins



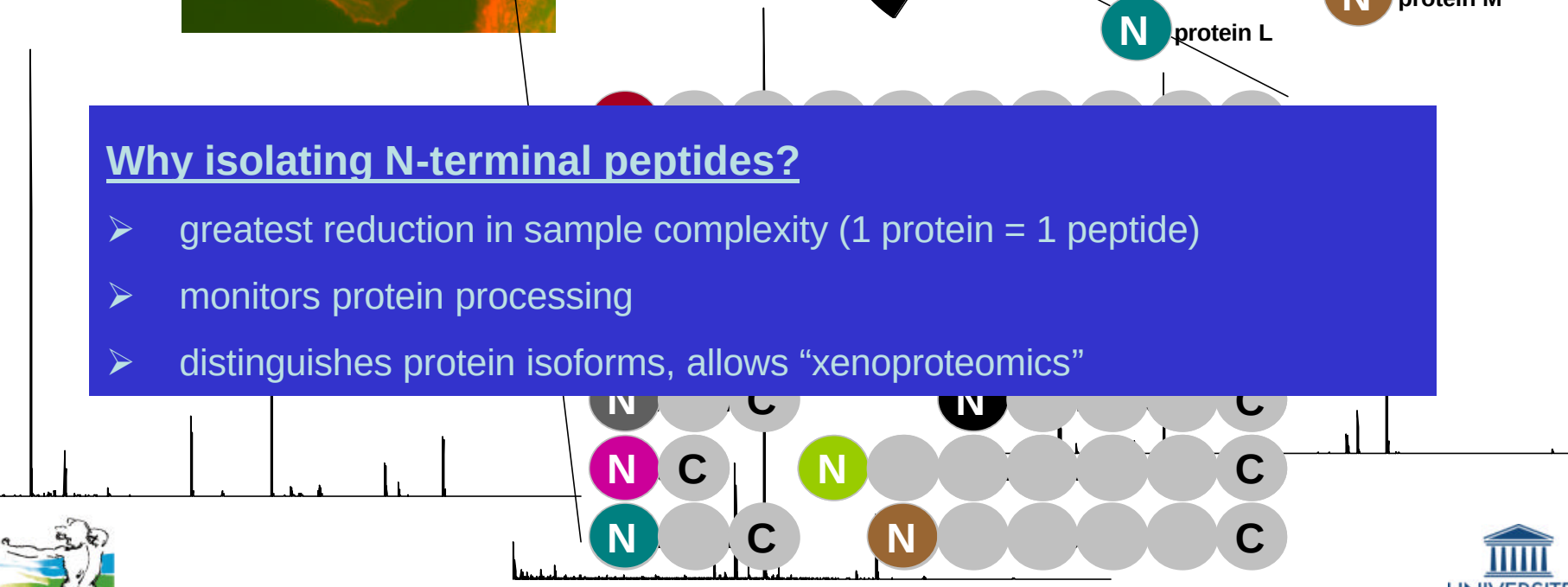
COFRADIC

N-terminal proteome of ends

N protein A
N protein B
N protein C
N protein G
N protein I
N protein L
N protein D
N protein E
N protein F
N protein J
N protein K
N protein M

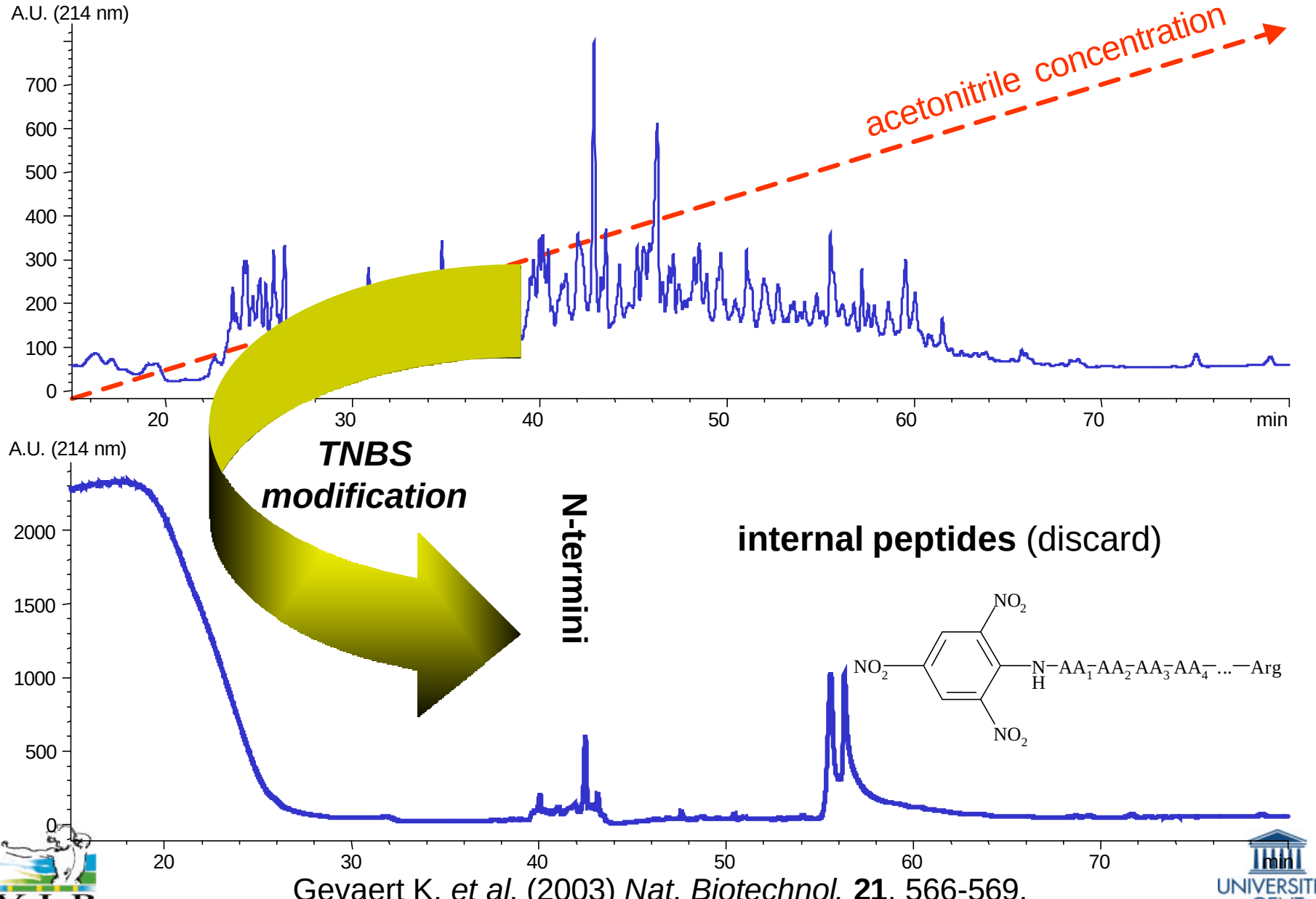
Why isolating N-terminal peptides?

- greatest reduction in sample complexity (1 protein = 1 peptide)
- monitors protein processing
- distinguishes protein isoforms, allows “xenoproteomics”



mass spectrometric analysis

COFRADIC isolation of N-terminal peptides

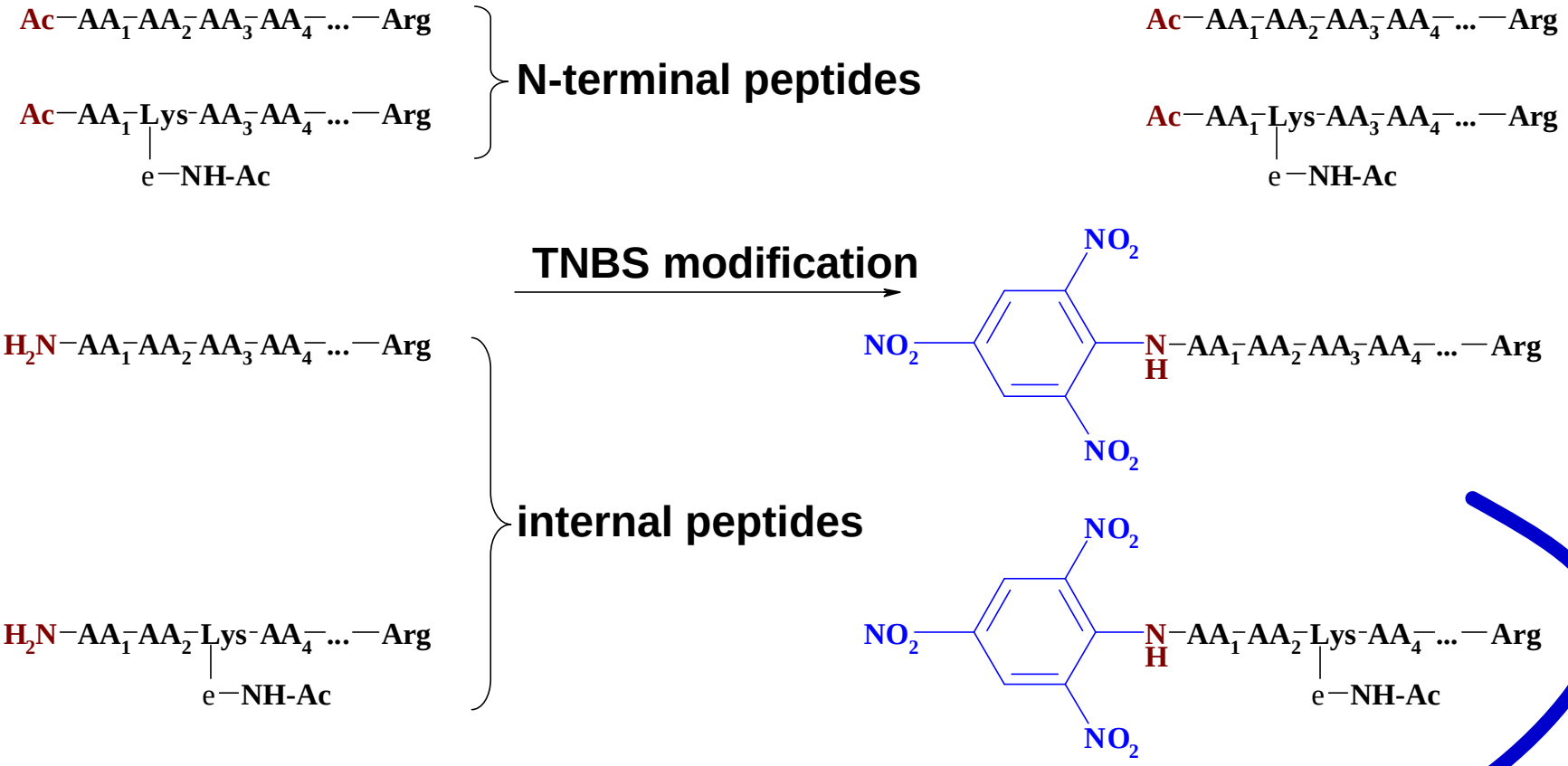


Gevaert K. *et al.* (2003) *Nat. Biotechnol.* **21**, 566-569.

Specific isolation of N-terminal peptides

primary run

secondary run

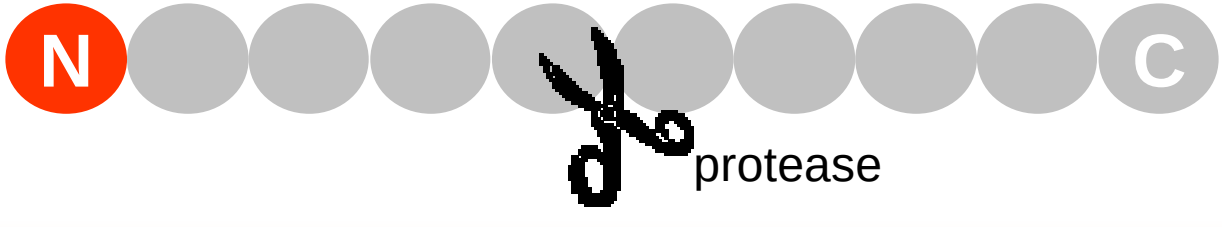


Gevaert K. *et al.* (2003) *Nat. Biotechnol.* **21**, 566-569.

Protein processing & Nter COFRADIC

“novel” N-termini:

- point to protein processing
- only present (e.g.) apoptotic cells



“mature” N-termini:

- do not point to protein processing
- present equal amounts in (e.g.) living and apoptotic cells

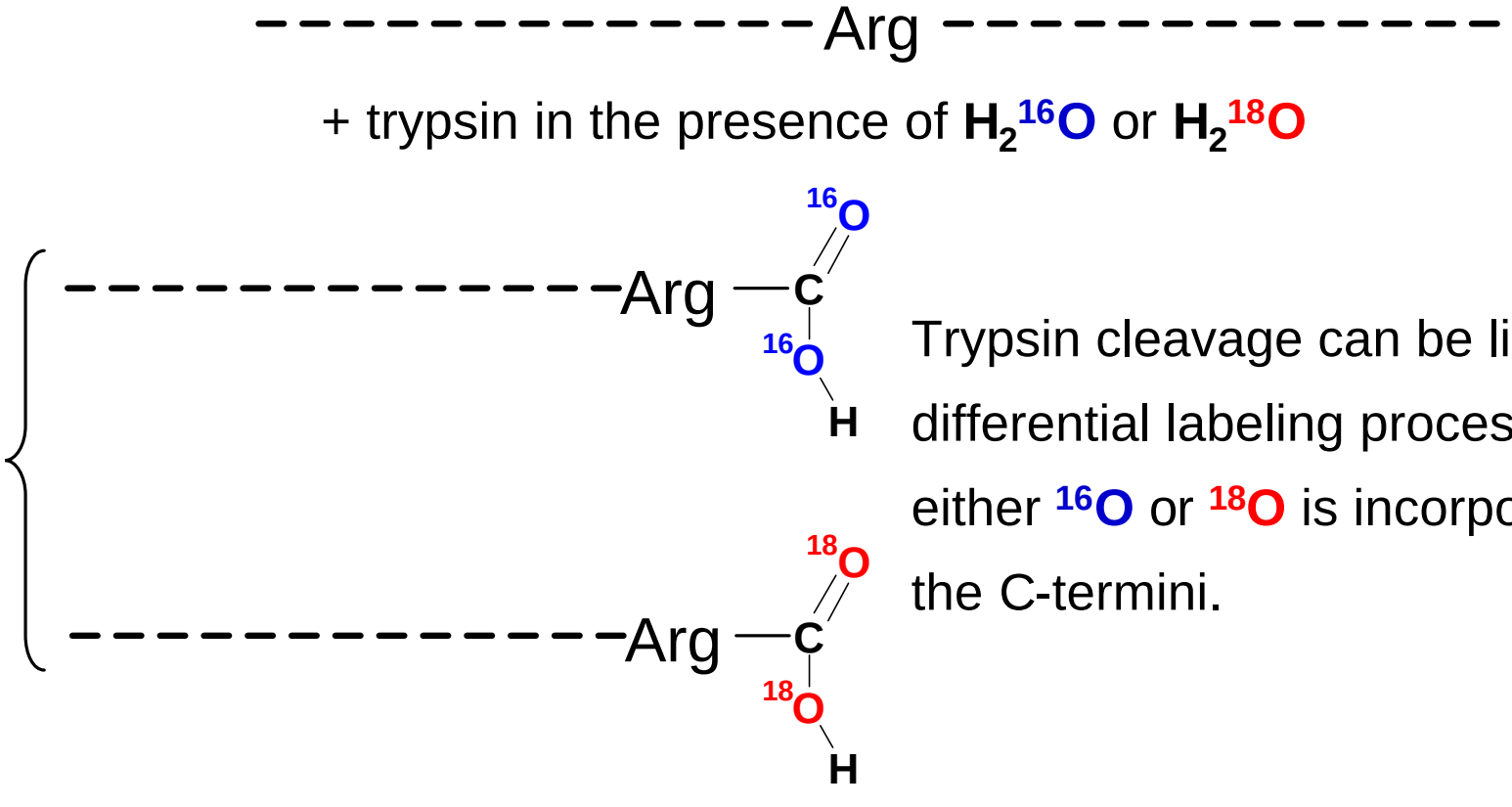
Van Damme P. *et al*. (2005) *Nat. Methods* **2**, 771-777.

Example 1: Fas-induced apoptosis in Jurkat T-cells

Experimental setup (1)

Van Damme P. *et al*. (2005) *Nat. Methods* **2**, 771-777.

Differential proteomics following oxygen-18 incorporation

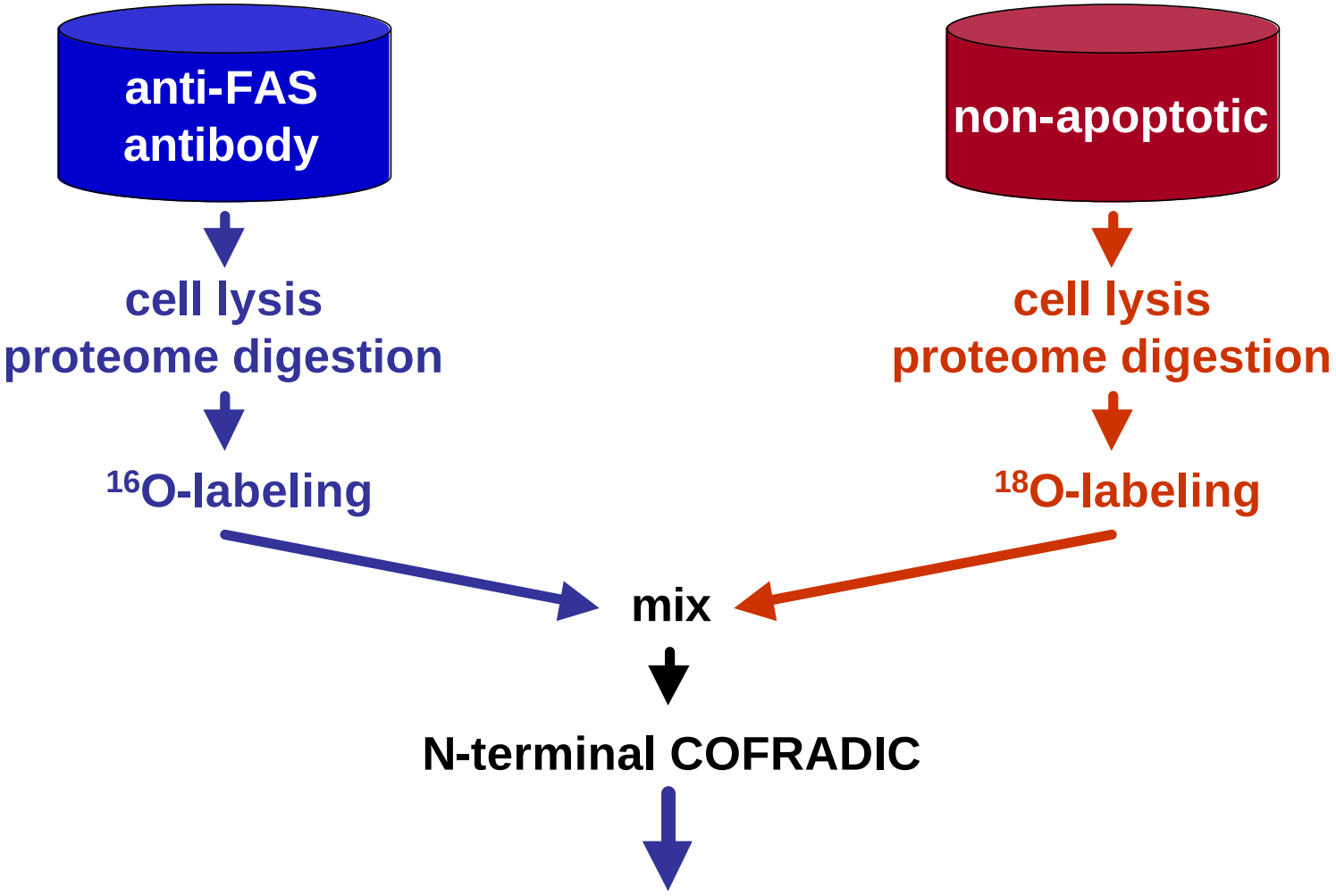


Trypsin cleavage can be linked to a differential labeling process by which either ^{16}O or ^{18}O is incorporated at the C-termini.

The chemically 'identical' ^{16}O and ^{18}O-derivatives behave identically during chromatography and ionization, but are separated by 4 amu's.

Staes A. & Demol H. *et al*. (2004) *J. Proteome Res.* **3**, 786-791.

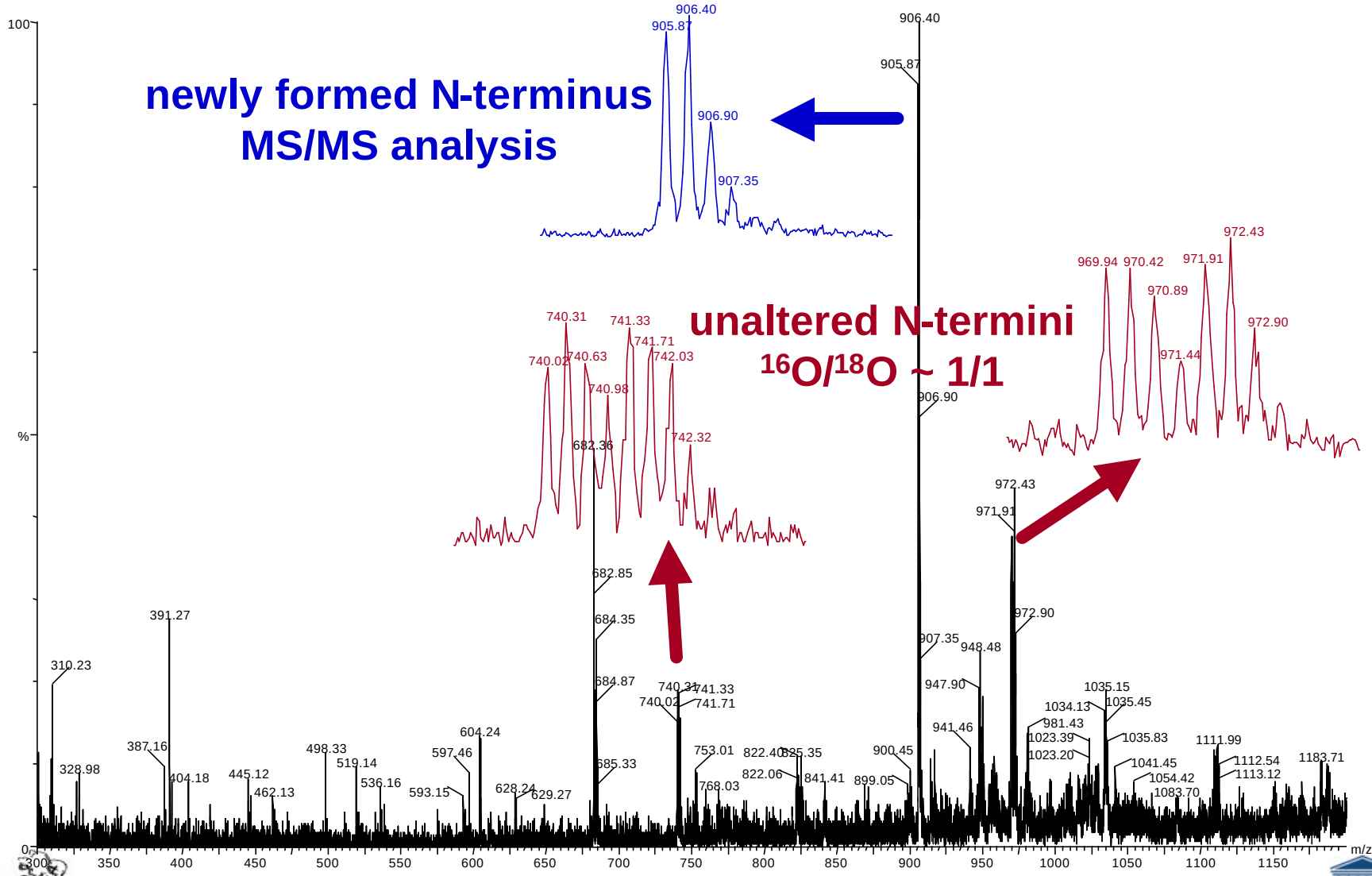
Experimental setup (2)



newly formed N-termini are from substrates of proteases and DO NOT contain ^{18}O-isotopes!

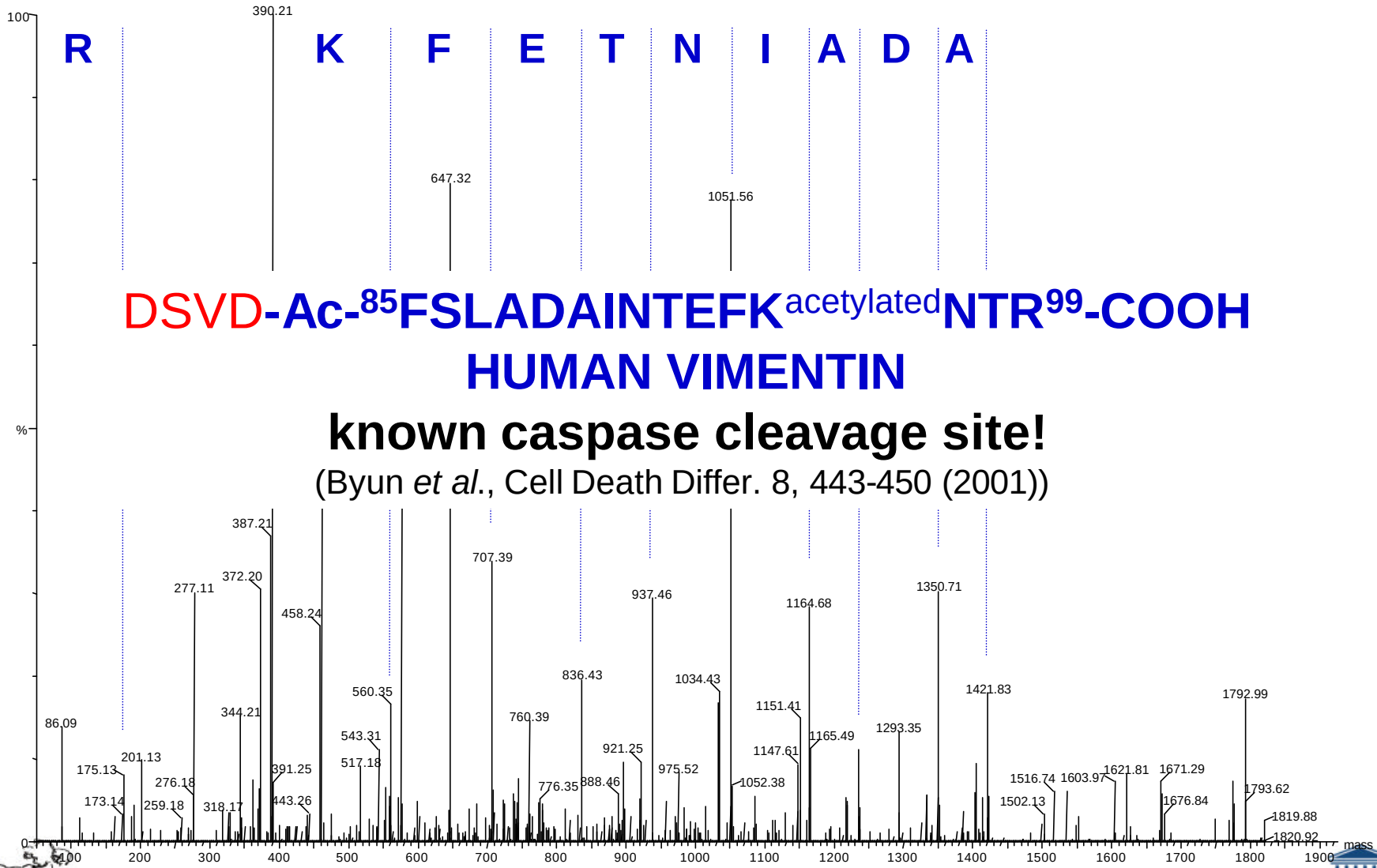
Van Damme P. *et al*. (2005) *Nat. Methods* **2**, 771-777.

(Q-TOF) MS-scan of a part of one secondary fraction



Van Damme P. *et al.* (2005) *Nat. Methods* **2**, 771-777.

(Q-TOF) MS/MS-spectrum of unlabeled 905.97 Th peptide



DSVD-Ac-^{85}FSLADAINTEFK$^{\text{acetylated}}$NTR^{99}-COOH

HUMAN VIMENTIN

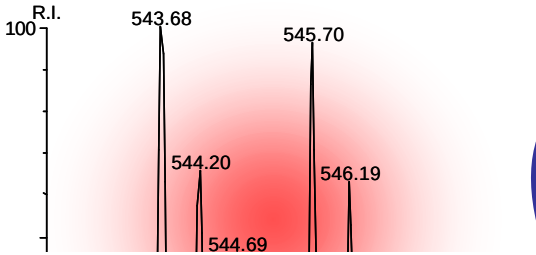
known caspase cleavage site!

(Byun *et al.*, Cell Death Differ. 8, 443-450 (2001))

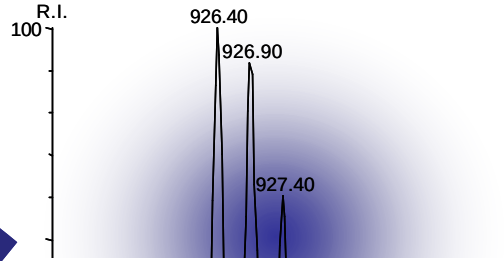
Van Damme P. *et al.* (2005) *Nat. Methods* **2**, 771-777.

Processing of SR-rich splicing factor U2AF 65kDa subunit at Asp-128

^{2}SDFDEFER^{9}

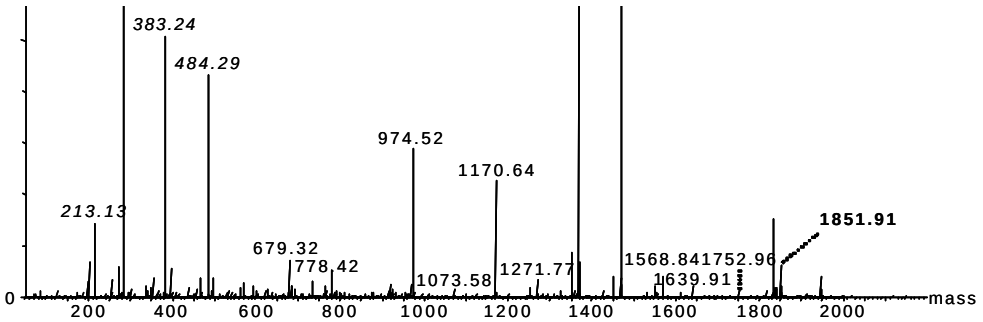


MTPD^{129}GLAVTPTPVPVVGSQMTR^{146}



MS/MS

MSDFDEFERQLNENKQERD[illegible]ENRHRKRSHSRSRSRDRKRRSRSRDRRNRDQRSASRDR
RRRSKPLTRGAKEEHGGLIR[illegible]PRHEKKKKVRKYWDVPPPGFEHITPMQYKAMQAAGQI
PATALLPT***MTPD?***GLAVTPTP[illegible]PVVGSQMTRQARRLYVGNIPFGITEEAMMDFFNAQM
RLGGLTQAPGNPVLAVQINQDKN[illegible]LEFRSVDETTQAMAFDGIIFQGQSLKIRRPHD
YQPLPGMSENPSVYVPGVVSTVVP[illegible]HKLFIGGLPNYLNDDQVKELLTSFGPLKAFN
LVKDSATGLSKGYAFCEYVDINVTDQAIAGLNGMQLGDKKLLVQRASVGAKNATLVSP
PSTINQTPVTLQVPGLMSSQVQMGGHPTEVLCLMNMVLPEELLDDEEYEEIVEDVRDE
CSKYGLVKSIEIPRPVDGVEVPGCGKIFVEFTSVFDCQKAMQGLTGRKFANRVVVTKY
CDPDSYHRRDFW

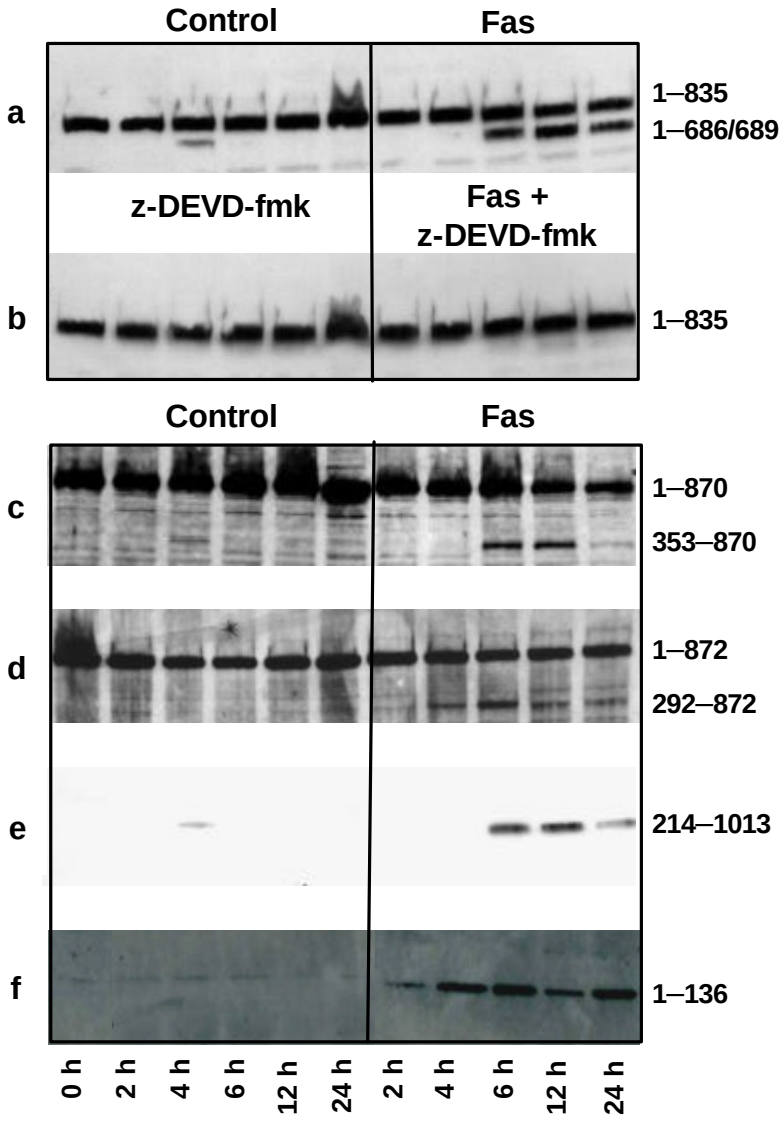


Caspase specific and non-specific *in vivo* protein processing during Fas-induced apoptosis.

Results:

- **1,834 proteins identified**
- **direct localization of 93 processing events in 71 proteins (86 NOVEL SITES)**
- **indirect evidence of processing in 21 other proteins**
- **thus: 92 protein processing events in Jurkat cells**
- **evidence for 58 caspase-specific processing events**
- **evidence for 35 processing events by other proteases (OMI?)**

Data validation: 1) by Western blotting



TIF1b

Dynamin 2

Subunit 2 of splicing factor 3B

PARP-1 (control)

Histone H3

Van Damme P. *et al.* (2005) *Nat. Methods* **2**, 771-777.

Data validation: 2) by peptide mimetics

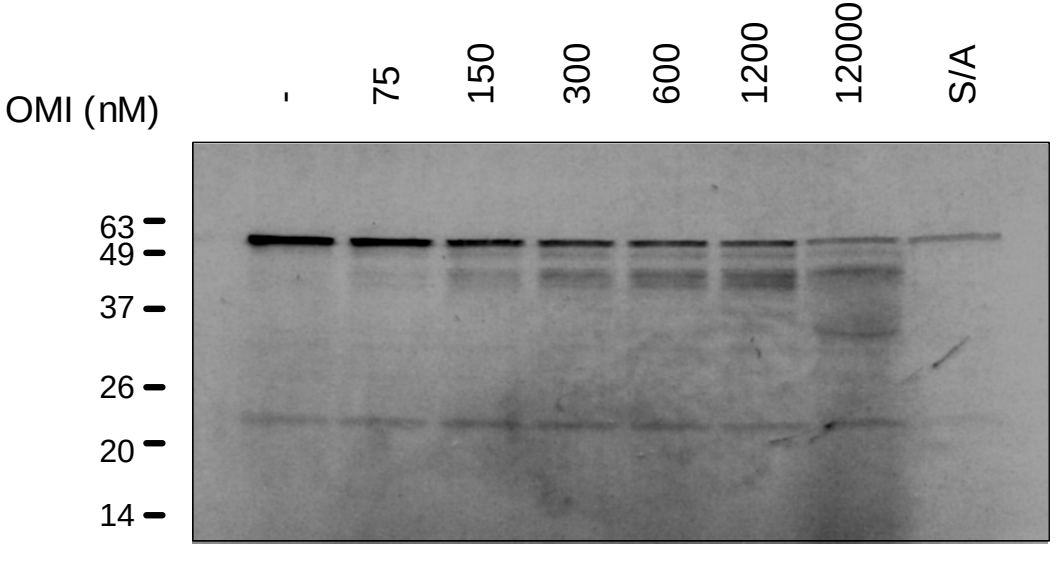
Hints to caspases that cleave protein in vivo!

	Protein Description	Site	Target	Caspase 3	Caspase 6	Caspase 7	Caspase 8
1	Apoptotic chromatin condensation inducer in the nucleus	VTLD?	Asp-68	50%	40%	20%	5%
2	*B-cell receptor-associated protein 31* ***(known)***	*AAVD?*	*Asp-163*	*45%*	*0%*	*33%*	*0%*
3	DnaJ homolog subfamily C member 7	AECD↓	Asp-8	0%	0%	0%	40%
4	GCIP-interacting protein p29 isoform 1	VLVD↓	Asp-11	40%	40%	0%	55%
5	Heterogeneous nuclear ribonucleoprotein A0	HAVD?	Asp-73	60%	0%	20%	0%
6	Lysyl-tRNA synthetase	VKVD?	Asp-12	65%	25%	0%	45%
7	*Proteasome activator complex subunit 3* ***(known)***	*DGLD?*	*Asp-80*	*100%*	*0%*	*33%*	*0%*
8	Ras GTPase-activating-like protein IQGAP1	DEVD↓	Asp-8	100%	5%	100%	40%
9	RNA-binding protein FUS	DWFD?	Asp-355	100%	0%	40%	0%
10	Splicing factor U2AF 65 kDa subunit	MTPD?	Asp-128	50%	0%	0%	0%
11	TAR DNA-binding protein-43	DETD?	Asp-89	100%	0%	65%	0%
12	Transcription intermediary factor 1-beta	DGAD?	Asp-688	20%	0%	0%	20%

Data validation: 3) by TnT assays & recombinant proteases

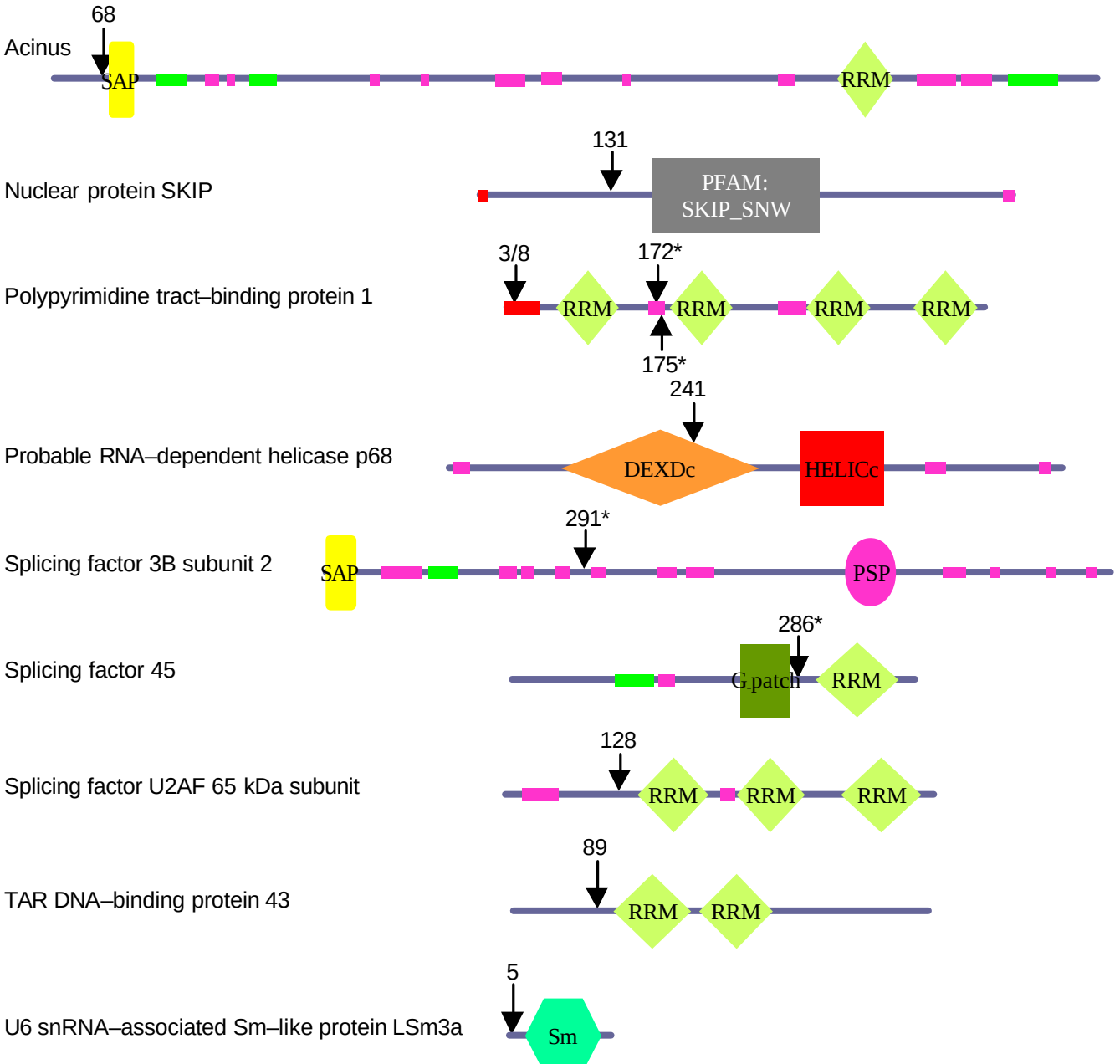
^{35}S Vimentin is cleaved by recombinant OMI

MSTRSVSSSSYRRMFGGPGTASRPSSSRSYVTTSTR***TYSL***?**GSALRPSTSR**SLYASSPGG
VYATRSSAVRLRSSVPGVRLLQDSVDFSLADAINTEFKNTRTNEKVELQELNDRFANYIDKV
RFLEQQNKILLAELEQLKGQGKSRLGDLYEEEMRELRRQVDQLTNDKARVEVERDNLAEDIM
RLREKLQEEMLQREEAENTLQSFRQDVDNASLARLDLERKVESLQEEIAFLKKLHEEEIQEL
QAQIQEQHVQIDVDVSKPDLTAALRDVRQQYESVAAKNLQEAEEWYKSKFADLSEAANRNND
ALRQAKQESTEYRRQVQSLTCEVDALKGTNESLERQMREMEENFAVEAANYQDTIGRLQDEI
QNMKEEMARHLREYQDLLNVKMALDIEIATYRKLLEGEESRISLPLPNFSSLNLRETNLDSL
PLVDTHSKRTLLIKTVETRDGQVINETSQHHDDLE

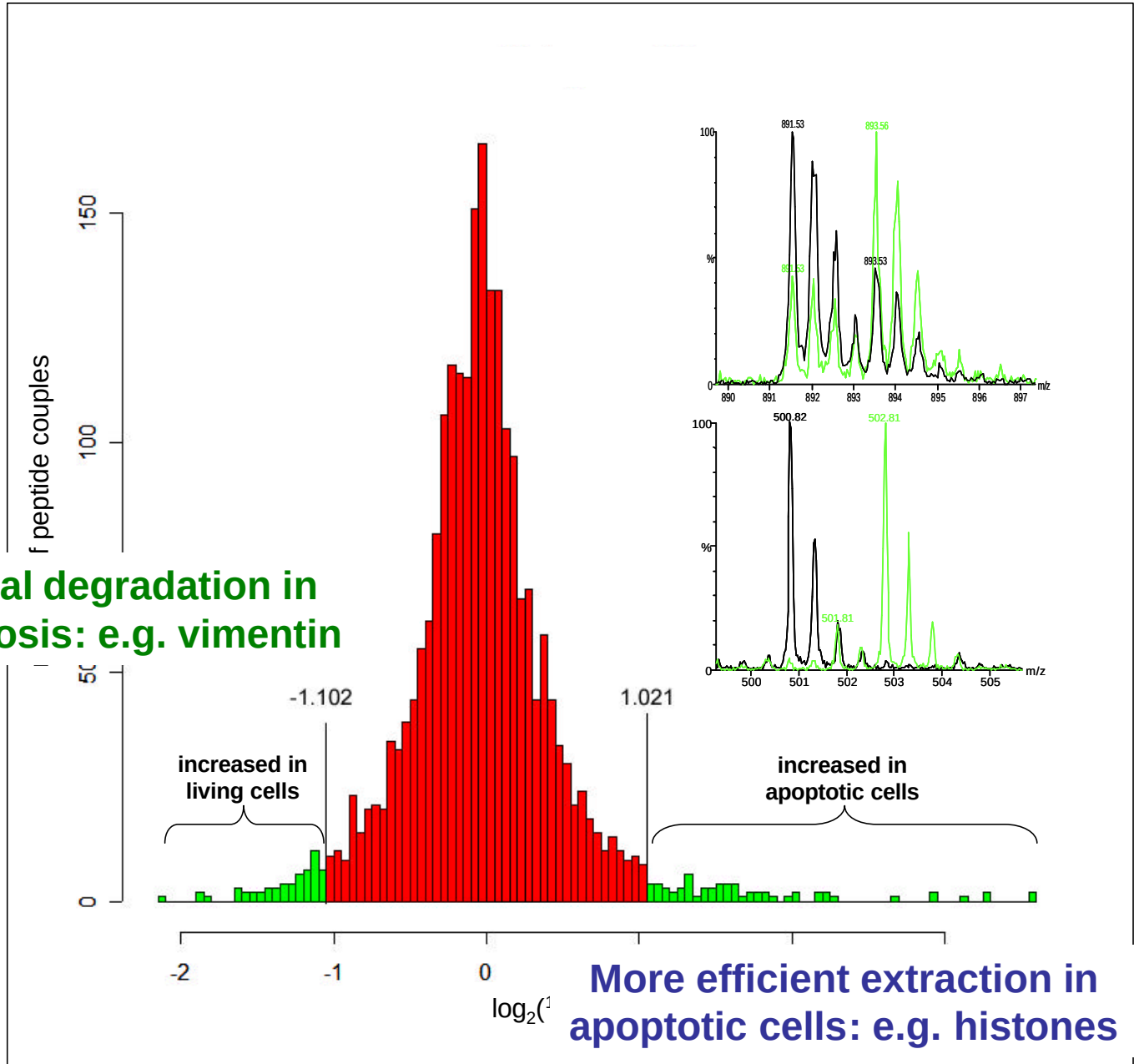


Vande Walle L. & Van Damme P. *et al*., in preparation.

Spliceosomal proteins are heavily processed (core proteins)



Enrichment of nucleosomal proteins in apoptotic cells



Partial degradation in apoptosis: e.g. vimentin

More efficient extraction in apoptotic cells: e.g. histones

Example 2: Granule-mediated cytotoxicity – substrates of granzyme B

Granule-mediated cytotoxicity

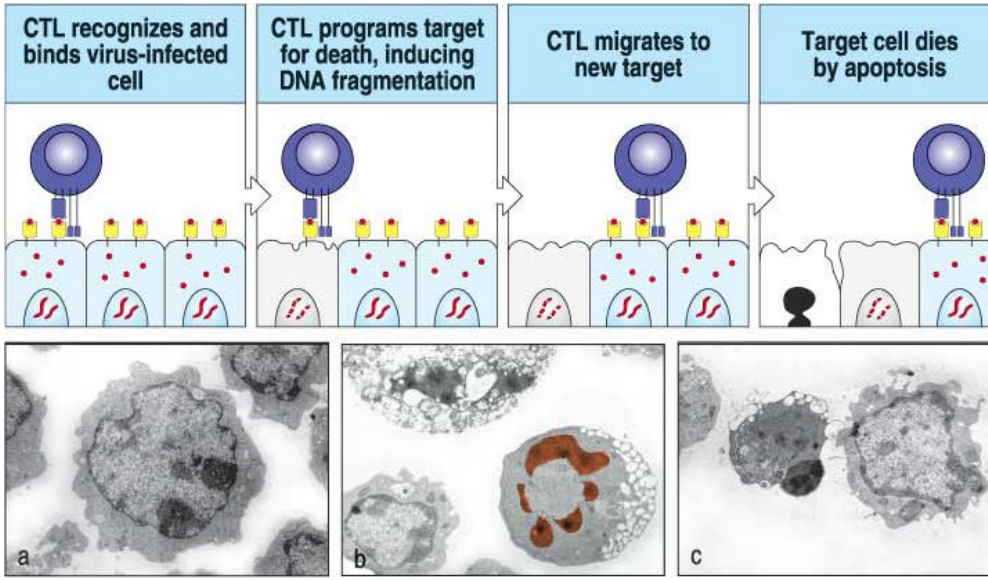
1. **Survival multicellular organism ?** **Protection by immune system**

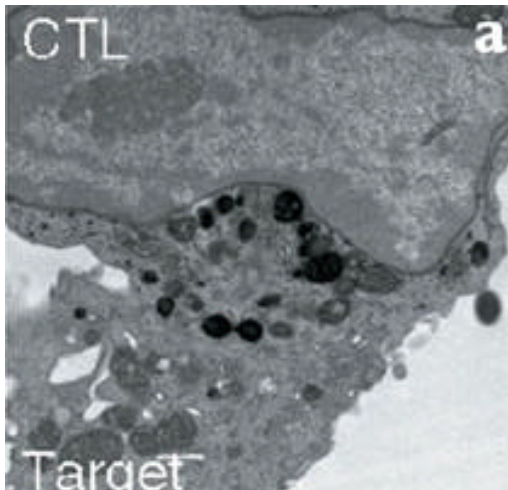
   Key cytotoxic effector cells:
   - **CTL**
   - **NK**

2. **Effector mechanisms: dual**

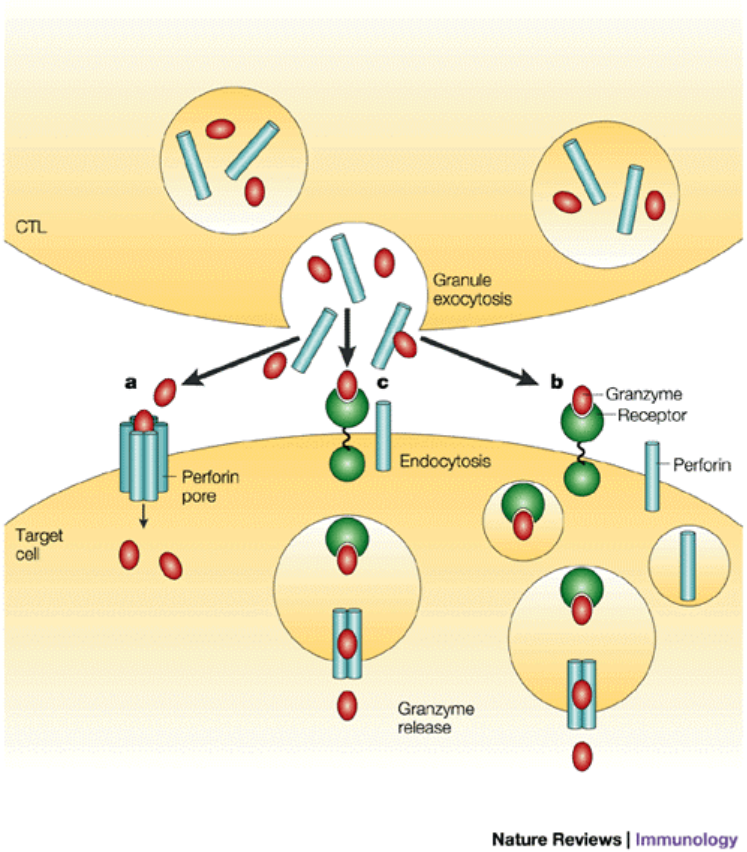
   - **Cytolytic molecules:**
     - **perforin/granzymes**
   - **Fas/FasL**
     - **apoptosis**

3. **Granule exocytosis model**





Perforin/Granzymes



Perforin:

- Pore-model
- Receptor-mediated endocytosis

Perforin-deficiency:

- Granular killing impaired

Protease	Specificity	Species
Granzyme A	Arg/Lys	Mouse, rat, human
Granzyme B	Asp	Mouse, rat, human
Granzyme C	Asn/Ser	Mouse, rat
Granzyme D	Phe/Leu	Mouse
Granzyme E	Phe/Leu	Mouse
Granzyme F	Phe/Leu	Mouse, rat
Granzyme G	Phe/Leu	Mouse
Granzyme H	Phe	Human
Granzyme J	?	Rat
Granzyme K	Arg/Lys	Mouse, rat, human
Granzyme M	Met/Leu	Mouse, rat, human

11 Granzymes:

- A, B, H & K
- A & B best characterized
- Different specificities

VIB

UNIVERSITEIT GENT

Granzyme B

Granzyme B specificity:

Serine-protease

Molecular modelling/combinatorial peptide libraries: cleavage after D (unique!)

- cfr. initiator caspase
- Cleft: 8 AA

[I/V - G/M/E- Xaa – D – Xaa - Gly] (Xaa: any amino acid)

X-ray crystal structure of granzyme B: predictions OK

Caspase-mediated apoptosis

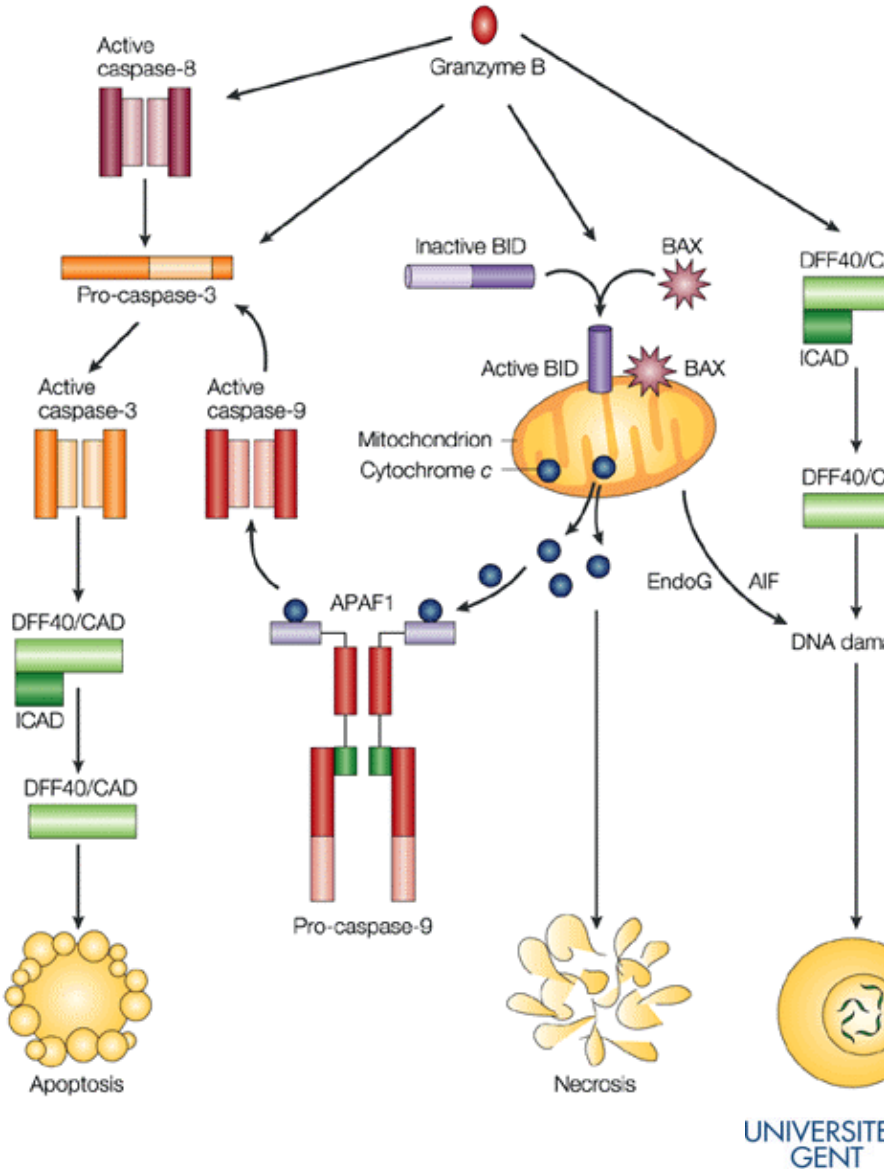
Caspase-independent cell-death

Substrates: ~ Caspase substrates

BID

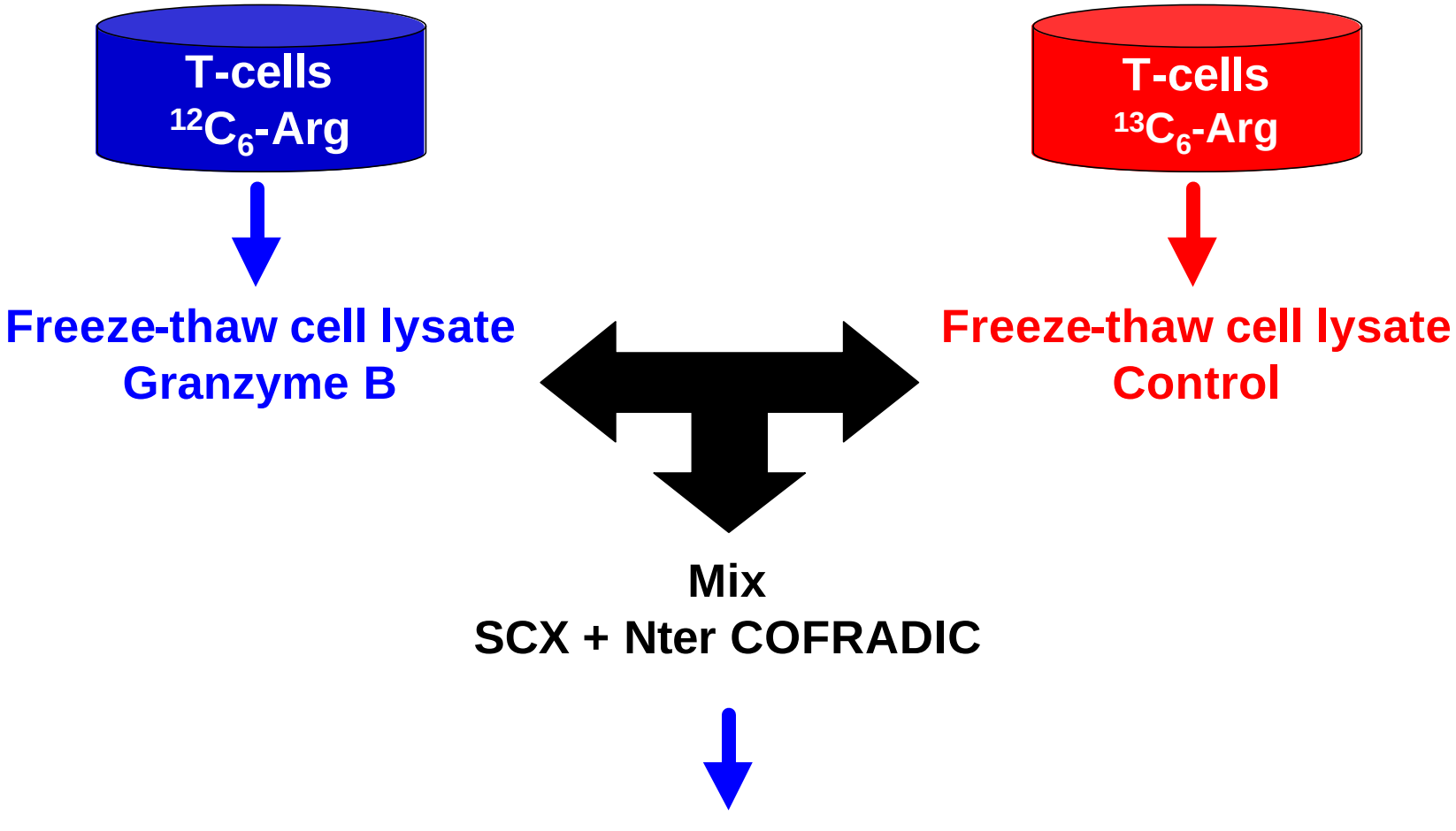
ICAD, PARP, Lamin B (Casp-6)

Cytoskeleton, ... ?



The quest for substrates of granzyme B

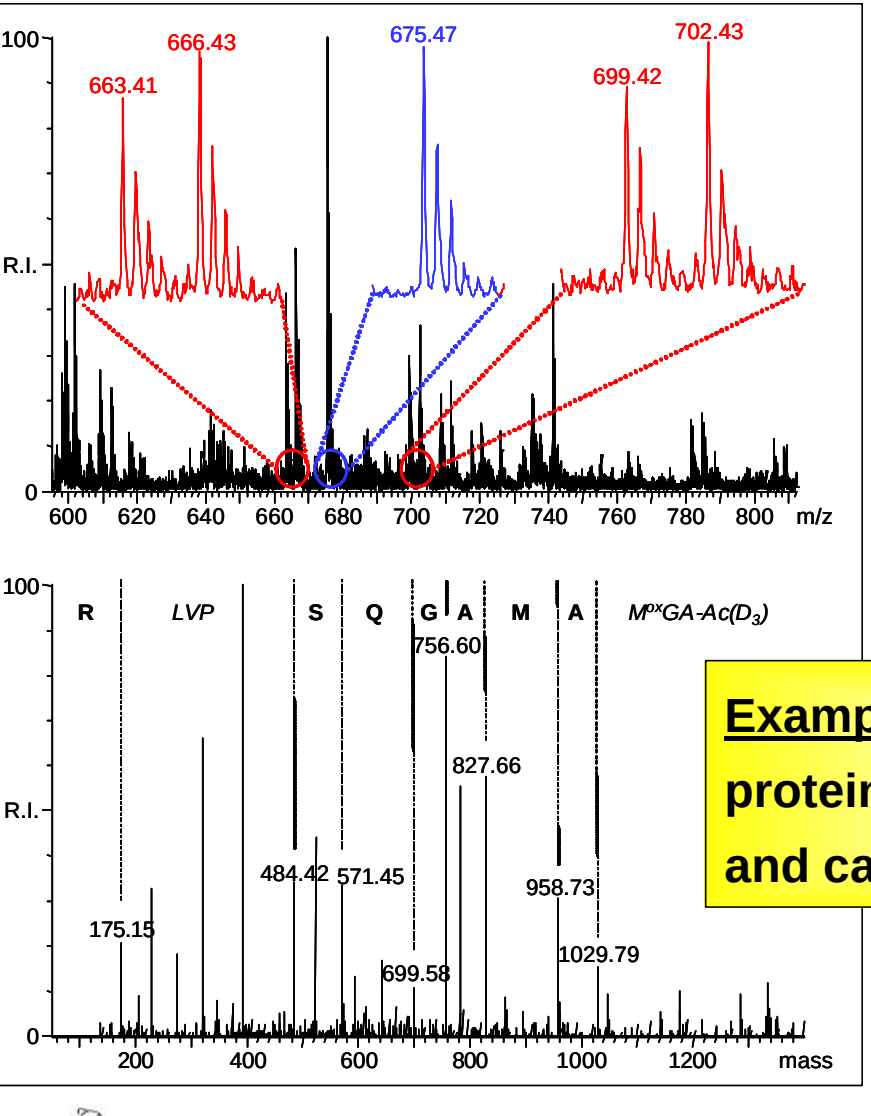
granule-mediated cytotoxicity



Granzyme B substrates => novel trideuteroacetylated peptides present in light form …

Van Damme P. *et al.*, unpublished results.

Why $^{12}C/^{13}C$-arginine?



a) All sorted peptides end on Arg.

b) Isotope label is very stable.

c) Increased "spacing" (6 Da)

<u>Example:</u> polypyrimidine tract-binding protein 1 (PTB) is processed by granzyme B and caspase-8 at Asp-172.

Gevaert K. *et al*. *BBA*, in press; Van Damme P. *et al*. (2005) *Nat. Methods* **2**, 771-777 & Van Damme P. *et al*., unpublished results.

Substrates of granzyme B (part 1)

➢ known substrates (and (some) known cleavage sites):

BID - BH3-interacting domain death agonist	IEAD	P55957	76	84	AcD3-SESQEDIIR
Chromodomain helicase-DNA-binding protein 4	VESD	Q14839	321	338	AcD3-FDDASINSYSVSDGSTSR
Heterogeneous nuclear ribonucleoprotein K	PAED	P61978	27	35	AcD3-MEEEQAFKR
Isoleucyl-tRNA synthetase	VTPD	P41252	983	992	AcD3-QSMVDEGMAR
Tubulin alpha-3 chain	VFVD	Q71U36	70	79	AcD3-LEPTVIDEVR

➢ unknown substrates and cleavage sites: more than 300 granzyme B cleavage sites (over 200 different proteins)

Proteins involved in apoptosis:

Bcl-2-associated transcription factor 1	VLAD	Q9NYF8	419	429	AcD3-QGKSFATASHR
BID	IEAD	P55957	76	84	AcD3-SESQEDIIR
Double-stranded RNA-specific adenosine deaminase	ESLD	P55265	704	714	AcD3-NLESMMPNKVR
MAP-kinase activating death domain-containing protein isoform d	MGMD	Q8WXG6	1343	1351	AcD3-QGPQEMIDR
Protein kinase C alpha type	VIQD	P17252	378	388	AcD3-DDVECTMVEKR
SON protein (Bax antagonist selected in saccharomyces 1)	LESD	P18583	154	172	AcD3-SFLKFDSEPSAVALELPTR
Zinc-finger protein ubi-d4 (Apoptosis response zinc fingerprotein)	ISQD	Q92785	116	124	AcD3-GSSLEALLR

Van Damme P. *et al.*, unpublished results.

Substrates of granzyme B (part 2)

Autoantigens:

60S ribosomal protein L11 (CLL-associated antigen KW-12)	xAQD	P62913	4	12	AcD3-QGEKENPMR
ATP-dependent DNA helicase 2 subunit 2 (Lupus Ku autoantigen protein p86)	PSGD	P13010	714	731	AcD3-TAAVFEEGGDVDDLLDMI
autoantigen RCD8	CSGD	Q6P2E9	58	67	AcD3-STSANKTGLR
Chromodomain helicase-DNA-binding protein 4 (Mi-2 autoantigen 218 kDa protein)	VESD	Q14839	321	338	AcD3-FDDASINSYSVSDGSTSR
DNA-directed RNA polymerase III 62 kDa polypeptide (systemic sclerosis SSc autoantigen)	RSSD	Q9BUI4	207	217	AcD3-EDAAGEPKAKR
Protein C9orf78 (Hepatocellular carcinoma-associated antigen 59)	PEVD	Q9NZ63	178	196	AcD3-LGIDAKIKNIISTEDAKAR
Serologically defined colon cancer antigen 10	AEHD	O60529	271	282	AcD3-EYIDGDEKNLMR
Striatin-3 (Cell-cycle autoantigen SG2NA)	KEFD	Q13033	307	321	AcD3-FLVTAEDGEGAGEAR

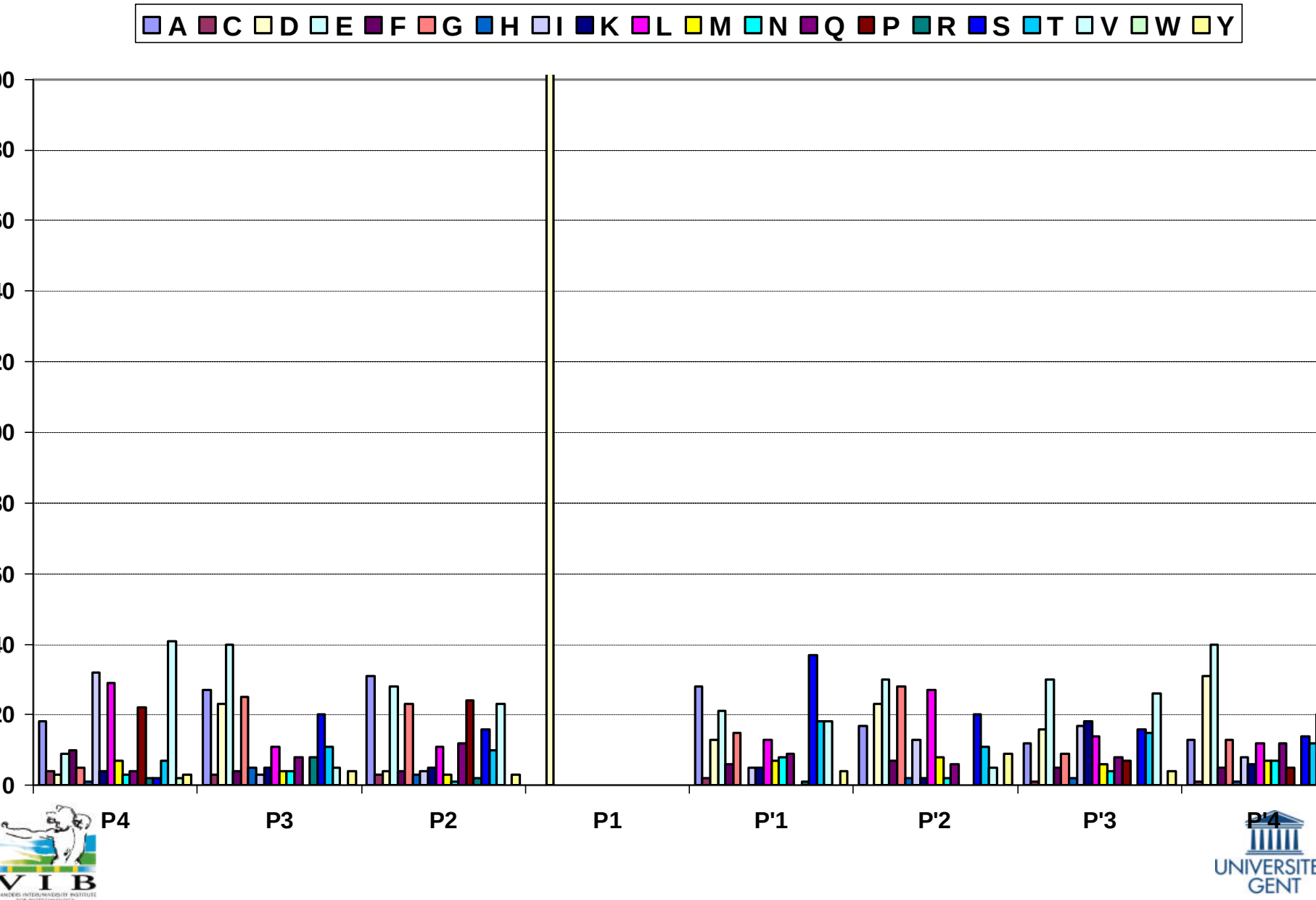
Proteins activated/inactivated during viral infection:

ATP-dependent RNA helicase DDX3X	LGLD	O00571	13	36	AcD3-QQFAGLDLNSSDNQSGGSTASKGR
Bcl-2-associated transcription factor 1	VLAD	Q9NYF8	419	429	AcD3-QGKSFATASHR
Double-stranded RNA-specific adenosine deaminase	ESLD	P55265	704	714	AcD3-NLESMMPNKVR
Eukaryotic translation initiation factor 4 gamma 1	CGPD	Q04637	666	675	AcD3-FTPSFANLGR
Interferon-induced, double-stranded RNA-activated protein kinase	INSN	P19525	219	233	AcD3-SDSLNSSSLLMNGLR

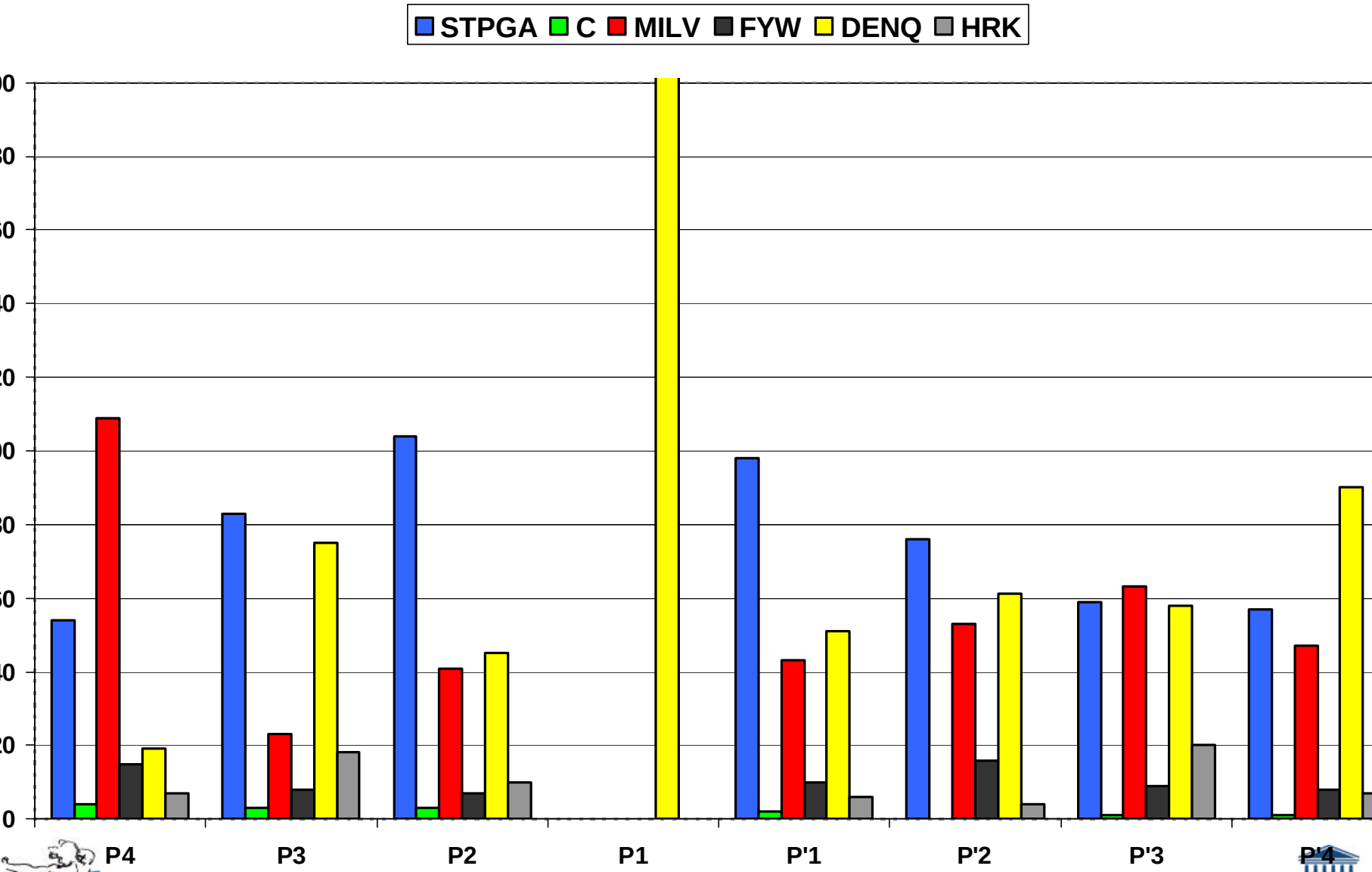
Other: ATP-binding proteins, cell proliferation, protein biosynthesis ...

Van Damme P. *et al.*, unpublished results.

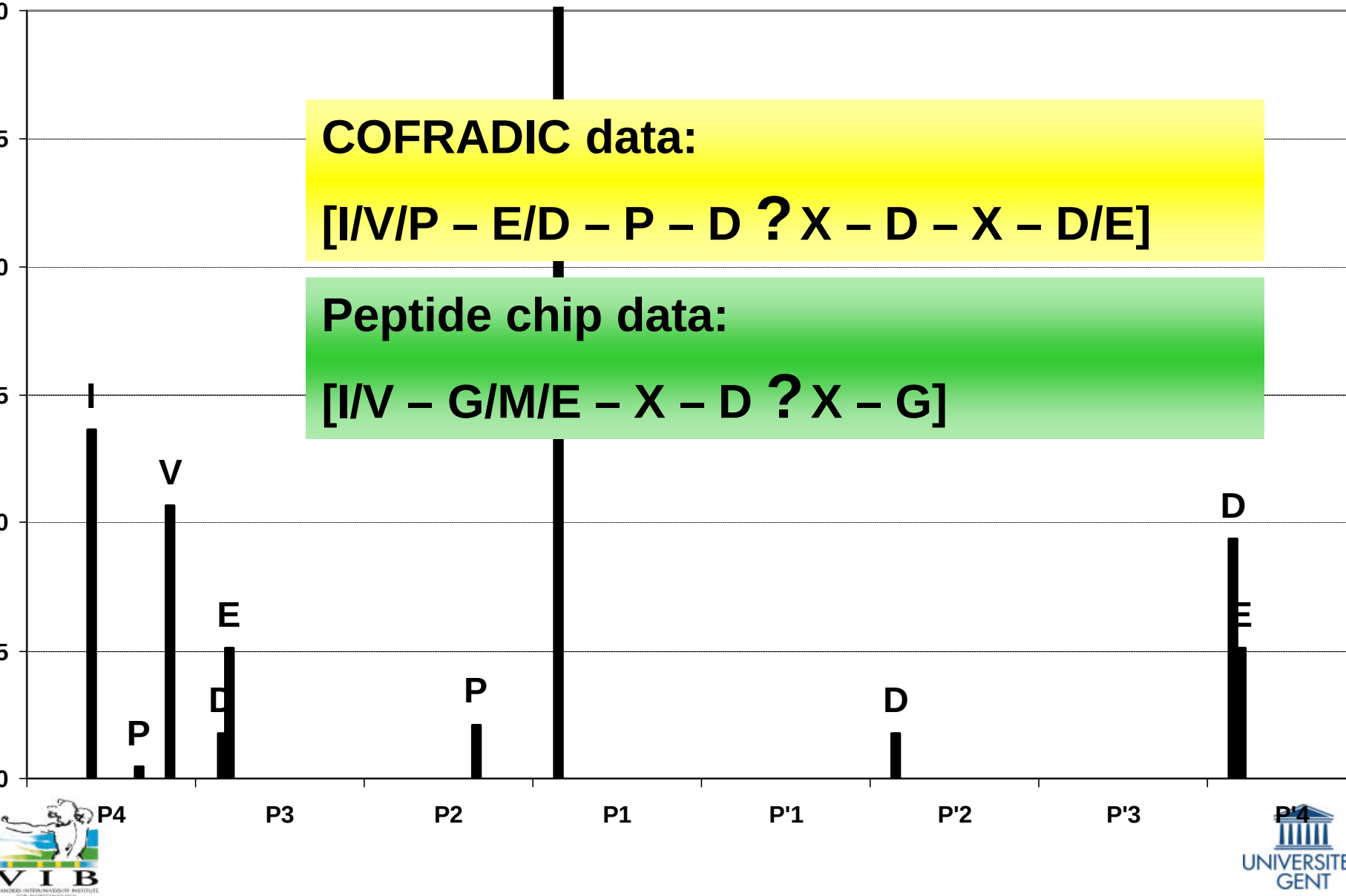
What about the recognition site? Absolute values (very complex)



What about the recognition site? Grouped amino acids (complex)



What about the recognition site? Correction for natural frequency …



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