

Characterization of the enzymatic activity of *Clostridium perfringens* TpeL

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Abstract: TpeL is a toxin produced by Clostridium perfringens which belongs to the large clostridial glucosylating toxin family. It was shown that TpeL modifies Ras using UDP-glucose or UDP-N-acetylglucosamine as cosubstrates (Guttenberg et al., 2012; Nagahama et al., 2011). We confirmed that TpeL preferentially glucosaminates the three isoforms of Ras (cH-Ras, N-Ras, and K-Ras) from UDP-N-acetylglucosamine and to a lower extent Rap1a and R-Ras3. In contrast to previous reports, we observed that Rac and Ral are no substrates of TpeL. In addition, we confirmed by in vitro glucosylation and mass spectrometry that TpeL modifies cH-Ras at Thr35.

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Highlights

- TpeL is a cytotoxin produced by *Clostridium perfringens* which belongs to the large clostridial glucosylating toxin family
- TpeL uses both UDP-glucose and UDP-*N*-acetylglucosamine as cosubstrates but preferentially this latter.
- TpeL modifies mainly cH-Ras, N-Ras, K-Ras and to a lower extent Rap1a, R-Ras3, and Rac1
- TpeL modifies cH-Ras at Thr35

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CHARACTERIZATION OF THE ENZYMATIC ACTIVITY OF *Clostridium perfringens* TpeL

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Abstract

TpeL is a toxin produced by *Clostridium perfringens* which belongs to the large clostridial glucosylating toxin family. It was shown that TpeL modifies Ras using UDP-glucose or UDP-*N*-acetylglucosamine as cosubstrates (Guttenberg *et al.*, 2012; Nagahama *et al.*, 2011). We confirmed that TpeL preferentially glucosaminates the three isoforms of Ras (cH-Ras, N-Ras, and K-Ras) from UDP-*N*-acetylglucosamine and to a lower extent Rap1a and R-Ras3, and very weakly Rac1. In contrast to previous report, we observed that Ral was not substrate of TpeL. In addition, we confirmed by *in vitro* glucosylation and mass spectrometry that TpeL modifies cH-Ras at Thr35.

1. Introduction

Clostridium perfringens is an ubiquitous anaerobic bacterium which is responsible for mild to severe diseases in humans (gas gangrene, gastrointestinal diseases) and animals (mainly gastrointestinal and enterotoxemic diseases). The pathogenicity of this microorganism is based on protein toxins, which are secreted in the culture medium, gastrointestinal tract or tissues (Popoff and Bouvet, 2009). *C. perfringens* is among the bacteria which produce the greatest number of toxins. According to the toxins, which are synthesized, *C. perfringens* strains are divided into several toxinotypes (Petit *et al.*, 1999). Recently, a new toxin, called TpeL (for toxin *C. perfringens* large cytotoxin), was identified in several strains of *C. perfringens* type B and C. TpeL was found to be lethal for mice and cytotoxic for Vero cells and shows a significant homology of its N-terminal region with the catalytic domain of the large glucosylating clostridial toxins which encompass *Clostridium difficile* toxin A (TcdA) and toxin B (TcdB), *Clostridium sordellii* lethal toxin (TcsL) and hemorrhagic toxin (TcsH), and *Clostridium novyi* alpha toxin (TcnA) (Amimoto *et al.*, 2007).

Large glucosylating clostridial toxins (LGCTs) contain at least three functional domains. The one third C-terminal part exhibits multiple repeated sequences (31 short repeats and 7 long repeats in TcdA), which are involved in the recognition of a cell surface receptor. The central part contains a hydrophobic segment and probably mediates the translocation of the toxin across the membrane. The enzymatic site characterized by the DxD motif surrounded by a hydrophobic region, and the substrate recognition domain are localized within the 543 N-terminal residues corresponding to the natural cleavage site in TcdB (Hofmann *et al.*, 1998; Hofmann *et al.*, 1997; Rupnik *et al.*, 2005). The overall structure of the enzymatic domains of TcdB, TcsL and TcnA is conserved and consists of a β -strain

1 central core (about 235 amino acids) forming an active center pocket surrounded by numerous
2 α -helices (Jank and Aktories, 2008; Reinert *et al.*, 2005; Ziegler *et al.*, 2008). LGCTs catalyze
3 the glucosylation of Rho- and/or Ras-GTPases from UDP-glucose, except TcnA, which uses
4 UDP-*N*-acetylglucosamine as cosubstrate. The glucosylating activity supports the main
5 biological activities of these toxins. But, LGCTs differ in their substrates. Thereby, TcdA and
6 TcdB glucosylate Rho, Rac and Cdc42 at Thr-37, whereas LcsT glucosylates Ras at Thr-35,
7 Rap, Ral and Rac at Thr-37 (Just and Gerhard, 2004; Popoff and Bouvet, 2009). The
8 structural basis of the different recognition of substrates is not well known. Differences in α -
9 helices, insertions-deletions, probably account for the substrate specificity of each toxin
10 (Reinert *et al.*, 2005; Ziegler *et al.*, 2008). Chimeric molecules between TcdB and TcsL have
11 been used to identify the sites of Rho-GTPase recognition. Amino acids 408 to 468 of TcdB
12 ensure the specificity for Rho, Rac and Cdc42, whereas in TcsL, the recognition of Rac and
13 Cdc42 is mediated by residues 364 to 408, and that of Ras proteins by residues 408 to 516
14 (Hofmann *et al.*, 1998; Jank *et al.*, 2007). Since TpeL seems to be a natural variant of large
15 glucosylating clostridial toxins, the characterization of its enzymatic activity and substrate
16 specificity would permit to better understand the structure/function of this group of toxins.
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29 In this study, we show that TpeL is a glucosylating toxin which targets Ras proteins
30 and to a lower extent Rap and R-Ras3, and preferentially uses UDP-*N*-acetylglucosamine as
31 cosubstrate. Similarly to *C. sordellii* TcsL, TpeL monoglucosylates Ras at Thr35. During the
32 progress of this work, related results have been published (Guttenberg *et al.*, 2012).
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38 **2. Materials and methods**

39 *2.1 Reagents*

40 Uridine-5-diphospho(UDP)-glucose, UDP-*N*-acetylglucosamine, UDP-galactose,
41 UDP-glucuronic acid, UDP-galacturonic acid, UDP-mannose, ADP-glucose, ADP-ribose,
42 CDP-glucose, GDP-glucose, TDP-glucose were from Sigma. Plasmids encoding Rho-, Rac-,
43 Cdc42-GST proteins were from A. Hall.
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51 *2.2 Production and purification of recombinant TpeL N-terminus (rN-TpeL)*

52 The DNA coding for the N-terminal part of TpeL was PCR amplified from *C.*
53 *perfringens* type B strain NCTC3181 with primers adding a *Bam*HI site at the 5' end and a
54 *Sal*I site at the 3' end. The PCR product was cloned into pCR2.1 vector (Invitrogen), and the
55 digested insert with *Bam*HI-*Sal*I was subcloned into pET28a (Novagen) at the corresponding
56 sites. The recombinant protein was fused to an N-terminal extension containing a 6-histidine
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motif from pET28.

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2 *E. coli* BL21 CodonPlus (DE3)-RIL (Stratagene) transformed with recombinant
3 plasmids respectively were grown in LB medium containing kanamycine (50 µg/ml) at 26° C
4 until a OD of 0.8. The expression was induced with IPTG (1 mM) and growth was continued
5 overnight at 26° C. The bacteria were harvested by centrifugation, suspended in lysis buffer
6 (50 mM Hepes, pH 7.5, 150 mM NaCl, and protease inhibitors (Calbiochem), and sonicated.
7 The cell debris were separated from the soluble fraction by centrifugation (18000g, 15 min).
8 The soluble fraction was applied on a cobalt column (Talon, Qiagen). The column was
9 washed with lysis buffer, and eluted with 2, 10 and 100 mM imidazole in the same buffer.
10 The fractions containing highly purified recombinant proteins were pooled and dialyzed
11 against 20 mM Hepes, pH 7.5, 150 mM NaCl.
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22 2.3 In vitro glucosylation

23 Glucosylation of small GTPases was achieved as followed according to (Popoff *et al.*,
24 1996). 2 µl of UDP-[¹⁴C]Glucose or UDP-[¹⁴C]-*N*-acetylglucosamine in ethanol (0.2 µCi,
25 300 mCi/mmol; DuPont NEN, les Ulis France) was dried under vacuum; 1 µg of GST-
26 GTPase protein in a final volume of 20 µl of glucosylation buffer (50 mM triethanolamine pH
27 7.5, 2 mM MgCl₂, 0.3 mM GDP) were added to the dried UDP-[¹⁴C]Glucose. One µl of rN-
28 TpeL (2 mg/ml) was added to start the reaction, which was carried out for 1 h at 37°C. The
29 reaction was stopped by adding 5 µl of 2 x SDS sample buffer, boiled and electrophoresed on
30 a 15% SDS-polyacrylamide gel. After staining with Coomassie blue, followed by distaining,
31 the gel was dried and autoradiographed.
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42 2.4 Peptide analysis by HPLC, sequencing and mass spectrometry

43 cH-Ras (5 µg) was incubated without or with rN-TpeL (10⁻⁷ M) in glucosylation
44 buffer containing UDP-*N*-acetylglucosamine (5 mM) for 3 h at 37°C. Ras was the subjected
45 to SDS-(14%)PAGE and stained with Amido Black. Ras bands were cut from the gel, washed
46 with distilled water, dried, and resuspended in 150 µl of 50 mM Tris-HCl (pH 8.6) containing
47 0.001% Tween 20 and 0.2 µg of trypsin (Promega). Following 18 h at 30°C, the preparations
48 were centrifuged, the pellets were washed with 100 µl of 50 mM Tris-HCl (pH 8.6)
49 containing 0.05% SDS and then with 100 µl 60% acetonitrile. The supernatants were
50 recovered and concentrated to 100 µl by Speed Vac. Samples were injected onto a DEAE-
51 HPLC column (Interchim Hema Bio 1000 DEAE, 33 x 1 mm) linked to a C18 reverse phase
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1 HPLC column (Interchim Uptisphere ODBD, 150 x 1 mm) eluted with 2-70% acetonitrile
2 gradient in 0.1% trifluoroacetic acid and monitored at 214 nm.

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4 Separated peptides (1-2 μ l on a ProteinChip gold array) were identified by their mass
5 using SELDI-TOF-MS (PCS4000) from Bio-Rad (focus mass = 4000 daltons; laser intensity
6 = 1100 nl). The matrix was α -cyano-4-hydroxycinnamic acid saturated in 50% acetonitrile,
7 0.5% trifluoroacetic acid.
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10 11 12 2.5 MS and MS/MS analysis

13 Purified HPLC fractions were diluted in 10 μ l of CHCA matrix (5 mg/ml in 70% ACN
14 / 30 % H₂O / 0.1 % TFA) and 0.5 μ l of them were spotted on the MALDI plate.
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17 MS spectra were acquired in reflector positive mode using the 4800 MALDI
18 TOF/TOF Analyzer (Applied Biosystems, USA) within a mass range of 800 to 4000 m/z. MS
19 calibration was first carried out to a final mass accuracy of 1 ppm with a mixture of 5 standard
20 peptides: des-Arg1 Bradykinin, $[M+H]^+=904.468$; Angiotensin, $[M+H]^+=1296.685$; Glu1-
21 Fibrinopeptide, $[M+H]^+=1570.677$; ACTH 1-17, $[M+H]^+=2093.087$; ACTH 18-39,
22 $[M+H]^+=2465.199$. MS spectra of the purified fractions were then acquired (3000 laser shots
23 were averaged) and calibrated using the external default calibration (10 ppm mass accuracy).
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26 MS/MS were performed in CID positive ion mode with a 2 kV collision energy of and
27 air as collision gas. MS/MS calibration was performed using 4 CID fragments of the Glu1-
28 Fibrinopeptide ($[M+H]^+=175.119, 684.346, 1056.475$ and 1441.634) (mass accuracy of 10
29 ppm). 4000 to 10 000 laser shots were averaged to acquired MS/MS spectra of the selected
30 precursors. Each spectrum was then calibrated using the external default calibration (mass
31 accuracy of 100 ppm).
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34 MS/MS queries were performed using the MASCOT search engine 2.1 (Matrix
35 Science Ltd., UK) and the SwissProt 57.4 database (470369 sequences; 166709888 residues)
36 with the following parameters: 50 ppm mass tolerance for the precursor and 0.3 Da for
37 MS/MS fragments, trypsin cleavage. Hexose-*N*-acetylglucosamine (HexNAc) and oxidation
38 of methionins were used as variable modifications. In these conditions, the minimal
39 MASCOT score was 28 for a peptide confidence index $\geq 95\%$.
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43 3. Results

44 3.1 *TpeL* is a glucosyltransferase which targets Ras proteins

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1 As previously found (Amimoto et al., 2007), TpeL shows a significant overall
2 sequence homology with the LCGTs and contains a conserved active enzymatic site (DxD) in
3 the N-terminal part, except that the total protein length is shorter (190 kDa) than that of the
4 glucosylating toxins from *C. difficile*, *C. sordellii* or *C. novyi* (250 to 300 kDa) (Jank and
5 Aktories, 2008; Just and Gerhard, 2004; Popoff and Bouvet, 2009; Rupnik and Just, 2006).
6 The N-terminal domain (amino acid 1 to 543) of LCGTs is the intracellular active domain,
7 which is released into the cytosol through an autoproteolytic process during the toxin
8 endocytosis (Jank and Aktories, 2008). Interestingly, the TpeL N-terminal domain shows a
9 high level of sequence similarity with that of the other LCGTs with an intermediate position
10 between the N-terminal domain of *C. novyi* alpha toxin and those of the *C. difficile* and *C.*
11 *sordellii* glucosylating toxins (Fig. 1). Therefore, we produced and purified the N-terminal
12 domain of TpeL as recombinant protein (Fig. 3) to check its enzymatic activity. In a first
13 series of experiments we used Rho, Rac, Cdc42 and cH-Ras, which are the representative
14 substrates of LGCTs, in a glucosylation assay with TpeL. As shown in Fig. 2B, TpeL N-
15 terminus glucosylated only cH-Ras and no RhoA, Rac1 or Cdc42. In addition, the
16 glucosylation of cH-Ras was more efficient by using UDP-*N*-acetylglucosamine than UDP-
17 glucose as cosubstrate. Thereby, recombinant TpeL N-terminal part contains a
18 glucosyltransferase activity specific of cH-Ras protein but not of Rho and Cdc42.
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3.2 *TpeL preferentially uses UDP-N-acetylglucosamine as cosubstrate*

34 In order to determine the cosubstrate requirement for TpeL (10^{-7} M) activity, kinetics
35 of cH-Ras glucosylation was performed in the presence of UDP- ^{14}C -glucose or UDP- ^{14}C -
36 *N*-acetylglucosamine (Fig. 3 A). An almost complete glucosylation of cH-Ras was obtained
37 with TpeL in the presence of UDP- ^{14}C -*N*-acetylglucosamine, whereas the reaction with
38 UDP- ^{14}C -glucose had longer kinetics (Fig. 3A and B). The level of cH-Ras glucosylation
39 was about 100 fold less with UDP-glucose compared to UDP-*N*-acetylglucosamine. Even
40 after longer incubation period (until 2 h), a 100-fold difference in enzymatic level was
41 observed between both cosubstrates (not shown).
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51 Then, we investigated if UDP-*N*-acetylglucosamine was the only efficient cosubstrate
52 of TpeL by a competition assay using cH-Ras, UDP- ^{14}C -*N*-acetylglucosamine, and various
53 non-radioactive hexose derivatives. As expected, non-radioactive UDP-*N*-acetylglucosamine
54 totally blocked the transfer of ^{14}C -*N*-acetylglucosamine from UDP- ^{14}C -*N*-
55 acetylglucosamine on cH-Ras, whereas *N*-acetylglucosamine was inefficient (Fig. 3C and D).
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1 UDP-glucose and UDP-mannose, a close isoform of UDP-glucose, were also as efficient as
2 UDP-*N*-acetylglucosamine to block the TpeL-dependent glucosylation of cH-Ras. The other
3 UDP derivatives (UDP-galactose, UDP-glucuronic acid, and UDP-galacturonic acid) also
4 inhibited the glucosylation of cH-Ras but to a lower extent. Among the other nucleoside-
5 diphosphate hexose derivatives, GDP-glucose partially prevented cH-Ras glucosylation. TDP-
6 glucose which contains a pyrimidine base as UDP, was not effective to compete the reaction.
7 This suggests that UDP is a major structure which is recognized by TpeL.
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13 3.3 *Ras is the main substrate of TpeL*

14 Next, we investigated the substrates of TpeL. Using a large set of recombinant small
15 GTPases and UDP-*N*-acetylglucosamine as cosubstrate, we checked the TpeL activity in the
16 *in vitro* glucosylation assay. As shown in Fig. 4A, Ras proteins including cH-Ras, K-Ras, and
17 N-Ras were the main substrates which were efficiently glucosylated by TpeL. In addition, R-
18 Ras3 and Rap1a, which also belong to the Ras family of proteins, were glucosylated by TpeL,
19 but to a lower extent. The glucosylation level of R-Ras3 was 16% from that of Ras, and that
20 of Rap1a 6% (Fig. 4B). No proteins from the Rho family nor Rab6 and Arf1, representative
21 proteins from the Rab and Arf families, were substrates of TpeL, except Rac1 which was very
22 weakly modified compared to cH-Ras (Fig. 4A). Thereby, TpeL contains a
23 glucosyltransferase activity targeting Ras proteins, and predominantly cH-Ras, K-Ras, and N-
24 Ras.
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38 3.4 *TpeL glucosylates cH-Ras at Thr35*

39 In an effort to identify the cH-Ras residue or residues that are modified by TpeL, cH-
40 Ras (5 µg) was incubated with TpeL (10^{-7} M), non-radioactive UDP-*N*-acetylglucosamine (10
41 mM) in glucosylation buffer during 3 h at 37°C and then analyzed by SELDI-TOF using a
42 Q10 chip (strong anionic exchanger). cH-Ras prepared in the same conditions but without
43 TpeL was used as reference. As shown in Fig. 5, cH-Ras treated with TpeL in the presence of
44 UDP-*N*-acetylglucosamine has an increase in mass of 205, which correlates with an addition
45 of a one HexNAc molecule (MW 203). This indicates that TpeL catalyzes the transfer of one
46 HexNAc molecule per cH-Ras molecule.
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54 Next, cH-Ras treated with rN-TpeL or untreated was run on a SDS-PAGE, trypsin
55 digested, and the resulting peptides were separated by HPLC. As shown in Fig. 6A, fraction
56 14 from rN-TpeL-treated cH-Ras has an increased retention time which corresponds to an
57 increased mass of 203 daltons compared to fraction 15 of untreated cH-Ras as determined by
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1 SELDI-TOF (Fig. 6B). N-terminal sequencing of fractions 14 and 15 showed that the peptides
2 with the sequence SALT and SALTIQ, respectively. This indicates that fraction 14 from rN-
3 TpeL treated cH-Ras and fraction 15 from untreated cH-Ras contained the same trypsin
4 cleavage peptide SALTIQNHVFVDEYDPTIEDSYR. These results show that the residue
5 modified by rN-TPEL and resulting from an addition of an HexNAc is part of the peptide
6 SALTIQNHVFVDEYDPTIEDSYR which contains two Thr residues.
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10 Fractions 14 and 15 were further analyzed by MS and MSMS followed by MASCOT
11 searching (Fig. 7 and supplementary Fig. 1 and 2). HexNAc modification was identified on
12 Thr19 of the peptide which corresponds to Thr35 of cH-Ras.
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18 **4. Discussion**

19 It is reported that TpeL is produced by several *C. perfringens* types including types A,
20 B and C and its gene localization on plasmid accounts for horizontal transfer between *C.*
21 *perfringens* strains (Amimoto et al., 2007; Sayeed et al., 2010). TpeL synthesis seems to be
22 dependent of the sporulation (Paredes-Sabja et al., 2011) and this novel toxin likely represents
23 an additional virulence factor involved in necrotic enteritis in poultry (Coursodon et al.,
24 2012). Indeed, TpeL is cytotoxic to various cell lines by inducing apoptosis via impairment of
25 the Ras signaling pathway (Guttenberg et al., 2012). However some *C. perfringens* strains,
26 like MC18, seems to contain a truncated *tpel* gene resulting in a protein lacking 128 C-
27 terminal amino acids which is not cytotoxic (Guttenberg et al., 2012). In our experience, only
28 a few *C. perfringens* strains type C isolated from necrotic enteritis in piglets, and a *C.*
29 *perfringens* type A strain isolated from food contain *tpel* gene.
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40 TpeL is related to the LCGTs based on amino acid sequence identity and shows the
41 highest similarity with TcnA. We have analyzed the enzymatic properties of TpeL, from
42 which most of them have been published during the progress of this work (Guttenberg et al.,
43 2012; Nagahama et al., 2011). Albeit confirmatory in a large part, our investigations bring
44 complementary data. TpeL is a glucosyltransferase, which retains the enzymatic site DxD of
45 LCGTs and with a global amino acid sequence identity more related to TcnA than to the other
46 LCGTs (Fig. 1). In addition, TpeL displays the same motif, A₃₈₃NQ₃₈₅, of co-substrate
47 specificity than TcnA, which uses UDP-*N*-acetylglucosamine as sugar donor, instead of
48 I₃₈₃NQ₃₈₅ in the other LCGTs which catalyzes the transfer of glucose from UDP-glucose
49 (Guttenberg et al., 2012). Using the recombinant catalytic domain (1-543), we showed that
50 TpeL exhibited both UDP-glucosylation and UDP-*N*-acetylglucosamination (GlcNAcylation)
51 activities towards cH-Ras, but with a 100 fold more efficiency of GlcNAcylation. UDP is the
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1 major binding determinant since the rN-TpeL GlcNAcylation was impaired by all UDP-
2 hexoses tested in competition assay (Fig. 3C and D). However, UDP-glucose and UDP-*N*-
3 acetylglucosamine were more efficient competitors than the other UDP-hexoses, indicating a
4 stringent specificity for these two cosubstrates. In contrast, Nagahama et al. report a TpeL
5 enzymatic competition only with UDP-glucose and UDP-*N*-acetylglucosamine and no with
6 other UDP-hexoses (Nagahama et al., 2011). The bases of these discrepant results are not
7 clear, in our study, the recombinant N-terminal TpeL 1-543 amino was used instead TpeL 1-
8 525 by Nagahama et al. (Nagahama et al., 2011).

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14 As previously found, the three isoforms of Ras including cH-Ras, N-Ras and K-Ras
15 are the main substrates of TpeL. Using a large panel of small GTPases, we observed that rN-
16 TpeL modified cH-Ras, N-Ras and K-Ras and to a lower extent Rap1a and R-Ras3. In
17 contrast to previous findings [Guttenberg, 2012 #3074;Nagahama, 2011 #3076], we showed
18 that Rac1 was a very poor substrate of TpeL in the presence of UDP-*N*-acetylglucosamine.
19 Only a weak glucosamination was evidenced after a long exposure of the autoradiographies
20 and no Rac glucosylation was observed with rN-TpeL and UDP-glucose. In agreement with
21 Guttenberg et al. (Guttenberg et al., 2012) we showed no modification of Ral by TpeL in the
22 presence of UDP-*N*-acetylglucosamine or UDP-glucose, whereas Ral has been reported to be
23 a TpeL substrate by Nagahama and coworkers (Nagahama et al., 2011). These different
24 observations might result from the distinct recombinant TpeL fragments used in each study.
25 We confirmed by *in vitro* glucosylation and mass spectrometry that TpeL modifies Ras at
26 Thr35. In our conditions, TpeL displays glucosyltransferase activity only with Ras proteins,
27 mainly the three isoforms of Ras and weakly Rap1a and R-Ras3, and is not active with Rho
28 proteins. This opens the way to delineate specific tools directed towards Ras for therapeutic
29 purpose and cell biology investigations.
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Figure legends

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4 **Figure 1.** Phylogenetic tree of the whole toxin sequence (A) and enzymatic domain (543 N-
5 terminal amino acids) (B) of TpeL (AB262081), TcdA (YP_001087137), TcdB (CAJ67492),
6 TcdBF (CAC19891), TcsL6018 (X82638) and TcnA (CAA88565.1) according to the UP-
7 GMA method. The percentage of replicate trees in which the associated taxa clustered
8 together in the bootstrap test (100 replicates) are shown next to the branches. The tree is
9 drawn to scale, with branch lengths in the same units as those of the evolutionary distances
10 used to infer the phylogenetic tree. The evolutionary distances were computed using the
11 Poisson correction method and are in the units of the number of amino acid substitutions per
12 site.
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22 **Figure 2.** TpeL N-terminal part glucosylates Ras but not Rho, Rac, and Cdc42. (A) SDS-
23 PAGE of recombinant N-terminal part of TpeL stained with Blue Coomassie R-250. (B)
24 Glucosylation of Ras, Rho, Rac, or Cdc42 (1 μ g recombinant protein) by TpeL N-terminal
25 part (10^{-7} M) in the presence of UDP- 14 C]Glucose or UDP- 14 C]-N-acetylglucosamine for 1 h
26 at 37°C. The proteins were run on a SDS-PAGE and autoradiographed.
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33 **Figure 3.** TpeL uses preferentially UDP-N-acetylglucosamine as cosubstrate. A kinetics of
34 Ras glucosylation by rN-TpeL. Ras (1 μ g) was incubated with rN-TpeL (10^{-7} M) for 1, 5, 10,
35 15 or 30 min at 37°C in glucosylation buffer containing 7 μ M UDP- 14 C]-N-
36 acetylglucosamine or UDP- 14 C]-glucose. The samples were electrophoresed on a SDS-
37 (14%)PAGE, autoradiographed, and band intensity was quantified by ImageJ (NIH, USA).
38 Experiments were done in triplicate. Means \pm standard deviation are shown. (B) Kinetics of
39 Ras glucosylation by rN-TpeL with UDP- 14 C]-glucose as in A but with a different scale of Y
40 axis. Note that Ras glucosylation with UDP- 14 C]-glucose is about 100 fold less than with
41 UDP- 14 C]-N-acetylglucosamine. (C) Competition assay of Ras glucosylation with various
42 hexose derivatives. Ras (1 μ g) was incubated in glucosylation buffer with rN-Tpel (10^{-7} M)
43 and each of the various hexose derivatives (5 mM) for 30 min at 37°C. Then UDP- 14 C]-N-
44 acetylglucosamine was added and the preparations were incubated at 37°C for additional 30
45 min and processed as described above. (D) Quantification of the competition assays.
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Figure 4. TpeL recognizes specifically Ras. (A) Glucosylation of recombinant small GPTases (1 μ g) with rN-Tpel (10^{-7} M) and UDP- 14 C]-*N*-acetylglucosamine was performed as described in Experimental Procedures for 1 h at 37°C. (B) Quantification of the glucosylated small GTPases. Glucosylation of Ras was defined as 100%.

Figure 5. Mono-*N*-acetylglucosaminylation of cH-Ras by TpeL. cH-Ras untreated and treated by rN-TpeL (10^{-7} M) in glucosylation buffer containing 5 mM UDP-*N*-acetylglucosamine for 3 h at 37°C was analyzed by SELDI-TOF mass spectrometry with a Q10 array. Ras treated with rN-TpeL shows an increased mass of 205 Da.

Figure 6. Identification of Ras peptide containing the TpeL modification. (A) Ras untreated and treated by rN-TpeL (10^{-7} M) in glucosylation buffer containing 5 mM UDP-*N*-acetylglucosamine for 3 h at 37°C was trypsin digested and analyzed by HPLC on a DEAE column linked to a C18 reverse phase column. Fraction 15 from untreated Ras and fraction 14 from rN-TpeL treated Ras were found to contain the same peptide SALTQLIQNHVDEYDPTIEDSYR by N-terminal sequencing. (B) SELDI-TOF mass spectrometry showing that the peptide from fraction 14 (rN-TpeL-treated Ras) has an increased mass of 203 Da versus that of the parental peptide (fraction 15) from untreated Ras.

Figure 7. TpeL mono-*N*-acetylglucosaminates Ras at Thr35. MS and MS-MS spectrum of fraction 14 from rN-TpeL-treated Ras (A) and fraction 15 from untreated Ras (B).

Supplementary Figure 1. Mascot search results of MS-MS residues of peptide from fraction 14 (rN-TpeL-treated Ras).

Supplementary Figure 2. Mascot search results of MS-MS residues of peptide from fraction 15 (untreated Ras).

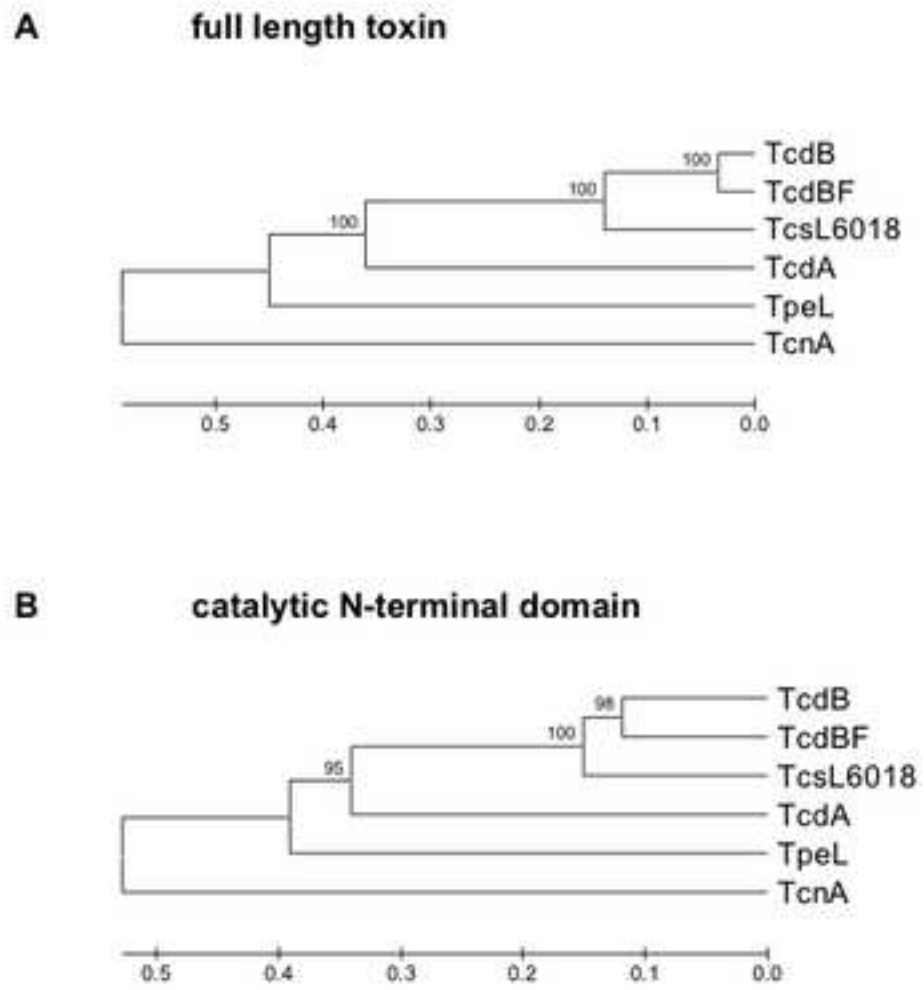


Figure 1

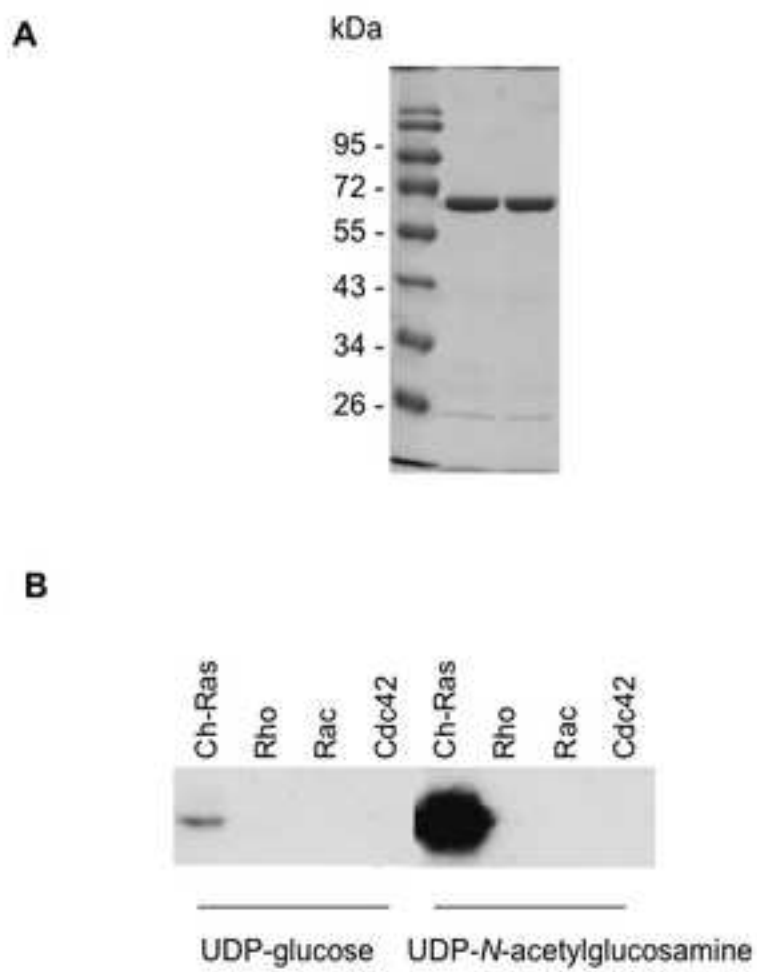


Figure 2

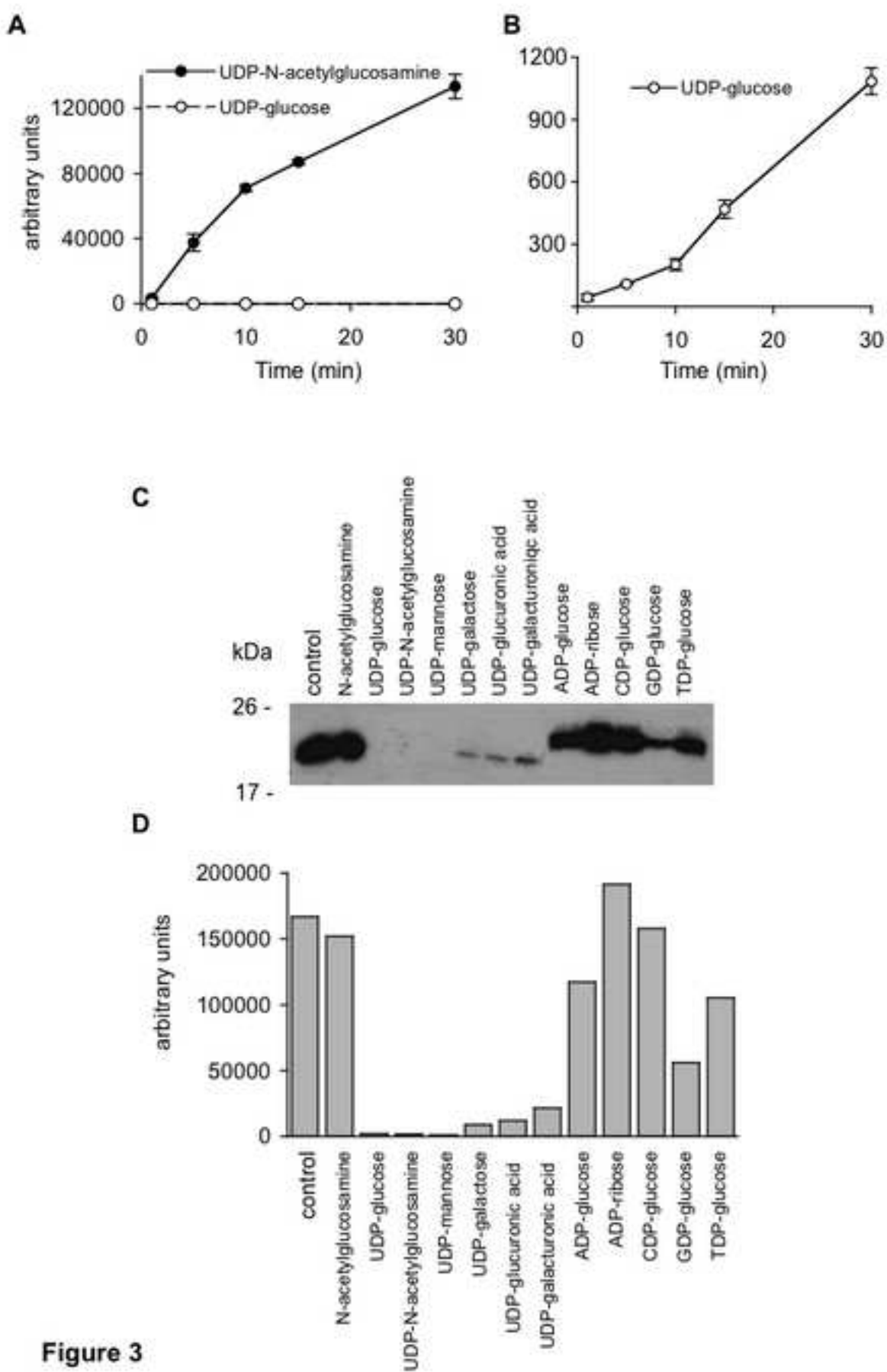


Figure 3

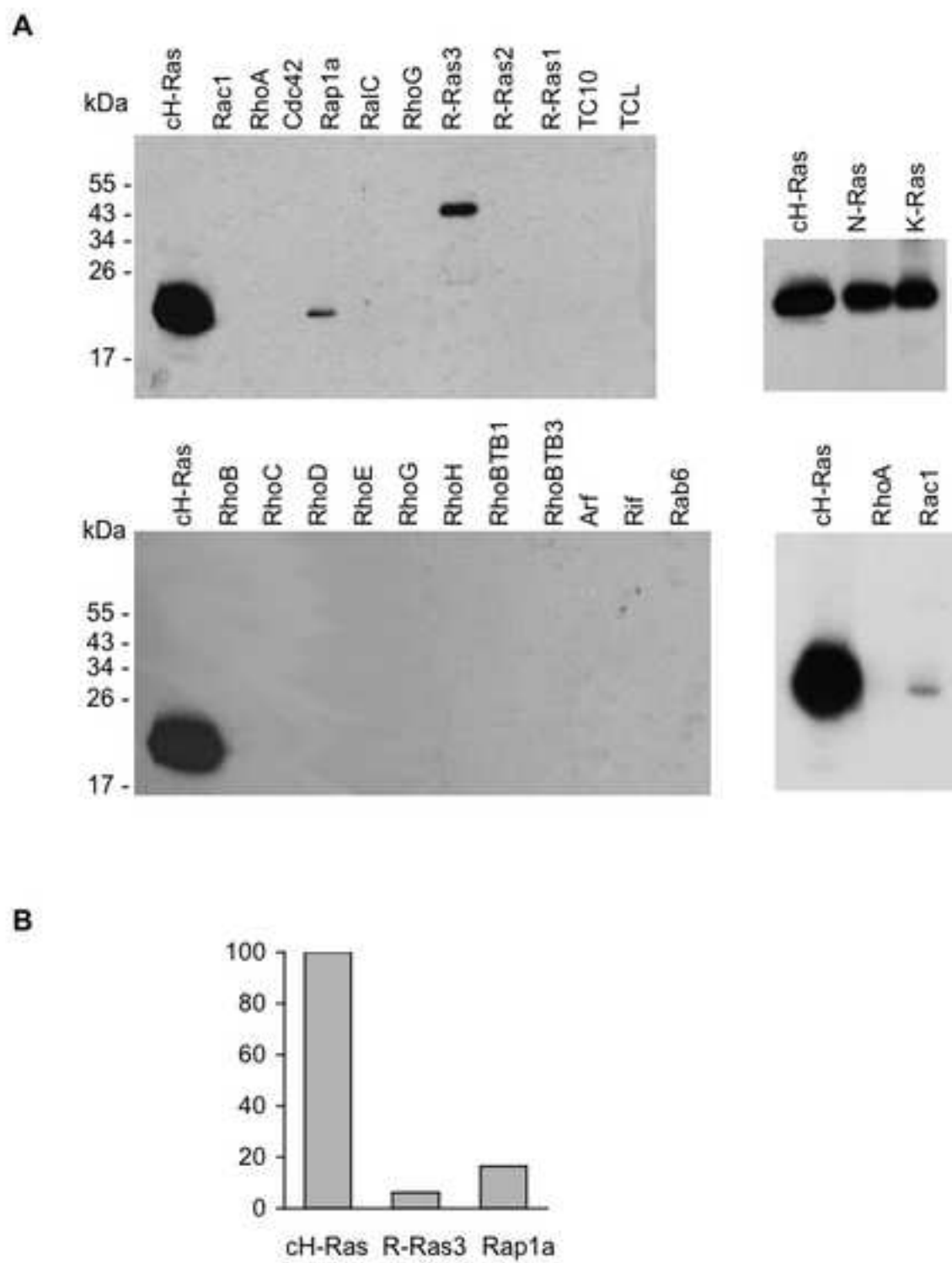
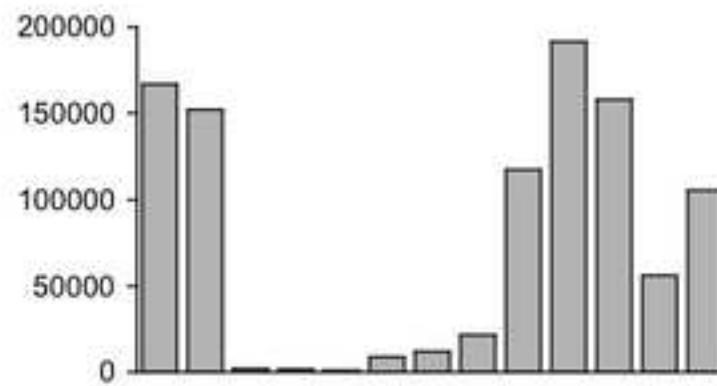
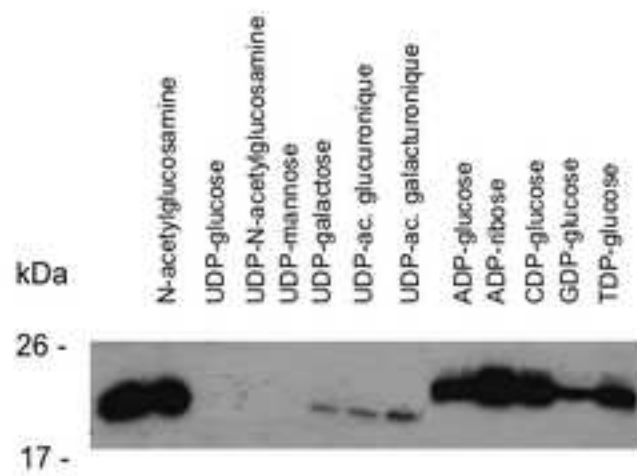


Figure 4

Figure

[Click here to download high resolution image](#)



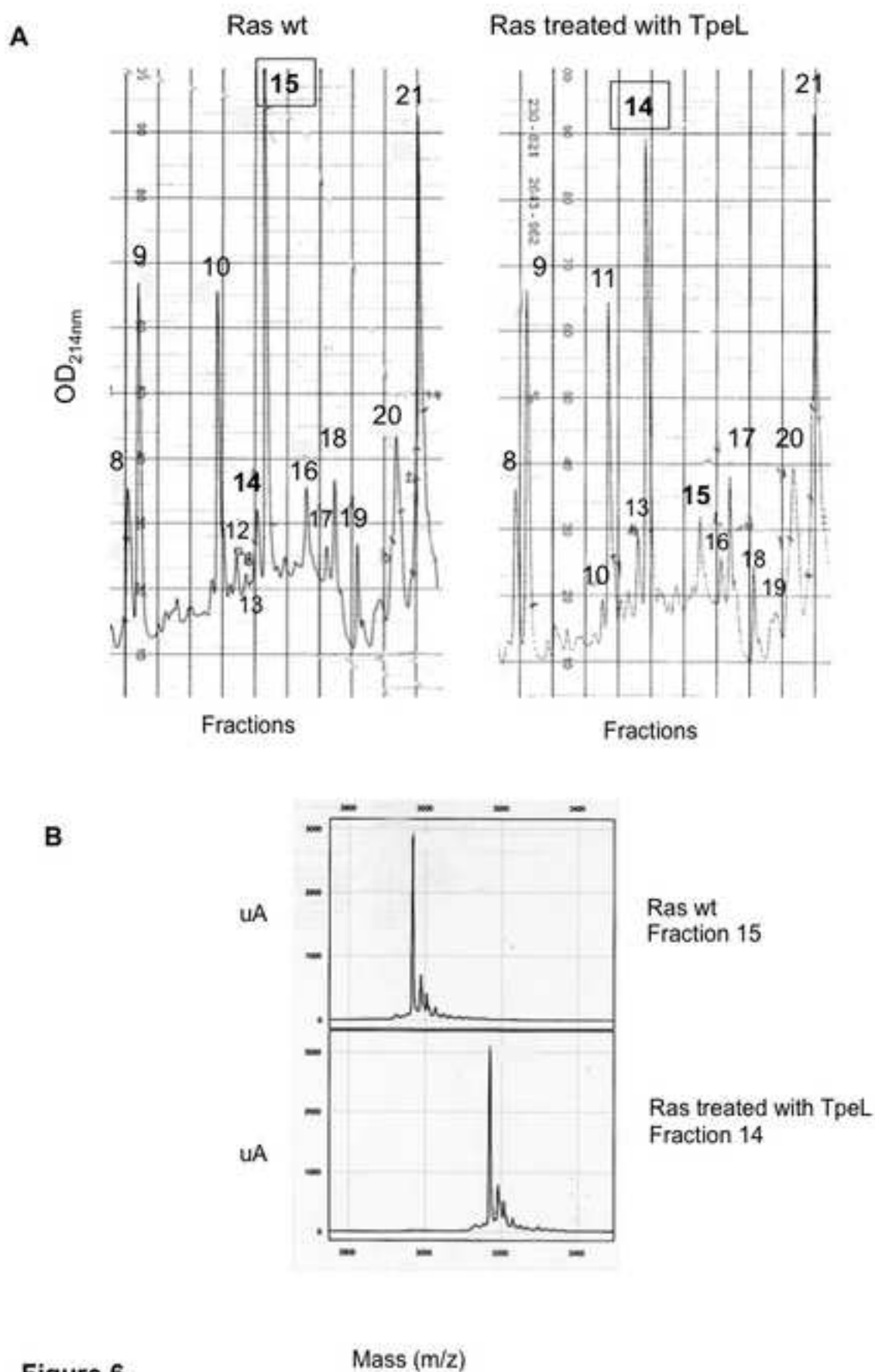


Figure 6

A

Ras treated with TpeL fraction 14

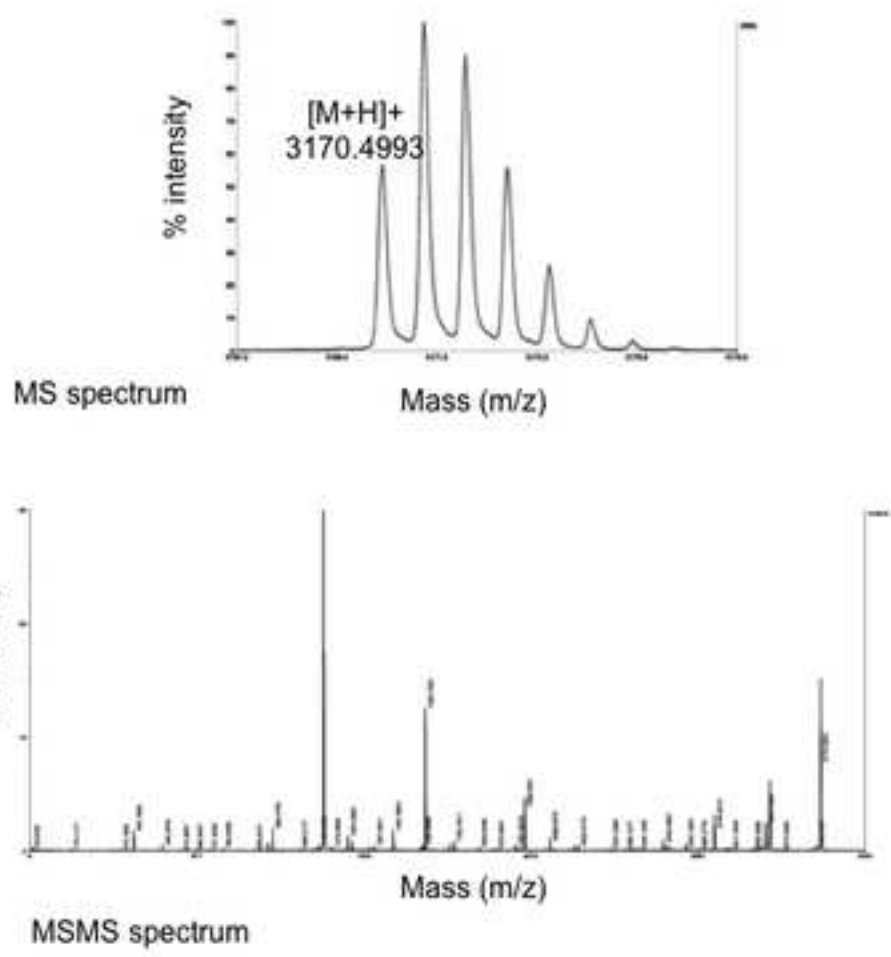


Figure 7

B

Ras wt fraction 15

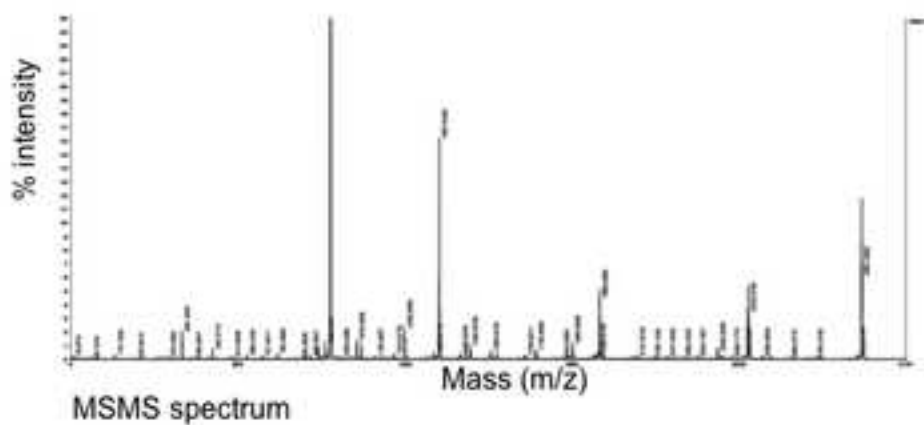
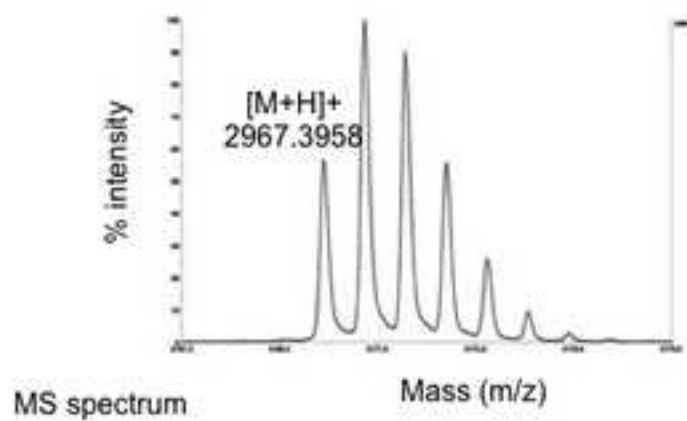


Figure 7 (followed)

Ras treated with TpeL fraction 14

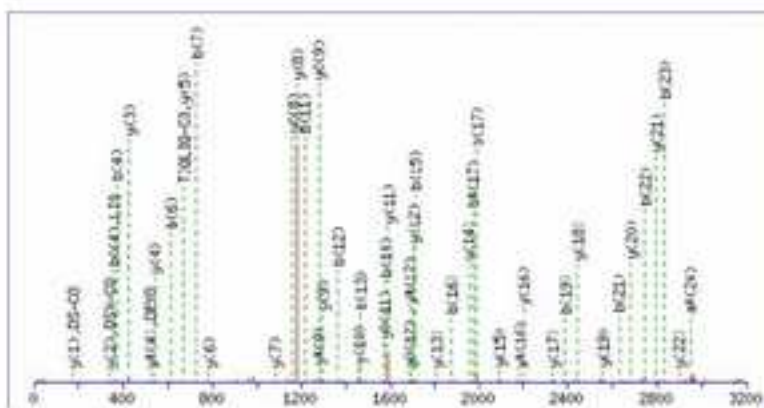
Mascot Search Results

Peptide View

MS/MS Fragmentation of **SALTIQLIQNHVFDEYDPTIEDSYR**

Found in **RASH_HUMAN**, GTPase HRas OS=Homo sapiens GN=HRAS PE=1 SV=1

Match to Query 1: 3169.491724 from(3170.499000,1+)



Monoisotopic mass of neutral peptide Mr(calc): 3169.5091

Variable modifications:

T19 : HexNAc (T)

Ions Score: 160 Expect: 3.3e-015

Matches (grey background): 54/439 fragment ions using 85 most intense peaks

#	Ion	a	a'	a''	b	b'	b''	Seq	v	w	w'	y	y'	y''	#
1	60.0444	60.0444		42.0338	88.0393		70.0287	S							25
2	44.0495	131.0815		113.0709	199.0764		141.0659	A				3083.4844	3066.4578	3065.4738	24
3	86.0964	244.1656		226.1550	272.1605		254.1499	L	2954.3690	2953.3738		3012.4473	2995.4207	2994.4367	23
4	74.0600	345.2132		327.2027	373.2082		355.1976	T	2853.3213	2866.3417	2868.3210	2999.3637	2882.3366	2881.3526	22
5	86.0964	438.2973		440.2867	486.2922		468.2817	F	2740.2373	2753.2577	2767.2733	2791.3133	2784.2898	2783.3058	21
6	101.0709	586.3599	569.3293	568.3453	614.3508	597.3242	596.3402	Q	2612.1787	2611.1834		2695.3113	2668.2849	2667.2209	20
7	86.0964	699.4399	682.4134	681.4294	727.4349	710.4083	709.4243	L	2499.0946	2498.0994		2573.1729	2546.1465	2545.1625	19
8	86.0964	832.5240	795.4975	794.5134	840.5189	823.4924	822.5084	F	2386.0106	2399.0310	2413.0466	2444.0929	2427.0623	2426.0783	18
9	101.0709	940.5826	923.5560	922.5720	968.5775	951.5509	950.5669	Q	2257.9520	2256.9567		2331.0308	2313.9782	2312.9942	17
10	87.0553	1054.6255	1037.5990	1036.6149	1082.6204	1065.5939	1064.6099	N	2143.9691	2142.9738		2217.9462	2200.9196	2199.9356	16
11	110.0713	1191.6844	1174.6579	1173.6739	1219.6793	1202.6528	1201.6688	H	2006.8302			2095.9132	2077.8767	2076.8927	15
12	120.0808	1338.7528	1321.7263	1320.7423	1366.7477	1349.7212	1348.7372	F	1859.7817			1947.8648	1929.8178	1928.8338	14
13	72.0608	1437.8212	1420.7947	1419.8107	1467.8162	1448.7896	1447.8056	V	1760.7133	1773.7337		1848.7799	1827.7484	1826.7654	13
14	88.0393	1552.8482	1535.8216	1534.8376	1579.8431	1563.8165	1562.8325	D	1645.6864	1644.6911		1709.7372	1692.6999	1691.7159	12
15	102.0150	1681.8908	1664.8642	1663.8802	1709.8857	1692.8591	1691.8751	E	1516.6438	1515.6486		1591.6948	1573.6540	1572.6700	11
16	136.0757	1844.9541	1827.9276	1826.9435	1872.9490	1855.9224	1854.9385	V	1353.5805			1411.6290	1404.6114	1403.6274	10
17	88.0393	1959.9810	1942.9545	1941.9705	1987.9760	1970.9494	1969.9654	D	1238.5535	1237.5583		1295.6121	1276.5713	1275.5873	9
18	70.0651	2077.0338	2060.0073	2059.0232	2085.0287	2068.0022	2067.0182	P	1141.5008	1140.5055		1193.5477	1176.5212	1175.5372	8
19	277.1394	2361.1609	2344.1343	2343.1503	2389.1558	2372.1292	2371.1452	F	837.3737	850.3941	852.3734	1096.4994	1089.4684	1088.4844	7
20	86.0964	2474.2449	2457.2184	2456.2344	2502.2399	2485.2133	2484.2293	F	724.2897	737.3101	751.3297	792.3678	785.3414	784.3574	6
21	102.0350	2603.2875	2586.2610	2585.2769	2631.2824	2614.2559	2613.2719	E	595.2471	594.2518		609.2636	602.2573	601.2733	5
22	88.0393	2718.3144	2701.2879	2700.3039	2746.3094	2729.2828	2728.2988	D	480.2201	479.2249		495.2317	488.2254	487.2414	4
23	60.0444	2805.3465	2788.3199	2787.3359	2834.3414	2816.3148	2815.3308	S	393.1881	392.1928		408.2037	401.1974	400.2134	3
24	136.0757	2968.4098	2951.3833	2950.3993	2996.4047	2979.3782	2978.3942	V	230.1248			245.1357	244.1417	243.1477	2
25	129.1135							R	74.0237	73.0284		173.1088	156.0924		1

Supplementary Figure 1

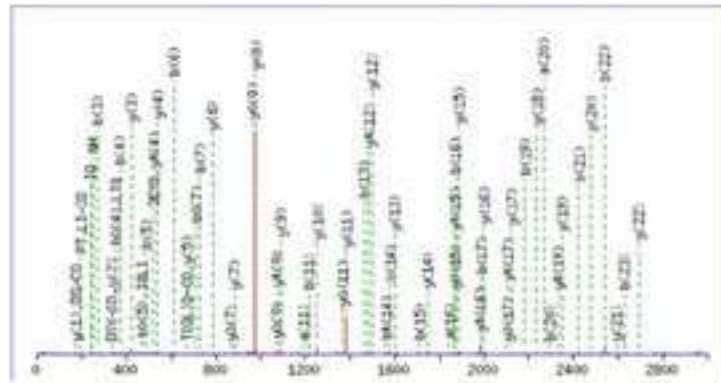
RasH wt fraction 15

Mascot Search Results
Peptide View

MS/MS Fragmentation of **SALTIQLIQNHVFDEYDPTIEDSYR**

Found in **RASH_HUMAN**, GTPase HRas OS=Homo sapiens GN=HRAS PE=1 SV=1

Match to Query 1: 2966.388724 from(2967.396000,1+)



Monoisotopic mass of neutral peptide Mr(calc): 2966.4297

ions Score: 191 Expect: 2.9e-018

Matches (grey background): 72/457 fragment ions using 83 most intense peaks

#	Ionmass	a	a*	a*	b	b*	b*	Seq	x	w	w'	y	y*	y*	#
1	60.0444	60.0444		42.0338	88.0393		70.0287	S							25
2	44.0495	131.0815		113.0709	159.0764		141.0659	A	2964.3737			2880.4090	2863.3784	2862.3944	24
3	86.0964	244.1656		226.1550	272.1605		254.1499	L	2751.2896	2750.2944		2699.3679	2792.3413	2791.3573	23
4	74.0600	348.2132		327.2027	373.2082		355.1976	T	2950.2420	2663.2624	2665.2416	2696.2639	2678.2573	2678.2733	22
5	86.0964	438.2973		440.2867	496.2922		488.2817	I	2537.1579	2550.1783	2564.1939	2599.2361	2578.2096	2577.2256	21
6	101.0709	586.3559	569.3293	568.3453	614.3508	597.3242	596.3402	Q	2409.0993	2408.1041		2492.1721	2465.1254	2464.1414	20
7	86.0964	699.4399	682.4134	681.4294	727.4349	710.4083	709.4243	L	2296.0153	2295.0200		2354.0933	2337.0670	2336.0829	19
8	86.0964	812.5240	795.4975	794.5134	840.5189	823.4924	822.5084	I	2182.9312	2193.9516	2208.9672	2231.0094	2223.9829	2222.9989	18
9	101.0709	940.5826	923.5560	922.5720	968.5775	951.5509	950.5669	Q	2054.8726	2053.8774		2127.9214	2110.8950	2109.9110	17
10	87.0553	1054.6255	1037.5990	1036.6149	1082.6204	1065.5939	1064.6099	N	1940.8297	1939.8344		1999.8669	1992.8403	1991.8562	16
11	110.0713	1191.6844	1174.6579	1173.6739	1219.6793	1202.6528	1201.6688	H	1803.7708			1892.8239	1885.7973	1884.8133	15
12	120.0808	1338.7528	1321.7263	1320.7423	1366.7477	1349.7212	1348.7372	F	1656.7024			1745.7555	1731.7384	1730.7544	14
13	72.0608	1437.8212	1420.7947	1419.8107	1465.8162	1448.7896	1447.8056	V	1557.6340	1570.6544		1601.6566	1584.6700	1583.6860	13
14	88.0393	1552.8482	1535.8216	1534.8376	1580.8431	1563.8165	1562.8325	D	1442.6070	1441.6118		1492.6291	1485.6016	1484.6176	12
15	102.0550	1661.8968	1644.8642	1643.8802	1689.8857	1672.8591	1671.8751	E	1313.5644	1312.5692		1367.6012	1370.5747	1369.5906	11
16	136.0757	1844.9343	1827.9276	1826.9435	1872.9489	1855.9223	1854.9383	V	1150.5011			1208.5396	1241.5121	1240.5480	10
17	88.0393	1939.9830	1942.9545	1941.9705	1987.9760	1970.9494	1969.9654	D	1033.4742	1034.4789		1095.4951	1078.4685	1077.4845	9
18	70.0651	2017.0138	2040.0073	2039.0232	2085.0287	2068.0022	2067.0182	P	938.4214	937.4261		998.4645	983.4418	982.4578	8
19	74.0600	2138.0815	2141.0549	2140.0709	2186.0764	2169.0498	2168.0658	T	837.3737	850.3941	852.3734	903.4116	886.3980	885.4140	7
20	86.0964	2371.1657	2254.1390	2253.1550	2299.1605	2282.1339	2281.1499	I	724.2897	737.3100	751.3257	782.3678	765.3414	764.3573	6
21	102.0550	2400.2081	2383.1816	2382.1976	2428.2030	2411.1765	2410.1925	E	595.2471	594.2518		649.2639	652.2373	651.2533	5
22	86.0393	2515.2351	2498.2085	2497.2245	2543.2300	2526.2034	2525.2194	D	480.2301	479.2249		540.2417	523.2147	522.2307	4
23	60.0444	2602.2671	2585.2406	2584.2565	2630.2620	2613.2355	2612.2515	S	393.1881	392.1928		425.2141	408.1876	407.2037	3
24	136.0757	2765.3304	2748.3039	2747.3199	2793.3254	2776.2988	2775.3148	V	250.1248			305.1523	321.1557		2
25	129.1135							R	74.0237	73.0284		175.1186	158.0924		1

Supplementary figure 2