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Author: Giona Sonego PhD Mélanie Abonnenc PhD David Crettaz Niels Lion PhD Jean-Daniel Tissot MD Michel Prudent PhD

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Irreversible oxidations of platelet proteins after riboflavin-UVB pathogen inactivation

Oxydations irréversibles des protéines de plaquettes après traitement d'inactivation des pathogènes UVB-riboflavine

Giona Sonego[1,2], PhD, Mélanie Abonnenc[1], PhD, David Crettaz[1], Niels Lion[1,2], PhD, Jean-Daniel Tissot[1,2], MD, Michel Prudent[1,2], PhD

[1]Laboratoire de Recherche sur les Produits Sanguins, Recherche et Développement Produits, Transfusion Interrégionale CRS, Epalinges, Switzerland

[2]Faculté de Biologie et de Médecine, Université de Lausanne, Lausanne, Switzerland

* Corresponding author. Dr. Michel Prudent, Transfusion Interrégionale CRS, Laboratoire de Recherche sur les Produits Sanguins, Route de la Corniche 2, CH-1066 Epalinges, Switzerland. *Phone:* +41 21 333 16 70. *E-mail address:* michel.prudent@itransfusion.ch

Abstract

Pathogen inactivation technologies are known to alter in vitro phenotype and functional properties of platelets. Because pathogen inactivation generates reactive oxygen species, oxidative stress is considered as one of the plausible cause at the origin of the platelet storage lesion acceleration after treatment. To date proteomics has been used to document the protein variations to picture out the impact. Here, platelet concentrates were prepared from buffy-coats in Intersol additive solution, leukoreduced and pathogen inactivated using a riboflavin/UVB treatment. At day 2 of storage the platelet proteomes of control (untreated) and treated platelet concentrates were investigated against the site specific oxidation by liquid chromatography coupled to tandem mass spectrometry in a shotgun experiment. The shotgun approach detected 9350 peptides (and 2534 proteins) of which 1714 were oxidized. Eighteen peptides were found exclusively oxidized in treated platelets whereas 3 peptides were only found oxidized in control. The present data evidenced an interference with several proteins involved in platelet aggregation and platelet shape change (such as talin and vinculin).

Keywords: Pathogen Inactivation, Platelet concentrate, Oxidation, Proteomics, Riboflavin, ROS, Shotgun

Résumé

Les traitements d'inactivation des pathogènes sont connus pour induire, in vitro, des modifications au niveau du phénotype et des fonctionnalités plaquettaires. De par le caractère oxydatif de ces traitements

photochimiques, la génération d'espèces réactives de l'oxygène est l'une des causes possibles du vieillissement accéléré des concentrés plaquettaires inactivés. Les analyses protéomiques ont montré jusqu'à présent les variations de certaines protéines suite à ces traitements. Dans la présente étude, des concentrés plaquettaires ont été préparés à partir de buffy-coat en solution Intersol, leucoréduits et traités pour l'inactivation des pathogènes à base de riboflavine/UVB. Deux jours après le don, des oxydations spécifiques du protéome des plaquettes traitées et non-traitées (contrôle) ont été étudiés par chromatographie liquide couplée à la spectrométrie de masse. L'approche « shotgun » utilisée a détecté 9350 peptides (pour 2534 protéines) dont 1714 étaient oxydés. Dix-huit d'entre eux ont été détectés exclusivement dans les plaquettes traitées et 3 exclusivement dans le contrôle. Ces données montrent l'impact de ces oxydations de protéines impliquées dans des processus d'agrégation plaquettaire et de changement de forme (telles la talin et la vinculin).

Mots clés : Concentré plaquettaire, Espères réactives de l'oxygène, Inactivation des pathogènes, Oxydation, Protéomique, Riboflavine, Shotgun

1 Introduction

Pathogen inactivation technologies (PITs) provide a higher level of safety in transfusion medicine but impact the quality of blood products [1]. They reduce platelets in vitro functionality [2-6], suggesting that in addition to early platelet storage lesions [7] there is an endogenous factor affecting their effectiveness. PITs enhance storage lesions and correlate with platelet aging and pre-activation [8, 9]. Hypotonic shock response highlighted lower deformability and flow cytometry evidenced increased pre-apoptotic markers, degranulation and integrins activation [3, 10]. In addition, glycolytic metabolism is accelerated resulting in medium acidification [6]. Despite these considerations, *in vitro* functionality tests are difficult to be translated *in vivo* and have not a clear clinical relevance to date [11-13].

PI produces ROS that can damage other molecules than DNA/RNA , such as peptides and proteins [14, 15]. For instance, ROS imbalance enhances protein carbonylation, a hallmark of oxidation, as recently shown in Mirasol-treated platelet concentrates (PCs). The production of hydrogen peroxide on days 5 and 7 was also significantly higher in treated samples, suggesting a decreased antioxidant cell defense and/or enhanced activation pathways due to second messengers. This was also evidenced in Intercept-treated platelets where antioxidant power was decreased [16] and where ROS-induced urate conversion into allantoin was observed [17]. At the protein level, the impact should mainly be post-translational such as oxidations [14, 15], since PI-platelets are able to synthesize proteins [18].

Considering that (i) proteomic data have reported protein damages [19], (ii) post-translational analyses are missing and (iii) functional properties (linked to protein functions) are altered (eg reduced response to hypothonic shock and increase adhesion to fibrinogen, Figure 1) [3], mass spectrometry (MS)-based proteomics were applied to look at oxidative damages. Although literature reports a relatively mild impact of PITs on platelet proteome [2, 7-9, 19-21], several protein-based pathways were found to be affected by PITs [19, 20, 22, 23]. In the present study, the impact of riboflavin/UVB on platelet proteome was investigated by a shotgun approach with a focus on irreversible oxidations.

2 Methods

2.1 Platelet concentrates

The experiments were composed by three biological replicates prepard as follows. Buffy coat-derived and leukoreduced PCs were prepared and treated as previously described in Intersol [3]. Then three ABO-matched PCs were pooled together in a 2L-Plasma bag, agitated for 5 min and split in three identical PCs: one control bag (untreated PC) was supplemented with 17.5 mL of Intersol, and one treated bag was completed with 17.5 mL of riboflavin and treated in home-made illuminator (addition of riboflavin followed by a UVB treatment at 5J/cm^2 applied in 20 min) [3]. PCs were stored overnight in a storage bag (1300 mL, PL2410 plastic) under agitation at 22°C, and sampling were done at day 2 post-donation. The third bag was not employed here.

2.2 Proteomics

Five µL aliquots of Prostaglandin E_1 (PGE_1) (> 98%, HPLC grade) from Sigma Aldrich (Steinheim, Germany) were diluted in 495 µL of Tyrode's buffer. Four mL of PCs were sampled in plastic tubes through sterile connection at day 2 and transferred to a 15-mL tube containing 40 µL of PGE_1 1%. PCs were incubated during 5 min at 37 °C under gentle rotation and followed by 10 min of centrifuge at 1'000*g* at room temperature (RT). The supernatant was carefully removed and the pellet was washed several times. Platelets were resuspended in 1 mL of Tyrode's buffer + 10 µL PGE_1 and samples were aliquoted at 50 µg. Aliquots were centrifuged (1'000*g*) at RT during 10 min and pellets were frozen at -80°C. 150 µL Laemmli 2x were added to the pellet for proteomics.

Proteins (8 µg) were separated on a 12% mini polyacrylamide gel (BioRad), excised in 6 gel bands (Supplementary Fig. S1) and digested with sequencing-grade Trypsin/LysC (Promega) [24]. Extracted peptides were vacuum-dried and resuspended in 0.05% trifluoroacetic acid, 2% (v/v) acetonitrile for LC-MS/MS analyses on a Dionex RSLC 3000 nanoHPLC system (Dionex, Sunnyvale, CA, USA) interfaced via a nanospray source to a high resolution mass spectrometer QExactive Plus (Thermo Fisher, Bremen, Germany) (see Supplementary Material for details).

2.3 Data analysis

Protein identification from MS raw files and label-free quantitation with MaxQuant version 1.5.3.30 [25] were realized as previously described [26], using the set of *Homo sapiens* proteome sequences downloaded from UniProt database (UP000005640_9606.fasta, December 2015 version, 21'026 sequences). Trypsin was used as the enzyme definition, allowing 2 missed cleavages. Database searches were carried out with a parent ion tolerance of 5 ppm after recalibration, and a fragment ion mass tolerance of 20 ppm. Variable modifications were specified as follows: N-terminal acetylation of protein, iodoacetamide derivative of cysteine, dioxidation and trioxidation of cysteine, oxidation and dioxidation of methionine and tryptophan, tryptophan oxidation to kynurenin. Both peptide and protein FDR rates were set at 1% as calculated against a decoy sequence database and the match between runs function was activated.

Protein pathways were analyzed on string-db.org (version 10.0, access on May 2016).

2.4 Statistical analysis

Oxidation levels were compared by combining moderated t-test (limma) with rank products on well-defined null hypothesis. A p-value < 0.05 is considered as significant.

3 Results

First of all, 2D gel-based proteomics was carried out as for Intercept-treated PCs [9]. It revealed a low impact on the proteome (in particular the overexpression of glyoxalase domain-containing protein 4 in treated samples) and identified proteins were not confirmed by western blot (see supplementary material).

Label free shotgun analysis of the samples at day 2 was carried out. A total of 2534 proteins (3700 are reported to date [27]) with a minimum of two peptides were detected (see Supplementary Table 1).

Although a considerable absolute number of oxidized peptides (1714) was detected, in general, identified proteins and relative peptides showed mitigate matching with our peptide-based oxidation database [14] and mainly contained methionine oxidation. Particularly, 18 peptides belonging to 17 proteins were found to be only oxidized in treated samples, while 3 proteins were only oxidized in control ones (Table 1, upper part). Protein-protein interactions analysis identified that the platelet aggregation was particularly affected by oxidation (unique biological process with a false discovery < 0.05, Table 2 and Supplementary Fig. S4).

Comparison of oxidation levels between treated and untreated PCs did not present differences, after classic statistical correction (i.e. Benjamini-Hochberg). However, treated samples displayed an oxidative tendency suggesting slight irreversible oxidation induced by PITs. Without the correction, several oxidized peptides (45) displayed higher intensity in treated samples and 8 oxidized peptides were more intense in control samples (p-value < 0.01). Therefore, to fully explored the dataset and to further gain statistical significance (considering the list of oxidized peptides > 1'000 rows and the presence of missing values [not all the peptides were detected in all samples]), moderated t-test (limma) with rank products on well-defined null hypothesis were combined according to Schwämmle et al. [28]. Hence, an increased significance for 18 peptides (allowing a maximum of two modifications per peptide) was obtained. Surprisingly, the majority of the significantly oxidized peptides (i.e. 11 peptides) belong to control PCs (Table 1, lower part).

4 Discussion

The present identification number of 2534 proteins is high which make difficult to explore all the potential modifications. Therefore, and this is one limitation of our approach, we have tp target on specific amino acids in order to find statistical variation. Therefore, three amino acids were preferentially selected. Methionine and cysteine are well known protein antioxidant and stabilize the protein structure [29]. In addition, they are involved to numerous biological processes as redox system. The choice of the third amino acid tryptophan was based on a previous study on PI-treated model peptides because of its high sensitivity to ROS [14].

Although a considerable absolute number of oxidized peptides were detected (1714 over 9350 detected peptides), only 18 and 3 oxidized peptides were found in treated and untreated samples, respectively (while the unmodified peptides were present in all replicates of both conditions) (Table 1, upper part). Such findings make us plaid for a slight increase of irreversible oxidation in riboflavin/UVB-treated platelet proteome. However, while in control PCs no significant enrichment was observed, at least 4 impacted proteins of treated PCs belong to the same biological process that is the aggregation. In addition, several proteins overlap cytoskeleton, focal adhesion and degranulation platelet apparatus. Of interest, talin and vinculin, involved in platelet shape change, were already reported to be affected by riboflavin/UVB treatment [19, 22] which might explain the decreased in platelet integrity as shown by hypotonic shock response (see Figure 1a) [3].

Looking for the oxidation of peptides present in both conditions, the analysis showed increased oxidation in control peptides (11 peptides versus 7). An intriguing subset of proteins is composed by 14-3-3Z and GPIbα. Following the binding of vWF to GPIb-IX-V, 14-3-3Z directly interacts with GPIb-IX-V cytoplasmic domain [30], which also showed an increased oxidation in treated samples. GPIb-IX-V is involved in platelet primary function by binding vWF. Protein oxidation could impair the response to vWF. Fibrinogen gamma

chain (FGG) was also up-oxidized in treated samples. FGG is secreted from platelets through alpha granules and carries the main binding site for αIIbβ3. In addition, it has to be taken into account that FGG is present in plasma, and oxidized FGG can originate from the plasma contamination. FGG in plasma is probably more exposed to the oxidative stress generated during the PI than the one present in the platelet cytoplasm (indeed, endogenous ROS are clearly attenuated when plasma concentration increase, data not shown). By the same way, myosin contains two different up-oxidized peptides in treated PCs. Interestingly, myosin tightly interacts with actin, which in turn is significantly down-oxidized. Indeed actin cluster is present with two different oxidized peptides. Actin oxidation is part of a complex system regulating the dynamic of F-actin assembly and disassembly. Actin irreversible oxidation is known to disassemble and inhibit the formation of new F-actin, favoring crosslinks, aggregates and disordered structures in different cells [31]. Inversely, actin reversible oxidation of cysteines (e.g. Cys272 and Cys374) constitutes an important oxidative stress sensor for apoptosis signaling inside cells and directly participate to F-actin elongation [32]. Here, by untargeted experiment, Cys217 was more oxidized in control PCs forming a sulfinic acid ($R\text{-}SO_2H$). This cysteine seems to participate in a lesser extent to F-actin formation, even though it was already detected in reversible oxidized form [33]. PF4 peptide AGPHCPTAQLIATLK is present in two different oxidized cysteine forms: $R\text{-}SO_2H$ and $R\text{-}SO_3H$. Interestingly such irreversible oxidation disrupts disulfide bond between Cys41 and Cys67. PF4 protein is released during aggregation and inhibits anticoagulant heparin effect. Here again it is surprising to find higher oxidation in untreated samples. Another intriguing case is GAPDH methionine carbonylation that could impair glycolysis even though direct conclusions are not feasible since the glycolysis is accelerated after PI [3, 6].

The impact of PI on platelet proteome was reported to be moderate [19] and the current data are in agreement with recently published analyses on Mirasol-treated PCs [34] and fill in previous data [19]. These irreversible protein oxidations cannot explain by themselves the cellular and functional lesions observed despite the focus on aggregation pathways (i.e. GPIb) and platelet shape change. Redox protein activities are known to be regulated via ROS. Indeed, reversible oxidations represent subtle changes that should be studied in details by proteomics [35], as in the case of the redox sensitive cysteines that can transiently be oxidized by second messengers during platelet signaling [36].

5 Conclusions

In vitro functional properties of platelets have been reported to be altered by riboflavin/UVB treatment, which migh be explained by protein modifications. The present shotgun experiment detected a low number of irreversibly oxidized proteins after riboflavin/UVB treatment. It evidenced an interference with several proteins involved in platelet aggregation and platelet shape change. The protein oxidations and modifications are part of the factors governing treatment-dependent PCs *in vitro* functionality and further in-depth investigations will be required such as redox signaling [35].

6 Acknowledgements

GS and DC ran the experiments. GS and MP designed the experiments, analyzed the data wrote the article. All the authors reviewed the data and the manuscript.

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7 Disclosure of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figure 1 caption

Fig. 1. Functional properties of riboflavin/UVB treated platelet compared to untreated platelets. (a) Hypotonic shock response is statistically decreased after treatment. (b) Static adhesion to fibrinogen is higher after treatment. * p-value < 0.05; *** p-value < 0.001. These data extracted from ref [3] were obtained with the same riboflavin/UVB procedure.

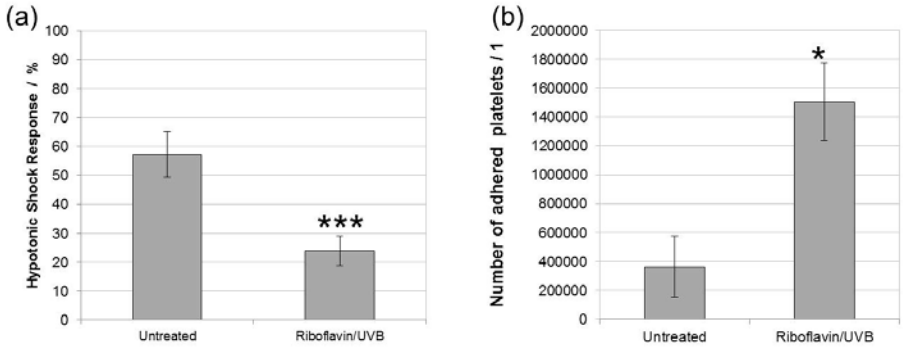

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Table 1: List of oxidized peptides in riboflavin/UVB-treated and untreated PCs at day 2. 1st part: oxidation observed only in one condition. 2nd part: differences in expression. The mean intensity differences are represented as "treated – control".

Presence only in one condition						
	Accession number	***Protein Name***	***Sequence***	***Modification***	***MS/MS count***	***Mean***
Control	P62820;Q9H0U4;Q92928	Ras-related protein Rab-1A;Ras-related protein Rab-1B;Putative Ras-related protein Rab-1C	QWLQEIDR	Kynurenin	2	na
	Q13418	Integrin-linked protein kinase	FALDMAR	Oxidation (M)	1	na
	P68032;P68133	Actin, alpha cardiac muscle 1;Actin, alpha skeletal muscle	YPIEHGIITNWDDMEKIWHHTFYNELR	2 Oxidation (W)	1	na
Treated	Q9Y490	Talin-1	LHTDDELNWLDHGR	Dioxidation (MW)	4	na
	P23229	Integrin alpha-6	LNYLDILMR	Oxidation (M)	4	na
	P60174	Triosephosphate isomerase	FFVGGNWK	Oxidation (W)	3	na
	Q86UX7	Fermitin family homolog 3	FIQAWQSLPDFGISYVMVR	Oxidation (W)	3	na
	Q00610	Clathrin heavy chain 1	LLEMNLMHAPQVADAILGNQMFTHYDR	Dioxidation (MW)	3	na
	P46109	Crk-like protein	YPSPPMGSVSAPNLPTAEDNLEYVR	Oxidation (M)	3	na
	Q13011	Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondrial	DHSVAESLNYVASWNMSMLQTQDLVK	2 Oxidation (M)	2	na
	O00429	Dynamin-1-like protein	LDLMDAGTDAMDVLMGR	Oxidation (M)	2	na
	Q9Y490	Talin-1	LHTDDELNWLDHGR	Kynurenin	2	na
	P14625	Endoplasmin	EAVEKEFEPLLNWMK	Oxidation (M)	1	na
	Q9Y696	Chloride intracellular channel protein 4	EMTGIWR	Oxidation (M)	1	na
	P50416	Carnitine O-palmitoyltransferase 1, liver isoform	GPLMVNSNYYAMDLLYILPTHIQAAR	Oxidation (M)	1	na
	P46459	Vesicle-fusing ATPase	GSMAGSTGVHDTVVNQLLSK	Oxidation (M)	1	na
	P18206	Vinculin	LANVMMGPYRQDLLAK	Oxidation (M)	1	na
	Q13418	Integrin-linked protein kinase	LNENHSGELWK	Oxidation (W)	1	na
	P54819	Adenylate kinase 2, mitochondrial	LVSDEMVVELIEK	Oxidation (M)	1	na
	P0DMV9;P0DMV8	Heat shock 70 kDa protein 1B;Heat shock 70 kDa protein 1A	MKEIAEAYLGYPVTNAVITVPAYFNDSQR	Oxidation (M)	1	na
	Q8WUM4	Programmed cell death 6-interacting protein	TMQGSEVVNVLK	Oxidation (M)	1	na
Difference in expression						
	Accession number	***Protein Name***	***Sequence***	***Modification***	***MS/MS count***	***Mean***
Control	Q9P1F3	Costars family protein	MNVDHEVNLLVEEIHR	Acetyl (Protein N-term);Oxidation (M)	4	-1.412
	Q13418	Integrin-linked protein kinase	MYAPAWVAPEALQK	Oxidation (M)	15	-1.487
	P10720;P02776	Platelet factor 4 variant;Platelet factor 4 variant(4-74);Platelet factor 4 variant(5-74);Platelet factor 4 variant(6-74);Platelet factor 4;Platelet factor 4, short form	AGPHCPTAQLIATLK	Trioxidation (C)	7	-1.501
	P10720;P02776	Platelet factor 4 variant	AGPHCPTAQLIATLK	Dioxidation (C)	4	-1.660
	P37802	Transgelin-2	YGINTTDIFQTVDLWEGK	Kynurenin (W)	6	-1.737
	Q96BM9;Q9NVJ2	ADP-ribosylation factor-like protein 8A;ADP-ribosylation factor-like protein 8B	EKDNIDITLQWLIQHSK	Dioxidation (MW)	1	-1.808
	P04406	Glyceraldehyde-3-phosphate dehydrogenase	VVDLMAHMASK	Oxidation (M)	14	-2.025
	P12814	Alpha-actinin-1	QFGAQANVIGPWIQTK	Dioxidation (MW)	3	-2.170
	P27105*	Erythrocyte band 7 integral membrane protein	VIAAEGEMNASR	Oxidation (M)	13	-2.519
	P63261	Actin, cytoplasmic 2	EKLCYVALDFEQEMATAASSSSLEK	Dioxidation (C)	2	-3.143
	P18206	Vinculin	LANVMMGPYR	2 Oxidation (M)	1	-4.265
Treated	P07359	Platelet glycoprotein Ib alpha chain	AMTSNVASVQCDNSDKFPVYK	Carbamidomethyl (C);Oxidation (M)	11	1.982

	P19105;P24844;O14950	Myosin regulatory light chain 12A;Myosin regulatory light polypeptide 9;Myosin regulatory light chain 12B	DGFIDKEDLHDMLASLGK	Dioxidation (MW)	5	2.058
	P02679	Fibrinogen gamma chain	MLEEIMK	Oxidation (M)	13	2.146
	P23284	Peptidyl-prolyl cis-trans isomerase B	VIKDFMIQGGDFTR	Dioxidation (MW)	1	2.218
	P19105;P24844;O14950	Myosin regulatory light chain 12A;Myosin regulatory light polypeptide 9;Myosin regulatory light chain 12B	ELLTTMGDR	Dioxidation (MW)	2	2.839
	P68032;P63267;P62736;P68133	Actin, alpha cardiac muscle 1;Actin, gamma-enteric smooth muscle;Actin, aortic smooth muscle;Actin, alpha skeletal muscle	YPIEHGIITNWDDMEK	Kynurenin	8	3.278
	P31946	14-3-3 protein beta/alpha;14-3-3 protein beta/alpha, N-terminally processed	TAFDEAIAELDTLNEESYKDSTLIMQLLR	Oxidation (M)	3	4.970

* Probable contamination from residual red blood cells.

Table 2: Protein-protein interactions analysis of the 17 proteins containing 18 oxidized peptides in riboflavin/UVB-treated PCs at day 2. Platelet aggregation was the only biological process with a false discovery < 0.05.

Biological Process			
#pathway ID	***pathway description***	***count in gene set***	***false discovery rate***
GO.0070527	platelet aggregation	4	2.98E-04
Cellular component			
#pathway ID	***pathway description***	***count in gene set***	***false discovery rate***
GO.0005925	focal adhesion	7	1.09E-05
GO.0030055	cell-substrate junction	6	1.52E-04
GO.0030054	cell junction	8	3.07E-04
GO.0070062	extracellular exosome	11	3.58E-04
GO.0044421	extracellular region part	12	5.48E-04
GO.0044430	cytoskeletal part	8	6.61E-04
GO.0005829	cytosol	11	9.65E-04
GO.0031988	membrane-bounded vesicle	11	1.96E-03
GO.0005576	extracellular region	12	2.23E-03
GO.0042995	cell projection	8	0.00223
GO.0005856	cytoskeleton	8	0.00546
GO.0042470	melanosome	3	6.96E-03
GO.0043034	costamere	2	9.66E-03
GO.0005911	cell-cell junction	4	0.0124
GO.0015629	actin cytoskeleton	4	2.16E-02
GO.0043198	dendritic shaft	2	3.09E-02