Platelet Proteomics: Identification of Potential Therapeutic Targets

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Key Words

Platelet · Proteomics · Mass spectrometry · Platelet phosphoproteome · Platelet releasate · Membrane lipid rafts

Abstract

The integration of proteomics into biochemical and biological investigation has proved a powerful tool in understanding the anucleate platelet. Over the past few years, various approaches have been used to document platelet proteins and here, we describe our efforts to analyse specific platelet subproteomes so as to capture biologically relevant proteins of low abundance. These include characterisation of the phosphotyrosine proteome, the platelet releasate and more recently, the proteome of membrane lipid rafts. These approaches have revealed a wealth of relevant platelet proteins, which may in the future prove suitable as therapeutic targets in atherothrombosis.

Proteomics can be defined as the qualitative and quantitative study of the protein complement (proteome) of a genome expressed in a cell type or organism at a given time [1]. Proteomics thus endeavours to provide a comprehensive overview of all biological processes by cataloguing the components of biological networks and mechanisms and by defining protein-protein interactions and protein complexes, even those that occur transiently. However, to catalogue all human proteins and ascertain their functions and interactions, presents challenges. These include the fact that the human proteome is a highly complex and dynamic protein network generated from 30,000 human genes. Indeed, there are estimates of up to one million different protein components expressed by the human genome [2]. In addition, the dynamic range of protein concentration in cells varies by estimates of up to 8 orders of magnitude, making the detection of low-abundance, biologically relevant, proteins a particular challenge. However, the ability to study expression, localisation, modification and interaction of proteins as well as monitoring changes that could be a cause or a consequence of disease processes, is a step beyond genomics and can complement and extend traditional single molecule analysis. Thus, proteomics will offer unprecedented opportunities to unlock the mystery of biological processes and is one of the technologies most likely to foster the translation of basic biological knowledge into clinical applications.

To characterize functional protein networks and their

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dynamic alteration during physiological and pathological processes, proteins have to be identified, sequenced, categorized and classified with respect to their function and interaction partners. This is achieved by the combination of high-resolution protein separation techniques with mass spectrometry and modern sequence database mining tools, which permit the routine identification of hundreds or even thousands of proteins in a single experiment [3]. Key technologies include protein separation of complex mixtures by two-dimensional electrophoresis (2-DE) or multi-dimensional chromatography and protein identification by mass spectrometry (MS). 2-DE uses isoelectric focusing and gel electrophoresis to separate proteins by charge and molecular mass [4], while the most common multi-dimensional chromatographic method separates peptides by charge and hydrophobicity using ion exchange and reverse phase chromatography [5]. This method, unlike 2-DE, is amenable to automation and detects proteins not well represented by 2-DE, such as transmembrane and basic proteins. Recent advances have made MS an unsurpassed protein identification technique, due to the accuracy of mass detection, the detection sensitivity, the capacity to deal simultaneously with mixtures of multiple proteins and the fact that it can be automated making it high throughput. MS instruments range

from matrix-assisted laser desorption ionisation-time of flight (MALDI-TOF) MS, which can provide precise peptide masses, to complex tandem MS/MS machines that also enable peptide sequence determination for unambiguous protein identification [6,7].

As anucleate cell particles, platelets do not lend themselves to analysis by traditional cell and molecular biology [8]. In contrast, proteomics offers the opportunity to comprehensively explore the proteins involved in the various pathways of platelet function, from the sub-second events following platelet activation and adhesion through to platelet aggregation and granule secretion. Despite the extensive global studies of platelet biology over the past twenty years, the number of proteins identified in platelets is relatively low. For a review of these studies see Maguire & Fitzgerald 2003, and Maguire et al 2004 [9,10]. While such expression maps have provided important insights into platelet biology, a more challenging goal is to define the low abundant, biologically relevant proteins that comprise specific cellular compartments, mediate signal transduction pathways or form functional units. Thus, proteomic analysis must be focused on specific functions or compartments for the optimal analysis of the function of the more than one hundred thousand proteins estimated to be present in

Fig. 1. The phosphotyrosine proteome of human platelets. The tyrosine-phosphorylated proteome from (a) resting and (b) thrombin-activated human platelets were isolated using the monoclonal antibody 4G10 and then separated out by two-dimensional gel electrophoresis. pI 3-10 IPGphor™ strips were used in the first dimension and the gels were silver stained. Characteristic phosphotyrosine proteomes for (a) control and (b) thrombin activated platelets are shown.

platelets. Here, we briefly describe three such specific examples of platelet subproteomics: (1) the characterisation of the phosphotyrosine proteome, (2) the platelet releasate and more recently, (3) the analysis of membrane lipid rafts.

Characterisation of the Phosphotyrosine Proteome:

Key targets for novel drug development are the elements involved in the signaling cascades regulating platelet activation and aggregation. These signaling cascades are controlled both positively and negatively by phosphorylation [11]. Indeed, many protein and lipid kinases, phosphatases, phospholipases and GTPases are tyrosine phosphorylated during platelet activation and spreading [11] including the extracellular signal-related kinase-2 (ERK-2) and cytosolic tyrosine kinases e.g., Syk and Src [12, 13, 14]. However, it is likely that additional protein phosphorylation events contribute to the dynamic changes involved in platelet activation and aggregation and proteomics studies provide a novel approach in providing an overview of these phosphorylation cascades. For a comprehensive review of these platelet phosphoproteomics studies see Maguire & Fitzgerald 2003 and Maguire et al 2004 [9, 10].

We employed a proteomic strategy to monitor tyrosine phosphorylation upon activation of platelets by thrombin, with the aim of identifying novel signalling proteins [15]. To capture all dynamic phosphotyrosine changes tyrosine phosphorylated proteins were prefractionated by immunoprecipitation using the monoclonal antibody 4G10, thus enriching relatively low abundant phosphoproteins.

Table 1. 16 of the identified proteins in the thrombin-activated platelet releasate using 2D LC-MS/MS. Spectra from the two-dimensional liquid-chromatography tandem mass spectrometry (2D-LC MS/MS) run were identified using the SEQUEST program and a composite mouse and human database (NCBI July 2002 release). The gene name and number of peptides identified for these proteins are indicated. For the full list of 300 proteins, see Coppinger et al, 2004 [23].

Name	Gene name	Matching peptides
Human serum albumin	ALBU_HUMAN	12
Platelet factor 4	PLF4_HUMAN	18
Fibrinogen Alpha chain	FIBA_HUMAN	17
Platelet basic protein	SZO7_HUMAN	108
Platelet proteoglycan	PGSG_HUMAN	3
Thrombospondin	TSP1_HUMAN	37
SPARC (secreted protein acidic and rich in cysteine)	SPRC_MOUSE	17
Transforming growth factor	LTBS_HUMAN	2
Coagulation Factor V	FA5_HUMAN	1
Vitamin K dependent Protein S	PRTS_HUMAN	1
Amyloid beta protein	A4_HUMAN	1
Multimerin	ECM_HUMAN	1
Thymosin beta four	TYB4_MOUSE	1
Secretogranin III	SG3_MOUSE	6
Cyclophilin A	CYPH_HUMAN	5
Calumenin	CALU_HUMAN	4

Tyrosine phosphorylated proteins from both control and activated platelets were then separated using 2-DE (Figure 1). In combination with MALDI-TOF MS and immunoblotting, 67 differences were reproducibly highlighted between control and thrombin-activated platelets and several of these proteins were identified, including FAK, Syk, mitogen-activated protein kinase (MAPKKK) and activin receptor-like kinase 4 (ALK-4), which contain the known tyrosine phosphorylation motifs DXXY and EXXY [15].

More recently, we have extended this phosphoproteomic analysis and have identified the non-muscle myosin heavy chain type IIA (NMMHC-IIA), not previously known to be regulated by tyrosine phosphorylation in platelets. This phosphorylation event appeared to be reversible, as treatment of platelets with abciximab, a α IIB β 3 antagonist, at a concentration that blocked platelet aggregation, prevented this dephosphorylation. We found that the NMMHC-IIA tyrosine phosphorylation was a feature of many agonists and dephosphorylation was in each case triggered by engagement of α IIB β 3. A previously undescribed serine phosphorylation site was mapped to the C-terminus (position 1943) of NMMHC-IIA while bioinformatics analysis revealed a strong candidate for tyrosine phosphorylation at position 158. This protein appears to be of particular importance to platelet function as five autosomal-dominant disorders including May-Hegglin anomaly and Sebastian Syndromes are all caused by mutations in the NMMHC-IIA gene, and akin to Bernard Soulier Syndrome these pathologies are characterised by giant platelets and thrombocytopenia [16, 17, 18]. Our data suggests NMMHC-IIA is also involved in the activity of adult platelets.

Characterization of the Platelet Releasate

Platelets contain a number of preformed, morphologically distinguishable storage granules - α -granules, dense granules and lysosomes - the contents of which are released upon platelet activation [19]. Many proteins released in this manner act in an autocrine or paracrine fashion to modulate cell signaling including the prothrombotic factor XIII and the immune modulator platelet basic protein [20, 21]. Interestingly, platelet-derived chemokines such as platelet factor-4 (PF4) are found in atherosclerotic plaques, and may contribute to the inflammation that is a hallmark of the disease [22]. Thus, the platelet releasate contains factors that conceivably contribute to the development of atherothrombosis, yet despite this biological significance, the complement of proteins comprising the platelet releasate is largely uncharacterized. Moreover, secreted proteins would prove suitable as therapeutic targets, given their extracellular localisation and indeed, neutralisation of platelet-derived

Relastase profile

Fig. 2. Secretory profile of the proteins found in the platelet releasate. 37% of these proteins were previously reported to be released from platelets, including thrombospondin, platelet factor 4 (PF4) and metalloproteinase inhibitor 1. A further 35% were previously known to be re-leaked from other secretory cells, including cofilin, profilin and 14-3-3 ϵ/δ from dendritic cells and cyclophilin A from smooth muscle cells. The remaining 28% of proteins have not been reported as released from any cell type and included many proteins of unknown function that mapped to expressed sequence tags (ESTs).

Fig. 3. Flow cytometry analysis of platelet calumenin, secretogranin III and cyclophilin A. Permeabilized washed platelets were dual stained with the platelet activation marker CD62P-selectin PE labelled and one of three indirectly FITC-labelled antibodies for the following proteins: calumenin, Secretogranin III (SgIII) and Cyclophilin A. Events in the upper right quadrant display dual staining for both FITC and PE fluorescence. The % positive values were calculated as a % of total CD62P positive events.

pro-inflammatory soluble factors may provide a novel therapeutic strategy in atherosclerosis without the risk of bleeding that complicates direct inhibition of platelet activation.

Using a multi-layered proteomics approach, we have identified over 300 proteins released by human platelets following thrombin activation [23]. Firstly, the activated platelet releasate was separated by 2-DE and twenty-two Coomassie Blue stained protein spots were excised, proteolytically digested and identified with the use of MALDI-TOF MS. The identified proteins included the known platelet secreted proteins osteonectin, thrombospondin, 1-antitrypsin and proteins previously unknown to be released including 14-3-3 protein ϵ/δ and actin. In addition, the released protein fraction from thrombin-activated platelets was digested with trypsin and the resulting peptides separated using multi-dimensional chromatography coupled to tandem mass spectrometry. This approach enabled the discovery of >300 proteins, 81 of which were observed in two or three experiments (Table 1). 37% of these proteins were previously reported to be released from platelets, including thrombospondin, platelet factor 4 (PF4) and metalloproteinase inhibitor 1. A further 35% were previously known to be released from other secretory cells, including cofilin, profilin and 14-3-3 ϵ/δ from dendritic cells [24] and cyclophilin A from smooth muscle cells [25]. The remaining proteins have not been reported as released from any cell type and included many proteins of unknown function that mapped to expressed sequence tags (ESTs) (Figure 2).

Three proteins, secretogranin-III, a potential monocyte chemoattractant precursor; cyclophilin A, a vascular smooth muscle cell growth factor; calumenin, an inhibitor of the vitamin K epoxide reductase-warfarin interaction; not previously attributed to platelets were examined further as they are of potential interest in the pathogenesis of atherosclerosis.

sis. These proteins were confirmed to localize in platelets (Figure 3) and to be released upon activation. Furthermore, while absent in normal vasculature, these proteins were identified in human atherosclerotic lesions. Therefore, these and other proteins released from platelets may contribute to atherosclerosis and to the thrombosis that complicates the disease.

Analysis of Membrane Lipid Rafts:

Lipid rafts are compositionally distinct ordered membrane microdomains consisting of dynamic assemblies of cholesterol and sphingolipids [26]. These domains are implicated as platforms for many important biological processes including signaling transduction and vesicle trafficking. Indeed, several platelet-signaling pathways are thought to be orchestrated through lipid raft domains including those mediated by GPVI and GPIb-IX/V [27,28]. Additionally, several platelet proteins are raft-associated, including CD36, LAT and the Src-family kinases. Moreover, intra- or extracellular stimuli can trigger reorganization to an altered subset of proteins in the DRM fraction. Indeed, binding of vWF to GPIb-IX-V recruits GPIb [28] into DRMs and upon activation of GPVI, the GPVI-FcR complex is mobilized to platelet DRMs [27]. However, the exact role of platelet lipid rafts remains to be clarified. Thus, we have undertaken a proteomic profiling of both control and von Willebrand Factor (VWF)-activated platelet lipid rafts in order to attain a more functional understanding of these domains. The standard biochemical method to isolate lipid rafts makes use of their insolubility in non-ionic detergents at 4°C. These detergent-resistant membrane proteins (DRM proteins) float in low-density fractions from sucrose gradients [29] and the majority of studies detailing lipid raft function have relied on such methods for effective lipid raft purification.

Following isolation of platelet DRM proteins using Triton X-100, individual protein bands were resolved by one-dimensional electrophoresis and in-gel digestion using trypsin. The resulting peptides were analysed by tandem mass spectrometry. Using this approach, we achieved a proteomic profile of over 80 proteins in control and over 100 proteins VWF/ristocetin-activated platelet lipid rafts. 43% of these proteins had previously been found in DRM protein fractions of other cells, such as flotillin-1, CD109 and one protein with unknown function [30]. At present, the other 57% are unique to the platelet DRM fraction, including the Ras GTPase activating protein 3 and the small GTPase rap 1b. Moreover, activation through GPIb-IX-V resulted in the recruitment of additional proteins to DRMs including GPIb, a glucose transporter, a number of hypothetical proteins and

CLP-36. As an adaptor protein, CLP-36 may interact with GPIb-IX-V through α -actinin, thereby facilitating signaling through lipid rafts. These changes in the proteome of platelet lipid rafts upon activation suggest that these dynamic membrane domains may act as concentrating platforms that co-cluster receptors and signalling molecules, thus ensuring efficient, co-ordinated platelet activation processes. Targeting proteins recruited to platelet lipid rafts upon activation may represent a novel therapeutic strategy in the prevention of thrombosis.

Future Outlook

The current explosion in proteomics and related technologies will have a major impact in understanding all biological processes and thus, in the discovery of new diagnostics and therapeutics for human disease control. This is particularly true for platelets, which are readily available and have a limited repertoire of proteins and where the proteome is largely independent of changes in gene expression, at least in vitro. However, proteomics is a young field and there are only a handful of published examples applying proteomics to platelet research. Nevertheless, advances in analytical methods such as automated protein quantitation coupled to tandem MS, promises the emergence of qualitative studies of whole proteomes that rival gene expression analysis [31,32,33]. Additionally, affinity tag purification of protein complexes will allow enrichment for enzymes and enzyme complexes generated during platelet activation [34, 35]. Application of these technologies to platelets is challenging, however, using these technologies, it will be possible to compare platelet proteins in patients with arterial thrombosis and those with stable arterial disease and so link changes in protein abundance to the risk of the disease. As the function of each platelet protein is understood and as mechanisms regulating protein modifications are unraveled, new potential protein drug targets will be discovered, forming the basis for the design of more potent anti-thrombotic drugs.

References

- 1 Williams KL, Hochstrasser DF: Introduction to the proteome; in Wilkins MR, Williams KL, Appel RD, Hochstrasser DF (eds): *Proteome Research: New Frontiers in Functional Genomics*. New York, NY, Springer, 1998, vol 1, pp 1-12
- 2 Hochstrasser DF, Sanchez JC, Appel RD: Proteomics and its trends facing nature's complexity. *Proteomics* 2002;2:807-812.
- 3 Griffin TJ, Aebersold R: Advances in proteome analysis by mass spectrometry. *J Biol Chem* 2001;276:45497-45500.
- 4 Gorg A, Obermaier C, Boguth G, Harder A, Scheibe B, Wildgruber R, Weiss W: The current state of two-dimensional electrophoresis with immobilized pH gradients. *Electrophoresis* 2000;21:1037-1053.
- 5 Link AJ, Eng J, Schieltz DM, Carmack E, Mize GJ, Morris DR, Garvik BM, Yates JR III: Direct analysis of protein complexes using mass spectrometry. *Nat Biotechnol* 1999;17:676-682.
- 6 Pandey A, Mann M: Proteomics to study genes and genomes. *Nature* 2000;405:837-846.
- 7 Yates JR III: Mass spectrometry: from genomics to proteomics. *Trends Genet* 2000;16:5-8.
- 8 Fox JE: Platelet activation: new aspects. *Haemostasis* 1996;26:102-31.
- 9 Maguire PB, Fitzgerald DJ: Platelet proteomics. *J Thromb Haemost* 2003;1:1593-1601.
- 10 Maguire PB, Moran N, Cagney G, Fitzgerald DJ: Application of proteomics to the study of platelet regulatory mechanisms. *Trends Cardiovasc Med* 2004; In Press.
- 11 Santos MT, Moscardo A, Valles J, Martinez M, Pinon M, Aznar J, Broekman MJ, Marcus AJ: Participation of tyrosine phosphorylation in cytoskeletal reorganization, alpha(IIB)beta(3) integrin receptor activation, and aspirin-insensitive mechanisms of thrombin-stimulated human platelets. *Circulation* 2000;102:1924-1930.
- 12 Kramer RM, Roberts EF, Striffler BA, Johnstone EM: Thrombin induces activation of p38 MAP kinase in human platelets. *J Biol Chem* 1995;270:27395-27398.
- 13 Clark EA, Shattil SJ, Ginsberg MH, Bolen J, Brugge JS: Regulation of the protein tyrosine kinase pp72syk by platelets agonists and the integrin alpha IIb beta 3. *J Biol Chem* 1994;269:28859-28864.
- 14 Saki A, Rendu F, Bachelot-Loza C: Platelet alpha IIb-beta 3 integrin induces the tyrosine phosphorylation of cb1 and its association with phosphoinositide 3-kinase and syk. *Biochem J* 2000;351:669-676.
- 15 Maguire PB, Wynne KJ, Harney DF, O'Donoghue NM, Stephens G, Fitzgerald DJ: Identification of the phosphotyrosine proteome from thrombin activated platelets *Proteomics* 2002;2:642-648.
- 16 Seri M, Cusano R, Gangarossa S, Caridi G, Bordo D, Lo Nigro C, Ghiggeri GM, Ravazzolo R, Savino M, Del Vecchio M, d'Apolito M, Iolascon A, Zelante LL, Savoia A, Balduini CL, Noris P, Magrini U, Belletti S, Heath KE, Babcock M, Glucksman MJ, Aliprandis E, Bizzaro N, Desnick RJ, Martignetti JA: Mutations in MYH9 result in the May-Hegglin anomaly, and Fechtner and Sebastian syndromes. *Nat Genet* 2000;26:103-105.
- 17 Heath KE, Campos-Barros A, Toren A, Rozenfeld-Granot G, Carlsson LE, Savige J, Denison JC, Gregory MC, White JG, Barker DF, Greinacher A, Epstein CJ, Glucksman MJ, Martignetti JA: Nonmuscle myosin heavy chain IIA mutations define a spectrum of autosomal dominant macrothrombocytopenias: May-Hegglin anomaly and Fechtner, Sebastian, Epstein, and Alport-like syndromes. *Am J Hum Genet* 2001;69:1033-1045.
- 18 Di Pumpo M, Noris P, Pecci A, Savoia A, Seri M, Ceresa IF, Balduini CL: Defective expression of GPIb/IX/V complex in platelets from patients with May-Hegglin anomaly and Sebastian syndrome. *Haematologica* 2002;87:943-947.
- 19 Fukami H, Holmsen H, Kowalska M, Niewiarowski S: Platelet. Secretion; in Colman RW, Hirsh J, Marder VJ, Clowes AW, George JN (eds): *Haemostasis and Thrombosis: Basic Principles and Clinical practice* 4th Ed. Philadelphia, PA, Lippincott Williams & Wilkins, 2001, pp 561-574
- 20 Angelillo-Scherer A, de Frutos P, Aparicio C, Melis E, Savi P, Lupu F, Arnout J, Dewerchin M, Hoylaerts M, Herbert J, Collen D, Dahlback B, Carmeliet P: Deficiency or inhibition of Gas6 causes platelet dysfunction and protects mice against thrombosis. *Nat Med* 2001;7:215-221.
- 21 Castor CW, Walz DA, Ragsdale CG, Hossler PA, Smith EM, Bignall MC, Aaron BP, Mountjoy K: Connective tissue activation. XXXIII. Biologically active cleavage products of CTAP-III from human platelets. *Biochem Biophys Res Commun* 1989;163:1071-1080.
- 22 Huo Y, Schober A, Forlow S, Smith DF, Hyman MC, Jung S, Littman DR, Weber C, Ley K: Circulating activated platelets exacerbate atherosclerosis in mice deficient in apolipoprotein E. *Nat Med* 2003;9:61-67.
- 23 Coppinger JA, Cagney G, Toomey S, Kislinger T, Belton O, McRedmond J, Cahill DJ, Emili A, Fitzgerald DJ, Maguire PB: Characterization of the proteins released from activated platelets leads to localization of novel platelet proteins in human atherosclerotic lesions. *Blood* 2004;103:2096-2104.
- 24 Thery C, Boussac M, Veron P, Ricciardi-Castagnoli P, Raposo G, Garin J, Amigorena S: Proteomic analysis of dendritic cell-derived exosomes: a secreted subcellular compartment distinct from apoptotic vesicles. *J Immunol* 2001;166:7309-7318.
- 25 Jin Z, Melaragno M, Liao D, Yan C, Haendeler J, Suh YA, Lambeth JD, Berk BC: Cyclophilin A is a secreted growth factor induced by oxidative stress. *Circ Res* 2000;87:789-796.
- 26 Simons K, Ikonen E: Functional rafts in cell membranes. *Nature* 1997;387:569-572.
- 27 Locke D, Chen H, Liu Y, Liu C, Kahn ML: Lipid rafts orchestrate signaling by the platelet receptor glycoprotein VI. *J Biol Chem* 2002;277:18801-18809.
- 28 Shrimpton CN, Borthakur G, Larrucea S, Cruz MA, Dong JF, Lopez JA: Localization of the adhesion receptor glycoprotein Ib-IX-V complex to lipid rafts is required for platelet adhesion and activation. *J Exp Med* 2002 ;196:1057-1066.
- 29 Brown DA, Rose JK: Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. *Cell* 1992;68:533-544.
- 30 Foster LJ, De Hoog CL, Mann M: Unbiased quantitative proteomics of lipid rafts reveals high specificity for signaling factors. *Proc Natl Acad Sci USA* 2003;100:5813-5818.
- 31 Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, Aebersold R: Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat Biotechnol* 1999;17:994-999.
- 32 Mirgorodskaya OA, Kozmin YP, Titov MI, Korner R, Sonksen CP, Roepstorff P: Quantitation of peptides and proteins by matrix-assisted laser desorption/ionization mass spectrometry using (18)O-labeled internal standards. *Rapid Commun Mass Spectrom* 2000;14:1226-1232.
- 33 Cagney G, Emili A: De novo peptide sequencing and quantitative profiling of complex protein mixtures using mass-coded abundance tagging. *Nat Biotechnol* 2002;20:163-170.
- 34 Uetz P, Giot L, Cagney G, Mansfield TA, Judson RS, Knight JR, Lockshon D, Narayan V, Srinivasan M, Pochart P, Qureshi-Emili A, Li Y, Godwin B, Conover D, Kalbfleisch T, Vijayadamodar G, Yang M, Johnston M, Fields S, Rothberg JM: A comprehensive analysis of protein-protein interactions in *Saccharomyces cerevisiae*. *Nature* 2000;403:623-627.
- 35 Giot L, Bader JS, Brouwer C, Chaudhuri A, Kuang B, Li Y, Hao YL, et al: A protein interaction map of *Drosophila melanogaster*. *Science* 2004;302:1727-1736.