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## The Red Blood Cell Proteome and Interactome: An Update

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Although a preliminary portrait of the red blood cell proteome and interactome has already been provided, the recent identification of 1578 gene products from the erythrocyte cytosol asks for an updated and improved view. In this paper, we exploit data available from recent literature to compile a nonredundant list of 1989 proteins and elaborate it with pathway and network analyses. Upon network analysis, it is intuitively confirmed that red blood cells likely suffer of exacerbated oxidative stress and continuously strive against protein and cytoskeletal damage. It also emerges that erythrocyte interaction networks display a high degree of maturity. Indeed, a series of core proteins were individuated to play a central role. A catalytic ring of proteins counteracting oxidative stress was individuated as well. In parallel, pathway analysis confirmed the validity of observations about the SEC23B gene role in CDA II in a fast and unbiased way.

**Keywords:** red blood cell • proteomics • interactomics • pathway analysis • storage

### 1. The “Selfish -Omics” or the Clash of the “Omics”

More than a decade has passed since the beginning of the genomic era.<sup>1</sup> However, 20–25 000 sequenced genes later,<sup>2</sup> no definitive advances have been made in the biological quest for the infinitely small, as Pascal would say. At the end of the beginning biological systems still stood fierce,<sup>2</sup> shrouded in as much mystery as when the race begun. It soon became evident that, when the genetic horizons had been reached, deeper and detailed information about its actual expression was still lacking. Proteomics<sup>3</sup> (and transcriptomics)<sup>4</sup> has become popular in this very phase, for bridging the gap toward the new horizon of the protein complement to the genome. As we approach this horizon as well, multiple directions are unveiled, yet unexplored and just as much stimulating: metabolomics, lipidomics,<sup>5</sup> PTMomics,<sup>6</sup> and last but not least, interactomics.<sup>7</sup>

The next goal is to determine interactions among the whole expressed gene products in each cell/tissue to retrieve biologically relevant information from the mapped relations. The “omics-centered” view of scientific evolution recalls the gene-centric view of evolution from Dawkins’s masterpiece “The selfish gene”,<sup>8</sup> as it holds that, if we ever have at disposal the final tool to delve into biological complexity, it will be a holistic, system complexity-oriented one.

This article is intended to give an updated view of the red blood cell (RBC) proteome and interactome.<sup>9</sup> RBCs play a pivotal role in gas transport (i.e., oxygen and carbon dioxide) and a minor, but not less important, role in a range of other functions, such as transfer of GPI-linked proteins<sup>10,11</sup> and transport of iC3b/C3b-carrying immune complexes.<sup>12</sup>

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In humans, the circulating mature RBC is the end stage of a developmental process that starts in the bone marrow, as hematopoietic stem cells differentiate to enucleated reticulocytes.<sup>13</sup> Being enucleated, erythrocytes display a meager amount of mRNA, as a limited heritage from their reticulocyte ancestors. After extrusion of nuclei and degradation of internal organelles and endoplasmic reticulum, reticulocytes emerge in the circulation, where they rapidly develop into mature RBCs.<sup>14,15</sup> Until the end of its life span of  $120 \pm 4$  days, with 120 miles of travel and  $1.7 \times 10^5$  circulatory cycles, the human RBC has successfully coped with a number of dangers, such as passages across narrow capillaries and splenic slits, periodic high turbulences and high shear stresses, along with extremely hypertonic conditions. Owing to its constant cytoskeleton rearrangement, RBCs are able to traverse passageways as narrow as  $1 \mu\text{m}$  in diameter, by changing their shape from a biconcave disk of  $8 \mu\text{m}$  diameter to a cigar shape.<sup>16</sup> In this perfectly balanced cellular carrier, hemoglobin accounts for more than 90% of the cellular dry weight and approximately 98% of the overall cytoplasmic protein content.

This biological/technical shortcoming has hitherto hampered a comprehensive analysis of the RBC proteome by altering the outcomes with minor, albeit inevitable, losses of information about a whole “hidden proteome”. Therefore, although RBCs have always been eligible targets for proteomics investigations, only in the past few years have new technical advancements tackling the dynamic range issue, such as prefractionation methods adopting hexapeptide combinatorial ligand libraries, allowed a substantial improvement in this field.<sup>17</sup>

Hereby we briefly resume a few milestones in the erythrocyte proteomics research and present an updated interactomics analysis of these newly available data.

**Proteomics of RBCs: a Brief Update.** The story of RBC proteomics is full of gradual constant progresses over the past

**Table 1.** Relevant RBC Proteomic Studies<sup>a</sup>

authors	ref	year	methods	proteins individuated	annotations
Rosenblum	<sup>19</sup>	1982	2-DE (IEF-SDS)	More than 600 spots	A proteomic survey on RBC membrane proteins of adults, neonates and patients with erythrocyte membrane disorders.
Low et al.	<sup>20</sup>	2002	1-DE or 2-DE (IEF- SDS), MALDI-TOF	102 overall proteins (59 distinct polypeptides, 43 isoforms)	First in-depth study of RBC membrane proteins.
Kakhniashvili et al.	<sup>21</sup>	2004	rp-HPLC, IT-MS/MS; Gel filtration - LC-MS/MS	181 unique proteins (91 membrane proteins, 91 cytosolic proteins)	Glycophorins. At first, proteasomal subunits were attributed to a small contamination of reticulocytes. Western blot and immune-assays demonstrated that these proteins are expressed by mature RBCs too.
Tyan et al.	<sup>22</sup>	2005	Gold enzyme chip for tryptic digestion of proteins, MudPIT (SCX+rp-HPLC, ESI-MS/MS analysis)	272 proteins (but only 30 by 2 unique peptides)	This manuscript introduces a novel technology for preliminary sample treatment.
Bruschi et al.	<sup>23</sup>	2005	2-DE (Immobiline gels instead of IPG strips + SDS) + MALDI-TOF	500 spots (but only a few new proteins)	Many filamentous proteins (spectrins and ankyrins) were detected.
Pasini et al.	<sup>15</sup>	2006	1-DE (SDS), in-gel digestion, LC-ESI-MS/MS (either Q-TOF and LTQ-FT MS)	566 (340 membrane proteins and 252 soluble proteins)	RBCs from control donors were analyzed after 72 and 96 h of storage. Thirty-nine proteins were found to anomalously migrate and displayed altered MW (degradation or incorrect maturation) were proposed as the likely causes.
Goodman et al.	<sup>9</sup>	2007	<i>In silico</i> analysis of yet existing databases	751 (review resuming previous studies and depicting a preliminary interactome)	Protein-protein interactions were graphed. The "Repair or destroy" (ROD) box was the fulcrum of the protein network. ROD was made up of chaperonines, heat shock proteins and proteasomal subunits. Being anucleated, erythrocytes almost do not synthesize new proteins. Thus ROD proteins may operate a pivotal role in refolding damaged ones.
D'Amici et al.	<sup>26</sup>	2007	2-DE (IEF-SDS), in-gel digestion, nanorp-HPLC-ESI-MS/MS (IT, Q-TOF)	392 (day 0)  487 (day 14) 447 (day 42)	Erythrocytes were analyzed after 0, 7, 14, or 42 days of 4 °C storage under aerobic or anaerobic (under helium) conditions, in presence or absence of protease inhibitors.
Bosman et al.	<sup>27</sup>	2008	1D-GE (SDS), in-gel digestion, nanoHPLC-ESI-Q/IT-FTICR; semiquantification with emPAI method (spectral counting exponentially modified protein abundance index)	257 (from membrane and vesicles during storage)	Storage reduced membrane-protein variability (less band 3, small G proteins, chaperones and components of the proteasome were observed) while increased the total number of microvesicle-isolated proteins (especially as it regarded Hb, band 3, CD47, complement proteins and metabolic enzymes); glucose transporter was found to increase in the membrane fraction of stored RBCs, suggestive of RBC resistance to storage lesions.
Simò et al. Bachi et al.	<sup>28,29</sup>	2008	Combinatorial Ligand Libraries (CLL) - ProteoMiner - 2-DE (IEF-SDS), nanoLC-ESI-MS/ MS	800 proteins	Preliminary sample fractionation through Proteominer technology allowed resolving proteins at a far higher resolution than ever before.
Roux-Dalvai et al.	<sup>31</sup>	2008	Combinatorial Ligand Libraries (CLL) - ProteoMiner - 2-DE (IEF-SDS), Orbitrap MS	1578 proteins in the cytoplasmic fraction	Peptide ligand libraries allowed in-depth proteomic analysis of RBCs and revealed a whole hidden proteome.

<sup>a</sup> Adapted from Liumentano et al., 2009.<sup>18</sup>

**Table 2.** RBC Interactome: Top 15 Pathways (Out of 850)

	category	function	function annotation	P-value	molecules	# molecules
1.	molecular transport	transport	transport of protein	$1.39 \times 10^{-22}$	AP1B1, AP1G1, AP1M1, AP2A1, AP2A2, AP2M1, AP3S1, AP4S1, ARCN1, ARF1, ARF6, ARFGAP1, ARFIP1, ASPSCR1, CALR, CFL1, CHMP5, DCTN1, DNAJA2, DNAJA4, EIF5A, ERP29, GDI2, HTT, IPO7, IPO9, IPO11, KPNA4, KPNA6, KPNB1, LGTN, MYH9, NAPA, NDE1, NEDD4, NPM1 (includes EG:4869), NRBP1, NUTF2, PDIA3, PEX5, PTPN11, RAB10, RAB13, RAB1A, RAB2A, RAB3GAP2, RAB4A, RAB7A, RAN, RANGAP1, RFFL, RHOB, SCAMP2, SCFD1, SEC22B, SEC23IP, SNX1, SNX9 (includes EG:51429), STX7, TMED10, TMX1, TRAPPc3, USE1, USO1, VAPA, VCP, VPS45, XPO1, XPO5, XPO7, YKT6, YWHAH, ZW10	73
2.	protein synthesis	metabolism	metabolism of protein	$2.67 \times 10^{-17}$	ABCF1, ACHE, ACO1, ADRM1, ANAPC5, ANPEP, APP, ARIH1, ARIH2, ATG7, ATG4A, ATG4B, BAG1, BAG2, BCL10, BLMH, CALR, CAPN1, CAPN2, CAPNS1, CASP3, CASP8, CAST, CKAP5, COP5, CTSG, CUL2, CUL3, DPP3, EEF2, EEF1A1, EEF1A2, EGLN2, EIF5, EIF2B1, EIF2B2, EIF2B3, EIF2B4, EIF2S1, EIF2S3, EIF3F, EIF3G, EIF3I (includes EG:8668), EIF3J, EIF4A1, EIF4A3 (includes EG:9775), EIF4B, EIF4E, EIF4G1, EIF5A, ELANE, FAF1, FBXO7, FLNA, FN1, GLMN, GSPT1, HBS1L, HDAC6, HGS, HNRNPK, <b>HSPB1</b> , HTT, IDE, IL18, IMPACT, INPP5D, KIAA0368, LNPEP, MAP2K3, MAPK1, METAP1, MTOR, MYH9, NACA, NCSTN, NEDD4, NFX1, PAIP1, PDIA2, PEPD, PIK3R1, PREP, PSMB3, PSMB5, PSMC2, PSMC4, PSMD14, PTBPI, RAD23A, RBM3, RNPEP, RPL8, RPL11, RPL22, RPL26, RPL30, RPL31, RPS2, RPS3, RPS5, RPS6, RPS9, RPS10, RPS11, RPS19, RPS12 (includes EG:6206), RPS17 (includes EG:6218), RPS3A, RPS4X, RPS6KA1, RPS6KB1, SERPINB1, SSB, STAT5B, THBS1, THOP1, TPP2, TSG101, UBA3, UBE2A, UBE2H (includes EG:7328), UBE2I, UBE2K, UBE2L3, UBE2N, UBE4B, UBR1, UFD1L, USE1, USP11, USP9X, VCP, XPNPEP1, XPO1	135
3.	cellular assembly and organization	transport	transport of vesicles	$2.83 \times 10^{-16}$	ACTR1A, AP1B1, AP1G1, AP1M1, AP1S1, AP2A1, AP2A2, AP2M1, AP3S1, AP4S1, APOA1, APOE, ARF1, ARF6, CHMP1A, CLINT1, COPZ1, CPNE1, CPNE3, CYTH1, DENND1A, EPN1, EPS15, GSN, HDAC6, HTT, ITSN1, M6PRBP1, MAP2K1, NAPA, NAPG, NDE1, PAFAH1B1, PICALM, RAB13, RAB1A, SCAMP2, SCFD1, SEC22B, SEC23B, SNAP23, SNCA, SOD1, SPTBN4, STX4, STX6, STX7, STX16, SYNJ1, USO1, VPS33B, VPS4A	52
4.	post-translational modification and protein folding	folding	folding of protein	$3.94 \times 10^{-14}$	AARS, BAG2, BAG5, CALR, CCT3, CCT7, CCT6A, DNAJA2, DNAJA4, DNAJB6, DNAJB2 (includes EG:3300), ERAF, ERP29, FKBP4, FKBP5, <b>HSP90AA1</b> , <b>HSP90AB1</b> , <b>HSPA5</b> , <b>HSPA8</b> , <b>HSPBP1</b> , PDIA2, PFDN2, PFDN4, PIN4, PPIA (includes EG:5478), RP2, RUVBL2, SH3GLB1, ST13, TBCA, TCP1, TXN, UGCGL1	33

**Table 2.** Continued

category	function	function annotation	P-value	molecules	# molecules
5. cellular assembly and organization	fusion	fusion of cellular membrane	$1.43 \times 10^{-12}$	ANXA1, ANXA7, ATG7, CTBP1, GCA, NAPA, NAPG, NPLOC4, NSFL1C, RABEP1, RAB1F, SNAP23, SNAP29, USO1, VAMP3, VAPA, VPS4B, VTI1A	18
6. immunological disease	acute allergic pulmonary	acute allergic pulmonary eosinophilia	$1.22 \times 10^{-10}$	ACTB, ALB, ALDOA, ARG1, ENO1, HNRNPAB, <b>HSPA5</b> , MYH9, P4HB, PDIA3, PRDX1, PRDX6, SELENBP1, STAT6, TKT, TPI1, TUBB	17
7. cellular function and maintenance	endocytosis	endocytosis	$1.02 \times 10^{-9}$	ACTN4, AP1S1, AP2A2, APP, ARF6, ARRB2, ATP5B, ATP6 V1H, CAP1, CD44, CD2AP, CDC42, CTTN, DENND1A, DNM2, EHD1, EPN1, EPS15, HGS, HTT, ITSN1, KRAS, NAE1, NECAP1, NEDD4, PICALM, RAB15, RAB22A, RAB7A, RABEP1, RAC1, REPS1, RHOA, RHOB, SCAMP2, SNX1, SNX2, SNX3, SYNJ1	39
8. protein degradation	catabolism	catabolism of protein	$1.27 \times 10^{-9}$	ANAPC5, ARIH1, ARIH2, ATG7, ATG4B, CAST, CUL2, CUL3, EGLN2, FAF1, FBXO7, FLNA, HDAC6, HGS, KIAA0368, LNPEP, MTOR, NCSTN, NEDD4, PSMB3, PSMC2, PSMD14, SERPINB1, UBE2A, UBE2H (includes EG:7328), UBE2I, UBE2K, UBE2L3, UBE2N, UBE4B, UBR1, UFD1L, USE1, USP11, VCP, XPO1	36
9. protein synthesis	synthesis	synthesis of protein	$2.24 \times 10^{-9}$	ABCF1, ACO1, APP, BCL10, CALR, CASP3, CKAP5, COPS5, DCTN2, EEF2, EEF1A1, EEF1A2, EIF5, EIF2B1, EIF2B2, EIF2B3, EIF2B4, EIF2S1, EIF2S3, EIF3F, EIF3G, EIF3I (includes EG:8668), EIF3J, EIF4A1, EIF4A3 (includes EG:9775), EIF4B, EIF4E, EIF4G1, EIF5A, ELANE, FN1, GLMN, GSN, GSPT1, HBS1L, HNRNPK, <b>HSPB1</b> , IL18, IMPACT, INPP5D, MAP2K3, MAPK1, METAP1, METAP2 (includes EG:10988), MTOR, NACA, NFX1, NPM1 (includes EG:4869), PAIP1, PIK3R1, PTBP1, PTPN11, RBM3, RPL8, RPL11, RPL22, RPL26, RPL30, RPL31, RPS2, RPS3, RPS5, RPS6, RPS9, RPS10, RPS11, RPS19, RPS12 (includes EG:6206), RPS17 (includes EG:6218), RPS3A, RPS4X, RPS6KA1, RPS6KB1, SSB, STAT5B, THBS1	76
10. cellular assembly and organization	development	development of cytoskeleton	$4.00 \times 10^{-9}$	ACTB, ADD1, AP1G1, APOE, ARF6, ARHGAP4, ARHGDI, ARPC5, CALR, CAP1, CDC42, CFL1, CNP, CORO1C, CRK, CSRP1, EPB49, FLG (includes EG:2312), FLNA, FLNB, FSCN1, GABARAP, KRAS, LSP1, MAEA, MAP1S, MYH10, PACSIN2, PAFAH1B1, PLEK2, RAC1, RAN, RHOA, ROCK1, ROCK2, SHC1, SHROOM3, TLN1, TPM1, TUBG1, VASP	41
11. cellular assembly and organization	formation	formation of vesicles	$5.56 \times 10^{-9}$	ANXA5, AP2A2, ARF1, ARFGAP1, ARFGEF2, ATG9A, C3, CAST, CLTC, EPS15, HGS, HTT, MTOR, NSF, PTPNA, PRKACA, ROCK1, ROCK2, YKT6	19
12. cellular assembly and organization	biogenesis	biogenesis of cytoskeleton	$5.96 \times 10^{-9}$	ADD1, AP1G1, APOE, ARF6, ARHGAP4, ARHGDI, ARPC5, CALR, CAP1, CDC42, CFL1, CNP, CORO1C, CRK, CSRP1, EPB49, FLG (includes EG:2312), FLNA, FLNB, FSCN1, GABARAP, KRAS, LSP1, MAEA, MAP1S, MYH10, PACSIN2, PAFAH1B1, PLEK2, RAC1, RAN, RHOA, ROCK1, ROCK2, SHC1, SHROOM3, TLN1, TPM1, TUBG1, VASP	40

**Table 2.** Continued

category	function	function annotation	P-value	molecules	# molecules
13. cell death	cell death	cell death of cell lines	$7.99 \times 10^{-9}$	ABCC1, ABCC4, ABCE1, ABCG2, ACHE, ACTB, ADRM1, AHSA1, AKT1S1, ALDH3A1, AP2A2, APOE, APP, ARHGDIA, ARRB2, ATG7, ATP2B1, ATXN3, BAD, BAG1, BAT3, BCL10, BCL2L1, BID, BTK, CALR, CAPNS1, CARD8, CASP3, CASP8, CAST, CAT, CCDC6, CCT2, CCT3, CCT5, CCT7, CCT8, CCT6A, CD44, CD47, CD55, CD59, CD99 (includes EG:4267), CDC42, CDKN2C, CHMP5, CIAPIN1, CIB1, CLU, COPSS1, CRK, CSNK1A1, CSNK2A1, CSNK2A2, CSTA, CTBP1, CTTN, CYB5R3, DCTN2, DDX3X, DFFA, DNAJB1, DNM1L, EEF1A1, EEF1A2, EIF4E, ENO1, FADD, FAF1, FASN, FKBP5, FN1, FNTA, FREQ, FTH1, FUBP1, G6PD, GAB1, GAPDH (includes EG:2597), GLO1, GLRX, GMFB, GNAS, GNB1, GNB2, GPI, GPX4, GSN, GSR, GSTP1, HCLS1, HNRNPA1, HNRNPC, <b>HSP90AB1, HSPA5, HSPA8, HSPB1, HTATIP2, HTT, HUWE1, IGHM, IL18, INPP5D, ITSN1, JUP, KRAS, LCMT1, LDHA, LGALS3, LGALS9, LSP1, MAP2K1, MAP2K4, MAPK1, MAPKAP1, MCTS1, MIB1, MSN, MTOR, NAMPT, NAPA, NFKBIB, NME1, NMNAT3 (includes EG:349565), NPM1 (includes EG:4869), NRAS, NUDCD3, P4HB, PA2G4, PAK2, PARK7, PCBP2, PDCD6IP, PDIA3, PEA15, PEBP1, PIK3R1, PIN1, PLSCR1, PML, PPIA (includes EG:5478), PPM1A, PPP2CA, PPP2R1A, PPP2R1B, PPP2R2A, PPP5C, PRDX1, PRDX2, PRKAA1, PRKACA, PRKAR1A, PRKAR2B, PTPN6, PTPN11, PURA, RABGGTA, RABGGTB, RAC1, RAD23B, RDX, RGS10, RHOA, RHOB, RNF7, RPLP0 (includes EG:6175), RPS6KA1, RPS6KB1, S100A4, S100A6, S100A8, S100A9, S100A11, SERPINB3, SERPINB5, SFN, SH3GLB1, SHC1, SIRT2, SLC2A1, SLK, SMAD2, SNCA, SOD1, SRPK1, STAT6, STAT5B, SYK, TAOK3, TCP1, TGM2, THBS1, TMX1, TPM1, TPP2, TRADD, TRAP1, TSG101, TUBA1A, TXN, TXNDC17, TXNRD1, UBA1, UBQLN1, USE1, VCP, VPS28, XRCC5, YARS, YWHAB, YWHAE, YWHAQ (includes EG:10971), ZMYND11)</b>	214
14. post-translational modification	modification	modification of protein	$1.29 \times 10^{-8}$	AADACL1, AARS, ACP1, ALDH1A1, ALDH3A1, APOA1, APOE, APP, ARAF, ARD1A, ARRB2, ATG3, ATG7, BAG2, BAG5, BCL10, BCL2L1, BSG, BTK, CALR, CAND1, CAPN1, CARM1, CAST, CAT, CCT3, CCT7, CCT6A, CD44, CD47, CD55, CDK2, CRK, CSNK2A1, CUL1, CUL2, CUL5, DNAJA2, DNAJA4, DNAJB6, DNAJB2 (includes EG:3300), ERAF, ERP29, FKBP4, FKBP5, FN1, FTH1, FTL, GSPT1, GYPC, HDAC6, <b>HSP90AA1, HSP90AB1, HSPA5, HSPA8, HSPB1</b> , HUWE1, IGHM, IMPACT, KEL, LCMT1, MAP2K4, MAPK1, METAP2 (includes EG:10988), MLST8, MOBKL1A, MTOR, NAE1, NCSTN, NEDD4, NME1, P4HB, PAK2, PARK7, PCMT1, PCNP, PDIA2, PDIA3, PFDN2, PFDN4, PIN4, PML, PPAP2A, PPIA (includes EG:5478), PPM1A, PPM1B, PPM1F, PPME1, PPP1CB, PPP2CA, PPP2R1A, PPP2R2A, PPP5C, PPP6C, PRDX1, PRDX6, PRKACA, PRKDC, PTPN6, PTPN7, PTPN11, RABGGTA, RFFL, RP2, RUVBL2, SET, SH3GLB1, SIRT2, SIRT5, SNCA, SPTBN1, ST13, STK38, STK38L (includes EG:23012), SYK, TAOK3, TBCA, TCEB1, TCEB2, TCP1, THBS1, TPP2, TSG101, TSTA3, TTN, TXN, UBA1, UBA3, UBE2H (includes EG:7328), UBE2I, UBE2L3, UBE2M, UBE2N, UBE3C, UBE4B, UBL4A, UGCGL1, USP7, WNK1	139

**Table 2.** Continued

category	function	function annotation	P-value	molecules	# molecules
15. cell death	cell death	cell death	$4.08 \times 10^{-8}$	AARS, ABCC1, ABCC4, ABCE1, ABCG2, ACHE, ACIN1, ACSL4, ACTB, ACTN4, ADRM1, AHSA1, AKT1S1, ALB, ALDH1A1, ALDH3A1, ALDOA, ALDOC, ANP32A, ANPEP, ANXA1, ANXA7, AP2A2, APEX1, APOA1, APOE, APP, APRT, ARF6, ARG1, ARHGDIA, ARRB2, ATG7, ATP1A1, ATP1A2, ATP2B1, ATP2B4, ATXN3, BAD, BAG1, BAG5, BAT3, BCL10, BCL2L1, BID, BLVRA, BRCC3, BRE, BSG, BTK, C3, CALR, CAPN1, CAPNS1, CARD8, CASP3, CASP8, CAST, CAT, CCDC6, CCT2, CCT3, CCT5, CCT7, CCT8, CCT6A, CD44, CD47, CD55, CD59, CD2AP, CD99 (includes EG:4267), CDC37, CDC42, CDK2, CDKN2C, CHMP5, CIAPIN1, CIB1, CLU, CNP, COPS5, CR1, CRADD, CRK, CSDA, CSE1L, CSNK1A1, CSNK2A1, CSNK2A2, CSTA, CTBP1, CTSG, CTTN, CUL1, CUL2, CUL3, CUL5, CUL4A, CYB5R3, DCTN2, DDX3X, DFFA, DNAJB1, DNAJB6, DNAJB2 (includes EG:3300), DNAJC5, DNM2, DNM1L, EEF1A1, EEF1A2, EEF1D, EEF1E1, EIF2S1, EIF4E, EIF5A, ELANE, ENO1, ERAF, EZR, FADD, FAF1, FASN, FIS1, FKBP5, FLNA, FN1, FNTA, FREQ, FTH1, FUBP1, G6PD, GAB1, GAPDH (includes EG:2597), GCLC, GCLM, GLO1, GLRX, GMFB, GNA13, GNAQ, GNAS, GNB1, GNB2, GPI, GPX4, GSN, GSPT1, GSR, GSTP1, HCLS1, HDGF, HIST1H1C, HMGB1 (includes EG:3146), HNRNPA1, HNRNPC, HPRT1, <b>HSP90AA1, HSP90AB1, HSPA2, HSPA5, HSPA8, HSPB1, HTATIP2, HTT, HUWE1, IGHG1, IGHM, IL18, INPP5D, IQGAP2, IRF3, ITSN1, JMJD6, JUP, KRAS, LCMT1, LDHA, LGALS3, LGALS9, LSP1, LYZ, MAEA, MAP1S, MAP2K1, MAP2K2, MAP2K3, MAP2K4, MAPK1, MAPKAP1, MCTS1, MDH1, MIB1, MSN, MTOR, NAE1, NAMPT, NAPA, NFKBIB, NME1, NMNAT3 (includes EG:349565), NP, NPM1 (includes EG:4869), NQO2, NRAS, NSF, NUDCD3, OPTN, P4HB, PA2G4, PAFAH1B1, PAFAH1B2, PAFAH1B3, PAK2, PARK7, PCBP2, PDCD5, PDCD6, PDCD6IP, PDIA2, PDIA3, PEA15, PEBP1, PIK3CB, PIK3R1, PIN1, PITPNA, PLSCR1, PML, PPIA (includes EG:5478), PPM1A, PPM1F, PPP2CA, PPP2R1A, PPP2R1B, PPP2R2A, PPP2R5A, PPP5C, PRDX1, PRDX2, PRDX5, PRDX6, PRG2 (includes EG:5553), PRKAA1, PRKACA, PRKAR1A, PRKAR2B, PRKDC, PSMB1, PSMG2, PTPN6, PTPN11, PURA, RABGGTA, RABGGTB, RAC1, RAD50, RAD23B, RDX, RGS10, RHOA, RHOB, RNF7, ROCK1, RPLP0 (includes EG:6175), RPS3, RPS6, RPS3A, RPS6KA1, RPS6KB1, S100A4, S100A6, S100A8, S100A9, S100A11, SEMA7A, SERPINB3, SERPINB5, SET, SFN, SH3BGRL3, SH3GLB1, SHC1, SIRT2, SLC2A1, SLC2A3, SLK, SMAD2, SNCA, SOD1, SRPK1, STAM, STAMBP, STAT6, STAT5B, STIP1, STK24, SWAP70, SYK, TAOK3, TCP1, TGM2, THBS1, THG1L, TMX1, TPM1, TPM3, TPP2, TRADD, TRAP1, TSG101, TUBA1A, TXN, TXNDC17, TXNL1, TXNRD1, UBA1, UBA3, UBE2K, UBE2M, UBE4B, UBQLN1, UBR4, USE1, USP7, VAMP3, VAPA, VAPB, VCL, VCP, VPS28, XRCC5, YARS, YWHAB, YWHAE, YWHAQ (includes EG:10971), ZMYND11</b>	337

**Table 3.** RBC Interactome: Top 50 Networks

ID	molecules in network	score	focus molecules	top functions
1	ACAP2, ANXA5, ARF6, C6ORF211, CIAPIN1, DDX17, EIF6, EPPK1, FIBP, GANAB, GLRX3, ILF3, IQSEC1, NCAPD2, NCAPG (includes EG:64151), NCAPH, NPEPPS, NPM1 (includes EG:4869), PFDN2, PGLS, PLS1, PLSCR1, PLSCR4, PTBP1, RAB3GAP2, RPS29, SAR1B, SBDS, SEC13, SEPT2, SHC1, SMC2, SMC4, TARS, TFG	43	35	DNA Replication, Recombination, and Repair, Cell Cycle, Cellular Assembly and Organization
2	ARL3, ASPSCR1, ATXN3, CUL3, FAF1, GLMN, GOLGA3, GOLGA7, Mapk, NFIA, NGLY1, NPLOC4, NSFL1C, PAAF1, PLAA, PSMC1, PSMD1, PSMD7, RAD23A, RAD23B, RASA2, RP2, RPL22, TP53I3, TSN, TSNAZ, UBA3, UBE4B, UBR1, UBR4, UBXN1, UBXN6, UFD1L, VCP, VCPIP1	41	34	Protein Degradation, Protein Synthesis, Cellular Assembly and Organization
3	Ap1, BLVRA, CCT2, CCT3, CCT5, CCT7, CCT8, CCT6A, CD58, CSNK2A2, HMBS, IGBP1, NACA, PACS1, PAFAH1B2, PBK, PP1/PP2A, PPP1CB, PPP1R7, PPP1R11, PPP2CA, PPP2R4, PPP2R1A, PPP2R1B, PPP2R2A, PPP2R5A, PPP2R5B, PPP2R5D, PPP4C, PPP4R1, PPP6C, SSSCA1, TCP1, TIPRL, TOM1	38	33	Cancer, Cell Death, Reproductive System Disease
4	ACAT2, ADD3, AKR1A1, AKR1B1, AKR7A2, Aldehyde reductase, ATG3, ATG7, ATG4A, ATG4B, FN3K, GABARAP, GABARAPL2 (includes EG:11345), GBAS, GOT1, HBB (includes EG:3043), HBD, HBE1, HBG1, HBQ1 (includes EG:3049), HBZ, HEBP1, IRGQ, LRSAM1, MDH1, NANS, NFE2, PDGF BB, PDLIM1, SLC29A1, SNX2, VAPA, VAPB, VPS35, YARS	38	33	Cell Morphology, Cellular Compromise, Small Molecule Biochemistry
5	APRT, ARFIP1, ARL6IP5, C6ORF108, COMMD9, COMMD10, EHBP1L1, FREQ, LIN7C, MIR1, MTM1, MTMR12, PAICS, PGM2, PGRMC2, PHGDH, Rab11, RAB14, RAB11B, RAB2B, RABGAP1L, RNH1, RPS10, RPS11, SARS, SLC2A4, SYNGR2, TKT, TMX1, TRAPPC3, TRAPPC2L, TRAPPC6B, TWF1, UGCGL1, WDR44	38	33	Genetic Disorder, Skeletal and Muscular Disorders, Carbohydrate Metabolism
6	ACTBL2, Alpha catenin, ANXA2, ARD1A, ARRB2, Cadherin, CCS, CLTA, CLTB, CPNE1, DDX27, EIF5A, FLNA, FLNC, HNRNPK, JUP, LGALS9, NAT13, PDIA2, PIK3RI, RPL30, RPS2, RPS5, RPS13, RPS19, RPS20, RPS23, RPS17 (includes EG:6218), RPS3A, Sapk, SNX8, SORBS1, TTN, TUBA1B, VPS13A	36	32	Protein Synthesis, Hematological System Development and Function, Hematopoiesis
7	ABCC1, ACIN1, ATP5B, ATP6 V0A1, ATP6 V0C, ATP6 V1A, ATP6 V1B2, ATP6 V1D, ATP6 V1F, ATP6 V1G1, ATP6 V1H, ATPase, BAT1, DDX19B, DHX15, H+-transporting two-sector ATPase, IDE, NSF, PSMB5, Psmb5-Psmb6-Psmb8-Psmb9, PSMC2, PSMC3, PSMC4, PSMC6, PSMD6, RAB6C, RP11-529I10.4, RPS15A, RUVBL1, RUVBL2, SKIV2L, SPAST, TRAP1, TXNL1, UBE3C	36	32	Molecular Transport, Cellular Compromise, Infectious Disease
8	ABCG2, ADK, APP, ATP7A, CAP1, CLIC1, EIF4E, FERMT3, FLNB, FLOT1, FLOT2, Flotillin, GBE1, GLRX, GLUL, GRHPR, GSR, ILF2 (includes EG:3608), LSM1, LSM2, LSM3, LSM4, LSM5, LSM6, LSM7, LSM8, NAGK, NARS, PGD, PLEKHF2 (includes EG:79666), PPME1, RTN3, TALDO1, UBE2K, ZNF259	36	34	RNA Post-Transcriptional Modification, Carbohydrate Metabolism, Small Molecule Biochemistry
9	C4, C3-Cfb, C4B, CALCOCO1, CAND1, CD55, COPS2, COPS3, COPS4, COPS5, COPS6, COPS8, COPS7A, COPS7B, CR1, CUL1, CUL2, CUL4A, CUL4B, CYB5R3, DDB1, GPS1 (includes EG:2873), HDGF, IQWD1, LSP1, NFKB (complex), OPTN, RBX1 (includes EG:9978), RNF7, STK10, TBC1D17, TRAFD1, UBXN7, WDR23, WDR26	34	32	Cell-To-Cell Signaling and Interaction, Hematological System Development and Function, Hematopoiesis
10	AGFG1 (includes EG:3267), ATP1A2, BSG, C10ORF97, Catalase, CSTA, DENR (includes EG:8562), DUSP23, EIF4A3 (includes EG:9775), EPS15L1, IFI35, IPO11, LPIN2, LPIN3, MAGOH, MAPK1, MCTS1, METAP2 (includes EG:10988), Na-k-atpase, NT5C3, PA2G4, Phosphatidate phosphatase, Pki, PPAP2A, RANBP3, RBM8A, RNF123 (includes EG:63891), RPL8, RPL27, RPL12 (includes EG:6136), RSU1, SLC16A1, SLC43A1, UBAC1, ZMYND11	34	31	Molecular Transport, Small Molecule Biochemistry, RNA Damage and Repair

**Table 3.** Continued

ID	molecules in network	score	focus molecules	top functions
11	BAG2, EEA1, GAPVD1, GDI1, GDI2, GNL1, M6PRBP1, NAE1, NAPA, NAPB, NAPG, P38 MAPK, RAB4, Rab5, RAB1A, RAB22A, RAB4A, RAB4B, RAB5B, RAB5C, RAB7A, RABEP1, RABGEF1, RABGGTA, RABGGTB, RILP, Snare, STX6, STX7, STX16, TXNDC17, VAMP3, VPS45, VTI1A, YKT6	34	31	Cellular Assembly and Organization, Molecular Transport, Protein Trafficking
12	ACO1, AGTRAP, ANXA11, BANF1, CDC2L5, CPNE3, FTH1, FTL, GABARAPL1, GAPDH (includes EG:2597), GSS, HCCA2, HIST1H1C, HNF4 $\alpha$ dimer, HNRNPH3, HPCAL1, Jnk, MOBKL1A, PDXK, PON1, POSTN, PRPS1, PRPS1L1, PRPSAP2, Ribose-phosphate diphosphokinase, RPS4X, S100A6, SERPINB3, SHMT1, STK24, STK38, STK38L (includes EG:23012), SYNCRI, TAOK3, Transferase	34	31	Cancer, Cellular Growth and Proliferation, Hematological Disease
13	BAT3, CD99 (includes EG:4267), CSDA, FCN1, FKBP2, FKBP3, FKBP4, FKBP5, FKBP15, Peptidylprolyl isomerase, peroxidase (miscellaneous), PIN1, PIN4, Pka, PPIA (includes EG:5478), PPIB, PPIL1, PPIL4, PRDX1, PRDX2, PRDX5, PRDX6, RALA, Rap, RAP1A, RAP1B, RAP2A, RAP2B, RIC8A, RPL13A, SEMG1 (includes EG:6406), UBL7, UBQLN1, UBQLN4, UROS	34	31	Molecular Transport, Small Molecule Biochemistry, Drug Metabolism
14	ADRM1, APOL3 (includes EG:80833), HDDC3, IL-2R, Immunoproteasome Pa28/20s, Interferon alpha, ISG20, MAPRE2, NIF3L1, NMI, NRBP1, PSMA, PSMA1, PSMA2, PSMA3, PSMA5, PSMA6, PSMA7, PSMB1, PSMB3, PSMB8, PSMB10, PSMD2, PSMD3, PSMD14, PSME1, PSME2, RP1A, STAT5B, TRIM21, TROVE2, TSC22D4, UBLCP1, VTA1, WARS	32	31	Protein Degradation, Dermatological Diseases and Conditions, Infectious Disease
15	CCDC6, CD2AP, CHMP5, CHMP7, CHMP1A, CHMP1B, CHMP2A, CHMP4A, CHMP4B, Clathrin, Endophilin, ERK, F11R, HGS, PDCD6, PDCD6IP, PEF1, PTPN23, ROCK1, SH3GLB2 (includes EG:56904), SNF8, STAM, STAMBP, TSG101, Tsg101-Vps28-Vps37, UBA5, VPS25, VPS28, VPS36, Vps22-Vps25-Vps36, VPS37A, VPS37B, VPS37C, VPS4A, VPS4B	32	30	Infection Mechanism, Molecular Transport, Protein Trafficking
16	Adaptor protein 1, AP1B1, AP1G1, AP1M1, AP1S1, AP1S2, AP2A1, AP2A2, AP2B1, AP2S1, ARF1, CLINT1, COP I, E3 HECT, ENaC, EPN1, EPS15, GGA3, LASP1, NECAP1, NECAP2, NEDD4, NEDD4L, OXSR1, PI3K, PICALM, SLC12A6, SLC12A7, SNAP91, SRP14, SWAP70, UBE3B, UBQLN2, WBP2, WNK1	32	30	Cellular Assembly and Organization, Cellular Function and Maintenance, Molecular Transport
17	Ap2a2-Cltc-Hd, BZW2, CUL5, DNAJ, DNAJA2, DNAJB1, DNAJC13, Hd-perinuclear inclusions, HSP90AB1, HSPA14, HSPA1L, HTT, HYPK, LANCL1, Ldh, LDHA, LDHB, Mre11, MRE11A, NPTN, OSTF1, PPA2, PTGES3 (includes EG:10728), RAD50, SEC22B, SRM, ST13, STIP1, TAGLN3, TERF2IP, TESC, USE1, XRCC5, ZFYVE19, ZW10	32	30	Drug Metabolism, Endocrine System Development and Function, Lipid Metabolism
18	AHS1A, ARIH2, C11ORF59, CARS, DARS, EEF2, EEF1A1, EEF1A2, EEF1B2, EEF1D, EEF1E1, EEF1G, EPRS, ERK1/2, FUBP1, KARS, LARS, MVD, PDCD5, POMP, Proteasome PA700/20s, Protein-synthesizing GTPase, PSMA4, PSMB4, PSMB6, PSMB7, PSMC, PSMD, PSMD11, RARS, SCAMP2, UGP2, VARS, WDR91, XPO5	30	30	Protein Synthesis, Molecular Transport, Nucleic Acid Metabolism
19	14-3-3 ( $\beta, \varepsilon, \zeta$ ), 14-3-3( $\beta, \gamma, \theta, \eta, \zeta$ ), 14-3-3( $\eta, \theta, \zeta$ ), ABCB6, CLNS1A, CTPS, DHRS12, ERAF, G6PD, Histone h3, Histone h4, JMJD6, KIF5B, KLC3, KLC4, LARP1, MARK3, OLA1, PARP10, PARP12, PCBD2, Poly ADP-ribose polymerase, PRMT5, RBBP7, RPS9, SAAL1, SEC31A, WDR1, WDR77, YWHAB, YWHAE, YWHAG, YWHAH, YWHAQ (includes EG:10971), YWHAZ	30	29	Protein Trafficking, Nucleic Acid Metabolism, Small Molecule Biochemistry
20	ABCE1, APEX1, BAG5, CIAO1, DNAJA4, DNAJB, DNAJB4, DNAJB6, DNAJB2 (includes EG:3300), DPP3, FAM96A, G3BP2, GTF3C5, Hdac, HELLS, Hsp27, Hsp70, HSPA2, HSPA5, HSPA6, HSPA8, HSPB1, HSPBP1, LRRC47, MAP3K7IP1, MAP3K7IP3, MMS19, NFKBIB, Nos, NUBP1, NUBP2, PSMD4, PSMD13, SNCA, Ubiquitin	30	29	Post-Translational Modification, Protein Folding, Cellular Function and Maintenance

**Table 3.** Continued

ID	molecules in network	score	focus molecules	top functions
21	Alcohol group acceptor phosphotransferase, BCL10, BTF3, C8, CAD, CARD8, Caspase, CD8, CK1/2, CRADD, CSNK1A1, CSNK2A1, ETF1, FADD, GLO1, GSPT1, HNRNPA1, HNRNPC, IFN TYPE 1, IPO7, NAMPT, NAP1L1, NF-kappaB (family), PAK2, PEA15, PEBP1, PKN1, PKP1, PRKAA1, RNF14, RPS3, RPS25, TARDBP, TRADD	28	28	Cancer, Cell Death, Embryonic Development
22	ANK3, ARIH1, C18ORF25, CARM1, Cbp/p300, Ctbp, DCUN1D1, ENG, EPB42, Ligase, MAPK1IP1L, PARK7, PEX19, Pias, PML, PURA, PURB, RANGAP1, RPL11, SENP8, Smad, SMAD2, Smad2/3, SPTAN1, SPTBN4, TES, Tgf beta, TGM2, TMCC2, TOLLIP, TOM1L2, TSSC4, UBA1, UBE2I, UBE2L3	28	28	Cellular Assembly and Organization, Gene Expression, Drug Metabolism
23	ABCC4, ACOT7, Aldehyde dehydrogenase (NAD), ALDH, ALDH16A1, ALDH1A1, ALDH9A1, ATL3, BLVRB, Cdc2, DHDH, DIS3L2, E2f, EGLN2, ENDOD1, FAH, FCHO2, G3BP1, GAS2L1, GOLT1B, KRAS, MIR124, NARG1, NASP, NAT5, OSBP2, Oxidoreductase, PPL, Rb, RP3–402G11.5, SYPL1, TMBIM1, TMEM109, USP7, VIM	28	28	Small Molecule Biochemistry, Drug Metabolism, Molecular Transport
24	ARHGDI1, ARHGDI1B, CNBP, eIF, Eif2, EIF1AY, eIF2B, EIF2B1, EIF2B2, EIF2B3, EIF2B4, EIF2C2, EIF2S1, EIF2S2, EIF2S3, Glucose Transporter, Gsk3, HNRNPA3, HNRNPA8, IL1, IMPACT, IPO4, IPO9, LYZ, PP1-C, PSMF1, RAB1B (includes EG:81876), RBMX, RNF126, RPL26, RPL31, RPL35A, RPS7, RPS16, SLC2A3	27	28	Protein Synthesis, Gene Expression, Genetic Disorder
25	ACTR3, ANAPC5, AQP1, CDC34 (includes EG:997), Cyclin B, DNA-directed RNA polymerase, E3 RING, FBXO4, FBXO7, GTF2A2, HEXIM1, HTATIP2, MLL2, PGK1, PKLR, PKP3, POLR2D, POLR2G, POLR2H, PRUNE, RNA polymerase II, Secretase gamma, SFN, SHROOM3, SKP1, SND1, STAT6, SUGT1, TAF15, TCEA1, TCEB1, TCEB2, Vegf, VHL-Cul2-Elongin-RBX1, WDR68	27	28	Carbohydrate Metabolism, Cellular Assembly and Organization, Gene Expression
26	Akt, AKT1S1, EIF3, EIF5, EIF3F, EIF3G, EIF3I (includes EG:8668), EIF3J, EIF3K, EIF4A, EIF4A1, EIF4B, EIF4F, Eif4g, EIF4G1, GPI, HNRNPD, HNRNPH1, KHSRP, MAPKAP1, MLST8, MTOR, MTORC1, mTORC2, NCBP2, NCBP1 (includes EG:4686), p70 S6k, PABPC1, PAIP1, PCBP2, PCBP1 (includes EG:5093), RPTOR, SEC14L2, TNPO2, TPP2	26	27	Protein Synthesis, RNA Post-Transcriptional Modification, Gene Expression
27	26s Proteasome, AKAP7, ANPEP, ARFGEF2, CARHSP1, CYFIP1, FH, FHOD1, Glycogen synthase, Ikb, Insulin, KIAA0368, LNPEP, MDH2, Membrane alanyl aminopeptidase, PKAR, PRKAC, PRKACA, PRKACB, PRKAG1, PRKAR1A, PRKAR2A, PRKAR2B, PSMB2, PSMC5, PSMD5, PSMD8, PSMD9, PSMD10, PSMD12, Rar, RNPEP, RPS6, RPS6KA1, VASP	26	27	Carbohydrate Metabolism, Lipid Metabolism, Small Molecule Biochemistry
28	ACLY, ALDOC, C12ORF30, CaMKII, CDK2, CKAP5, COMT, EEFSEC, HNRNPA2B1, HUWE1, Ikk (family), MTHFD1, Ndpk, NME1, PKM2, PP1, Pp2c, PPM1A, PPM1B, PPM1F, Proteasome, Pyruvate kinase, RAB35, S100A8, SELENBP1, SET, TAGLN2, TBCA, UBA6, UBE2, UBE2A, UBE2H (includes EG:7328), UBE2M, UBE2N, UBE2R2 (includes EG:54926)	26	27	Post-Translational Modification, Nervous System Development and Function, Cell Signaling
29	ABCFL1, ACTA1, ATP1A1, ATP8A1 (includes EG:10396), BID, BLMH, C1q, CALR, CNDP2, Collagen(s), Cpla2, CTSG, ELANE, GMPS, GYG1, GYS1, HPRT1, LAP3, LTA4H, LTF, NCSTN, NUDT5, NUTF2, PEPD, peptidase, PLP2, PREP, PRKAB1, S100A4, SERPINB1, SSB, SSR4, THBS1, THOP1, UFM1	26	31	Protein Synthesis, Protein Degradation, Cell-To-Cell Signaling and Interaction
30	ANP32A, Apoptosome, AVEN, CASP3, Caspase 8/10, CSE1L, CYTH1, DFF, DFFA, FNTA, FNTB, Importin alpha, Importin alpha/beta, Importin beta, IPO5, KPNA1, KPNA3, KPNA4, KPNA6, KPNB1, Lamin b, LMNA, LOC389842, MIB1, NAP1L5, NUP50, NUPL1, RAN, RFFL, SCFD1, SLK, SRP19, Tap, TNPO1, USO1	25	27	Molecular Transport, Protein Trafficking, Amino Acid Metabolism

**Table 3.** Continued

ID	molecules in network	score	focus molecules	top functions
31	Alpha Actinin, ANKRD28, Calpain, CAPN1, CAPN2, CAPN5, CAPNS1, CAST, CIRBP, DPYSL2, FAK, FCGR1A/2A/3A, Filamin, FN1, Integrin alpha 3 beta 1, Integrin $\beta$ , Lxn, Myosin Light Chain Kinase, PANK2, RBM3, RPS21, RPSA, SAPS1, SAPS2, SAPS3, SEC23B, SNAP23, STOM, Talin, TLN1, TLN2, TNS1, TSTA3, VAT1, VCL	25	26	Cell-To-Cell Signaling and Interaction, Cellular Assembly and Organization, Cellular Function and Maintenance
32	ACTR2, ACTR3B, ADD1, ADD2, AHCY, Alpha actin, ANK1, ANXA7, Arp2/3, ARPC2, ARPC5, ARPC5L (includes EG:81873), CORO1B, DSTN, F Actin, Fascin, FSCN1, G-Actin, GCA, GSN, LCP1, Myosin, PFN, PITPNNA, PITPNB, Pkc(s), SPTA1, SPTB, SPTBN1, SRI, TMOD1, TPM1, TPM3, TPM4, Tropomyosin	24	26	Cell Morphology, Cellular Assembly and Organization, Cellular Movement
33	Adaptor protein 2, Ap2 alpha, AP2M1, Arf, ASAP1, BCCIP, Beta Arrestin, CAPZA1, CAPZA2, CAPZB, Caveolin, CLTC, DBNL, DNM2, DNM1L, DNPEP, DRG2, Dynamin, EHD1, Epsin, FIS1, FLJ11506, GABAR-A, KRT31, PACSIN2, PDAP1, Pdgf, PFN1, RPL23, RWDD1 (includes EG:51389), SH3GL1, SH3GLB1, SNX9 (includes EG:51429), SYNJ1, TWF2	24	26	Cell Morphology, Cellular Assembly and Organization, Cell Cycle
34	ACTB, ACTR10, ACTR1A, ACTR1B (includes EG:10120), AKR1C1, ALDOA, BCL2L1, Caspase 3/7, CCNDBP1, CDKN2C, Creatine Kinase, Cyclin D, DCTN1, DCTN2, DCTN3, DCTN4, DCTN6, DR1, DRAP1, ENO1, Enolase, FGA, Fibrin, MAPRE1, Nuclear factor 1, RPLP1, RPLP2, RPLP0 (includes EG:6175), RPS12 (includes EG:6206), RPS24 (includes EG:6229), Scf, SRPK1, Stat3-Stat3, T3-TR-RXR, VDAC3	24	26	Cellular Assembly and Organization, Hematological Disease, Immunological Disease
35	14-3-3, ATP2B1, ATP2B4, C20ORF27, Calcineurin A, CALML5, Calmodulin, Ck2, CNP, Dynein, Girk, GPHN, HDAC6, Hexokinase, HMGB1 (includes EG:3146), KCNN4, LYPLA2, NDE1, NUDCD3, PAFAH1B1, PFK, PFKL, PFKM, PHKB, Pmca, RAB8B, RGS10, SIRT2, TUBA1A, TUBA1C, TUBB, TUBB1, TUBB2C, TUBG1, Tubulin	21	25	Cancer, Reproductive System Disease, Cardiovascular Disease
36	ARCNI, ARFGAP1, ARHGAP1, ARHGAP4, ARHGAP17, ARHGEF6/7, CD47, CDC42, COPA, COPG, COPZ1, Dgk, DGKA, Ephb, Ephb dimer, Integrin alpha V beta 3, IQGAP, IQGAP1, IQGAP2, ITSN1, NWASP, Phosphatidylinositol4,5 kinase, Rac, RAC1, RHAG, RHCE, RHD, RhoGap, SACM1L, SLC4A1, SNX5, TMED7, TMED9, TMED10, WASP	21	24	Genetic Disorder, Hematological Disease, Cellular Assembly and Organization
37	AADACL1, APEH, APOBEC3B, APOE, BAG1, CPPED1, CPT1A, FASN, GC-GCR dimer, GCLC, GCLM, HDHD1A, HDL, Hydrolase, IDH1, IRF3, JINK1/2, KIAA0174, LDL, N-cor, NCOR-LXR-Oxysterol-RXR-9 cis RA, Nr1h, PTMS, PTPN7, REXO2, RPS14, Rxr, SAA@, SNX1, SNX6, SNX15, TAF9, THTPA, Thyroid hormone receptor, VPS29	20	24	Drug Metabolism, Amino Acid Metabolism, Small Molecule Biochemistry
38	Actin, CD44, CFL1, Cofilin, DAAM1, EPB41, Erm, EZR, GNA13, GYPC, HSPH1, Mlc, MlcP, Mrlc, MSN, MYH9, MYH10, MYL3, MYL4, MYL6, MYL12B, Myosin phosphatase, Pak, PDE6D, PDXP (includes EG:57026), RAB13, RAB18, Ras homologue, RDX, RHOA, Rock, ROCK2, RPS6KA3, S100A11	19	25	Cellular Assembly and Organization, Cellular Compromise, Cell Morphology
39	ATG9A, BPGM, C7ORF64, CCDC90B, CDC42EP3, CYP7A1, DERA, DLST, FDPS, FNTB, FTSJ1, GIPC2, HNF4A, NP, NRBF2, NUDT2, OTUD6B, PAAF1, PHPT1, PPARGC1B, PSMC4, PSMD1, PSMD7, PSMD8, PSMD10, PYGL, RIF1, RPL18A, RPRD1B, RRM1 (includes EG:6240), RTCD1, SEMA7A, SETDB1, SREBF2, UMPs	18	22	Gene Expression, Genetic Disorder, Metabolic Disease
40	ALB, AMPK, APOA1, ARG1, BTK, C3, CA2, CA3, CA8, CA1 (includes EG:759), Calcineurin protein(s), Carbonic anhydrase, Cytochrome c, FSH, GIPC1, GYPA, IGHG1, IGHM, IgM, ITPR, KCMF1, NFAT (complex), Nfat (family), NMDA Receptor, PCMT1, PGM2L1, PI4K2A, PTP4A1, QDPR, RAB33B, SAFB2, SLC2A1, SNAP29, SOD1, STX4	18	25	Cardiovascular Disease, Metabolic Disease, Genetic Disorder

**Table 3.** Continued

ID	molecules in network	score	focus molecules	top functions
41	Androgen-ARA55-AR-ARA70-HSP40-HSP70-HSP90, AR-HSP40-HSP70-HSP90, ATIC, CAT, CHORDC1, CLU, DNAJC, DNAJC5, DNAJC9, DNAJC17, FKBPL, G protein beta gamma, Glutathione peroxidase, Glutathione transferase, GMFB, GOT, GPX4, GST, GSTO1, GSTP1, GSTT1, HSP, Hsp22/Hsp40/Hsp90, HSP40-HSP70-HSP90, HSP90AA1, HSPA4, IL12 (complex), PEX5, RAB10, RAB15, RAB8A, RABIF, SERPINB5, SGTA, Sod	17	22	Drug Metabolism, Endocrine System Disorders, Small Molecule Biochemistry
42	APRT, ARHGAP18, BMP6, CER1, CISD2, DDT, DNAJB6, DNAJC8, EIF2S3, EIF3M, FAM49B, GH1, GLOD4, IKBKE, ISYNA1, LPHN2, LTA4H, MIR298 (includes EG:723832), MTAP (includes EG:4507), NCDN, NFYB, NUDT5, PDHB, PFDN5, PIP4K2C, PRKCSH, RAB21, SESN1, SLC43A2, SLC8A2, SLTRK2, SORD, TKT, TTLL12, TXNDC12	17	21	Cardiovascular System Development and Function, Cellular Function and Maintenance, Embryonic Development
43	ACHE, AChR, AMPD3, AP1B1, ATP2B1, C8ORF55, CFTR, CLTC1L, COLQ, DAZAP1, FAT1, IFIT5, KCNJ1, L-carnitine, LGTN, MAPKAP1, MAT2A, MAT2B, Methionine adenosyltransferase, MUC2, NOS1, PDDC1, protoporphyrin IX, PRSS23, PSME2, RASD1, S100A7, SH3BGRL2, SLC9A3, SP2, TMEM222, TNF, TPD52L2, VAC14, ZNF330	14	19	Small Molecule Biochemistry, Amino Acid Metabolism, Nucleic Acid Metabolism
44	AGPAT6, C12ORF34, CSE1L, DCD, DSG1, EFHD2, GFI1B, GRIN1, GRIN3A, HLCS, INPP5K, KALRN, KLHL18, MATR3, MIR20A (includes EG:406982), MIR210 (includes EG:406992), MYH14, NELF, OTUB1, PCCA, PCCB, PPP2R2B, R3HCC1, RAD51L3, RAN, RANBP10, RANGRF, RTN1, STT3B, SYNGR1, ULK3, XPO5, XPO7, XPOT	12	17	Molecular Transport, RNA Trafficking, Genetic Disorder
45	ALDH3A1, ARAF, ATYPICAL PROTEIN KINASE C, BAD, BCAM, Complement component 1, Creb, Cyclin A, Cyclin E, Fcer1, hCG, ICAM4 (includes EG:3386), IgE, IgG, Integrin, IVL, KLC1, Laminin, LGALS3, MAP2K1, MAP2K2, MAP2K3, MAP2K4, MAP2K1/2, MAP3K, Mek, MVP, Pkg, PLEK2, PP2A, PPA1, RAB2A, Rap1, SPAG9, TSHR	12	18	Cardiac Hypertrophy, Cardiovascular Disease, Cellular Development
46	7-dehydrocholesterol, amino acids, ASNA1, BMPR2, Ck2, CTBP1, FLAD1 (includes EG:80308), GMFB, GMFG, LANCL2, LCMT1, MAP2K5, MAP3K1, MAP3K6, MAP4K2, MERTK, MIR193A, MOS, MYLK2, MYO1D, NAP1L4, NOP2, PHKG2, phosphatase, PI4KA, PIP4K2A, PMM2, RABGGTA, RPL10A (includes EG:4736), SIRT5, SRPK1, TRIM23, TWF1, UBAP1, ZNF516	12	17	Amino Acid Metabolism, Post-Translational Modification, Small Molecule Biochemistry
47	BAT2, BAT3, BMI1, C11ORF67, C11ORF73, C14ORF133, C9ORF64, CHCHD3, CHCHD6, CRYZL1, FN3KRP, GSTK1, HBS1L, HNF4A, IMMT, INTS4, KIF22, MRPL44, PNPO, RAP2C, RNF113A, SAMM50, SEC23A, SEC23IP, SLC7A6OS, SREBF1, TXNDC9, VPS33B	11	15	Cell Morphology, Cellular Assembly and Organization, Cellular Function and Maintenance
48	ALP, BMP2K (includes EG:55589), CK1, CMPK1, COASY, CTTN, DDX3X, Fgf, Fibrinogen, Growth hormone, HCLS1, HS1BP3, Ifn, IFN Beta, Ifn gamma, Igf, IL18, II12 (family), IRAK, IRAK4, MHC Class II, Mmp, Na+,K+ -ATPase, NGF, PLC gamma, PRG2 (includes EG:5553), RPS6KB1, SF3B4, STAT, STAT5a/b, Tlr, TXN, TXNRD1, WBP4, XPO1	11	16	Cell Morphology, Cell-mediated Immune Response, Cellular Growth and Proliferation
49	ANP32E, BMF, C20ORF3, CD70, DBI, DYNC1I1, DYNLL2, ERRFI1, FKBP5, GLCCI1, HECTD3, HOXB7, HSD17B12, MAEA, MIR195, MIR26A1, MIR26A2, NfkB-RelA, NFKBIA, NKIRAS2, NOD2, NR3C1, OSBPL9, PDCL3, PIR, PPCDC, PSMG2, RAB28, SLC1A6, SLC1A7, TNFAIP8, TNFSF14, TRAF3IP2, WIBG, ZNF346	11	16	Amino Acid Metabolism, Small Molecule Biochemistry, Cell-To-Cell Signaling and Interaction
50	ADPRHL2, ASCC2, ATP5G3, ATP6 V0A4, ATP6 V0D1, ATP6 V1C1, ATP6 V1C2, ATP6 V1E1, ATP6 V1E2, ATP6 V1G2, ATP6 V1G3, C8ORF30A, CORO1C, ERK, ETNK2, FAM63B, GBP6, GUK1, H+-exporting ATPase, IFNB1, IgG, MAP3K3, Mg2+, NDUFB2, PFDN4, PFDN5, POLA2, RNF114, RPUSD1, TBCB, UBC, UBL4A, URM1, VBP1, XPNPEP1	11	16	Molecular Transport, Antigen Presentation, Cell-To-Cell Signaling and Interaction



**Figure 1.** Top ten toxicity pathways. As it emerges from this analysis, oxidative stress likely plays a crucial role in the toxicity toward RBCs. This “quasi-truism” is useful to correlate the oxidative stress (3 out of 4 top pathways) with the regulation of apoptotic and antiapoptotic signaling.

decade, as it has been recently reviewed (Table 1).<sup>18</sup> Nonetheless, early proteomics investigations on the RBC membrane proteome date back to 1982, when Rosenblum and co-workers first outlined the 2-DE profiles of normal adults, neonates, and patients with erythrocyte membrane disorders.<sup>19</sup> However, more in-depth proteomic studies on RBC membranes had to wait the advent of mass spectrometry. In 2002, Low and colleagues individuated overall 102 proteins (59 distinct polypeptides, 43 isoforms, mostly membranaceous) by means of 2-DE and MALDI-TOF analysis.<sup>20</sup>

In 2004, Kakhniashvili investigated the RBC proteome by means of an IT-MS/MS coupled online with a RP-LC, which yielded identification of 182 proteins, equally distributed between the cytosolic and the membrane fractions.<sup>21</sup>

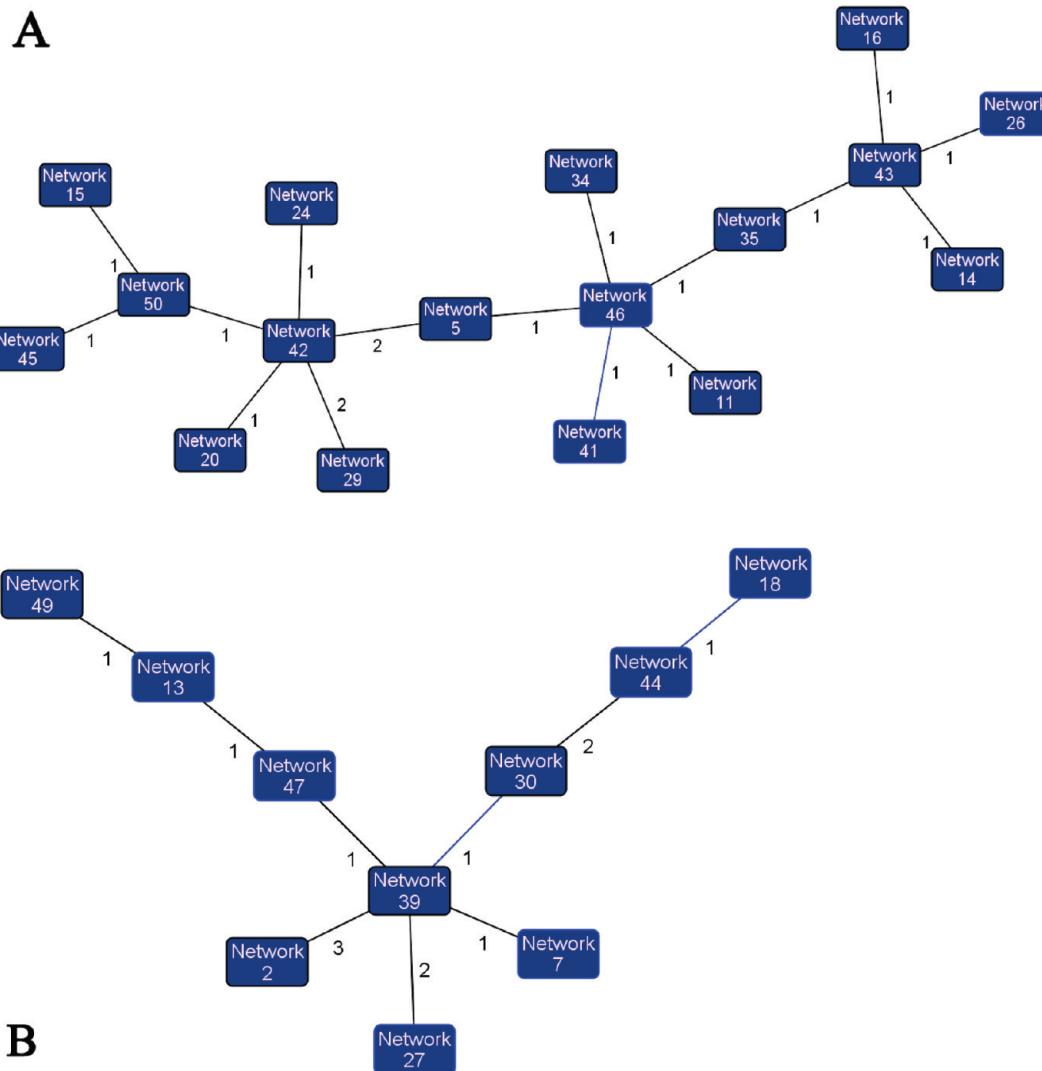
In a recent study, Tyan and colleagues adopted a special proteolytic chip, consisting of 11-mercaptopundecanoic acid bonded on self-assembled monolayers of alkanethiols onto gold surfaces.<sup>22</sup> Subsequent 2D-nano-HPLC and MS/MS analyses enabled the identification of 272 proteins from erythrocyte samples.

In 2005, Bruschi and co-workers individuated 500 spots with soft Immobiline gels,<sup>23</sup> among which was the presence of high levels of filamentous proteins (e.g., alpha-spectrin and ankyrins) or integral membrane proteins (e.g., band 3, band 4.1, and band 4.2). Both classes of proteins are not usually displayed or barely present in maps exploiting immobilized pH gradients in the first dimension, whereas they were revealed through this

alternative approach. Indeed, several drawbacks limit the use of 2-DE for proteomics research. Although 2-DE has many benefits, the technique does not lend itself to large-scale, high-throughput proteomic analyses due to a series of major shortcomings. For example, not all types of proteins are well resolved in this system, while proteins bearing extremes of size, hydrophobicity, or charge fail to enter the gel and are poorly represented.<sup>24</sup> Soft Immobiline gels replacing IPG strips in the first dimension of 2-DE allowed to partially overcome these obstacles, especially as far as it regarded high molecular mass proteins.<sup>27</sup>

One of the most recent and complete studies on the RBC proteome dates back to 2006, when Pasini et al. carried out a thorough analysis consisting of 1-DE (SDS-PAGE) followed by in-gel digestion and LC-ESI-MS/MS (either Q-TOF and FT-ICR).<sup>15</sup> The overall analyses helped the authors compile a final list of 340 membrane and 252 soluble proteins.

Recently debated clinical retrospective<sup>25</sup> studies have prompted the transfusion medicine establishment to wonder about the safety and efficiency of long-stored blood components and, in particular, of erythrocyte concentrates. In this scenario, proteomics has revealed an innovative instrument to re-establish quality criteria, first of all addressing the molecular changes at the protein level that accompany RBCs as they age. Zolla's<sup>26</sup> and Bosman's<sup>27</sup> group papers, though fundamentally addressing the storage issue, have nonetheless provided a detailed



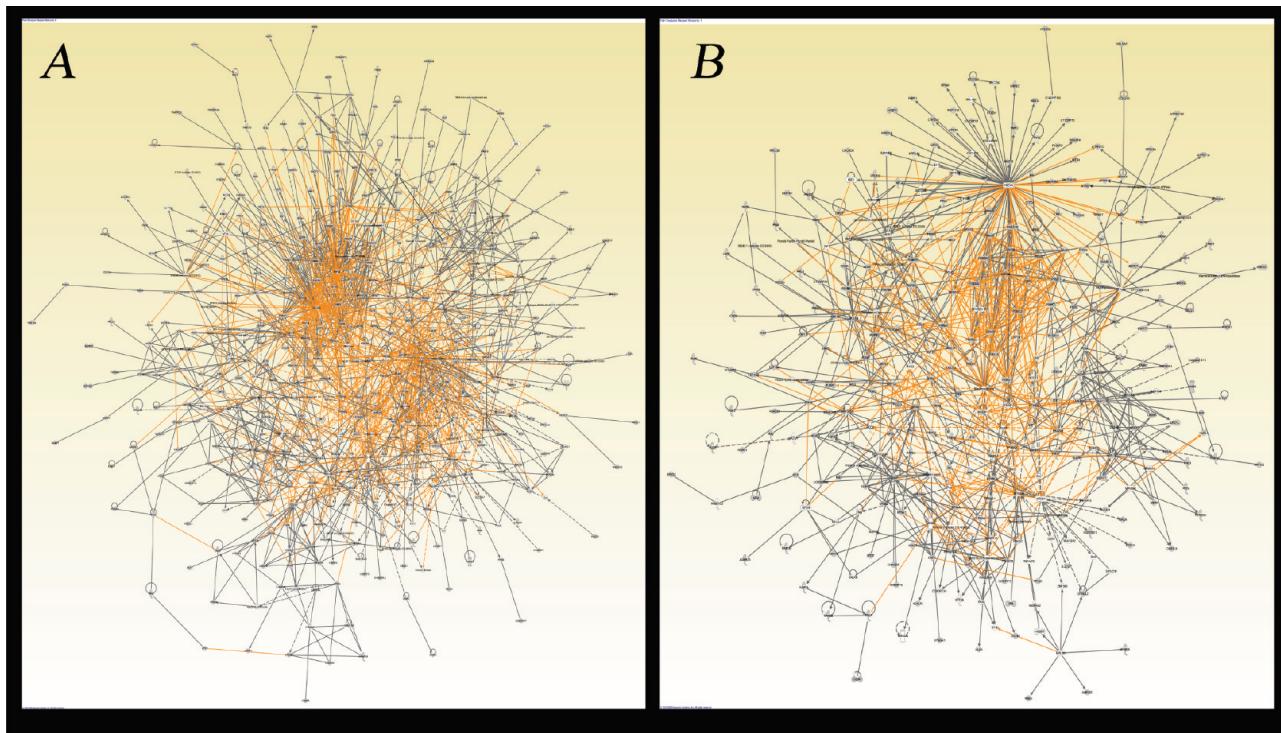
**Figure 2.** Representation of the individuated “ultra-networks”. They were created by calculating networks with function affinity which shared at least one node. (A) Networks 6, 14–16, 20, 24, 26, 29, 34, 35, 42, 43, 46, 50 and regroups those networks which play physiological functions related to protein control (protein synthesis, folding, post-translational modifications, degradation, trafficking, molecular transport, cell function and maintenance, cellular assembly and organization). (B) Networks 2, 7, 13, 18, 27, 30, 39, 44, 47, 49 and regroups proteins involved in molecular transport and metabolic activities (cellular assembly and organization, molecular transport, small molecule biochemistry, carbohydrate, amino acid and lipid metabolism, cell morphology).

portrait of the membrane and secreted micro- and nanovesicle proteomes as well (257 proteins).

A substantial stride in the field of RBC proteomics has been favored by the introduction of the “ProteoMiner Technology”, which is a method enabling the capture of all species present in a proteome, but at much reduced protein concentration differences. It consists on a combinatorial library of hexapeptide ligands coupled to spherical porous beads of polymethacrylate. When these beads are contacted with proteomes of widely differing protein composition and relative abundances, they are able to “normalize” the protein population, by sharply reducing the level of the most abundant components while simultaneously enhancing the concentration of the most dilute species.

In the joint study by Bachi et al.<sup>28</sup> and Simò et al.<sup>29</sup> individual amino acids or peptides of different length (from 2 to 6 amino acids) were packed as stationary phases of chromatographic affinity columns, and used as baits for capturing the low-abundance cytoplasmic proteome of RBCs. Aminoacids were

named “petit catchers” and “grand catchers” after their capacity to interact and retain proteins. This protocol allowed the identification of approximately 800 proteins upon 2-DE (SDS-IEF) and by nanoLC-ESI-MS/MS.<sup>28,29</sup> Being repeatedly eluted from each one of the 16 affinity columns, a group of 72 proteins represented the “maximum common denominator”. It is worthwhile to underline that better outcomes were obtained with hydrophobic aminoacids, as polarity seemed to be a negative influencing-factor for interactions with the stationary phase. Simultaneously and independently, Roux-Dalvai et al.,<sup>30</sup> through the refinement of the ProteoMiner Technology,<sup>31</sup> and the adoption of the fast and high-throughput ORBITRAP MS analysis, have utterly lead to the astonishing identification of 1,578 cytosolic proteins.<sup>30</sup> The striking conclusion was that, in an RBC lysate where hemoglobin alone constitutes 98% of the total proteins, the remaining 2% proteome is constituted by an incredible array of unique gene products. However, the debate is still open about their role as actually bioactive



**Figure 3.** Graphic representations of the individuated “ultra-networks”. (A) Ultranetwork 1 (including networks networks 6, 14–16, 20, 24, 26, 29, 34, 35, 42, 43, 46, 50); (B) ultranetwork 2 (including networks 2, 7, 13, 18, 27, 30, 39, 44, 47, 49). They were created by calculating networks with function affinity which shared at least one node. Ultranetworks representations were obtained by merging subnetworks in each case. It is not relevant that nodes ID could not be read in this compressed version of the graph. What is notable is that, although containing hundreds of nodes, these ultranetworks display a quite ordered disposition, especially the ones in B.

molecules or simple remnants of degraded/under degradation proteins inherited from reticulocyte ancestors.

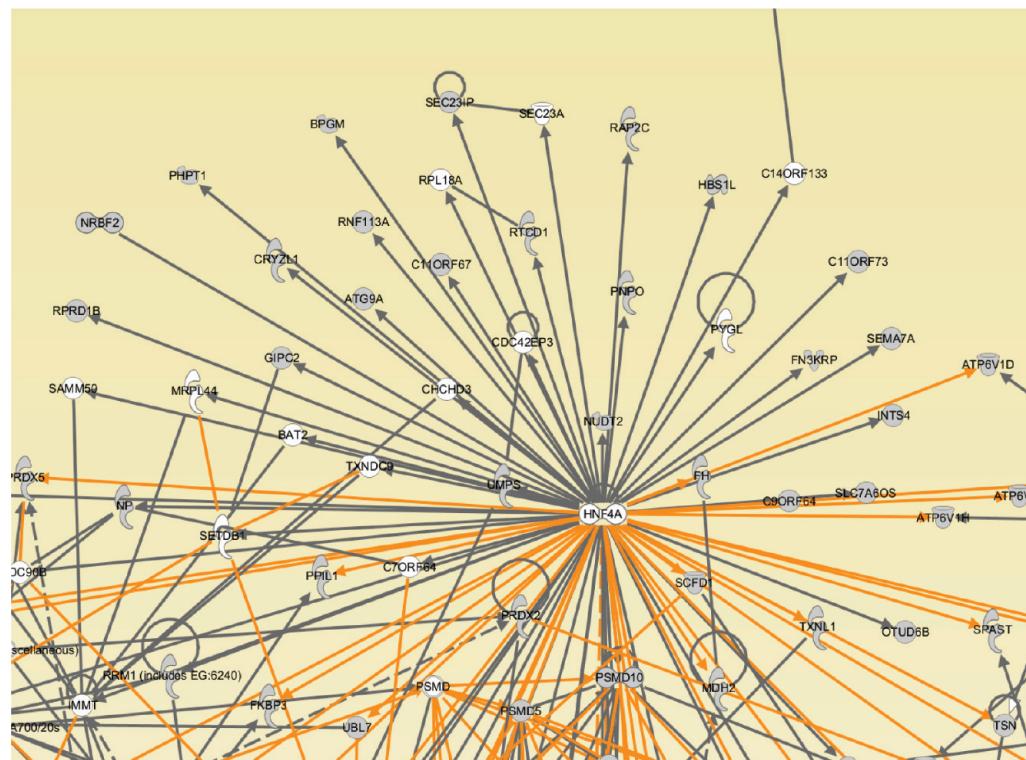
**From Proteomics to Interactomics: Fostering Complexity.** Although a preliminary attempt to dive into the *mare magnum* of the RBC interactome has been recently made,<sup>9</sup> new advancements in the field of RBC proteomics claim for an update of the map of the erythrocyte complexity.<sup>30</sup> In 2007, Goodman and colleagues elaborated an *in silico* analysis of the interactome of RBCs based on a summary of overall 751 proteins from previously published studies.<sup>9</sup> However, out of those 751 gene products from the original data set, only 279 could be represented as nodes interacting among each others, while the remaining were omitted. In particular, a series of biologically relevant information could be retrieved from this analysis. Strikingly, the “interactome” scheme orbited around a central fulcrum of expressed proteins. This central core of expression encompassed a series of proteins involved in physiological responses to oxidative stress and unfolding (e.g., peroxiredoxins, catalases, chaperonines, heat shock proteins, and proteasomal subunits). Thus, the authors named it the repair or destroy (ROD) box after the biological role of these classes of proteins. These findings highlighted the molecular behavior of RBCs, which are enucleated and base their survival on the maintenance and repair of the existing proteome instead of on the synthesis of new proteins.<sup>9</sup>

During the last 2 years, new software platforms have become available as the interactomics discipline has started attracting a growing deal of interest. We exploited what is perhaps the most promising one for performing the present network and pathway analysis.

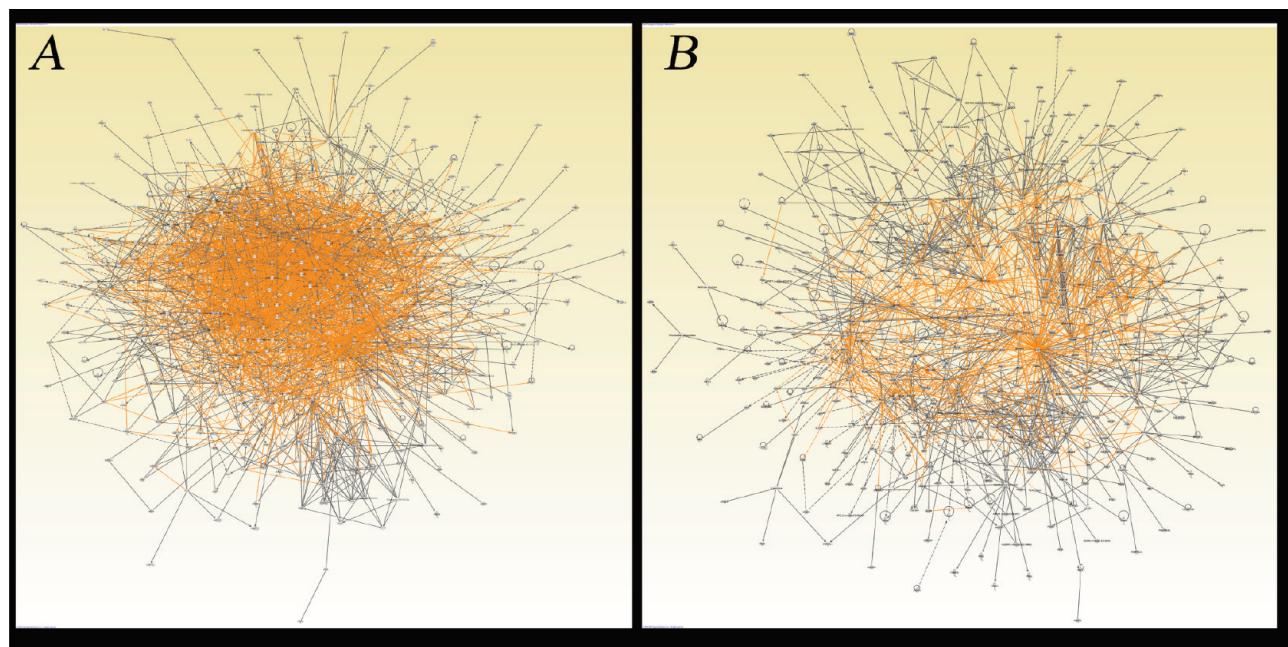
A list of 2086 proteins reporting gene IDs was created merging the data from a series of proteomics papers available

from literature.<sup>15,26,29–32</sup> This data set was submitted for elaboration of pathway and network analyses to the Ingenuity Pathway Analysis software (Ingenuity Systems, [www.ingenuity.com](http://www.ingenuity.com)).<sup>33</sup>

Each gene identifier from the submitted list was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. Redundant proteins were excluded, while isoforms of the same proteins were maintained to reach a total of 1989 distinct gene products (Supporting Information 1). A total of 1574 proteins had a match in the database and were eligible for network analysis (79.18%), while only 1374 (69.08%) for pathway analysis. The significance of the association between the data set and the canonical and disease/toxicity pathways was measured in 2 ways: (1) A ratio of the number of proteins from the data set that map to the pathway divided by the total number of proteins that map to the canonical pathway is displayed. (2) Fischer’s exact test was used to calculate a *p*-value determining the probability that the association between the proteins in the data set and the canonical pathway is explained by chance alone. Highest scores are proportional to a lower probability of casual association. In the end, the software determines and graphs unbiased networks, in which gene products are represented as nodes, and the biological relationship between two nodes is represented as an edge (line). All edges are supported by at least 1 reference from the literature, from a textbook, or from canonical information stored in the Ingenuity Pathways Knowledge Base. Nodes are displayed using various shapes that represent the functional class of the gene product. Gray nodes represent the proteins from the submitted data set which have a match in the canonical pathway from the database, while white nodes represent gene products that the software attributed to the



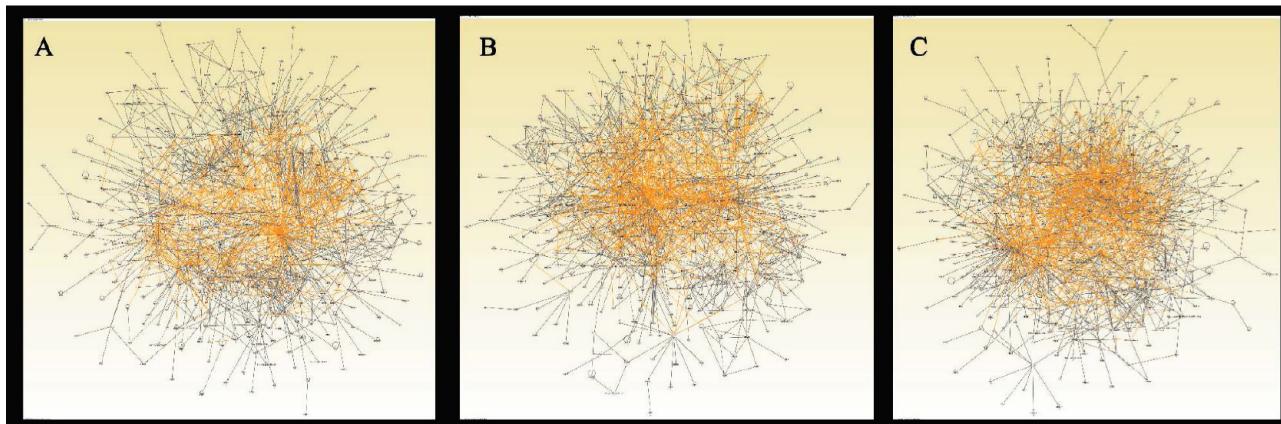
**Figure 4.** Detail of Figure 3B. When encountering a white node surrounded by gray nodes, it is very likely that it is a missing piece toward which future research should be oriented, or it is an inducer of the expression of those gene products in the sample tissue/cell while being expressed elsewhere, as it is for HNF4A, which is expressed by hepatocytes and regulates erythropoiesis from E 11.5 onward.<sup>40</sup>



**Figure 5.** Merging the first 500 nodes from top network analysis of the whole data set when (A) excluding Roux-Dalvai et al. from the original list or (B) not. The networks merged in B display a more ordered shape: pivotal nodes are more evident and a center of the map could be located. On the other hand, the lower number of original data accounts for an increased “disorder” in the map in A, which still provides information about specific nodes while being impossible to read as a whole. From comparison of A and B it clearly emerges the role of the newly gained insights in red blood cell proteomics, which justify this updated overview of the red blood cell interactome.

same networks, although they were not present in the elaborated data set. Continuous lines (edges) represent direct interactions, while indirect ones are represented by interrupted

lines. Circular lines around one node describe a feed-back loop of activity of that node on itself (e.g., by self-modulating its activity or expression). Gray edges represent interactions within



**Figure 6.** Schematic view of the maps obtained by progressively merging groups of 14 networks based on their scores. (A) Networks 1–14 maps 490 nodes, as well as (B) networks 15–28, while (C) networks 29–42 harbors 487 nodes. It is worthwhile to underline that, even if the number of the nodes is almost identical in each case, the shape of each network group differs consistently with the diminution of the networks scores. Lower score networks include a series of proteins that are absent from the original data set and are introduced by the software to fill up the determined network, even if this reduces the “interaction noise” thus making the map more difficult to read.

a single network, while orange edges cross-link nodes from multiple interacting networks. The program could either graph single networks alone or merged together to stress their interactions.

The Ingenuity Pathway Analysis software allowed us to perform an unbiased elaboration of the available data, to focus subsequent analyses and discussions on the pivotal pathways and networks which are revealed upon the elaboration phase.

**Pathway Analysis.** Software elaboration of the submitted data set identified 69 main canonical pathways which could be further divided into 850 different subpathways, ranging from a minimum probability of  $1.39 \times 10^{-22}$  (highest score) to a maximum of  $9.76 \times 10^{-3}$  (lowest score). A list of the pathways is fully reported in the Supporting Information 2, and Table 2 reports the top 15 canonical pathways, pointing out their functions, relative scores, and protein entries. On the other hand Figure 1 reports the top 10 disease/toxicity pathways, which enlist a series of proteins accounting for specific stresses/pathologies.

From these analyses, it clearly emerges that oxygen has a role in the toxicity toward RBCs, as it is intuitive. Being the main RBC function, oxygen transport exposes the RBC to a dramatic dose of continuous oxidative stress. Three out of four top toxicity pathways are clearly related to oxidative stress, as expected. However, these pathways are likely related to regulation of apoptosis and antiapoptotic signaling, as the three “apoptosis”, “anti-apoptosis”, and “Nfk $\beta$  signaling” pathways explicitly suggest. This is in agreement with the mainstream theory about erythrocyte aging *in vivo* and *in vitro* being closely related to apoptosis, as Lang’s group has resumed the well-known RBC-related senescence phenomena under the name of *eryptosis*.<sup>34</sup> Similar considerations could be made when analyzing the list of top canonical pathways. Indeed, being constantly exposed to oxidative stresses while being enucleated and thus incapable of synthesizing new proteins, RBCs seem to be primarily devoted to protect their accumulated protein machinery from oxidative stresses, as Goodman and colleagues had suggested.<sup>9</sup> Most of the top canonical pathway functions involve protein (synthesis, folding, post-translational modifications, protein transport, and metabolism/degradation) or cell death, almost meaning that RBC philosophy could be resumed

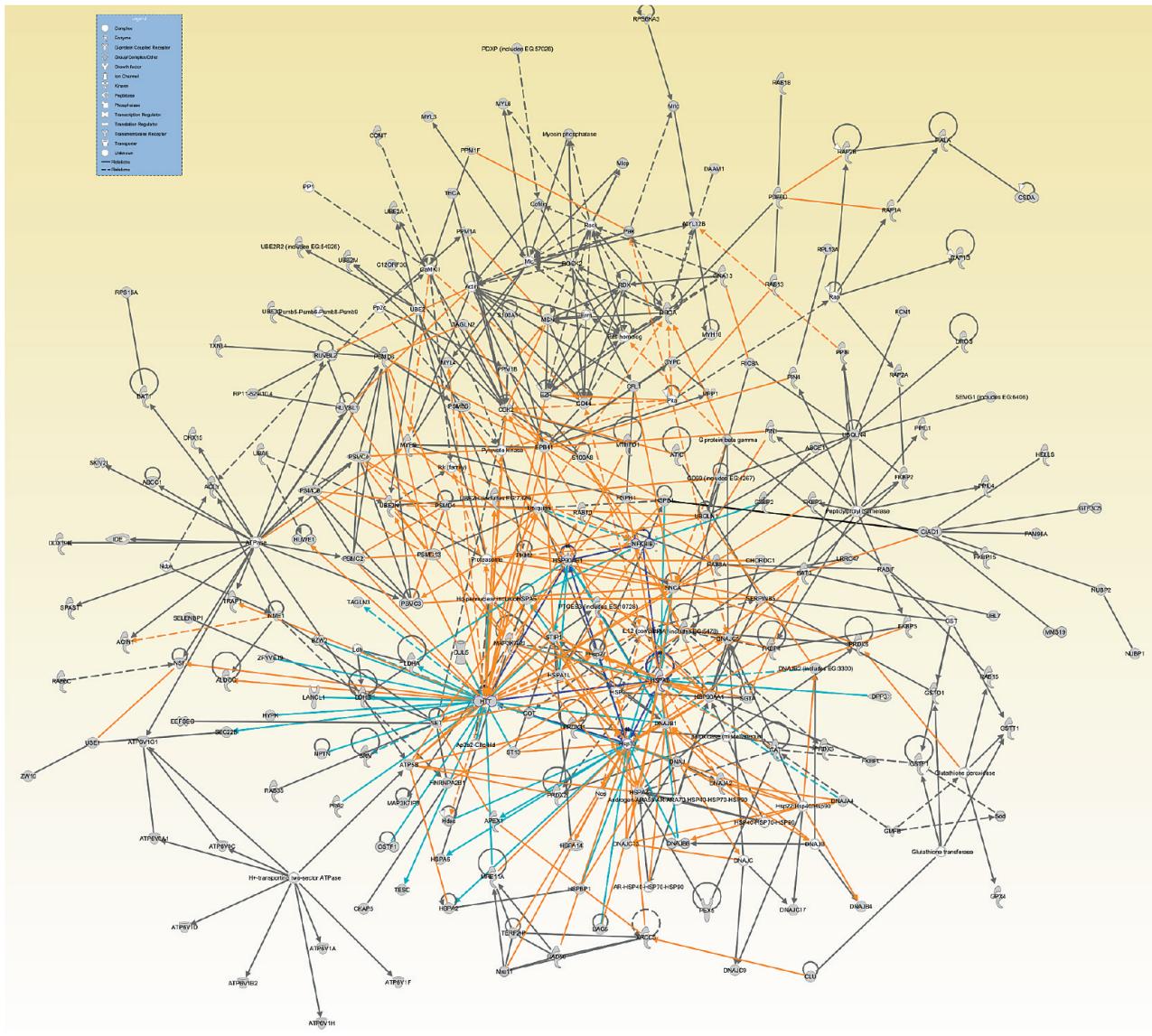
with a simple “*save or sacrifice*”, whether they fail to counteract oxidative stresses.

Cellular assembly and organization also play a fundamental role, as 6 out of 15 top canonical pathways include proteins involved in vesiculation (transport of vesicles, fusion of cellular membrane, endocytosis) or cytoskeleton formation and maintenance. Once again, this recalls either the need for structural elasticity to exert their biological function even in peripheral districts or the senescence-induced vesiculation events. The latter end up determines the spherocytosis phenotype and accounts for the gradual elimination of not yet viable, terminally denatured proteins through membrane blebbing of micro- and nanovesicles as a sort of self-protecting mechanism.<sup>35</sup>

Strikingly, although being enucleated and devoid of protein synthesis, RBCs still display an actual arsenal of proteins involved in translational activities, such as a series of elongation factors and other proteins listed in table 2 in the protein synthesis canonical pathway. These proteins could be vestigial remnants of the very last translational activity of the yet enucleated reticulocyte, which still inherits meager amounts of mRNA from its nucleated ancestors.<sup>36</sup> However, it should be highlighted that most of these proteins are actually fragmented, degraded or under-degradation, sometimes represent actual remnants of cellular organelles (although RBCs loose organelles during maturation), while their functionality is controversial.

**Pathway Analysis and CDA II.** The introductory observations of the statistical pathway analyses allowed us to assess that this kind of approach could be useful for a general description of the system under analysis. We now wanted to evaluate pathway analyses when adopted to gain insights of a more targeted issue.

The greatest portion of the entries included in our data set derived from the paper by Roux-Dalvai et al.<sup>30</sup> This huge data set had recently been exploited to perform a proteomics and genomics integrated approach to glean insight of the molecular candidates which could be likely responsible of the congenital dyserythropoietic anemia type II (CDA II).<sup>37</sup> On the basis of the list of 1578 proteins and a linkage analysis, the authors were able to determine a list of 17 candidates whose gene products were encoded on the incriminated region on chromosome 20:



**Figure 7.** An updated representation of the repair or destroy box (ROD) from the paper by Goodman et al.<sup>7</sup> Merged networks 7, 10, 13, 17, 20, 28, 38, and 41 are graphed together. Notably, the central core is represented by a series of proteins, which are known to counteract protein unfolding (HSPs), oxidative stress (PRDX1), and apoptotic signaling (NFKB1B), forming a sort of catalytic ring, which could guarantee a strengthened response to denaturing stresses. Gray nodes, proteins from the data set having a match in the database; White nodes, proteins from the database which were not identified (if present) upon the experimental phase; Gray edges, interactions within a network; Orange edges, interactions between networks; Light-blue edges, interactions involving HTT, HSP70, HSP90AA1, HSPA8, NFKB1B, and HSP90AB1.

SNX5, SEC23B, DTD1, NAT5, GINS1, BCL2L1, MAPRE1, CHMP4B, EIF2S2, AHCY, ACSS2, GSS, EIF6, CPNE1, EPB41L1, RPRD1B-C20orf77, and TGM2. From thereon, a progressive exclusion was performed by the Authors on the basis of a series of acute observations, as to restrain the list to 4 candidates only: SNX5, MAPRE1, CPNE1, and SEC23B. Among these four proteins, SEC23B was individuated as the eligible candidate, since it encodes for a protein possibly involved in ER-to-Golgi vesicle-mediated transport and in vesicle budding from the ER, in line with the recent evidence of a defect affecting cis- to trans-Golgi processing in CDAII erythroblasts.<sup>38</sup>

We expected to find that, whether our pathway analysis reflected the biological complexity of the sample under analysis, we could have individuated SEC23B in one of the top score pathways and with a p-value lower (higher probability) than each one of the other 16 candidates. Therefore, we performed

a protein-by-protein search in our pathway analysis datasheet. Among the 850 canonical pathways, SEC23B mapped only in the “Cellular assembly and organization—transport of vesicle” pathway, which was the third top score pathway in the whole list (italics in Table 2).

It is worthwhile to underline that each one of the other eligible candidates had a far lower score than SEC23B, while an exception could be made for CPNE1, which mapped in the same pathway as SEC23B. Notably, CPNE1 was one of the 4 candidates of the restrained list proposed by the authors, and it was not excluded until the very last phase, on the basis of the genetic observation of the logarithm of the odds (LOD) score in CDAII changing from 20q to 20p11.<sup>39</sup>

It is therefore possible to conclude that pathway analyses could be exploited for targeted evaluation of proteomics data

and allows unbiased results which guarantee as much credibility as the acute and subtle reasoning by expert Authors.

**Network Analysis.** Network analysis is perhaps the most intuitive and well-known aspect of interactomics. It allows mapping a series of molecules depending on their connections among each other. Our analysis has been focused on the top 50 networks which could be individuated by the software (Table 3). This limitation permitted us to consider only 1336 nodes out of the 1574 proteins eligible for network analysis instead of focusing on the whole list of 1989 distinct gene products from the original data set. For 238 proteins, the program did not attribute a score which allowed them to enter the top 50 network list and thus they were apparently lost in the process. This is true to some extent, since when performing complementary analyses on these specific proteins, further 36 networks were individuated. However, half of these networks had an extremely low score (1 on average), while the first half presented high scores which were on the other hand biased by this separated analyses (Supporting Information 3). Therefore, we decided not to include them in the main body of the article.

At a rapid glance, network analysis agrees with the observations from canonical and toxicity pathway analyses. Top networks include proteins mainly devoted to gas transport (such as the series of hemoglobins in the “small molecule biochemistry - Network 3” - bold), oxidative stress response (catalase in “Network 10”, peroxiredoxins in “Network 13”), protein control (folding, metabolism, degradation, protein trafficking, post-translational modification; Networks 2, 6, 11, 14, 15, 19, 20, 24, 26, 28–30, 46), cellular assembly and organization (cytoskeleton formation and cell morphology, molecular transport, vesiculation and vesicular trafficking; Networks 1, 2, 7, 11, 16, 20, 22, 25, 31, 32, 33, 34, 36, 38, 47, 48, 50) and cell death/apoptosis/antiapoptotic signaling (Networks 1, 3, 21, 36, 42).

**Meta-Analysis.** A more in-depth observation of the networks pointed out two main ultranetworks, cross-linking a series of networks sharing similar functions and at least one node (Figure 2). The first one includes networks 6, 14–16, 20, 24, 26, 29, 34, 35, 42, 43, 46, 50 and regroups those networks which play physiological functions related to protein control (as previously mentioned) (Figure 3A). The second one includes networks 2, 7, 13, 18, 27, 30, 39, 44, 47, 49 and regroups proteins involved in molecular transport activities (Figure 3B). These ultranetworks, although containing hundreds of nodes, display a well-ordered structure which focuses around the activity of a reduced number of key nodes. Some of these key nodes represent pivotal points in the interaction network, although some of them were absent from the submitted data set and thus graphed as white nodes. For example, in the second network HNF4A, absent from the data set, interacts with a series of proteins which have been individuated during the primitive experimental phase (gray nodes) (Figure 4). Indeed HNF4A is abundantly expressed by fetal liver hepatocytes and plays a crucial role in the regulation of early erythropoiesis.<sup>40</sup> Therefore it is plausible that, although absent, its interacting/induced partners in the RBC are both present in the data set and graphed in the same network. *Vice versa*, this could account for the quality of the elaborated networks, as it suggested proteins that should be there (or are somewhat related), but have not been actually identified.

As far as the shapes of the networks are concerned, we noted that the “order” of the networks increases (less cross-interac-

tions, individuation of crucial nodes) proportionally to the increase of the “grey nodes/white nodes” ratio (the number of the gene entries from the original data set which have a match with the database). A simple analysis supports this statement. We elaborated data from all the aforementioned studies,<sup>15,26,29,30,32</sup> and excluded from the broadest list available.<sup>30</sup> A final data set of 508 nonredundant entries was determined out of which 466, having a match in the database, were elaborated through networks analysis, merged and graphed (Figure 5A). The complexity (disorder) of this graph far overwhelms the one of the graph obtained by merging the top 14 networks of the complete data set (including Roux-Dalvai et al.<sup>30</sup>) (Figure 5B). This observation prompts us to consider that the quality of a network and the number of information that could be retrieved from it are likely proportional to the quality (number of entries) of the original experimental data set.

Analogously, the analysis of a maximum of 500 nodes restrained our chances to obtain a comprehensive map of the whole interactome. Thus we decided to perform a progressive analysis, including 14 networks at a time. In particular, network 1 to 14 included 490 nodes; network 15 to 28 included 490 nodes as well; network 29 to 42 included 487 nodes, while network 43 to 50 only 271 nodes. When excluding the latter, due to an extremely lower number of networks and corresponding nodes, we could underline a particular trend in the complexity of those networks, since the map became difficult to read as we progressively merged lower score networks (Figure 6). This could be explained as an effort of the program to bias for those networks which appear to be incomplete. A number of proteins are absent in the original data set, but the program includes them since it calculates that they should take part in the individuated network. As a result, the program includes a series of “white nodes” which end up completing the network on the one hand, although at the expenses of the “noise” (improper connections) on the other. A technical caveat could be represented by the adoption of a higher cutoff for choosing molecules eligible for the analyses. This is particularly meaningful when performing comparative/quantitative analyses among several data sets (this is not the case), in which the cut off represents the fold-change value for the same proteins among replicates (a 3 cutoff value is more stringent than 2, since it implies a 3 fold-change variation among samples). This is often the case of analyses performed on microarray data.<sup>41</sup> This strategy allows decreasing the total number of genes to be analyzed and thus the likelihood of possible false-positives.<sup>42</sup>

**Repair or Destroy Box: Goodman Revisited.** In the previous section, we focused on the meta-analysis of the elaborated protein networks. One of the ultranetworks individuated harbored a series of proteins involved in protein folding and degradation control, as well as in response to oxidative stress.

Notably, it is becoming increasingly accepted that oxidative stress is the main cause of the reduced viability of long-stored RBC concentrates.<sup>43–45</sup> In parallel, accumulating evidence has recently suggested for a peripheral component to schizophrenia, mainly due to an increased susceptibility to oxidative injuries.<sup>46</sup>

Interestingly, in their proteomics and interactomics review, Goodman et al.<sup>9</sup> had already located a core of interacting proteins, which they named the repair or destroy box (ROD) after the biological activity of many of its nodes. The ROD box contained proteins that utilize the energy of ATP hydrolysis to fold nascent proteins or refold damaged proteins (heat shock proteins and chaperonins). As mature RBCs are thought not

to synthesize nascent proteins only, the latter function was considered to be relevant. The ROD box also contained proteins involved in the proteasomal degradation of ubiquitinated proteins (e.g., proteasomal subunits).

We wanted to investigate and graph an updated version of the ROD. To this end, we merged networks 7, 10, 13, 17, 20, 28, 38, and 41 and analyzed the obtained map (Figure 7). Although displaying few peripheral subgraphs, this merged network presented a central core of a few pivotal nodes. These nodes were evidenced (light blue) as to highlight their edges. A counterclockwise list includes: huntingtin (HTT), heat shock protein 70 kDa (HSP70), heat shock protein 90 kDa alpha class A member 1 (HSP90AA1), heat shock 70 kDa protein 8 (HSPA8), nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor beta (NFKBIB), and heat shock protein 90 kDa alpha class B member 1 (HSP90AB1).

These proteins are involved in cell protection to denaturing stress and folding control (HSPs),<sup>47</sup> vesiculation (HTT),<sup>48</sup> and apoptotic signaling cascades (NFKBIB).<sup>49</sup> As postulated by Capra,<sup>50</sup> a series of proteins/enzymes playing partially overlapped biological functions could potentially act as a catalytic ring. A catalytic ring is a closed number of interactors that induce the activity of the subsequent node in the row, up until the last one interacts with the first so as to close a hypothetical ring. Within this ring, the activity of each gene is reinforced in every cycle. The catalytic ring hereby individuated could exploit the basis of this principle to counteract oxidative stress (PRDX1 intermediate) and protein damage (HSPs) or, when this no longer possible, activate vesiculation events (HTT) to remove damaged proteins, or trigger apoptosis (NFKBIB). Such an oversimplified view of the biological complexity of these merged networks conveys the idea that, although it is relevant to determine and classify protein actors through proteomics, it is still fundamental to determine if and how these proteins finally interact to comprehensively understand their relevance.

## Conclusion

Recent technical advancements in the field of proteomics and interactomics have provided us with the basilar tools for this updated overview. A list of 1989 nonredundant proteins has been compiled on the basis of data available from literature.<sup>15,26,29–32</sup>

Although it has always been intuitive that RBCs suffer from an increased oxidative stress, pathway analyses of the canonical and toxicity pathways produced an unbiased score-based evaluation (Figure 1).

Network analysis provided a list of 50 networks among which top score networks were mainly devoted to protein protection to folding damage (Table 3). In particular, an organized web of interactions involving a handful of proteins has been individuated and could likely constitute a catalytic ring to enhance the Repair or Destroy box activity (Figure 7) that Goodman and colleagues proposed in 2007.<sup>9</sup> Network analyses also pinpoint a series of proteins that appear to be the likely trigger of central molecular cascades, even of some proteins that have not yet been individuated experimentally, thus suggesting further research directions or indicating crucial molecules that induce signaling events in the sample tissue/cell, albeit being produced elsewhere (HNF4A).

Although conveying valuable new insights on the biology of RBC response to oxidative stress, our analysis supports recent proteomics and genomics integrated studies on the likely

relation of SEC23B with CDA II, in a faster and unbiased way which does not necessarily rely on the acuteness of the authors.<sup>37</sup>

Interactomics certainly holds a future ahead, which will be disclosed as newly spreading software platforms will allow progressively more in-depth analyses on a broader data set, without numerical or graphical limitations (for example: 50 networks threshold and the maximum limit of 500 nodes to be represented at the same time).

We are still in that early phase in which a dictionary is being written, when a biological meaning is attributed to a specific interactomic profile (networks shape complexity, subgraph localization, catalytic ring, “interactomics noise”, crucial interactors). When this phase is over, we will be confidently relying on the study of the whole proteome profile (genome, transcriptome, PTMome and other “omes” as well), instead of focusing on specific molecules or specific pathways alone.

**Supporting Information Available:** Supplementary tables. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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