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5 ***Plasmodium falciparum* infection induces dynamic changes in the erythrocyte**
6 **phospho-proteome**

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31 **Abstract**

32 The phosphorylation status of red blood cell proteins is strongly altered during the
33 infection by the malaria parasite *Plasmodium falciparum*. We identify the key
34 phosphorylation events that occur in the erythrocyte membrane and cytoskeleton
35 during infection, by a comparative analysis of global phospho-proteome screens
36 between infected (obtained at schizont stage) and uninfected RBCs. The meta-
37 analysis of reported mass spectrometry studies revealed a novel compendium of 495
38 phosphorylation sites in 182 human proteins with regulatory roles in red cell
39 morphology and stability, with about 25% of these sites specific to infected cells. A
40 phosphorylation motif analysis detected 7 unique motifs that were largely mapped to
41 kinase consensus sequences of casein kinase II and of protein kinase A / protein
42 kinase C. This analysis highlighted prominent roles for PKA / PKC involving 78
43 phosphorylation sites. We then compared the phosphorylation status of PKA (PKC)
44 specific sites in adducin, dematin, Band 3 and GLUT-1 in uninfected RBC stimulated
45 or not by cAMP to their phosphorylation status in iRBC. We showed cAMP-induced
46 phosphorylation of adducin S59 by immunoblotting and we were able to demonstrate
47 parasite-induced phosphorylation for adducin S726, Band 3 and GLUT-1,
48 corroborating the protein phosphorylation status in our erythrocyte phosphorylation
49 site compendium.

50

51

52 **Abbreviations:**

53 CK1 and CK2, casein kinases I and II; GO, gene ontology; GLUT-1, glucose
54 transporter 1; iRBC, infected red blood cell; LC-MS/MS, liquid chromatography
55 tandem mass spectrometry; LFQ, label free quantification; PTMs, post-translational
56 modifications; PKA, protein kinase A; PKC, protein kinase C

57

58 **Keywords:** Erythrocyte; *P. falciparum*; Protein phosphorylation; cAMP / protein
59 kinase A; GLUT-1; Cytoskeleton

60

61 **1.1 Introduction**

62 Mammalian erythrocytes have been widely studied for cytoskeleton structure,
63 membrane composition and transport properties. Over the last decade, numerous
64 studies have incorporated mass spectrometry techniques to mine and quantify the
65 proteins expressed in red blood cells (RBCs) and to link these proteins functionally to
66 various physiological or pathophysiological situations. However, even if these cells
67 have a simple structural organization and specific function, proteomics studies have
68 proven difficult to get a good overview of the diversity of proteins in RBCs for several
69 reasons: (i) the abundance of ultra-majoritarian proteins in the cytosol (haemoglobin,
70 up to 98% of cytosolic proteins at an unrivalled concentration: 5 mM – 340 g/l) and in
71 the membrane (Band 3, 1 M copies/cell) and (ii) the tight links between membrane
72 and sub-membranous cytoskeleton. With the progression of techniques, the number
73 of proteins identified has raised to an unexpected number of 1578 unique proteins in
74 the cytosol [1], and a total number of 2289 unique proteins in the RBC identified so
75 far [2].

76 The next challenge now is to link these proteins to physiological processes, and one
77 key feature is to elucidate the regulatory role of various post-translational
78 modifications (PTMs). These modifications include oxidation effects, glycosylation,
79 palmitoylation and most of all protein phosphorylation. Indeed, kinase activities have
80 been described as a key regulatory mechanism in RBC and deregulation of their
81 activities seems to be implicated in multiple diseases [3] including malaria, the focus
82 of this study. Human RBCs in circulation harbour various active protein kinases,
83 including protein kinase C (PKC), protein kinase A (PKA), casein kinases I and II
84 (CK1 and CK2), Syk, Lyn, Hck-Fgr, and Fyn, as reviewed by Pantaleo et al. [4].
85 Activity of these kinases has been mostly studied regarding their effects on
86 cytoskeleton or membrane proteins in various physiological or pathophysiological
87 contexts.

88 Spectrin phosphorylation by casein kinase has been associated with membrane
89 destabilization [5] and enhanced spectrin phosphorylation is linked to hereditary
90 elliptocytosis and pyropoikilocytosis [6]. The horizontal junctional complex
91 components are also phosphorylated; the combined phosphorylation of adducin and
92 protein 4.1 by PKC decreased their binding to spectrin and actin, resulting in weaker

93 membrane stability [7]; dematin is known to be phosphorylated by PKA, leading to
94 disruption of actin/spectrin binding [8]. Regarding vertical complexes and integral
95 membrane proteins, Band 3 PTMs have been widely studied (in regard to its pivotal
96 role in membrane transport and structure). Notably, Band 3 tyrosine phosphorylation
97 triggers its dissociation from ankyrin, consequently releasing Band 3 from
98 spectrin/actin cytoskeleton [9].

99 The present study focusses on the changes in RBC protein phosphorylation resulting
100 from infection with the human malaria causing parasite *Plasmodium falciparum*.
101 During its complex life cycle, the parasite invades RBCs, where it is largely hidden
102 from the host immune system. Inside erythrocytes it multiplies via the process of
103 schizogony every 48 h to form up to 32 new merozoites. After rupture of infected
104 RBCs, merozoites are released to invade fresh red cells and to complete a new
105 cycle of asexual development. During intra-erythrocytic development, the parasite
106 remodels the red blood cell surface, notably by exporting proteins to the host cytosol
107 and membrane and by forming knobs that mediate interactions with host endothelial
108 cells to escape clearance of infected RBCs (iRBC) by the spleen. The process of
109 sequestration and cytoadherence of iRBCs results in clogging of blood vessels in
110 various organs and it contributes to the clinical symptoms of malaria. Host cell
111 permeability [10] or deformability [11] are also largely modified upon infection.
112 Various exported parasite proteins play crucial roles in this process, and lately it's
113 becoming clear that an involvement of host proteins is also required for parasite-
114 mediated host cell remodelling. Multiple host proteins see their phosphorylation
115 status altered during infection, which was demonstrated by the pioneering study of
116 Wu et al. [12], who in 2009 showed increased phosphorylation during parasite
117 infection by immunoblotting and mass spectrometry. With the emergence of liquid
118 chromatography tandem mass spectrometry, several studies [13—16] followed with
119 phospho-proteome analyses of iRBCs, but remarkably largely ignored published
120 data on phosphorylation sites identified in human red blood cell proteins. This study
121 summarizes our current knowledge on RBC phosphorylation by comparing published
122 phosphorylation sites measured in normal and *P. falciparum* infected RBCs and
123 experimentally validating some of the major findings.

124

125 **2. Material and methods**

126 *2.1. Compendium of red blood cell phosphorylation sites*

127 Phospho-peptide sequences identified in large scale RBC phospho-proteome LC–
128 MS/MS studies with data taken from normal [17] and *P. falciparum*-infected RBCs at
129 the schizont life cycle stage [13–16] were remapped to the UNIPROT protein
130 database using the software tool Protein Coverage Summarizer
131 (<http://omics.pnl.gov/software/protein-coverage-summarizer>) in order to generate a
132 uniform format for comparing phosphorylation sites across data sets. The
133 compendium of phospho-sites included all sites detected in normal RBCs from
134 Soderblom et al. [17], and sites from infected RBCs that were detected at least twice
135 in the four independent studies [13–16].

136 *2.2. Phosphorylation motif analyses*

137 Phosphorylation sites were categorized by their chemical properties as acidic, basic,
138 proline-directed, tyrosine or other by a decision tree method described previously [14]
139 as follows: 1) get the 6 neighbouring amino acids before and after the
140 phosphorylation site; 2) pY at position 0 then classify as “Tyrosine”; 3) P at+1 then
141 classify as “Proline-directed”; 4) positions +1 to +6 contain more than one D and E
142 residues then classify as “Acidic”; 5) K or R at position –3 then classify as “Basic”; 6)
143 D or E at +1,+2, or +3 then classify as “Acidic”; 7) between –6 and –1 more than 2 K
144 or R residues then classify as “Basic”; 8) remaining peptides classify as “Other”.
145 Phosphorylation motifs were identified using MotifX [18] with the following
146 parameters: phosphorylation motif window = 13 amino acids, p-value threshold = $1 * 10^{-4}$
147 for S and T residues, $1 * 10^{-3}$ for Y residues, motif fold increase ≥ 2 , a motif
148 frequency N5, and a background of all RBC proteins identified. The analysis was
149 repeated for a degenerate amino acid set with conservative amino acid substitutions
150 within the motif window according to: A = AG, D = DE, F = FY, K = KR, I = ILVM, Q =
151 QN, S = ST, C = C,H=H, P=P,W=W. When different motifs were found for a peptide
152 by the analyses with different amino acid residues, priority was given to the motif
153 with the highest MotifX score. Sequence logos were generated with Weblogo 3 [19]
154 from <http://weblogo.threeplusone.com/create.cgi>. The motifs were matched to known

155 protein kinase target motifs using PhosphoMotifFinder [20] and matches were
156 considered as potential links between phosphorylation motifs and protein kinases.

157 2.3. Gene ontology (GO) analysis

158 GO enrichment analyses of lists of membrane-associated phosphoproteins from
159 infected and normal RBCs were carried out using the web tool Database for
160 Annotation Visualization and Integrated Discovery (DAVID, version 6.7.
161 <http://david.abcc.ncifcrf.gov/>) [21,22] with a background set of all human proteins.
162 Enrichment of GO FAT terms was considered statistically significant when corrected
163 for multiple testing by the Benjamini–Hochberg method with adjusted p-values lower
164 than 0.05. Overlap between enriched GO terms was visualised in a network with the
165 Cytoscape plugin Enrichment Map [23].

166 2.4. Cell preparation and Western blotting

167 Human RBCs were drawn from healthy volunteers under informed consent, washed
168 several times in RPMI, kept in culture medium (see below) and used within one week
169 of collection. *P. falciparum* NF54 was grown in RBCs as described previously, using
170 0.5% Albumax II® (Invitrogen) instead of human serum [52]. Parasites were
171 synchronized twice using 5% w/v sorbitol solution according to Lambros and
172 Vanderberg [53]. Schizont-stage parasite cultures were then enriched to N95% by
173 centrifugation on a 70% Percoll/sorbitol solution as described in [24]. Parasite growth
174 and development of schizont stages was monitored by Giemsa-stained thin blood
175 smears. Uninfected RBC controls were incubated for 48 h in culture medium at 37 °C
176 and kept in the presence or absence of 50 µM dibutyryl-cAMP (Sigma Aldrich), a
177 cell-permeable non-hydrolysable cAMP analogue that activates protein kinase A, for
178 the last 30 min before harvesting. Cell counts were determined by using a
179 Cellometer Mini (Nexcelom Bioscience) automated cell counter following
180 manufacturer's recommendations.

181 Western blotting of uninfected RBCs and *P. falciparum*-infected RBC (1 × 10⁷)
182 samples was performed using erythrocyte ghosts. Briefly, RPMI-washed
183 erythrocytes were incubated in 20 volumes of ice-cold hypotonic buffer 5P8 (5 mM
184 NaH₂PO₄, pH 8.0) containing 1 mM phenylmethylsulfonyl fluoride, protease inhibitor
185 mix (1 µg each of chymostatin, leupeptin, antipain, and pepstatin and 8 µg aprotinin

186 per ml), and phosphatase inhibitors (10 mM Na fluoride, 2mMβ-D glycerophosphate,
187 1 mM Na orthovanadate), then washed several times in 5P8 containing protease and
188 phosphatase inhibitors (30 min centrifugation 14,000 ×g at 4 °C). Membrane proteins
189 were solubilized in lysis buffer (150 mM NaCl, 10 mM KCl, 1 mM MgCl₂, 20mM Tris–
190 HCl pH 7.5, 1% Triton X-100) in the presence of protease and phosphatase
191 inhibitors. *P. falciparum*-infected RBC samples were further lysed by sonication
192 followed by centrifugation at 10,000 ×g. Dephosphorylation of proteins was achieved
193 by incubation with λ-protein phosphatase in 1× NE Buffer (New England BioLabs,
194 Inc.) supplemented with 1 mM MnCl₂ for 30 min at 30 °C. A Bradford protein assay
195 kit (Bio-Rad Laboratories) was used to determine protein concentrations. Protein
196 samples diluted in reducing Laemmli buffer were separated using NuPAGE 10% Bis-
197 Tris (Invitrogen) SDS-PAGE. Western blotting was performed using standard
198 methods. Antibodies used to probe RBC membrane antigens were rabbit monoclonal
199 anti-α-adducin phospho Ser59 (Abcam ab76251) 1/500; rabbit polyclonal anti-α-
200 adducin phospho Ser726 (Santa Cruz sc101627) 1/200; mouse monoclonal anti-α-
201 adducin (Abcam ab54985) 1/1000; rabbit polyclonal anti-dematin phospho Ser 403
202 (Sigma-Aldrich SAB4504167), 1/500; mouse polyclonal anti-dematin (Abcam
203 ab89161) 1/500; horseradish peroxidase-conjugated goat anti-rabbit IgG (H + L)/goat
204 anti-mouse IgG (H + L) (Bio-Rad Laboratories) 1/3000. Pierce ECL 2 Western
205 blotting detection reagent (Thermo Scientific) was used and fluorescent signals were
206 captured by Typhoon fluorescence imager (GE Healthcare Life Sciences).

207 For immunoprecipitation, uninfected RBCs and *P. falciparum* infected RBC (1×10^8)
208 lysates were incubated with rabbit monoclonal anti-phospho-Akt substrate RxxS*/T*
209 (110B7E) antibody conjugated to sepharose beads (9646 Cell Signalling) for 4 h at
210 4 °C, then washed 3 times with lysis buffer containing protease and phosphatase
211 inhibitors. Samples were eluted by boiling the beads in reducing Laemmli buffer,
212 then subjected to SDS-PAGE and Western blotting using standard methods.
213 Membranes were probed with mouse monoclonal anti-Band 3 (BIII-136, Sigma
214 B9277) 1/20,000 and mouse monoclonal anti-GLUT-1 (Abcam ab40084) 1/2500.

215

216 2.5. Mass spectrometry

217 Ghost preparations of uninfected RBCs and Percoll-purified schizont stage *P.*
218 *falciparum*-infected RBCs (N95% enrichment, 100 µg) were digested in solution by
219 trypsin using the FASP procedure [25], and tryptic peptides were purified by STAGE
220 tips [26]. Liquid chromatography tandem mass spectrometry analysis of tryptic
221 peptides (500 ng) was performed on the Orbitrap Velos Pro mass spectrometer
222 (Thermo Fisher, Bremen, Germany) equipped with the Ultimate 3000 UPLC system
223 (Thermo Fisher, Germany) as previously described in [27]. Protein identification at 1%
224 FDR and label free quantification (LFQ) were performed using MaxQuant [28] with
225 settings that were described in [27].

226 3. Results

227 3.1. Compendium of protein phosphorylation sites in red blood cells

228 Changes in phosphorylation events at serine, threonine and tyrosine amino acid
229 residues of human red cell proteins induced by infection with the malaria parasite *P.*
230 *falciparum* were established by a comparative analysis of the four published
231 phospho-proteomes of parasite-infected cells [13–16] with normal red cells [17].
232 Large scale *P. falciparum* phospho-proteome studies were primarily focussed at the
233 schizont intra-cellular life cycle stage identifying more than 9000 parasite
234 phosphorylation sites at various confidence levels [29]. We therefore required
235 detection by at least two independent studies for inclusion of human red blood cell
236 phosphorylation sites in this study. The uninfected red cell phosphorylation sites
237 were taken from the work of Soderblom et al. as part of their investigation of the
238 ERK1/2-mediated human phosphorylation changes in sickle red blood cell
239 membrane [17]. Taken together, we generated a novel information source
240 comprising 495 phosphorylation sites in 182 human proteins (Table S1) that
241 combines 380 sites found in normal red cells and 274 sites described for infected red
242 cells with 158 phospho-sites in common, as depicted in the Venn diagram (Fig. 1A).
243 The distribution of phosphorylation sites by amino acid in infected cells and normal
244 RBCs (Fig. 1B) is similar to a previous large scale phosphorylation study in humans
245 reporting 6600 sites [30]. We find similar distributions of phospho-sites between
246 infected cells and normal cells for serine (79.9% vs 76.3%) and threonine (18.2% vs

247 17.9%) and higher tyrosine phosphorylation for normal cells with 5.8% compared to
248 1.8% in infected cells.

249 We supplemented the phosphorylation information with knowledge from additional
250 sources about these sites and found that 17 sites have been reported in the review
251 by Pantaleo et al. [4] about phosphorylation changes in red cell membranes of
252 normal and diseased red cells, and that 359 sites (72.5%) are stored in the Kinexus
253 PhosphoNET database [31] including 267 sites with experimental evidence for
254 detection in various cell types and 92 bioinformatically predicted sites. We also
255 compared our compendium of phosphorylation sites with results from small scale
256 protein phosphorylation studies [4,12] by two dimensional gel electrophoresis
257 combined with mass spectrometry in normal RBCs and *P. falciparum* trophozoite-
258 infected RBCs. Wu et al. [12] reported 34 human protein phosphorylation sites of
259 which 25 are present in our compendium, and Pantaleo et al. [4] identified 22
260 phospho-sites during parasite growth including 3 reported here. Finally, we added
261 gene ontology cellular localisation annotation to highlight phosphorylation sites in the
262 red cell cytoskeleton. In total we find 94 RBC cytoskeleton phospho-sites specifically
263 detected in infected cells, and 143 specific sites in the membrane of normal RBCs.

264 3.2. Phosphorylation motif analysis

265 Our compendium of 495 phosphorylation sites detected in normal and *P. falciparum*-
266 infected red blood cells enabled us to identify shared phosphorylation motifs and to
267 predict associated protein kinase activity in red cells. Firstly, we compared
268 phosphorylation motif classes between infected and normal red cells (Fig. 1C), with
269 motif classes based on their chemical properties previously described in the schizont
270 phosphoproteome [14]. Almost identical distributions of the acidic and proline-
271 directed classes are found between normal and infected RBCs, whereas a 3.25-fold
272 upregulation of basic-directed motifs and a 3-fold downregulation in tyrosine-directed
273 motifs are observed in iRBCs. Secondly, phosphorylation sites were mapped to
274 motifs with the phosphorylation motif finding algorithm MotifX [18], and we identified
275 7 phosphorylation motifs (Fig. 1D): 4 acidic-directed motifs (motif 1: [pS/pT]xx[E/D], 4:
276 [pS/pT]x[E], 6: [pS/pT] [E/D] [E/D],[E/D], 7: [pS/pT]x[E/D][E/D]), 2 proline-directed
277 motifs (motif 2: [pS/pT][P], 5: [R/K]xx[pS/pT][P]) and 1 basic-directed motif (motif 3:
278 [R/K]xx[pS/pT]). All these phosphorylation motifs were observed in proteins from

279 normal and infected red cells (Fig. 1D). Phosphorylation sites specifically detected in
280 normal RBCs include all 7 motifs, while sites identified exclusively in infected RBCs
281 include 5 motifs with the exceptions of motifs 6 and 7 that were not mapped on
282 specific sites in iRBCs. Thirdly, the 7 motifs were associated with kinase target sites
283 by PhosphoMotifFinder [20], where we consider primarily human protein kinases
284 which have been detected in red blood cells by mass spectrometry (Table S2)
285 [32,33]. This linked casein kinase II to 133 phosphorylation sites (motifs 1,4, 6 and 7)
286 and protein kinase A/protein kinase C to 78 sites with motifs 3 and 5. Motif 2
287 [pS/pT][P] is mapped to several protein kinases (ERK1,ERK2, CDPK5 and GSK3)
288 with 67 sites, where all four kinases have escaped detection by mass spectrometry
289 in RBCs so far [32,33].

290 *3.3. Functional Implications of RBC phospho-proteomes*

291 The functional implications of the phospho-proteome to RBC physiology were
292 assessed by GO enrichment analyses of the 123membrane associated RBC
293 phospho-proteins compared to all human proteins using DAVID [21,22]. The
294 enrichment results for cellular component, molecular function and biological process
295 ontologies are depicted as a histogram plot with bars representing terms for infected
296 and normal RBCs (Fig. 2A). The strongest signals are found for the spectrin-
297 associated cytoskeleton with 170-fold enrichment for infected RBCs and 115-fold
298 enrichment in normal red cells, followed by spectrin-binding with 154-fold enrichment
299 in infected RBCs and 114-fold enrichment in normal RBCs. This trend of stronger
300 enrichment in iRBCs than in normal RBCs is observed for all cytoskeleton-related
301 GO terms. All GO terms functionally associated with the cytoskeleton and surface
302 membrane of infected RBCs were organized and visualised in a network (Fig. 2B)
303 using the Cytoscape plugin Enrichment Map [23], where each term is represented by
304 a node with edges showing degree of overlap between GO term sets. The
305 cytoskeleton subnetwork of infected RBC GO terms – depicted as cluster A in Fig.
306 2B – is composed of 35 terms out of 43, with 8 terms specific for iRBCs and 27
307 terms showing higher enrichment in iRBCs.

308 Most terms relate to cytoskeleton organization and regulatory processes, e.g. for
309 actin filament polymerization and protein complex assembly, and thereby highlighting
310 associated proteins with roles in orchestrating red cell shape and deformability. The

311 filament proteins making up the ordered meshwork forming the cytoskeleton in red
312 cells are observed phosphorylated in infected and normal red cells (Fig. 3A).
313 Spectrins, actin, protein 4.1, adducin, dematin, tropomyosin and tropomodulin show
314 multiple phosphorylation sites presumably regulating cytoskeleton organization.
315 Several transmembrane glycoproteins with transbilayer domains (Band 3,
316 glycophorin C) anchoring the cytoskeleton via their cytoplasmic domains are
317 regulated by kinase activity via multiple phosphorylation sites.

318 The phosphorylation status in our compendium for the main erythrocyte cytoskeleton
319 proteins, transporters and various membrane associated proteins in normal and
320 infected RBCs is listed in Fig. 3B, which shows that *P. falciparum* infection likely
321 induces specific changes in RBC phosphorylation status. For the cytoskeleton
322 protein 4.2, spectrin alpha chain, the glucose transporter 1 (GLUT-1), and for
323 membrane associated proteins we observe strong upregulation of *P. falciparum*
324 induced sites with the detection of more than 50% specific sites.

325 *3.4. Wet-bench validation of some phospho-sites and their cAMP dependence*

326 Given that infection of RBC by *P. falciparum* leads to a rise in intra-erythrocyte cAMP
327 levels [34–37] combined with our identification of two potential PKA phosphorylation
328 motifs we decided to ask how many of the phospho-sites detected in infected
329 erythrocyte proteins are phosphorylated by a cAMP-dependent kinase. To this end,
330 we compared the phosphorylation status of red blood cell adducin, dematin, Band 3
331 and GLUT-1 in uninfected RBC stimulated or not by cAMP-stimulation to their
332 phosphorylation status in iRBC (Fig. 4). With adducin we focussed on two phospho-
333 sites; namely, Ser59 and Ser726 and for dematin we examined Ser403, as for both
334 proteins antibodies against these specific phospho-sites are commercially available
335 (Fig. 4A). Phosphorylation of Ser59 in adducin can be observed in uninfected ghost
336 lysates (lane 1), where the degree of phosphorylation increases following cAMP-
337 stimulation (lane 2) and is completely ablated by phosphatase treatment (lane 3).
338 Thus, Ser59 is a bona fide cAMP-dependent kinase site. Ser59 is also observed in
339 iRBC ghost lysates and the phospho-signal is ablated by phosphatase treatment
340 (lane 5). However, comparing lanes 1 and 4 leads to the conclusion that - although
341 Ser59 is a cAMP dependent site - the level of phosphorylation in iRBC is similar to
342 non-infected erythrocytes i.e. infection doesn't appear to increase phosphorylation of

343 Ser59. By contrast, Ser726 of adducin is phosphorylated only in iRBC, and in non-
344 infected erythrocytes phosphorylation of Ser726 is not sensitive to cAMP stimulation
345 (compare lanes 2 and 4). Phosphorylation of Ser726 in iRBC is ablated by
346 phosphatase treatment, but this site does not appear to be a substrate for a cAMP-
347 dependent kinase. Ser726 occurs within a motif 3 context (Fig. 1D) that was
348 ambiguously mapped to PKA or PKC and the data in Fig. 4A suggests that in
349 infected RBC it is PKC rather than PKA that phosphorylates Ser726 of adducin.

350 Ser403 in dematin can be observed weakly phosphorylated in erythrocyte ghost
351 lysates (lane 1) and its phospho-status increases under the influence of cAMP (lane
352 2) and is significantly diminished by phosphatase treatment (lane 3). In infected
353 erythrocytes one can observe a strong band that is insensitive to phosphatase
354 treatment (lanes 4 and 5) indicating a likely non-specific cross-reactivity of the
355 pSer403 antibody to an unknown parasite protein present in the iRBC ghost
356 preparation.

357 The apparent lower molecular weights of 48 and 52 kDa dematin isoforms following
358 phosphatase treatment of iRBCs sample (Fig. 4A; lane 5 lower panel) suggest that
359 dematin phosphorylation is maintained at other phosphorylatable residues during *P.*
360 *falciparum* infection.

361 We next turned our attention to Band 3 and GLUT-1 to which there are no available
362 phospho-specific serine or threonine antibodies, so we exploited the availability of
363 commercial antibodies to sites phosphorylated in the context of an RxxS*/T* motif.
364 We recall that the RxxS*/T* motif is present in cAMP-dependent PKA motif following
365 our analysis of RBC proteins detected phosphorylated in vivo (Fig. 1D). First, the
366 levels of both Band 3 and GLUT-1 were determined for RBC (lane 1), RBC
367 stimulated by cAMP (lane 2) and for iRBC (lane 3). We note the drastic reduction in
368 GLUT-1 levels observed in iRBC (lane 3) and that GLUT-1 appears highly
369 glycosylated as judged by the smear-like signal (lanes 1 and 2). Both α -adducin and
370 dematin levels were used as a loading control (input). Following immunoprecipitation
371 with the sepharose-linked anti-RxxS*/T* antibody the amounts of Band 3 and GLUT-
372 1 in the precipitate were estimated by Western blot (top two panels; IP anti-
373 RxxS*/T*). Band 3 is readily detected phosphorylated at an RxxS*/T* site in iRBC
374 (lane 3) and weakly detected in non-infected erythrocytes (lane 1). However, the

375 phosphorylation level is not increased following cAMP stimulation (lane 2) indicating
376 that the strong signal observed following infection (lane 3) is likely to be due to
377 erythrocyte PKC that like PKA can phosphorylate residues in the context of the
378 RxxS*/T* motif.

379 The low levels of GLUT-1 observed by Western Blot for iRBC (input, lane 3) and the
380 observed smeared signal that has been reported to be the result of protein
381 glycosylation [38] lead us to estimate the amount of GLUT-1 present in our ghost
382 preparations by mass spectrometry analysis of digested protein samples using label
383 free quantification LFQ [39] (Fig. 3C). Here, glycosylation does not interfere with the
384 quantification measurement and similar GLUT-1 relative protein abundances were
385 determined in infected and normal RBCs, which was expected due to the high levels
386 of glucose consumption by iRBC [40]. After immunoprecipitation with anti-RxxS*/T*,
387 however, the signal in iRBCs (lane 3) appears stronger than in non-infected cells,
388 stimulated or not by cAMP (lane 1 and 2). As for Band 3, this suggests an increase
389 in GLUT-1 phosphorylation for this motif upon infection.

390 **4. Discussion**

391 In this study we used a comparative analysis of phosphorylation sites between
392 normal and *P. falciparum*-infected red blood cells and showed that the main host
393 proteins determining cell shape, rigidity/ deformability or permeability are
394 differentially phosphorylated/dephosphorylated upon infection. The compendium of
395 human sites was compiled from published phospho-proteome studies generated by
396 liquid chromatography tandem mass spectrometry focussed at the schizont life cycle
397 stage during asexual development in RBCs. These sites were reported in
398 supplementary tables as 'by-products' to *P. falciparum* phosphorylation sites without
399 further functional interpretation and have gone largely unnoticed.

400 Our phosphorylation analysis is quite stringent, as it required that a given phospho-
401 site be detected in at least 2 independent studies done in separate laboratories. This
402 identified 495 sites in 182 human RBC proteins of which 379 sites in 153 proteins
403 can be detected in normal RBC, and 274 sites (91 proteins) detected in iRBC of
404 which 158 in 57 proteins are in common. This indicates that 116 sites belonging to
405 50 proteins appear to be specifically phosphorylated upon *P. falciparum* infection,
406 and that 221 sites belonging to 113 proteins are likely be subjected to phosphatase

407 activities in iRBCs. Strikingly, in iRBCs tyrosine phosphorylation appears less
408 frequent (5.8% down to 1.8%) suggesting that infection has induced tyrosine
409 phosphatase activity. The parasite lacks any gene encoding classical tyrosine
410 kinases [41] and consequently, all tyrosine phosphorylation events in erythrocyte and
411 parasite proteins likely derive from erythrocyte tyrosine kinase activity. We consider
412 the alternative explanation of incorrect phosphorylation localisation in normal RBCs
413 less likely than increased phosphatase activity in iRBCs given that 62.5% of the sites
414 have been confirmed experimentally, or predicted [31]. Amongst the 22 pY sites in
415 normal RBCs we find a site in a protein phosphatase (PTPRD_HUMAN Y274)
416 potentially involved in switching on phosphatase activity in infected RBCs. Other
417 candidate tyrosine phosphatases in red cells are ACP1 and PTPRC (CD45) that
418 have been detected in red cells by mass spectrometry [32,33] (Table S2).

419 Previous small scale phosphorylation studies in iRBCs of the trophozoite life cycle
420 stage by Wu et al. [12] and Pantaleo et al. [4] analysed respectively 34 and 18 spots
421 (Table S3) isolated from two dimensional protein gels. The majority of the sites
422 (73.5%) detected by Wu et al. are present in our compendium that is covering the
423 schizont asexual life cycle stage, but not the trophozoite stage, while most sites
424 detected by Pantaleo et al. (86%) are absent in our compendium. These studies
425 highlighted the role of protein phosphorylation in the interactions between the
426 parasite and its human erythrocyte host and reported phosphorylation sites in key
427 cytoskeletal proteins, e.g. spectrin beta, ankyrin 1, Band 3, alpha and beta adducin,
428 Band 4.1, and dematin. All these proteins are found phosphorylated in our
429 compendium, although some sites have escaped detection. Dematin pS403 is an
430 example that has been detected by peptide NELKKKApSLF that allows for three
431 miscleaved tryptic sites in the identification searches. In our compendium most
432 studies utilised standard identification settings including 2 miscleavages and thereby
433 likely excluded the identification of this peptide.

434 The role of protein phosphorylation in alterations of red cell membrane and
435 cytoskeleton is evident from the GO enrichment analysis of the 123 membrane-
436 associated RBC phosphoproteins (Fig. 2). This analysis shows that spectrin-
437 associated cytoskeleton is 170-fold enriched in iRBC up from 114-fold in normal
438 RBC, which supports the findings of trophozoite-infected red blood cells [4,12].
439 Generally, it is consistent with infection particularly inducing phosphorylation of

440 spectrin-associated functions like actin filament polymerization and protein complex
441 assembly highlighting parasite-induced orchestration of red cell shape and
442 deformability. For example, following *P. falciparum* infection more than 50% of the
443 specifically induced sites were in protein 4.2, spectrin alpha chain and the glucose
444 transporter GLUT-1 (Fig. 3).

445 We identified 7 short linear consensus sequences around phosphorylated residues
446 determining the phosphorylation motif for protein kinase activities for 56% of the
447 phosphorylation site compendium. The phosphorylation motifs are observed in
448 normal and infected RBCs suggesting that human kinase activity is primarily
449 responsible for phosphorylation events involving these sites in infected RBCs. Global
450 proteome studies of red cells [32,33] reveal a limited repertoire of 16 human
451 serine/threonine protein kinases (Table S2) potentially recognising these 7
452 phosphorylation consensus site motifs. The limited number of protein kinases
453 detected by mass spectrometry in RBCs facilitates linking protein kinase activities
454 with phosphorylation motifs by eliminating protein kinases that are absent in RBCs
455 from ambiguous bioinformatics predictions. This revealed prominent roles for casein
456 kinase II (CK2) activity in red cells with 133 predicted sites, and agrees well with the
457 reported involvement of CK2 in phosphorylating cytoskeletal proteins (e.g. spectrin,
458 adducin, ankyrin, and protein 4.1) [42,43], in phosphorylation of the blood group
459 antigens Kell and Kx [44], in regulation of spectrin binding by phosphorylated ankyrin
460 [45] and in regulating cytoadherence of *P. falciparum*-infected red blood cells [46].

461 Although all CK2 sites were observed in normal and infected RBCs, the 34 CK2 sites
462 mapping to motifs 6 and 7 (Fig. 1D) are absent in the sites exclusively observed in
463 infected RBCs. This suggests that parasite infection induces phosphatase activities
464 that dephosphorylate CK2-positive sites to modulate red cell morphology. Protein 4.1,
465 which is a major structural element of the red cell cytoskeleton and regulates
466 mechanical stability, is dephosphorylated upon infection at pS104 and pS95, and
467 ankyrin – which forms the bridge between the integral membrane protein Band 3 and
468 spectrin – is dephosphorylated at pS817.

469 The motif analysis highlighted prominent roles for PKA/PKC involving 78
470 phosphorylation sites. For this reason, we compared the phosphorylation status of
471 specific sites in adducin, dematin, Band 3 and GLUT-1 in uninfected RBC stimulated

472 or not by cAMP to their phosphorylation status in iRBC. With adducin we focussed
473 on two phospho-sites; namely, Ser59 and Ser726 and for dematin we examined
474 Ser403, as both proteins can be phosphorylated at these sites by cAMP-dependent
475 PKA [47–50], and phosphosite-specific antibodies are commercially available. When
476 we examined Ser59 of adducing in normal RBC we observed an increase in its
477 phosphorylation status following cAMP-stimulation. We point out that cAMP-
478 stimulation was performed using intact RBCs, right before ghost preparation.
479 However with this caveat, *P. falciparum* infection that leads to increased cAMP levels
480 and greater PKA activity [34–37] does not lead to a detectable increase in Ser59
481 phosphorylation in iRBC. By contrast, Ser726 is detected as a doublet by the
482 phospho-specific antibody only in iRBC, but its phosphorylation in uninfected RBC
483 isn't induced following cAMP-stimulation implying that in iRBC Ser726
484 phosphorylation is not by cAMP-dependent PKA, but perhaps by another host or
485 exported parasite AGC-like kinase. There are two isoforms of dematin in normal
486 RBC [32,33] and Ser403 phosphorylation is induced up cAMP-stimulation [47–49],
487 but neither isoform is observed phosphorylated in iRBC, suggesting that infection
488 has led to dephosphorylation of S403 in dematin. Surprisingly, the commercial
489 phospho-Ser403 antibody strongly detected a signal band in iRBC that likely
490 represents a cross-reactive parasite protein present in the ghost preparation.

491 As there are no specific antibodies to phosphorylated serines and threonines in Band
492 3 and GLUT-1 we exploited a commercial sepharose-linked antibody that reacts with
493 residues phosphorylated in the context of RxxS*/T* that resembles closely the
494 enriched PKA motifs (3 & 5 in Fig. 1D). Following immunoprecipitation the
495 precipitates were probed with specific antibodies to Band 3 and GLUT-1. *P.*
496 *falciparum*-infection of RBC induces phosphorylation of Band 3 at one or more
497 RxxS*/T* sites and careful inspection of human Band 3 reveals the presence of 3
498 sites (RyqS*(S349); KpdS*(S356) and KasT*(T746)) all of which have been detected
499 phosphorylated in iRBC [14,16]. However, cAMP-stimulation of normal RBC did not
500 induce phosphorylation at any RxxS*/T* site implying that in iRBC infection-induced
501 phosphorylation at S349, S356, or T746 is by cAMP-independent kinase.

502 The situation with GLUT-1 is very intriguing, as LC–MS/MS measurements showed
503 that GLUT-1 protein levels are equal between uninfected and infected red blood cells,
504 while phosphorylated GLUT-1 appears enriched in anti-RxxS*/T* immunoprecipitates

505 from iRBC (Fig. 4B). This result is consistent with the five GLUT-1 phospho-peptides
506 detected specifically in iRBC (Fig. 3). Direct detection with anti-motif 4 (RxxS*/T*) of
507 phosphorylated GLUT-1 by probing immunoblots of anti-GLUT-1 immunoprecipitates
508 is difficult due to the glycosylation smear and reduced GLUT-1 signal in iRBCs. We
509 propose that upregulation of GLUT-1 phosphorylation may be essential for the
510 heightened glucose uptake of iRBC that fuels the parasite's energy metabolism and
511 leads to high lactate output. Further experiments are required to investigate whether
512 phosphorylation of GLUT-1 does indeed underpin increased glucose uptake by iRBC
513 as inhibiting GLUT-1 kinase(s) could impact negatively on parasite growth.

514 **5. Conclusion**

515 In this study we generated a comprehensive compendium of protein phosphorylation
516 sites in erythrocyte membrane proteins from normal and *P. falciparum*-infected
517 RBCs. We demonstrated that the compendium is a new information source for
518 exploration of phosphorylation sites involved in regulating red cell morphology and
519 metabolite transport.

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527

528 **References**

- 529 [1] F. Roux-Dalvai, A. Gonzalez de Peredo, C. Simó, L. Guerrier, D. Bouyssié, A.
530 Zanella, A. Citterio, O. Bulet-Schiltz, E. Boschetti, P.G. Righetti, B.Monsarrat,
531 Extensive analysis of the cytoplasmic proteome of human erythrocytes using the
532 peptide ligand library technology and advanced mass spectrometry, *Mol. Cell.*
533 *Proteomics* 7 (2008) 2254–2269.
- 534 [2] S.R. Goodman, O. Daescu, D.G. Kakhniashvili, M. Zivanic, The proteomics and
535 interactomics of human erythrocytes, *Exp. Biol. Med. (Maywood)* 238 (2013) 509–
536 518.
- 537 [3] A. Pantaleo, L. De Franceschi, E. Ferru, R. Vono, F. Turrini, Current knowledge
538 about the functional roles of phosphorylative changes of membrane proteins in
539 normal and diseased red cells, *J. Proteomics* 73 (2010) 445–455.
- 540 [4] A. Pantaleo, E. Ferru, F. Carta, F. Mannu, G. Giribaldi, R. Vono, A.J. Lapedda, P.
541 Pippia, F. Turrini, Analysis of changes in tyrosine and serine phosphorylation of red
542 cell membrane proteins induced by *P. falciparum* growth, *Proteomics* 10 (2010)
543 3469–3479.
- 544 [5] S. Manno, Y. Takakuwa, K. Nagao, N. Mohandas, Modulation of erythrocyte
545 membrane mechanical function by β -spectrin phosphorylation and
546 dephosphorylation, *J. Biol. Chem.* 270 (1995) 5659–5665.
- 547 [6] S. Perrotta, E.M. del Giudice, A. Iolascon, M. De Vivo, D. Di Pinto, S. Cutillo, B.
548 Nobili, Reversible erythrocyte skeleton destabilization is modulated by beta-spectrin
549 phosphorylation in childhood leukemia, *Leukemia* 15 (2001) 440–444.
- 550 [7] S. Manno, Y. Takakuwa, N. Mohandas, Modulation of erythrocyte membrane
551 mechanical function by protein 4.1 phosphorylation, *J. Biol. Chem.* 280 (2005) 7581–
552 7587.
- 553 [8] E. Ferru, K. Giger, A. Pantaleo, E. Campanella, J. Grey, K. Ritchie, R. Vono, F.
554 Turrini, P.S. Low, Regulation of membrane-cytoskeletal interactions by tyrosine
555 phosphorylation of erythrocyte band 3, *Blood* 117 (2011) 5998–6006.

- 556 [9] S. Baumeister, P. Gangopadhyay, U. Repnik, K. Lingelbach, Novel insights into
557 red blood cell physiology using parasites as tools, *Eur. J. Cell Biol.* 94 (2015) 332–
558 339.
- 559 [10] K. Kirk, A.M. Lehane, Membrane transport in the malaria parasite and its host
560 erythrocyte, *Biochem. J.* 457 (2014) 1–18.
- 561 [11] S. Sanyal, S. Egee, G. Bouyer, S. Perrot, I. Safeukui, E. Bischoff, P. Buffet, K.W.
562 Deitsch, O. Mercereau-Puijalon, P.H. David, T.J. Templeton, C. Lavazec,
563 *Plasmodium falciparum* STEVOR proteins impact erythrocyte mechanical properties,
564 *Blood* 119 (2012) e1–e8.
- 565 [12] Y. Wu, M.M. Nelson, A. Quaile, D. Xia, J.M. Wastling, A. Craig, Identification of
566 phosphorylated proteins in erythrocytes infected by the human malaria parasite
567 *Plasmodium falciparum*, *Malar. J.* 8 (2009) 105.
- 568 [13] M.O. Collins, J.C. Wright, M. Jones, J.C. Rayner, J.S. Choudhary, Confident and
569 sensitive phosphoproteomics using combinations of collision induced dissociation
570 and electron transfer dissociation, *J. Proteomics* 103 (2014) 1–14.
- 571 [14] E. Lasonder, J.L. Green, G. Camarda, H. Talabani, A.A. Holder, G. Langsley, P.
572 Alano, The *Plasmodium falciparum* schizont phosphoproteome reveals extensive
573 phosphatidylinositol and cAMP-protein kinase A signaling, *J. Proteome Res.* 11
574 (2012) 5323–5337.
- 575 [15] L. Solyakov, J. Halbert, M.M. Alam, J.P. Semblat, D. Dorin-Semblat, L.
576 Reininger, A.R. Bottrill, S. Mistry, A. Abdi, C. Fennell, Z. Holland, C. Demarta, Y.
577 Bouza, A. Sicard, M.P. Nivez, S. Eschenlauer, T. Lama, D.C. Thomas, P. Sharma, S.
578 Agarwal, S. Kern, G. Pradel, M. Graciotti, A.B. Tobin, C. Doerig, Global kinomic and
579 phospho-proteomic analyses of the human malaria parasite *Plasmodium falciparum*,
580 *Nat. Commun.* 2 (2011) 565.
- 581 [16] M. Treeck, J.L. Sanders, J.E. Elias, J.C. Boothroyd, The phosphoproteomes of
582 *Plasmodium falciparum* and *Toxoplasma gondii* reveal unusual adaptations within
583 and beyond the parasites' boundaries, *Cell Host Microbe* 10 (2011) 410–419.
- 584 [17] E.J. Soderblom, J.W. Thompson, E.A. Schwartz, E. Chiou, L.G.
585 Dubois, M.A. Moseley, R. Zennadi, Proteomic analysis of ERK1/2-mediated human

586 sickle red blood cell membrane protein phosphorylation, Clin. Proteomics 10 (2013)
587 1.

588 [18] D. Schwartz, S.P. Gygi, An iterative statistical approach to the identification of
589 protein phosphorylation motifs from large-scale data sets, Nat. Biotechnol. 23 (2005)
590 1391–1398.

591 [19] G.E. Crooks, G. Hon, J.M. Chandonia, S.E. Brenner, WebLogo: a sequence logo
592 generator, Genome Res. 14 (2004) 1188–1190.

593 [20] R. Amanchy, B. Periaswamy, S. Mathivanan, R. Reddy, S.G. Tattikota, A.
594 Pandey, A curated compendium of phosphorylation motifs, Nat. Biotechnol. 25 (2007)
595 285–286.

596 [21] W. Huang da, B.T. Sherman, R.A. Lempicki, Systematic and integrative analysis
597 of large gene lists using DAVID bioinformatics resources, Nat. Protoc. 4 (2009) 44–
598 57.

599 [22] W. Huang da, B.T. Sherman, Q. Tan, J.R. Collins, W.G. Alvord, J. Roayaei, R.
600 Stephens, M.W. Baseler, H.C. Lane, R.A. Lempicki, The DAVID Gene Functional
601 Classification Tool: a novel biological module-centric algorithm to functionally analyze
602 large gene lists, Genome Biol. 8 (2007) R183.

603 [23] D. Merico, R. Isserlin, G.D. Bader, Visualizing gene-set enrichment results using
604 the Cytoscape plug-in enrichment map, Methods Mol. Biol. 781 (2011) 257–277.

605 [24] A. Radfar, D. Mendez, C. Moneriz, M. Linares, P. Marin-Garcia, A. Puyet, A.
606 Diez, J.M. Bautista, Synchronous culture of *Plasmodium falciparum* at high
607 parasitemia levels, Nat. Protoc. 4 (2009) 1899–1915.

608 [25] J.R. Wisniewski, A. Zougman, N. Nagaraj, M. Mann, Universal sample
609 preparation method for proteome analysis, Nat. Methods 6 (2009) 359–362.

610 [26] J. Rappsilber, Y. Ishihama, M. Mann, Stop and go extraction tips for matrix-
611 assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample
612 pretreatment in proteomics, Anal. Chem. 75 (2003) 663–670.

613 [27] L. Zhou, J. Lyons-Rimmer, S. Ammoun, J. Muller, E. Lasonder, V. Sharma, E.
614 Ercolano, D. Hilton, I. Taiwo, M. Barczyk, C.O. Hanemann, The scaffold protein

615 KSR1, a novel therapeutic target for the treatment of merlin-deficient tumors,
616 *Oncogene* (2015).

617 [28] J. Cox, M. Mann, MaxQuant enables high peptide identification rates,
618 individualized p.p.b.-range mass accuracies and proteome-wide protein
619 quantification, *Nat. Biotechnol.* 26 (2008) 1367–1372.

620 [29] E. Lasonder, M. Treeck, M. Alam, A.B. Tobin, Insights into the *Plasmodium*
621 *falciparum* schizont phospho-proteome, *Microbes Infect.* 14 (2012) 811–819.

622 [30] J.V. Olsen, B. Blagoev, F. Gnad, B. Macek, C. Kumar, P. Mortensen, M. Mann,
623 Global, in vivo, and site-specific phosphorylation dynamics in signaling networks,
624 *Cell* 127 (2006) 635–648.

625 [31] <http://www.phosphonet.ca/>, PhosphoNET.

626 [32] T. Hegedus, P.M. Chaubey, G. Varady, E. Szabo, H. Saranko, L. Hofstetter, B.
627 Roschitzki, B. Stieger, B. Sarkadi, Inconsistencies in the red blood cell membrane
628 proteome analysis: generation of a database for research and diagnostic
629 applications, *Database (Oxford)* 2015 (2015), bav056.

630 [33] <http://rbcc.hegelab.org>, Red Blood Cell Collection.

631 [34] A. Dawn, S. Singh, K.R. More, F.A. Siddiqui, N. Pachikara, G. Ramdani, G.
632 Langsley, C.E. Chitnis, The central role of cAMP in regulating *Plasmodium*
633 *falciparum* merozoite invasion of human erythrocytes, *PLoS Pathog.* 10 (2014),
634 e1004520.

635 [35] A. Merckx, M.P. Nivez, G. Bouyer, P. Alano, G. Langsley, K. Deitsch, S. Thomas,
636 C. Doerig, S. Egee, *Plasmodium falciparum* regulatory subunit of cAMP-dependent
637 PKA and anion channel conductance, *PLoS Pathog.* 4 (2008), e19.

638 [36] G. Ramdani, B. Naissant, E. Thompson, F. Breil, A. Lorthiois, F. Dupuy, R.
639 Cummings, Y. Duffier, Y. Corbett, O. Mercereau-Puijalon, K. Vernick, D. Taramelli,
640 D.A. Baker, G. Langsley, C. Lavazec, cAMP-signalling regulates gametocyte-
641 infected erythrocyte deformability required for malaria parasite transmission, *PLoS*
642 *Pathog.* 11 (2015), e1004815.

643 [37] C. Syin, D. Parzy, F. Traincard, I. Boccaccio, M.B. Joshi, D.T. Lin, X.M. Yang, K.
644 Assemat, C. Doerig, G. Langsley, The H89 cAMP-dependent protein kinase inhibitor
645 blocks *Plasmodium falciparum* development in infected erythrocytes, Eur. J.
646 Biochem. 268 (2001) 4842–4849.

647 [38] J.F. Flatt, H. Guizouarn, N.M. Burton, F. Borgese, R.J. Tomlinson, R.J. Forsyth,
648 S.A. Baldwin, B.E. Levinson, P. Quittet, P. Aguilar-Martinez, J. Delaunay, G.W.
649 Stewart, L.J. Bruce, Stomatin-deficient cryohydrocytosis results from mutations in
650 SLC2A1: a novel form of GLUT1 deficiency syndrome, Blood 118 (2011) 5267–5277.

651 [39] J. Cox, M.Y. Hein, C.A. Luber, I. Paron, N. Nagaraj, M. Mann, Accurate
652 proteome-wide label-free quantification by delayed normalization and maximal
653 peptide ratio extraction, termed MaxLFQ, Mol. Cell. Proteomics 13 (2014) 2513–2526.

654 [40] M. Urscher, R. Alisch, M. Deponte, The glyoxalase system of malaria parasites
655 — implications for cell biology and general glyoxalase research, Semin. Cell Dev.
656 Biol. 22 (2011) 262–270.

657 [41] D. Miranda-Saavedra, T. Gabaldon, G.J. Barton, G. Langsley, C. Doerig, The
658 kinomes of apicomplexan parasites, Microbes Infect. 14 (2012) 796–810.

659 [42] G. Clari, V. Moret, Phosphorylation of membrane proteins by cytosolic casein
660 kinases in human erythrocytes. Effect of monovalent ions, 2,3-bisphosphoglycerate
661 and spermine, Mol. Cell. Biochem. 68 (1985) 181–187.

662 [43] T. Wei, M. Tao, Human erythrocyte casein kinase II: characterization and
663 phosphorylation of membrane cytoskeletal proteins, Arch. Biochem. Biophys. 307
664 (1993) 206–216.

665 [44] F. Carbonnet, C. Hattab, J.P. Cartron, O. Bertrand, Kell and Kx, two disulfide-
666 linked proteins of the human erythrocyte membrane are phosphorylated in vivo,
667 Biochem. Biophys. Res. Commun. 247 (1998) 569–575.

668 [45] S. Ghosh, F.C. Dorsey, J.V. Cox, CK2 constitutively associates with and
669 phosphorylates chicken erythroid ankyrin and regulates its ability to bind to spectrin,
670 J. Cell Sci. 115 (2002) 4107–4115.

671 [46] R. Hora, D.J. Bridges, A. Craig, A. Sharma, Erythrocytic casein kinase II
672 regulates cytoadherence of *Plasmodium falciparum*-infected red blood cells, J. Biol.
673 Chem. 284 (2009) 6260–6269.

674 [47] L. Chen, J.W. Brown, Y.F. Mok, D.M. Hatters, C.J.McKnight, The
675 allostericmechanism induced by protein kinase A (PKA) phosphorylation of dematin
676 (band 4.9), J. Biol.Chem. 288 (2013) 8313–8320.

677 [48] L. Chen, J.W. Brown, Y.F. Mok, D.M. Hatters, C.J.McKnight, The
678 allostericmechanism induced by protein kinase A (PKA) phosphorylation of dematin
679 (band 4.9), J. Biol.Chem. 290 (2015) 17808.

680 [49] I. Koshino, N. Mohandas, Y. Takakuwa, Identification of a novel role for dematin
681 in regulating red cell membrane function by modulating spectrin–actin interaction,
682 J.Biol. Chem. 287 (2012) 35244–35250.

683 [50] Y. Matsuoka, C.A. Hughes, V. Bennett, Adducin regulation. Definition of the
684 calmodulin-binding domain and sites of phosphorylation by protein kinases A and C,
685 J. Biol. Chem. 271 (1996) 25157–25166.

686 [51] E.S. Zuccala, T.J. Satchwell, F. Angrisano, Y.H. Tan, M.C. Wilson, K.J. Heesom,
687 J. Baum, Quantitative phospho-proteomics reveals the Plasmodium merozoite
688 triggers pre-invasion host kinase modification of the red cell cytoskeleton, Sci. Rep. 6
689 (2016) 19766.

690 [52] D. Walliker, I.A. Quakyi, T.E. Wellems, T.F. McCutchan, A. Szarfman, W.T.
691 London, L.M. Corcoran, T.R. Burkot, R. Carter, Genetic analysis of the human
692 malaria parasite *Plasmodium falciparum*, Science 236 (1987) 1661–1666.

693 [53] C. Lambros, J.P. Vanderberg, Synchronization of *Plasmodium falciparum*
694 erythrocytic stages in culture, J. Parasitol. 65 (1979) 418–420.

695

696 **Figure Legends**

697 **Fig. 1. Compendium of protein phosphorylation sites in human red blood cells**

698 Compendium of protein phosphorylation sites in human red blood cells. A. Venn
699 diagram depicting phospho-peptide counts in normal and *P. falciparum*-infected
700 RBCs with data collected by Soderblom et al. [17] (normal RBCs) and by several
701 independent studies of the schizont life cycle stage [13–16]. B. Distribution of amino
702 acid phosphorylation for serine, threonine and tyrosine in phospho-proteomes. C. Pie
703 chart showing the distribution of phosphorylation motif classes in RBC proteins for
704 normal and infected red cells. The phosphorylated residue is located at the central
705 position within a sequence window of 13 amino acids. Classes were defined by the
706 chemical properties of the sequence window peptide as acidic, basic, proline-
707 directed, tyrosine-directed or other by a decision tree method described earlier [14].
708 D. Table of phosphorylation motifs in normal and infected RBCs identified by Motif-X
709 [18] analysis with phosphorylation motifs depicted as sequence logos. Putative
710 protein kinases associated with these motifs were predicted by PhosphoMotifFinder
711 [20].

712 **Fig. 2. Functional annotation of red blood cell phospho-proteomes**

713 Functional annotation of red blood cell phospho-proteomes. A. GO enrichment of
714 membrane-associated phosphoproteins in normal and infected RBCs for the
715 ontologies molecular function (red bars), cellular component (green bars), and
716 biological processes (blue bars). Fold enrichment in the phospho-proteome relative
717 to the human proteome is displayed at the xaxis. B. Network visualisation of the
718 overlap between enriched GO terms in infected RBCs by Cytoscape plugin
719 Enrichment Map. Nodes represent GO terms and edges display overlap between
720 terms with line thickness corresponding to degree of overlap. Node numbers refer to
721 GO terms listed in the left column in A.

722

723 **Fig. 3. Phosphorylation of RBCmembrane and cytoskeleton proteins**

724 A. Schematic representation of a cross section from the red cell membrane shows
725 protein phosphorylation of the key players spectrin, actin, tropomyosin and Band 4.1,
726 which are forming a meshwork laminating the inner surface of the membrane.
727 Linkage to the lipid bilayer membrane via ankyrin occurs with the transmembrane
728 protein Band 3 and glycophorin C, the surface glycoprotein CD47, Rh and Rh-
729 associated glycoprotein (RhAG) establishing the bridges with the cytoskeleton
730 meshwork. Proteins are provided with numbers representing counts of
731 phosphorylation sites specifically detected in infected RBCs and counts of all sites. B.
732 Table highlighting the phosphorylation status of the main erythrocyte cytoskeleton
733 proteins, transporters and various membrane-associated proteins in normal and
734 infected RBCs. *Proteins with more than 50% of infected specific sites. The majority
735 of proteins in this figure (eg glycophorin A&C, ankyrin, spectrins, adducins, band 3,
736 dematin, protein 4.1, GLUT1, CA1&2, actin, nucleoside transporter 1) have recently
737 been reported phosphorylated following merozoite binding to the RBC surface
738 (Zuccala ES et al, Sci. Rep. 6:19766 [51]).

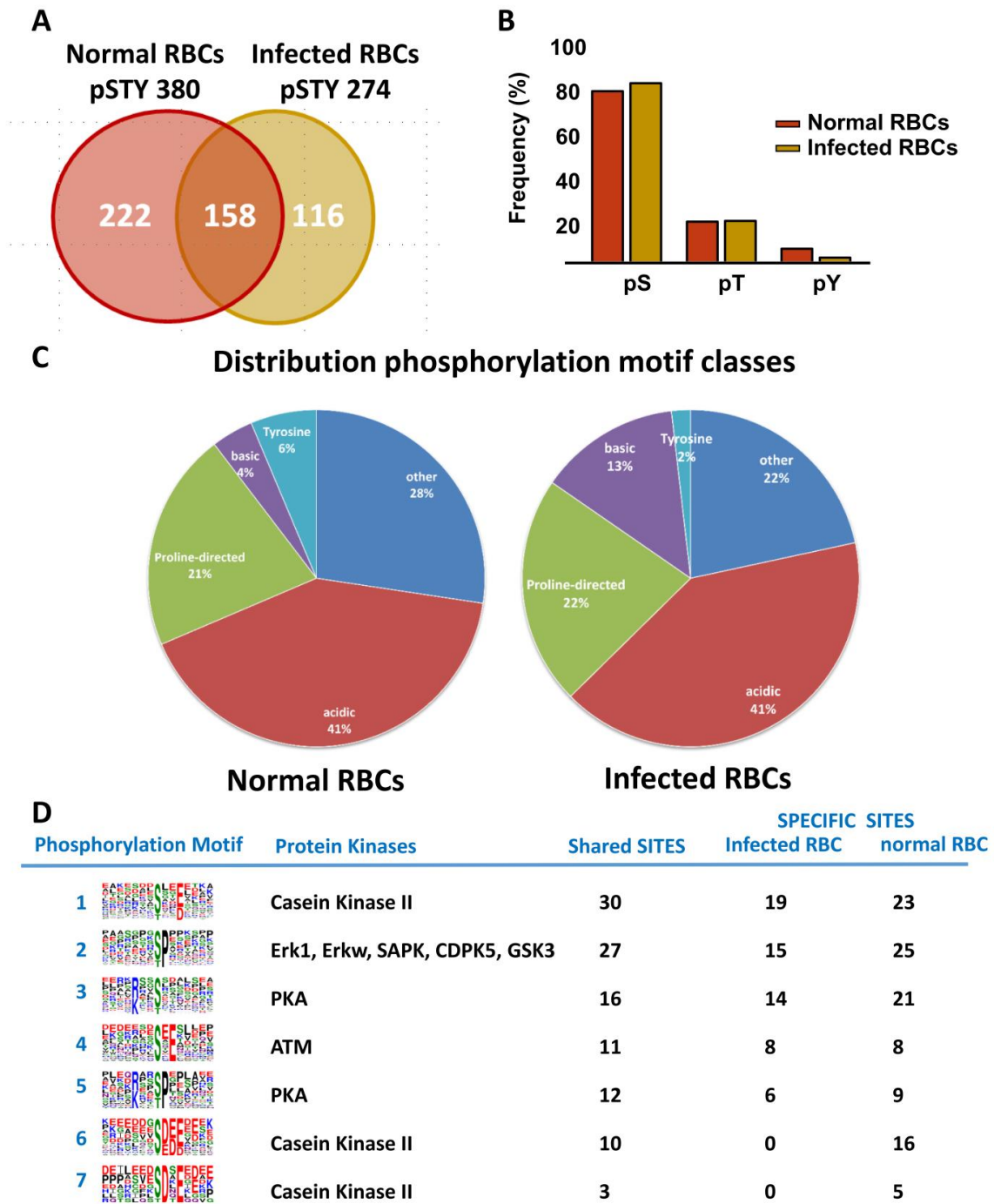
739 **Fig. 4. Wet-bench validation of phospho-sites and their cAMP-dependence**

740 A. Immunoblots showing phospho-Ser59, phospho-Ser726 and α -adducin (81 kDa),
741 left panel; phospho-Ser403 and α and β dematin (48 and 52 kDa), right panel; using
742 ghost lysates obtained from uninfected RBCs (lanes 1–3) and N95%-enriched
743 schizont stage of *P. falciparum* (NF54)-infected RBCs (lanes 4, 5); uninfected RBCs
744 incubated 30 min at 37 °C in the presence of 50 μ M dibutyryl-cAMP (lane 2);
745 dephosphorylated ghost lysates prepared by incubation with λ -protein phosphatase
746 (lanes 3, 5). 1×10^7 uninfected RBCs and *P. falciparum* (NF54)-infected RBCs
747 representing 5 μ g and 30 μ g total proteins, respectively. B. Immunoprecipitation/
748 immunoblot analysis of ghost lysates from uninfected RBCs and N95%-enriched
749 schizont stage of *P. falciparum* (NF54)-infected RBCs (1×10^8). Immunoblots of total
750 ghost lysates, where lane 1 = uninfected RBC; lane 2 = RBC stimulated with cAMP;
751 lane 3 = iRBC, showing levels of Band 3 (95 kDa) and GLUT-1 (54 kDa), with α -
752 adducin and dematin levels taken as loading controls (input). Top two panels show
753 amounts of Band 3 and GLUT-1 immunoprecipitated by the anti-RxxS*/T* antibody
754 (IP anti-RxxS*/T*). Note the drastically reduced Western blot signals of GLUT1

755 detected in total ghost lysates from iRBC (compare lanes 1, 2 with 3). The low-
756 amount of GLUT-1 in the anti-RxxS*/T* precipitate contrasts with phospho-Band 3
757 that is readily detected (lane 3, top panel). C. Measurement of GLUT-1 protein
758 abundance in isolated ghosts from infected and uninfected RBCs analysing 500 ng
759 tryptic digests by liquid chromatography tandem mass spectrometry using label free
760 quantification in triplicate runs. Similar GLUT-1 protein levels are observed in iRBC
761 and RBC.

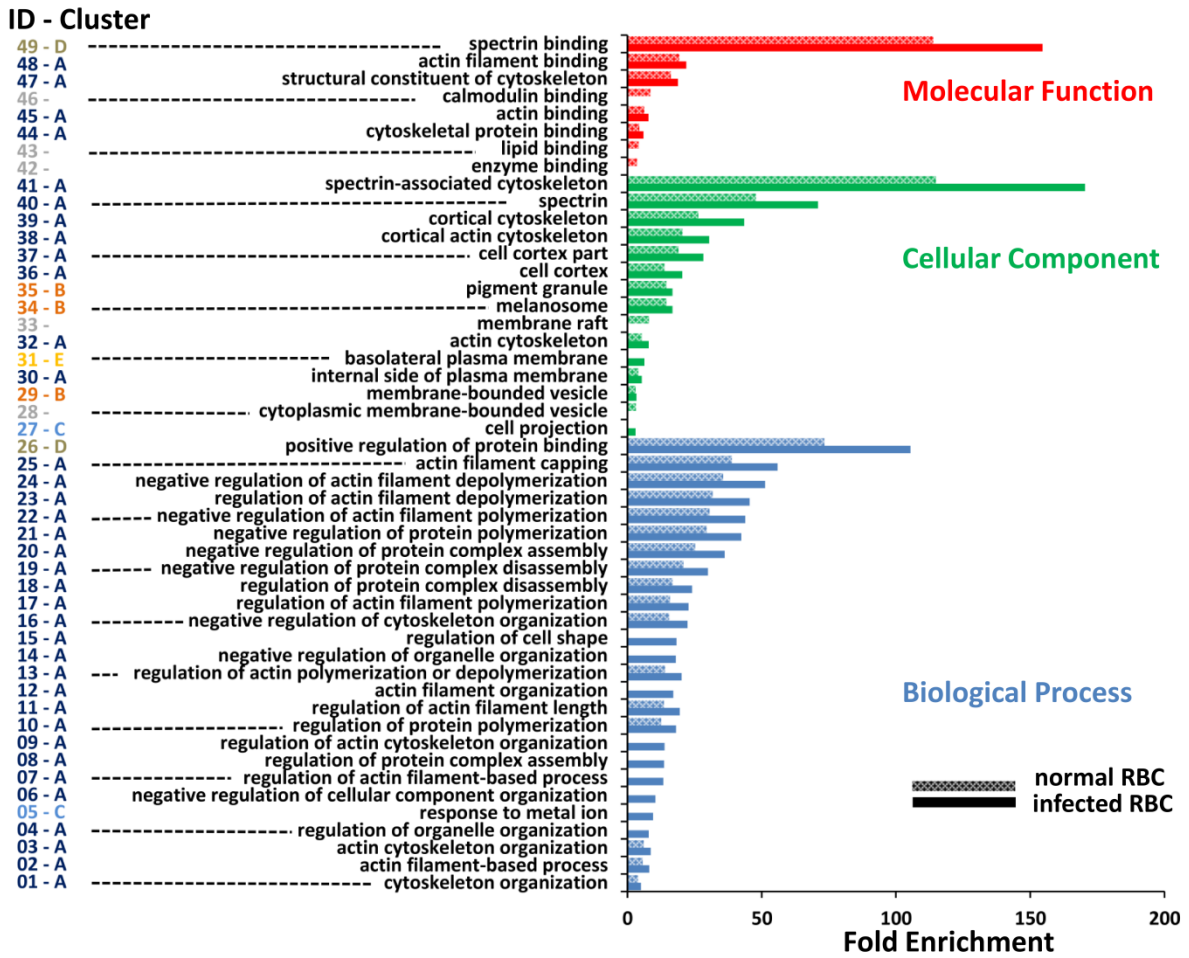
762

763 **Figure 1**

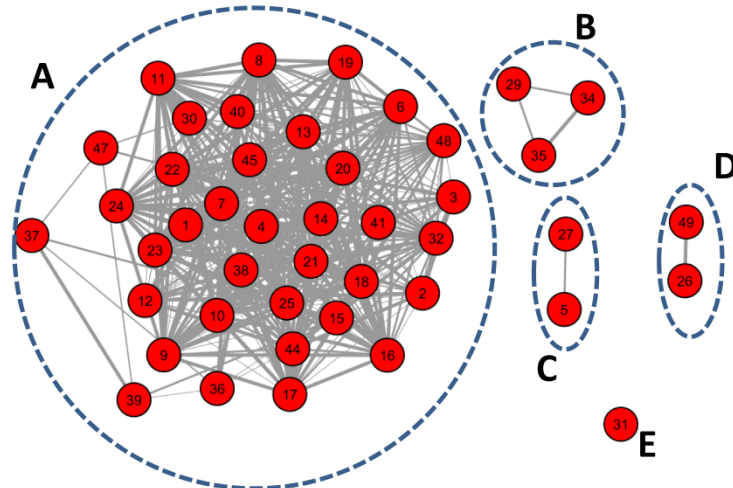


764

A: Gene Ontology Enrichment



B: GO networks infected RBCs

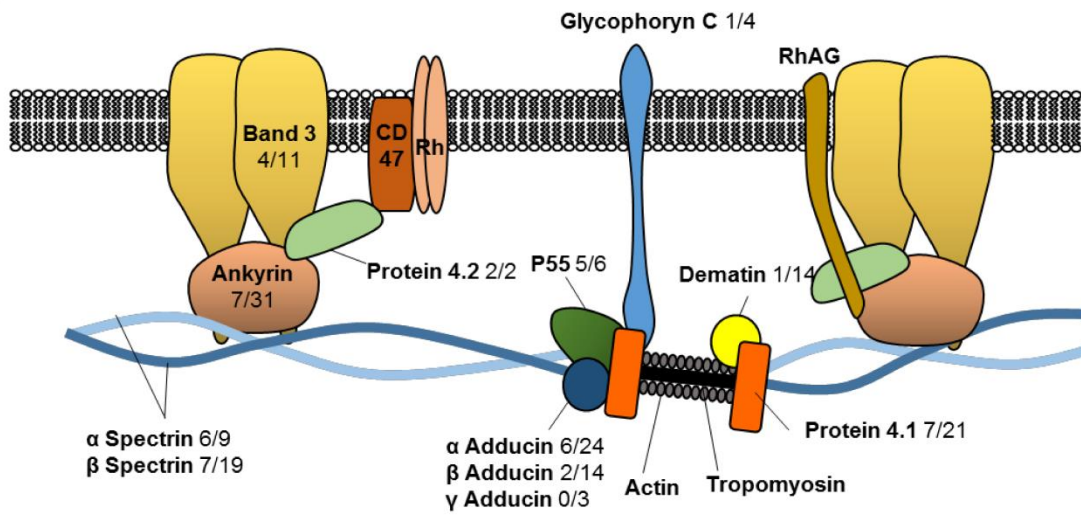


766

767

768

A



B

	Protein name	all sites	Infected RBC specific sites	RBC specific sites	Common sites
cytoskeleton	Protein 4.1	21	7	7	7
	Protein 4.2 *	2	2	0	0
	Alpha adducin	24	6	7	11
	Beta adducin	14	2	4	8
	Gamma adducin	3	0	1	2
	Ankyrin-1	31	7	3	21
	Dematin	14	1	4	9
	Spectrin alpha chain *	9	6	0	3
	Spectrin beta chain	19	7	1	11
	Stomatin	3	1	0	2
transporters	PMCA	1	0	1	0
	Aquaporin-1	3	1	0	2
	Band 3	11	4	1	6
	GLUT-1 *	7	5	0	2
	Eq.nucleoside transporter 1	5	2	0	3
Membrane associated	Carbonic anhydrase 1 *	2	2	0	0
	Carbonic anhydrase 2 *	1	1	0	0
	CD44 antigen *	2	2	0	0
	Cytochrome b reductase 1 *	2	2	0	0
	P55 *	6	5	0	1
	GAPDH *	1	1	0	0

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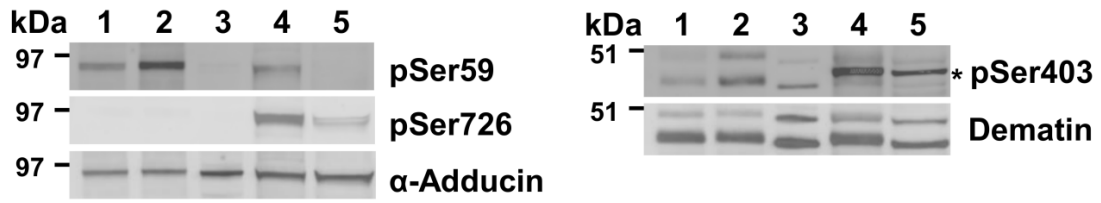
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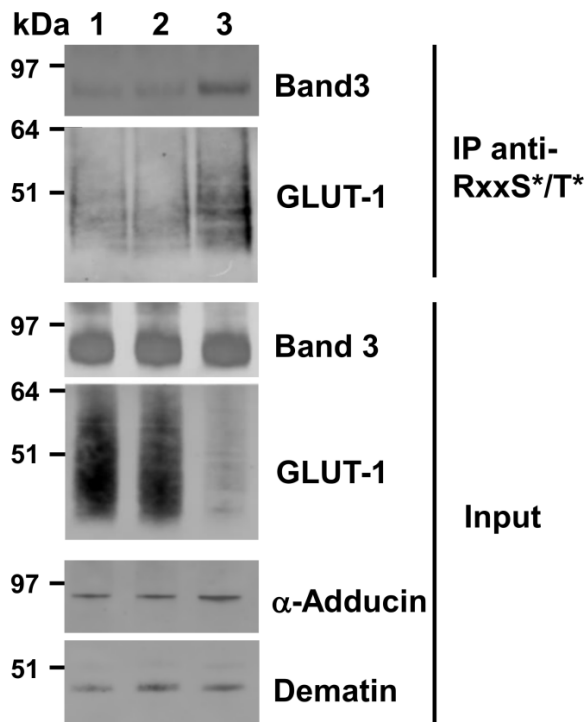
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A



B



C

