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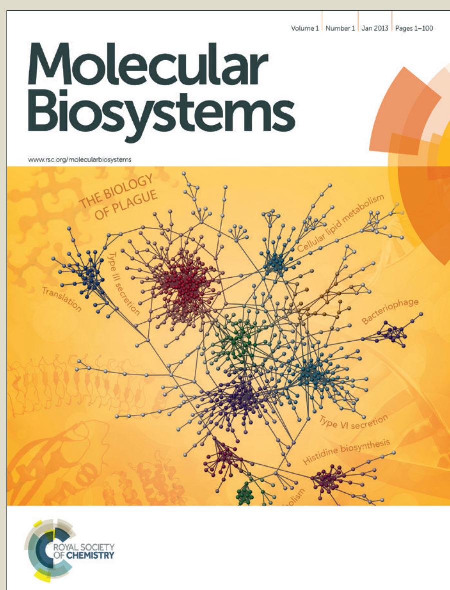

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1 # Unexpected similarities between the *Schizosaccharomyces* and human blood metabolomes, and novel human metabolites
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15 **Running title:** Human blood and fission yeast metabolomes

16 **Abbreviations:** RBC, red blood cell;

17 **Keywords:** red blood cell, erythrocyte, leukocyte, metabolome, fission yeast, *S.*
18 *pombe*

19

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1 **Abstract**

2 Metabolomics, a modern branch of chemical biology, provides qualitative and
3 quantitative information about the metabolic states of organisms or cells at the
4 molecular level. We here report non-targeted, metabolomic analyses of human blood,
5 using liquid chromatography-mass spectrometry (LC-MS). We compared the blood
6 metabolome to the previously reported metabolome of the fission yeast,
7 *Schizosaccharomyces pombe*. The two metabolomic datasets were highly similar:
8 101 of 133 compounds identified in human blood (75%) were also present in
9 *S. pombe,* and 45 of 57 compounds enriched in red blood cells (RBCs) (78%), were
10 also present in yeast. The most abundant metabolites were ATP, glutathione, and
11 glutamine. Apart from these three, the next most abundant metabolites were also
12 involved in energy metabolism, anti-oxidation, and amino acid metabolism. We
13 identified fourteen new blood compounds, eight of which were enriched in RBCs:
14 citramalate, GDP-glucose, trimethyl-histidine, trimethyl-phenylalanine, trimethyl-
15 tryptophan, trimethyl-tyrosine, UDP-acetyl-glucosamine, UDP-glucuronate, dimethyl-
16 lysine, glutamate methyl ester, *N*-acetyl-(iso)leucine, *N*-acetyl-glutamate, N_2-acetyl-
17 lysine, and N_6-acetyl-lysine. Ten of the newly identified blood metabolites were also
18 detected in *S. pombe*, and ten of them were methylated or acetylated amino acids.
19 Trimethylated or acetylated free amino acids were also abundant in white blood cells.
20 It may be possible to investigate their physiological roles using yeast genetics.

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1 ## Introduction

2 Metabolomics is a rapidly growing branch of comprehensive, post-genomic,
3 quantitative chemical biology[1-5]. It aims to profile small molecules present in living
4 organisms, and is now recognized as an important tool for studying metabolic
5 regulation in a synthetic way, together with transcriptomic and proteomic analyses[6].
6 Mass spectrometry (MS), a highly sensitive molecular detection method, can reveal
7 thousands of intracellular metabolites over a very broad range of concentrations.
8 However, the number of identified, named compounds is surprisingly small. One
9 reason is the paucity of commercially available standards. Second, it is difficult to
10 identify unknown ions, due to the limited structural information that can be obtained
11 using MS. . Third, the concentrations of many metabolites present in biological
12 samples are very low, and they tend to undergo changes during sample preparation.
13 Hence fewer than several hundred relatively abundant compounds are usually
14 reported in comprehensive metabolomic studies[7-9].
15 Red blood cells (RBCs or erythrocytes), deliver oxygen to tissues and remove
16 carbon dioxide. In humans, approximately 5 L of blood circulate through the whole
17 body per min[10]. In healthy individuals, RBCs comprise 45% of the blood volume,
18 and the remainder is mostly plasma, with a tiny additional fraction of leucocytes, or
19 white blood cells (WBCs). Plasma contains proteins (e.g. albumins, globulins,
20 fibrinogen, etc.), electrolytes, hormones, glucose, and other metabolites. Mammalian
21 RBCs exclude nuclei, mitochondria, and other prominent cell organelles, such as
22 lysosomes, endoplasmic reticulum, and Golgi bodies during erythropoiesis in the bone
23 marrow. Mature, simplified and specialized for gas transfer, RBCs have no
24 demonstrable protein synthesis, nor tricarboxylic acid (TCA) cycle activity[11].
25 Nevertheless, RBCs produce adenosine triphosphate (ATP) glycolytically, maintain

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1 redox homeostasis, and osmoregulate[12]. Human RBCs have a relatively long life span
2 of about 120 days[13]. When senescent, they are captured by the spleen for degradation.
3 Numerous blood metabolomic analyses have been previously reported, mostly
4 of serum and plasma[14-19]. However, metabolomics of whole blood or RBCs have
5 been less well investigated, except for several reports on long-term stored blood[20-23],
6 blood of disease patients[24, 25], or blood marker compounds of food intake[26]. Reports
7 on the WBC metabolome are scarce[27, 28]. This study focuses on the metabolome of
8 blood cells, mainly RBCs, separated from plasma by low speed centrifugation. We
9 also briefly investigated the metabolome of WBCs prepared by Ficoll gradient
10 centrifugation, to compare it with that of RBCs.
11 The fission yeast, *Schizosaccharomyces pombe,* a eukaryotic microbe with a
12 genome encoding only ~5,000 genes[29, 30], is an excellent model organism to study
13 nutritional control of the transition between proliferation and quiescence (e.g.
14 Yanagida et al.[31]). We have employed metabolomic analysis of *S. pombe* cells in
15 order to understand cellular metabolic states under different nutritional regimes and as
16 governed by different genotypes. Initially, we reported the identification of 123
17 compounds using liquid chromatography-mass spectrometry (LC-MS) and their
18 changes following heat stress and genetic perturbations[8]. We described the
19 accumulation of antioxidants, glutathione, and ergothioneine in a proteasome
20 regulatory subunit mutant *mts3-1*[32]. Also we made targeted measurements of
21 intermediates of the coenzyme A biosynthetic pathway from pantothenate[33],
22 investigated changes in the cellular metabolome upon glucose[34] and nitrogen
23 starvation[35], and accumulation of various metabolites in the quiescence-defective
24 Δ*klf1* mutant[36]. These studies showed that *S. pombe* may be used as a model

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1 organism to study comprehensive metabolic patterns under defined genetic and
2 physiological conditions.
3 *S. pombe* cells grow and divide in culture media containing only
4 0.08% glucose, as rapidly as in regular synthetic culture media containing 2%
5 glucose; however, their division rate is impaired if extracellular glucose drops below
6 0.08%[34] (4.4 mM), a concentration that is roughly equivalent to that in the blood of
7 healthy humans before breakfast. That the glucose concentration required to sustain
8 life is roughly equivalent between such diverse eukaryotes, is remarkable. In this
9 study we report further significant conservation in metabolites between evolutionarily
10 distant human RBCs and *S. pombe* vegetative cells.
11

12 **Results**

13
14 **Preparation and analysis of human blood metabolomic samples**
15 Blood samples (4-5 mL) for metabolomic analysis were collected from healthy
16 volunteers before breakfast (schematized in Fig. 1A, Materials and Methods). WBCs
17 stained with Giemsa (Fig. 1B) were infrequently observed (<1%) in blood. WBCs
18 were not removed, since rapid quenching of metabolic reactions was necessary to
19 obtain reproducible, quantitative data (Ficoll centrifugation to separate WBCs is a
20 time-consuming step.). Low-molecular-weight metabolites were isolated by filtration
21 with a 10 kDa cut-off filter at 4° C, concentrated on a rotary evaporator, re-suspended
22 in 40 μL 50% acetonitrile, and stored at -80° C until analysis.
23 For LC-MS analysis, metabolites were first separated by hydrophilic
24 interaction chromatography (ZIC-pHILIC column; Merck SeQuant)[37], and detected
25 using an LTQ Orbitrap MS (Thermo Fisher Scientific), in full scan mode (100-

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1 1000 m/z, ratio of mass-to-charge) with both positive and negative electrospray
2 ionization. LC-MS data contain semi-quantitative information about thousands of
3 compounds in human blood. For compound analysis and quantification, we employed
4 basically the same procedures used in previous analyses of *S. pombe* metabolites[8]
5 (Fig. 1C). For quantification, we integrated peak curves, obtaining peak areas in
6 arbitrary AU units. ATP and glutathione are RBC-enriched, meaning that peak areas
7 in the RBC-fraction were at least 2-fold higher than corresponding peaks in plasma
8 (See 'Fifty-seven RBC-enriched compounds' below). It is difficult to obtain
9 reproducible quantitative data on reduced glutathione (GSH) due to its auto-oxidation
10 during sample preparation. For that reason, only levels of oxidized glutathione
11 (GSSG) are reported in the present study.
12
13 **Thirty-two compounds identified in human blood were not detected in yeast**
14 MZmine 2 software[38] was used for data processing and identification of blood
15 metabolites. We employed an in-house database of m/z and RT values of compounds
16 previously identified in fission yeast studies[8 and others]. For peaks not in the database,
17 we performed a search using online databases HMDB[39], KEGG[40], or ChemSpider[41].
18 Whenever possible, identified compounds were verified using purchased standards.
19 In some cases, isomers (e.g. *N*-acetyl-leucine, *N*-acetyl-isoleucine; paraxanthine,
20 theobromine, theophylline) could not be clearly distinguished by LC and were
21 designated by more general names (*e.g., N*-acetyl-(iso)leucine or dimethyl-xanthine,
22 respectively). To identify metabolites for which standards were not available, we
23 performed MS/MS analysis. Methyl-lysine, trimethyl-phenylalanine, and trimethyl-
24 tyrosine were tentatively identified and described (Supplemental Fig. 1A-C). We
25 were able to identify 133 compounds in blood, representing 14 categories (Table 1).

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1 To compare compounds present in *S. pombe* with those in human blood,
2 *S. pombe* cells were cultivated at 26° C in synthetic, minimal EMM2 medium with
3 0.1% glucose (5.6 mM; roughly the same glucose concentration found in blood).
4 Among 133 identified blood metabolites, 32 were not found in fission yeast (Table 1).
5 They include three nucleoside bases and derivatives, one coenzyme precursor (4-
6 aminobenzoate), one nucleotide-sugar derivative (UDP-glucuronate), one sugar
7 derivative (1,5-anhydroglucitol), two organic acids (chenodeoxycholic acid, glyceric
8 acid), six methylated amino acids including dimethyl-proline, eight other amino acid
9 derivatives including creatine, creatinine, and taurine, and ten carnitines. Thus, 24 of
10 32 compounds are derived from three categories, namely, methylated amino acids,
11 other amino acids, and carnitines. These three categories illustrate the major
12 difference between human blood and *S. pombe* metabolomes. Some compounds may
13 play specific roles in higher eukaryotes. For example, creatine and phosphocreatine
14 are stored in muscle as an energy source, and creatinine is the metabolic byproduct of
15 creatine phosphate[42]. Carnitines are produced in liver and stored in muscle for
16 consumption and transport of fatty acids[43]. Urate is a purine metabolic byproduct,
17 high blood concentrations of which can cause gout[44], while caffeine is a xanthine
18 alkaloid and of dietary origin[45, 46]. *S. pombe* does not produce caffeine, but can
19 become hypersensitive or resistant to caffeine by certain mutations[47, 48].
20
21 **Fourteen newly identified blood compounds**
22 To our knowledge, 14 metabolites have not hitherto been reported in human
23 blood, based on a recent report of detected blood metabolites[49] and literature database
24 searches (Table 1). These new blood metabolites include <u>**citramalate**</u>, <u>dimethyl-</u>
25 <u>lysine</u>, **GDP-glucose**, <u>glutamate methyl ester</u>, <u>*N*-acetyl-glutamate</u>, *N*-acetyl-

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(iso)leucine, N_2-acetyl-lysine, N_6-acetyl-lysine, **trimethyl-histidine**, **trimethyl-phenylalanine**, **trimethyl-tryptophan**, **trimethyl-tyrosine**, **UDP-acetyl-glucosamine** and **UDP-glucuronate.** The eight compounds in boldface were enriched in RBCs, while ten underlined compounds were also found in *S. pombe*. Ten of the 14 novel blood metabolites are methylated or acetylated amino acids.

Quantification of blood metabolite peaks

Each blood sample produced thousands of peaks in positive and negative ionization modes with a broad range (10^4~10^9 AU) of peak areas. We quantified compounds on the basis of their peak areas: High (H, over 10^8 AU), Medium (M, 10^7-10^8 AU) and Low (L, <10^7 AU). In blood samples, L, M, and H groups comprised 92, 7, and 1% of all peaks, respectively.

Quantitative reproducibility of peak areas was examined by collecting two blood samples independently from the same person at 1h intervals. Each pair of samples (#1 and #2) of blood, plasma, and RBCs was compared in a scatter plot (Fig. 2A, and Supplemental Fig. 2A). In all cases, 85-87% of peak areas varied less than 2-fold (0.5 – 2.0x). Fission yeast samples obtained under identical conditions showed similar reproducibility[8]. Very small peaks (area <10^6 AU) showed larger deviations. For 133 compounds identified in blood, plasma or RBCs, however, 97% of peaks in the compared samples changed less than 2-fold (Fig. 2B, Supplemental Fig. 2B). Thus, in both, blood and fission yeast metabolomes, quantitative reproducibility was better for identified peaks[8].

Highly abundant metabolites form various adducts or fragments, resulting in multiple MS peaks. For quantification, we used singly charged proton adducts in positive $[M+H]^+$ and negative modes $[M-H]^-$. ATP produced these two peaks as its

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1 highest signals (Fig. 2C). ATP also produced 16 additional peaks (6 in positive and
2 10 in negative mode). Since their retention time (RT) was basically identical to that
3 of the corresponding primary peak, we suspect that these additional peaks were
4 produced during ionization in the MS. For ergothioneine, 17 peaks were identified in
5 addition to the primary peaks (Supplemental Fig. 2C).
6 In blood samples we were able to identify 37 (74%) peaks in group H, 118
7 (33%) peaks in group M, and 518 peaks (11%) in group L. The total number of
8 assigned peaks (673) is much larger than that of actually identified compounds (133),
9 due to the fact that many metabolites produced multiple peaks. A number of peaks
10 were also produced by electrolytes such as NH_4Cl, originating from NaCl in blood
11 samples. While several thousand peaks were obtained by LC-MS, the actual number
12 of compounds that can be detected in blood may be much less, possibly ~1,000.
13
14 **Fifty-seven RBC-enriched compounds**
15 To determine the degree of compound enrichment in RBCs *vs* plasma, samples
16 of both were prepared from the same blood donor several times. We designate RBC-
17 enriched compounds as those having an RBC:plasma ratio more than 2.0 (Figure 2D
18 and Table 1; detailed annotation of all peaks in Supplemental Fig. 3).
19 ATP and glutathione showed particularly large peak areas (>10^8 AU) in the
20 RBC sample, but much smaller in the plasma sample (RBC:plasma ratios of 81 and
21 1900, respectively). In contrast, carnitine and urate showed RBC:plasma ratios of
22 0.85 and 0.69, respectively, even though their peak areas were large (>10^8 AU) in
23 both samples.
24 Fifty-seven compounds were enriched in RBCs (Table 1). Most metabolites
25 highly enriched in RBC-fractions (RBC:plasma ratio >30) were nucleotides (ADP,

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1 AMP, ATP, GDP, GTP, IMP) sugar phosphates (6-phosphogluconate, diphospho-
2 glycerate, fructose-1,6-diphosphate, glyceraldehyde-3-phosphate, pentose-phosphate,
3 phosphoenolpyruvate, phosphoglycerate), vitamins (NAD^+, NADH, $NADP^+$,
4 NADPH), antioxidants (ergothioneine, glutathione disulfide (GSSG), ophthalmic
5 acid), methylated compounds (*S*-adenosyl-homocysteine, *S*-methyl-ergothioneine,
6 tetradecanoyl-carnitine, trimethyl-histidine) and *N*-acetyl-D-glucosamine. There was
7 no evidence of leakage of these highly enriched compounds from RBCs, confirming
8 that cells were not damaged during handling. Metabolites moderately enriched in
9 RBCs (RBC:plasma ratio between 2 and 30) contain compounds from the categories
10 mentioned above, as well as nucleotide-sugar derivatives and at least one or two
11 compounds from other categories (except antioxidants and choline derivatives).

12 Many RBC-enriched compounds, such as nucleotides and sugar-phosphates,
13 are involved in RBC metabolic pathways[23]. Others such as acetyl-carnitine[50] and
14 trimethyl-lysine[51] were previously reported to be enriched in RBCs, but their origins
15 and roles in RBCs are unknown. Interestingly, eight of the fourteen newly discovered
16 blood components (nucleotide-sugar derivatives and methylated amino acids) were
17 also enriched in RBCs.

18 In contrast to RBC-enriched compounds, adenosine, guanosine, and inosine
19 were scarcely detected in RBCs. Thus, brief centrifugation to sediment RBCs in
20 combination with LC-MS quantification enabled us to classify metabolites into
21 several groups based upon their RBC:plasma ratios (>30 highly RBC-enriched, <30,
22 >2 RBC-enriched; <2 present in both plasma and RBC).
23

24 **Comparison of semi-quantified metabolites between blood and *S. pombe***

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1 Some compounds exhibited similar abundance in blood and *S. pombe* cells
2 (Table 1). ATP (H), ADP (M), AMP (M) and GTP (M) were similar in both, whereas
3 UTP was abundant in *S. pombe* (H), but not in blood (L). Except for UTP, these
4 nucleotides are enriched in RBC; thus RBCs evidently have a low requirement for
5 UTP, since RNA synthesis does not occur in RBCs. Diphospho-glycerate, an
6 allosteric regulator of hemoglobin present at mM concentrations in RBCs[52], was
7 highly abundant (H) in blood, but not in *S. pombe* (L). Pentose-phosphate and
8 sedoheptulose-7-phosphate were more abundant in *S. pombe* (M) than in blood (L),
9 indicating that growing and dividing yeast cells require greater amounts of pentose
10 phosphate pathway intermediates.
11 Seventeen regular and seven acetylated amino acids were commonly present
12 in blood and yeast. Fourteen methylated amino acids were significantly different
13 between blood and *S. pombe*, while seven methylated amino acids (betaine, dimethyl-
14 arginine, dimethyl-lysine, methyl-histidine, methyl-lysine, trimethyl-histidine,
15 trimethyl-lysine) were commonly present in both, and seven others (butyro-betaine,
16 dimethyl-proline, *S*-methyl-ergothioneine, trimethyl-phenylalanine, -tryptophan, -
17 tyrosine) were detected only in human samples. Three anti-oxidants, glutathione,
18 ergothioneine, and ophthalmic acid, were present both human and yeast samples.
19
20 **Eighteen *S. pombe* compounds not detected in human blood**
21 Eighteen compounds present in *S. pombe* were not detected in RBCs or in
22 plasma (Supplemental Table 1). Compounds that control fast cell division and
23 growth, such as cyclic AMP (an activator of protein kinase A[53]), AICAR (5'-
24 phosphoribosyl-5-amino-4-imidazolecarboxamide; an activator of AMP-dependent
25 protein kinase, AMPK[54]) and SAICAR (succinylaminoimidazolecarboxamide ribose-

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1 5'-phosphate, an activator of pyruvate kinase PKM2[55]), PRPP (phosphoribosyl
2 pyrophosphate, involved in nucleotide metabolism[56]) are present in *S. pombe*, but not
3 in blood. Acetyl-CoA, biotin, Coenzyme A, FAD (flavine adenine dinucleotide),
4 HMG-CoA may be required for rapid cell division. Ferrichrome is a cyclic
5 hexapeptide present in lower eukaryotes, such as *Schizosaccharomyces* and *Ustilago*,
6 which forms a complex with iron[57]. Trehalose is a disaccharide having an α,α-1,1-
7 glucosidic bond implicated in anhydrobiotic (anti-desiccant) and anti-oxidant
8 mechanisms[58]. Trehalose is not synthesized in the human body. Trehalose-6-
9 phosphate has been implicated in plant flowering[59], but no function in fission yeast
10 has been proposed. Saccharopine is an intermediate of lysine metabolism[60].
11
12 **Compounds detected in WBCs in comparison with those from RBCs and**
13 ***S. pombe***
14 We then examined metabolites of WBCs isolated by Ficoll gradient
15 centrifugation (Supplemental Table 2). While the metabolic profile of WBCs was
16 similar to that of RBCs, seven metabolites, 3'-5'-cAMP, acetyl-CoA, CMP, CoA,
17 FAD (flavin adenine dinucleotide), HMG-CoA, and PRPP, were detected only in
18 WBCs (Supplemental Table 1). Differences between the two are mainly the types of
19 vitamins and coenzymes. 4-aminobenzoate was detected in blood and RBCs, but not
20 in WBCs. Acetyl-CoA, biotin, FAD, and HMG-CoA, described above, belong to the
21 coenzyme category. Seven compounds, which seem to be mainly involved in cell
22 growth and division in eukaryotes, were found in both WBCs and *S. pombe*
23 (Supplemental Table 1).
24
25 **Summary and categorization of detected blood compounds**

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1 Human blood metabolites identified in this study are summarized in Fig. 3.
2 Three nucleosides, adenosine, guanosine and inosine, may be restricted to plasma, as
3 RBC:plasma ratios are close to zero (Table 1). *cis*-Aconitate (0.3), citrate (0.3),
4 indoxyl-sulfate (0.4), kynurenine (0.4), *N*-acetyl-arginine (0.3), and quinolinic acid
5 (0.4) appear to be found primarily in plasma. In contrast, $NADP^+$, *S*-adenosyl-
6 homocysteine, phosphoenolpyruvate, glutathione disulfide (GSSG), diphospho-
7 glycerate, fructose-1,6-diphosphate were highly enriched in RBC fractions. Their
8 RBC:plasma ratios are rather large (>1,000). RBC compounds of intermediate
9 abundance (ratio >50 - <1,000) included 6-phosphogluconate, ADP, AMP, ATP,
10 ergothioneine, GTP, IMP, NAD^+, NADPH, ophthalmic acid, phosphoglycerate, *S*-
11 methyl-ergothioneine, tetradecanoyl-carnitine, and trimethyl-histidine. All other
12 compounds are presumably present in both RBCs and plasma.
13
14 **Energy and anti-oxidant metabolites abundant in human blood and fission yeast**
15 Some compounds (ATP, glutathione and glutamine) were highly abundant
16 in both RBCs and *S. pombe* (Fig. 4). Most compounds in the High and Medium
17 categories are implicated in energy metabolism, anti-oxidation, or amino acid
18 metabolism. In the WBC metabolome (Supplemental Table 2), ATP, glutathione, and
19 glycerophosphocholine (instead of glutamine) were abundant. Some medium-level
20 compounds (e.g. NAD^+ and UDP-glucose) are required for production of high-energy
21 compounds such as ATP, GTP, and UTP. Ergothioneine and ophthalmic acid are
22 thought to be anti-oxidants. It thus appears that energy metabolites, anti-oxidants, and
23 amino acid metabolites may be the most highly conserved in eukaryotes.
24
25 **Discussion**

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1 In the present study we performed LC-MS-based metabolomic analysis of
2 human blood, plasma, and RBCs in comparison with previously published
3 metabolomic results from the fission yeast, *S. pombe*[8, 34]. Analysis of the *S. pombe*
4 metabolome was performed simultaneously, and results were consistent with those of
5 previous reports. This comparative study enabled us to learn which metabolomic
6 features are conserved between these distantly related cellular systems. The LC
7 column we employed can separate hydrophilic compounds with high resolution, but is
8 not appropriate for separating hydrophobic compounds; therefore, our data contain
9 limited information on lipids, except for two choline derivatives, CDP-choline and
10 glycerophosphocholine. For this reason, conclusions regarding metabolite
11 conservation should be restricted to hydrophilic compounds.
12 Thirty-two compounds were found in blood, but not in *S. pombe* (Fig. 3).
13 Metabolite compositions of blood and fission yeast are unexpectedly similar, with
14 75% of identified compounds present in both. The WBC metabolome is also highly
15 similar to those of RBCs and yeast. However, metabolites mostly belonging to three
16 categories (ten carnitines, six methylated amino acids, eight other amino acid
17 derivatives) were not detected in *S. pombe* (Table 1). If we compare metabolites in
18 the remaining 11 categories, only 8 of 89 compounds were not present in *S. pombe*
19 (caffeine, dimethyl-xanthine, urate, 4-aminobenzoate and UDP-glucuronate, 1,5-
20 anhydroglucitol, chenodeoxycholic acid, and glyceric acid), therefore the overall
21 similarity between the two metabolomes is 90% (81/89×100). Differences are mostly
22 restricted to carnitines and amino acid derivatives.
23 Among the fifteen high abundance blood compounds, however, *S. pombe*
24 lacks six (40%) metabolites (acetyl-carnitine, carnitine, creatine, dimethyl-proline,
25 trimethyl-tryptophan and urate), indicating that the most abundant compounds in

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1 blood are quite different from those of *S. pombe*. Conversely, among the 14 high
2 abundance *S. pombe* compounds, only 2 (trehalose, ferrichrome) were not present in
3 human blood, indicating that 86% are also found in human blood. We presume that
4 the high abundance metabolites of *S. pombe* may be more 'fundamental' than those of
5 blood, since *S. pombe* is a single-celled eukaryote. Blood contains a multitude of
6 specialized metabolites. Carnitine is not strictly a metazoan compound, as it is also
7 synthesized from lysine in *Neurospora*[61]. However, a great variety of carnitine
8 derivatives (9) carrying different fatty acids might be a higher eukaryotic feature.
9 The high similarity of these two metabolomes raises the possibility that
10 *S. pombe* genetics might be useful to understand the role of certain metabolites, such
11 as small anti-oxidants (ophthalmic acid, ergothioneine, and glutathione), which are
12 enzymatically synthesized in *S. pombe*. *S*-adenosyl-homocysteine (SAH) and *S*-
13 adenosyl-methionine (SAM), coenzymes involved in the methionine cycle, were also
14 enriched in RBCs. *S*-adenosyl-methionine synthase has been reported in RBCs[62]. In
15 RBCs, SAM has been shown to act as a coenzyme for protein carboxyl methylation[63],
16 synthesis of phosphatidylcholine[64], and N-, O- and S-methyltransferase activities
17 (reviewed by Weinshilboum et al.[65]). It remains to be determined how SAH and
18 SAM in RBCs are involved in the production of methylated compounds. We are
19 particularly interested in determining whether levels of free methylated amino acids
20 are controlled by SAM and SAH. To this end, we isolated a number of *S. pombe*
21 mutants of methionine cycle enzymes and are examining their metabolic profiles
22 (T. Hayashi et al., unpublished results).
23 We found 14 novel human blood compounds, eight of which were
24 methylated or *N*-acetylated amino acids. To our knowledge, there has been no report
25 describing these as blood components. Blood data presented in this report came from

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1 four healthy volunteers. We further extended our analysis to more than 30 people
2 (Chaleckis et al., manuscript in preparation), and these compounds were universally
3 present, suggesting that their occurrence is neither accidental nor specific to certain
4 dietary customs. Interestingly, eight of these compounds (UDP-acetyl-glucosamine,
5 citramalate, dimethyl-lysine, trimethyl-histidine, *N*-acetyl-glutamate, N_2-acetyl-lysine,
6 N_6-aceltyl-glutamate and glutamate methyl ester) were also present in *S. pombe*.
7 Their physiological roles can thus be further investigated using *S. pombe* as a model.
8 Seven of the eight compounds are RBC-enriched. RBCs may require UDP-acetyl-
9 glucosamine, a nucleotide sugar and a coenzyme, as a signaling molecule of sugar
10 metabolism, like in other eukaryotic cells, including *S. pombe*[66]. UDP-glucuronic
11 acid is synthesized in liver, binds to hormones or toxic compounds, and is also used
12 for the synthesis of glucuronic acid-containing polysaccharides[67, 68]. It is not present
13 in lower eukaryotes. Citramalate is an intermediate in bacterial glutamate
14 degradation[69]. Citramalate and glutamate methyl-ester are also present in *S. pombe*,
15 but their physiological role is not understood. Two acetylated amino acids N_2-acetyl
16 and N_6-acetyl-lysine, are present in plasma and RBCs, as well as in *S. pombe*, but
17 their biological role is little understood. *N*-acetyl-glutamate is involved in the
18 removal of waste from the body in the urine as it is an allosteric cofactor of carbamyl
19 phosphate synthetase I, the first enzyme in the urea cycle[70].
20 Five novel blood compounds are methylated amino acids, four (histidine,
21 phenylalanine, tryptophan, tyrosine) of which are trimethylated and enriched in
22 RBCs. Trimethyl-histidine (hercynine) is a precursor of ergothioneine, but humans
23 do not synthesize ergothioneine; therefore this compound might be of dietary origin or
24 possibly a degradation product of ergothioneine[71], whereas *S. pombe* can produce
25 them[34, 35]. Trimethyl-tryptophan (hypaphorine) was highly abundant in RBCs. It has

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1 been reported that this compound has soporific[72] and anti-glycemic effects in mice[73].
2 Hypaphorine was reported in human milk following maternal consumption of
3 legumes[74].
4 ATP and glutathione were selectively enriched in RBCs (scarcely present in
5 plasma), while glutamine was found in both plasma and RBCs in roughly equal
6 amounts. Eleven sugar phosphate compounds required for sugar and energy
7 metabolism were all found in RBC-enriched fractions and also in *S. pombe*.
8 Similarly, all twelve nucleotides, four nucleotide-sugar derivatives, and five
9 coenzyme NAD-related compounds were selectively enriched in RBCs. Fifty-six
10 percent of RBC-enriched compounds are energy-related; these compounds are also
11 found in *S. pombe*. Three anti-oxidant compounds, glutathione, ergothioneine, and
12 ophthalmic acid, were enriched in RBCs and abundant in *S. pombe*. Glutathione and
13 ophthalmic acid may be synthesized in RBCs, as the synthetic enzymes encoded by
14 the human genes are present in RBCs[75]. Aspartate and glutamate were selectively
15 enriched in RBCs. Both are excitatory neurotransmitters. Inhibitory transmitters,
16 GABA and glycine, are difficult to measure using our method. Glutamate may be
17 partly utilized for the synthesis of glutathione[76]. These energy and anti-oxidant
18 compounds are most likely essential for maintaining RBCs during their relatively long
19 lifespan of 120 days, and these compounds are also common to *S. pombe.*
20

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25

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10

11 ## Disclosure statement

12 The authors declare that they have no competing interests.

13

14 ## Materials and Methods

15 ### Chemicals and reagents

16 Standards for metabolite confirmation were obtained from various vendors
17 (Supplemental Table 3), depending on the compound. Ergothioneine and *S*-methyl-
18 ergothioneine were a kind gift from Tetrahedron, France.

19

20 ### Human subject characteristics

21 Three healthy male and one female volunteer (23-33 years old) participated in this
22 study. Blood samples for metabolomic analysis were taken in the morning and
23 subjects were asked not to eat breakfast to ensure at least 12 h of fasting prior to
24 sampling.

25

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1 **Ethics statement**

2 Written informed consent was obtained from all donors in accordance with the
3 Declaration of Helsinki. All experiments were performed in compliance with relevant
4 Japanese laws and institutional guidelines. All protocols were approved by the
5 Ethical Committee on Human Research of Kyoto University Hospital and by the
6 Human Subjects Research Review Committee of the Okinawa Institute of Science and
7 Technology Graduate University (OIST).

8

9 **Blood sample preparation for metabolomic analysis**

10 Blood samples for metabolomic analysis were drawn by venipuncture into 5 mL
11 heparinized tubes (Terumo). Immediately, 0.2 mL blood (8-12$\times10^8$ RBC) were
12 quenched in 1.8 mL −40° C 55% methanol. The remainder of each blood sample was
13 centrifuged at 120 x *g* for 15 min at room temperature to separate plasma and RBCs.
14 After centrifugation, 0.2 mL of separated plasma and RBCs (14-20$\times10^8$ RBC),
15 respectively, were quenched in 1.8 mL −40°C 55% methanol. Ten nmol each of
16 HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, and PIPES, piperazine-
17 N,N′-bis(2-ethanesulfonic acid) were added to each sample to serve as standards.
18 After brief vortexing, samples were transferred to Amicon Ultra 10 kDa cut-off filters
19 (Millipore, Billerica, MA, USA) to remove proteins and cell debris. Following
20 sample concentration by vacuum evaporation, each sample was re-suspended in 40 μL
21 of 50% acetonitrile and 1 μL (equivalent to 5 μL of initial sample) was used for each
22 injection into the LC-MS system.

23

24 **Isolation of leucocytes by Ficoll gradient**

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1 For isolation of leucocytes we employed Ficoll gradient centrifugation. Blood of two
2 donors, 20 mL each, was collected into heparinized tubes (Terumo) and subsequently
3 diluted with the same volume of PBS buffer. Diluted blood was carefully layered
4 onto the same volume of Lympholite-H (Cosmo Bio, Tokyo, Japan) and centrifuged
5 at room temperature for 20 min at 800 x *g*. Separated leucocytes were washed 3x in
6 PBS (1 min, 350 x *g*, 4° C). After dilution, 0.2 mL of leucocytes (~5×10^7 cells) were
7 quenched in 1.8 mL −40° C 55% methanol. After addition HEPES and PIPES,
8 samples were processed as described above. Metabolites were isolated from 10 μL of
9 leukocyte cell volume (assuming an individual leukocyte cell volume of 200 fL[77] and
10 an estimated 5×10^7 cells per sample). One μL of the metabolome sample was injected
11 into the LC-MS system, corresponding to ~0.25 μL leukocyte intracellular volume.
12
13 **Fission yeast growth conditions and preparation for metabolomics analysis**
14 The wild-type heterothallic haploid 972 h^- *S. pombe* strain[78] was used for
15 metabolomic experiments. Cells were cultivated at 26° C in minimal synthetic
16 medium EMM2[79] with 0.1% glucose (5.6 mM) content. Limited glucose media were
17 prepared by mixing regular EMM2 (2% glucose) medium with EMM2-G (0%
18 glucose) in an appropriate ratio. Preparation of fission yeast samples was done as
19 described previously[8]. Metabolites were isolated from 30 μL of total *S. pombe* cell
20 volume (*S. pombe* cell diameter 3.5 μm[80]; cells grown at 5.6 mM glucose have lengths
21 of 13 μm[34], thus using a formula from Mitchison[80], mean cell volume is 120 fL; total
22 number of cells per sample 2.5×10^8). LC-MS sample injections (1 μL) corresponded
23 to ~0.75 μL *S. pombe* intracellular volume.
24
25 **LC-MS analysis**

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1 LC-MS data were obtained using a Paradigm MS4 HPLC system (Michrom
2 Bioresources, Auburn, CA, USA) coupled to an LTQ Orbitrap mass spectrometer
3 (Thermo Fisher Scientific, Waltham, MA, USA) as previously described[8]. Briefly,
4 LC separation was performed on a ZIC-pHILIC column (Merck SeQuant, Umeå ,
5 Sweden; 150 mm × 2.1 mm, 5 µm particle size). Acetonitrile (A) and 10 mM
6 ammonium carbonate buffer, pH 9.3 (B) were used as the mobile phase, with gradient
7 elution from 80% A to 20% A in 30 min, at a flow rate of 100 µL mL^{-1}. Peak areas
8 for metabolites of interest were integrated using MZmine 2 software version 2.10[38].
9 Detailed data analytical procedures and parameters were described previously[8].
10 Metabolites were initially identified by searching their m/z values in on-line databases
11 (see text) or by predicting chemical formulae from mass spectra[81]. Identified peaks
12 were verified by analyzing pure standards (STD category) to confirm their retention
13 times, or in cases where pure standards were not available, by analyzing their
14 fragmentation patterns (MS/MS category).
15
16 **Supplemental Material**
17 Raw LC-MS data in mzML format were submitted to the MetaboLights repository
18 (URL: http://www.ebi.ac.uk/metabolights). The accession number for the fission
19 yeast and human blood metabolome comparison is MTBLS87, while that for
20 metabolomic samples of a single individual's blood donated 4 times within 24 hr to
21 determine RBC:plasma metabolite distribution is MTBLS88.

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1 **Legends for Figures**

2 **Fig. 1. Preparation and analysis of blood metabolomic samples**

3 **A**. Metabolic compounds were extracted in 50% MeOH at -40° C from whole blood,
4 plasma, and RBCs (Materials and Methods). Extracted metabolites were isolated with
5 a 10-kDa cut-off filter, concentrated by a rotary evaporator, and analyzed on an LC-
6 MS system, as illustrated. **B**. Blood cells stained with Giemsa solution under a
7 microscope. Arrows indicate WBC. **C**. Raw LC-MS data 3D plots of plasma fraction
8 (top) and RBC fraction (bottom) obtained in positive ionization mode are shown: X-
9 axis, retention time (RT, min); Y-axis, m/z; Z-axis, signal intensity. Twenty
10 identified peaks are shown as examples. Peaks 1-10 are detected in both plasma and
11 RBCs. These are amino acids, creatine, carnitine, dietary metabolites (caffeine,
12 dimethyl-xanthine), and compounds introduced during sample preparation (HEPES as
13 internal standard, NH_4Cl formed in the LC-MS system). Peaks 11-20 are enriched in
14 RBC samples. Many compounds are involved in energy production, anti-oxidation,
15 and amino acid metabolism (see text).

16

17 **Fig. 2. Quantification of peak area reproducibility examined by scatter plot**

18 Blood was donated twice by the same person in 1h, and the two blood samples were
19 processed separately (samples #1 and #2). **A**. Scatter plot of all peaks detected in
20 both blood samples (positive and negative ionization modes combined). 87 % of
21 peaks differed less than 2-fold. Less than 15% of these peaks could be assigned to a
22 known compound (assigned peaks marked yellow). **B**. A scatter plot of 129 identified
23 compounds detected in blood samples #1 and #2. Approximately 97% of these peaks
24 were found within 2-fold change. **C.** Multiple peaks are produced by abundant
25 compounds such as ATP. In addition to the primary single-charged ions (indicated by

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1 arrows), compounds produce multiply charged ions, fragments of the molecule,
2 adducts with salts, and complexes with other compounds eluting at similar retention
3 times. We were able to identify 18 peaks related to ATP, listed in the inset table. **D**.
4 RBC and plasma samples were prepared from the same person, and the peak areas
5 were determined for identified compounds, and compared in a scatter plot.
6
7 **Fig. 3 Human blood compounds identified and characterized in the present study**
8 Compounds that were either RBC-enriched (57) or not (76), based on whether the
9 ratios of their RBC:plasma peak areas, were either >2 or <2, respectively (Table 1).
10 Abundance of compounds classified by peak area size, indicated by color, red (high),
11 green (medium) and blue (low). Compounds with the statue symbol are not present in
12 *S. pombe*. See text for detail.
13
14 **Fig. 4 Relatively high-abundance compounds in human blood and fission yeast**
15 Compound abundance in human blood and *S. pombe*. For example, “High-High”
16 indicates that ATP, glutathione, and glutamine are highly abundant in both blood and
17 *S. pombe*. See text.
18
19 **Supplemental Fig. 1**
20 **MS/MS analysis of peaks identified as methyl-lysine (A), *N*-trimethyl-**
21 **phenylalanine (B) and *N*-trimethyl-tyrosine (C)**
22 Identification of any peak by MS/MS analysis in the absence of a standard requires
23 the m/z value, fragmentation pattern, and retention time (RT). Similar RTs were
24 found in cases of amino acids and their methylated counterparts confirmed by
25 standard compounds (e.g. *N*-trimethyl-tryptophan 6.0 min and tryptophan 8.7 min).

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1 **A**. A peak with an m/z value of 161.128 Da in positive ionization mode matched the
2 calculated value for the methyl-lysine positive ion with a hydrogen adduct
3 (161.129 Da). The RT is 20.3 min while that of lysine is 23.3 min. The MS/MS
4 fragmentation pattern matches the methyl-lysine structure; the 130.086 Da fragment
5 corresponds to loss of $NH_2(CH_3)$ (31.043 Da), the 84.080 Da fragment to loss of
6 $NH_2(CH_3)$ (31.043) and CO_2H_2 (46.005 Da). While lysine has two amino-groups, we
7 could not determine which of the amino groups is methylated; thus, we tentatively
8 identified the compound as methyl-lysine. **B**. A peak with an m/z value of
9 208.133 Da in positive ionization mode matched the calculated value for the
10 trimethyl-phenylalanine positive ion with a hydrogen adduct (208.133 Da). The RT is
11 5.3 min while that of phenylalanine is 7.3 min. The MS/MS fragmentation pattern
12 matches the *N*-trimethyl-phenylalanine structure; the 149.059 Da fragment
13 corresponds to loss of of $N(CH_3)_3$ (59.074 Da), the 131.049 Da fragment to loss of
14 $N(CH_3)_3$ (59.074 Da) and H_2O (18.010 Da), and the 103.054 Da fragment to loss of
15 $N(CH_3)_3$ (59.074 Da) and CO_2H_2 (46.005 Da). Therefore we tentatively identified the
16 peak as *N*-trimethyl-phenylalanine. **C**. A peak with an m/z value of 224.128 Da in
17 positive ionization mode matched the calculated value for the trimethyl-tyrosine
18 positive ion with a hydrogen adduct (224.128 Da). The RT is 8.0 min while that of
19 tyrosine is 10.3 min. MS/MS fragmentation pattern matches the *N*-trimethyl-tyrosine
20 structure; the 165.054 Da fragment corresponds to loss of $N(CH_3)_3$ (59.074 Da), the
21 147.044 Da fragment to loss of $N(CH_3)_3$ (59.074 Da) and H_2O (18.010 Da). We thus
22 tentatively identified the peak as *N*-trimethyl-tyrosine.
23
24 **Supplemental Fig. 2**

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1 **Quantification of plasma and RBC metabolites and ergothioneine derivative**

2 **peak areas**

3 Blood samples were obtained as described in Fig. 2. **A**. A scatter plot comparison of

4 plasma and RBC samples #1 and #2, as described in Fig. 2A. 85 % of peaks were

5 within a 2-fold change in both plasma and RBC samples. **B**. Scatter plot comparison

6 of identified compounds in plasma and RBC samples as described in Fig. 2B. In both

7 plasma and RBC samples, approximately 96% of the peaks were within a 2-fold

8 range. **C.** Multiple peaks are ergothioneine derivatives. Arrows indicate primary

9 peaks. We were able to identify 19 peaks derived from ergothioneine.

10

11 **Supplemental Fig. 3**

12 **Scatter plot comparison of compounds quantified in both RBCs and plasma**

13 Peak areas of each compound identified in RBCs and plasma, plotted in a scatter plot.

14 Each compound is indicated with the number with the compound name in the table

15 inset. Purple number and name represent the compounds enriched in RBC (the ratio

16 RBC:plasma is more than 2-fold).

17

18 **Tables**

19 **Table 1. List of 133 identified metabolites in blood[a]**

Category / Compound	Status	In fission yeast	New blood component	RBC enriched[b]	Ratio RBC:plasma ± standard deviation[c]	Peak area[d] Blood	Peak area[d] Fission yeast
Nucleotides							
ADP	STD	○		R	64.0 ± 15.0	M	M
AMP	STD	○		R	52.0 ± 6.8	M	M
ATP	STD	○		R	81.0 ± 13.0	H	H
CDP	STD	○		R	4.6 ± 2.1	L	L
CTP	STD	○		R	2.2 ± 0.48	L	M
GDP	STD	○		R	32.0 ± 5.5	L	M
GMP	STD	○		R	3.5 ± 0.29	L	L
GTP	STD	○		R	84.0 ± 14.0	M	M

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IMP	STD	o		R	**180.0**	±	**48.0**	L	L
UDP	STD	o		R	**2.3**	±	0.5	L	M
UMP	STD	o		R	**19.0**	±	**3.6**	L	L
UTP	STD	o		R	**4.1**	±	1.6	L	H
Nucleosides, nucleobases and derivatives									
1-Methyl-adenosine	STD	o			1.3	±	0.14	M	M
1-Methyl-guanosine	STD	o			1.1	±	0.36	L	L
Adenine	STD	o			**3.4**	±	0.055	L	M
Adenosine	STD	o			0.045	±	0.012	L	L
Caffeine	STD				0.77	±	0.057	M	ND
Cytidine	STD	o			**2.1**	±	0.22	L	L
Dimethyl-guanosine	STD	o			0.77	±	0.1	L	L
Dimethyl-xanthine	STD				0.88	±	0.044	M	ND
Guanosine	STD	o			0.12	±	0.077	L	L
Hypoxanthine	STD	o			1.4	±	0.22	L	L
Inosine	STD	o			0.059	±	0.12	L	L
Uracil	STD	o			1.0	±	0.041	L	L
Urate	STD				0.69	±	0.22	M	ND
Uridine	STD	o			1.0	±	0.027	L	L
Vitamins, Coenzymes									
4-Aminobenzoate	STD				**2.5**	±	0.86	L	ND
NAD^+	STD	o		R	**130.0**	±	**30.0**	M	H
NADH	STD	o		R	**80.0**	±	**68.0**	L	M
$NADP^+$	STD	o		R	**7200.0**	±	**3000.0**	L	L
NADPH	STD	o		R	**340.0**	±	**220.0**	L	L
Nicotinamide	STD	o		R	**26.0**	±	**5.7**	M	M
Pantothenate	STD	o			**3.3**	±	1.1	L	M
Nucleotide-sugar derivatives									
GDP-glucose	STD	o	o	R	**20.0**	±	**3.6**	L	M
UDP-acetyl-glucosamine	STD	o	o	R	**11.0**	±	1.4	L	M
UDP-glucose	STD	o		R	**35.0**	±	**6.6**	M	H
UDP-glucuronate	STD		o	R	**37.0**	±	**6.2**	L	ND
Sugar phosphates									
6-Phosphogluconate	STD	o		R	**150.0**	±	**25.0**	L	L
Diphospho-glycerate	STD	o		R	**1700.0**	±	**350.0**	H	L
Fructose-1,6-diphosphate	STD	o		R	**1400.0**	±	**360.0**	M	M
Fructose-6-phosphate	STD	o		R	**23.0**	±	**2.2**	L	M
Glucose-6-phosphate	STD	o		R	**32.0**	±	**4.1**	M	M
Glyceraldehyde-3-phosphate	STD	o		R	**910.0**	±	**1100.0**	L	L
Glycerol-2-phosphate	STD	o		R	**3.7**	±	1.1	L	M
Pentose-phosphate	STD	o		R	**39.0**	±	**12.0**	L	M
Phosphoenolpyruvate	STD	o		R	**1000.0**	±	**780.0**	L	M
Phosphoglycerate	STD	o		R	**150.0**	±	**17.0**	M	M
Sedoheptulose-7-phosphate	STD	o		R	**3.6**	±	0.76	L	M

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Sugars and derivatives									
1,5-Anhydroglucitol	STD				0.95	±	0.14	M	ND
Gluconate	STD	o		R	**16.0**	±	1.8	M	L
Glucosamine	STD	o			0.89	±	0.13	M	L
Glucose	STD	o			0.87	±	0.12	M	L
myo-Inositol	STD	o			1.3	±	0.39	L	L
N-Acetyl-D-glucosamine	STD	o		R	**35.0**	±	1.4	M	L
Quinic acid	STD	o			**2.1**	±	2.0	L	M
Organic acids									
2-Oxoglutarate	STD	o			0.94	±	0.16	L	L
Chenodeoxycholate	STD				0.49	±	0.14	M	ND
cis-Aconitate	STD	o			0.33	±	0.11	L	L
Citramalate	STD	o	o	R	**3.2**	±	0.87	L	M
Citrate	STD	o			0.28	±	0.069	M	M
Fumarate	STD	o		R	**5.6**	±	0.9	L	L
Glutarate	STD	o			0.94	±	0.072	L	L
Glycerate	STD				0.69	±	0.18	L	ND
Malate	STD	o		R	**4.6**	±	0.6	L	L
Succinate	STD	o			0.92	±	0.21	L	L
Standard amino acids									
Arginine	STD	o			0.57	±	0.072	H	H
Asparagine	STD	o			1.6	±	0.18	L	L
Aspartate	STD	o		R	**6.3**	±	0.91	L	M
Glutamate	STD	o		R	**3.4**	±	0.74	M	H
Glutamine	STD	o			0.56	±	0.053	H	H
Histidine	STD	o			0.93	±	0.054	M	H
Isoleucine	STD	o			0.95	±	0.14	M	L
Leucine	STD	o			1.1	±	0.098	M	L
Lysine	STD	o			0.91	±	0.15	L	L
Methionine	STD	o			0.76	±	0.099	M	L
Phenylalanine	STD	o			0.93	±	0.16	H	M
Proline	STD	o			0.92	±	0.054	H	L
Serine	STD	o			1.1	±	0.19	L	L
Threonine	STD	o			1.1	±	0.12	M	M
Tryptophan	STD	o			0.47	±	0.054	M	M
Tyrosine	STD	o			1.1	±	0.083	M	M
Valine	STD	o			0.87	±	0.07	M	L
Methylated amino acids									
Betaine	STD	o			1.2	±	0.11	H	L
Butyro-betaine	STD			R	**5.3**	±	0.52	M	ND
Dimethyl-arginine	STD	o			0.95	±	0.12	L	L
Dimethyl-lysine	STD	o	o		0.51	±	0.09	L	L
Dimethyl-proline (stachydrine)	STD			R	**4.9**	±	0.66	H	ND
Methyl-histidine	STD	o			0.92	±	0.056	L	M

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Methyl-lysine	MS/MS	○			0.67	±	0.11	M	L
S-Methyl-ergothioneine	STD			**R**	**1500.0**	±	**2300.0**	L	ND
Trimethyl-histidine (hercynine)	MS/MS	○	○	**R**	**110.0**	±	**140.0**	L	M
Trimethyl-lysine	STD	○		**R**	**6.3**	±	1.5	M	L
Trimethyl-phenylalanine	MS/MS		○	**R**	**14.0**	±	**9.0**	L	ND
Trimethyl-tryptophan (hypaphorine)	STD		○	**R**	**6.1**	±	1.7	H	ND
Trimethyl-tyrosine	MS/MS		○	**R**	ND*			L	ND
Acetylated amino acids									
N-Acetyl-(iso)leucine	STD	○	○		1.8	±	0.39	L	L
N-Acetyl-arginine	STD	○			0.34	±	0.047	L	M
N-Acetyl-aspartate	STD	○			0.84	±	0.2	L	L
N-Acetyl-glutamate	STD	○	○		0.82	±	0.18	L	M
N-Acetyl-ornithine	STD	○			**2.6**	±	1.2	L	L
N_2-Acetyl-lysine	STD	○	○		**2.1**	±	0.3	L	L
N_6-Acetyl-lysine	STD	○	○		0.88	±	0.26	L	M
Other amino acid derivatives									
2-Aminoadipate	STD	○			0.91	±	0.073	L	L
Arginino-succinate	STD	○			0.69	±	0.46	L	M
Citrulline	STD	○			0.97	±	0.19	M	M
Creatine	STD			**R**	**7.0**	±	1.6	H	ND
Creatinine	STD				0.97	±	0.079	H	ND
Glutamate methyl ester	STD	○	○		1.5	±	0.28	L	L
Hippurate	STD				0.54	±	0.083	M	ND
Histamine	STD	○			1.0	±	0.5	L	L
Indoxyl-sulfate	STD				0.43	±	0.092	M	ND
Kynurenine	STD				0.41	±	0.081	L	ND
Ornithine	STD	○			1.2	±	0.16	L	M
Phosphocreatine	STD			**R**	**3.0**	±	0.95	L	ND
Quinolinic acid	STD				0.35	±	0.04	L	ND
S-Adenosyl-homocysteine	STD	○		**R**	**2100.0**	±	**1600.0**	L	M
S-Adenosyl-methionine	STD	○		**R**	**57.0**	±	**62.0**	L	L
Taurine	STD				0.92	±	0.18	M	ND
Carnitines									
Acetyl-carnitine	STD			**R**	**4.0**	±	0.58	H	ND
Butyryl-carnitine	STD				0.95	±	0.2	M	ND
Carnitine	STD				0.85	±	0.1	H	ND
Decanoyl-carnitine	STD				0.42	±	0.11	M	ND
Dodecanoyl-carnitine	STD				0.51	±	0.072	L	ND
Hexanoyl-carnitine	STD				0.68	±	0.087	L	ND
Isovaleryl-carnitine	STD				0.94	±	0.25	L	ND
Octanoyl-carnitine	STD				0.45	±	0.086	M	ND
Propionyl-carnitine	STD			**R**	**5.3**	±	0.82	M	ND
Tetradecanoyl-carnitine	STD			**R**	**11.0**	±	**15.0**	L	ND
Choline derivatives									

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CDP-choline	STD	○			0.98	±	0.37	L	L
Glycerophosphocholine	STD	○			1.5	±	0.24	M	H
Antioxidant									
Ergothioneine	STD	○		**R**	**100.0**	±	**4.8**	**H**	**M**
Glutathione disulfide (GSSG)	STD	○		**R**	**1900.0**	±	**430.0**	**H**	**H**
Ophthalmic acid	STD	○		**R**	**310.0**	±	**150.0**	**L**	**M**

[a] One hundred thirty-three identified metabolic compounds detected in human blood metabolome samples by LC-MS. Status of the compounds was either confirmed by comercially available standard standard (STD) or MS/MS analysis (MS/MS). Compounds not detected in fission yeast (32 compounds) and compounds not reported as blood metabolites (14 compounds) are marked by ○ in respective columns.

[b] on average in multiple persons.

[c] Ratios between RBC and plasma samples calculated from four blood samples donated by the same person within 24 hours. Values and standard deviations rounded to 2 significant numbers. ND - not detected. Values >2 shown in bold.

[d] Peak areas defined as H, high (>10^8 AU); M, medium (10^7-10^8 AU); L, low (<10^7 AU); ND - not detected. In case of blood, equivalent of ~5µl of blood was injected into the LC-MS system, for fission yeast ~0.75µl of internal cell volume (see Materials and Methods).

Table S1. List of 18 identified metabolites detected in fission yeast, but not in blood.

Table S2. Compounds detected in fission yeast and blood

Table S3. List of analyzed standard compounds

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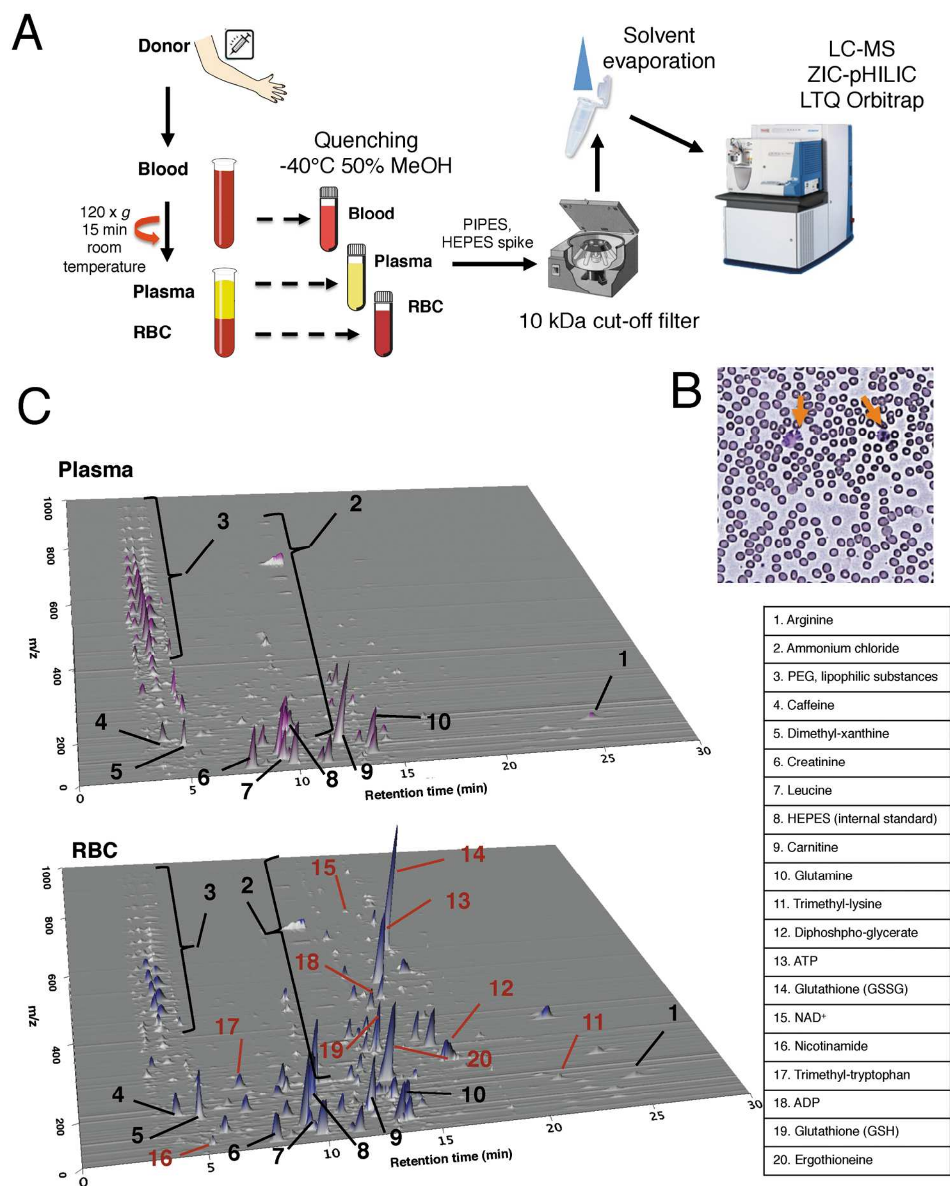

Fig. 1. Preparation and analysis of blood metabolomic samples
A. Metabolic compounds were extracted in 50% MeOH at -40° C from whole blood, plasma, and RBCs (Materials and Methods). Extracted metabolites were isolated with a 10-kDa cut-off filter, concentrated by a rotary evaporator, and analyzed on an LC-MS system, as illustrated. B. Blood cells stained with Giemsa solution under a microscope. Arrows indicate WBC. C. Raw LC-MS data 3D plots of plasma fraction (top) and RBC fraction (bottom) obtained in positive ionization mode are shown: X-axis, retention time (RT, min); Y-axis, m/z; Z-axis, signal intensity. Twenty identified peaks are shown as examples. Peaks 1-10 are detected in both plasma and RBCs. These are amino acids, creatine, carnitine, dietary metabolites (caffeine, dimethyl-xanthine), and compounds introduced during sample preparation (HEPES as internal standard, NH4Cl formed in the LC-MS system). Peaks 11-20 are enriched in RBC samples. Many compounds are involved in energy production, anti-oxidation, and amino acid metabolism (see text).
171x214mm (300 x 300 DPI)

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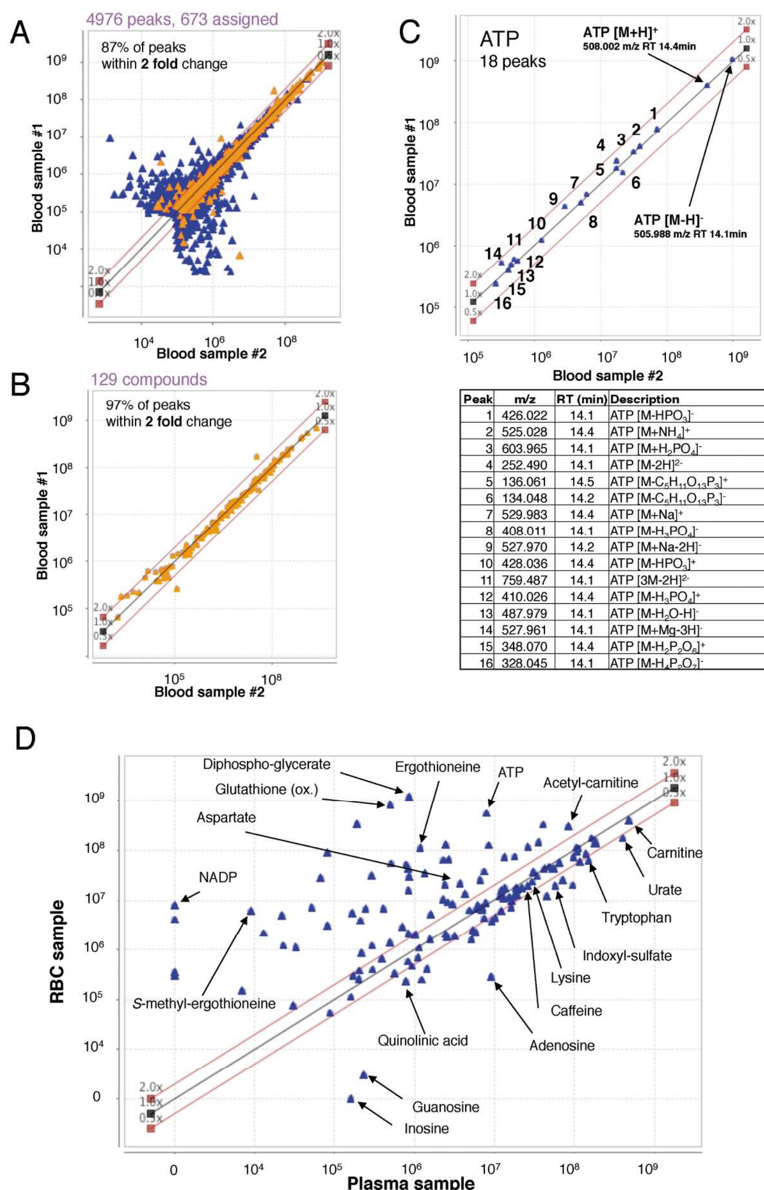

Peak	m/z	RT (min)	Description
1	426.022	14.1	ATP $[M-HPO_3]^-$
2	525.028	14.4	ATP $[M+NH_4]^+$
3	603.965	14.1	ATP $[M+H_2PO_4]^-$
4	252.490	14.1	ATP $[M-2H]^{2-}$
5	136.061	14.5	ATP $[M-C_5H_{11}O_{13}P_3]^+$
6	134.048	14.2	ATP $[M-C_5H_{11}O_{13}P_3]^-$
7	529.983	14.4	ATP $[M+Na]^+$
8	408.011	14.1	ATP $[M-H_3PO_4]^-$
9	527.970	14.2	ATP $[M+Na-2H]^-$
10	428.036	14.4	ATP $[M-HPO_3]^+$
11	759.487	14.1	ATP $[3M-2H]^{2-}$
12	410.026	14.4	ATP $[M-H_3PO_4]^+$
13	487.979	14.1	ATP $[M-H_2O-H]^-$
14	527.961	14.1	ATP $[M+Mg-3H]^-$
15	348.070	14.4	ATP $[M-H_2P_2O_6]^+$
16	328.045	14.1	ATP $[M-H_4P_2O_7]^-$

Fig. 2. Quantification of peak area reproducibility examined by scatter plot
Blood was donated twice by the same person in 1h, and the two blood samples were processed separately (samples #1 and #2). A. Scatter plot of all peaks detected in both blood samples (positive and negative ionization modes combined). 87 % of peaks differed less than 2-fold. Less than 15% of these peaks could be assigned to a known compound (assigned peaks marked yellow). B. A scatter plot of 129 identified compounds detected in blood samples #1 and #2. Approximately 97% of these peaks were found within 2-fold change. C. Multiple peaks are produced by abundant compounds such as ATP. In addition to the primary single-charged ions (indicated by arrows), compounds produce multiply charged ions, fragments of the molecule, adducts with salts, and complexes with other compounds eluting at similar retention times. We were able to identify 18 peaks related to ATP, listed in the inset table. D. RBC and plasma samples were prepared from the same person, and the peak areas were determined for identified compounds, and compared in a scatter plot.
155x233mm (300 x 300 DPI)

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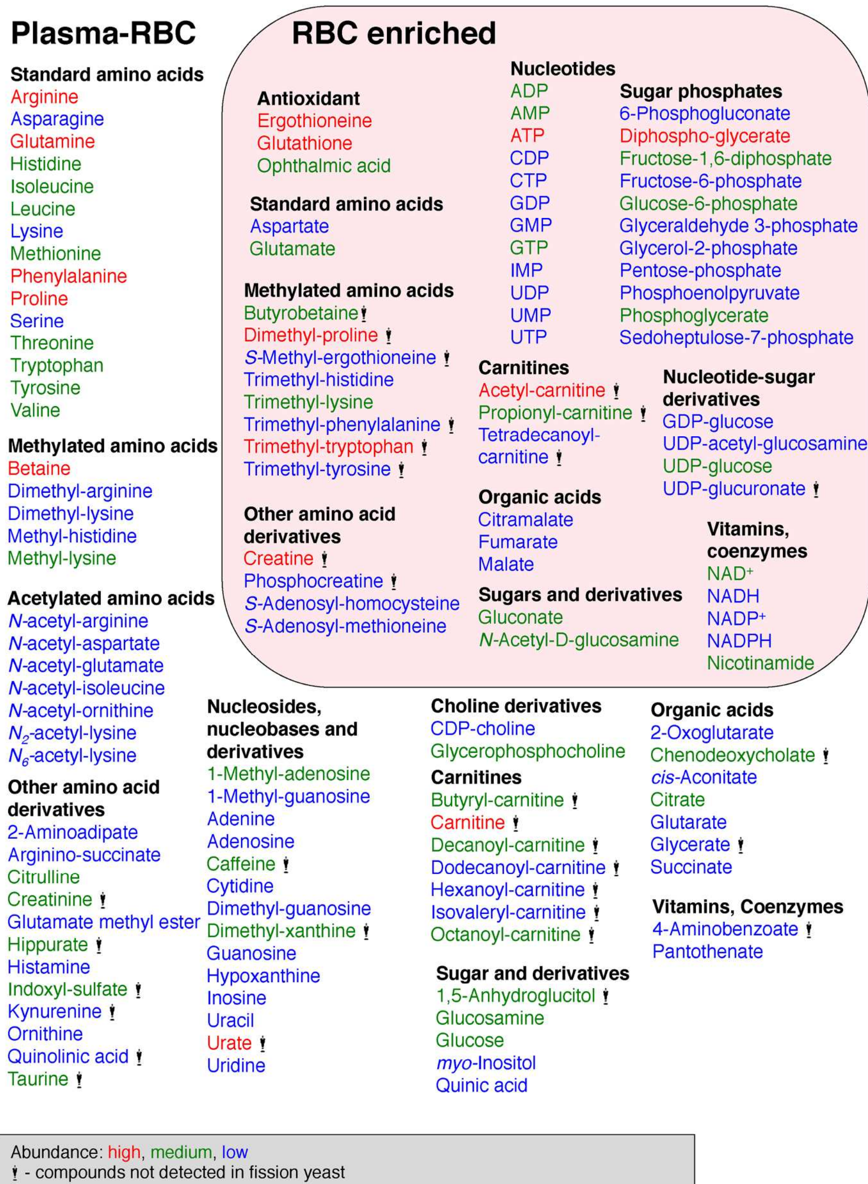

Fig. 3 Human blood compounds identified and characterized in the present study
Compounds that were either RBC-enriched (57) or not (76), based on whether the ratios of their RBC:plasma peak areas, were either >2 or <2, respectively (Table 1). Abundance of compounds classified by peak area size, indicated by color, red (high), green (medium) and blue (low). Compounds with the statue symbol are not present in S. pombe. See text for detail.
171x228mm (300 x 300 DPI)

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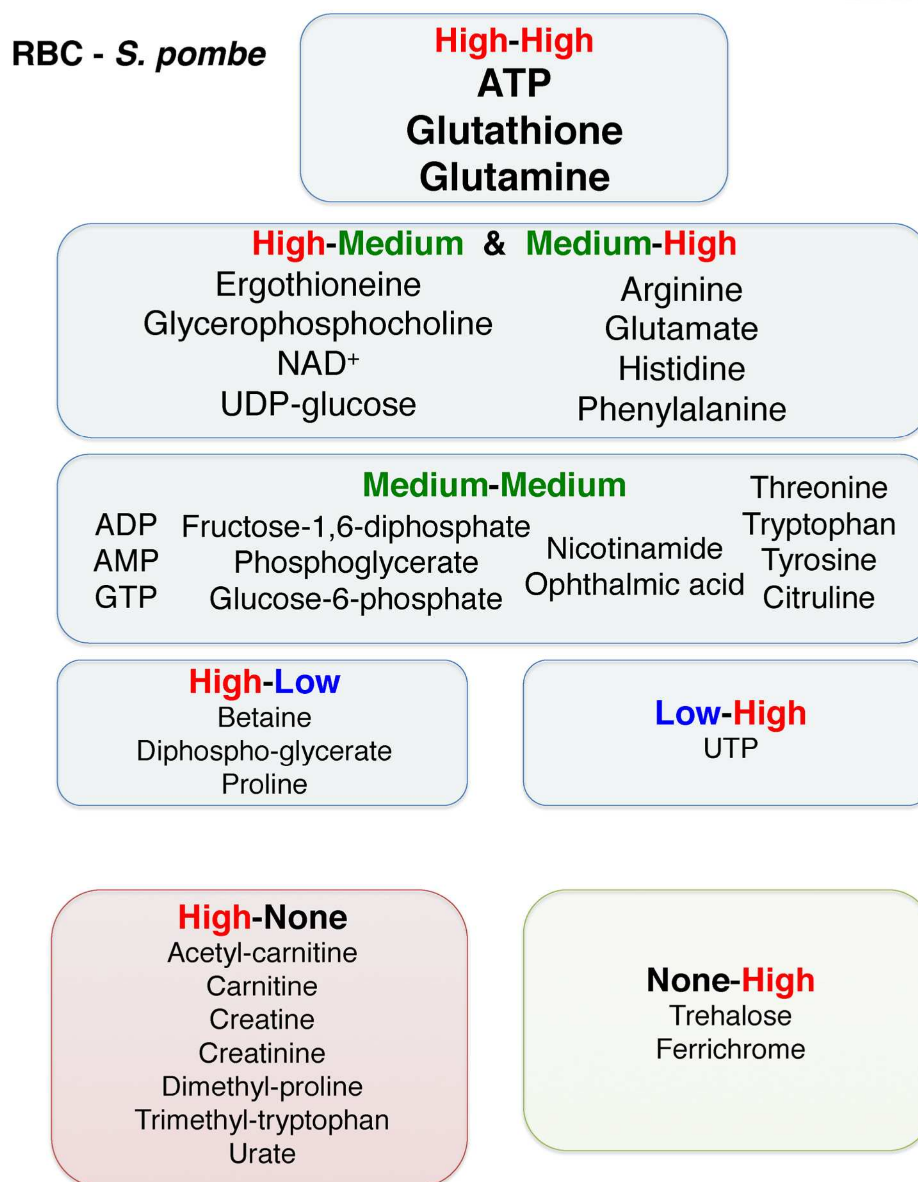

Fig. 4 Relatively high-abundance compounds in human blood and fission yeast
Compound abundance in human blood and S. pombe. For example, "High-High" indicates that ATP, glutathione, and glutamine are highly abundant in both blood and S. pombe. See text.
171x217mm (300 x 300 DPI)

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