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The deep thioredoxome in *Chlamydomonas reinhardtii*: new insights into redox regulation

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Running title: 1000 targets of thioredoxin in *Chlamydomonas*

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proteomics, redox regulation, thioredoxin targets

20 **Abbreviations**

21	At	<i>Arabidopsis thaliana</i>
22	Cr	<i>Chlamydomonas reinhardtii</i>
23	MS/MS	Tandem mass spectrometry
24	NTR	NADPH-dependent thioredoxin reductase
25	PTM	Post-translational modification
26	RNS	Reactive nitrogen species
27	ROS	Reactive oxygen species
28	TCEP	Tris(2-carboxyethyl)phosphine hydrochloride
29	Trx	Thioredoxin

ABSTRACT

Thiol-based redox post-translational modifications have emerged as important mechanisms of signaling and regulation in all organisms and thioredoxin plays a key role by controlling the thiol-disulfide status of target proteins. Recent redox proteomic studies revealed hundreds of proteins regulated by glutathionylation and nitrosylation in the unicellular green alga *Chlamydomonas reinhardtii* while much less is known about the thioredoxin interactome in this organism.

By combining qualitative and quantitative proteomic analyses, we have investigated comprehensively the *Chlamydomonas* thioredoxome and 1188 targets have been identified. They participate in a wide range of metabolic pathways and cellular processes. This study broadens not only the redox regulation to new enzymes involved in well-known thioredoxin-regulated metabolic pathways, but also sheds light on cellular processes for which data supporting redox regulation are scarce (aromatic amino-acid biosynthesis, nuclear transport,...). Moreover, we characterized 1052 thioredoxin-dependent regulatory sites and showed that these data constitute a valuable resource for future functional studies in *Chlamydomonas*.

By comparing this thioredoxome with proteomic data for glutathionylation and nitrosylation at the protein and cysteine levels, this work confirms the existence of a complex redox regulation network in *Chlamydomonas* and provides evidence of a tremendous selectivity of redox post-translational modifications for specific cysteine residues.

INTRODUCTION

Living cells rely on a complex interplay among thousands of different molecules that maintain cellular integrity and morphology and perform numerous biological functions. Although protein functions are encoded in genes, the actual regulation of protein structure and function is generally executed by specific post-translational modifications (PTMs) that enable a gigantic heterogeneity and diversity of gene products. Cell and tissue types as well as environmental stimuli influence the way proteins are post-translationally modified. Thus, cell-specific patterns of PTMs will determine protein structure, subcellular localization, protein function and interactions with other proteins. To cope with the numerous environmental challenges they may encounter, living cells have developed complex signaling networks and adaptive responses for which PTMs are key players (Choudhary et al., 2014). Consequently, studying the nature and mechanisms of signaling events is a large and crucial part of biological and medical research. A major challenge of contemporary biology is to map out, understand and model in quantifiable terms the topological and dynamic properties of the protein networks that control the behavior of the cell (Barabasi and Oltvai, 2004; Kandpal et al., 2009; Pieroni et al., 2008).

Redox PTMs have emerged as important mechanisms of signaling and regulation in all organisms. Indeed, the thiol moiety of cysteine residues can evolve toward reversible redox states (e.g. sulfenic acid, intra or inter-subunit disulfide bond, S-cysteinylation, S-glutathionylation, S-nitrosylation) or can be irreversibly damaged into sulfinic and sulfonic acids (Couturier et al., 2013; Go et al., 2015). These thiol-based redox PTMs constitute molecular switches regulating protein functions and are themselves under the control of small oxido-reductases called thioredoxins (Trxs) and glutaredoxins (Go et al., 2015; Hancock, 2009; Paulsen and Carroll, 2010).

Trxs are evolutionary conserved proteins exhibiting a characteristic three dimensional structure denominated “Trx fold” composed of five β -strands surrounded by four α -helices (Martin, 1995). Trxs contain a canonical disulfide active site (-WCG(P)PC-) which is at the origin of their thiol/disulfide oxidoreductase activity. Since its discovery in 1964 as hydrogen donor for ribonucleotide reductase in *Escherichia coli* (Laurent et al., 1964; Sengupta and Holmgren, 2014), the Trx system has been extensively studied and recognized as having multiple roles in cellular processes and human diseases (Buchanan et al., 2012; Hanschmann et al., 2013; Lee et al., 2013; Toledano et al., 2013). Non-photosynthetic organisms contain a limited number of Trxs

79 (2 in *E. coli*; 3 in *Saccharomyces cerevisiae* and 2 in *Homo sapiens*) that are localized in the
80 cytosol and mitochondria and are reduced by the NADPH-dependent flavoenzyme thioredoxin
81 reductase (NTR). In photosynthetic organisms, Trxs are part of a large multigenic family (4 Trxs
82 in *Synechocystis*, 21 in the plant model *Arabidopsis thaliana* and 10 in *C. reinhardtii*). They are
83 classified according to their subcellular localization: f-, m-, x-, y- and z- type Trxs are
84 chloroplastic while o-type and h-type are found in mitochondria and cytosol (Lemaire et al.,
85 2007; Meyer et al., 2012; Serrato et al., 2013). Whereas cytosolic and mitochondrial Trxs are
86 reduced by NTRs, chloroplastic Trxs are specifically reduced by the ferredoxin-thioredoxin
87 reductase which derives electrons from ferredoxin and the photosynthetic electron transfer chain
88 (Balsera et al., 2014; Jacquot et al., 2009; Michelet et al., 2013; Schürmann and Buchanan, 2008).
89 In photoautotrophic organisms, Trxs were originally highlighted for their capacity to modulate
90 the activity of chloroplastic enzymes involved in carbon metabolism such as the Calvin-Benson
91 cycle (CBC) enzyme fructose-1,6-bisphosphatase (FBPase) (Wolosiuk and Buchanan, 1977),
92 NADP malate dehydrogenase (NADP-MDH) (Jacquot et al., 1978) or glucose-6-phosphate
93 dehydrogenase (Scheibe and Anderson, 1981). In the following years, a few additional proteins
94 were also recognized as Trx-regulated targets such as proteins involved in the CBC or in its
95 regulation, in light-dependent ATP production or in diverse carbon metabolism pathways
96 (Lemaire et al., 2007; Schürmann and Buchanan, 2008). All these enzymes are regulated by light
97 through Trx-dependent reduction of disulfide bonds. Trxs were also recognized as electron
98 donors for the regeneration of major antioxidant enzymes such as peroxiredoxins or methionine
99 sulfoxide reductases (Kaya et al., 2015; Lu and Holmgren, 2014; Perez-Perez et al., 2009; Sevilla
100 et al., 2015). The mechanisms of these Trx-dependent processes were investigated in detail at the
101 molecular and structural level in different model systems. However, during the last fifteen years,
102 the development of proteomic studies considerably expanded the repertoire of Trx target proteins.
103 These studies were initiated within the field of plant biology and are mostly focused on
104 photosynthetic organisms. We and other groups participated to this effort by developing
105 proteomic approaches that led to the identification of hundreds of putatively redox-regulated
106 proteins (Buchanan et al., 2012; Lindahl et al., 2011; Montrichard et al., 2009). Two main
107 approaches were employed. The first one relies on our knowledge of the reaction mechanism of
108 thiol-disulfide interchange occurring between reduced Trx and its oxidized target protein. It has
109 been established that the N-terminal and most reactive cysteine of the Trx active site performs an

110 initial nucleophilic attack on the disulfide bond of the target protein leading to the formation of a
111 transient mixed-disulfide, *i.e.* a covalent disulfide-bonded heterodimer between the Trx and its
112 target (Brandes et al., 1993; Holmgren, 1995). This heterodimer is further resolved by a second
113 nucleophilic attack involving the C-terminal Trx active site cysteine. This attack allows releasing
114 the oxidized Trx and the reduced target protein. Therefore, mutation of the second active site
115 cysteine (resolving cysteine), into serine or alanine, allows to stabilize the heterodimer. Such a
116 monocysteinic Trx variant can be used as bait to trap covalently bound targets. This strategy was
117 used to purify Trx targets *in vivo* in yeast (Verdoucq et al., 1999) and more recently in human
118 cells (Wu et al., 2014) and *E. coli* (Arts et al., 2016). The monocysteinic Trx can also be grafted
119 on a chromatographic resin and Trx-bound targets specifically eluted by adding a chemical
120 reductant like DTT and identified by mass spectrometry (MS) (Figure 1A). This approach was
121 applied to numerous protein extracts from different photosynthetic prokaryotes (Lindahl and
122 Florencio, 2003; Pérez-Pérez et al., 2006; Pérez-Pérez et al., 2009) and eukaryotes (Alkhalfioui et
123 al., 2007; Balmer et al., 2003; Balmer et al., 2004a; Balmer et al., 2006b; Balmer et al., 2004b;
124 Bartsch et al., 2008; Goyer et al., 2002; Hall et al., 2010; Hosoya-Matsuda et al., 2005; Lemaire
125 et al., 2004; Marchand et al., 2006; Marchand et al., 2010; Motohashi et al., 2001; Wong et al.,
126 2004; Yamazaki et al., 2004; Yoshida et al., 2013). The second main strategy for proteomic
127 analysis of Trx targets is based on the *in vitro* reconstitution of the enzymatic Trx system
128 (NADPH, NTR, Trx) within a cell-free extract (Figure 1B). This system allows *in vitro* reduction
129 of Trx-regulated cysteines that are labeled with thiol-specific probes allowing detection of Trx-
130 targeted proteins and their identification by MS. Initially developed by Buchanan and co-workers
131 on proteins from peanut seeds using the monobromobimane fluorescent probe (Yano et al.,
132 2001), this strategy was rapidly applied to different total or subcellular soluble protein extracts
133 from different land plants (Alkhalfioui et al., 2007; Balmer et al., 2006a; Balmer et al., 2006b;
134 Hall et al., 2010; Maeda et al., 2004; Maeda et al., 2005; Marx et al., 2003; Wong et al., 2003;
135 Wong et al., 2004; Yano and Kuroda, 2006) and more recently to Archaea (Susanti et al., 2014).
136 This strategy was also extended in terms of detection to fluorescent (Maeda et al., 2004),
137 radioactive (Marchand et al., 2004) or biotinylated probes (Marchand et al., 2006). More recently,
138 quantitative adaptations of this approach were developed for MS analyses based on isotopic
139 labeling with cleavable Isotopic-Coded Affinity Tag reagents (cICAT) (Hagglund et al., 2014;
140 Hagglund et al., 2008) or with cysteine-reactive Tandem Mass Tag (Cys-TMT) (Zhang et al.,

2016). Both of these cysteine-specific reagents allowed the enrichment of cysteine-containing peptides by affinity or immuno-purification, respectively, and the identification of Trx-targeted cysteines.

In the green biflagellate microalga *Chlamydomonas reinhardtii*, we previously identified 55 putative targets of Trx by coupling the monocysteinic Trx-based approach with protein separation on 2D-gels and identification by MALDI-TOF MS (Lemaire et al., 2004). Considering the number of Trx targets identified later in other photosynthetic organisms and the results from our own studies in *Chlamydomonas* that identified 225 proteins regulated by S-glutathionylation (Michelet et al., 2008; Zaffagnini et al., 2012a) and 492 nitrosylated proteins (Morisse et al., 2014b), the number of 55 Trx targets appears likely underestimated. Taking advantage of the latest improvements of mass spectrometry sensitivity and capacity to deal with complex samples (Doll and Burlingame, 2015; Hebert et al., 2014; Riley et al., 2016) we not only reinvestigated comprehensively the “thioredoxome” in *Chlamydomonas* but also performed an in-depth characterization of Trx-reduced cysteines by combining qualitative and quantitative mass spectrometry analyses. This study broadens the landscape of redox regulation in photosynthetic organisms by identifying more than 1000 Trx targets and sheds light on the underlying molecular mechanisms thereby providing a valuable resource for future functional studies. Moreover, this work confirms the existence of a complex redox regulation network and provides evidence of a tremendous selectivity of redox PTMs for specific cysteine residues.

EXPERIMENTAL PROCEDURES

Detailed descriptions of experimental and analytical approaches are found in SI Materials and Methods.

All mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (<http://proteomecentral.proteomeexchange.org>) *via* the PRIDE partner repository (Vizcaino et al., 2013) with the data set identifiers PXD006097 (Reviewer account Username: reviewer97461@ebi.ac.uk; Password: pM7i6HgK) and PXD006116 (Reviewer account Username: reviewer69485@ebi.ac.uk; Password: YuEMZT17).

RESULTS - DISCUSSION

Identification of Trx-targets by affinity chromatography

To broaden the landscape of proteins targeted by Trx, we took advantage of the enrichment capacity of the affinity chromatography approach. As in our pioneering study in *Chlamydomonas* (Lemaire et al., 2004), we grafted the monocysteinic variant of the cytosolic Trx, CrTrxh1-C39S, on a Sepharose chromatographic resin. *Chlamydomonas* contains only two cytosolic Trx but CrTrxh1 is by far the most highly expressed and the most abundant (Lemaire et al., 2003a; Lemaire et al., 2003b; Lemaire and Miginiac-Maslow, 2004). Moreover, CrTrxh1 does not contain any extra-cysteine in its primary sequence meaning that covalent binding between the C39S mutant Trx and a target protein occurs exclusively through the nucleophilic cysteine. Finally, CrTrxh1 exhibits a broad substrate specificity as it is able, *in vitro*, to reduce efficiently most known cytosolic and chloroplastic Trx targets including antioxidant enzymes and Calvin-Benson cycle enzymes (Fischer et al., 2009; Goyer et al., 2002; Rivera-Madrid et al., 1995; Stein et al., 1995). Soluble protein extracts from two independent *Chlamydomonas* cultures grown under standard laboratory growth conditions in Tris-Acetate-Phosphate medium were subjected to the classical workflow depicted in Figure 1A. For each biological replicate, soluble proteins retained on the column were selectively eluted with DTT and further analyzed in duplicate by nLC-MS/MS without any prior separation on SDS-PAGE or 2D-gels. Moreover, to go deeper into this sub-proteome and identify less-abundant Trx targets, flow-through fractions were kept, concentrated and loaded again onto the column. Raw MS data from analytical replicates were concatenated before identification and the results obtained from successively eluted protein fractions of the same biological replicate were merged. Each biological replicate allowed us to identify 1322 and 1225 cysteine-containing proteins, respectively. To generate robust data, we considered only proteins identified in both biological replicates restricting the landscape of Trx targets to almost one thousand proteins (980 proteins) (Figure 2A).

From the identified Trx-target proteome only 31 supplemental proteins lacking cysteine in their primary NCBI sequence were also identified by this approach. Using the primary sequence of these 31 proteins, we performed a BLAST search against the latest *Chlamydomonas* genome annotation available at the Phytozome website (<https://phytozome.jgi.doe.gov/pz/portal.html>). Seven of these NCBI sequences corresponded to partial sequences or wrong gene models since

the corresponding proteins contain at least one cysteine in their Phytozome full-length sequences. Therefore, they were finally reintegrated in our Trx-target dataset. For the 24 remaining cysteine-lacking proteins, we hypothesized that their presence likely reflects their natural abundance in our soluble proteins extracts rather than a lack of specificity. To confirm this hypothesis, we experimentally characterized the soluble proteome of *Chlamydomonas* using extracts from cells grown under conditions similar to those employed for identification of Trx targets. The 24 proteins were almost all identified among the most abundant proteins (according to their sequence coverage) (Supplemental Table 2). This suggests that, under our experimental conditions, false positive contaminants were limited to a very small number of highly abundant proteins.

The affinity chromatography approach allowed us to finally identify 980 putative Trx targets (Figure 2A) with low contamination (Supplemental Table 1). We also noted that 142 proteins identified as putative Trx targets by affinity chromatography were not identified in the soluble proteome confirming the enrichment capacity of this approach. Among these 980 proteins, we found 54 of the 55 previously identified Trx-targets (Lemaire et al., 2004). In fact, the missing CPN20 chaperonin was identified in only one of the two biological replicates and thus was not taken into account in our dataset. Moreover, almost all proteins known to be regulated by Trx in other photosynthetic organisms, but not yet established in *Chlamydomonas*, were identified by this approach. These include methionine sulfoxide reductases (Tarrago et al., 2009; Vieira Dos Santos et al., 2005), ACCase (Hunter and Ohlrogge, 1998; Kozaki et al., 2001; Sasaki et al., 1997), APS reductase (Bick et al., 2001), DAHP synthase (Entus et al., 2002), glucan water dikinase (Mikkelsen et al., 2005), uricase (Du et al., 2010), magnesium chelatase CHLI subunit (Ikegami et al., 2007), cytosolic NAD-MDH (Hara et al., 2006) or ADP-glucose pyrophosphorylase (Ballicora et al., 2000). This suggests that other proteins identified in this dataset are also relevant. Thus, the Trx-affinity strategy combined to modern mass spectrometry is a powerful method to identify proteins forming mixed-disulfides with thioredoxins. However, one major drawback of this approach is that the exact cysteine targeted by Trx within the protein remains undetermined. Therefore, in addition to the Trx-affinity chromatography, we developed a quantitative reductome approach in order to gain further insights into the thioredoxome of *Chlamydomonas*.

Identification of Trx-targeted cysteines by the reductome approach

Combination of the reductome approach with differential labeling was proven to be efficient to characterize cysteines targeted by Trxs at a proteome scale (Hagglund et al., 2008; Marchand et al., 2006; Zhang et al., 2016). In this study, we used cICAT reagents, commercially available in light (ICAT_L) and heavy (ICAT_H) forms, to label cysteines of soluble proteins after *in vitro* incubation in the presence or absence of the Trx system composed of NADPH, AtNTR-B and cytosolic CrTrxh1 (Figure 1B). The cICAT labelled proteins were digested and the corresponding peptides were recovered after avidin affinity purification and acidic cleavage of the biotin tag. The peptide mixtures were then analyzed by nLC-MS/MS. For each cysteine-containing peptide, the determination of cICAT ratio by quantitative MS can be assimilated to a reduction level allowing discrimination between truly Trx-reduced proteins and false positives.

First, we sought to determine whether the acidic cleavage of the biotin moiety of cICAT labeled peptides was required since this supplementary step was a potential source of material loss. For this purpose, *Chlamydomonas* protein extracts were labeled either with light or heavy cICAT reagents and mixed together in equal ratio. After trypsin digestion and avidin affinity purification, eluted peptides were analyzed by MS on a Q-Exactive instrument before or after cleavage of the biotin moiety by acidic treatment. The base peak chromatogram revealed that retention times of biotinylated peptides were clearly delayed toward the end of the gradient, suggesting that biotinylated peptides are more hydrophobic (Supplemental Figure 1A). Moreover, fragmentation spectra of cICAT-labeled peptides generated by HCD with a normalized collision energy of 27 appeared dominated by an intense fragmentation pattern (m/z 167.07; 232.15; 258.17; 284.14; 515.29 Da) due to the cICAT moiety rendering them much less informative about the peptide sequence (Supplemental Figure 1B and 1C). By comparison, fragmentation spectra of peptides for which the biotin moiety was removed are much more informative. Finally, the presence of the biotin moiety also appeared to alter the elution profiles of heavy and light peptides and could therefore significantly alter MS quantification. Indeed, the extracted ion chromatograms for heavy and light peptides differed significantly when the biotin moiety was present whereas co-elution of isotopes was achieved when the biotin tag had been removed (Supplemental Figure 1D). Altogether, these multiple issues led, under our experimental MS conditions, to dramatic losses in terms of identification (data not shown) and we decided to systematically cleave the biotin moiety for subsequent experiments.

To establish thresholds below and above which a cICAT ratio determines that cysteines are Trx-targeted, we reduced a protein extract either chemically, using Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) as strong reducing agent, or enzymatically, with the thioredoxin system. In each case, protein extracts were divided equally in two fractions for labeling with the light or heavy form of cICAT and mixed again in equal ratio. After digestion, avidin affinity purification, and removal of the biotin tag, peptides were analyzed and quantified by MS. For both experiments, logarithmized ICAT_H/ICAT_L ratio showed a symmetric distribution centered on a value close to zero as expected for samples mixed in equal ratio (Supplemental Figure 2A and 2B). Moreover, calculated standard deviations for both conditions were similar suggesting that the workflow was reproducible. To generate results with high confidence, we calculated logarithmized thresholds based on the global mean ratio and three standard deviations as shown in the following equation:

$$\text{Log}_2(\text{Thresholds}) = \pm |\text{Global Mean Ratio}| \pm 3 \times \text{Standard Deviation}$$

Application of this formula to our experimental dataset revealed that peptides having ICAT_H/ICAT_L ratios below 0.46 and above 2.18 could be considered as having a cysteine whose redox state is significantly reduced (ratio above 2.18) or oxidized (ratio below 0.46) by Trx. We have to notice that both distributions failed the Shapiro statistical test indicating that they do not strictly follow a Normal distribution. Thus, a precise false positive rate for these thresholds cannot be statistically extrapolated but was estimated to remain below 1.5% based on our experimental data (1.27% for TCEP reduction and 1.37% for enzymatic reduction).

Finally, we decided also to check whether an isotopic effect could be observed in our workflow. We therefore performed, on the same biological sample, two reductome experiments in parallel that were similar in all aspects except that the heavy and light forms of cICAT reagents were interchanged. We performed comprehensive MS analyses (five technical replicates) associated to stringent criteria for MS identification (peptide FDR < 0.1%) and quantification (peptide quantified at least 3 times over the 5 replicates). Frequency profiles of cICAT ratios from both experiments appeared similar indicating that enzymatically reduced and control protein samples can be indifferently labeled by light or heavy cICAT (Supplemental Figure 3).

The quantification results from the two experimental datasets obtained after incubation in the presence or absence of the Trx system (Figure 1B) were taken into consideration and concatenated. Among peptides identified in our analysis, only around 6% did not contain cysteine

in their sequence (data not shown) underlining the specificity of our approach. Moreover, among cysteine-containing peptides, 977 peptides had a significant ICAT_H/ICAT_L ratio (Supplemental Table 3). Actually, only one peptide belonging to the β -subunit of the T-complex protein 1 had inconsistent quantitative data and thus was not included in the final list. Almost all identified cysteines showed a higher reduction level and only 25 cysteines appeared unexpectedly oxidized after the Trx reduction treatment. The appearance of oxidized proteins after Trx reduction was previously observed by Zhang and colleagues using a Cys-TMT approach (Zhang et al., 2016). These cysteines belong to fourteen different proteins. For two of these, at least another peptide having a Trx-reduced cysteine was also identified and eight proteins were also retained on the affinity chromatography column. Altogether, this suggests that detection of such oxidized redox state in the presence of the Trx system could originate from complex mechanisms involving conformational changes and/or disulfide bond isomerization as established for chloroplastic NADP-malate dehydrogenase (Ruelland et al., 1997). Thus, we decided to consider these fourteen proteins as putative Trx targets. Almost all peptides exhibiting a significant H/L ratio were proteotypic and only 18 peptides could map to more than one possible protein. As these proteins are closely related since they are encoded by duplicated genes such as tubulin α , they were grouped together and counted for only one isoform. Overall, these 977 peptides allowed us to identify 1052 Trx-targeted cysteines spread over 603 proteins.

Validation of Trx-reducible cysteines identified by the reductome approach

This fruitful identification of putative Trx-targeted cysteines prompted us to evaluate the relevance of the cysteines identified. Interestingly, the two active site cysteines of CrTrxh1, CrNTR3 and AtNTR-B were identified. To validate our data, we focused on eight other Chlamydomonas enzymes known to be activated by Trx and for which we have structural information about Trx-dependent cysteines (Figure 3).

Chloroplastic ATP-synthase— Chloroplast ATPase (CF₀CF₁) is activated both by the electrochemical proton potential difference (Strotmann and Bickel-Sandkötter, 1984) and by reduction of a disulfide bond located in the γ -subunit of the CF₁ domain (Moroney et al., 1984; Yu and Selman, 1988) under the control of m- and f-type thioredoxin (He et al., 2000; Schwarz et al., 1997). In Chlamydomonas, the regulatory disulfide occurs between Cys198 and Cys204

whose mutation to serine completely abolished redox regulation (Ross et al., 1995). In this study, we identified Cys204, the cysteine corresponding to the second regulatory cysteine.

NADP-malate dehydrogenase (NADP-MDH)– This enzyme is involved in the export of reducing power from the chloroplast to the cytosol through the malate valve. Chloroplastic NADP-MDH from land plants are redox regulated through two disulfide bonds present in N- and C-terminal extensions (Issakidis et al., 1996). By contrast, *Chlamydomonas* NADP-MDH contains only one C-terminal Trx-regulated disulfide bond and a potential second internal structural disulfide bridge with no regulatory function (Lemaire et al., 2005). Interestingly, in the present study we identified the two C-terminal Cys involved in the regulatory disulfide bond but not the cysteines involved in the structural disulfide bond, suggesting that the reductome approach is selective for redox-modified cysteines.

Phosphoglycerate kinase (PGK)– This Calvin-Benson cycle enzyme is not known to be redox regulated in land plants. By contrast, a Trx-dependent redox activation was reported in *Chlamydomonas*, *Synechocystis* and *Phaeodactylum tricornutum* (Bosco et al., 2012; Morisse et al., 2014a; Tsukamoto et al., 2013). In *Chlamydomonas* chloroplastic PGK, the regulatory disulfide bond was shown to involve the two C-terminal cysteines (Morisse et al., 2014a), one of which, Cys412, was identified in our reductome approach.

CP12/Phosphoribulokinase (PRK)/GAPDH complex– In photosynthetic organisms, Trxs are known to modulate independently PRK and GAPDH activities but also to initiate the disassembly of the (A₄-GAPDH)₂-CP12₄-PRK₂ complex through the reduction of CP12 (Lopez-Calcano et al., 2014; Marri et al., 2009). CP12 contains four well-conserved cysteines that are involved in two Trx-controlled disulfide bonds (Lopez-Calcano et al., 2014) (Avilan et al., 2000). In the present study, CP12 was retained on the Trx column and the four cysteines were clearly identified as reduced by Trx, thereby validating the two approaches. In PRK, the two N-terminal cysteines form a Trx-regulated disulfide bond in higher plants (Brandes et al., 1996; Milanez et al., 1991; Porter et al., 1988) and in *Chlamydomonas* (Lebreton et al., 2003). Surprisingly, instead of these two cysteines, the reductome approach identified two other well-conserved cysteines, Cys243 and Cys249. Interestingly, these cysteines were suggested to participate in a disulfide whose formation alters the physical interaction of PRK with GAPDH and CP12 (Thieulin-Pardo et al., 2015). A₄-GAPDH is likely regulated by glutathionylation (Zaffagnini et

al., 2007) but was not shown to be controlled by Trx. Intriguingly, we identified the A₄-GAPDH active-site cysteine Cys149 and its vicinal cysteine Cys153 as both labeled by cICAT reagents suggesting that they form a Trx-reduced disulfide which was also detected in human GAPDH (Rinalducci et al., 2015). Moreover, we also identified ADK3, an adenylate kinase having a C-terminal domain similar to that of CP12 (Thieulin-Pardo et al., 2016). Chloroplastic ADK3 was identified by both approaches and we identified the two cysteines of the disordered CP12-like domain, suggesting that ADK3 could be regulated by chloroplastic Trxs through its CP12 tail.

Peroxiredoxins (Prxs)– They constitute a family of enzymes reducing peroxides and peroxynitrites and present in the chloroplast, mitochondria and cytosol (Dietz, 2011). Oxidized Prxs can be regenerated in their active form by different electron donors such as Trxs, glutaredoxins, glutathione and ascorbate. In *Chlamydomonas*, the reductome approach identified CrPrx2, a 2-cys type Prx located in cytosol or flagella and CrPrx4, which belongs to the group of type II Prxs and is localized in mitochondria (Dayer et al., 2008). CrPrx1, the chloroplastic counterpart of Prx2, has been shown to be dependent on Trxs for its regeneration (Goyer et al., 2002). Interestingly, in both Prx2 and Prx4, we identified the peroxidatic cysteine responsible for the reactivity of Prxs with peroxides. For Prx4, we also identified Cys57 as a cysteine putatively reduced by Trxs.

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco)– This Calvin-Benson cycle enzyme catalyzes the first step of photosynthetic CO₂ fixation through its carboxylase activity. Despite the fact that Rubisco was systematically identified among putative Trx targets in redox proteomic studies performed in photosynthetic cells (Michelet et al., 2013), a direct effect of Trx on Rubisco has not yet been reported. In *Chlamydomonas* Rubisco large subunit, seven of the twelve cysteines are highly conserved among photosynthetic eukaryotes (Moreno et al., 2008) and among them, oxidation of Cys172, Cys192, Cys449 and Cys459 was reported to play a prominent role either in Rubisco inactivation and degradation through conformational modulation (Garcia-Murria et al., 2008; Marin-Navarro and Moreno, 2006) or chloroplastic mRNA binding (Uniacke and Zerges, 2008; Yosef et al., 2004). Interestingly, we identified Cys459 as putative Trx target. Cys449 and Cys459 are located close to the surface and in a flexible region since the distance between the two residues seems to fluctuate (Garcia-Murria et al., 2008). Moreover, Cys459 was recently identified as nitrosylated in *Chlamydomonas* (Morisse et al., 2014b). Altogether, these

data are compatible with the proposed role of Cys449 and Cys459 as redox sensor (Garcia-Murria et al., 2008) in which chloroplastic Trxs could play a direct regulating role.

Other proteins— Numerous other enzymes of the dataset were also studied at the molecular level in other species but not in *Chlamydomonas*. In this case, comparison of the position of target cysteines is much less informative. Indeed, redox regulatory mechanisms may significantly vary between species. For example, the activity of the autophagy cysteine protease Atg4 is regulated by Trx in both yeast (Perez-Perez et al., 2014) and *Chlamydomonas* (Perez-Perez et al., 2016) but through distinct disulfide bonds, *i.e.* located in completely different regions of the enzyme. The fact that *Chlamydomonas* Atg4 was not identified among Trx targets in the present study may be linked to its low abundance as suggested by its absence from our soluble proteome of *Chlamydomonas* cells (Supplemental Table 2) but also to the fact that this protease is regulated by Trx under conditions of autophagy induction while our extracts were prepared from non-autophagic conditions. Some enzymes may also be regulated only in some species as established for GAPDH (Trost et al., 2006) or PGK (Morisse et al., 2014a). Another typical example is Rubisco activase (RCA). RCA is a molecular chaperone of the AAA+ family that uses the energy from ATP hydrolysis to release tight binding inhibitors from the active site of Rubisco (reviewed in (Portis, 2003; Portis et al., 2008)). *Chlamydomonas* only contains a short β isoform of RCA that does not contain TRX-regulated cysteines found in α isoforms (Zhang and Portis, 1999; Zhang et al., 2001) but still exhibit light dependent regulation of RCA activity (Gontero and Salvucci, 2014; Michelet et al., 2013; Salvucci and Anderson, 1987). Nevertheless, *Chlamydomonas* RCA contains four cysteines (Cys148, Cys196, Cys255, Cys289) that are highly conserved in all photosynthetic organisms and were all identified by our reductome approach. This suggests that Trx may also play some role in the regulation of β isoforms of RCA. Interestingly, the α isoform of Arabidopsis RCA was detected as nitrosylated in Arabidopsis leaves (Lindermayr et al., 2005) and Cys196 and Cys148 from respectively *Chlamydomonas* and Arabidopsis β -RCA, were detected as S-nitrosylated (Fares et al., 2011; Morisse et al., 2014b). Altogether, these data suggest that the redox regulation of RCA is likely more complex than currently established and reminds, for some aspects, the redox regulation of A-type and B-type chloroplastic GAPDHs. Nevertheless, for some proteins the position of cysteines reduced by Trx is conserved between land plants and algae and were confirmed by our *Chlamydomonas*

reductome. This is for example the case of the Calvin-Benson cycle enzymes FBPase and SBPase (Chiadmi et al., 1999; Gutle et al., 2016; Jacquot et al., 1997; Rodriguez-Suarez et al., 1997), chloroplastic magnesium chelatase CHLI (Ikegami et al., 2007), 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase (Entus et al., 2002) or glucose-6-phosphate dehydrogenase (Nee et al., 2014; Wenderoth et al., 1997). For some of these enzymes the reductome approach suggests, in addition to known target cysteines, the existence of additional sites of redox regulation. Interestingly some of these sites were found to undergo glutathionylation (Zaffagnini et al., 2012a) or nitrosylation (Morisse et al., 2014b).

Altogether, the *Chlamydomonas* reductome recapitulates most of the previously established sites of Trx-dependent regulation, strongly suggesting that other sites identified in our dataset are also relevant. Therefore, this study unravels the existence of hundreds of new Trx-dependent regulatory sites.

Chlamydomonas thiorodoxome: more than 1000 targets, more than 1000 cysteines.

Mass spectrometers have been tremendously improved over the last ten years in terms of mass accuracy, resolution, sensitivity and scan speed and they are now able to deal with highly complex biological samples even without fractionation (Doll and Burlingame, 2015; Hebert et al., 2014; Riley et al., 2016). The use of such up-to-date instruments is surely at the origin of our fruitful harvest of Trx-regulated proteins, but the development of complementary biochemical approaches performed at the protein and peptide levels contributed too. In this report, the two strategies, namely the Trx affinity column and the reductome, allowed the identification of 973 and 603 Trx targets, respectively, with a global overlap of ca. 33% (Figure 2B). This indicates that the two methods are complementary. A similar limited overlap between these two approaches was previously reported in wheat (Wong et al., 2004) and *Arabidopsis* (Marchand et al., 2006), two studies exclusively conducted at the protein level, or in *Chlamydomonas* for S-nitrosylation where both proteins and cysteinyl-peptides were identified (Morisse et al., 2014b). In the present study, the combination of both approaches allowed us to identify 1188 putative Trx targets in *Chlamydomonas*.

This number may seem high but only represents, at the proteome scale, 6.5% of cysteine-containing proteins, a value consistent with previous studies (Arts et al., 2016; Morisse et al., 2014b; Susanti et al., 2014). Moreover, distribution of *Chlamydomonas* proteins according to

their number of cysteines does not show a significant enrichment for proteins containing a higher number of cysteines (Figure 4). In the reductome approach, 92% of identified peptides contain only one cysteine and 7.5% contain two cysteines in their sequence. When compared to the theoretical proteome (Supplemental Figure 4), this distribution suggests that peptides containing at least 2 cysteines could be underestimated, a limitation that may be linked to the MASCOT identification software (Garcia-Santamarina et al., 2014).

The depth of proteomic analyses dedicated to post-translational modifications is generally hampered by both modification levels and protein relative abundance. Without use of robust depletion/equalization or enrichment methods, low-abundant proteins are often missed (Doll and Burlingame, 2015; Riley et al., 2016). Interestingly, 18.6% of Trx targets present in our dataset (142 and 118 proteins in the affinity chromatography and the reductome approaches, respectively) are likely low-abundant proteins. Indeed, these proteins were not identified in the soluble proteome but were identified as Trx targets most likely thanks to the enrichment capacity of our approaches (Supplemental table 4). However, other Trx-regulated proteins remain probably to be identified in *Chlamydomonas*, especially those that are induced under specific growth conditions (strictly photoautotrophic conditions, nutrient starvations, light/dark cycles) or those that are expressed at very low levels, such as nuclear proteins for which dedicated and exhaustive analyses of Trx targets will be required. Nevertheless, by comparison with all previously published data, our study considerably broadens the landscape of redox regulation in photosynthetic organisms since our *Chlamydomonas* thioredoxome contains 1188 Trx targets and 1052 redox regulated cysteines.

Subcellular localization

The subcellular distribution of the 1188 proteins identified was determined using the PredAlgo software, a multi-subcellular localization prediction tool dedicated to algae (Tardif et al., 2012). As shown in Supplemental Figure 5A, Trx targets are predicted to be distributed for 30% to the chloroplast, 9% to mitochondria, 6% to the secretory pathway and 55% to other compartments. Such a distribution is not surprising despite the fact that we used a cytosolic Trx. Indeed, it is recognized that redox proteomic studies performed *in vitro* lack specificity and similar targets are identified regardless of the Trx isoform employed (Buchanan et al., 2012; Montrichard et al., 2009) and this is all the more true for CrTrxh1 that is able to reduce *in vitro* most cytosolic and

chloroplastic Trx targets (Fischer et al., 2009; Goyer et al., 2002; Rivera-Madrid et al., 1995; Stein et al., 1995).

Proteins present in the *Chlamydomonas* soluble proteome (Supplemental Figure 5B) exhibited, in percentage, a subcellular distribution similar to that of Trx targets while a slight enrichment for chloroplastic proteins was observed with the total theoretical proteome encoded by the three genomes (Supplemental Figure 5C). These data suggest that the redox regulation mediated by Trx plays an important role in the different subcellular compartments and that the chloroplast is a metabolic hub for redox regulation even under mixotrophic growth conditions where photosynthesis is dispensable for *Chlamydomonas* cells. These results are consistent with the established importance of thioredoxins in multiple subcellular compartments, organs and developmental stages of photosynthetic organisms (for detailed reviews see: (Balsera et al., 2014; Delorme-Hinoux et al., 2016; Gelhaye et al., 2005; Hagglund et al., 2016; Kang and Wang, 2016; Konig et al., 2012; Lemaire et al., 2007; Meyer et al., 2012; Michelet et al., 2013; Nikkanen and Rintamaki, 2014; Rouhier et al., 2015; Sevilla et al., 2015; Traverso et al., 2013; Zaffagnini et al., 2013a)).

Functional annotation

Functional annotation according to the Kyoto Encyclopedia of Genes and Genomes for *Chlamydomonas* revealed that Trx-targeted enzymes participate in a wide variety of metabolic pathways and cellular processes (Figure 5). The major categories correspond to proteins involved in metabolic functions (30.3%), genetic information processing (22.1%), cellular processes (8.3%) stress response and redox homeostasis (4.2%), environmental information processing (4.2%) and for 30.9% their functions are still unknown.

The implication of Trxs in metabolic functions (essentially carbon metabolism, energy metabolism and lipid metabolism) is not surprising. Initially discovered in plant chloroplasts as light-dependent activators of Calvin-Benson cycle enzymes, the functional role of Trxs was rapidly extended to other metabolic pathways such as ATP synthesis, starch synthesis and degradation, lipid and amino-acid synthesis (Geigenberger and Fernie, 2014; Geigenberger et al., 2005; Lemaire et al., 2007; Meyer et al., 2009). Interestingly, some metabolic pathways appear highly controlled by Trxs. Among these, it is not surprising to find the Calvin-Benson cycle, for which all 11 enzymes were identified in the present study. Redox regulation of the Calvin-

Benson cycle by Trxs has been well established for five of the 11 enzymes and all enzymes were also detected as glutathionylated or nitrosylated in *Chlamydomonas* and other species (Michelet et al., 2013; Morisse et al., 2014b). This tight redox regulation may be required to allow a fine-tuning of the Calvin–Benson cycle for a transient redistribution of the energy (in the form of ATP) and reducing power (in the form of NADPH) within chloroplasts to cope with stress conditions (Lemaire et al., 2007; Michelet et al., 2005). Redox PTMs may also divert these abundant enzymes to moonlighting functions, *i.e.* new functions unrelated to their metabolic role in carbon metabolism as well established for glycolytic GAPDH (Zaffagnini et al., 2013a). The biosynthesis pathway for aromatic amino acids also appeared as a major Trx target, since almost all the enzymes of the pathway (14 out of 20) were identified in the present study, including 7 out of 8 enzymes of the shikimate pathway. In *Arabidopsis*, the first enzyme of this pathway, DAHP synthase, was suggested to be redox-regulated since its activity was shown to be Trx f-dependent *in vitro* (Entus et al., 2002). For all other enzymes, very little is known about the functional role of oxidative modifications on their regulations (Maeda and Dudareva, 2012). It should be noted that some of these enzymes were also detected as glutathionylated and nitrosylated in *Chlamydomonas* (Morisse et al., 2014b; Zaffagnini et al., 2012a). Interestingly, higher contents of tyrosine and phenylalanine were observed in illuminated poplar leaves compared to darkened ones (Noctor et al., 1998) or under biotic and abiotic oxidative stress conditions (Maeda and Dudareva, 2012; Sadeghnezhad et al., 2016). Altogether, these data suggest that redox regulation of the biosynthesis pathway for aromatic amino-acids should be carefully studied and our putative Trx-targeted sites could be a good starting point to evaluate the redox-dependency of these enzymes.

A total of 263 proteins are functionally annotated as participating in genetic information and processing. Among these, 41 proteins (16%) are involved in DNA-related functions: transcription, replication and repair, retrotransposon element. By contrast, 222 proteins (84%) participate in protein metabolism: translation, folding, sorting and degradation. This suggests that, in *Chlamydomonas*, Trx-dependent regulation of protein expression might occur mainly at the translational and post-translational levels. The 64 proteins involved in cell motility (5.4%) mainly correspond to flagellar proteins, a result consistent with the existence of two specific Trx isoforms in the flagella, absent in *Arabidopsis* (Patel-King et al., 1996). These Trxs were proposed to participate in a redox-regulatory pathway that affects outer dynein arm function and

influences flagellar motility (Harrison et al., 2002; Wakabayashi and King, 2006). Among transport and catabolism proteins (35 proteins, 2.9%), we identified several proteins involved in nuclear transport, mitotic spindle formation, and nuclear envelope assembly (reviewed in (Quimby and Dasso, 2003)), such as the RAN1 protein, also identified in our pioneer study (Lemaire et al., 2004). This underscores the importance of redox regulation of nuclear protein trafficking and the nuclear functions of Trxs that have recently emerged in both photosynthetic and non-photosynthetic eukaryotes (Delorme-Hinoux et al., 2016; Go et al., 2015). The identification of 47 proteins (4.0%) involved in signaling is consistent with the central role of oxidative post-translational modifications of cysteine residues in plant signal transduction (Waszczak et al., 2015). The two subunits of the circadian RNA-binding protein CHLAMY1 were also recovered among Trx targets, consistent with the suggested existence of a close coupling of redox processes and the circadian clock in *Chlamydomonas* (Filonova et al., 2013; Voytsekh et al., 2008). Similarly, 50 proteins (4.2%) are not surprisingly involved in stress responses and redox homeostasis. Most of these proteins correspond to established partners of Trx including peroxiredoxins, glutathione peroxidases or methionine sulfoxide reductases (Dietz, 2011; Fischer et al., 2009; Navrot et al., 2006; Tarrago et al., 2009). Finally, the most prominent category corresponds to the 368 proteins of unknown function that represent 30.9% of the Trx targets. This high proportion of proteins of unknown function illustrates our limited understanding of biological systems as recently illustrated by studies on the minimal genome by the Craig Venter Institute that assembled the smallest genome of any self-replicating organism that contains 473 genes including 149 genes (31.5%) of unknown function (Hutchison et al., 2016). The percentage in our dataset is however lower than in the theoretical proteome where more than 50% of the proteins have unknown functions, suggesting that Trx targets primarily map to major functional networks.

Protein and cysteine specificity in a complex network of redox modifications

Emerging data indicate that redox networks coordinate large numbers of redox elements involved in a multitude of pathways and cellular processes to allow resistance and adaptation to environmental challenges (Go et al., 2015). These networks involve multiple redox post-translational modifications, the best studied being oxidoreduction of disulfide bonds, glutathionylation, nitrosylation and sulfenylation (Couturier et al., 2013; Yang et al., 2016). This

cysteine proteome (Cys proteome) can be considered as an interface between the functional genome and the external environment (Go and Jones, 2014). It is a highly dynamic network of protein thiols with flexible reactivities (Paulsen and Carroll, 2013; Poole and Schoneich, 2015; Weerapana et al., 2010). Therefore, combinations of multiple redox PTMs act in concert throughout the cell and act as a network rather than as insulated elements. Gaining insights into the functioning of redox networks will require to unravel the determinants of the specificity of the diverse redox PTMs for specific proteins and cysteines.

Numerous proteins are known to be regulated by multiple redox post-translational modifications. A typical example is the *E. coli* transcription factor OxyR that can be nitrosylated, glutathionylated, sulfenylated and undergoes reversible disulfide bond formation under the control of Trx (Choi et al., 2001; Kim et al., 2002; Seth et al., 2012). The Calvin-Benson cycle constitutes an extreme example since all 11 enzymes of the pathway were identified as glutathionylated (Zaffagnini et al., 2012a), nitrosylated (Morisse et al., 2014b) and were also all found here regulated by Trx. However, such multiple redox targeting may not be necessarily true for other proteins. In other words, we can wonder whether multiple redox PTMs occur on a limited number of proteins containing reactive cysteines or if each modification targets a distinct redox network. The redox proteomic datasets we have generated in *Chlamydomonas* provide a unique opportunity to further explore the specificity of redox PTMs at the proteome scale. Indeed, besides the extended thioredoxome reported here, we have previously identified 492 nitrosylated proteins (Morisse et al., 2014b) and 225 glutathionylated proteins (Michelet et al., 2008; Zaffagnini et al., 2012a). These studies were performed on the same strain of *Chlamydomonas* using highly similar growth conditions, *i.e.* starting from a comparable proteome. Although some proteins are clearly targeted by multiple PTMs, the overlap appears limited since 68.8% appear regulated by a single type of modification (Figure 6). This result may appear surprising considering that *Chlamydomonas* Trxh1 was reported to catalyze denitrosylation (Berger et al., 2016) and deglutathionylation (Bedhomme et al., 2012). However, they are consistent with the fact that i) Trx targets were analyzed in conditions where nitrosylation and glutathionylation are limited or absent (Morisse et al., 2014b; Zaffagnini et al., 2012a), ii) Trx-dependent denitrosylation is considered to affect a limited fraction of nitrosylated proteins since a vast majority (more than 80%) of nitrosylated proteins are denitrosylated by GSH rather than Trx (Benhar et al., 2010; Paige et al., 2008; Romero and Bizzozero, 2009; Zaffagnini

et al., 2013b); (iii) the mechanisms of denitrosylation and deglutathionylation in photosynthetic organisms are considered to yield Trx-SNO and Trx-SSG rather than a mixed disulfide between the Trx and its target (Bedhomme et al., 2012; Kneeshaw et al., 2014; Zaffagnini et al., 2012c) suggesting that the Trx affinity column identifies proteins containing a Trx-reducible disulfide rather than nitrosylated or glutathionylated proteins. A high specificity, comparable to that found in *Chlamydomonas* (Figure 6), was already observed when comparing 193 sulfenylated proteins with previously identified targets of disulfide bond formation, nitrosylation and glutathionylation (Leonard et al., 2009). This limited overlap suggests that the different redox modifications are specific toward different subsets of the proteome.

Moreover, when a given protein is regulated by multiple PTMs, this does not necessarily imply that the same cysteine is targeted. The ability of a given cysteine to undergo a specific type of redox PTM is governed by multiple factors. Some cysteines undergo multiple redox modifications, as demonstrated for Cys178 of *Chlamydomonas* isocitrate lyase (Bedhomme et al., 2009) and Cys149 of *Arabidopsis* GAPC1 (Bedhomme et al., 2012) that both undergo nitrosylation and glutathionylation. In the case of other enzymes, such as the *Chlamydomonas* Calvin-Benson cycle enzymes fructose-1,6-bisphosphatase and transketolase (Michelet et al., 2013); *E. coli* OxyR (Choi et al., 2001; Kim et al., 2002; Seth et al., 2012) or human Trx (Benhar, 2015; Casagrande et al., 2002; Hashemy and Holmgren, 2008; Sengupta and Holmgren, 2012), the different modifications occur on distinct cysteine residues. The specificity primarily depends on the biochemical properties of the cysteine residue that are largely linked to its microenvironment within the folded protein which can notably influence the accessibility, the acidity and the nucleophilicity of the residue (Reddie and Carroll, 2008; Winterbourn and Hampton, 2008; Zaffagnini et al., 2012b). Our *Chlamydomonas* proteomic datasets allow exploring the site-specificity of the different redox PTMs at the proteome scale. These comparisons revealed a strikingly high specificity of each modification for specific cysteine residues. Indeed, considering all cysteines experimentally identified as redox-regulated, more than 75% undergo a single modification while less than 1% are regulated by nitrosylation, glutathionylation and disulfide bond formation (Figure 7A). To avoid any bias due to performances of mass spectrometers used in these three studies, a second comparison was performed by restricting this cysteine dataset to *Chlamydomonas* proteins undergoing at least two different redox PTMs and again, similar proportions were observed (Figure 7B). These results

indicate that the Cys proteome does not represent a small subset of highly reactive cysteines that are modified through indiscriminate interaction with the molecules they encounter but represent a complex organized network of redox PTMs. The different redox PTMs appear to control different subnetworks that are strongly interconnected. Strikingly, a recent analysis of 1319 mouse liver proteins and four cysteine modifications (nitrosylation, glutathionylation, sulfenylation and S-acylation) also revealed a very high specificity of redox PTMs with limited overlap (Gould et al., 2015). These results suggest that the different redox modifications are specific toward distinct interconnected protein networks.

Our proteomic datasets provide a unique and comprehensive map of this network, the Cys proteome, in the model alga *Chlamydomonas reinhardtii* (Figure 6). However, this view is rather static while the redox network probably involves spatial and temporal regulation of several redox post-translational modifications on hundreds of proteins in a highly dynamic manner. This complexity is likely providing the robustness and specificity required to allow a limited number of simple molecules such as reactive oxygen and nitrogen species (ROS/RNS) to play a signaling role. The redox network is likely a major component of signal integration and constitutes the molecular signature of the ROS/RNS crosstalk whose importance in cell signaling has been recognized (Foyer and Noctor, 2016; Frederickson Matika and Loake, 2014; Gross et al., 2013; Mock and Dietz, 2016; Sevilla et al., 2015). Understanding this complex network requires to determine the stoichiometry and dynamics of multiple redox PTMs under diverse physiological conditions or in different genetic backgrounds. This should be favored in the future by the development of sensitive and accurate redox quantitative mass spectrometry approaches combined with the development of new selective chemical probes (Yang et al., 2016). In addition, computational structural genomic approaches will be required to integrate the Cys proteome at the structural level in order to get insights into the molecular mechanisms and the structural determinants governing each type of redox modification.

Besides redox PTMs, the integration of the signal implicates a myriad of other molecules and processes acting at the translational, transcriptional and post-transcriptional levels (Mastrobuoni et al., 2012). In photosynthetic organisms, numerous redox PTMs are clearly linked to signaling pathways controlled by hormones (Feng et al., 2013; Kneeshaw et al., 2014; Terrile et al., 2012; Wang et al., 2015; Waszczak et al., 2015; Yu et al., 2012) or calcium (Trapet et al., 2015) and in

652 mammals, nitrosylation was reported to interfere with signaling processes mediated by
653 phosphorylation, ubiquitylation, sumoylation, acetylation or palmitoylation (Held and Gibson,
654 2012; Hess and Stamler, 2012). An intensive effort is therefore required to integrate redox
655 signaling networks with other signaling pathways and to analyze their impacts on the cellular
656 responses at multiple levels. This will require large-scale systems biology approaches and
657 development of innovative computational methods to analyze and explore the massive datasets
658 generated. Such approaches will certainly be crucial to unravel how environmental challenges are
659 encoded into a biochemical signal than can be exploited to trigger the appropriate responses in
660 terms of localization, duration and intensity, at the genome, transcriptome, proteome and
661 metabolome level to allow adaptation and survival.

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AUTHOR CONTRIBUTIONS

S.D.L. and C.H.M. designed the research. M.E.P.P., A.Mauries, M.H. and C.H.M. performed research. A.Maes and N.J.T. performed bioinformatic analyses. M.E.P.P., A.Mauries, S.D.L. and C.H.M. analyzed data. S.D.L and C.H.M. wrote the article with input from the other authors.

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FIGURE LEGENDS

Figure 1. Schematic overview of the experimental workflow used to identify both thioredoxin targets and their redox-regulated cysteines. The experimental workflow proceeds through two procedures. (A) Monocysteinic Trx column approach: identification of Trx-targets using the affinity chromatography approach with the cytosolic CrTrxh1-C39S mutant (B) Reductome approach: identification of the redox-regulated cysteines after *in vitro* reduction by the cytosolic CrTrxh1 system and labeling of reduced cysteines with cleavable ICAT.

Figure 2. Distribution of thioredoxin targets. (A) Venn diagram showing the distribution of proteins identified by the monocysteinic Trx column approach between two independent biological samples. (B) Venn diagram showing the distribution of proteins identified by the two proteomic approaches used in this study.

Figure 3. Redox-regulated cysteines in Chlamydomonas enzymes. Eight redox-regulated proteins are schematically and linearly represented: ATP synthase γ -subunit (Cre06.g259900); PGK (chloroplastic phosphoglycerate kinase, Cre22.g763250); NADP-MDH (chloroplastic NADP-malate dehydrogenase, Cre09.g410700); CP12 (chloroplastic protein 12, Cre08.g380250); PRK (chloroplastic phosphoribulokinase, Cre12.g554800); PRX2 (cytosolic 2-cys peroxiredoxin; Cre02.g114600); PRX4 (Type-II peroxiredoxin; Cre02.g080900); Rubisco LSU (chloroplastic ribulose biphosphate carboxylase/oxygenase; rbcL). The numbering corresponds to the full-length sequence. Lines between cysteines indicate confirmed (plain) or suggested (dashed) disulfide bonds in Chlamydomonas enzymes. Cysteines identified as nitrosylated (Morisse et al., 2014b) and glutathionylated (Zaffagnini et al., 2012a) by proteomic approaches in Chlamydomonas are labeled with NO (yellow) and SG (green), respectively.

Figure 4. Distribution of Chlamydomonas proteins according to the number of cysteines. Distributions of Chlamydomonas Trx targets identified in this work (black bars) and proteins in the total proteome derived from the genome sequences (white bars) according to the number of cysteines present in their sequence.

Figure 5. Functional annotation of the Chlamydomonas thioredoxome. Proteins were classified according to the Kyoto Encyclopedia of Genes and Genomes for Chlamydomonas and using a limited number of Gene Ontology annotation levels. Nodes are labeled either with the Gene name or with the UniprotKB identifier. Proteins are grouped according to their role in metabolic pathways or cellular processes with the following color code: metabolism, green;

genetic information processing, brown and tawny; cellular processes, red and light red; environmental information processing, purple; stress response/redox homeostasis, light blue; unknown functions, grey.

Figure 6. Global network of redox post-translational modifications in *Chlamydomonas* cells. *Chlamydomonas* proteins are represented as nodes in a network depending of proteins targeted by thioredoxins (S-S) (this study and (Lemaire et al., 2004)), S-nitrosylation (S-NO) (Morisse et al., 2014b) or S-glutathionylation (S-SG) (Michelet et al., 2008; Zaffagnini et al., 2012a) using an in-house software and D3.js.

Figure 7. Specificity of redox post-translational modifications in *Chlamydomonas*. (A) Venn diagram showing the distribution of cysteines experimentally identified as reduced by thioredoxins (Trx; this study) or modified by S-nitrosylation (S-NO; (Morisse et al., 2014b)) or by S-glutathionylation (S-SG; (Zaffagnini et al., 2012a)). (B) Venn diagram showing the distribution of cysteines experimentally identified as redox-modified after dataset restriction to *Chlamydomonas* proteins undergoing at least two of these three redox modifications (Trx reduction, S-nitrosylation, S-glutathionylation).

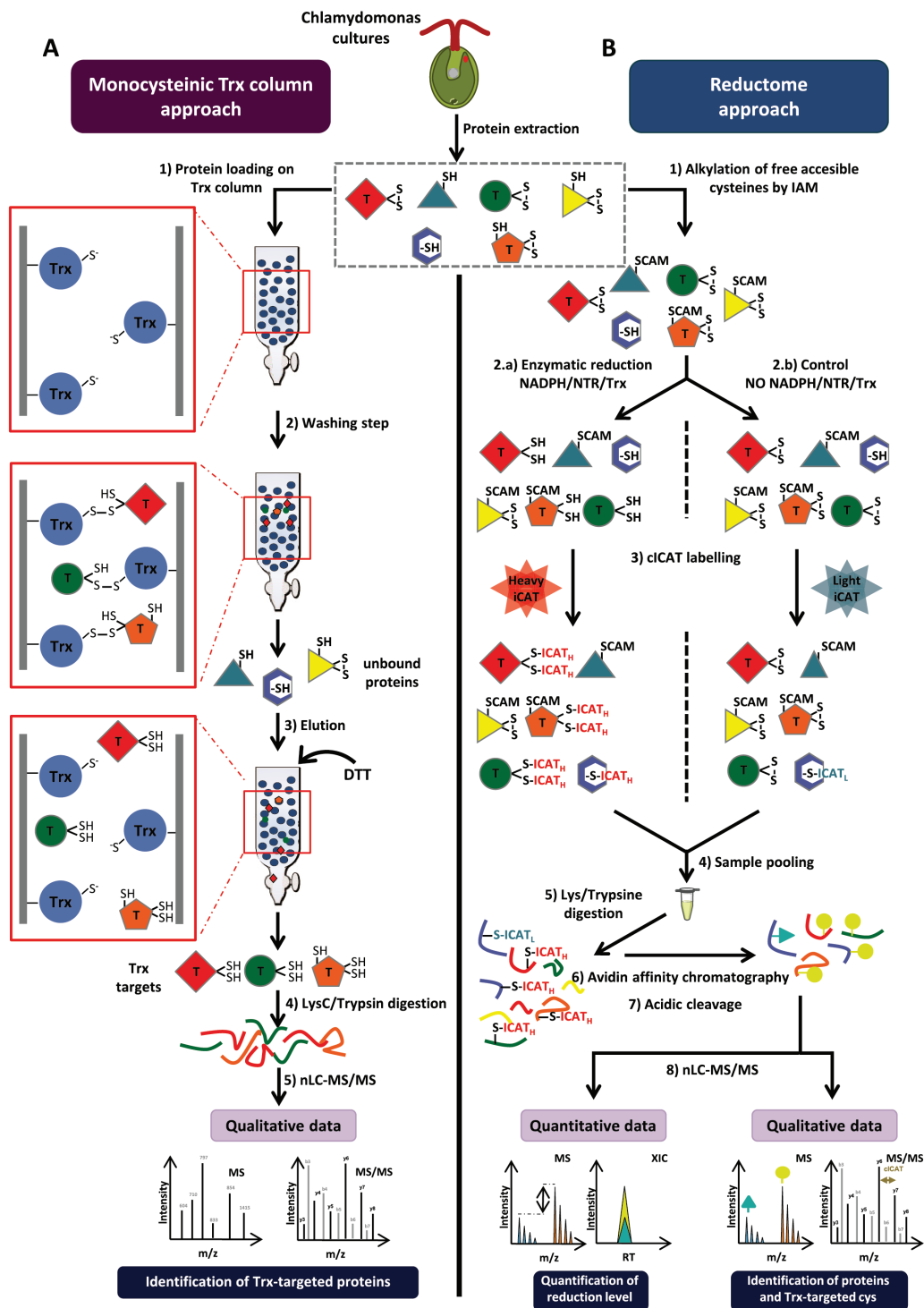


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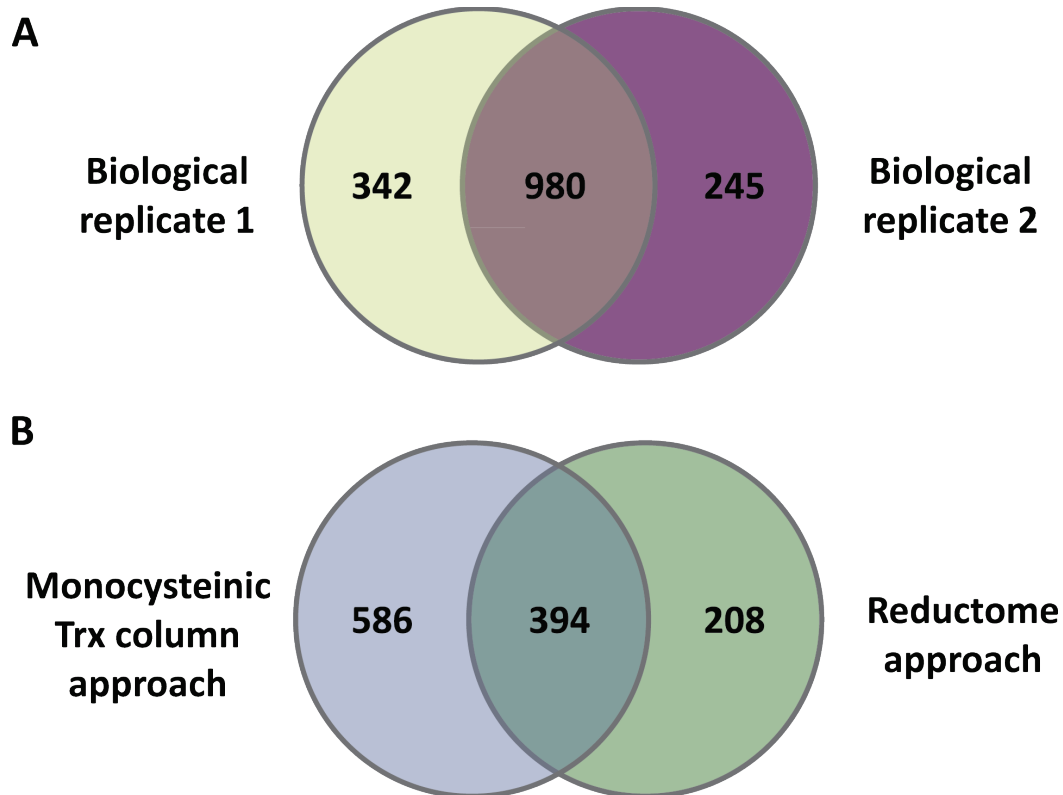


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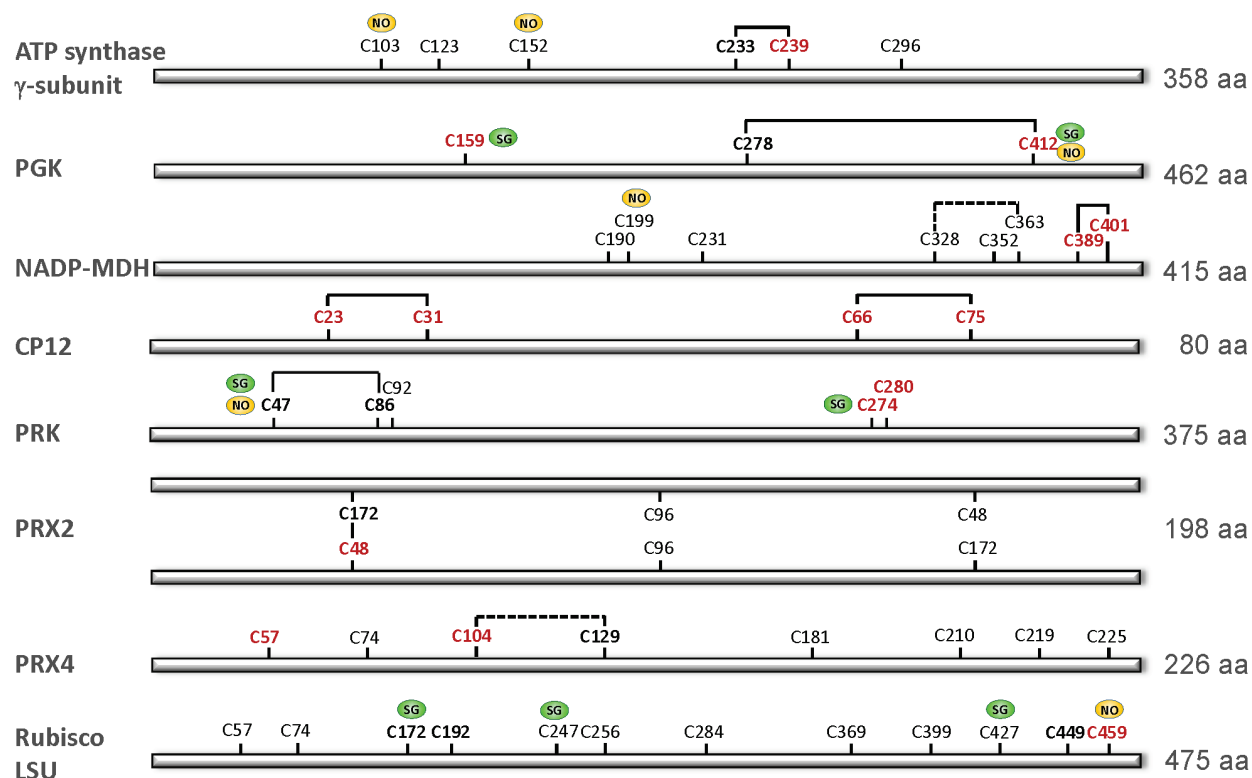


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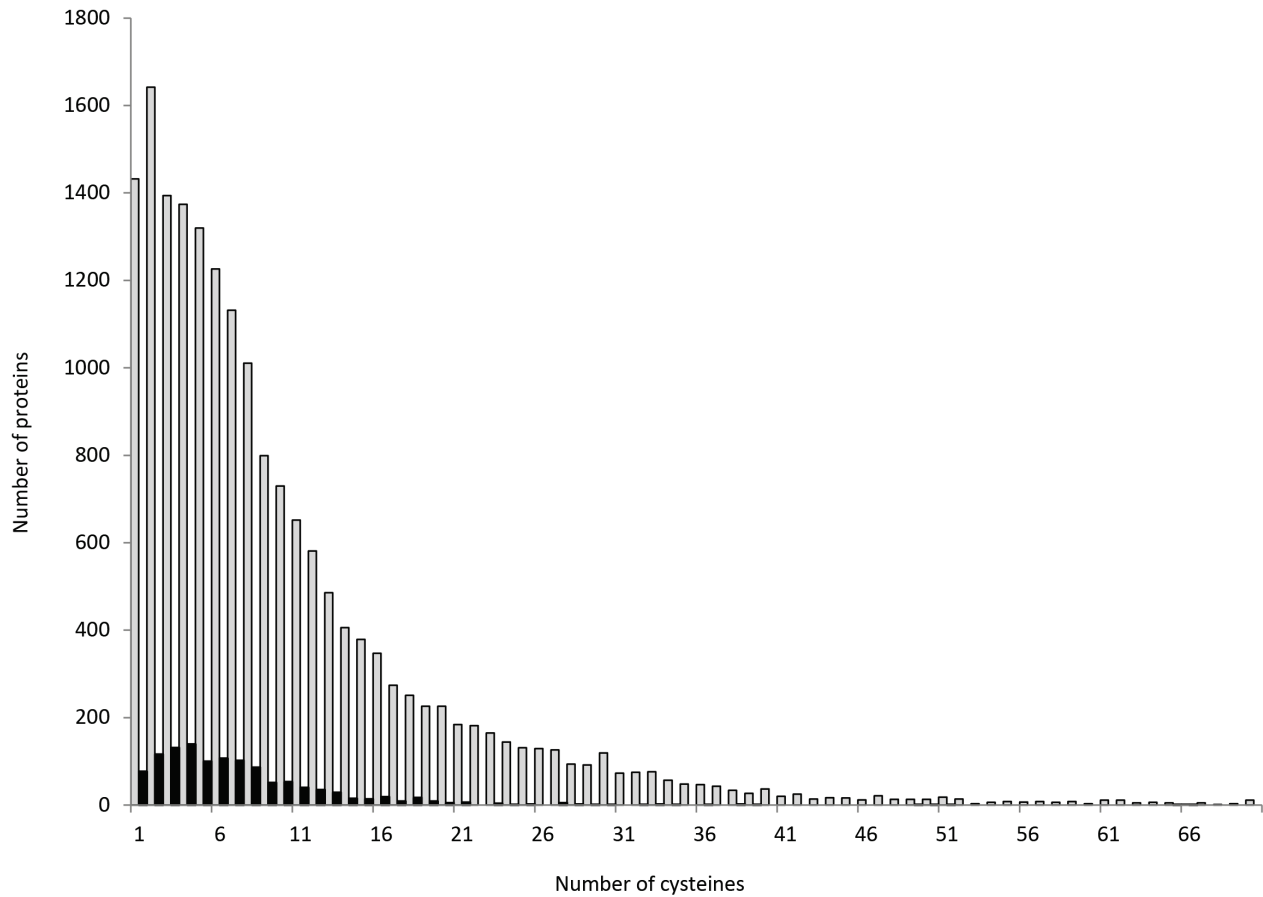


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METABOLISM

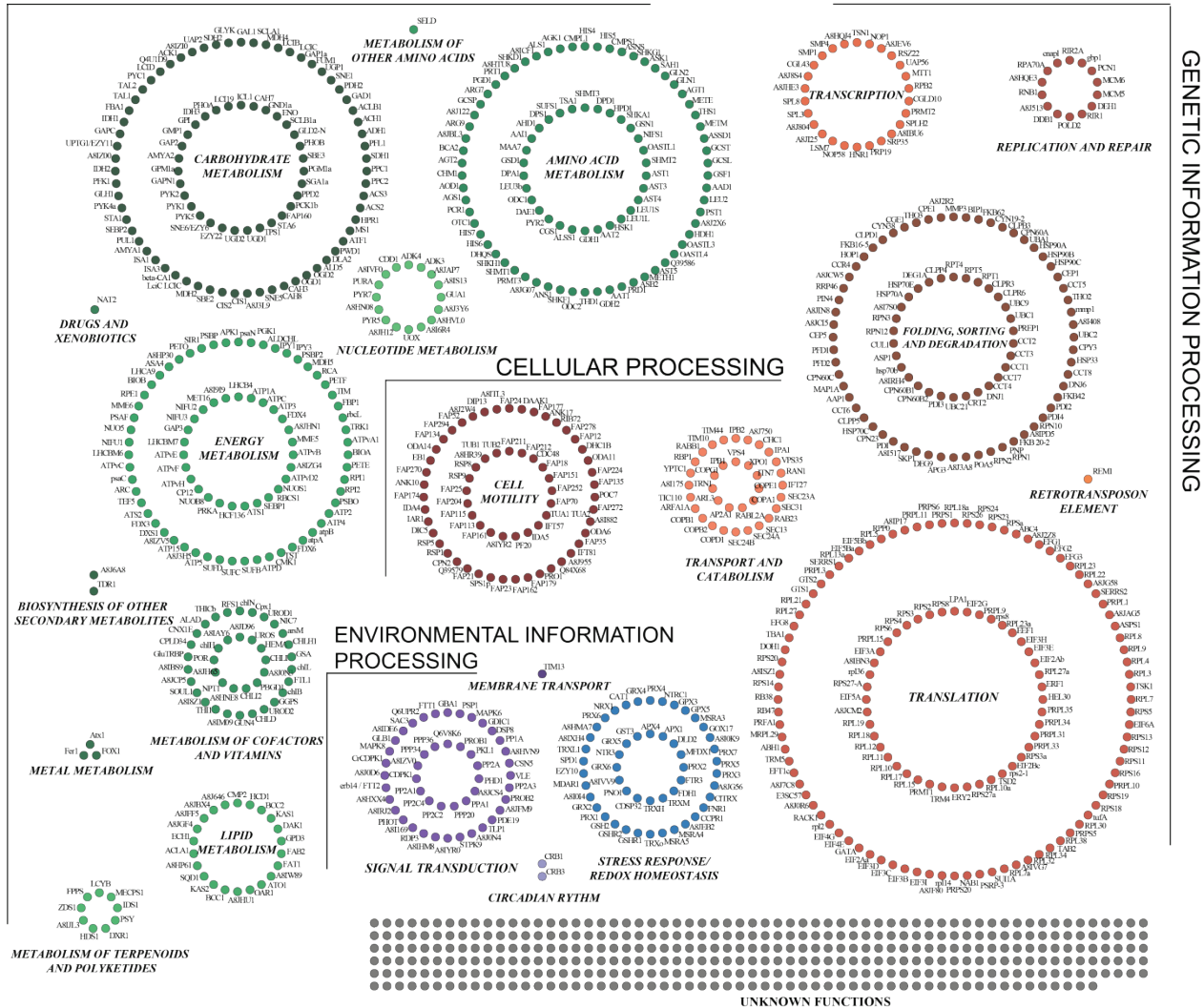


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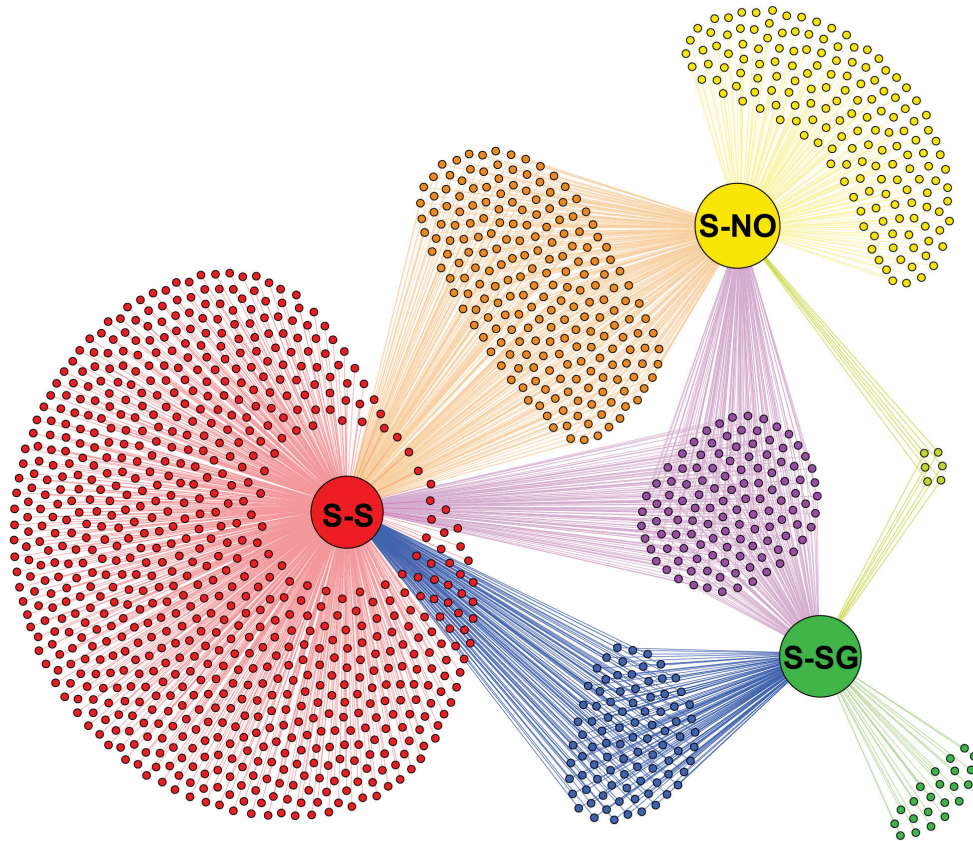


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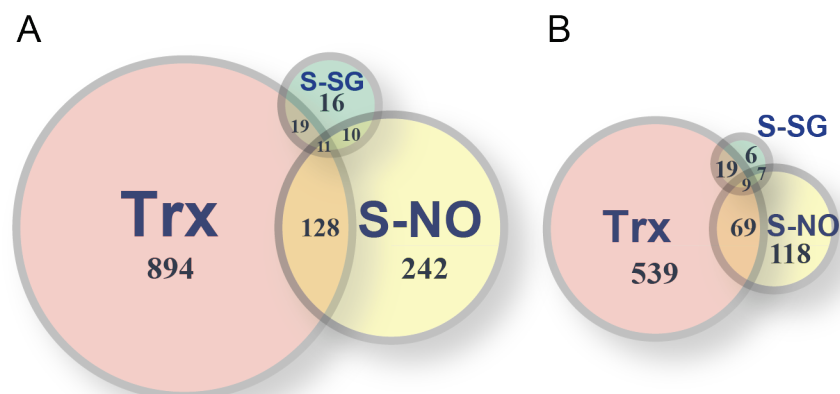


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