Short title: Chronic iron limitation induces resistance to ROS

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Chronic iron limitation confers transient resistance to oxidative stress in marine diatoms

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One sentence summary:
The physiological and proteomic profiles induced during iron limitation indicate a trade-off between growth and susceptibility to oxidative stress in a marine diatom.

Author Contributions
S.G.v.C, S.R., A.V. designed all the experiments, analyzed the data and wrote the article with contributions of all the authors. S.G.v.C, S.R., conducted all experimentation and the bioinformatics work and Y.L. performed and analyzed the shotgun proteomics experiment.

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Abstract
Diatoms are single celled photosynthetic bloom-forming algae that are responsible for at least 20% of global primary production. Nevertheless, more than 30% of the oceans are considered “ocean deserts” due to iron limitation. We used the diatom *Phaeodactylum tricornutum* as a model system to explore diatom’s response to iron limitation and its interplay with susceptibility to oxidative stress. By analyzing physiological parameters and proteome profiling, we defined two distinct phases: short-term (< 3 days, phase I) and chronic (> 5 days, phase II) iron limitation. While at phase I no changes in physiological parameters were observed, molecular markers for iron starvation, such as ISIP and flavodoxin, were highly upregulated. At phase II, down regulation of numerous iron-containing proteins was detected in parallel to reduction in growth rate, chlorophyll content, photosynthetic activity, respiration rate and antioxidant capacity. Intriguingly, while application of oxidative stress to phase I and II iron limited cells similarly oxidized the GSH pool, phase II iron limitation exhibited transient resistance to oxidative stress, despite the down regulation of many antioxidant proteins. By comparing proteomic profiles of *P. tricornutum* under iron limitation and metatranscriptomic data of an iron enrichment experiment conducted in the Pacific Ocean, we propose that iron limited cells in the natural environment resemble the phase II metabolic state. These results provide insights into the trade-off between maximal growth rate and susceptibility to oxidative stress as a possible key determinant in the response of diatoms to iron quota in the marine environment.

Introduction
Phytoplankton are single celled photosynthetic microorganisms that live in the upper, illuminated layer of the oceans. Like plants in terrestrial ecosystems, phytoplankton are the basis of marine food webs. High turnover, estimated to be weekly based, due to high growth and death rates mediated mainly by availability of nutrients, grazing, and viral infections, make phytoplankton very responsive to climate change (Falkowski et al., 1998; Bidle and Falkowski, 2004; Chavez et al., 2011; Behrenfeld and Boss, 2014; Bidle,
Diatoms dominate phytoplankton communities and are responsible for at least 20% of global primary production (Nelson and Brzezinski, 1997; Armbrust, 2009; Arrigo et al., 2012). They are abundant in well-mixed coastal, upwelling regions, and near the sea-ice edge, where they form massive blooms (Arrigo et al., 2012). Diatoms are thus central to the biogeochemical cycling of important nutrients such as carbon, nitrogen, iron and silica (Field et al., 1998; Morel and Price, 2003).

Algal growth is highly dependent on iron (Fe) bioavailability (Behrenfeld, 1999), due to its involvement in fundamental enzymatic and metabolic reactions such as photosynthesis (photosystem I, ferredoxin, cytochrome b6f), TCA cycle (aconitase, fumarase) and nitrate assimilation (nitrate and nitrite reductases) (Milligan and Harrison, 2000; Behrenfeld and Milligan, 2013). Iron-limited diatoms have reduced chlorophyll content, photosynthetic efficiency (Greene et al., 1991; Marchetti and Harrison, 2007) and nitrate assimilation rate (Milligan and Harrison, 2000), all of which lead to limited growth rate. More than a decade of mesoscale open ocean experiments revealed that iron is limiting primary productivity in about 30% of the ocean ecosystems (Behrenfeld and Milligan, 2013; Moore et al., 2013). These observations support “The iron hypothesis” which claims that iron is the limiting factor for phytoplankton blooms in high nitrate low chlorophyll ocean areas (Behrenfeld et al., 1996; Behrenfeld et al., 2006), therefore, artificial addition of iron to these areas will induce blooms which would potentially activate the biological pump and reduce atmospheric CO2 (Martin, 1990). Natural and artificial iron enrichments lead to rapid increase in phytoplankton biomass, where addition of dissolved iron to a body of water with less than 0.05 nM iron led to diatom-dominated blooms and enhanced carbon export to the deep sea (Boyd et al., 2007; Marchetti et al., 2012; Smetacek et al., 2012; Xiu et al., 2014).

Diatoms developed diverse strategies to cope with low and fluctuating iron quota, such as high-affinity iron uptake systems, iron storage capacity, substitution of iron-containing proteins with non-ferrous functional equivalents, and mechanisms to mitigate the risk of damage from reactive oxygen species (ROS) produced in the presence of this redox-active metal (Allen et al., 2008; Lommer et al., 2012; Marchetti et al., 2012; Morrissey and Bowler, 2012). Iron uptake systems in diatoms include a high-affinity reductive
system, containing ferric reductase that dissociates Fe$^{3+}$ from organic ligands, a multicopper oxidase that oxidizes the released Fe$^{2+}$ to Fe$^{3+}$, and an iron permease that receives Fe$^{3+}$ for translocation across the cell membrane (Maldonado and Price, 2001; Maldonado et al., 2006; Kustka et al., 2007). Recently, a copper-independent, non-reductive mechanism was reported to directly bind and concentrate Fe$^{3+}$ by Iron Starvation Induced Protein 2a (ISIP2a) (Morrissey et al., 2014). After acquiring the iron, intracellular concentrations must be tightly regulated to avoid oxidative damage. Ferritin, an iron-storage protein used by plants, animals and microorganisms to concentrate and store iron (Theil, 2004), is present in all diatom classes but is probably used to buffer iron concentrations and not for long-term storage (Marchetti et al., 2009; Groussman et al., 2015; Pfaffen et al., 2015). During iron limitation diatoms can substitute the common iron-sulfur redox protein ferredoxin with flavodoxin, a functionally equivalent, non-iron-containing protein (La Roche et al., 1993; McKay et al., 1997; Erdner et al., 1999; McKay et al., 1999). Induction of carotenoids biosynthesis, tocopherols, dehydroascorbate and alternative oxidase may all compensate for down-regulation of iron containing antioxidants such as heme peroxidase, while proteorhodopsins may partially compensate for impaired iron-rich photosystem I (Allen et al., 2008; Marchetti et al., 2015).

Despite the advantage of iron-based metabolism, iron might be cytotoxic for living cells as the production of harmful ROS such as hydroxyl radical can be triggered by free iron (Fe$^{2+}$) in the Fenton/Haber-Weiss reaction (Kolthoff and Medalia, 1949). Therefore, iron uptake sequestration is tightly controlled to avoid the accumulation of potentially dangerous free iron in the cell. In contrary, iron limitation can lead to oxidative stress as a result of inefficient activity of the photosynthetic electron transport chain that use iron as an essential co-factor, and reduction in iron containing antioxidants (Niyogi, 1999). In the centric diatom *Thalassiosira pseudonana* iron limitation led to oxidative stress and further induction of hallmarks of programmed cell death (PCD) (Thamatrakoln et al., 2012; Luo et al., 2014). In plants, long-term iron limitation can lead to H$_2$O$_2$ accumulation, indicating iron deficiency-induced oxidative stress (Le et al., 2016). Due to their toxic effects, ROS levels are tightly controlled by the cellular antioxidant network (Mittler et al., 2004). Iron is an important co-factor in numerous ROS-degrading enzymes.
suggesting an important interplay between ROS and iron metabolism. Iron based metabolism can make the cells more susceptible to oxidative stress as high ROS level can damage Fe-S-containing proteins, impairing their biochemical activity and leading to the release of Fe^{2+} which can trigger the Fenton reaction. The aim of the present study was to examine the effect of iron limitation on the response to oxidative stress in the model diatom *P. tricornutum*. We measured physiological parameters and conducted proteome profiling during different phases of iron limitation in which the susceptibility to oxidative stress changed markedly.
**Results**

**Physiological response to iron starvation.**

In order to investigate the effect of iron limitation on cell physiology and growth, we monitored *P. tricornutum* growth under iron limitation in the presence of the iron chelator desferrioxamine B (DFB) as described in the methods (Hutchins et al., 1999). Iron starvation reduced growth rate and chlorophyll autofluorescence per cell (Fig. 1A and B), together with decrease in PSII photosynthetic efficiency, measured by variable fluorescence (Fv/Fm) and oxygen production rate (Supplemental Fig. S1 A and B). Respiration rates as measured by oxygen consumption in the dark were stable during the early stages and significant reduction was found only on day 7 of iron limitation (Supplemental Fig. S1 C). In order to examine how iron limitation affects antioxidant capacity, we monitored the cellular GSH pool of the same cultures during iron limitation by the fluorescent stain Monochlorobimane which stains reduced thiols of small molecules, and detected a decrease in the GSH pool (Fig. 1C). At day 7 of iron limitation, growth rates, chlorophyll autofluorescence per cell, GSH content, Fv/Fm, oxygen production and respiration were 38%, 49%, 43%, 18%, 25%, and 3% of replete cultures respectively (Fig. 1 A - C, Supplemental Fig. S1 A - C). Upon resupply of 1850 nM (as in replete) or 18.5 nM Fe to iron-limited cultures at day 7, growth rates recovered within 4 days (Fig. 1A). Ten days following resupply of 1850 nM or 18.5 nM Fe (day 17), GSH levels were 120% and 72% of cells under Fe replete conditions respectively, while under iron depletion the GSH pool was 51% of replete (Supplemental Fig. S2). We further used *P. tricornutum* cells expressing the redox-sensitive GFP (roGFP) targeted to the chloroplast, mitochondria or nucleus (Rosenwasser et al., 2014; Graff van Creveld et al., 2015) to examine the effect of iron limitation on the GSH redox potential. H$_2$O$_2$-dependent cell death was similar to WT cell death in the roGFP transformant lines (Supplemental Fig. S3). No remarkable changes in roGFP oxidation were detected in iron-limited cultures compared to control cells (<10% oxidation, p-values between replete and iron limitation >0.09, Fig. 1D). A decrease in the GSH pool without oxidation of the roGFP probe suggests that the glutathione pool was smaller in size, but was retained in a highly reduced state during 7 days of iron limitation.
In order to examine how the observed decrease in the GSH pool may remodel the susceptibility to oxidative stress during iron limitation, we treated the cells with 30 or 150 µM H$_2$O$_2$ and measured cell death after 24 hours by using Sytox Green DNA-binding fluorescent stain, which is used to assess membrane integrity and phosphatidylserine externalization (Annexin V) both considered as a good proxy for induction of cell death (Graff van Creveld et al., 2015). No cell death was detected in response to 30 µM H$_2$O$_2$ in replete or in iron limited cells, while application of 150 µM H$_2$O$_2$ induced both cell death as measured by fluorescence markers for membrane permeability, and phosphatidylserine externalization in replete cultures (Fig. 2A and B). Quantification of phosphatidylserine externalization at day 6 of iron limitation, exhibited 47% positive...
Figure 2. Chronic iron limitation confers resistance to oxidative stress in *P. tricornutum*.

A, Cell death assessed by Sytox staining, measured 24 hours after treatment with 30 or 150 µM H$_2$O$_2$. The time (days) in the X axis represents the duration of iron limitation, similar to the time depicted in Fig. 1. B, Phosphatidylserine externalization measured by Annexin V stain at day 6 of iron limitation. Cells treated with 0 and 150 µM H$_2$O$_2$ were stained with Annexin V at 5 and 8 hours post treatment. C, Cell death assessed by Sytox positive cells, measured 24 hours after treatment with 150 µM H$_2$O$_2$. Application of H$_2$O$_2$ was done 0.5, 1.5, 16, 24 and 168 hours following iron resupply at day 7 of iron limitation. Flow cytometry analysis is based on fluorescent measurements of at least 5000 cells per sample. Error bars represent standard error of biological triplicates.

cells at 8 hours post 150 µM H$_2$O$_2$ treatment (Fig. 2B), at this time point there were less than 12% Sytox positive cells (Supplemental Fig. S4). Intriguingly, whereas the iron-limited cultures showed similar sensitivity to oxidative stress during the first four days of limitation, they exhibited a profound resistance to H$_2$O$_2$ from day 5 of iron limitation (Fig. 2A). This resistance to H$_2$O$_2$ treatment was transient, with 4% Sytox positive cells at 24 hours post treatment, but 92% Sytox positive cells within 48 hours, as in the replete cells (Supplemental Fig. S5A). The same treatment at day 6 of iron limitation led to 2% Sytox positive cells 24 hours post treatment, preceded to 37% and 97% Sytox positive cells at 48 and 102 hours respectively (Supplemental Fig. S5B). The observed delay in cell death suggests that the chronically iron-starved cells were in a metabolic and physiological state that enabled them to survive oxidative stress.
Accordingly, we defined two physiological phases in iron limited cultures, “phase I” in which $\text{H}_2\text{O}_2$ sensitivity was similar to iron-replete cells (days 1-3 of iron limitation) and “phase II” in which chronically iron-limited cells (> 5 days of iron limitation) acquired resistance to $\text{H}_2\text{O}_2$. Oxidation of organelle-specific roGFP in response to 150 $\mu$M $\text{H}_2\text{O}_2$ was comparable under replete Fe conditions and during phase I (day 1) and phase II (day 7) of iron limitation (Fig. 1D), indicating that $\text{H}_2\text{O}_2$ permeability and GSH oxidation in response to $\text{H}_2\text{O}_2$ in the specific organelles was not modified under iron limitation. In order to examine the reversibility and dependence of $\text{H}_2\text{O}_2$-susceptibility on iron availability, iron-limited cells (at day 7) were resupplied with 18.5 nM (1% of replete) or 1850 nM (100% of replete) FeCl$_3$ (Fig. 2C). Growth was resumed after 4 days of resupply, either of 18.5 or 1850 nM (Fig. 1A). We subsequently examined susceptibility to oxidative stress by treating the cells with $\text{H}_2\text{O}_2$ (150 $\mu$M) at 0.5, 1.5, 16, 24 and 168 hours after iron resupply and cell death was monitored 24 hours post treatment. Resupply of 1850 nM Fe reversed the $\text{H}_2\text{O}_2$ susceptibility within 24 hours while following resupply of 18.5 nM Fe, cells remained $\text{H}_2\text{O}_2$ resistant for 168 hours (Fig. 2C). In summary, resistance to oxidative stress in diatom cells was closely associated with low iron quota and availability.

**Decoupling between early oxidation and subsequent induction of cell death under chronic iron limitation**

We previously demonstrated that early oxidation of mitochondrial roGFP, which represent the mitochondrial GSH redox potential, is an early perturbation event in the subsequent induction of a cell death biochemical cascade (Graff van Creveld et al., 2015). In line with these findings, we detected threshold levels in oxidation in the mitochondria (70%) or nucleus (40%) within 3 hours of $\text{H}_2\text{O}_2$ treatment under iron replete conditions. These thresholds were indicative of subsequent induction of PCD-like process 24 hours post-treatment in replete cells (Fig. 3A). Iron limited cells in phase I (days 1-3) displayed a similar trend (Fig. 3B). In contrast, no correlation between mitochondrial or nuclear roGFP oxidation and subsequent induction of cell death was detected in phase II (day 5 onward) iron-limited cells (Fig. 3C, Supplemental Table S1). Cells that were oxidized above threshold, did not undergo cell death within 24 hours, pointing towards a decoupling between early roGFP oxidation and subsequent induction of cell death. This
indicates that cells under chronic iron limitation (phase II) exhibit a distinct metabolic state, characterized by transient resistance to H$_2$O$_2$.

**Global proteomic profiling and functional categorization of differentially expressed proteins**

In order to better characterize the metabolic states of replete (day 3 of replete), phase I (day 3 of iron limitation) and phase II (day 5 of iron limitation) cells, we conducted a comparative liquid chromatography–mass spectrometry (LC-MS) based proteomic experiment which provide protein expression profiles of cells in different iron limitation phases. Principle component analysis (PCA) clearly shows that phase II (resistant to oxidative stress) is clustered distinctively from phase I and replete cultures (Fig. 4A).
of 2191 identified proteins, ~10% were upregulated (fold change > 1.5, p value <0.05) in phase I (day 3 of iron limitation, compared to the replete culture) or in phase II (day 5 compared to day 3 of iron limitation), while 2% and 30% of the identified proteins were down regulated (fold change < -1.5, p value <0.05) in phase I and phase II, respectively (Fig. 4B).

Functional analysis of enriched biological terms was performed for each treatment using Gene Ontology (GO) analysis. In phase I (day 3 of iron limitation compared to replete culture), GO terms related to cell redox homeostasis, oxidoreductase activity, and NADP metabolic process (GO: 0045454, 0016668, 0006739) were significantly enriched (fold enrichments: 1.76, 3.59 and 2.61 respectively) in up-regulated proteins while Fe-S...
binding and protein phosphorylation (GO: 0051537, 0006468) were enriched (fold enrichments: 6.69, 3.79) in down-regulated proteins (Fig, 4C, and Supplemental Table S2). In phase II (day 5 compared to day 3 of iron limitation), proteins related to meiosis and vesicle trafficking (GO: 0051321, 0030136) were significantly enriched in up-regulated proteins. While photosynthesis, photosystem I and Fe-S binding proteins (GO: 0015979, 0009522, 0051536) were significantly enriched in down-regulated proteins (Fig. 4C, and Supplemental Table S2), light harvesting proteins (GO: 0009765), such as fucoxanthin chlorophyll binding protein, (protein ID 219110471, 219129382) were upregulated (Supplemental Table S4) which may mediate tolerance to photo-oxidative stress under iron limited conditions (Lommer et al., 2012).

The iron starvation induced proteins (ISIP’s) and the canonical iron starvation protein flavodoxin, which substitutes the iron-containing ferredoxin, were induced during iron limitation (Fig. 5A). These proteins were highly upregulated in phase I and further induced in phase II, corroborating our experimental setup, indicating existence of iron-limitation already in phase I. Ferredoxins, heme binding, Fe-binding and Fe-S binding proteins (defined by GO terms) were down-regulated mainly in phase II (Fig. 5A, and Supplemental Table S3).

ROS metabolism under iron limitation
Since we could phenotypically categorize the different phases during iron-limited conditions based on susceptibility to oxidative stress, we were motivated to explore how proteins related to ROS metabolism were modulated during iron limitation in the two phases. Antioxidant proteins were generally un-changed or slightly up-regulated in phase I and down-regulated in phase II (Fig. 5A). While peroxiredoxin (Prx), Cu/Zn superoxide dismutase (Cu/Zn-SOD) and thioredoxin (Trx) were slightly upregulated and 17 antioxidants were unchanged in phase I, 12 antioxidant enzymes were significantly down-regulated in phase II, with the exception of one glutathione-S-transferase (GST), and 2 Trxs (Fig. 5A and Supplemental Table S3). Interestingly, bacterial-like Ni-SOD was upregulated in phase II (Fig. 5A and Supplemental Table S3). The general reduction
in antioxidant capacity during phase II (Fig. 5A), and the major decrease in GSH content (Fig. 1C) emphasizes the paradox in the observed resistance to oxidative stress, displayed by the cells in this phase of iron limitation.

To conclude, we detected two phases of response to iron limitation. In the first phase we observed induction of cellular strategies to cope with iron deficiency (as ISIP’s and flavodoxin) with no decrease in growth rate or photosynthetic efficiency, while in chronic iron limitation (phase II) we observed a decrease in growth rate, chlorophyll content, photosynthetic efficiency, glutathione content and a large proteomic shift (about 40% of the proteome) that were coupled with resistance to oxidative stress.
Iron limited cells in the ocean resemble phase II metabolic state

Based on various physiological parameters, response to oxidative stress and proteome profiling, we defined two distinct cellular states representing early (phase I) and late (phase II) cellular responses to iron limitation. We were encouraged to investigate whether any of the phases is reflected in natural diatoms population in the ocean. We compared the *P. tricornutum* proteome with metatranscriptomic data from Ocean Station Papa in the Pacific Ocean (50°N, 145°W), where iron enrichment (addition of 4 nM to oceanic water with less than 0.05 nM Fe) led to induction of phytoplankton growth, dominated by diatoms of the genera *Pseudo-nitzschia* and *Fragilariopsis* (Marchetti et al., 2012). A recent study described a coordinated transcriptome and proteome patterns in diatoms response to phosphorus limitation (Dyhrman et al., 2012). Nevertheless, since quantitative proteomics and transcriptomics data are not necessarily comparable (Bertrand et al., 2012), we examined the trend similarities between the proteome and the metatranscriptome studies by comparing between lists of differentially expressed proteins and transcripts (up or down regulation). This qualitative comparative approach can provides a straightforward way to determine the similarity in expression profiles between datasets retrieved using different methodologies. Diatom-specific gene expression profiles were retrieved from (Marchetti et al., 2012), and compared to the *P. tricornutum* proteome using best hit blast. A total of 1190 proteins were detected in both datasets. For example, Fe-containing ferredoxins which were down regulated in the iron limited proteome were induced in iron resupply metatranscriptome as expected, however Fe-free flavodoxin, which is known to replace ferredoxin under low iron conditions was further induced upon iron resupply (Fig. 5A). Interestingly, 98 hours upon iron resupply, diatoms relay on Fe-free flavodoxin and plastocyanin and not on Fe-containing ferredoxin and cytochrome C6, despite the existence of the genes encoding for Fe-containing enzymes in the genome of the associated diatoms species (Marchetti et al., 2012). This data raises fundamental questions about diatom’s response to variation in iron quota and suggests possible benefits of the iron-free metabolic state. Interestingly ISIP2a, ISIP3 and flavodoxin were also induced upon iron enrichment (Fig. 5A). A general induction of antioxidant proteins was detected as well, for example, ascorbate peroxidase, glutathione peroxidase, glutathione reductase, GST, Prx, and SOD were induced in the environmental
iron enrichment (Fig. 5A), indicating the importance of antioxidants enzymes in iron-rich environment.

Venn diagrams showing iron-limitation induced proteins (defined as proteins with fold change above 1.5 in phase I or phase II, or genes with fold change below -1.5 in Papa Station), and iron-limitation down-regulated proteins (defined as proteins with fold change below -1.5 in phase I or phase II, or genes with fold change above 1.5 in Papa Station) suggests that iron limitation in the ocean is more similar to phase II than to phase I in culture experiments (Supplemental Fig. S6). Out of 1192 proteins down-regulated in either phase I, II or Papa Station, 8 were common between phases I and II, 11 between phase I and Papa Station and 284 between phase II and Papa Station (Supplemental Fig. S6). For example, ascorbate peroxidase, glutathione peroxidase, GST and Cu/Zn-SOD are down-regulated in response to iron limitation in phase II and in Papa Station, but not in phase I (Fig. 5A). Gene to gene comparison of 116 genes that were significantly altered (p-value <0.05, fold change either above 1.5 or below -1.5) in phase I and phase II and had homologous genes in Papa Station metatranscriptome, revealed that in phase I 8% of the genes respond in the same trend as in Papa Station (either induced in phase I and down-regulated in Papa Station or vice versa), while 62% of the genes exhibited the same trend in phase II and in Papa Station (Fig. 5B). Out of 51 iron-binding encoded proteins identified in the *P. tricornutum* proteome, 31 were identified in the metatranscriptome as well, 5 were down-regulated, and 26 were upregulated upon iron resupply, indicating that chronic iron limitation conditions led to major remodeling of diatom’s metabolism under low iron quota that is similar to phase II in our lab experiments.
Discussion

The increase in abundance of iron containing proteins during evolution, despite the decrease in iron bioavailability due to ocean oxygenation, points to the great advantage of iron-based metabolism in promoting cellular growth (Lawrence and Alm, 2011). Iron is an ideal transition metal for redox reactions in biological systems during photosynthesis, respiration, and diverse enzymatic reactions involved in nitrogen assimilation, pigment synthesis, fatty acid saturation and DNA synthesis. Nevertheless, recent metatranscriptomic experiment suggests a possible benefit of low-iron metabolism, where fraction of the metatranscriptome encoded proteins remained iron-free, although iron was added to the seawater samples (Marchetti et al., 2012). By examining sensitivity to oxidative stress, as a result of H$_2$O$_2$ treatment, during iron limitation in parallel to protein expression profiles in the model diatom *P. tricornutum*, we detected two distinct metabolic phases in the response of diatoms to iron limitation. While phase I is characterize by up-regulation of several antioxidant proteins and sensitivity to oxidative stress, phase II cells exhibited resistance to oxidative stress along with a decrease in the cellular GSH pool, antioxidant proteins, iron containing proteins, photosynthetic efficiency, respiration rate and growth rate. Importantly, our data indicate that the differential sensitivity to oxidative stress between phase I and phase II was neither due to differential H$_2$O$_2$ permeability nor due to differential oxidization of the GSH pool, which was highly comparable between the two phases (Fig. 1D). These results suggest a tradeoff between high growth rates, achieved by the iron based metabolism, and resistance to environmental stress conditions (i.e. high light, nitrogen limitation and infochemicals) that can induce organelle specific oxidative stress that is comparable to external application of H$_2$O$_2$ (Rosenwasser et al., 2014; Graff van Creveld et al., 2015). Furthermore, early oxidation of mitochondrial roGFP was found to predict subsequent cell death in cells under natural environmental stress conditions and in response to H$_2$O$_2$ (Fig. 3, A, B and Graff van Creveld et al., 2015). The only exception we detected so far is in the response of chronically iron limited (phase II) cells to H$_2$O$_2$ (Fig. 3C). This implicates that diatom survival in response to external stress is defined by their internal iron quota.
The ability to donate and accept electrons makes iron not only crucial co-factor in enzymes that catalyze redox reactions, but also a producer of oxidative damage, especially when free Fe$^{2+}$ react with H$_2$O$_2$ in the Fenton reaction. Consequently, oxidative stress and iron metabolism are tightly connected, and regulated cell death from iron-dependent oxidative injury was described as “ferroptosis” in mammalian cells (Dixon et al., 2012; Reed and Pellecchia, 2012). While iron-chelators can directly prevent cell death in cancer and neurodegenerative diseases, the direct iron-containing, peroxide sensitive target is still unknown (Antunes et al., 2001; Seiler et al., 2008; Weinreb et al., 2010; Hamacher-Brady et al., 2011; Dixon and Stockwell, 2014). To the best of our knowledge, ferroptosis was never described in photosynthetic organisms, nevertheless, free Fe$^{2+}$ have a crucial role in plant PCD (Swidzinski et al., 2004). In contrast to mammalian cells, many diatoms are adapted to low-iron environments and have large internal storage of iron (Sutak et al., 2012). In this light, it is not surprising that diatoms resistant to oxidative stress are observed only in long term, chronic iron limitation and following application of a strong chelator, when the internal storage of iron is completely depleted.

Due to its high reactivity, iron homeostasis has to be tightly controlled, restricting iron availability only to specific targeted proteins (Finney and O’Halloran, 2003). In order to avoid the toxicity of free iron, ferritin is used in all kingdoms to safely store iron (Andrews et al., 1992). In plants, ferritin is crucial in order to utilize iron while avoiding iron-induced oxidative damage and toxicity (Ravet and Pilon, 2013). In the absence of iron the ferritin mutant plants do not exhibit any macroscopic phenotype, but have elevated ROS levels and display induction of antioxidants. However, under high iron conditions, wild-type plants induced growth while the ferritin mutant is overwhelmed by oxidative damage (Ravet et al., 2009). Ferritin was found in all diatoms classes (Groussman et al., 2015), and it is important in maintaining high growth rates in sporadic availability of iron (Marchetti et al., 2009; Pfaffen et al., 2013). Intriguingly, the role of the diatom ferritin is probably to buffer the iron quota rather than to store it, as this ferritin is not optimized for iron mineralization, enabling rapid release of iron when it is required (Pfaffen et al., 2015).
Upon iron limitation, algae (red and green linages) and cyanobacteria replace the iron-containing ferredoxin with the iron-free flavodoxin, which is absent in genomes of land plants (Tognetti et al., 2006). Flavodoxin and especially the ratio between flavodoxin and ferredoxin were suggested as in situ markers for iron limitation in the marine environment, due to induction of flavodoxin in iron limitation and its downregulation upon iron-resupply (La Roche et al., 1995; La Roche et al., 1996; Erdner et al., 1999; Chappell et al., 2014). Ectopic expression of flavodoxin in land plants not only improves fitness during iron starvation, but also confers multiple stress-tolerance, including tolerance to oxidative stress (Tognetti et al., 2006; Tognetti et al., 2007; Lodeyro et al., 2012). Part of the effect may be due to replacement of ferredoxin functions, as ferredoxin is downregulated under diverse stress conditions. Nevertheless, overexpression of ferredoxin did not induce stress tolerance as flavodoxin did (Ceccoli et al., 2011). The induction in flavodoxin expression during iron limitation, cannot by itself explain the H$_2$O$_2$ resistance that was detected in phase II, as flavodoxin was highly induced already during phase I, and the decrease in ferredoxin was mainly in phase I (Fig. 5A). Remarkably, in the metatranscriptomic experiment on natural populations, 4 days after iron enrichment there was some induction of ferredoxin, and 10 fold higher induction in flavodoxin (Fig. 5A). High flavodoxin levels may contribute to general stress tolerance or as cellular preparation for fluctuating iron availability in the ocean. Another possible explanation is that 4 days following iron enrichment (4 nM Fe) iron is mainly consumed by nitrogen assimilation pathway and photosynthesis proteins, therefore hindering induction of ferredoxin (Marchetti et al., 2012). This exemplifies the strength of proteomic approach in increasing the detection resolution of the cellular response to varying iron concentrations and enables better characterization of the two phases of iron limitation.

In addition to the regulation of iron homeostasis in the cells, the cellular antioxidant network which is composed of antioxidant enzymes and small molecules serves in detoxifying harmful ROS and prevent their interaction with free iron. Indeed, in phase II of iron limitation, we detected a general decrease in antioxidant proteins such as ascorbate peroxidase, catalase, dehydroascorbate reductase, glutathione peroxidase, glutathione reductase and Prx (Fig. 5A), suggesting the role of these antioxidants in iron...
replete environments. Upregulation of antioxidant proteins, such as glutathione peroxidase, Prx, Trx and SODs, was detected following iron enrichment in Ocean Station Papa (Fig. 5A). In contrast, few antioxidants were upregulated in chronic iron limitation, including two Trxs, one out of four GSTs and Ni-SOD (Fig. 5A). Although two Trxs are induced in phase II, thioredoxin reductase, which is essential to reduce Trx is induced in phase I and down regulated in phase II (fold changes 2.1, -7.4 respectively, p values <0.05, Supplemental Table S4). The specific induction of one GST may induce glutathionylation and protection of specific target proteins that are crucial for the resistance to oxidative stress. However, as there is no complete growth arrest in phase II, there is still a demand for antioxidant enzymes to detoxify normal byproducts of photosynthesis and respiration.

Diatoms under iron limitation were shown to substitute Fe-SOD with other Fe-free isoforms to minimize oxidative stress under low iron availability (Allen et al., 2008). In our proteome we identified 3 SODs: Cu/Zn-SOD, Ni-SOD and Mn/Fe-SOD (Supplemental Table S3). Cu/Zn-SOD exhibited downregulation in phase II (fold change -1.53, p value 0.002) while the Ni-SOD was induced in phase II (fold change 1.63, p value 0.045), to our knowledge, this is the first time that Ni-SOD shown do have a role in diatoms response to iron limitation. Ni-SOD is prevalent in open ocean species, where iron concentrations are low, and considered to be acquired via horizontal gene transfer, concomitant to the loss of Fe-SOD (Dupont et al., 2008; Dupont et al., 2010).

Comparison of *P. tricornutum* proteome to a metatranscriptome of a naturally iron-limited population (Marchetti et al., 2012) suggested that natural populations of diatoms exhibit a metabolic state which is more similar to phase II than to phase I of iron limitation (Fig. 5B), suggesting that diatom cells in natural low-iron conditions may benefit from low-iron metabolism by resistance to oxidative stress as in phase II of iron limitation (Fig. 2A). Remarkably, following resupply of a low amount of iron (18.5 nM), chronically iron starved cells (phase II) can maintain high growth rates while still displaying resistance to oxidative stress (Fig. 1A and 2C), suggesting the existence of metabolic state in which cells benefit from iron based metabolism in parallel to resistance to stress conditions. In such conditions, when only low concentrations of iron are
available, prioritizing the iron distribution between different metabolic pathways would be critical. Indeed, based on metatranscriptomics analysis during iron enrichment in Ocean Station Papa, pennate diatoms (i.e. *Pseudo-nitzschia* and *Fragilariopsis*) probably shunt the iron to the nitrogen assimilation pathway while other metabolic pathways were kept partially iron free (Marchetti et al., 2012). Notably, in *P. tricornutum*, upregulation of ferritin was detected (Supplemental Table S3), enabling safe iron storage. In agreement, upon iron resupply to the ocean the blooming species are usually pennate diatoms that encode for ferritin (Marchetti et al., 2012; Morrissey and Bowler, 2012; Groussman et al., 2015). As ferritin is crucial for iron utilization and avoiding iron toxicity, it is important to note that some pennate, and many centric diatoms, do not have the gene encoding for ferritin (Groussman et al., 2015). These diatoms may be subjected to high oxidative damage in response to natural or artificial iron enrichments. Taken together, these metabolic and physiological consequences should be considered when suggesting to artificially fertilize the ocean with iron in order to enhance biological productivity and remove atmospheric CO₂. Here we revealed a possible cost of iron based metabolism as high-sensitivity to oxidative stress. While the scale of the iron-induced blooms and the deposition to the deep ocean are widely investigated, our data suggests that physiological tradeoff and metabolic remodeling should be further investigated in higher resolution. Defining the physiological and metabolic states under iron limitation, based on hundreds of co-expressed proteins, provides a sensitive systems approach to reveal a gradient in phenotypic strategies employed by marine algae in response to stress in the marine environment.

**Materials and Methods**

**Culture growth:**

*P. tricornutum*, accession Pt1 8.6 (CCMP2561 in the Provasoli-Guillard National Center for Culture of Marine Phytoplankton) cells were batch grown in f/2 medium at 18 °C under a 16: 8 light:dark cycles and light intensity of 80 μmol photons·m⁻²·sec⁻¹ supplied by cool-white LED lights. Exponentially growing cells (1·10⁶ cells ml⁻¹) were harvested by centrifugation (4000 g, 10 min), washed 3 times by resuspension in 0.2 μm filtered and autoclaved seawater (FSW) and centrifugation, and used to inoculate triplicate
cultures of either replete f/2 medium (replete) or f/2 medium without added iron and with
the efficient iron chelator, 1 µM DFB (Fe-limited, or -Fe) at a cell concentration of 2·10^5
cells·ml^{-1}. DFB may compete with cell surface binding sites for available Fe^{2+} (Shaked et
al., 2005). In order to keep iron the only limiting factor, we diluted the cultures every few
days. Dilutions were done into fresh media with the same properties (replete or -Fe
media), cells were counted prior to dilution in order to dilute replete and -Fe cells to the
same cell density. Seawater used for media formulations was collected from coastal Israel
Mediterranean Sea, iron concentration was less than 0.005 mg·l^{-1}.

Iron resupply: cultures were centrifuged (4000 g, 10 min), washed 3 times in FSW, “-Fe”
cultures were divided into three: one part was resuspended in replete f/2 media (+Fe,
1850 nM), a second was resuspended in f/2 media with 18.5 nM iron (+Fe, 18.5 nM) and
a third part was kept without iron, with DFB (-Fe). “Replete” cultures were resuspended
in replete f/2 media.

Samples were removed daily and monitored for cell abundance, photosynthetic
efficiency, respiration, chlorophyll autofluorescent per cell, GSH contents, roGFP
oxidation and sensitivity to oxidation stress.

Monitoring physiological response to iron limitation:
Cell abundance was determined using the Multisizer 4 Coulter Counter (Beckman
Coulter, Fullerton, CA, USA).

Oxygen evolution and respiration was measured using a Clark-type oxygen electrode at
20 °C (Oxy-Lab, Hansatech Instruments, King’s Lynn, UK). For each measurement cells
were concentrated by 10-minute centrifugation at 4000 g to a final concentration of 10^7
cells·ml^{-1} and dark-adapted for 20 minutes. Respiration was measured in the dark, O_2
evolution was measured at 1000 µmol photon·m^{-2}·s^{-1} provided by light-emitting diodes
with an emission maximum around 650 nm.

Photosynthetic efficiency was measured using an imaging PAM system (Heinz Walz).
Cells were concentrated by 10-minute centrifugation at 4000 g to a final concentration of
10^7 cells·ml^{-1} and were dark-adapted for 20 minutes. Photosynthetic efficiency was
determined as Fv/Fm, calculated according to Maxwell and Johnson, 2000, where Fm is
the maximum fluorescence emission level in the dark measured with a saturating pulse of light (emission peak at 450 nm, 2700 μmol photons·m⁻²·s⁻¹, 800 ms) and Fv = Fm - F₀.

GSH content was measured by the fluorescence dye Monochlorobimane (Sigma), solubilized in DMSO at final concentration of 1 μM. Samples were incubated at room temperature in the dark for 40 minutes before analysis by flow cytometry (ex: 405 nm, em: 455/50 nm). Leakage of chlorophyll autofluorescence to the blue channel was subtracted by measurement of unstained samples.

Degree of oxidation was measured using transformant lines expressing roGFP in the chloroplast, mitochondria or nucleus as described in (Rosenwasser et al., 2014, Graff van Creveld et al., 2015). All the experiments were preformed simultaneously in WT and roGFP expressing lines and all the examined physiological parameters such as growth rates, chlorophyll auto fluorescence per cell and H₂O₂ resistance (e.g. Sytox green stain for cell death assay) were similar between the WT and roGFP expressing transformants. Basically, roGFP was measured by flow cytometry in the green channel (525/50 nm) following excitation at 405 nm (oxidized) and 488 nm (reduced), leakage of autofluorescence to the green channel was subtracted by measurement of WT cells in parallel to the roGFP transformants. Fluorescent ratios (ex: 405/488), arbitrary units were normalized to oxidation degree by comparing the measurements to calibration of fully oxidized and fully reduced samples.

Membrane permeability was determined by Sytox Green (Invitrogen) at a final concentration of 1 μM. Samples were incubated at room temperature in the dark for 30 minutes prior to measurement. Phosphatidylserine externalization was estimated using Annexin-V-Alexa Fluor 488 (Invitrogen). 10⁶ cells were harvested via centrifugation, washed in cold PBS, resuspended in 150 μl of Annexin binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl₂, pH 7.4) and stained with 7.5 μl of Annexin V for 15 min at room temperature in the dark, after which 600 μl of Annexin binding buffer was added. After staining, cells were pelleted via centrifugation, washed once with PBS and measured. Sytox Green and Annexin V staining were measured by flow cytometry in the green channel (ex: 488 nm, em: 525 nm).
All flow cytometry measurements (chlorophyll autofluorescent per cell, GSH contents, Sytox, Annexin V stains and roGFP oxidation) were obtained using the Eclipse iCyt flow cytometer (Sony Biotechnology Inc., Champaign, IL, USA), equipped with 405 nm and 488 nm solid state air cooled lasers, both with 25 mW on the flow cell and with standard filter set-up. At least 5000 cells were measured in each sample, with at least 3 biological replicated.

**Protein extraction:**
Proteins were extracted from *P. tricornutum* cells at day 3 of replete and day 3 and 5 of iron limitation, using 10% TCA-acetone. For protein precipitation, cells were collected by centrifugation of 250 ml cultures (10 minutes, 4000 g, 4 °C) and resuspended in 500 µl of 10% TCA-acetone. Then, cells were sonicated gently and kept at -20 °C for 1 hour. After centrifugation (10 minutes, 10000 g) the supernatant was discarded and the pellet was washed twice with 5% TCA-acetone and stored at -20 °C for 1 hour. Precipitated proteins were collected by centrifugation (10 minutes, 10000 g, 4 °C) and the pellet was dried under nitrogen flow. Pellets were solubilized using 8 M urea (Sigma). Samples were then diluted to 1.6 M urea and proteins reduced using 5 mM dithiothreitol (Sigma) for 30 minutes in 56 °C. This was followed by alkylation using 10mM iodoacetamide for 30 minutes in the dark at room temperature. Trypsin was then added at a ratio of 50:1 (protein:trypsin) at 37 °C overnight. Digestion was stopped using 1% formic acid. Samples were desalted using Oasis HLB (Waters). Samples were frozen in -80 °C until mass spectrometry analysis.

**Liquid Chromatography:**
ULC/MS grade solvents were used for all chromatographic steps. Each sample was loaded using split-less nano-Ultra Performance Liquid Chromatography (10 kpsi nanoAcquity; Waters, Milford, MA, USA) in high-pH/low-pH reversed phase (RP) 2 dimensional liquid chromatography mode. 15 µg of digested protein from each sample were loaded onto a C18 column (XBridge, 0.3x50 mm, 5 µm particles, Waters). The following two buffers were combined: (A) 20 mM ammonium formate, pH 10 and (B) acetonitrile (ACN). Peptides were released from the column using a step gradient: 6.9% B, 10.4% B, 12.1% B, 13.5% B, 14.7% B, 15.9% B, 17.3% B, 18.8% B, 20.9% B and
65% B. Each fraction flowed directly to the second dimension of chromatography. The buffers used in the low pH RP were: (A) $\text{H}_2\text{O} + 0.1\%$ formic acid and (B) ACN + 0.1% formic acid. Desalting of samples was performed online using a reverse-phase C18 trapping column (180 $\mu$m i.d., 20 mm length, 5 $\mu$m particle size, Waters). Then the peptides were separated using a C18 HSS T3 nano-column (75 $\mu$m i.d., 150 mm length, 1.8 $\mu$m particle size, Waters) run at 0.4 µl·min$^{-1}$. Finally, peptides were eluted from the column and loaded onto the mass spectrometer using the following protocol: 3% to 30% B over 60 minutes, 30% to 95% B over 5 minutes, 95% maintained for 7 minutes (and then back to initial conditions).

**Mass Spectrometry:**

The nanoLC was coupled online through a nanoESI emitter (7 cm length, 10 mm tip; New Objective; Woburn, MA, USA) to a quadrupole ion mobility time-of-flight mass spectrometer (Synapt G2 HDMS, Waters) tuned to 20000 mass resolution (full width at half height). Data were acquired using Masslynx version 4.1 in HDMSE positive ion mode, in which the quadrupole was set to transfer all ions. The ions were separated in the T-Wave ion mobility chamber and transferred into the collision cell. Collision energy was alternated from low to high throughout the acquisition time. In low-energy (MS1) scans, the collision energy was set to 5 eV and this was ramped from 27 to 50 eV for high-energy scans. For both scans, the mass range was set to 50-2000 Da with a scan time set to 1 second. A reference compound (Glu-Fibrinopeptide B; Sigma) was infused continuously for external calibration using a LockSpray and scanned every 30 seconds.

**Data Processing, Searching and Analysis:**

Quantification of protein expression was conducted using MS1 intensity based label-free quantification as described in (Levin et al., 2011). Raw data were imported into Rosetta Elucidator System version 3.3 (Rosetta Biosoftware, Seattle WA USA). Elucidator was used for retention time alignment and extraction of MS1 feature intensities. In parallel, database searching was performed using ProteinLynx Global Server (IdentityE) version 2.5. Database searching was carried out using the Ion Accounting algorithm described by Li et al., 2009. Trypsin was set as the protease, one missed cleavage was allowed and
fixed modification was set to carbamidomethylation of cysteines. Variable modifications included oxidation of methionine.

Data were searched against a *P. tricornutum* protein database which was built by combining three publically available protein databases: The National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/protein/?term=tricornutum), Doe Joint Genome Institute (JGI, http://genome.jgi.doe.gov/Phatr2/Phatr2.download.html) and TrEMBL (http://www.ebi.ac.uk/uniprot/database/download.html) as in Rosenwasser et al., 2014. A target-decoy strategy was performed using reversed sequences. The criteria for protein identification were set to minimum of three fragments per peptide, five fragments per protein and minimum peptide score of 6.7, which corresponds to the false identification rate (FDR) of 1%. The approach for setting the minimum identification score is based on reports by Keller et al, and termed Peptide Prophet (Keller et al., 2002; Nesvizhskii et al., 2003). Identifications were imported automatically into Elucidator for annotation of features, applying a ‘match between runs’ approach of propagating identifications between samples. Protein quantification inference was conducted using the Hi-3 method (Silva et al., 2005). A Student’s T Test was used for statistical evaluation after logarithmic transformation of protein intensities. Fold changes were determined by dividing the arithmetic mean of the three biological replicates in each group. When a protein was detected only in one of the condition a fold change value of +/-1000 was assigned. All mass spectrometry data, including raw data, processed spectra and identifications have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner (Vizcaino et al., 2013) with the dataset identifier PXD004694.

**Protein functional annotation and enrichment analysis:**

GO enrichment was assessed utilizing the Ontologizer application (http://compbio.charite.de/contao/index.php/ontologizer2.html) (Bauer et al., 2008).

**Comparison to metatranscriptomic data:**
Diatoms-derived expression datasets from Papa station metatranscriptomes (Marchetti et al., 2012) were obtained from Adrian Marchetti in order to compare with the *P. tricornutum* proteome. Diatoms sequences were retrieved using Uniport databases (http://www.uniprot.org/). To obtain orthologous protein pairs these proteins were blasted against the *P. tricornutum* databases (Rosenwasser et al., 2014) using BlastP matched based on best hit best hit (e-value <0.05).

**Supplemental Material**

**Supplemental Figure S1.** Photosynthetic efficiency and respiration during iron limitation.

**Supplemental Figure S2.** GSH content during iron limitation and resupply.

**Supplemental Figure S3.** Cell death 24 hours after H$_2$O$_2$ treatment as measured on roGFP transformant lines.

**Supplemental Figure S4.** Cell death assessed by Sytox staining, measured 5 and 8 hours after treatment with 0 and 150 µM H$_2$O$_2$ at day 6 of iron limitation.

**Supplemental Figure S5.** Cell death following H$_2$O$_2$ treatment.

**Supplemental Figure S6.** Venn diagrams comparing between *P. tricornutum* proteomic profiles and Ocean Station Papa metatranscriptome during iron limitation and enrichment.

**Supplemental Table S1.** Number of samples in each cluster presented in Figure 3; ‘reduced and live’, ‘reduced and dead’, ‘oxidized and live’ or ‘oxidized and dead’, in replete, phase I and phase II.

**Supplemental Table S2.** Functional analysis of enriched biological terms was performed for phase I (day 3 of iron limitation compared to replete) and phase II (day 5 compared to day 3 of iron limitation) using Gene Ontology (GO). This data is visualized in Fig 4C.

**Supplemental Table S3.** Fold changes and p values in phase I, phase II and 98 hours following iron enrichment in Ocean Station Papa (Marchetti et al., 2012).
**Supplemental Table S4.** Fold changes and p values in phase I, Phase II and 98 hours following iron enrichment in Ocean Station Papa (Marchetti et al., 2012).

**Supplemental Figure S1. Photosynthetic efficiency and respiration during iron limitation.** A, Time course photosynthetic efficiency (Fv/Fm) during iron limitation. B, Maximal oxygen evolution rates during iron limitation. C, Maximal respiration rates during iron limitation. D, Cell death assessed by Sytox staining, measured 24 hours after treatment with 0, 150 µM H₂O₂ for the specific experiment described in A-C.

**Supplemental Figure S2. GSH content during iron limitation and resupply.** Quantification of the reduced GSH pool per cell as percent of replete cells (treatment/replete) during iron limitation and resupply at day 7, measured as monochlorobimane fluorescence using flow cytometry (ex: 405 nm, em: 455 nm). Flow cytometry analysis is based on fluorescence measurements of at least 5000 cells per sample. Error bars represent standard error of biological triplicates.

**Supplemental Figure S3. Cell death 24 hours after H₂O₂ treatment as measured on roGFP transformant lines.** Cell death of transformant lines expressing roGFP in the mitochondria (Mit) or the chloroplast (Chl) assessed by Sytox staining, measured 24 hours after treatment with 150 µM H₂O₂. The time (days) in the X axis represents the duration of iron limitation, similar to the time depicted in Fig. 1 and 2. Flow cytometry analysis is based on fluorescent measurements of at least 5000 cells per sample.

**Supplemental Figure S4. Cell death assessed by Sytox staining, measured 5 and 8 hours after treatment with 0 and 150 µM H₂O₂ at day 6 of iron limitation.** Flow cytometry analysis is based on fluorescence measurements of at least 5000 cells per sample. Error bars represent standard error of biological triplicates.

**Supplemental Figure S5. Cell death following H₂O₂ treatment.** A, Cell death assessed by Sytox staining, measured 24, 48, 72 and 126 hours after treatment with 0-500 µM H₂O₂ at day 5 of iron limitation. B, Cell death assessed by Sytox staining, measured 24,
48, 102 and 144 hours after treatment with 0-500 µM H₂O₂ at day 6 of iron limitation.

Flow cytometry analysis is based on fluorescence measurements of at least 5000 cells per sample. Error bars represent standard error of biological triplicates.

**Supplemental Figure S6.** Venn diagrams comparing between *P. tricornutum* proteomic profiles and Ocean Station Papa metatranscriptome during iron limitation and enrichment. Venn diagrams showing iron-limitation induced proteins (fold change > 1.5 in phase I, phase II proteomics, < -1.5 in Papa Station), and iron-limitation down-regulated proteins (fold change < -1.5 in phase I, phase II proteomics, > 1.5 in Papa Station).

**Supplemental Table S1.** Number of samples in each cluster presented in Figure 3; ‘reduced and live’, ‘reduced and dead’, ‘oxidized and live’ or ‘oxidized and dead’, in replete, phase I and phase II. A sample was defined ‘dead’ when > 50% of the cells were Sytox positive 24 hours post H₂O₂ treatment. A sample was defined ‘oxidized’ when nucleus roGFP oxidation was above 40% or above 70% in oxidation of mitochondrial roGFP. The numbers in brackets indicate the percentage of samples in each cluster.

**Supplemental Table S2.** Functional analysis of enriched biological terms was performed for phase I (day 3 of iron limitation compared to replete) and phase II (day 5 compared to day 3 of iron limitation) using Gene Ontology (GO). This data is visualized in Fig 4C.

**Supplemental Table S3.** Fold changes and p values in phase I, phase II and 98 hours following iron enrichment in Ocean Station Papa (Marchetti et al., 2012). Data presented only for the proteins presented in Fig 5A. Protein codes represent *P. tricornutum* GI numbers. Fold change < -1.5 presented in green, fold change >1.5 presented in red and p value >0.5 presented in grey background.

**Supplemental Table S4.** Fold changes and p values in phase I, Phase II and 98 hours following iron enrichment in Ocean Station Papa (Marchetti et al., 2012). Data presented all the detected proteome. Protein codes represent *P. tricornutum* GI numbers. Fold change < -1.5 presented in green, fold change >1.5 presented in red and p value >0.5 presented in grey background.
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Figure legends

Figure 1. Physiological response to iron limitation by the model diatom *P. tricornutum*. A, Time course of cell abundance showing the growth of batch cultures incubated in replete Fe conditions (black circles, f/2 media, 1850 nM), or in -Fe (blue triangles, f/2 without iron, with 1 µM DFB). After 7 days Fe-limited cultures were supplemented with either18.5 nM (green squares) and 1850 nM (purple diamonds). Arrows indicate culture dilutions into fresh media as described in the method section. Grey background represents iron resupply at day 7. B, Chlorophyll autofluorescence per cell measured by flow cytometry (ex: 488 nm, em: 665 nm) during iron limitation. C, Quantification of the reduced GSH pool per cell during iron limitation measured as monochlorobimane fluorescence using flow cytometry (ex: 405 nm, em: 455 nm). D, roGFP oxidation in the chloroplast (Chl), mitochondria (Mit) and nucleus (Nuc), during iron limitation, 30 minutes after addition of 0 and 150 µM H2O2. Oxidation was measured by flow cytometry (ex: 405 nm or 488 nm, em: 525 nm). In all experiments, flow cytometry analysis is based on fluorescent measurements of at least 5000 cells per sample. Error bars represent standard error of biological triplicates.

Figure 2. Chronic iron limitation confers transient resistance to oxidative stress in the *P. tricornutum*. A, Cell death assessed by Sytox staining, measured 24 hours after treatment with 30 or150 µM H2O2. The time (days) in the X axis represents the duration of iron limitation, similar to the time depicted in Fig. 1. B, Phosphatidylserine externalization measured by Annexin V stain at day 6 of iron limitation. Cells treated
with 0 and 150 μM H$_2$O$_2$ were stained with Annexin V at 5 and 8 hours post treatment. C, Cell death assessed by Sytox positive cells, measured 24 hours after treatment with 150 μM H$_2$O$_2$. Application of H$_2$O$_2$ was done 0.5, 1.5, 16, 24 and 168 hours following iron resupply at day 7 of iron limitation. Flow cytometry analysis is based on fluorescent measurements of at least 5000 cells per sample. Error bars represent standard error of biological triplicates.

**Figure 3.** Decoupling between early oxidation and subsequent induction of cell death under chronic iron limitation. Scatter plots of degree roGFP oxidation in *P. tricornutum* cells targeted to the nucleus (3 hours post H$_2$O$_2$ treatment) (circles) and mitochondria (0.5 hours post H$_2$O$_2$ treatment) (triangles) in response to 0-150 μM H$_2$O$_2$, and level of cell death measured by Sytox positive cells 24 hours post treatment. A, Replete. B, Phase I (days 1-4 of iron limitation). C, Phase II (since day 5 of iron limitation). Each graph present at least 130 samples.

**Figure 4.** Global proteomic profiling in *P. tricornutum* response to iron limitation. A, Principal component analysis (PCA) of the proteomic data derived from Fe replete (replete, day 3, grey circles), phase I (day 3 of Fe limitation, purple squares) and phase II (day 5 of Fe- limitation, green triangles) cultures. B, Number of differentially expressed proteins (fold change $<-1.5$ or $>1.5$, $p<0.05$) in phase I (purple, Fe-limited cells on day 3 compared to cells in Fe-replete on day 3) and phase II (green, Fe-limited cells on day 5 compared Fe-limited cells on day 3) cultures. C, Significantly enriched GO terms (hypergeometric test, $p<0.05$, down regulated $<-1.5$ fold change, up regulated $>1.5$ fold change) related to each phase for up or down regulated proteins. X axis represents -log10 of p value, size represents number of genes in each GO term.

**Figure 5.** Comparison between *P. tricornutum* proteomic profiling under iron limitation and metatranscriptome study at Ocean Station Papa. A, Fold change of iron responsive and antioxidants proteins in phase I (purple) and phase II (green). 98 hours following iron enrichment in Ocean Station Papa (striped black) is presented on secondary Y axis (Marchetti et al., 2012). Gene numbers represent *P. tricornutum* GI numbers. B, Gene to gene comparison of proteins present in both *P. tricornutum* phase I or phase II proteomic data (p value $<0.05$), and Papa Station, total of 116 genes. Genes
with the same trend (induced in iron limitation proteomics in *P. tricornutum* and reduced in iron fertilization metatranscriptome, or the opposite) are marked in blue. Genes with the opposite trend (induced in iron limitation proteomics in *P. tricornutum* and in iron fertilization metatranscriptome, or the opposite) are marked in red. Genes with fold change between -1.5 to 1.5 marked in white.


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