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Supplementary Discussion

There is an extensive history demonstrating that iron-sulfur clusters (ISCs) are particularly sensitive to O_2 [1]. Approximately 3 billion years ago when the concentration of O_2 in the Earth's atmosphere was minimal [2], one of the most important reactions for all future life on Earth was the fixation of N_2 to NH_3 or other molecules, a reaction carried out by the enzyme nitrogenase [3]. A key feature of nitrogenase is that it contains ISCs required for its function. Indeed, NFS1 (homolog of Nitrogen-Fixing bacteria S) was named for the role of NFS1 homologs in supporting nitrogen fixation [4]. Nitrogenase activity is highly sensitive to the concentration of O_2 [3, 5]. Therefore when the earth's atmosphere underwent an increase in O_2 concentration approximately 2.5 billion years ago, microbial life likely evolved mechanisms to protect the nitrogenase from damage [2]. This phenomenon can be observed in cyanobacteria, where the nitrogenase reaction is carried out largely in heterocysts [6, 7], specialized structures that exclude O_2 [5, 8].

Another interesting system which demonstrates the sensitivity of ISCs to O_2 is the E. coli enzyme fumarate and nitrate reductase (FNR). FNR acts as a physiologic O_2 sensor with an ISC switch: in hypoxic conditions FNR contains an ISC, permitting nucleic acid binding and the transcriptional activation or repression of hundreds of oxygen responsive genes, analogous to the mammalian HIFs (reviewed in [9]). Importantly, in a high oxygen environment, the FNR ISC directly interacts with O_2, resulting in cluster degradation [10]. These experiments, and others, have led scientists to conclude that the FNR cluster degrades according to the following chemical equation [9]:

$$[4Fe-4S]^{2+} + O_2 \rightarrow [2Fe-2S]^{2+} + Fe^{2+} + Fe^{3+} + 2S^{2-} + O_2^-$$

Moreover, ISCs are well-established to be oxidized by O_2 *in vitro* and *in vivo*. Biophysical methods such as EPR, UV, and circular dichroism spectroscopy on isolated proteins have been used to demonstrate that O_2 can degrade ISCs [9, 11, 12]. Despite these important examples, one can still imagine a scenario by which O_2 is converted to another reactive oxygen species which then leads to cluster degradation. While the antioxidant defenses of the cell severely curtail the presence of H_2O_2 and O_2^-, several studies also directly address the differential ability of O_2 and other reactive oxygen species to degrade ISCs. Quoting from [1]:

> Importantly, [the enzyme] PFOR is more easily oxidized by oxygen than by superoxide [13], in marked contrast to the [4Fe-4S] dehydratases. Hydrogen peroxide and superoxide oxidize metals by inner-sphere mechanisms [14], which require that the oxidant directly bind the cluster in order to receive electrons from it. In contrast, it is likely that electrons can hop from the slightly buried PFOR cluster to nearby molecular oxygen, as they normally do to the cluster of ferredoxin. In this way molecular oxygen may oxidize a fully coordinated cluster, while H_2O_2 and O_2^- cannot.

A similar sentiment is expressed by a different set of authors in [15]:

> Redox active Fe-S clusters, like in ferredoxin, do not need to keep the fourth position open for ligation with substrates or cofactors. Ferredoxins may have four buried protein ligands and be protected by the protein matrix. Consequently, ROS will not react the same way, or at all, with each type of Fe_4S_4 cluster. It has been proposed that buried Fe_4S_4 clusters with four protein

ligands are less sensitive to oxidation by superoxide and hydrogen peroxide. Nevertheless they can still be oxidized by molecular oxygen, most probably via outer-sphere transfer…

Therefore, for ISCs coordinated by four cysteines, O_2 may be the only oxygen species capable of easily oxidizing the cluster. Indeed, of the top ten ISC proteins rated most essential by high throughput screens (Extended Data Figure 5b), nine are coordinated by four cysteine ligands based on UniProt annotations. Thus, the most essential ISC proteins are of the class described above which are predicted to be relatively resistant to oxidation by H_2O_2 and O_2^-. Reactive oxygen species can have a role in degrading iron-sulfur clusters, particularly *in vitro*. However, cellular antioxidant defenses largely neutralize these highly reactive species, while permitting O_2 access to important cellular enzymes requiring it as a substrate. Indeed, we observed that culture of cells in 21% oxygen decreases aconitase activity, but treatment of tbHP does not (Figure 3f); and culture of cells in 21% oxygen increases TFRC expression, but treatment of tbHP does not (Figure 3h). These same tbHP conditions increase cellular ROS, whereas these same O_2 conditions do not increase cellular ROS, as measured by ROS active dyes (Figure 3g). These observations are consistent with O_2 playing a greater role than other ROS in degrading ISCs in cells with an intact antioxidant defense.

Several studies have demonstrated that cancer cells alter cellular iron concentrations via regulation of iron uptake, storage and efflux pathways [16-18]. For example, cirrhotic patients with primary hemochromatosis, an iron overload disorder, exhibit a higher occurrence of hepatic cancer [19], and correlation between iron overload and with other cancer types has been suggested [20, 21]. Given its possible role in tumorigenesis, iron depletion has been proposed as a potential therapeutic approach for cancer in preclinical studies. However, clinical trials utilizing iron chelators such as DFO showed only mixed results [22, 23].

Although several studies suggest a link between cancer and iron [24], less is known about how tumors utilize iron to support neoplastic growth. Several investigators have proposed that iron could be neoplastic via upregulating the activity of iron-dependent enzymes. Excess iron could also contribute to tumor initiation via iron-dependent ROS production [25]. Indeed, alteration of genes involved in iron metabolism can affect ROS levels [26], and disruption of iron metabolism can induce sensitivity to chemotherapeutic agents [27].

Alternatively, studies have attempted to increase intracellular free iron to induce cellular toxicity. Ascorbate, a potent reducing agent, increases available cellular iron by releasing insoluble Fe^{3+} from the iron storage protein ferritin reducing it to Fe^{2+}, thereby increasing susceptibility to ROS-producing agents specifically in cancer cell lines expressing high levels of ferritin [28-30]. Therefore, oral administration of ascorbate has been tested in clinical trials, but this treatment failed to prevent cancer growth due to poor absorption and transport [31, 32]. In contrast, intravenous ascorbate injections have enhanced chemo-sensitivity in some cancers, renewing interest in the potential of ascorbate treatment, although targeting iron metabolism is not cited as the rationale for current applications [33-35]. The synergistic effect of high cellular iron to induce ROS is also exploited by targeting cancer cells with iron-containing micelles with promising pre-clinical results [36-38]. However, targeting the iron starvation response to predispose tumors to oxidative damage has not been previously proposed. Activation of the iron starvation response is expected to increase cellular labile iron levels by both increasing iron import via the transferrin receptor while releasing iron from intracellular stores via ferritinophagy and decreased ferritin expression. Therefore, altering iron metabolism to enhance existing ROS sensitivity presents an unexplored therapeutic potential to target cancer cells.

We propose that elevated oxygen level exerts a selective metabolic pressure on incipient lung adenocarcinomas. While prior work demonstrates that tumour hypoxia selects for cells that are more migratory and metabolically adapted [39], available evidence points to the strong influence of hyperoxia on normal cell biology and tissue physiology. *In vitro* oxygen levels increase DNA damage and impact the time to senescence [40], and in ischemia/reperfusion injury tissue reoxygenation induces damage via reactive oxygen species [41, 42]. The lung exists in an oxygen-rich environment requiring a heightened control of redox balance and oxidative damage [43]. Defective anti-oxidant or redox management has been linked to pulmonary fibrosis [44, 45], and oxidative damage is exacerbated in cystic fibrosis [46], as pulmonary mucus protects lung epithelium from oxidative damage [47, 48]. Interestingly, low elevation correlates significantly with the development of neoplasms in the lung, but not other tissues, an observation attributed to higher atmospheric oxygen levels driving tumourigenesis [49]. Indeed, pathways that support the anti-oxidant response, such as NRF2/KEAP1, are frequently altered in lung cancer [50].

The ISC biosynthetic enzyme NFS1 is an exemplar of a gene whose cellular requirement is impacted by environmental oxygen level. *NFS1* lies in a region of genomic amplification under positive selection in lung adenocarcinoma. This amplification is required for anchorage independent growth and optimal growth in atmospheric oxygen conditions. Our data support the notion that high oxidative stress present in the lung renders acquisition of a robust anti-oxidant defense a key alteration in lung tumourigenesis, and are consistent with the hypothesis that robust NFS1 expression supports the early phases of lung adenocarcinoma development. By increasing NFS1 levels, lung tumour cells protect ISCs, cofactors that are particularly sensitive to molecular oxygen and are required for several cell-essential enzymes. Moreover, ISCs trigger the iron starvation response when damaged, increasing the free iron pool. Intracellular iron level has been linked to the propensity for exogenous oxidants to damage cells and tissues [51], resulting in ferroptosis due to oxidative damage to polyunsaturated fatty acids [52-54]. However, because *in vivo* ferroptosis markers have not been identified, we are unable to determine whether ferroptosis occurs in human tumours or xenograft models.

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Figure 1F

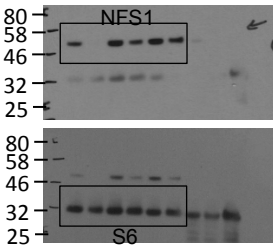


Figure 2D

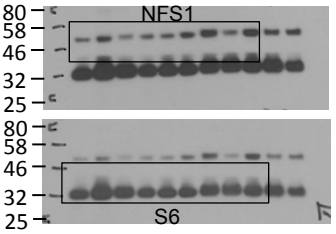


Figure 2E

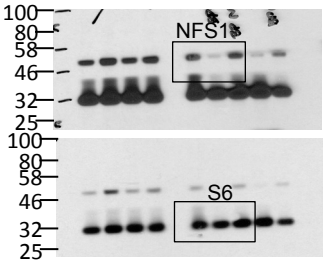


Figure 2H

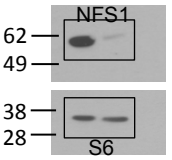
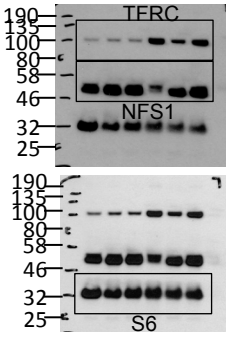


Figure 3C



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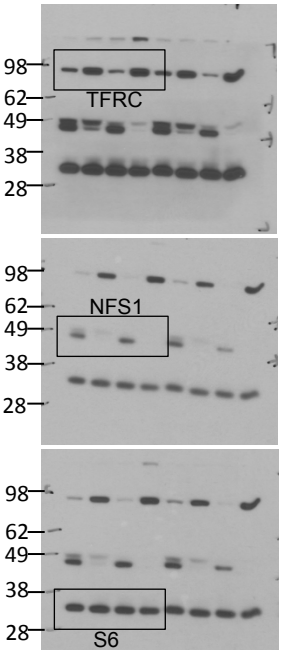
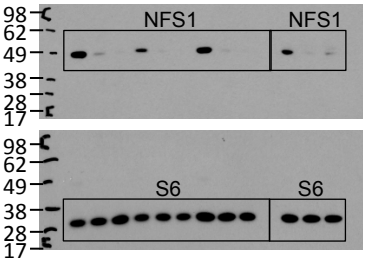
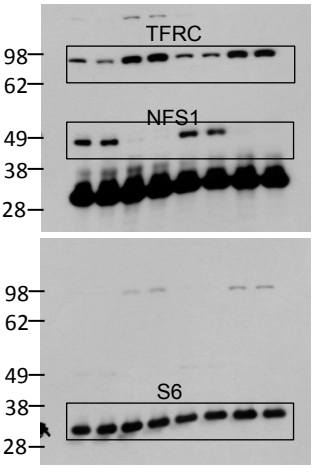


Ext Data Figure 2C, 5A





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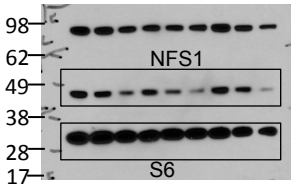

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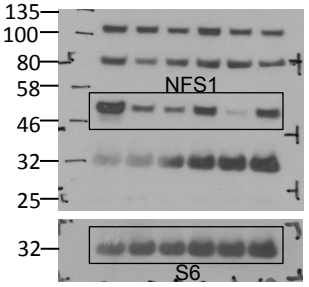

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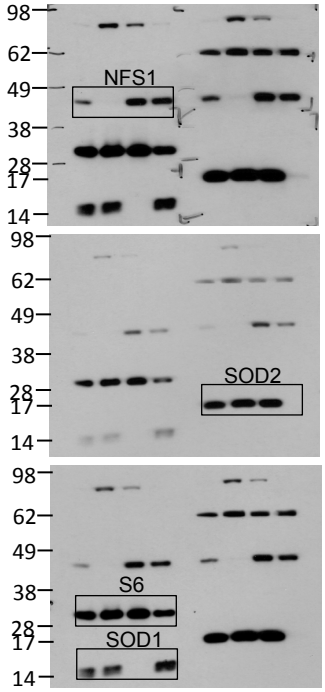

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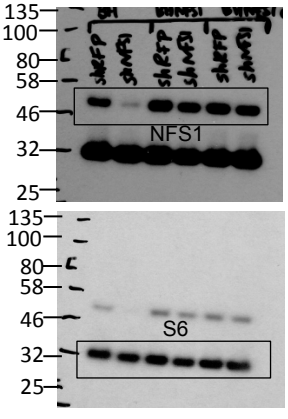

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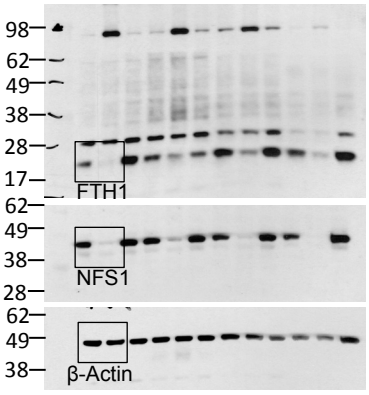

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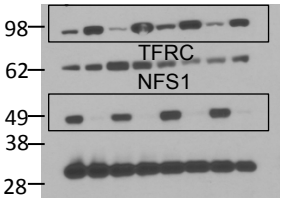
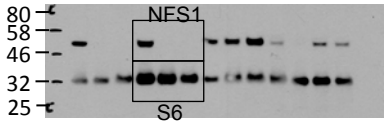
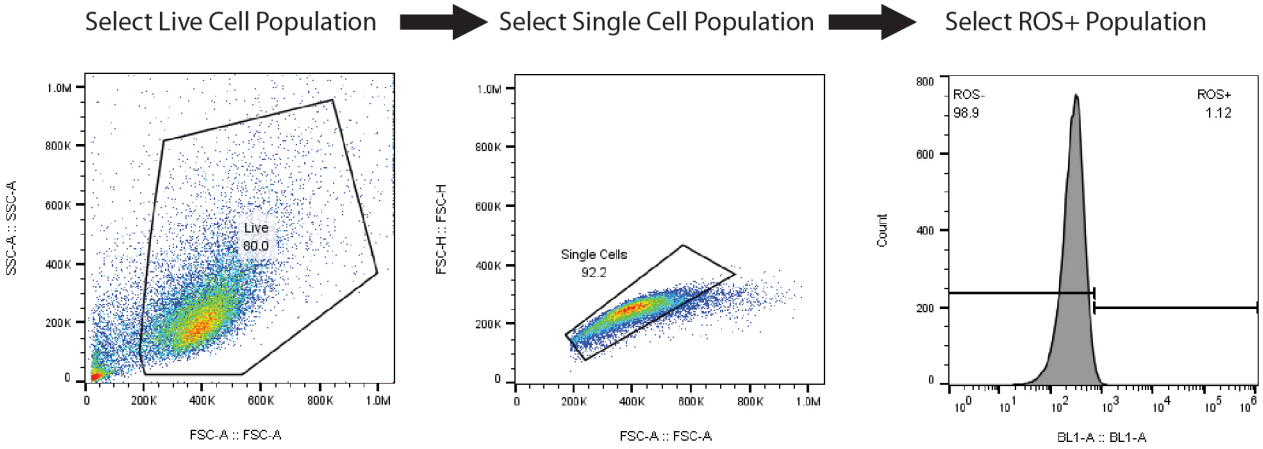


Rev 2:Figure 1E




Select Live Cell Population
Select Single Cell Population
Select ROS+ Population
SSC-A :: SSC-A
FSC-A :: FSC-A
Live
80.0
FSC-H :: FSC-H
FSC-A :: FSC-A
Single Cells
92.2
Count
BL1-A :: BL1-A
ROS-
98.9
ROS+
1.12