The Essence of Nature Can be the Simplest (1)–– Warburg Effect: Transition from Intracellular ATP to Extracellular Fenton Chemistry

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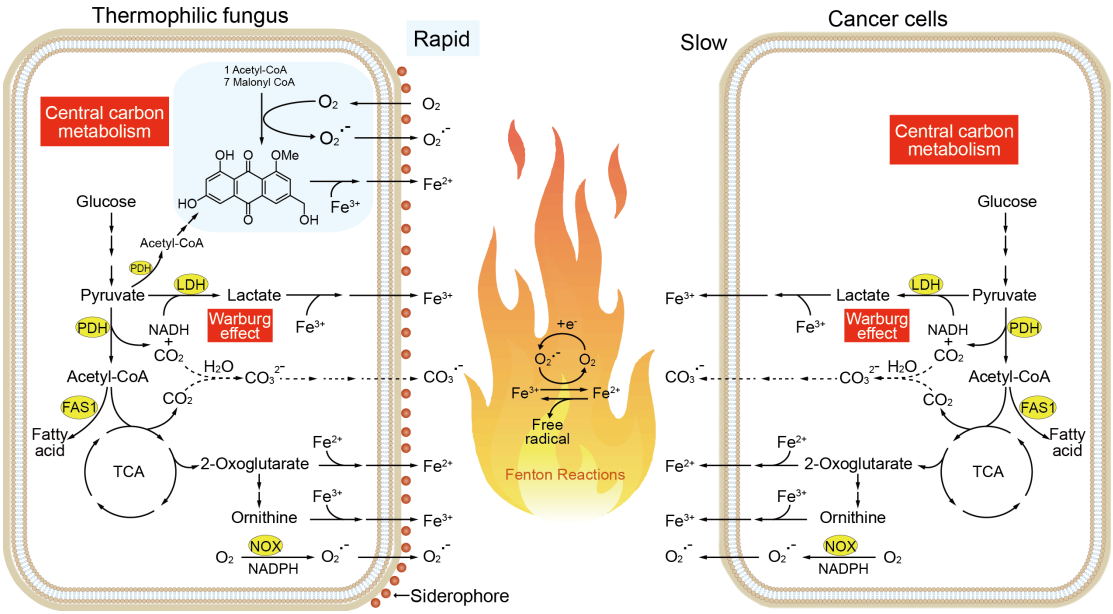

Figure 1. Warburg effect of aerobic glycolysis in tumor cells is due to the large amount of oxygen used by tumor cells for extracellular Fenton reactions. The Warburg effect is a universal phenomenon in nature. The development of extracellular Fenton reaction can be divided into rapid eruption and slow formation. The rapid extracellular Fenton reactions occurred predominantly in fungi, bacteria and plant that contained the key biosynthetic genes *PKS*, *NRPS* and *TPS*, while slow extracellular Fenton reactions occurred mainly in endotherms. Cold stress is a key factor in inducing the Warburg effect in fungi. PKS or TPS-derived aromatic metabolites can initiate and control the extracellular Fenton reactions and NRPS-derived siderophores can sequester iron near cell wall and protect the host from extracellular Fenton reactions.

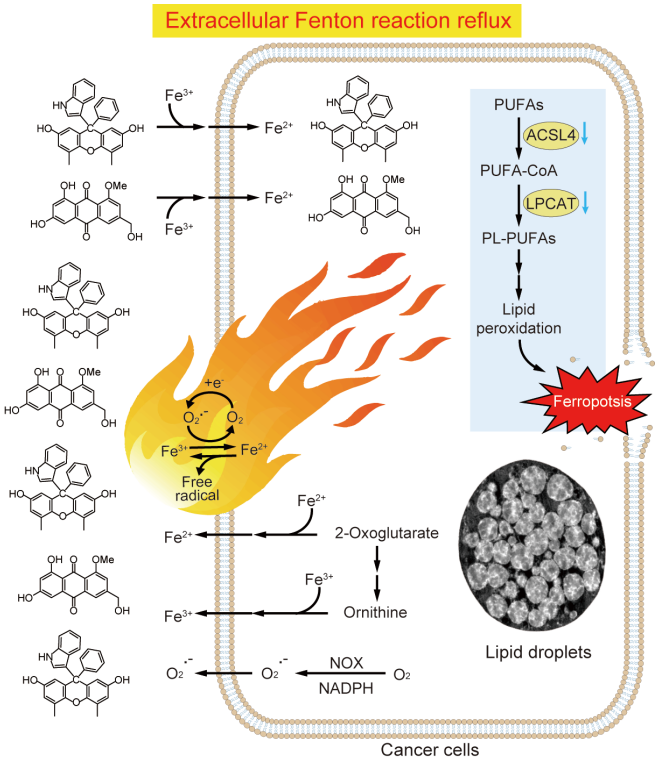

Figure 2. Polycyclic aromatic metabolites counteract extracellular Fenton reactions of cancer cells by facilitating the transport of iron into cancer cells, a phenomenon we term "extracellular Fenton reaction reflux".

Abstract: In this paper, we propose a new theory based on previous research results, and provide a mechanism behind the Warburg effect, which is the most critical mystery in cancer cells and has been unsolved for a hundred years. Warburg effect of aerobic glycolysis in tumor cells is due to the large amount of oxygen used by tumor cells for extracellular Fenton reactions. The Warburg effect is a universal phenomenon in nature. The development of extracellular Fenton reaction can be divided into rapid eruption and slow formation. The rapid extracellular Fenton reactions predominantly occurred in organisms that contained the key biosynthetic genes *PKS*, *NRPS* and *TPS*, while endotherms like mammals have a limited capacity for slow extracellular reactions due to lack of these critical genes. PKS or TPS-derived aromatic metabolites can initiate and regulate the extracellular Fenton reactions and NRPS-derived siderophores can sequester iron near cell wall and protect the host from extracellular Fenton reactions. Cold stress is a critical factor in inducing the Warburg effect. Exogenous polycyclic aromatic metabolites can inhibit cancer cells by counteracting their extracellular Fenton reactions through facilitating the transport of iron into cancer cells, a phenomenon we term "extracellular Fenton reaction reflux".

Key words: Warburg effect, aerobic glycolysis, thermophilic fungus, cancer cells, extracellular Fenton reaction, *PKS*, *NRPS*, *TPS*, Cold stress

Introduction:
Otto Warburg's observations in the 1920s revealed that tumors consume glucose and secrete lactate even in the presence of oxygen—a phenomenon well known as aerobic glycolysis or the Warburg effect [1,2]. Subsequent studies have shown that oxygen is essential for tumor growth [3], yet cancer cells typically generate significantly less ATP than normal cells. This raises a fundamental question: why is the Warburg effect advantageous for tumor growth? While glycolysis requires minimal oxygen, the substantial oxygen consumption associated with the Warburg effect warrants further investigation. Additionally, the tumor microenvironment is often nutrient-depleted, yet it remains highly complex, dynamic, and heterogeneous. What factors contribute to this complexity? Tumor initiation and progression are closely linked to the metabolic reprogramming of cancer cells [4]. Metabolic transformation is recognized as a hallmark of cancer and a promising target for therapeutic intervention [5]. The aberrant accumulation of specific metabolites can drive tumorigenesis, influencing cancer progression as well as the function and fate of immune and stromal cells. However, reliable diagnostic metabolites for tumorigenesis have yet to be identified. In addition, understanding the factors behind the Warburg effect remains a major challenge in cancer research.

Rationale:
Oxygen and iron, the two most abundant elements on Earth, play a crucial role in planetary formation. These elements are also central to the Fenton reaction, a powerful energy-producing process that degrades organic matter into water (H_2O) and carbon dioxide (CO_2) [6]. The Fenton reaction is implicated in excessive iron accumulation and lipid peroxidation, which drive a unique form of programmed cell death known as ferroptosis [7]. While most studies on Fenton reaction-induced ferroptosis focus on intracellular processes in mammals, extracellular Fenton reactions have been observed primarily in fungi, such as the brown rot wood-decaying fungus *Gloeophyllum trabeum* and the ectomycorrhizal fungus *Paxillus involutus* [8, 9]. These fungi are thought to utilize extracellular Fenton chemistry to decompose organic matter through metabolites such as hydroquinones or catechols. Despite of these insights, the mechanisms by which fungi manipulate iron and oxygen for extracellular Fenton chemistry remain poorly understood. Recent studies from our lab revealed that thermophilic fungi, dominant in various composting systems, can carry out extracellular Fenton reactions by employing anthraquinones or naphthalones to reduce and export iron while increasing superoxide ($O_2^{\cdot-}$) production [10]. Thermophilic fungi harness the energy from the extracellular Fenton reaction for their survival and growth under cold-stress conditions [10,11]. Intriguingly, we found that the absence of anthraquinones that initiated and control the extracellular Fenton reactions induced a significant fungal metabolic shift. The thermophilic fungus switched from a state of reduced ATP production in the presence of oxygen to a state of increased ATP generation in self-containment [10].

Since 2008, our research group has focused on investigating the biological and ecological roles of natural products produced by thermophilic fungi [12-19]. These unique eukaryotes thrive at elevated temperatures ranging from 45°C to 60°C and are common mycoflora in both natural and artificial composting systems. Phylogenetically, the genus *Thermomyces* is closely related to mesophilic fungi such as *Aspergillus* and *Penicillium* species. Within *Thermomyces*, two species—*T. lanuginosus* and *T. dupontii* (formerly classified as *Penicillium dupontii* and *Talaromyces thermophilus* until 2014)—are notable for their relatively compact genomes (18.9 Mb and 19.2 Mb,

respectively, compared to the larger genomes of *Aspergillus* and *Penicillium*, ranging from 36 Mb to 45 Mb) [20]. Intriguingly, both *Thermomyces* species develop significantly darker colonies at a low temperature of 37°C, contrasting with their optimal growth temperature of 45°C [10,11].

Using a combination of chemical investigation, chromatography, Mass and NMR spectrometry to analyze culture broths from two *Thermomyces* species, we discovered that *T. lanuginosus* accumulates polyketide-derived dihydroxynaphthalenes (TDN), key precursors for fungal melanin synthesis [11,17]. Meanwhile, *T. dupontii* produces a polyketide-derived yellow anthraquinone pigment, carvionlin A (CA), along with a series of tryptophan-derived cyclodipeptides, both prenylated and non-prenylated, named talathermophilins A–F (TTPs) [10,18,19]. Through mutational and functional analyses, supported by transmission electron microscopy, we found that TDN in *T. lanuginosus* reduces iron and lipid accumulation, lowers $O_2^{\cdot-}$, reactive oxygen species (ROS), and lipid peroxidation, and strengthens fungal cell walls. These functions collectively inhibit iron-dependent ferroptosis induced by cold stress [11]. Initially, we inferred that cold stress induced intracellular Fenton reactions in *T. lanuginosus*, as TDN deficiency resulted in a distinct ferroptosis phenotype. This phenotype was marked by extensive rupture of mitochondrial membrane and cristae in the mutant Δ*TDN* mycelia.

To investigate the roles of various metabolites in fungal responses to cold stress and determine whether similar ferroptosis phenomena occur in other strains, we further investigated *T. dupontii*, the sister strain of *T. lanuginosus*. Initially, the prominent peaks corresponding to the six talathermophilins (TTPs) in the metabolic profile of *T. dupontii* drew our attention, particularly in contrast to the inconspicuous and partially obscured peak for the major anthraquinone CA [18,19]. Unexpectedly, the absence of TTPs in *T. dupontii* resulted in significant reductions in both Fe^{2+} and total iron levels, as well as decreased ROS and lipid droplets in the Δ*TTP* mutant. However, Fe^{3+} and $O_2^{\cdot-}$ levels were elevated in the Δ*TTP* mutant [10,18]. These findings partially contradicted our initial hypothesis that the loss of TTPs would increase Fe^{3+} levels, thereby raising total free iron levels in the Δ*TTP* mutant, given that TTPs are known to sequester Fe^{3+} [19]. Furthermore, the contrasting trends in $O_2^{\cdot-}$ and ROS levels between the Δ*TTP* mutant and the wild-type (WT) strain added complexity to our observations. We hypothesized that additional compounds in the Δ*TTP* mutant might compensate for the absence of TTPs by reducing intracellular Fe^{2+}, ROS, and lipid droplets and increasing $O_2^{\cdot-}$. These compounds could play a protective role, shielding the mutant from iron-dependent lipid peroxidation triggered by cold stress—specifically, intracellular ferroptosis.

Detailed analysis of the metabolic profiles revealed that more anthraquinones and derivatives accumulated in the Δ*TTP* mutant compared to the WT strain [11]. Previous studies have demonstrated that anthraquinone metabolites possess strong ROS scavenging activity while also promoting $O_2^{\cdot-}$ formation [21]. Notably, $O_2^{\cdot-}$ facilitates the reduction of Fe^{3+} to Fe^{2+}, a critical step in catalyzing the Fe^{2+} cycling involved in the Fenton reaction. Through mutational and functional analyses, we confirmed that anthraquinones in *T. dupontii* play a central role in reducing free iron levels by converting Fe^{3+} to Fe^{2+}, exporting Fe^{2+}, and inducing TTP formation, while significantly increasing $O_2^{\cdot-}$ levels [10]. Unexpectedly, in the Δ*An* mutant with anthraquinone deficiency, we observed a unique state of fungal self-isolation mediated by oxygen-free

ergosterenes [10]. This discovery, supported by transcriptional and metabolic analyses, chemical investigations, and transmission electron microscopy (TEM), highlighted a novel adaptive response. Remarkably, the Δ*An* mutant exhibited a substantial increase in ATP levels compared to the WT strain, suggesting that the WT strain generates significantly less ATP under normal oxygen conditions. These findings collectively suggest that *T. dupontii* utilize an extracellular Fenton reaction to produce powerful energy, enabling resistance to cold stress by exporting large quantities of iron while consuming minimal oxygen inside the fungus. Ultimately, our results indicate that *T. dupontii* is capable of transition from generating ATP-based energy internally to harnessing more powerful energy externally.

A review of the literature revealed only sporadic reports of fungi utilizing extracellular Fenton reactions to degrade organic matter, including the brown rot wood-decaying fungus *Gloeophyllum trabeum* and the ectomycorrhizal fungus *Paxillus involutus* [8,9]. Metabolites with reducing properties, such as hydroquinones and catechols, are known to play a critical role in recycling iron in Fenton reactions. However, the precise mechanisms by which these fungi perform extracellular Fenton reactions remain poorly understood. Notably, there is no well-characterized physiological excretion mechanism for iron in eukaryotes, although iron export is reported in certain mammalian cells—such as hepatocytes, enterocytes, and macrophages—and in some bacteria via specialized proteins, including divalent metal transporters, ferroportin-1, heme carrier protein-1, and duodenal cytochrome-b [22-24]. Traditionally, such functions are attributed to proteins or enzymes, the idea that fungi could export iron using metabolites initially seemed implausible, which has limited research on extracellular Fenton reactions of these organisms. However, small molecular metabolites have distinct advantages: their rapid formation, ability to chelate iron, and capacity to cross membranes make them ideally suited to the metabolic pattern changes of fungi adapted to fluctuations in ambient temperature.

Results:

1. Warburg effect also occurred in thermophilic fungus under cold stress

In the thermophilic fungus *T. dupontii*, the transition from high ATP levels in the Δ*An* mutant to low ATP levels in the WT strain, mediated by anthraquinones, mirrors the Warburg effect observed in cancer cells [25]. The Warburg effect describes a metabolic shift in cancer cells from high ATP production, characteristic of normal cells, to low ATP production despite the presence of oxygen. Transcriptional analysis using KEGG pathway-based gene set enrichment analysis (GSEA) revealed that central carbon metabolism in cancer pathways was significantly activated in the WT strain compared to the self-contained Δ*An* mutant (Figure S1A). Notably, lactate biosynthesis was upregulated in the WT strain (Figure S1B), corroborating its reliance on aerobic glycolysis even under oxygen-rich conditions, confirming that the fungal WT strain employs the Warburg effect to distribute large amounts of oxygen to the more oxygen-consuming extracellular Fenton reactions. This raises the intriguing possibility that cancer cells utilizing the Warburg effect might employ a similar strategy to support their rapid growth. Specifically, cancer cells could perform extracellular Fenton reactions, degrading organic matter indiscriminately—akin to a fire—leaving behind uncertain residues. This concept may shed new light on the highly complex, dynamic, and heterogeneous nature of the tumor microenvironment.

2. Metabolites in cancer cells for Extracellular Fenton reactions

A critical step in extracellular Fenton reactions is the export of iron outside the cell, raising the question: which metabolites in tumor cells facilitate iron excretion? In bacteria and fungi, metabolites that chelate iron and aid in its transport across membranes are known as siderophores. These siderophores are typically classified into three main categories based on their chelating groups: hydroxamate-type, catecholate-type, and lactate-type [26]. Lactate has long been shown to bind ferric iron, forming a monochelate complex [27,28]. Hydroxamate-type siderophores are generally composed of hydroxylated and/or alkylated ornithines, while catecholate-type siderophores are derived from aromatic amino acids. Our recent research identified hydroxylated ornithine, a siderophore precursor, as a participant in ferric chelation in the carnivorous fungus *Arthrobotrys oligospora* [29]. Furthermore, in the thermophilic fungus *T. dupontii*, we observed significant upregulation of the biosynthetic pathways for ornithine and aromatic amino acids in the WT strain compared to the Δ*An* mutant (Figure S1A and C). Combined with the increased lactate biosynthesis observed in the WT strain, our findings suggest that all three types of siderophore biosynthesis are activated in response to cold stress in *T. dupontii*. Intriguingly, similar upregulations of ornithine and aromatic amino acids biosynthesis have been documented in cancer cells [30,31], further highlighting potential parallels in their metabolic adaptations.

Numerous studies have demonstrated that elevated intracellular α-ketoglutarate plays a critical role in tumor progression [32,33]. As a key metabolite in the TCA cycle, α-ketoglutarate is known to bind ferrous iron and activate molecular oxygen in various biochemical reactions [34]. Among the α-ketoglutarate/iron-dependent oxygenases, the most well-studied are prolyl and lysyl hydroxylases, which catalyze the post-translational hydroxylation of collagen. In our research, we observed a significant upregulation of α-ketoglutarate biosynthesis in the WT strain compared to the Δ*An* mutant [10]. This finding suggests that major metabolites, such as α-ketoglutarate, may not only play a role in cellular metabolism but also possess the capacity to chelate iron and facilitate its transport across membranes in tumor cells, paralleling the similar mechanisms observed in the fungus.

To investigate the above inference, we compared extracellular Fenton reactions of human breast cancer cell lines Hcc1806 and Hcc1937 with normal MCF10A cells using chemical dyes specific for Fe^{2+} (Rhonox-1), $O_2^{\cdot-}$ (Hydroethidine), and H_2O_2 (Peroxyfluor 1), and calcium ions (Ca^{2+}) were assessed as a reference. Exactly as expected, no significant difference was observed in fluorescence change with the Ca^{2+} dye (Figure S2). Notably, both the tumor cells lines Hcc1806 and Hcc1937 exhibited tremendously stronger fluorescence intensity for Fe^{2+} than the normal MCF10A cells (Figure S3). These two cancer cells also showed significantly strong fluorescence for $O_2^{\cdot-}$ (Figure S4), but the intensity is not as high as the fluorescence intensity for Fe^2. For the H_2O_2 dye, the fluorescence intensity of Hcc1937 cancer cells increased significantly compared to MCF10A normal cells, while the fluorescence intensity of Hcc1806 cancer cells did not increase significantly compared to MCF10A normal cells (Figure S5). These findings confirmed that cancer cells export much more Fe^{2+} than normal cells, suggesting that cancer cells have a greater ability to conduct extensive extracellular Fenton reactions than normal cells. Meanwhile, $O_2^{\cdot-}$ played a key role in extracellular Fenton reactions, while H_2O_2 participation depends on the biological system, consistent with a previous study [35]. Collectively, these results suggest that tumor cells exhibit significantly stronger extracellular Fenton reactions compared to normal cells.

3. Metabolites that determine the rapid transition are lost in mammals

By comparing the transcriptionally altered genes and pathways in the *T. dupontii* fungal WT strain and tumor cells, we found strikingly similar trends in their primary changes (Figure 1). The most distinct difference, however, lies in the production of anthraquinones and TTPs in the fungus, which are not produced by tumor cells. Notably, the key biosynthetic gene *An*, responsible for anthraquinone biosynthesis, encodes a polyketide synthase (PKS), while the critical gene *PIA* involved in TTP biosynthesis encode a nonribosomal peptide synthase (NRPS). These two types of genes are widely distributed in fungi, bacteria and plants, but are absent in mammals. Interestingly, the anthraquinones that initiate and regulate extracellular Fenton reactions of the thermophilic fungus *T. dupontii* represent the largest group of quinoid natural pigments. These compounds are widely distributed in various fungi, medicinal plants from diverse families, lichens, and marine organisms [36,37]. This suggests that the rapid utilization of extracellular Fenton reactions to degrade surrounding materials and generate heat and energy is a common phenomenon among non-homeothermic organisms in nature.

In addition, we identified another class of polycyclic aromatic metabolites derived from terpenoid synthase (TPS), distinct from PKS, which can also transport iron [10]. One notable example is Tanshinone IIA, the primary active pharmaceutical ingredient in Danshen (*Salvia miltiorrhiza* Bunge), a Lamiaceae medicinal plant used for centuries in traditional Chinese medicine to treat cardiovascular and cerebrovascular diseases [38,39]. The precise mechanisms underlying Danshen's therapeutic effects have remained largely unknown. Our experiments demonstrated that Tanshinone IIA can import ferrous iron into the mycelia of *T. dupontii* and HepG2 carcinoma cells [10, 25], indicating that the highly reducing properties of polycyclic aromatic metabolites, such as Tanshinone IIA and its analogues, can also facilitate extracellular Fenton reactions of their producing plants.

In 2016, Shabuer et al. reported that the obligate anaerobic plant-pathogenic bacterium *Clostridium puniceum*, which infects potatoes and causes annual yield losses of 30–50%, produces a polyketide metabolite known as clostrubin to survive in oxygen-rich plant environments [40]. Since clostrubins are polycyclic aromatic metabolites, it is reasonable to deduce that *C. puniceum* utilizes clostrubins to initiate extracellular Fenton reactions, enabling its growth on potatoes. Because Fenton reactions occur extracellularly, the bacterium does not require oxygen to penetrate its cells. Instead, it releases electron-rich clostrubins, ferrous iron, and other metabolites to drive the extracellular Fenton reactions. This mechanism provides a compelling explanation for how anaerobic microorganisms can thrive under aerobic conditions.

4. Metabolites that protect against extracellular Fenton reactions

As an old Chinese saying goes, if you have a spear, you have a shield. Since the rapid utilization of extracellular Fenton reactions is a common strategy among non-homeothermic organisms, defending against such indiscriminate attacks from other organisms must also be a priority for their survival. Consequently, these organisms have evolved various iron-chelating agents to sequester and store iron. In bacteria and fungi, siderophores are the predominant iron chelators reportedly used for iron uptake and storage. Most siderophores are derived from amino acid condensation mediated by NRPS. As mentioned above, the thermophilic fungus *T. dupontii* utilizes TTPs for iron chelation during extracellular Fenton reactions under cold stress. Our recent studies

revealed that the loss of TTPs led to a tremendous transformation of the fungus's robust penicillin-like structures into small, abnormal conidium-like structures at low temperatures [18]. This finding suggests that TTPs play a critical role in protecting fungal cell walls from damage caused by extracellular Fenton reactions. Notably, TTP analogues are commonly found in filamentous fungi, particularly in the genera *Penicillium* and *Aspergillus* of Ascomycota. Examples include astechrome from *Aspergillus terreus* and hexadehydroastechrome from the human pathogen *Aspergillus fumigatus*. We propose that these iron chelators may share similar protective functions against extracellular Fenton reactions. Generally speaking, any substance capable of chelating iron could contribute to safeguarding organisms from the harmful effects of extracellular Fenton reactions.

Our findings suggest that the metabolites derived from PKS, TPS, and NPRS play pivotal roles in facilitating the rapid transition of the fungus under cold stress from intracellular ATP-based energy production to extracellular Fenton reaction-mediated thermal energy generation (Figure 1). We propose that fungi, plant and bacteria retain these metabolites derived from PKS, TPS, and NPRS because they are not maintained at a constant temperature like mammalian cells. Instead, these fungi, plant and bacteria inhabit variable natural environments where temperatures can fluctuate rapidly on a daily basis, compounded by seasonal variations. In contrast, mammalian cells may have lost these key biosynthetic genes as they adapted to a more stable and constant temperature environment, reducing the selective pressure associated with low temperatures. On the other hand, we suggest that normal mammalian cells may initiate the Warburg effect and transform into tumor cells in response to cold and/or nutrient stresses. These stresses likely compel cells to generate additional energy to ensure survival. Furthermore, we hypothesize that such stresses are often persistent and long-lasting, driving the metabolic reprogramming characteristic of cancer. Data can be obtained by comparing the number of these key biosynthetic genes in gut microbes with those microbes in harsh environments.

5. Advantages of extracellular Fenton reactions in controlling intracellular iron

Our recent studies have shown that iron levels in fungi, including thermophilic and carnivorous species, are regulated by ambient temperatures [10,11,19,41]. Interestingly, under cold-stress, the two strains of *Thermomyces* fungi showed distinct morphologies without the PKS metabolites that initiates and controls the extracellular Fenton reaction, but both showed the elevated risk of intracellular Fenton reaction, thus leading to ferroptosis. For example, lack of anthraquinones in the thermophilic fungus *T. dupontii* induced the loss of the Warburg effect in the self-sealed Δ*An* mutant mycelia with abnormal mitochondria, in which the genes *acsl4* and *lpcat* primarily associated with facilitating lipid peroxidation [42,43], were significantly up-regulated (Figure S6). Meanwhile, in *T. lanuginosus*, TDN deficiency caused the organelle membranes in the mycelia of the mutant Δ*TDN* to almost disappear, leaving only large black granules [11]. Additionally, the mutant Δ*TDN* mycelia developed abnormal and almost dead conidial cells with accumulation of large amounts of giant lipid droplets, a phenomenon not typically associated with ferroptosis. Surprisingly, all lipid droplets in the mutant Δ*TDN* conidial cells displayed unknown large grey dense streak areas. Initially, we inferred these giant lipids as a protective response to cold stress due to the absence of TDN-mediated cell wall reinforcement. However, this hypothesis presented a paradox: If there is already plenty of lipid droplets to protect these conidia cells against cold, why is the spore germination rate so low. This unorthodox phenomenon can be

explained by the concept that the abundant lipid droplets appear to serve as a mechanism to carry increased intracellular iron induced by cold stress and prevent iron aggregation. The large grey dense streak areas observed on the surface of the giant lipid droplets may represent iron particles. The larger the lipid droplet, the larger the surface, the more ions it can hold.

All the our results showed that iron excretion for extracellular Fenton reaction was obviously superior to intracellular ATP production for the fungal survival and growth under cold stress, which can not only obtain powerful energy, but also reduce the risk of ferroptosis caused by the increase of intracellular iron contents. Similar elevations in iron levels have been frequently reported in tumor cells, which we hypothesize may also represent a response to cold stress. The tumor cells under cold stress may adopt the similar strategy as thermophilic fungi to export iron for extracellular Fenton reaction and to reduce the risk of intracellular ferroptopsis. This hypothesis aligns with the principles of traditional Chinese medicine, which advocates "warming Yang and avoiding cold" [44]. Taken together, our findings suggested that cold stress might be a key factor in contributing to tumor development.

Perspective:

1 Diverse radicals and self-protection mechanisms

It is well established that free radicals generated by Fenton reactions can indiscriminately damage and degrade organic matter. In 2021, Meyerstein proposed that, under physiological conditions, the carbonate radical ($CO_3^{\cdot -}$) is likely the primary oxidizing intermediate in Fenton reactions, whereas hydroxyl radicals ($OH^{\cdot}$) are formed only under specific conditions and are unlikely to occur under normal physiological conditions despite their common association with these reactions [35]. The identification of $CO_3^{\cdot -}$ as the dominant free radical aligns with the observed excess production of CO_2 in tumor cells, suggesting a critical role for CO_2 in Fenton reactions—a function previously dismissed as merely a byproduct. Meyerstein further emphasized that the mechanisms of Fenton and Fenton-like reactions are highly dependent on several factors, including the nature of the central cations, their ligands, the types of peroxides used, the pH, and the substrates present. Consequently, the mechanism of each system must be studied individually, as it cannot be assumed to behave identically to other systems described in generalized reaction models [35].

Our results indicate that Fe^{2+} and $O_2^{\cdot -}$ are essential for the extracellular Fenton reaction in both the Hcc1937 and Hcc1806 cancer cell lines, while H_2O_2 appears to be dispensable in Hcc1806, aligning with Meyerstein's findings. Different tumor cells may rely on distinct substances to perform and harness extracellular Fenton reactions, depending on their unique structural and compositional characteristics. Furthermore, during the export of these highly reactive substances, cancer cells must safely isolate Fe^{2+} and $O_2^{\cdot -}$, and transport them across the cell membrane for extracellular release. This complex process likely contributes to the diversity of tumor cell types and their behaviors. Proteins and associated components known to be involved in anti-ferroptotic mechanisms may play a critical role in eliminating or mitigating the free radicals generated during the isolation and transport of Fe^{2+} and $O_2^{\cdot -}$. Additionally, these proteins may facilitate the formation of protective barriers or transport vesicles, safeguarding the cell from the damages from Fenton reactions during this process.

2. Landmark elements and metabolites for tumorigenesis

Our experiment demonstrated that chemical dyes assessing extracellular Fe^{2+} and $O_2^{\cdot-}$ levels can quickly differentiate between normal and cancer cells, offering a potential method for tumor diagnosis. Interestingly, we observed that the thermophilic fungus *T. dupontii* WT strain, capable of performing extracellular Fenton reactions, accumulated large amounts of oxygen-containing ergosterols, including peroxyergosterol. In contrast, the Δ*An* mutant, which lacks the ability to perform extracellular Fenton reactions, predominantly produced oxygen-free ergosterenes localized in the cell membrane [10]. Peroxyergosterol is commonly identified as a major sterol in fungi [45, 46], although the mechanism underlying its abundant production remains unclear. Our results suggest that peroxyergosterol is formed when $O_2^{\cdot-}$ is "hijacked" by ergosterenes as large amounts of $O_2^{\cdot-}$ pass through the cell membrane. Notably, peroxycholesterols, the mammalian analogs of peroxyergosterol, have been associated with tumor cells [47]. This observation raises the possibility that tumor occurrence and progression could be diagnosed by analyzing the oxidation states and compositions of steroids in affected cells.

3. Rapid alternation of heat and cold

Since iron content in organisms is inversely proportional to temperature, when the temperature rises, the high content of intracellular iron at low temperatures needs to be reduced. Interestingly, we observed that the carnivorous fungus *Arthrobotrys oligospora* develops a phenotypic system, commonly referred to as an "iron detoxification trapping device," under elevated temperatures [41]. Genomic analysis revealed that all carnivorous fungi lack the crucial Ccc1-mediated vacuolar iron detoxification mechanism, which is conserved in most other fungi. Bayesian relaxed molecular clock analysis further suggested that the loss of Ccc1-mediated vacuolar iron storage occurred during the longest Late Paleozoic Ice Age. Inserting the *Ccc1* gene, cloned from *Saccharomyces cerevisiae*, into *A. oligospora* significantly reduced the formation of trapping devices [41]. All the results indicated that the increase in temperature causes the fungi to also need to lower the intracellular iron levels. We propose that fluctuating temperatures may prompt mammalian cells lacking proper iron detoxification mechanisms to adapt by altering their metabolic processes to deal with the control of intracellular iron content. The elimination of intracellular excess iron in cells induced by temperature fluctuations through exporting Fe^{2+} may enable cells to continuously strengthen their ability to conduct the extracellular Fenton reaction, eventually leading to tumorigenesis.

4. Converting extracellular Fenton reactions into intracellular one

Many anthraquinones and melanin precursors are well-known for their applications in food, cosmetics, and pharmaceuticals [48,49]. The polycyclic aromatic scaffolds have been extensively studied for its pharmacological properties, including antifungal, antiviral, antimalarial, antimicrobial, antiparasitic, anticancer, antiplatelet, antidiabetic, neuroprotective, laxative, and other therapeutic effects [50]. However, the mechanisms underlying the broad biological activity of these aromatic metabolites remain unclear. Our research demonstrated that CA inhibits the proliferation of human hepatocellular carcinoma HepG2 cells by transporting Fe^{2+} into the cells, thereby increasing intracellular ferrous levels [10]. Further experiments showed that mitoxantrone, an anthraquinone-based anticancer drug, and tanshinone IIA also strongly inhibited the growth of HepG2 cells by elevating intracellular Fe^{2+} levels, total iron content, and the Fe^{2+}/Fe^{3+} ratio. These findings suggest that polycyclic aromatic metabolites may counteract extracellular Fenton reactions of cancer cells by facilitating the transport of

iron into tumor cells, a phenomenon we term "extracellular Fenton reaction reflux" (Figure 2). Broadly speaking, any organism performing extracellular Fenton reactions, such as pathogenic fungi and bacteria, is likely to encounter extracellular Fenton reaction reflux induced by polycyclic aromatic metabolites. This concept may shed light on the broad biological activity of anthraquinones.

Traditional Chinese medicine (TCM) categorizes foods based on their thermal properties, classifying them as cold (寒), cool (凉), warm (温), hot (热), and other characteristics [51]. These thermal properties may be associated with the regulation of metal ions, such as iron (Fe), copper (Cu), cobalt (Co), and manganese (Mn), as well as their oxidation states [52] and the presence of reactive oxygen species like O_2^-, NO and $CO_3^{\cdot-}$. These factors can influence the promotion or inhibition of extracellular Fenton reactions in individuals consuming these foods. Plant-based foods and beverages often contain bioactive compounds such as flavonoids, small molecular benzenoids, and coumarins, which may contribute to inducing "extracellular Fenton reaction reflux" in cancer cells, as well as in pathogenic fungi and bacteria.

5. Features of extracellular Fenton reaction reflux

Our previous work demonstrated that *Escherichia coli* strains fed with toluquinol produced an unprecedented class of fluorescein-like arthrocolins with a multi-aryl carbon core through an oxygen-mediated free radical reaction [53-55]. Among these, arthrocolins A–C exhibited broad-spectrum antitumor activity against 13 tested cancer cell lines, including the paclitaxel-resistant A549/Taxol cell line [56], as well as against fluconazole resistant *Candida albicans* and methicillin-resistant *staphylococcus*. Notably, they displayed the strongest effect on human ovarian cancer cells (A2780), with IC_{50} values ranging from 0.4 μM to 0.8 μM, while normal 293T cells showed IC_{50} values of approximately 8 μM. Interestingly, A2780 cancer cells treated with arthrocolins exhibited distinct ferroptosis features, such as abnormal mitochondria with damaged cristae and membranes. Similarly, super large lipid droplets were observed in these cancer cells. Furthermore, levels of Fe^{2+} and $O_2^{\cdot-}$ were significantly elevated compared to the solvent control, while ROS and lipid peroxidation showed only slight increases. Notably, the genes *acsl4* and *lpcat3*, which facilitate lipid peroxidation, were strongly down-regulated in A2780 cells treated with arthrocolins. Among five genes associated with anti-ferroptosis mechanisms, three—*GSS*, *GPX4*, and *FSP1*—were significantly up-regulated, while *GCH1* and *DHODH* were down-regulated [56]. These findings suggest that arthrocolins may induce extracellular Fenton reaction reflux in tumors (Figure 2). Combined with results from the thermophilic fungus *T. lanuginosus*, we inferred that the extracellular Fenton reaction is characterized by elevated Fe^{2+} and $O_2^{\cdot-}$ levels and without significant transcriptional activation of *ACSL4* and *LPCAT3*, which are traditionally associated with ferroptosis. Whether the accumulation of giant lipid droplets is an important indicator remains to be verified, but it can be inferred that products that can chelate and sequester Fe^{2+} should rise, such as α-ketoglutarate.

In summary, tumor cells can perform extracellular Fenton reactions that consume significant amounts of oxygen, contributing to the Warburg effect, characterized by aerobic glycolysis in cancer cells. Fe^{2+} and $O_2^{\cdot-}$ are the primary components driving these extracellular Fenton reactions. This phenomenon is ubiquitous in nature, as Fenton reactions represent one of the earliest sources of energy and heat on Earth. Organisms such as fungi, bacteria and plants are capable of rapidly conducting extensive extracellular Fenton reactions due to the preservation of the key biosynthetic

genes *PKS*, *TPS*, and *NRPS*. In contrast, endotherms like mammals have a limited capacity for these extracellular reactions because they lack these critical genes. Metabolites derived from PKS and TPS in fungi and other organisms play an essential role in initiating and regulating the extracellular Fenton reactions of their natural hosts. Meanwhile, metabolites from NRPS and PKS primarily contribute to iron chelation, protecting organisms from extracellular damage. However, environmental stressors such as prolonged temperature fluctuations, dietary influences on iron regulation, and genetic defects in iron chelation can trigger excessive extracellular Fenton reactions of mammalian cells, potentially leading to tumorigenesis. We propose that bioactive metabolites from plants and other organisms could be leveraged to induce extracellular Fenton reaction reflux in tumors, thereby inhibiting their growth.

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Competing interests
The author declares no competing interests

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Supporting Information

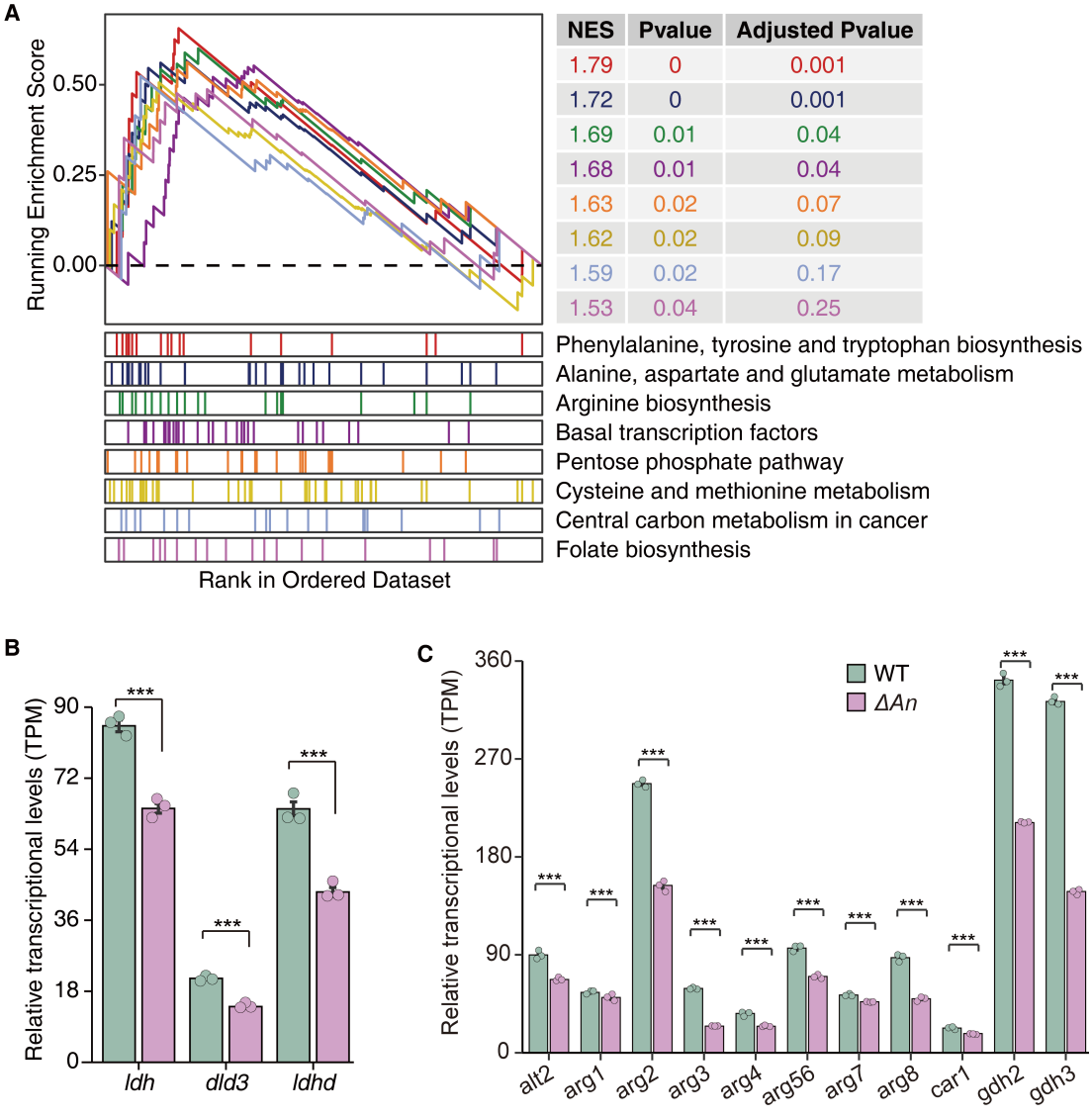
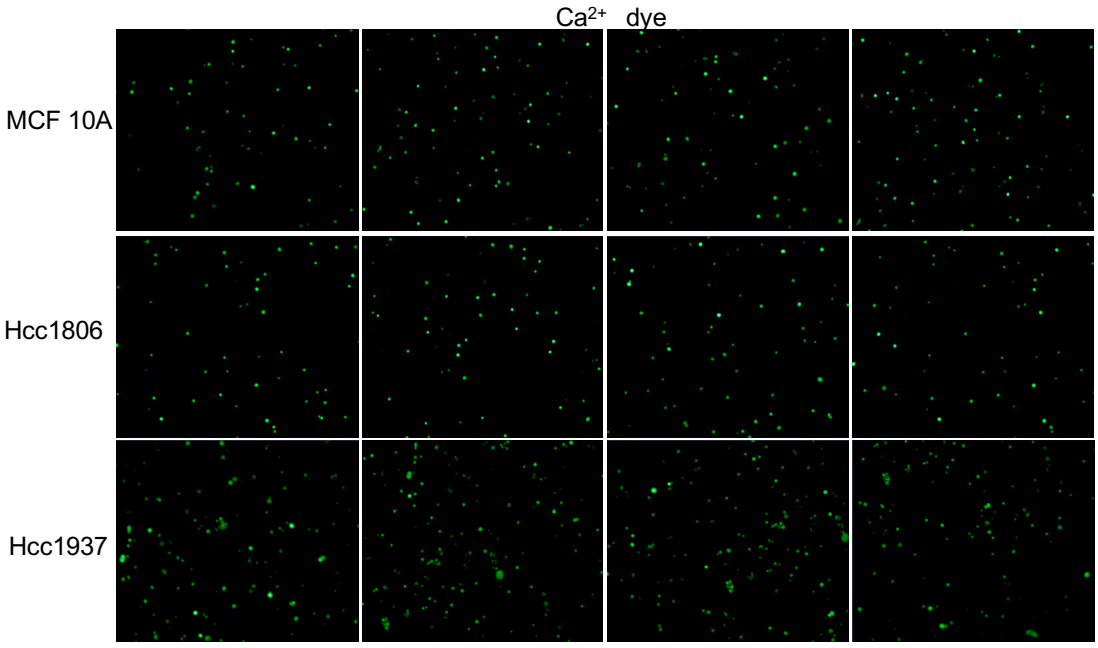


Figure S1. Transcriptional expression changes of *T. dupontii* WT compared to *ΔAn* under 37°C. (A) Significant upregulated pathways identified through KEGG-based GSEA analysis in WT (vs. *ΔAn*). The pathways shown are ordered by their enrichment scores (NES), with the top pathways displaying the most significant upregulation. Left panel shows the top-ranked pathways with normalized enrichment scores (NES) greater than 1.5. Right panel shows detail statistical values (NES, pvalue and adjusted pvalue) of each enriched pathway. Colored vertical lines in bottom panel display the positions of each gene in the pathway within the ranked gene list. (B) and (C) Transcriptional levels of genes involved in lactate and ornithine biosynthesis. Significance was tested using wald test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).



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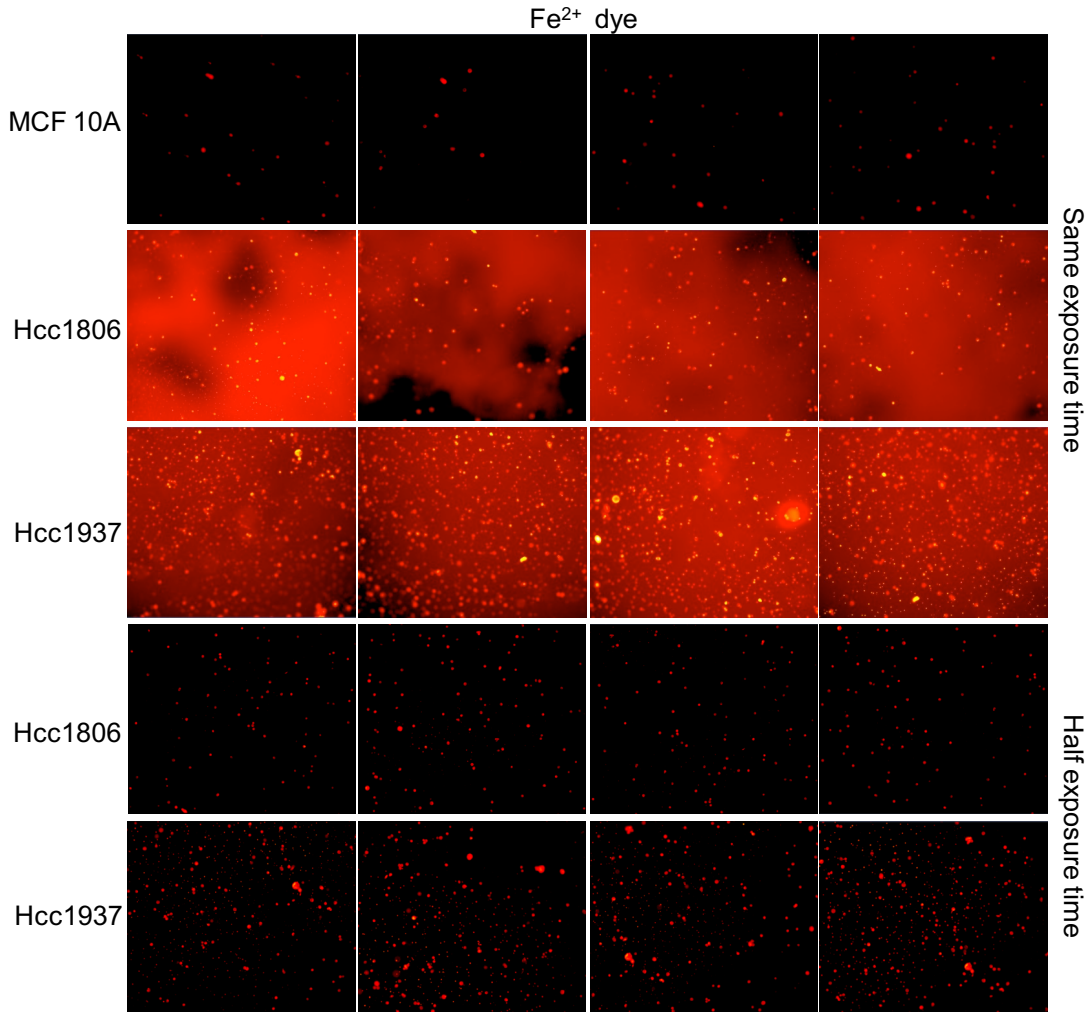
683 Figure S2. Comparisons of fluorescence response of human breast cancer cell lines
684 Hcc1806 and Hcc1937 with normal MCF10A cells to 5μM Fluo-3 for Ca^{2+}.

685



Fiugre S3. Comparison of fluorescence responses of human breast cancer cell lines Hcc1806 and Hcc1937 with normal MCF10A cells to 5μM RhoNox-1 for Fe^{2+}.

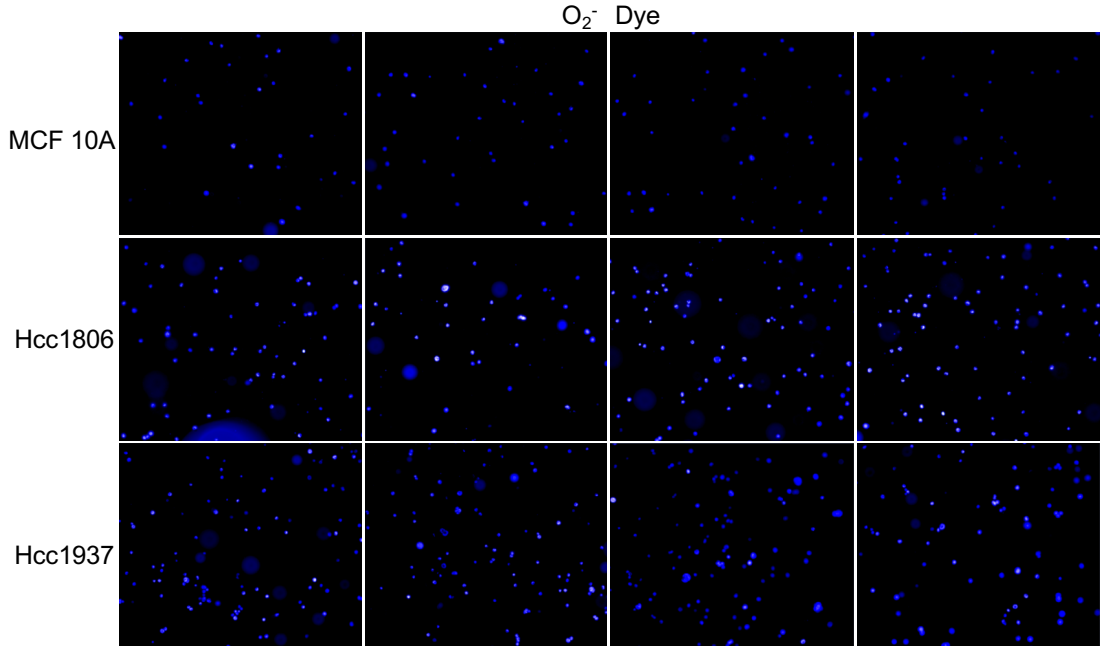


Figure S4. Comparison of fluorescence responses of human breast cancer cell lines Hcc1806 and Hcc1937 with normal MCF10A cells to 5μM Dihydroethidium for $O_2^{\cdot -}$.

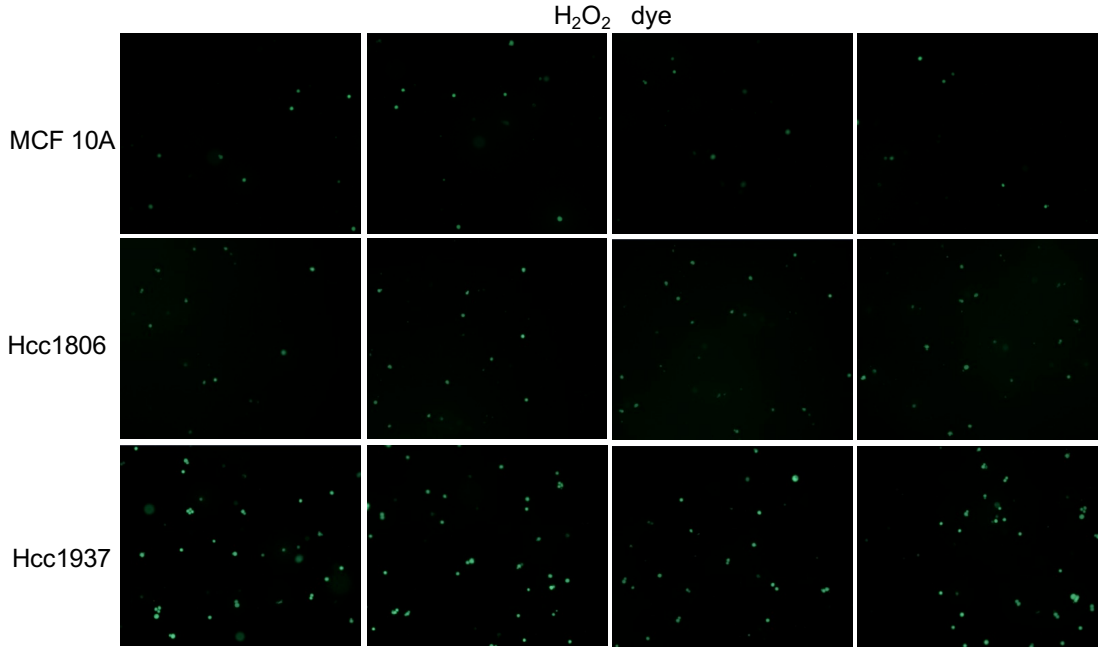


Figure S5. Fluorescence response of human breast cancer cell lines Hcc1806 and Hcc1937 with normal MCF10A cells to 5μM Peroxyfluor 1 for H_2O_2.

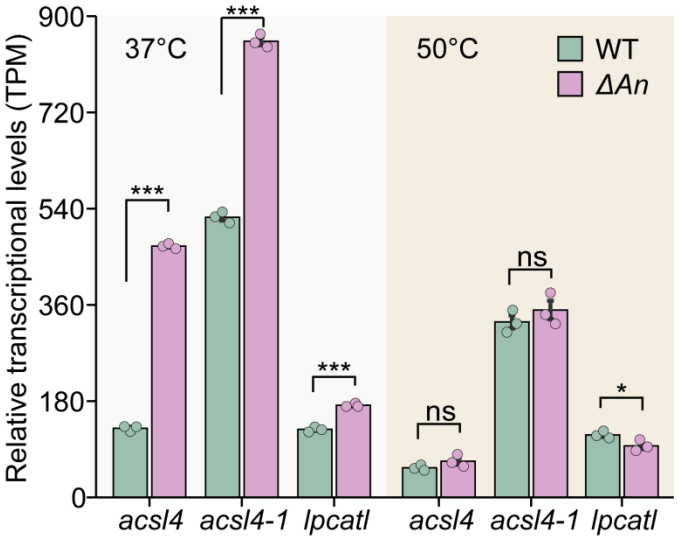


Figure S6. **Transcriptional levels of lipid peroxidation-related genes in WT and mutant *ΔAn* under at high (50°C) and low (37°C) temperatures**. Significance was tested using wald test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Method

Bioassay for the evaluation of Ca^{2+}, Fe^{2+}, $O_2^{\cdot-}$ and H_2O_2 were carried out according to previous studies [1-4]. RhoNox-1, Peroxyfluor 1, Dihydroethidium, and Fluo-3, AM were stored in a deep freezer at −80 °C to maintain stability. Each compound was dissolved in dimethyl sulfoxide (DMSO) to prepare a 1 mM stock solution. Prior to use, the stock solution was diluted at a ratio of 1:200 in 10 mM phosphate-buffered saline (PBS, pH 7.4) to achieve a final working concentration of 5 μM. The working solution was freshly prepared on the day of the experiment and used immediately to ensure reagent activity. Human breast cancer cell lines (Hcc1806 and Hcc1937) and normal MCF10A cells were harvested by centrifugation at 500 x g for 5 minutes, and the supernatant was discarded, separately. The cell pellets were washed twice with PBS (5 minutes per wash) to remove any residual media or debris. The final cell density was adjusted to 1 x 10^6 cells/mL using PBS. To perform staining, 1 mL of the prepared working solution (5 μM) was added to the cell suspensions. The mixture was incubated at room temperature for 5–30 minutes to allow sufficient uptake and reaction of the dye. Following incubation, cells were centrifuged at 400 x g for 3–4 minutes, and the supernatant was carefully removed. The cell pellets were washed twice with PBS (5 minutes per wash) to eliminate unbound dye. After the final wash, the stained cells were resuspended in 1 mL of PBS. The cell suspension was immediately subjected to fluorescence microscopy for observation. Images were captured under appropriate excitation and emission wavelengths specific to the fluorescent dye used. All observations were performed within a single experimental session to avoid signal degradation.

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