Nrf1 acts as a highly-conserved determinon for maintaining robust redox homeostasis in the eco-evo-devo process of life histories

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Date: 2024-05-24T00:00:00+00:00

Abstract

Differential and even opposing functions of two major antioxidant transcription factors Nrf1 and Nrf2 (encoded by Nfe2l1 and Nfe2l2, respectively) are determined by distinctions in their tempospatial positioning, topological repartitioning, proteolytic processing, and biochemical modification, as well as in their shared evolutionary origin. As a matter of fact, the allelopathic potentials of Nrf1 and Nrf2 (both resembling two entangled 'Yin-Yang' quanta that comply with a dialectic law of the unity of opposites) are fulfilled to coordinately control redox physiological homeostasis so as to be maintained within the presetting thresholds. By putative exponential curves of redox stress and intrinsic anti-redox capability, there is inferable to exist a set point at approaching zero with the 'Golden Mean' for the healthy survival (i.e., dubbed the 'zero theory'). A bulk of the hitherto accumulating evidence demonstrates that the set point of redox homeostasis is dictated selectively by multi-hierarchical threshold settings, in which the living fossil-like Nrf1 acts as a robust indispensable determinon, whereas Nrf2 serves as a versatile chameleon-like master regulon, in governing the redox homeodynamic ranges. This is attributable to the facts that Nrf2 has exerted certain 'double-edged sword' effects on life process, whereas Nrf1 executes its essential physiobiological functions, along with unique pathophysiological phenotypes, by integrating its 'three-in-one' roles elicited as a specific triplet of direct sensor, transducer and effector within multi-hierarchical stress responsive signaling to redox metabolism and target gene reprogramming. Here, we also critically reviewed redox regulation of physio-pathological functions from the eco-evo-devo perspectives, through those coding rules (redox code, stress-coping code, and topogenetic code). The evolving concepts on stress and redox stress were also further revisited by scientific principles of physics and chemistry, apart from two novel concepts of 'oncoprotists' and 'reverse central dogma' being introduced in this interdisciplinary and synthetic review.

Full Text

Preamble

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Abstract

The differential and even opposing functions of the two major antioxidant transcription factors Nrf1 and Nrf2 (encoded by *Nfe2l1* and *Nfe2l2*, respectively) are determined by distinctions in their spatiotemporal positioning, topological repartitioning, proteolytic processing, and biochemical modification, as well as by their shared evolutionary origin. In fact, the allelopathic potentials of Nrf1 and Nrf2—both resembling two entangled 'Yin-Yang' quanta that comply with a dialectical law of the unity of opposites—are fulfilled to coordinately control redox physiological homeostasis within presetting thresholds. Through putative exponential curves of redox stress and intrinsic anti-redox capability, one can infer the existence of a set point approaching zero with the 'Golden Mean' for

healthy survival (i.e., dubbed the 'zero theory'). A substantial body of accumulating evidence demonstrates that the set point of redox homeostasis is dictated selectively by multi-hierarchical threshold settings, in which the living fossil-like Nrf1 acts as a robust indispensable determinon, whereas Nrf2 serves as a versatile chameleon-like master regulon in governing redox homeodynamic ranges. This is attributable to the fact that Nrf2 exerts certain 'double-edged sword' effects on life processes, whereas Nrf1 executes its essential physiobiological functions, along with unique pathophysiological phenotypes, by integrating its 'three-in-one' roles elicited as a specific triplet of direct sensor, transducer, and effector within multi-hierarchical stress-responsive signaling to redox metabolism and target gene reprogramming. Here, we also critically review redox regulation of physio-pathological functions from eco-evo-devo perspectives, through those coding rules (redox code, stress-coping code, and topogenetic code). The evolving concepts of stress and redox stress are further revisited through scientific principles of physics and chemistry, apart from two novel concepts of 'oncoprotists' and 'reverse central dogma' being introduced in this interdisciplinary and synthetic review.

Keywords: Nrf1, Nrf2, homeostasis, determinon, topogenetics, redox stress, eco-evo-devo, redox code, stress-coping code, oncoprotist, 'zero theory', and 'reverse central dogma'

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1. Introduction

A search of PubMed-cited literature on reductive and oxidative (collectively termed redox, but dictated by gain or loss of electrons and/or hydrogens, respectively) and anti-redox (antioxidant and anti-reduction) reveals an overwhelmingly large number of publications, with at least 2,177,092 entries in this library (as of December 18, 2023). As shown in Figure 1 [Figure 1: see original paper], this constitutes an exponentially expanding field with broad implications for physiology, pathophysiology, biomedical and life sciences—except for its slow start over a century after the first experimental report in 1807 by Dispan on the gaseous oxide of azote and the second in 1811 by Wollaston on cystic oxide, a new species of urinary calculus. Nonetheless, the great majority of redox studies have focused disproportionately (95%) on oxidative and antioxidant topics, whereas research on reductive and anti-reduction topics accounts for less than 0.5% of the literature (Figure 1, top box). This distinction demonstrates that (anti-)reductive research has been largely neglected for a considerable time. Only recently has the crucial importance of (anti-)reductive studies begun to be gradually recognized in the redox research field, though this still requires further emphasis.

The concept of oxidative stress was first proposed by Paniker et al. in 1970 and later formulated by Helmut Sies in 1985, then further redefined by Dean P. Jones in 2006. Although this continuously heated topic on redox stress (including reductive stress) and redox defense response seems to have thoroughly penetrated

the cell stress zeitgeist, it remains open to great concern from workers in distinct fields and represents one of the most persistently intractable problems to be addressed for health and disease, particularly across vast varieties of changing environmental conditions. Among its merits elicited by evoking biological stress responses, a steady-state redox balance is constantly maintained within a homeodynamic threshold range by cell respiration, aerobic metabolism, and redox switches governing redox stress defense responses. Meanwhile, the pitfalls of oxidative stress could lead to another indiscriminate use of this term as a global concept without clear relation to 'real redox chemistry' per se in particular cases. At the molecular level, the major roles for antioxidant and anti-reductive defense systems are fulfilled predominantly by redox-controlling biomolecules such as key proteins, enzymes, transcription factors, and co-factors involved in cellular redox biochemical and metabolic processes, as well as by the adaptive reprogramming of relevant gene expression profiles in normal physiological and even pathophysiological responses to redox stress during distinct eco-evo-devo stages of life histories.

In this synthetic review, we first introduce the past and present of redox-based systems in the 'redox central theory' (distinct from but correlative to the generally nonspecific 'stress-coping system'), along with their pivotal roles during life's origin and ensuing evolution. Second, we critically review redox regulation of physio-pathological functions from eco-evo-devo perspectives, through distinct mechanisms and obligate coding rules (e.g., redox code, stress-coping code, and topogenetic code, all selected by nature). Third, the evolving concepts of stress and redox stress are further revisited through scientific principles of physics and chemistry, together with quantitative stratification of redox stress and anti-redox response through the 'zero theory', in addition to two novel concepts of 'oncoprotists' and 'reverse central dogma' proposed via interdisciplinary synthesis. Fourth, we present a host of convincing evidence revealing that the antioxidant Nrf1 (nuclear factor, erythroid 2-related factor 1, encoded by *Nfe2l1*) is a living fossil transcription factor that is considerably closer than Nrf2 to their common ancient orthologues arising from the eco-evo-devo process of life histories. Notably, Nrf1 also exhibits unique pathophysiological phenotypes that are distinctive or absent from Nrf2 (nuclear factor, erythroid 2-related factor 2, encoded by *Nfe2l2*). This is attributable to the unique topogenetic folding of Nrf1 and dynamic topovectorial repositioning of this CNC-bZIP factor across endoplasmic reticulum (ER) membranes to dislocate to the nucleus before transcriptional regulation of cognate target genes. Finally, we critically review the uniquely differentiated yet integrated roles of both Nrf1 and Nrf2 in governing cellular redox, energy, and metabolism homeostasis and organ integrity during distinct life processes, in which activation of Nrf1 is evoked by distinct regulatory mechanisms. Overall, the allelopathic potentials of Nrf1 and Nrf2 (both dubbed as two entangled 'Yin-Yang' quanta abiding by a dialectical law of the unity of opposites) are fulfilled to coordinately control redox physiological homeostasis within certain homeodynamic threshold ranges, which are perpetually maintained for normal healthy survival.

2. The far past and present of redox-based systems

2.1 'Central redox theory' refined by nature

From the origin of life and its ensuing evolution to the modern present of the 21st century, all cellular life forms have experienced vast challenges within diversely changing environments, a major one of which is spawned predominantly by cellular stress—particularly redox stress—threatening normal homeostasis of a life system. This is because life fundamentally depends on the free energy (ΔG) provided by electrochemical disequilibrium between reduced (electron-donating) and oxidized (electron-accepting) environmental substrates and possesses a crucial ability to further convert such redox environmental disequilibria into intracellular disequilibria, as defined by Schrödinger. Redox (i.e., electron-exchanging) reactions existed early in both the chemical and metabolic worlds during the origin of life and have persisted in all life processes throughout the entire evolutionary course until now. Notably, reductive stressors likely provided a primordial force sufficient to meet essential bioenergetic needs for the origin of life and its long-term evolution and have been embodied and embedded in present-day life processes. This remains accompanied by an ancient anti-reduction strategy for fitness to prevent excessive reduction of intrinsic compounds by undergoing reduction themselves or by removing ambient dihydrogen (H_2, which enables reduction of sulfate to H_2S, along with oxidation of certain organic materials). However, much less attention has been paid to such anti-reductive responses to reductive stress relative to their counterparts in the overwhelming majority of studies on prevailing antioxidant responses to oxidative stress.

A bulk of accumulating evidence has demonstrated that the hitherto known free radicals and reactive species of sulfur (RSS), nitrogen (RNS), oxygen (ROS), carbonyl (RCS) and halogen (RHS), along with transition metals (e.g., Fe, Cu, Mn, Zn, Ni, Co, Mg) and minerals (e.g., Se, As), can all be collectively construed as 'Redox X species' that serve as constantly electron-exchanging key players in distinct series of multi-hierarchical redox reactions, relevant interactions, and/or relationships with biomolecules. In this process, a considerably large portion of these active reactants and redox products per se have a Janus face, being both reduced and oxidized as 'redoxidants' (for example, as shown in Figure 2A [Figure 2: see original paper]). It is crucial to notice that redox balance between redox stress and anti-redox response is maintained for healthy life processes in a certain robust homeostatic state, as summarized in the 'redox code' with a particular conceptual proclivity for ROS-based oxidative stress and antioxidant protection. The inaccurate scenario of 'central redox theory' biased toward the oxidative stress defense system should have been established on the foundation of the so-called 'ox-tox' hypothesis, as a commonly accepted antioxidant mechanism to detoxify the burgeoning production of O_2 and ROS. This is exemplified by the statement believing that activation/deactivation cycles involving O_2 and H_2O_2 are "richly elaborated in the oxygen-dependent life where they contribute to spatiotemporal organization for differentiation, development, and adaptation

to the environment" and where "disruption of the organizational structure during oxidative stress represents a fundamental mechanism in system failure and disease."

2.2 The evolving redox-based systems

Cellular life began in an anoxic ferrous ocean approximately 3.8 billion years ago (Ga) and then experienced nearly seven-eighths of its evolutionary course under almost no or less (< 0.1% PAL) oxygenized environments. As such, the appearance of those so-called 'classic antioxidant' enzymes—including catalase (Cat), superoxide dismutases (SODs), glutaredoxin (Grx), thioredoxin (Trx), peroxiredoxin (Prx), sulfiredoxin (Srx), and other oxidoreductases—seems to be rather coincidentally and closely aligned with the origin of anoxygenic photosynthesis (i.e., H_2S with CO_2 is reduced to yield methane and oxidized polysulfides) over a billion years prior to the great oxidation event (GOE, arising from incrementing oxygenic photosynthesis where H_2O with CO_2 is oxidized to yield O_2 and methane). Such O_2/ROS-detoxified defense systems, together with early respiratory nitric oxide (NO) reductases (NORs, structurally related to present-day respiratory O_2 reductases, alias cytochrome/quinol oxidases or Complex IV), were further corroborated to exist in 'obligate O_2/ROS-tolerant anaerobes' created at early stages of life's evolution, and even emerged in primordial populations of protocells known jointly as the last universal common ancestor (LUCA) of all life forms on Earth. Importantly, several lines of experimental evidence have validated that such 'classic antioxidant' enzymes (e.g., Cat, SOD, Trx, Grx, Prx) are essentially involved in the redox metabolism of H_2S and polysulfides (H_2Sn, n=2-8), which are collectively referred to as RSS and viewed as a physiologically relevant O_2-sensing mechanism. Altogether, it is inferable that these 'antioxidant detoxification' systems should be lent and also evolutionarily extended from coping with prior sulfur-based redox reactions in anti-redox signaling response to RSS, possibly via RNS, toward dealing with nowadays prevailing oxygen-based redox reactions in relevant cytoprotective response to ROS (Figure 2B). This notion was also proposed elsewhere, albeit it remains truly hard to distinguish disparate causal efficacy of RSS from ROS in relevant redox stress responses by means of hitherto established methodologies, whereby RSS was rather measured more sensitively than ROS per se.

Clearly, it was conjectured from mounting evidence that the onset of life was driven by redox disequilibria resulting from the evolution of nascent terrestrial niches, followed by co-evolution with its surviving environments during a long-term transition from highly reducing (H_2S/RSS) to gradually oxygenated (O_2/ROS) status. Such biological evolution of life takes place abiding by (pre)Darwinian law of natural selection for novelty to ensure that its functional information will increase within the possibility space of configurations, which are also preferentially selected based on function and enable all those growing functions to be efficiently performed during the redox-driving process (Figure 2C). Hence, the origin of life and its ensuing evolution appears to

be 'open-ended,' forging adaptations to changing environmental challenges and constructing expectable functional configuration spaces, all of which are selected in a stochastic manner. Collectively, the redox-driving force (provided by redox recycling reactions and interactions) should play a central role for the evolving life system to be selectively self-organized, self-replicated, and self-maintained. In the redox central theory, redox code remains as a quintessence of conserved functional traits achieved from adaptive co-evolution of life with its environments.

Yet, such 'redox code' should also be refined by encompassing H_2S/RSS-based redox and anti-redox principles, because this ever-existing H_2S/RSS-based system exerts certain pivotal and indispensable effects to be stored for 'redox memory' and then reused for 'redox recycling,' which are substantially disparate from those 'one-off' effects of the O_2/ROS-based redox system. More importantly, a considerable key portion of the O_2/ROS-based redox and anti-redox effects within the 'originally-defined redox code' are de facto transferred to the H_2S/RSS-based redox system and hence indirectly executed by this reactive thiol-switching system.

3. Redox regulation of physio-pathological functions in the nature eco-evo-devo process

3.1 Redox regulation by distinct mechanisms

As an evolving self-organizing system, life is simply dichotomously defined to comprise two parts (selected for adaptation and variation) as a whole, by Darwinian evolutionary law, along with another law of incrementing functional information, albeit as its complexity is gradually increased. The major stably selected portion of life should be made of those predominantly statically persistent systems with stronger robustness. By stark contrast, the remaining variated part of life should be principally composed of those dynamically persistent systems with versatile plasticity to explore novelty. This variating novelty is determined by newly generated organic codes—an arbitrary set of principally-coding rules from a geological point of view that amounts to a sudden event—which could yet be predicted by experience-building memory (storage of functional information acquired by sensing and measuring) and/or by a memory-independent fashion. Thereby, it is plausible that those stable configurations of life and its selected physiological functions should be maintained and perpetuated by robust homeostatic mechanisms and further remodeled by two distinct and even opposing mechanisms: homeorhesis and allostasis, respectively.

In this regulatory process, redox and anti-redox systems certainly play vital determinant roles in orchestrating the configurations of life during morphogenesis and regulating the architecture of its physiological functions at normal morphostasis. This is because redox and anti-redox systems have inherently evolved to establish a set of lingua franca for life, unifying the entire eco-evo-devo (i.e., ecological, evolutionary, and developmental biological) process. Besides the 're-

dox code,' there also exist innately codifying biological mechanisms executed directly by redox electrochemical reactions and relevant specific biochemical modifications. As aforementioned, most of these (but not all) enable storage for 'redox memory' within the redox-derived lingua franca dictionary and, if later required for biological needs, reuse for 'redox recycling' to directionally switch redox regulation of physiological and pathophysiological functions. These redox-reactive species and redox-bearing carriers also enable transport throughout intracellular and extracellular (interstitial) fluids, allowing efficient redox signaling communications between different subcellular organelles, distinct cell lineages, and diverse tissues and organs.

Such redox communications yet remain, to certain extents, confined by inherent 'kinetic' barriers that frustrate their immediate diffusion and free dissipation to reach some (electrochemical) equilibrium, leading to disparate redox-compartmentalized distributions throughout intracellular and extracellular contexts. Consequently, discrete 'redox potential gradients' with selective modularity are established in diverse topovectorial processes and 'phase spaces' that are redoxokinetically compartmented to give rise to distinct redox potential energy capacitors (Figure 2D), as evinced by distinct status of 'redox phenotypes.' Such 'redox gradients' have been shown to act as an internal impetus to embryogenesis and ensuing physiological development and healthy growth during life processes. Conversely, 'aberrant redox gradients' have also been implicated in aging and pathogenesis of relevant diseases.

In addition to redox reactions and interactions with all other regulatory elements (e.g., organic codes, topogons, minimotifs) and signaling molecules in the redox interactome and systems biology, redox electrochemical reactive systems also enable arousal of certain extents of their intrinsic quantum biological effects on relevant physio-pathological regulation by virtue of their unique quantum mechanical mechanisms. Thus, it is inferable that such redox-reactive quanta (along with putative quantum effects exerted possibly via a system of 'meridians' recognized in the long history of traditional Chinese medicine) could play an essential role in critically unifying all systems networks to regulate diverse physiological and pathophysiological functions across all distinct strata of life from molecular and subcellular levels to the whole body.

3.2 'Redox code' acquired and refined from the eco-evo-devo process

The self-organizing life appears to be also conjectured as a self-manufacturing artifact by self-copying and self-codifying according to those pre-existing templates and/or coding rules, all of which are selected by (pre)Darwinian evolutionary law during its natural eco-evo-devo process. All distinct types of constituents of life (e.g., components, adaptors, topogons, minimotifs, templates, and codes) are stereochemically patterned and further self-assembled in certain topovectorial phase spaces to yield diverse topoforms (each with specific physiological functions and unique behaviors) of molecular machines, subcellular apparatus, body-planned cells, tissues, organs, or organismic individuals (Figure 2D). All

of these have been fulfilled according to their cognately coding rules in combination with their topological orders, biochemical relationships, and ontological status. In reality, most components and adaptors of living systems should be plastically changed in the long run of eco-evo-devo processes, but only a few of them (including codes, templates, minimotifs, topogons) must be absolutely conserved to gain the robustness of living systems. Notably, the arbitrary coding rules are also highly conserved because they serve as a universally governing mechanism that Nature has constantly employed in the course of life's origin and ensuing evolution. From the eco-evo-devo scenario, it is inferable that 'redox-based coding rules' should predate the establishment of the commonly accepted genetic code and other organic codes, because the 'redox code' (defined by Jones & Sies) originally took an early leading place, particularly in transforming the inanimate to animate worlds. This notion is based on the finding that far ancient genetic templates are likely executed by living fossil-like 'basic-region zipper minimotifs,' albeit with low infidelity, which were later evolutionarily replaced by polynucleotide templates (i.e., RNAs or DNAs).

According to the general 'code theory' modeled by Barbieri, redox code is referred to as a set of consensus rules for codifying all redox-based spatiotemporal systems and their interactions with other biological systems in the body-planning networks of life, tightly governing its intrinsic anti-redox responsive mechanisms to cell stress. Such 'refined redox code,' like those organic codes, is also reckoned to have gone through five possible phases from its origin to complete establishment. In the beginning, its 1st version emerged possibly as a means of performing a series of particular redox-relevant functions by diverse redox reactive cascades with organic macromolecules, but with certain necessary ambiguity to enable closer linkage of the inanimate to animate worlds during life's origin. Such ambiguity was, in the 2nd phase of redox code, steadily reduced by gradually improved compartmentalization of redox-based systems to yield distinct gradients and stereotyped capacitors with different redox potential energy, enabling life to effectively distinguish its internal milieu from surrounding environments while allowing certain interchanges between internal and external environments across its membrane-based platform system. In the 3rd phase, the redox code was optimized by selecting its specificity to monitor particular physiological functions by gradient redox potential energies within distinct redox compartments and across redox-configured microdomains, e.g., by NAD(P)H/$NAD(P)^+$-driven redox metabolisms and O_2/H_2O_2-, NO/RNS-, or H_2S/RSS-leading redox signaling networks, together with relevant (bio)chemical modifications. In the 4th phase, its major transition is most likely yielded by redox switching of putative physio-pathological functional activation or inactivation of some molecular machines and subcellular apparatuses primarily by thiol-active and/or -reactive redox (re)cycling networks, especially upon cellular redox sensing to signaling responses with interactomes. The stress-leading damage repair and disposal mechanisms are also likely embedded in this major transformation phase, manifested by certain changed configurations. In the last 5th phase, conservation of redox code is de facto embodied by either program-

ming or reprogramming of redox signaling to cellular redox metabolism and relevant gene expression profiling critical for genetic, epigenetic (and topogenetic) responses to diverse redox stress. The inheritable conservativity of redox code may be enforced by intra- or inter-generational incorporation of selenocysteine (Sec), persulfidated cysteine (CysSnH, n $\geq$ 2), and other redox-active not-yet-identified amino acids (e.g., HO-Pro) into redox proteomes during alternative translation of potential redox-responsive gene transcripts. Notably, those known basic-region (zipper) superfamily transcription factors—HIF1α, Nrf2 (encoded by *Nfe2l2*) and Nrf1 (encoded by *Nfe2l1*, Figure 3A [Figure 3: see original paper]), together with NF- B and FOXO—are identified to act as conserved players in redox programming or reprogramming responses and further govern proteostasis of the redox proteome during coping with stress.

3.3 'Redox kinetic barriers' dictated by membrane topogenesis (via 'topogenetic code')

Differential positioning of redox gradient distributions in distinct topovectorial phase spaces and subsequent repartitioning of redox-based systems across diverse topovectorial spaces are all dictated predominantly by redox-compartmentalized membranes serving as certain 'kinetic' barriers (including redoxsome). More importantly, such membranes have fulfilled an irreplaceable pivotal role in transmitting architectural orders during self-organization of cellular life. This is precisely because cell membranes possess an indispensable topological property: they are never constructed *de novo*, enabling them to always grow from pre-existing membranes and pass down from one generation to the next in an uninterrupted chain of descent. Since this remarkable innate property of cell membranes has been defined as chromosome-like 'membrane heredity,' it is inferable to be determined by its ever-existing but not-yet-identified 'topogenetic code' for manufacturing distinct types of membrane topogenesis and further orchestrating most of the self-assembling topobiological structures of cellular life in real spatiotemporal phases on diverse membrane-based platforms to perform accurate physiological functions in orderly organization. Such place (i.e., position in space)-dependent morphogenesis endowed with unique physiological functions during the eco-evo-devo process is dictated by membrane-associated topology and topobiology.

Initially, topobiology was only referred to as the place-dependent interactions of differential cell adhesion to extracellular matrix (ECM) via membrane-based platforms to drive morphogenesis in the developing embryo and the origin of living systems. This paradigm was later revised to include force-dependent molecular switches, cell and tissue tension, and reciprocal interactions with microenvironments through conserved decision-making modules (switch, connector, capacitor, transistor, and topogon) to specify morphogenesis of multiscale forms with unique functionality. Morphostasis is maintained by spatial segregation and organization of those anchored proteins and secreted factors through emergent properties of tissues, including tension fields and energy op-

timization. The concept of topobiology, as an important key decision-making mechanism accounting for the eco-evo-devo process, has been further refined by applying original mathematical and physical laws of topology into life science and medicine, aiming to unlock real Gordian knots (e.g., of redox electrochemical and quantum systems) in biology. Collectively, such inheritable membranes cannot only determine compartmentalization of redox microdomains and their kinetic systems but also dictate orderly morphogenesis of self-organizing life and its morphostasis (of a given topoform with specified physiological function).

On evolving membrane-based platforms, the redox-reactive system of H_2S/RSS together with iron sulfide (FeS) plays a vital role in morphogenesis and morphostasis of early life. This is supported by molecular evolution of ancient ferredoxin (Frx), which consists of only 23 aa with four Cys residues enabling a [4Fe-4S] cluster to be anchored on a positively charged mineral surface and thus mediating electron transfer in the primordial membrane system prior to the origin of species diverged from LUCA. Subsequent evolutionary respiratory NO to O_2 reductases, possibly with Frx, contribute to energy metabolism by distinct species' membrane systems but also to the yield of H_2O_2/ROS, NO/RNS, and H_2S/RSS in electron transferring processes.

The membrane-tethered NAD(P)H oxidases [NOX, also called NAD(P)H-dependent reductases] cannot only reduce O_2 to yield most of ROS (i.e., $O_2 \bullet^-$ and H_2O_2) and remove O_2 to water (H_2O). Furthermore, O_2/Fe^{2+}-dependent prolyl hydroxylases (PHDs, as transmembrane enzymes governing HIF1 turnover but conversely induced by HIF1), together with Cu^{2+}-dependent lysyl oxidases (LOXs) of collagens (secreted from the endoplasmic reticulum to the ECM), dictate rigid formation of their stereotyped 'tensor networks' by multiple crosslinking with cell adhesive molecules, transmembrane-tethering proteins (e.g., integrins and fibronectins), and consecutively associating with membrane-enclosed cytoskeleton and karyoskeleton, together with all subcellular organelles connected with one another through their membrane extensions (e.g., stromules, peroxules, and matrixules). Collectively, these membrane-based topobiological mechanisms (possibly via 'topogenetic code' rules for body plan) cannot only enhance compartmentalization of redox microdomains and their kinetic systems in all distinct intracellular and extracellular matrix contexts but also determine distinct topoforms of cell lineages (by their interaction forces), tissues, organs, and even individual beings in distinct positioning sizes of topovectorial phase spaces.

3.4 Coordinated control of redox responsive mechanisms by Nrf1 and Nrf2

Overall, such diverse topogenetic phenotypes are also modulated by distinct redox status, dubbed 'redox phenotypes,' which are maintained at certain physiological homeostasis during normal conditions. Once such homeostasis (along with morphostasis) is markedly disrupted by excessive redox stimulation for a long term, this results in relevant pathophysiological deterioration and

pathogenic phenotypes of many chronic diseases including cancer, diabetes, atherosclerosis, and neurodegenerative diseases. Hence, to combat excessive redox stimulation, all cellular life forms have been evolutionarily armed with a series of innate powerful anti-redox defense systems. Among these is a set of essential anti-redox, detoxification, and cytoprotective mechanisms governed by the cap 'n' collar and basic region leucine zipper (CNC-bZIP) family of transcription factors. Strikingly, Nrf1 and Nrf2 are two principal CNC-bZIP factors in vertebrates that finely tune transcriptional expression of cognate genes by binding consensus antioxidant or electrophile response elements (AREs/EpREs) in their promoter regions. In fact, we have unraveled that the allelopathic potentials of Nrf1 and Nrf2 (both resembling two entangled 'Yin-Yang' quanta that comply with a dialectical law of the unity of opposites, as illustrated in Figure 3A) are exerted to coordinately govern redox physiological homeostasis maintained within presetting threshold ranges.

To date, numerous studies on Nrf2 have revealed that it functions as a master regulator of antioxidant response and relevant redox signaling. However, such versatile Nrf2 acts de facto as a promiscuous but not essential player for optimal ARE-binding to most of its target genes. This supports the conclusion that Nrf2 is dispensable for normal growth and development, with no obvious pathological phenotypes manifested in its global knockout mice. As a matter of fact, Nrf1, rather than Nrf2, is a living fossil with ancestral properties because it shares evolutionary conservativity with SKN-1, Cnc, and Nach factors. Like its ancient homologues, Nrf1 is topologically integrated within the endoplasmic reticulum (ER), then repartitioned and dislocated across ER membranes to enter extra-ER subcellular compartments, where it is processed to yield a mature N-terminally truncated factor, similar to Nrf2, before transactivating its cognate target genes.

Collectively, such a highly conserved, indispensable role is fulfilled by Nrf1 (with a unique topobiological feature), but not by Nrf2, for maintaining the steady-state threshold of normal redox homeostasis. This likely further dictates morphostasis of distinct topogenetic phenotypes with healthy physiological functions, because loss of its function results in severe endogenous oxidative stress, accompanied by disruption of cellular lipid, glucose, and protein homeostasis and organ integrity, leading to cancer development and malignancy, even though Nrf2 is aberrantly accumulated in such Nrf1α-deficient cells (Figure 3B) [115].

4. Scientific conceptual evolution of redox stress from physics and chemistry to biomedicine

4.1 Axiomatic concepts of stress and relevant parameters revisited by principles of physics

4.1.1 Physics-based concepts of stress and parameters in biology Although contemporary concepts regarding 'stress' employed conventionally across scientific disciplines have evolved over the past 2½ millennia, they remain a hot subject of distinct scientific debates. In fact, the word 'stress' originates

etymologically from the common proto-Indo-European root 'str,' historically associated with exertion of pressure. Until 1660, 'stress' was introduced in mechanical physics and accurately formulated by Hooke's law of elasticity ($F = \Delta L \cdot k$); the relationship of 'stress' ($\sigma = F/A = \Delta L \cdot k/A$, a ratio of the internal force brought into play when a substance is distorted to the area over which the force acts, whereas the external force producing the distortion was defined as 'load') over the resulting 'strain' ($= \Delta L/L_0$, a ratio of such distorted change in size or shape to their original ones) was further scientifically defined by Thomas Young's elastic modulus ($Em = \sigma/ = L_0 \cdot k/A$) in 1807. Such original meaning of stress per se was also used to denote the internal force generated within a living body by any force that leads to strain (or distortion) of the body. Hooke's law was recently extended by Dietmar Kültz for application in biological systems, aiming to properly and unambiguously refine the concept of stress by its original physical principles, which was hence restated as $F = \Delta Hc \cdot k$. Here, k is a constant describing the phenotype of a biological system at the time of exposure to force F arising from stress, while ΔHc represents the extent of dysregulation of this system differing from its set-point of the homeostatic norm (H) of the most critical and limiting physiological variables c.

As $F = f(\Delta Hc) \cdot k$ was refined here, this is because a maximally informative yet minimally complex set of variables ΔHc may be modeled by a certain function (f) of their core endogenous network and further integrated ( ) in ontogenetics and phylogenetics, the topology of which would accurately reflect the amount of strain (deformed) on this system during stress. Since 'stress σ' and 'strain ' were restated as $\sigma = F/R = f(\Delta Hc) \cdot k/R$ and $= \Delta Hc/Hc$, respectively, this system's elasticity (or plasticity) may be formulated as $P = [f(\Delta Hc)]/\Delta Hc \cdot Hc \cdot k/R$, and further simplified as $P \quad Hc \cdot k/R$, only if $f(\Delta Hc) = \Delta Hc$, in which R represents the robustness of a living system (i.e., cell or organism) and acts as an inherent property of this evolving, complex dynamic system. Moreover, if the stress potential energy is stated as $Es = ½ k \cdot (\Delta Hc)^2$, it may be rewritten as $Es = ½ k \cdot (\Delta G + \Delta S \cdot T)^2$, because free energy ($\Delta G$) is formulated by $\Delta G = \Delta Hc - \Delta S \cdot T$. Herein, the stress-reactive entropy is represented by ΔS, due to the nature of stress at the atomic level in dense polymer systems (of cell or organism) being classically viewed as molecular, based on the 'entropic spring' concept, stating the intrinsic monomer stress by contribution of the individual monomer to the macroscopic stress referred to a local moving coordinate system in which its backbone bonds attached to the monomer are fixed. ΔG equals the responsive work, i.e., $W = \Sigma(WCSR+WCHR+WOHR)$, arousing a counteracting impetus of the host against stress by distinct coping mechanisms. Collectively, stress, strain, plasticity, and robustness, along with other relevant parameters, can all be subjected to precision evaluation and axiomatic demarcation by principles of physics.

4.1.2 Non-physics-based concepts of stress response evolving in biomedicine However, it should also be noted that all those other evolving definitions of stress, as known so far, seem largely irrelevant to the original

meaning of its physics. As such, multi-faceted stress and relevant responses remain partially delineated by their evolving concepts for nearly a century after being introduced into biology, revealing the reaction and interaction of diverse changing environmental (including social and psychosomatic) stimuli with all life systems from distinct aspects of bioscience and medicine (Figure 4, A & B).

From the fluid matrix of life's body demarcated as 'milieu interieur' by Claude Bernard to its steady state named 'homeostasis' by Walter Cannon, the stress and strain of homeostasis were first described in a biological system 'in terms of a homeostatic index (H_0/Hc), which states the measured ability to react, without disturbance of the fluid matrix, to a group of standard stresses that may readily disturb it' or cause 'an excessive strain upon the protective agencies.' Later, stress was defined by Hans Selye as a 'nonspecific deviation from the normal resting state; it is caused by function or damage,' but also 'stimulates repair.' This is due to 'the facts that stress is not necessarily the result of damage but can be caused by physiological function, and that it is not merely the result of a nonspecific action but also comprises the defense against it.' Thereby, the remaining portion, after subtraction of all specific changes caused by distinct stimuli and their reacting targets, is considered as 'the general-adaptation syndrome (GAS) produced by diverse nocuous agents.' The GAS was determined by such nonspecific commonly shared responses to all types of stress, with all relevant activities (e.g., affecting behavior, temperament, physiological homeostasis, and anatomic morphostasis) being harmoniously integrated by the hormonal and nervous systems of organisms. The autonomic neuroendocrine responsive systems (e.g., HPA and SAM predominantly) and extended immune systems to any stress were collectively referred to as the 'stress response system.' By contrast with physiological protective responses for the purpose of fit healthy resilience, prolonged or exaggerated stress is rather accompanied by a continuum from inappropriate adaptive to maladaptive responses, ultimately leading to development of a series of pathological phenotypes and even chronic diseases (i.e., 'stress-led syndromes,' including psychosomatic disorders).

4.2 Ontological concepts of redox stress revisited by principles of chemistry

4.2.1 Scientific basis for redox stress in chemistry Redox stress is integrated in terms of a Janus-faced parameter of redox electrochemical potential disequilibrium arising from 'biologically inappropriate' reactive imbalances amongst all oxidative, antioxidant, reductive, and anti-reductant agents involved during interaction of adequate stimulus with the host and ensuing transformation to its adaptive responses. This collective definition of whether it is prone to favor oxidative or reductive stress depends on the principle of which stress changes the nature of redox potential energy. This may be accurately determined by the Nernst-Peters derived equation $Eh = E_0 + 0.03 \cdot \log_{10}[\text{oxidant/reductant}]$. Hence, $\Delta Eh = Eh - E_0$ was calculated by a logarithmic ratio of [oxidant] to [reductant] ($\Delta Eh = 0.03 \cdot \log_{10}[\text{oxidant/reductant}]$; their

relationship was also shown in a sigmoid buffering curve). The term 'near equilibrium' is defined to describe reactions in which there is essentially zero free energy ($\Delta G = 0$) available from putative electrons (and/or hydrogens) being transferred or exchanged because this reaction is maintained so very close to an equilibrium of redox distribution between reactants and products. That is, $\Delta G = \Delta Eh = 0$, such that [oxidant] is equally balanced with [reductant].

Therefore, it is inferable that such a redox equilibrium of biological systems in cells or organisms should be preserved only by tightly governing a ratio of Σ[oxidants] to Σ[reductants] balanced to near zero extents (as theoretically shown in Figure 4C). In such a case of imbalanced redox status, whether an onset of oxidative or reductive stress occurs depends on the antioxidant or antireductant capacity of life, respectively, together with its detoxifying cytoprotective capability in its emergency response system. This poses a challenging question of how cells and organisms deal with a mounting pool of redox equivalents to properly balance the generation of 'reactive X species' (including ROS, RNS, and RSS) and their elimination, and optimize all those redox buffer couples (e.g., NADH/NAD^+, NADPH/$NADP^+$, GSH/GSSG, CysS/CysSX, O_2/H_2O_2, and H_2S/RSSn), particularly during cellular stress. To address this question, it is required to clearly figure out the scientific distinctions amongst oxidative (oxidant) stress, reductive (reductant) stress, and redox stress, just because their previously confounded terms have been employed disparately by biologists but distinguishably by chemists.

4.2.2 Debating concepts of oxidative stress in biology The term 'oxidative stress' was initially used in 1970 by Paniker et al. for a study of erythrocytic glutathione metabolism affected by glutathione reductase (GSR) deficiency, which was determined by measuring a ratio of GSH to GSSG in the cell response to exogenous H_2O_2 (viewed as a stimulus of oxidative stress imposed on the cell). At the end of 1970, intracellular H_2O_2 changes were first discovered in eukaryotes by Helmut Sies and Britton Chance through pioneering collaboration using spectrophotometry of isolated hemoglobin-free perfused rat livers. Later, this phenomenon of 'oxidative stress' was originally formulated by Sies H. in 1985 as 'a disturbance in the prooxidant-antioxidant balance in favor of the former, leading to potential damage to intact cells and organs.' Since that time, oxidative stress has inspired numerous investigations across all relevant fields as a mechanism for inducing a unique set of adaptive homeostatic responses in cells and organisms (i.e., CHR or OHR, in Figure 4B). This notion has also evolved in biomedical research as an experimental method for exploring adaptive redox responses and as a putative determinant of aging and other pathological processes. In this process, 'oxidative stress' was further refined by Jones D.P. in terms of 'an imbalance between oxidants and antioxidants in favor of the former oxidants, leading to a disruption of redox signaling and control and/or molecular damage.'

Quite recently, the concept of oxidative stress was updated by Lushchak VI and

Storey KB, who revisited it in terms of 'a transient or long-term increase of steady-state ROS levels, disturbing cellular metabolic and signaling pathways, particularly ROS-based ones, and leading to oxidative modifications of an organism's macromolecules that, if not counterbalanced, may culminate in cell death via necrosis or apoptosis.' Overall, as to biologists, oxidative stress implies that a cell or organism produces or is exposed to an excess of highly reactive molecules (i.e., RXS), predominantly oxygen- and/or nitrogen-centered ROS/RNS, which exceeds its endogenous antioxidant capacity (endowed on the organism with a host of all those oxidoreductases, reductases, and small-molecule antioxidants, as illustrated in Figure 4D).

4.2.3 Major challenges for precision definition of oxidative or reductive stress by chemistry Herein, it should be noted that a critical pitfall of oxidative stress may lead to indiscriminate use of this term as a global concept with certain ambiguity and circularity but without clear relation to redox chemistry in particular cases. Accordingly, by reevaluating those previously debating concepts of oxidative stress from the chemical perspective, it was found that most definitions by biologists appear 'too vague and imprecise to recognize explicitly what the oxidants are in a given set of intracellular and extracellular circumstances and how their reactivity is countered, controlled, or quenched, and most importantly, what the reductants really are to which the oxidants must be coupled for the redox reactions to proceed.' Rather, to achieve a precision definition of oxidative stress by redox chemical ontology in complex biological systems, certain unavoidable challenges exist for the following reasons: (i) It is very hard to identify or quantify each specific species of ROS/RNS (not as a global index) in a hitherto incomplete picture of all their sources, sinks, and fluxes. (ii) Their chemical reactions, particularly with the very short-lived free radicals involved in a wide range of redox potential propagating to terminating reaction cascades, are often too rapid to be accurately determined within the availably measured time scales of presently established techniques. (iii) The redox compartmental heterogeneity of ROS/RNS in their gradient distributions varies with changing status of intracellular and extracellular contexts, making them difficult to trace in real-time topovectorial space by relevant reporters—an ideal molecule that can react sufficiently and rapidly with each species of ROS/RNS with exquisite specificity at low enough concentrations so as not to affect the steady-state level of the indicated species. (iv) Many current ROS/RNS estimation methods may have actually and more sensitively detected RSS, such that they are rarely distinguished from each other. In fact, the ratio of ROS to RSS in cells was examined to be considerably less than three (ROS/RSS $<$ 3), because H_2S/RSS can be co-produced with H_2O_2/ROS (e.g., by SOD, Cat, respiratory chain), so its production may well exceed ROS yield on most occasions. As such, the term 'oxidation' exemplifies this ingrained bias as there is no equivalent term 'reduction.' But, as a biological consequence of oxidative stress, it is cognized only in light of the reductants that have been oxidized in this process and the effects of those coupled redox reactions on the biological func-

tional pathways in which the redox-active molecules are involved. Collectively, it is inferable that this complicated redox biochemical process cannot be well generalized by an overly simplistic biological principle nor determined by another chemical precision algorithm applied for a statistically complex ensemble of such redox reactions.

Intriguingly, most important biological oxidants (connoting oxidative stress) are derivatives from O_2 (with a proclivity for electrons), but they exist in partially reduced forms, including superoxide anion ($O_2\bullet^-$) and H_2O_2, from a simple chemical perspective. Coincidentally, these 'Janus-faced redoxidants' can oxidize some molecules of appropriately matched negative redox potential ($\Delta Eh < 0$), accompanied by reducing other molecules of more positive redox potential ($\Delta Eh > 0$). For instance, $O_2\bullet^-$ can reduce disulfides (to yield reduced thiol forms) and simultaneously oxidize α-tocopherol, leading per se to its oxidation to O_2 and its reduction to H_2O_2, respectively. Similarly, H_2O_2 can reduce ferrylhemoglobin (to yield O_2-carrying Fe^{2+}Hb) and oxidize methionine or thiol-active proteins, leading per se to its oxidation to $O_2\bullet^-$ and its reduction to H_2O, respectively. As a consequence, certain disulfide linkages are constructed by oxidation of thiol-active proteins to facilitate their proper folding to gain functional configuration or crosslinking with other proteins in the more oxidizing endoplasmic reticulum and extracellular environments. From these points, a certain excess of $O_2\bullet^-$ and H_2O_2 is not necessary for leading to simply 'oxidative stress.' Conversely, this should just as well be viewed as imposing a 'reductive stress' potential on biological systems from a chemical perspective, since partially reduced forms of ROS involve redox reactions in which they reduce those molecules of more positive redox potential ($\Delta Eh > 0$). Therefore, 'misleading definitions' of oxidative stress by biologists appear to focus only on the oxygen-centered nature of ROS without regard to the precision chemistry of redox-coupled reactions in which they can be involved and/or to the precise changes in redox compartments where they are generated or controlled. That is, on the contrary, a properly scientific definition of 'oxidative or reductive' stress by a chemical principle should be dictated by the flow of those electrons (or hydride anions) exchanged in the redox-coupled reactions in which all reactive species have been implicated.

4.2.4 Proper definition of redox stress as 'a generic term' by biological chemistry Reductive stress was originally observed in hypoxic hepatocytes by Gores et al.; this phenomenon caused by respiratory inhibition favors formation of toxic oxygen species (i.e., hydroperoxide), as was accelerated during aerobic but not anaerobic chemical hypoxia. This stress was further found to contribute to lethal cell injury, which was greater during aerobic, as compared with anaerobic, chemical hypoxia, but delayed by desferrioxamine or cyanidanol, rather than by SOD $\pm$ Cat, during intermittent or incomplete oxygen deprivation. In this setting, reductive stress is only recognized, from a biological perspective, 'as a complement of oxidative stress in virtue of providing an excess of reducing equivalents' that cannot be adequately quenched by endogenous oxidoreductases

and accommodated by existing oxygen counterparts in local environments (due to oxygen being a terminal electron acceptor in the respiratory chain electron transport and redox reactions in living systems). However, such a definition of 'reductive stress,' if interrogated from a chemical perspective, can also pose the same misleading and confusing issues as the aforementioned 'oxidative stress.' For instance, even in the absence of re-oxygenation by reperfusion, sustained hypoxia leads to an increase in ROS, albeit as oxidative stress from the biological angle, whilst these species are de facto partially reduced forms of O_2 that can evolve in this setting to hierarchically increase reducing equivalents, with their consequences depending on the redox-coupled reactions in which they are involved. Taken together, given the 'oxidative and reductive' complexity as yet oversimplified previously with chemical imprecision, 'redox stress' should be commonly accepted as 'a generic term' to describe 'disturbances in the oxidation-reduction reactions arising from an excess of oxidants or reductants, with their functional consequences in a biological system.' Also, it is critically important 'to be as explicit as possible about the particular molecular species (most of which are Janus-faced and varied within distinct ambient contexts) involved in the redox reactions that modify biological phenotypes.'

4.3 'Stress-coping code' acquired by evolving cellular life responses

Generally, a physiological or pathophysiological functional consequence of redox stress depends on the extent and duration of whether there is a mismatch between the excess of oxidants or reductants in the redox biological systems of organisms and its intrinsic anti-redox capacity to counteract or mitigate their potential damaging effects in the (mal)adaptive responses to those reactive species during the life process (Figure 4, B to F). Thereby, such redox stress has been recognized, from the eco-evo-devo perspective, as a powerful mechanistic mediator of life history trade-offs between its traits (which arise and reflect constraints imposed by the environment and physicochemical laws during the evolutionary process). If an organism can appropriately recognize and respond to changing environmental stress challenges with minimal costs, this represents an important physiological attribute with great adaptive value (Figure 4, C vs E). Conversely, if this organism is unable to do so appropriately, with greater costs, it fits into a pathophysiological or (pre)pathological attribute with another great maladaptive value (Figure 4, D & F). Such striking distinctions in those costs and benefits arising from diverse mechanisms that selectively enable organisms to cope with changing stresses experienced from predictable and controllable to unpredictable and uncontrollable status are manifested to distinctive extents at different life stages and can thus serve as strong selective pressure to drive evolution of life histories.

In the life process, a 'stress-coping code' is acquired by natural selection for a set of arbitrary rules accounting for discrete organisms to cope with distinct types of stress (including primary and secondary redox stress, and even psychogenic stress) instigated by all challenges from internal and external environments.

Such a proper code for life to cope with stress by 'the nonspecific response of its body to any demand,' as early conjectured by Hans Selye, should be constructed 'primarily based on the laws of nature, all of which must be accepted' within the ready-made machine. That is based on how the body works (and/or how it should work) within a stress-coping code founded on natural laws (which was figured out as a basic necessity of living systems, especially work whose fruits can be accumulated), as with the 'genetic code' containing the receipt of all those traits inherited in chemical language. However, such putative stress-coping code does not yet appear to be unveiled heretofore, albeit animal adaptive stress responses fall into two coding styles (i.e., proactive hawk and reactive/passive dove) of their behavior, physiological health, and relevant pathological diseases.

Following natural principles applied to code biology, 'stress-coping code' could be defined as a series of set rules that enable distinct cellular life forms to cope with various stresses caused by changing internal and external environments. Such 'stress-coping codes' have experienced five phases, as have other organic codes. In the beginning phase, the first version of coding rules appears in a living system as means of performing a particular function to cope with stress by governing all relevant adaptors and components, which were orchestrated into an ensemble of both 'stress-sensing' (e.g., physicochemical, electrochemical, or quantum chemical) reactive cascades and its 'strain-leading' biochemical metabolisms with responsive signaling pathways. The certain ambiguity of this code in its second phase is evolutionarily reduced insomuch as to steadily improve its particular functional specificity to cope with stress, resulting in distinct subsets of multi-hierarchical stress-coping physicochemical reaction cascades in their real spatiotemporal orders coupled with multi-dimensional signaling responsive transduction pathways, comprising a complex gradient-ordered 'tensor' network. Such code in its third phase is further optimized by distinct mechanisms (such as hormesis, homeorhesis, homeostasis, allostasis, and morphostasis, as in Figures 4, B and G). The consequence enables a living life to establish two major classes of 'stress-coping responses,' i.e., cellular homeostatic response (CHR) and cellular stress response (CSR), both of which are also extended to organismal homeostatic response (OHR) and organismal stress response (OSR), as described by Dietmar Kültz. In the fourth phase, this optimized code is further subjected to a major transition (by integral functional and structural means across distinct strata from molecular, subcellular, and cellular to tissue, organismal, and even population levels), so far as to construct a perfect responsible set of 'stress-coping systems' coordinately regulated by distinct mechanisms (as proposed by two groups: Kültz D. and Chrousos G.P.). In the last fifth phase, such stress-coping code continues to evolve by selective programming and further reprogramming with requisite specificity. So ultimately, it becomes an intact set of highly conserved inheritable rules that can tightly monitor all relevant responsive behavior, physiological and pathophysiological adaptation or even maladaptation to various stresses (e.g., two distinct coping styles), apart from being virtually transmitted by genetics, epigenetics, and topogenetics to descendants. For example, CSR is one of the most highly evolutionarily con-

served responses inherent in all cells, representing a prerequisite for evolution of the neuroendocrine stress response (NESR). This NESR cannot only integrate the response to stress in metazoans but also, in turn, is another prerequisite for evolution of psychological and/or emotional stress response (PESR) in vertebrates to its preeminence in humans. Overall, stress is not something to be avoided, no matter what happens; there thus arises a demand for stress-coping code to provide necessary principles for the spatiotemporal order to perform tasks required to maintain homeostasis of cellular life and also resist and adapt to changing influences from its internal and external environments (including redox stress).

5. Quantitative stratification of redox stress and anti-redox responses

In 1977, the stress of life was simply qualified by Hans Selye to be good eustress or bad distress and also quantified as hyperstress (overstress) or hypostress (sustress); all four basic variations are deciphered in Figure 4(G). Three decades later, Nel A. et al. presented a hierarchical redox stress model in which Tiers 1 to 3 were first defined by distinct extents of oxidative stress with inverse ratios of GSH/GSSG, but Tier 0 at normal physiological conditions was left blank. This blank space was filled by our work focused on Nrf1. Moreover, oxidative (and reductive) stress was classified by relevant dose-effect curves based on its intensity and time course. Oxidative eustress and distress were quantitatively distinguished by Helmut Sies and colleagues based on extracellular and intracellular H_2O_2 concentrations (i.e., $[H_2O_2]$ex & $[H_2O_2]$in) (as shown in Figure 5A [Figure 5: see original paper]).

5.1 Updated hierarchical modelling of redox stress and anti-redox capability

As illustrated in Figure 5A, a natural exponential curve was drawn from data of $[H_2O_2]$ex reported by Sies, H. et al. The X-axis represents changes from predictability and controllability of eustress to unpredictability and uncontrollability of distress challenged at distinct oxidative status (Tiers 0 to 3), while the Y-axis shows the life-threatening intensity of $[H_2O_2]$ex (μM). On this basis, according to the natural law of symmetry (as a fundamentally driving force), we thus conjecture that a robust steady-state redox system should be, at least in theory, demarcated by four dynamic curves representing oxidative stress, reductive stress, antioxidant capability, and anti-reductive capability (of intrinsic reactive components), respectively (Figure 5B). In such parameter spaces, it is inferable that there exists a highly conserved 'habitable Goldilocks Zone' in which all essential redox and anti-redox biochemical elements should be tightly governed within certain homeodynamic threshold settings for normal physiological hemostasis. The redox homeostasis could also be represented by a 'Golden Center' of healthy living systems, just as proposed by Ursini, F., et al. Equally importantly, a set point approaching zero (P_0) co-exists within this 'Golden

Mean' for healthy living beings. This may be dubbed the 'zero theory,' similar to that of a mathematical function, f(x) (± CR, a robust constant). Taken altogether, this conserved symmetry of redox stress and anti-redox responsive system closely adheres to Noether's theorem (which states that symmetry of their action corresponds to a conservation law, also with a conserved quantity).

The point of limit (PL) is represented to allow for resilience of this system to its normal archetype by proper responses to eustress, albeit this can lead to a novel eustasis for fitness. Conversely, further deterioration of redox distress could result in a severe malfunction status of this response system (called cacostasis), with its maladaptation or even exhaustion (Figure 5B). These can be reflected by more explicit Figure 5C, which was obtained from simple generalization by a base-10 logarithm of the above-curved parameters (e.g., $\log_{10}[H_2O_2]$ex), fully in accord with the Weber-Fechner law (S = k logc X). Moreover, it should be noted that those aberrant redox set-points (Pox or Pre) may emerge during malignant transition to cacostasis and are monitored possibly by allostatic mechanisms.

5.2 Differential involvement of Nrf1 and Nrf2 in redox stress responses stratified by physio-pathology

As shown in Figure 6A [Figure 6: see original paper], we further refined distinct tiers of redox stress responses by optimally stratifying them from molecular and subcellular to cellular levels, established on the basis of previous multi-hierarchical models. Notably, Tier 0 is defined under normal physiological (healthy) status, which is predominantly dictated by Nrf1 (and its long isoform TCF11), together with its unique subset of target genes responsible for anti-redox, detoxifying, and cytoprotective roles in maintaining cell homeostasis and organ integrity. This is due to ever-accumulating facts that loss of Nrf1/TCF11's function results in severe endogenous oxidative stress; this is also accompanied by significant spontaneous pathological phenotypes as reviewed in detail. By contrast, loss of Nrf2 enables resulting deficient cells and/or animals to become more susceptible and vulnerable to redox stressors or carcinogens but is not accompanied by any obvious spontaneous phenotypes. These demonstrate that Nrf1 is essential for cell homeostatic response (CHR), whereas Nrf2 is not required for CHR but still for cell stress response (CSR) as defined by Kültz.

At Tier 1, which is generally accepted as a redox eustress status with hormesis, the anti-redox, detoxifying, and cytoprotective defense system is aroused predominantly by Keap1-sensing redox signaling to Nrf2-mediated target genes (driven by those cis-regulatory consensus antioxidant and/or electrophile response elements, called AREs/EpREs, within their promoter regions) in cellular responses to redox stress, particularly acute stress (Figure 6A). At Tier 2, viewed as a major pathophysiological transition switching from subhealthy status into onset of certain pathology, responses become complicated if redox stress has deteriorated. In addition to overstimulation of those (nonspecific) stress signaling networks responsible for activation of pathophysiological switching, the

inflammatory responsive pathway mediated by NF- B is critically activated and even hyper-activated, leading to onset of certain pathologies and some pathogenesis of chronic diseases. When redox stress further deteriorates from Tier 2 to Tier 3 (as a distressed status), severe cytotoxicity along with accumulating damage signaling to cell processes, including mitochondrial dysfunction, leads to metabolic, epigenetic, and genetic reprogramming, topogenetic remodeling, and even cell and/or organ reshaping, with aberrant immunopathology. As a consequence, this results in cell apoptosis and aging-related chronic diseases, or otherwise aberrant proliferative carcinogenesis overstimulated by this distress. Collectively, the underlying molecular and cellular details in response to distinct tiers of redox stress (Figure 6B) require further elucidation. However, the major role of antioxidant and anti-reductive defense systems (aiming to prevent, intercept, repair, and eliminate) is fulfilled predominantly by redox-controlling enzymes, key transcription factors (e.g., Nrf1 and Nrf2), and co-factors involved in cellular redox biochemical and relevant metabolic processes, as well as for long-term adaptation principally by reprogramming of their regulatory gene expression profiles in physiological and pathophysiological responses to distinct severity of redox distress.

As shown in Figure 6C, stably expressed Nrf2 is rapidly induced within 2 h and quickly reaches its maximal peak at 6 h of induction by a tetracycline-inducible HEK293 cell system (as described elsewhere), but thereafter gradually diminishes and even extinguishes. By sharp contrast, Nrf1α and its long isoform TCF11, along with its N-terminally truncated isoform TCF11ΔN, are gradually induced by this tetracycline-inducible system to reach maximal activation from 8 h to 12 h of induction and then maintained at high levels until experiments stopped. Additional two shorter Nrf1β and Nrf1γ (as a dominant negative form) are stably induced by tetracycline (Figure S1). Notably, both Nrf1α and TCF11 are required for N-linked glycosylation and then deglycosylation before being selectively processed to remove their N-terminal portion and thus give rise to a mature active CNC-bZIP factor, such as TCF11ΔN (Figure S2, A & C). All these distinct isoforms of Nrf1/TCF11, with differential half-lives of their protein stability (Figure S2), enable them to exert distinct or even opposing roles in transcriptional regulation of their target genes. As such, Nrf1/TCF11 is innately endowed to fulfill a unique indispensable function distinctive from that exerted by Nrf2 in response to distinct tiers of redox stress. Hence, it is inferable that Nrf1α/TCF11 exerts an irreplaceable and pivotal role in governing cellular homeostatic response (CHR), whilst Nrf2's role in handling such rapid emergency response (CSR) has provided a way of 'buying time' for the robust homeostatic response mediated by Nrf1α/TCF11, insomuch as to coordinately cope with redox stress threatening cell homeostasis and organ integrity of living systems.

5.3 Adaptive landscapes accounting for distinct responsive phenotypes to redox stress

Based on mathematical models of Darwinian evolutionary laws and systems biology developed by Ao's group, it is inferable that discrepant responsive physiopathological phenotypes (and genotypes) to distinct tiers of redox stress should be determined predominantly by coordinated control of Nrf1/TCF11 and Nrf2, along with their differential but yet integral transcriptional regulation of cognate target genes responsible for anti-redox, detoxifying, and cytoprotective defenses, as deciphered by distinct adaptive landscapes (Figure 7 [Figure 7: see original paper], A to D). This is because distinct phenotypic (and/or genotypic) steady states are dictated by different profiling of those key differential expression genes at the distinct status of each multi-hierarchical robust endogenous molecular-cellular network. Therein, differential expression of such a minimum set of key genes at distinct strata is de facto exhibited at their abundances, activities, and topoforms at different topovectorial phase transitions, along with their intricate interactions between those core modular molecules in different subcellular, intracellular, and extracellular contexts.

A healthy redox homeostasis is maintained within a certain homeodynamic threshold range around P_0 preset by Nrf1/TCF11-leading CHR to normal redox physiological fluctuations, in a robust basic redox metabolic network with cell respiration and aerobic energy metabolism (Figure 7A). According to the homeodynamic model (Figure S3) based on theory for self-organization of dynamic systems by Lloyd D. et al., homeostasis is only viewed as an exclusive mode of operation emphasizing stability of the internal milieu toward perturbation, tightly governed by a series of feedback mechanisms controlling the normal physiological steady state. That is to say, it is a relatively balanced (i.e., redox and anti-redox) stage of dynamics with monotonic states (fixed points). Since biological systems are indeed endowed with dynamic capability to be self-referenced, self-organized, and self-maintained in the eco-evo-devo process, they are placed as a steady state with high activity, particularly at certain phase transitions (of saddle points as a bistable redox switch at the excitable bifurcation, or contingently as a spontaneous oscillator with chaotic potentials so as to lose stability but rapidly retake resilience). Such processes could proceed on different spatiotemporal scales from those very rapid reactive processes between redox and anti-redox molecules within membrane-compartmented spaces to other very slow evolutionary changes of, e.g., 'redox-coded homeostasis,' in life histories.

When redox homeostasis is threatened by stress, the response of life experiences at least three phases: (i) alarm reaction, (ii) adaptation with resistance, and (iii) exhaustion leading to 'general adaptation syndrome.' The time course of such redox adaptation was also classified into instant (emergency) response, short-term (metabolic), and long-term (transcriptional) adaptations. In addition to homeostatic feedback regulations, multi-hierarchical homeorhetic regulations are also required from molecular and cellular to organismal strata (Figure 6B). Once

homeostatic loss of stability at the redox excitable bifurcation reaches a certain extent, heterostasis and even allostasis mechanisms are switched to reset a new point of Pox or Pre. The robustness of homeostatic regulation is based on high-gain integral feedback mechanisms, while heterostasis could be associated with low-gain integral feedback processes when organisms are submitted to unitary-step disturbances or changes of the set-point (Pox/Pre) for the novel feedback loop at the entrance of subhealthy eustasis or pathological cacostasis, which depends on whether such new Pox/Pre are prescribed within acceptable or even unacceptable ranges (as shown in Figure 7, B to D). Notably, regulatory mechanisms for redox responsive adaptations are dependent on crucial roles of those key intrinsic reactive compounds (IRC), including important functional signaling molecules, metabolic enzymes, and transcription factors (e.g., Nrf1, Nrf2, HIF1, HSF, and NF- B) in reprogramming their critical signaling cascades, metabolism networks, and target gene expression profiles insomuch as to dictate distinct redox physio-pathological phenotypes. If too much loss of homeostatic stability enables it to be gradually exhausted, so that its adaptive resilience is not fully recovered, or otherwise allostasis is overloaded, even to reach cacostasis, certain pathophysiological maladaptive responses are triggered, consequently leading to putative pathogenic switches to chronic diseases, particularly when IRC is almost largely consumed or even exhausted, of which Nrf1 is diminished or abolished (as deciphered by adaptive landscapes in Figure 7, C to D). Moreover, another explicit interpretation by a geometric landscape of redox potential energy coordinated with redox responses to distinct redox states of different physio-pathological phenotypes is presented (in Figure 7E), consistent with the model proposed by Lekaa Hussain.

6. Nrf1 is a living fossil's transcription factor, that is closer than Nrf2 to the ancient orthologues arising from the eco-evo-devo process of life histories

6.1 Nrf1 is much closer than Nrf2 to the ancient orthologues emerged during evolution of life

Due to certain limitations existing in the past three decades, the origin of CNC-bZIP proteins seems to have been traced back only to vertebrates, although their highly conserved CNC domain was first identified in the *Cnc* gene from *Drosophila melanogaster* and thereafter unraveled in *Skn-1* from *Caenorhabditis elegans*. Another noteworthy topic of such CNC-bZIP study is disproportionately focused on Nrf2 (also called NFE2L2), a stress-emergency responsive gene enabling rapid examination by available experimental tools along with necessary and powerful preference for its non-lethal knockout animals. The consequence was positively misleading to the putative state that priority of Nrf2, together with its negative regulator Keap1 (Kelch-like ECH-associated protein 1), was evolutionarily selected so as to enable it to serve as a predominant and essential determinant responsible for antioxidant, detoxification, and cytoprotection against a variety of cellular stress. Nonetheless, such is not an accurately true

case as a matter of objective facts that should be clarified hereby.

A neighbor-joining phylogenetic tree (Figure 8A [Figure 8: see original paper]) appears fan-shaped with three distinct clades and a bundle, clarifying the phylogenetic relationship of all selected CNC-bZIP family members by analyzing evolutionary conservation of their amino acid sequences. Among these, a major clade comprises Nrf1 (also called NFE2L1, along with a long isoform TCF11 and another short form LCR-F1 or Nrf1β), Nrf3 (also called NFE2L3), and Nach (i.e., Nrf and CNC homology) proteins from distinct metazoan species, which are much closer to their founding members Cnc and Skn-1 (Figure 8A, right panel). By contrast, Nrf2 and NFE2 p45 are closely clustered in the second clade, whilst the third clade is made up of transcription repressors Bach1 and Bach2 (Figure 8A, left panel). These CNC-bZIP family members are also further gathered closer to both the sMaf/Maf and Jun subfamilies within a relatively larger phylogenetic tree constructed from distinct families of 495 bZIP proteins (Figure S4), as described previously in detail. This early-originated basic-region superfamily of transcription factors also includes those containing the basic helix-loop-helix zipper (bHLH-ZIP, e.g., SREBP1/2) or the bHLH-PAS domain (e.g., HIF1α). Such basic-region minimotifs, conjectured as primordial peptide-replicated templates albeit with rather lower fidelity, have been retained divergently in most viral, bacterial, and archaeal reigns and all eukaryotic kingdoms of cellular life since the origin of their last common ancestor (LUCA, Figures S4 & S5).

These CNC proteins except Skn-1 can heterodimerize with partners sMaf or other bZIP proteins (e.g., Jun, c-Fos, Fra1, ATF2, ATF4) by physical interaction of their bZIP domains before binding cognate target genes involved in antioxidant, detoxification, and cytoprotection against oxidative and other stresses. Besides the bZIP region, the unique DNA-binding CNC domain is highly conserved in all family members, including *Drosophila melanogaster* Cnc, *Caenorhabditis elegans* Skn-1, and other metazoan proteins Nach1 to Nach8, which emerged at early evolutionary stages of life histories (Figure 8B), but none of their orthologues are identified in plants and fungi. Notably, such a novel subgroup of Nach1-8 with high homology to all vertebrate CNC-bZIP proteins (e.g., NFE2p45, Nrf1, Nrf2, and Nrf3) are identified to be predominantly present in Echinodermata, Mollusca, Actiniaria, Placozoa, Porifera, and bacteria, respectively (Figure 8, A & B).

Collectively, these findings demonstrate that CNC-bZIP proteins originated from marine bacteria to multicellular organisms. However, a perplexing gap appears to exist between marine bacteria and multicellular metazoa (e.g., *Amphimedon queenslandica*), because none of their orthologues have been identified in unicellular protozoans. Further alignment of multiple amino acid sequences (Figure 8C) revealed that there still exists a conserved basic-adjoining region homologous with the CNC domain and another extended homology region (EHR, within the Maf/sMaf family) in the filasterean bZIP protein (XP_{004343898}.1) from *Capsaspora owczarzaki* and also in the choanoflagellate bZIP protein (XP_{001744453}.1) from *Monosiga brevicollis*. Moreover,

this is likely represented by a CNC-like Smc domain in the cyanobacteria bZIP protein (PHJ76996) from *Nostoc linckia* z2 (Figures 8C, bottom line). Together with marine bacterial Nach 1/2, these suggest they were endowed with such ancestral conservativity from bacteria to protozoa, commonly shared by all four families of CNC, Maf/sMaf, ATF2, and XBP1, albeit with certain variations. In addition, a bZIP protein (KRG21159.1) is yielded by *Candidatus Berkiella aquae*, a γ-proteobacterium that exists as an obligate intranuclear endosymbiont of freshwater amoebae. This suggests putative horizontal gene transfer (HGT) may have occurred during protozoan evolution.

Notably, only one or two Nach proteins are found in each species such as ascidian, sea urchin, octopus, fly, and hydra, with no exception of CNC/EHR-like bZIP proteins in the aforementioned protozoan organisms. This indicates that expansion and diversification of the CNC-bZIP family seem to take place only in vertebrates, leading to yield of 4 to 10 homologues encoded by distinct genes. Further comparison of structural domains in these CNC-bZIP proteins (as deciphered in Figure 8B) revealed that functional distinctions between Nrf1 and Nrf2 are dictated predominantly by an extra membrane-binding NTD (N-terminal domain, which contains an ER-targeting NHB1 signal peptide and another protease-processing NHB2 motif) and another N-glycosylated transactivation (NST) domain in Nrf1, but not in Nrf2. Such ER-associated NTDs are also present in Nach3, Nach5, Nach6, Nach7, CncC, Skn-1A, and Nrf3, implying they should be endowed with similar topobiological features to those conferred upon Nrf1. By contrast, the stability of Nrf2 and its transcriptional activity are principally negatively regulated by Keap1, a redox-sensing adaptor targeting the former CNC-bZIP protein to the ubiquitin-mediated proteasomal degradation system. The negative regulation of Nrf2 by Keap1 is dictated primarily by physical interaction of the DGR domain of Keap1 with the N-terminal Neh2 domain of Nrf2. Similar Keap1-binding Neh2L domains are represented in Nach3, Nach4, Nach6, Nach7, and Nach8, as well as in CncC and Nrf1, but not Skn-1. Yet, Keap1 is also absent in sponges, placozoans, sea anemones, or nematodes, although putative Keap1-like orthologues were found in some invertebrates such as ascidian, sea urchin, octopus, and fruit fly.

Taken altogether, these demonstrate that Nrf1 rather than Nrf2 is much closer to its ancient orthologues (e.g., Nach, Skn-1, and Cnc) of this CNC-bZIP family that emerged during early evolution of life. This notion is also supported by the facts that distinct length isoforms of CNC or SKN-1, like Nrf1, are yielded from alternative splicing of various transcripts of their single genes, ensuring alternative translation and post-translational processing of these proteins. An N-terminally truncated isoform (similar to Nrf1ΔN or TCF11ΔN) functions as does Nrf2 (Figure 7, B & C), whilst their full-length isoforms (CncC and Skn-1A) evince similarity to intact Nrf1α/TCF11 (Figure 8B). Therefore, it is inferable that vertebrate Nrf2 is evolutionarily selected by genetic duplication of a putative Nrf1-like gene that has been trimmed off the negative regulatory NTD, enabling it to rapidly mediate an efficient emergency response to stress and thus acquire a 'buying time' function for the robust homeostatic response

mediated by Nrf1/TCF11.

6.2 Nrf1 is a living fossil of the CNC-bZIP transcription factors emerged in the redox eco-evo-devo process

From the origin of life and its co-evolution with the surrounding atmosphere (consisting of N_2, O_2, CO_2, CH_4) and ocean ($SO_4{}^{-2}$, P, and Fe^{+2}-containing) redox environments of life histories to give rise to diversity of modern biomes on Earth, at least nine major historical events of life occurred (as indicated on the top) throughout the entire eco-evo-devo process. Nearly seven-eighths of this process was dominantly placed in sulfur-based reductive environments, whereas only the recent one-eighth of life histories was principally based on oxygen-centered oxidative environments. In such distinct challenging conditions, the intracellular redox homeostasis of almost all life forms has steadily been maintained within a homeodynamic threshold range by evolving their intrinsic anti-redox responsive mechanisms (CHR, CSR, OHR, and OSR), which are differentially monitored by the CNC-bZIP family transcription factors, as described above (in Figures 3, 4B, and 6A).

6.2.1 Co-evolutionary histories of life with its ambient redox environments The redox evolution of Earth's atmospheric environments depended on changes in the abundance of key small molecular gases, including H_2/H_2S, NO/N_2, O_2/H_2O_2, and CO_2/CH_4. Notably, only the most recent one-eighth of life's history has relied on oxygen-based redox conditions, as illustrated by the atmospheric O_2 curve (Figure 9a). The classical "two-step" model of atmospheric evolution proposes an initial O_2 level of <0.1% present atmospheric level (PAL) between 1.8 and 0.8 Ga [40, 212], followed by a second rise to 10–40% PAL around 0.54 Ga [213]. This O_2 curve merges with CO_2 proxy estimates from 0.42 Ga to the present [215], alongside various Precambrian pCO_2 estimates [216–220]. A schematic methane (CH_4) history derived from a biogeochemical box model coupled to photochemistry [81] aligns with plausible biological CH_4 fluxes into atmospheres with rising O_2 levels during the Great Oxidation Event (GOE) and the Neoproterozoic Oxygenation Event (NOE). In contrast, the preceding seven-eighths of life's history depended on sulfur-based redox environments, with early energy metabolism deriving from H_2S/FeS and respiratory NO pathways [23, 37, 105]. NO is produced primarily through the $NO_3{}^- \rightarrow NO_2{}^- \rightarrow NO$ pathway, which is reduced to yield N_2O; the latter undergoes successive cascaded reduction by $NO_3{}^- \rightarrow NO_2{}^-$ to generate N_2 (as shown in Figure 2).

The origin and subsequent evolution of life were fundamentally shaped by changing ocean redox environments [237, 238]. The earliest evidence for life appears in the oldest stromatolites dating to ~3.7–4.1 Ga (Figure 9f) [242, 243], placing life's origins in anoxic, reducing (H_2/H_2S) environments dominated by ferrous iron (Fe^{2+}/FeS) in ferruginous oceans. This suggests that the first organisms (i.e., LUCA) were likely sulfur-metabolizing. Approximately 200 million

years later, anoxygenic photosynthesis emerged, enabling organisms to escape chemotrophic existence by using H_2S to reduce CO_2 to organic carbon (e.g., CH_4) while oxidizing sulfur to polysulfide (S) (Figure 9, top). In this anoxygenic process, CO_2 may be partially reduced to HCO_3^- while H_2O is oxidized to yield minor H_2O_2. Around 3.0 Ga, as light-harvesting antenna efficiency increased, H_2O became the primary reductant for oxygenic photosynthesis (first appearing in cyanobacteria). The evolution of oxygenic photosynthesis permitted episodic O_2 accumulation ("oxygen oases") along late Archean ocean margins, with widespread surface ocean oxygenation accompanying the GOE and a slight increase in atmospheric O_2 levels. This modest atmospheric O_2 increase enabled sulfur oxidation to sulfate (SO_4^{2-}), which was then washed into the sea. This sulfur was subsequently reduced, forming massive euxinic oceanic zones that were anoxic and sulfidic (H_2S/RS^-) (Figure 9e). Elevated microbial sulfide production at sites of high organic carbon export further developed euxinic conditions at mid-depths and in restricted basins, though deep oceans remained predominantly ferruginous [239]. Expansion of ferruginous deep oceans occurred during a major crustal growth event at 1.88 Ga and during severe Neoproterozoic glaciations. Widespread ocean oxygenation commenced by the Ediacaran Period [82, 240, 241], though significant oscillations in oceanic redox-stratified conditions persisted until the Devonian Oxygenation Event (DOE) led to permanent ocean ventilation, except for sporadic transient anoxic events [82].

From the evolutionary history of life (Figure 9f), oxygenic photosynthesis must have been present directly before the GOE [244] or as early as 3.0 Ga [40, 55]. The earliest convincing evidence for eukaryotic life appears in euxinic environments at ~1.6–1.7 Ga [245], with plants emerging shortly thereafter, though the origin of eukaryotes could have been much earlier [246]. Records of multicellular eukaryotes appear as early as ~1.2–1.6 Ga [247, 248]. Biomarker evidence for early sponges indicates the first animal appearance during the Cryogenian "Snowball Earth" episode [249]. The NOE occurred when substantial O_2 production by plants (in addition to cyanobacteria) gradually rose to present-day levels, allowing sufficient ocean oxidation and sulfide disappearance before major biological innovations. These innovations included the appearance of new biological and ecological strategies: the first metazoans [249, 250], the first large and architecturally complex organisms during the Ediacaran [251], the Cambrian Explosion with subsequent rapid species diversification [252, 253], and the emergence of mobility and predation [254, 255].

6.2.2 Nrf1 is a living fossil of the CNC-bZIP factors arising from distinct evolutionary stages of life All known CNC-bZIP and CNC-like transcription factors across species are mapped according to the evolutionary tree of life (Figure S6, adapted from https://evogeneao.s3.amazonaws.com/images/tree_{{{of}}*{{life}}}/tree-of-life*{2000}.png). The emergence of distinct evolutionary stages correlates markedly with ecological co-evolution of relevant ambient environments (Figure 9, bottom). Since the oldest stromatolites represent an intriguing fossil record

of prokaryotic life first emerging ~3.7–4.1 Ga ago [242, 243], the presence of Nach1 and Nach2 alongside ancient CEBP in marine γ-proteobacteria [114], with CNC-bZIP domains highly conserved and closely related to early animal Nach4, Nach5 (both in sponges), Nach6 (in anemones), and Nach3 (in placozoans) (Figure 8C), demonstrates that the CNC-bZIP family originated at least from marine bacteria (Figure 9, bottom). Furthermore, vertebrate Nrf1—rather than Nrf2 or other homologs—is evolutionarily conserved closer to metazoan CNC-bZIP factors such as Skn-1 (lacking Keap1 in nematodes), CNC (in fruit flies), Nach7 (in octopus), and Nach8 (in sea urchin) (Figures 8 and 9). Additionally, two bacterial transmembrane CNC-like transcription activators, CadC and ToxR [257, 258], were identified in *Escherichia coli* and *Vibrio cholerae*, respectively (Figures 8C and 10). Together, these findings demonstrate that transmembrane-associated Nrf1 is a living fossil-like CNC-bZIP factor, much closer to ancient orthologs arising from distinct stages of evolutionary history compared to the water-soluble Nrf2 factor.

From an evo-evo-devo perspective, euxinic oceanic reductive environments (H_2S/RSS without O_2) led to the appearance of the first eukaryotes—unicellular protozoans—after the GOE triggered by oxygenic photosynthesis in cyanobacteria (with CNC-like Smc domain-containing bZIP protein PHJ76996). Coincidentally, two protozoan CNC/HER-like bZIP proteins (XP_{004343898}.1 and XP_{001744453}.1) exist in the filasterean *Capsaspora owczarzaki* and the choanoflagellate *Monosiga brevicollis*, respectively, and are highly conserved with the CNC, Maf/sMaf, ATF2, and XBP1 families (Figure 8C). According to endosymbiotic theories for eukaryote origin [259, 260], the birth of the chimeric eukaryote accompanied nucleus origin from the karyomastigont in amitochondriate protists. This notion is further supported by an obligate intranuclear endosymbiont of a γ-proteobacterium in the freshwater amoeba *Candidatus Berkiella aquae* (encoding bZIP protein KRG21159.1) [202]. Thus, we postulate that bacterial CNC/bZIP-encoding genes underwent horizontal gene transfer (HGT) to be retained in the earliest eukaryotes (unicellular protists) and subsequently differentiated to yield various ancient progenitors shared by distinct families of versatile bZIP transcription factors that emerged in later-evolving protozoans and even multicellular metazoans.

Equally important, transmembrane CNC-bZIP transcription factors (Nach5) emerged alongside the first animals, as indicated by biomarker evidence for early sponges [249]. Similar ancient transmembrane-bound orthologs Nach3, Nach6, and Nach7 are present in placozoans, anemones, and octopus, respectively, alongside CncC and Skn-1 (Figure 8). Additionally, two bacterial transmembrane CNC-like transcription activators, CadC and ToxR [257, 258], were found in *Escherichia coli* and *Vibrio cholerae*, respectively. Hence, we infer that a putative fusion gene encoding a transmembrane-associated polypeptide fused with a CNC-bZIP protein portion likely arose through homologous recombination of an endosymbiont transmembrane CNC-like gene with a host CNC-bZIP gene, enabling efficient expression during the origin of multicellular metazoans. The appearance of such membrane-tethered CNC-bZIP factors allowed ancient

animals to adapt to H_2S/RSS-based euxinic oceanic environments.

Thereafter, expansion and diversification of this CNC-bZIP family occurred only in vertebrates, yielding 6–10 homologs (e.g., Nrf1, Nrf2, Nrf3, p45NFE2, Bach1, and Bach2) encoded by distinct genes that were duplicated and differentiated during evolution [114]. This contributed to efficient response to the NOE caused primarily by plant photosynthesis, which led to the gradual appearance of the first large, architecturally complex organisms (e.g., vertebrates) and subsequent rapid increases in life species diversity [251–253].

Overall, transmembrane-bound Nrf1—rather than water-soluble Nrf2—is a living fossil-like CNC-bZIP factor highly conserved with the aforementioned ancient orthologous members emerging at distinct evolutionary stages. These objective facts demonstrate that Nrf1 is innately endowed to fulfill unique, intrinsic, and indispensable biophysiological functions distinct from those of Nrf2 in maintaining cell homeostasis and organ integrity [87]. Conversely, loss of Nrf1 function inevitably results in severe redox stress and pathological phenotypes [87, 174]. For example, conditional knockout of Nrf1 in mouse livers leads to spontaneous non-alcoholic steatohepatitis (NASH) and subsequent malignant transformation into hepatoma [261, 262]. Full-length Nrf1α-specific knockout by two distinct gene-editing techniques in human HepG2 cells leads to redox stress, lipid deposition, metaflammation [263, 264], and cancer malignancy [265, 266]. Collectively, these indicate that Nrf1, particularly Nrf1α/TCF11, is intrinsically conferred to act as a potent cancer suppressor [123, 167].

6.2.3 Cancer is likely defined as 'oncoprotists' by the eco-evo-devo ontology of its origin

From an integrated eco-evo-devo perspective [267–269], cancer can be defined by its "nature scientific essence" as an atavistically specialized unicellular life form (like protists) that selectively survives, originating from host tissues comprising collective multicellular sets in harmonious cooperation with distinct cell lineages. Generally, cancer arises early from an oncogenically specialized cell that becomes derailed from the integrated multicellular collective control system maintaining host tissue homeostasis, subsequently undergoing adaptive selection pressure through Darwinian evolutionary dynamics under ecological conditions of redox stress damage and/or long-term inflammatory infection within host tissues, particularly upon loss of Nrf1. Ultimately, such a specialized oncogenic cell-proliferating population undergoes an exclusive continuum of retrogressive and degenerative processes through reprogramming of cellular metabolism, genetics, epigenetics, and topogenetics during intragenerational proliferation, reverting to its original unicellular life status. This ontological essence represents a single-cellular protozoan, hence designated as "oncoprotists"—some exhibiting primitive characteristics of polyploid giant cancer cells (PGCC) [270]. This notion is evidenced by experimentally establishing nearly all cancer unicellular lines and relevant tumor-transplanted xenograft models. From this perspective, the most critical core question in current cancer research is how to

decode the origin and evolution of oncoprotists (i.e., cancer life); nothing in this core makes sense except in the light of cancer biology. Thereby, a vital key for cancer prevention strategy is deduced: preventing dedifferentiation of host cells (N$→1)*togeneratean"offbeat"oncogenicorganism*(*i.e.*, *oncoprotist*)*whilesimultaneouslyinducingoncoprotist*

7. Unique topogenetics of Nrf1 and its dynamic dislocation across membranes to the nucleus before regulating target genes

The basic concepts of topogenetics—membrane heredity, membrane system platforms, and relevant coding rules in topobiology—were introduced above (Section 3.4). Briefly, the early origin of almost all cellular life forms was dictated predominantly by the topogenesis of primordial membrane platforms and subsequent evolving membrane systems with inheritance. These cellular membrane-based frameworks are multi-hierarchically cross-linked with intracellular and extracellular matrix-scaffolding networks to selectively self-assemble an adaptive "toposkeleton" preset for the body plan to self-organize a topoform-specific life. Thus, the membrane system platform essentially lays a solid foundation for the origin of life and its ensuing evolution. Such membrane-relevant topogenetics should be tightly governed by key transmembrane molecular machinery, which critically encompasses membrane-bound transcription factors to serve especially as essential controls of topogenetic transformation, e.g., between epithelial and mesenchymal cellular states.

7.1 The peptide minimotifs evolving from primitive templates to specific DNA-binding transcription factors

According to the Central Dogma (DNA→RNA→Protein), the diversity of almost all cellular life's identifications and behaviors, with vast varieties of topoforms and relevant physio-pathological statuses, should be predominantly determined by this dogma-governed gene expression profiling. Such gene-centered determinism has dominated life sciences for over 70 years and seems almost unquestioned. Although the genetic codes for the Central Dogma were selected by Nature during long-term eco-evo-devo processes, it is impossible that the first formation of such DNA-encoding genes occurred de facto in primordial worlds (e.g., "chemical world," "metabolic world," "lipid world," "peptide world," and "RNA world"), all likely contained within membrane-based vesicles at the early beginning of cellular life. Hence, we conjecture that primeval replicating templates in reproducing pre-cells or proto-cells were likely executed by living fossil-like minimotifs (e.g., "basic-region zipper") [83–85], albeit with low infidelity (yielding multiple paraorthologous polypeptides through direct self-replication). Additionally, natural selection from such genetic minimotifs of peptides could enable "reverse translation" to give rise to the first single-stranded nucleotides (RNAs and/or DNAs) of life's origin [271]. This reversed flow of genetic information from peptide minimotifs as templates to yield primitive RNA/DNA (i.e., peptide → RNA/DNA), alongside another flow of known genetic information

from RNA to DNA or topologically folding chromatin [272], should collectively be referred to as the "Reverse Central Dogma"—an obligate rule de facto followed in prion propagation [273], though not central to biology. Moreover, the route of information transfer from protein to genome might not be completely blocked; thus, the strict validity of the Central Dogma could be questioned [272], possibly through convergent foundational discoveries from synthetic biology.

Conversely, in ensuing molecular evolutionary processes, a host of such fissile-like minimotif-containing polypeptides (e.g., the "basic-region zipper" superfamily) were ultimately selected as distinct sets of specific DNA-binding transcription factors, innately endowed to fulfill essential functions in controlling cognate genes. Notably, a portion of such trans-acting transcription factors was further subjected to distinct topogenetic folding to be integrated and positioned within and around membrane-based platforms (Figure 10 [Figure 10: see original paper]).

Figure 10. Unique topogenetic folding of Nrf1 and its dynamic dislocating across the ER membranes to the nucleus.

A. A set of transmembrane transcription factors selected from all distinct kingdoms of life on Earth. These life species include prokaryotes (bacteria) and unicellular and multicellular eukaryotes (yeasts, algae, plants, and complex animals). Most functional domains of Nrf1, Nrf1D, and others have been identified (in relevant references) except the viral Gag.

B. Several highly conserved structures of DNA-binding domains (e.g., CNC-bZIP, bHLH-ZIP) from indicated key transcription factors, adapted from αFoldDB (https://www.uniprot.org/).

C. By membrane-topogenetic folding of identified transmembrane transcription factors, they are divided into five distinct classes. Notably, Nrf1 is distinctive because it is endowed with unique membrane-topogenetic folding and dynamic repositioning across membranes before selective proteolytic processing of this CNC-bZIP factor and subsequent dislocation from the ER to the nucleus before regulating cognate genes.

7.2 Distinct topological folding of transmembrane transcription factors existing in all kingdoms of life

Such membrane-spanning transcription factors were computationally predicted to exist in all kingdoms of life and even in viruses [274]. Among them, transmembrane-associated CNC-ZIP transcription factors (Nrf1, Nrf1D, Nrf3, Skn-1A, CncC, Nach3, Nach5, Nach6, and Nach7 in Figure 7B) were identified by conserved NHB1 signal sequences targeting the endoplasmic reticulum (ER) and anchoring ER membranes [114, 203, 275]. In bacteria, a group of membrane-spanning transcription factors (e.g., CadC, ToxR, OmpR) with distinct sensors for pH, toxin, and osmotic changes across membranes (Figure 10A) were identified to regulate target genes through direct DNA-binding

wHTH (winged helix-turn-helix) domains [257, 258], which appear topologically folded similarly to the CNC-like domain (Figure 10B). One extensively studied pH-responsive system is CadC, a genetic transmembrane one-component sensor for dynamic regulation of D-xylonic acid accumulation [276]. The monotonic membrane-spanning ToxR regulon is also linked to lipid-remodeling gene expression profiles in *Vibrio cholerae* [277]; its life cycle is controlled by regulated proteolysis of ToxR, allowing rapid adaptation to stress [278].

NanH was identified as a bitonic membrane-spanning HTH transcription regulator that binds promoters of genes involved in sialic acid metabolism in the anaerobic pathogen *Clostridium perfringens* [279, 280], where sialidase mediates early-life colonization by pioneering gut commensals [281]. This is attributable to NanH facilitating commensal resilience and recovery after antibiotic treatment in defined microbial communities, revealing a co-evolutionary mechanism with microbiota through host-derived glycans to promote stable colonization. Furthermore, by genetically dissecting functions of regulatory proteins and enzymes responsible for catechol metabolism in the human gut bacterium *Eggerthella lenta*, another widespread family of polytonic membrane-spanning LuxR-type transcriptional regulators was revealed to be topologically folded similarly to DadR, HcdR, and CadR [282].

In yeasts, two homologous transmembrane NF- B-related transcription factors, Spt23 and Mga2, are selectively processed near the ER through internal proteasomal cleavage within ANK repeat domains (retaining a voltage-dependent potassium channel) to be released as mature trans-activators [283] (Figure 10, B & C2). Both factors play distinctive roles in activating the Δ9 fatty acid desaturase gene *OLE1* (crucial for de novo biosynthesis of unsaturated fatty acids as lipid building blocks) in *Saccharomyces cerevisiae* [284], though fatty acid-mediated regulation of Mga2 activity is independent of its proteolytic processing to yield a soluble transcription activator. Notably, Mga2p was originally identified as the first eukaryotic sensor for low temperature and oxygen to induce *OLE1* expression [285] through low oxygen response elements (LOREs) in promoter regions, though this gene is repressed by unsaturated fatty acids [286]. The OLE pathway is generally accepted as the best-characterized eukaryotic sense-and-control system regulating membrane lipid saturation by Spt23 and Mga2 [287]—two key factors determining maintenance of fluid lipid bilayers, membrane integrity, and even cell viability. This is based on the fact that membrane fluidity and phase behavior are dictated by proper proportions of saturated and unsaturated acyl chains in membrane lipid composition.

In almost all eukaryotes from yeast to humans, another link exists between ER membrane lipid sensing by a juxta-membrane amphipathic helix within the transmembrane domain of inositol-requiring enzyme 1 (IRE1, acting as both kinase and endoribonuclease) and resultant IRE1 signaling activation by detecting lipid bilayer stress to trigger the unfolded protein response (UPR) mediated by XBP1 (along with its homolog HAC1 in yeasts and bZIP60 in plants) [288]. This fissile-like IRE1-XBP1-UPR axis [289] is induced by lipid disequilibrium to coun-

teract membrane stress-induced cell death by reprogramming protein homeostasis and maintaining membrane functions without affecting composition [290]. For this, expression of different responsive genes to lipid bilayer stress and/or unfolded protein stress is regulated by spliced XBP1/HAC1/bZIP60 through the IRE1 sensor [291]. However, unspliced XBP1u mRNA and its protein are targeted to the ER membrane [292–294]. Under normal conditions, prototypic XBP1u protein is anchored within membranes through its C-terminal transmembrane (TMc) region (Figure 10C2) in a topological fashion similar to C-terminal Nrf1D before eliciting its unique function as a transcriptional repressor against its spliced effects [114]. Upon ER stress exposure, IRE1-mediated splicing of XBP1u mRNA transcripts to remove 26 nucleotides gives rise to an open reading frame-shifting variant XBP1s (Figure 10B), which lacks an available TM-targeting peptide to directly translocate to the nucleus and regulate UPR target genes [289]. Similarly, the TMc peptide of prototypic DMRT2u (226 aa) enables membrane anchoring (Figure 10C2), but alternative splicing of its transcripts in the stop codon-containing exon 4 gives rise to a fusion protein (DMRT2s) with an additional 328-aa polypeptide sharing 58% identity with Terra, critical for somitogenesis and sex determination [295].

Another classic ER stress signaling axis to UPR is monitored by the transmembrane sensor ATF6, which folds to adapt its initial membrane topology within and around the ER (Figure 10C3). If stimulated by ER stress, it is transported to the Golgi apparatus, where this bZIP protein undergoes progressive two-step processing by Site-1 and Site-2 proteases (S1P and S2P) to yield cleaved ATF6n factor [296, 297]. ATF6n is then released to enter the nucleus and activate target genes driven by either UPR elements or ESRE (ER stress response element) within promoter regions. Similar topological folding and processing occur for OASIS (Figure 10C3). By contrast, although sterol regulatory element binding protein 1 (SREBP1) contains two TM domains with distinct local topologies integrated within and around the ER, only its TM1 is folded similarly to ATF6. When target genes are required for cholesterol and other lipid synthesis, SREBP1 undergoes transfer similar to ATF6 and then dislocates from the ER through the Golgi to the nucleus (Figure 10C3). This processing is attributed to intramembrane proteolysis by S1P and S2P successively in the Golgi apparatus to generate cleaved activator SREBP1n [297, 298]. An additional evolutionarily conserved transmembrane-bound transcription factor, MYRF (myelin regulatory factor), was identified in invertebrates to vertebrates and is subject to proteolytic self-processing into an active factor required for myelin development and relevant diseases [299–302]. Overall, intracellular redox, protein, and lipid homeostasis are robustly maintained and tightly governed by all aforementioned transmembrane proteins together with other canonical sensors and transducers that increased during evolution from yeasts to vertebrates in response to distinct stress types.

7.3 Unique topological processing of Nrf1 to yield an active CNC-bZIP factor along with short isoforms

The unique membrane-topogenetic folding of Nrf1, its post-synthetic modifications, and proteolytic processing near the ER were comprehensively reviewed in detail [87, 167]. Briefly, the NHB1 signal peptide of Nrf1 enables its TM1 to be integrally anchored within ER membranes co-translationally [203, 303] and determines topological folding of adjacent domains (and amphipathic helix-adjoining domains) to be selectively partitioned into either the luminal side (e.g., transactivation domains) or cytoplasmic side (e.g., DNA-binding CNC-bZIP domains) of membranes [275, 304] (Figure 10C5). In the ER lumen, Nrf1 undergoes N-linked glycosylation in its NST domain by oligosaccharyltransferase to yield an inactive glycoprotein [203, 305, 306].

When biological cues require it, dynamic repositioning of the luminal-resident transactivation domain (TAD) of this intact CNC-bZIP factor is driven by p97-fueled retrotranslocation machinery through the Hrd1-leading retrotranslocon across membranes to enter the cytoplasmic side [275]. Once Nrf1's NST domain is dislocated out of the ER, the protein undergoes deglycosylation by N-glycanase 1 (NGLY1), re-editing its amino acid sequence from glycosylated asparagines to deglycosylated aspartates, thus potentiating its transactivation activity [275, 307]; similar work was confirmed for Skn-1 [308]. In cytoplasmic subcellular compartments, Nrf1 is also likely modified by O-GlcNAcylation in its Neh2L region of TADs [309] and its Neh6L adjacent to the CNC-bZIP domain [310, 311]. Such similar but differential modifications can bidirectionally (negatively and positively) monitor Nrf1 protein stability and transactivation activity. In extra-ER subcellular compartments, this CNC-bZIP protein is further subjected to selective proteolytic processing by cytosolic proteases DDI1/2 or proteasomes to yield an active N-terminally truncated CNC-bZIP factor (e.g., Nrf1/TCF11ΔN) together with distinct short isoforms (e.g., Nrf1β and Nrf1γ) [122, 312–314]. These distinctive functional isoforms are released from membranes and translocated into the nucleus, where each Nrf1 isoform can only form a functional heterodimer with its partner sMaf or other bZIP proteins before exerting different transcriptional regulation of target genes [87].

Collectively, different spatiotemporal modifications and selective proteolytic processing of Nrf1 are dominantly dictated by dynamic topovectorial folding and trafficking of this CNC-bZIP protein within and around the ER to dislocate to the nucleus, which determines its protein stability and transcriptional activity to regulate cognate genes [87, 315]. This notion is further supported by experimental evidence from Martinon's group [316], who found that both Rad23A and Rad23B are required for proteolytic processing of Nrf1 by DDI2, which was promoted by HRD1-mediated ubiquitination of this CNC-bZIP protein [316]. However, our experimental evidence revealed that Nrf1 ubiquitination is not a prerequisite for its proteolytic processing; instead, it is further activated by deubiquitination by USP19, enabling Nrf1 to be rescued from ER-associated degradation (ERAD) by proteasomes [317]. Like Rad23 and Dsk2, DDI2 can

also serve as a shuttling factor, but it contains a retroviral protease domain that influences binding of ubiquitylated proteins (e.g., Nrf1) through its N-terminal ubiquitin-like domain (UBL) and subsequent proteasomal degradation [318]. Altogether, these indicate that DDI2 determines selective proteolytic processing of Nrf1 to either yield a mature active factor (by this protease) or target it for proteasomal degradation (via this shuttling factor). However, Nrf1 is indeed also likely processed by other DDI1/2-independent proteolytic pathways [319]. More importantly, rapid recovery of proteasome activity from sublethal proteasome inhibitors is DDI2-independent, occurring before Nrf1-upregulated transcription of proteasomal genes but requiring protein translation [320]. The N-terminal domain of Nrf1 (containing NHB1 and NHB2) was further predicted to fold as an atypical UBL [122], similar to equivalents in DDI1, DDI2, Rad23, and Dsk2 (Figure S7). Overall, selective proteolytic processing of Nrf1 by DDI1/2 and/or proteasomes depends on dynamic spatiotemporal (re)positioning of NHB2 adjoining its putative UBL domain in different topovectorially localized subcellular compartments [122, 321], even though unique topogenetic folding of Nrf1 is dictated by NHB1-adjoining TM1 orientation within and around the ER.

Importantly, under unstressed conditions, only a fraction of Nrf1 undergoes proteolytic degradation mediated by the ubiquitin-proteasome system, because most of this CNC-bZIP protein is in situ protected by ER membranes and ferried through the ER-associated endomembrane network to reach the inner nuclear membrane (INM) [305], while its functional domains are repositioned in nucleoplasmic compartments, enabling it to exert unique physio-biological functions. In such topogenetic dislocation of Nrf1 from ER to nucleus, it likely undergoes topovectorial processing by another DDI1/2-independent pathway to yield a mature CNC-bZIP factor that activates cognate genes responsible for maintaining normal physiological homeostasis and organ integrity.

Additionally, a specific variant Nrf1D arises from alternative splicing of mRNA transcripts causing a reading frameshift mutation, leading to constitutive substitution of intact Nrf1's C-terminal 72-aa residues (covering the second half of its zipper motif to the C-terminal Neh3L domain) by another extended 80-aa stretch folded into a redox-sensitive transmembrane domain (TMc, Figure 10A), enabling tight integration within ER membranes beyond its NHB1-adjoining TM1 region [322]. Furthermore, Nrf1D was hitherto identified as the first candidate secretory transcription factor, though its precursor was predicted to fold as an integral transmembrane-bound CNC-bZIP protein entailing dynamic topologies within and across ER membranes [322]. However, it remains unknown how it is proteolytically processed by a yet-unidentified protease within the TMc-adjoining domain before being unleashed from the ER and secreted into the blood.

8. Unique pathophysiological phenotypes of Nrf1 that are distinctive from Nrf2

From the molecular phylogenetic evolution of the CNC-bZIP family described above (Figure 8), its members diverged upon vertebrates [114]. Notably, Nrf1 and Nrf2 are two major principal CNC-bZIP members in mammals, where Nrf1 appears to act de facto as an ortholog possibly arising through gene vertical transfer from ancestors, while Nrf2, together with other homologs, should be viewed as a redundant paralog (i.e., para-ortholog). This reasoning is convincingly evidenced by gene-targeting experiments revealing that global Nrf2$^{-}/^{-}$ knockout (KO) mice are viable and fertile, with no obvious defects or typical pathological phenotypes (e.g., spontaneous cancer) during embryonic development and postnatal growth [118, 323]. This demonstrates that Nrf2 is not necessary for normal development and healthy growth [324], though Nrf2$^{-}/^{-}$ mice were observed to be more susceptible than wild-type mice to chemical carcinogens [325]. By contrast, Nrf1 is indispensable for determining cell homeostasis and organ integrity because it is innately endowed with unique remarkable features distinctive from Nrf2 [87, 167] (Figure 11 [Figure 11: see original paper], A and B). This is based on discoveries from gene-targeting strategies for Nrf1 knockout that created distinct animal models with significant pathological phenotypes (Figure 11C) [261, 262, 326–329].

Figure 11. Unique and significant pathophysiological functions of Nrf1 that are distinguishable from Nrf2.

A. Schematic diagram of inter-regulation between Nrf1 and Nrf2, exerting uniquely differentiated yet integrated roles in governing intrinsic anti-redox compounds within multi-hierarchical signaling networks to mediate adaptive cytoprotective responses against various cellular stresses.

B. Functional distinctions between Nrf1 and Nrf2 evident in at least 15 different aspects. Nrf1 manifests unique physio-pathological functions that are distinctive or even absent from Nrf2. By sharp contrast, Nrf2 acts as a versatile chameleon-like master regulon and often plays certain “double-edged sword” effects.

C. Schematic representation of different tissue-specific loss of Nrf1 function in animal models, all significantly manifested with distinct pathophysiological phenotypes. Rather, constitutive activation of Nrf1 (to gain function in Nrf1-Tg:MGRD mice) can also give rise to observable phenotypes induced by a high-fat diet.

8.1 Global knockout of Nrf1 results in embryonic lethality

Apart from elevated expression in the heart, midbrain, and head mesenchyme between embryonic day (E) 8 and 9, the single *Nrf1/Nfe2l1* gene is ubiquitously expressed with constant mRNA transcript levels detected in all other tissues from E7.5 to E17.5 [330]. Conversely, global KO of Nrf1$^{-}/^{-}$ in mice leads to embryonic lethality at E6.5 to E14.5, resulting from severe oxidative

stress damage [326–328]. This presages that loss of Nrf1 cannot be compensated by Nrf2, even though both factors can elicit similar overlapping functions in regulating ARE-driven gene expression [331], as confirmed by their double knockout (Nrf1^{-}/$^{-}$:Nrf2^{-}/$^{-}$) mouse model [332]. This fact demonstrates that Nrf1, rather than Nrf2, fulfills unique and indispensable functions for embryonic development.

8.1.1 Genetic deletion of Nrf1's aa 172-741 causes embryonic lethality by blocking mesoderm formation The first global Nrf1 KO mutant (i.e., *Lcrf1tm1uab*, directly deleting 3.5 kb of its gene sequence with loss of aa 172-741) resulted in embryonic lethality during early gastrulation on Black Swiss outbred backgrounds [327]. Nrf1^{-}/$^{-}$ embryos developed to the late egg cylinder stage indistinguishable from wild-type counterparts, but thereafter arrested at E6.5 and most died before E7.5. This is attributable to failure to form primitive streak mesoderm with no Brachyury (T) gene expression, even though ectoderm and visceral endoderm layers appeared normal. However, the homozygous Nrf1^{-}/$^{-}$ defect was rescued after injection into wild-type blastocysts, and such mutant embryonic stem cells (ESCs) still contributed to all cell lineages in examined chimeras [327]. Overall, this Nrf1^{-}/$^{-}$ defect, despite being non-cell-autonomous, demonstrates that Nrf1's unique function is essentially required for transcriptional expression of a subset of critical genes constitutively involved in controlling mesoderm formation (principally by EMT [333–335]).

8.1.2 Targeted disruption of Nrf1's bZIP domain causes embryonic death from anemia The second global Nrf1 knock-in mutant (i.e., *Nrf1rPGK-neo*, generated by reversely inserting a phosphoglycerate kinase-neomycin cassette into the 5 -end of Nrf1's bZIP-coding region to disrupt Nrf1) resulted in embryonic lethality at mid-to-late gestation from E13.5 to E18.5 on C57BL/6J blastocyst background [328]. Nrf1^{-}/$^{-}$ embryos died in utero from decreased definitive enucleated red cells and ensuing anemia, resulting from impaired maturation of erythroid progenitors in fetal liver microenvironments but without increased apoptosis of hematopoietic cells [328]. Despite this lack of cell autonomy, Nrf1 serves as an essential gene for red cell maturation because no compensatory functions were efficiently executed by hematopoiesis-specific NF-E2 p45, Nrf2, or other CNC-bZIP factors expressed at high levels [336–338]. Furthermore, the hepatocyte-specific function of Nrf1 in fetal and adult livers is not rescued by putative compensatory function of Nrf2 [172]. These suggest certain overlapping functions and potential redundancy between the two CNC/bZIP factors in early embryogenesis and development. This is supportively evidenced by double KO Nrf1^{-}/$^{-}$:Nrf2^{-}/$^{-}$ leading to relatively earlier embryonic death at E10.5, attributable to extensively increased apoptosis and severe oxidative stress-induced growth retardation [332].

Mechanistic studies revealed that p53-Noxa-mediated apoptosis was markedly induced by elevated ROS (measured as hydrogen peroxide and singlet oxygen), resulting from severely impaired expression of antioxidant defense genes (e.g.,

Mt-1, *Gclm*, *Gclc*, *Ferritin H*, *Ho-1*, *Nqo1*, but not *Sod1/2*) compared to individual Nrf1^{-}/$^{-}$ or Nrf2^{-}/$^{-}$ [332]. This implies at least partial overlapping functions of Nrf1 and Nrf2 in regulating genes essential for intracellular redox homeostasis during early embryogenesis, possibly determined by both their co-expression patterns [337–339] and amino acid sequence similarity beyond CNC/bZIP domains [87]. Additionally, MG132-induced transcriptional expression of proteasome (PSM) genes was abrogated in Nrf1^{-}/$^{-}$ mouse embryonic fibroblasts (MEFs) but not Nrf2^{-}/$^{-}$ [340]. Lastly, the disparity in obvious pathophysiological phenotypes between *Nrf1rPGK-neo* [328] and *Lcrf1tm1uab* [327] is likely attributable to different strategic gene manipulations, because the former *Nrf1rPGK-neo* mice still allowed varying expression of certain Nrf1 isoforms (e.g., a C-terminally truncated negative mutant) [326, 328, 340–342].

8.1.3 Genetic ablation of Nrf1's bZIP domain leads to hepatocyte apoptosis in late gestation An additional lethal phenotype in homozygous Nrf1^{-}/$^{-}$ embryos was obtained by deleting a 2.2-kb genomic fragment encoding the bZIP domain to yield *Nrf1lacZ* mice [172], similar to *Nrf1rPGK-neo*; their livers were smaller, lighter, and hypoplastic, prominently by E13.5–E14.5 [328]. However, such developmental arrest cannot be interpreted as impaired erythropoiesis in fetal liver, because these erythropoietic cells still grew normally in vitro and Nrf1^{-}/$^{-}$ ESCs efficiently contributed to erythroid cells in chimeric mice generated with wild-type blastocysts [328]. Several chimeric embryos also exhibited anemia occurring before detection of cell death in liver parenchyma [172], suggesting that anemia is secondary to failure to sustain hematopoiesis in the impaired liver microenvironment.

To circumvent embryonic lethality, alternative chimeric mice were generated by injecting positive Nrf1^{-}/$^{-}$ ESC clones with 129/Sv background into C57BL/6J mouse blastocysts where Nrf1 is normally expressed [172]. Characterizing these chimeric mice at 8–16 weeks revealed that loss of Nrf1 impaired contribution of such Nrf1^{-}/$^{-}$ mutant ESCs to adult (rather than fetal) hepatocytes, even though they still contributed to other tissues (e.g., lung, kidney, muscle, heart) in adult animals where Nrf1 is highly expressed under normal genetic conditions [172]. Significantly, although Nrf1^{-}/$^{-}$ ESCs contributed to fetal liver development at E14.5, hepatocytes in chimeric embryos underwent widespread apoptosis in late gestation [172]. Further examination revealed that hepatocyte apoptosis resulted from increased oxidative stress and decreased expression of antioxidant genes (e.g., *Gclc*, *Gclm*, *Gpx1*, *Ho-1*, and *Mt-1/2*). These findings demonstrate a cell-autonomous role for Nrf1 in protecting hepatocytes from apoptosis and enabling their healthy survival during late fetal development, primarily by maintaining normal redox homeostasis.

8.2 Liver-specific knockout of Nrf1 causes spontaneous development of NASH and hepatoma

Given massive cell death and degeneration in livers of chimeric embryos derived from Nrf1^{-}/$^{-}$ ESCs [172], such models cannot determine Nrf1's critical role in hepatocyte maturation. To bypass this obstacle, conditional KO models of Nrf1 in mouse livers were created using a specific Cre-loxP transgenic system to determine Nrf1's unique essential function in adult hepatocytes beyond embryonic development [261, 262].

8.2.1 Adult hepatocyte-specific deletion of Nrf1's aa 296-741 results in NASH and hepatoma Liver-specific KO of Nrf1 (deleting the fourth exon encoding aa 296-741, i.e., LCR-F1/Nrf1β) in adult mice resulted in a typical pathologic phenotype resembling NASH and hepatic neoplasia, including hepatocellular adenomas and carcinomas that spontaneously developed as early as 4 months after birth [261]. Before cancer development, Nrf1^{-}/$^{-}$ livers also exhibited interrelated steatosis, apoptosis, necrosis, inflammation, and fibrosis.

In addition to such precancerous lesions, loss of Nrf1^{-}/$^{-}$ per se may directly contribute to tumorigenesis by promoting chromosome missegregation [343]. Tamoxifen-inducible KO of Nrf1^{-}/$^{-}$ (from *Nrf1flox/flox:Cre-ERT2*) increased abnormal nuclei and micronuclei numbers 3-fold higher than controls. Such Nrf1^{-}/$^{-}$-led genetic instability appears closely associated with decreased expression of kinetochore genes *Ndc80*, *Nuf2*, and *Spc25*, as well as the spindle assembly checkpoint gene *Sgol1* [343]. Together, these suggest that Nrf1 could function as a tumor suppressor in hepatocytes.

To gain insight into the molecular pathological basis for NASH caused by Nrf1^{-}/$^{-}$ in livers, further experiments revealed that Nrf1-deficient hepatocytes acquired increased susceptibility to oxidative stress and relevant damage, along with down-regulation of some ARE-battery genes (e.g., *Gstm3*, *Gstm6*, and *Gstp2*) but up-regulation of *Cyp4A* genes [261]. Loss of Nrf1 function resulted in significantly increased intracellular ROS levels, generated at least in part by ω-oxidation of fatty acids using proliferated microsomal CYP4A (i.e., in the ER). Increased ROS levels were also associated with elevated lipid and other pathogenesis leading to NASH.

Similar pathological damages were observed in mouse livers of another hepatocyte-specific Nrf1^{-}/$^{-}$ model [262]. Such pathophysiological phenotypes demonstrate that Nrf1 acts as a unique vital player in mediating expression of critical genes for maintaining intracellular redox and lipid homeostasis in livers. Further experimental evidence revealed that lipid accumulation in Nrf1^{-}/$^{-}$ livers at 5 weeks resulted from up-regulation of 1,500 genes and down-regulation of 1,700 genes [344]. Genes involved in lipid metabolism (e.g., *Lipin1*, *PGC-1β*, *PPARα*), amino acid (e.g., methionine) metabolism, the TCA cycle, mitochondrial respiration, and 26S proteasomes were decreased, whereas genes responsible for cell cycle and DNA replication were increased; these

Nrf1-target genes were unaffected by loss of Nrf2 or Keap1.

A bulk of ubiquitinated and/or oxidatively damaged proteins, besides lipids, accumulated in Nrf1^{-}/$^{-}$ hepatocytes, leading to ER stress-associated steatosis [345]. This is attributed to impaired transcription of PSM genes and increased expression of ER stress response genes (e.g., *Atf4*, *Atf6*, *Bip*, *Chop*, *Gadd45β*, *Herp*), accompanied by phosphorylation of PERK-eIF2α signaling [345]. Further microarray analysis revealed down-regulation of 52 Nrf1-dependent genes (i.e., *Mt1/2*, *Clrf*, *Gcn20*, *Gadd45γ*, *Mfsd3*, *Pdk4*, and *Spp3*) by $ $3-fold.

Intriguingly, adaptive activation of 20 Nrf2-target genes by single KO of Nrf1^{-}/$^{-}$ was abolished by double KO of Nrf1:Nrf2 [262]. This demonstrates that Nrf1 exerts an essential physiological function required for basal constitutive expression of a subset of cytoprotective responsive genes against endogenous (redox, proteotoxic, and lipotoxic) stresses (that trigger Nrf2 activation). Rather, no significant changes in prototypic Nrf2-target genes (e.g., *Gclc*, *Gclm*, *Gss*, *Nqo1*) were observed upon acute hepatic loss of Nrf1 in 3-methylcholanthrene-inducible KO mice (*Nrf1flox/flox:Cyp1A1-Cre*), which also led to profound NASH but without obvious oxidative stress [346]. Collectively, these demonstrate that Nrf1 regulates a separate battery of genes distinct from those regulated by Nrf2.

8.2.2 Cholesterol-led NASH is deteriorated by hepatocyte-specific loss of Nrf1's bZIP domain Hepatocyte-specific KO of Nrf1 (deleting a fragment spanning exons 4 and 5 encoding its DNA-binding domain in homozygous mice obtained by crossbreeding C57BL/6N-A/a chimeric males with C57BL/6J females) substantially aggravated cholesterol-led pathological phenotypes in liver resembling human hepatosteatosis and NASH [347]. Cholesterol-fed Nrf1^{-}/$^{-}$ mice developed dramatically heavier fatty livers, showing massive lipid accumulation and indications of hepatocyte ballooning and damage, though without increased body weight compared to wild-type controls [347]. Cholesterol-exposed Nrf1^{-}/$^{-}$ mice exhibited a substantial rise in cholesterol esters accounting for >50% of total liver lipids. Such elevated cholesterol, cholesterol ester, and cardiolipin, while other classes remained unchanged, coincided with a greater cholesterol-to-phospholipid ratio in livers. These findings indicate a critical physiological role for Nrf1 in protecting hepatocytes and livers from excessive cholesterol. This notion is further evidenced by subsequent experiments revealing that Nrf1 controls a transcriptional program for hepatocyte adaptation to cholesterol as a means of averting stress and limiting cholesterol accumulation, though repression of such Nrf1 activity is indeed evoked by cholesterol [347]. This repression of Nrf1 by cholesterol is further aggravated by directly sequestering this CNC-bZIP factor within ER membranes through its cholesterol recognition amino acid consensus (CRAC) motifs [122, 275, 304, 347], such that connecting (PEST-)degrons can be buried in the lumen and escape proteolytic processing, forming a negative regulatory feedback circuit on Nrf1-target genes.

Conversely, Nrf1-deficient livers develop severe pathological problems because they are relatively refractory to transcriptional changes induced by cholesterol

challenge: only 93 differentially expressed genes (DEGs, 0.91%) out of 10,174 detectable genes, whereas 826 DEGs (8.12%) were measured in cholesterol-fed Nrf1 wild-type mice [347]. Further, 790 DEGs identified in wild-type cases were absent from Nrf1^{-}/$^{-}$ livers; these were defined as Nrf1-dependent genes regulated by cholesterol and enriched by gene ontology clustering for acute inflammatory response, lipid transport, and sterol metabolic process [347]. Further experimental analysis of Nrf1^{-}/$^{-}$ livers with cholesterol-led increased H_2O_2 and phosphorylated JNKs revealed that Nrf1 suppresses cholesterol accumulation and stress-triggering inflammation (NASH) by down-regulating *CD36* (and *AbcA1*, *AbcG1*, *Gltp*, *ApoC2*, *F4/80*, *C1Q8*, *Orm2*, *Saa2*), while promoting cholesterol excretion by up-regulating *Cyp7a1*, *Cyp7b1*, and *Cyp8b1* (besides *Ces1f* and *Insig1*), compared to wild-type controls. Notably, induction of *Srebp1c*, *AbcA1*, and *AbcG1* by cholesterol or liver X receptor (LXR) agonist GW3965 in Nrf1^{-}/$^{-}$ primary hepatocytes was blunted by ΔNT-Nrf1, which acts as a constitutively active but cholesterol-insensitive Nrf1 isoform but could not rescue Nrf1-deficient livers. This implies likely interference of nuclearly located Nrf1 factor on LXR (and/or SREBP1), consistent with LXR's role as a multifaceted countermeasure against excess cholesterol. Besides, two ER membrane-spanning transcription factors—Nrf1 and SREBP2 (Figure 10)—likely co-evolved as a "Yin-Yang" counterbalance whereby SREBP2 promotes cholesterol production while Nrf1 promotes cholesterol removal (Figure 12 [Figure 12: see original paper]), achieving cholesterol homeostasis and stabilizing relevant metabolic activity.

Figure 12. Comparison of liver-specific Nrf1^{-}/$^{-}$ phenotypes with those of constitutive active Nrf1-Tg:MGRD mice.

A. Schematic representation of collective pathophysiological phenotypes of liver-specific Nrf1^{-}/$^{-}$ mice, manifested with spontaneous development of progressive fatty liver diseases such as hepatosteatosis, nonalcoholic steatohepatitis (NASH) or metabolism-associated steatohepatitis (MASH), and even hepatoma, also accompanied by reduced glycemia (hypoglycemia) in some cases. Notably, a major pathogenic mechanism accounting for these phenotypes results from disrupting the "Yin-Yang" counterbalance between Nrf1 and SREBP2/LXR [347].

B. Constitutive activation of Nrf1-Tg:MGRD mice to gain function also results in a lean phenotype with insulin resistance and even diabetes mellitus development, more obviously induced by high-fat diet [329] but ameliorated by heterozygous Nrf1^{+}/$^{-}$ mice.

8.2.3 The pathogenesis of NASH is accelerated by combined deficiency of Nrf1 and Nrf2 in hepatocytes Reduced growth of Nrf1- but not Nrf2-deficient mice (with 80% knockout by floxed Nrf1/Nrf2-encoding alleles at 14 days after infecting liver-targeting adeno-associated virus expressing Cre recombinase via thyroxine-binding globulin promoter) was observed, though both groups exhibited normal appearance and behavior [348]. By contrast, substantial weight loss and morbidity (e.g., poor grooming, slow movement) were caused by combined Nrf1:Nrf2 deficiency; these effects were so severe that 46%

(5/11) of males and 27% (3/11) of females did not survive 28 days [348]. This is attributable to increased lipids (triglyceride and cholesterol) deposited in livers of combined Nrf1:Nrf2-deficient mice, along with sex-dependent increases in plasma cholesterol, low-density lipoprotein (LDL), and very low-density lipoprotein (VLDL) cholesterol in males but not females, apart from reduced liver VLDL secretion in Nrf1- or Nrf1:Nrf2-deficient mice.

Histopathological phenotype of modestly increased steatosis and inflammation (i.e., steatohepatitis) was manifested in Nrf1- but not Nrf2-deficient livers, and combined deficiency caused significant steatohepatitis resulting in hepatocyte ballooning [348]. This was accompanied by increased mRNA levels of chemokine ligand 2 (*ccl2*), *TNFα*, *TGFβ*, and collagens (*col1a1*, *col1a2*, *col3a1*), and increased plasma alanine transaminase (ALT) in Nrf1:Nrf2- or Nrf1- but not Nrf2-deficient livers. Nrf1 deficiency-caused steatohepatitis was also featured by p62-enriched Mallory-Denk bodies, intracellular hyaline inclusion bodies or aggresomes, aside from lipid droplets. Cholesterol crystals, a hallmark of steatohepatitis and hepatic cholesterol overload [349, 350], were determined in Nrf1- but not Nrf2-deficient livers and significantly increased by combined deficiency of both factors in both sexes [348]. Collectively, these demonstrate complementary roles of Nrf1 and Nrf2 in guarding against hepatic cholesterol overload, increased triglyceride storage, cholesterol crystallization, and diet-induced steatohepatitis—roles vital for whole-body viability. Conversely, combined hepatocyte Nrf1:Nrf2 deficiency accelerated steatohepatitis pathogenesis, accompanied by increased cholesterol accumulation and crystallization, altered bile acid metabolism, and decreased biliary cholesterol.

Such severe steatohepatitis in nutrient-overloaded livers was much more likely ameliorated by combined induction of Nrf1 and Nrf2 as therapeutic co-targets for effectively treating metabolic-associated steatohepatitis (MASH). This notion is further supported by experimental evidence revealing that therapeutic effects of the Nrf2-activating drug bardoxolone methyl require Nrf1 as a key contributor due to their co-regulation of immunometabolic stress defense genes that eliminate cholesterol overload and mitigate oxidative stress damage and inflammation [348]. The complementary gene programming of Nrf1 and Nrf2 can thus counteract a pathological progressive continuum of cholesterol-associated fatty liver diseases (including NASH). As such, the modest pathological phenotype caused by Nrf1 deficiency rather than Nrf2 deficiency cannot be rescued by the latter's compensatory increased expression (~40% higher mRNA levels) in Nrf1-deficient livers [348]. This strongly demonstrates that Nrf1 exerts an essential role—irreplaceable by Nrf2—in hepatic pathophysiological processes (Figure 12).

Further RNA-sequencing analysis revealed that functions related to cholesterol and bile acid synthesis, transport, and metabolism were inhibited by altered retinoid X receptor (RXR)-dependent pathway activity [348]. RXR-linked control of immunometabolism was disrupted by hepatocytic deficiency of Nrf1 and Nrf2. Their co-target gene-binding activity was also confirmed by ChIP to *txn1*

(thioredoxin 1, for redox stress defense), *lonp2* (lon peptidase 2, for peroxisomal proteostasis defense), and *abcg8* (ATP-binding cassette subfamily G member 8, for cholesterol excretion); all three were reduced by combined Nrf1:Nrf2 deficiency to a greater degree than by single deficiency of Nrf1 or Nrf2. By sharp contrast, Nrf1-specific targets *Psma1* and *Psmc2* were reduced to similar extents by deficiency of Nrf1 or Nrf1:Nrf2, while Nrf2-target *Ces1g* (carboxylesterase 1G) and *Gstm1* (glutathione S-transferase mu 1) in biotransformation and phase 2 detoxification were reduced to similar extents by deficiency of Nrf2 or Nrf1:Nrf2. Together, these overlapping and exclusive genome sites enable Nrf1 and Nrf2 to interact for cognate target gene regulation in the liver.

Combined Nrf1:Nrf2 deficiency also caused greater reduction of *PPARα* (peroxisome proliferator-activated receptor α) and *CAR* (constitutive androstane receptor), two known transcription factors regulating lipid, bile acid, and peroxisome metabolism, as well as liver detoxification and ROS defense, along with reduced *BAAT* (bile acid-coenzyme A amino acid N-acyltransferase), *AbcG5*, *PRDX1* (peroxiredoxin 1), and *Cat* (catalase), compared to single deficient cases [348]. Conversely, greater increases in two known NASH-drivers *IHH* (Indian hedgehog) and *tnfrsf12a* (TNF receptor superfamily member 12A) were caused by combined Nrf1:Nrf2 deficiency, accompanied by increased expression of 4-hydroxynonenal (4HNE)-conjugated proteins, as well as *TAZ* (taffazin) and *YAP1* (yes-associated protein 1), which drive IHH expression and steatohepatitis [351, 352]. Collectively, these demonstrate a co-regulatory role for Nrf1 and Nrf2 in hepatobiliary detoxification of cholesterol and bile acids.

Recently, significantly reduced HDL (high-density lipoprotein) cholesterol and apolipoprotein A1 (ApoA1) levels were found to be caused by combined deletion of Nrf1:Nrf2 but not by single deletions [353]. This was also accompanied by decreased HDL capacity to accept cholesterol efflux from macrophages and counteract TNFα-induced inflammatory effects on endothelial cells. This coincided with substantial alterations in the HDL-resident proteome, which fully correlated with liver gene expression profiles of corresponding proteins (e.g., ApoA1, ApoA2, ApoA4, ApoE, C3, C9, ApoJ, Cpn2, Gpld1, Itih2, and Itih4) responsible for mediating reverse cholesterol transport, reducing inflammation, and abrogating oxidative damage. Their modest changes were also observed from Nrf1 but not Nrf2 deficiency [353]. These reveal coordinating control of HDL functioning by Nrf1 and Nrf2 to improve hepatoprotective and even atheroprotective actions, in which Nrf1 likely exerts a vital role.

8.2.4 Hepatocyte-specific deficiency of Nrf1 results in reduced glycemia (hypoglycemia) Although both Nrf1 and Nrf2 serve as stress-defense transcription factors, they also exert complementary but exclusive regulatory roles in programming genes responsible for hepatocytic glucose metabolism and even systemic glucose homeostasis [354–356]. Conversely, reduced glycemia (hypoglycemia) was caused by hepatocyte-specific loss of Nrf1 or Nrf1:Nrf2 but not Nrf2 in mice (fed a mild stressful fat-fructose-cholesterol

diet for 1–3 weeks), but this phenotype did not occur in leptin-deficient obese and diabetic mouse models (*leptinob/ob:Nrf1flox/flox*) [356]. This indicates an Nrf1-supporting defense for hepatocytes to counteract hypoglycemia but not promote hyperglycemia or diabetes. Consistently, Nrf1 deficiency resulted in reduced hepatic glycogen and glycogen synthase (e.g., *GYS2*) expression, accompanied by decreased insulin and insulin-like growth factor-1 (IGF1) and increased growth hormone (GH), with no marked alterations in other examined glycemia-influencing hormones (e.g., glucagon, ghrelin, cortisol, triiodothyronine, thyroxine, and gastric inhibitory polypeptide) [356]. Although Nrf1-deficient livers' capacity for gluconeogenesis appeared unaffected [356], glucose starvation-induced rapid death of human Nrf1α- but not Nrf2-deficient hepatoma cells results from fatal defects in redox metabolism reprogramming [354]. Altogether, these reveal an essential role for Nrf1 in modulating glucose homeostasis for steady-state euglycemia through the GH-IGF1 signaling axis to liver glycogen storage (Figure 12). In turn, reduced expression of GH receptor (sensing GH to stimulate liver IGF1 secretion) contributed to reduced glycemia caused by Nrf1 or Nrf1:Nrf2 deficiency but not Nrf2 deficiency [356].

8.3 Gain-of-function of Nrf1 loses body weight but gains risk of diabetes with insulin resistance

The physio-pathological function of Nrf1 seems somewhat uninterpretable from evidence obtained in above-mentioned loss-of-function mutants, which manifested severe liver dysfunction and metabolic disorders. To bypass this, gain-of-function transgenic mice (i.e., Nrf1-Tg:MGRD) were created by engineering overexpression of Nrf1-3×Flag driven by the MafG-regulated promoter domain [329]. Consequently, increased Nrf1 expression leads to body weight loss in Nrf1-Tg:MGRD mice fed normal and even high-fat diets. Thereafter, high-fat diet-induced obesity model mice further developed pathology resembling human diabetes mellitus, with markedly higher blood glucose levels after insulin injection [329]. Pancreatic islets of Nrf1-Tg:MGRD mice were much larger than those of wild-type mice, with increased insulin-positive areas, implying constitutive activation of insulin secretion leading to systemic insulin resistance. Similar insulin resistance also occurred in normal-fed Nrf1-Tg:MGRD mice, positively correlating with increased Nrf1 expression.

Induction of insulin resistance appears interpretable as disruption of cell signaling to metabolic perturbation by forced Nrf1 expression, evidenced by supportive findings [329]: (i) insulin-induced phosphorylation of Akt-Ser473 was obviously prevented in liver and skeletal muscle but not white adipose tissue of Nrf1-Tg:MGRD mice, implying Nrf1 suppresses insulin signaling to Akt phosphorylation; (ii) Nrf1-Tg:MGRD mice showed increased liver weights but not skeletal muscle or white adipose tissue weights; such increased liver weights were further augmented by high-fat diet with altered hepatic metabolism; (iii) both glucose utilization and production in livers were impaired by Nrf1-Tg:MGRD repressing insulin-regulated glycolysis-related genes (e.g., *GCK*, *AldoB*, *PGK1*, and *PK*),

gluconeogenesis-related genes (e.g., *Fbp1* and *Pck1*), and glucose transporter 2 (*Glut2*), particularly under high-fat diet conditions; (iv) Nrf1-Tg:MGRD suppressed glucose entry into the glycolytic pathway, leading to marked decreases in glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P) levels, accompanied by increased pyruvate and reduced lactate, such that TCA cycle entry was increased with elevated acetyl coenzyme A (CoA), citrate, and ATP levels in livers of high-fat diet-fed Nrf1-Tg:MGRD mice; (v) hepatic CoA utilization to generate β-hydroxybutyrate was increased from other fuels (fatty acids) beyond glucose and amino acid catabolism. Lastly, such impaired glucose metabolism was also obviously ameliorated by decreased Nrf1 expression in heterozygous $Nrf1^{+}/^{-}$ mice compared to wild-type mice, even with high-fat diet feeding. Collectively, impaired insulin signaling and dysregulated glucose metabolism by constitutive Nrf1-Tg:MGRD induction results in diabetes mellitus development triggered by high-fat diet (Figure 12). However, except that insulin-induced Akt phosphorylation was repressed by forced Nrf1 expression, no obvious changes in major insulin signaling pathway component expression levels were determined by microarray analysis of liver and skeletal muscle from Nrf1-Tg:MGRD mice [329]. This indicates insulin resistance is triggered by Nrf1 through a pathway distinct from transcriptional repression of insulin signaling components.

Additionally, a single nucleotide polymorphism (SNP) rs3764400 located in the 5 -flanking region of the human *Nrf1* gene represents a risk factor for obesity with body mass index (BMI, $P = 5 \times 10^{-6}$) [357]. Reporter gene activity showed that rs3764400, as a regulatory SNP of Nrf1 expression with ~2.2-fold higher level, was driven by the risk C allele compared to control T allele within the 2.1-kb promoter region upstream from the first transcription start site [329]. The major T allele of rs3764400 (5 -CAGT/CT-3 [357]) composes a consensus c-Myb-binding sequence 5 -CAGTT-3 (reversed by 5 -AACNG-3) [358], while the minor C allele (enabling Nrf1 induction [329]) cannot allow c-Myb-related factors to bind the altered motif. Thus, c-Myb likely acts as an upstream regulator of Nrf1 for transcriptional control of glucose metabolism, but its dysfunction leads to increased Nrf1 expression contributable to obesity pathophysiology and relevant glucose metabolic dysregulation. Moreover, differential regulation of glucose metabolism by Nrf1 and Nrf2 is supported by further experimental evidence revealing that blood glucose levels were increased by gain-of-function of Nrf1 [329] but decreased by loss-of-function of Nrf1 but not Nrf2 [356], as well as decreased by gain-of-function of Nrf2 [359, 360].

8.4 Pancreatic loss of Nrf1 causes impaired insulin secretion and glucose metabolism leading to diabetes

Failure of pancreatic β-cells to secrete insulin sufficiently to meet increasing glucose metabolic demand is a major contributor to type 2 diabetes with secondary β-cell loss. Impaired β-cell function reducing glucose-stimulated insulin secretion (GSIS) is a critical pathophysiological event in diabetes. Coincidentally, β-cell-specific Nrf1-KO mice exhibit severe fasting hyperinsulinemia accompa-

nied by reduced GSIS and glucose intolerance [361]. The pathophysiological phenotype results from deficiency of almost all Nrf1 isoforms in mouse islets and MIN6 β-cells, leading to markedly increased basal insulin release with reduced GSIS—a β-cell phenotype reminiscent of early-stage type 2 diabetes. Hyperinsulinemia has been accepted as a compensatory response to insulin resistance, and its prolonged status can deteriorate insulin resistance induction and even obesity [362, 363]. Thus, the pathological phenotype of pancreatic Nrf1-KO mice likely accounts for ensuing glucose intolerance.

Further biochemical examinations of Nrf1-deficient islets and β-cells revealed substantially increased NADPH/NADP and ATP/ADP ratios attributable to enhanced glycolysis and mitochondrial metabolism (e.g., β-oxidation) rather than oxidative phosphorylation [361]. Silencing Nrf1 up-regulated expression of glucose transporter 2 (*Glut2*) and glycolytic proteins such as lactate dehydrogenase 1 (*Ldh1*), glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*), and hexokinase 1 (*HK1*), but down-regulated glucokinase (*Gck*). Interestingly, such impaired glycolysis and GSIS resulting from Nrf1 loss can also be rescued by *HK1* knockdown. However, no changes in expression profiles of mitochondrial protein complexes I, II, cytochrome c, ATP synthase F1, or mitochondrial biogenesis-related *PGC-1α*, *PGC-1β*, and nuclear respiratory factor 1 (called aPalNRF1 [364]) were determined in Nrf1-deficient cells compared to wild-type controls [361].

Collectively, β-cell-specific depletion of Nrf1 leads to a shift from oxidative phosphorylation to aerobic glycolysis for energy, a consequence similar to the Warburg effect in cancer cells [365, 366]. Furthermore, altered expression levels of p53, phosphorylated AKT, and AMPKα were observed in Nrf1-deficient cells [265, 355] or constitutive active Nrf1-Tg:MGRD mice [329], implying involvement of these signaling molecules in altered glucose metabolism. Altogether with evidence provided by [367], we infer that coupled metabolic reprogramming switches with signaling responses to endogenous redox stress status may exist in Nrf1-deficient cells.

8.5 Adipose-specific loss of Nrf1 disrupts its plasticity, adaptive thermogenesis, and metabolic homeostasis

Adipocytes possess remarkable adaptive capacity to respond to nutrient excess, fasting, or cold exposure, with distinct adipocyte types maintaining proper metabolic health. Brown adipose tissue (BAT) is a crucial metabolic organ in facultative thermogenesis (acute response), with great plasticity to respond to long-term cold adaptive thermogenesis. BAT thermogenic capacity depends on neurohumoral response to sympathetic stress stimulation [368]. Such BAT is distinguishable from white adipose tissue (WAT) because it is richly innervated by the sympathetic nervous system (SNS, where norepinephrine released at nerve endings activates adrenergic receptors on brown adipocytes) and highly vascularized in brown adipocytes, with several small lipid vacuoles and many large mitochondria [369]. In addition to critical roles in maintaining thermal

and energy homeostasis, BAT is involved in plasma triglyceride clearance and glucose homeostasis [370, 371], demonstrating essential functionality in combating obesity and metabolic diseases (e.g., diabetes and cardiovascular disease). By contrast, WAT comprises a population of specialized white adipocytes for energy storage (as triacylglycerols) and mobilization (as fatty acids), long considered an inactive tissue primarily serving thermal insulation. Lipid and glucose metabolism in white adipocytes also confers a pivotal role to WAT in whole-body homeostasis, particularly energy homeostasis [372]. Conversely, dysfunction in white adipocyte metabolism is a cardinal event in developing insulin resistance and associated disorders (e.g., type 2 diabetes mellitus). In such distinct adipose tissues, specific knockout of Nrf1 as a key thermogenic factor in mice resulted in different pathophysiological phenotypes [373–375].

8.5.1 BAT-specific KO of Nrf1 in mice that cannot adapt to rising thermogenic activity Conditional deletion of Nrf1 expression was created using homozygous floxed *Nfe2l1* alleles (coding its DNA-binding domain) under control of the *Ucp1* promoter-driven Cre (to yield *Nfe2l1*Δ*BAT* mice) or *Ind.Nfe2l1*$^{-}/^{-}$ mice raised by tamoxifen-inducible Cre under control of chicken β-actin promoter-enhancer coupled with cytomegalovirus immediate-early enhancer (CAGGCre-ERTM) [373] to investigate linkage of cold adaptive response to target proteasome in BAT. This is due to ER-associated protein degradation (ERAD) removing unfolded, damaged, or dispensable proteins by the ubiquitin-proteasome system (UPS) in this UPRER, and it was also found from adipocyte-specific deficiency of IRE1α (encoded by *Ern1*) or XBP1 in mice that this conserved canonical branch of UPRER is dispensable for BAT-mediated homeostasis [373].

Compared to 30°C, cold adaptation caused markedly increased Nrf1 expression in wild-type BAT (aside from minor expression in inguinal WAT). Cold exposure induction of Nrf1 in brown adipocytes yielded higher levels of ER-localized Nrf1 protein and its cleaved, transcriptionally active form in BAT. By contrast to WT controls, markedly lower Nrf1 mRNA and protein levels were observed in cold-adapted *Nfe2l1*Δ*BAT* mice, implying thermoneutrality is a state of natural Nrf1 deficiency [373]. Thermogenesis requires cold-inducible proteasomal functionality transcriptionally driven by Nrf1, because cold adaptation of BAT induces Nrf1 to increase proteasomal activity crucially for maintaining ER homeostasis and cellular integrity, particularly when cells are in high thermogenic activity states. Under such thermogenic conditions, BAT-specific Nrf1 deletion enabled *Nfe2l1*Δ*BAT* mice to enter a hyper-ubiquitinated state, resulting in ER stress, metainflammation, markedly diminished mitochondrial function, and BAT whitening. This suggests Nrf1 is required for BAT activity to maintain metabolic adaptation besides ER homeostasis, supported by *Nfe2l1*Δ*BAT*-leading hyper-ubiquitination of 228 proteins most notably enriched in mitochondria and organelle homeostasis, particularly related to ER proteins and energy metabolism. Thus, Nrf1 in thermogenic fat cells acts as a metabolic guardian preventing tissue stress and inflammation, independently of

BAT differentiation, mass, or expandability.

Mice lacking Nrf1 in brown adipocytes are born normal, but *Nfe2l1ΔBAT* BAT alterations develop as a function of cold and age. At thermoneutrality, where BAT activity is minimal, Nrf1 was lowered in BAT, but in this environment neither proteasome inhibition nor genetic Nrf1 deficiency is more deleterious for the tissue. This indicates Nrf1-mediated transcriptional programming is required for adaptation to rising thermogenic activity. This is also corroborated by the phenotype of tamoxifen-induced Nrf1 deletion in adult *Ind.Nfe2l1*$^{-/-}$ mice [373]. BAT thermogenic activation following cold adaptation or treatment with CL316243 (an adipocyte-specific $3 − *adrenergicagonist*[376])*atthermoneutralitydefactorequiresNrf*1 − *mediatedincreasedproteasomeactivitytofulfillitsmetabolicpotential*, *butsuchproteasomalactivityisimpaired* *mediatedNrf*1*expressionorproteasomeactivatorPA*28 $ expression to enhance proteostasis in BAT resulted in obviously improved insulin sensitivity. Altogether, Nrf1 emerges as a novel guardian of brown adipocyte function, providing enhanced proteometabolic quality control for thermogenic adaptation to cold or obesity.

Another recent study revealed that adipocyte-specific Nrf1-KO [*Nfe2l1(f)-KO*] mice develop age-dependent whitening and shrinking of BAT, with signatures of down-regulated proteasome, impaired mitochondrial function, reduced thermogenesis, pro-inflammation, and elevated regulatory cell death [377]. This is attributable to Nrf1 deficiency in brown adipocytes predominantly causing down-regulated expression of lipolytic genes (*PNPLA2*, *ATGL*, *HSL*, *MGL*, *PLIN1*), decelerating lipolysis so BAT cannot fuel thermogenesis and thus leading to brown adipocyte hypertrophy (enriched with larger lipid droplets and swollen mitochondria), inflammation related to regulatory cell death, and consequently cold intolerance. Further single-nucleus RNA-sequencing of BAT unraveled that Nrf1 deficiency caused significant transcriptomic reprogramming and aberrant expression of various genes involved in thermogenesis (*Ucp1*), lipid metabolism (e.g., *Prdm16*, *Elovl3*, *Cpt1b*, *Pparα*), mitochondrial (respiratory) stress (*Cox8b*, *Cox7a1*, *PGC1α*, *COXIV*, *NDUFS4*, *FIS1*, *OPA1*), inflammatory response (*Adgre1*, *Ifng*, *IL1b*, *Cd68*, *F4/80*), and regulatory cell death (*Casp1*, *Nlrp3*, *Pycard*), besides proteasome, in distinct brown adipocyte subpopulations [377]. Overall, these demonstrate that Nrf1 functions as a key transcription factor determining thermogenesis, subsequent cell fate, and BAT heterogeneity in mice by controlling lipidometabolic and proteometabolic homeostasis.

8.5.2 Adipocyte-specific KO of Nrf1 disrupts WAT plasticity and metabolic homeostasis *Nfe2l1(f)-KO* mice were generated by crossing mice bearing a floxed *Nfe2l1* allele [262] with mice expressing adipocyte-specific Cre recombinase under control of the adiponectin gene promoter (*Adipoq-Cre*) [374]. *Nfe2l1(f)-KO* mice exhibited abnormal fat distribution, with dramatically reduced subcutaneous adipose tissue mass but slightly increased gonadal WAT mass. Importantly, Nrf1-deficient mice also displayed glucose intolerance, in-

sulin resistance, adipocyte hypertrophy, and severe adipose inflammation [374].

Further mechanistic studies revealed that Nrf1 deficiency caused significantly diminished expression of genes involved in adipogenesis, lipogenesis, lipolysis, and general adipocyte function in inguinal WAT, accompanied by dramatically increased expression of macrophage markers and inflammatory response genes such as *Adgre1* (adhesion G protein-coupled receptor E1), *Cd68*, *Ifng* (interferon γ), *IL1b* (interleukin 1β), and pyroptosis-related genes (e.g., *Casp1* [caspase 1], *Nlrp3* [NLR family pyrin domain containing 3], and *Pycard* [apoptosis-associated speck-like protein containing a CARD]) [374]. Notably, adipogenic gene expression (*Pparg2* and *Cebpb*) was significantly increased in gonadal WAT of *Nfe2l1(f)-KO* mice, but no marked changes in mitochondrion-related *Ppargc1a* and *Cox8b* were observed compared to controls. Collectively, these demonstrate that Nrf1 deficiency results in aberrant expression of lipolysis-related genes in WAT, leading to adipocyte hypertrophy followed by inflammation, pyroptosis, and even insulin resistance. This reveals a vital role for Nrf1 in regulating adipose tissue plasticity and energy homeostasis.

In-depth examination of the stromal vascular fraction isolated from WAT of *Nfe2l1(f)-KO* mice revealed augmented adipogenesis with elevated adipogenic marker expression and lipid accumulation [378]. Enhanced and accelerated adipogenic differentiation was further corroborated by stable Nrf1 knockdown in 3T3-L1 preadipocytes. Conversely, dexamethasone-methylisobutylxanthine-insulin (DMI)-induced adipogenesis was substantially attenuated by forced overexpression of Nrf1α but not its shorter isoforms in 3T3-L1 cells [378]. Mechanistic investigation revealed that Nrf1α negatively regulates transcription of peroxisome proliferator-activated receptor γ ($PPAR\gamma$), particularly *PPAR$ 2 $*(amasterregulatorofadipogenesis), aswellas *Cebp*, *Cebp*, *aP2*, *CD36*, and *Glut4* *.TheseindicatethatNrf1 $ likely acts as a critical negative regulator of adipogenesis by suppressing PPARγ, while Nrf1β may play a contrary role in basal PPARγ expression.

The crucial constitutive roles of Nrf1 in adipocytes were further evaluated by treating juvenile *Nfe2l1(f)-KO* mice with CL316243, a $ 3 − *adrenergicagonistpromotinglipolysiseffectsonadiposeinflammation*[379].*Four − week − oldjuvenileadipocyte − specificNrf1 − KOmicedisplayednormalfatdistributionbutexhibitedreducedfa Nfe2l1(f) − KO ∗ mice*[379].*Alteredexpressionofinflammation − andpyroptosis − relatedgenes, aswellasmacrophageinfiltrationinWAT, wasdramaticallyalleviatedafter*7 − *dayCL*316243*treatment.However, ∗Nfe2l1(f) − KO ∗ phenotypewasaggravatedafter*3 − *weekrosiglitazonetreatment*(*athiazolidinedionePPAR* $ agonist for type 2 diabetes treatment by stimulating triglyceride storage genes), accompanied by increased expression of inflammation- and pyroptosis-related genes in shrunken WAT [380]. Collectively, these highlight that since Nrf1 fundamentally regulates lipolytic gene expression and defends against relevant metainflammation, it may thereby be considered a potential therapeutic target to improve adipose plasticity, lipid homeostasis, and whole-body energy homeostasis.

8.6 Skeletal myocyte-specific KO of Nrf1 results in an oxidative lean phenotype with muscle remodeling

Skeletal myocyte-specific Nrf1-mKO mice were generated by crossing floxed-*Nfe2l1* mice [373] with mice expressing *Acta1-Cre* (skeletal muscle-specific Cre recombinase under control of the actin-$ $1 promoter) [381]. mKO mice displayed lowered body weights predominantly caused by slightly lower lean mass but not fat mass. A prominent reduction in gastrocnemius muscle size and weight was also associated with lower grip strength in Nrf1-mKO mice compared to wild-type controls [381]. A major phenotypic change in gastrocnemius muscle of Nrf1-mKO mice revealed a shift toward a more oxidative profile. Such phenotypic switch toward more oxidative fibers was also supported by myosin heavy chain gene expression analysis, indicated by higher slow-twitch *Myh7* and lower fast-twitch *Myh2* levels. Notably, perinatal myosin heavy chains *Mhy3* and *Mhy8* were markedly higher in Nrf1-mKO mice, implying presence of regenerative muscle fibers. Transmission electron microscopy ultrastructural insight revealed that Nrf1 loss leads to aberrant myofibrillar organization in Nrf1-mKO muscle tissues, where mitochondria appeared bulged and disrupted with less coenzyme Q:cytochrome c reductase, a component of oxidative phosphorylation Complex-III [381]. Reduced respiration after ADP and succinate addition, as well as reduced maximal respiration capacity, was detected in isolated muscle fibers of Nrf1-mKO mice compared to wild-type controls. Collectively, these demonstrate a fiber-type switch from fast- to slow-twitch fibers with aberrant mitochondrial bioenergetics in gastrocnemius muscle of Nrf1-mKO mice.

Nrf1 knockdown by siRNA led to lower proteasomal activity and subunit gene expression (*Psma1*, *Psmb1*) in differentiated C2C12 cells but higher ubiquitylated protein levels after proteasome inhibition by bortezomib [381]. Similarly, genetic deletion of myocytic Nrf1 resulted in markedly lower proteasome subunit gene expression in Nrf1-mKO muscle tissues, blunting ex vivo proteasomal activity and leading to markedly higher ubiquitylation levels [381]. Rather, trypsin-like activity remained higher in Nrf1-mKO muscle tissue, possibly indicating dynamic remodeling of proteasomal activity independently of Nrf1. Overall, these demonstrate the importance of myocyte Nrf1 for proteasomal function and UPS in skeletal muscles. This is further evidenced by proteomic hyperubiquitylation (of 2,832 differentially expressed proteins) in Nrf1-mKO muscles, highlighting energy metabolism and filament organization as major pathways [381]. RNA-seq analysis of soleus and gastrocnemius muscles from matched Nrf1-mKO mice uncovered the imperative role of Nrf1 in energy metabolism and UPS, also pointing toward impacts on respiration, ATP metabolic processes, and muscle cell differentiation [381]. Gastrocnemius muscle metabolomics revealed strong differences in metabolites related to glucose metabolism, the Warburg effect, and anti-oxidative characteristics. Therefore, the critical role of Nrf1 for UPS and proteostasis in skeletal muscle dictates muscle function and energy metabolism, but Nrf1 loss causes profound phenotypic changes in gastrocnemius muscle while impact on soleus was rather mild.

Linear regression modeling of the relationship between energy expenditure and body weight revealed higher energy expenditure in Nrf1-mKO mice, which displayed increased food intake to fuel elevated energy expenditure but no evident physical activity differences compared to wild-type controls [381]. This indicates that systemic energy metabolism for the whole animal body is regulated by myocyte Nrf1 in vivo. Conversely, loss of myocytic Nrf1 function protects mice from body-weight gain and metabolic imbalance caused by high-fat diets, because Nrf1-deficient mice displayed hormetic energy metabolism and resistance to high-fat diet-induced obesity, associated with a lean phenotype and muscle fiber type switching [381]. Distinct metabolic conditions of obesity were analyzed by multi-omics to be associated with UPS recalibration in muscle. Overall, these define an adaptive role for fine-tuning proteasome toward UPS governed by Nrf1 in remodeling muscle proteome and biological function.

8.7 Cardiomyocyte-specific knockout of Nrf1 prevents neonatal heart regeneration and repair

Cardiomyocyte-specific Nrf1-cKO mice (generated by crossbreeding *Nrf1fl/fl* mice with αMHC-Cre transgenic mice) exhibited no cardiac morphology or function aberrations at 2 months of age [382]. However, following myocardial infarction (MI), Nrf1-cKO hearts showed decreased cardiac function (measured as fractional shortening, ejection fraction, and myocardial wall motion) with increased fibrotic scarring. This is due to greater myocardial loss at 1-day post-MI and markedly decreased cardiomyocyte proliferation at 3-day post-MI in Nrf1-cKO hearts underlying impaired heart regeneration, accompanied by notably reduced proteasomal activity [382], albeit with an enhanced inducible cardiomyocyte subpopulation responding to injury by apoptosis and hypertrophy.

During heart regeneration, the oxidative phosphorylation (OXPHOS) pathway was downregulated in wild-type cells but upregulated (inhibiting cardiomyocyte proliferation) in Nrf1-cKO hearts, leading to significantly increased ROS along with upregulated expression of two cardiac stress markers *Nppa* and *Nppb* in Nrf1-cKO MI hearts [382]. By contrast, downregulated genes in Nrf1-cKO hearts were enriched in actin filament-based processes, PI3K-AKT and Wnt signaling pathways, aside from known Nrf1-target genes (*Hmox1* and *Psmd1*). Such Nrf1 loss prevented neonatal cardiomyocytes from initiating the transcriptional response required for heart regeneration.

After ischemia/reperfusion injury, mice pre-injected with Nrf1-expressing adeno-associated viral vector (AAV9-Nrf1) showed significantly reduced infarct area (from 52% to 40%) and improved cardiac function. Additional increases in left ventricular diastolic and systolic volumes observed in control MI hearts were profoundly attenuated in AAV9-Nrf1-treated mice, which showed reduced cardiac dilation and remodeling, as well as reduced fibrotic scarring [382]. These indicate that Nrf1 overexpression in vivo confers a cardioprotective effect in adult hearts following ischemic injury. This is also supported by further experimental evidence from neonatal rat ventricular myocytes (NRVMs) overexpressing Nrf1,

revealing consistent protective effects against all examined cardiotoxins (e.g., H_2O_2, peroxynitrite, doxorubicin, elastin). Notably, such protective effects of Nrf1 were much greater than those of Nrf2 in response to all these cardiotoxin treatments [382], though Nrf2 was also viewed as a master regulator conferring cardioprotection [383]. This fact highlights an important role of Nrf1 as a key regulator of cellular stress in the heart. Fully consistent with this, significantly increased proteasome-mediated proteolyses, such as proteasome protein catabolism and ERAD pathways, but specifically downregulated ECM organization, were determined in NRVMs overexpressing Nrf1 but not Nrf2, though both shared antioxidant and anti-inflammatory responses. To clarify potential redundant functions between Nrf1 and Nrf2, cardiomyocyte-specific Nrf2-cKO or Nrf1:Nrf2-dKO mice were generated [382]. Consequently, expression levels of proteasome (*Psma1*, *Psmd1*, and *Psmb3*) and antioxidant target genes (*Hmox1*, *Sod1*, and *Cat*) were reduced to greater degrees in Nrf1-cKO compared to Nrf2-cKO hearts, but importantly not further reduced in Nrf1:Nrf2-dKO hearts, implying Nrf1 plays a predominant role in regulating these genes in the heart.

Analysis of human heart ventricles from 431 donors (age 20–79) in the Genotype-Tissue Expression (GTEx) database revealed varying Nrf1 expression levels (differing by >70-fold) among individuals, with significant decline in the elderly demographic (age 60–79) [382]. In these samples, 1,167 genes were identified by co-expression analysis as highly correlated with Nrf1 (Spearman correlation coefficient >0.8, adjusted $p < 0.00001$). Apart from known Nrf1-targets *PSMC1*, *PSMD7*, *SOD1*, and *CAT*, Nrf1 co-expressed genes are clustered with key metabolic and energy production processes in hearts (e.g., fatty acid degradation, electron transport chain, mitochondrial organization, muscle contraction) and known Nrf1 downstream pathways (ubiquitination, proteasome degradation, autophagy). From these, we infer that Nrf1 expression degree correlates with metabolic and contractile heart properties and likely affects cardiac function. Conversely, significant reduction of Nrf1 transcript levels in heart ventricles from three independent patient cohorts with heart failure ($n = 28$) was revealed by further analysis of published datasets [382]. Doxorubicin-induced cardiomyopathy in cancer patients carries poor prognosis and is frequently fatal. However, Nrf1 overexpression enables human induced pluripotent stem cell-derived cardiomyocytes (hPSC-CMs) to be sufficiently protected from doxorubicin-induced cardiotoxicity (and other cardiotoxins). Altogether, these unravel that the adaptive responsive mechanism mediated by Nrf1 enabling proteasome activation and redox homeostatic balance is required for neonatal heart regeneration and confers cardioprotection in adult hearts. Therefore, reactivating this mechanism in adult hearts could represent a potential therapeutic approach for cardiac repair.

8.8 Bone-specific deficiency of Nrf1 impairs osteoclast differentiation and bone formation

Mechanistic insight into osteoblast differentiation induced by redox inducer vitamin C (ascorbic acid) [384, 385] revealed significant inducible increase in osterix (*Osx*) expression—a key osteoblast-specific transcription factor principally regulated by Nrf1 binding to its ARE sequence—in impaired bone marrow stromal cells [386]. These mutant cells were originally derived from spontaneous fracture mice lacking the gulonolactone oxidase gene, leading to vitamin C deficiency [387]. Although all three Nrf members can potentially bind the ARE sequence in the *Osx* promoter, its expression induced by Nrf1 was 50- and 500-fold higher than those induced by Nrf2 and Nrf3, respectively; the latter two CNC-bZIP factors were also unaffected in vitamin C-treated bone marrow stromal cells [386]. Thus, Nrf1 is essential for transcriptional regulation of *Osx*-mediated gene expression profiling, further supported by Nrf1 knockdown showing decreased *Osx* expression, impaired osteoblast differentiation, and reduced mineralized nodule formation from bone marrow stromal cells [386]. By sharp contrast, Nrf2 was considered to negatively regulate differentiation of osteoblasts and chondrocytes [388, 389].

The in vivo physiological function of Nrf1 in regulating bone formation was further determined using osteoblast-specific KO mice generated by crossbreeding *Nrf1flox/flox* mice [390] with transgenic mice bearing *Col1$ 2 − $iCre$ ∗ [$whereCrerecombinaseisundercontrolofcollagen − 1 2(∗Col1 2∗)regulatorysequence][391].$Osteoblast − specificNrf1 − KOmiceshowedobviousreductionsinbones$ $Osx ∗ expression, reducedcelldifferentiation, andimpairedboneformation.Intriguingly, Nrf1deficiencyena$ $regulationby40^{\{-\}/}\{-\}$ mice to butylated hydroxytoluene [392], and their glycoproteins were co-localized and integrated within and around the ER in similar membrane-topological folding fashion [275, 305, 393, 394].

Knockout of all Nrf1 transcript isoforms specifically in the myeloid lineage in *Nfe2l1(M)-KO* mice (generated by crossing floxed *Nfe2l1* allele mice with *LysM-Cre*-expressing mice) caused increased osteoclast activity, decreased bone mass, and worsening of osteoporosis induced by ovariectomy and aging [395]. However, no significant effects of Nrf1 loss on osteoblasts and osteocytes were observed in *Nfe2l1(M)-KO* mice. Further investigations of bone marrow cells and RAW 264.7 macrophages revealed that Nrf1 deficiency leads to accelerated and elevated osteoclast differentiation, attributable to enhanced ROS accumulation in early osteoclast differentiation and expression of *Nfatc1α* (encoding nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 1α) [395]. Such Nrf1-regulated osteoclast differentiation and transcriptional expression of multiple antioxidant genes, as well as *Nfatc1α*, were isoform-specific. Full-length Nrf1α likely functions as an accelerator of osteoclast differentiation with induction of *Nfatc1α* and antioxidant genes, while Nrf1β serves as a brake controlling the accelerator effect of Nrf1α. This indicates differential and even opposing roles of multiple distinct Nrf1 isoforms in osteoclast genesis and differentiation, bone remodeling, and metabolism homeostasis, providing understanding

of pathogenesis in various bone disorders including osteoporosis and arthritis.

In undifferentiated odontoblasts, physical interaction of Nrf1 with C/EBPβ through their bZIP domains—where binding efficiency depends on PKA phosphorylation at Ser569 (i.e., Ser599 situated on the boundary between Neh6L and CNC domains in human TCF11)—directs specificity of CNC-bZIP function to repress basal constitutive expression of the *DSPP* (dentin sialophosphoprotein) gene encoding two specific markers DPP (dentin phosphophoryn) and DSP (dentin sialoprotein) [396]. Conversely, loss of such Nrf1-C/EBPβ interaction in fully differentiated odontoblasts increased *DSPP* transcriptional expression to yield DPP and DSP. These demonstrate coordinated control of odontoblast differentiation by both bZIP factors to switch *DSPP* on or off.

8.9 Brain-specific knockout of Nrf1 leads to pathogenesis of neurodegenerative diseases

Nrf1 expression is also found in neuronal systems, and its evident function in the central nervous system has been proven in a few studies [390, 397, 398]. Only two groups have worked on neuronal tissue-specific Nrf1 KO in vivo. Neuronal-specific Nrf1 KO was achieved using Nestin as a neural stem cell marker in the Cre-lox system [397, 398]. Resulting Nrf1-deficient mice displayed notable phenotypes showing growth retardation starting at seven days postnatal and mortality by three weeks. Behavioral abnormalities such as motor ataxia and feeding difficulties were evident, along with hindlimb clasping reflex indicative of neurodegenerative conditions.

Interestingly, similar phenotypes are also observed in small Maf-deficient mice (i.e., *MafG*$^{-/-}$:*MafK*$^{-/-}$) [399, 400], suggesting the importance of cooperation between Nrf1 and small Maf proteins in neuronal functions. Histological analysis highlighted neuronal degeneration in the hippocampus CA3 region and spinal cord. Notably, Nrf1-deficient neurons exhibited severe oxidative stress in the spinal cord, with ubiquitinated protein accumulation in cortical pyramidal neurons, other brain regions, and spinal cord. Nestin expression is seen beyond neural stem cells [401]; therefore, more specific Cre drivers are needed to ensure Nrf1's role in the neuronal system. Nevertheless, Nrf1 knockout using the CaMK2cre system demonstrated that Nrf1 loss in the brain resulted in accumulation of ubiquitinated proteins and reduced expression of proteasomal subunits including *PsmB6* [390]. Forebrain atrophy and decreased cortex and hippocampus thickness were observed, with significant neuronal loss and hindlimb clasping reflexes at 3–4 months, suggesting essential involvement of Nrf1 deficiency in neurodegenerative pathogenesis and highlighting its causal role in neurodegeneration.

Nrf1 loss in the brain, as demonstrated by CaMK2Cre-mediated deletion, has unveiled significant implications: neurodegeneration and impaired proteasome function due to down-regulation of various proteasomal genes, whereas NestinCre-mediated Nrf1 deletion exhibits similar phenotypes without reduced

proteasome gene expression. Although NestinCre-mediated Nrf1 deletion shows ubiquitinated protein accumulation, only CaMK2Cre-mediated Nrf1 loss showed reduced proteasome gene expression, possibly due to differences in mouse models. The NestinCre model entails global neuronal Nrf1 knockout, whereas CaMK2Cre specifically targets cortex and hippocampus. Additionally, while CaMK2Cre demonstrates neural damage via apoptosis without oxidative stress, no neural cell apoptosis evidence was observed in the NestinCre model. More importantly, NestinCre-mediated Nrf1 knockout unveiled loss of ubiquitin-specific proteases (USPs), with *USP9x* identified as one Nrf1 target gene [398]. Because USPs play crucial roles in regulating neural activities—for instance, a mutation in *USP14* in an ataxia mouse model leads to severe tremors and premature death [402], and reduction of *Usp9x* promotes degradation of Survival Motor Neuron protein, thereby improving Spinal Muscular Atrophy [403]—*USP9x* expression is notably high in specific brain regions including layer V of neocortex, certain hippocampal subfields, and Purkinje cells in cerebellum of adult mice [404]. Higher expression is found in CA3 than CA1 region in hippocampal pyramidal cells, suggesting co-localization with Nrf1. Notably, *Usp9x* knockdown induces toxic α-synuclein inclusion formation in SH-SY5Y cells upon proteolytic inhibition [405], indicating its neuroprotective role, but its dysfunction may trigger Parkinson's disease. Therefore, reduced *USP9x* expression resulting from Nrf1 loss may lead to polyubiquitinated protein accumulation, with implications for neurodegenerative pathogenesis processes, though further in-depth molecular assessments are warranted to identify specific proteins accumulating in ubiquitinated states upon Nrf1 function loss in the neuronal system.

Additionally, no single KO of other CNC-bZIP factors (i.e., *Nfe2p45*, *Nrf2*, *Nrf3*, *Bach1*, and *Bach2*) besides Nrf1 gave rise to any apparent neuronal phenotypes [323, 406–409]. From this, we infer that Nrf1 is the only candidate within the CNC-bZIP family responsible for neuronal homeostasis [398]. Nrf1 contributes to central nervous system development after birth, as evidenced by conditional Nrf1 KO in brains [390, 397], although global Nrf1 KO did not cause apparent neuronal deficits in mouse embryonic stages before death [172, 326].

9. Uniquely differentiated yet integrated roles of Nrf1 and Nrf2 in governing cell homeostasis during distinct life processes

From an eco-evo-devo view, Nrf1 is a highly conserved fossil-like indispensable CNC-bZIP transcription factor with ancient properties, while the relatively younger Nrf2 likely arose from ancient gene differentiation and emerged in later-evolved vertebrates. As two major principal CNC-bZIP factors in mammals, Nrf1 and Nrf2 often resemble two entangled "Yin-Yang" quanta, complying with a dialectic law of unity of opposites in biosciences (Figures 3 & 11A). Such allelopathic potentials of Nrf1 and Nrf2 are sufficiently evoked for their coordinated control of adaptive responsive protective mechanisms to maintain

cell homeostasis and organ integrity within certain preset physiological thresholds. This notion is evidenced by supportive experiments comparing combined Nrf1:Nrf2-deficient versus respective single-deficient mice [332, 348]. Therefore, it is crucial to gain insights into the uniquely differentiated yet integrated roles of Nrf1 and Nrf2 in governing robust physiological homeostasis and healthy survival during distinct life processes.

9.1 Distinct regulation of Nrf1 and Nrf2 in redox responsive signaling against cancer development

The tissue-specific Nrf1 knockout mice described above exhibit distinct pathological phenotypes that closely resemble human non-alcoholic steatohepatitis and hepatoma [**?**, **?**], type-2 diabetes mellitus [**?**], and neurodegenerative diseases [**?**, **?**]. These findings demonstrate that mouse Nrf1 (and its derived isoforms) performs indispensable functions in regulating critical target genes required for maintaining robust physiological development and growth under normal homeostatic conditions. In stark contrast, Nrf2 knockout mice are viable and fertile, displaying no typical pathological phenotypes [**?**, **?**], which suggests that Nrf2 is not essential for normal development and growth. Nevertheless, Nrf2 is widely recognized as a master regulator of antioxidant response element (ARE)-driven gene expression [**?**, **?**]. This designation stems from observations that Nrf2-deficient mice show increased susceptibility to chemical carcinogens compared to wild-type animals [**?**], and that Nrf2 induction (activating ARE-driven genes) represents a potential chemopreventive and therapeutic target against carcinogenesis and malignant progression [**?**, **?**, **?**]. However, hyperactive Nrf2 activity is also being reconsidered as a potent oncogenic driver that promotes tumor growth, progression, metastasis, and even therapy resistance [**?**, **?**]. These dual "double-edged sword" effects of Nrf2 in cancer prevention versus progression have prompted serious consideration of how Nrf2's opposing activities are tightly constrained by Nrf1, as illustrated in Figure 13 [Figure 13: see original paper].

Figure 13. Inter-regulation between Nrf1 and Nrf2 in the opposing control of cancer progression.

A. Different phenotypes observed in xenograft tumor-bearing model mice inoculated with various mutant or restored cell lines, compared to their progenitor wild-type (WT, Nrf1/2^{+/+}) human hepatoma HepG2 cells. These tumors were obtained from relevant references [**?**, **?**, **?**, **?**]. Notably, Nrf1 acts as a potent tumor suppressor, whereas Nrf2 functions as a potential tumor promoter, although both CNC-bZIP factors regulate each other.

B. The tumor-promoting effect of Nrf2 is tightly constrained by robust Nrf1 expression through its negative regulator Keap1 and the Nrf1-targeted proteasome-mediated degradation pathway, although Nrf1 transcription is itself controlled by Nrf2.

These inter-regulatory roles enable Nrf1 and Nrf2 to oppositely yet coordinately

govern multiple signaling networks that strictly control cancer development and progression, in addition to mediating antioxidant, detoxification, and cytoprotective responses against oxidative stress and associated damage.

9.1.1 Distinct regulation of Nrf1 and Nrf2 with Keap1-proteasomal and p62-autophagic disposing systems Nrf2 functions as a master redox regulon and antioxidant transcription factor that is activated during adaptive hormesis responses to oxidative stress, thereby facilitating maintenance of cellular redox homeostasis, particularly when cells are challenged by oxygenated environments [**?**, **?**, **?**, **?**, **?**, **?**]. Under non-stressed conditions, Nrf2 transcriptional activity is rigorously constrained by its negative regulator Keap1, a redox-sensing adaptor that sequesters this CNC-bZIP factor in the cytoplasm (by binding its Neh2 domain) and enables its ubiquitination by the Cul3-Rbx1 E3 cullin-RING ligase (CRL3) complex, targeting it for proteasomal degradation. However, Keap1 does not appear to exert similar negative effects on Nrf1, even though a putative Keap1-binding Neh2-like domain is present in Nrf1 [**?**]. This is because the Neh2L domain is topologically sequestered within the ER lumen, preventing Nrf1 from accessing extra-ER Keap1 [**?**, **?**]. If the Neh2L domain of Nrf1 is retrotranslocated across ER membranes into cytoplasmic compartments, it can bind Keap1 and be stabilized by this redox-sensing adaptor, which would represent a distinct regulatory mechanism from Nrf2 [**?**], though such stabilization of Nrf1 by Keap1 has been difficult to validate experimentally [**?**, **?**].

Surprisingly, specific knockout of human Nrf1α (i.e., Nrf1α^{-/-} with its long isoform TCF11) in HepG2 cells led to substantial diminishment of Keap1, while Nrf2 knockout (with genomic deletion of its transactivation domains) caused obvious increases in basal Keap1 expression compared to wild-type controls [**?**, **?**]. This demonstrates a bidirectional regulatory profile in which Keap1 is positively regulated by Nrf1α and negatively regulated by Nrf2. Such regulation likely involves the autophagy adaptor p62 and its role in lysosomal degradation of the relatively long-lived Keap1 protein [**?**]. The p62 protein (also called sequestosome 1, SQSTM1) is transcriptionally induced by oxidative stress through direct Nrf2 binding to ARE sequences in the p62 promoter [**?**]. When p62 accumulates (as occurs when autophagy is impaired), increased p62 directly binds Keap1 and targets it for autophagy-mediated lysosomal degradation, resulting in reduced proteasomal turnover of Nrf2 via a feed-forward loop during chronic redox stress signaling. By contrast, p62 expression was basally enhanced in Nrf1α^{-/-} cells, implying negative regulation by Nrf1α [**?**], though the detailed mechanisms remain to be elucidated.

The autophagy-lysosome and ubiquitin-proteasome systems represent two primary cellular pathways for degrading misfolded or damaged proteins to maintain intracellular protein homeostasis (proteostasis). When proteasome function is impaired, cells compensate by activating selective autophagy to eliminate ubiquitinated or damaged protein aggregates. Studies of these cross-talking mechanisms revealed that treatment with proteasome inhibitors for 4

hours causes rapid, dramatic, and selective induction of GABARAPL1 (but not other autophagy genes) and p62 (which bridges ubiquitinated proteins with GABARAPL1 on autophagosomes), promoting cell survival before full autophagy activation [**?**]. Conversely, knockdown of p62 or GABARAPL1 reduces cell survival upon proteasome inhibition. Nrf1-mediated transcriptional induction of p62 enhances survival primarily by sequestering ubiquitinated proteins in nuclear inclusions and perinuclear aggresomes [**?**], while simultaneously inducing proteasome gene expression (the proteasome bounce-back response) [**?**, **?**]. Nrf1's transcriptional activation of aggrephagy is further corroborated by direct targeting of autophagy-related p62/SQSTM1 and GABARAPL1 (an ATG8 family gene) by this CNC-bZIP factor [**?**]. Interestingly, Nrf1 was also found to be indispensable for formation of p62-positive puncta and their co-localization with ULK1 and TBK1; both kinases may activate p62 via phosphorylation, as Nrf1 knockdown substantially reduced p62 phosphorylation at Ser403 [**?**]. Such selective GABARAPL1 upregulation by Nrf1 also facilitates clearance of ubiquitinated proteins, particularly when proteasome function is impaired [**?**]. However, prolonged proteasome inhibitor treatment for 20 hours activates autophagy and expression of most autophagy genes through a potentially Nrf1-independent mechanism [**?**], although Nrf1 has recently been confirmed to regulate proteotoxic stress-induced autophagy [**?**].

The conserved proteasome bounce-back response was originally observed in *Saccharomyces cerevisiae* [**?**], where chemical or genetic proteasome inhibition induces new proteasome synthesis promoted by the stress-regulated transcription factor RPN4 (with its DNA-binding C2H2 zinc-finger domain). A similar transcriptional feedback loop exists between the proteasome and SKN-1 in *Caenorhabditis elegans* [**?**], revealing that proteasomal dysfunction activates SKN-1, which is tied to the protein-degradation machinery and produces a selective oxidative (proteotoxic) stress response. Increased expression of 20S proteasomes was validated to be mediated by SKN-1 and CncC in worms and flies, respectively [**?**]. Such compensatory recovery of proteasome activity induced by limited proteasomal inhibition was also observed in mammals [**?**] and identified to be predominantly activated by Nrf1 (and its long isoform TCF11, but not Nrf2) via an ERAD-dependent feedback loop [**?**, **?**]. Under non-inducing conditions, Nrf1/TCF11 within and around ER membranes is targeted to ERAD requiring the E3 ubiquitin ligase Hrd1 and the AAA ATPase p97. Upon exposure to lower doses of proteasome inhibitors, accumulation of oxidant-damaged proteins promotes nuclear translocation of Nrf1/TCF11 from the ER, permitting activation of proteasome gene expression through ARE binding in promoter regions. The Nrf1-driven transcriptional feedback loop regulating proteasomal subunits, co-factors, and p97 was further confirmed by other groups [**?**, **?**]. Overall, Nrf1/TCF11, rather than Nrf2, serves as the key essential regulator for activation of the highly conserved proteasome bounce-back response for 26S proteasome formation, compensating for reduced proteolytic activity and mediating innate cytoprotective responses against proteotoxic stress caused by proteasome inhibition.

Enhanced autophagy-lysosome pathway activity occurs in response to graded proteasome dysfunction caused by RPN10 mutation in *Caenorhabditis elegans* [**?**]. In this model, enhanced resistance to aggregation-prone proteins depends on autophagy genes (*atg-13*, *atg-16.2*, *lgg-1*, *bec-1*, and *prmt-1*), though animals become particularly sensitive to lysosome inhibition via RNAi or chemical means. Such moderate proteasome dysfunction can be leveraged to improve proteostasis capacity, organismal health, survival, and longevity through activation of compensatory mechanisms regulated by SKN-1A/Nrf1 (and ELT-2/GATA), which mediate increased expression of genes encoding proteasome subunits (as a conserved bounce-back response) and those mediating anti-oxidative and heat-stress adaptive responses [**?**, **?**]. Proteasome regulation by SKN-1A/Nrf1 also enables innate immune responses to be kept in check in a tissue-specific manner against natural pathogens of *C. elegans*. Conversely, constitutive expression of immune response programs against pathogens is triggered by loss-of-function mutants of SKN-1A and its activating enzymes DDI1 and PNG1, leading to proteasome inhibition [**?**].

Additionally, the ER-anchored ubiquitin-specific protease USP19 acts as a novel mechanistic modulator of Nrf1 (but not Nrf2), directly interacting with Nrf1 near the ER and functioning topologically as a deubiquitinating enzyme to remove ubiquitin moieties, thereby rescuing Nrf1 from the putative ubiquitin-directed ERAD pathway [**?**]. In turn, transcriptional expression of endogenous USP19 and its promoter-driven reporter genes is differentially regulated by Nrf2 and Nrf1 at distinct layers within a complex hierarchical regulatory network. Recently, Keap1 was discovered to be a crucial multifunctional player in governing and maintaining robust proteostasis, as it contributes not only to ubiquitin-mediated proteasomal degradation by interacting with numerous ubiquitination enzymes (including Cul1 to Cul5) along with 26S proteasomal core and regulatory subunits, but also enables novel contributions to protein stability (e.g., Smad2/3) through an additional deubiquitination system [**?**].

9.1.2 Inter-regulation of Nrf1 and Nrf2 with differential redox signaling to cancer metabolism Aberrant accumulation of hyperactive Nrf2 was observed in human Nrf1α^{-/-} HepG2 cells [**?**], attributable to substantially diminished expression of its negative regulator Keap1 and Nrf1-targeted proteasome dysfunction. Despite Nrf2 hyperactivity, malignancy and metastasis of Nrf1α^{-/–}driven xenograft tumors were enhanced [**?**, **?**]. Similar results were obtained from knockdown of all Nrf1 isoforms (Nrf1-KD) [**?**]. Conversely, Nrf2 knockdown suppressed Nrf1α^{-/–}derived tumors to a similar extent as wild-type controls (Figure 13A). In sharp contrast, inactive Nrf2 dramatically repressed Nrf2^{-/–}driven tumors, almost completely abolishing them in xenograft model animals [**?**], whereas constitutively active Nrf2 (caNrf2) expression did not significantly alter caNrf2-derived tumors compared to wild-type controls (Figure 13A). These observations demonstrate that Nrf2 likely acts as a tumor promoter, while Nrf1 serves as a tumor suppressor. This was further evidenced by experiments showing that restored expression of ectopic Nrf1 or

TCF11 factors convincingly corroborated remarkable tumor-repressing effects on hepatocellular carcinoma [?]. Therefore, the tumor-promoting effect of Nrf2 appears to be stringently confined by Nrf1 through indispensable braking control of Keap1 and proteasome function in distinct adaptive responses. Additionally, Nrf2 transcriptional expression is inhibited by Nrf1, as both basal and inducible Nrf2 mRNA levels were upregulated to varying extents in tissue-specific Nrf1^{-/-} mice [?, ?] and human Nrf1α^{-/-} hepatoma cells [?, ?]. Conversely, Nrf2 deficiency substantially reduced Nrf1 transcription, implying that Nrf1 is positively regulated by Nrf2 and also by itself, as revealed by promoter-driven reporter assays [?]. Overall, such inter-regulation between Nrf1 and Nrf2 occurs at multiple levels, from gene transcription to post-synthetic protein disposal, and is further corroborated by in vivo mouse models expressing the Nrf1-MafG heterodimer, which revealed that strongly induced Nrf1 can activate canonical Nrf2 target cytoprotective genes [?].

Crucially, severe endogenous oxidative stress caused by loss of Nrf1 function cannot be compensated by hyperactive Nrf2 accumulated in deteriorated Nrf1α^{-/-} hepatoma cells [?, ?, ?, ?, ?, ?]. Similarly, oxidative stress was observed in Nrf1^{-/-} mouse models [?, ?, ?, ?] and was further aggravated by combined Nrf1 and Nrf2 deficiency, leading to fatal defects with typical pathologies [?, ?]. These findings demonstrate that Nrf1 acts as an indispensable determinant for robust redox homeostasis, although Nrf2 is accepted as a master regulon of antioxidant, detoxification, and cytoprotective genes in this process [?, ?]. Silencing Nrf2 can rescue glucose deprivation-induced rapid death of Nrf1α^{-/-} cells, as severe endogenous oxidative stress arising from aberrant redox metabolism is ameliorated by Nrf2 knockdown, similar to the rescue effect observed with catalase [?]. This indicates that existing endogenous oxidative stress in Nrf1α^{-/-} hepatoma cells is further augmented by hyperactive Nrf2, particularly upon glucose starvation, suggesting a possible Nrf2-dependent pathway (e.g., KLF9) that stimulates increased ROS production, as described by Zucker et al. [?]. Such switching of redox signaling by key modular molecules (e.g., GPX, PRDX) dictates cell fate decisions through Nrf2-mediated dual opposing responses for adaptation or maladaptation, specifically when Nrf1 is lost.

To defend against diverse environmental challenges, an evolutionarily selected set of antioxidant, detoxification, and cytoprotective systems are predominantly regulated by Nrf1 and Nrf2 for coordinated redox control to maintain cell homeostasis and organ integrity during healthy survival. Loss of full-length Nrf1α causes dramatic increases in intracellular ROS levels and oxidative damage in resulting Nrf1α^{-/-} cells, and this increase is not eliminated by drastically elevated Nrf2, although antioxidant systems are substantially enhanced by hyperactive Nrf2 [?]. Further experiments revealed that increased ROS production in Nrf1α^{-/-} cells resulted from marked impairment in the mitochondrial oxidative respiratory chain and its gene expression profile, which is regulated by two nuclear respiratory factors (called αPalNRF1 and GABPNRF2, which are non-homologous nuclearly-controlled transcription factors) (Figures S8 and 9) [?, ?, ?]. In addition to intrinsic antioxidant capacity, aerobic glycolysis was

greatly augmented (indicative of the Warburg effect) by aberrantly elevated Nrf2 to partially relieve energy demands in Nrf1α^{-/-} cells, but this heavily aggravated mitochondrial stress (yielding evident UPR^{mito}, which is similar to but distinct from UPR^{ER}) [**?**, **?**]. ROS generation was also differentially regulated by Nrf1 and Nrf2 via the miR-195 and/or miR-497-mediated UCP2 pathways [**?**]. Upon glucose starvation of Nrf1α^{-/-} cells, the altered gluconeogenesis pathway was greatly aggravated, accompanied by weakened pentose phosphate pathway function and dysfunction of serine-to-glutathione synthesis, leading to ROS accumulation and severe oxidative damage such that intracellular reduced equivalents (i.e., GSH, NADPH, TRX) were exhausted [**?**]. Thus, glucose starvation leads to acute death of Nrf1α^{-/-} (but not Nrf2^{-/-}) hepatoma cells due to fatal defects in redox metabolism reprogramming. This is likely attributable to distinct requirements of Nrf1 and Nrf2 for regulating constructive and inducible expression of key genes involved in redox metabolic reprogramming during cancer development and malignancy (Figure 14 [Figure 14: see original paper]) [**?**, **?**].

Figure 14. Differential but integral roles of Nrf1 and Nrf2 in regulating responsive genes.

A. Transcriptomic analysis of Nrf1α^{-/-} and Nrf2^{-/-} cell lines compared to wild-type (WT) cells. Differentially expressed genes (DEGs) were clustered by functional annotation into distinct signaling pathways principally governing cancer development and progression.

B. Inter-regulatory roles of Nrf1 and Nrf2 in uniquely yet coordinately controlling redox signaling, energy metabolism, and other target gene networks. These graphs with relevant big data analyses were adapted from [**?**, **?**].

9.1.3 Distinct regulation of Nrf1 and Nrf2 in cancer metabolism reprogramming Strikingly, Nrf1 can serve as a dual sensor and regulator of glucose homeostasis, as glycosylation of Nrf1α enables it to sense energy status [**?**]. Dysfunction of this energy sensor leads to glucose metabolism reprogramming, markedly aggravating the Warburg effect in Nrf1-silenced hepatoma cells and causing resulting mitochondrial damage. These glucose reprogramming effects are driven primarily by uncontrollable signaling mediated by AMP-activated protein kinase (AMPK) in Nrf1-deficient cells, because this CNC-bZIP factor negatively regulates this nutrient-sensing master kinase through direct physical interaction [**?**]. Crucially, recent studies further revealed that loss of Nrf1 function also leads to lipid metabolism disorders, with severe accumulation of ROS and lipids deposited in lipid droplets of Nrf1α^{-/-} cells [**?**, **?**]. This is attributable to upregulation of the cellular lipid synthesis pathway by JNK-Nrf2-AP1 signaling, while the lipid decomposition pathway is downregulated by nuclear receptor PPAR-PGC1 signaling. By sharp contrast, Nrf2 knockout decreases lipid synthesis and uptake capacity, indicating that Nrf1 and Nrf2 contribute to significant differences in cellular lipid metabolism profiles and relevant pathological responses. Further studies uncovered that lipid uptake and

deposition in Nrf1α^{-/-} cells resulted from CD36 upregulation via activation of the PI3K-AKT-mTOR signaling pathway, consequently leading to aberrantly activated inflammatory responses [**?**, **?**]. Interestingly, lipid droplet formation in Nrf1α^{-/-} cells was strikingly alleviated by 2-bromopalmitate [**?**], accompanied by substantially abolished expression of endogenous CD36 and critical inflammatory cytokines. This finding provides a potential strategy for cancer prevention and treatment through precision targeting of Nrf1, Nrf2, or both.

Metabolic reprogramming is recognized as a central hallmark of cancer and plays a pivotal role in malignant tumor occurrence, metastasis, and even drug resistance [**?**, **?**]. Based on the aforementioned findings, Nrf1 and Nrf2 are differentially and integrally required for multifaceted crosstalk between redox-responsive signaling and metabolic pathways to coordinately control numerous signaling molecules and metabolic enzymes involved in cancer metabolism, aiming to sustain robust redox and metabolic homeostasis (metabostasis, including glucose, lipid, cholesterol, and protein homeostasis). In-depth insights revealed that genetic deletion of Nrf1 and Nrf2 resulted in distinct metabolic reprogramming in human hepatoma cells [**?**]. Specifically, loss of Nrf1α led to enhanced glycolysis, reduced mitochondrial oxygen consumption, enhanced gluconeogenesis, and activated pentose phosphate pathway in hepatocellular carcinoma cells. By contrast, loss of Nrf2 attenuated glycolysis and gluconeogenesis pathways but had no significant effects on the pentose phosphate pathway. Nrf1α knockout caused fat deposition and increased amino acid synthesis and transport, particularly serine synthesis [**?**], whereas Nrf2 deficiency did not cause fat deposition but attenuated amino acid synthesis and transport. Such distinct metabolic programming between Nrf1α^{-/-} and Nrf2^{-/-} cells was further revealed to result from substantial activation of the PI3K-AKT-mTOR signaling pathway triggered by Nrf1 loss, leading to increased expression of critical genes for glucose uptake, glycolysis, pentose phosphate pathway, and de novo lipid synthesis, whereas Nrf2 deficiency resulted in the opposite phenomenon by blocking this pathway [**?**]. Downstream transcription factors HIF1 and SREBP1/2 were further corroborated as key players in such distinct metabolic reprogramming between Nrf1α^{-/-} and Nrf2^{-/-} cells, as previously described [**?**, **?**]. Notably, Nrf1α^{-/–}enhanced activation of HIF1 signaling led to increased expression of glycolysis rate-limiting hexokinases HK1/2 and glucose transporters (e.g., SLC2A1, also called Glut1) [**?**, **?**, **?**].

In addition to significantly increased lipid uptake by CD36 [**?**, **?**], loss of Nrf1 resulted in enhanced de novo lipid synthesis via activated SREBP1/2 through the PI3K-AKT-mTOR pathway [**?**]. Crucially, Nrf1 acts as a direct ER membrane sensor critical for cholesterol and lipid homeostasis through SREBP1/2 and LXR [**?**]. Such cholesterol-dependent homeostasis can further finely tune regulation of very long-chain sphingolipid synthesis to maintain plasma membrane lipid homeostasis and cell integrity [**?**]. Together, these findings indicate a Yin-Yang relationship between Nrf1 and SREBP1/2 for sustaining cholesterol and lipid homeostasis. Another recent report by Xu's group showed that SREBP activity is inhibited by promoting degradation of SREBP-cleavage activating protein

(SCAP, a canonical cholesterol sensor) through the ubiquitin E3 ligase RNF5-dependent proteasomal pathway; the ligase is recruited to a direct Nrf1 target, the ER-resident transmembrane protein TMEM33 [**?**]. TMEM33 serves as a downstream effector of pyruvate kinase isoform 2 (PKM2), which coordinates with p97/VCP to tightly control selective processing of Nrf1 and SREBPs and their bidirectional regulatory capability to dictate lipid metabolism and homeostasis [**?**]. Because Nrf1 and SREBP1 manifest distinct topogenetic behaviors around membranes [**?**], both should be endowed with disparate topovectorial spatiotemporal partitioning to exert their respective unique biological functionality, which occurs only after dislocation from the ER and Golgi into the nucleus to access cognate target genes (Figure 10). These findings demonstrate that Nrf1 contributes to negative regulation of lipid metabolism and homeostasis through the PKM2/p97-Nrf1-TMEM33-RNF5-SCAP-SREBPs axis. Collectively, along with an additional experimental report [**?**], these studies have convincingly confirmed that Nrf1 is not a direct target of SREBP1, although both are integrated into the rapamycin-responsive signaling network, and this conclusion does not support the relevant view presented in Manning's work [**?**].

9.2 Unique and overlapping roles of Nrf1 and Nrf2 in integrating multi-hierarchical signaling networks for genetic adaptive reprogramming

Considerable experimental evidence (including that described above) has unveiled the unique and overlapping roles of Nrf1 and Nrf2, as well as their differential positive and negative regulatory effects, all integrated to elaborately govern cellular homeostasis and organ integrity while tightly preserving robust status within certain preset physio-pathological threshold ranges. Such robust intrinsic status should be determined by unifying all relevant endogenous molecular-cellular-organismal networks and shaped by switching their physio-pathological functionality [**?**, **?**]. Within this framework, Nrf1 plays a predominant, indispensable role, while Nrf2 also acts as a master regulator in determining such robust physio-pathological states.

This notion is solidly supported by gene-targeting experiments revealing unique pathophysiological phenotypes in Nrf1 (but not Nrf2) knockout mice, as evidenced in distinct tissue-specific knockout models (see Section 8) and specific knockout cell lines from human hepatoma (Figure 13A). Accordingly, double knockout of both Nrf1 and Nrf2 in mice results in worsened pathological phenotypes compared to single Nrf1 knockout mice [**?**, **?**]. From these findings, it is inferable that such unique and overlapping biological roles of Nrf1 and Nrf2 are fulfilled by integrating all multi-hierarchical signaling networks into genetic adaptive reprogramming.

Analysis of chromatin immunoprecipitation (ChIP)-sequencing combined with RNA-sequencing data identified 31 differentially expressed genes (DEGs) as co-targets of Nrf1, Nrf2, and Nrf3 (i.e., doxycycline-inducible FLAG-tagged Nrf1ΔN1-121, Nrf2, and Nrf3ΔN1-173 in human osteosarcoma U2OS cell lines)

[?]. Among these, 18 genes were upregulated and 9 genes downregulated by all three factors, while only 4 genes were differentially regulated by at least one of the three. Additionally, 84, 84, and 22 genes were specifically up- or down-regulated by Nrf1, Nrf2, and Nrf3, respectively [?]. Scrutiny of ARE-containing ChIP peaks revealed that Nrf1 prefers strict binding to AREs flanked by AT-rich regions (e.g., to drive metabolism genes), whereas Nrf2 prefers more loose binding to canonical AREs adjoined by GC-rich regions [?]. Furthermore, another overexpression system using FLAG-tagged Nrf1ΔN1-84, Nrf2, and Nrf3ΔN1-112 in HEK293T cells was subjected to RNA-sequencing-based transcriptomics combined with quantitative proteomics to delineate overlapping and differential genetic programs mediated by these three factors [?]. Nrf1-specific targets were identified to include several chaperones (e.g., HSPA4, HSPA8, HSPA9, DNAJC1, DNAJA2), the chaperonin TCP complex (e.g., CCT2, CCT5, CCT8), and a set of heat shock response genes in addition to the proteasomal bounce-back response [?]. This implies a crucial role for Nrf1 in cellular protein quality control through enhanced protein folding and increased proteasomal degradation to augment proteostasis-based repair. However, it should be noted that these N-terminally truncated Nrf1 and Nrf3 constructs may represent artificial artifacts of this CNC-bZIP family [?]. Importantly, evaluation of the tethered Nrf1-MafG heterodimer function in mouse models further revealed that this heterodimer can in vivo activate transcription of proteasome subunits and proteostatic stress response genes (involved in ERAD, chaperone, and ubiquitin-mediated degradation pathways) by specifically binding to ARE-related sequences in proximity to these genes [?].

Figure 15 [Figure 15: see original paper]. Distinctive or even opposing regulation of target genes by Nrf1/TCF11 and Nrf2.

A. Combined transcriptome and proteome sequencing analysis of two distinct cell model systems. First, transcriptomic sequencing of Nrf1α^{-/-} and Nrf2^{-/-} cell lines, plus a tetracycline-inducible HEK293 cell system stably expressing Nrf1, TCF11, or Nrf2, compared to corresponding controls. Second, proteome analysis of Nrf1α^{-/-} and Nrf2^{-/-} cell lines versus control cells. Results revealed differential or even opposing expression of genes specifically regulated by either Nrf1/TCF11 or Nrf2.

B. Such specific gene regulatory networks are bidirectionally governed by Nrf1/TCF11 and Nrf2.

C. Schematic representation of Nrf1/TCF11- or Nrf2-specific gene networks, along with their shared co-target genes, selected as described previously [?].

The unique transmembrane-topogenetic behavior of Nrf1 makes its functions more complicated than Nrf2, enabling alternative splicing of its transcript and selective processing of its protein to yield distinct isoforms of varying lengths (e.g., TCF11, Nrf1α, Nrf1β, Nrf1γ) [?, ?]. Tetracycline-inducible stable expression of these isoforms, together with TCF11ΔN2-156 and Nrf2, was established using the Flp-In T-Rex HEK293T system. Transcriptomic analysis revealed that

although Nrf1α and TCF11 have similar yet different regulatory profiles, both contribute primarily to positive regulation of their co-targets, which differ from those regulated by Nrf2 (Figures 15C and S10), whereas mutant TCF11ΔN2-156 appears to resemble Nrf2 in structure and function [**?**] (Figures 6C and S10C). Disparities in genes regulated by Nrf1 and Nrf2, along with those of TCF11, Nrf1α, Nrf1β, and Nrf1γ, were validated by comprehensive functional annotation of specific and common target genes (Figure S10) and combined RNA-sequencing analysis of Nrf1α^{-/-} and Nrf2^{-/-} cell lines versus wild-type controls [**?**]. Notably, Nrf1α/TCF11-specific genes focus on nutrient uptake, cellular metabolism, protein folding, sorting and degradation, plus DNA replication and repair, while Nrf2-specific genes are heavily weighted in developmental processes [**?**].

Opposing regulation of 108 genes by Nrf1 and Nrf2 was clustered into cancer-related pathways, including PI3K-AKT, p53, VEGF, JAK-STAT signaling, ECM-receptor interacting EMT process, plus antioxidant and inflammatory responses (Figure 14A). From these, 52 genes were selected by combined transcriptomic and proteomic analysis of Nrf1/2-indicated stably expressing and deficient cell lines (Figure 15A) to build a gene regulatory network (Figure 15B). Some of these distinct genes, mapped with metabolome analysis (Figure S11), were annotated to cellular sphingolipid, inositol phosphate, glutathione, and purine metabolisms, as well as phosphatidylinositol (e.g., PI3K-AKT) signaling systems.

These findings are consistent with previously reported results revealing opposing effects of Nrf1 and Nrf2 on PTEN (phosphatase and tensin homologue) [**?**], which negatively regulates intracellular phosphatidylinositol-3,4,5-trisphosphate (PIP3) levels and functions as an upstream tumor suppressor by negatively regulating PI3K-AKT-mTOR signaling (Figure 13B). Such negative and positive regulation of PTEN by Nrf1 and Nrf2, respectively, on PI3K-AKT-mTOR signaling toward HIF1, AMPK, and SREBP led to adaptive reprogramming of glucose and lipid metabolisms, along with certain amino acid metabolism reprogramming [**?**, **?**, **?**, **?**]. In addition to PTEN, similar opposing regulatory effects of Nrf1 and Nrf2 were observed on p53 signaling [**?**, **?**], the WNT-β-catenin signaling pathway, and the EMT process [**?**, **?**]. Furthermore, Nrf1 and Nrf2 bidirectionally contribute to coordinated control of arachidonic acid metabolism enzymes COX1, COX2, and ALOX5 to confine inflammatory stimulation (Figure 13B) [**?**]. Upon loss of Nrf1α, uncontrollable inflammatory responses are spontaneously aroused, leading to NF- B signaling activation and increased inflammatory cytokine expression, accompanied by Nrf2-driven JNK-JUN (AP-1)-mediated stress responses and aberrant accumulation of ROS and lipids [**?**, **?**, **?**]. The EMT process in Nrf1α^{-/-} cells is further activated by putative ROS-stimulated signaling pathways via MAPK, HIF1α, NF- B, PI3K, and AKT, all players involved in cancer development and progression. As a collective consequence, inflammatory malignant transformation and cancer progression occur in Nrf1-deficient cases, where hyperactive Nrf2 acts as a potent tumor promoter, because malgrowth of Nrf1α^{-/–}driven tumors in xenograft

model mice was significantly suppressed by Nrf2 silencing [**?**].

Figure 16 [Figure 16: see original paper]. A proposed model for better understanding of key transcription factor inter-regulatory networks.

Differential but integral regulation of Nrf1, Nrf2, and their target HIF1 signaling controls the biomarker STC2's role in mediating distinctive phenotypes between Nrf1α^{-/-} and Nrf2^{-/-}. The distinction in their pathophysiological phenotype status is determined by such a robust endogenous molecular-cellular network. Conversely, STC2 can also serve as a potent feedback regulator of Nrf2 through Keap1 and/or p62. These critical protein expression levels are tightly monitored by Nrf1-targeted proteasome and/or autophagy-lysosomal disposal systems, in addition to transcriptional responsive reprogramming. For detailed explanations, see the relevant text and cited references [**?**].

To gain insights into mechanisms underlying distinct pathologic phenotypes between Nrf1α^{-/-} and Nrf2^{-/-}, transcriptome data from liver cancer in the TCGA database were mined to establish a prognostic model and calculate predicted risk scores for each cell line. Results revealed that Nrf1α knockout markedly increased risk scores in liver cancer, while Nrf2 knockout reduced risk scores [**?**]. In the prognostic model, stanniocalcin 2 (STC2), a potential biomarker highly expressed in hepatocellular carcinoma tissues with reduced overall survival, was significantly upregulated in Nrf1α-deficient cells but strikingly downregulated in Nrf2-deficient cells [**?**]. Negative regulation of STC2 by Nrf1α depends on Nrf2 and HIF1α (as a direct upstream regulator of STC2 transcription [**?**, **?**]). In turn, positive regulation of STC2 by Nrf2 may also be direct and independent of HIF1α (Figure 16). STC2 may regulate Nrf2 via a putative calcium-triggering Keap1-antagonized signaling pathway to form a positive feedback regulatory circuit. Further studies revealed that STC2, like Nrf2, functions as a dominant tumor promoter, because STC2-driven increases in hepatoma cell clonogenicity and xenograft tumor growth were almost completely abolished in STC2^{-/-} cells [**?**]. This indicates that STC2 is also a potential therapeutic target for liver cancer, in addition to being a diagnostic marker.

9.3 Differential yet integral roles of Nrf1 and Nrf2 in cellular homeostatic and adaptive responses

Upon exposure of cells to homeostasis-threatening stress, particularly redox stress, a series of intrinsic anti-redox cytoprotective mechanisms (established during evolutionary life histories) are activated to defend against such challenges, giving rise to adaptive and homeostatic responses that promote cellular resilience and maintain physiological homeostasis. In these responses (i.e., CHR and CSR, as defined by Kültz [**?**]), Nrf1 plays essential, predominant, and indispensable roles in cellular homeostatic response (CHR), while Nrf2 is involved in but not essential for CHR, although it is required for cellular stress-adaptive response (CSR). This conclusion is based on their distinct time-dependent re-

sponses driven by inducible expression of Nrf1 and Nrf2 with disparate half-lives (Figures 6 & S2) and accumulated experimental evidence described in previous sections. Thus, although Nrf1/TCF11 exerts an irreplaceable and pivotal role in governing CHR, Nrf2's involvement in rapid emergency response and CSR provides an invaluable way of "buying time" for transition to the lagging CHR mediated by Nrf1/TCF11, thereby coordinately coping with redox stress threatening cell homeostasis and organ integrity (Figure 17 [Figure 17: see original paper]).

When stimulated by stress, besides direct reactions, an acute emergency response is rapidly initiated by sensing stress signals that trigger redistribution of oxygen, nutrients, and energy, followed by short-term metabolic reprogramming and subsequent long-term adaptive genetic reprogramming to restore original homeostasis or gain adaptive or even maladaptive tolerance. This depends on stress severity and overall capacity of intrinsic cytoprotective responses (Figures 5 and 6). In these cellular homeostatic and adaptive responses, both Nrf1 and Nrf2 fulfill differential yet integral physiobiological roles by governing all relevant multi-hierarchical cellular-molecular signaling networks in distinct topovectorial and spatiotemporal ways to shape adaptive metabolic and genetic reprogramming (Figures 14B, 17, and S12).

Figure 17. A proposed model for better understanding of the major mechanism by which Nrf1 controls cell homeostasis.

The membrane-tethered Nrf1 factor is selectively processed and activated to determine cellular redox and energy metabolism homeostasis, as well as mitochondrial biogenesis, respiratory chain function, and homeostatic integrity. Importantly, Nrf1 not only governs two key nuclear respiratory factors (αPalNRF1 and GABPNRF2) essentially required for mitochondrial function and homeostasis (see Figures S8, S9, and S12), but also finely monitors the mitochondrial stress response through protein quality control (i.e., UPR^{mito}). Such unique biological functions of Nrf1 are distinguishable from those of Nrf2, because Nrf2 protein stability and activity are tightly confined by Nrf1. However, once the unique functions of Nrf1 are lost in deficient cells or organs, resulting dyshomeostasis of redox, glucose, lipid, and protein metabolism, as well as mitochondrial dysfunction with severe endogenous oxidative stress, could ultimately lead to unresolvable inflammation and malignant transformation into cancer, rapid aging (e.g., non-infectious inflammatory aging), and age-related neurodegenerative diseases. This graph was partially adapted from [**?**, **?**]. It should be noted that two non-homologous nuclear respiratory factors (also abbreviated as NRF1/2) should not be confused with Nrf1/2 abbreviated from nuclear factor, erythroid 2-related factor 1/2, and are thus designated αPalNRF1 and GABPNRF2 (see Figure S8, as discussed in references [**?**, **?**]).

Cellular redox homeostasis is determined by balancing production of all redox equivalents (R_o) and their elimination (R_e), as formulated by $\Delta H = H\_S - H\_0 = [R\_o - R\_e] \cdot k$, where H_0 and H_S represent basal redox homeostatic and stress-stimulated redox robust states, respectively. Substantial research

has revealed that differential yet integral roles of Nrf1 and Nrf2 in governing production and elimination of redox equivalents are exerted as two entangled "Yin-Yang" defenders that coordinately regulate anti-redox and cytoprotective transcriptional responses to maintain robust redox homeostasis within a preset threshold range (Figures 3 and 11A). Nrf1 has been identified as a robust indispensable determinant for cellular redox homeostasis (and mitochondrial homeostasis) under normal physiological conditions, whereas Nrf2, as a master regulon, plays a versatile role specifically in mediating adaptive hormetic effects induced by stress. Notably, loss of Nrf1 function leads to severe oxidative stress with obvious pathological phenotypes that cannot be compensated by hyperactive Nrf2 in Nrf1-deficient mice and cell lines [**?**, **?**, **?**, **?**]. In such cases, permanently hyperactive Nrf2 is more likely to cause reductive stress [**?**, **?**]. Altogether, this may create a novel maladaptive redox homeostatic status that ultimately leads to pathogenesis of cancer and other degenerative diseases.

Brown adipocyte-specific Nrf1 knockout mice that cannot acquire cold adaptation through increased thermogenic activity revealed Nrf1 as a master regulator of thermogenesis by controlling transcriptional expression of UCP1 [**?**]. Another uncoupling protein, UCP2, was differentially regulated by Nrf1 and Nrf2 via miR-195/497 [**?**]. This implies that Nrf1 and Nrf2 have differential and even opposing or combinational roles in governing cellular energy metabolism homeostasis (i.e., Intake - Expenditure = Storage), as evidenced experimentally in gene-manipulated model mice and cell lines [**?**, **?**, **?**, **?**, **?**, **?**]. For instance, energy uptake is controlled by Nrf1-driven transcriptional expression of glucose transporters (e.g., Glut1/2) [**?**, **?**] and lipid transporter CD36 [**?**, **?**]. Energy storage in fat mass and glycogen is also affected by Nrf1 presence or absence. Reduced glycemia in hepatocyte-specific Nrf1^{-/-} mice resulted from reduced hepatic glycogen and glycogen synthase GYS2 levels, together with decreased insulin and IGF1 [**?**]. Intriguingly, two similar yet distinct lean phenotypes with lowered body weight but not fat mass were manifested in skeletal myocyte-specific Nrf1^{-/-} mice [**?**] and constitutively active Nrf1-Tg:MGRD mice [**?**]. However, large amounts of fat mass were markedly stored in liver-specific Nrf1^{-/-} mice [**?**, **?**, **?**] and cell lines [**?**, **?**]. Energy expenditure was also altered by adaptive reprogramming of cellular glucose and lipid metabolisms, oppositely controlled by Nrf1 and Nrf2 through PTEN-controlled PI3K-AKT-mTOR signaling cascades toward HIF1/AMPK- and SREBP1/2-mediated transcriptional responses, respectively [**?**]. Additionally, Nrf1 functions as a direct energy sensor to negatively regulate AMPK, a master kinase regulator of glucose metabolism reprogramming [**?**]. Overall, these findings demonstrate that Nrf1 also functions as a robust determining factor for energy metabolism homeostasis (including glucose, lipid, and nutrient homeostasis), in addition to its roles in redox homeostasis and proteostasis.

In mediating adaptive transcriptional responses, Nrf1 and Nrf2 are differentially and integrally activated upon stimulation by distinct types of cellular stress, including tert-butylhydroquinone (tBHQ, a pro-oxidative stressor) [**?**], dithiothreitol (DTT, a reductive stressor) [**?**], classic ER stressors tunicamycin

(TU) [?] and thapsigargin (TG) [?, ?], and cisplatin (a platinum-based alkylating anticancer drug) [?], in addition to limited proteasomal inhibitors and other agents reviewed previously [?, ?, ?, ?, ?]. Beyond antioxidant, detoxifying, and cytoprotective responses, Nrf1 was identified as a vital and irreplaceable player in mediating mitochondrial stress response (i.e., UPR^{mito}) through ATF4/5-CHOP signaling [?], as well as governing mitochondrial biogenesis and homeostasis through two nuclear respiratory factors (αPalNrf1 and GABPNrf2) and two mitochondrial transcription factors (TFAM and TFBM) (Figures 17, S8, S9, and S12). Importantly, Nrf1, rather than Nrf2, is essentially required for DNA damage repair response through the H2AX-XPC pathway [?], while hyperactive Nrf2 in Nrf1α^{-/-} cells shows strong correlation with chemoresistance to cisplatin, although mechanistic details remain elusive.

10. Activation of Nrf1 is evoked by distinct topovectorial regulatory mechanisms

10.1 Activation of Nrf1 is dictated by its topogenetic folding and retro-translocation across membranes

As illustrated in Figure 18 [Figure 18: see original paper], the membrane-bound Nrf1 transcription factor is integrally anchored within and around the ER and conditionally sequestered by membranes, because its transactivation domains (TADs, including AD1, NST, and AD2) are co-translationally positioned on the ER luminal side, whereas its DNA-binding CNC-bZIP domains reside on the cytoplasmic side [?, ?, ?, ?, ?]. Subsequently, retro-translocational repositioning and selective proteolytic processing of full-length Nrf1α (including its transcripts, as described in Section 7) can generate various polypeptide isoforms with differential or even opposing functions, such as mature N-terminally truncated Nrf1ΔN (and TCF11ΔN), relatively less active Nrf1β, and dominant-negative Nrf1γ (Figures 6C and S1A) [?, ?, ?, ?]. Such unique topovectorial regulation of Nrf1 can also dictate specific post-synthetic modifications and transcriptional activity to fulfill its unique physiobiological functions (as described in Sections 8 and 9) [?, ?, ?, ?, ?].

Figure 18. Nrf1 acts as a robust determinant for cell homeostasis by integrating its “three-in-one” roles in topobiology.

The upper panel shows that Nrf1 serves, by integrating its “three-in-one” role, as a specific triplet of direct sensor (for redox, energy, cholesterol, and proteotoxic changes), signaling transducer, and effector in stress defense responses to challenges from changing environments. The lower left panel shows that unique topogenetic folding of Nrf1 and its dynamic retrotranslocation repositioning across ER membranes dictate topovectorial regulation through specific modification and selective processing to a mature active CNC-bZIP factor and other shorter isoforms, before regulating particular subsets of target genes, some of which (down-regulated genes in green, up-regulated genes in black) are listed in the lower right panel and are responsible for fulfilling unique biophysiological

functions as deciphered herein.

In this context, membrane-topological regulation of Nrf1 by Hrd1/p97-driven retro-translocation of its glycosylated NST-adjoining TADs from ER luminal to cytoplasmic sides [**?**, **?**, **?**] is finely tuned by membrane lipid patterning, particularly within ordered microdomains composed primarily of cholesterol and sphingomyelin (including ceramide). This is attributable to the presence of at least five cholesterol recognition amino acid consensus (CRAC) motifs within Nrf1, enabling this CNC-bZIP protein to sense changes in membrane cholesterol density and become tightly anchored in cholesterol/ceramide-enriched microdomains [**?**, **?**]. Thus, dynamic topogenetic folding and subsequent retro-translocation of Nrf1, along with functional domain repartitioning, followed by selective proteolytic processing to yield distinct isoforms, could all be confined by cholesterol and/or ceramide overloading in distinct topovectorial processes. However, detailed mechanisms by which cholesterol and/or ceramide monitor such topobiological regulation through luminal-to-cytosolic retro-translocation of glycosylated NST-adjoining regions (to become functional transactivation domains) remain to be elucidated, although sphingomyelin metabolism is significantly affected by loss of Nrf1 function (Figure S11).

10.2 Activation of Nrf1 depends on its specific modification and selective processing

First, Nrf1 activation is induced by tunicamycin (TU) [**?**, **?**], generally accepted as a classic ER stressor that acts as a specific inhibitor of oligosaccharyl transferases (OSTs) catalyzing N-linked glycosylation of ER-resident proteins, resulting in unfolded or misfolded proteotoxic stress. Such Nrf1 induction by TU yields enhanced expression of non-glycosylated protein and facilitates its proteolytic processing and subsequent dislocation from the ER to the nucleus before target gene regulation. Increased Nrf1 transcriptional expression at the mRNA level is likely activated by TU-stimulated PERK-Nrf2 and IRE1-XBP1 signaling pathways, as well as by Nrf1 itself [**?**, **?**, **?**, **?**]. However, it should be noted that TU-stimulated effects on Nrf1 were misinterpreted by a misleading pathway [**?**] (with suspected attention on PUBPEER, Figure S13) and further misled by another short commentary on Nrf1 unleashed from the ER [**?**], based on incorrectly designed Nrf1 topogenetic experiments [**?**].

Second, Nrf1 activation involves deglycosylation catalyzed by peptide:N-glycosidase (PNG-1, encoded by *NGLY1*), which occurs only after ER luminal-resident N-glycosylated NST-adjoining TADs are dynamically retro-translocated across membranes to the cytoplasmic side [**?**, **?**, **?**, **?**, **?**, **?**]. In addition to removing N-linked glycans from Nrf1, NGLY1-mediated deglycosylation leads to amino acid sequence re-editing of glycosylated asparagines to deglycosylated aspartates, potentiating acidic transactivation capacity [**?**]. This was convincingly corroborated by two additional laboratories working on Nrf1/Nfe2l1 and Skn-1 [**?**, **?**]. Conversely, Nrf1 N-glycosylation and transactivation activity are significantly suppressed by NGLY1-specific inhibitors

[?, ?] or genetic loss of function [?]. Upon blockage of Nrf1 deglycosylation, glycosylated proteins accumulate and become abnormally ubiquitinated by the sugar-recognizing ubiquitin ligase SCFFBS2 in NGLY1-deficient cells, resulting in retention of aberrantly modified Nrf1 in cytoplasmic compartments before proteolytic degradation [?], accompanied by proteasome dysfunction and proteotoxic stress. Importantly, NGLY1 mutant patients manifest a congenital disorder of deglycosylation [?, ?], leading to congenital autosomal recessive disorder [?] and even death from adrenal insufficiency [?]. Like brain-specific Nrf1^{-/-} pathological phenotypes, Ngly1^{-/-} animals develop neurodegenerative phenotypes and pathological abnormalities in peripheral and central nervous systems [?]. Similar to hepatocyte-specific Nrf1^{-/-} cases, liver-specific Ngly1 deletion causes abnormal nuclear morphology and lipid metabolism, particularly under food stress [?]. Collectively, these pathological phenotypes of NGLY1-deficient patients and animal models result predominantly from defective Nrf1 function, with consequences of impaired proteostasis, proteotoxic stress-induced cell death, and downregulation of genes (e.g., *GCLC* and *GCLM*) responsible for glutathione synthesis, specifically in lymphoblastoid cells [?].

Third, Nrf1 activation is conferred by selective proteolytic processing of this CNC-bZIP factor by cytosolic proteases DDI1/2 or activity-limited proteasomes to yield mature N-terminally truncated active forms Nrf1ΔN (and TCF11ΔN) (Figures 6C, 17, and 18) [?, ?, ?, ?]. Mammalian DDI2 is conserved with but distinct from its yeast ortholog DDI1 in structure and function [?], originally acting as a shuttling factor for proteasomes [?]. For instance, DDI1/2 is required for removal of replication termination factor 2 (RTF2) from stalled forks by proteasomal degradation to maintain genome integrity [?]. DDI1/2 contains a highly conserved retroviral protease domain that influences direct binding of ubiquitinated proteins via its ubiquitin-like domain and proteasomal degradation [?]. Thus, selective proteolytic activation of Nrf1 by DDI1/2 or its turnover by proteasomes is dictated by ER-trafficking configurations in distinct topovectorial phases [?]. Such DDI2 protease activity is required for controlling embryonic development and inflammation by Nrf1/TCF11 [?], as DDI2^{-/-} mouse embryos die at E12.5 with severe developmental failure resulting from insufficient proteasome expression, proteotoxic stress, and imbalance between innate immune and autoinflammatory responses. Furthermore, activation of Nrf1 signaling toward the ubiquitin-proteasome system by DDI2, together with its anti-redox response, protects cells from ferroptosis [?, ?]. In the "bounce-back" response of Nrf1 to sublethal proteasomal inhibitors, Nrf1 undergoes selective proteolytic processing by DDI1/2 to yield an active CNC-bZIP factor with reduced degradation by activity-limited proteasomes [?, ?, ?]. This is accompanied by enhanced Nrf2 protein expression, which augments transcriptional expression of Nrf1 and Nrf1-targeted proteasomes, although Nrf2 is not a direct upstream regulator of proteasome transcription.

Fourth, Nrf1 is stabilized to facilitate its transactivation activity and promote proteasome gene expression through deubiquitination by USP19 [?], USP7 [?],

and/or USP15 [**?**] (which can also negatively regulate Nrf2 by deubiquitinating Keap1 [**?**]). Such Nrf1 deubiquitination not only rescues it from ERAD targeting but also favors subsequent selective proteolytic processing to yield an active CNC-bZIP factor that transactivates cognate genes (e.g., proteasome subunits and chaperones). It should be noted that Hrd1 can serve not only as a critical E3 ubiquitin ligase for Nrf1/TCF11 ubiquitination [**?**, **?**, **?**] but also as an essential retrotranslocon for this CNC-bZIP factor [**?**]. Whether and how retro-translocation of Nrf1 in its ER-trafficking is affected by deubiquitinating enzymes remains to be further elucidated.

10.3 The transcriptional activation of Nrf1 in the defense response to distinct types of stress

In addition to directly sensing cholesterol, energy, and proteotoxic stress (as described above), membrane-tethered Nrf1 can directly sense redox changes between the most oxidizing ER lumen and relatively reducing extra-ER cytoplasmic and nuclear compartments through its thiol-active groups, particularly at Cys341 and Cys360 residues (situated in its glycosylated NST domain and DNA-binding basic region, respectively) [**?**]. Such distinct stress types are directly sensed and transformed into various cellular signals and transduced to Nrf1 (with aid from Nrf2) to mediate transcriptional responses regulating cognate target genes. Thus, Nrf1's integrated "three-in-one" roles manifest as a highly efficient triplet of specific sensor, transducer, and effector in diverse stress defense responses (illustrated in Figure 18, upper panel). This is dictated predominantly by Nrf1's unique topogenetic folding and topovectorial regulation through dynamic retro-translocational repositioning from the ER lumen across membranes to extra-ER compartments before nuclear dislocation, where it regulates transcriptional expression of unique subsets of antioxidant, detoxification, and cytoprotective genes (Figure 18, lower right panel).

Transcriptional activation of Nrf1 to enhance its mRNA expression levels is triggered under redox stress conditions and regulated by Pitx2/3 and αPalNRF1 [**?**, **?**], Nrf2 and itself [**?**], and other transcription factors rather than SREBP1/2 [**?**, **?**]. Additionally, Nrf1 transcriptional activation is monitored by heterodimerizing partners (e.g., sMafs, ATF4, Jun) [**?**] and several transcriptional co-factors, including CtBP2 (C-terminal binding protein 2) [**?**] and HCF-1 (host cell factor 1) [**?**, **?**].

11. Concluding remarks and future work

From a holistic eco-evo-devo perspective, we have presented extensive evidence revealing that Nrf1 is a "living fossil" transmembrane transcription factor that is evolutionarily closer than the water-soluble Nrf2 to their shared ancient orthologues (e.g., Nach, Cnc, Skn-1) that emerged during distinct evolutionary stages of life histories. Such highly conserved Nrf1 acts as a robust indispensable determinant, while Nrf2 serves as a versatile chameleon-like master regulon, in maintaining cellular redox, energy, and metabolism homeostasis and organ integrity

within preset homeodynamic ranges. Conversely, loss of Nrf1's unique functions in distinct tissues leads to obvious pathophysiological phenotypes that are distinctive or even absent upon Nrf2 loss. Such distinctions between Nrf1 and Nrf2 are attributable to Nrf1's unique topogenetic folding and dynamic topovectorial repositioning by Hrd1/p97-driven retrotranslocation across ER membranes to dislocate to the nucleus before transcriptional regulation of cognate target genes (Figures 10 and 18). In distinct topovectorial processes, Nrf1 undergoes specific reversible modifications (e.g., N-linked or O-linked glycosylation and deglycosylation, ubiquitination and deubiquitination) and selective proteolytic processing by cytosolic proteases DDI1/2 and/or limited proteasomes to yield an active N-terminally truncated CNC-bZIP factor and several shorter isoforms with distinctive or even opposing functions. Collectively, such uniquely differentiated yet integrated roles of both Nrf1 and Nrf2 coordinately govern cell homeostasis and organ integrity during distinct life processes, manifesting allelopathic potentials that virtually resemble two entangled "Yin-Yang" quanta abiding by the dialectical law of the unity of opposites (Figures 3, 11, and 18).

Crucially, full-length Nrf1α executes its essential physiobiological functions by integrating its "three-in-one" roles as a specific triplet of direct sensor (for redox, energy, cholesterol, and proteotoxic changes), signaling transducer, and effector in stress defense responses to challenges from changing oxygenated environments (Figure 18). Such highly efficient Nrf1α activation is evoked by distinct topovectorial regulatory and processing mechanisms to mediate transcriptional responses through subsets of antioxidant, detoxification, and cytoprotective genes against inflammatory and degenerative diseases (e.g., cancer, aging, and aging-related neurodegenerative diseases). This provides a potential strategy for chemoprevention and treatment of cancer and other degenerative diseases through precision targeting of Nrf1 alone or in combination with Nrf2 [**?**, **?**]. However, several open questions remain: (i) How is the full-length Nrf1 mRNA transcript selectively processed to generate distinct isoforms? (ii) Is the yield of distinct Nrf1 transcripts monitored by alternative 5 - and/or 3 noncoding regions within its gene locus? (iii) What mechanisms account for alternative translation of Nrf1 to enable selective production of distinct polypeptide forms? (iv) What are the distinct isoform-specific physio-pathological functions exerted in vivo?

Additionally, it remains unclear which Nrf1/Nfe2l1 isoforms elicited unexpected tumor-promoting roles in very few recently reported but unconfirmed cases [**?**]. Putative promotion of triple-negative breast cancer was suggested to arise through Nrf1-mediated proteasomal cytoprotective responses resisting proteasomal inhibitor treatments [**?**] and/or Nrf1-driven enhanced expression of programmed death ligand 1 (PD-L1) for immune evasion [**?**]. Ferroptosis of oral squamous cell carcinoma was also restrained by Nrf1-targeted holiday junction recognition protein (HJURP), leading to cancer progression [**?**]. These tentative and preliminary investigations require further substantiation through elaborate mechanistic studies. Nrf1 is also essentially required for antitumor immune responses that block immune evasion by mediating transcriptional expression of

TNFSF9/41BBL (a tumor necrosis factor superfamily transmembrane cytokine that functions as a bidirectional signal transducer and ligand for the costimulatory receptor 41BB in T lymphocytes) [**?**]. Conversely, Nrf1 dysfunction in NGLY1- or DDI2-deficient cells and animals leads to activation of immune-related genes by both cGAS-STING and MDA5-MAVS pathways, resulting in immune dyshomeostasis accompanied by inflammatory responses [**?**, **?**]. Similarly imbalanced immune activation and inflammation were revealed in experimental autoimmune encephalomyelitis, potentially caused by combination of proteasomal subunit displacement and reduced Nrf1 expression [**?**]. Interestingly, transcriptional expression of immunoproteasome (i-20S) and its activators PA28$\alpha\beta$ (which function predominantly in antigen presentation and regulation by γ-interferon rather than protein degradation) was substantially suppressed in proteasomal inhibitor-stimulated "bounce-back" responses mediated by Nrf1 [**?**], even though canonical ARE sequences do not exist in these gene promoters.

Altogether, such debating results presage a promising start for exploring potential roles of Nrf1/Nfe2l1 in immune homeostasis along with anti-tumor immune and anti-inflammatory responses.

Moreover, differential yet integral roles of Nrf1 and Nrf2 in redox regulation of physio-pathological functions determine distinct robust steady states (i.e., health, subhealth, pathogenesis, and disease) that may be quantitatively stratified by extents of redox stress and anti-redox defense responses based on "zero theory" (at P_0). Such distinct robust states are likely demarcated through different mechanisms accounting for homeostasis, morphostasis, homeorhesis, eustasis, allostasis, and cacostasis (ΔH = H_S - H_0), along with relevant coding rules (e.g., redox code, stress-coping code, and topogenetic code), all selected by natural eco-evo-devo processes. Since stress and redox stress should be parsed according to real scientific principles of physics and chemistry, oxidative or reductive stress with anti-redox responses can be determined by redox biochemical ontology in cancer development as a unicellular "oncoprotist"-like life form, not simply as a disease. This definition is based on the law of Darwinian evolutionary dynamics of life from a holistic eco-evo-devo perspective [**?**]. The robust state is further dictated by multi-hierarchical molecular-cellular-organismal signaling cascades toward metabolic networks, key gene regulatory profiles, and their reprogramming [**?**]. All these components comprise a large complex endogenous "tensor" network that can be mathematically modeled with stochastic effects [**?**, **?**] and precisely quantified by equations for network dynamics using nonequilibrium potential function approaches [**?**, **?**]. For example, as two key transcription factors governing cellular energy metabolic processes and homeostasis, Nrf1 and Nrf2 constitute a minimum regulatory network (Figure 13B). From a quantitative and dynamical perspective, mathematical modeling can be established. This minimum network is embedded in a larger complex network viewable from two sides: it is part of the large regulatory and signal transduction network extensively studied as endogenous networks [**?**, **?**], and it also controls metabolic networks. One of us discussed dynamics with various approaches in 2005 [**?**], where considerations were discussed, and later addressed

how to handle realistic experiments in more controlled systems [**?**]. After series of efforts, quantitative modeling was summarized [**?**]. Dynamics of corresponding endogenous and metabolic networks should be studied together because biologically they influence each other, as explored in combined studies from the modeling perspective [**?**].

Acknowledgements: We thank the many researchers who contributed to understanding Nrf1 and other CNC-bZIP family members and their relevance to (redox) stress, and apologize to those whose work we could not cite due to space restrictions and unintentional oversight. This work was supported by the National Natural Science Foundation of China (NSFC, grant number 82073079) awarded to Prof. Yiguo Zhang (Chongqing University); the funding source had no direct involvement in the research.

Author contributions: Y.Z. designed this work, conceptualized novel terms, analyzed all relevant data, prepared all figures with cartoons, and wrote and revised the paper. M.W. collected and analyzed transcriptome, proteome, and metabolome data and prepared relevant network figures, while Y(-ping) Z. performed bioinformatics analysis and constructed the phylogenetic tree with structural cartoons. C.L. and W.S. wrote the section on co-evolutionary histories of life with ambient redox environments and made related figures. P.A. provided invaluable discussion on theoretical physics, Darwinian evolution law, and network dynamics. H.T. wrote and revised the neuroscience section. X.C. and Z.Z. collected relevant bioinformatics data, edited the paper, and polished the English language. All co-authors read and agreed to the published version of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

Supplementary Materials: The supporting information includes thirteen supplemental figures.

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