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SYMPOSIUM

Stress-Free Evolution: The Nrf-Coordinated Oxidative Stress Response in Early Diverging Metazoans

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Synopsis Environmental stress from ultraviolet radiation, elevated temperatures or metal toxicity can lead to reactive oxygen species in cells, leading to oxidative DNA damage, premature aging, neurodegenerative diseases, and cancer. The transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2) activates many cytoprotective proteins within the nucleus to maintain homeostasis during oxidative stress. In vertebrates, Nrf2 levels are regulated by the Kelch-family protein Keap1 (Kelch-like ECH-associated protein 1) in the absence of stress according to a canonical redox control pathway. Little, however, is known about the redox control pathway used in early diverging metazoans. Our study examines the presence of known oxidative stress regulatory elements within non-bilaterian metazoans including free living and parasitic cnidarians, ctenophores, placozoans, and sponges. Cnidarians, with their pivotal position as the sister phylum to bilaterians, play an important role in understanding the evolutionary history of response to oxidative stress. Through comparative genomic and transcriptomic analysis our results show that Nrf homologs evolved early in metazoans, whereas Keap1 appeared later in the last common ancestor of cnidarians and bilaterians. However, key Nrf–Keap1 interacting domains are not conserved within the cnidarian lineage, suggesting this important pathway evolved with the radiation of bilaterians. Several known downstream Nrf targets are present in cnidarians suggesting that cnidarian Nrf plays an important role in oxidative stress response even in the absence of Keap1. Comparative analyses of key oxidative stress sensing and response proteins in early diverging metazoans thus provide important insights into the molecular basis of how these lineages interact with their environment and suggest a shared evolutionary history of regulatory pathways. Exploration of these pathways may prove important for the study of cancer therapeutics and broader research in oxidative stress, senescence, and the functional responses of early diverging metazoans to environmental change.

Introduction

Cells are constantly subjected to stressors from both their external environment and their own metabolites. The formation of reactive oxygen species (ROS) can cause stress resulting in oxidative damage as a consequence of exposure to xenobiotics, heavy metals, high temperatures, and ultraviolet (UV) radiation. High levels of ROS can result in premature aging and a variety of cancers and neurodegenerative diseases. ROS are also important for cell signaling during development (Lacher et al. 2018). To combat the threat of DNA damage and cancerous growth, cells developed a system of enzymes and antioxidant stress proteins to act as cytoprotectants (Itoh et al. 1999). In mammalian cells, many genes that encode for cellular defenses are regulated by the Nuclear factor (erythroid-derived 2)-like 2 (Nrf2)–Kelch-like ECH-associated protein 1 (Keap1) system, an ancient system of reactionary feedback to cellular stress (Fuse and Kobayashi 2017). Nrf2 is a transcription factor of the Cap'n'Collar (CnC) basic region leucine zipper

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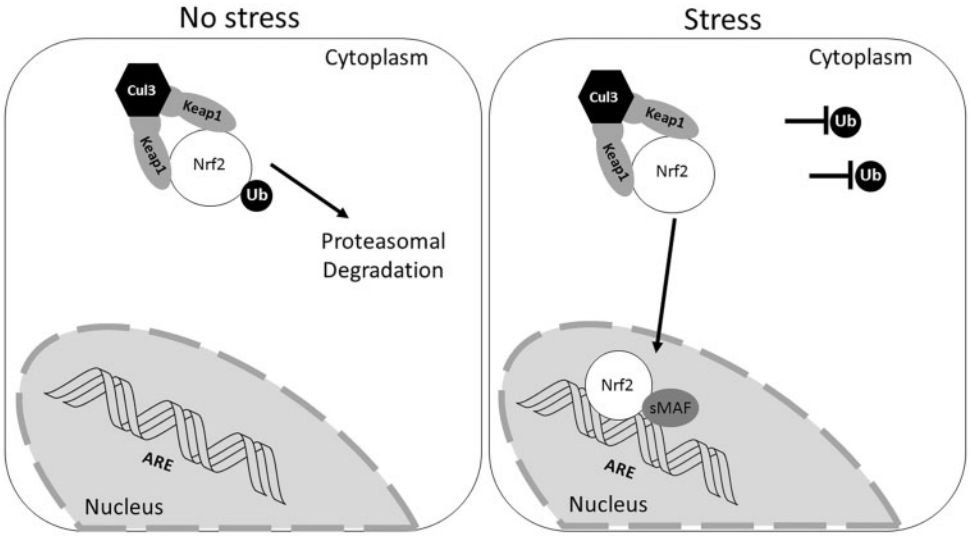

Fig. 1 Schematic of the Nrf2-Keap redox pathway in bilaterian cells. During periods of low ROS abundance (No Stress), Cul3/Keap1 and Nrf2 are in a complex in the cytoplasm. In the presence of ROS (), a conformational change of Keap1 prevents the ubiquitination of Nrf2 which builds up in the cytosol and subsequently migrates to the nucleus and binds to ARE in key genes to affect a response to the stressor (ROS). Cul3, Cullin3; Ub, ubiquitin.

(bZIP) family (Nguyen et al. 2009). Nrf2 is one of four Nrf-like proteins in vertebrates, the others being Nrf1, Nrf3, and NF-E2 (Nuclear factor, erythroid-derived 2) (Fuse and Kobayashi 2017). Nrf1 also regulates the transcription of antioxidants, however, Nrf1 activity is regulated by glycosylation in a different manner than the Nrf2 system (Bugno et al 2015), as discussed below. Both Nrf1 and Nrf3 are tethered to the ER (endoplasmic reticulum) membrane at their N-terminus. NF-E2 is expressed in hematopoietic tissues and knockout studies have demonstrated high mortality in NF-E2 negative mice (Shivdasani and Orkin 1995). Invertebrates for the most part possess only a single Nrf protein (Fuse and Kobayashi 2017). In non-stressful conditions, Nrf2 levels are maintained by the ubiquitinating complex of Keap1–Cullin3 which marks Nrf2 for degradation in the proteasome. Nrf2 has been implicated in many cellular reactions, including xenobiotic metabolism and subsequent excretion, however, it was first found to be associated with antioxidant response in mouse (Itoh et al. 1997). In the presence of high levels of electrophiles, the ubiquitination function of Keap1 is inhibited, allowing newly synthesized Nrf2 to translocate into the nucleus where it interacts with another group of bZIP transcription factors—small musculoaponeurotic fibrosarcoma proteins (sMafs). These Nrf2/sMaf heterodimers bind to a cis-regulatory antioxidant response element (ARE) in the upstream enhancers of target genes (Fig. 1). These target genes encode proteins that in turn deactivate ROS.

The regulation of Nrf2 function, as described earlier, relies on binding with the protein Keap1. Keap1 is part of a large family of Kelch proteins that are active in many varied pathways within the cell (Dhanoa et al. 2013). Keap1 proteins have high numbers of cysteine residues, three of which have experimental evidence of being oxygen sensitive in mouse (Kansanen et al. 2013). These Keap1 proteins have three characteristic domains: (1) Broad complex, Tram track, and Bric a Brac (BTB) which is responsible for its dimerization; (2) a glycine repeat region (5–6 repeats) that interacts with Nrf2, and; (3) IVR (intervening region) that aids the ubiquitination of Nrf2 (Li et al. 2008). Keap1 is localized close to the nucleus via binding to the actin cytoskeleton of the cell. It is possible that this cytoskeleton network facilitates rapid translocation of Nrf2 to the 26S proteasome for degradation (Kang et al. 2004). During oxidative or electrophilic stress, the oxidation of the thiols in Keap1's hinge region causes a conformational change preventing the ubiquitination and degradation of Nrf2, allowing newly synthesized Nrf2 to accumulate and translocate to the nucleus where it activates cytoprotective proteins (Dinkova-Kostova et al. 2002).

The origin of Nrf proteins has been linked to the rising oxygen levels on Earth during the Paleoproterozoic era (Gacesa et al. 2016). Nrf homologs are found within yeast (*Yap1*), *Drosophila* (CnC), and in Skn-1 in *Caenorhabditis elegans* (Fuse and Kobayashi 2017). Keap1 has not been found in *C. elegans* and Skn-1 has been shown to

be regulated by a separate mechanism involving glycogen synthase kinase 3β (GSK3-B) signaling (An et al. 2005). It has been suggested that Keap1 evolved in an ancestor of bilaterians, with further gene duplication events creating paralogues in some lineages (McMahon et al. 2010). Almost all that we know about the Nrf–Keap1 interaction and the activation of AREs for cytoprotection by Nrf homologs is based on bilaterian model animal systems (Fuse and Kobayashi 2017). However, oxidative stress response has an ancient origin and therefore a similar stress response is likely to exist in other groups including early branching metazoans and the largest sister group to Bilateria—the Cnidaria.

Cnidarians comprise a diverse phylum of animals that include free-living and parasitic taxa (Zhang 2013; Okamura et al. 2018). Free-living cnidarians are found from polar to tropical regions and in the deep sea to shallow waters. Many undergo an alternation of generations as benthic polyp and pelagic medusa stages and many sustain endosymbiont populations within their tissues. Some free-living cnidarians have invaded freshwaters, including the model animal *Hydra* (Technau and Steele 2011). Parasitic cnidarians exploit marine, freshwater, and terrestrial hosts with the largest group (the Myxozoa) characterized by complex life cycles that involve vertebrate and invertebrate hosts. The Myxozoa represent an extensive and under-sampled radiation of endoparasitic cnidarians whose species diversity may rival that of free-living cnidarians (Okamura et al. 2018). Cnidarians are also capable of extensive regeneration following dormancy and associated transdifferentiation of tissues (Okamura et al. 2015). This plasticity in life history and development has enabled cnidarians to inhabit a broad range of habitats where they can be expected to be exposed to challenges ranging from temperature extremes and UV radiation to host environments and immune responses. Stress response genes and proteins that have been identified previously in cnidarians include enzymes involved in thermal change and xenobiotic metabolism (Goldstone 2008; Dunlap et al. 2013). Corals are often studied for their reaction to extreme heat and UV stress due to concern about coral health and climate change (Voolstra et al. 2011; Weston et al. 2015; Cziesielski et al. 2018) yet these are often clouded by endosymbiont stress responses (Weis 2008; Tchernov et al. 2011; Weston et al. 2012). Cnidarians ability to deal with stress can also be observed in their longevity and resistance to age related degradation, with extensive regeneration and the implication of "immortality" in some species (Petralia et al. 2014). The mechanisms used by cnidarians and other non-bilaterians to deal with stressors are poorly understood and are often inferred by extrapolation from mammalian model systems.

Nrf and putative Keap1 proteins as well as antioxidant response genes have been identified in only a few free-living cnidarian species to date. This study expands our understanding of stress response proteins by utilizing genomic and transcriptomic datasets from parasitic cnidarians (Myxozoa and the monotypic sister clade to Myxozoa, *Polypodium hydriforme*) and in other early diverging Metazoa in order to examine variation in the regulation of the oxidative stress response pathway in non-bilaterians. We were particularly interested to gain insights in pathways used by parasitic taxa because parasites commonly are shown to have lost many of their ancestral genes (Tsai et al. 2013; Sun et al. 2018) and because myxozoans are suggested to have one of the smallest metazoan genomes (Chang et al. 2015). We aimed to compare key proteins in the known system of Nrf2–Keap1 antioxidation to explore what aspects of this ancient regulatory pathway are shared despite large divergences in biology, environment, and life history in early diverging metazoans.

Materials and methods

Identification of Nrf, Keap1, and Maf homologs in early branching metazoans

Putative homologs of Nrf, Keap1, and Maf proteins were searched for in a number of representative species (Table 1) from the four extant phyla of early diverging metazoans, using a combination of sequence similarity and conserved domain/amino acid architecture. We searched for homologs in the following publicly available datasets: Cnidaria (free-living)— *Hydra vulgaris* (Chapman et al. 2010), *Acropora digitifera* (Shinzato et al. 2011); Cnidaria (Myxozoa—all taxa belonging to the derived Myxosporea)—*Enteromyxum leei*, *Sphaeromyxa zaharoni*, *Kudoa iwatai*, *Myxobolus cerebralis*, (Chang et al. 2015), *Thelohanellus kitauei* (Yang et al. 2014); Ctenophora—*Mnemiopsis leidyi* (Ryan et al. 2013), *Pleurobrachia bachei* (Moroz et al. 2014); Placozoa—*Trichoplax adhaerens* (Srivastava et al. 2008); Porifera—*Sycon ciliatum* (Fortunato et al. 2014), *Amphimedon queenslandica* (Srivastava et al. 2010). In-house draft transcriptomic datasets for the staurozoan *Calvadosia cruxmelitensis*, the parasitic cnidarian *P.* hydriforme, and the early diverging malacosporean myxozoan *Buddenbrockia plumatellae* were also mined (unpublished, these

Table 1 Summary of the number of Nrf2, Maf, and Keap1 proteins in early diverging metazoans

Species	Nrf	Maf-like	Keap1	Other KLHL
Cnidaria (free-living)				
Hydra vulgaris	1	2	1	9
Acropora digitifera	1	3	1	22
Calvadosia cruxmelitensis	1	2	1	17
Cnidaria (parasitic)				
Polypodium hydriforme	1	1	–	5
Buddenbrockia plumatellae	1	1	–	2
Enteromyxum leei	1	–	–	–
Sphaeromyxa zaharoni	–	1	–	–
Myxobolus cerebralis		1	–	–
Kudoa iwatai	–	–	–	–
Thelohanellus kitauei	–	–	–	–
Placozoa				
Trichoplax adhaerens	1	2	–	7
Ctenophora				
Mnemiopsis leidyi	1	1	–	11
Pleurobrachia bachei	1	1	–	3
Porifera				
Amphimedon queenslandica	1	1	–	10
Sycon ciliatum	2	3	–	7

datasets will be presented in detail in a future publication). Nrf, Keap1, and Maf protein homologs from a number of vertebrate and invertebrate species were used as initial queries in BLASTP and TBLASTN searches with an E-value cut-off of 1e-03 using the basic local alignment search tool (blast+) suite (v.2.6.0) (Camacho et al. 2009). Results were imported into Geneious v.10 (https://www.geneious.com/) and redundant sequences/alternative transcripts were removed. Protein domains were predicted using Interproscan (Jones et al. 2014). To distinguish Nrf-like and Maf-like proteins from other bZIP family transcription factors, only sequences with a predicted "Bzip_Maf" domain (PFAM accession: PF03131) were retained. In order to find putative Keap1 proteins, only sequences containing BTB (PF00651), BACK (PF07707), and 5–6 Kelch repeats (PF01344), characteristic of the KLHL subfamily of Kelch proteins of with Keap1 is a member (Dhanoa et al. 2013), were kept for downstream analyses.

Phylogenetic analysis of Nrf, Maf, and KLHL proteins

Phylogenetic analyses were carried out on both Nrf/Maf proteins and KLHL family proteins. For the Nrf/Maf phylogeny, "Bzip-maf" domains were aligned to several metazoan proteins from bilaterian species (Supplementary Table S2) using the Geneious plugin for MAFFT (Katoh et al. 2002) with default settings. For KLHL analysis, the BTB, BACK, and Kelch domains from the early branching metazoan datasets were aligned with MAFFT (using the E-INS-i algorithm for aligning sequences with multiple domains) to previously published KLHL (kelch like proteins) proteins from human and *Drosophila melanogaster* (Prag and Adams 2003; Dhanoa et al. 2013) (Supplementary Table S2). A maximum likelihood phylogenetic analysis was carried out with RAxML (v.8.2.9) (Stamatakis 2014) with the default rapid hill-climbing mode executing 10 starting randomized maximum parsimony trees using the following command (raxmlHPC-PTHREADS-AVX2 -p 12345 -s alignment.phy -T 10 -f d -m PROTGAMMAILG -N 10 -n tree.out). PROTTEST (v.3.4.2) (Darriba et al. 2011) was used to determine the best evolutionary model for these analyses. About 1000 bootstrap replicates were performed, and these were applied to the tree with the best likelihood score. Trees were imported into Figtree (v.1.4.3) for visualization and were subsequently edited in Inkscape (https://inkscape.org/).

Characterization of Nrf/Keap1 interaction sites

Putative Nrf protein sequences from early branching metazoans were examined for Keap1 interacting amino acids. Nrf proteins were aligned to determine the presence of conserved Neh domains using MAFFT (Katoh et al. 2002) with default settings, and the alignment was subsequently manually inspected. Putative Keap1 domains were aligned to bilaterian Keap1 homologs and examined for characteristic oxygen sensing cysteine residues as well as highly conserved amino acids known to interact with mammalian Nrf2 during binding. Along with our phylogenetic analysis, these conserved amino acids were also used to distinguish Keap1 proteins in our datasets from other KLHL family proteins.

Conservation of putative Nrf targets in cnidarians

To determine whether Nrf downstream targets were present in the last common ancestor of cnidarians and bilaterians, we searched our cnidarian datasets for several antioxidant response genes whose transcription is regulated by Nrf homologs. A number of Nrf targets from several vertebrate and invertebrate species were used as queries for BLASTP and TBLASTN searches against each cnidarian database (E-value cut-off 1e-03). Results for free-living and parasitic cnidarians were compared and a presence/

absence table was compiled (Supplementary Table S1). Homologous Nrf target proteins were mapped to the *H. vulgaris* genome (https://research.nhgri.nih.gov/hydra/) using BLAT (v.0.35) (Kent 2002). The putative 1 kb genomic promoter region upstream of each successfully mapped gene was excised. The program "Find individual motif occurrences" (FIMO), part of the Multiple Em for Motif Elicitation suite of motif discovery tools (v.5.0.2) (Bailey et al. 2009), was used to predict putative Nrf-binding sites in our genomic promotor regions. Position weight matrices (PWMs) for Nrf/CnC transcription factor binding sites generated for human, mouse, and *Drosophila* (MA050.1, MA0150.2, and MA0530.1, respectively) were downloaded from the JASAPAR database (jaspar.genereg.net). For each PWM, FIMO was run with default settings except that the background model was generated from the input motif (argument: –bgfile –motif). Results from the three runs were concatenated and the redundant matches removed.

Results and discussion

Nrf, Maf, and Keap1 proteins appeared early in animal evolution

Using a combination of sequence similarity, domain architecture, and phylogenetics, we found homologs for both Nrf and Maf-like proteins in early diverging metazoans (Fig. 2A). Free-living cnidarian species (*H. vulgaris*, *C. cruxmelitensis*, and *A. digitifera*), ctenophores (*M. leidyi* and *P. bachei*), the sponge *S. ciliatum*, and the placozoan *T. adhaerens* all possessed a single Nrf protein (Table 1). These findings are consistent with previous studies showing most bilaterian invertebrate species possessing one ancestral copy of Nrf (Fuse and Kobayashi 2017) with subsequent duplications in vertebrate lineages, possibly due to a series of genome duplication events. Interestingly, the sponge *A. queenslandica* was found to have two Nrf-like proteins, which may represent a species-specific expansion (Table 1). Our examination of the parasitic cnidarians detected Nrf homologs in *P. hydriforme*, in the malacosporean *B. plumatellae*, and in the myxosporean *E. leei*. We could not find any homologs of Nrf in the datasets of the other four myxosporean species examined (*S. zaharoni*, *T. kitauei*, and *K. iwatai*, *M. cerebralis*) (Table 1). Previous studies have demonstrated rapidly evolving genes, genome reduction and gene loss within the Myxozoa (Kent et al. 2001; Chang et al. 2015; Fiala et al. 2015) and as a result Nrf may have been independently lost in several myxozoan lineages.

At least one Maf-like protein was identified in each major lineage of early branching metazoans (Fig. 2A, Table 1). A single Maf-like protein was found in the ctenophores *M. leidyi* and *P. bachei* as well as the sponge *A. queenslandica*. Each of the free-living cnidarians we examined had at least two *Maf* genes. One *Maf* gene was also found in *P. hydriforme* and *B. plumatellae*. We found a Maf-like protein in two myxosporeans (*S. zaharoni* and *M. cerebralis*); however, both proteins appeared highly derived with long branches in our phylogenetic tree. Although we recovered two potentially interesting protein sequences in cnidarians, these did not appear to group with sMaf families, the proteins that are known to bind to AREs with Nrf (Fig. 2A). Similarly, almost none of the Maf proteins found in other non-bilaterian lineages clustered with sMaf proteins. Exceptions were two sequences ("Polypodium_18594_c0_g1_i3" and "Myxobolus_GBKL01025194") from parasitic cnidarians *P. hydriforme* and *M. cerebralis*. Two sequences clustered basal to the bilaterian sMaf clade in our phylogenetic analysis. However due to the long branch associated with both proteins and the absence of any defining motifs, we believe this to be an artifact, possibly due to the low phylogenetic signal associated with short sequence alignment. Previous studies found two sMaf-like proteins in the sea anemone *Nematostella vectensis*, but no phylogenetic analysis was carried out to confirm their relationship to other Mafs (Goldstone 2008). Based on our phylogeny, we surmise that although early branching metazoans possessed Maf proteins, they have undergone independent expansion and are not homologous to the expansion of small and large Maf proteins in bilaterians. This evidence is supported by the single copy Maf in ctenophores and the poriferan *Amphimedon*, and what appear to be independent expansions in the sponge *S. ciliatum* as well as in cnidarians and placozoans (Fig. 2A, Table 1). Furthermore, our data agree with evolutionary studies of the bZIP transcription factor family which have also shown that small and large Maf proteins diverged in bilaterians from ancestral Mafs (Amoutzias et al. 2007; Jindrich and Degnan 2016).

Phylogenetic analysis recovered a total of 15 KLHL families conserved between bilaterians and early diverging metazoans (Fig. 3, Supplementary Fig. S1). Although several KLHL family proteins were found in each lineage, we only found Keap1 homologs in free-living cnidarians (Fig. 3, Table 1). Based on phylogenetic analysis and conservation of key binding sites (Fig. 3, Supplementary Fig. S2), we did not detect a Keap1 homolog in sponges,

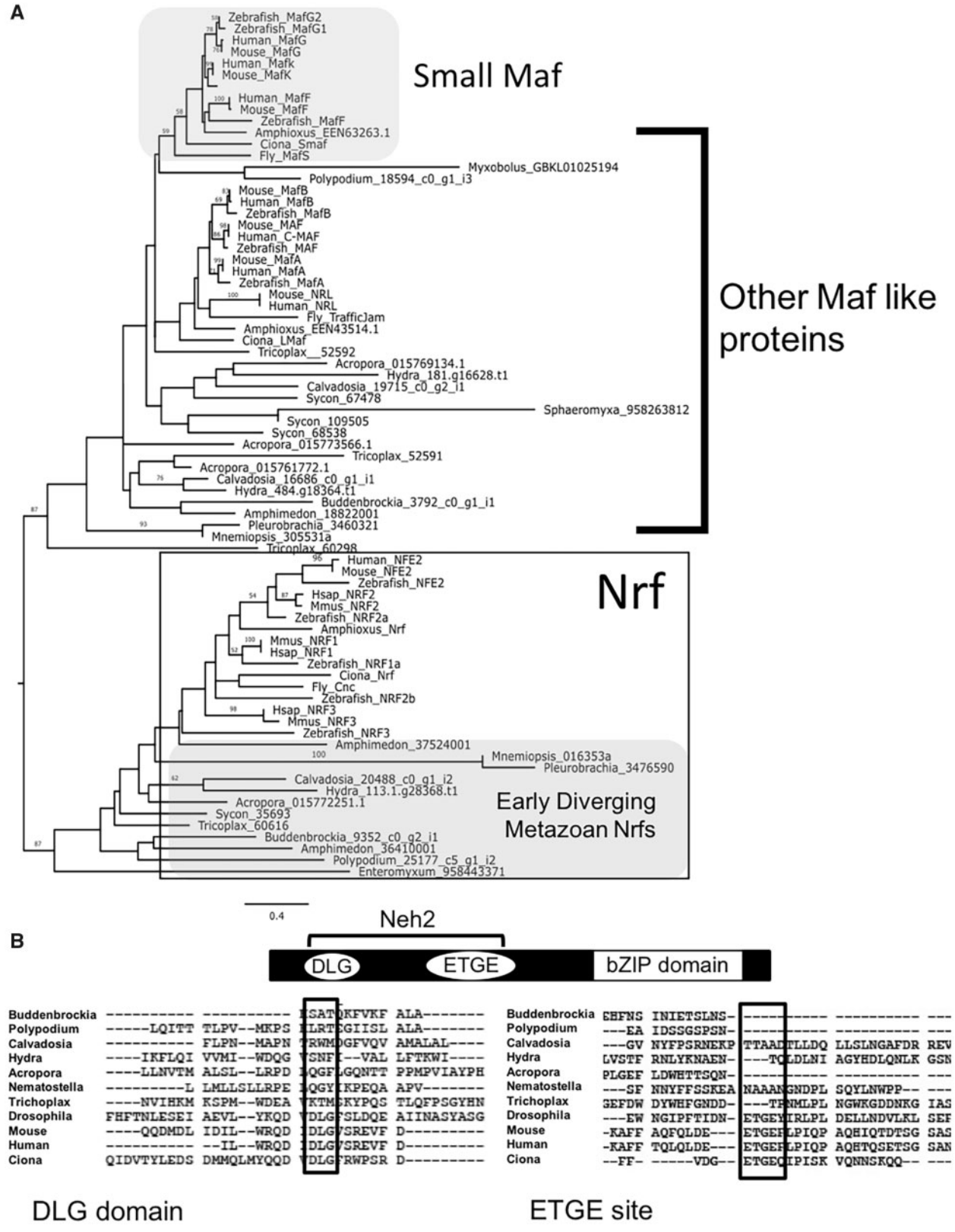

Fig. 2 Evolution of Nrf2 and Maf-like proteins. (**A**) Maximum likelihood phylogenetic analysis of proteins from bilaterians and early diverging metazoans with bZIP domains showing the Nrf2 and Maf-like clades. Tree has been midpoint rooted for display purposes. Bootstrap values >50% displayed. (**B**) Diagram representing the placement of the defining domains of Nrf2 with alignment Neh2 domain. DLG (site of ubiquitination) and ETGE (Keap1 interaction) and the residues conserved amongst bilaterians and other metazoans (black box highlighting essential amino acids). Genus names in tree refer to the following: *A. digitifera, A. queenslandica*, Amphioxus (*Branchiostoma floridae*), *B. plumatellae, C. cruxmelitensis, Ciona intestinalis, E. leei*, Fly (*Drosophila melanogaster*), Human (*Homo sapiens*), *H. vulgaris, M. leidyi*, Mouse (*Mus musculus*), *M. cerebralis*, *N. vectensis, P. bachei, P. hydriforme, S. zaharoni, S. ciliatum, T. adhaerens*, Zebrafish (*Danio rerio*). Accession numbers for tree can be found in Supplementary Table S2.

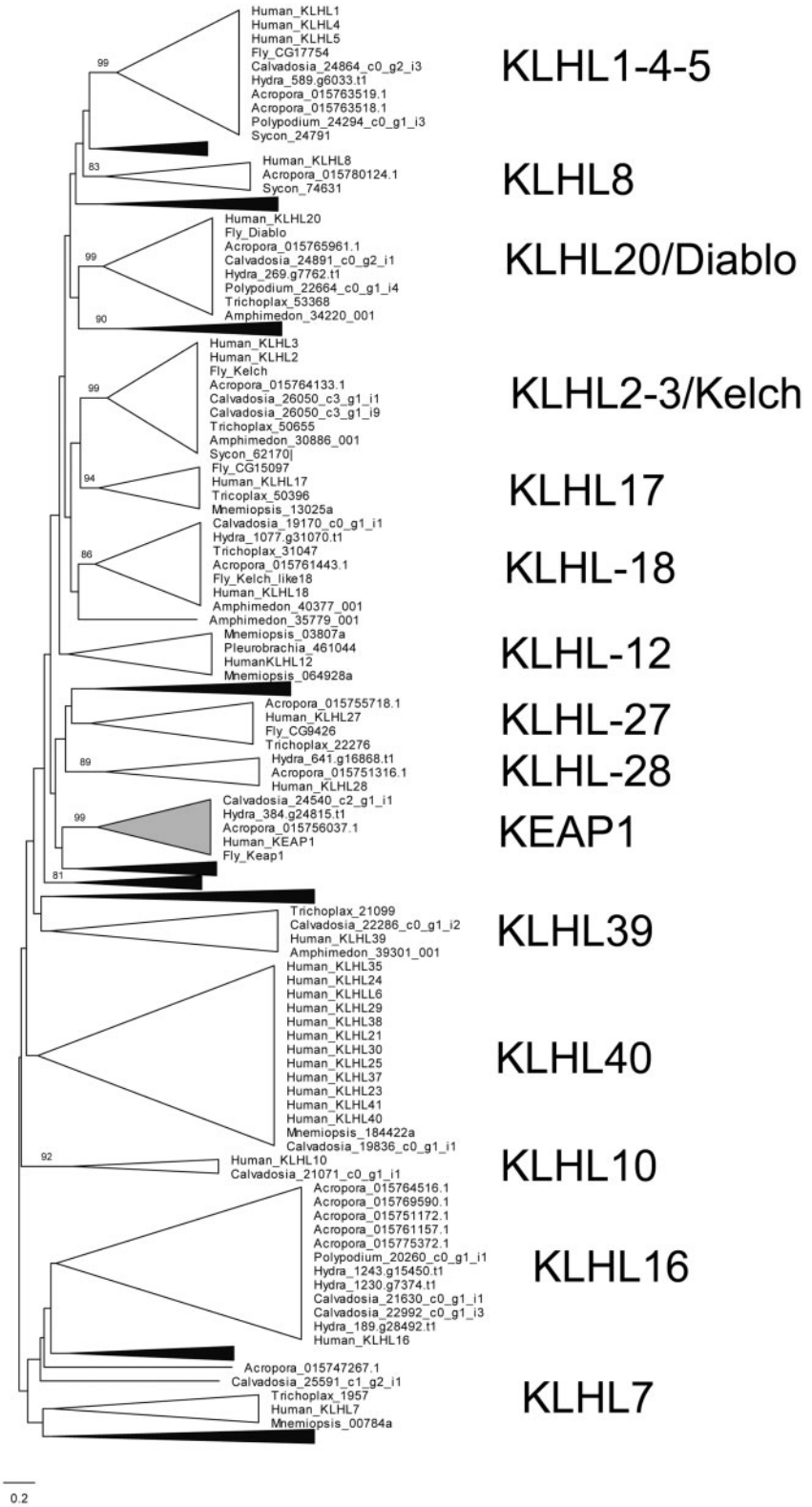

Fig. 3 Phylogenetic analysis of KLHL proteins. Maximum likelihood phylogenetic tree showing the 15 KLHL families conserved between bilaterians and early diverging metazoans. Keap1 is conserved in cnidarians and bilaterians. Black collapsed clades represent KLHL families not conserved between bilaterians and EDMs. Bootstrap values >50% displayed. Tree is midpoint rooted and main clades collapsed for display purposes. Genus names in tree refer to *A. digitifera, A. queenslandica, B. plumatellae, C. cruxmelitensis*, Fly (*Drosophila melanogaster*), Human (*Homo sapiens*), *H. vulgaris, M. leidyi, P. bachei, P. hydriforme, S. ciliatum, T. adhaerens*. Full uncollapsed tree can be viewed in Supplementary Fig. S1. Accession numbers used in tree in Supplementary Table S2.

ctenophores, or placozoans. The number of KLHL family proteins was also reduced or absent in parasitic cnidarians. We found no proteins similar to Keap1 in Myxozoa or *P. hydriforme*, suggesting loss of Keap1 in these two lineages.

Taken together, our data indicate that several aspects of the bilaterian Nrf/Keap1 pathway are conserved in different lineages of early branching metazoans. Nrf homologs appeared early in metazoan evolution, being present in all pre-bilaterian taxa. Keap1 evolved from other KLHL proteins at the stem of cnidarian/bilaterians and appears to have been lost in parasitic cnidarians. Finally, during the radiation of Bilateria, small and large Mafs evolved from ancestral Maf-like proteins which followed a separate expansion in non-bilaterian lineages (Fig. 4).

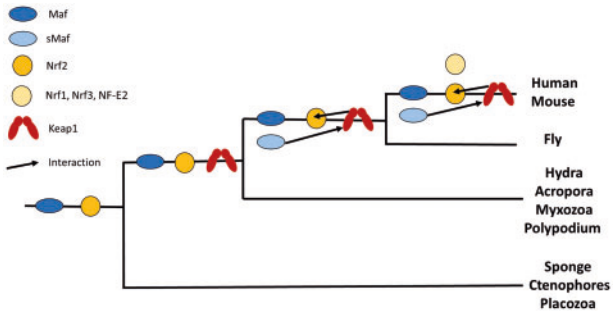

Fig. 4 Schematic representation of oxidative-stress response evolution in metazoans. Nrf and Maf-like proteins appeared at the root of metazoans before the split of early diverging lineages (sponges, ctenophores and placozoans). Keap1 appeared in the last common ancestor of cnidarians and bilaterians. sMaf proteins diverged from Maf-like proteins in early bilaterians. Keap/Nrf2/sMaf was present in the protostome/deuterostome ancestor. Ancestral Nrf duplicated multiple times in vertebrate lineages giving rise to Nrf1, Nrf2, Nrf3, and NF-E2.

Do Nrf/Keap1 proteins interact in cnidarians?

Although an Nrf homolog appears to be present at the root of all animals, our data strongly suggest that Keap1 first evolved in the common ancestor of cnidarians and bilaterians (Fig. 3). The exact amino acids in the double glycine repeat region of Keap1 responsible for binding and the maintenance of structural integrity with Nrf2 are known from exhaustive structural analyses in mouse (Tong et al. 2007). A number of highly conserved cysteine residues in Keap1 also act as oxygen sensors (Yamamoto et al. 2008; Saito et al. 2016). Keap1 activity is hampered in the presence of reactive species binding these cysteine residues, thus preventing the ubiquitination of Nrf2 (Zhang and Hannink 2003). Consequently, we compared the sequence similarity of bilaterian and cnidarian Keap1.

Sequence alignment confirms that Keap1 homologs in cnidarians possess most of the amino acids necessary to interact with Nrf (Supplementary Fig. S2). However, although several cysteine residues were conserved between bilaterian and cnidarian Keap1, oxygen sensing cysteines were not conserved in cnidarian Keap1 proteins (Supplementary Fig. S2). Only one of the three experimentally confirmed oxygen sensing cysteines were conserved in the coral *A. digitifera* (equivalent to mouse C288) and none were found in the medusozoans *H. vulgaris* or *C. cruxmelitensis*. Therefore, it appears that although Keap1 appeared early in the cnidarian/bilaterian ancestor and possesses the requisite domains and conserved binding sites capable of interacting with Nrf, it critically lacks several cysteine residues in the BTB and IVR regions which would allow it to function as an oxygen sensor. This function appears to have evolved later, after the split of bilaterians and cnidarians. Furthermore, dimerization of Keap1 is required for sequestering and degradation of Nrf2, and *in vitro* studies have shown that Ser-104 is essential for this dimerization (Zipper and Mulcahy 2002). This serine residue was not conserved in either *H. vulgaris* or *C. cruxmelitensis* but was conserved in *A. digitifera* (Supplementary Fig. S2). It is possible that Keap1 homologs in these medusozoans do not dimerize and thus cannot interact with Nrf. It is worth noting that a number of other serine residues are present surrounding the missing S-104 in the BTB domain of these cnidarian sequences and it is possible that these also aid in dimerization (Supplementary Fig. S2). Therefore, more research is required to determine if cnidarian Keap1 proteins can form homodimers and thus successfully interact with Nrf targets.

Examination of Nrf proteins in early branching metazoans also reveals loss of conservation in the Neh2 domain, responsible for interaction with Keap1. Two important Keap1 interacting motifs, the ETGE and DLG motifs, are missing in non-bilaterian Nrf proteins (Fig. 2B). Given the lack of the ETGE motif in cnidarian Nrf proteins, as well as the loss of the DLG motif, it is possible that cnidarian Keap1 and Nrf cannot interact in a canonical fashion, raising questions about the early evolution and conservation of the Nrf/Keap1 pathway. Other Neh domains are highly conserved in the Nrf homologs of early diverging metazoans (Supplementary Fig. S3). These include the Neh1 domain, which is important for DNA binding and dimerization to sMaf proteins, and the Neh3 domain, required for transactivation of downstream ARE-dependent targets (Supplementary Fig. S2) (Nioi et al 2005; Fuse and

Kobayashi 2017). Previous investigation of the *Hydra* Nrf protein has also shown conservation of the N-terminal Homology Box (homologous to the ER-binding domain in mouse Nrf1) and the Neh6 domain, which plays a role in regulation of Nrf2 through phosphorylation (Fuse and Kobayashi 2017). Conservation of these domains may indicate the importance of invertebrate Nrf in control of downstream ARE-containing antioxidants as well as an alternative and perhaps more ancient Keap1 independent regulation system.

Minimal conservation of the Nrf–Keap1 interacting pathway in non-bilaterians points to a more ancient system

Nrf2 is known as the master regulator of response to oxidative stress in bilaterians. Its antagonist Keap1 plays a vital role in both sensing rising levels of oxidative stress and keeping Nrf2 at acceptable levels during homeostasis. Functional homology of these two proteins has been shown in both *Drosophila* and vertebrates (Sykiotis and Bohmann 2008; Fuse and Kobayashi 2017), suggesting this stress response system is at least as old as bilaterians. However, the utilization of this system in earlier animal lineages is now questionable. Within the Cnidaria, Keap1 and Nrf do not seem to interact, lacking important binding sites that would allow them to do so. Earlier diverging lineages, the sponges and ctenophores, also appear to lack any Keap1 homolog. These findings raise several questions.

Was Nrf the master regulator of oxidative stress response prior to the expansion of bilaterians? Although functional studies are required to fully answer this question, several lines of evidence suggest Nrf still plays an important role in oxidative stress response. At least within the cnidarians, several antioxidant and detoxifying proteins that are known downstream mammalian Nrf2 targets were found to be conserved (Supplementary Table S1). Many of these proteins are retained in several myxozoan lineages, possibly reflecting a selective pressure in maintenance of antioxidant proteins. Motif analysis of the 1 kb promoter regions of Nrf targets in the *H. vulgaris* genome found highly conserved AREs in putative promoter regions of two genes, glutathione-S-transferase and superoxide dismutase (Supplementary Fig. S4). Our preliminary genome wide analysis has also found many ARE-containing gene promoter regions in *Hydra* (data not shown). The protein Yap1 in yeast (a putative distant homolog of Nrf-like bZIP proteins) switches on downstream antioxidant elements (called Yap-1 response elements) in response to stress (Schnell and Entian 1991; Rodrigues-Pousada et al. 2010). The presence of Nrf-like proteins in yeast acting as regulators of O_2 stress response, as well as the conservation of AREs in the *Hydra* genome suggest an ancient role for these proteins in mediation of antioxidant regulation.

The putative lack of sMaf proteins in early diverging metazoan taxa also poses an interesting question. In vertebrate systems Nrf2 couples with sMaf proteins to activate downstream targets by binding to the ARE. Cnidarians, placozoans, sponges, and ctenophores do not appear to possess bilaterian sMaf protein homologs and it is unclear whether Maf-like proteins in these basal lineages function in a similar manner to sMafs in higher animals. Nrf2 can be found in association with other proteins besides sMafs to activate downstream targets via the ARE pathway. In mammalian cells, the bZIP transcription factor activating transcription factor 4 (ATF4) forms dimers with Nrf2 to upregulate genes involved in the electrophilic stress response and unfolding protein response (Lange et al. 2008). Similarly, Jun bZIP transcription factors bind with Nrf2 to upregulate downstream antioxidant genes such as NAD(P)H Quinone Dehydrogenase 1 in response to stress (Li and Jaiswal 1992; Venugopal and Jaiswal 1996). Jun and ATF4 proteins are conserved in at least cnidarians and sponges (Jindrich and Degnan 2016) and these may serve as an alternate source of dimerization with Nrf2 if Maf-proteins have evolved as of yet unknown functions in non-bilaterians.

Finally, the question of what (if anything) regulates cnidarian Nrf in the absence of Keap1 needs further study. Normally, Keap1 is required to maintain Nrf2 at low levels in cells and indeed, disregulation of the Nrf2/Keap1 pathway can lead to oncogenesis. Accordingly, mutations of Keap1 leading to a loss of expression have been shown in many types of cancers (Singh et al. 2006; Shibata et al. 2008a, 2008b). High abundance of Nrf2 can also lead to the expression of anti-apoptotic genes such as *Bcl-2* and *Bcl-xL* (Niture and Jaiswal 2012; 2013). How then do early branching metazoans deal with Nrf regulation? Several alternative mechanisms of Nrf regulation have been discovered in other metazoans. Negative regulators of Nrf2 such as the Src subfamily A proteins are found in the cell nucleus (Niture et al. 2011). These proteins are capable of phosphorylating Nrf2 at Tyr568 which causes export of Nrf2 from the nucleus and subsequent degradation. In *C. elegans*, the Nrf orthologous protein Skinhead-1 (Skn-1) is regulated by an alternative mechanism, as *C. elegans* lacks a Keap1 homolog (Fuse and Kobayashi 2017). Here, the WD-40/

DDB1 complex targets Skn-1 for ubiquitination. GSK3-B and p38 also negatively regulate Skn-1 levels via phosphorylation and phosphorylation-based regulation of Nrf2 can be seen in mammalian cells (Salazar et al. 2006). Such regulation may represent a more ancient mechanism of Nrf control.

Early branching metazoans may utilize a more primitive method of regulation of oxidative stress. However, articles in this volume also provide evidence that these animals can also employ sophisticated chemical repertoires (e.g., Leys and Kahn 2018; Dimos et al. 2019). Furthermore, many cnidarians, such as corals (Roark et al. 2009; Bythell et al. 2018) and the hydrozoan *H. vulgaris*, are known to have long lifespans (Martinez and Bridge 2012). The extent that such prolonged longevity relies on effective cellular stress responses merits further investigation. The mechanisms of Nrf transcriptional regulation in organisms where Keap1 control is apparently absent, may provide useful insights into the mammalian pathways that evolved later, potentially advancing the development of interventions to improve human health and wellbeing. Insights into the mechanisms involved in the regulation of oxidative stress may also enable us to understand the functional responses of early diverging metazoans to stressors that can be expected to increase in frequency and severity as a result of increased exposure to high temperatures and UV radiation in our changing world.

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Supplementary data

Supplementary data are available at *ICB* online.

References

Amoutzias GD, Veron AS, Weiner J 3rd, Robinson-Rechavi M, Bornberg-Bauer E, Oliver SG, Robertson DL. 2007. One billion years of bZIP transcription factor evolution: conservation and change in dimerization and DNA-binding site specificity. Mol Biol Evol 24:827–35.

An JH, Vranas K, Lucke M, Inoue H, Hisamoto N, Matsumoto K, Blackwell TK. 2005. Regulation of the *Caenorhabditis elegans* oxidative stress defense protein SKN-1 by glycogen synthase kinase-3. Proc Natl Acad Sci U S A 102:16275–80.

Bailey TL, Boden M, Buske FA, Frith M, Grant CE, Clementi L, Ren J, Li WW, Noble WS. 2009. MEME SUITE: tools for motif discovery and searching. Nucleic Acids Res 37:W202–8.

Bugno M, Daniel M, Chepelev NL, Willmore WG. 2015. Changing gears in Nrf1 research, from mechanisms of regulation to its role in disease and prevention. Biochim Biophys Acta 1849:1260–76.

Bythell JC, Brown BE, Kirkwood T. 2018. Do reef corals age? Biol Rev Camb Philos Soc 93:1192–202.

Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K, Madden TL. 2009. BLAST+: architecture and applications. BMC Bioinformatics 10:421.

Chang ES, Neuhof M, Rubinstein ND, Diamant A, Philippe H, Huchon D, Cartwright P. 2015. Genomic insights into the evolutionary origin of Myxozoa within Cnidaria. Proc Natl Acad Sci U S A 112:14912–7.

Chapman JA, Kirkness EF, Simakov O, Hampson SE, Mitros T, Weinmaier T, Rattei T, Balasubramanian PG, Borman J, Busam D, et al. 2010. The dynamic genome of Hydra. Nature 464:592–6.

Cziesielski MJ, Liew YJ, Cui GX, Schmidt-Roach S, Campana S, Marondedze C, Aranda M. 2018. Multi-omics analysis of thermal stress response in a zooxanthellate cnidarian reveals the importance of associating with thermotolerant symbionts. Proc Biol Sci 285:1877.

Darriba D, Taboada GL, Doallo R, Posada D. 2011. ProtTest 3: fast selection of best-fit models of protein evolution. Bioinformatics 27:1164–5.

Dhanoa BS, Cogliati T, Satish AG, Bruford EA, Friedman JS. 2013. Update on the Kelch-like (KLHL) gene family. Hum Genomics 7:13.

Dimos BA, Butler CC, Ricci CA, MacKnight NJ, Mydlarz LD. 2019. Responding to Threats both Foreign and Domestic: NOD-like Receptors in Corals. Integr Comp Biol (doi:10.1093/icb/icz111).

Dinkova-Kostova AT, Holtzclaw WD, Cole RN, Itoh K, Wakabayashi N, Katoh Y, Yamamoto M, Talalay P. 2002. Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. Proc Natl Acad Sci U S A 99:11908–13.

Dunlap WC, Starcevic A, Baranasic D, Diminic J, Zucko J, Gacesa R, van Oppen MJ, Hranueli D, Cullum J, Long PF. 2013. KEGG orthology-based annotation of the predicted proteome of *Acropora digitifera*: zoophyteBase - an open access and searchable database of a coral genome. BMC Genomics 14:509.

Fiala I, Bartošová-Sojková P, Whipps CM. 2015. Classification and phylogenetics of *myxozoa*. In: Okamura B, Gruhl A, Bartholomew JL, editors. Myxozoan evolution, ecology and development. Cham: Springer International Publishing. p. 85–110.

Fortunato SAV, Adamski M, Ramos OM, Leininger S, Liu J, Ferrier DEK, Adamska M. 2014. Calcisponges have a

ParaHox gene and dynamic expression of dispersed NK homeobox genes. Nature 514:620.
Fuse Y, Kobayashi M. 2017. Conservation of the Keap1-Nrf2 System: an evolutionary journey through stressful space and time. Molecules 22:436.
Gacesa R, Dunlap WC, Barlow DJ, Laskowski RA, Long PF. 2016. Rising levels of atmospheric oxygen and evolution of Nrf2. Sci Rep 6:27740.
Goldstone JV. 2008. Environmental sensing and response genes in Cnidaria: the chemical defensome in the sea anemone *Nematostella vectensis*. Cell Biol Toxicol 24:483–502.
Okamura B, Hartigan A, Naldoni J. 2018. Extensive uncharted biodiversity: the parasite dimension. Integr Comp Biol 58:1132–45.
Itoh K, Chiba T, Takahashi S, Ishii T, Igarashi K, Katoh Y, Oyake T, Hayashi N, Satoh K, Hatayama I, et al. 1997. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochem Biophys Res Commun 236:313–22.
Itoh K, Wakabayashi N, Katoh Y, Ishii T, Igarashi K, Engel JD, Yamamoto M. 1999. Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. Genes Dev 13:76–86.
Jindrich K, Degnan BM. 2016. The diversification of the basic leucine zipper family in eukaryotes correlates with the evolution of multicellularity. BMC Evol Biol 16:28.
Jones P, Binns D, Chang HY, Fraser M, Li W, McAnulla C, McWilliam H, Maslen J, Mitchell A, Nuka G, et al. 2014. InterProScan 5: genome-scale protein function classification. Bioinformatics 30:1236–40.
Kang MI, Kobayashi A, Wakabayashi N, Kim SG, Yamamoto M. 2004. Scaffolding of Keap1 to the actin cytoskeleton controls the function of Nrf2 as key regulator of cytoprotective phase 2 genes. Proc Natl Acad Sci U S A 101:2046–51.
Kansanen E, Kuosmanen SM, Leinonen H, Levonen AL. 2013. The Keap1-Nrf2 pathway: mechanisms of activation and dysregulation in cancer. Redox Biol 1:45–9.
Katoh K, Misawa K, Kuma K, Miyata T. 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res 30:3059–66.
Kent ML, Andree KB, Bartholomew JL, El-Matbouli M, Desser SS, Devlin RH, Feist SW, Hedrick RP, Hoffmann RW, Khattra J, et al. 2001. Recent advances in our knowledge of the Myxozoa. J Eukaryot Microbiol 48:395–413.
Kent WJ. 2002. BLAT–the BLAST-like alignment tool. Genome Res 12:656–64.
Lacher SE, Levings DC, Freeman S, Slattery M. 2018. Identification of a functional antioxidant response element at the HIF1A locus. Redox Biol 19:401–11.
Lange PS, Chavez JC, Pinto JT, Coppola G, Sun CW, Townes TM, Geschwind DH, Ratan RR. 2008. ATF4 is an oxidative stress-inducible, prodeath transcription factor in neurons in vitro and in vivo. J Exp Med 205:1227–42.
Leys SP, Kahn AS. 2018. Oxygen and the energetic requirements of the first multicellular animals. Integr Comp Biol 58:666–76.
Li L, Kobayashi M, Kaneko H, Nakajima-Takagi Y, Nakayama Y, Yamamoto M. 2008. Molecular evolution of Keap1. Two Keap1 molecules with distinctive intervening region structures are conserved among fish. J Biol Chem 283:3248–55.
Li Y, Jaiswal AK. 1992. Regulation of human NAD(P)H: quinone oxidoreductase gene. Role of AP1 binding site contained within human antioxidant response element. J Biol Chem 267:15097–104.
Martinez DE, Bridge D. 2012. Hydra, the everlasting embryo, confronts aging. Int J Dev Biol 56:479–87.
McMahon M, Lamont DJ, Beattie KA, Hayes JD. 2010. Keap1 perceives stress via three sensors for the endogenous signaling molecules nitric oxide, zinc, and alkenals. Proc Natl Acad Sci U S A 107:18838–43.
Moroz LL, Kocot KM, Citarella MR, Dosung S, Norekian TP, Povolotskaya IS, Grigorenko AP, Dailey C, Berezikov E, Buckley KM, et al. 2014. The ctenophore genome and the evolutionary origins of neural systems. Nature 510:109.
Nguyen T, Nioi P, Pickett CB. 2009. The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress. J Biol Chem 284:13291–5.
Nioi P, Nguyen T, Sherratt PJ, Pickett CB. 2005. The carboxy-terminal Neh3 domain of Nrf2 is required for transcriptional activation. Mol Cell Biol 25:10895–906.
Niture SK, Jain AK, Shelton PM, Jaiswal AK. 2011. Src subfamily kinases regulate nuclear export and degradation of transcription factor Nrf2 to switch off Nrf2-mediated antioxidant activation of cytoprotective gene expression. J Biol Chem 286:28821–32.
Niture SK, Jaiswal AK. 2012. Nrf2 protein up-regulates antiapoptotic protein Bcl-2 and prevents cellular apoptosis. J Biol Chem 287:9873–86.
Niture SK, Jaiswal AK. 2013. Nrf2-induced antiapoptotic Bcl-xL protein enhances cell survival and drug resistance. Free Radic Biol Med 57:119–31.
Okamura B, Gruhl A, Reft AJ. 2015. Cnidarian origins of the *Myxozoa*. In: Okamura B, Gruhl A, Bartholomew JL, editors. Myxozoan evolution, ecology and development. Cham: Springer International Publishing. p. 45–68.
Petralia RS, Mattson MP, Yao PJ. 2014. Aging and longevity in the simplest animals and the quest for immortality. Ageing Res Rev 16:66–82.
Prag S, Adams JC. 2003. Molecular phylogeny of the kelch-repeat superfamily reveals an expansion of BTB/kelch proteins in animals. BMC Bioinformatics 4:42.
Roark EB, Guilderson TP, Dunbar RB, Fallon SJ, Mucciarone DA. 2009. Extreme longevity in proteinaceous deep-sea corals. Proc Natl Acad Sci U S A 106:5204–8.
Rodrigues-Pousada C, Menezes RA, Pimentel C. 2010. The Yap family and its role in stress response. Yeast 27:245–58.
Ryan JF, Pang K, Schnitzler CE, Nguyen A-D, Moreland RT, Simmons DK, Koch BJ, Francis WR, Havlak P, Smith SA, et al. 2013. The genome of the ctenophore *Mnemiopsis leidyi* and its implications for cell type evolution. Science 342:1242592.
Saito R, Suzuki T, Hiramoto K, Asami S, Naganuma E, Suda H, Iso T, Yamamoto H, Morita M, Baird L, et al. 2016. Characterizations of three major cysteine sensors of Keap1 in stress response. Mol Cell Biol 36:271–84.
Salazar M, Rojo AI, Velasco D, de Sagarra RM, Cuadrado A. 2006. Glycogen synthase kinase-3beta inhibits the

xenobiotic and antioxidant cell response by direct phosphorylation and nuclear exclusion of the transcription factor Nrf2. J Biol Chem 281:14841–51.

Schnell N, Entian KD. 1991. Identification and characterization of a *Saccharomyces cerevisiae* gene (Par1) conferring resistance to iron chelators. Eur J Biochem 200:487–93.

Shibata T, Kokubu A, Gotoh M, Ojima H, Ohta T, Yamamoto M, Hirohashi S. 2008a. Genetic alteration of Keap1 confers constitutive Nrf2 activation and resistance to chemotherapy in gallbladder cancer. Gastroenterology 135:1358–68.

Shibata T, Ohta T, Tong KI, Kokubu A, Odogawa R, Tsuta K, Asamura H, Yamamoto M, Hirohashi S. 2008b. Cancer related mutations in NRF2 impair its recognition by Keap1-Cul3 E3 ligase and promote malignancy. Proc Natl Acad Sci U S A 105:13568–73.

Shinzato C, Shoguchi E, Kawashima T, Hamada M, Hisata K, Tanaka M, Fujie M, Fujiwara M, Koyanagi R, Ikuta T, et al. 2011. Using the *Acropora digitifera* genome to understand coral responses to environmental change. Nature 476:320–3.

Shivdasani RA, Orkin SH. 1995. Erythropoiesis and globin gene expression in mice lacking the transcription factor NF-E2. Proc Natl Acad Sci U S A 92:8690–4.

Singh A, Misra V, Thimmulappa RK, Lee H, Ames S, Hoque MO, Herman JG, Baylin SB, Sidransky D, Gabrielson E, et al. 2006. Dysfunctional KEAP1-NRF2 interaction in non-small-cell lung cancer. PLoS Med 3:e420.

Srivastava M, Begovic E, Chapman J, Putnam NH, Hellsten U, Kawashima T, Kuo A, Mitros T, Salamov A, Carpenter ML, et al. 2008. The Trichoplax genome and the nature of placozoans. Nature 454:955.

Srivastava M, Simakov O, Chapman J, Fahey B, Gauthier MEA, Mitros T, Richards GS, Conaco C, Dacre M, Hellsten U, et al. 2010. The *Amphimedon queenslandica* genome and the evolution of animal complexity. Nature 466:720.

Stamatakis A. 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30:1312–13.

Sun GL, Xu YX, Liu H, Sun T, Zhang JX, Hettenhausen C, Shen GJ, Qi JF, Qin Y, Li J, et al. 2018. Large-scale gene losses underlie the genome evolution of parasitic plant *Cuscuta australis*. Nat Commun 9:2683.

Sykiotis GP, Bohmann D. 2008. Keap1/Nrf2 signaling regulates oxidative stress tolerance and lifespan in Drosophila. Dev Cell 14:76–85.

Tchernov D, Kvitt H, Haramaty L, Bibby TS, Gorbunov MY, Rosenfeld H, Falkowski PG. 2011. Apoptosis and the selective survival of host animals following thermal bleaching in zooxanthellate corals. Proc Natl Acad Sci U S A 108:9905–9.

Technau U, Steele RE. 2011. Evolutionary crossroads in developmental biology: Cnidaria. Development 138:1447–58.

Tong KI, Padmanabhan B, Kobayashi A, Shang C, Hirotsu Y, Yokoyama S, Yamamoto M. 2007. Different electrostatic potentials define ETGE and DLG motifs as hinge and latch in oxidative stress response. Mol Cell Biol 27:7511–21.

Tsai IJ, Zarowiecki M, Holroyd N, Garciarrubio A, Sanchez-Flores A, Brooks KL, Tracey A, Bobes RJ, Fragoso G, Sciutto E, et al. 2013. The genomes of four tapeworm species reveal adaptations to parasitism. Nature 496:57–63.

Venugopal R, Jaiswal AK. 1996. Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD(P)H: quinone oxidoreductase1 gene. Proc Natl Acad Sci U S A 93:14960–5.

Voolstra CR, Sunagawa S, Matz MV, Bayer T, Aranda M, Buschiazzo E, DeSalvo MK, Lindquist E, Szmant AM, Coffroth MA, et al. 2011. Rapid evolution of coral proteins responsible for interaction with the environment. PLoS One 6:e20392.

Weis VM. 2008. Cellular mechanisms of Cnidarian bleaching: stress causes the collapse of symbiosis. J Exp Biol 211:3059–66.

Weston AJ, Dunlap WC, Beltran VH, Starcevic A, Hranueli D, Ward M, Long PF. 2015. Proteomics links the redox state to calcium signaling during bleaching of the scleractinian coral *Acropora microphthalma* on exposure to high solar irradiance and thermal stress. Mol Cell Proteomics 14:585–95.

Weston AJ, Dunlap WC, Shick JM, Klueter A, Iglic K, Vukelic A, Starcevic A, Ward M, Wells ML, Trick CG, et al. 2012. A profile of an endosymbiont-enriched fraction of the coral *Stylophora pistillata* reveals proteins relevant to microbial-host interactions. Mol Cell Proteomics 11:M111.015487.

Yamamoto T, Suzuki T, Kobayashi A, Wakabayashi J, Maher J, Motohashi H, Yamamoto M. 2008. Physiological significance of reactive cysteine residues of Keap1 in determining Nrf2 activity. Mol Cell Biol 28:2758–70.

Yang Y, Xiong J, Zhou Z, Huo F, Miao W, Ran C, Liu Y, Zhang J, Feng J, Wang M, et al. 2014. The genome of the myxosporean *Thelohanellus kitauei* shows adaptations to nutrient acquisition within its fish host. Genome Biol Evol 6: 3182–98.

Zhang DD, Hannink M. 2003. Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by chemopreventive agents and oxidative stress. Mol Cell Biol 23:8137–51.

Zhang Z-Q. 2013. Animal biodiversity: an update of classification and diversity in 2013. Zootaxa 3703:5–11.

Zipper LM, Mulcahy RT. 2002. The Keap1 BTB/POZ dimerization function is required to sequester Nrf2 in cytoplasm. J Biol Chem 277:36544–52.