

AGEING AND THE MICROBIOME – LESSONS FROM NON-SENESCENT MODELS

BENEDIKT M. MORTZFELD and THOMAS C.G. BOSCH

Zoological Institute, Christian-Albrechts-University Kiel, Kiel, Germany

SUMMARY

Research on the biological processes of ageing is at the brink of a revolution with respect to our understanding of its underlying mechanisms as well as our ability to prevent and cure a wide variety of age-related pathologies. Ageing is ordinarily recognized as a process that results from the combined influence of genetic and epigenetic determinants, life-style associated factors and external events. Studies using model organisms such as budding and fission yeasts, the nematode *Caenorhabditis elegans*, zebrafish and mice have generated significant insight into (epi)genetic pathways involved in ageing. However, developing an integrated understanding of these diverse processes and, thereby, achieving insight into the causes and mechanisms of the ageing process, remain a challenge. Here we review recent studies in non-senescent *Hydra* which demonstrate that a continuously high activity of the transcription factor FoxO contributes to continuous stem cell proliferation and at the same time also supports robust colonization of epithelia with a stable microbiome.

THE SIGNATURE OF AGEING

Numerous studies show that ageing is not a series of stochastic defects but a directed biological program that has a conserved genetic background (*Hitt et al.*, 1999; *Kenyon*, 2010; *Jeck et al.*, 2012; *López-Otín et al.*, 2013) and is dependent on both genetic and environmental factors (*López-Otín et al.*, 2013; *Dato et al.*, 2017). Remarkably, only two proteins have been consistently associated with human ageing and longevity – Apolipoprotein E (Apo-E) and forkhead-box protein O3 (FoxO3). The genes were initially discovered in candidate gene studies of long-lived French (Apo-E) (*Schächter et al.*, 1994) and long-lived Japanese/Okinawans in Hawaii (FoxO3) (*Willcox et al.*, 2008), by comparing selected candidate gene frequency between

long-lived cases (mainly centenarians) and shorter-lived controls. These findings were subsequently replicated in numerous populations worldwide (*Morris et al.*, 2015). Genome-wide association studies (GWAS) utilizing larger numbers of cases and controls confirmed these genes as impacting human longevity but no other candidates have emerged with widespread replication (*Broer et al.*, 2015). Apo-E plays an important role in clearance of cholesterol and lipoproteins from the circulatory system in vertebrates (*Phillips*, 2014). The gene consists of three alleles – the ‘protective’ allele $\epsilon 2$, the most common ‘neutral’ allele $\epsilon 3$ and the ‘risk’ allele $\epsilon 4$. While $\epsilon 2$ is associated with reduced risk for CVD and dementia as well as with increased life

span, $\epsilon 4$ contributes to opposite effects (Schächter et al., 1994; Bennet et al., 2007; Nebel et al., 2011; Lindahl-Jacobsen et al., 2013). FoxO is a conserved transcription factor that shuttles between cytoplasm and nucleus (Van Der Heide et al., 2004) and binds directly to the regulatory sequences of its target genes (Webb et al., 2016). Four mammalian members of the FoxO family are described, FoxO1, FoxO3, FoxO4 and FoxO6. While FoxO6 is rather specific for neurons, the other FoxOs are expressed in most tissues and are causally linked to cell survival, cellular proliferation and DNA damage repair response (Monsalve and Olmos, 2011). In humans, a 1.5 Kb region of chromosome 6, in or near intron 2, contains dozens of non-coding *foxO3* single nucleotide polymorphisms (SNPs), in high linkage disequilibrium (close association) (Donlon et al., 2012). These *foxO3* SNPs vary in frequency by ethnicity, but one or more SNPs are directly associated with life span in all populations. This finding has been observed in case-control studies of centenarians and shorter-lived controls as well as cohort studies of older individuals (Broer et al., 2015; Santos-Lozano et al., 2016). Furthermore, since the FoxO protein and its target sites are highly conserved, analyses of human, mouse, *C. elegans* and *Drosophila* homologs recently uncovered FoxO as being a central hub in regulating a network of ageing-related genes (Webb et

al., 2016). Functional analyses in model organisms verified that the level of FoxO expression is indeed directly linked to life span without detectable costs for the individuals (Kenyon et al., 1993; Hwangbo et al., 2004; Schaible and Sussman, 2013). Several human tissues experience synchronized changes in gene expression during the ageing process which were shown to be a direct cause of age-related diseases (Yang et al., 2015). Furthermore, also the immune system is affected by the ageing process. “Immunosenescence” describes the deteriorating function of the immune system which results in increased susceptibility of elderly populations to infection, autoimmune diseases and cancer (Pawalec, 1999; Castle, 2000). The breakdown of mechanical barriers like the epithelia of lung, skin or intestinal tract leads to increased incidences of pathogenic invasion (Gomez et al., 2005). Combined with the declined functionality of immune cells and dysregulation of central components of innate immunity, elderly individuals observe a lower effectiveness in coping with the increased inflammation status, consequently resulting in higher morbidity and mortality (Franceschi et al., 2005; Rosenstiel et al., 2008). Although the understanding of FoxO signalling antagonizing ageing appears crucial to explain life span and health span in humans, the causative role of FoxO can only be addressed in model organisms.

MICROBES MATTER DURING THE AGEING PROCESS

All living beings are metaorganisms, associated with myriads of viruses, archaea and bacteria (Zilber-Rosenberg and Rosenberg, 2008; Bosch and McFall-Ngai, 2011). High throughput sequencing studies on humans showed that all epithelia are colonized with

different, distinct microbial communities (Eckberg et al., 2005; Grice and Segre, 2011). Furthermore, many severe and chronic diseases, e.g. irritable bowel syndrome, inflammatory bowel disease, allergy or asthma, are associated with an altered bacterial

composition on the affected site (Khosravi and Mazmanian, 2013; Fujimura and Lynch, 2015). Studies in model organisms show that symbiotic bacteria can provide essential amino acids to the host (Sandström and Moran, 1999; Shigenobu et al., 2000), detoxify harmful substances (Chaucheyras-Durand et al., 2010) or degrade complex carbohydrates and thereby make them accessible to the host (Warnecke et al., 2007; Keeney and Finlay, 2011). Additionally, the composition of the gut microbiota regulates the nutrition uptake and is involved in weight control in mice and humans (Ley et al., 2005, 2006). Therefore, an organism's health status is reliant on an intact gut microflora (Goodman et al., 2009; Blottière et al., 2013; Khosravi and Mazmanian, 2013). Recent research also underlines the importance of bacterial colonization on host behaviour (Vuong et al., 2017) as well as fundamental developmental processes involving cell proliferation and immune system maturation (Rakoff-Nahoum et al., 2004; Mazmanian et al., 2005; Bates et al., 2006; Cheesman et al., 2011; Hill et al., 2016).

Interestingly, the human microbiome is not stable throughout life but experiences significant changes with age. First inoculation occurs during birth as new-borns are colonized with birth canal specific taxa (Dominguez-Bello et al., 2016). Disruption of this transmission via caesarean section or prenatal use of antibiotics may increase

the risk of coeliac disease, asthma or obesity (Couzin-Frankel et al., 2010; Decker et al., 2010; Ajslev et al., 2011; Metsälä et al., 2015) and therefore have a huge impact on the individual's health. The intestinal microbiota resembles an adult stage already at the age of three (Yatsunen et al., 2012), while the community of the skin microbes still undergoes drastic changes in puberty before it reaches a robust state (Oh et al., 2012). Over adulthood the bacterial communities are very stable but start to change again in elderly cohorts. The ageing process affects the structure of the human gut microbiota which results in a decrease in species diversity and a higher risk of pathogenic infections (Keller and Surawicz, 2014). In model organisms the structure of the bacterial community is discussed to have a direct influence on the ageing process as it can be protective against pathogens, shapes the nutrient landscape and affects the inflammation status (Heintz and Mair, 2014). In the short-lived African turquoise killifish the microbial community of young donors is able to extend life span in older individuals by preventing the decrease in microbial diversity associated with host ageing (Smith et al., 2017). Concordantly, latest research in *C. elegans* describes the positive impact of certain bacterial genotypes on host life span and health as non-essential bacterial compounds, e.g. colanic acid, are able to regulate mitochondrial dynamics and unfolded protein response (UPR^{mt}) (Han et al., 2017).

NON-SENESCENT MODEL ORGANISMS TO STUDY THE INTERACTION BETWEEN FOXO AND MICROBES

While most ageing research has been done in short-living models, some animals show no ageing phenotype and are considered as non-senescent. The

freshwater polyp *Hydra* (Figure 1A) has an estimated life span that exceeds 1400 years (Jones et al., 2014). It belongs to the class of *Hydrozoa* within

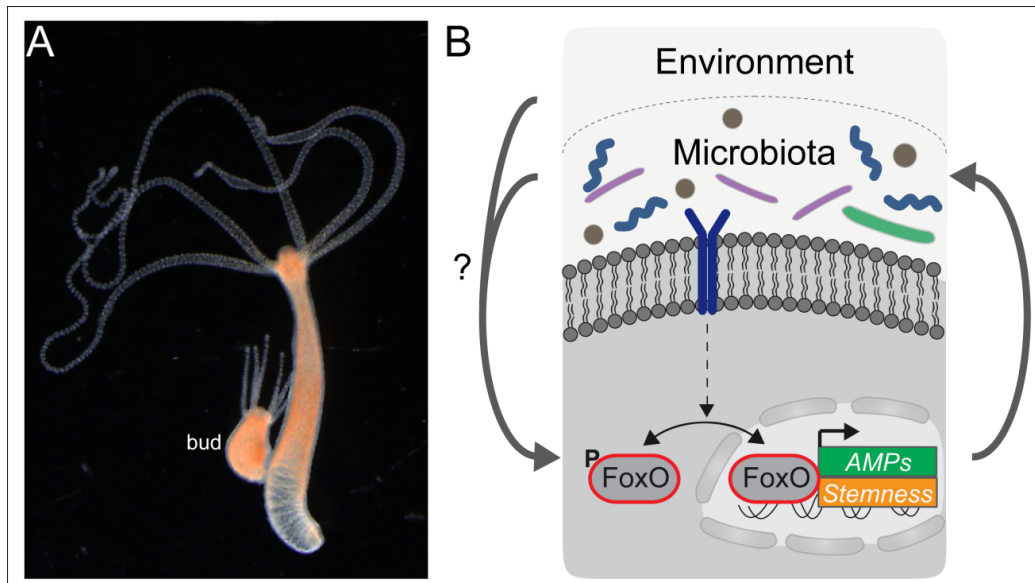


Figure 1: Conserved transcription factor FoxO controls non-senescence and innate immunity in Hydra. (A) Non-senescent Hydra. Continuous proliferation of the stem cell compartment provides the potential of an unlimited life span and clonal growth by asexual budding. (B) Model depicting the transcription factor FoxO as the key regulator of tissue maintenance and as link to the innate immune system. In response to environmental or bacterial signals FoxO can be shuttled between the transcriptionally inactive state in the cytoplasm and the active form in the nucleus. FoxO targets include genes of tissue maintenance and AMPs that directly loop back to the associated microbes. Therefore, FoxO is not only an ageing antagonist but could also be involved in maintenance of the metaorganism (from: *Mortzfeld and Bosch, 2017*).

the phylum of *Cnidaria*, the sister group of bilaterians, and therefore holds a basal position in the animal tree of life (*Martindale et al., 2002*). Due to three everlasting stem cell lineages that give rise to about 20 cell types (*Bosch, 2009*), the animals are able to continuously reproduce asexually via budding (Figure 1A). A single homolog of the longevity gene *foxO* was found to be expressed in all three stem cell lineages in *Hydra* (*Hemmrich et al., 2012*). While earlier *Hydra* studies associated the transcription factor with apoptosis (*Lasi et al., 2010*) and stress resistance (*Bridge et al., 2010*), functional analyses uncovered FoxO's role in both stem cell regulation and innate immunity (*Boehm et al., 2012*). *In situ* hybridizations of the cnidarians *Clytia* and *Hydra* revealed *foxO* expression in the

area of cell proliferation (*Chevalier et al., 2006; Boehm et al., 2012*). Epithelial FoxO deficiency for the latter resulted in a significant increase of differentiated cell mass in relation to the stem cell compartment which resembles the ageing phenotype in senescent animals. Inversely, an overexpression in the interstitial stem cell lineage caused not only an increase in cell proliferation, but also expression of stem cell marker genes in terminally differentiated cells (*Boehm et al., 2012*). FoxO's capacity in other model organisms [*Drosophila* (*Hwangbo et al., 2004*) and *C. elegans* (*Kenyon et al., 1993*)] to delay the onset of age-related processes and to extend life span underlines its conserved potential in stem cell control and tissue maintenance, especially in the absence of classical stem cell

factors like Oct-3/4 or SOX2 (Chapman et al., 2010). Remarkably, the changes in tissue homeostasis were accompanied by altered expression of antimicrobial peptides (AMPs), effectors of the innate immune system.

Microbial colonization has been studied intensively in cnidarians as they have a huge impact on marine ecology but also offer a great opportunity to study bacteria-host interactions in organisms of lower complexity. Intracellular photosynthetic dinoflagellates (e.g. *Symbiodinium*) allow corals to populate nutrient-poor environments (Muscatine and Porter, 1977), however, recent research has shown that basal metazoans in addition to eukaryotic photobionts also harbour extracellular, species-specific bacterial communities (Rohwer et al., 2002; Littman et al., 2009; Franzenburg et al., 2013a). In the case of *Hydra* even closely related species are colonized by specific microbiomes that mirror the phylogenetic relatedness between their host species (phylosymbiosis; Brooks et al., 2016). The species-specific microbiomes in cnidarians are extremely stable and still resemble the ones of animals from the wild even after years of

culturing under laboratory conditions involving artificial water and feeding (Fraune and Bosch, 2007; Franzenburg et al., 2013a; Mortzfeld et al., 2016). The acquisition of bacterial symbionts occurs during early development and establishes in a robust, reproducible pattern (Franzenburg et al., 2013b; Mortzfeld et al., 2016). Specific bacterial taxa may not only be acquired horizontally (Apprill et al., 2009; Sharp et al., 2010) but in some cases also via vertical transmission (parent to offspring) (Fraune et al., 2010; Sharp et al., 2012). Certain taxa of free-living bacteria can even be attracted by the host using chemotactic gradients (Tout et al., 2015). Underlining the importance of the microbiome, a study with germ-free animals showed that *Hydra* is protected from fungal infections by its bacterial colonizers. Interestingly, recolonization experiments proofed that a combination of the two most abundant bacteria of the naturally occurring microbiota [*Curvibacter sp.* 75% and *Duganella sp.* 10% (Franzenburg et al., 2013a)] was most protective, while monoassociations resulted in insufficient effects (Fraune et al., 2015).

AGEING ANTAGONIST FOXO CONTROLS BOTH STEM CELLS AND MICROBES

Besides its well-known conserved function as major tissue regulator and ageing antagonist, FoxO modulates the innate immune system in various model organisms including mouse (Seiler et al., 2013), *Drosophila* (Becker et al., 2010), *C. elegans* (Libina et al., 2003) and *Hydra* (Boehm et al., 2012). In mice FoxO signalling has been shown to reduce susceptibility to bacterial infections by reducing oxidative stress and induction of inflammatory cytokines (Joseph et al., 2016).

Furthermore, FoxO transcription factors directly regulate TLR3-mediated innate immune responses as well as the expression of AMPs and thereby contribute to pathogen clearance in the respiratory tract (Seiler et al., 2013). Also in *Drosophila* AMPs are well-known effector molecules of the innate immune system and important regulators of the bacterial colonizers. Here, oral bacterial infection induces FoxO activity in the intestine, while impaired FoxO signalling decreases resistance to

intestinal infections. The inability to raise the expression level of AMPs leads to an elevated bacterial load and a decline in survival (Fink et al., 2015). In *Hydra*, the microbiome is selectively assembled by a species-specific combination of AMPs which are predominantly expressed in epithelial cells (Fraune et al., 2010; Franzenburg et al., 2013a). FoxO-deficient *Hydra* polyps show in addition to defects in stem cell maintenance a severe change of the immune status and drastically altered expression of AMPs (Boehm et al., 2012). Remarkably, loss of tissue homeostasis as well as AMP-deficiency compromise the ability to select for microbial communities resembling the polyps' native microbiota (Fraune et al., 2009; Franzenburg et al., 2013a). Interestingly, a recent study indicates that FoxO signalling may also be involved in the establishment of symbiosis in the early developmental phase of the sea anemone *Aiptasia* (Wolfowicz et al., 2016).

Taken together, the conserved transcription factor FoxO appears to combine two functions crucially involved in ageing and health in metazoans (Figure 1B): FoxO is responsible for stem cell regulation, including tissue maintenance and renewal, and controls the innate immune system. In response to environmental (including bacterial) signals, FoxO switches between a transcriptionally inactive state in the cytoplasm and an active form in the nucleus thereby serving as an intracellular control board for environmental signals. The capabilities of the FoxO transcription factor to extend life span and control effectors of the immune system

demonstrate a strong and unique mechanism of cross-regulation of tissue homeostasis and innate immunity.

By exploring epithelial FoxO loss-of-function mutants, we recently made two important discoveries (Mortzfeld et al., 2018). First, deficiency in FoxO signalling leads to dysregulation of multiple AMP families. Most genes encoding epithelially expressed AMP families including Hydramacin, Kazal and Arminin respond with downregulation to FoxO-deficiency. Only one gene (contig 45266) was found to be upregulated in FoxO deficient animals, suggesting a mainly activating function of FoxO signalling on AMP expression and innate immunity. Second, FoxO loss-of-function polyps were more susceptible to colonization of foreign bacteria and impaired in selection for bacteria resembling the native microbiome. Therefore, FoxO-induced decrease in AMP expression is correlating with differences in microbial colonization and highlights the inhibitory action of AMPs against non-commensal bacteria. In a state of intact FoxO signalling secretion of numerous AMP families provides a highly selective milieu and shapes the microbial composition in a species-specific manner (Figure 1B). FoxO-deficiency reduces the expression of AMPs, which results in a decreased selection pressure on colonizing taxa and in establishment of higher abundances of foreign bacteria in the community. Consequently, especially during the process of colonization, reduced expression of FoxO compromises the resilience of the microbiome.

CONCLUSIONS

Stem cell maintenance and immunity are two of the prominent areas in

ageing research. Observations in different organisms indicate that, in contrast

to the essentially static genome, the microbiome is rather dynamic throughout life history. Our observations in non-senescent *Hydra* add support to the view that (i) there is a need to consider the holobiotic nature of an organism when thinking about longevity, that (ii) the microbial environment matters in the context of senescence and contributes to complex processes such as aging; and that (iii) the hub regulator

FoxO presents a direct link between age-related processes and microbial colonization. In the newly discovered world of metaorganisms, stem cell proliferation and immunity are part of a global program, which seeks to fuse stem cell biology with ecological concepts and the rules governing the interactions between an organism and its microbial environment (Mortzfeld and Bosch, 2017; Mortzfeld et al., 2018).

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