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GIT2 – a keystone in ageing and age-related disease

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Highlights

- Highly complex physiological processes are controlled by nuanced systemic protein networks
- GIT2 acts as an integrator protein for hallmark ageing processes
- GIT2 genomic deletion models show multiple signs of ageing and age-related disorders
- Regulation of GIT2 expression/functionality may allow reversal of age-related disorders

ABSTRACT

Since its discovery, G protein-coupled receptor kinase-interacting protein 2, GIT2, and its family member, GIT1, have received considerable interest concerning their potential key roles in regulating multiple inter-connected physiological and pathophysiological processes. GIT2 was first identified as a multifunctional protein that is recruited to G protein-coupled receptors (GPCRs) during the process of receptor internalization. Recent findings have demonstrated that perhaps one of the most important effects of GIT2 in physiology concerns its role in controlling multiple aspects of the complex ageing process. Ageing can be considered the most prevalent pathophysiological condition in humans, affecting all tissue systems and acting as a driving force for many common and intractable disorders. The ageing process involves a complex interplay among various deleterious activities that profoundly disrupt the body's ability to cope with damage, thus increasing susceptibility to pathophysiologies such as neurodegeneration, central obesity, osteoporosis, type 2 diabetes mellitus and atherosclerosis. The biological systems that control ageing appear to function as a series of interconnected complex networks. The inter-communication among

multiple lower-complexity signaling systems within the global ageing networks is likely coordinated internally by keystones or hubs, which regulate responses to dynamic molecular events through protein-protein interactions with multiple distinct partners. Multiple lines of research have suggested that GIT2 may act as one of these network coordinators in the ageing process. Identifying and targeting keystones, such as GIT2, is thus an important approach in our understanding of, and eventual ability to, medically ameliorate or interdict age-related progressive cellular and tissue damage.

ABBREVIATIONS

AD: Alzheimer's disease
ArfGAP: ADP-ribosylation factor GTPase-activating proteins
AT: adipose tissue
ATM: Ataxia Telangiectasia Mutated
ATP: Adenosine tri-phosphate
BRCA1: Breast cancer type 1 susceptibility protein
CNS: central nervous system
DDR: DNA damage response
DSB: Double-strand break
EGF: endothelial growth factor
GIT2: G protein coupled receptor kinase interacting protein 2
GPCR: G protein-coupled receptor
Ins: Insulin
IGF1: insulin growth factor 1
IL: interleukin
NF- κ B: Nuclear factor NF-kappa-B
OB: osteoblast
OC: osteoclast
PARP: Poly (ADP-ribose) polymerase
PIX: PAK (p21-activated kinase) Interacting exchange factor
RANK: receptor activator of nuclear factor kappa B
ROS: Reactive oxygen species
RUSC2: RUN and SH3 Domain Containing 2
T2DM: Type 2 Diabetes Mellitus
TLR: Toll-like receptor
TNF α : Tumor necrosis factor-alpha
WT: wild-type

Keywords: Keystones, Ageing, Age-related disorders

1. INTRODUCTION

Mammalian GIT1 was first identified as a binding partner for G protein-coupled receptor kinases (GRKs), and thus named GRK-interacting protein 1 (GIT1) (Premont et al., 1998). GIT1 and GIT2 comprise the GIT protein family, which share enzymatic function as GTPase-activating proteins (GAPs) for the ADP-ribosylation factor (Arf) small GTP-binding proteins (Premont et al., 1998; Vitale et al., 2000). GIT proteins function to limit the activity of Arf proteins, and are members of the larger family of ArfGAPs (Kahn et al., 2008). Arf proteins have no intrinsic GTPase activity, and thus require GAPs to convert the GTP bound to active Arf to GDP, causing deactivation (Randazzo et al., 1994). Both GIT proteins were originally identified as regulators of GPCR internalization through the influence they exert on the Arf GTP-binding proteins (Claing et al., 2000; Premont et al., 1998; Vitale et al., 2000). Purified GIT proteins are linked functionally to plasma membrane protein Arf6 (Claing et al., 2000; Di Cesare et al., 2000; Jones et al., 2009; Meyer et al., 2006; Miura et al., 2009), but inactivate all subtypes of Arf proteins (Vitale et al., 2000).

GIT proteins and their primary interaction partners, the PIX (PAK (p21-activated kinase)-interacting exchange factor) proteins, can function together as signaling scaffolding proteins with their multiple domains binding to many protein partners (Zhou et al., 2016). The most well described binding partners of GIT proteins are p21-activated kinase-interacting exchange factors α -PIX and β -PIX (Bagrodia et al., 1998; Premont et al., 2000; Premont et al., 2004; Zhao et al., 2000). GIT proteins act as part of this scaffold complex to link signaling molecules to distinct sites of action in the cell and within many distinct signaling networks. Over 100 GIT-associated proteins and dozens of direct interactors, many of which have been first identified in the brain, have been described (Table S1) including liprin- α , piccolo, and huntingtin (Hoefen and Berk, 2006; Zhou et al., 2016). GIT proteins have been implicated in the regulation of cognition, where loss of GIT1 resulted in severe learning and memory deficiencies in three distinct murine knockout models (Hong and Mah, 2015; Menon et al., 2010; Schmalzigaug et al., 2009a; Won et al., 2011), and microcephaly due to a neuron size reduction (Hong and Mah, 2015), while GIT2-KO mice exhibit anxiety-like behavior and advanced ageing (Lu et al., 2015; Schmalzigaug et al., 2009b).

The GIT proteins have been implicated in multiple cellular processes, including cell migration (Zhao et al., 2000), dendritic spine formation (Zhang et al., 2003; Zhang et al., 2005), T-cell activation (Phee et al., 2005), huntingtin aggregation (Goehler et al., 2004) and centrosome dynamics (Zhao et al., 2005). Brain tissues from Huntington's disease patients have been shown to display the accumulation of a C-terminal proteolytic fragment of GIT1 (Goehler et al., 2004). GIT1 localizes in both pre- and post-synaptic terminals in hippocampal neurons (Zhang et al., 2003) and downregulation or mislocalization of GIT1 leads to disrupted dendritic spine and synapse formation (Zhang et al., 2003; Zhang et al., 2005). Additionally, GIT1 facilitates AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor targeting in primary neurons of the hippocampus (Ko et al., 2003) and mediates ephrin B signaling during spine formation (Segura et al., 2007). In contrast, less is known about the neuronal functions of GIT2, despite the large expression overlap between GIT1 and GIT2 throughout the brain (Schmalzigaug et al., 2007b).

Mammals express GIT1 and GIT2, whereas zebrafish express three GIT proteins, since they have 2 GIT2 genes (*git2a* and *git2b*) (Yu et al., 2011). While GIT1 has only two splice variants in humans and mice, GIT2 undergoes extensive alternative splicing (Premont et al., 2000). GIT1 and GIT2 share a conserved domain architecture, including the N-terminal zinc finger ArfGAP domain, three Ankyrin repeats, a Spa2-homology domain/Src homology 2 domain-containing transforming protein D (SHD), a coiled-coil (CC) domain, a poorly conserved linker region and a focal adhesion targeting (FAT) domain (Fig. 1A&B) (Zhou et al., 2016). The α -PIX and β -PIX interacting partners bind to the SHD domain. The coiled-coil domain allows dimerization of GIT proteins through formation of a parallel coiled-coil (Premont et al., 2004; Schlenker and Rittinger, 2009). The FAT domain acts as the binding site for the focal adhesion

adaptor protein paxillin (Schmalzigaug et al., 2007a; Zhang et al., 2008). GIT2-short is a truncated variant of GIT2 that is missing the FAT domain (Fig. 1C) and is highly expressed in immune cells. The importance of GIT2 splicing remains unclear, though GIT2-short displays an inability to bind to paxillin in the same manner as GIT2 (Premont et al., 2000) and both GIT1 and GIT2 are able to regulate Arf6-dependent GPCR sequestration. The direct comparison between GIT1 and GIT2 suggests that GIT2 binds to paxillin with much lower affinity than GIT1 (Premont et al., 2000). Another difference is that GIT1 tyrosine phosphorylation is unaffected by cell adhesion, while GIT2 is transiently phosphorylated during attachment (Shikata et al., 2003). The high homology seen between GIT1 and GIT2, both in structure and function as well as the strong homo- and heterodimerization of these proteins, suggests the presence of some redundancy *in vivo*, implying that these exert different functions. To analyze the individual GIT proteins in a cellular context, Schmalzigaug et al. investigated tissue- and cell-specific expression patterns (Schmalzigaug et al., 2007b). While their research in mice confirmed the broad distribution of the two GIT genes seen in human and rat, it also revealed underlying expression patterns. GIT2 appears to be nearly ubiquitously expressed, whereas GIT1 expression distribution is much more restricted. Both GIT1 and GIT2 are co-expressed throughout most of the brain, except for the cerebellum, where only GIT2 can be found in the granule cells. While GIT1 expression is restricted mainly to the vasculature in liver and lung, GIT2 is expressed in most cell types (Schmalzigaug et al., 2007b). Furthermore, GIT1 and GIT2 genes are regulated in a cell maturation-dependent manner in testes, where GIT2 expression is turned on in early-stage spermatogonia cells but is turned off as these cells mature; GIT1 expression shows the opposite pattern. This suggests a developmental shift in expression between the two isoforms. Even though both GIT1 and GIT2 expression is prominent in testes, deficiency in these genes does not cause male infertility, indicating that neither GIT gene is absolutely required for normal sperm development and function (Schmalzigaug et al., 2007b). Taken together, while both exert functions in the brain, GIT1 is mainly involved in brain development and GIT2 possibly has a more prominent role in neurodegeneration due to ageing (Goehler et al., 2004; Hong and Mah, 2015; Lu et al., 2015).

1.1 Molecular Mechanisms of Ageing

Ageing is one of the most complex and interconnected biological processes known, characterized by a progressive loss of physiological integrity that leads to impaired functionality and increased vulnerability to morbidity and in the end mortality (Lopez-Otin et al., 2013). This process also represents one of the highest risk factors for many major human disorders, *i.e.* neurodegeneration, osteoporosis, and Type 2 Diabetes Mellitus (T2DM) (Lopez-Otin et al., 2013). While the ageing process itself is not considered a disease, it is a condition that facilitates or perhaps even induces the occurrence of disease in many elderly persons (Rattan, 2014) and can as such be considered a risk factor for the development of most age-related disorders (Collier et al., 2011; Niccoli and Partridge, 2012). While ageing does not conform to the traditional characteristics of a disease, treating ageing as a disease makes the road to developing treatments for complex ageing-related pathologies a much easier one to take.

Investigating the cause of a disorder often starts at the genome. Several genes have been identified to be important in regulating lifespan in animal models, where genetic manipulation of a number of genes that are involved in the growth hormone/insulin-like growth factor 1 axis strongly affect lifespan (de Magalhaes, 2011). At the molecular signaling/functional level, however, there are three main characteristics involved in determining survival ability and thus lifespan: *i*) tissue/cell damage control; *ii*) stress response and *iii*) consistent molecular remodeling and adaptation. It is the progressive diminution of these somatic repair and adaptation functionalities that defines the rate of the ageing process (Rattan, 2014). Each of these reactive and productive processes require effective energy metabolism. Current data has demonstrated that optimal regulation of energy use in both the central nervous system (CNS) and the peripheral nervous system facilitates healthy ageing (Cai et al., 2012; de la Monte, 2014; de la Monte et al., 2017; Janssens et al., 2014). The hypothalamus is one of the key organs in the body responsible for maintaining an efficient interaction between energy balance and neurological activity. It is thus a vital region of the brain in the

ageing process, since it trophically coordinates both central and peripheral functions (Cong et al., 2012; Stranahan et al., 2012a; Wang et al., 2010; Zhang et al., 2013). Complex physiological systems, encompassing both nervous and endocrinological modalities, are moderated by intricate and inter-dependent networks of genes and proteins rather than just any single unitary factor (Chadwick et al., 2012) – this dimensional complexity however can be practically reduced to a smaller group of trophic regulatory factors that help maintain network integrity and the ability to adapt quickly to perturbations.

1.2 Molecular Networks and Keystones

It has become increasingly clear that biological systems function as complex integrated networks rather than simplistic linear molecular cascades (Barabasi and Oltvai, 2004). Functional biological networks are, at the most basic level, composed of clusters of self-reinforcing protein-protein interactions. These protein-protein networks can then regulate signaling cascades, transcriptional responses and cell-cell communication systems to control processes from the single cell to the level of the whole body (Fraser, 2005). The higher the degree of interconnectivity of a protein in these networks, the more biologically important it may be considered to be (Albert et al., 2000; Jeong et al., 2001).

These highly-connected proteins are called ‘keystone’ proteins or ‘hubs’ (Han et al., 2004; Schadt, 2009; Schadt et al., 2009). The presence of these keystones allows the network to become highly resistant to random (peripheral) node failure, thus generating network robustness. In addition, these keystone factors allow response and adaptation to changes in external and internal conditions, without losing normal network functionality and integrity (Wolfson et al., 2009). Highly-connected keystones are especially important for multicellular/multi-tissue processes, such as ageing, as they significantly improve the efficiency of communication across these multi-tissue hyper-complex systems (Watts and Strogatz, 1998). In times of stress, when multi-tissue coordinated responses are vital, an additional level of physiological coordination may be necessary, *i.e.* keystone-keystone communication. Interactions between different keystones may be therefore important to coordinate life-preserving stress responses, and are most likely responsible for bridging different classes of cellular pathways, *e.g.* linking metabolic integrity to DNA repair. In normal physiological functions, these domain-crossing complex interactions of trophic control mechanisms allow for adaptation to sudden needs and unpredictable changes (Lipsitz and Goldberger, 1992). The current data suggests that in a global sense that there is a progressive loss of these complex interactions linking network integrity and flexibility during the ageing process (Balasubramanian and Nagaraj, 2016; Lipsitz and Goldberger, 1992; Morrison and Newell, 2012; Sleimen-Malkoun et al., 2014), causing a loss of dynamic range in physiological function and thus a reduced ability to adapt to stress. This has been seen in multiple processes such as: 1) cardiovascular control; 2) pulsatile hormone release; and 3) electroencephalographic potentials (Lipsitz and Goldberger, 1992).

Maintaining the complex interactions that occur between different biological systems through keystone functionality may be a promising approach to attenuate the severity of age-related disorders. Hence, targeting keystone functionality/expression could significantly control the ageing process in a minimally-invasive and self-reinforcing manner, *i.e.* modulation of the keystone itself may create remedial synergistic ‘ripples’ across the desired physiological network. Remedial regulation of keystone factors may engender a subtle, but highly effective, therapeutic response, as not one single signaling system is targeted directly thus reducing the opportunity for rapid reflexive tachyphylaxis at a simple pathway level, *e.g.* desensitization. Most currently used prescription drugs have been rationally designed to induce their therapeutic efficacy by regulating only a small number of factors/proteins within a signaling cascade. However, these small, localized effects may then be counteracted by other distal components of the ‘disease’ network; in contrast, effective control over a keystone factor would likely generate an effect that spreads across the network in a self-reinforcing manner.

One such target protein in such complex ageing networks is GIT2 (Chadwick et al., 2012). GIT2 was identified as an important protein linked to several aspects of the complex ageing process, including energy metabolism and neurophysiological regulation. Using unbiased Latent Semantic Indexing (LSI) approaches, GIT2 was shown to be strongly linked to the greatest number of functional aspects of protein networks within the ageing hypothalamus. GIT2 potentially plays a multidimensional role in linking neuronal and energy-regulatory functions in ageing (Chadwick et al., 2012). Supporting the keystone role of GIT2, Chadwick et al. (2012), showed a strong elevation of GIT2 expression with advancing age in a wide variety of CNS tissues (hypothalamus, brainstem, cerebellum, cortex, and pituitary), as well as in multiple peripheral tissues associated with somatic energy metabolism (pancreas, liver, skeletal muscle and adipose tissue). This age-related increase in GIT2 expression suggested a fundamental role in the ageing/energy regulation process for this scaffolding protein (Chadwick et al., 2012). Further investigation into the scaffold protein GIT2, revealed that it interacts with many proteins involved in multiple ‘distinct’ signaling pathways (Fig. 2 and Table S1) (Zhou et al., 2016). Many of these physical interaction partners are involved in age-related disorders, including Ataxia Telangiectasia Mutated (ATM), DNA repair associated Breast cancer type 1 susceptibility protein (BRCA1), tumor suppressor protein p53 (p53), insulin receptor and insulin receptor substrate 2 (Lu et al., 2015; Martin et al., 2015). The GIT2 protein may thus play a pivotal role in the organization of complex molecular signaling networks, through its capacity to exert control over signaling on a more trophic level (Han et al., 2004; Mirzarezaee et al., 2010).

2. GIT2 and the HALLMARKS OF AGEING

While the molecular intricacies of the ageing process are unique to every individual, there are several common denominators of ageing, called hallmarks (Fig. 3). Here we will discuss how GIT2 functionality intersects with many of these hallmarks, such as DNA damage, oxidative stress, metabolic decline, inflammation and fat deposition.

2.1 GIT2 controls DNA damage response factors

With constant exposure to environmental as well as internal metabolic/endocrine stresses, somatic and mitochondrial genetic damage occurs naturally throughout life (Moskalev et al., 2013). Underlining the importance of DNA damage in ageing, the prototypic premature ageing disorders, such as Werner, Bloom and Hutchinson–Gilford progeria syndromes, are caused by an increased DNA damage accumulation (Burtner and Kennedy, 2010). DNA stability and integrity is continuously challenged by exogenous and endogenous threats, such as DNA replication errors and reactive oxygen species (ROS) (Hoeijmakers, 2009). To consolidate resistance to these insults, organisms have developed a complex network of DNA repair mechanisms that are collectively able to contend with most types of DNA lesions (Lord and Ashworth, 2012). The DNA damage response (DDR) involves recognition of DNA damage, DNA repair, and transcriptional reprogramming. Interestingly, current evidence suggests that these responses are gated by circadian clock mechanisms (Sancar et al., 2010). This clock is a molecular system which confers daily rhythmicity to physiological functions (Hastings et al., 2003; Reppert and Weaver, 2002; Sancar, 2004; Takahashi et al., 2008), allowing the organism to survive (Uchida et al., 2010). The core components of this circadian clock have been implicated in cell cycle and DDR regulation. Moreover, multiple intracellular signaling cascades, important for cell cycle and DDR, are also essential for clock regulation (Uchida et al., 2010).

GIT2 appears to promote DNA repair through multiple mechanisms; *i.e.* stabilization of BRCA1, upregulation of repair proteins as well as regulation of poly (ADP-ribose) polymerase (PARP) activity. The first indication of a role for GIT2 in DNA repair was the elevated GIT2 expression seen in the nucleus after DNA double-strand break (DSB) induction in neuroblastoma cells (Lu et al., 2015). Ectopic overexpression of GIT2 promotes the repair of DNA damage and increases expression levels of associated DDR proteins, while GIT2 silencing by siRNA causes the opposite effect. GIT2 has been shown to be highly synergistic

with the expression of multiple DDR-related factors, *i.e.* HMGB1 (High mobility group protein B1), MDC1 (Mediator of DNA damage checkpoint protein 1), and FANCI (Fanconi anemia group I protein) (Lu et al., 2015). Further investigation has demonstrated that GIT2 also functionally associates with multiple DDR complex proteins, such as γ -H2AX (a marker for DNA DSB sites), MDC1, p53 binding protein 1 (p53BP1), the p53 tumor suppressor itself, and ATM upon exposure to DNA damaging agents, suggesting that GIT2 might form part of a contextually-sensitive multiprotein complex consisting of DDR-related proteins (Lu et al., 2015). As mentioned previously, GIT2 appears to promote the activity of DNA damage response proteins; PARP1 and -2. Ectopic expression of GIT2 promoted Poly-ADP ribose (PADR) synthesis by PARP1 and PARP2, in ionizing radiation-exposed cells, indicating that GIT2 can affect DNA repair by facilitating the activity of PADR polymerases. Furthermore, this genomic deletion of GIT2 resulted in the accumulation of CNS DNA damage and accelerated ageing (Lu et al., 2015). Lastly, immunohistochemical staining of cortical sections obtained from GIT2KO mice demonstrated the presence of significantly more γ -H2AX foci in the cellular nuclei of young GIT2KO mice than in old wild-type (WT) mice, indicating an advanced ageing phenotype, potentially due to the reduced DNA repair capacity (Lu et al., 2015). In addition to this strong role of GIT2 in maintaining the physical components of DDR complexes, recent data has also suggested that genomic GIT2 deletion can alter cellular functions related to the circadian rhythm, such as diurnal/nocturnal metabolic shifts (Martin et al., 2015) and immune cell activity (Siddiqui et al., 2017).

Not only can cellular clock mechanisms regulate chronological ageing in cellular tissues, but these mechanisms can also strongly regulate the rate/extent of metabolic disruption, telomere stability and DNA damage during the ageing process (Collis and Boulton, 2007; Kagawa, 2012; Khapre et al., 2010). It is not surprising therefore that cellular clock functionality has now been linked to multiple age-related disorders including dementia (Musiek et al., 2013), glycemic/adiposity disorders such as Metabolic Syndrome (Bruce et al., 2016; Vieira et al., 2014) and premature pathophysiological ageing associated with attenuated DNA damage repair (Kowalska et al., 2013; Vaziri et al., 1997). It is interesting to note that a strong evolutionary synergy between clock genes and proteins involved in the DDR process has been proposed (Uchida et al., 2010). Multiple intracellular signaling proteins that are functionally linked to GIT2, *e.g.* ATM, p53, and BRCA1, are involved in stress-responsive cascades and play important roles in both cell cycle/DDR control and circadian clock regulation (Gery et al., 2006; Kowalska et al., 2013; Miki et al., 2013; Storcelova et al., 2013; Vieira et al., 2014). Siddiqui et al. (2017) recently demonstrated that across multiple immunological tissues, GIT2 genomic deletion led to significant co-reductions in multiple clock-related mRNA transcripts that are also associated with premature ageing and DNA damage repair functions (Siddiqui et al., 2017). GIT2 therefore may serve as a functional bridge between cellular senescence, clock regulation and DNA damage, and thus could possess the capacity to potentially alter the accumulation of age-related cellular damage. As age-related accumulation of DNA damage and metabolic dysfunction appear to synergize to accelerate the onset of ageing-related disorders, it is interesting to note that therapies targeting clock regulation mechanisms are currently showing promise for the treatment of ageing-related diseases (Blackburn et al., 2015; Dankel et al., 2014; He et al., 2016).

2.2 GIT2 expression is modulated by oxidative stress

Low levels of oxidative stress by ROS appear to be beneficial to organisms, as it may prolong lifespan in yeast and *C. Elegans* (Doonan et al., 2008; Mesquita et al., 2010), suggesting that ROS may play a role in triggering cell proliferation and survival in response to normal physiological signals and stress conditions (Sena and Chandel, 2012). However, as ageing continues, cellular stress and damage increases as do the levels of ROS, overwhelming oxidative stress response systems, leading to increasing oxidative stress and age-associated damage (Hekimi et al., 2011). The free radical theory of ageing suggests that progressive mitochondrial dysfunction with increasing age results in increased ROS production, which in turn causes global cellular oxidative damage (Harman, 1965). Long-term exposure of human neuronal cells to non-cytotoxic levels of oxygen radicals can replicate many of the neurophysiological deleterious effects

of the ageing process *in cellula*, e.g., reduced neurotrophin sensitivity, disrupted cellular calcium buffering and attenuated glucose uptake and utilization. This long-term exposure therefore effectively mimics the low levels of constant stress experienced by normal ageing cells or the early stages of neurodegenerative disorders (Chadwick et al., 2010b). Chadwick et al. found a significant elevation of both lamin A (connected to progeric laminopathy conditions, e.g. Hutchinson-Gilford Progeria Syndrome) and GIT2 in this *in cellula* model of CNS ageing (Chadwick et al., 2010b). When this *in cellula* research was translated into actual aged CNS tissues, profound increases in GIT2 expression in the CNS of mice, rats, non-human primates (Rhesus Macaque) and humans with increased age (Chadwick et al., 2010b) were discovered. Given the role of GIT2 in regulating DNA damage, it is likely that this age-dependent increase reflects a reflexive cellular protection mechanism attempting to attenuate potential ROS-induced DNA damage and other cellular stressors.

2.3 GIT2 controls metabolic functionality

It has long been established that glucose metabolism is progressively impaired with ageing (Davidson, 1979). The role of the insulin/insulin growth factor-1 (Ins/IGF1) pathway in the regulation of lifespan is perhaps one of the most investigated molecular ageing scenarios (across multiple organisms) to date (Lopez-Otin et al., 2013; Piper et al., 2008). Forty percent of proteins of the Ins/IGF1 pathway have been identified as longevity-related proteins (Wolfson et al., 2009). Consistent with this, caloric restriction-induced remediation of Ins/IGF1-related dysglycemia has been shown to increase lifespan or “healthspan” (*i.e.* the period of life in which no significant disease pathologies are evident) in all investigated eukaryotic species (Colman et al., 2009; Fontana et al., 2010; Mattison et al., 2012; Speakman and Mitchell, 2011). Furthermore, pharmacological manipulations mimicking a state of limited nutrient availability, e.g. rapamycin or resveratrol, have been shown to extend the lifespan and/or healthspan of both mice and non-human primates (Harrison et al., 2009). As mentioned previously, with age there is a system-wide decrease in an organism’s ability to cope with stress, and this is partially due to a decline in efficiency of the primary glyceic energy-generating metabolic system (Barzilai et al., 2012; Daum et al., 2013; Terman, 2006). Primary energy synthesis via classical mitochondrial oxidative phosphorylation provides the most efficient mechanism of adenosine triphosphate (ATP) production using glucose catabolism, and alternative energy-generating mechanisms, e.g. via lipid or protein utilization, exert greater physiological stress than oxidative phosphorylation and generate less ATP per unit of catabolite used. Changes in primary energy-generating catabolite usage can be quantified using the respiratory exchange ratio (RER), *i.e.* ratio of oxygen consumption to carbon dioxide production. A high RER (~0.9-1) indicates that carbohydrates are being predominantly used, whereas a lower RER suggests lipid oxidation (~0.8) or protein degradation (~0.7-0.8) (Pendergast et al., 2000; Simonson and DeFronzo, 1990). Recent epidemiologic analysis has demonstrated that human ageing rates may be strongly linked to early-life metabolic ‘trajectories’ that can be measured using classical metabolic analyses (Belsky et al., 2015).

Due to the gradual reduction of metabolic efficiency with age the body becomes more susceptible to multiple pathophysiology linked to energy insufficiency (Chadwick et al., 2012) including neurodegeneration, insulin resistance and metabolic disorders such as T2DM as well as chronic inflammation (Chadwick et al., 2012; Ford et al., 2002; Morley, 2008). This age-related susceptibility can be largely associated with: *i*) the increased production of ROS per ATP synthesized, *ii*) reduction of non-essential protein degradation activity leading to excessive protein aggregation, and *iii*) attenuation of the capacity of cellular damage repair processes. Therefore, it is likely that mammalian healthspan is tightly linked to the organism’s capacity to maintain peak metabolic efficiency. The use of less efficient energy-generating catabolic processes is therefore likely to be an effective indicator of imminent age-related health decline. Related to this, it has been demonstrated that relatively young (4-6 month-old) GIT2KO mice show a significantly reduced respiratory exchange ratio (RER) compared to WT mice, indicating a switch towards adipose (and potentially protein) catabolism to generate energy, instead of glucose (Martin et al., 2015). This somatic energy decline was shown to be driven by the presence of a strong hypothalamic molecular

signature promoting age-related metabolic dysfunction. Underscoring the system-level functionality of GIT2, it also was shown that loss of GIT2 significantly affected the structure and activity of pancreatic islets, resulting in decreased insulin production capacity, loss of β -cell mass and α -cell islet involution (Martin et al., 2015). These structural pancreatic pathologies in the GIT2KO mice were associated with significantly higher plasma glucose levels in fasting conditions, reduced plasma insulin and insulin resistance, compared to age-matched controls. Indicative of the connectivity between ageing of the organism and metabolic status, it was also shown that while GIT2 can serve as an ageing biomarker, this function is determined by the prevalent metabolic state of the organism. Hence *db/db* mice, a model for diabetes and obesity, demonstrated a premature elevation in GIT2 expression levels (Martin et al., 2015). In accordance with the effects of metabolic disruption on human ageing trajectories (Sancar, 2004). Interestingly, it appears that the intersection between the Ins/IGF1 system and GIT2 occurs at the physical association/scaffolding level, through the physical association of GIT2 with the Insulin Receptor and with Insulin receptor substrate 2. Furthermore, the association of GIT2 with these two components of the Ins/IGF1 system was disrupted in the pancreas of *db/db* mice, suggesting metabolic regulation of these interactions (Martin et al., 2015).

2.4 GIT2 functionality is associated with T-cell activity and immune response capacity

During the ageing process, there is a progressive dysfunction of multiple receptor signaling systems of which Insulin receptor resistance is by far the most commonly studied. In addition to progressive metabolic disruption, alterations in inflammation-related receptor signaling systems lead to a progressive increase in low-grade chronic inflammation. This phenomenon has recently been defined as 'inflammaging' (Franceschi et al., 2000; Franceschi and Campisi, 2014; Franceschi et al., 2007; Frasca and Blomberg, 2016; Monti et al., 2017; Stranahan et al., 2012b). Pervasive human inflammaging is thought to occur primarily in the absence of overt exogenous infection (Franceschi et al., 2000; Frasca and Blomberg, 2016). Inflammaging is linked to elevated levels of inflammatory biomarkers such as C-reactive protein and interleukin (IL)-6. Elevation of these factors engender further deleterious changes in body composition, energy production and utilization, metabolic homeostasis, obesity, immune senescence, atherosclerosis and neuronal health (Barzilai et al., 2012; Tabas, 2010).

Inflammation associated with the ageing process can have multiple causes: *i*) the accumulation of tissue damage due to inflammation, *ii*) immune system failure, leading to an inability to effectively clear pathogens and dysfunctional host cells, *iii*) the tendency of senescent cells to secrete pro-inflammatory cytokines, *iv*) the enhanced activation of the ageing-related NF- κ B transcription factor, and *v*) defective autophagy responses (Salminen et al., 2012).

The molecular consequences of inflammaging are seen in both innate and adaptive immune responses (Deeks, 2011). Ageing of the immune system likely aggravates the somatic ageing phenotype due to the failure of the immune system to efficiently clear infectious agents, infected cells and cells undergoing malignant transformation (Lopez-Otin et al., 2013). The multifunctional character of GIT2 extends to its involvement in adaptive immunity, where this protein is required for positive selection of thymocytes, a process essential for thymocyte maturation and commitment to either CD4⁺ or CD8⁺ T cell lineage (Hogquist et al., 2015; Klein et al., 2014; Phee et al., 2010). GIT2KO thymocyte migration is defective *in vivo* in GIT2KO mice, which is proposed to be due to incorrect responses to local chemokine gradients, thus trapping the thymocytes near sources of chemokines in the cortex and hindering the scanning of thymic epithelial cells during positive selection (Phee et al., 2010). Moreover, regulatory T cell function is controlled, in-part, by the GIT2- α PIX-PAK complex, which is located at focal adhesion complexes (Kong et al., 2014). Interestingly, GIT2 levels are decreased in α -PIX deficient lymphocytes, suggesting that GIT2 is unstable in the absence of a PIX partner. These α -PIX and GIT2-deficient B- and T-lymphocytes show reduced antigen stimulated proliferation (Missy et al., 2008), a defect also observed in ageing. A substantial amount of research shows a role for GIT2 in bottleneck features of the ageing immune system, such as

neutrophil function, pro-inflammatory cytokine production and thymocyte selection and regulation (Mazaki et al., 2006). T-cell activity and immune responses are also associated with GIT2 functionality, as T cell maturation is significantly disrupted due to the premature thymic involution found in GIT2KO mice (Siddiqui et al., 2017).

2.5 GIT2 regulates fat deposition

Adipose tissue (AT) is composed primarily of adipocytes, and can act as a connective tissue, energy storage depot, thermal insulator and mechanical pad (Snyder et al., 1975). Fat, on the other hand, is mainly composed of lipids in the form of triglycerides (Wang et al., 1992). With ageing, AT becomes dysfunctional due to an impairment of the differentiation of pre-adipocytes to mature adipocytes. This results in the generation of dysfunctional adipocytes that are unable to store fat, which is subsequently redistributed aberrantly to ectopic sites (Pararasa et al., 2015). During ageing, an increase in body fat, visceral AT and ectopic fat deposition, *i.e.* storage at sites that are normally not associated with fat deposition, can occur. This alteration in fat deposition is strongly related to diminished health in the elderly and has been also linked to metabolic dysfunctions (Pararasa et al., 2015; Zamboni et al., 2014). The increase in total adiposity that occurs with normal ageing can be independent of alterations in body weight, due to the simultaneous decrease in muscle mass with ageing, an age-related disorder called sarcopenia (Prentice and Jebb, 2001). The alterations in AT distribution and quality are correlated to a higher risk of developing diabetes, hypertension, dyslipidemia and cardiovascular disease in the elderly (Zamboni et al., 2005).

GIT2 expression has been shown to increase with age in organs involved in energy metabolism, *i.e.* pancreas, liver, skeletal muscle, and AT (Chadwick et al., 2012). Reinforcing this association of GIT2 with aberrant AT activity, GIT2 genomic deletion induces a dysfunctional skeletal bone phenotype associated with altered adipose deposition (Wang et al., 2012). This phenotype is associated with a pattern of mesenchymal stem cell differentiation favoring an adipocytic, rather than an osteoblastic, lineage, and with bone marrow AT accumulation (Wang et al., 2012). Further investigations of GIT2KO mice have also demonstrated that deletion of GIT2 attenuates the extent of subcutaneous and visceral fat accumulation compared to age-matched control mice, when fed with a high fat diet - thus displaying a greater resistance to high fat-induced obesity. In addition, GIT2KO mice exhibit increased energy expenditure, and elevated expression of gene transcripts involved in fatty acid oxidation and thermogenesis (*unpublished data*).

3. GIT2 AND AGE-RELATED PATHOPHYSIOLOGY

3.1 GIT2 interactors BRCA1 and ATM are involved in development of neurodegenerative disorders

Neurodegenerative disorders and pathophysiological ageing are caused by a functional interplay among a variety of diverse biological systems including neurological, sensory, endocrine, and metabolic activities (Chadwick et al., 2010a; Chadwick et al., 2012; Chadwick et al., 2011; Chadwick et al., 2010b; Lu et al., 2015; Martin et al., 2008a; Martin et al., 2008b), many of which are functionally integrated within one crucial CNS organ - the hypothalamus (Moheet et al., 2015). The hypothalamus acts as a trophic master-controller of the endocrine system and possesses neuronal projections to several autonomous and higher centers of the brain. In this manner, it provides a vital connection between ageing and age-related disorders such as dementia (Janssens et al., 2014).

The process of neurodegeneration encompasses the progressive loss of structure and function of neurons, causing a deficiency of coordinated neural function (Fig. 4). The major neurodegenerative diseases present in our population, *i.e.* Alzheimer's disease (AD) and Parkinson's disease, are strongly

controlled/generated by many of the hallmarks of ageing, such as DNA damage, oxidative stress, metabolic dysfunction, and inflammation (Cannon and Greenamyre, 2011). The mammalian brain consumes as much as 1/5 of total oxygen intake, leading to exposure of neurons to ROS byproducts (Barja, 2004; Hirano et al., 1996; Kaneko et al., 1996; Nakae et al., 2000). The brain is also thought to have a decreased anti-oxidant to pro-oxidant enzyme ratio (Canugovi et al., 2013). When antioxidants are depleted in the brain, due to either physical stress or metabolic dysfunction, neurons become more susceptible to ROS-induced DNA damage (Barja, 2004; Hirano et al., 1996; Kaneko et al., 1996; Nakae et al., 2000). ROS alone have the ability to generate more than 100 different oxidative DNA base modifications and these alterations have a high mutagenic potential (Iyama and Wilson, 2013). This oxidative damage to neuronal cells may be of importance in the development of neurodegenerative disorders, as suggested by reports showing that the base excision repair (BER) system, utilized to repair such oxidative adducts, is important in preventing neurodegeneration (Bosshard et al., 2012; Lillenes et al., 2013; Sheng et al., 2012). Accumulation of DNA damage may be especially prevalent in the CNS owing to the low DDR capacity in these tissues (Maynard et al., 2015). The vulnerability of post-mitotic neurons to DNA damage, coupled with a gradual decline in the activities of DDR mechanisms, could lead to the accumulation of DNA damage with age, contributing to brain ageing and neurodegeneration (Madabhushi et al., 2014). Defective DNA repair has been linked with age-associated neurodegenerative disorders such as AD, Parkinson's Disease and also amyotrophic lateral sclerosis (Madabhushi et al., 2014). DDR has been shown to be important during both neural development and in mature neurons. Mutations in core DDR factors are either incompatible with life or, when tolerated, can manifest in severe neurodevelopmental disorders. An example of such a DDR protein is BRCA1, which has been implicated in the development of AD (Suberbielle et al., 2015). The number of DSBs are increased in disorders such as AD and amyotrophic lateral sclerosis (Adamec et al., 1999; Martin, 2001; Mullaart et al., 1990), and in several mouse models of neurodegeneration (Dobbin et al., 2013; Kim et al., 2008; Suberbielle et al., 2013).

GIT2KO mice have revealed a possible role for GIT2 in neurobehavioral and neurobiological functions (Caffrey et al., 2011; Lu et al., 2015). Lu et al. demonstrated that cortical neurons from GIT2KO mice contained more DNA damage compared to age-matched WT mice, suggesting a crucial role for GIT2 in CNS DNA damage *in vivo*. They further identified the *in silico* potential for ATM-based phosphorylation of GIT2, and confirmed and GIT2 association with active ATM at DSB sites (Lu et al., 2015). The ATM kinase is crucial for the initiation of signaling pathways following DNA damage and DSBs, allowing their repair (Banin et al., 1998). In addition, Lu et al. also showed that GIT2 controls the temporal stability of BRCA1 (Fig. 4), a DDR protein recently implicated in the development of AD, within dynamic DSB complexes (Lu et al., 2015; Suberbielle et al., 2015). GIT2 depletion using siRNA prevented proper recruitment of BRCA1 to DSBs. In addition to stabilizing DDR complexes, GIT2 was also crucial for the regulation of DSB-related PARP1, which assists in the creation and stabilization of the initial DDR complex components (Lu et al., 2015).

In addition to the recent linkage of DDR to advancing neurodegeneration, it has become clear in recent years that metabolic dysfunction (especially with respect to mitochondrial energy generation) is a strong promoter of neurodegenerative disease (Lu et al., 2015). Metabolic Syndrome (MetS), abdominal obesity, glucose intolerance, hypertension, hyperinsulinemia, and elevated fasting plasma glucose all have been shown to be risk factors for AD (Accardi et al., 2012; Razay et al., 2007; Vanhanen et al., 2006). With advancing age, alterations in brain glucose/energy metabolism have been consistently observed (Hoyer, 1998). In 2003, Watson et al. found an association between AD and hyperglycemia, which was supported by the later study of Razay et al. in 2007, that showed an association among AD, hyperglycemia and insulin resistance (Razay et al., 2007; Watson and Craft, 2003) (Fig. 4). Martin et al. demonstrated that GIT2KO mice display metabolic dysfunction, where these mice show increased fasting plasma glucose and insulin resistance (Martin et al., 2015). Therefore, GIT2 may be a protective factor against T2DM, which is now considered one of the strongest risk factors for AD. Furthermore, GIT2 has been associated with MetS,

where GIT2KO mice exhibited a gender-specific protection from high-fat diet induced obesity, compared to WT mice under the same conditions (*unpublished data*).

Along with a pervasive link to metabolic dysfunction, a highly atypical innate immune response develops in the ageing brain dominated by activated microglia, the resident tissue macrophages (Dheen et al., 2007). This innate immune response can be triggered by misfolded and aggregated proteins, typical in AD, which bind to pattern recognition receptors on microglia and astroglia (Heneka et al., 2015; Lunnon et al., 2011) to affect release of inflammatory mediators that contribute to AD progression and severity (Heneka et al., 2015). Wei et al. (2014) showed that GIT2KO mice show a substantial increase in pro-inflammatory cytokines, and that GIT2 is a crucial terminator of Toll-like receptor signaling (Wei et al., 2014) (Fig. 4), indicating a crucial role for GIT2 in the innate immune system during ageing and for the development of neurodegenerative disorders.

3.2 GIT2's role in DNA damage response and obesity suggest involvement in atherosclerosis development

Major vessel atherosclerosis is a well-known age-related pathophysiology, where sclerotic and stenosing lipid deposits form within the intima layer of the vessels at branch points with turbulent flow, and eventually develop into proinflammatory atherosclerotic plaques (Ross, 1999). Endothelial dysfunction, leading to a chronic deficit in nitric oxide bioavailability, is thought to be the first step in the development of atherosclerosis (Libby, 2002; Penn et al., 1986; Ross, 1999). Chronic inflammation, ('inflammaging'), is now well recognized to promote atherosclerosis due to its propensity to damage DNA by forming ROS (Fig. 4) and reactive nitrogen species (Libby, 2002). Atherogenic lesions can be initiated by mutational events in a manner similar to benign tumors (Penn et al., 1986; Trosko and Chang, 1980) and increasing evidence has shown that there is an accumulation of DNA damage in vascular smooth muscle cells and inflammatory cells within atherosclerotic plaques. vascular smooth muscle cells and macrophages within plaques express DNA damage markers, including phosphorylated forms of ATM and γ -H2AX, which increase with disease severity (Gray and Bennett, 2011). Interestingly, it has recently been shown that oxidized low-density lipoprotein also downregulates enzymes that take part in BER (Chen et al., 2000). The progression of atherosclerosis also appears to be strongly influenced by the clustering of unbalanced metabolic processes with the vasculature itself. Recently, the regulation of cellular energy homeostasis has emerged as a promising approach in cardiovascular pharmacology (Wang and Lopaschuk, 2007), since hyperglycemia has been identified as a promotor of atherosclerosis (Aronson and Rayfield, 2002). Furthermore, it was found that Mildronate treatment to inhibit γ -butyrobetaine hydroxylase, which catalyzes the formation of an essential cofactor for mitochondrial import and oxidation of long-chain fatty acids, decreases the formation of atherosclerotic plaques, supporting the role for energy metabolism regulation in treating atherosclerosis (Vilskersts et al., 2009). While no current reports exist explicitly linking GIT2 and atherosclerosis, its role in immune cell 'inflammaging' activity, oxidative stress and DDR suggest a likely role for GIT2 in atherosclerosis development (Fig. 4) (Chadwick et al., 2010b; Lu et al., 2015). It is clear in multiple cell lineages that GIT2 can act as a molecular sensor of oxidative stress (Chadwick et al., 2010b) as well as a strong pro-survival factor during stress periods where DNA damage is occurring (Lu et al., 2015). GIT2 association with DDR complexes is also likely controlled by its physical interactions with ATM and γ -H2AX (Fig. 4) (Lu et al., 2015). These two proteins have been shown to be present within atherosclerotic plaques and have been implicated in disease severity (Penn et al., 1986), thus revealing a possible link between GIT2 and atherosclerotic plaque development. Furthermore, as mentioned previously, genomic deletion of GIT2 in can be protective against obesity (*unpublished data*), which is one of the largest risk factors for the development of cardiovascular disorders such as atherosclerosis (Roever et al., 2016). While GIT2 has not been directly associated with atherosclerosis as yet, GIT1 has been shown to activate endothelial cell nitric oxide synthetase, the enzyme which produces NO in endothelial cells. GIT1 has been identified as a molecular scaffold which facilitates NO production, through the association with endothelial cell nitric oxide synthetase, which is regulated by Src and Akt kinase activity (Liu et al., 2014).

3.3 GIT2KO mice show decreased bone mineral density and bone volume

Osteoporosis, the most common metabolic bone disease, is characterized by reduced bone mass and bone mineral density (Rachner et al., 2011) and is mostly associated with advanced age (Cheung et al., 2010). In 2005, Yalin et al. revealed a negative correlation between superoxide dismutase (SOD) and lumbar bone mineral density levels in male osteoporotic patients (Yalin et al., 2005). This data suggested that oxidative stress plays an important role in the pathophysiology of primary male osteoporosis (Yalin et al., 2005). In women, however, one of the most intriguing hypotheses considers the ability of sex hormones, *i.e.* estrogen, to protect bone against oxidative stress by acting as antioxidants (Cervellati et al., 2013; Di Gregorio et al., 2001; Lean et al., 2003), and this has subsequently been confirmed both *in vitro* and *in vivo* (Lean et al., 2003). Estrogen loss after menopause alters ROS generation and the antioxidant defense capacity of cells (Fig. 4) (Lean et al., 2003). This leads to an accumulation of oxidant species, which in turn are able to stimulate osteoclast formation and resorption, thus increasing the severity of the disorder (Cervellati et al., 2013; Hodge et al., 2011; Lean et al., 2004). A causal role for DNA damage in osteoporosis and other skeletal defects has also been suggested through the observation that mutations in genes encoding for DDR proteins can lead to compromised bone development and/or deregulation of bone (Chen et al., 2013). Chen et al. showed that knocking out the DDR protein Excision Repair Core Complementary group-1 in mice lead to persistent DNA damage, leading to premature cellular senescence and reduced osteoblast proliferation. These mice demonstrated a failure to repair DNA damage and thus suffered from accelerated ageing and spontaneously developed osteoporosis (Chen et al., 2013). Furthermore, ATM-deficient mice (ATMKO) have been proposed as an osteoporosis model. ATMKO mice possess reduced bone mass, especially at the trabecular bones, and this is accompanied by a decrease in bone formation rate and defective osteoblast differentiation. However, osteoprogenitor and osteoblast number are unaltered in ATMKO mice. ATMKO mice also demonstrate a marked elevation in osteoclastogenesis and bone resorption, although ATM does not appear to exert cell-autonomous effects on osteoclast differentiation and resorption (Rasheed et al., 2006).

Osteoporosis has long been linked to hyperparathyroidism, where excessive parathyroid hormone (PTH) removes too much calcium from bones, making them thin and brittle. Hyperparathyroidism also disrupts normal glucose metabolism (Chiu et al., 2000; Procopio et al., 2002) and is associated with hyperglycemia, and hyperlipidemia (Ring et al., 2012; Silverberg et al., 2009; Walker et al., 2012; Walker and Silverberg, 2008). Hyperglycemia, which can be experimentally induced by GIT2 deletion, may induce osteoporosis and bone fractures by negatively regulating the normal functioning of osteoblasts, and simultaneously positively regulating osteoclast function (Lampropoulos et al., 2012). Recent evidence gathered from humans suffering from diseases such as postmenopausal osteoporosis indicates a crosstalk between the immune system and bone. It has been suggested that bone loss, induced by estrogen withdrawal in menopause, is a complex effect of a multitude of pathways and cytokines that cooperate to regulate osteoclast- and osteoblastogenesis. Amongst these cytokines, receptor activator of nuclear factor κ -B ligand and Tumor necrosis factor- α (TNF α) appear to play particularly important roles in inducing osteoclast formation and activity. Additionally, IL-17 can promote bone loss by favoring osteoclast production and inhibiting osteoblast differentiation (Faienza et al., 2013). The osteoclasts themselves are members of the monocyte-macrophage family and are derived from the fusion of marrow-derived mononuclear phagocytes, *i.e.* osteoclast precursors, which differentiate under the influence of macrophage colony stimulating factor and RANK ligand (Massey and Flanagan, 1999). RANK ligand itself is produced by osteoblasts, and binds to its receptor RANK (receptor activator of nuclear factor kappa B) on osteoclasts (Pacifci, 1996). These factors increase bone resorption by increasing the number of pre-osteoclasts in bone marrow, thus inducing osteoporosis (Satpathy et al., 2015). Obesity increases the expression of several chemical messengers that exert their effects by modulating the signaling pathways in bone and muscle. These messengers (*e.g.* TNF α , IL-6, leptins, advanced glycation end products (AGE)) are modulated as a result of obesity, and have been

shown to act as negative regulators of osteoblasts, and osteocytes, while acting as positive regulators of osteoclasts, thus ultimately increasing the risk for osteoporosis (Roy et al., 2016).

Dynamic cytoskeletal reorganization plays an important role in both osteoblast differentiation and osteoclast polarity maintenance during bone resorption, and GIT2 was suggested to play a role in osteoporosis development through regulation of the cytoskeleton (Wang et al., 2012). Adult GIT2KO mice have decreased bone mineral density and bone volume in both the trabecular and cortical compartments, which was associated with defects in osteoblast maturation. Interestingly these mice showed an increase in RANK ligand mRNA expression, suggesting a compensatory response to this decline in osteoblastic activity. In the absence of GIT2, differentiation of mesenchymal stem cells favors the adipocyte lineage over the osteoblast lineage, leading to a reduction in osteoblast number and function (Fig. 4) (Wang et al., 2012). How GIT2 is involved in the development of osteoporosis-like symptoms, remains unclear. We can hypothesize, however, that this may be through its association with ATM (Fig. 4) (Lu et al., 2015). ATMKO mice have been introduced as a mouse model for osteoporosis, displaying reduced bone mass, bone formation rate and defective osteoblast differentiation (Rasheed et al., 2006). The GIT2-ATM interaction may explain the association between GIT2 and osteoporosis development, but requires further investigation.

3.4 GIT2 expression increases in aged skeletal muscle reminiscent of Sarcopenia

Sarcopenia is the loss of muscle mass and function, which is most often related to age. The pathogenesis of sarcopenia is multifaceted and encompasses lifestyle habits, systemic factors (*e.g.* chronic inflammation and hormonal alterations), local environmental perturbations (*e.g.* vascular dysfunction), and intramuscular-specific processes (Marzetti et al., 2013). In aged muscle tissue, an increased presence of oxidative damage adducts have been observed (Fulle et al., 2004; Ji, 2001; Mecocci et al., 1999). ROS overproduction causes oxidative damage, activating intracellular signaling pathways involved in sarcopenia (Bonetto et al., 2009) and disrupts the balance that allows for continuous degradation and resynthesis of skeletal muscle proteins (Koopman and van Loon, 2009). Studies on Super Oxide Dismutase 1 (SOD1) KO mice have revealed a sarcopenic phenotype, suggesting a role for mitochondrial dysfunction and oxidative stress in sarcopenia development and progression (Jang et al., 2010). Oxidative stress has also been shown to induce increased levels of chronic low-grade inflammation, which is detrimental to skeletal muscle in humans (Howard et al., 2007) and in animal models (Siu et al., 2008). Moreover, ROS appear to function as second messengers for TNF α in skeletal muscle by either directly or indirectly activating the redox-sensitive transcription factor, Nuclear factor NF-kappa-B (NF- κ B) (Reid and Li, 2001). This factor can induce inflammation and mediates age-related upregulation of IL-6, and TNF α (Meng and Yu, 2010) and is critical for bone and muscle homeostasis (Huang et al., 2014). The combination of increased oxidative stress and inflammation in sarcopenia appears causal to the induction of NF- κ B (Kim et al., 2007). The NF- κ B pathway is the most important one linked to the wasting of skeletal muscle in normal and pathophysiological ageing (Li et al., 2008). This pathway is essential for myoblast proliferation and maintenance in an undifferentiated state (Guttridge et al., 1999; Mitin et al., 2001). The activation of NF- κ B, p38 mitogen activated protein kinase (MAPK) and p53 pathways have been implicated in skeletal muscle atrophy (Guttridge, 2004).

Furthermore, it has been suggested that mitochondrial DNA mutations and deletions can also contribute to the development of sarcopenia (Kujoth et al., 2005). Mutations in mitochondrial DNA have been shown to be causal in sarcopenia by affecting the assembly and function of electron transport chain complexes. Hiona et al. demonstrated that a lack of these complexes induces a decrease in oxidative phosphorylation, in the absence of oxidative stress, ultimately causing muscle apoptosis and sarcopenia (Hiona et al., 2010). Lastly, sarcopenia and obesity share several pathophysiological mechanisms, and collectively named sarcopenic obesity (Choi, 2016). The complex interplay of common pathophysiological mechanisms, such as an increase in proinflammatory cytokines, oxidative stress, insulin resistance, and a

decreased physical activity underlie the close relationship between sarcopenia and obesity. This causes a vicious cycle between the accumulation of ectopic fat and the loss of skeletal muscle mass, which have a reciprocal influence on each other (Kim and Choi, 2015). Sarcopenia leads to a reduction in physical activity, which leads to an increased risk of obesity (Zamboni et al., 2008), while obesity induces inflammation that contributes to the development of sarcopenia (Gregor and Hotamisligil, 2011; Lutz and Quinn, 2012). In this context, it is therefore likely that, via its ability to connect both ROS sensitivity and DDR processes (Fig. 4), GIT2 is likely to play a role in age-related muscular degeneration. Supporting this, a potential compensatory age-related elevation in GIT2 has been observed in skeletal muscle obtained from wild-type mice (Chadwick et al., 2012; Martin et al., 2015). As mentioned previously, an increase in oxidative damage in muscle can be observed with the development of sarcopenia and with ageing, which could be associated with the increase in GIT2 expression with oxidative stress and ROS (Chadwick et al., 2010b). Furthermore, as mentioned above, sarcopenia and inflammaging are associated (Lutz and Quinn, 2012), indicating again a possible connection with GIT2 function (Siddiqui et al., 2017). GIT2 has previously been identified as a negative regulator of NF- κ B signaling through control of Toll-like receptor (TLR) (Wei et al., 2014) and through interaction with several other NF- κ B regulatory proteins (Fig. 4) (Wang et al., 2011). Lastly, GIT2 has been shown to be involved in the development of central obesity (*unpublished data*), and inflammation and obesity have been shown to work together in contributing to the development of sarcopenia (Gregor and Hotamisligil, 2011; Lutz and Quinn, 2012).

3.5 GIT2KO mice show a metabolic decline reminiscent of Type 2 Diabetes Mellitus

T2DM results from an inadequacy of pancreatic islet β -cells to secrete insulin in response to glucose ingestion, resulting in increased plasma glucose promoting further insulin resistance and obesity (Pandey et al., 2015). Oxidative stress has long been identified as a major player in the pathogenesis and complications of diabetes (Matough et al., 2012). In this context, mitochondrial overproduction of ROS has been indicated to be one of the most important upstream events in this pathological process (Giacco and Brownlee, 2010). T2DM patients possess an imbalance between oxidant and antioxidant systems, enhanced flux of glucose both increases oxidant production and impairs antioxidant defenses (Jakus, 2000). It has been proposed that high levels of serum glucose, common in diabetes patients, can enhance intracellular levels of glucose, and thus induce an increased rate of glycolysis. This would then increase substrate delivery to the mitochondria and increase accumulation of ROS (Fig. 4) (Ola et al., 2006). The research of Aouacheri et al. suggests that hyperglycemia in T2DM patients may lead to the inhibition of antioxidant activities, which could lead to more oxidative stress, decreased insulin sensitivity and impaired secretory response, exacerbating the disorder (Aouacheri et al., 2015; Kocic et al., 2007). The oxidative stress can, also in this case, result in DNA damage and this is one of the mechanisms implicated in the pathogenesis of diabetic complications (Pacal et al., 2011; Prasad et al., 2015; Shin et al., 2001; Slatter et al., 2000). Prasad et al. showed that the frequency of DNA damage is significantly higher in T2DM patients compared to controls (Prasad et al., 2015); the mechanism however is not yet known.

T2DM patients additionally display pro-inflammatory phenotypes characterized by the presence of cytokines, apoptotic cells, immune cell infiltration, amyloid deposits and fibrosis. The inflammation is a manifestation of disease, where it may exacerbate T2DM by tissue destruction due to inflammatory mediators and ROS (Donath et al., 2008). Activated innate immunity and inflammation are important factors in the pathogenesis of diabetes. IL-1 β , a pro-inflammatory cytokine, shows increased mRNA expression in T2DM patient samples (Donath et al., 2008; Navarro and Mora, 2006). Furthermore, C-reactive protein, TNF α , and IL-6 have been positively correlated with measures of insulin resistance (Festa et al., 2000; Muller et al., 2002; Temelkova-Kurktschiev et al., 2002). TNF α , IL-1 and IL-6 are associated with body fat mass and can be synthesized and released by adipose cells (Navarro and Mora, 2006). The significant association between increased body fat mass, obesity and risk for T2DM has already been established (Abdullah et al., 2010; Carey et al., 1997; Hu et al., 2001; Wang et al., 2015). Adipocytes secrete

adipocyte hormones and adipokines, which could increase the risk of diabetes via several pathways, *i.e.* insulin resistance (Bray, 2004).

As mentioned in section 3.3, GIT2KO mice demonstrate a significantly reduced RER, indicating a shift away from glucose utilization to generate usable energy, towards the utilization of fat and potentially protein. GIT2 is expressed in both pancreatic α - and β -islet cells (Martin et al., 2015). Genomic deletion of GIT2 resulted in a disruption of the relative distribution of these cell types within islets in a manner consistent with other experimental models of diabetes, where the mice displayed a significantly reduced islet area and β -cell percentage, with a significant increase in α -cell percentage. There was also an evident α -cell involution into the β -cell mass – a facet indicative of diabetic pathologies in murine paradigms (Chen et al., 2012; Martin et al., 2015). Furthermore, GIT2 was found to be prematurely (in an ageing sense) upregulated in diabetic *db/db* mice compared to WT control mice, both in the hypothalamus and the pancreas. Lastly, a physical association was found between GIT2 and multiple proteins vital to the glucose metabolic/insulin-regulatory system in pancreatic tissues, *i.e.* insulin receptor and insulin receptor substrate 2 (Fig. 4). The interaction between GIT2 and these proteins was specifically disrupted in the *db/db* pathological state (Martin et al., 2015). Therefore, it is highly likely that the observed age-dependent increases of GIT2 expression may represent a response mechanism to control oxidative cellular damage, DNA repair, and islet β -cell function, as well as the physical receptor integrity of the Ins/IGF1 system (Fig. 4).

3.6 GIT2 expression is associated with a progressive increase in body weight, possibly leading to central obesity

Central obesity, *i.e.* the increase in adipose deposition around the major visceral organs, is a pivotal and indispensable component of MetS and pathological ageing (Mathieu et al., 2010). AT possesses relatively high levels of antioxidant defensive enzymes, in order to manage the high ROS production occurring with lipid accumulation and the addition of free fatty acids (Matsuda and Shimomura, 2013). Increased fat accumulation is generally associated with an elevated risk of the development of metabolic disorders. Compared to subcutaneous fat, visceral fat accumulation is known to release more pro-inflammatory factors, leading to insulin resistance and oxidative stress (Funahashi and Matsuzawa, 2007; Nikolopoulou and Kadoglou, 2012). Demirbag et al. discovered that there is an increase in DNA damage in pre-obese/obese compared to normal-weight subjects (Demirbag et al., 2006), and related these changes to metabolic abnormalities occurring with obesity (De Lorenzo et al., 2007; Demirbag et al., 2006; Di Renzo et al., 2010; Khan et al., 2006; Vassalle et al., 2009).

Matsuzawa et al. have demonstrated that a substantial proportion of adipocytokines are involved in inflammatory stimulation and response, either pro-inflammatory or anti-inflammatory (Matsuzawa et al., 2004). TNF α has come to be known as an important adipocytokine, and TNF α levels in plasma and adipose tissues are increased in humans with obesity (Ho et al., 2005). In addition, IL-1 β has also been shown to be secreted from AT and be linked to inflammation (Ouchi et al., 2003).

Studies have shown that GIT2 acts as a keystone protein in age-related metabolic alterations, where an age-dependent increase in GIT2 expression in organs associated with energy metabolism was linked to a progressive increase in body weight and leptin levels (Fig. 4), along with a decrease in circulating adiponectin (Chadwick et al., 2012; Martin et al., 2015). Further research showed that GIT2KO mice developed a gender-associated protection from high-fat diet-induced obesity. The GIT2KO mice showed reduced fat accumulation, elevated metabolic rate, increased energy expenditure, lower fasting glucose and insulin levels, and healthier plasma lipid profiles (*unpublished data*). Reinforcing this role of GIT2 in controlling obesity-related mechanisms, GIT2 is identified as a GWAS risk allele identified in a screen for MetS (Fig. 4) (Zabaneh and Balding, 2010).

3.7 GIT2 disruption is associated with premature thymic dysfunction

Immunosenescence and ‘inflammaging’ cause a profound age-dependent dysregulation of the immune system (Deleidi et al., 2015). Immunosenescence refers to the detrimental loss of the efficiency of immune architecture and pathways with ageing (Pera et al., 2015), characterized by various anomalies such as an inverse CD4/CD8 ratio, loss of efficient B and T cell migration to secondary immune tissue (Montecino-Rodriguez et al., 2013) and decline in the frequency of well-functioning natural killer cells (Albright and Albright, 1985). Immunosenescence is usually accompanied by ‘inflammaging’, in which there is a severe accumulation of inflammatory mediators in several tissues (Coppe et al., 2010).

Oxidative stress is recognized as a major player in determining and maintaining the low-grade inflammation observed in ageing and age-related disorders (‘inflammaging’) (De la Fuente and Miquel, 2009). In recent years, data has revealed a tight cause-effect link between oxidative stress and inflammaging, where oxidative stress has been shown to affect both innate and adaptive immune response (Cannizzo et al., 2011). The oxidative burst associated with the innate immune response upregulates the formation of ROS and the overall oxidative stress response, decreasing cellular antioxidant capacity. The free radicals that are overproduced will react with membrane lipids and proteins, impairing their function and creating a circular loop of TLRs and Nalp3 inflammasome activation (Gill et al., 2010). The increased inflammatory response seen with age has also been linked to DNA damage and DDR. DDRs have been demonstrated to be of importance in inflammatory mediator production, following cellular senescence (Rodier et al., 2009).

GIT2 has been implicated in multiple diverse immune functions, such as thymocyte positive selection, cell motility and neutrophil directional sensing through the regulation of small GTP-binding proteins ARF and Rac1 (Wei et al., 2014). At a relatively young age (12 months), GIT2KO mice present a prematurely distorted thymic structure and T-cell related dysfunction (Fig. 4), compared to age-matched 12 month-old WT mice (Siddiqui et al., 2017). Generic thymic dysfunction is widely accepted to represent one of the most cross-species conserved hallmarks of ageing (Brelinska, 2003; Brelinska et al., 2008; Fabris et al., 1982). GIT2KO mice demonstrate temporally advanced decreases in T cell precursor levels (with 3 month-of-age GIT2KO being similar to 12-month-old WT mice), an advanced-age reduction in double positive/CD4⁺/CD8⁺ cells (Fig. 4), and total deficits in thymic structure and key functional regulators. (Siddiqui et al., 2017). These mice are also more susceptible to dextran sodium sulfate-induced colitis, *Escherichia coli* infection or endotoxin-shock challenge, and a dramatic increase in proinflammatory cytokines could be observed in GIT2KO mice and their macrophages. Recently GIT2 was identified as a negative regulator of TLR-induced NF- κ B signaling (Fig. 4), terminating TLR-induced NF- κ B and MAPK signaling by recruiting the deubiquitinating enzyme cylindromatosis to inhibit the ubiquitination of TNF receptor associated factor 6 (TRAF) (Wei et al., 2014). The ubiquitination of TRAF6 is critical for the activation of NF- κ B. The susceptibility of GIT2KO mice to dextran sodium sulfate-induced colitis has furthermore been shown to be dependent on TLR signaling. Thus, GIT2 has been identified as an essential terminator of TLR signaling, and loss of GIT2 can lead to uncontrolled inflammation and severe organ damage (Wei et al., 2014).

3.8 GIT2 interacts with RUSC2 in lung cancer cells

A first step in carcinogenesis is permanent modification of genetic material resulting from ionizing radiation, mutagenic compounds or oxidative damage. The redox imbalance occurring in various cancer cells is caused by oxidative stress, and has led to the hypothesis that oxidative stress may be related to oncogenic stimulation. Increased levels of oxidative DNA lesions have been noted in various tumors. During carcinogenesis, the level of ROS in cancer cells is increased, while antioxidants are depleted (Gao et al., 2003; Hwang and Bowen, 2007). ROS production can further allow tumor progression by stimulating hypoxia-inducible factor-1 α (HIF1- α) and signaling proteins such as vascular endothelial growth factor

(VEGF), which support angiogenesis, allowing tumor growth. ROS also alters the expression of the p53 suppressor gene - a key player in apoptosis (Barrera, 2012; Matsuzawa and Ichijo, 2008; Nguyen et al., 2009; Wiemer, 2011).

DNA damage, either due to environmental factors or normal metabolic processes inside the cell, can compromise a cell's ability to carry out its original functions (Bartkova et al., 2005; Hanahan and Weinberg, 2000; Lengauer et al., 1998). DDR has been shown to be critical in protection against cancer, with an increasing number of studies reporting that cancers arise in part from germ-line mutations in DDR genes (Hoeijmakers, 2001; Wyman and Kanaar, 2006). Regarding activation of DDR proteins, increased autophosphorylation of ATM has been reported in early-stage tumors, suggesting that DDR may serve as a barrier to tumor progression (Bartkova et al., 2005; Gorgoulis et al., 2005). Furthermore, overexpression of DNA damage response proteins BRCA1, PARP1, and Excision Repair Core Complementary group-1 has also been observed in various cancers (Fig. 4) (Klauke et al., 2012; Squires et al., 2013; Taron et al., 2004). BRCA1 plays an important role in the repair of DSBs, the mutation of BRCA1 causes a higher susceptibility for carcinogenesis (Taron et al., 2004), while its overexpression reduces DNA damage and enhances DNA repair mechanisms (Feng et al., 2015). Excision Repair Core Complementary group-1 is a key component of the nucleotide excision repair pathways, excising DNA adducts, which could hinder cellular replication (Squires et al., 2013). PARP1 is a key sensor for the repair of DNA single-strand breaks, inhibition of which leads to the accumulation of single-strand breaks, and subsequently DSBs after cell division (Klauke et al., 2012). One of the main interactors of GIT2, p53, is one of the most frequently mutated genes in sporadic cancers in humans (Fig. 3&4) (Hosoya and Miyagawa, 2014; Lu et al., 2015).

Inflammation can have several underlying origins which in turn can cause cancer; 1) infection, such as *Helicobacter pylori* causing gastric carcinoma in humans (De Falco et al., 2015), 2) autoimmunity, e.g. inflammatory bowel disease causing colon cancer (Francescone et al., 2015) or 3) the environment, like smoke pollution (Cohen and Pope, 1995). The microenvironments of chronic inflammatory conditions and of tumors have many similarities, supporting the role for inflammation in tumor progression (Raposo et al., 2015). Once a tumor is established, chronic inflammation is reinforced by the tumor microenvironment (Hanahan and Weinberg, 2011). This is also linked to oxidative stress and DNA damage, the former being produced by inflammatory cells enhancing the mutation rate in cells and increases genomic instability by inducing DNA damage (Waris and Ahsan, 2006).

Cancer cells require a high amount of energy to maintain their limitless replication potential (Marahatta et al., 2005; Warburg et al., 1927) causing an energy metabolism dysfunction. Warburg et al. [301] (Warburg et al., 1927) noted that the increased intake of glucose and production of lactate occurs even in the presence of oxygen (aerobic glycolysis) in tumor cells, now named the "Warburg effect", which has been implicated in cell transformation, immortalization and proliferation during tumorigenesis (Oliveira et al., 2015). These metabolic changes alter the microenvironment and surroundings of the cell, making them more conducive to cancer cell proliferation (Milosevic et al., 2014; Navratilova et al., 2013). Lastly, in 2002, the International Agency for Research on Cancer (IARC) concluded that breast, renal, colon, and endometrial cancer and esophageal adenocarcinoma could be prevented by avoiding weight gain (Gonzalez Svatetz and Goday Arno, 2015). The mechanisms by which cancer are caused can vary depending on the type of cancer, but many cases are related to insulin resistance and the resulting chronic hyperinsulinemia and chronic inflammation, where obesity has been reported to play a causal role (Gonzalez Svatetz and Goday Arno, 2015).

GIT2 is important in the control of cell polarization and direction-dependent regulation of the Golgi through RUN and SH3 Domain Containing 2 (RUSC2; Fig. 4). RUSC2 is an interactor of GIT2 through its SHD phosphorylation site. In various lung cancer cells, RUSC2 stabilizes GIT2 by inhibiting its degradation and increasing its phosphorylation (Mazaki et al., 2006; Yu et al., 2009). GIT2KO mouse neutrophils show a partial loss of directionality and defective chemotaxis toward chemoattractants (Mazaki

et al., 2006). Previous studies have shown that GIT2 interacts with PIX, MAP kinase/ERK kinase 1 (MEK1), phospholipase-C gamma (PLC γ) and paxillin to regulate cell polarization and motility through the control of cytoskeletal dynamics, membrane trafficking and focal adhesion turnover (Brown et al., 2002; Hoefen and Berk, 2006). Duan et al. demonstrated an important role for the interaction between RUSC2 and GIT2 in regulating EGF-stimulated RAB35-dependent directional cell migration in non-small cell lung cancer cells (Duan et al., 2016). Dysregulation of the EGF-receptor can lead to increased intracellular pathway activity, resulting in direct or indirect cell proliferation, angiogenesis, invasion, and metastasis (Ciardiello et al., 2004). Thus, Duan et al. 2016 provides a line of evidence that the RAB35/RUSC2/GIT2 pathway is central for EGF receptor-induced directional migration in lung cancer cells (Duan et al., 2016).

4. DISCUSSION

In this review, we have discussed the highly complex ageing process, a near-universal occurrence in biological systems, characterized by a progressive loss of physiological integrity, leading to impaired function and eventually to death (Lopez-Otin et al., 2013). We postulate that such a highly complex process could perhaps be controlled by a keystone protein, such as GIT2.

Age-related disorders are caused by a multitude of underlying processes or ‘hallmarks’, and GIT2 appears to be involved in each one of the previously-discussed processes. While not all links have been proven, the supporting research is encouraging. GIT2 plays an important role in stress sensitivity (Chadwick et al., 2010b; Lu et al., 2015; Schmalzigaug et al., 2009b), ageing (Chadwick et al., 2012), and somatic energy management (Martin et al., 2015), indicating that this protein might provide a novel target for therapies designed to minimize pathological ageing and age-related disease.

It thus stands to reason that GIT2-based therapies may potentially aid in alleviating some of the pathological effects caused by ageing. Considering the data obtained from GIT2KO mice, which age more rapidly than the WT age-matched controls, we hypothesize that the increase in GIT2 expression occurring with age is the body’s attempt to counter the ageing process, which in the end is insufficient to overcome the damage (Lu et al., 2015; Martin et al., 2015). If we assume that the ageing process can (to a certain degree) be termed a ‘GIT2-opathy’, strengthening GIT2 function could potentially reinforce network resiliency, and hence allow age-related pathological processes to be reversed.

Even though a considerable body of current ageing research is being performed on the genomic level, even if target genes can be identified, we must still replace and/or alter our cells (*i.e.* modify the genome) to avoid pathological ageing (de Magalhaes, 2014). Employing gene therapy reveals potential for regenerative medicine, but it has been shown that other life-extending interventions, such as caloric restriction, show larger and more promising effects (Shen et al., 2011). Even if we understand the full functional ramifications of the somatic genetic code, this does not imply the possibility to therapeutically edit the human genome. A simpler option might be to target at the protein level, using a keystone protein such as GIT2, and thus exploiting the cell’s own repair mechanisms to develop ageing therapies.

While GIT2 may represent a crucial therapeutic target for ageing disorders, drug targets are preferably receptors, ion channels, kinases or phosphatases. GIT2, which acts as a scaffolding protein, does not represent an ideal drug target. To control GIT2, the identification of another protein that could control its expression would be the simplest approach. One such candidate is the PIX protein family. PIX proteins bind tightly to GIT2 (as well as GIT1), and mice lacking α -PIX in immune cells also display a dramatic deficit in GIT2 expression (Missy et al., 2008; Pararasa et al., 2015). It thus appears that GIT2-PIX association stabilizes both proteins, so it will be important to understand whether age-related increases in GIT2 are accompanied by increases in PIX as well, and whether manipulating PIX expression can alter GIT2 levels and activity. Secondly, recent work has demonstrated that GPCRs can effectively regulate the

expression of multiple signaling proteins (Gesty-Palmer et al., 2013), suggesting that GPCRs might be used to regulate the expression of specific signaling proteins of interest. To potentially control this ‘ageing system’, a GPCR with a strong functional link to GIT2 will have to be identified, which should show a strong co-expression relationship with GIT2 in desired tissues. This GPCR should next be targeted using a ligand that possesses a capacity to control GIT2 expression. We believe that GIT2 may represent an important target for both therapeutic development for and diagnostic research of age-related disorders, and justifies further research into means of altering keystone functions of GIT2, to reduce the burdens associated with ageing.

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Figure Legends

Fig. 1: Domain-based structure of GIT family proteins. GIT1 (A) and GIT2 (B) share a common amino-terminal (N) ArfGAP (ADP ribosylation factor (Arf) GTPase-activating protein) domain, three ankyrin repeats (3x Ank), and the Spa homology domain (SHD) – indicated with two Spa2-like repeats - a coiled-coil (CC) dimerization domain and a C-terminal focal adhesion-targeting (FAT) domain. The ArfGAP domains facilitate the GTP hydrolysis on Arf1 and Arf6; Ankyrin repeats promote the integration and stabilization of multiple transmembrane proteins; the SHD regions mediates PIX, FAK, MEK, Piccolo binding; the CC domain is likely associated with regulating transcriptional functions; the FAT domain mediates localized targeting to plasma membrane-bound integrin complexes. In contrast to GIT1, GIT2 has been shown to exist as multiple isoforms, which are produced due to alternative splicing of five internal in-frame regions (indicated with A, B, C, D, E). Additionally, a distinct exon (D') exists which leads to GIT2-short (C), a truncated isoform where the FAT domain is absent. For GIT1, alternative splicing of a single exon can occur at the start of the SHD. Prominent post-translational modification phosphorylation sites (tyrosine – Y) are shown in red.

Fig. 2: The functional spectrum of curated GIT2 interactors. GIT2 in-part acts as a vital scaffolding protein as well as a, ADP-ribosylation factor GTPase-activating protein (ArfGAP). As protein scaffolding has been shown in recent years to be a vital component of intracellular signaling cascades, *e.g.* the assembly of mitogen-activated protein kinase pathways by adapters such as β -arrestin, the interactomic profile of proteins such as GIT2 are likely to be a strong indicator of the eventual functionality of the specific protein in a cellular system. The radial diagram centered upon GIT2 indicates a comprehensive assessment of GIT2 published interacting proteins identified to date. The presented information is based upon literature and data from Zhou et al. (2016), and Lu et al. (2016). All of the known and validated GIT2-interacting proteins are rationally grouped (via color matching with the groups radially indicated) based on their biological functions – thus facilitating an indication of the potential interactomic ramifications of GIT2 protein-protein binding activity. Red rings around the specific interacting proteins are used to identify GIT2-interacting proteins belonging to more than one functional signaling group, *e.g.* SRC (Proto-oncogene tyrosine-protein kinase Src) interaction with GIT2 demonstrates the role of this molecular interaction with immune responsivity regulation, cell cycle regulation and osteogenesis. The most commonly found and interconnected GIT2 interactors are indicated via enlarged protein identification text, *i.e.* ATM (ataxia telangiectasia mutated), SRC (Proto-oncogene tyrosine-protein kinase Src), TP53 (tumor protein p53) and GIT1 (ARF GTPase-activating protein GIT1). With further interactomics-based research the complexity and connectivity of these associated scaffolding factors – and how they eventually regulate GIT2-associated biology – will be further elucidated, allowing the potential for therapeutic interventions based on these reactions. All previously identified GIT2 interactors can be found in supplementary Table S1.

Fig. 3: Parallel effects of the major ageing hallmarks during lifespan. The complex multifactorial process of ageing is likely both initiated and controlled by the potential simultaneous interaction between several pathological domains. The generation, potentially in early middle-age, *i.e.* 30-35, of an individual's ageing trajectory is likely an index of both the degree and temporal co-occurrence of multiple pro-ageing molecular pathologies. In this simplistic model we propose that initial metabolic dysfunctions that interfere with

optimal glucose usage as the primary energy source will likely force the individual to enhance the usage of lipid-based metabolism for supplementary energy generation. This switch will likely require increased storage of lipids in tissues to act as an energy reserve – this abnormal/premature lipid infiltration and storage in tissues may then induce a greater pro-inflammatory state in the individual. The combination therefore of reduced energy generation efficiency and enhanced inflammatory markers is likely to be coincident with an increased oxidative radical burden (due to the move away from efficient oxidative phosphorylation) which in turn will increase the risk for cellular protein, lipid and nucleic acid damage. It is highly likely however that many of these events will occur in most individuals during the aging process but the relative magnitude of each event and their temporal relationships will likely vary, resulting in an individualistic aging trajectory in each person.

Fig. 4: The multitude of GIT2 functions in age-related disorders. GIT2 represents a multiple domain, multiply-spliced multidimensional functional scaffold that possesses an ability to interact with G protein-coupled receptors and vesicular trafficking proteins. Taking together this highly versatile profile it is unsurprising that the functional activities of GIT2 are associated with a broad spectrum of functional and pathophysiological processes. From our data and that of other researchers this highly-connected nature of GIT2 suggests that it could be considered one of the factors that interconnects multiple domain-based activities, enabling the systems-wide coordination of both biological functions and also pathological ones, *e.g.* deleterious aging-related diseases. It is therefore highly likely that GIT2 potentially has the role of keystone protein in ageing and age-related disorders. To illustrate the multidimensional role of GIT2 in such a complex process, we have selected several GIT2-interacting partners and functions associated with ageing-related disease. Our posit of the keystone status of GIT2 is reinforced by its evidential ability to coordinate and regulate profound domains of related pathophysiology, *e.g.* inflammation (NF- κ B, TLR), DNA damage response (BRCA1, ATM, p53) and Type 2 Diabetes Mellitus (InsR, Irs2, Ins). These global pathological domain activities can then promote the development of age-related disorders such as neurodegeneration (*e.g.* Alzheimer's disease: associated with GIT2 via DDR/BRCA1, InsR interactions), atherosclerosis (associated with GIT2 links to ROS sensitivity and DDR processes), osteoporosis (associated with GIT2 via its role in osteoblast function and fat deposition), central obesity, cancer, and sarcopenia. For each pathology box indicated in the radial diagram (excl. chronic inflammation and cancer) the normal condition (left) is compared to the pathology (right). Adipocyte level (adipocyte); DNA damage response (DDR); inflammation (Inf); insulin (Ins); Insulin receptor (InsR); Insulin receptor substrate 2 (Irs2); metabolic syndrome (MetS); osteoblast level (OB); Reactive oxygen species (*i.e.* oxidative stress) (ROS).





