Investigations on the role of GILZ in statin therapy and the influence of glucocorticoid metabolism in age-related inflammation

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To the memory of my Abuelita

I love you, my angel

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Abbreviations and Symbols

11-DHC	11-dehydrocorticosterone
aa	amino acids
ACTH	adrenocorticotropic hormone
Akt	RAC-alpha serine/threonine-protein kinase
ALT	alanine transaminase
ANOVA	analysis of variance
AP-1	activator protein 1
AST	aspartate transaminase
ATCC	American Type Culture Collection
AVP	arginine vasopressin
BCA	bicinchoninic acid
BMM	bone marrow-derived macrophages
bp	base pairs
BSA	bovine serum albumin
$Ca(NO_3)_2$	calcium nitrate
$CaCl_2$	calcium chloride
Cas9	CRISPR associated protein 9
CBG	corticosteroid-binding globulin
cDNA	complementary DNA
ChIP	chromatin immunoprecipitation
CI	confidence interval
CRH	corticotropin-releasing hormone
CRISPR	clustered regularly interspaced short palindromic repeats
CRP	C-reactive protein
CVD	cardiovascular disease
DAPI	4′,6-diamidino-2-phenylindole
DHEA	dehydroepiandrosterone
DM	differentiation medium
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPBS	Dulbecco's Phosphate-Buffered Saline
ECACC	European Collection of Authenticated Cell Cultures
ECGM	endothelial cell growth medium
ECL	enhanced chemiluminescence

EDTA	ethylene diamine tetracetic acid
ELISA	enzyme-linked immunosorbent assay
FBS	foetal bovine serum
FDB	*flexor digitorum brevis*
FHRE	forkhead response element
FoxO3a	forkhead box O3
FPP	farnesyl pyrophosphate
GADPH	glyceraldehyde 3-phosphate dehydrogenase
GC	glucocorticoid
gDNA	genomic DNA
GGPP	geranylgeranyl pyrophosphate
GILZ	glucocorticoid-induced leucine zipper
GM	growth medium
GR	glucocorticoid receptor
GRE	glucocorticoid response element
GTP	guanosine triphosphate
H_3BO_3	boric acid
HCl	hydrochloric acid
HEPES	hydroxyethyl piperazineethanesulfonic acid
HESI	heated electrospray ionization
HIV	human immunodeficiency virus
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HPA	hypothalamic-pituitary-adrenal
hpf	hours post-fertilization
HRP	horseradish peroxidase
HSD	hydroxysteroid dehydrogenase
HUVEC	human umbilical endothelial vein cells
ICE	inference of CRISPR Edits
IF	immunofluorescence
IGF-1	insulin-like growth factor-1
IgG	immunoglobulin G
IL	interleukin
IQR	interquartile range
IRE	insulin response element
KCl	potassium chloride
KH_2PO_4	potassium dihydrogen phosphate
$KHCO_3$	potassium bicarbonate
KLF2	Krüppel-like factor 2
KD	knockdown

KO	knockout
LB	lysogeny broth
LC-HRMS/MS	liquid chromatography-high resolution mass spectrometry
LDL-C	low-density lipoprotein cholesterol
LPS	lipopolysaccharide
mAb	monoclonal antibody
Mcl-1	induced myeloid leukaemia cell differentiation protein
M-CSF	macrophage colony-stimulating factor
MDA	malondialdehyde
$MgSO_4$	magnesium sulphate
MHC	major histocompatibility complex
MHC	myosin heavy chain
MMP	matrix metalloproteinase
MO	morpholino
MOI	multiplicity of infection
MRF	myogenic regulatory factor
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MVA	mevalonic acid
Na_2HPO_4	sodium hydrogen phosphate
NaCl	sodium chloride
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NaH_2PO_4	sodium dihydrogen phosphate
NCE	normalised collision energy
NF-κB	nuclear factor kappa B
NH_4Cl	ammonium chloride
NHEJ	non-homologous end joining
NO	nitric oxide
NOS	nitric oxide synthase
PBL	peripheral blood leukocytes
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PER	proline/glutamate-rich
PGC-1α	peroxisome-proliferator-activated receptor coactivator-1α
PI3K	phosphoinositide 3 kinase
PM	peritoneal macrophages

PRR	pattern recognition receptors
PVDF	polyvinylidene difluoride
RBB	Rockland blocking buffer
RIPA	radio Immunoprecipitation Assay
RNA	ribonucleic acid
RNase	ribonuclease
ROS	reactive oxygen species
RT-qPCR	reverse transcription – quantitative PCR
SAMS	statin-associated muscle symptoms
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEM	standard error of the mean
sgRNA	single guide RNA
shRNA	short hairpin RNA
SINE	short interspersed elements
siRNA	small interfering RNA
TBARS	thiobarbituric acid reactive substances
TBE	Tris/Borate/EDTA
TGF-β	transforming growth factor β
TNF-α	tumour necrosis factor α
tSIM	targeted single ion monitoring
TU	transducing units
UA	uric acid
UHPLC	ultra-high-performance liquid chromatography
VP	viral particles
WT	wildtype

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Abstract

The glucocorticoid-induced leucine zipper (GILZ) is an immunomodulatory, ubiquitously expressed protein, with multiple roles in different pathophysiological processes. Aim of the present study was to evaluate the role of GILZ and glucocorticoid (GC) metabolism in two different settings:

Statins, the most prescribed class of drugs for prevention of cardiovascular disease, exert beneficial lipid-lowering independent effects, as well as muscle-related adverse effects, by mechanisms not fully understood. The role of GILZ in these mechanisms was investigated. Statins were able to induce GILZ expression in skeletal muscle, endothelial cells, and macrophages. Moreover, using *in vitro*, *ex vivo* and *in vivo* approaches, we demonstrated that GILZ is an important mediator of statin-induced muscle damage.

The second part of this study focused on aging, which is characterised by a chronic, low-grade inflammatory state —termed "inflammaging"— that contributes to age-related pathogenesis. The elucidation of the mechanisms that modulate this state is of interest to geroscience. Evaluation of the age-associated changes in the myeloid compartment of mice, focusing on glucocorticoid metabolism, showed reduced levels of circulating GCs that, together with perturbations in GC pre-receptor metabolism in aged macrophages, result in dysregulation of the anti-inflammatory networks in these innate immune cells and, thus, might promote the inflammaging phenotype.

Zusammenfassung

Der Glukokortikoid-induzierte Leucin Zipper (GILZ) stellt ein ubiquitär exprimiertes Protein dar und spielt eine Rolle in verschiedenen pathophysiologischen Prozessen. Ziel der vorliegenden Studie war es, die Beteiligung von GILZ und des Glukokortikoid (GC)-Metabolismus in zwei Ansätzen zu untersuchen.

In der Prävention kardiovaskulärer Erkrankungen stellen Statine die meistverordnete Substanzklasse dar. Neben ihrer Lipid-senkenden Wirkung zeichnen sie pleiotrope Effekte sowie muskelschädigende Nebenwirkungen aus. Zunächst wurde die Hypothese überprüft, dass GILZ für die Vermittlung dieser Prozesse eine Rolle spielt, die nicht in direktem Zusammenhang mit der Lipid-Senkung stehen. Statine führten zu einer Erhöhung der GILZ-Expression in Muskelzellen, Endothelzellen und Makrophagen. Darüber hinaus konnte gezeigt werden, dass GILZ als wichtiger Mediator in der Statin-induzierten Muskelschädigung agiert.

Der zweite Teil der Studie fokussierte auf Alterungsprozesse, die sich durch eine chronische, Entzündung auszeichnen. Die Untersuchung alterungsbedingter Änderungen im myeloiden Kompartiment von Mäusen zeigte im Hinblick auf deren GC Metabolismus reduzierte Spiegel zirkulierender GCs. Zusammen mit Störungen im GC Prä-Rezeptor Metabolismus, wie er in alten Mäusen zu finden ist, führt dies zu einer Dysregulation des anti-inflammatorischen Netzwerks dieser Immunzellen, welches zum entzündlichen Phänotyp des Alterns beitragen könnte.

1. Introduction

1.1 Cardiovascular disease

The term cardiovascular disease (CVD) comprises a group of disorders of the heart, brain vasculature, and blood vessels, such as ischaemic heart disease (or coronary artery disease), stroke, and hypertension (Mendis et al., 2011). CVD is the leading cause of mortality and disability worldwide, accounting for an estimated 31% of global deaths per year (World Health Organization, 2018).

The main pathophysiological condition leading to CVD is atherosclerosis, a chronic inflammatory process that occurs in medium and large blood vessels (Mendis et al., 2011). The pathogenesis of atherosclerosis involves lipid deposition, activation of dysfunctional endothelium, and release of pro-inflammatory cytokines and chemoattractant molecules; subsequent leukocyte recruitment, activation and transmigration (primarily monocyte-derived macrophages that later form foam-cells, and T-cells); platelet aggregation; and smooth-muscle cell migration and proliferation as a result of cytokine and growth factor secretion (Tousoulis et al., 2014). Among the most important risk factors for atherosclerosis and CVD are advancing age and high atherogenic lipid burden, *i.e.* cholesterol and its lipoprotein carriers (Catapano et al., 2016; Grundy et al., 2018; Mendis et al., 2011).

1.2 Statins

Statins are the first line of treatment in the management of hyperlipidaemias and prevention of CVD (Catapano et al., 2016; Stone et al., 2014). Since their introduction in 1987, statin prescription has risen, as shown by several studies in different populations (Nolte et al., 2010; Salami et al., 2017; Vancheri et al., 2016). Thus, statins are positioned among the most prescribed drug classes worldwide, in a tendency that seems to be maintained.

1.2.1 Pharmacological and pharmacokinetic aspects

Statins are competitive inhibitors of the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase enzyme, the rate-limiting enzyme in the mevalonate biosynthetic pathway. As a consequence, they block the biosynthesis of cholesterol in the liver, enhancing clearance of circulating low density lipoprotein (LDL)-cholesterol (Sirtori, 2014). Inhibition of the mevalonate pathway by statins also impairs biosynthesis of its other products, such as isoprenoid metabolites, important for the post-translational modifications of several proteins, and ubiquinone (Figure 1.1 A).

First discovered as fungal secondary metabolites (Endo, 2010), statins are either classified according to their origin in natural/semi-synthetic (lovastatin, simvastatin and pravastatin), and synthetic statins (atorvastatin, rosuvastatin, fluvastatin, pitavastatin, and cerivastatin); or

according to their lipophilicity. They are administered as the active hydroxy-acid, except for lovastatin and simvastatin, which are lactone prodrugs (Gazzerro et al., 2012; Sirtori, 2014). The chemical structure and lipophilic properties of statins are summarised in Figure 1.1 B.

A
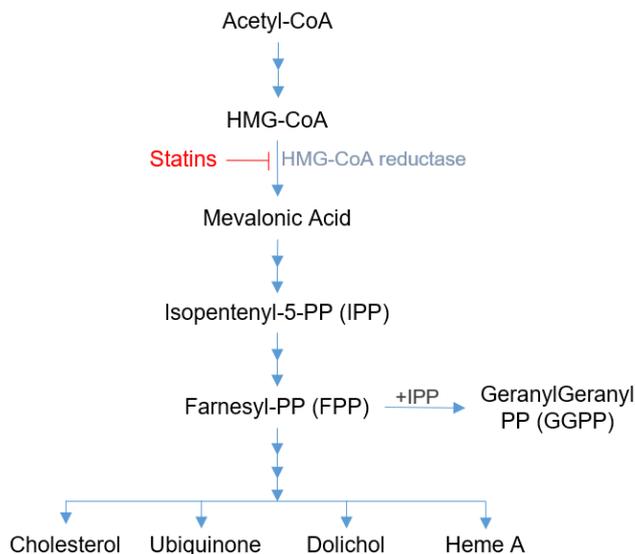
B
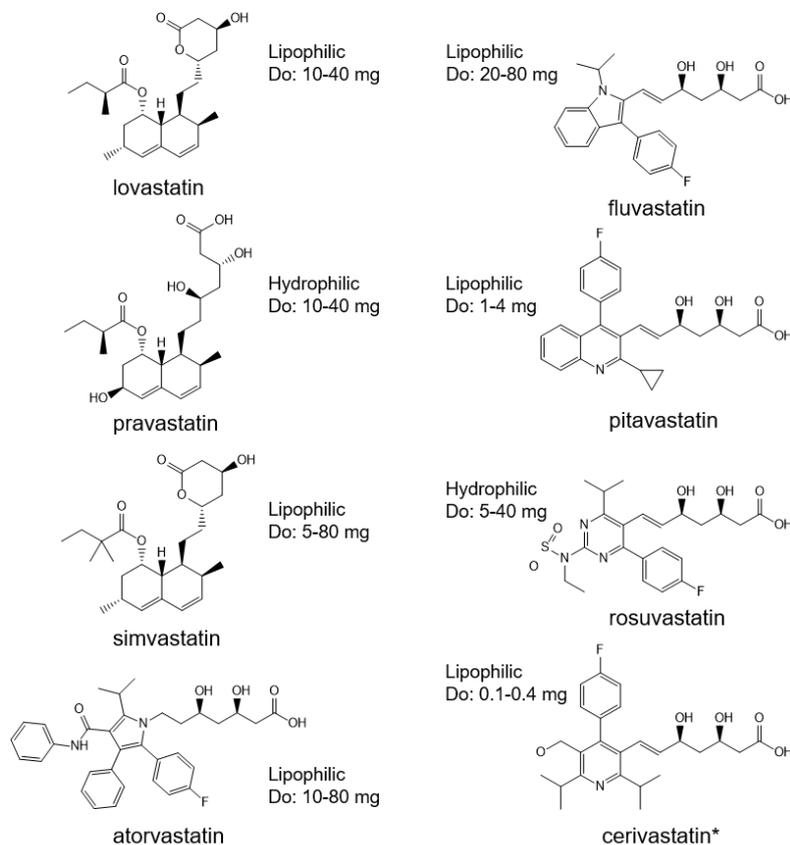
Figure 1.1. General aspects of statins. The mevalonate pathway, simplified schema (**A**). Statins inhibit HMG-CoA reductase, the rate limiting enzyme of the pathway. PP, pyrophosphate. The structure of the 8 therapeutically approved statins (**B**). The lipophilic properties and standard daily dose are specified. *Withdrawn from the market.

1.2.2 Pleiotropic effects of statins

Extended research has shown that statins exert beneficial effects independent from their lipid-lowering action. In the cardiovascular system, these so called "pleiotropic" effects include improvement of endothelial function, inhibition of vascular inflammation and thrombogenesis, and atherosclerotic plaque stabilisation (Almeida and Budoff, 2019). Furthermore, statins showed immunomodulatory and antioxidant actions, neuroprotective effects, and beneficial effects in lipid-independent diseases, such as rheumatoid arthritis, multiple sclerosis, bone loss, and cancer (Gazzerro et al., 2012; Margaritis et al., 2014; Oesterle et al., 2017).

Several cellular and molecular mechanisms underlie statin pleiotropy, the majority of them attributed to inhibition of Ras, Rho and Rac small GTPase function, as a result of reduced isoprenylation (Huacuja Álvarez et al., 2006). Studies have shown that statins increase the expression of the atheroprotective transcription factor Krüppel-like factor 2 (KLF2) and improve nitric oxide (NO) activity in the vascular endothelium, inhibit nuclear factor (NF)-κB transcriptional activity in different cell types, inhibit cell proliferation, decrease reactive oxygen species (ROS) generation, and inhibit the major histocompatibility complex (MHC) II in T-cells, among other effects (Gazzerro et al., 2012; Margaritis et al., 2014; Oesterle et al., 2017).

1.2.3 Statin-associated muscle symptoms

It is generally considered that the benefits of statin therapy largely outweigh the risks of adverse effects (Collins et al., 2016), of which the most relevant are newly diagnosed type 2 diabetes mellitus, hepatotoxicity, and statin-associated muscle symptoms (SAMS) (Newman et al., 2019). SAMS are a class effect, dose-dependent and not correlated to the hypolipidemic effect of the statin (Kobayashi et al., 2008; Vaklavas et al., 2009). They constitute one of the principal causes of non-adherence to treatment, in fact, muscle toxicity was the reason for cerivastatin withdrawal from the market in 2001 (Taha et al., 2014). With a prevalence of 5–29% in clinical practice, SAMS can range from mild myalgia without elevation of creatine kinase (CK) to, in very rare cases, fatal rhabdomyolysis (Grundy et al., 2018; Stroes et al., 2015).

The molecular mechanisms that lead to muscle impairment are still not fully understood. Several HMG-CoA-related and non-related mechanisms have been proposed (recently reviewed by du Souich et al., 2017): isoprenoid depletion (particularly of GGPP) cause apoptosis and proteolysis mediated by inhibition of the protein kinase Akt and the transcriptional coactivator peroxisome-proliferator-activated receptor coactivator (PGC)-1α; direct impairment of mitochondrial respiration, ROS production, and calcium homeostasis in the myocyte also trigger apoptosis. Moreover, reduced resting chloride channel conductance and decreased lactate efflux could cause muscle fatigue. In addition to their myotoxicity, statins impair muscle regeneration *via* GGPP depletion, and impaired insulin-like growth factor-1

(IGF-1) signalling (Baba et al., 2008; Ogura et al., 2007; Trapani et al., 2012). Further research in the field can improve the understanding of these mechanisms and allow for identification of risks and biomarkers associated with SAMS (Muntean et al., 2017).

1.3 The glucocorticoid-induced leucine zipper (GILZ)

GILZ was first identified as a dexamethasone-inducible gene (*Tsc22d3*) encoding for a 137-amino acid (aa) protein in murine thymocytes (D'Adamio et al., 1997). Later, other splice variants of murine GILZ were described, encoding for proteins of 201 aa, 43 aa, 80 aa (Soundararajan et al., 2007), and 234 aa (Bruscoli et al., 2010). Human GILZ is a 134-aa protein with 97% similarity in the coding region with its murine orthologue (Cannarile et al., 2001). Different transcript variants coding for other isoforms are also annotated for the *GILZ* gene and have been observed experimentally (NCBI "Gene"; Köberle et al., 2012).

GILZ consists of three domains: The N-terminal domain (residues 1–75), a central leucine zipper (LZ) domain (residues 76–97), and the C-terminal domain, which includes a proline/glutamate-rich (PER) region (residues 98–137). The N- and C-terminal regions are responsible for the interaction of GILZ with its targets, while the LZ-domain mediates the protein homodimerization (Figure 1.2 Ayroldi et al., 2014; Fan and Morand, 2012a).
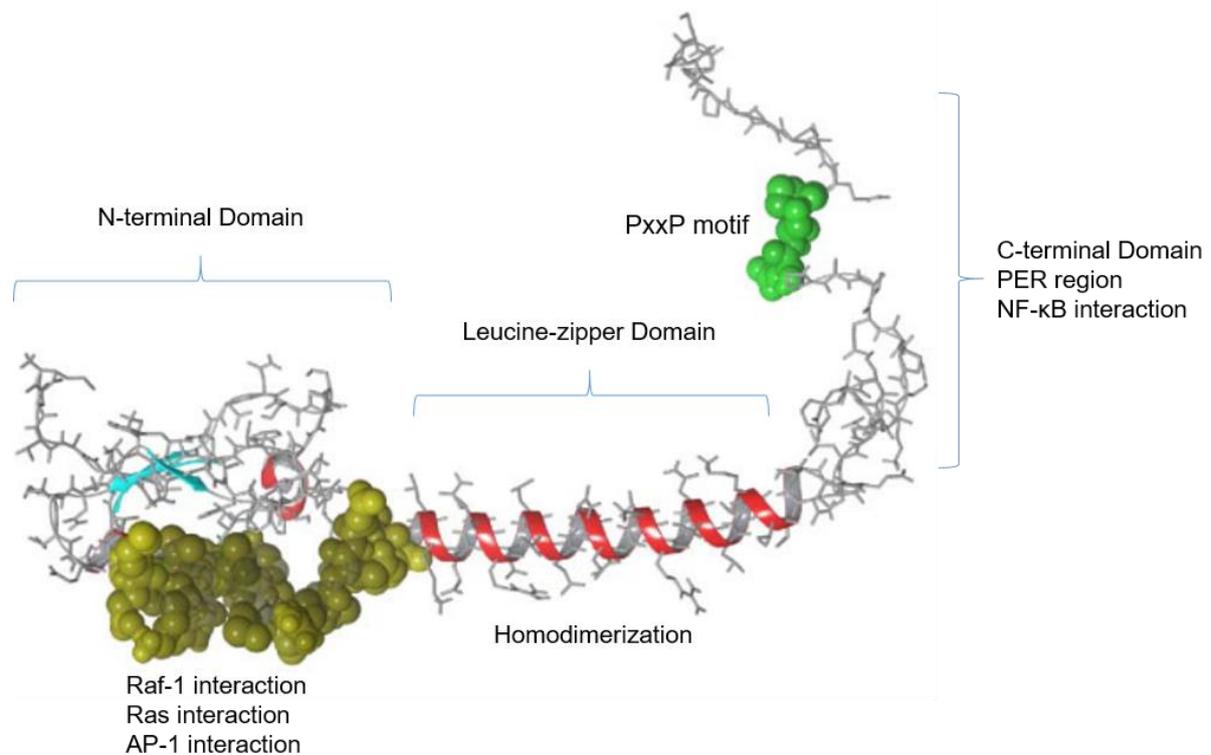
Figure 1.2. GILZ structure and functional domains. Modified from (Ayroldi et al., 2014).

GILZ is ubiquitously expressed in human and mouse tissues (Cannarile et al., 2001; Soundararajan et al., 2007). Besides its immunomodulatory function, it plays multiple roles in other glucocorticoid (GC)-mediated as well as non-GC-mediated cellular processes. The anti-inflammatory action of GILZ is mainly exerted *via* inhibition of NF-κB transcriptional activity, as well as interaction with activator protein (AP)-1. Anti-proliferative, anti-oncogenic, pro-apoptotic and anti-apoptotic functions of GILZ in different cell types have also been reported (reviewed by Ayroldi et al., 2014).

1.4 Aging and age-related diseases

Aging, defined as the functional decline that occurs during the lifespan of an organism, constitutes a risk factor for several human pathologies (López-Otín et al., 2013). For instance, the prevalence and mortality associated with CVD is expected to grow exponentially, as the world population continues to age (Costantino et al., 2016). The recent field of geroscience intends to understand the mechanisms driving aging and age-related chronic disease, how they interact and overlap, in order to provide therapeutic opportunities to extend human lifespan by targeting aging itself (Kennedy et al., 2014). In this context, seven pillars of aging have been identified (Figure 1.3). These are not independent factors, but rather constitute an integrated network that, interestingly, converge on inflammation (Franceschi et al., 2018; Kennedy et al., 2014). Indeed, longitudinal studies have shown a correlation between inflammation and longevity, capability, and cognition in the aged population (Arai et al., 2015).
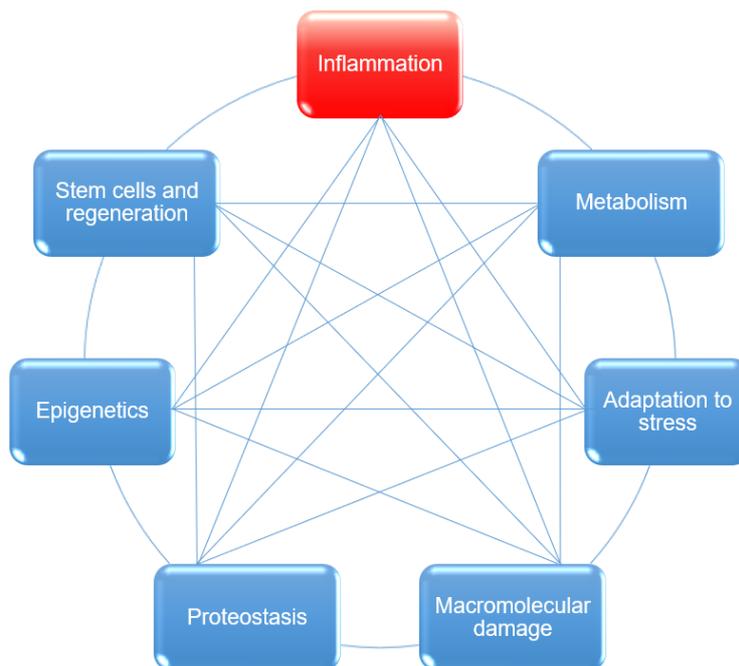
Figure 1.3. The seven pillars of aging. After Kennedy et al., 2014.

1.4.1 Inflammaging

The changes in the immune system associated with aging, globally known as immunosenescence, affect both innate and adaptive immunity. Rather than solely detrimental, such changes represent an adaptive/remodelling response that results in dysregulated homeostasis not only of immunity, but of other systems that influence and are influenced by the immune system, like the nervous and endocrine systems (Fulop et al., 2018).

The chronic, low-grade systemic inflammatory state observed with advancing age, termed "Inflammaging" (Franceschi et al., 2000), involves functional alteration of immune cells as a consequence of different mechanisms, including cellular senescence, oxidative stress, mitochondrial dysfunction, defective autophagy and mitophagy, inflammasome activation, and dysbiosis (Franceschi et al., 2018). Population studies have shown that aged individuals have elevated circulating levels of pro-inflammatory mediators/markers, such as tumour necrosis factor α (TNF-α), interleukin (IL)-6 and C-reactive protein (CRP), that correlate with increased risk of morbidity and mortality (Bandaranayake and Shaw, 2016; Minciullo et al., 2016). The global pro-inflammatory state, however, is not the only determinant of successful/unsuccessful aging, as a compensatory, anti-inflammatory response, is observed in parallel to inflammaging, particularly in very long-lived individuals (centenarians). The mediators associated with anti-inflammaging include transforming growth factor (TGF)-β, IL-10, dehydroepiandrosterone (DHEA), and cortisol (Baylis et al., 2013; Minciullo et al., 2016). It is considered that, ultimately, the ability of the anti-inflammatory network to cope with and modulate chronic inflammation is determinant to either attain healthy aging and longevity, or for the onset of chronic inflammatory diseases.

1.5 Glucocorticoid function and metabolism

1.5.1 Regulation of glucocorticoid response and function

Endogenous GCs are master regulators of several physiological functions, including metabolism, development, cardiovascular function, response to stress, and immune function (Kharwanlang and Sharma, 2017). The synthesis of GCs is regulated in a circadian manner, and in response to stressors (including pro-inflammatory cytokines), by the hypothalamic-pituitary-adrenal (HPA) axis, a component of the neuroendocrine system (Gupta and Morley, 2014). Activation of the HPA axis starts with release of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) from the hypothalamus, which stimulate the secretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland. ACTH enters the circulation and stimulates synthesis of GCs through steroidogenesis in the adrenal cortex. Secreted GCs, in turn, cause a decrease in the release of CRH and ACTH, in a negative feedback loop (Figure 1.4 A). GCs circulate bound in a 95% to corticosteroid-binding globulin (CBG).

Free GCs can diffuse through cell membranes and exert their action by binding to the widely expressed glucocorticoid receptor (GR) through genomic mechanisms (transactivation, transrepression, and composite glucocorticoid response element (GRE) binding), or non-genomic effects (Cain and Cidlowski, 2017).

While the HPA axis controls systemic GC release, the bioavailability and activity of GCs is controlled at the intracellular, pre-receptor level, by two enzymes: 11β-hydroxysteroid dehydrogenase (11β-HSD) 1 and 2, that catalyse the interconversion of active cortisol and inactive cortisone (Chapman et al., 2013). 11β-HSD1 functions mainly as a reductase, catalysing the NADPH-dependent reduction of cortisone to cortisol in the endoplasmic reticulum, although it can also act as an NADP-dependent dehydrogenase, inactivating cortisol. 11β-HSD2, on the contrary, functions solely as a NAD-dependent reductase (Figure 1.4 B).
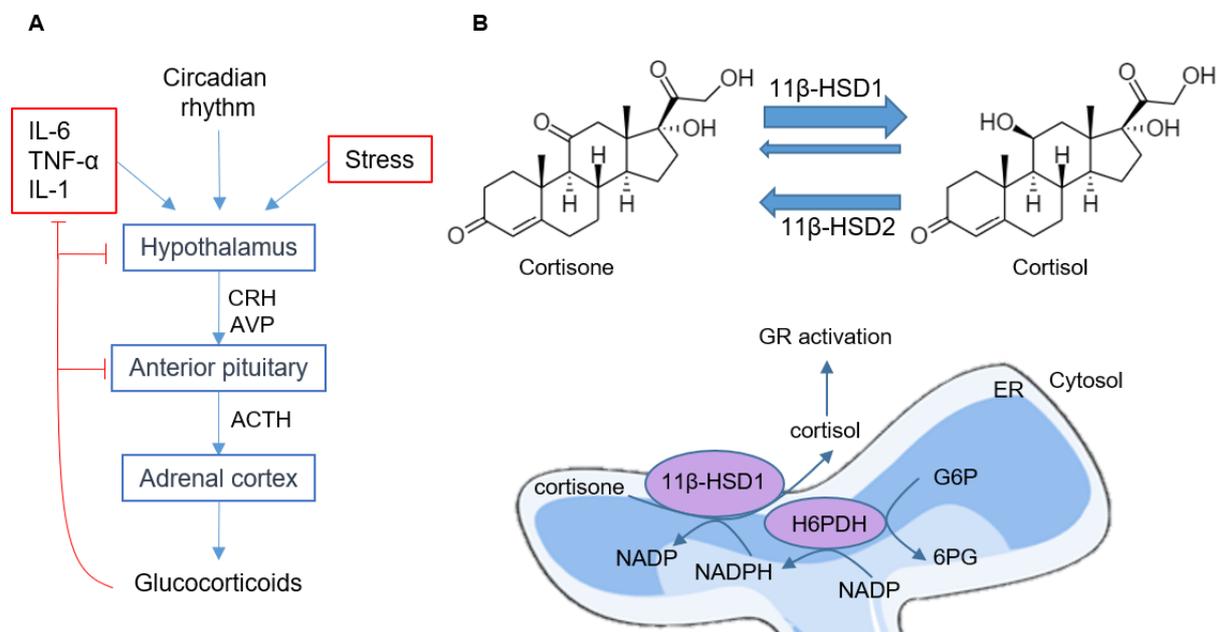
Figure 1.4. Regulation of cortisol levels. The HPA axis, see text for details (**A**). Intracellular levels of cortisol are controlled by the interconversion of cortisone and cortisol by 11β-HSD isozymes (**B**). In the endoplasmic reticulum (ER), 11β-HSD1 reduces cortisone to cortisol using the NADPH generated by hexose 6-phosphate dehydrogenase (H6PDH) as cofactor. H6PDH catalyses the conversion of glucose-6-phosphate (G6P) to 6-phosphogluconate (6GP).

1.5.2   Glucocorticoids in inflammation

Although GCs are well established anti-inflammatory agents in pharmacological therapy, they cannot be defined as strictly immunosuppressive in normal physiology. The anti-inflammatory action of GCs occurs at all stages of inflammation. During the alarm phase, GCs attenuate the secretion of pro-inflammatory mediators by inhibiting NF-κB and AP-1 activity, decrease histamine release by mast cells, and reduce vascular permeability. In the mobilization phase,

they attenuate leukocyte extravasation by reducing the secretion of chemokines and adhesion molecules. Finally, during the resolution phase, they promote an anti-inflammatory, phagocytic phenotype in macrophages, to allow for tissue clearance (Cain and Cidlowski, 2017). On the other hand, GCs can also increase the expression of genes associated with the detection of insult and initiation of the inflammatory response, *i.e.* pattern recognition receptors (PRRs), chemokine/cytokine receptors, and complement factors, in what is thought to be a mechanism to sensitise immune cells so that they respond rapidly and effectively to insult. Once the inflammatory response is triggered, elevated concentrations of GCs (in response to stress) restrain the extent of inflammation, suppress the adaptive immune response, and help restore homeostasis (Busillo and Cidlowski, 2013; Cain and Cidlowski, 2017). This biphasic immunostimulant/immunosuppressive action highlights the main role of endogenous GCs in regulation of immunity, and how the dysregulation of GC secretion and function (*e.g.* during chronic stress associated with aging) might directly impair immune function.

1.6 Aim of the present work

Statins are the cornerstone of CVD prevention and treatment. As CVD remains the leading cause of morbidity and mortality worldwide, and a growing portion of the population already is or will be under statin treatment, the study of the molecular mechanisms that mediate their beneficial and deleterious effects remains of importance. In the first part of this work, we aimed to elucidate such mechanisms, focusing on the role of GILZ. First, its potential role in the onset of SAMS, the most common adverse effect associated with statin use, was investigated. Secondly, its potential role as an anti-inflammatory mediator of the pleiotropic effects of statins in vascular inflammation was evaluated.

The aging population is at high risk of suffering from cardiovascular events, as well as other age-related diseases. Chronic inflammation is one of the pillars that underlie the functional decline associated with age, thus, identification of the pathways that control/fuel the so called "inflammaging" process is relevant for the design of preventive and therapeutic strategies for age-related diseases. Aim of the second part of this work was to evaluate the age-associated changes in gene expression and glucocorticoid metabolism across tissues, as well as in the myeloid compartment of mice, to improve the current understanding of the molecular mechanisms that modulate inflammaging.

2. Materials and Methods

2.1 Chemicals and reagents

Cell-culture grade dimethyl sulfoxide (DMSO), atorvastatin, simvastatin, cerivastatin, pravastatin, and mevalonic acid lithium salt were obtained from Sigma-Aldrich (St Louis, MO, USA). Simvastatin sodium salt, geranylgeranyl pyrophosphate (GGPP), and farnesyl pyrophosphate (FPP) were obtained from Cayman Chemicals (Ann Arbor, MI, USA). 20 mM statin stock solutions were prepared in DMSO and stored at -20 °C. Unless stated otherwise, experiments performed with simvastatin refer to the active acid form of the drug. 100 mM stock solution from mevalonic acid was prepared in sterile water and stored at -20 °C. Foetal bovine serum (FBS), horse serum, trypsin-EDTA, accutase, high glucose DMEM, and RPMI 1640 were from Sigma-Aldrich. Penicillin/streptomycin and glutamine were from PAA (Pasching, Austria). MCDB 131 medium was from Thermo Fisher Scientific (Waltham, MA, USA). Endothelial Cell Growth Medium (ECGM) and endothelial cell growth medium SupplementMix were obtained from PromoCell (Heidelberg, Germany). DNA oligos and Sanger sequencing services were provided by Eurofins Genomics (Ebersberg, Germany). All other chemicals were obtained either from Sigma Aldrich or Carl Roth (Karlsruhe, Germany), unless stated otherwise.

2.1.1   General Buffers

PBS: 2.7 mM KCl, 1.8 mM KH_2PO_4, 137 mM NaCl and 10 mM Na_2HPO_4 in distilled water. The pH was adjusted to 7.4 and the solution was adjusted to a final volume of 1 l. PBST was prepared by adding 0.1% tween-20 to PBS.

TBS: 20 mM Tris and 137 mM NaCl in distilled water. The pH was adjusted to 7.6 and the solution was adjusted to a final volume of 1 l. TBST was prepared by adding 0.1% tween-20 to TBS.

TBE: 90 mM Tris, 90 mM H_3BO_3, 2 mM EDTA disodium in distilled water, in a final volume of 1 l.

Sodium phosphate buffer, 1 mM, pH 5.6: 65.05 mg $NaH_2PO_4 \cdot H_2O$, 4.05 mg Na_2HPO_4 in distilled water. The pH was adjusted to 5.6 and the solution was adjusted to a final volume of 500 ml.

Electrophoresis buffer: 25 mM Tris, 192 mM glycine, 0.1% SDS in distilled water, to a final volume of 1 l.

Blotting buffer: 25 mM Tris, 192 mM glycine, and 5% methanol in distilled water, to a final volume of 1 l.

2.2 Cell culture

2.2.1 Cell lines

C2C12 cells (ECACC 91031101) were maintained in high glucose DMEM (growth medium, GM) at a maximum confluency of 65%, HEK-293T cells (ATCC® CRL-3216) were maintained in high glucose DMEM, RAW 264.7 cells (ATCC® TIB-71) were maintained in RPMI 1640, and HMEC-1 cells (ATCC® CRL-3243) in MCDB 131 medium. All cell culture media were supplemented with 10% FBS, penicillin/streptomycin (100 U / 100 µg/ml), and 2.0 mM glutamine, unless stated otherwise. For inducing differentiation of C2C12 into myotubes, confluent cell layers were cultured in high glucose DMEM supplemented with 2% horse serum, penicillin/streptomycin and glutamine (differentiation medium, DM), with medium change every other day. All cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2. Cells were subcultured according to the ATCC recommendations. Briefly, cells were washed with PBS and detached with trypsin-EDTA. Digestion was stopped with culture medium, the cell suspension was centrifuged for 5 minutes at 200 x g and resuspended in medium. The resulting suspension was used for seeding cells for experiments, and appropriate aliquots were used for passaging.

2.2.2 Cell freezing / thawing

For freezing, cells were washed and detached as for splitting. The cell suspension was centrifuged for 5 minutes at 200 x g and cells were resuspended in freezing medium (FBS with 10% DMSO). Cells were aliquoted into cryovials, frozen at -80 °C for 2 days, and transferred to liquid nitrogen for long-term storage.

For thawing, frozen cells were warmed up in a 37 °C water bath, and quickly transferred into pre-warmed medium. The resulting suspension was centrifuged for 10 minutes at 200 x g, the cell pellet was resuspended in culture medium and cells were cultured as described above.

2.2.3 Primary cells

Isolation of primary cells in the laboratory was performed with technical assistance from TheoRanßweiler, Dr. Jessica Hoppstädter, and Anna Dembek.

Human umbilical endothelial vein cells (HUVECs): HUVECs were isolated from umbilical cords as described previously (Weber et al., 2003). In brief, the umbilical cord vein was digested with a 0.01% collagenase A solution (#10103586001, Roche, Basel, Switzerland) for 45 minutes. Cells were suspended in ECGM supplemented with endothelial cell growth medium SupplementMix, 10% FBS, penicillin/streptomycin (100 U / 100 µg/ml), and kanamycin (50 µg/ml), and plated in a 25 cm^2 cell culture flask. On the next day, cells were washed twice

with PBS, the medium was renewed, and cells were cultured as described above. Cells were used for experiments in passages 2–3.

Murine bone marrow-derived macrophages (BMMs): BMMs were isolated from male C57BL/6J wildtype (WT) or GILZ knockout (KO) mice, following a previously published method (Hoppstädter et al., 2015). In brief, femurs and tibias were flushed with RPMI 1640 medium supplemented with penicillin/streptomycin. After centrifugation for 10 minutes at 200 x g, the cell pellet was resuspended in hypotonic buffer (155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA disodium in distilled water, to a final volume of 1 l) for erythrocyte lysis, during 3 minutes in a 37 °C water bad. Cells were centrifuged and resuspended in RPMI 1640 medium supplemented with 10% FBS (PAN Biotech, Aidenbach, Germany), penicillin/streptomycin, glutamine, and 50 ng/ml M-CSF (#99906, Biomol, Hamburg, Germany), plated in a 75 cm^2 cell culture flask, and allowed to adhere overnight. Non-adherent cells were then transferred into a 175 cm^2 cell culture flask and cultured for another 5 days before use in experiments.

Murine peripheral blood leukocytes (PBL): whole blood from young and aged C57BL/6 mice was collected in PBS-EDTA (5 mM EDTA in PBS) containing tubes, and centrifuged for 20 minutes at 500 x g and 4 °C. The cell pellet was resuspended in 1 ml erythrocyte lysis buffer and incubated for 15 minutes on ice. After 5 minutes of centrifugation at 500 x g and 4 °C, the supernatant was discarded and the PBL pellet was frozen at -80 °C.

Murine peritoneal macrophages (PMs): PMs were isolated from young and aged C57BL/6J mice by washing the peritoneal cavity with cold PBS-EDTA. The fluid was collected and centrifuged for 10 minutes at 350 x g and 4 °C. Cells were resuspended in RPMI-1640 medium supplemented with 10% FBS (PAN Biotech), penicillin/streptomycin, and glutamine, seeded in a 35 mm cell culture dish, and allowed to adhere for 2 hours. After this time, non-adherent cells were removed by washing with PBS and PMs were detached with accutase. The cell suspension was centrifuged, and the pellets were frozen at -80 °C. When used for treatment, cells were resuspended and plated in RPMI-1640 medium without FBS and, after removing the non-adherent cells at the end of 1 hour, treated as specified below.

Flexor digitorum brevis **(FDB) muscle fibres:** FDB fibres were isolated from WT or GILZ KO C57BL/6J mice by Dr. Jessica Hoppstädter in the laboratory of Professor Carlo Riccardi (Department of Medicine, University of Perugia), following a protocol adapted from the literature (Keire et al., 2013; Park et al., 2014; Weisleder et al., 2012). The FDB muscles were dissected under a stereo microscope and digested in a 0.2% collagenase A solution in DMEM for 60 minutes. Then, the myofibres were carefully separated under a stereo dissecting microscope, dispersed in DMEM containing 10% horse serum by drawing through a series of

pipette tips with gradually decreasing diameter, and purified by sedimentation steps. Pure fibres were plated in laminin (#L2020, Sigma Aldrich)-coated cell culture dishes, and cultured in DMEM supplemented with 20% serum replacement 2 (#S9388, Sigma Aldrich), 1% horse serum, penicillin/streptomycin, and glutamine.

Murine primary myoblasts: Primary myoblasts were isolated from hindlimbs of male 3-day-old WT or GILZ KO C57BL/6J mice by Dr. Jessica Hoppstädter in the laboratory of Professor Carlo Riccardi (Department of Medicine, University of Perugia). The muscle was dissected and shredded, washed in PBS and digested with trypsin-EDTA for 60 minutes. After filtering through a 70 µm cell strainer, the cell suspension was diluted in DMEM supplemented with 10% FBS and centrifuged for 20 minutes at 200 x g and 4 ℃. The resuspended cells were pre-plated for 1 hour to allow fibroblast adhesion. Non-adherent cells were collected, centrifuged for 10 minutes at 200 x g and 4 ℃, resuspended in F-10-based primary myoblast growth medium (Ham's F-10 nutrient mixture, #N6013, Sigma-Aldrich, supplemented with 20% FBS, penicillin/streptomycin, and glutamine), and plated onto collagen I (#C3867, Sigma-Aldrich)-coated cell culture dishes. Further enrichment of the myoblasts was achieved by dislodging and pre-plating the cells onto collagen-coated dishes every fourth day for 1-2 weeks. Afterwards, myoblasts were cultured in growth medium (40% DMEM, 40% Ham's F-10 nutrient mixture, 20% FBS), supplemented with penicillin/streptomycin and glutamine.

2.3 Bacterial culture

The following *E. coli* strains were used as host organisms for plasmid amplification:

> TOP10 (Invitrogen, Carlsbad, CA, USA). Genotype: F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(StrR) endA1 λ-

> GT116 (Invivogen, San Diego, CA, USA). Genotype: F⁻ mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 endA1 Δdcm ΔsbcC-sbcD.

> Plasmids obtained from Addgene (Cambridge, MA, USA) were received and cultured in DH5α strain. Genotype: F- Φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rk-, mk+) phoA supE44 thi-1 gyrA96 relA1 λ-.

Liquid cultures of bacteria were prepared in lysogeny broth medium (LB, 10% tryptone, 5% yeast extract, 5% NaCl, in water, pH 7.5), with or without antibiotic selection marker (ampicillin 100 µg/ml, Sigma-Aldrich), and cultured at 37 °C in an incubator shaker. Agar plates were prepared by adding 30% of agar to the culture medium.

2.3.1 Generation of competent E. Coli

Competent *E. coli* were generated using the calcium chloride method. Briefly, 5 ml of an overnight culture were diluted to 100 ml with LB and allowed to grow to an $OD_{600} = 0.4$. The culture was then placed on ice for 30 min, centrifuged at 2,000 x g and 4 °C, and resuspended in 10 ml ice cold $CaCl_2$ solution (75 mM $CaCl_2$, 15% glycerol, in water). After 30 minutes of incubation on ice, cells were centrifuged at 2,000 x g and 4°C, resuspended in 2.5 ml $CaCl_2$, aliquoted, and stored at -80 °C.

2.3.2 Plasmid isolation

Plasmids were isolated from overnight liquid cultures using either the High Pure Plasmid Isolation Kit (#11754777001, Roche), or the QIAprep Spin Miniprep Kit (#27106 Qiagen, Hilden, Germany), following the manufacturer's instructions.

2.4 Mice

Mice were housed in a 12/12 hours light/dark circle with food and water ad libitum. GILZ KO mice were generated as previously described (Bruscoli et al., 2012; Hoppstädter et al., 2015). For aging studies, young (10 weeks) or aged (80–100 weeks) C57BL/6 mice were either non-treated or treated with one intraperitoneal injection of 5 mg/kg LPS (#tlrl-smlps, InvivoGen, San Diego, CA, USA) or vehicle (DPBS) for 4 hours before sacrifice (approval number from animal welfare committee GB 3-2.4.2.2-06/2016). Tissues were removed and stored at -80 °C for RT-qPCR analysis. Serum, BMMs, PBLs, and PMs were also collected for analysis

2.5 RNA isolation and reverse transcription

Total RNA from cultured cells was isolated using the High Pure RNA Isolation Kit (#11828665001, Roche), following the manufacturer's instructions. The RNA concentration was measured using a NanoDrop™ Lite spectrophotometer (Thermo Fisher Scientific). Samples with an A260/A280 ratio higher than 1.8 were used for further analysis.

Total RNA from murine tissue was isolated using the QIAzol lysis reagent (#79306, Qiagen) following the manufacturer's instructions. Residual genomic (g) DNA contamination was removed using the DNA-free™ DNA Removal Kit (#AM1906, Thermo Fisher Scientific). To verify the absence of gDNA, a SINE-PCR was performed using the GenScript Taq DNA polymerase (#E00007, GenScript Inc., Piscataway, NJ, USA). The primer sequences were: forward 5'-CTTCTGGAGTGTTTGAAGAC-3', reverse 5'-CTGGAACTCACTCTGAAG AC-3'. The reaction was carried out in a T100 thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) using the following program:

Denaturation	5 min 94 °C	
Denaturation	1 min 94 °C	
Annealing	1 min 56 °C	30 cycles
Elongation	1 min 72 °C	
Final elongation	10 min 72 °C	

RNA was considered free of gDNA contamination when no product was detected by agarose gel electrophoresis. A 2% agarose gel containing 0.04% ethidium bromide was used for detection. Upon addition of a suitable volume of 10 x loading buffer (40 mM EDTA disodium, 0.05% bromophenol blue, 0.05% xylene cyanol, 70% glycerol, in water ad 50 ml), DNA was loaded onto a gel and separated in TBE at 100 V. To determine the DNA fragment sizes, a 50 bp ladder (#SM0372, Thermo Fisher Scientific) was used. The DNA bands were detected using a UV transilluminator and the software ArgusX1 (Biostep, Stollberg, Germany).

RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (#4368813, Thermo Fisher Scientific), in the presence of RNase inhibitor (RNaseOUT™, #10777019, Thermo Fisher Scientific), following the manufacturer's instructions.

2.6 Quantitative Polymerase Chain Reaction (qPCR)

qPCR was performed on cDNA samples using the 5xHotFirePol EvaGreen qPCR Mix (no ROX, #08-25-00020, Solis BioDyne, Tartu, Estonia) in a 20 µl format. The primer sequences and annealing temperatures for each transcript are detailed in table 2.1. The reaction was conducted in a CFX96 touch™ Real-Time PCR detection system running the CFX Manager 2.1 software (Bio-Rad Laboratories), using the following program:

Denaturation	15 min 95 °C	
Denaturation	0:15 min 94 °C	
Annealing	0:20 min	
Elongation	0:20 min 72 °C	40 cycles
Plate read		
Melt curve	65 °C – 95 °C	

Data were analysed either by absolute quantification, using a standard curve of the PCR product cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA), or with the comparative $\Delta\Delta$Ct method. Housekeeping genes were chosen based on the literature, or after evaluating the expression stability of at least three candidate genes under the experimental conditions, using the geNorm, NormFinder, and BestKeeper Software tools. Absolute amounts of transcript were normalised to the corresponding housekeeping genes.

Table 2.1. Primer sequences and annealing conditions for the transcripts evaluated in this study

Gene	NCBI Accession number	Forward primer sequence 5´-3´	Reverse primer sequence 5´-3´	µl primer [10 µM] / reaction	Annealing T (°C)
Human					
ACTB	NM_001101.3	TGCGTGACATTAAGGAGAAG	GTCAGGCAGCTCGTAGCTCT	0.5	60
KLF2	NM_016270.2	AGACCACGATCCTCCTTGAC	AAGGCATCACAAGCCTCGAT	0.5	60
NOS3 (eNOS)	NM_00603.4	CCTCACCGCTACAACATCCT	ACGTTGATTTCCACTGCTGC	0.5	60
TSC22D3 (GILZ)	NM_004089.3	TCCTGTCTGAGCCCTGAAGAG	AGCCACTTACACCGCAGAAC	0.5	60
Mouse					
Anxa1	NM_010730.2	TGGAAAGCCCTTGGATGAAGT	AAGTCCCTTCATGGCACCAC	0.5	60
Cdkn1a	NM_007669.5	GACCAGCCTGACAGATTTCTA	TGGGCACTTCAGGGTTTTCT	0.5	60
Cdkn2a	NM_009877.2	CGGGGACATCAAGACATCGT	GCCGGATTTAGCTCTGCTCT	0.5	60
Cebpa	NM_001287523.1	TTCGGGTCGCTGGATCTCTA	TCAAGGAGAAACCACCACGG	0.5	60
Cebpb	NM_001287739.1	GGAGACGCAGCACAAGGT	AGCTGCTTGAACAAGTTCCG	0.5	60
Csnk2a2	NM_009974.3	GTAAAGGACCCTGTGTCAAAGA	GTCAGGATCTGGTAGAGTTGCT	0.8	60
GR-β	HM236293.1	AAAGAGCTAGGAAAAGCCATTGTC	CTGTCTTTGGGCTTTTGAGATAGG	0.5	61
H19	NR_130973.1	CAGAGGTGGATGTGCCTGCC	CGGACCATGTCATGTCTTTCTGTC	0.5	60
H6pd	NM_173371.4	ATAGATGCGGAAGGTCGGGC	ACCAGCGTGAGGATCTCAGT	0.5	60
Hdac3	NM_010411.2	GCATTCGAGGACATGGGGAA	TTTCGGACAGTGTAGCCACC	0.5	60
Hsd11b1	NM_008288.2	GGAACCCAGGAAGGAAGATCA	CAGGCAGGACTGTTCTAAGAC	0.5	60
Hsd11b2	NM_008289.2	AACCTCTGGGAGAAACGCAAG	GGCATCTACAACTGGGCTAAGG	0.5	60
Il1b	NM_008361.3	CCAAAAGATGAAGGGCTGCTT	GGAAGGTCCACGGGAAAGAC	0.5	60
Il6	NM_031168.2	AAGAAATGATGGATGCTACCAAACTG	GTACTCCAGAAGACCAGAGGAAATT	0.4	60
Klf2	NM_008452.2	CCTTGCACATGAAGCGACAC	ACTTGTCCGGCTCTGTCCTA	0.5	60
Mkp1	NM_013642.3	CTCCAAGGAGGATATGAAGCG	ACTAGTACTCAGGGGGAGGC	0.5	60
Mmp9	NM_013599.3	GCCGACTTTTGTGGTCTTCC	TACAAGTATGCCTCTGCCAGC	0.5	60
Nr3c1	NM_008173.3	AAAGAGCTAGGAAAAGCCATTGTC	TCAGCTAACATCTCTGGGAATTCA	0.5	61

Ppia	NM_008907.1	GGCCGATGACGAGCCC	TGTCTTTGGAACTTTGTCTGC	0.5	58
Rn18s	NR_003278.3	AGGTCTGTGATGCCCTTAGA	GAATGGGGTTCAACGGGTTA	0.5	61
Sgk1	NM_001161845.2	GAGGCCATGTGTCAATCATGC	TTCTTTCACTTCACACCCAGGTT	0.5	60
Sirt1	NM_019812.3	TGGAGCAGGTTGCAGGAATC	GGCACCGAGGAACTACCTGAT	0.5	60
Tnf	NM_013693.2	CCATTCCTGAGTTCTGCAAAGG	AGGTAGGAAGGCCTGAGATCTTATC	0.5	60
Tsc22d3	NM_010286.4	GGGATGTGGTTTCCGTTAAACTGGA	TGCTCAATCTTGTTGTCTAGGGCCA	0.4	61
Tsc22d3	NM_010286.4	GCTGCTTGAGAAGAACTCCCA	GAACTTTTCCAGTTGCTCGGG	0.5	60
Zebrafish					
actb2	NM_181601.4	AAATTGCCGCACTGGTT	ACGATGGATGGGAAGACA	0.5	60
elf1	NM_131159.2	CTCAAATGGCATGGATGTTGCCCA	GGTCTTGGTTTGCGCACTTTGGTT	0.5	60
fbxo32	NM_200917.1	GGAGCACCAAAGAGCGTCAT	AGTTGGGACTTGGCGATGAG	0.5	60
hatn10	NA	TGAAGACAGCAGAAGTCAATG	CAGTAAACATGTCAGGCTAAATAA	0.5	60
rn18s	KY486501.1	CCGCTAGAGGTGAAATTCTTG	CAGCTTTGCAACCATACTCC	0.5	60
tsc22d3	NM_200569.2	AACAACCAGCTGGAGCGCGAA	GCAGAGCCCGTGCTGCTGTATT	0.5	60

2.7 Western blot

Protein lysates were prepared either in SB lysis buffer (50 mM Tris-HCl, 1% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.004% bromophenol blue, in water) or RIPA buffer (50 mM Tris-HCl, 1% triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 150 mM NaCl, in water), supplemented with a protease inhibitor cocktail (cOmplete® Mini, # 04693124001, Roche). Samples were sonicated for 5 s and stored at -80 °C for further analysis. The protein concentration in RIPA lysates was measured using the Pierce™ BCA Protein Assay Kit (#23225, Thermo Fisher Scientific).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using polyacrylamide gels (4% stacking gel and 12% resolving gel) and the Mini PROTEAN system (Bio-Rad Laboratories). Samples were thawed on ice and denatured at 95 °C for 5 minutes before loading into the gel. For samples prepared in RIPA buffer, an appropriate volume of loading buffer (Roti®-Load 1, #K929, Carl-Roth) was added before denaturation. To estimate protein size, a prestained protein ladder was run in parallel to the samples (PageRuler™, #26616, Thermo Fisher Scientific). Samples were transferred onto an Immobilon®-FL PVDF membrane (#IPFL00010, Merck, Darmstadt, Germany) using a Mini Trans-Blot® cell (Bio-Rad

Laboratories). After blotting, the membrane was allowed to air-dry. Afterwards, it was soaked in methanol and rinsed with distilled water, before continuing with the immunodetection.

2.7.1 Near-Infrared detection

The membrane was blocked for 1 to 2 hours in blocking buffer for fluorescent western blotting (RBB, #MB-070, Rockland Immunochemicals, Limerick, PA, USA) before incubation with primary antibody at 4 °C overnight. Antibody sources and dilutions are indicated in table 2.2. The membrane was washed 4 times for 5 minutes with PBST (or TBST) and incubated with the appropriate IRDye-conjugated secondary antibody, at room temperature for 90 minutes. After washing, the signal was detected using an Odyssey® Near-Infrared Imaging System and software (LI-COR Biosciences, Lincoln, NE, USA).

2.7.2 Chemiluminescent detection

Chemiluminescent immunodetection was performed in the laboratory of Professor Carlo Riccardi (Department of Medicine, University of Perugia), using the HRP-coupled secondary antibodies listed in table 2.2 and the Clarity™ Western ECL Blotting Substrate (Bio-Rad Laboratories), according to the manufacturer's instructions.

Table 2.2. Antibodies used in this study for western blot

Antibody	Dilution	Cat. Number	Supplier
Mouse anti α-tubulin [DM1A] mAb	1:1,000 in 5% milk powder – PBST	T 9026	Sigma-Aldrich
Polyclonal goat anti 11β-HSD1	1:400 in RBB	AF3397	R&D Systems, Minneapolis, MN, USA
Rat anti GILZ [CFMKG15] mAb	1:1,000 in 5% milk powder – TBST	14-4033-80	Thermo Fisher Scientific
Mouse anti GADPH [OTI2D9]	1:2,000 in 5% milk powder – TBST	TA802519	OriGene Technologies, Rockville, MD, USA
Mouse anti Akt (pan) [40D4] mAb	1:2,000 in 5% milk powder – TBST	2920	Cell Signalling Technology Danvers, MA, USA
Rabbit anti phospho-Akt (Ser473) [D9E)] XP® mAb	1:2,000 in 5% milk powder – TBST	4060	
Polyclonal rabbit anti phospho-FoxO3a (Ser253)	1:2,000 in 5% milk powder – TBST	9466	
Mouse anti FoxO3a [D12] mAb	1:1,000 in gelatine buffer (0.75% gelatine A, 0.1% tween-20, 20 mM Tris, 137 mM NaCl, pH 7.5)	sc-48348 X	Santa Cruz Biotechnology Dallas, TX, USA
Mouse anti myogenin [5FD] mAb	1:200 in TBST	sc52903	Santa Cruz Biotechnology
Polyclonal rabbit anti cleaved Caspase-3	1:1,000 in TBST	ALX-210-807-C100	Enzo Life Sciences Farmingdale, NY, USA

IRDye® 680RD goat anti rabbit IgG	1:10,000 in RBB	926-68071	LI-COR Biosciences, Lincoln, NE, USA
IRDye® 800CW goat anti mouse IgG	1:10,000 in RBB	926-32210	
IRDye® 800CW donkey anti goat IgG	1:10,000 in RBB	926-32214	
Goat anti rat IgG (H+L), HRP	1:5,000 in TBST	31470	Thermo Fisher Scientific
Goat anti mouse IgG (H+L), HRP	1:5,000 in TBST	31430	
Goat anti rabbit IgG, HRP	1:10,000 in TBST	12-348	Merck

2.8 Cytotoxicity measurement

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay was used to determine cell viability after statin treatment. Cells were seeded in 96-well plates at a density of $1x10^4$ cells/well (or $2x10^4$ cells/well for C2C12 differentiation), allowed to adhere overnight, and treated with test compounds at the indicated concentrations. Cells incubated without any treatment were used as growth controls, cells incubated with solvent at the maximum concentration present in the assay (0.25%) served as negative controls, and wells with treatment but without cells were used as blank. At the end of treatment, cells were incubated for 3 hours with MTT solution (0.5 mg/ml in medium) and lysed. The absorbance was measured at 550 nm in a microplate reader (XFluor4 Sunrise^{TM}, TECAN), using a reference wavelength of 690 nm. The relative viabilities were calculated related to the negative control; the comparison between the viability of growth control and negative control showed that the solvent had no statistically significant effect on cell viability (data not shown).

For evaluating the cell viability in FDBs treated with statin, fibres isolated from 2 mice were pooled and treated with test compounds at the indicated concentrations. At the end of treatment, the number of living and non-living myofibres was evaluated using the trypan blue exclusion method.

2.9 Effects of statins on C2C12 differentiation

C2C12 myoblasts were seeded at a density of $1.0x10^5$ cells/well in 24-well plates and induced to differentiate in the absence or presence of non-toxic concentrations of atorvastatin (5 µM), pravastatin (50 µM), simvastatin (1 µM) and cerivastatin (0.1 µM) for 5–6 days. Myotube formation was evaluated *via* Jenner-Giemsa staining and IF of myosin heavy chain. Similarly, primary myoblasts of WT and GILZ KO mice, as well as stable C2C12^{scr} or C2C12^{shGILZ} cells (see 2.15) were induced to differentiate in the absence or presence of statins.

2.10 Jenner-Giemsa staining

To measure the myogenic differentiation of C2C12 myotubes Jenner-Giemsa staining was performed, following a previously published protocol (Veliça and Bunce, 2011). In brief, cells were fixed in ice-cold methanol for 5 minutes, air-dried, and stored at 4 °C until analysis. For staining, wells were incubated with Jenner's stain solution (Santa Cruz Biotechnology; diluted 1:3 in 1 mM sodium phosphate buffer pH 5.6) for 5 minutes at room temperature, washed with distilled water, and subsequently incubated with Giemsa solution (Carl Roth, diluted 1:20 in the same buffer) for 10 minutes at room temperature. Wells were observed in a phase contrast microscope equipped with a digital camera (ZEISS Axiovert 40 CFL with Canon EOS 400D), and each well was photographed in 3 randomly selected regions. Images were analysed using the free image-processing software Fiji ("Fiji Is Just ImageJ", Schindelin et al. 2012).

2.11 Immunofluorescence

Myosin heavy chain immunofluorescence (MHC IF) was performed on C2C12 myotubes. Cells were cultured and differentiated onto glass coverslips that were previously treated for 10 minutes in a 1:1 mixture of 70% ethanol and 0.1 N HCl (Andrés and Walsh, 1996). At the end of treatment, cells were pre-fixed by adding paraformaldehyde solution (4% in PBS) directly into the culture medium. After 2 minutes, the pre-fixation culture medium was replaced with paraformaldehyde solution and cells were fixed for 15 minutes at room temperature. Cells were washed with PBS and permeabilized with an 0.2% triton X-100 solution in PBS for 10 minutes at room temperature, washed, blocked in a 5% BSA solution in PBS for 1 hour at room temperature, and incubated with primary monoclonal anti-myosin heavy chain antibody (MF20, deposited by Donald A. Fischman to the Developmental Studies Hybridoma Bank, The University of Iowa, Department of Biology, Iowa City, IA, USA) diluted 1:50 in dilution buffer (1% BSA in PBS), at 4 °C overnight. After washing, cells were incubated with Alexa Fluor® 488 goat anti-mouse secondary antibody (#A11017, Thermo Fisher Scientific) diluted 1:800 in dilution buffer, for 1 hour at room temperature. Then, cells were washed and counterstained with DAPI (#D9542, Sigma-Aldrich) for 15 minutes at room temperature, mounted with FluorSave™ (Merck) and observed with an Axio Observer Z1 epifluorescence microscope, equipped with an AxioCam Mr3 and AxioVision software (Zeiss, Oberkochen, Germany). Photographs from randomly selected regions were analysed using the Fiji software, and the fusion index was calculated as the percentage of total nuclei incorporated into myotubes, defined as myosin-expressing cells containing 3 or more nuclei.

2.11.1 Whole-zebrafish immunofluorescence

Whole zebrafish embryos were stained following a protocol based on the literature (Cao et al., 2009). Zebrafish embryos were fixed in paraformaldehyde solution at 4 °C overnight, and then stored in methanol at -20 °C for at least four hours. Embryos were permeabilized in acetone for 30 minutes at -20 °C, washed, blocked in a 1% BSA solution in PBST for 2 hours at room temperature, and incubated with primary monoclonal anti-myosin heavy chain antibody (F59, deposited by Frank E. Stockdale to the Developmental Studies Hybridoma Bank, The University of Iowa, Department of Biology, Iowa City, IA, USA) diluted 1:100 in blocking buffer at 4 °C overnight. After washing in PBST, the embryos were incubated with Alexa Fluor® 594 goat anti-mouse secondary antibody (1:200; # A-11005 Thermo Fisher Scientific) at room temperature for 4 hours, washed again, and observed on a Leica SP8 Confocal microscope using the 40X magnification.

2.12 GILZ induction under statin treatment

To evaluate the ability of statins to induce GILZ in different cell types, HUVECs and C2C12 cells were seeded in 12-well plates at a density of 1.5×10^5 and 1.0×10^5 cells/well, respectively, and BMMs were seeded at 2.5×10^5 cells/well in 24-well plates. Cells were treated with 3 µM atorvastatin and frozen at different time points for RT-qPCR analysis.

In a second set of experiments, C2C12 or primary murine myoblasts were seeded at 2.0×10^5 cells/well in 12-well plates and induced to differentiate in the absence or presence of non-toxic concentrations of atorvastatin (5 µM), pravastatin (50 µM), simvastatin (1 µM) and cerivastatin (0.1 µM) for up to 72 hours. Cells were frozen at different time points for RT-qPCR or western blot analysis.

For evaluating the effect of toxic concentrations of statins on GILZ expression levels, C2C12 myoblasts (5×10^4 cells/well) and 72-hour myotubes (1×10^5 cells/well) seeded in 24-well plates were treated with 50 µM statins for 6 hours, in the absence or presence of mevalonate (100 µM). Gene expression was evaluated *via* RT-qPCR, and protein *via* western blot. Primary murine myoblasts were similarly treated with atorvastatin and harvested for western blot analysis. In an analogue experiment, C2C12 myoblasts were treated with statin in the absence or presence of GGPP and FPP (10 µM).

HUVECs were seeded in 24-well plates at 7×10^4 cells/well and treated with statins in the absence or presence of mevalonate (100 µM) for 24 hours. HMEC-1 cells were seeded in 24-well plates at a density of 1×10^5 cells/well and treated with statins for 24 hours. Cells were frozen for RT-qPCR analysis.

2.13 Gene expression in zebrafish embryos under statin treatment

Zebrafish embryos from the AB wildtype strain were obtained from the laboratory of Professor Rolf Müller (Helmholtz Institute for Pharmaceutical Research Saarland). The developmental stage was determined by embryo morphology, in hours post fertilization (hpf, Kimmel et al. 1995) by observation under a stereo microscope. To examine the effects of statins, embryos at 19–22 hpf were sorted and placed at a density of 2–3 embryos/cm^2 in 6-well plates, and incubated at 28 °C in Danieu's solution (17 mM NaCl, 2.0 mM KCl, 1.5 mM HEPES pH 7.1–7.3, 1.8 mM Ca(NO$_3$)$_2$, 1.2 mM MgSO$_4$, 1.2 µM methylene blue, in water) containing 1 µM statin or solvent control. 12 hours after treatment, 10–20 embryos were pooled, flash-frozen in liquid nitrogen, and stored at -80 °C for RNA isolation and RT-qPCR analysis.

2.14 Chromatin immunoprecipitation (ChIP)

The ChIP assay was performed by Dr. Jessica Hoppstädter in the laboratory of Professor Carlo Riccardi (Department of Medicine, University of Perugia), using the EZ-ChIP™ Kit (#17-371, Merck) according to the manufacturer's instructions. In brief, C2C12 cells were fixed in 1% paraformaldehyde for 10 minutes, lysed, and sonicated on ice. Precleared lysates from 1×10^6 myoblasts in a volume of 100 µl were incubated overnight at 4 C° with 10 µg monoclonal anti-FoxO3 antibody (D12, #sc-48348 X, Santa Cruz Biotechnology) or 10 µg normal mouse IgG (#12-371B, Merck). Immunocomplexes were purified, and qPCR analysis was performed using the SYBR® Select Master Mix (#4472908, Thermo Fisher Scientific) and The Applied Biosystems 7300 qPCR system to determine the abundance of specific *Gilz* promoter regions. The primers used to quantify forkhead response elements (FHRE)-containing sequences and a non-FHRE sequence within the *Gilz* promoter are listed in table 2.3.

Table 2.3. Primers used in the ChIP assay

	Forward primer sequence 5´-3´	Reverse primer sequence 5´-3´
FHRE1	TGGCCCAGTTAAACCACATCC	GCTGAACTGTTTACAGTCCCTGA
FHRE2	TAACCGTGTAAACAGGAGCCAG	GGAACTCCTGGGGAAATCCTA
FHRE3	AGCATGGGCAGAAAAAGGAATAAG	CTGGTTTGGTTGGTGTAAACAGT
FHRE4 (IRE, insulin-response element,)	AGAGCTTTCTTGGTCTGAGAGAAT	AATTTTGAGGTGAGTAGCAGTAGT
non-FHRE	GTATTCGGCCTTCTCCTTGC	CTGCTGCGTGGTGAAAAACA

2.15 Lentiviral transductions and stable cell line generation

All lentiviral plasmids were obtained from Addgene. The packaging plasmid psPAX2 (#12260), and the envelope plasmid pMD2.G (#12259), were a gift from Didier Trono. FUW-tetO-loxP-hKLF2 was a gift from Rudolf Jaenisch (#60850, Theunissen et al., 2014). The pLKO.1-TRC cloning vector was a gift from David Root (#10878, Moffat et al., 2006). pLKO.1-scramble shRNA was a gift from David Sabatini (#1864, Sarbassov et al., 2005). LentiCas9-Blast was a gift from Feng Zhang (#52962, Sanjana et al. 2014).

Two different sequences of short hairpin (sh) RNA targeting murine GILZ were used for generating lentiviral particles. shGILZ1 (5'-GGAGTACTGACTGGTCTCTTA-3') was designed using the siRNA Wizard™ v3.1 software tool (InvivoGen). shGILZ2 (5'-ACAGCTT CACCTGACAATG-3') was previously published (Bruscoli et al., 2010). Sequences were cloned into the pLKO.1-TRC cloning vector, following the protocol available from Addgene (Addgene, 2006), with minor modifications. In brief, the cloning vector was digested with AgeI-HF and EcoRI-HF (New England BioLabs, Ipswich, MA, USA) and purified from an agarose gel using the NucleoSpin® Gel and PCR Clean-up Kit (#740609, Macherey-Nagel, Düren, Germany). The annealed oligos were then ligated into the vector using T4 DNA ligase (#M0202, New England BioLabs) overnight at room temperature. The ligation mix was transformed into GT116 *E. coli*, and ampicillin-resistant clones were screened for inserts by sequencing, using the pLKO.1 sequencing primer (5'-GACTATCATATGCTTACCGT-3').

2.15.1 Lentivirus generation

Lentiviral particles were produced following Addgene's protocol. Briefly, $5x10^5$ HEK-293T cells plated onto 6-cm cell culture dishes in medium without antibiotics were transfected at 50–80% confluency with the transfer, packaging and envelope plasmids (1000:750:250) using the fuGENE® 6 Transfection Reagent (#E2691, Promega). 12–15 hours after transfection, the medium was replaced with complete DMEM. The lentivirus-containing media were then harvested at 24 and 48 hours, pooled, centrifuged at 200 x *g* for 5 minutes, aliquoted, and stored at -20°C.

2.15.2 Lentiviral titre

Non-biological titration of the lentiviral vectors was performed by determining the viral particle content in the harvested supernatants, using a protocol adapted from the literature (Geraerts et al., 2006; Scherr et al., 2001). Genomic viral RNA was purified from 200 µl of freshly harvested lentivirus stock using the High Pure Viral RNA Kit (#11858882001, Roche), following the manufacturer's instructions. After digestion of residual DNA with the DNA-free™ DNA Removal Kit (Thermo Fisher Scientific), 10-fold serial dilutions of the purified viral RNA were

reverse transcribed and amplified *via* qPCR, using primers for the 5'LTR region (forward 5'-AGCTTGCCTTGAGTGCT TCA-3', reverse 5'-TGACTAAAAGGGTCTGAGGGA-3'), and the 5'-end of the *gag* gene (forward 5'-GGAGCTAGAACGATTCGCAGTTA-3', reverse 5'-TGTAGCTGTCCCAGTATTTGTC-3'). The copy number (viral particles, VP) contained in the supernatant was calculated from comparison against a plasmid standard curve, and the titre calculated as viral particles per ml of supernatant (VP/ml).

The biological titration of the vectors was performed either *via* limiting dilution, following the protocol developed by the MISSION® RNAi Team (Sigma-Aldrich); or by qPCR, following a procedure modified from the protocol of the Laboratory of Virology and Genetics at the EPFL (*École Polytechnique Fédérale De Lausanne*), as follows: HEK-293T cells were seeded in 12-well plates at a density of $8x10^4$ cells/well and transduced with increasing amounts of lentiviral supernatant in the presence of 8 µg/ml polybrene (#H9268, Sigma-Aldrich). 96 hours after transduction, gDNA was extracted using the GenElute Mammalian gDNA Miniprep Kit (#G1N70, Sigma Aldrich) according to the manufacturer's recommendations, and qPCR of 5'LTR and *gag* was performed, using *ACTB* as housekeeping gene. For each dilution, the HIV copy number integrated per genome was calculated as the ratio of 5'LTR or *gag* copies to *ACTB*, and the viral titre was determined as the number of transducing units per ml of supernatant (TU/ml).

For each lentivirus, the ratio from biological to non-biological titre was established and used for estimation of the functional titre (TU/ml) for all further preparations that were only titrated *via* the gRNA method. This functional titre was then used to determine the volume of viral supernatant needed for infecting cells at a given multiplicity of infection (MOI, defined as the number of TU per cell).

2.15.3 Antibiotic kill curve

The optimal concentration of antibiotic needed for selection of transduced cells was determined by performing an antibiotic kill curve. In brief, cells seeded at the same relative densities used for transduction in 24-well plates, were treated with increasing antibiotic concentrations. Cells were cultured with medium change every-other day and observed daily. The minimum concentration of antibiotic that resulted in complete cell death after 3–5 days was chosen for selection in the experiments.

To determine whether the cells were able to grow starting as single cells, the kill curve was performed on a 96-well plate, in a matrix that combined decreasing concentrations of antibiotic (in rows) with decreasing cell numbers (in columns), from 1,000 to 1 cell/well. Cells were

considered to be able to grow as single clones when growth was observed after 10 days in the antibiotic control wells containing less than 4 cells on average.

2.15.4 Generation of C2C12shGILZ stable cell lines

Stable, polyclonal C2C12 cell lines were generated by reverse transducing 7.5×10^4 cells in 6-well plates with scramble shRNA, shGILZ1, or shGILZ2 lentiviral particles at an MOI of 10 in the presence of polybrene, following the protocol for generation of stable cell lines available from Addgene. 24 hours after transduction the medium was changed, and 48 hours after infection puromycin (2 µg/ml, #540411, Merck) was added to select for transduced cells. The puromycin resistant cells were expanded for 2 to 3 weeks, after which they were harvested for analysis of gene and protein expression, and cell stocks were frozen. Further culture and experiments were done in 1 µg/ml puromycin-containing medium. A non-transduced control was run in parallel to confirm no viable cells were present after selection.

2.15.5 KLF2 overexpression

The FUW-tetO-loxP-hKLF2 transfer vector uses the Tet-On system to allow for doxycycline-inducible overexpression of human KLF2. For overexpression in HUVECs, cells were seeded at 2×10^4 cells/well in a 24-well plate and transduced with hKLF2 lentivirus at an MOI of 10 and 20 in the presence of polybrene. 24 hours after transduction, medium containing 2 µg/ml doxycycline (#ab141091, Abcam, Cambridge, UK) was added to the cells, to induce expression of the transgene. 72 hours after doxycycline addition, cells were harvested for RT-qPCR analysis.

2.15.6 Generation of RAW 264.7^{Cas9} cells

A RAW 264.7 macrophage cell line stably expressing Cas9 was generated for further use in CRISPR/Cas9 mediated gene editing experiments (see 2.18), following a protocol adapted from the literature (Joung et al., 2017). 5×10^5 cells were placed in a 6-well plate, Cas9 lentiviral particles were added at an MOI of 0.4 in the presence of polybrene, and cells were spinfected by spinning the plate at 1,000 x g for 2 hours at 33 °C. After 24 hours the medium was changed, and 48 hours after infection blasticidin S (12.5 µg/ml, #10264913, Fisher Scientific) was added to select for transduced cells. The blasticidin resistant cells were expanded for 2 to 3 weeks, after which cells stocks were frozen. A non-transduced control was run in parallel to confirm no viable cells were present after selection.

2.16 Short interfering (si) RNA-mediated gene knockdown

For silencing KLF2 expression in HUVECs, 5×10^4 cells were transfected in a 24-well format with an siPOOL™ targeting the KLF2 gene, or a negative control siPOOL™ (siTools Biotech, Planegg/Martinsried, Germany), using the riboxxFECT transfection reagent (Riboxx, Radebeul, Germany). A reverse transfection protocol, according to the manufacturer's instructions, was followed. The final concentration of siRNA was 3–10 nM.

2.17 Morpholino-mediated Gilz silencing and mRNA-mediated overexpression in zebrafish

The gene silencing/overexpression experiments in zebrafish were performed by the research group of Professor William K.F. Tse (Faculty of Agriculture, Kyushu University, Fukuoka, Japan). Zebrafish from the AB wildtype strain were raised and staged as previously described (Kimmel et al., 1995). All experimental procedures were approved by the Animal Experimental Committee, Kyushu University.

2.17.1 Morpholino (MO) design and mRNA synthesis

The Gilz MO and mRNA experiments were performed as previously published (Tse et al., 2013). The sequence of *tsc22d3* MO, targeting the ATG site, was 5′-GATTTTTGAACATCTC CGTGCTCAT-3′ (Gene Tools, Philomath, OR, USA). Its efficiency and specificity were confirmed by splicing MO, mRNA rescue, and Western blot analysis.

tsc22d3 mRNA was amplified from full-length cDNA using Pfu DNA polymerase (Agilent Technologies, Santa Clara, CA, USA) and the following primers: forward 5′-CCGAATTCATGAGCACAGAGATGTTCAA-3′, reverse 5′-CCCTCGAGTTATACAGCAG AGCCCCGT-3′, that contain EcoRI and XhoI restriction sites, respectively. The digested PCR products were ligated into the pCS2+ vector to generate the pCS2+tsc22d3 expression construct. The construct vector was linearized by digestion with NotI, and capped RNA was *in vitro* transcribed with the mMESSAGE mMACHINE™ SP6 Transcription Kit (#AM1340, Thermo Fisher Scientific), following the manufacturer's instructions.

2.17.2 MO and mRNA Injection

Two pmol of *tsc22d3* MO or 1,200 pg of mRNA were injected into 1- or 2-cell stage embryos. Embryos from 4 different fish pairings were used for each injection. At 24 hpf, embryos were collected and fixed for whole-mount immunofluorescence staining, as described under 2.11.

2.18 CRISPR/Cas9-mediated GILZ knockout

The clustered regularly interspaced short palindromic repeats (CRISPR) / CRISPR-associated (Cas) 9 technique was used to disrupt the GILZ gene in HMEC-1 and C2C12 cells, using a transient transfection and clonal expansion approach. Validated synthetic TrueGuide™ single guide (sg) RNAs were obtained from Thermo Fisher Scientific. Target sequences were for *TSC22D3* 5'-ACTTACACCGCAGAACCACC-3', and for *Tsc22d3* 5'-GATGTACGCTGTG AGAGAGG-3'.

Cells at 30–70% confluency were transfected in a 12-well format with the synthetic sgRNA and a recombinant Cas9 protein (TrueCut™ Cas9 Protein v2, #A36496, Thermo Fisher Scientific), using the Lipofectamine™ CRISPRMAX™ Cas9 transfection reagent (#CMAX00001, Thermo Fisher Scientific), following the manufacturer's instructions. 48 hours after transfection, cells were detached and counted. A fraction of the cells was used for clonal expansion, following the limiting dilution cloning procedure for HMEC-1 (seeding cells at 0.8 cells/well in 96-well plates), or using cloning discs for isolation of single colonies from C2C12 seeded at low density (50, 250, 1,000 and 5,000 cells) in 6 cm cell culture dishes (Domann and Martinez, 1995). The remaining cells were used for gDNA extraction and verification of editing efficiency. The surviving clones after clonal expansion were harvested for gDNA extraction, PCR amplification of the edited region and sequencing analysis, amplified and kept either in culture or frozen, until confirmation of the knockout was achieved.

2.18.1 Verification of editing efficiency

The T7E1 endonuclease assay was performed on gDNA from control and transfected cells to verify the CRISPR genome editing in the mixed population. The assay is based on the ability of T7E1 to cleave mismatched DNA heteroduplexes, formed between WT and CRISPR-mutated DNA strands after denaturation and reannealing of a PCR product amplified from the genomic target region. To this end, the Alt-R™ Genome Editing Detection Kit (#1075931, Integrated DNA Technologies, Skokie, IL, USA) was used according to the manufacturer's instructions. The primers used for PCR amplification of the edited region were: *TSC22D3* forward 5'-GCTTGGTGTTACTAGGCCCC-3', reverse 5'-AGGATAGAGCTTCAGGCA CAA-3', *Tsc22d3* forward 5'-ACAGAAGGCTGACTTGGCTC-3', reverse 5'-GGCCAC GATGGCTAAGGAAT-3'. The annealing temperature for the PCR, run with the 5xHotFirePOl EvaGreen qPCR Mix under the program described in 2.6, was set at 64.4 °C.

2.18.2 Sequencing analysis

gDNA was extracted with QuickExtract® DNA Extraction Solution (#101094, Biozym, Hessisch Oldendorf, Germany). PCR of the edited region was performed as mentioned above,

the PCR products were purified and sent for analysis *via* Sanger sequencing. The sequencing primer sequences were: *TSC22D3* 5'-GCCACGATGGCTAAGGAATG-3', *Tsc22d3* 5'-GAT AGAGCTTCAGGCACAATATAG-3'. Analysis of the sequencing files for indel detection was performed with assistance of the CRISP-ID web application (Dehairs et al., 2016), and the ICE analysis tool (Synthego, Redwood City, CA, USA).

2.19 Serum Analysis

Serum collected from young and aged mice was sent to the "*Zentrallabor des Universitätsklinikums des Saarlandes*" for clinical chemistry analysis. The determination of corticosterone and 11-dehydrocorticosterone (11-DHC) levels in serum was performed by the research group of Professor Markus Meyer (Centre for Molecular Signalling (PZMS), Saarland University), as described under 2.22.

2.20 Thiobarbituric acid reactive substances (TBARS) assay

Products of lipid peroxidation were measured in liver tissue from young and aged mice, following a previously described protocol (Simon et al., 2014). The total TBARS are expressed as µmol malondialdehyde (MDA) equivalents per µg of total protein, quantified using the Pierce™ BCA Protein Assay Kit.

2.21 Determination of 11β-HSD1 activity in peritoneal macrophages

The determination of 11β-HSD1 activity was performed by Rebecca Linnenberger and Dr. Jessica Hoppstädter. The activity of 11β-HSD1 in PMs from young and aged mice was measured incubating cells with 0.1 µM or 1 µM cortisone-D_8 for 24 hours. At the end of treatment, supernatants were collected and analysed as described under 2.22 to measure cortisone-D_8 and cortisol-D_8 levels. The conversion to cortisol-D_8 was expressed as percentage of the total steroid measured, and is presented per µg of total protein, quantified using the Pierce™ BCA Protein Assay Kit.

2.22 Determination of steroid levels via LC-HRMS/MS

The determination of steroid levels was performed by the research group of Professor Markus R. Meyer (Centre for Molecular Signalling (PZMS), Saarland University), using liquid chromatography–high resolution mass spectrometry (LC-HRMS/MS). Samples were mixed 1:1 with an internal standard (cortisol-D_4, 100 µg/ml in acetonitrile containing 0.1% formic acid), and centrifuged for 10 min at 18,407 x *g* and -10 °C. The supernatant was transferred into an MS vial and a volume of 5 µl was used for analysis.

Chromatographic separation of the analytes was carried out on a Dionex Ultimate UHPLC System (Thermo Fisher Scientific) using an Accucore^{TM} Phenyl Hexyl LC column (100 mm x 2.1 mm, 2.6 µm) heated to 40 °C. Mobile phase A was water with 0.1% formic acid and ammonium formate, mobile phase B was acetonitrile with 0.1% formic acid. The LC gradient was as follows: starting with 2% solvent B over 0.1 min, the gradient was increased to 98% solvent B until 5 min, maintained at 98% until 7.5 min, and decreased to 2% until 10 min. The flow rate was set to 600 µl/min. Detection of analytes was achieved *via* high resolution mass spectrometry on a ThermoFisher Q-Exactive Plus equipped with a heated electrospray ionization (HESI)-II source. The HESI-II source conditions were as follows: sheath gas, nitrogen, at 55 arbitrary units; auxiliary gas, nitrogen, at 15 arbitrary units; temperature, 450 °C; spray voltage, 3.50 kV; ion transfer capillary temperature, 275 °C; and S-lens RF level, 55.0. Mass spectrometry was done in positive polarity mode using targeted single ion monitoring (tSIM) mode. The settings for tSIM mode were as follows: resolution, 35,000; microscans, 1; AGC target, 5e4; maximum IT, 200 ms; isolation window, 2.0 m/z; normalised collision energy (NCE), 35; scan range, m/z 150–900; spectrum data type, profile; and underfill ratio, 0.5%. The peak areas of the analytes were normalised using the internal standard peak area ratio.

2.23 Statistics

Results are expressed as mean ± SEM (standard error of the mean). Statistically significant differences between means were determined using the GraphPad Prism 6.0 or Origin 2019 software. Unless stated otherwise, an unpaired Welch's *t*-test was performed for the comparison of two groups, and the comparison of three or more groups was carried out by one or two-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc analysis for individual differences. Where specified, median comparison of two groups was performed using Mann Whitney U Test; and median comparison of three or more groups was carried out by Kruskal-Wallis test followed by Dunn's post-hoc analysis. Results were considered significant at $P<0.05$.

3. Chapter I

The glucocorticoid-induced leucine zipper (GILZ) mediates statin-induced muscle damage

3.1 Introduction

Statins are the first line of treatment in the management of hyperlipidaemia and prevention of CVD (Catapano et al., 2016; Grundy et al., 2018). These drugs are inhibitors of the HMG-CoA reductase enzyme, preventing the biosynthesis of cholesterol in the liver by blocking the mevalonate pathway and, as a consequence, enhancing clearance of circulating LDL-cholesterol (Sirtori, 2014). Since their introduction in 1987, statin prescription rates have risen, as shown by several studies in different populations (Nolte et al., 2010; Salami et al., 2017; Vancheri et al., 2016), positioning them among the most prescribed drug classes worldwide. Given the high prevalence of CVD, and the favourable data on CVD prevention by statins (Heller et al., 2017; Taylor et al., 2013), this tendency seems to be maintained.

Statins have a satisfactory safety profile, their most relevant adverse effect being skeletal muscle toxicity (Grundy et al., 2018). SAMS have an incidence of 5–29% in clinical practice (Grundy et al., 2018; Stroes et al., 2015), can range from mild myalgia to, in rare cases, fatal rhabdomyolysis (Moßhammer et al., 2014), and are a frequent cause for non-adherence to treatment or its discontinuation (Laufs et al., 2017). Although several studies have been conducted, and a number of risk factors that contribute to the onset of SAMS have been described, such as sex, pharmacokinetic differences, or genetic factors (Needham and Mastaglia, 2014; du Souich et al., 2017), the molecular mechanisms leading to myopathy are still not fully understood.

GILZ was first described as a dexamethasone-induced, immunomodulatory protein (D'Adamio et al., 1997). Since then, it has been shown that GILZ expression is not restricted to immune cells but is extended to several tissues and cell types, including skeletal muscle (Cannarile et al., 2001). GILZ plays multiple roles in both, glucocorticoid and non-glucocorticoid-mediated cellular processes (Ayroldi and Riccardi, 2009) beyond its immune-modulating function. For instance, studies demonstrated a role for GILZ in inhibition of adipocyte differentiation (Shi et al., 2003), in sodium homeostasis in the kidney, where it is robustly induced by aldosterone (Rashmi et al., 2017; Soundararajan et al., 2005), and in spermatogenesis, where lack of GILZ leads to hyperactivity of Ras signalling pathway and apoptosis during meiosis (Bruscoli et al., 2012). Of particular interest is the role described for GILZ in the regulation of skeletal muscle differentiation: it was shown that GILZ is strongly induced after dexamethasone treatment. In this setting, GILZ mediated dexamethasone anti-myogenic action *via* inhibition of the transcriptional activity of an early myogenic regulatory factor (MRF), MyoD (Bruscoli et al., 2010).

We analysed different publicly available gene expression profiling datasets that suggest statins were able to induce GILZ in different cell types (GEO accession numbers GDS2987, GSE32547, GSE4883). Interestingly, the analysis of a transcription profiling of human *quadriceps femoris* muscle following statin treatment suggested a moderate increase in *GILZ* expression in biopsies from patients receiving atorvastatin and simvastatin for 8 weeks, compared with those receiving placebo (ArrayExpress dataset E-TABM-116 (Laaksonen et al., 2006), Figure 3.1). In the present study, we aimed to test the hypothesis that GILZ plays a role in the onset of SAMS, focusing on both, myotoxic and anti-myogenic effects.
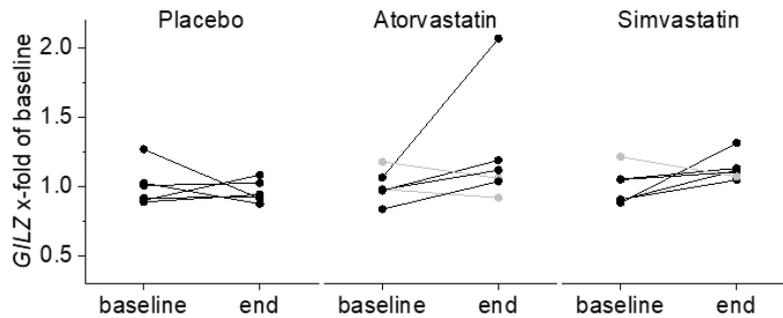
Figure 3.1. Analysis of the publicly available ArrayExpress dataset E-TABM-116: transcription profiling of human quadriceps femoris muscle following statin treatment. *GILZ* mean expression (NCBI RefSeq NM_004089.3; scan REFs GI_37622900-A, GI_37622900-I) is shown as fold of baseline. Connected data points represent patients before and after intervention.

3.2 Results

3.2.1 *Statins are toxic towards skeletal muscle cells*

We used the MTT assay to measure cell viability of C2C12 myoblasts and differentiating myotubes treated with increasing concentrations of atorvastatin, cerivastatin, simvastatin in its lactone and acid forms, and pravastatin, for 24 hours. All statins were toxic towards myoblasts, although higher concentrations of the hydrophilic statin pravastatin were needed to cause a significant decrease in cell viability (Figure 3.2 A). In myotubes, neither pravastatin nor simvastatin acid was significantly toxic at the evaluated concentrations (Figure 3.2 B).
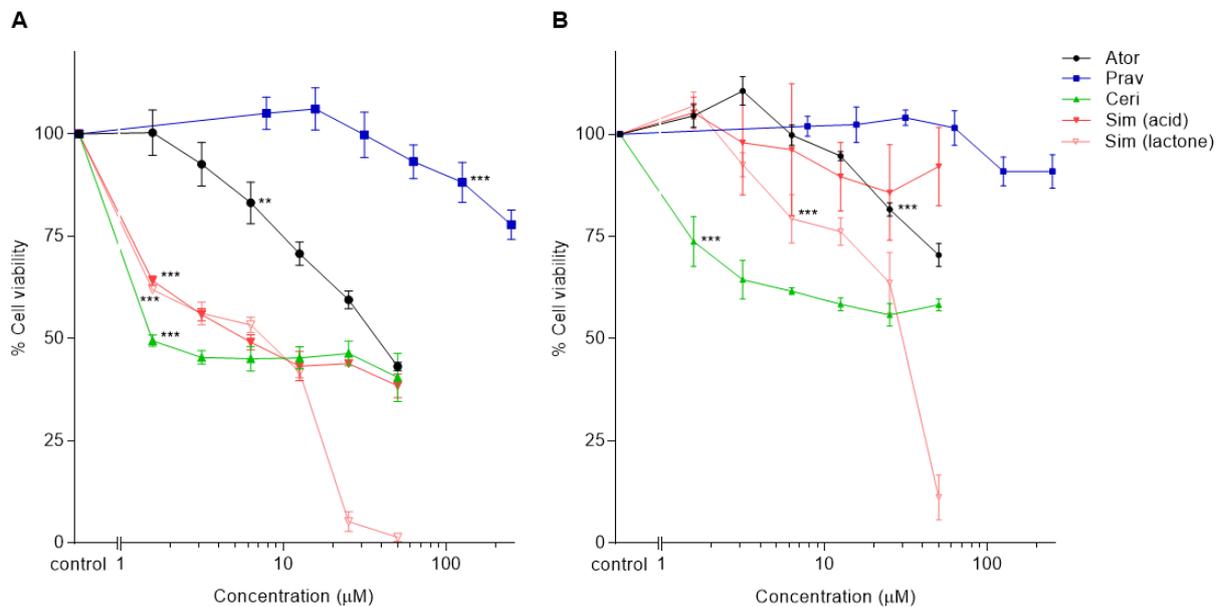
Figure 3.2. Statin cytotoxicity towards muscle cells. C2C12 myoblasts (**A**) or 48-hours myotubes (**B**) were treated with increasing concentrations of atorvastatin (Ator), cerivastatin (Ceri), pravastatin (Prav), simvastatin (Sim) acid or simvastatin lactone for 24 hours. The cell viability was measured via MTT assay. Data show the mean of three independent experiments performed in replicates ± SEM. **$P < 0.01$, ***$P < 0.001$ for the lowest concentration that caused a significant decrease in viability relative to the control.

In accordance with the literature (Skottheim et al., 2008; Taha et al., 2016), comparison of the dose-response curves also showed that the lactone form of simvastatin was more toxic towards muscle cells than the active acid form of the drug [statin main effect, myoblasts $F_{(1,24)}=115.8$, $P<0.001$; myotubes $F_{(1,24)}=21.16$, $P<0.001$].

3.2.2 *Non-toxic concentrations of statin induce GILZ in skeletal muscle cells*

To first evaluate whether GILZ expression was modified in muscle cells in the presence of statins, we treated C2C12 myoblasts with a non-toxic concentration of atorvastatin (3 µM) for up to 24 hours and measured the levels of *Gilz* mRNA at different time points. A significant

increase compared with the control was detected after 8 hours of treatment, to a level of induction that was maintained until the end of treatment (Figure 3.3).
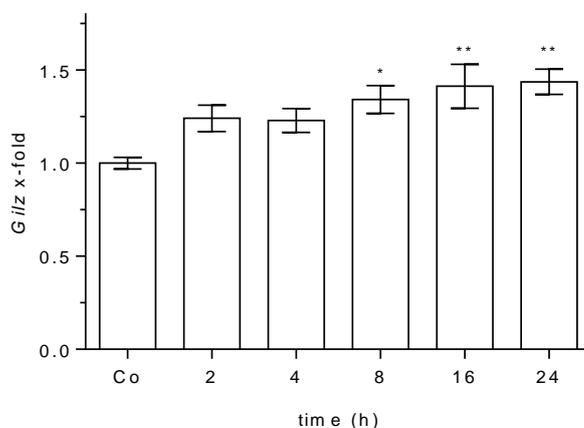
Figure 3.3. Effect of statin treatment on *Gilz* mRNA expression in C2C12. C2C12 myoblasts were treated with atorvastatin (3 µM) for up to 24 hours. *Gilz* expression was normalised against the housekeeping gene (*Rn18s*) and is presented as fold change of control. Data show the mean of 3 independent experiments performed in replicates ± SEM. *P<0.05, **P<0.01, relative to the control.

3.2.3   Toxic concentrations of statin induce GILZ expression

Next, we treated myoblasts and myotubes with 50 µM atorvastatin, simvastatin, or cerivastatin. At this concentration all statins were cytotoxic after 24 hours, but not at 6 hours (Figure 3.2 and data not shown). We detected an increase in *Gilz* mRNA expression after 6 hours of treatment with all statins, which was reversed by the addition of mevalonate (100 µM) to the medium, indicating that the mechanism of GILZ induction relates to the inhibition of HMG-CoA reductase (Figure 3.4 A–B). In myotubes, the increase in *Gilz* expression was modest compared with that of myoblasts. Western blot analysis showed an elevated expression of GILZ protein in both, C2C12 and primary murine myoblasts treated with toxic concentrations of statins (Figure 3.4 C–F).

3.2.4   GILZ induction can be reversed by geranylgeranyl pyrophosphate

The inhibition of HMG-CoA by statins not only impairs cholesterol biosynthesis, but also other biosynthetic pathways. Of main importance is the inhibition of protein prenylation resulting from decreased synthesis of the isoprenoid derivatives GGPP and FPP. Thus, we evaluated whether GILZ induction could be reversed by the addition of any, or both, of these mediators to the medium. Co-treatment of C2C12 myoblasts with 10 µM GGPP in addition to simvastatin completely reversed *Gilz* induction, while FPP had no effect (Figure 3.4 G).
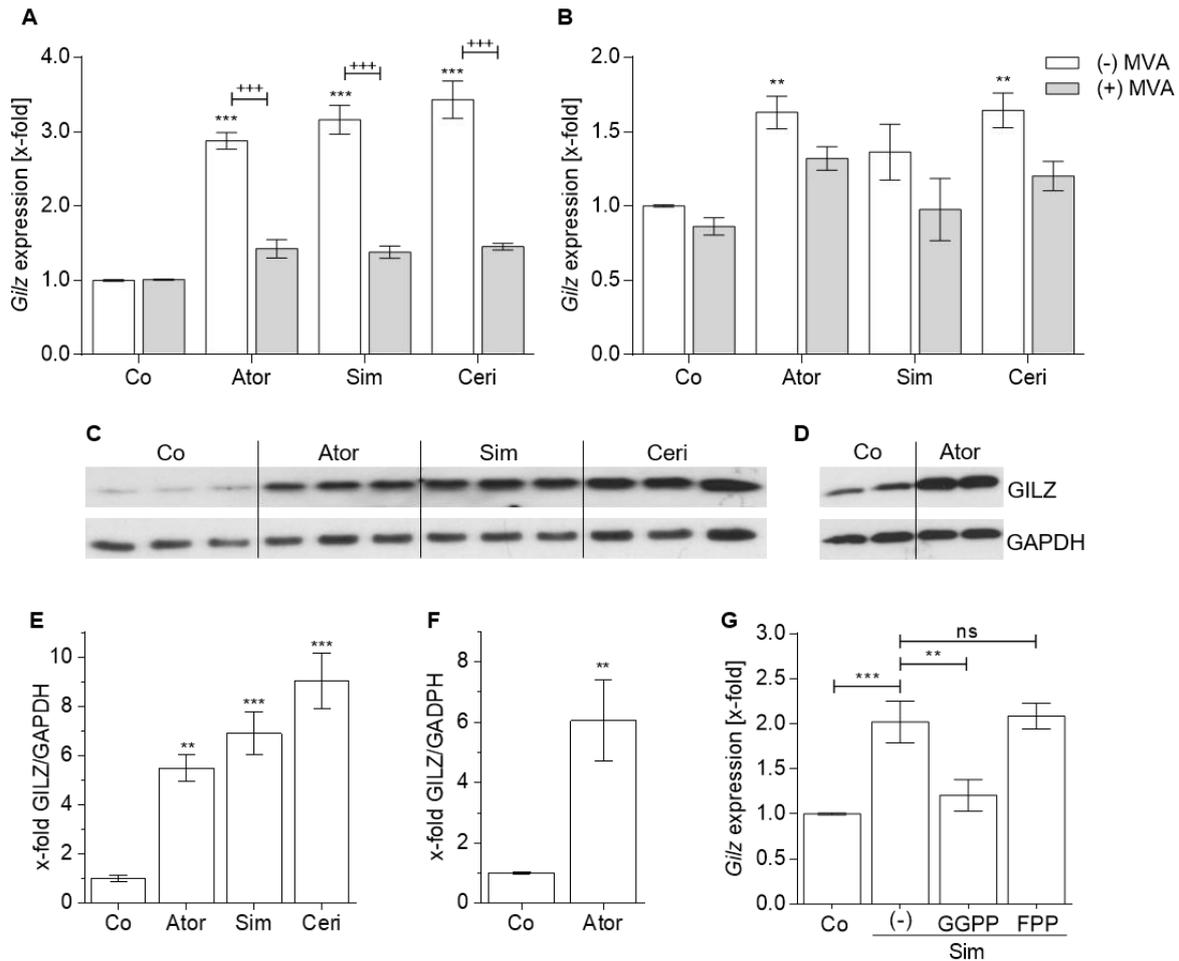
Figure 3.4. Effect of statin treatment at toxic concentrations on GILZ expression in muscle cells. C2C12 myoblasts (**A**) or 72-hour myotubes (**B**) were treated with 50 µM statin in the absence or presence of mevalonate (MVA, 100 µM) for 6 hours, *Gilz* mRNA expression was measured. GILZ protein expression was measured in C2C12 (**C**) and primary murine (**D**) myoblasts after treatment with 50 µM statin for 6 hours. One representative blot is shown. Densitometric analysis, normalised to the housekeeping protein (**E,F**). C2C12 myoblasts were treated with 50 µM simvastatin in the absence or presence of GGPP and FPP (10 µM) for 6 hours, *Gilz* mRNA levels were measured (**G**). mRNA expression was normalised against the housekeeping gene (*Csnk2a2*) and is presented as fold change of control. Data show the mean of at least 2 independent experiments performed in replicates ± SEM. **$P<0.01$, ***$P<0.001$ relative to the corresponding control, +++ $P<0.001$ relative to the non-MVA condition.

3.2.5   Statin-induced impairment of myogenesis is accompanied by GILZ induction

Myogenesis is a multistep, tightly regulated process that leads to the formation of skeletal muscle, both during embryonic development as well as in adult life, to maintain muscle homeostasis and repair muscle after injury (Bentzinger et al., 2012). Statins are not only toxic towards skeletal muscle fibres but might also impair the muscle regeneration process (Baba et al., 2008; Ogura et al., 2007; Trapani et al., 2012). To examine these effects, we induced differentiation of C2C12 myoblasts by switching confluent cell layers to differentiation medium (DM) in the absence or presence of statins, and evaluated myotube formation by Jenner-Giemsa staining and myosin heavy chain (MHC) immunofluorescence. We observed that treatment of C2C12 cells with sub-toxic concentrations of statins during differentiation resulted in less

myotube formation, and a significantly reduced fusion index after 6 days, where fully differentiated myotubes were visible in the control (Figure 3.5).
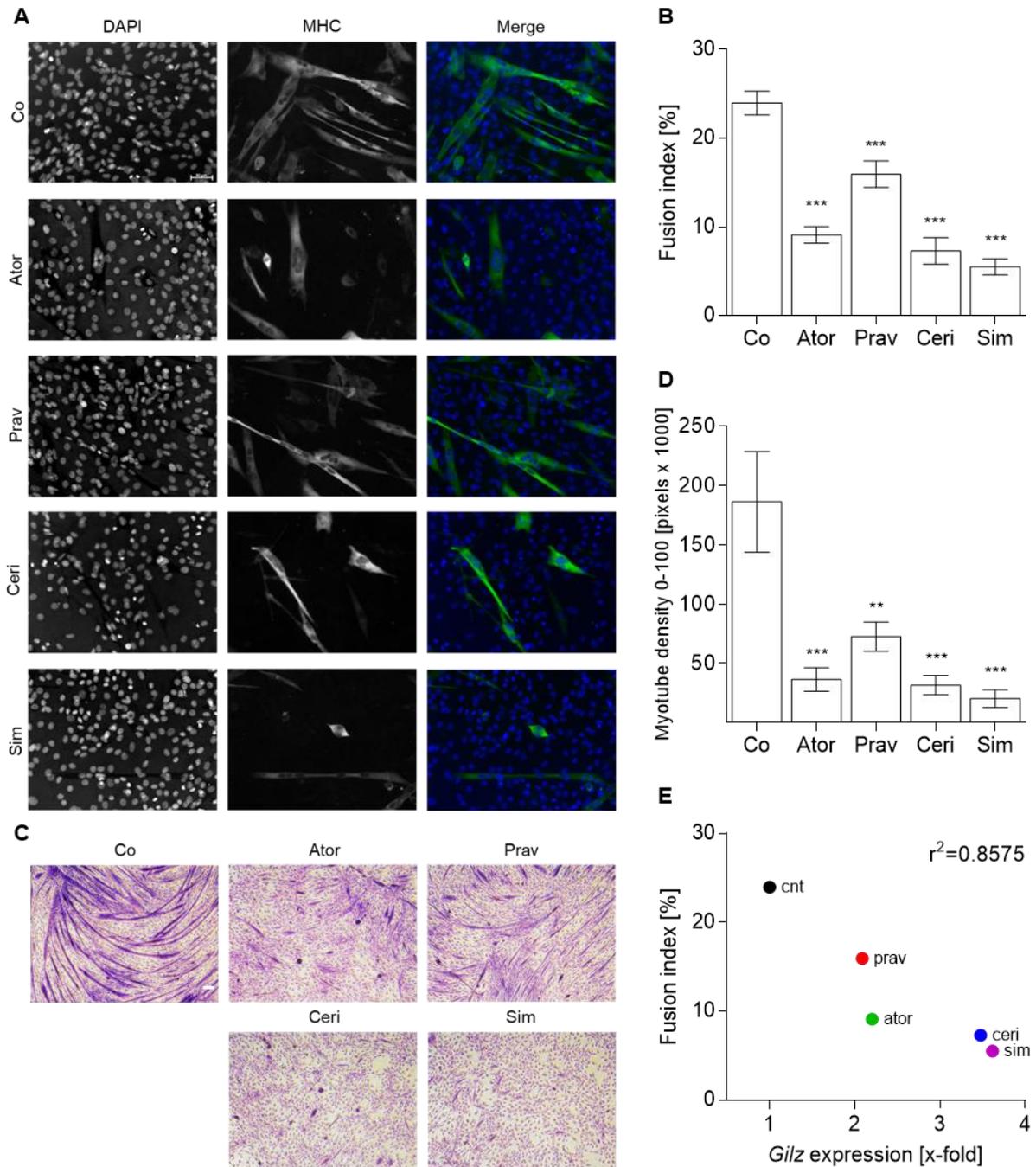
Figure 3.5. Effect of statins on C2C12 differentiation. C2C12 were induced to differentiate for 6 days in the absence or presence of atorvastatin (5 µM), pravastatin (10 µM), cerivastatin (0.1 µM), and simvastatin (1 µM), and subjected to MHC IF (**A**, scale bar, 50 µm) or Jenner-Giemsa staining (**C**, scale bar, 100 µm). Representative images are shown. The degree of differentiation was quantified by measuring the fusion index in IF images (**B**), and myotube density in Jenner-Giemsa images (**D**). Correlation between the degree of *Gilz* mRNA induction after 72 hours of statin stimulation (Figure 3.6 A) and fusion index (**E**). Data show the mean of 3 independent experiments performed in replicates ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ relative to the control.

We analysed GILZ expression levels in differentiating C2C12 and primary murine myotubes treated with statins, finding that the impairment in myogenesis was also accompanied by GILZ induction on both, mRNA and protein level (Figure 3.6). Pearson correlation analysis showed a negative correlation between the fold increase in *Gilz* mRNA levels, and the fusion index (r^2=0.8575). Since GILZ has been shown to mediate the anti-myogenic effects of dexamethasone (Bruscoli et al., 2010), we hypothesised that it might also be involved in the effects observed after statin treatment.
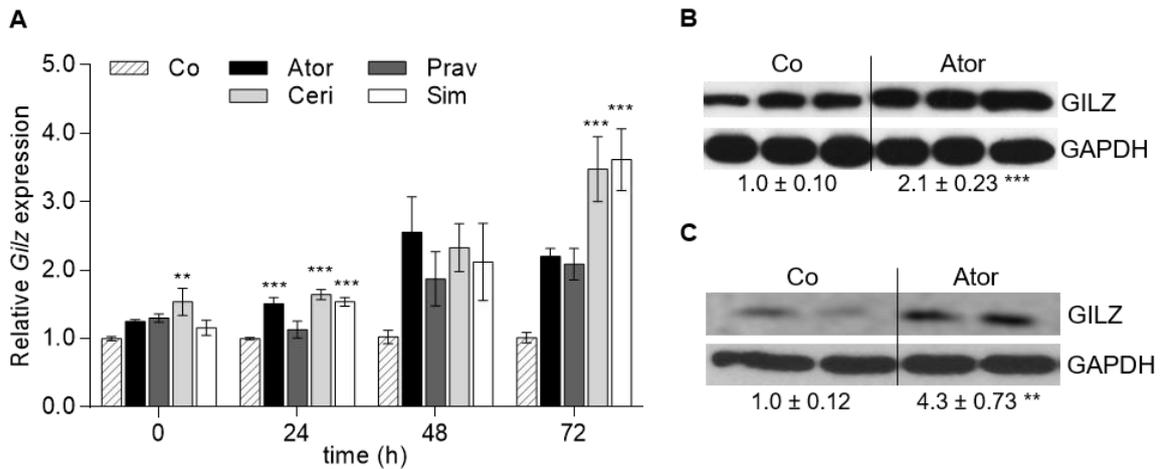
Figure 3.6. Effect of statins on GILZ expression during myogenesis. C2C12 were induced to differentiate for 72 hours in the absence or presence of atorvastatin (5 µM), pravastatin (10 µM), cerivastatin (0.1 µM), and simvastatin (1 µM). *Gilz* expression data was normalised against the housekeeping gene (*Csnk2a2*) and is presented as fold change of control (**A**). GILZ protein levels in C2C12 (**B**) and primary murine (**C**) myotubes treated with atorvastatin (5 µM) during myogenesis. Representative blots are shown. Data show the mean of 3 independent experiments ± SEM. *P<0.05; **P<0.01, ***P<0.001 relative to the control.

3.2.6   GILZ knockout abolishes the cytotoxic effects of statins

To assess whether loss of GILZ could rescue statin-induced cytotoxicity, we isolated primary myoblasts from WT and GILZ KO mice, treated them with increasing doses of statins, and measured cell viability after 24 hours. Comparison of the dose-response curves obtained showed significant differences between genotypes: GILZ KO myoblasts were significantly less sensitive towards atorvastatin, cerivastatin, and simvastatin treatment than their WT counterparts [cell type main effect $F_{(1,124)}$=10.06, P=0.002; $F_{(1,124)}$=26.24, P<0.0001; and $F_{(1,116)}$=24.18, P<0.0001, respectively, Figure 3.7 A–C].

Due to the importance of the PI3K/Akt signalling pathway in statin-induced myotoxicity (Bonifacio et al., 2015; Mallinson et al., 2009), we hypothesised that the resistance to cell death from GILZ KO myoblasts might be related to modulation of the Akt phosphorylation status. Indeed, atorvastatin treatment induced dephosphorylation of Akt and activated the apoptotic pathway, as observed by cleaved caspase-3 detection, in WT myoblasts.

On the other hand, Akt phosphorylation levels were restored to that of the control in statin-treated GILZ KO cells, and the active form of caspase-3 was undetectable (Figure 3.7 D–E).
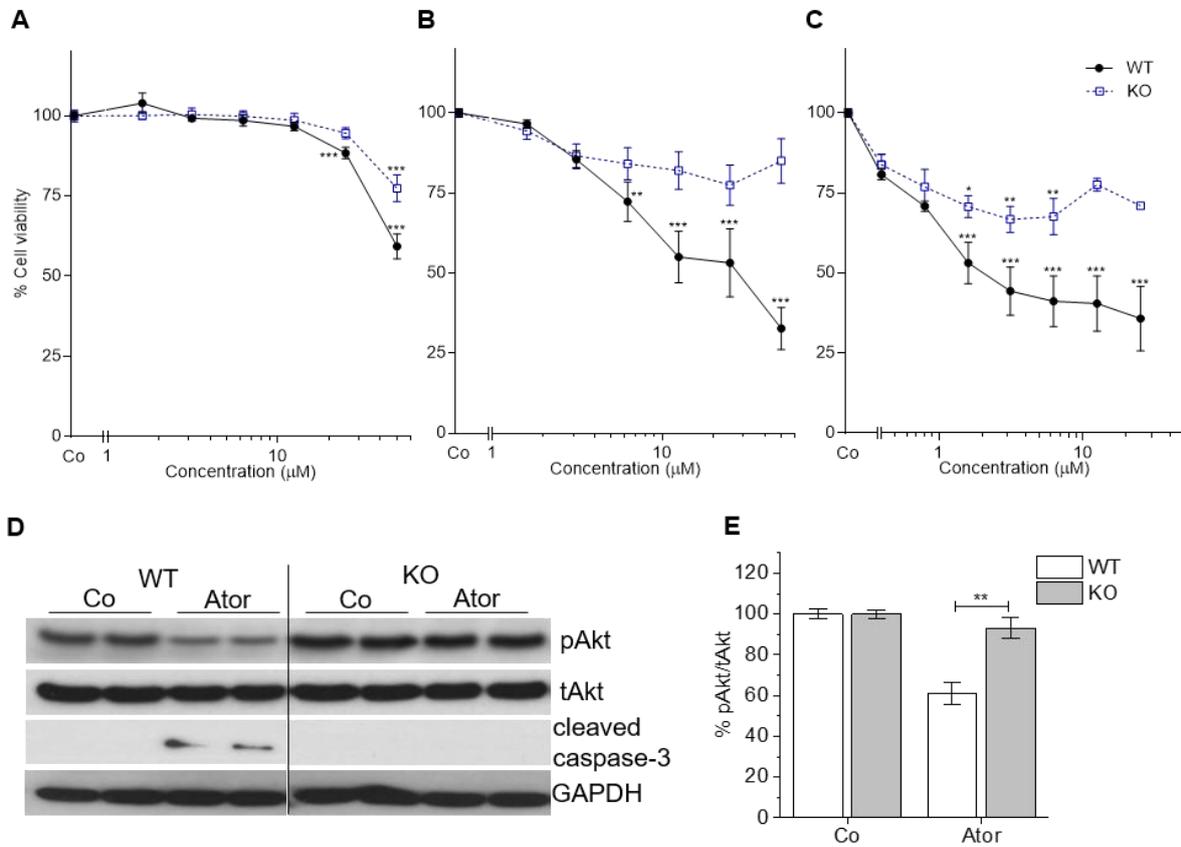
Figure 3.7. Effect of GILZ knockout on statin-induced myotoxicity. WT and GILZ KO primary murine myoblasts were treated with atorvastatin (**A**), simvastatin (**B**) or cerivastatin (**C**) in increasing concentrations for 24 hours. The cell viability was measured *via* MTT assay and differences between curves were analysed by two-way ANOVA. Myoblasts were treated with atorvastatin (50 µM) for 6 hours, Akt phosphorylation and caspase-3 activation were measured by western blot. One representative blot is shown (**D**). The ratio of phosphorylated to total Akt was measured by densitometric analysis and normalised to the corresponding control (**E**). Data show the mean of at least 3 independent experiments performed in replicates ± SEM. *P<0.05, **P<0.01, ***P<0.001 relative to the control.

As an *ex vivo* model for the evaluation of statin myotoxicity, we used *flexor digitorum brevis* myofibres isolated from WT and KO animals. These short fibres can be isolated, dissociated, and cultured, representing a more mature system for evaluation of statin effects than cell culture systems, since myoblasts *in vitro* can differentiate to myotubes but are unable to form fully differentiated myocytes and, therefore, do not exactly resemble the features of mature muscle (Ravenscroft et al., 2007; Sakamoto and Kimura, 2013). Treatment of FDB fibres from WT mice with atorvastatin, simvastatin, and cerivastatin induced vacuolation at 72 hours. After 5 days the sarcomere structures were no longer visible, the fibres were swollen, ruptured, and blebs appeared (Figure 3.8 A and data not shown). Viability analysis using the trypan blue exclusion method after 5 days indicated that statins induced fibre death in a dose-dependent

manner. Loss of GILZ prevented the morphological changes indicative of myotoxicity and made the fibres resistant towards statins: in KO fibres, there were no significant differences in viability between the control and treatments at any of the concentrations evaluated (Figure 3.8 A–B).
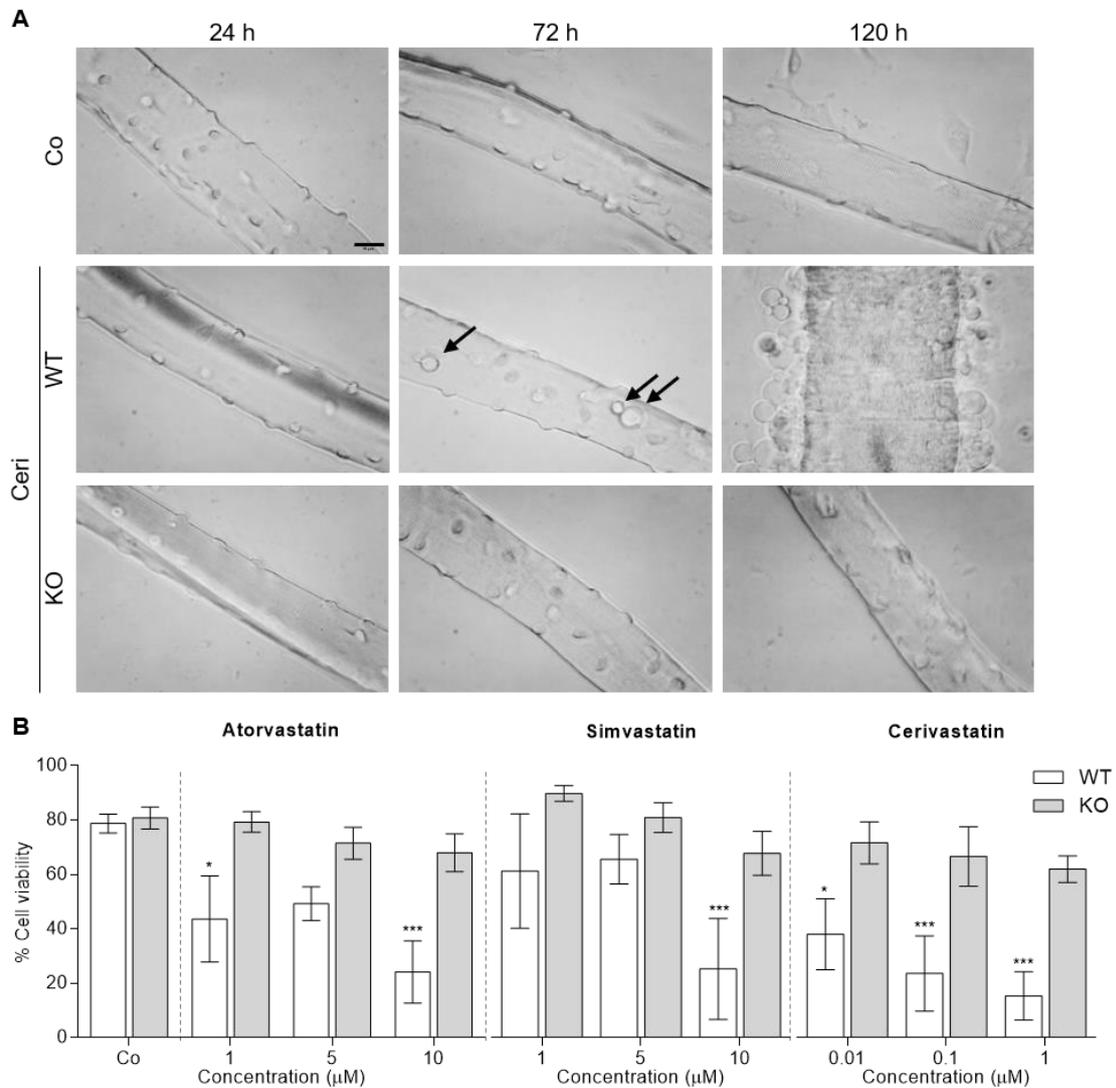
Figure 3.8. Effect of GILZ knockout on statin-induced FDB myofibre toxicity. WT and GILZ KO FDB myofibres were treated with cerivastatin (1 µM) for up to 120 hours and imaged for morphological analysis, representative pictures are shown (**A**). Scale bar, 20 µm. Cell viability was measured after 120 h of treatment, using the trypan blue exclusion method (**B**). Data show the mean of 4 independent experiments performed in replicates ± SEM. *P<0.05, ***P<0.001 relative to the corresponding control.

3.2.7   GILZ contributes to statin-induced inhibition of myogenesis

To investigate the contribution of GILZ to statin-induced inhibition of myogenesis, we differentiated WT and GILZ KO myoblasts in the presence of statins and evaluated the expression of the MRF myogenin, which is required in cells committed to the myogenic program for driving their fusion and terminal differentiation (Bentzinger et al., 2012). We observed a decrease in myogenin protein levels in WT primary murine myoblasts induced to differentiate for 72 hours in the presence of atorvastatin. By contrast, the expression of myogenin in atorvastatin treated GILZ KO cells was restored to that of the control (Figure 3.9), providing evidence that GILZ acts as a mediator of the anti-myogenic effects of statins.
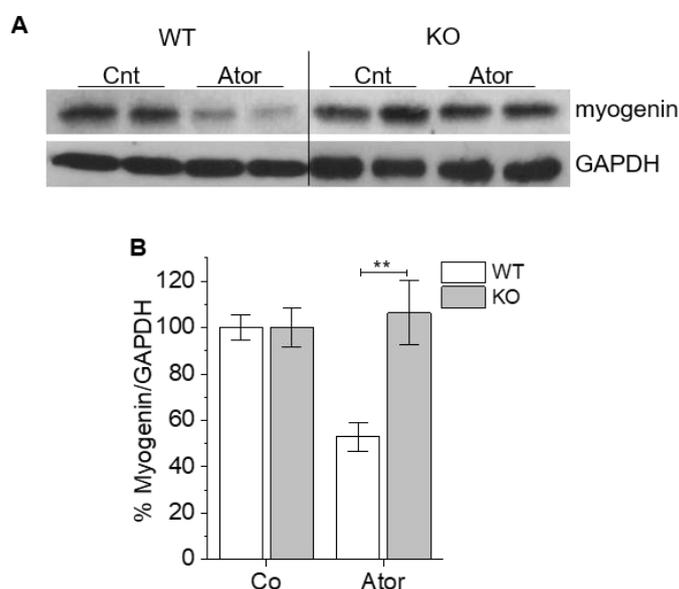
Figure 3.9. Effect of GILZ knockout on statin-induced anti-myogenic effects. Primary murine myoblasts from WT and GILZ KO animals were induced to differentiate for 72 hours in the absence or presence of atorvastatin (5 µM). Myogenin levels were measured *via* western blot, one representative blot is shown (**A**). Densitometric analysis (**B**). Data show the mean of 3 independent experiments ± SEM. **P<0.01 relative to the control.

To further analyse the role of GILZ in these effects, we generated C2C12 cell lines stably expressing scrambled or GILZ shRNA constructs by lentiviral transduction. The C2C12^{shGilz1} and C2C12^{shGilz2} cell lines showed a degree of gene knockdown of approximately 70% and 60%, respectively, whereas in the scrambled control cell line, C2C12^{scr}, *Gilz* expression was unaffected (Figure 3.10).
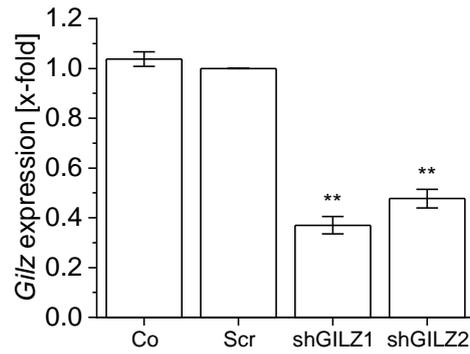
Figure 3.10. *Gilz* expression in stable C2C12[shGilz] cell lines. Scrambled and shGILZ-silenced C2C12 cell lines were generated by lentiviral transduction, *Gilz* mRNA expression was normalised against the housekeeping gene (*Csnk2a2*) and is presented as fold change of the control. Data show the mean of 3 independent experiments ± SEM. **$P<0.01$ relative to scrambled cells.

We hypothesised that, as a result of restored myogenin expression, *Gilz* silencing should reinstate the ability of the myoblasts to differentiate in the presence of statins. Importantly, we found that C2C12[shGilz1] and C2C12[shGilz2] cells displayed terminally differentiated myotubes already after 4 days in DM, whereas C2C12[scr] cells were not fully differentiated yet (Figures 3.11 A and 3.12 A, upper panels). GILZ knockdown, however, could only partially reverse the impairment in differentiation caused by statins: although Jenner-Giemsa staining showed myotube formation in the C2C12[shGilz] lines (Figure 3.12, lower panels), these cells were not differentiated to the same extent as their controls. Nevertheless, fusion index analysis of MHC-stained cells showed an improvement in myotube formation from C2C12[shGilz] cells compared with the C2C12[scr] line (Figure 3.11 B).
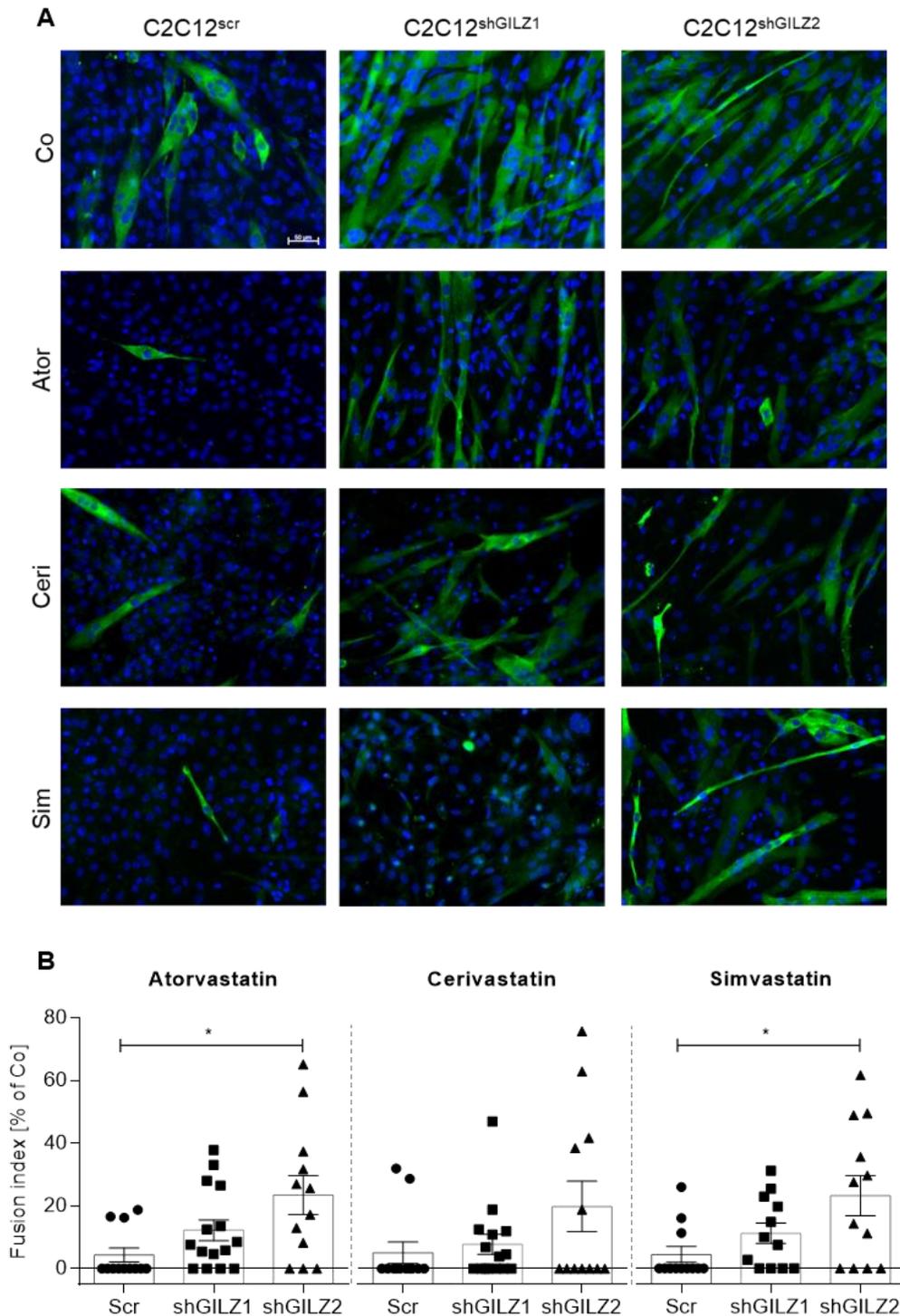
Figure 3.11. Effect of GILZ absence on statin-induced anti-myogenic effects, as measured by MHC IF. Scrambled (Scr)- and shGILZ-C2C12 cell lines were induced to differentiate for 4 days in the absence or presence of atorvastatin (5 µM), cerivastatin (0.1 µM), or simvastatin (1 µM), and subjected to MHC IF, representative images are shown (**A**). Scale bar, 50 µm. The degree of differentiation was quantified by measuring the fusion index (**B**) and is presented relative to the controls. Bars show the mean of at least 3 independent experiments performed in replicates ± SEM, data points correspond to each quantified field. Comparison was performed using Kruskal-Wallis test followed by Dunn's post-hoc analysis. *P<0.05.
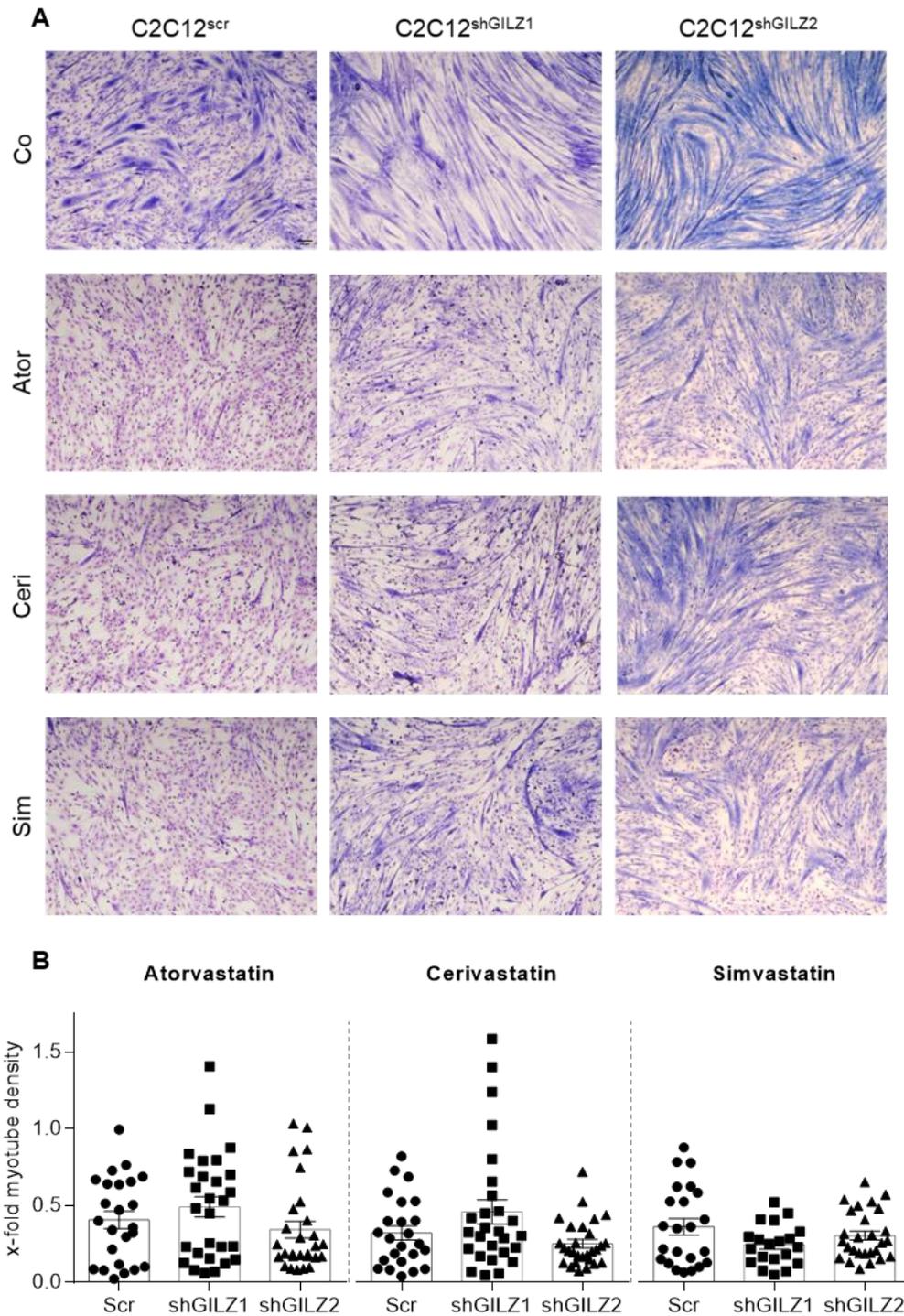
Figure 3.12. Effect of GILZ absence on statin-induced anti-myogenic effects, as measured by Jenner-Giemsa staining. Scrambled (Scr)- and shGILZ-C2C12 cell lines were induced to differentiate for 4 days in the absence or presence of atorvastatin (5μM), cerivastatin (0.1 µM), or simvastatin (1 µM), and subjected to Jenner-Giemsa staining, representative images are shown (**A**). Scale bar, 100 µm. The degree of differentiation was quantified by measuring the myotube density and is presented as fold of the corresponding controls (**B**). Bars show the mean of at least 3 independent experiments performed in replicates ± SEM, data points correspond to each quantified field.

3.2.8   FoxO3 mediates GILZ induction by statins in muscle

In the search for potential upstream regulators of GILZ expression after statin treatment in muscle, we focused on the Forkhead Box O3 (FoxO3) protein. FoxO3 is a direct phosphorylation target of Akt, and our observations in skeletal muscle tissue from FoxO3 KO mice, where *Gilz* expression levels were significantly lower than in WT animals, hinted towards FoxO3 as a transcriptional regulator of GILZ in this tissue (Figure 3.13 A). Like in primary myoblasts, we observed that treatment of C2C12 myoblasts with statins led to Akt dephosphorylation and, in line with this, to reduced levels of phosphorylated FoxO3 protein, in parallel to GILZ induction (Figure 3.13 B–D). Hence, we performed chromatin immunoprecipitation to evaluate whether FoxO3 activated *Gilz* transcription in myoblasts by binding to the forkhead responsive elements (FHRE) in its promoter following statin treatment. Indeed, we found sequences corresponding to three of the four FHRE present in the *Gilz* promoter in immunoprecipitates from atorvastatin-treated myoblasts, indicating that GILZ induction in muscle follows dephosphorylation, nuclear translocation, and activation of FoxO3 (Figure 3.13 E).

3.2.9   Statins induce Gilz in zebrafish embryos, and deregulation of Gilz expression impairs somitogenesis

The zebrafish is a powerful and versatile *in vivo* model for the study of developmental and physiological processes that has been used for the elucidation of statin effects on muscle development (Campos et al., 2016) and homeostasis (Hanai et al., 2007; Huang et al., 2011; Pasha and Moon, 2017). Given that the zebrafish expresses a GILZ orthologue (Tse et al., 2013), we chose it as an *in vivo* model to study the effects of statins on GILZ expression.

In line with our *in vitro* findings, treatment of zebrafish embryos at 20 hpf with statins at concentrations that have been described to cause major muscle damage, led to an up-regulation of *gilz* mRNA (Figure 3.14 A). To characterise the effects of increased Gilz expression in zebrafish muscle development, we performed myosin IF in Gilz overexpressing embryos. Compared with control animals, we found a diffuse MHC staining with loss of septa, a clear sign that Gilz impairs embryonic muscle development. To causally link Gilz expression with statin-induced muscle damage, we considered performing gene silencing *via* MO injection to evaluate the effects of statin treatment in WT *vs* morphant embryos. *gilz* morphants, however, showed similar features to that of the overexpressing embryos in muscle, as observed by myosin staining (Figure 3.14 B), indicating that Gilz plays an important role in the regulation of somitogenesis.
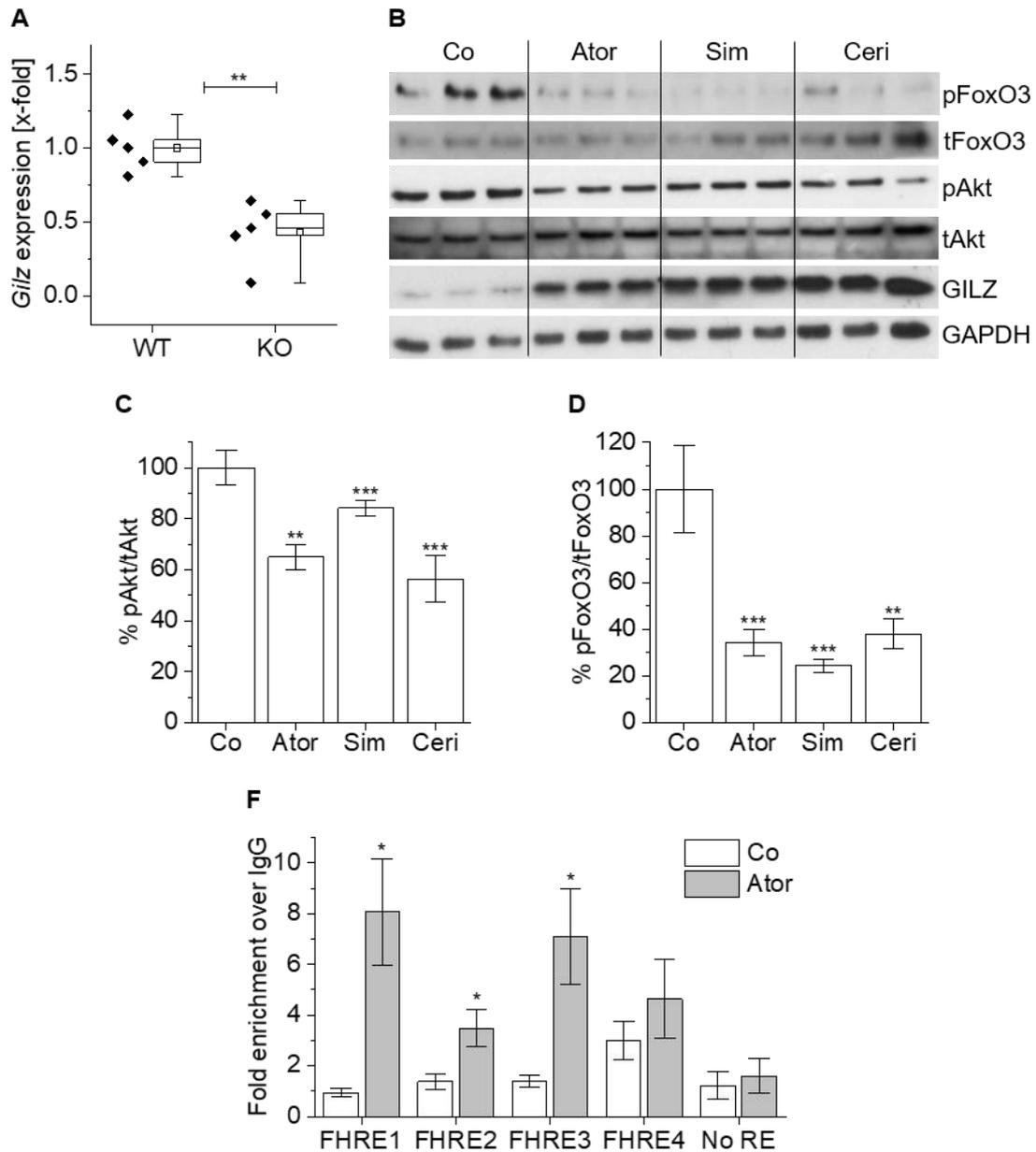
Figure 3.13. Involvement of FoxO3 in statin-induced GILZ expression. *Gilz* expression on skeletal muscle from FoxO3 KO mice (**A**). mRNA expression data was obtained from WT and KO mice (n=5), normalised against the housekeeping gene (*Csnk2a2*), and is presented as fold change of WT in box-plots showing the 25–75th percentiles, mean (square), median (line) and range (whiskers). Western blot analysis C2C12 myoblasts treated with 50 µM statin for 6 hours, a representative blot is shown (**B**). Densitometric analysis of ratio of phosphorylated to total FoxO3 (**C**). ChIP analysis was used to detect the binding of FoxO3 to the *Gilz* promoter region (**D**). Expression data is presented as fold enrichment over IgG. Data show the mean of 3 independent experiments ± SEM. *P<0.05; **P<0.01, ***P<0.001 relative to the control.
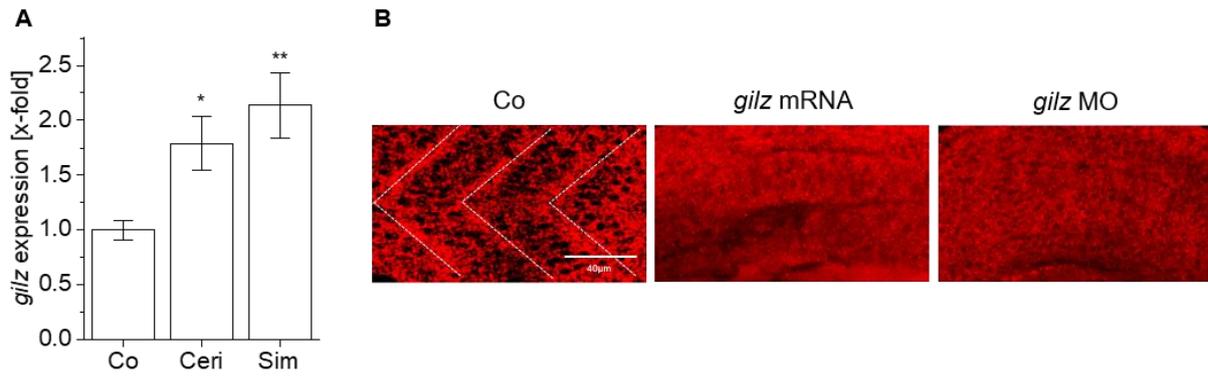
Figure 3.14. Effect of statins on gene expression in zebrafish, and effect of *gilz* deregulation in zebrafish muscle development. Zebrafish embryos at 20 hpf were treated with cerivastatin or simvastatin lactone (1 µM) for 12 hours (**A**). mRNA expression was normalised against the housekeeping gene (*actb2*) and is presented as fold change of control. MHC IF of *gilz*-overexpressing and *gilz*-morphant zebrafish embryos at 24 hpf, representative pictures are shown (**B**). Panels are side views, anterior, left. White dotted lines in Co outline the V-shape pattern of somites. Bar, 40 µm. Data show the mean of 3 independent experiments performed in replicates ± SEM. *P<0.05, **P<0.01, relative to the control.

3.3 Discussion

More than 30 years after their introduction to the market, statins remain the cornerstone of the pharmacological management of hyperlipidaemia and CVD prevention. In light of their importance and extended use, the understanding of the mechanisms underlying the onset of SAMS is of highest relevance (Stroes et al., 2015). In the present study, we report a role for GILZ as a pivotal mediator of the myotoxic and anti-myogenic effects of statins. We first demonstrate that treatment of murine myoblasts with different statins, at concentrations typical for *in vitro* studies on SAMS (Bouitbir et al., 2012; Hanai et al., 2007; Schirris et al., 2015), induces GILZ expression. Since SAMS are a class effect, and several of the mechanisms underlying muscle toxicity are directly related to the inhibition of HMG-CoA (Osaki et al., 2015; Trapani et al., 2012), we evaluated whether GILZ expression depended on this pathway as well. Indeed, we found GILZ induction to be mevalonate-dependent, more specifically, on geranylgeranylation.

The significantly lower myotoxicity of the hydrophilic statin pravastatin (Gadbut et al., 1995; Kaufmann et al., 2006; Kobayashi et al., 2008; Schirris et al., 2015), might be the result of reduced cellular uptake in our *in vitro* setting, that also accounts for the rather modest levels of *Gilz* induction observed in comparison to the other statins. The hydrophilicity of the molecules is also of relevance when comparing the cytotoxic effects of the lactone *vs* hydroxy acid forms of statins towards muscle, given that the former are significantly more lipophilic than the latter (Skottheim et al., 2008). It has been reported that patients with atorvastatin-induced myopathy have increased levels of the lactone form of the drug in plasma (Hermann et al., 2006), and *in vitro* studies also demonstrated that physiological conditions that favour the conversion of statins to their lactone form, such as acidosis, increase statin myotoxicity (Taha et al., 2016).

Several molecular mechanisms of statin-induced myotoxicity have been proposed, describing their deleterious effects on mitochondrial function, calcium homeostasis, and cell survival in the myocyte (du Souich et al., 2017). In our hands, statins caused Akt dephosphorylation and activation of the apoptotic cascade in undifferentiated myoblasts. This effect agrees with previous findings in cultured myotubes (Bonifacio et al., 2015; Johnson et al., 2004). Our observations suggest a crucial role for GILZ in mediating this action. First described in thymocytes as an anti-apoptotic protein (D'Adamio et al., 1997), GILZ can exert anti- or pro-apoptotic effects depending on the cell type: for instance, GILZ has been shown to promote apoptosis through Mcl-1 downregulation in neutrophils (Espinasse et al., 2016), or by inhibition of the Akt/mTOR signalling pathway, in myeloma cells (Joha et al., 2012).

There are discrepancies, however, in the mechanism by which statins induce cell death in cultured myoblasts/myotubes vs mature fibres. For instance, statins trigger apoptosis in cultured

cells, whereas mature skeletal muscles show necrotic features (Sakamoto and Kimura, 2013). Hence, instead of using terminally differentiated cultured myotubes, we chose isolated FDB myofibers as an *ex vivo* model to evaluate the effects of the absence of GILZ in statin toxicity towards mature muscle. We noticed that treatment of murine FDB fibres with atorvastatin, simvastatin, and cerivastatin caused cell death with similar features as those previously described for fluvastatin-treated rat FDB fibres (Sakamoto et al., 2011). Furthermore, in accordance with our observations in proliferating myoblasts, GILZ was of crucial importance in mediating statin-induced fibre breakdown.

FoxO3 is a transcription factor involved in different aspects of muscle homeostasis, like regulation of mitochondrial metabolism, activation of protein breakdown *via* the ubiquitin-proteasome and autophagy pathways, and inhibition of muscle precursor cell proliferation (Sanchez et al., 2014). In statin-induced myopathy, reports have shown that FoxO3 activation results in expression of the muscle atrophy-related protein MAFbx/atrogin-1 and other genes implicated in muscle proteolysis *in vitro* and *in vivo* (Hanai et al., 2007; Mallinson et al., 2009). Moreover, FoxO3 has been reported as a transcriptional regulator of GILZ in T cells, where it drives IL-2 withdrawal-induced GILZ expression (Asselin-Labat et al., 2005). We found statin-induced GILZ expression in muscle to be FoxO3-dependent and, as mentioned above, GGPP dependent. These results are in accordance with previous studies that linked SAMS to reduced geranylgeranylation, but not farnesylation, of different small GTPases like Rac1, Rap1, and Rab1 (Baba et al., 2008; Cao et al., 2009; Johnson et al., 2004; Sakamoto et al., 2007). Our findings suggest that the inhibition of protein geranylgeranylation by statins downregulates the Akt signalling pathway, leading to FoxO3-driven GILZ induction, which in turn further decreases Akt phosphorylation and promotes apoptosis.

The fact that GILZ is expressed in skeletal muscle, as observed by us and reported in earlier studies (Cannarile et al., 2001), might denote a role in muscle tissue homeostasis. Indeed, an earlier study showed that GILZ and its longer isoform, L-GILZ, modulate myogenesis in the absence of pharmacological intervention, and mediate glucocorticoid-induced inhibition of myogenesis by decreasing MyoD-mediated myogenin transcription, thus impairing myoblast fusion (Bruscoli et al., 2010). For this reason, we investigated the role of GILZ in the anti-myogenic effects of statins. We found that all statins were able to induce GILZ transcription, an effect that seemed to correlate with the anti-myogenic effect of the statin used. A previous study reported no differences in myogenin mRNA levels in simvastatin-treated C2C12 (Baba et al., 2008). We, however, found myogenin protein expression to be impaired in primary differentiating myoblasts treated with atorvastatin. Moreover, our results in GILZ KO myoblasts indicate that statin-induced GILZ expression, and the consequent myogenin transcriptional repression, is a mechanism by which this class of drugs impairs myogenesis.

GILZ silencing by shRNA caused the resulting C2C12^{shGilz} cell lines to differentiate considerably faster than the C2C12^{scr} control, even to a higher degree than observed for WT cells after terminal differentiation. This supports the importance of GILZ itself as a modulator of myogenesis and corresponds to our observations in zebrafish embryos, where Gilz overexpression severely impaired somitogenesis. Importantly, zebrafish embryos represent a frequently used *in vivo* model for the mechanistic study of SAMS (Campos et al., 2016, 2015; Huang et al., 2011; Pasha and Moon, 2017). The regulatory role played by Gilz in zebrafish embryonic development is rather complex: the study that first described the presence of a GILZ orthologue in zebrafish, showed that manipulation of Gilz expression in this model, either *via* MO silencing or mRNA overexpression, causes significant defects in embryonic development, altering the dorsoventral patterning, segmentation, and brain development processes (Tse et al., 2013). In rodents, however, GILZ-independent regulatory pathways are most likely involved in the modulation of skeletal muscle development, since GILZ KO mice do not exhibit altered muscle features (unpublished observations).

Even though myogenin expression was rescued in the absence of GILZ, we could not observe a complete recovery in myotube formation after statin treatment in C2C12^{shGilz} cells. This might be related to residual GILZ expression in the silenced cells, or to additional pathways that mediate the anti-myogenic effects observed, such as the IGF-1/PI3K/Akt pathway (Ogura et al., 2007). Our observations indicate that GILZ is critical for statin-induced inhibition of myogenin, an MRF crucial for myoblast fusion and terminal differentiation (Asfour et al., 2018). Additional factors, however, may also contribute to the inhibition of muscle regeneration by statins.

Taken together, our data point towards GILZ as an essential mediator of the molecular mechanisms leading to statin-induced muscle damage and impairment of muscle regeneration. This study contributes to a better understanding of the molecular mechanisms underlying statin-induced myopathy, a necessary step towards the development of prevention strategies and safer therapy approaches for a class of drugs that remains a pillar in the treatment of cardiovascular disease.

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The Glucocorticoid-Induced Leucine Zipper (GILZ) Mediates Statin-Induced Muscle Damage

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4. Chapter II

A potential role for GILZ in the pleiotropic effects of statins

4.1 Introduction

Statin therapy improves adverse outcomes in CVD patients (Almeida and Budoff, 2019), and is beneficial in primary prevention of CVD (Taylor et al., 2013). It has been suggested that the overall benefit of statin use is attributable not only to reduction of LDL-cholesterol but also to a plethora of pleiotropic effects, exerted on different cell types involved in cardiovascular homeostasis (Oesterle et al., 2017). Although the clinical significance of these effects is still debated (Labos et al., 2018), it is clear that statins reduce systemic inflammation, as demonstrated by different trials where reductions in high-sensitivity CRP are observed in patients with or without concomitant hyperlipidaemia (Ridker et al., 2008).

Atherosclerosis, the main pathophysiological condition driving CVD, is considered a chronic inflammatory disease (Mendis et al., 2011; Tousoulis et al., 2014). Indeed, the key role of inflammation in this pathology could be confirmed with the results from the CANTOS trial, where therapeutic targeting of the pro-inflammatory cytokine IL-1β with canakinumab led to a significantly lower rate of recurrent cardiovascular events (Ridker et al., 2017). Development of the atherosclerotic plaque involves endothelial dysfunction, monocyte and T-cell recruitment and activation, lipid accumulation, and smooth muscle cell proliferation (Novikova et al., 2018; Ross, 1999). Statins have shown different beneficial, anti-inflammatory effects in these cell types: for example, reduction in expression of adhesion molecules in endothelial and smooth muscle cells (Chung et al., 2002), in matrix metalloproteinase (MMPs) secretion in macrophages and smooth muscle cells (Luan et al., 2003), or reduced inflammasome activation in endothelial cells (Wang et al., 2017). These effects are attributed to inhibition of Rho GTPase-mediated pro-inflammatory activity, of oxidative stress, and the activity of pro-inflammatory transcription factors, like NF-κB (Huacuja Álvarez et al., 2006; Oesterle et al., 2017; Tousoulis et al., 2014).

GILZ is an immunomodulatory, anti-inflammatory protein (Ronchetti et al., 2015). Altered endogenous GILZ expression has been reported in different inflammation-related conditions, like obesity (Lee et al., 2016; Robert et al., 2016), rheumatoid arthritis (Beaulieu et al., 2010), endotoxin tolerance (Hoppstädter et al., 2015) and, interestingly, vascular inflammation (Hahn et al., 2014). Studies in different cell types indicate that GILZ exerts its anti-inflammatory activity *via* inhibition of NF-κB transcriptional activity (Ayroldi et al., 2001; Cheng et al., 2013; Yang et al., 2008). Of notice, apart from glucocorticoids, GILZ can be induced in response to other anti-inflammatory compounds like curcumin in macrophages (Hoppstädter et al., 2016), as well as by vascular protective conditions, such as laminar shear stress in endothelial cells (Hahn et al., 2014). We aimed at evaluating the hypothesis that statins can induce GILZ in two cell types involved in vascular inflammation, *i.e.* endothelial cells and macrophages, and that this protein plays a role in the anti-inflammatory, pleiotropic effects of statins.

4.2 Results

4.2.1 Statin cytotoxicity towards endothelial cells and macrophages

Evaluation of statin toxicity towards endothelial cells and macrophages was performed by treatment of HUVECs and BMMs with increasing concentrations of atorvastatin, cerivastatin, simvastatin in lactone and acid form, and pravastatin. The effects on cell viability were measured *via* MTT assay. Similar to the findings in skeletal muscle (Chapter I), pravastatin was the least toxic of statins, while the other statins were toxic already at low concentrations (Figure 4.1). For this reason, concentrations in the nanomolar–low micromolar range were used for further experiments. The viability of BMMs was not affected by statin treatment at the evaluated concentrations (data not shown).
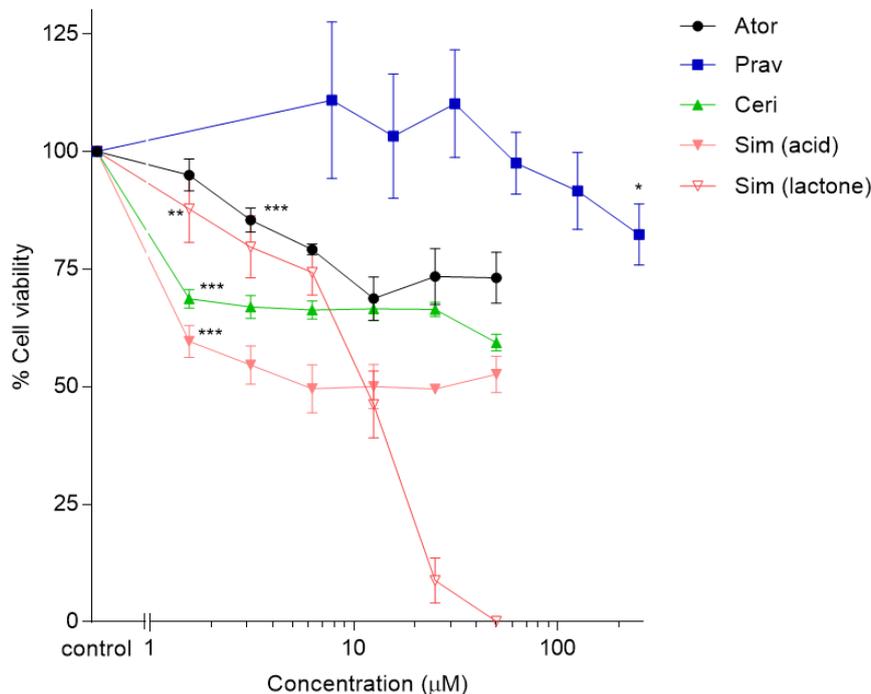
Figure 4.1. Statin cytotoxicity towards endothelial cells. HUVEC were treated with atorvastatin, cerivastatin, pravastatin, simvastatin lactone or simvastatin sodium salt in increasing concentrations for 24 hours. The cell viability was measured via MTT assay. Data show the mean of three independent experiments ± SEM. *P<0.05, **P<0.01, ***P <0.001 for the lowest concentration that caused a significant decrease in viability relative to the control.

4.2.2 Atorvastatin induces GILZ expression in endothelial cells and macrophages

To assess the effects of statin treatment on GILZ expression in endothelial cells and macrophages, HUVECs and BMMs were treated with a non-toxic concentration of atorvastatin (3 µM) for up to 24 hours, to measure the levels of *GILZ* mRNA at different time points. Atorvastatin induced *GILZ* expression in endothelial cells already at 2 hours of treatment, the induction was significant at 8 hours and continued to increase until the end of treatment (Figure

4.2 A). Furthermore, a moderate increase in *Gilz* expression was observed in BMMs, statistically significant at 16 hours (Figure 4.2 D). The shear stress-inducible, atheroprotective transcription factor *KLF2*, is known to be upregulated by statins (Parmar et al., 2005; Tuomisto et al., 2008), and endothelial nitric oxide synthase (*eNOS*) is one of the key targets of statins in vascular endothelium (Margaritis et al., 2014); both mRNAs were also measured as positive controls. As expected, atorvastatin treatment induced *KLF2* and *eNOS* expression in a time-dependent manner (Figure 4.2 B–C, E).
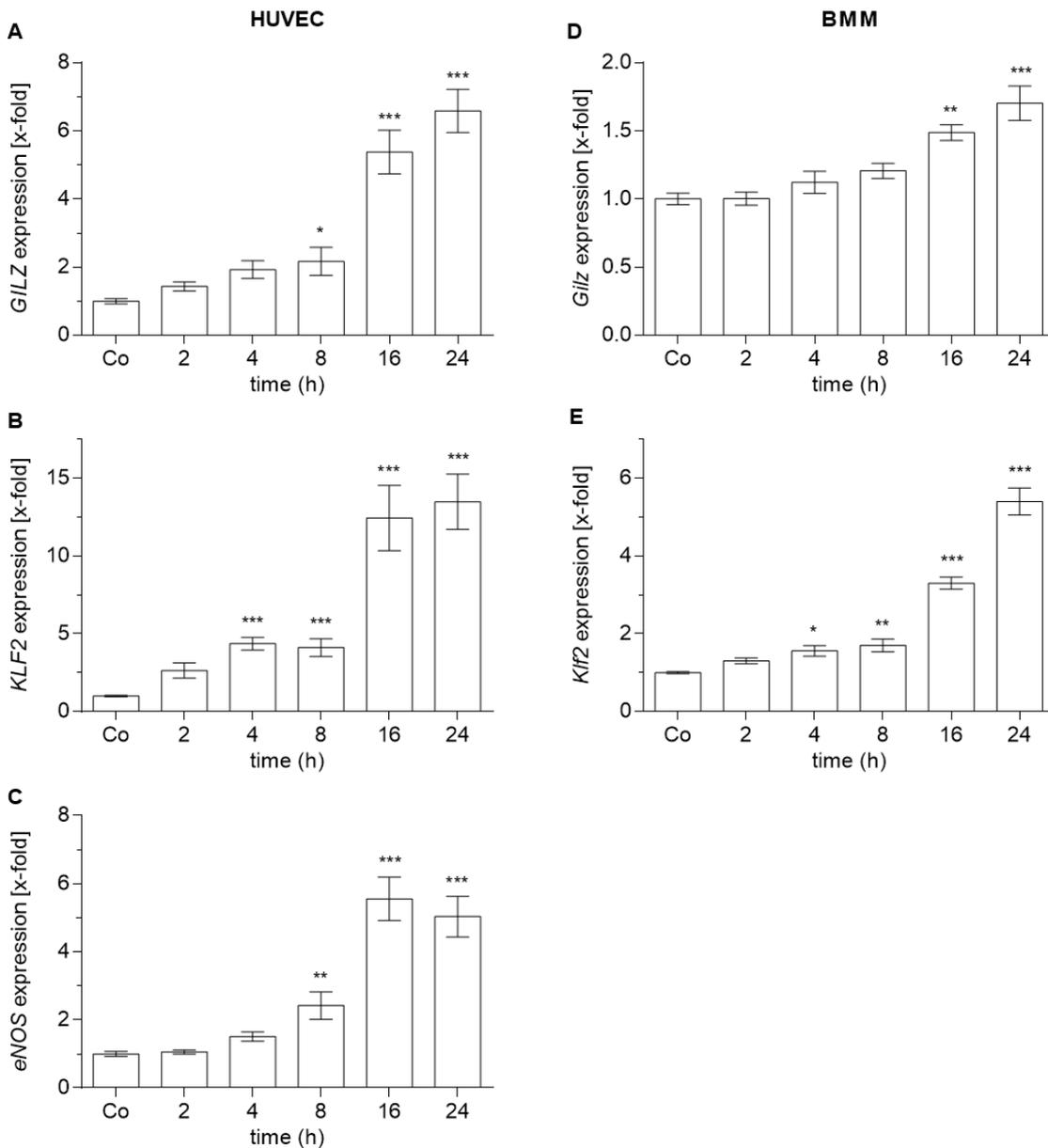
Figure 4.2. Effect of statin treatment on mRNA expression in endothelial cells and macrophages. HUVECs (**A–C**) and BMMs (**D–E**) were treated with atorvastatin (3 µM) for up to 24 hours, mRNA expression levels were measured. Gene expression was normalised against the housekeeping gene (A–C *ACTB*; D–E *Rn18s*) and is presented as fold change of control. Data show the mean of 3 independent experiments performed in replicates with cells derived from different donors ± SEM. *P<0.05, **P<0.01, ***P<0.001 relative to the control.

4.2.3 Statin-induced GILZ expression in HUVECs is mevalonate-dependent

To evaluate the dependence of GILZ induction on HMG-CoA inhibition, HUVECs were treated with non-toxic concentrations of statins in the absence or presence of mevalonate. As shown in Figure 4.3, all statins induced *GILZ* expression after 24 hours of treatment. The effect was reversed by coincubation with mevalonate.
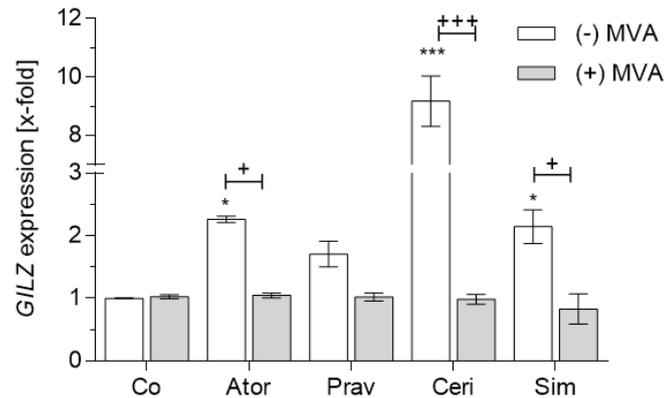
Figure 4.3. Effect of mevalonate on statin-induced *GILZ* expression in endothelial cells. HUVECs were treated with atorvastatin (1 µM), pravastatin (50 µM), cerivastatin (100 nM), or simvastatin (200 nM) in the absence or presence of mevalonate (100 µM) for 24 hours. *GILZ* expression was normalised against the housekeeping gene (*ACTB*) and is presented as fold change of control. Data show the mean of 2 independent experiments performed in replicates with cells derived from different donors ± SEM. *$P<0.05$, **$P<0.01$, ***$P<0.001$ relative to the control. + $P<0.05$ +++ $P<0.001$ relative to the non-MVA condition

4.2.4 KLF2 *is not involved in the regulation of* GILZ *expression in endothelial cells*

Given the main role played by KLF2 as mediator of the cardiovascular beneficial effects of statins, and the parallel upregulation of both genes observed upon atorvastatin treatment, it was of interest to evaluate whether regulation of *KLF2* expression influenced *GILZ* expression in endothelial cells. The notion of KLF2 as transcriptional activator of *GILZ* expression was supported by *in silico* analysis of the murine *Gilz* promoter, where KLF2-binding sites were found (data not shown). Knockdown of the *KLF2* gene in HUVECs was performed using an siRNA approach. In addition, overexpression was achieved by means of a doxycycline-inducible lentiviral expression system. siRNA knockdown resulted in a 50% decrease in gene expression (Figure 4.4 A) but had no effects on baseline expression of *GILZ* in transfected HUVECs, compared with the scramble controls (Figure 4.4 B). Similarly, lentiviral overexpression at two different MOIs resulted in a 10–15-fold induction of *KLF2* expression but had no effects on *GILZ* (Figure 4.4 C).
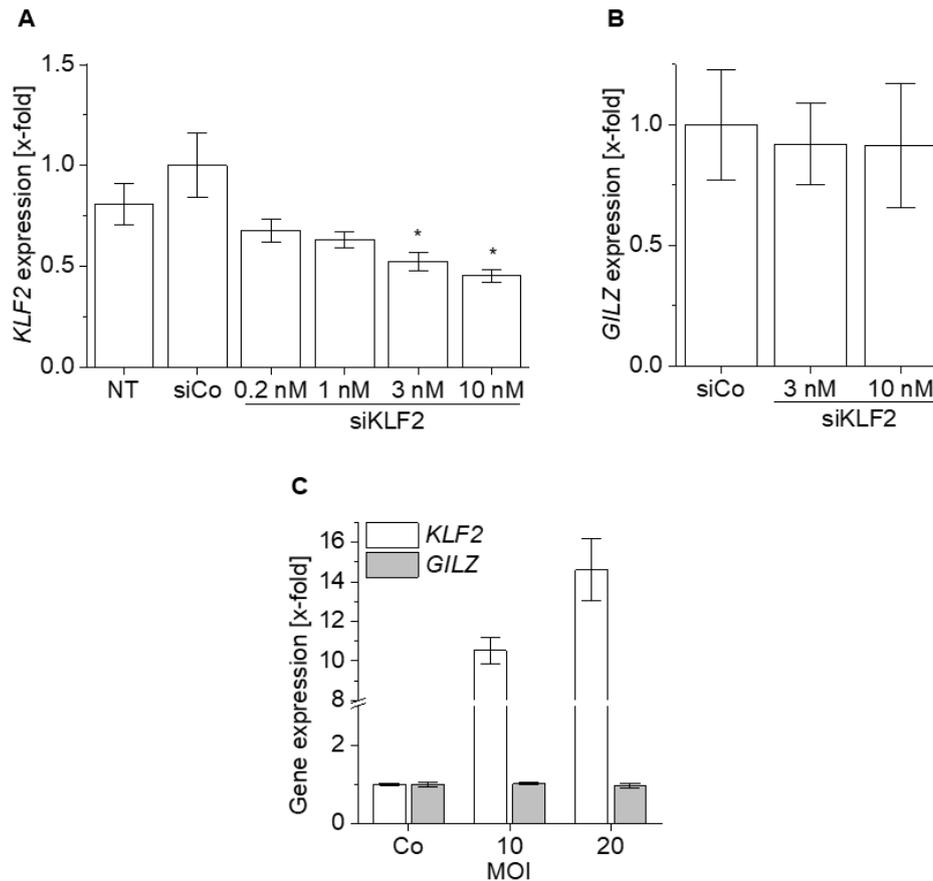
Figure 4.4. Effect of KLF2 knockdown and overexpression on GILZ expression in endothelial cells. HUVECs were transfected with siRNA targeting *KLF2* at different concentrations for 24 hours, *KLF2* levels were determined (**A**). *GILZ* levels were measured at the concentrations where significant KD was found (**B**). HUVECs were transduced with hKLF2 lentivirus and allowed to overexpress KLF2 for 72 hours before harvesting for mRNA analysis (**C**). mRNA expression was normalised against the housekeeping gene (*ACTB*) and is presented as fold change of control. Data show the mean of 3 independent experiments performed in replicates with cells derived from different donors (A–B) or a single experiment performed in duplicate (C) ± SEM. *P<0.05 relative to the control.

4.2.5   Development of a GILZ-KO endothelial cell line model via CRISPR/Cas9 editing

For analysis of the functional implications of GILZ induction by statins in vascular endothelium, a cell model with GILZ loss of function would be of interest. To this end, CRISPR/Cas9 technology, a highly versatile and efficient tool for gene editing (Boettcher and McManus, 2015), was used for the establishment of an endothelial GILZ-KO cell line. HUVECs are primary cells, hence not suitable for stable cell line generation. Therefore, the human dermal microvascular endothelium cell line HMEC-1 was chosen for CRISPR/Cas9 mediated GILZ deletion. Statin treatment significantly induced *GILZ* expression in these cells to a similar extent as in primary cells (Figure 4.5). To avoid the caveats of plasmid cloning, transfection/transduction, and expression of the CRISPR components in the cells, a system that involved direct delivery of Cas9 recombinant protein and the synthetic sgRNA was chosen. The

gene editing experiment was also performed in C2C12 myoblasts and murine RAW 264.7 macrophages, in parallel to HMEC-1 cells.
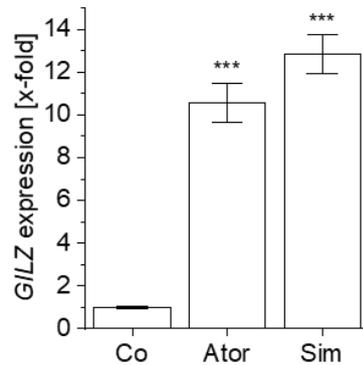
Figure 4.5. Effect of statin treatment on GILZ expression in an endothelial cell line. HMEC-1 cells were treated with simvastatin or atorvastatin at 1 µM for 24 hours, *GILZ* mRNA expression was normalised against the housekeeping gene (*ACTB*) and is presented as fold change of control. Data show the mean of 2 independent experiments performed in replicates ± SEM. ***P<0.001 relative to the control.

The *GILZ* gene is located on the X chromosome. For HMEC-1 and RAW264.7 cells, lines of male origin, this means only one allele needs to be edited by non-homologous end joining (NHEJ). C2C12, on the other side, is a female cell line with a hypo tetraploid karyotype — including 4 copies of the X chromosome— (Casas-Delucchi et al., 2011), which makes it difficult to obtain a clone with complete knockout. The validated murine and human sgRNA sequences were located in the last exon of their corresponding gene (Figure 4.6 A-B). Off-target analysis performed with the GT-scan software tool (O'Brien and Bailey, 2014) showed no predicted off-target effects in exonic regions of the genomes, thus, specific editing could be expected. The T7E1 mismatch assay performed in the bulked cell population after transfection indicated cutting by Cas9 in C2C12 and HMEC-1, but not in RAW 264.7 (Figure 4.6 C). After clonal expansion, none of the 20 C2C12 clones screened had indels in all alleles, while the 3 HMEC-1 clones that survived showed successful genomic 1 and 34-bp deletions (Figure 4.7). The growth of these clones, however, was impaired. For this reason, the cells could not be further expanded for characterization. As for the RAW264.7 macrophage model, the lack of editing could be due to poor transfection efficiency, common for this cell line. Hence, to facilitate the screening for functional sgRNAs and the generation of KO cell lines, a Cas9-expressing cell line was developed by a lentiviral approach (data not shown).
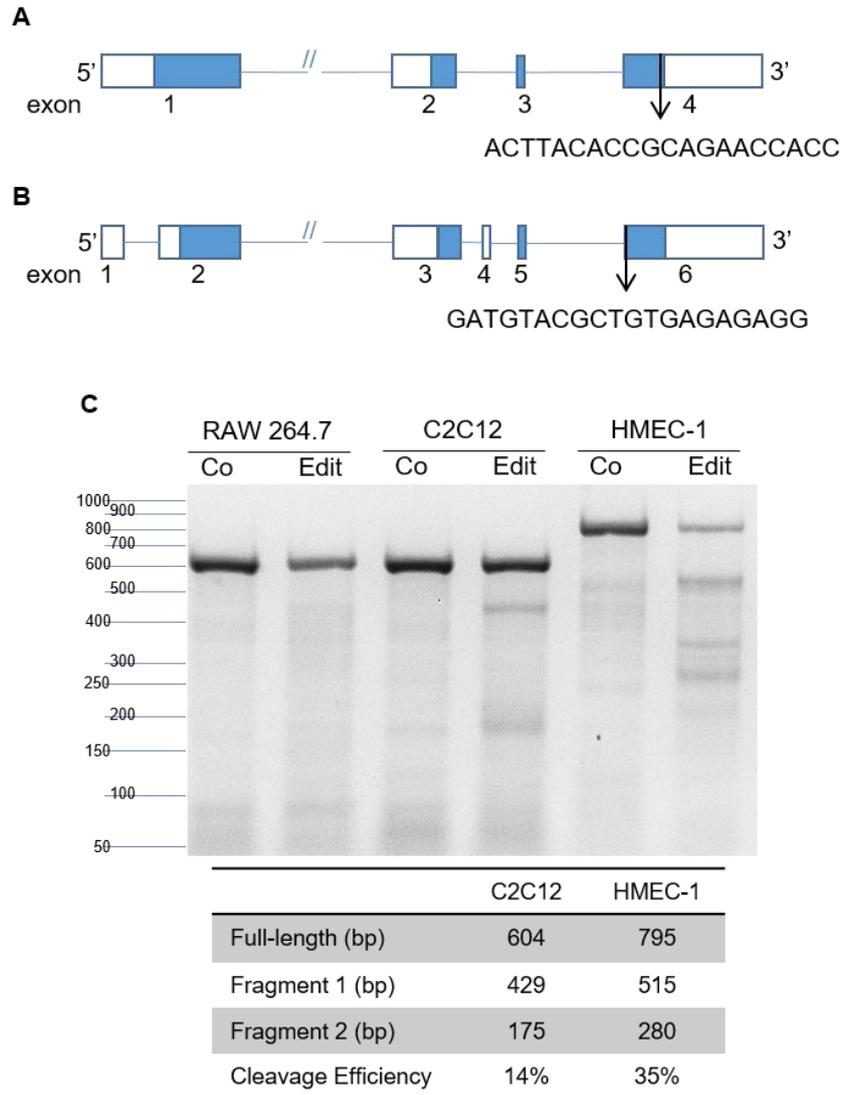
Figure 4.6. CRISPR/Cas9 – mediated GILZ knockout in cell lines. Schematic representation of the human (**A**) and murine (**B**) GILZ genes, black arrows indicate the approximate position of the sgRNAs. Genome editing efficiency in bulked population of edited RAW 264.7, C2C12, and HMEC-1 cells (**C**). The target genomic region from control and transfected cells was amplified by PCR and subjected to mismatch T7E1 assay, products were separated on an agarose gel for analysis. Cleavage efficiency was estimated from the intensities of the parental and cleaved bands.
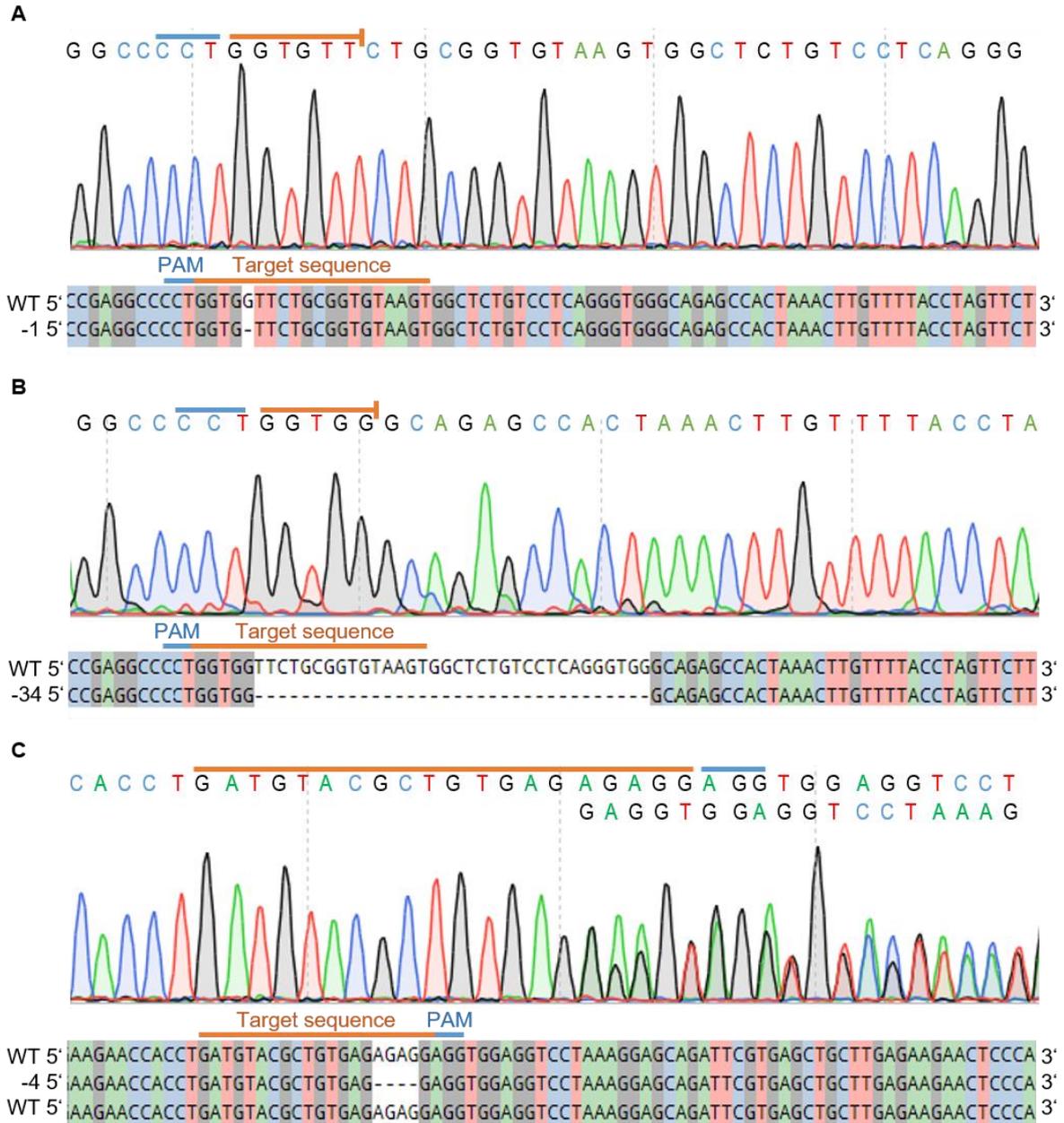
Figure 4.7. Validation of CRISPR/Cas9-mediated genome editing in HMEC-1 and C2C12 clones *via* Sanger sequencing. Sequence of three edited HMEC-1 clones was obtained, chromatogram analysis shows a single allele sequence. Clones 1 and 5 have the same 1 bp deletion (**A**) clone 2 has a 34 bp deletion (**B**). Example sequence of a non-pan-allelic edit obtained in C2C12 clone 17, two sequences are observed in the chromatogram, one corresponding to the WT sequence, and one with a 4 bp deletion (**C**). Sequences were deconvoluted using the CRISP-ID web application, first rows correspond to the reference, WT cells, and bottom rows to the observed sequencing results.

4.3 Discussion

The benefits of statin treatment on CVD are indisputable. Nevertheless, further study of the molecular mechanisms underlying the actions of statins in vascular inflammation are still needed (Almeida and Budoff, 2019). Here it is demonstrated that statins induce the anti-inflammatory protein GILZ in two cell types of critical importance in atherogenesis (Novikova et al., 2018), *i.e.* endothelial cells and macrophages. The induction was mevalonate-dependent, that is, specific to their mechanism of action. Based on the results in muscle cells (chapter I), it might be possible that statin-induced GILZ expression occurs *via* inhibition of protein prenylation in these cell types as well. The role of GILZ in inflammation has been characterised in different tissues and cell types, and more recently it has been shown that GILZ peptide analogues have therapeutic potential for treatment of chronic inflammatory diseases in the central nervous system (Gu et al., 2018; Srinivasan et al., 2016). Therefore, one might expect GILZ induction to be at least partially responsible for the pharmacological effects of statins in vascular inflammation.

A well-recognised mediator of the pleiotropic effects of statins is KLF2 (Parmar *et al.*, 2005; Sen-Banerjee *et al.*, 2005; Tuomisto *et al.*, 2008), a transcription factor that can also be induced by glucocorticoid receptor activation in macrophages, in what has been proposed to be an important cooperation for the control of inflammatory processes (Chinenov et al., 2014). Although the induction patterns of *KLF2* and *GILZ* after atorvastatin treatment were very similar in HUVECs, the exploratory findings presented here do not support the hypothesis that KLF2 acts as an upstream regulator of GILZ expression in endothelium. Whether GILZ might act as an upstream regulator of KLF-2 expression remains to be investigated.

It was possible to successfully introduce genomic edits in the *GILZ* gene in HMEC-1 cells by using CRISPR/Cas9 technology, establishing a protocol for generation of knockouts in our laboratory that can be of use to evaluate the functional aspects of GILZ induction by statins. The three HMEC-1 clones with validated deletions exhibited impaired growth, which might be indicative of a role for GILZ in endothelial cell proliferation. Since the validated target sgRNA sequence was in the last exon of the gene, constitutive to all transcript variants but at the C-terminal portion of the protein, it is likely that the clones did not have abolished expression of GILZ. For CRISPR/Cas9 mediated KO generation it is recommended targeting portions near the N-terminus of the protein, or 5' constitutive exons, to increase the probability of a frameshift mutation that leads to nonsense-mediated mRNA decay or a non-functional protein product (Doench et al., 2014; Joung et al., 2017). The deletions obtained here, however, may cause alterations in the C-terminal portion of GILZ, responsible for interaction with NF-κB (Di Marco et al., 2007). Further work in generation of GILZ KO HMEC-1 cells might clarify this issue.

Although still a matter of discussion, a body of evidence points towards pleiotropic effects of statins as major contributors to their beneficial effects on the cardiovascular system. The findings presented in this study open the opportunity to investigate the potential role of GILZ as mediator of the anti-inflammatory actions of statins in the cardiovascular system.

5. Chapter III

Macroph-aging: role of glucocorticoid metabolism

5.1 Introduction

Aging is characterised by a gradual decline of the immune function that results in a chronic, low-grade systemic inflammatory state, termed "Inflammaging" (Franceschi et al., 2000). This chronic inflammation, fuelled by continuous exposure to a number of exogenous and endogenous antigens during lifespan, is considered by geroscience one of the seven pillars of aging, and largely determines the onset of age-related diseases (Franceschi et al., 2018).

Different factors, like cell senescence, endocrine, and metabolic alterations, contribute to sustain inflammaging (Bandaranayake and Shaw, 2016). Among these, the macrophage is considered a key cell type driving age-related inflammation. Indeed, since its origins, the theory of inflammaging has been intertwined with "macroph-aging", that is, the chronic activation of macrophages with age (Franceschi et al., 2000; Prattichizzo et al., 2016). Macrophages are a widely distributed, heterogeneous, plastic cell population, with central roles as effector and mediator of the innate and adaptive immune response (Janeway et al., 2001; Wynn et al., 2013). Several studies in humans and animals have reported age-related alterations in different macrophage functions, such as phagocytic activity, pro-inflammatory cytokine secretion, and antigen presentation (Jackaman et al., 2017; Sebastián et al., 2005). Furthermore, loss of macrophages has been associated with improved inflammatory-induced pathology and survival after systemic immunostimulation (Bouchlaka et al., 2013), and with reduced neurodegeneration (Yuan et al., 2018) in murine models of aging.

Endogenous GCs are main regulators of the immune function (Cain and Cidlowski, 2017). GC secretion is regulated in a circadian manner, and in response to stress, by the HPA axis, the function of which is dysregulated with advancing age (Gupta and Morley, 2014). Although sometimes contradictory, literature reports regarding altered cortisol secretory profiles in humans often describe elevations in cortisol levels in aged subjects that correlate with negative health outcomes (Gaffey et al., 2016). Noticeably, increased cortisol levels seem to be a phenotypic feature of centenarians, subjects who are able to cope with inflammation and have achieved longevity by mounting an effective anti-inflammaging response (Franceschi et al., 2007; Genedani et al., 2008; Sergio, 2008). Thus, the balance between pro- and anti-inflammatory mediators is what ultimately determines successful aging. Because of their role in modulation of the inflammatory response (Busillo and Cidlowski, 2013), one might hypothesise that alterations in GC metabolism affect the function of immune cells, including macrophages, during aging.

Previous work in our group found a pro-inflammatory phenotype in aged (85 weeks) C57BL/6 mice compared with their young (10 weeks) counterparts. This phenotype was characterised by higher levels of circulating pro-inflammatory cytokines, like TNF-α, and lower levels of total

corticosteroids in serum (Figure 5.1 A–B). Likewise, flow cytometry analysis showed decreased expression of the GC-inducible, anti-inflammatory protein GILZ in the myeloid compartment of different organs, in PBLs, and in PMs from aged animals (Data not shown and figure 5. C). Given the importance of macrophage activation and GC response in the aged immune system, we aimed to further evaluate the age-associated changes in gene expression and GC metabolism across tissues, as well as in the myeloid compartment of mice, to improve the current understanding of "macroph-aging".
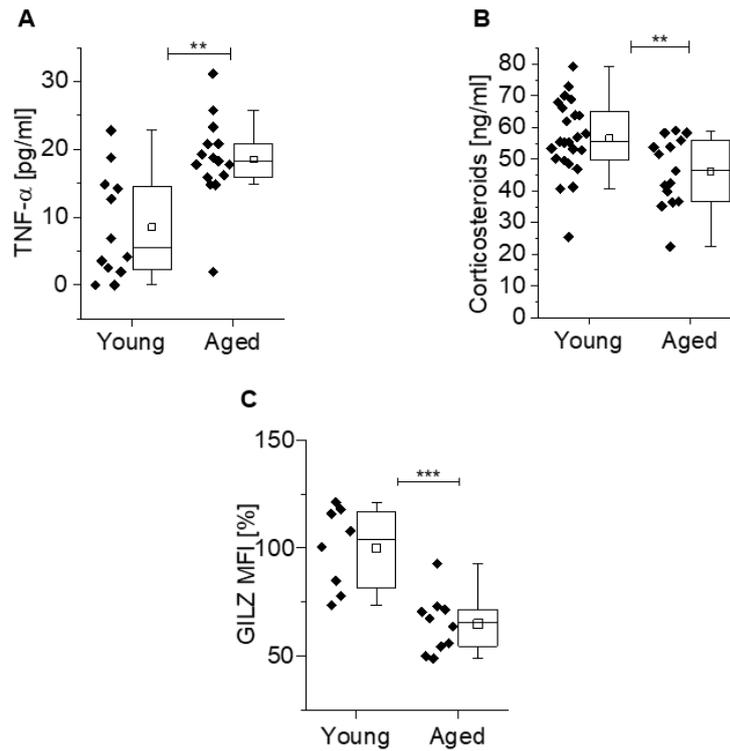
Figure 5.1. Aged mice show a pro-inflammatory phenotype. Levels of TNF-α (**A**) and total corticosteroids (**B**) in serum from young (10 weeks) and aged (85 weeks) C57BL/6 mice (n=12–15), as determined by ELISA. Flow cytometry analysis of GILZ levels in PMs isolated from young and aged mice (**C**, n=8–10). Box-plots show the 25–75th percentiles (Box), mean (square), median (line) and range within 1.5 IQR (whiskers). **P<0.01, ***P<0.001 relative to young mice.

5.2 Results

5.2.1 Lipid peroxidation and clinical chemistry analysis

To further characterise the aged-related changes of C57BL/6 mice, TBARS assay was performed to measure the oxidative stress levels of young and aged animals in liver tissue. Contrary to what could be expected, there were lower levels of malondialdehyde in livers from aged mice, compared with the young controls (Figure 5.2). Serum analysis of aspartate and alanine transaminases, triglycerides, cholesterol, albumin, and uric acid levels did not reveal age-related significant differences among groups (Table 5.1).
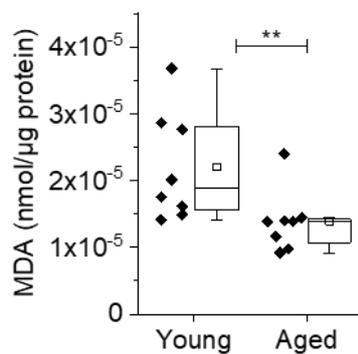
Figure 5.2. Oxidative stress levels in livers from young and aged mice. TBARS assay was performed in liver tissues from young (10 weeks) and aged (85 weeks) C57BL/6 mice (n=8). MDA concentrations were normalised to total protein content in the sample and are presented in Box-plots showing the 25–75th percentiles (Box), mean (square), median (line) and range within 1.5 IQR (whiskers). **P<0.01 relative to young mice.

Table 5.1. Clinical chemistry analysis in serum from young and aged C57BL/6 mice.

	Female		Male	
	Young (n=3)	Aged (n=6)	Young (n=9)	Aged (n=6)
AST (U/l)	1005 (420-1450)	555 (390-800)	1015 (450-1205)	592.5 (515-890)
ALT (U/l)	95 (50-135)	107.5 (60-190)	175 (70-425)	132.5 (75-320)
Triglycerides (mg/dl)	105 (90-110)	82.5 (65-110)	140 (135-175)	102.5 (95-135)
Cholesterol (mg/dl)	60 (60-65)	60 (-)	75 (65-80)	65 (60-70)
Albumin (g/l)	35 (-)	37.5 (35-40)	30 (30-35)	35 (30-35)
Uric acid (mg/dl)	7.5 (5-8)	8 (7-8)	7 (6-9.5)	7.5 (7-8)

Data are presented as median (IQR). Comparison was performed with Mann Whitney U Test. No significant differences were detected.

5.2.2 Cytokine expression in tissues from aged mice

Measurement of mRNA expression of three pro-inflammatory mediators, namely *Tnf*, *Il1b*, and *Il6*, was performed at baseline and after treatment with 5 mg/kg LPS, in different tissues from young and aged mice. There were no consistent differences in the expression of these mediators in tissue that correlated with the elevated serum cytokine levels observed by ELISA in previous experiments. At baseline, *Tnf* levels were significantly elevated in lung but decreased in lymph nodes of aged mice, while *Il1b* expression was increased in both, liver and lymph nodes (Figure 5.3 A,C). After LPS stimulation, significant differences between groups were only found for *Tnf* expression in liver and spleen (Figure 5.3 B,D). *Il6* expression was very low in all tissues, what impedes to draw significant conclusions from the data obtained (Appendix).

5.2.3 Altered glucocorticoid homeostasis in myeloid cells from aged mice

Further analysis focused on changes in gene expression of PMs and PBLs isolated from young and aged mice, given that no major differences between groups could be detected in whole tissues. The expression of GC-responsive genes other than GILZ was evaluated in these cells, finding significantly decreased levels of the dual specificity phosphatase 1 (*Dusp1*) gene, but not of *Anxa1* or *Sgk1* (Figure 5.4). The reduced levels of GILZ protein and *Dusp1* gene expression might be indicative of a decreased ability of myeloid cells to respond to GC stimulation. Since altered regulation of genes responsible for GC homeostasis within cells could account for dysregulated GC-responsiveness in aging, the expression of such genes was investigated.
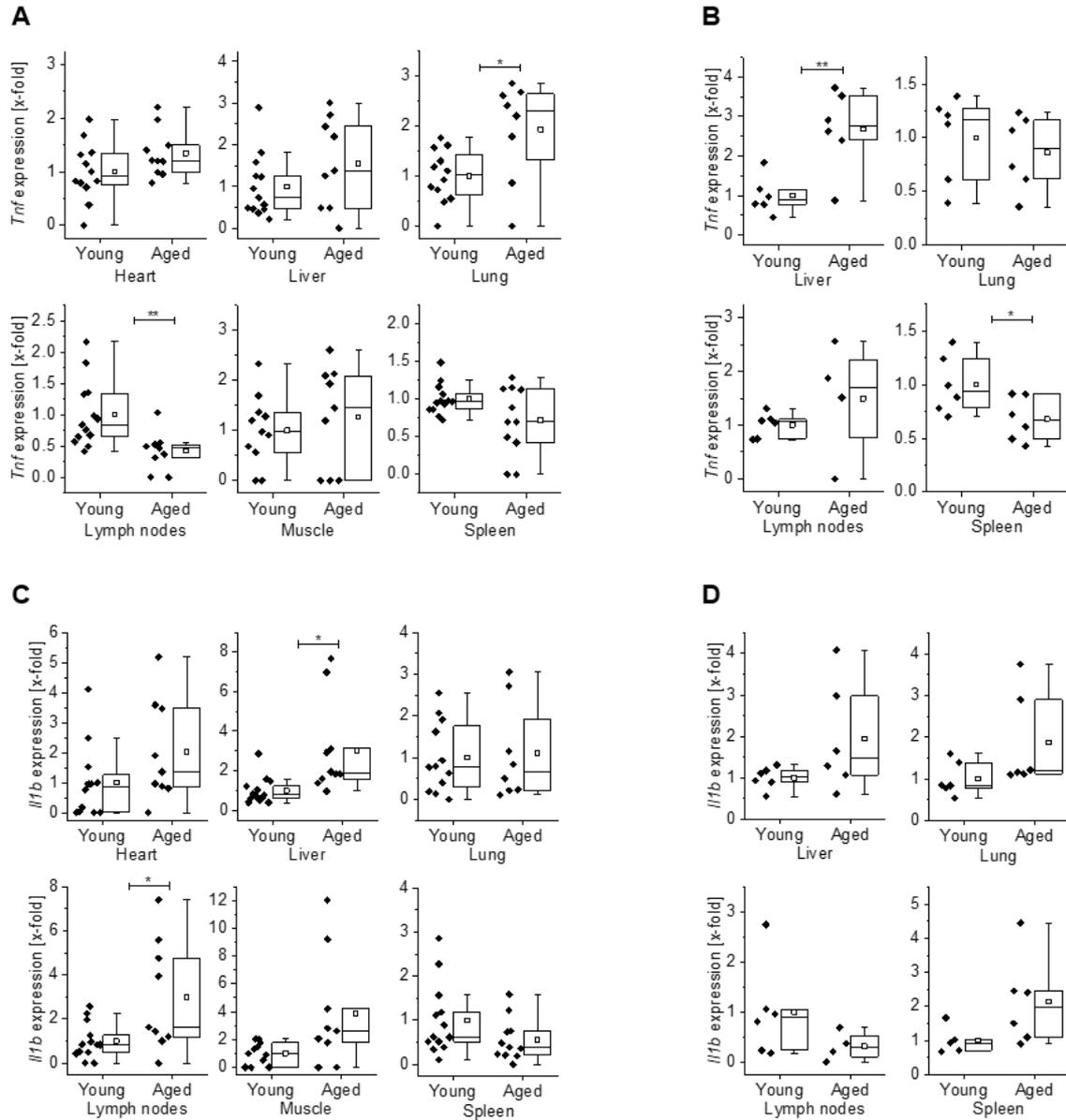
Figure 5.3. Expression of pro-inflammatory cytokines in tissues from young and aged mice. mRNA expression of *Tnf* and *Il1b* was measured in heart, liver, lung, lymph nodes, skeletal muscle, and spleen tissues from young (10 weeks) and aged (80–100 weeks) C57BL/6 mice at baseline (**A**,**C**, n=9–13), or in liver, lung, lymph nodes, and spleen after treatment with 5 mg/kg LPS for 4 hours (**B**,**D**, n=4–6). mRNA expression was normalised against the housekeeping gene (*Ppia*) and is presented as fold change of young. Box-plots show the 25–75th percentiles (Box), mean (square), median (line) and range within 1.5 IQR (whiskers). *P<0.05, **P<0.01 relative to equally treated young mice.
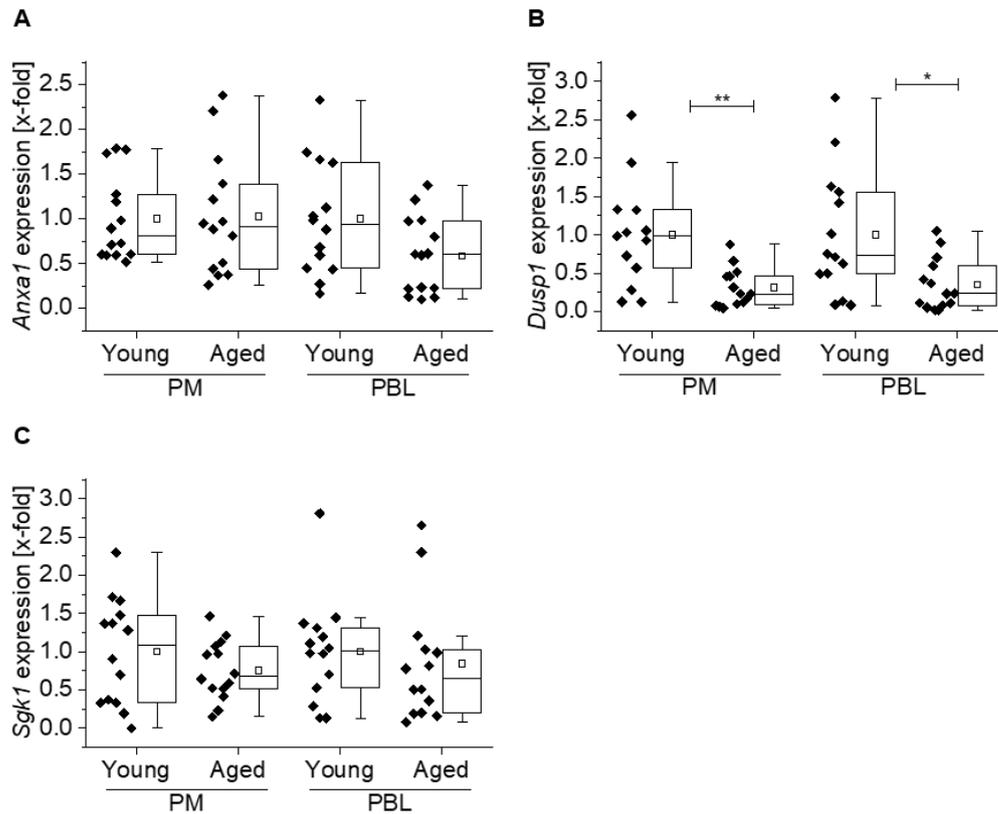
Figure 5.4. Expression of GC-responsive genes in PM/PBL from young and aged mice. mRNA expression of *Anxa1*, *Sgk1* and *Dusp1* was measured in PMs and PBLs from young (10 weeks) and aged (80–100 weeks) C57BL/6 mice (n=13–14). Gene expression was normalised against the housekeeping gene (*Ppia*) and is presented as fold change of young. Box-plots show the 25–75th percentiles (Box), mean (square), median (line) and range within 1.5 IQR (whiskers). *P<0.05, **P<0.01 relative to young mice.

GCs exert their action through binding to the ubiquitously expressed glucocorticoid receptor (GR). Therefore, changes in cellular GR expression determine GC-responsiveness (Oakley and Cidlowski, 2013; Ramamoorthy and Cidlowski, 2013). mRNA expression analysis, however, indicated that GR levels remained unchanged with aging in PMs and PBLs (Figure 5.5 A). At pre-receptor level, the intracellular action of GCs is largely determined by the activity of the two isozymes 11β-HSD1 and 11β-HSD2, that catalyse the interconversion of active cortisol/corticosterone and inactive cortisone/11-dehydrocorticosterone (11-DHC), thereby modulating GC availability (Tomlinson et al., 2004). *Hsd11b1* expression was significantly decreased in aged mice compared with young controls, not only in PMs and PBLs, but also in liver, where the enzyme is most abundant (Figure 5.5 B–C). The findings at mRNA level were confirmed at protein level in PMs and bone-marrow derived macrophages (BMM, Figure 5.5 D–F). Expression of the hexose-6-phosphate dehydrogenase gene (*H6pd*) was upregulated in aged PMs and PBLs. In the cell, H6PDH is the source of NADPH, needed as cofactor for 11β-HSD1 reductase (cortisone to cortisol) activity (Atanasov et al., 2004). On the other hand, the gene expression of *Hsd11b2*, the isozyme that exerts dehydrogenase (cortisone to cortisol) activity, remained unchanged in PMs and PBLs from aged mice, compared with young controls

(Figure 5.5 G–H). These findings are indicative of alterations in intracellular GC metabolism in the macrophages of aged mice.
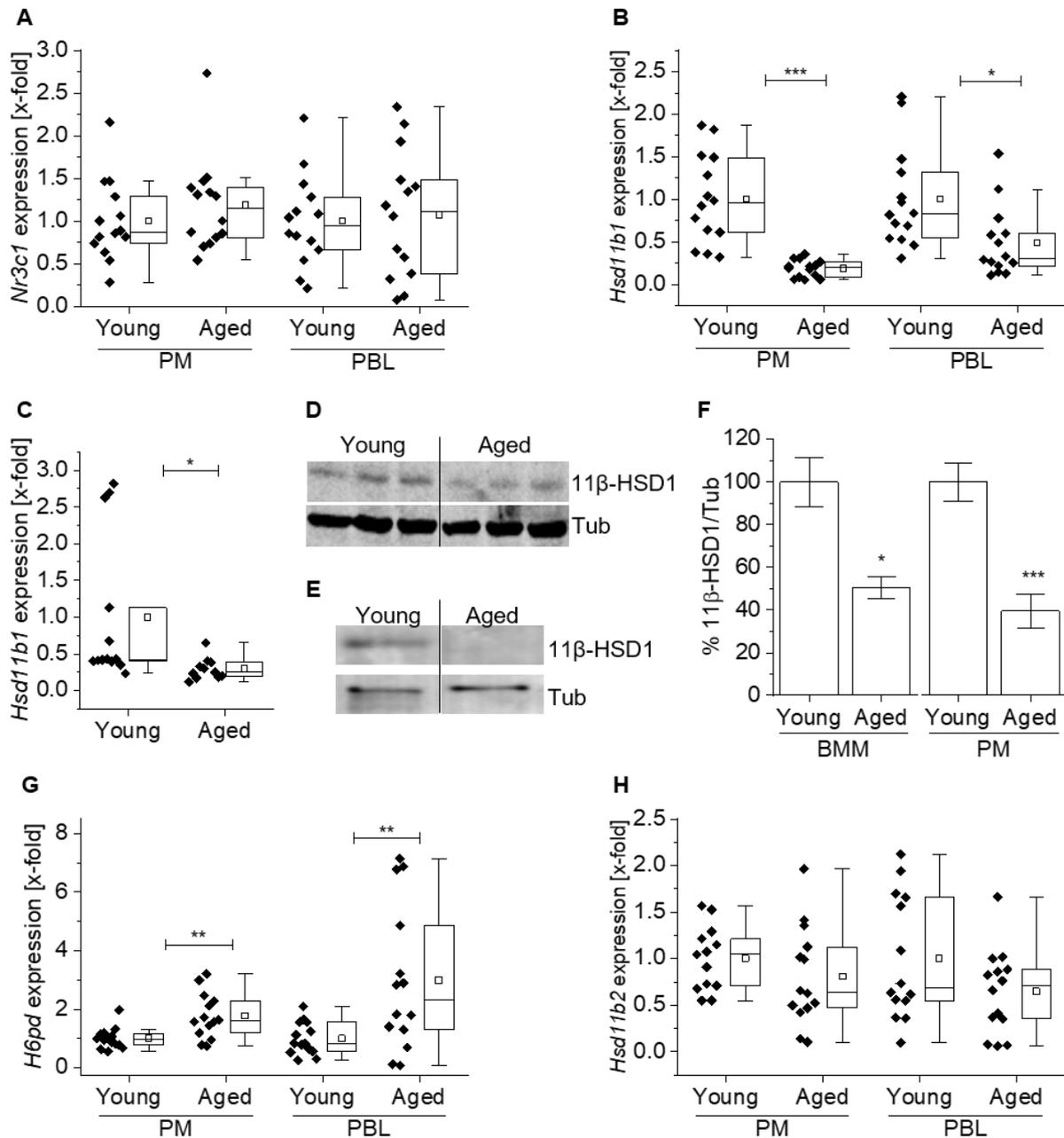
Figure 5.5. Alterations in glucocorticoid metabolism in aged mice. Expression of GR mRNA (*Nr3c1*) in PMs and PBLs from young (10 weeks) and aged (80–100 weeks) C57BL/6 mice (n=13–14, **A**). *Hsd11b1* expression in PMs, PBLs, and liver tissue from young and aged mice (**B–C**). 11β-HSD1 protein expression was measured in BMMs (**D**, n=4) and PMs (**E**, n=11–12). Representative blots are shown. Densitometric analysis, normalised to the housekeeping protein (α-Tubulin, **E**). mRNA levels of *H6pd* and *Hsd11b2* were also measured in PMs and PBLs (**G–H**). Gene expression was normalised against the housekeeping gene (*Ppia*) and is presented as fold change of young. Box-plots show the 25–75th percentiles (Box), mean (square), median (line) and range within 1.5 IQR (whiskers). Bars show the mean ± SEM. *P<0.05, **P<0.01, ***P<0.001 relative to young mice.

5.2.4 Reduced corticosterone levels and 11β-HSD1 activity in aged mice

Analysis of the functional implications of reduced 11β-HSD1 expression was first performed by measurement of the levels of corticosterone and 11-DHC, the main GCs in rodents (Gong et al., 2015), in serum from young and aged mice. In line with the previous ELISA findings, aged mice displayed reduced levels of both corticosteroids in serum (Figure 5.6 A–B). The ratio of active corticosterone to inactive 11-DHC, however, remained unchanged in both groups (data not shown).

Since 11β-HSD1 regulates the intracellular availability of active GCs, alterations in 11β-HSD1 reductase activity were evaluated by measuring the conversion rate of deuterated cortisone to cortisol in isolated young and aged PMs. As could be expected, the downregulation of the enzyme resulted in less intracellular conversion to cortisol (Figure 5.6 C). Hence, one might hypothesise, in the *in vivo* setting reduced 11β-HSD1 expression translates into less corticosterone available to activate the GR in aging macrophages.
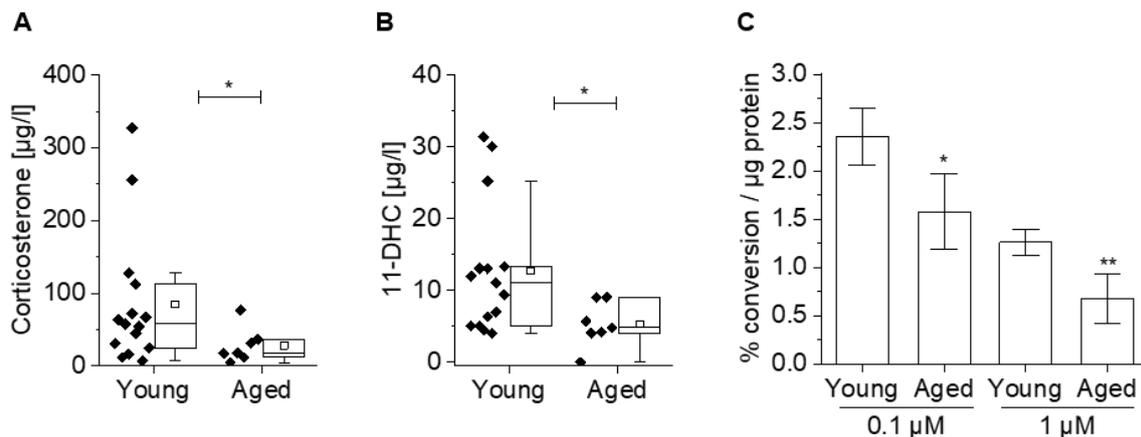
Figure 5.6. Differences in serum glucocorticoids and 11β-HSD1 activity in young and aged mice. Serum levels of corticosterone and 11-DHC from young (10 weeks, n=15) and aged (80–100 weeks, n=7) male C57BL/6 mice were measured by LC-HRMS/MS (**A**,**B**). PMs isolated from young and aged animals (n=10) were incubated for 24 hours with 0.1 μM and 1 μM cortisone-D$_8$, the conversion to cortisol-D$_8$ was measured in supernatants by LC-HRMS/MS (**C**). Conversion percentages were normalised to total protein content in the sample. Box-plots show the 25–75th percentiles (Box), mean (square), median (line) and range within 1.5 IQR (whiskers). Bars show the mean ± SEM. *P<0.05, **P<0.01, relative to young mice.

5.2.5 Upstream regulators of 11β-HSD1 are downregulated in aged macrophages

Transcription of *Hsd11b1* is regulated by members of the CCAAT/enhancer binding protein (C/EBP) family of transcription factors (Chapman et al., 2013). As a first insight into the mechanisms leading to 11β-HSD1 downregulation in aged PM and PBL, mRNA levels of C/EBPα and C/EBPβ were measured. Both genes were significantly downregulated in PMs

from aged animals. In PBLs, a similar tendency for *Cebpa* expression was observed, while the expression of *Cebpb* remained unchanged (Figure 5.7).
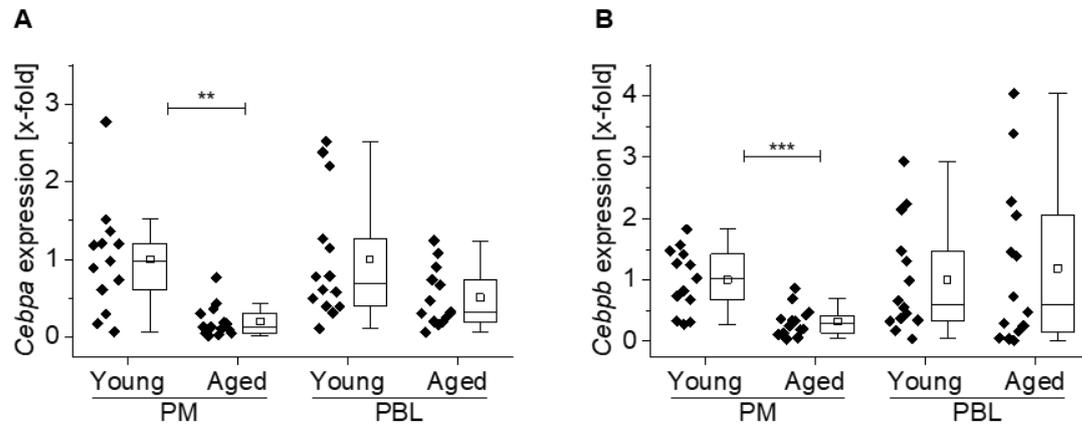
Figure 5.7. Gene expression of upstream regulators of 11β-HSD1. *Cebpa* (**A**) and *Cebpb* (**B**) mRNA levels were measured in PMs and PBLs from young (10 weeks) and aged (80–100 weeks) C57BL/6 mice (n=13–14). Gene expression was normalised against the housekeeping gene (*Ppia*) and is presented as fold change of young. Box-plots show the 25–75th percentiles (Box), mean (square), median (line) and range within 1.5 IQR (whiskers). **P<0.01, ***P<0.001 relative to young mice.

5.3 Discussion

The aging process represents a gradual deterioration of the function and homeostasis of different systems that are closely linked with the onset of chronic diseases, and ultimately determine survival. The present work shows evaluation of the phenotype of aging C57BL/6 mice, and reports alterations in the intracellular GC homeostasis of macrophages and PBLs from these mice.

Oxidative stress has been signalled as a determinant factor that contributes to age-related inflammation (De la Fuente and Miquel, 2009). In serum (Balkan et al., 2002) and erythrocytes (Kawamoto et al., 2005) of aged subjects, increased levels of endogenous lipid peroxidation, indicative of oxidative stress, have been reported. On the other hand, the literature about lipid peroxidation on mammal tissues is conflicting, and the results are species-, sex-, and tissue-dependent (Khansari et al., 2009). In this setting, age was associated with reduced hepatic lipid peroxidation —and thus oxidative stress— similar to previous reports in 22-month C57BL/6 mice (Selman et al., 2008). The reduced MDA levels found in aged mice may be explained by conserved or improved antioxidant capacity in the liver, as previously shown for old female Fischer 344 rats (Rikans et al., 1991). This aspect, however, was not evaluated in the present study.

The involvement of GC signalling and metabolism in chronic inflammation and stress-related disorders, two phenomena that occur with aging, has been a topic of interest for the last decades (Herriot et al., 2017). Literature reports indicate associations between both, higher and lower GC levels, and negative health outcomes (Gaffey et al., 2016; Raison and Miller, 2003). In rodents, increase (Kizaki et al. 2002), no differences (Morano et al., 1994) and, more recently, decrease (Zambrano et al., 2015) of serum corticosterone with age has been reported. Given that GCs have an anti-inflammatory and immunosuppressive function, it is logical to think that the reduced total circulating GCs, and particularly corticosterone, found in our study might contribute to the pro-inflammatory phenotype observed in aged mice. Nevertheless, cellular GC-responsiveness is ultimately determined by the intracellular availability of active GCs, as well as the expression of GRs. Aged PMs and PBLs, indeed, showed reduced levels of two GC-inducible genes with important roles in the regulation of the inflammatory response: GILZ downregulation has been associated with increased pro-inflammatory activation of macrophages (Hoppstädter et al., 2015), and DUSP-1 has been shown to reduce their pro-inflammatory cytokine expression upon LPS-activation (Abraham et al., 2006; Chen et al., 2002).

Although a previous study reported elevated GR mRNA expression in peritoneal exudate cells from aged mice (Kizaki et al., 1998), our findings suggest that alterations in GC-homeostasis

from PMs are not associated with altered GR expression but with reduced intracellular activation of 11-DHC by 11β-HSD1. There might be different functional implications of blunted 11β-HSD1 signalling in aged macrophages: it has been shown that 11β-HSD1-defficient mice are more susceptible to endotoxemia, and macrophages derived from these mice are hyperresponsive to LPS stimulation (Zhang and Daynes, 2007). Loss of 11β-HSD1 in macrophages has also been associated with delayed phagocytic capacity (Gilmour et al., 2006).

Various studies have addressed the changes in 11β-HSD1 expression and activity in different tissues and cell types with aging. For example, elevated expression in hippocampus from aged C57BL/6J mice was related to impaired cognitive function (Holmes et al., 2010). Similarly, protein expression was elevated in skeletal muscle from aged female volunteers, and correlated with reduced grip strength and insulin resistance (Hassan-Smith et al., 2015). Age-related elevations in 11β-HSD1 have also been reported for brown adipose tissue (Doig et al., 2017), skin (Tiganescu et al., 2013), and CD4+ T lymphocytes (Zhang et al., 2005). Interestingly, 11β-HSD1 was regulated in the opposite fashion not only in PMs, but also in PBLs, a mixed population of granulocytes, lymphocytes, and monocytes. To our knowledge, this is the first report on age-induced changes in 11β-HSD1 expression in macrophages.

In summary, the present data provide insight into the alterations in GC metabolism in macrophages during aging. The reduced circulating corticosterone levels, together with a decreased availability of active GC within cells as a consequence of reduced 11β-HSD1, might contribute to the imbalance between pro- and anti-inflammatory signalling in macrophages from aged mice, thus promoting the inflammaging phenotype.

The results presented in this chapter are part of the following manuscript, in preparation:

Macroph-aging: Role of glucocorticoid metabolism

J. Vanessa Valbuena Perez; Anna Dembek; Rebecca Linnenberger; Carlo Riccardi, Ph.D.; Stefano Bruscoli, Ph.D.; Markus R. Meyer, Ph.D.; Alexandra K. Kiemer Ph.D.; and Jessica Hoppstädter. Ph.D.

6. Summary and Conclusions

Statins are essential in the pharmacotherapy for prevention of the most important cause of death globally, *i.e.* cardiovascular disease. These drugs are generally considered safe and exert a plethora of beneficial effects in the vasculature. SAMS, however, still represent an important reason for non-adherence to treatment. Because of their widespread use, even a low incidence in SAMS would represent a substantial number of patients affected. In the present study, GILZ was identified as an essential mediator of the molecular mechanisms underlying statin-induced muscle damage and impairment of muscle regeneration. We confirmed the importance of impaired protein geranylgeranylation, Akt signalling, and FoxO3 activation in statin-induced myotoxicity, and demonstrated that loss of GILZ can prevent statin-induced cell death.

The effects of statins in muscle differentiation have been less investigated than their myotoxicity. This work demonstrated that GILZ induction by statins is also involved in their deleterious effects on myoblast differentiation, since GILZ depletion was able to partially rescue myogenesis in the presence of statins. Moreover, the involvement of endogenous GILZ in the regulation of skeletal muscle development and regeneration appears as an interesting topic for further research.

The functional implications of GILZ induction by statins are, nonetheless, not only negative, since GILZ is also induced in vascular endothelial cells and macrophages, two cell types in which the anti-inflammatory function of this protein is well characterised. To which extent GILZ contributes to the pleiotropic, anti-inflammatory actions of statins, remains to be investigated.

In conclusion, the first part of this study contributes to a better understanding of the molecular mechanisms underlying statin-induced myopathy, a valuable step towards the development of prevention strategies and safer therapy approaches for this class of drugs. Furthermore, the findings presented here open the opportunity to investigate the potential role of GILZ as a mediator of the anti-inflammatory actions of statins in the cardiovascular system.

The onset of CVD and other chronic inflammatory diseases is largely predicted by age. Indeed, the field of geroscience considers that aging and age-related diseases share a molecular basis, in which inflammation plays a key role. The comprehensive investigation of the mechanisms that fuel, maintain, and modulate inflammaging is therefore necessary to understand its potential role in age-related pathogenesis and identify points for intervention. Macrophages are the central cell type driving the inflammatory response, and maladaptive function of aged macrophages largely contributes to inflammaging. The second part of this work investigated whether GC regulation was altered with age in this cell type, given that GCs are main modulators of the immune function and stress response. The data obtained provide insight into the alterations in GC metabolism in macrophages during aging. Reduced circulating GC levels,

together with a decreased availability of active GC within cells as a consequence of reduced 11β-HSD1, might contribute to the imbalance between pro- and anti-inflammatory signalling in macrophages from aged mice, thus promoting the inflammaging phenotype.

7. References

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Appendix

Appendix 1

Gene expression in tissues from young and aged WT mice. *Gilz, Il6, and* GR *(Nr3c1)* mRNA expression levels were measured in heart, liver, lung, lymph nodes, skeletal muscle, and spleen tissues from young (10 weeks) and aged (80–100) weeks C57BL/6 mice at the baseline (**A,C,E,** n=9–13), or in liver, lung, lymph nodes, and spleen after treatment with 5 mg/kg LPS for 4 hours (**B,D,F**, n=4–6). Gene expression was normalised against the housekeeping gene (*Ppia*) and is presented as fold change of young. Box-plots show the 25–75th percentiles (Box), mean (square), median (line) and range within 1.5 IQR (whiskers). Levels below LOD in A and B were assigned as zero-points for illustrational purposes. **P<0.01 relative to equally treated young mice.
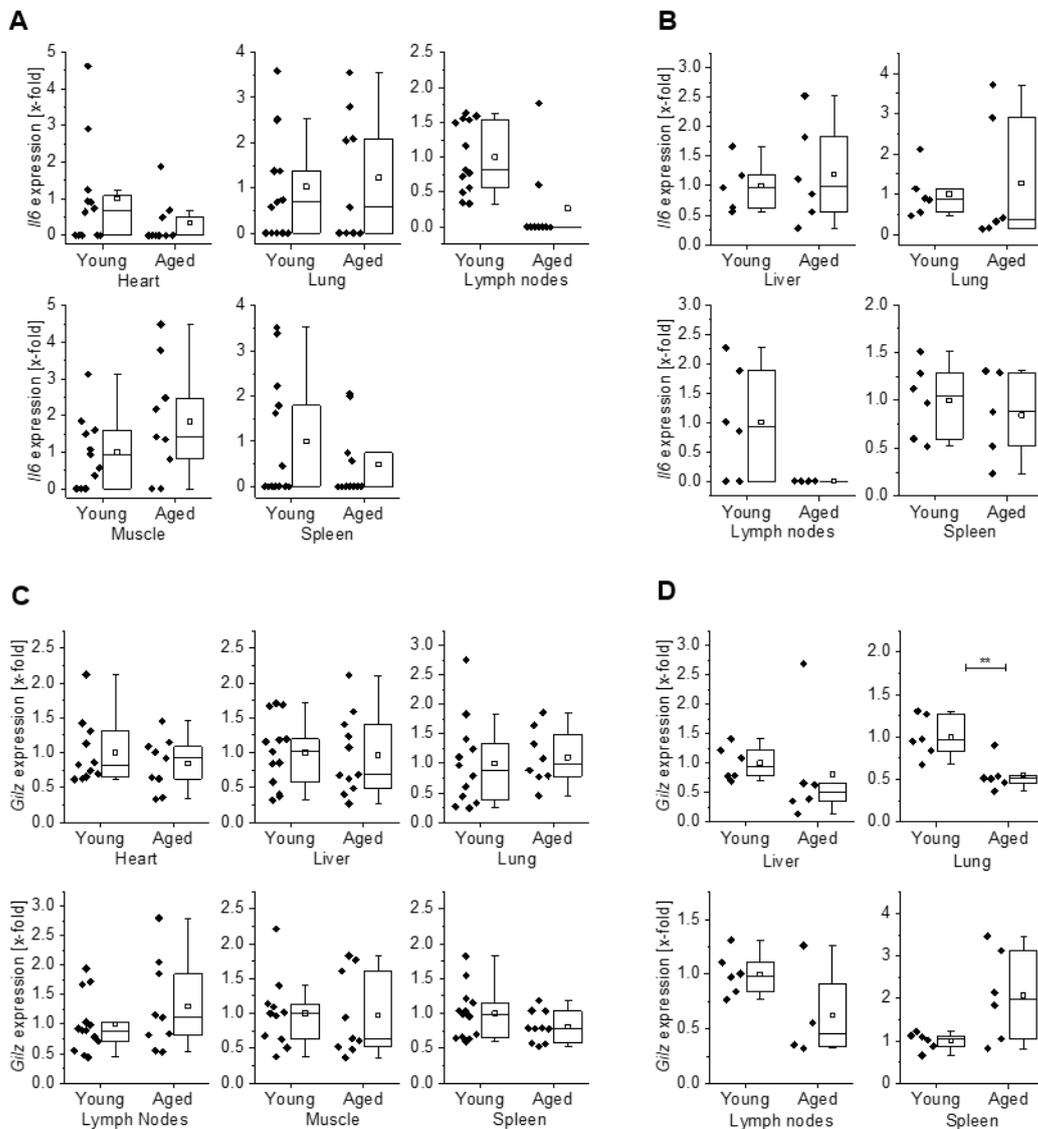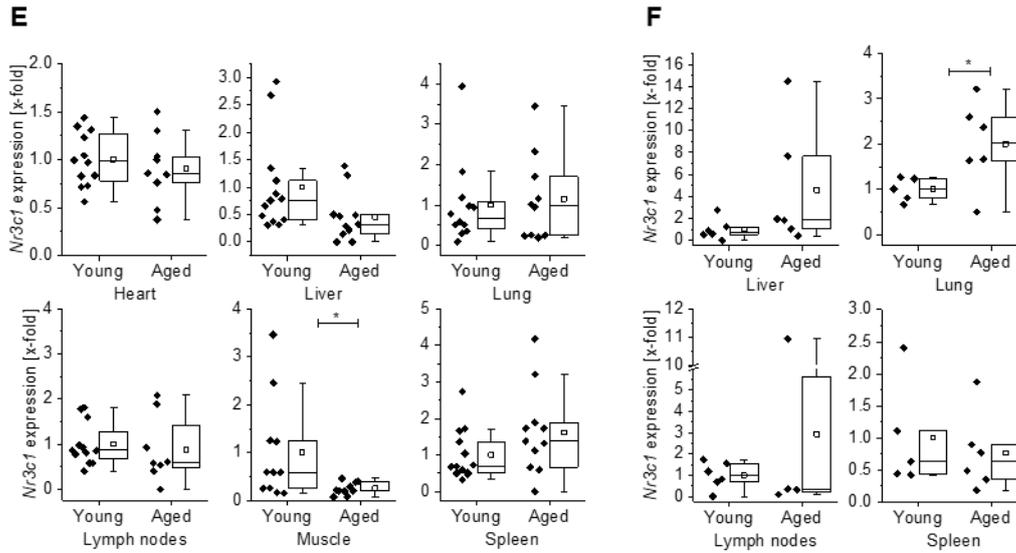
Gene expression in tissues from young and aged GILZ KO mice. mRNA expression was measured in tissues after treatment with 5 mg/kg LPS for 4 hours (n=4–6), normalised against the housekeeping gene (*Ppia*) and is presented as fold change of equally treated young WT. Data show mean (95% CI). *P<0.05 relative to equally treated young WT mice, as determined by Welch's t-test.

		Relative mRNA expression [x-fold]			
		Tnf	*Il1b*	*Il6*	*Nr3c1*
Young KO	Liver	2.16 (0.73-3.58)	1.27 (0.43-2.10)	0.92 (0.02-1.82)	1.72 (0.17-3.26)
	Lung	0.81 (0.38-1.24)	1.05 (0.39-1.72)	1.45 (-0.25-3.15)	2.05 (0.92-3.19)*
	Lymph nodes	2.24 (0.98-3.50)	1.23 (0.44-2.02)	0.88 (-0.41-2.17)	1.27 (0.15-2.39)
	Spleen	0.63 (0.38-0.89)*	2.48 (0.07-4.90)	0.98 (-0.10-2.06)	0.79 (0.28-1.30)
Aged KO	Liver	3.51 (1.52-5.49)	1.30 (0.34-2.26)	1.49 (0.98-2.00)	11.8 (9.53-14.2)
	Lung	0.85 (0.28-1.42)	1.95 (0.39-3.51)	0.75 (-0.04-1.53)	1.22 (0.65-1.78)
	Lymph nodes	0.45 (0.27-0.63)	0.84 (0.33-1.36)	12.9 (-1.13-26.9)	1.76 (0.55-2.97)
	Spleen	0.56 (0.36-0.76)	1.60 (-0.08-3.27)	2.60 (0.36-4.83)	1.06 (0.53-1.60)

Appendix 2

Gene expression in PM/PBL from young and aged WT mice. mRNA expression levels were measured in PMs and PBLs from young (10 weeks) and aged (80–100 weeks) C57BL/6 mice (n=13–14). Gene expression was normalised against the housekeeping gene (*Ppia*) and is presented as fold change of young. Box-plots show the 25–75th percentiles (Box), mean (square), median (line) and range within 1.5 IQR (whiskers). ***P<0.001 relative to young mice.
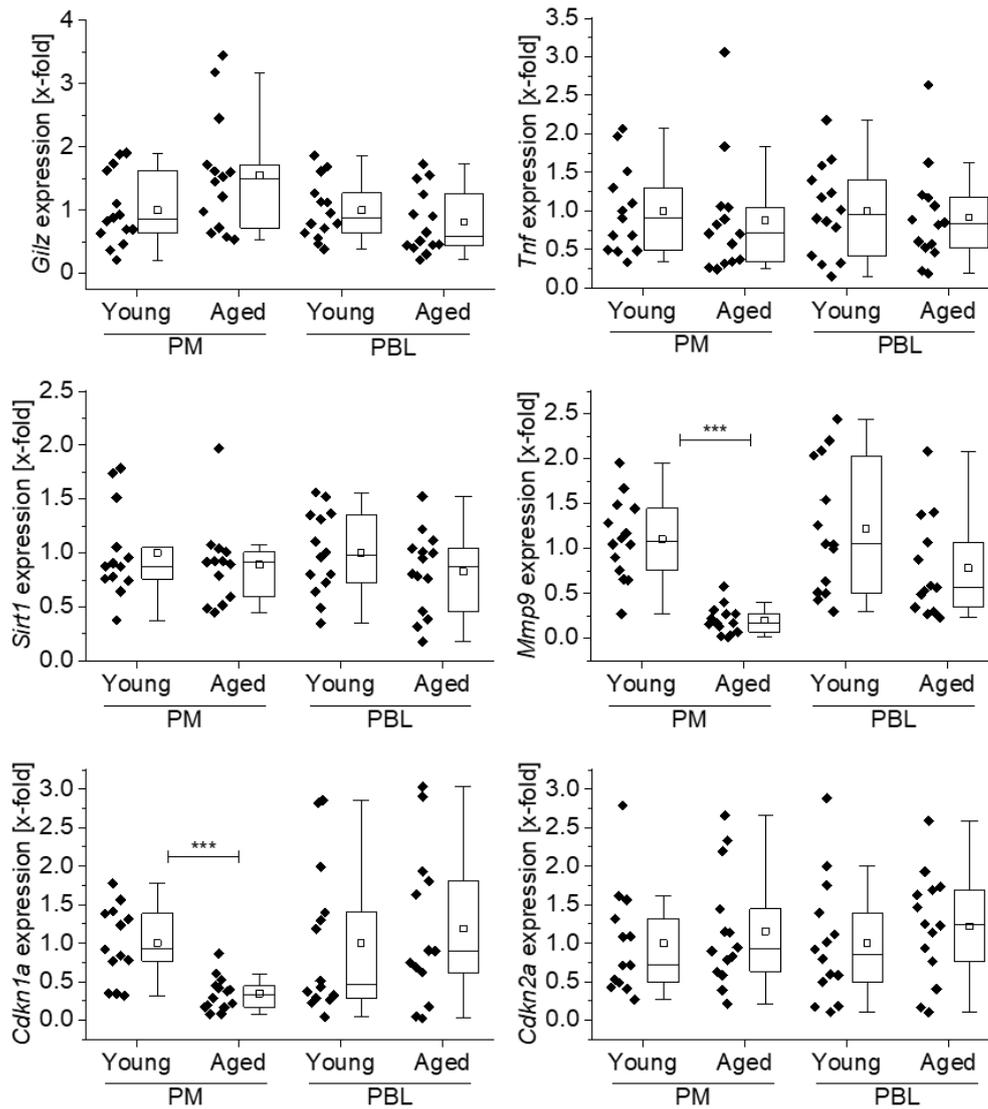
Gene expression in PM/PBL from young GILZ KO mice. mRNA expression levels were measured in PMs and PBLs from young (10 weeks) GILZ KO mice (n=11). Gene expression was normalised against the housekeeping gene (*Ppia*) and is presented as fold change of young WT. Data show mean (95% CI). **P<0.01, ***P<0.001 relative to young WT mice, as determined by Welch's t-test.

Gene	PM	PBL
Tnf	1.26 (0.32-2.20)	0.66 (0.53-0.80)
Mmp9	1.31 (0.89-1.73)	1.31 (1.03-1.60)
Nr3c1	0.86 (0.73-1.00)	1.73 (1.37-2.10)**
Anxa1	1.66 (1.28-2.04)**	3.16 (1.88-4.44)**
Dusp1	1.38 (0.63-2.13)	1.04 (0.68-1.40)
Sgk1	1.63 (1.19-2.07)	0.91 (0.50-1.32)
Hsd11b1	0.62 (0.36-0.89)	0.96 (0.77-1.15)
Hsd11b2	1.14 (0.70-1.57)	1.12 (0.78-1.46)
H6pd	0.23 (0.18-0.28)***	1.06 (0.64-1.47)
Cebpa	0.85 (0.49-1.22)	1.10 (0.62-1.59)
Cebpb	0.98 (0.70-1.26)	1.23 (0.76-1.69)
Cdkn1a	1.02 (0.67-1.37)	1.37 (0.63-2.11)
Cdkn2a	1.49 (0.98-2.00)	1.89 (0.92-2.87)
Sirt1	0.96 (0.64-1.29)	0.87 (0.60-1.15)

Appendix 3

Gene expression in skeletal muscle from young and aged mice. mRNA expression levels were measured in skeletal muscle tissue from young (10 weeks) and aged (80–100 weeks) C57BL/6 mice (n=13–14). Gene expression was normalised against the housekeeping gene (*Ppia*) and is presented as fold change of young. Box-plots show the 25–75th percentiles (Box), mean (square), median (line) and range within 1.5 IQR (whiskers).
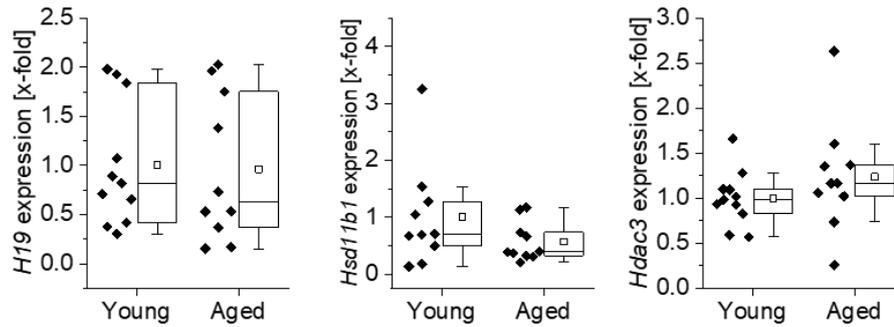
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