

2014

The human Klotho VS variant: focus on the processing and function of the V, S and VS isoforms

<https://hdl.handle.net/2144/13168>

"Downloaded from OpenBU. Boston University's institutional repository."

BOSTON UNIVERSITY
SCHOOL OF MEDICINE

Dissertation

**THE HUMAN KLOTHO VS VARIANT: FOCUS ON THE PROCESSING
AND FUNCTION OF THE V, S AND VS ISOFORMS**

by

TRACEY BETH TUCKER ZHOU

B.S., University of Rochester 2007

Submitted in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

2014

Approved by

First Reader

Carmela R. Abraham, Ph.D.
Professor of Biochemistry

Second Reader

Shelley J. Russek, Ph.D.
Professor of Pharmacology and Experimental Therapeutics,
Director BU Graduate Program in Neuroscience

© Copyright by
TRACEY BETH TUCKER ZHOU
2014

ACKNOWLEDGEMENTS

First and foremost, I would like to thank Dr. Carmela Abraham for being a wonderful, kind and caring mentor throughout my years in her lab and for providing a great atmosphere for me to learn and develop.

Secondly, I would like to thank all of my committee members for their support throughout my time as a student here. Dr. Benjamin Wolozin, thank you for being the Chair of my committee and for keeping everyone in line. Dr. Shelley Russek, thank you for being my second reader and for your positive attitude throughout the whole process. Dr. Susan Leeman, thank you for being the unofficial third reader. You provided a lot of needed guidance in my preparations for the defense. Dr. Konstantin Kandrор, thank you for your constructive criticisms—it really helped make sure my data was of a good quality.

I would also like to thank all the past and present members of the Abraham Lab for their support both in and out of the lab. This includes Dr. Rong Fan, Jane Hsu, Dr. Christina Khodr, and Jen Liang. Dr. Cidi Chen, thank you for your help in getting this project started and with the all the cloning work. Dr. Gwendalyn King, thank you for all your guidance and really making me get my butt in gear. I would not be in the same place without you. Tricia Brazee, thank you for your help with experiments and also for being an awesome friend. Also, a big thank you to my fellow graduate students in my time here –Dr. Pauline So and Mike Nagle –you all have been great friends and really made working in this lab a lot of fun.

All the students in both the Pharmacology and Biochemistry Departments deserve a big thank you as well, for their support and for always being there to commiserate. This includes my awesome classmates Dr. Jennifer Parker Duffen and Zhuting Li as well as all the others including but not limited to: Cathy Wei, Yvette Joon Ying Boon, Brian Honeyman and Terri Mills. Also Dr. Joerg Kumbrink, you are not a student, but you've also been an awesome friend throughout the years and have always been there to listen to my scientific problems. I would also like to thank the staff of both the Pharmacology and Biochemistry Department for all their help throughout the years. They really do a great job of keeping all the students in line.

Last but not least, I could not have accomplished any of this without the support of my family and friends. They have helped me get through this as well as so many other obstacles in my life. Of course, I have to thank my parents for infecting me with the science bug and for their years of seemingly infinite support. Also, thank you to my big brother Darren and my sister-in-law Cat for their support and providing distractions in the form of my new nephew Leo (and Alfie). And of course, thank you Yuan for putting up with me through all of this. I wouldn't have made it through without you.

**THE HUMAN KLOTHO VS VARIANT: FOCUS ON THE PROCESSING
AND FUNCTION OF THE V, S AND VS ISOFORMS**

TRACEY BETH TUCKER ZHOU

Boston University School of Medicine, 2014

Major Professor: Carmela R. Abraham, Ph.D., Professor of Biochemistry

ABSTRACT

Klotho (KL), an anti-aging protein, attracted interest in the aging field because of the dramatic phenotype of KL deficient mice and its connection to signaling pathways implicated in aging. The KLVS variant consists of the F352V (KLV) and C370S (KLS) substitutions. It was detected in genome wide association studies (GWAS) that linked it to alterations in longevity and disease risk. The molecular mechanism(s) underlying these associations are unknown. To understand how KL increases the risk of age-related diseases, the studies in this dissertation investigated whether expression of the KLVS variant, when compared to wildtype (KLWT), displays differences in processing, protein-protein interactions and enzymatic activity.

Differences in processing were evaluated by studying changes in shedding, half-life and plasma membrane localization of KL variants. The decrease in KLV shedding, as measured by the intracellular: extracellular ratio,

were explained by a decreased half-life. This decreased half-life is potentially due to decreased KLV plasma membrane localization, which is attenuated by co-expression of dominant negative dynamin, suggesting a role of endocytosis in these differences.

To assess whether there are changes in KLVS protein-protein interactions, differences in dimerization were measured by Blue Native gel electrophoresis and cross-linking. KLV dimerization was increased while KLS and KLVS variants decreased dimerization. Co-immunoprecipitation of tagged KL assessed whether these changes were due to alterations in homodimerization. The presence of KLVS in dimers decreased the levels of immunoprecipitated KL suggesting KLVS decreases homodimerization.

Changes in heterodimerization of KLVS with fibroblast growth factor receptor (FGFR) 1c were also investigated through co-immunoprecipitation. KLVS increased heterodimerization with FGFR1c. Addition of FGF23, for which KL is a co-receptor, showed that KLVS increases FGF signaling downstream of FGFR1c.

To determine differences in enzymatic activity of KLVS, 4-methylumbelliferyl-beta-D-glucuronide was used to measure alterations in glucuronidase activity. Results showed that KLVS had decreased enzymatic activity compared to KLWT.

These findings are the first to show that KLVS leads to differences in function as demonstrated by decreased homodimerization and enzymatic activity

and increased heterodimerization with FGFR1c. Given the association of KLVS with disease and longevity, these results suggest that these functions are integral in KL's anti-aging role in humans.

TABLE OF CONTENTS

Title.....	i
Copyright Page.....	ii
Reader’s Approval Page.....	iii
Acknowledgements.....	iv
Abstract.....	vi
Table of Contents.....	ix
List of Figures.....	xvii
List of Abbreviations.....	xx
Chapter 1: Introduction.....	1
Theories of Aging.....	1
General Introduction.....	1
Evolutionary Theories.....	2
Aging Theory of Natural Selection.....	2
Disposable Soma Hypothesis.....	3
Oxidative Stress And Aging.....	5
Free Radical Theory.....	5
Mitochondrial Theory.....	7
DNA Damage And Aging.....	8
Gene Regulation Theory.....	8
Senescence/Telomere Theory.....	11

System Based Theories Of Aging.....	13
Network Theory.....	13
Inflammation Hypothesis.....	15
Neuroendocrine Theory.....	16
Neuroendocrine-Immuno Theory.....	18
Conclusions.....	19
Klotho And Aging	19
Discovery Of Klotho.....	19
Characterization Of Klotho.....	20
Structure Of Klotho.....	20
Expression Of Klotho Protein.....	22
Klotho Gene Regulation.....	24
Klotho Protein Processing.....	25
Klotho Deficient Mouse.....	26
General Properties	26
Hypogonadism.....	26
Premature Thymic Involution.....	27
Tissue Calcification.....	28
Impaired Bone Mineralization.....	28
Skin Atrophy.....	29
Pulmonary Emphysema.....	29
Neural Deficits.....	30

Hearing Loss.....	32
Cardiovascular Deficits.....	32
Blood Chemistry.....	33
Rescue Of Phenotypes By Klotho Expression.....	33
Klotho Overexpressing Mouse.....	35
Phenotype Of KL Overexpression.....	35
Protective Effects Of KL Overexpression.....	35
Klotho Functions.....	36
Klotho And Fibroblast Growth Factor Signaling.....	37
Klotho Regulation Of Ion Channels.....	41
Klotho Inhibits Insulin/IGF Signaling.....	43
Klotho Inhibition Of Other Growth Factors.....	45
Klotho And Oxidative Stress.....	46
Klotho’s Anti-Inflammatory/Immune System Function.....	47
Klotho And Senescence.....	48
Klotho And Nitric Oxide Synthesis.....	49
Summary.....	51
Klotho And Disease.....	51
Chronic Kidney Disease.....	52
Epilepsy.....	54
Acromegaly.....	54
Cancer.....	55

Cardiovascular Disease.....	57
Diabetes.....	58
Case Studies And Polymorphisms.....	59
Case Study: Increased Circulating Klotho.....	59
Case Study: H193R Polymorphism.....	62
Polymorphism Of The Promoter: G395A.....	63
Polymorphisms In Non-Coding Regions.....	65
Polymorphisms In KL2.....	66
Polymorphisms In KL1.....	68
Hypothesis And Specific Aims.....	71
Significant Aim 1: Investigate Whether KLVS Alters Kl Processing.....	72
Significant Aim 2: To Determine Whether The KLVS Variant Leads To Altered Function.....	72
Chapter 2: Methods.....	73
Plasmid DNA Cloning.....	73
Plasmid Constructs.....	73
KLVS Variant Constructs.....	73
Truncated Klotho Constructs.....	73
Klotho Constructs With GFP Tag.....	74
Klotho Constructs With V5 Tag.....	74
Site Directed Mutagenesis.....	75
Restriction Enzyme Digest.....	75

DNA Ligation.....	76
Agarose DNA Gel Electrophoresis.....	76
DNA Gel Extraction.....	77
DNA Sequencing.....	77
Plasmid DNA Preparation.....	77
Bacterial Transformation.....	77
Minipreps.....	78
Midipreps.....	78
DNA Spectrophotometry.....	79
Cell Culture.....	79
Maintenance Of Cell Cultures.....	79
Human Embryonic Kidney (HEK) Cells.....	79
HEK Stable Cells.....	80
Transfection.....	82
Sample Preparation.....	82
Preparation Of Cell Lysates.....	82
Preparation Of Media Samples.....	83
Protein Analysis.....	84
Bicinchoninic Acid (BCA) Assay.....	84
Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis.....	84
Protein Transfer.....	85
Western Blots.....	85

Western Blot Antibodies.....	86
Analysis Of Klotho Processing.....	87
Shedding Assay.....	87
Gaussia Luciferase Assay.....	87
MT-1 Chemical Treatment.....	88
Analysis Of Klotho Half-Life.....	88
Membrane Biotinylation	89
SORLA Effects On Kl Trafficking.....	89
Immunofluorescence.....	90
Klotho Functional Assays.....	92
Live Cell Chemical Crosslinking.....	92
Blue Native Gel Electrophoresis.....	92
Co-Immunoprecipitation.....	93
FGF 23 Assay.....	94
Enzymatic Assay: β -Glucuronidase Activity.....	95
Data Analysis And Statistics.....	96
Chapter 3: Results.....	98
Aim 1: Changes In Kl Processing Due To KLVS Variant.....	98
Klotho Variant Constructs.....	98
Expression Of KLV Variant Leads To Decreased Shedding.....	100
Kl Transmembrane Domain Necessary For Decrease In KLV Shedding..	101
KLV Shedding Decrease Is Not Due To Misfolding.....	103

MT-1 Increases Kl Shedding Irrespective Of Variant Expression.....	103
Glycerol Treatment Can Not Rescue KLV Shedding Differences.....	105
Screening For Modulators Of Kl Shedding.....	107
Designing Screen To Discover Modulators Of Kl Shedding.....	107
Igf-2 Increases Kl Shedding.....	109
KLV Expression Results In Decreased Half-Life.....	111
Membrane Localization Of Kl Variants.....	113
Kl Isoforms Result In Altered Membrane Localization.....	113
Kl Variants Alteration Of Membrane Localization Attenuated By Inhibition Of Endocytosis.....	115
SORLA Does Not Alter KLVS Processing.....	115
Altered Subcellular Localization Of Kl Variants In Endosomes.....	117
Aim 2: KLVS Variant Leads To Altered Kl Function.....	119
KLVS Variant Alters Kl Dimerization.....	119
Alteration In Dimerization Assessed By Crosslinking.....	119
Differences In Dimerization As Shown By Blue Native Page.....	122
Homodimerization Decreased By KLVS And Implications For Heterozygote KLVS Carriers.....	124
KLVS Increases Heterodimerization With FGFR1c.....	126
KLVS Increases Downstream Signaling Through FGFR1c.....	129

KLVS Has Decreased Enzymatic Activity.....	131
Chapter 5: Discussion.....	139
Klotho And Aging.....	139
Klotho Polymorphisms.....	140
Alterations In Processing Of KLVS Isoforms.....	141
KLV Expression Leads To Altered KI Processing.....	141
KLS Alters Endosomal Trafficking.....	145
Intragenic Complementation Of KLV And KLS Result In Like Normal KLVS Processing.....	146
KLVS Variant Alters KI Function.....	147
KLVS Decreases Homodimerization.....	147
KLVS Increases Heterodimerization With FGFR1c.....	152
Effect Of KLVS On Enzymatic Activity Of KL.....	154
Summary.....	154
Future Directions.....	155
Concluding Remarks.....	156
List of Journal Abbreviations.....	159
References.....	169
Curriculum Vitae.....	219

LIST OF FIGURES

Figure	Title	Page
1	Interconnection of Aging Theories.....	21
2	Schematic representation of KL	23
3	Klotho Control of FGF23 Signaling.....	39
4	Regulation of Ion Channels by KL-mediated Removal of Sialic Acid.....	42
5	Klotho Inhibits the Insulin/IGF Pathway.....	44
6	Klotho’s Activity in Ion Homeostasis and Beyond.....	50
7	Location of KL Polymorphisms in Coding and Non-Coding Regions.....	60
8	Association of KLVS with Disease Risk.....	69
9	Diagram of KL Constructs.....	99
10	KLV Dramatically Decreases Shedding.....	102
11	Truncation of KL Attenuates Shedding Differences.....	104
12	MT-1 Increases KL Shedding Irrespective of Variant Expression.....	106
13	Glycerol Treatment Can Not Rescue KLV Shedding Differences.....	108
14	Designing Screen to Discover Modulators of KL Shedding	110

15	Validating GLucKL as Screening Platform.....	112
16	IGF-2 Increases KL Shedding.....	114
17	KLV Decreases Protein Half-life.....	116
18	Blocking internalization differentially affects KL V and S variant membrane localization.....	118
19	SORLA Does Not Affect KLVS Processing.....	120
20	Altered Subcellular Localization of KL Variants in Endosomes.....	121
21	Crosslinking Reveals Differences in KLVS Dimerization.....	123
22	KL Variants Alter the Levels of Both Monomeric and Dimeric KL in Cells.....	125
23	Diagram of KL-KL Co-Immunoprecipitation.....	127
24	KL homodimerization is altered by the presence of the VS variant.....	128
25	Control Co-immunoprecipitation of KLGFP and KLV5.....	130
26	KLVS enhances interaction with FGFR1c.....	132
27	Control Co-immunoprecipitation Shows No Non-Specific Pulldown of FGFR1c/KL Complex.....	133
28	KLVS enhances FGF23 signal transduction.....	135
29	KLVS Leads to Decreased Enzymatic Activity.....	137
30	Immunodepletion of KL Eliminates KL Specific Enzymatic Activity.....	138

31	Correlation Between Shedding and Half-life Changes in KL Variants.....	143
32	Shedding and Dimerization Changes in KL Variants are Inversely Correlated.....	149
33	Half-Life and Dimerization Changes in KL Variants are Inversely Correlated.....	151
34	Interactions of Different KL Functions on the Plasma Membrane.....	158

LIST OF ABBREVIATIONS

[S ₀]	Initial Substrate Concentration
4Mu-GlcU	4-methylumbelliferyl- β -D-glucuronide
8-OHdG	8-Hydroxydeoxyguanosine
ADAM	A Disintegrin and Metalloproteinase
AKI	Acute Kidney Injury
BCA	Bicinchoninic Acid
bFGF	basic Fibroblast Growth Factor
BME	β -mercaptoethanol
BMI	Body Mass Index
BRCA1	Breast Cancer 1, Early Onset
BSA	Bovine Serum Albumin
cAMP	Cyclic AMP
CASR	Calcium Sensing Receptor
CD4	Cluster Of Differentiation 4
cDNA	complementary DNA
CKD	Chronic Kidney Disease
CSF	Cerebrospinal Fluid
CVD	Cardiovascular Disease
Cyp2b1	Cytochrome P450 2B1
DMEM	Dulbecco's Modified Eagle's Media
DMP1	Dentin Matrix Phosphoprotein 1

DMSO	Dimethyl Sulfoxide
DN	Dominant Negative Dynamin
DPBS	Dulbecco's Phosphate Buffered Saline
DSP	Dithiobis[succinimidylpropionate]
Egr1	Early Growth Response Protein 1
EMT	Epithelial to Mesenchymal Transition
ER	Endoplasmic Reticulum
ERK	Extracellular Signal-Related Kinase
EV	Empty Vector
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factors
FGF23	Fibroblast Growth Factor 23
FGFR	Fibroblast Growth Factor Receptor
FOXO	Forhead Box O
FRS2 α	Fibroblast Growth Factor Receptor Substrate 2 α
G418	Geneticin
GALNT3	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 3
GFAP	Glial Fibrillary Acidic Protein
GFP	Green Fluorescent Protein
GSK3 β	Glycogen Synthase Kinase 3 β
GWAS	Genome Wide Association Studies

HDL	High-Density Lipoprotein
HEK	Human Embryonic Kidney
HPA	Hypothalamus Pituitary Axis
HRP	Horseradish Peroxidase
HS	Hippocampal Sclerosis
HUVECs	Human Umbilical Vein Endothelial Cells
IGF	Insulin Like Growth Factor
IRI	Ischemia Reperfusion Injury
KL	Klotho
KL1	1 st Extracellular Domain of Klotho
KL2	2 nd Extracellular Domain of Klotho
KLWT	Wildtype KL
K_m	Michaelis-Menten Constant
LacNAc	N-acetyllactosamine
Late Endo.	Late Endosomes
Lyso.	Lysosome
M6P	Mannose-6-Phosphate
MAP2	Microtubule-associated Protein 2
MAPK	Mitogen Activated Protein Kinase
mIgG	mouse IgG
MMP-9	Matrix Metalloproteinase-9
NaPi-2a	Sodium-Phosphate Co-Transporter Type 2a

NaPi-2b	Sodium-Phosphate Co-Transporter Type 2b
NCBI	National Center for Biotechnology Information
NEB	New England Biolabs
NF κ B	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
NO	Nitric Oxide
p21	Protein 21
p53	Protein 53
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
pERK	Phosphorylated ERK
PHEX	Phosphate-Regulating Neutral Endopeptidase, X-Linked
PI3K	Phosphoinositide 3-Kinase
PKA	Protein Kinase A
PKC	Protein Kinase C
PM	Plasma Membrane
PTH	Parathyroid Hormone
RAAS	Renin-Angiotensin-Aldosterone System
Rel A	Reticuloendotheliosis oncogene cellular homolog A
RER	Rough Endoplasmic Reticulum
RFU	Relative Fluorescence Units
RIG-1	Retinoic Acid Inducible Gene-1
RNAi	RNA interference

ROMK1	Renal Outer Medullary Potassium Channel 1
SASP	senescence-associated secretory phenotype
SCID	Severe Combined Immunodeficiency
SDS	Sodium Dodecyl Sulfate
SIRT1	sirtuin1
SNPs	Single Nucleotide Polymorphisms
SOD2	Mitochondrial Manganese Superoxide Dismutase
sp1	Specificity Protein 1
TCA	Trichloroacetic Acid
tERK	Total ERK
TGF β	Transforming Growth Factor B
TIMP-1	Tissue Inhibitor Of Matrix Metalloproteinase-1
TLE	Temporal Lobe Epilepsy
TNF- α	Tumor Necrosis Factor- α
TRPC	Transient-Receptor Potential Canonical
TRPV	Transient Receptor Potential Vallinoid
v_0	Initial Velocity
VEGF	Vascular Endothelial Growth Factor
VEGFR2	Vascular Endothelial Growth Factor Receptor 2
v_{max}	Maximum Velocity
VSMCs	Vascular Smooth Muscle Cells
WGA	Wheat Germ Agglutinin

CHAPTER I: INTRODUCTION

1. THEORIES OF AGING

1.1 General Introduction

Over the years, advances in preventative medicine and progress in the treatment of the major killers such as heart disease, stroke and cancer, have led to an increase in the percentage of the United States population over the age of 65. From the years 1960 to 1990 alone, there was an 86.67% increase in this group compared to the 38.7% overall increase in the population. When this comparison is confined to people who are over 85, the difference is even greater. This group saw an increase of 225.2% over this 30-year period (1). Despite our increased ability to prolong life and delay the onset of many diseases, we still do not have a good understanding of the underlying causes of aging. Until we have a better grasp of what causes aging, we will be able to delay, but not prevent the many diseases for which aging is a major risk factor. Although we have found ways to prolong the healthy years, eventually most people succumb to disease, which leads to a heavy strain on healthcare and other systems that have to support the people that can no longer care for themselves. Although disease prevention is an admirable goal, targeting just one disease at a time will only lead to small incremental differences in lifespan. Even curing cancer, which is one of the leading causes of death in the US(2), would only lead to a 3-year extension of life expectancy. By targeting and understanding the underlying mechanisms of

aging instead, we could better grasp the underlying causes of these diseases leading to a healthier aging population.

Intense study of the mechanisms of aging is fairly new, but despite that, many have attempted to create theories in order to explain why organisms age. In fact, in the relatively short time since the first theory was posited in 1889, there have been more than 300 theories of aging (3). Most have merit and explain an important aspect of aging, but due to the complexities of aging, the answer will not be found solely in one theory. Developing a global understanding of the posited theories from past and present help to not only understand how the field has matured, but also to appreciate how they come together to help us reach towards the ultimate goal of elucidating how and why organisms age.

1.2. Evolutionary Theories

1.2.1 Aging Theory of Natural Selection

One of the first theories of aging posited in the 1940's built on ideas from natural selection. This theory suggested that forces of natural selection decline with age resulting in selection of genes that are beneficial for early survival and reproduction but detrimental for longevity. This theory was first advanced after observations on the pervasiveness of Huntington's disease. It was thought that a dominant lethal gene such as Huntingtin should be selected against, but because of its later onset (after child-bearing age) there was no selective pressure against it (4). Another contemporary scientist expounded upon this idea leading to the

Mutation Accumulation Theory of aging. This theory suggested senescence and aging associated pathology may be caused by the accumulation of late-acting detrimental mutations that escape natural selection due to time of onset (5). This was further built upon by G.C. Williams, who came up with the idea of antagonistic pleiotropy, he proposed that late-acting detrimental mutations could be selected for if they caused beneficial pleiotropic effects in early life (6). Although the mutation accumulation theory is not currently widely backed (7), these early theories spurred a plethora of research into the role natural selection plays in aging.

Research that was built upon the early theories of aging has shown that natural selection can play a role in how organisms age. Not only does selection of offspring derived from longer-lived parents lead to an increase in lifespan in *Drosophila* (8,9), but different environmental conditions of opossums also altered their lifespan. When compared to opossums that live in areas of high predation, island opossums that were protected from predators lived longer and aged more slowly than their mainland counterparts (10). This suggests that natural selection due to predation and environmental conditions can affect the longevity of organisms.

1.2.2 Disposable Soma Hypothesis

One hypothesis that expanded upon the idea of antagonistic pleiotropy, the disposable soma hypothesis, suggested that selection favors genes that

increase reproductive fitness regardless of its effect on long-term survival (11). It posited that in the tradeoff between energy invested into reproduction, somatic maintenance and survival, selection favors the traits that allow for increased reproductive fitness. More recent theories that have examined the origin of aging suggest that asymmetric cell division in unicellular organisms leads to an uneven distribution of accumulated mutations. By unevenly distributing structures into one progeny, the second progeny must make new structures that do not contain any of the accumulated damage resulting in the creation of one 'rejuvenated' offspring and one 'parent' cell that contains all the damage. If damage accumulation is high enough, this asymmetrical division can lead to progeny with a higher chance of survival than those obtained by symmetrical division. This asymmetrical division leads to the aging 'parent' population that has slowed division and growth rate and the 'rejuvenated' offspring population. Before this research it was thought that aging arose after the origin of eukaryotes, but this research suggests that it may have arose earlier and might be the underpinnings of the evolutionary reasons for aging (12).

These theories focus on the genetic origins of aging and longevity, which help us to understand why aging may have developed, but lack mechanistic insight into how the changes in genes lead to the phenotypes we see.

1.3 Oxidative Stress and Aging

1.3.1 Free Radical Theory

The free radical theory of aging was posited shortly after the debut of the first evolutionary theories in the 1950's. Inspired by Gerschman's observations on oxygen poisoning (13), Harman hypothesized that environmental and genetic factors lead to the accumulation of harmful oxygen radicals in cells producing the damage that causes aging and eventually death of organisms (14). This theory was further modified in lieu of new information about the importance of mitochondria in the initiation of most free radical reactions (15,16). The ubiquitous nature of free radicals makes the free radical theory a popular explanation of how and why organisms age. In eukaryotes specifically, mitochondrial respiration, which is necessary for energy production, is responsible for leaking free radical intermediates from the electron transport chain (17). Not only is the presence of free radicals and the scavengers responsible for their clearing universal, but the targets of free radicals are also widespread (18). Oxidative damage can affect most of the major players in cells: DNA, protein and lipid molecules (19) and this damage has been shown to accumulate in protein (20-22) and DNA (23-25) with age.

Despite evidence that oxidative damage accumulates with age, much of the evidence of its involvement in the aging process is limited to lower eukaryotes. *Drosophila* with increased superoxide dismutase expression led longer lives (26,27) and flies selected for longer lifespan showed increased levels of this

enzyme (28). Studies in *C. elegans* have also shown long-lived mutants are resistant to oxidative stress and have increased activity of superoxide scavengers with age (29). *C. elegans* longevity can also be increased artificially by compounds that mimic superoxide dismutase or other antioxidant molecules (30).

Unlike lower eukaryotes, modification of lifespan by regulation of oxidative stress in mammals has not been as straightforward. Overexpression of superoxide dismutase was not capable of modifying lifespan (31) and dietary administration of antioxidants to rodents has led to inconclusive results on its ability to modify lifespan (32). The unclear results of antioxidant administration could be due to differences in length of treatment (33) or differences in the outcome measured. Although treatments do not always have a direct effect on maximum lifespan, they can have an effect on quality of life as measured by organ and organelle health (32,34-39).

Oxidative damage is widely accepted as a contributing factor in the aging process despite the lack of effective treatments using antioxidants. Many groups have shown oxidized protein accumulate with age, which supports its connection to aging (40). Many pathways that protect against organism aging, which are discussed in detail later, also act by protecting against oxidative stress further implicating oxidative stress as a key player in the aging phenotype.

1.3.2 Mitochondrial Theory

Stemming from the reactive oxygen theory of aging, the mitochondrial theory concentrates on the importance of mitochondria not only in the generation of reactive oxygen species, but also on its role in maintenance of cell health and physiology (41). Despite the crucial role mitochondria play in cell health, they are susceptible to damage by reactive oxygen species. It is estimated that levels of oxidation in mitochondrial DNA are 10 to 20 fold higher than that of nuclear DNA due to the mitochondria's role in oxygen metabolism as well as its lack of histones and less efficient DNA repair (24,42). This higher rate of mutation creates a vicious cycle because of the mitochondria's role in the electron transport chain. Not only is it the source of many oxygen radicals, but mitochondrial DNA is responsible for encoding many of the proteins that make up the electron transport chain as well as proteins necessary for their synthesis (43). This means that damage to these genes can lead to further disruption of the electron transport chain potentially leading to additional alterations in levels of reactive oxygen species.

There is a lot of indirect evidence linking mitochondria to aging, including findings by the Framingham Heart Study that have shown lifespan is more strongly correlated with maternal than paternal death, suggesting that mitochondria may play a role in lifespan determination (44). Other studies have reported a correlation between mitochondrial DNA polymorphisms and longevity (45-47). Some groups have also shown that increasing rates of mutation in

mitochondrial DNA leads to the display of features suggestive of premature aging (48-50). Decreasing mitochondrial damage due to oxidative stress leads to the opposite effect, increasing the lifespan of mice overexpressing catalase in mitochondria (51). This research supports an indirect role of mitochondrial function in the aging process through its regulation of electron transport and metabolism. Despite these connections, more information is necessary to determine how intricately involved the mitochondria is in the process of aging.

1.4 DNA Damage and Aging

1.4.1 Gene Regulation Theory

The gene regulation theory of aging posits that senescence is the product of changes in gene expression that occur over time (52). Microarray studies have found many genes that are differentially expressed with age (39,53-60).

Reminiscent of the evolutionary theory of aging, it has been suggested that the genes that are differentially regulated by age are not likely to be genes that directly regulate senescence, but actually those that regulate survival (61). This distinction is necessary to help remind us that aging itself is not a programmed process, but is stochastic and not directly regulated. Despite this, twin studies in humans suggest there is a small albeit significant genetic component of lifespan (62,63). Studies in centenarians have also shown some genetic associations (64-66) and recently a locus of chromosome 4 has been linked to the longevity effects seen in this group (67).

It is known that genes are altered both directly and epigenetically in aging and it is possible that these alterations can lead to changes in their expression thus causing more age-related effects. Werner's syndrome, which causes progeria, gives insight into how gene recombination leads to changes in gene expression that can regulate aging. The WRN gene that is altered in this syndrome suppresses genome instability and homologous recombination (68,69) suggesting that dysregulation of recombination can lead to aging symptoms. Further evidence for the importance of gene recombination comes from mouse models that have defects in genome maintenance systems that protect against large genome rearrangements. Mice deficient in these areas have high levels of recombination and present with a variety of accelerated aging phenotypes (70,71). Levels of DNA methylation (72-74) and patterns of methylation have both been reported to change with age (75-78) and these changes can lead to alterations in the amount of specific genes transcribed (75,77-80). Changing the expression levels of genes could lead to alterations in cell function and contribute to the senescent phenotype seen with age.

Identification of the insulin/insulin like growth factor (IGF) 1 pathway's involvement in aging further suggests that gene regulation can affect aging. This pathway was first implicated in *C. elegans* where it was shown that worms with mutations in the insulin-like dauer formation pathway, lived longer (81). This research was followed by studies in mice showing that heterozygous knockdown of the IGF-1 receptor led to an increase in lifespan (82). This increase in lifespan

is thought to be connected to the regulation of transcription factors such as p53 and Forkhead box O (FOXO), which can also regulate lifespan in mice (83) (84). These transcription factors lead to the upregulation of antioxidant proteins showing a connection between the gene regulation theory and the free radical theory of aging.

Despite the connections to the insulin/IGF-1 pathway, many models of increased mouse longevity, including the fairly robust model of caloric restriction, are shown to have increased insulin sensitivity suggesting that there may be more Insulin/IGF-1 signaling (85). Although this further implicates this pathway in longevity and aging, the direction of influence is counter-intuitive given the effects of inhibiting the insulin pathway. This suggests that the interaction of insulin and IGF-1 with aging is more complex than is currently appreciated and must be studied further to understand the relationship.

The studies into the Gene Regulation Theory of aging provide multiple series of evidence suggesting that regulation of genes is involved in the aging process, but our understanding of aging and natural selection remind us that aging may not be directly regulated by these changes in gene expression. The full complexity of the relationship between gene expression changes and aging is not completely understood as small differences in gene transcription can lead to large downstream changes in protein expression. More investigation of the Gene Regulation Theory will elucidate how the changes in gene expression tie in to other theories of aging leading to the global effect seen in aging organisms.

1.4.2 Senescence/Telomere Theory

The theory of senescence stemmed from early observations in cultured cells demonstrating that these cells had a replicative limit (86,87). Hayflick and colleagues expanded upon these findings proposing what is now referred to as the senescence theory of aging. He described senescence through his observations that human cells in culture undergo a limited number of divisions after which cell growth was arrested and the physiology of the cells was altered.

Further research determined that the replicative senescence described is often due to shortening of telomeres, specialized DNA repeats found at the end of chromosomes. In human cells, telomeres are shortened during mitosis due to suppression of telomerase expression that is responsible for maintaining telomere length (88). Shortening of telomeres eventually leads to a DNA damage response that initiates senescence (89-93). Conversely, cells lines that express telomerase do not senesce, but continue to proliferate normally (94-96). Senescence can also be initiated by stress responses that include inappropriate nutrient conditions and oxidative stress (97-100). In most cases these are not associated with the same DNA damage response that occurs with telomere-associated senescence, but there are reports of oxidative damage causing telomere shortening (101). The development of the senescence growth arrest phenotype upon cell stress or DNA damage supports the role of antagonistic pleiotropy in aging. While it is thought to have arisen as anti-tumorigenic mechanism, it also has an affect in aging organisms.

Evidence for involvement of senescence in aging can be seen both in animal models and human tissues. Mice that express a constitutively active truncated protein 53 (p53) display a number of aging phenotypes culminating in a shortened lifespan (102,103). The transcription factor p53 is known to be a master regulator of senescence (104-107) and cells in vivo and in vitro from mice expressing the truncated form of the protein senesced rapidly (103,108), suggesting that the alteration in senescence is responsible for the aging effects. Other mice models that induce senescence also lead to pathologies resembling advanced aging (109-111). There is also evidence from human tissues showing that increased age of the tissue donor is correlated with a decreased capacity for population doubling (112-118). This suggests that older tissues have accelerated rates of senescence even when transplanted into younger hosts, although other studies show that parabiosis of young and old mice can influence the apparent age of both brain and heart tissue arguing that circulating factors are capable of modulating the aging phenotype (119,120). Further support for the involvement of senescence in human aging comes from data showing that shortened telomeres accumulate with age in human cell lines and tissues (121-123). Levels of β -galactosidase, a marker of *in vitro* senescence, also had increased expression with age (124) further pointing to an increase of senescence with age. All of this points to a role of senescence, either through telomere shortening or other means in the aging process.

1.5 System Based Theories of Aging

1.5.1 Network Theory

The network or immune theory of aging builds upon previous theories concentrating on the network of defense mechanisms in the cell that indirectly control aging (125). This network consists of a variety of mechanisms, namely DNA repair, production of heat shock and other stress proteins, enzymatic and non-enzymatic antioxidant defense systems, poly (ADP-ribose) polymerase activation and the immune system that have developed to protect cells from internal and external stressors (125). This theory explains differences in lifespan by the fact that the aforementioned mechanisms involved in the network are controlled by genes that have varying inter-species and intra-species expression levels (126). This intertwines the immune theory with previously mentioned theories suggesting a role of gene expression in aging.

The immune system, in particular macrophages, are highly implicated as playing a central role in the network of defense mechanisms (127). Macrophages' ability to respond to a multitude of different stress signals by releasing many different factors including cytokines, biogenic amines and neuropeptides make it a key regulator of the immune system and by proxy, the aging process (127). The results of many studies suggest that macrophage function and ability to respond to tissue-derived signals is impaired during aging (128-133), leading to an overall impaired immune response (134). Besides macrophages, other immune cell types have been implicated in aging. Lymphocytes, for example, are chronically

stimulated leading to an expansion of memory cells, decrease of naïve cells and shrinkage of the T cell repertoire with age (135-138).

To support the theory that the immune system is an important regulator of aging, it has been shown that maintenance of a healthy immune system is correlated with increased longevity (139). Additional evidence comes from studies in centenarians and long-lived individuals that demonstrate these populations have immune system comparable to young controls versus the decrease in function seen in normal elderly subjects (139-143), further implicating the immune system in the regulation of aging.

The importance of the network/immune theory is strengthened by its connections with other theories, including the free radical theory. Antioxidants are found in higher concentrations in immune cells suggesting dysregulation of this system with age could result in more oxidative stress due to fewer scavengers present. Antioxidants are also important for maintenance of proper immune system function (144) so it is possible that the increases in free radicals with age could lead to the dysfunction of the immune system. The network/immune theory also ties back to the evolutionary theory of aging because of the importance of the immune system in survival. Like many genes/ pathways that affect aging, the immune system is important for survival in early age, but in dysregulation in later stages it can be detrimental to the organism.

1.5.2 Inflammation Hypothesis

The inflammation hypothesis is directly connected to the network/immune theory of aging, but emphasizes the upregulation of inflammation in later life that leads to the chronic inflammatory state associated with many diseases (145-148). This “inflamm-aging”, as it has been coined (127), is the result of environmental stimuli and innate aging processes that lead to increased levels of pro-inflammatory cytokines (149-152) upsetting the balance between pro- and anti-inflammatory signals. The effects of polymorphisms that act upon these pathways demonstrate that altering levels of pro- and anti-inflammatory cytokines can alter longevity (153-156).

Like the network/immune theory of aging, this hypothesis ties in many different theories to start making a more coherent picture of how these multiple changes may lead to the global phenotype of aging. One theory to which the inflammation hypothesis is closely tied is the senescence/telomere theory. Senescent cells secrete pro-inflammatory cytokines in a process known as the senescence-associated secretory phenotype (SASP). This suggests that the increase in senescence associated with age can also lead to an increase in inflammation, which may be related to the inflammatory phenotype seen in aging. This theory, along with the network/immune theory, show how interconnected the different aging hypotheses are and highlight the importance of the immune system and inflammation in the aging process.

1.5.3 Neuroendocrine Theory

As the master regulator of the body, the brain is intrinsically linked to many of the systems that are suggested to be involved in aging. The neural and endocrine systems together are responsible for communicating and responding to the external environment by regulating internal responses to keep the body in an optimal functional state for reproduction and survival (157). Through these regulations, the neuroendocrine system is responsible for controlling evolutionarily important systems such as reproduction, growth and development as well as regulation of stress adaptation (157).

Many early hypotheses regarding the neuroendocrine involvement in aging focused on the role of biological clocks in controlling stages of life. The 'aging clock' was purported to be located in the hypothalamus, pineal gland, or thymus where it regulated the aging process through modification of hormone signaling (158-162). Other groups suggested that there is a defect of the hypothalamic pituitary axis, which controls stress responses throughout life. Many studies have shown that the hypothalamus is non-uniformly less sensitive with increased age and that metabolism and concentrations of neurotransmitters are changed as well (163-171). These alterations lead to secondary changes in the peripheral endocrine system creating the disruption of homeostasis that is thought to be the cause of aging (171-174). Many studies have focused on the decline in growth hormone secretion by the pituitary and associated levels of IGF-1 (175).

Despite the evidence suggesting a role of the neuroendocrine system in aging, attempts to treat individuals with hormone replacement therapy have met with controversial results. Many of the treatments seem successful, but are often fraught with side effects. This is true for dehydroepiandrosterone treatment (176-179) which has beneficial effects on body composition and muscle strength, but can lead to declines in the levels of some thyroid globulins and insulin resistance (176). Studies of the treatment of growth hormone deficient elderly patients with recombinant growth hormone showed positive changes in many features associated with aging and popularized the idea of using recombinant growth hormone therapy in healthy aging individuals (175,180). Unfortunately, follow up studies on the first group of treated individuals as well as others has shown that treatment with recombinant human growth hormone can lead to multiple serious side effects (181). Melatonin has been shown to successfully slow aging in mice, but increases tumor growth simultaneously (182,183). Estrogen replacement therapy has multiple beneficial effects (184-186), especially for postmenopausal women, but can also increase risk of breast and ovarian cancer as well as heart disease (187-189). Overall, this suggests that targeting the neuroendocrine system through hormone replacement therapy is potentially beneficial, but side effects occur due to the complex nature of the endocrine system.

1.5.4 Neuroendocrine-Immuno Theory

The neuroendocrine and immune system are tightly linked due to their overlap in function. These systems have shared components, most notably the shared use of ligands and receptors of “classical” hormones and immunoregulatory mediators (190). These shared components allow for bidirectional communication between the two systems (191-193). Through this communication, cytokines from the immune system can regulate the secretory phenotype of the hypothalamus pituitary adrenal axis (HPA) (191,192). This results in a cytokine-induced increase in glucocorticoids, which then feeds back to the immune system to down-regulate expression and stability of the cytokines (194,195). This feedback loop creates a homeostatic mechanism that can regulate both systems to modulate the inflammatory and stress responses (196).

Since both the neuroendocrine system and the immune system are independently implicated in aging, it is not a far stretch to see that the interactions between the two systems may be important in regulating the aging process. This theory suggests that it is the disruption of the normal communications between the two systems that leads to the altered function. This theory highlights the importance of finding a theory that can unify the multiple complex systems that play a role in the aging process.

1.6 Conclusions

As discussed above, there are a multitude of theories on aging that have been presented over the past century. Many of them provide insight into possible mechanisms of aging, but it is only after looking at them together that a clearer picture of what may cause aging emerges. When taken together, it is possible to see how many of the theories are intertwined emphasizing different pieces of the complex aging puzzle that focus on different levels of complexity from the cellular to whole body systems (Figure 1).

Despite the major inroads that have been made towards understanding the process of aging, it is clear that there is much left to discover. Efforts to understand the aging process will help lead to a population undergoing “successful aging” in which not only is disease avoided, but mental and physical capacities are maintained and sustained engagement with life is possible (197). This goal cannot be achieved purely by analysis of diseases related to aging. It requires a deeper understanding of the processes that happen within the aging organism.

2. KLOTHO AND AGING

2.1 Discovery of Klotho

The serendipitous discovery of klotho (KL) occurred in 1997 during efforts to overexpress a sodium-proton exchanger in mice. In the attempts to make a transgenic mouse, approximately 10 copies of the sodium-proton exchanger were

inserted in tandem into a single locus of chromosome 5 resulting in disruption of the KL promoter (198). The disruption of the KL gene resulted in a mouse displaying a variety of aging phenotypes that were characterized and discussed in the seminal paper on KL. This mouse sparked a lot of interest due to its close approximation of normal aging. In the 16 years since its discovery, much has been discovered about the many functions of KL that might help us understand the aging process.

2.2 Characterization of Klotho

2.2.1 Structure of Klotho

The KL gene consists of 5 exons spanning over 50 kilobases on chromosome 13q12 (198-200). From this transcript there are two alternative RNA splice variants encoding a membrane and secreted form of KL, which consists of exons 1-3 (199). Despite higher mRNA expression of the secreted isoform of KL in humans (199), this splice variant has been identified as a candidate for nonsense mediated decay (201). The full length KL gene encodes a single pass type I transmembrane protein of 1014 amino acids (198). It consists of a small (10 amino acids) intracellular domain of unknown function and a large extracellular domain with two internal repeats (Figure 2).

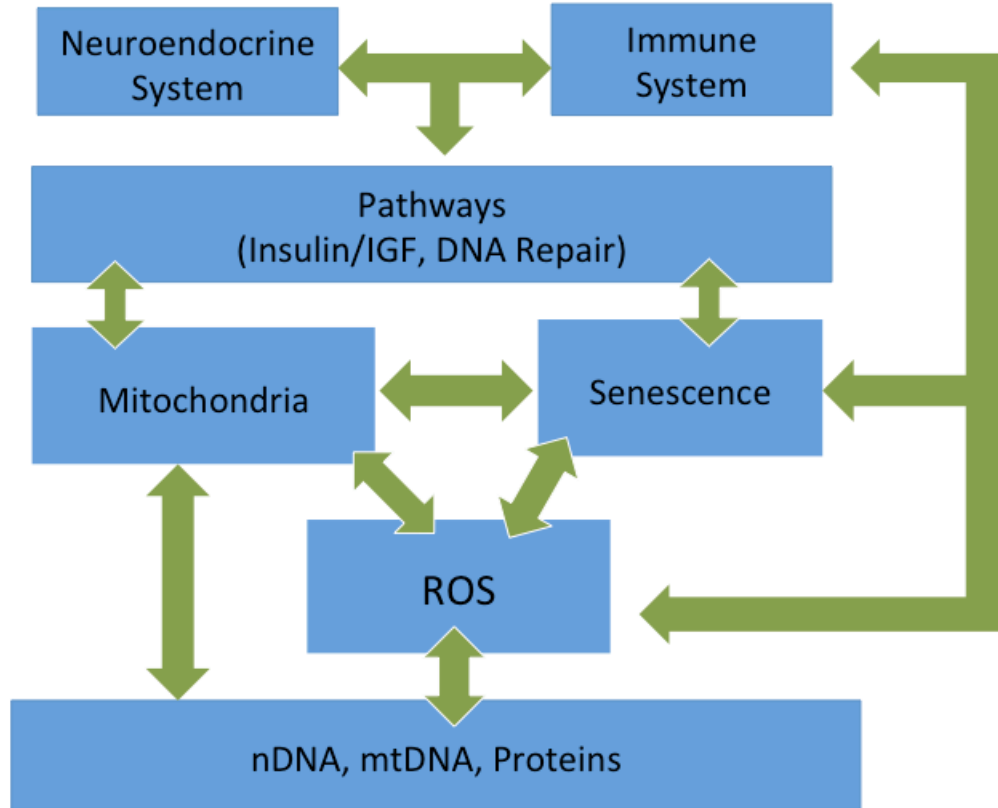


Figure 1. Interconnection of Aging Theories. A diagram of the different aging theories presented in a top to bottom hierarchy of system complexity. This demonstrates how interconnected the different theories are and how disruption of one pathway can lead to the damage of another suggesting the theories should not be taken as contrasting ideas, but as part of the same puzzle viewed from another angle.

The two domains of the extracellular portion of KL (KL1 and KL2) have about a 21% similarity in amino acids and share homology with family I glycosidases including β -glucosidases of bacteria and plants(202) as well as mammalian lactase phlorizin-hydrolase (198,203,204).

2.2.2 Expression of Klotho Protein

KL levels are most highly expressed in the distal convoluted tubules of the kidney and choroid plexus of the brain (198). Kuro-o and colleagues also found lower levels of KL mRNA in the pituitary gland, placenta, skeletal muscle, urinary bladder, aorta, pancreas, testis, ovary, colon and thyroid gland (198). These results spurred further research from other groups expanding upon these preliminary findings. These studies examined the location of KL in more detail finding that KL location in the choroid plexus was mainly isolated to the apical surface of ependymal cells. They also showed that localization in the sex organs was restricted to the inner regions of the seminiferous tubules of the testis as well as the oocytes of Graafian follicles in the ovaries, which are both the most mature forms of germ cells in their respective organs (205). Further investigation of KL localization in the brain showed staining not only in the choroid plexus but also in Purkinje cells of the cerebellum with a previously unreported perinuclear localization in both cell types (206). KL is also expressed in the parathyroid gland of rats (207) as well as in the stria vascularis and spiral ligament of the

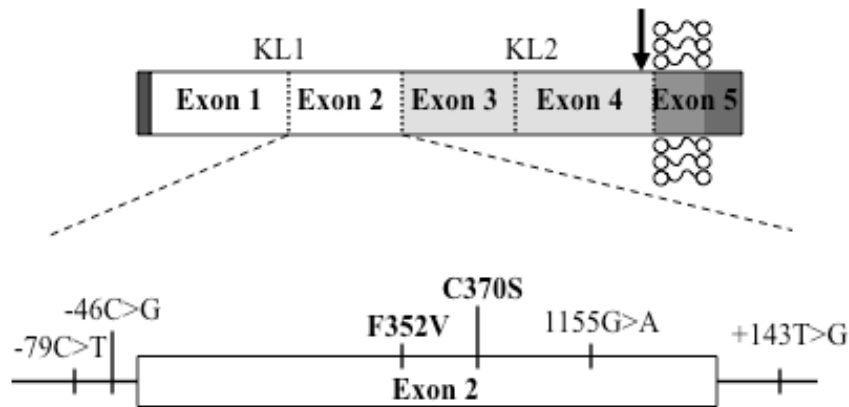


Figure 2. Schematic representation of KL. The KL protein is generated from 5 exons that make up the full length protein. It is a type I transmembrane protein with a short intracellular region. Shown are the relative locations of the known domains (KL1 and KL2) as well as known cleavage site of ADAM 10/17 (arrow). The signal sequence is depicted as a dark gray portion of KL preceding exon 1. Magnification of exon 2 depicts the approximate location of the six single nucleotide polymorphisms (SNP) that encode for the KLVS variant, including 3 SNPs located in the intron surrounding exon 2, two SNPs encoding for amino acid changes at phenylalanine 352 and cysteine 370 (in bold letters), and one SNP in exon 2 that does not change the amino acid sequence.

inner ear of mice (208). In human cell lines and tissues, KL expression has been found in breast tissue (209), thoracic aorta (210) and human colorectal cancer cell lines (211). KL is expressed in a variety of tissues, but many of the organs that are affected by lack of KL do not express it at high levels. This suggests that shed KL is necessary for its age-associated effects and functions as a circulating hormone.

2.2.3 Klotho Gene Regulation

Early on, the putative KL promoter was identified and found to be heavily GC rich (199). This lends it to regulation by methylation, which can determine its tissue specificity (212). The GC rich region has also been associated with alterations in KL expression with age (213) as well as with diseases such as cancer and chronic kidney disease (211,214-219). The KL promoter also has binding sites for transcription factors such as specificity protein 1 (Sp1) (199) and early growth response protein 1 (Egr1) (220), which can regulate its expression. External factors such as oxidative stress (221), inflammation via nuclear factor kappa-light-chain-enhancer of activated B cells (NF κ B) (222), and dehydration through increased antidiuretic hormone and aldosterone (223) can all lead to decreases in KL expression. Conversely studies have shown that Gentian root, statins and other compounds are capable of increasing KL promoter activity (224-226) and that sirtuin 1 (SIRT1) and vitamin D can increase KL mRNA levels (227,228). Recent studies have also identified microRNAs that are capable of

decreasing KL levels in vitro (229). Research in KL gene regulation still has many stones to upturn. Although these are promising results, there is still not a full picture of how KL is regulated.

2.2.4 Klotho Protein Processing

Klotho, like many transmembrane proteins, trafficks through the endoplasmic reticulum and golgi to reach the plasma membrane and in this process it is glycosylated (230). Once it is at the membrane, it can be cleaved by A Disintegrin and Metalloproteinase (ADAM) 10 and 17 or β -secretase to release the KL1 and KL2 domains into the extracellular space (231,232). This cleavage is modulated by insulin and IGF-1 signaling (231). The leftover membrane-bound portion is also a substrate for γ -secretase, which may aid in the removal of the remaining C-terminal fragment from the membrane (232). The released extracellular domain can be found in the blood and cerebrospinal fluid (CSF) of humans and mice. It was shown by size exclusion gel filtration that this shed portion is often found in dimers or higher order oligomers (230). Further co-immunoprecipitation as well as metabolic labeling experiments supported this finding showing that KL molecules associated with each other within the cell (230,231). Due to the nature of KL, understanding how it is processed and trafficked to the membrane is important. Processing at the membrane is fairly well understood, but the events preceding and proceeding it are still being discovered.

2.3 Klotho Deficient Mouse

2.3.1 General Properties

Before 3 to 4 weeks of age, KL mice are indistinguishable from their wild type littermates, but around this time growth retardation starts to become apparent (198). Except for the aforementioned smaller body size and display of kyphosis, the gross appearance of the KL mice is relatively normal (198). After the onset of growth retardation at 3 to 4 weeks, the knockout mice gain almost no weight and slowly become lethargic and malnourished until their death at around 8-9 weeks of age (198). Unfortunately, the exact cause of death could not be determined because the multitude of disorders present in the mice were not fatal within themselves (198).

2.3.2 Hypogonadism

KL deficient mice never reach sexual maturity and are infertile likely due to their atrophic internal and external genitalia. The atrophic testes do not contain any mature sperm as the maturation of spermatocytes is halted at the pachytene stage (198). Observations that levels of dynamin 2 and 3 are reduced in the testes of KL deficient mice suggest that alterations in endocytosis may explain the decreased spermatogenesis in these animals (233). The ovaries are also atrophied containing primary and secondary follicles, but not Graafian follicles or corpus lutea (198). The ovarian atrophy is likely due to defects in hypothalamus and pituitary function. Not only are levels of lutenizing hormone

and follicle stimulating hormones low in KL deficient mice, but normal ovarian maturation can be restored by gonadotrophin treatment. Ovaries from KL deficient mice can also function normally when transplanted into a wildtype female suggesting the atrophy is due to alterations in circulating hormones (234).

2.3.3 Premature Thymic involution

The thymus of KL deficient mice develops normally until 3 to 4 weeks of age at which point it starts to atrophy, becoming almost undetectable by death at 6-9 weeks (198). Hematopoietic stem cells from KL deficient mice are capable of differentiating into normal lymphoid cells if transplanted into severe combined immunodeficiency (SCID) mice suggesting that there is no intrinsic defect in the lymphohematopoietic progenitors (235). It is thought the atrophy may be caused in a similar manner to normal aging (236) in which there is a defect in the thymic endothelial cells that are necessary for support and proliferation of thymocytes (237-240). This is supported by the fact that injection of keratinocyte growth factor induces proliferation of thymic endothelial cells and is capable of improving thymopoiesis and restoring thymic degeneration in both KL deficient mice and aged wild-type mice (235). Taken together, evidence suggests that thymic involution in the KL mouse resembles the thymic phenotype seen in normal aging supporting the use of KL deficient mice as an accelerated aging model.

2.3.4 Tissue Calcification

Ectopic calcification presents in KL deficient mice around 4 weeks and progresses with age (198). It can be seen in many soft tissues most notably in the gastric mucosa, trachea, renal tubules, small arteries of the kidney, middle-sized muscular arteries, and larger renal and aortic arteries (198). The vascular calcification present does not result in intimal thickening or accumulation of foam cells associated with typical disease associated arteriosclerosis (198). Instead, the vascular calcification more resembles Mönckeberg-type arteriosclerosis, which is observed in aged individuals as well as patients suffering from diabetes or chronic kidney disease (198). Further studies suggested that the calcification may be secondary to changes in calcium, phosphate and vitamin D levels (241,242).

2.3.5 Impaired Bone Mineralization

KL deficiency in mice results in osteopenia with cortical bone thickness of the tibia, femur and vertebrae decreasing between 20 and 40% (198,243). The mice have significant reductions in both bone formation and resorption, but the reductions in formation are greater resulting in a net bone loss with low turnover (198). This pathology is similar to estrogen independent osteoporosis seen in aging individuals (244). Despite the cortical bone loss, there is an increase in trabecular bone mass in the vertebrae and the metaphysis of tibia and femur of KL deficient mice (243,245,246).

2.3.6 Skin Atrophy

KL deficient mice had more sparse hair than wild-type mice (198). Further histological examination showed that there is a reduction in the number of hair follicles(198), similar to what is found in old age (247). Other features common in old age were also found in the mice such as decreases in dermal and epidermal thickness as well as almost undetectable levels of subcutaneous fat (198).

2.3.7 Pulmonary Emphysema

At around 5 weeks of age, KL deficient mice start to develop enlarged air spaces distal to the terminal bronchiole and destruction of alveolar walls (198). This is concomitant with an increase in levels and activity of matrix metalloproteinase 9 (MMP-9) and decreased levels of tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) in the lung, suggesting the imbalance of these enzymes may be contributing to the onset of emphysema (248). There is also an increase in the amount of apoptotic cells accompanied by a decrease in lung elastic recoil (249). At later stages, calcium deposits in type I collagen fibers of alveolar septa are present and type II pneumocytes start to degenerate (250). Functionally, the mice had longer expiration times and higher dynamic compliance than wild-type mice, which is similar to patients with emphysema (250). A computational study comparing the pulmonary damage seen in KL deficient mice to damage from smoking-related emphysema concluded that the

KL mouse phenotype more closely resembled systemic damage due to old age rather than local damage caused by smoking or other artificial means (251).

2.3.8 Neural Deficits

Kuro-o and colleagues first reported changes in the neural function of KL deficient mice in the seminal paper that described the KL phenotype (198). They found the mice have a parkinsonian-like gait that is accompanied by loss and degeneration of Purkinje cells (198). There is also loss of mesencephalic dopaminergic cells in the substantia nigra and ventral tegmental area, which is also associated with Parkinson's disease. Further studies showed that KL deficient mice have decreased cognitive capacities as assessed by novel-object recognition and fear conditioning tests (252). Changes in DNA and lipid peroxide levels as well as increases in apoptotic markers in the hippocampus suggest that increases in oxidative stress may be the cause of the cognitive deficits (252). Other researchers also found evidence of neurodegeneration in cellular organelles of hippocampal cells (253). The importance of oxidative stress is supported by data showing administration of α -Tocopherol, an antioxidant, prevented the cognitive deficits while simultaneously lowering the oxidative stress levels and number of apoptotic cells (252). KL deficient mice also have decreased amounts of anterior horn cells in the spinal cord, which is seen in amyotrophic lateral sclerosis (254). These neurons have decreased levels of ribosomal RNA gene transcription and a concomitant reduction in cytoplasmic

RNA and rough endoplasmic reticulum (RER) (254). This study, along with others, also showed increases in the amount of glial fibrillary acidic protein (GFAP)(253,254), which is associated with increased age of an organism (255-257). An exhaustive immunohistochemical analysis of the brains of KL knockout mice expanded upon previous findings showing that lack of KL increases levels and phosphorylation of neurofilaments, decreases microtubule-associated protein 2 (MAP2) expression and increases lysosomes and autophagy related structures and markers (253). These changes seen with KL mice are all independently associated with normal aging. Neurofilament levels are increased in aging animals and in age-related neurodegenerative diseases (258-264). Levels of neurofilament phosphorylation, which have also been reported to change in KL mice by two other groups (254,265), are enhanced in neurodegenerative and/or aging conditions (258,259). Studies by Chen and colleagues show that neurons are not the only brain derived cell to be affected by KL deficiency. Myelination is also altered in the optic nerve and corpus callosum of these mice due to decreased levels of mature oligodendrocytes showing that KL is also important for proper oligodendrocyte maturation and myelination (266). Overall, KL has a variety of functions on the nervous system most likely due to its humoral effects that allow it to have far-reaching targets.

2.3.9 Hearing Loss

Klotho expression in the ear is localized to the stria vascularis and spiral ligament of the inner ear(208). In KL deficient mice, there are no obvious morphological abnormalities of the ear, but the threshold for the auditory brainstem response was significantly higher and wave I latencies were prolonged (208). This difference may be regulated by KL's effect on ion homeostasis through which it could modulate endolymph fluid levels. This is supported by changes in the levels of ion channels regulated by KL in the inner ear with age (267).

2.3.10 Cardiovascular Deficits

KL expression in the heart is limited to the sinoatrial node, which acts as the pacemaker for the rest of the circulatory system (268). Investigation into the effect of KL deficiency on the heart showed that KL deficient mice had higher rates of sudden death after long periods of restraint stress (268). This difference is due to an abnormal stress response that led to a longer recovery time in the KL deficient mice leading to sudden death (268). Further investigation of the cardiovascular system of KL deficient mice showed that they also suffer from impaired angiogenesis and vasculogenesis (269). These changes may be due to KL's regulation of nitric oxide synthesis.

2.3.11 Blood Chemistry

Alterations in the blood chemistry of KL deficient mice consist of increased levels of serum phosphorus, calcium and active vitamin D (241) and decreased levels of glucose (270). Further studies showed that despite decreases in glucose, insulin levels were also decreased (270). These seemingly conflicting results are due to increased insulin sensitivity in the KL deficient mice (270). The ratio of lymphocytes to leukocytes was also decreased probably due to thymic involution (198). Other measurements such as creatinine, total protein, albumin, cholesterol and triglyceride levels were unchanged (198). The changes in blood chemistry seen in KL deficient mice are present before the onset of symptoms, suggesting that they are important in the age-related effects of these mice.

2.3.12 Rescue of Phenotypes by Klotho Expression

Thirty-seven transgenic mouse lines were developed under the control of the EF-1 α promoter in order to test whether exogenous expression of KL in the KL deficient mice could rescue the aging phenotypes (198). Two of these lines have detectable levels of KL measured by RNase protection assays and are able to rescue most of the phenotypes. The rescued mice have an appearance almost indistinguishable from wild type and also have close to normal thymus and genital organ sizes and composition, which resulted in fertile mice. Blood chemistry values are near normal and calcification, including atherosclerosis, is markedly decreased. Cortical bone thickness is normal and osteopenia is

improved. Other measurements taken, such as emphysema and skin changes, are ameliorated. Overall, rescue of KL expression markedly improves the aging phenotype of the klotho deficient mice. Interestingly, only one of the lines resulted in KL overexpression in all tissues examined (kidney, brain, lung, ovaries, testes etc.). The other transgene that is able to rescue could only be found in the brain and testes. This suggests that many of the aging phenotypes associated with KL are regulated by these areas or that KL's ability to act as a humoral factor is crucial for its effect on aging. The ability of parabiosis of wild-type mice with KL deficient mice to restore endothelial function suggests that it is KL's humoral function that is responsible for the wide-ranging effects (271). Another independently created model of KL rescue relied on zinc dependent induction of KL in KL deficient mice (272). This showed that KL expression was able to rescue the age-related phenotypes of KL deficient mice even after the onset of symptoms. Turning off KL gene expression led to a resurgence of the symptoms further illustrating that changes in KL levels are responsible and sufficient to modify the aging phenotype of the mice. This suggests that increasing KL levels in aging individuals could be enough to slow or reverse the phenotypes present.

2.4 Klotho Overexpressing Mouse

2.4.1 Phenotype of KL overexpression

The two transgenic mouse lines that were first made to rescue the KL deficient phenotype were further studied to look at their effects independent of KL deficiency (273). Both lines led to increases in lifespan of 20-30% in males and approximately 19% in females. This increase in lifespan could not be explained by decreased caloric intake or body size, but the overexpressing mice did have smaller litters than wild-type controls. Despite normal caloric intake, these mice have a similar increase in lifespan as is seen by inhibition of the insulin/IGF pathway. These mice had normal blood glucose levels, but higher circulating insulin levels. This was explained by the fact that the transgenic mice displayed insulin and IGF-1 resistance, although insulin resistance was not shown in female transgenic mice. The differences in the insulin/IGF pathway suggest that KL's inhibition of insulin/IGF signaling may be important for its longevity effects. This would tie in with the independent studies showing associations between inhibition of this pathway and longevity (274).

2.4.2 Protective Effects of KL Overexpression

Further studies showed that transgenic mice overexpressing KL had decreased levels of oxidative stress as measured by 8-Hydroxydeoxyguanosine (8-OHdG) urinary excretion and were protected from a lethal dose of the herbicide paraquat that generates reactive oxygen species (275). Both of these

results suggest that KL overexpression protects against oxidative stress. KL overexpression was also protective in models of renal and cardiovascular damage as well as in a multiple risk factor syndrome (276-279). Another group examined KL expression in 20 different inbred mouse strains and discovered that one of the strains had high expression levels of KL mRNA. This strain had better hearing and reduced risk of arteriosclerosis compared to the other strains with less KL expression (280). This shows that higher levels of KL expression, even when not artificially induced, are protective against age-related phenotypes. KL's relation to decreased oxidative damage ties back to the free radical theory of aging, giving credence to both the theory and KL's anti-aging effects, suggesting they may be linked to its ability to control oxidative stress.

2.5 Klotho Functions

KL deficiency results in a global phenotype that affects a wide range of tissue types culminating in decreased longevity. Due to shed KL's humoral activity, it is possible for KL to have many functions on a variety of tissue types. Although it is still unclear what functions of KL are direct and which may be secondary effects of a different function, KL has been implicated to regulate many pathways through which it can have its wide-ranging age related effects.

2.5.1 Klotho and Fibroblast Growth Factor Signaling

The first function of KL was discovered when it was realized that the phenotype of fibroblast growth factor 23 (FGF23) knockout mice heavily overlapped with mice deficient in KL (198,281). Beside the predicted phenotypes of hyperphosphatemia and soft tissue calcification, the mice also demonstrated a shortened lifespan, growth retardation, hypogonadism, premature thymic involution, osteopenia, sacropenia, skin atrophy and pulmonary emphysema which are all reminiscent of the KL deficient mice (198,281-283). Supporting the role of KL in FGF23 signaling, the double knockout of KL and FGF23 has identical phenotypes to their single knockout counterparts. Injection of FGF23 could not rescue the phenotypes as it does for FGF23 knockout mice alone (284) implicating KL in FGF23 signaling. KL mice also had very high serum levels of FGF23 suggesting that KL deficiency results in severe FGF23 insensitivity (285) further supporting the role of KL in FGF23 signaling. In fact, FGF23 and two other fibroblast growth factors (FGF), FGF19 and 21, were found to be atypical members of the FGF superfamily (286,287). Unlike the canonical FGFs that have autocrine or paracrine actions, these members of the family act as endocrine factors because of the low heparin binding affinity attributed to their unique structure (288,289). This unique structure allows them to travel past the heparin sulfate rich extracellular matrix to have more endocrine like effects, but the affinity of FGFs for heparin sulfate is critical for their binding to Fibroblast Growth Factor Receptors (FGFR) (290). To this end, FGF23 is not capable of

activating cultured cells even when there is endogenous expression of FGFRs (291) suggesting that it requires a cofactor for proper activity. It was shown that KL was capable of binding FGFR and acting as the obligate co-receptor for FGF23 binding and signaling (285,291). Through this signaling, the complex activates Fibroblast Growth Factor Receptor Substrate 2 α (FRS2 α) leading to downstream extracellular signal-related kinase (ERK) 1/2 phosphorylation and eventually leading to increased expression Egr1 mRNA, suggesting the signaling leads to transcriptional regulation of this transcription factor (285,291).

In the kidney, FGF23 released from bone and KL promote phosphate excretion into urine by down-regulating the amount of sodium-phosphate co-transporter type 2a (NaPi-2a) present on the apical brush border membrane of proximal tubules (292-296). They also regulate levels of 1,25(OH)₂D, the active form of vitamin D, by inhibiting the activity of 1- α -hydroxylase (292,297,298). Reduction of vitamin D synthesis also leads to decreased phosphate absorption in the intestine further promoting decreased plasma phosphate levels (299). The active form of vitamin D completes the negative feedback loop between FGF23, KL and itself by independently up-regulating the FGF23 and KL genes (Figure 3) (227,298)

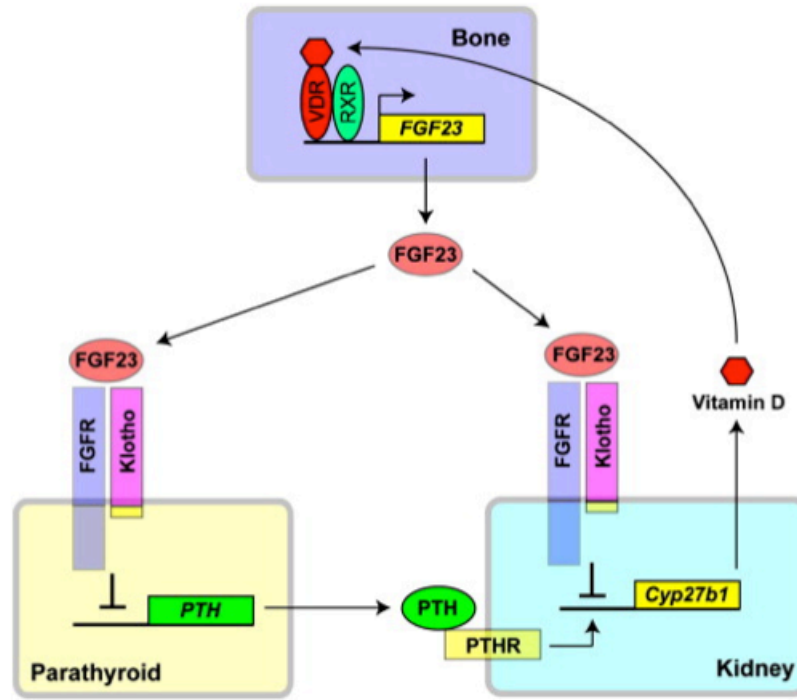


Figure 3. Klotho Control of FGF23 Signaling. Regulation of vitamin D metabolite is maintained by klotho and FGF23 signaling. In the bone, active vitamin D binds to the vitamin D receptor (VDR), which makes a complex with the nuclear receptor RXR. The formed complex binds to a region of the FGF23 promoter leading to increased expression of FGF23. FGF23 secreted from the bone acts on the klotho-FGFR complex in the kidney and the parathyroid gland. In the kidney, downstream signaling of FGF23 suppresses expression of *Cyp27b1* gene that encodes 1α -hydroxylase and closes a negative feedback loop for vitamin D homeostasis. In the parathyroid gland, FGF23 signaling suppresses expression of PTH. Since PTH is a potent inducer of *Cyp27b1* gene expression, suppression of PTH by FGF23 reduces expression of *Cyp27b1* gene as well as serum levels of 1,25-dihydroxyvitamin D₃, which closes another negative feedback loop for vitamin D homeostasis. Klotho and FGF23 are indispensable for the regulation of vitamin D metabolism, because defects in either Klotho or FGF23 cause hypervitaminosis D. Diagram from Kuro-o, 2009 .

FGF23 and KL also act in the parathyroid gland to regulate phosphate homeostasis as well as calcium metabolism. FGF23/KL signaling can down-regulate parathyroid hormone (PTH)(207,300), which much like the FGF23/KL complex leads to phosphaturia (301,302), but increases serum levels of active vitamin D (Figure 3) (303). This modulation of hormones in the endocrine system by KL hearkens back to the theories positing a role of the endocrine system in aging. Since the KL and FGF knockout mice show that this function is important for the anti-aging effects of KL, it adds strength to the theory that the endocrine system plays a crucial role in aging.

The importance of FGF23 signaling in the aging-related effects of KL is highlighted by the similarities of the two knockout mice (273,282,283,295). The importance of this pathway is further illustrated by the ability of a low vitamin D diet to rescue the altered serum phosphate and calcium levels in both FGF and KL knockout mice preventing the expression of the age related phenotypes (241,304). A similar rescue occurs when the vitamin D receptor gene or the cytochrome P450 2B1 (Cyp2b1) gene that helps transcribe 1- α -hydroxylase is ablated (305-307) leading to a similar decrease in the amount of active vitamin D. A low phosphate diet is also able to rescue the shortened lifespan and vascular calcification in both mice models (304,308). All of this points to the importance of the regulation of phosphate and vitamin D homeostasis by KL and FGF23 signaling.

2.5.2 Klotho Regulation of Ion Channels

KL knockout mice have altered phosphate and calcium ion levels suggesting that KL plays a role in ion homeostasis (198). One way that KL may control ion homeostasis is through its regulation of the membrane localization of ion channels. The shed extracellular domain of KL is capable of modulating membrane levels of transient receptor potential vullinoid (TRPV) channels, TRPV5 and TRPV6, leading to their increased localization at the membrane (309,310). Both channels are important in regulating calcium levels: TRPV5 is mainly located in the distal convoluted tubules and connecting tubules where it mediates renal transcellular transport leading to calcium reabsorption while TRPV6 mediates calcium absorption in the intestine (311). Investigation into the mechanism of KL induced membrane localization showed that this process was mediated by KL's enzymatic activity, which may be linked to its weak homology to β -glycosidases (312). KL cleaves off an N-terminal sialic acid from the glycan chain, which exposes the N-acetyllactosamine (LacNAc) (313). The exposed LacNAC can then bind to galectin-1, which prevents TRPV endocytosis leading to increased presence of channels on the plasma membrane (Figure 4) (313). KL is also able to regulate the membrane localization of renal outer medullary potassium channel 1 (ROMK1) in a similar manner (314). In the case of ROMK1, increased membrane localization leads to a rise in potassium reabsorption by the kidney and more urinary excretion of potassium (314). The renal phosphate transporter NaPi-2a and NaPi 2b are also regulated by KL's enzymatic activity,

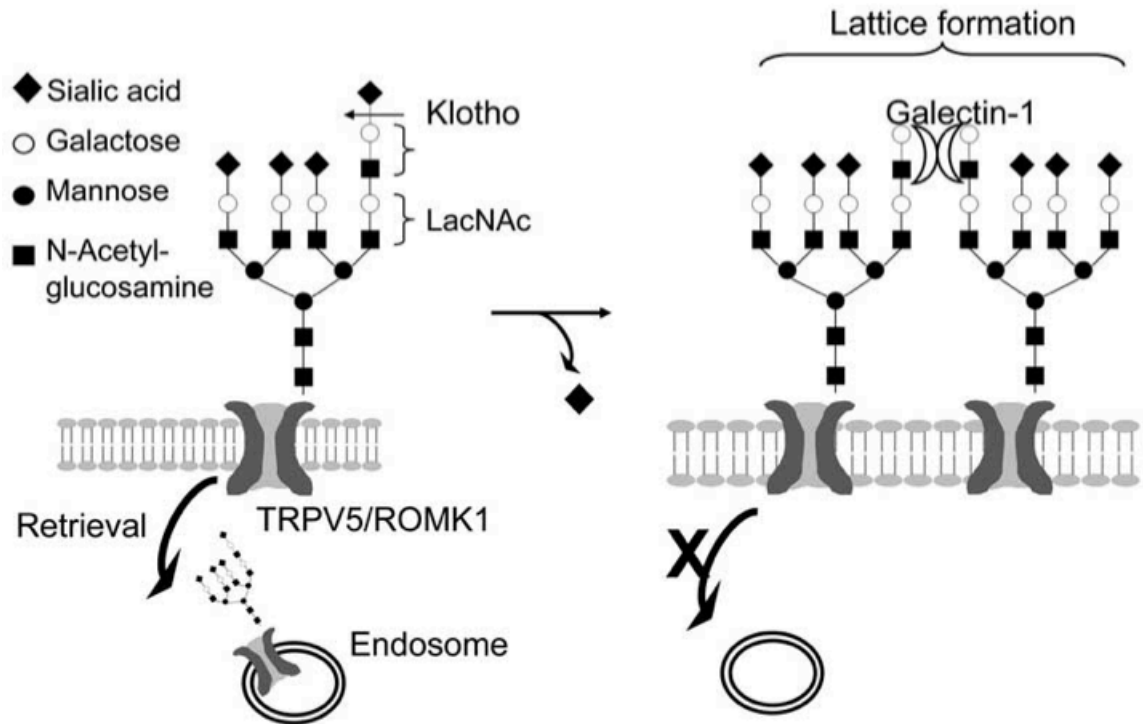


Figure 4. Regulation of Ion Channels by KL-mediated Removal of Sialic Acid. When located at the plasma membrane, ion channels such as TRPV5 and ROMK1 undergo regular endocytosis to maintain an equilibrium at the membrane. The extracellular domain of KL is capable of regulating the membrane localization of such ion channels by manipulating the glycan chain on these channels. Klotho cleaves off an N-terminal sialic acid from the glycan chain, which exposes the LacNAc. The exposed LacNAc can then bind to galectin-1, which effectively stabilizes the ion channels on the plasma membrane. Diagram from Huang CL, 2011 (317).

but in this case KL leads to a decrease of cell surface expression instead of the increase seen with the other ion channels (315,316).

KL also regulates the membrane localization of transient-receptor potential canonical calcium channel (TRPC) 1 and TRPC6, but the mechanism of action is not through KL's enzymatic activity (276,318). TRPC1 is involved in vascular endothelial growth factor (VEGF) mediated calcium entry and KL is able to bind to the complex of TRPC1 and vascular endothelial growth factor receptor 2 (VEGFR2) to strengthen it and promote the complex internalization upon VEGF stimulation (318). Klotho can also interact with TRPC6 through which it can down-regulate the receptor. This action is cardioprotective as excess TRPC6 levels in mice lead to spontaneous cardiac hypertrophy that is ameliorated by soluble KL expression (276). It is possible that KL regulates TRPC6 by a different mechanism than TRPC1 because Kusaba and colleagues could not pull down a KL-TRPC6 complex (318).

2.5.3 Klotho Inhibits Insulin/IGF Signaling

While KL deficient mice present with hypoinsulinemia, hypoglycemia and extreme insulin sensitivity (198,270), KL overexpressing mice are insulin/IGF-1 resistant despite maintaining normal fasting blood glucose levels and not developing diabetes (273). The prevalence of insulin/IGF-1 related symptoms in the KL mice models suggest that KL plays a role in regulating insulin/IGF

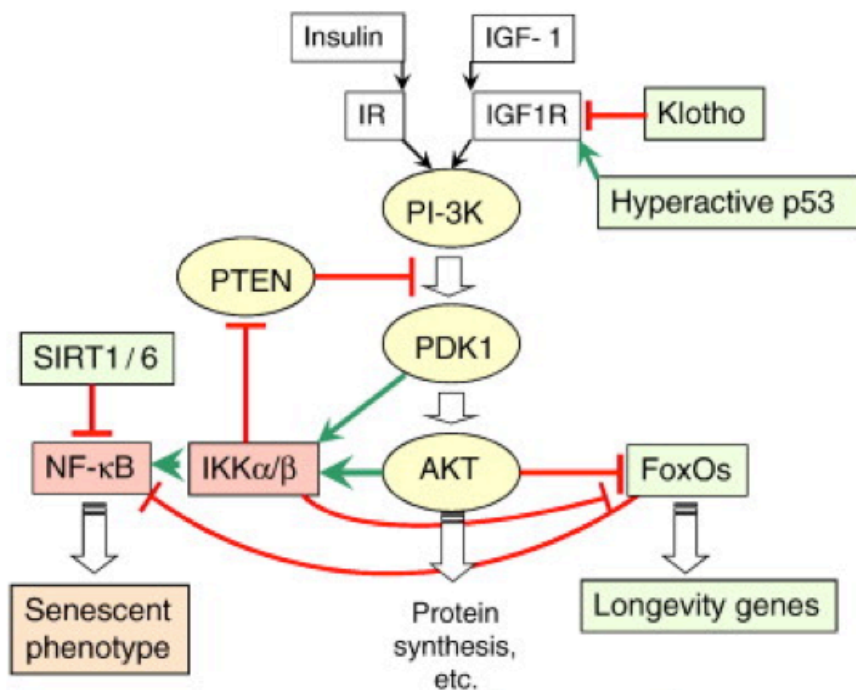


Figure 5. Klotho Inhibits the Insulin/IGF Pathway. Diagram depicting the interactions of KL with the insulin/IGF pathway, which is independently implicated in aging. PI3K signaling downstream of IGF1R can lead to activation of AKT and subsequent phosphorylation and inactivation of FOXOs. When activated, FOXO's can upregulate the expression of genes associated with longevity. Through PDK1, PI3K signaling can also activate NFκB, which leads to a senescent phenotype. Klotho can inhibit downstream signaling through the insulin/IGF pathway by an unknown mechanism. Through this inhibition, KL can upregulate longevity associated genes and inhibit the senescence phenotype. Diagram from Salminen *et al.*, 2010{Salminen, 2010 #852}.

signaling. This is supported by KL's ability to inhibit insulin signaling in cultured cells (209,273). Although the mechanism by which KL inhibits insulin signaling is unclear, it is possible it is through KL's enzymatic activity. N-glycosylation of insulin receptors can affect its function and localization (319) and sialidase expression has been linked to development of insulin resistance in cultured cells and mouse models (320,321). There are findings that suggest the action of KL on insulin signaling may not be direct (322), but evidence from *C. elegans* suggests that KL inhibition of insulin/IGF signaling is necessary for its effect on longevity (323). The involvement of insulin/IGF in KL's longevity effect is supported by the fact that insulin/IGF signaling has been independently linked to longevity (Figure 5) (81,82,324-328). The link between these two pathways further bolsters the hypothesis that they are involved in the aging process and emphasizes the interconnections between different pathways involved in aging.

2.5.4 Klotho Inhibition of Other Growth Factors

Although more attention is paid to KL's association with the insulin/IGF pathway due to its close ties to aging, KL also regulates the activities of other growth factors. KL also binds to wnt ligands including Wnt1, Wnt3, Wnt4 and Wnt5a (329). This interaction inhibits wnt signaling, which could explain the increase in wnt activity seen in *klotho* deficient mice (329). Wnt can regulate bone mass suggesting that KL regulation of this pathway could explain the increased bone mass seen in certain bones of KL deficient mice. The wnt

pathway is also involved in the kidney injury that is associated with loss of KL (330) and has been implicated in cancer progression as well as oligodendrocyte maturation that KL is known to affect (331-333). Another growth factor, transforming growth factor β (TGF β), forms a negative feedback loop with KL. TGF β is capable of down-regulating KL expression in the kidney (330,334) and KL is capable of binding directly to the type II TGF β receptors disrupting the binding of its ligand and therefore inhibiting downstream signaling (335). Klotho also inhibits VEGF signaling by promoting the internalization of the VEGFR2-TRPC1 complex (318). There is also a strong negative correlation between levels of VEGF and KL in kidney disease (318) and there is also decreased staining for VEGF in the aorta of KL heterozygote mice (336) further implicating KL in VEGF inhibition. It is possible that KL plays a major role in regulation of the epithelial to mesenchymal transition (EMT) because of its inhibition of the many growth factors discussed above that promote it (337,338). Since EMT is associated with many diseases, it is possible that KL may regulate disease risk through this pathway (339-341).

2.5.5 Klotho and Oxidative Stress

Evidence from both KL deficient mice and overexpressing mice suggest that KL plays a role in oxidative stress. While deficient mice show increased levels of DNA and lipid oxidative stress in the hippocampus (252), overexpressing mice showed low levels of oxidative stress accompanied by increased levels of

superoxide dismutase and resistance to lethal levels of oxidative damage (275). KL's regulation of oxidative stress could be through its inhibition of insulin/IGF signaling. This pathway negatively regulates FOXO transcription factors by leading to their phosphorylation and degradation (342). When activated, FOXOs lead to upregulation of antioxidant proteins including mitochondrial manganese superoxide dismutase (SOD2) (343). This protein is also upregulated in muscles by KL overexpression potentially through KL's dis-inhibition of FOXOs (275). Expression of KL is also regulated by SIRT1 (228), another gene that has been independently associated with protection from oxidative stress (344) further supporting KL's involvement in oxidative stress. The involvement of both FOXOs and SIRT1 suggest that KL's ability to protect against oxidative stress may be related to its inhibition of insulin/IGF signaling as these molecules are known to be inhibited by insulin/IGF signaling.

2.5.6 Klotho's Anti-Inflammatory/Immune System Function

KL's role in the immune system was first suggested when it was found that KL deficiency can lead to premature thymic involution (198). KL deficient mice also have higher rates of sepsis-induced T-lymphocyte apoptosis as well as higher levels of cytokines (345). KL is also downregulated in cluster of differentiation 4 (CD4) positive T-lymphocytes of healthy elderly individuals and this downregulation is even stronger in individuals with rheumatoid arthritis (346). KL may also have an indirect effect on B-lymphocytes as evidenced by the early

impaired B cell development in bone marrow of KL deficient mice (347). KL not only regulates lymphocyte development and response, but it also regulates tissue response to released cytokines (348). Pre-incubation with KL inhibits the effects of TNF- α in human umbilical vein endothelial cells resulting in a decreased induction of adhesion molecules as well as reduced levels of NF- κ B (348). Further investigation revealed this mechanism is dependent on phosphorylation of reticuloendotheliosis oncogene cellular homolog A (Rel A) (349). KL can also protect from senescence-induced inflammation through down-regulation of retinoic acid inducible gene-1 (RIG-1) (350). Together, these studies show KL can regulate the immune system in two ways: by regulating expression and function of immune cells such as lymphocytes and by regulating tissue response to cytokines and other inflammatory signals. Regulation of the immune system is postited to play a role in the aging process by the Immune Hypothesis and it is possible that KL's regulation of this system is important for its age-related effects.

2.5.7 Klotho and Senescence

KL's links to protection from oxidative stress and inhibition of inflammation make it a strong candidate for regulation of senescence. The overlap of these conditions can be seen by KL's aforementioned ability to inhibit senescence-associated inflammation (350). KL is able to protect from oxidative stress induced senescence (351,352), cytokine induced senescence and replicative senescence (353). KL can also protect against stress-induced telomere

shortening (353), which often leads to senescence. Levels of senescence also increase in epidermal cells of KL deficient mice (329). Downregulation of KL can lead an increase in senescence as demonstrated by expression of KL RNA interference (RNAi) or by administration of KL lowering compounds (354,355). Multiple studies show that KL inhibition of senescence is through a mitogen activated protein kinase (MAPK)/ERK dependent down-regulation of p53 and protein 21 (p21) (351,352,354). Senescence is often used as a marker for aging so KL's regulation of this event, which is linked to its ability to regulate oxidative stress and inflammation, may play a role in its age related functions.

2.5.8 Klotho and Nitric Oxide Synthesis

KL deficient mice have impaired acetylcholine-induced endothelium-dependent vasodilation of aorta and arterioles (271). This finding suggested that KL plays a role in nitric oxide (NO) synthesis because of the importance of NO in vascular tone. Further studies showed responses to norepinephrine and superoxide dismutase were also altered in these mice (336). Angiogenesis and vasculogenesis, which are also reliant on NO signaling, are impaired in KL deficient mice (269). This decrease in NO synthesis of KL deficient mice is systemic as evidenced by the decrease in urinary excretion of NO metabolites (271). Gene delivery of KL is able to rescue endothelial dysfunction through increased NO synthesis in a mouse model of multiple atherogenic risk factors (278). Evidence from heterozygous KL deficient mice also suggests that

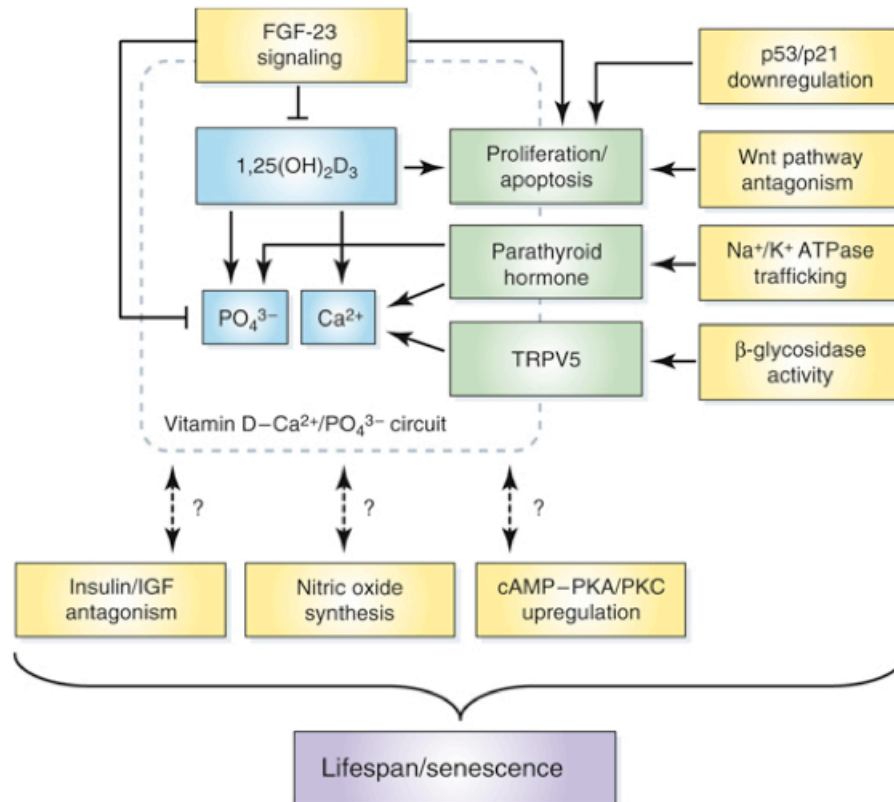


Figure 6. Klotho's Activity in Ion Homeostasis and Beyond. KL can function through various pathways in an autocrine, endocrine or paracrine fashion. It can regulate downstream $\text{Ca}^{2+}/\text{PO}_4^{3-}$ homeostasis by negatively regulating $1,25(\text{OH})_2\text{D}_3$ synthesis, and adjusting PO_4^{3-} directly, bypassing the vitamin D pathway. Klotho can also indirectly affect the vitamin D- $\text{Ca}^{2+}/\text{PO}_4$ circuit through other mediators including PTH and TRPV5. Many other pathways regulated by KL may also influence the vitamin D- $\text{Ca}^{2+}/\text{PO}_4$ circuit through unknown mechanisms, but together it is known that the pathways KL regulates lead to increased longevity when activated. Diagram from Wong F, and Q. Xu, 2009 (356).

differences in NO synthesis are not due to alterations in phosphate or vitamin D homeostasis because changes in ion homeostasis are not present in these mice. The amelioration of symptoms through parabiosis with a wildtype mouse also suggests that the alterations in NO synthesis are due to the humoral effects of the shed form of KL (271).

2.5.9 Summary

KL is able to regulate many age-related pathways resulting in a protein that is heavily implicated in the aging process. It is still unclear whether KL directly regulates all of these pathways or whether a few functions of KL mediate secondary effects creating a large web of functional changes associated with altering levels of KL. So far, KL's regulation of FGF and other growth factors is highly implicated as a primary effect of KL necessary for its aging phenotype. Through FGF signaling and other means, KL can regulate ion and vitamin D homeostasis, which seems to be another key function of KL. KL acts through these mechanisms to decrease levels of oxidative stress, prevent premature senescence and lead to a protein that can increase longevity (Figure 6).

2.6 Klotho and Disease

KL's association with multiple processes of aging indicate that it may regulate risk factors previously implicated in age-related disorders. Evidence

from the KL deficient mice further suggest KL is a regulator of age-related diseases. Implications from model organisms as well as recent advances in measuring KL levels in serum have led to an increase in the amount of research studying KL levels in human diseases. These studies, which are described below, show that KL is altered in many diseases. It is possible, as suggested by KL studies in model organisms, that these changes in KL could play a direct role in the pathology of the discussed diseases. Investigations of KL's role in human disease can help elucidate what function KL is playing in human physiology.

2.6.1 Chronic Kidney Disease

The high levels of KL found in mice kidney suggests that it has an important function in this organ. This is further substantiated by the results of studies that show angiotensin II induces down-regulation of KL (357). Inhibition of angiotensin II and the related renin-angiotensin-aldosterone system (RAAS) is a major target of chronic kidney disease (CKD) therapy (358) and can lead to increases in KL levels (359). This suggests that KL down-regulation may lead to some of the symptoms seen in CKD. Studies in mouse models of kidney injury show that levels of KL can affect the disease outcome. While higher levels of KL can protect against acute kidney injury (AKI) and ischemia reperfusion injury (IRI), lower KL levels worsen the outcome (360-362). This action as a renoprotective agent suggests that decreases in KL could lead to kidney disease. Several articles report that KL levels in the kidney are decreased in many mouse

models of kidney disease (278,279,363) as well as human cases of chronic kidney disease (364,365). Measurements of KL in the urine of CKD patients also show decreases that start in the early stages of CKD and are positively correlated with the severity of disease (361,366). In mouse models of CKD, increased KL levels can be protective, suggesting that targeting KL may be able to alter disease outcome in humans (277,279,315,367). These experiments as well as KL's ability to protect against damage even after the insult in IRI suggests it could be used as a therapeutic and that it is more than just a biomarker (360).

Evidence from mouse models and human patients make it clear that KL is involved in CKD, but does not explain what functions of KL are necessary for its renoprotection. Further studies suggested that it is KL's ability to regulate the FGF23-vitamin D axis that affects kidney function. Through this regulation, it can maintain phosphate homeostasis and prevent hyperphosphatemia, which is associated with accelerated CKD complications and higher mortality rates (368-371). Downregulation of KL can lead to decreased FGF23 sensitivity leading to higher levels of circulating FGF23, causing further hyperphosphatemia (372) and more severe disease progression (373). KL's role in CKD disease progression most likely comes from its control of phosphate homeostasis that is dysregulated by angiotensin II-induced KL downregulation (357). Gene transfer of KL is also capable of protecting against angiotensin II-mediated renal damage (357). Overall, KL is highly implicated in the progression of CKD and this association may be relevant to the aging phenotype of KL deficient mice (374).

2.6.2 Epilepsy

Temporal lobe epilepsy (TLE) is characterized by excessive neuronal excitation, usually caused by excessive release of glutamate leading to calcium overload and eventually cell dysfunction and death. One of the main pathological findings in patients that have suffered from TLE is hippocampal sclerosis (HS). HS is identified by increased astrocyte numbers associated with the destruction of nearby neurons namely, astrogliosis. Recently, inflammation has been suggested to have a crucial role in epileptogenesis (375) and also has ties to increases in astrogliosis (376). In particular tumor necrosis factor- α (TNF- α) has been identified as a regulator of neuroinflammation and cell death in epilepsy (375,377,378). KL has been shown to protect the hippocampus against stress-induced toxicity and can also inhibit TNF- α signaling. These connections suggested that KL might play a role in TLE. Examination of post-mortem tissue of TLE patients with HS showed that there was indeed up-regulation of TNF- α in the hippocampus and a concomitant decrease in KL levels (379). It is possible that down-regulation of KL may play a role in the pathogenesis of epilepsy due to its roles in controlling inflammation or potentially by its regulation of calcium homeostasis via control of membrane localization of calcium channels.

2.6.3 Acromegaly

Acromegaly is a growth disorder most commonly caused by pituitary adenomas that result in increased production of growth hormone and subsequent

increases in IGF-1 (380). This slow-moving disorder eventually leads to organ overgrowth and physical deformity over the course of decades. Metabolically, it leads to hyperphosphatemia, hypercalcemia, insulin resistance, high levels of FGF and vitamin D, and hyperglycemia (381-383). The incidence of cardiovascular, cerebrovascular, metabolic and respiratory comorbidities lead to a decreased life expectancy of approximately 10 years (384,385). Until recently, there was no known connection between KL and acromegaly despite the multiple connections between KL and the pathways affected in the disease. Recent studies have shown that serum KL levels are high in patients with acromegaly and that these levels decline after removal of the tumor (386-388). Despite rises in serum KL levels, staining for KL in the pituitary is not altered suggesting that it is not increased expression by the tumor itself and may be secondary to the systemic actions of growth hormone or IGF-1 (387). In fact, IGF-1 is known to increase shedding of full length KL (231), which could, in part, lead to the differences in KL. Although the importance of KL in the pathogenesis of acromegaly is yet to be determined, these studies suggest that it may be a biomarker of disease presence and severity, which could aid in the development of better treatment.

2.6.4 Cancer

Despite the lack of spontaneous tumor growth in KL deficient mice, low levels of KL are associated with many different cancer types. KL has been associated with cancers of the breast, ovary, cervix, lung, pancreas, intestine,

colon, skin, stomach and kidney (209,211,216-219,335,389-400). While most studies have found lower KL expression associated with increased severity, one study found KL levels increased with disease progression and death in epithelial ovarian cancer (389). This seemingly controversial study may be due to an unknown alteration in these cells that leads to decreased KL sensitivity. This could explain the higher levels of KL as well as insulin that would increase KL cleavage as a compensatory mechanism to try to maintain normal KL levels. All other studies have found low levels of KL associated with tumor progression and increased cancer cell growth (209,211,216-219,335,390,391,393,395-400). Conversely, increased levels of KL are associated with positive outcome after surgery in patients with lung cancer (392,394). Many groups have reported the reason for decline of KL levels in cancer is because of hypermethylation of the promoter. This has been found in breast, cervical, gastrointestinal and colorectal cancer (211,216-219). This decline in expression blocks KL from having its tumor suppressing activity through inhibition of growth factors (209,219,335,390,391,396-400). Although KL's inhibition of multiple growth factors has been implicated in its tumor suppressing activity, the association with the insulin/IGF pathway has been the most researched (209,390,397,400). KL's tumor suppressing activity has also been independently associated with its inhibition of the wnt pathway via its inhibition of Filamin A cleavage (219,391,396) and TGF- β signaling by inhibiting induction of the mesenchymal phenotype (335). All of these pathways can regulate similar downstream signals,

namely phosphoinositide 3-kinase (PI3K)/Akt/glycogen synthase kinase 3 β (GSK3 β)/snail signaling, which is associated with KL's tumor growth inhibition (398,399). Coupled with the fact that KL levels are often associated with the severity and invasiveness of the cancer, it is possible that KL mediates the epithelial to mesenchymal transition through its regulation of growth factors leading to its inhibitory effects on cancer.

2.6.5 Cardiovascular Disease

The first studies in KL deficient mice noted that these mice presented with atherosclerosis as one of the phenotypes (198). KL also has a role in maintaining endothelial wall homeostasis and health of the vasculature through its regulation of nitric oxide (271,401,402). Endothelial dysfunction, which is often correlated with decreased bioavailability of NO, is an early step in the development of cardiovascular disease (CVD)(403) suggesting that down-regulation of KL may result in increased risk for CVD. Studies in mice and rats support this hypothesis showing that KL protects against cardiovascular injuries through a NO dependent mechanism (278,348). KL's rescue of NO production is also capable of protecting human umbilical vein endothelial cells (HUVECs) from expression of adhesion molecules involved in the pathogenesis of cardiovascular disease (348).

Furthermore, evidence from analysis of mouse and human vascular smooth muscle cells (VSMCs) show that KL can reduce vascular calcification through its regulation of FGF23 signaling (361,404). It has also been suggested that KL can

protect from spontaneous cardiac hypertrophy and remodeling in mice overexpressing TRPC6 by down-regulating TRPC6 membrane localization therefore decreasing TRPC6 dependent currents (276). Most notably, a recent study has shown that low plasma KL levels are independently associated with CVD (405). All of these studies suggest that KL is involved in CVD through its regulation of many pathways. This involvement may be intertwined with its association in CKD, which can lead to development of CVD (406).

2.6.6 Diabetes

Klotho's regulation of insulin/IGF pathways suggests it may play a role in controlling glucose metabolism. However, despite insulin resistance in KL overexpressing mice, there was no increase in blood glucose levels (273). In a chemically induced rat model of diabetes, it was shown that KL levels are decreased in the kidney, which was associated with poor renal function(407). These phenotypes were recoverable by treatment with blood glucose lowering insulin or phloridzin, or tiron, an antioxidant (407). Both mechanisms of improving KL levels and renal function lowered levels of reactive oxygen species, suggesting that it was a decrease in oxidative stress that was responsible for rescuing KL (407). The reduction in KL levels after chemically induced diabetes was confirmed in rats as well showing that the effect was not species specific (407). It was also found that levels of renal KL in patients with diabetic nephropathy were reduced (408). Immunoassay for KL levels confirmed the

lower levels of KL in human diabetic patients (409). Whether these lower levels of KL are just a biomarker of disease or are involved in the pathology is uncertain, but it is possible that they are caused by the pro-inflammatory state associated with diabetes (410).

2.7 Case Studies and Polymorphisms

Large alterations in KL levels are not often seen in humans except in the case of late stage diseases making it difficult to ascertain the functional significance of KL in normal human function. Examinations of infrequent occurrences such as case studies and polymorphisms in KL allow for a better understanding of KL disease risk in humans. While case studies offer insight into how extreme differences in KL levels can affect human pathologies, studies of polymorphisms, which can be found in coding and non-coding regions of KL (Figure 7), allow more information to be gathered on how smaller changes in KL function can affect risk of diseases in a larger population.

2.7.1 Case study: Increased Circulating Klotho

At 13 months of age, a child presented with poor linear growth and increasing head size and was diagnosed with a nutritional form of rickets (411). After unsuccessful treatment with vitamin D₂, further investigations revealed hypophosphatemia, increased phosphatase activity and elevated PTH (411). The diagnosis was changed to hypophosphatemic rickets due to inappropriate renal

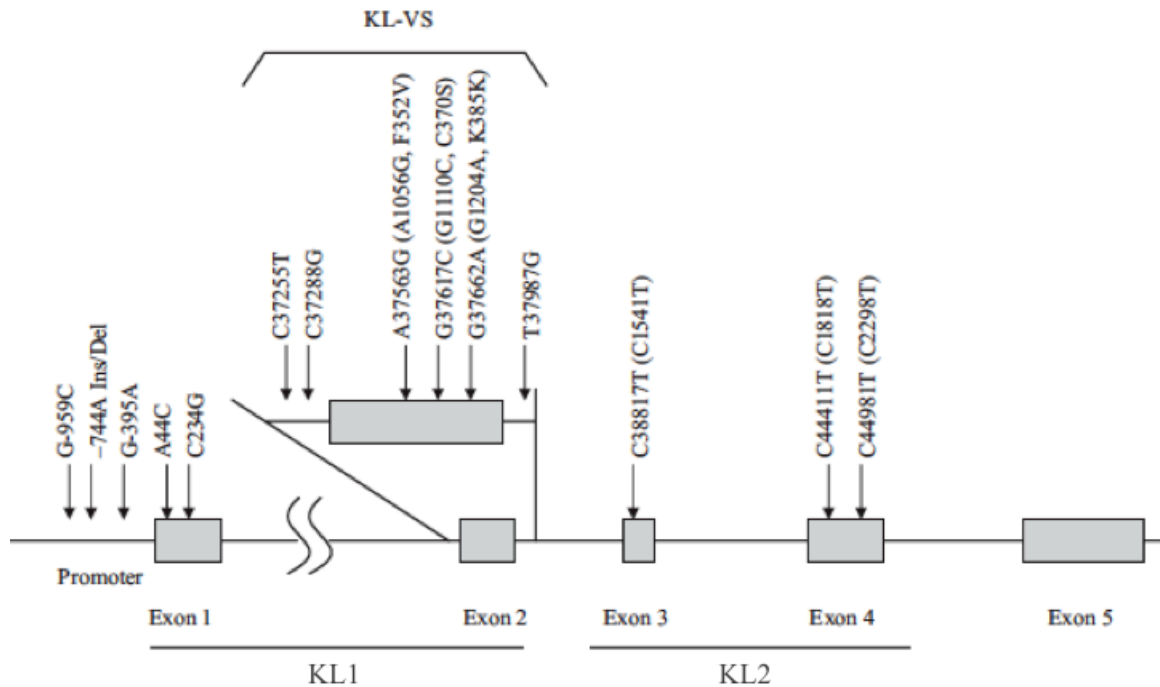


Figure 7. Location of KL Polymorphisms in Coding and Non-Coding Regions. Polymorphisms in KL that are associated with disease risk have been identified in the promoter and exons 2, 3 and 4. With the exception of KLVS, they consist of one SNP independently associated with one or more diseases. The KLVS is comprised of 6 SNPs that span exon 2 and the surrounding introns. Diagram from *Shimoyama et al.*, 2009 (412).

phosphate losses and the patient was given 1,25(OH)₂ vitamin D supplemented with phosphate salt, which improved her condition. Despite improvements, by the age of 7 it was evident that PTH levels were increasing leading to hypercalcemia. Surgical investigation of the neck revealed four-gland parathyroid hyperplasia, which prompted removal of 3.5 glands. The patient remained stable until 19 years of age at which time more of the parathyroid gland, which had enlarged again, was removed. Despite sequencing of FGFR1, phosphate-regulating neutral endopeptidase, X-linked (PHEX), dentin matrix phosphoprotein 1 (DMP1) and FGF23, which are highly involved in pathways that regulate these symptoms no mutations were found in these genes. Further investigations of the chromosomes showed that there was a translocation between chromosome 9 and 13. A closer look at the translocation identified KL as the closest gene distal to the breakpoint on chromosome 13. Since KL is able to regulate phosphate, calcium and vitamin D levels, it was a likely candidate as the affected gene. Further studies showed that plasma levels of KL were highly elevated compared to age and gender-matched controls (411). This case study shows that marked up-regulation of KL can be just as detrimental as down-regulation most likely due to its important role in ion homeostasis. This suggests that targeting KL as a potential therapy may only be useful in diseases where KL levels are measurably declined and may require monitoring of KL levels to make sure they fall within normal parameters.

2.7.2 Case study: H193R Polymorphism

A 13-year-old female presented with elevated blood serum levels of calcium, phosphate, 1,25(OH)₂-vitamin D and PTH (413). Surgical examination revealed 4-gland hyperplasia and subsequently, part of the excess gland was removed. The subtotal parathyroidectomy normalized PTH and calcium levels, but production of calcium-phosphorus continued to be elevated. Further analysis revealed diffuse osteopenia, patchy sclerosis in the hands, feet, long bones and calvariae, and metacarpal periosteal reaction. Calcification was present intracranially in the dura and carotid arteries but not in the parenchyma and was also present in the Achilles tendon (413). Presence of tumoral calcinosis prompted examination of FGF23 levels, which were 150 to 550 fold higher than normal. Mutational analysis to find the gene that was altered showed no differences in the calcium sensing receptor (CASR), UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 3 (GALNT3) or FGF23, which have all been independently associated with similar symptoms. Further analysis led to the discovery that there was a nucleotide substitution in the KL gene leading to replacement of a histidine for an arginine at amino acid 193 (413). Examination of the H193R substitution revealed that this polymorphism led to decreased amounts of mature KL and less shed KL in the media. It was found that this polymorphism reduced the formation of the KL-FGFR-FGF23 signaling complex. Interestingly, KL still interacted with FGFR1c at close to wild-type levels, but did not co-immunoprecipitate with FGF23 (413).

These findings suggest that it is the lack of functional KL-FGFR-FGF23 complex and subsequent downstream signaling that is responsible for the severe tumoral calcinosis presented in this case study.

Of note, some of the phenotypes present in this case study of decreased functional KL parallel the phenotypes seen in the case study of increased KL levels. Specifically, both patients suffered from increased levels of PTH and parathyroid hyperplasia even though other blood serum counts trended in opposite directions. It is also worth noting that in both cases parathyroidectomy was capable of regulating symptoms. Increases in PTH in the presence of decreased KL are expected because of the inhibitory effect of KL on PTH secretion. Increases of PTH in the presence of increased levels of KL, however, suggest that the regulatory mechanisms for this pathway are more complicated than currently understood.

2.7.3 Polymorphism of the Promoter: G-395A

Evidence from hypermethylation of the KL promoter support the hypothesis that alterations in KL promoter activity can lead to disease. Therefore, it is not surprising that a polymorphism in the promoter is associated with many changes in disease risk. One polymorphism has been identified and its effect on KL function has been well studied.

Many studies have analyzed the effect of the G-395A substitution on the risk of CVD and found that it leads to alterations in many risk factors associated

with CVD such as high blood pressure (414). This increased association with risk factors leads to higher incidences of many types of CVD including coronary artery stenosis (415), essential hypertension (416), atherosclerotic coronary heart disease (417) and cardioembolisms in individuals with this polymorphism (418). Despite all of these increased risks for CVD, one study found that in older individuals (over 60 years) there was actually a lower incidence of coronary artery disease (419). This suggests that age may be a factor in the effect of this polymorphism.

The G-395A polymorphism has also been linked to disease progression and mortality in IgA nephropathy (420), which is closely linked to its affect in the cardiovascular system because it is also associated with early dysfunction of vascular access in hemodialysis patients (421). Patients that have undergone hemodialysis also have higher levels of uric acid if the G-395A polymorphism is expressed (412).

Interestingly, the G-395A polymorphism is associated with higher bone mineral density especially in postmenopausal Japanese women (422). This polymorphism has also been associated with osteocalcin levels (423). On the contrary, other studies have found an association of this variant with decreased bone mineral density in women 65 and older (424) and with increased risk of knee osteoarthritis in women (425). These differences could be due to variations in other polymorphisms or in the age of the patients studied.

Further studies suggest that there is also a link between G-395A and risk of diabetes. Not only is the incidence of diabetes increased with the expression of this substitution (426), there are also alterations in lipid metabolism (427) and increased body mass index (BMI) (428) which are associated with risk of diabetes. G-395A is also associated with secondary characteristics related to sickle cell anemia such as leg ulcers and priapism (429,430). Lastly, this polymorphism can also lead to increased risk of kidney stones (431).

The variety of diseases that the G-395A polymorphism is associated with is reminiscent of the klotho deficient mice that have a global phenotype affecting many different tissue types. Evidence in HEK293 cells suggests that this polymorphism alters the DNA-protein interaction, which could lead to alterations in KL expression (424). It is possible that individuals with this variant have consistently lower levels of KL leading to the variety of effects reported.

2.7.4 Polymorphisms in Non-coding regions

Many of the polymorphisms in intron 1 of KL are not well studied either due to low frequency in the population or ethnic specificity of the variant. One such polymorphism is strongly linked with hemodialysis mortality despite the fact that it is not located near an exon (432). It is known that the first intron often serves a regulatory function, which could explain this effect. Another possibility is that it is associated with other polymorphisms in the area creating a secondary effect due to other polymorphisms (432). Despite the unknown

mechanism by which this polymorphism is acting, it is shown that expression of the polymorphism does lead to decreased levels of KL in cultured cells known to express the variant (432). This suggests there is a direct correlation between KL levels and hemodialysis survival. A separate set of polymorphisms found in intron 1 and the surrounding 3' untranslated region are associated with sickle cell osteonecrosis (433). This further supports the idea that polymorphisms in this region are capable of regulating KL expression.

A series of polymorphisms were also identified downstream of the last exon in the CA repeat region. Although the associations were weak, there were age-related links to the amount of spondylosis in postmenopausal women. This suggests the region downstream of exon5 can regulate KL's expression and/or activity leading to these changes in disease risk seen in the polymorphisms (434).

2.7.5 Polymorphisms in KL2

Although many of the functions of KL have been associated with the KL1 domain, the KL2 domain has been associated with KL's regulation of TRPC1/VEGF membrane localization. It is possible that KL2 is important in other functions of KL, but due to the mRNA splice variant consisting of KL1, this portion of the extracellular domain is less studied.

Despite the lack of information on the importance of the KL2 domain, there are polymorphisms in this domain that are associated with risk of disease development and disease outcome. Many studies have linked one polymorphism

in particular, C1818T, with increased risk for a variety of diseases. For instance, this polymorphism is linked to sickle cell anemia as it is associated with increased prevalence of secondary illnesses (429) (430). C1818T is also more highly expressed in individuals with metabolic disorder (435) and has also been associated with changes in metabolism that are concomitant with this disease such as higher levels of fasting glucose (414), high body fat ratios and low levels of high-density lipoprotein (HDL) (427).

There are also studies linking this polymorphism to CVD showing that there is actually a lower prevalence of coronary artery disease in carriers under the age of 60 (419). This beneficial effect of the C1818T is confirmed by an association with higher levels of nitric oxide in younger individuals (436) and leads to lower levels of low density lipoprotein in hemodialysis patients (412). Despite these positive effects on cardiovascular health, this variant is also associated with higher systolic blood pressure in younger individuals (427). Despite all of these associations with disease risk, there are no studies investigating how the C1818T changes KL protein function.

Other polymorphisms in KL2 are less frequently studied, but have also been associated with disease risk. A polymorphism found in exon 4, C2998T, is associated with increased risk of knee osteoarthritis in both men and women in a Greek population (425). A polymorphism in exon 3, rs3752472, is linked to increased calcium oxalate urolithiasis leading to an increased risk of kidney stones in a Han population from Eastern China (437). Another polymorphism in

the 4th intron is associated with carotid atherosclerosis in hypertensive patients (438) and severity of end stage renal disease (439). These polymorphisms are not well studied, which could be due to their low frequency in the population or their isolation to specific ethnic groups. Despite this, they still add valuable information about how alterations in KL can lead to increased risk for a variety of diseases.

2.7.6 Polymorphisms in KL1

The first extracellular domain of KL has been associated with many of the functions attributed to KL. It is highly implicated in KL's tumor suppressing functions as well as its ability to inhibit growth factor signaling. Despite its importance, there is only one polymorphism cluster in KL1 that has been associated with altered risk of disease (Figure 8).

The KLVS variant consists of 6 single nucleotide polymorphisms (SNPs) that are always found together. Three SNPs lie in introns and do not alter splicing and another substitution at nucleotide 1155 causes no change in amino acid designation. However, the changes from phenylalanine to valine at amino acid 352 (F352V) and cysteine to serine at amino acid 370 (C370S) result in substitutions that could alter protein function (Figure 2).

Multiple studies show that humans heterozygous for the KLVS allele have an increased chance of survival by approximately 2 fold over the age of 75 (440-442).

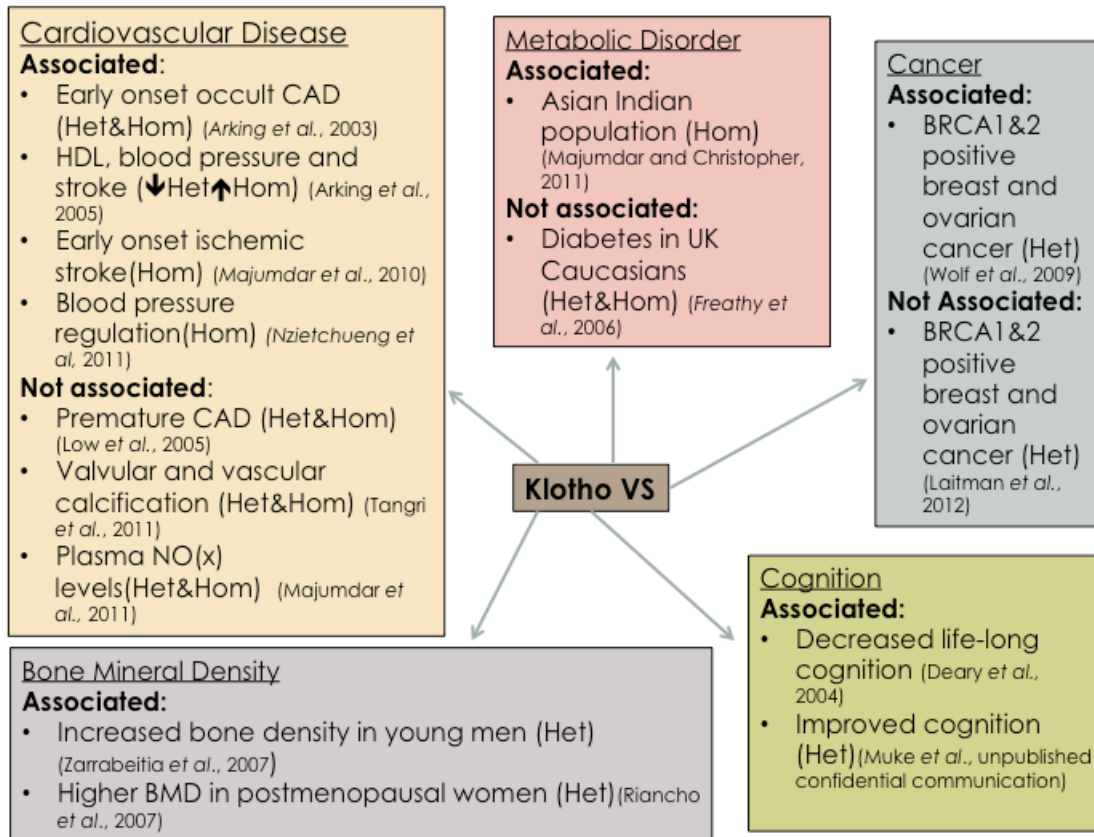


Figure 8. Association of KLVS with Disease Risk. Diagram showing outlining the associations of the KLVS variant with increased risk for different diseases. Within diseases, studies are split into those showing an association and not. Whether individuals are homozygous (hom) or heterozygous (het) for the variant is also depicted. It can be seen from the outlined studies that many of the associations with increased risk of disease are limited to individuals homozygous for KLVS.

One study also found a reduced representation of KLVS homozygotes after this age (442). Further characterization of the variant revealed protective effects of heterozygosity after 81, despite reduced frequency of heterozygotes in the population before this age (441). In independent studies, the KLVS variant has also been linked to an increased risk of cardiovascular disease and metabolic syndrome with a similar pattern of advantageous KLVS heterozygosity and increased disease risk associated with homozygosity (435,441,443,444). This could be explained by KLVS's regulation of factors that independently increase risk of these diseases such as HDL-Cholesterol and systolic blood pressure (441). There are also age-dependent increases in bone mineral density in both male and female heterozygotes, but presence of a homozygote disadvantage could not be determined due to the low frequency of that genotype (445,446). Contrary to the other effects, heterozygosity for KLVS increases the risk of breast cancer 1, early onset (BRCA1) associated breast and ovarian cancer and decreases the risk in homozygous patients from an Ashkenazi Jewish population, but not in a larger cohort of European ancestry (447,448).

Unlike most other variants, there are studies suggesting that KLVS does change protein function. The first studies showing a difference in function suggested that the KL1 domain containing KLVS had a slight increase in secretion. When isolated, the V substitution led to a large decrease in secretion of the KL1 domain, while the S substitution led to a marked increase (442). The

intermediate phenotype of the KLVS suggested that there was intragenic complementation between the two substitutions correcting for the drastic decrease caused by the V variant (442). The same study showed that a homolog of KLV led to decreased enzymatic activity suggesting that the V substitution may affect KL's enzymatic function(442). Further studies investigated the V variant's ability to alter KL's tumor suppressing activities. These findings suggested that expression of the V substitution suppressed KL's ability to inhibit colony formation and tumor growth (447). This suggests that the VS polymorphism may lead to a drastic change in KL function. Despite these preliminary studies, it is of note that most of the drastic changes have been found in the KLV variant alone, but are absent or have not been studied in the physiologically relevant KLVS polymorphism. These studies also lack a mechanistic explanation of what pathways are altered by the KLVS leading to the increased risk of age-related diseases and longevity.

3. HYPOTHESIS AND SPECIFIC AIMS

As the percentage of the population over 65 increases, there is growing pressure to better understand the aging process so that we may target the pathways that lead to an increase development of many chronic diseases. Finding a way to attenuate the aging process and allowing a longer, and more importantly, healthier lifespan will lessen the amount of healthcare costs that go to treating these chronic diseases and make the later years of life more vibrant

and fulfilling. Investigation into the function of KL has provided insight into a protein that regulates many of the pathways associated with aging and holds promise into elucidating how these various pathways could work together to cause the aging phenotype. Our work will aid in this greater goal by providing mechanistic clarity of the function of KLVS. *We hypothesize that the KLVS variant leads to differences in the basic biochemical characteristics of the KL protein that lead to differences in the function of KL resulting in increased risk of disease.* In order to investigate this hypothesis, the following specific aims were developed.

3.1 Significant Aim 1: Investigate Whether KLVS Alters KL Processing

This aim follows up on previous data suggesting that KLVS leads to alterations in the shedding of secreted KL to elucidate whether there are differences in the processing of full-length KLVS or in the individual substitutions it contains.

3.2 Significant Aim 2: To Determine Whether The KLVS Variant Leads to Altered Function

The results from this aim will elucidate whether the substitutions that comprise KLVS result in a KL with altered function as determined by its ability to homodimerize, form complexes with FGFR and FGF23 and have enzymatic activity.

CHAPTER 2: METHODS

1. PLASMID DNA CLONING

1.1 Plasmid Constructs

1.1.1 KLVS Variant Constructs

The full length KL complementary DNA (cDNA) in pcDNA 3.1 was kindly provided by Dr. Hal Dietz from Johns Hopkins University School of Medicine, Baltimore MD. To construct polymorphic variants containing F352V, C370S, or both, site directed mutagenesis was performed per manufacturer's instructions (Agilent Technologies Inc., Santa Clara CA) using the following sense and antisense primers (Integrated DNA Technologies):

KL C370S:

5'- AGGTGGGTCCAAAGCTAAGAGCAAAAAGTC-3' and

5'- GACTTTTTTGCTCTTAGCTTTGGACCCACCT-3'

KL F352V:

5'- TCTCAGATTCAGTAACATCAGGCAGAATAGA-3' and

5'- TCTATTCTGCCTGATGTTACTGAATCTGAGA-3'

1.1.2 Truncated Klotho Constructs

To create constructs of the KL variants (V, S, VS) without the transmembrane and cytoplasmic domains, the KL variant constructs and a previously created KL980 construct that lacks the transmembrane and

cytoplasmic domains were digested with restriction enzymes *ClaI* and *AgeI*. The resulting pcDNA3.1 plasmid containing the majority of the KL variant was ligated to the smaller fragment lacking the C-terminal end of KL. Successful ligation was confirmed by sequencing (Tufts DNA Sequencing Core).

1.1.3 Klotho Constructs with GFP Tag

To create constructs of KL variants with a C-terminal green fluorescent protein (GFP) tag both the created KL variant constructs (V, S or VS) and a previously created KLGFP construct (231) were digested with restriction enzymes *AgeI* and *XhoI*. The excised fragment from the KLGFP construct containing GFP cDNA was ligated into the linearized KL variant constructs. Confirmation of correct product was performed by sequencing (Tufts DNA Sequencing Core).

1.1.4 Klotho Constructs with V5 Tag

To create constructs of the KL variants (V, S, VS) with a C-terminal V5 tag, the KL variant constructs and a previously created KLV5 construct (231) were digested with restriction enzymes *ClaI* and *AgeI*. The resulting pcDNA3.1 plasmid containing the majority of the KL variant was ligated to the small fragment containing the truncated C-terminal end of KL containing a V5 tag. Successful ligation was confirmed by sequencing (Tufts DNA Sequencing Core).

1.2 Site Directed Mutagenesis

Site directed mutagenesis was used to introduce point mutations into exon 1 of the KL gene. The mutagenesis experiments were carried out using QuikChange Mutagenesis Kit (Stratagene) according to the manufacturer's protocol. This procedure included using a denaturing temperature of 95°C and an elongation time of 16 minutes at 68°C. After mutagenesis, the samples were digested with *Dpn1* endonuclease at 37°C for 1 hour to remove any remaining parental DNA. Digestion was followed by transformation into competent cells to screen for DNA containing the desired mutation.

1.3 Restriction Enzyme Digest

Restriction enzymes and accompanying buffers were acquired from New England Biolabs (NEB). These enzymes were used to excise vectors and inserts from DNA for ligation and to confirm that plasmids contained the correct cDNA. Buffer conditions were chosen based on information gathered from the NEB website Double Digest Finder. Approximately 4µg of DNA was mixed with 1µL of the appropriate restriction enzymes and 2µL of 10x buffer. The mixture was brought up to 20µL with HyClone HyPure Molecular Biology Grade water (Thermo Scientific) and incubated at 37°C overnight for complete digestion.

1.4 DNA Ligation

Ligations were performed to join excised inserts and vectors together using the Fast-Link Ligation Kit (Epicentre) per manufacturer's protocol. In the process of troubleshooting, some ligations were performed at 16°C overnight instead of 15 minutes at room temperature. After ligation, the products were transformed into competent bacterial cells for selection of successful ligation products.

1.5 Agarose DNA Gel Electrophoresis

Samples were run on agarose gel electrophoresis in order to separate restriction enzyme products by molecular size. One percent agarose gels were prepared from Genepure LE Quick Dissolve Agarose (ISC BioExpress) and TBE buffer (108g tris-base, 55g boric acid, 40mL 0.5M EDTA in 5L Millipore H₂O). The mixture of agarose and TBE is heated in a microwave to ensure that all the agarose is dissolved. Afterwards, approximately 0.5mg/μL Ethidium bromide is added to the solution to allow for visualization of DNA fragments. The resulting mixture is poured into a gel casting tray until hardened. DNA samples of 1-2μg mixed with 6x Blue/Orange Loading Dye (Promega) were loaded into each well and separated by electrophoresis for approximately 30 minutes at 100V. A 1 kb ladder (Promega) was run alongside the samples in order to allow for identification of the molecular weights of the bands. The bands were visualized under a UV light box.

1.6 DNA Gel Extraction

Gel extraction was used to retrieve restriction enzyme digested DNA fragments that had been separated out on agarose gels. To this end, the StrataPrep DNA Gel Extraction Kit (Stratagene) was used according to manufacturer's protocol. After elution, DNA was stored in elution buffer at -20°C until used.

1.7 DNA Sequencing

The sequences of all cloned plasmids were verified at the Tufts University Core Facility DNA Sequencing Lab, Boston, MA.

2. PLASMID DNA PREPARATION

2.1 Bacterial Transformation

Bacterial transformation was used to introduce plasmid DNA into competent bacterial cells. This was done to screen for successfully cloned plasmids containing antibiotic resistance genes that allow their growth on antibiotic supplemented plates. Transformation was also done to amplify plasmids in order to obtain large amounts of DNA for experiments. For transformation, plasmid DNA was transformed into NEB Turbo Competent *E. coli* (High Efficiency) cells (New England Biolabs) using the heat shock method. Fifty microliters of competent cells were incubated with 2-5µg of plasmid DNA on ice for 15min, and then the samples were heated at 42°C for 45 seconds, and then

put back on ice for 2min. Nine hundred microliters of LB-media (MP Biomedicals) was then added to the cells and they were incubated at 37°C for 1 hour in an environmental shaker at 200 rpm. Afterwards, the cells were quickly centrifuged for a few seconds at 16,873xg (Eppendorf 5418 Microcentrifuge), and the supernatant was removed. The cells were resuspended in 100µL of fresh LB- then the cell suspension was spread onto 0.5mg/mL ampicillin agar plates prepared using LB-Agar (MP Biomedicals) and incubated in a 37°C incubator overnight to allow colony formation.

2.2 Minipreps

Minipreps were used to extract and purify plasmid DNA at a small scale. Transformed bacterial colonies were selected from agar plates and were transferred into 4mL of LB broth containing 0.5mg/mL ampicillin. The resulting inoculations were incubated at 37°C and 200 rpm in an environmental shaker. After overnight incubation, DNA was purified from the bacterial culture using the QIAprep Spin Miniprep kit (Qiagen) according to manufacturer's protocol. After elution, DNA concentration was determined and the resulting DNA was stored at -20°C until used.

2.3 Midipreps

Midipreps were used to extract and purify plasmid DNA at a larger scale. Transformed bacterial colonies were selected from agar plates and were

transferred into 100mL of LB broth containing 0.5mg/mL ampicillin. The resulting inoculations were incubated at 37°C and 200 rpm in an environmental shaker overnight. The next day, DNA was purified from the bacterial culture using the Wizard Plus Midiprep Purification System (Promega) in accordance with manufacturer's protocols. Resulting DNA concentration was determined and the samples were stored at -20°C until used.

2.4 DNA Spectrophotometry

Quantification of purified DNA was determined by use of an Eppendorf Biophotometer. Briefly, the purified DNA was diluted 1:50 with Millipore H₂O and absorbance values were read at 260 and 280 nm. The measurement at 260nm was used to measure the concentration of DNA and measurement at 280 measured the concentration of protein contaminants and the 260/280 ratio was used to estimate the purity of the DNA. Samples were considered pure if the ratio was between 1.8 and 2.0.

3. CELL CULTURE

3.1 Maintenance of Cell Cultures

3.1.1 Human Embryonic Kidney (HEK) 293 Cells

HEK293 cells were purchased from the American Type Culture Collection (ATCC). Purchased cells were cultured in 10cm plates in Dulbecco's modified Eagle's media (DMEM) (CellGro) supplemented with 10% fetal bovine serum

(FBS) (Atlanta Biologicals) and 1% penicillin-streptomycin (CellGro) at 37°C and 5% CO₂. Cells are grown until they reach 70-80% confluency at which point they are split (approximately every 3 to 4 days). To split, cells are washed with Dulbecco's phosphate buffered saline (DPBS) and then cell adhesion proteins are removed by incubation with 0.25% trypsin (CellGro) for 5 minutes at 37°C. Afterwards, 9mL of growth media is added to cells and pipetted up and down to dislodge cells. The cell suspension is transferred to a 15mL centrifuge tube (BD Falcon) and centrifuged at 259g in a Beckman Coulter Allegra 21R centrifuge for 5min. The supernatant is removed, and the cells are re-suspended in 10mL of growth media. One milliliter of the re-suspended cells is then added to a 10cm plate to propagate as the next passage of cells in 10mL of growth media. To freeze cells for storage, the same process of trypsinization and spinning is undertaken, but instead of resuspension in growth media, the cells are resuspended in a freezing media containing 70% DMEM, 20%FBS and 10% dimethyl sulfoxide (DMSO). After resuspension, 1 mL aliquots are transferred to cryogenic tubes (Corning) and then put in an isopropanol bath at -80°C overnight. The next day, the cells are transferred to a liquid nitrogen tank for permanent storage until used.

3.1.2 HEK Stable Cells

HEK293 cells stably overexpressing KL with a N-terminal Gaussia luciferase tag (provided by Dr. Cidi Chen) or overexpressing KLWT or VS 980

were made by the filter selection method. To do so, HEK293 cells were split into 2-3 10-centimeter plates, enough to prepare the stable cell line and one extra for a control plate. The day after splitting, the appropriate plasmids were transfected into separate plates at 10 µg DNA each. Two days after transfection, the media was changed to 10mL of growth media supplemented with 800µg/mL Geneticin (G418) (CellGro). Cells are monitored over time and are maintained and split as needed until all of the cells on the control plate that do not contain G418 resistance are dead. At this time, all remaining cells should contain the plasmid, which confers G418 resistance. The remaining 10 cm plates are then split at multiple dilutions (1:50, 1:100, 1:200, 1:500, 1:1000) and analyzed for formation of single colonies once the cells have attached. Once single colonies have formed, they are picked from the plate using filter paper soaked in .25% trypsin (CellGro) and placed in a 6-well plate containing 2 mL of growth media supplemented with G418. At least 10 colonies are picked to ensure that at least one colony is stably transfected with the appropriate plasmid. Cells are maintained in G418 supplemented growth media until they reach confluency at which point they are trypsinized and transferred to a 10 cm plate. Once the 10 cm plate has reached 70-80% confluency, the cells are harvested to test for proper insertion of the plasmid of interest by western blotting. The clones that tested positive were selected and expanded. Frozen stocks are prepared and stored in liquid nitrogen as described above.

3.2 Transfection

Transient transfection was used to introduce plasmid DNA into mammalian cells. During cell division, the plasmid will amplify and large quantities of the desired DNA product will be synthesized. For the experiments, plasmid DNA was transfected into cells using the Nanofect (Qiagen) polymer as a carrier. Cells were split with 0.25% trypsin-EDTA (CellGro) into 6-well or 12-well tissue plates (Corning) at 1×10^5 cells per 6 well or 1×10^3 cells per 12 well the day before transfection. The next day, a transfection mix consisting of 100 μ L Opti-MEM, 6 μ L Nanofect and 2 μ g of total DNA for each 6-well or 50 μ L Opti-MEM, 3 μ L Nanofect and 1 μ g DNA for each 12-well was prepared. The mix was incubated for 15min at room temperature for the formation of transfection complexes. After 15min, the transfection mix was added to the cells. Twenty-four hours after transfection, cells and media were harvested for experiments.

3.3 Sample Preparation

3.3.1 Preparation of Cell Lysates

Cell lysates were collected and prepared for protein analysis in various experiments. Transfected cells were washed 1x with DPBS and then lysed with either 250 μ L (6-well) or 150 μ L (12-well) of RIPA lysis buffer (150 mM NaCl, 1% Triton X-100, 0.5 % Deoxycholate, 0.1 % SDS, 50 mM Tris pH 7.5) containing protease inhibitors (Roche) and phosphatase inhibitors (Roche) when needed. Lysates were pipetted up and down to facilitate complete lysis and were

transferred into 1.5 mL conical tubes. Resulting samples were centrifuged at 16,000xg for 15 minutes after which the supernatant was collected and stored at -20°C until use.

3.3.2 Preparation of Media Samples

Media samples were collected and prepared to analyze proteins in the extracellular space of transfected cells. Media was collected and debris was removed by centrifugation at 2,000xg after which the supernatant was moved to a new 1.5 mL tube. Bovine serum albumin (BSA) was added to the samples in order to adjust to a final protein concentration of 10µg/mL. Cold (4°C) trichloroacetic acid (TCA) was added to a final concentration of 25% then the mixture was incubated at -20°C for 5 minutes and then at 4°C overnight. After incubation, the samples were centrifuged at 16,000xg and the supernatant was removed. The resulting pellet was washed 3 times with ice-cold acetone with 5-minute centrifugations. The pellet was dried at 98°C for 5 minutes and then dissolved in 2x Laemmli sample buffer. After resuspension half of the mixture was used for Sodium Dodecyl Sulfate (SDS)- Polyacrylamide Gel Electrophoresis (PAGE) separation and the remaining sample was stored at -20°C until use.

4. PROTEIN ANALYSIS

4.1 Bicinchoninic Acid (BCA) Assay

Protein concentration of cell lysates was measured by BCA analysis following the BCA protein assay kit (Pierce). This colorimetric assay relies on the reduction of copper ions to cuprous ions by proteins in an alkaline media. The cuprous ions can then be detected by their chelation with two molecules of BCA, which forms a purple colored product detectable at a wavelength of 562 nm. This allows for the amount of protein present in a sample to be detected by analyzing the intensity of the readout at 562 nm. The 562 nm absorbance of samples and standards was read in the Promega Glomax Multi Detection System plate reader.

4.2 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Sodium Dodecyl Sulfate (SDS) Polyacrylamide Gel Electrophoresis (PAGE) was used to separate proteins by molecular weight. Ten percent Tris-Glycine gels were prepared with the Bio-Rad Mini-Protean Tetra System according to the recipe calculated by the SDS-PAGE website (<http://www.changbioscience.com/calculator/sdspc.htm>). The gels were prepared using electrophoresis grade water (Fisher), 30% UltraPure Protogel (National Diagnostics), 10% Sodium Dodecyl Sulfate (Fisher), 10% Ammonium Persulfate (Sigma), TEMED (Sigma) and Tris-HCl (Fisher). Protein samples were denatured in 5x Laemmli buffer (250mM Tris-HCl (pH 6.8), 10% SDS, 30% Glycerol, 5% beta mercaptoethanol, 0.02% bromophenol blue) and heated at

98°C for 5 minutes. Twenty micrograms of protein samples were loaded into each lane of the Tris-Glycine gels and proteins separated in 1x Tris-Glycine SDS-PAGE running buffer at 150 volts for 90 minutes. The Fisher Bioreagent EZ-Run Protein Marker was loaded as a protein ladder in order to estimate the molecular weight of the visualized protein bands.

4.3 Protein Transfer

Proteins separated by SDS-PAGE were transferred to nitrocellulose membranes for further analysis via western blotting. Proteins were transferred from the Tris-Glycine gels to 0.4 μ M Immobilon Hybridization nitrocellulose membranes (Millipore) at 100 V for 120 minutes in 1x Transfer Buffer (25mM Tris Base, 192mM glycine, 20% Methanol). After transfer, membranes were stained with PonceauS (0.1% PonceauS in 5% glacial acetic acid) to confirm successful transfer.

4.4 Western Blots

Western blot analysis was performed on separated proteins present on nitrocellulose membranes. The membranes were incubated twice for 15 minutes in 5% non-fat dry milk powder in TBS/T (50 mM Tris, 150 mM NaCl and 0.1% (v/v) Tween 20). After blocking, the membrane was incubated in primary antibody overnight. After incubation, the membranes were washed three times for 10 minutes with TBS/T, followed by the addition of a secondary antibody for 1

hour. After the secondary antibody incubation, the membranes were washed four times for 15min each with TBS/T. Finally, the Supersignal West Pico Chemiluminescent Substrate (Thermo scientific) or the Immobilon Western Chemiluminescent HRP Substrate (Millipore) was added to the membrane for 5min, depending on the intensity of the signal desired. The blots were then developed on Phoenix Research Products F-BX810 Blue X-Ray Films.

4.5 Western Blot Antibodies

Primary antibodies used for western blots were prepared in TBS-T with 0.03% sodium azide and 3% bovine serum albumin (BSA). For detection of KL, the rat monoclonal antibody KM2076 (Cosmo Bio Co., Ltd., Japan) was used at a dilution of 1:2000. Mouse anti- β -tubulin (1:10,000) (Invitrogen, Grand Island NY) was used to stain tubulin, which served as a loading control, and dynamin was detected with mouse anti-Dynamin1 (1:10,000)(Abcam, Cambridge MA). FGFR1C-V5 was detected using a mouse monoclonal antibody against the V5 tag (1:5000)(Sigma) and phosphorylated ERK (1:10,000) and total ERK (1:1000) were detected using antibodies obtained from Sigma Aldrich and Cell Signaling Technologies, respectively. Appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from KPL and were used at a dilution of 1:10,000.

5. ANALYSIS OF KLOTHO PROCESSING

5.1 Shedding Assay

The shedding assay was used to analyze the amount of total KL protein that is shed into the media. To accomplish this, cells were transfected with full-length wildtype KL (KLWT) or KL containing either one or both of the mutations. Twenty-four hours after transfection, cells were washed with Dulbecco's Phosphate Buffered Saline (Mediatech Inc.) and incubated in serum-free DMEM for 24 hours. Media was collected as described above and ran out on SDS-PAGE for western blotting analysis. After the media collection, lysate was collected as described above and proteins were separated on SDS-PAGE for western blotting.

5.2 Gaussia Luciferase Assay

In order to look for compounds that alter KL shedding, HEK 293 cells stably overexpressing KL with a C-terminal Gaussia luciferase tag were tested for alterations in luciferase activity in both media and lysates of treated cells using the BrightGlo Luciferase Assay System (Promega). Briefly, One hundred microliters of lysate or media collected from 6-well plates were added to a 96-well white plate. Directly before reading, 100 μ L of the BrightGlo Luciferase Substrate was added and then the plate was analyzed for changes in levels of luminescence on the Promega Glomax Multi Detection System plate reader.

5.3 MT-1 Chemical Treatment

To determine whether polymorphism changes are due to protein misfolding, transfected cells were treated with MT-1, (5 μ M; provided by Dr. Gal Bitan at UCLA) or control media immediately after transfection. MT-1 acts as a chemical chaperone to help correct proteins misfolding and restore normal trafficking. After 48 hours lysate and media were collected and analyzed by western blotting (as above).

5.4 Analysis of Klotho Half-life

Half-life analysis was performed to analyze the effects the variants have on the degradation and processing of KL. Use of biotinylation to measure half-life was first described by Fimbel *et al.* in an in vivo model (449). This assay was developed in order to use biotin to measure half-life in a cell-based system. To achieve this, HEK 293 cells were transfected as described above. Twenty-four hours later, cells were washed twice with DPBS and incubated with a 1mM solution of cell permeable NHS-SS-Biotin (Pierce) containing 0.1% DMSO for 30 minutes at room temperature. Afterwards, biotinylation was quenched by washing the cells twice with serum-free DMEM. Cells were returned to DMEM containing serum and incubated at 37°C for the indicated time points. At each time point, the media was aspirated from the cells and cell lysates were collected as described above. After collection and centrifugation, the BCA Protein Assay (Pierce) was used to determine the protein concentration of the samples. Then

100 µg of protein from each sample was incubated at 4°C with neutravidin beads (Thermo Scientific) overnight in order to pull down all biotinylated proteins. The bound protein was then eluted from the beads by incubating in 2x Laemmli sample buffer and separated on 10% acrylamide SDS-PAGE.

5.5 Membrane Biotinylation

Cells were co-transfected with pcDNA3.1 empty vector (EV) or dominant negative dynamin (DN) and one of the KL variants. After 24 hours, the cells were put on ice, washed twice with ice-cold DPBS and incubated with a 1 mM solution of cell impermeable Sulfo-NHS-SS-Biotin (Pierce) for 30 minutes at 4°C. After collection and centrifugation, the BCA Protein Assay (Pierce) was used to determine the protein concentration of the samples. Then 100 µg of protein from each sample was incubated at 4°C with neutravidin beads (Thermo Scientific) overnight in order to pull down all biotinylated proteins. The bound protein was then eluted from the beads by incubating in 2x Laemmli sample buffer and separated on 10% acrylamide SDS-PAGE.

5.6 SORLA Effects on KL Trafficking

If the V variant leads to increased endocytosis, it may be possible to rescue its decreased shedding by directing the endocytosed protein towards recycling endosomes instead of to the late endosomes where it is targeted for degradation by the lysosomes. To test this, HEK 293 cells were co-transfected with KLWT or

the variants and SORLA, a sorting receptor that regulates trafficking and processing of proteins sending them towards the recycling endosome. The media and lysate were collected as described previously for analyzing changes in shedding and processed for western blotting.

5.7 Immunofluorescence

Immunofluorescence was performed to elucidate and compare the subcellular location of the variants. HEK 293 cells were grown and transfected onto autoclaved, circular 18mm diameter, No.1 (0.13-0.16mm thick), cover glasses (Fisher) in 6-well plates with either KLWT-GFP or KL-GFP expressing the variants. Forty-eight hours after transfection, the cells were washed twice with DPBS and fixed with ice cold 100% ethanol for 10 min. After fixation, cells were again washed twice with 1x DPBS for 5min. Cells were blocked and permeabilized with blocking media (0.5% BSA, 0.1% Triton X-100 in 1x DPBS) for 10min except when cells were to be stained for plasma membrane. Plasma membrane samples were not permeabilized, and so were blocked with the antibody diluent, which is the blocking media without Triton X-100. After blocking, cells were incubated with primary antibody for one hour. Primary antibodies were diluted in antibody diluent at the following dilutions: Plasma membrane, wheat germ agglutinin (WGA), 1:200; endoplasmic reticulum (ER), calnexin, 1:200; Golgi, Giantin, 1:500; Lysosome, LAMP1, 1:500; and early endosome, EEA1, 1:500. The amount of antibody used was just enough to cover

the entire coverslips. All antibodies were purchased from Abcam. Following primary antibody incubation, cells were washed twice with 1x DPBS for 5min. Then, cells were incubated in the dark with the secondary antibody, 1:200 AlexaFluor 633, for 40min due to the light sensitivity of the antibody. After incubation with the secondary antibody, cells were again washed twice with 1x DPBS for 5min. Then, 300nM of the nuclear stain DAPI diluted in phosphate buffered saline (PBS) (Invitrogen) was added to the cells for 10 minutes, followed by two more washes with 1x DPBS for 5min. A few drops of mounting media (90% glycerol in 100mM Tris (pH 8.0)), or alternatively the Prolong Gold Anti-Fade Mounting Media (Invitrogen), were then placed onto Gold seal Rite-On Frosted Microslides (Thermo Scientific) with tweezers. A little bit of pressure was applied to the cover glasses to ensure that the mounting solution covers the whole area without bubbles, after which, they were sealed with nail polish. Samples were kept at 4°C until ready for viewing under a microscope.

For obtaining images, the Nikon deconvolution wide-field epifluorescence system and then Zeiss LSM710-Live Duo Scan were used, which are located at the Boston University Core Laboratories & Facilities Cellular Imaging Core. The excitation and emission wavelengths of the different fluorophores are as follows: GFP– 488/509nm (green), AlexaFluor 633 – 621/639 (red), and DAPI – 372/456 (blue). To excite the fluorophores, the lasers used include the 488nm FITC laser for KL-GFP green emission, 594nm Texas Red laser for the Alexa 633 sub-cellular localization markers emission, and 405nm UV laser for the nuclear DAPI

emission. ImageJ was used to make a composite image of all three emissions and overlap between green and red, indicating overlap in localization, became yellow.

6. KLOTHO FUNCTIONAL ASSAYS

6.1 Live Cell Chemical Crosslinking

Crosslinking was used to detect and identify protein-protein interactions between KL molecules. The crosslinker used was dithiobis[succinimidylpropionate] (DSP, Thermo Scientific). This is a thiol-cleavable and amine reactive agent. For the experiments outlined below, live cell crosslinking was carried out as previously published (450). Briefly, transiently transfected cells in 12 well plates were washed 2x with DPBS and then incubated with 100 μ M DSP for 30 minutes at room temperature. Afterwards, the crosslinking solution was removed and the reaction was quenched by incubating with 50mM Tris buffer (pH 7.4) for 15 minutes. After quenching, cells were briefly washed with DPBS and collected for western blotting as described above except for use of Laemmli buffer without β -mercaptoethanol to preserve crosslinked interactions.

6.2 Blue Native Gel Electrophoresis

Blue Native gel electrophoresis was performed to separate proteins without denaturation. This was done in order to determine whether the KL variants altered KL dimerization. The Invitrogen NativePAGE Novex 4-16% Bis-

Tris Gel system was used in accordance to the manufacturer's protocol for the outlined experiments. Briefly, HEK 293T cells transfected as described above were solubilized in NativePAGE sample buffer containing 1% digitonin and complete protease inhibitor without EDTA (Roche) at 4°C. After determining protein concentration, an equal amount of protein from each sample was prepared for separation by mixing with 5% Coomassie G-250 (Invitrogen) to reach a final concentration of 1% Coomassie G-250. Pre-chilled anode and cathode buffers (dark blue and light blue) were used to run the gel. The dark blue cathode buffer was used until the dye front had moved through 1/3 of the gel and then was exchanged for the light blue cathode buffer until the completion of the run. After separation, the proteins were transferred onto an Immobilon 0.4µM PVDF membrane (Millipore). To fix the proteins, the resulting PVDF membranes were incubated in 8% acetic acid and then the membranes were destained with methanol. In parallel, the same samples were run in reducing conditions on 10% acrylamide SDS-PAGE gels in order to compare total expression of the transfected proteins. Afterwards, both membranes were processed for western blotting as described above.

6.3 Co-immunoprecipitation

Co-immunoprecipitation studies were undertaken in order to determine whether the KLVS variant altered the protein interactions between KL and itself or other binding partners. To achieve this, HEK 293 cells were co-transfected

with combinations of KLWT and KLVS containing a V5 or GFP tag or with combinations of FGFR1c-V5 and EV, KLWT-GFP or KLVS-GFP. Twenty-four hours after transfection, cells were washed twice with ice-cold DPBS and collected in IP lysis buffer (1% Triton X-100, 0.01M Tris-HCl, 0.01M EDTA, 0.05M NaCl, 0.05M NaF, pH 7.2) containing protease inhibitors. After protein concentration was measured by BCA as described above, 100 µg of protein was loaded onto pre-washed V5 conjugated sepharose beads (Sigma, St. Louis MI) and incubated overnight at 4°C. An identical set of lysates containing equal protein were incubated with anti-GFP (Roche) for 1 hour at 4°C before adding pre-washed protein A/G beads (Pierce) and incubating overnight 4°C. After incubation, beads were washed and processed for SDS-PAGE as described above.

6.4 FGF23 Assay

To determine whether differences in KL-FGF interactions led to differences in downstream signaling, FGF23 assays were performed. HEK 293 cells were transfected with EV, KLWT or KLVS. Twenty-four hours after transfection, cells were incubated in serum free media for two hours and then either bFGF (basic fibroblast growth factor) or FGF23 (R&D Systems) was added to the wells to reach a concentration of 100ng/mL or 10ng/mL, respectively. The cells were incubated for 15 minutes at 37°C and then were immediately washed in DPBS and lysed in RIPA containing protease and phosphatase inhibitors (Roche). After lysis, samples were prepared for SDS-PAGE as described above.

6.5 Enzymatic Assay: β -Glucuronidase Activity

The glucuronidase assay was used to determine whether the VS variant led to changes in the enzymatic activity of KL. Cells stably overexpressing the 980 form of KLWT or KLVS or HEK 293 EV control cells were grown to confluency on 10 cm plates. Once confluency was reached, the media was aspirated, cells were washed with DPBS and serum free media was placed on the cells. Twenty-four hours later, the media was collected and concentrated by use of Amicon Ultra 15 centrifugal filters with a molecular weight cutoff of 50 kDa. The filters were pre-washed by centrifugation at 5000xg for 8 minutes with 10 mL of Millipore water. Afterwards, the collected media, which had already been spun to remove cell debris was added on to the filter and spun for 10 minutes at 5000xg. After centrifugation, the concentrated media was collected and the process was repeated with the same filter for media from a total of 3 10cm plates. This concentrated media was then analyzed by western blot to assure equal concentrations of KLWT and KLVS in their respective media.

After concentration was determined, an equal amount of KLWT or KLVS was incubated with varying concentrations of 4-methylumbelliferyl β -D-glucuronide ranging from 0.0125 to 3.2 mM. This compound is a substrate for the extracellular domain glucuronidase activity of KL whose hydrolysis results in a fluorescent product. Readings were taken at 30min time points from 0- 2.5 hours. Fluorescence was measured using a fluorometer at excitation wavelength of 360nm and emission wavelength of 470nm. Fluorescence readouts measured

in relative fluorescence units (RFU) were converted to μM concentration of 4-methylumbelliferone product by analyzing fluorescence readouts for a serial dilution of 4-methylumbelliferone product. The resulting information was plotted and the linear correlation between the RFU and μM product were determined so that the RFU values for the experimental conditions could be converted to μM 4-methylumbelliferone allowing for further analysis.

7. DATA ANALYSIS AND STATISTICS

Statistical analyses were performed with Graphpad Prism software version 5.0. Before further analysis, data sets were analyzed for significant outliers using the Grubbs Outlier Test. Data sets containing 3 or more groups were analyzed using 1-way ANOVA. Post-hoc Dunnet tests were performed when significance of $p < 0.05$ was achieved. Data sets containing only two groups were analyzed by student t-tests.

Half-life data was analyzed by using the extra sum-of-squares F test to compare lines of best fit generated from a one-phase decay model. Data was considered significant when a global comparison of Y_0 , K and plateau resulted in a rejection of the null hypothesis with a $p < 0.05$.

For analysis of glucuronidase data, first the concentration of 4-methylumbelliferone product in each sample was determined. To accomplish this, fluorescence readouts measured in RFU were converted to μM concentration of 4-methylumbelliferone product by analyzing fluorescence

readouts for a serial dilution of 4-methylumbelliferone product. The resulting information was plotted and the linear correlation between the RFU and μM product were determined so that the RFU values for the experimental conditions could be converted to μM 4-methylumbelliferone allowing for further analysis. After this conversion, the concentration of 4-methylumbelliferone product was plotted over reaction time for EV, WT and VS and the initial velocity (v_o) of each reaction was determined for every initial substrate concentration ($[S_o]$) by calculating the slope of the resulting graphs. $[S_o]$ and v_o were then plotted to obtain the Lineweaver-Burke plot for the samples. From this plot, the Michaelis-Menten constant (K_m) and maximum velocity (v_{max}) were calculated using Graphpad Prism software.

CHAPTER III: RESULTS

1. AIM 1: CHANGES IN KL PROCESSING DUE TO KLVS VARIANT

1.1 Klotho Variant Constructs

Constructs containing point mutations representing the substitutions present in the KLVS variant were used to further study how these two amino acid substitutions might affect the trafficking and function of KL. To this end, constructs were generated that expressed the V substitution (KLV) and S substitution (KLS) each alone, as well as one that expressed both V and S substitutions (KLVS). Constructs were also made that represent the extracellular domain of KL referred to as KL980 containing the same substitutions (V, S and VS) (Figure 9A). This recombinant engineering allowed us to further investigate how the absence of the transmembrane and intracellular domains of KL may affect its function in a manner specific to these amino acid substitutions. Furthermore, constructs of KLV, KLS and KLVS were made with GFP fused at the C-terminus of the protein in order to allow for easy visualization of the protein with microscopy (Figure 9B). This approach allowed for a detailed investigation of the subcellular location of KL and how it might be affected by the presence of substitutions. Constructs were also made containing a V5 tag at the KL C-terminus (Figure 9C). This tag allowed for the isolation and detection of KL without a specific antibody and aided in co-immunoprecipitation, as well as depletion of KL from conditioned media. These constructs helped us to identify alterations in the trafficking and function of KL associated with the VS variant.

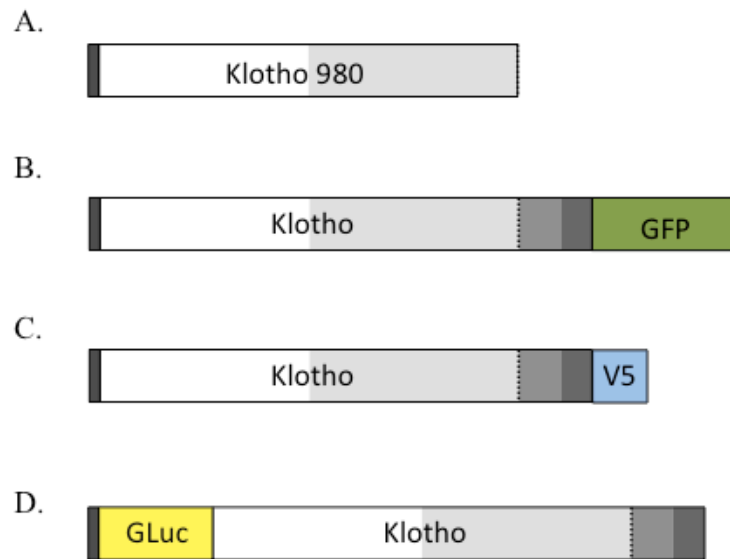


Figure 9. Diagram of KL Constructs. Diagram of the KL constructs prepared outlining location of tags and presence of truncations. (A) Constructs were made to represent the extracellular domain of KL lacking the intracellular C-terminus and membrane-spanning fragment. The resulting construct consists of 980 amino acids leading to it being referred to as KL980. (B) Constructs of KL were made containing a C-terminal GFP tag (KLGFP) to allow for easy visualization of the protein via microscopy. (C) Constructs containing a C-terminal V5 tag were prepared to allow for isolation and detection of KL without a specific antibody to the KL protein. D) A construct was prepared containing an N-terminal Gaussia Luciferase (GLuc) tag located after the signal sequence (depicted as dark grey portion on N-terminus) in order to visualize the KL shed into the media.

1.2 Expression of KLV Variant Leads to Decreased Shedding

In previous studies, insertion of the VS polymorphism in a construct consisting solely of the KL1 portion of the extracellular domain revealed differential secretion (442) when compared to wildtype. However, the effect of the two amino acid substitutions on the shedding of full-length KL was not investigated. Since the full length 135kDa transmembrane protein is detected in cells and the 130kDa shed protein including both the KL1 and KL2 domains is detected in both blood and CSF, we reasoned that these protein fragments are likely to be the most physiologically relevant to investigate (230).

To assess whether there are differences in the shedding of the transmembrane form, dependent upon the variant, HEK 293 cells were transfected with transmembrane KLWT, KLV, KLS or KLVS. Shedding was measured by collecting conditioned serum free media and lysates from the transfected cells over a 24-hour period. To correct for differences in KL construct expression, shedding was expressed as a ratio of extracellular (media) to intracellular (lysate) KL levels. Compared to KLWT, the KLV variant displayed a 46% decrease in the amount that was shed into the extracellular space (n=5, $p < 0.05$) (Figure 10), similar to the difference reported previously in the literature (442). Conversely, neither the KLS nor KLVS variant showed a significant difference in shedding from wild type. The absence of a VS specific phenotype suggests that there is intragenic complementation in cis between the two

substitutions. Expression of the KLS substitution can correct for the reduced shedding phenotype of KLV leading to a KLVS that is shed normally.

1.3 KL Transmembrane Domain Necessary for Decrease in KLV Shedding

To determine if transmembrane trafficking of KL through the trans golgi network is necessary for the shedding changes seen in the KL variants, we generated additional constructs of KL variants that lacked the intracellular and transmembrane domains. These constructs, consisting of the N-terminal 980 amino acids (KL980), resemble the extracellular portion that is usually shed from the membrane. To determine whether there are still differences in secretion, KLWT 980 or KL980 containing one or both of the variants were transfected into HEK 293 cells. Twenty-four hours after transfection, the media was changed to DMEM without serum and the resulting media and cell lysates were collected after 24 hours. The samples were analyzed for amounts of KL in lysate and media to obtain levels of intracellular and extracellular KL respectively. The extracellular: intracellular ratio was calculated as a means to determine differences in secretion. Unlike the full-length transmembrane KL, there is no difference in the secretion of KLV 980 compared to KLWT 980 (n=5) (Figure 11). This suggests its attachment to the membrane is necessary for the difference in shedding seen in the full-length protein. The lack of transmembrane domain does not significantly alter the secretion of any other variants, suggesting

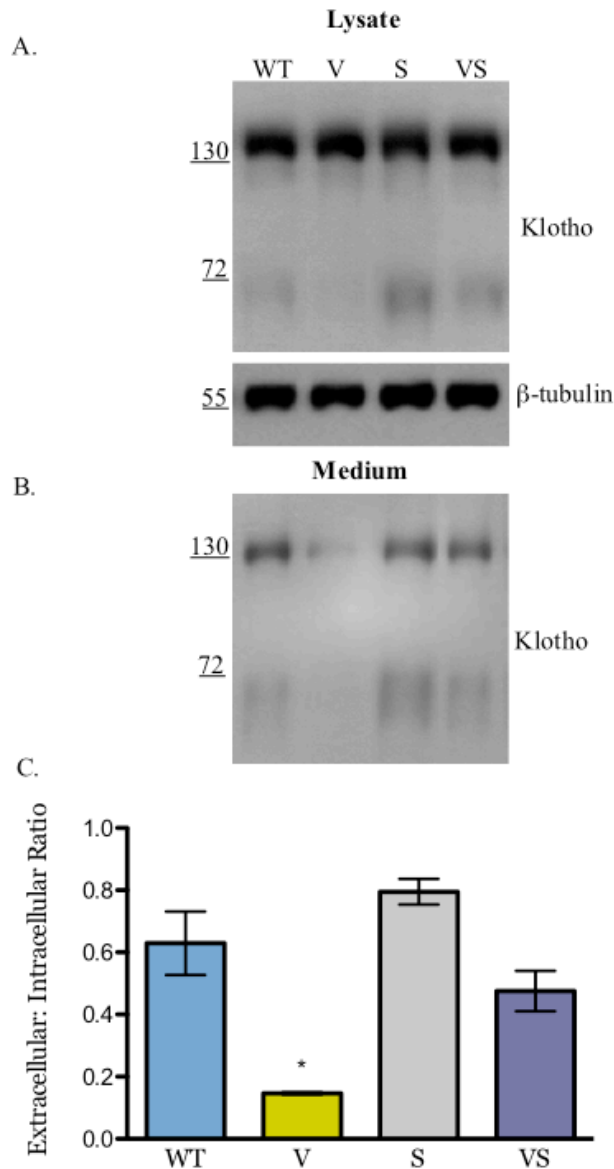


Figure 10. KLV Dramatically Decreases Shedding. Shedding was examined by transfecting HEK 293 cells with wildtype (WT) KL or KL containing one or both of the amino acid substitutions in KLVS (V, S or VS). Shedding differences were measured by western blot as change in the extracellular:intracellular KL protein ratio. (A) Representative blot showing KL expression and β -tubulin loading control in the lysate (intracellular), and KL levels in the (B) media. (C) Quantification of band intensity was measured across multiple independent experiments. Graph represents the mean fold change of extracellular:intracellular KL (error bars are \pm SEM; n=5; *p<0.05, ANOVA).

that the effect on the KLV is specific to the phenotype of this variant alone.

It is known that absence or presence of a transmembrane domain can lead to alterations in glycosylation of proteins, which can alter their trafficking and function (451). Coupled with the results above, our data suggests that the phenotype associated with the KLV variant requires transmembrane-dependent processing of KL. It is also possible that lack of membrane anchoring changes the binding partners of KLV that usually lead to increased KLV internalization. For example, KL's complex with TRPC1 and VEGFR2 leads to internalization of all the components including KL (318). If the KLV has increased affinity for this complex, it could explain the increase in internalization. Together, these results show that loss of KL's transmembrane localization attenuates the shedding phenotype seen with the KLV, but does not alter other variants showing that it is not a non-specific effect.

1.4 KLV Shedding Decrease is not Due to Misfolding

1.4.1 MT-1 Increases KL Shedding Irrespective of Variant Expression

MT-1 is a small molecule that acts as a chemical chaperone to help correct protein misfolding and restore normal trafficking. If the KLV variant leads to misfolded protein, treatment with MT-1 should restore normal trafficking of the protein and lead to a correction of the altered shedding seen with this variant. To test this, HEK 293 cells transfected with KLWT or KLV were treated with 5 μ M MT-1 for 48 hours and then assessed for differences in shedding. While MT-1 did

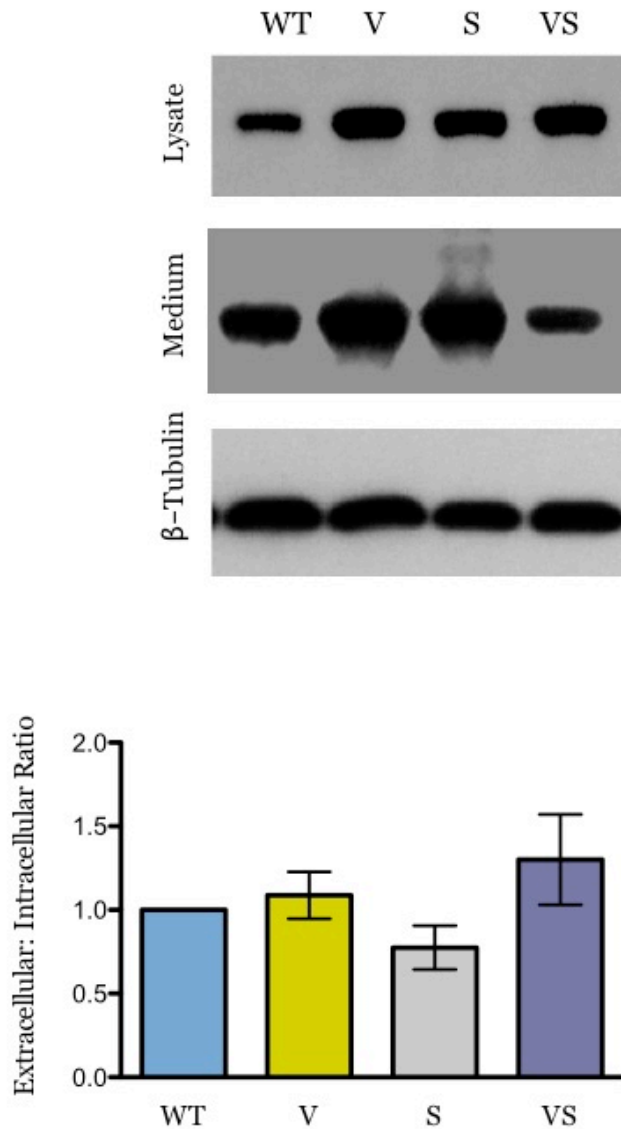


Figure 11. Truncation of KL Attenuates Shedding Differences.

Shedding was evaluated by transfecting HEK 293 cells with the truncated extracellular KLWT980, or KL980 containing one or both of the amino acid substitutions in KLVS (V, S or VS). Secretion differences were measured by western blot as a change in the extracellular: intracellular KL protein ratio. (A) Representative blot showing KL980 expression and β-tubulin loading control in the lysate (intracellular), and KL980 levels in the (B) media. (C) Quantification of band intensity was measured across multiple independent experiments. Graph represents the mean fold change of extracellular:intracellular KL (error bars are ± SEM; n=3).

significantly increase KLV shedding, it also increased KLWT shedding by a similar magnitude ($p < 0.05$, $n = 3$) (Figure 12). The results suggest that MT-1 is affecting the shedding of KL by a secondary mechanism unrelated to the amino acid change caused by the KLV substitution. This could be due to a stabilization of KL folding unrelated to the KLV variant or stabilization of another protein that is involved in KL trafficking.

1.4.2 Glycerol Treatment Can Not Rescue KLV Shedding Differences

Previous studies have shown that glycerol treatment is capable of reversing the misfolding phenotype of mutated protein (452), therefore treatment with glycerol should allow normal trafficking of KLV if the protein is misfolded. To determine whether glycerol could correct the abnormally trafficked KLV, HEK 293 cells transfected with either KLWT or KLV were treated with media containing 10% glycerol for 24 hours. Afterwards, alterations in shedding were assessed by calculating the extracellular: intracellular ratio. These calculations revealed that there was no effect of glycerol on the aberrant shedding of KLV ($n = 3$) (Figure 13). However, glycerol treatment did have an effect on overall KL expression. It led to a decrease in the amount of KL present in the lysate and media of both KLWT and KLV ($n = 3$, $p < 0.05$, 2-way ANOVA). Decreased KL expression suggests there may be cytotoxic effects of this dose of glycerol on HEK 293 cells that alter the results or that glycerol acts on another protein that alters KL processing.

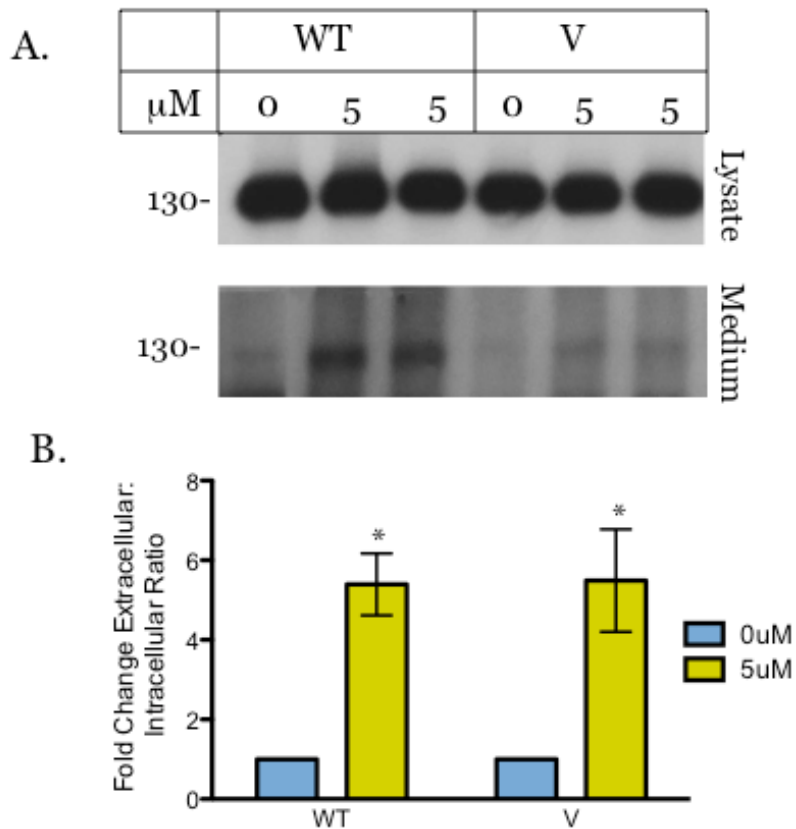


Figure 12. MT-1 Increases KL Shedding Irrespective of Variant Expression. Shedding was evaluated after treatment of HEK293 cells transfected with KLWT or KL containing the V substitution with 5 μM MT-1 or control media for 48 hours. Shedding was evaluated by western blot as a change in the extracellular: intracellular KL protein ratio. (A) Representative blot showing KL expression in the lysate (intracellular) and media (extracellular) in the presence of 0 or 5 μM MT-1. (B) Quantification of band intensity was measured across multiple independent experiments. Graph represents the mean fold change of the extracellular:intracellular KL ratio (error bars are \pm SEM; n=3, *p<0.05, ANOVA) Treatment of MT-1 led to an increase in KL shedding of KLWT and KLV irrespective of presence of the variant.

1.5 Screening for Modulators of KL Shedding

1.5.1 Designing Screen to Discover Modulators of KL Shedding

KL shedding into the extracellular space is necessary for many of the functions of KL. Therefore it is important to maintain normal trafficking and shedding of the protein. It is possible that mediators of KL shedding could also correct the detrimental phenotype of the KLV variant. To this end, we created a stable cell line overexpressing a KL molecule with a N-terminal Gaussia Luciferase tag following the signal sequence (GLucKL) (Figure 9D). This construct allowed for visualization of the amount of KL shed into the media. In the end, two stable lines were tested for expression of the construct by assessing luciferase activity in the lysate and media of the cells (n=3) (Figure 14). Both cell lines expressed KL in the lysate and in the media suggesting they were good candidates for use in screening of compounds.

Since insulin is a known mediator of KL shedding (231), it was used as a positive control to assess the validity of the GLucKL assay. To do this, GLucKL stable cells were treated with 0.2 $\mu\text{g}/\text{mL}$ insulin in serum free media for 0, .5, 1, 2, 4 or 6 hours or with serum free media without insulin for the same time. After treatment, both media and lysate were collected and tested for luciferase activity. During this time period, there was a linear relationship between the amount of GLucKL in the media and time cells were incubated with insulin (n=3) (Figure 15). Although this relationship is expected to plateau at some time point, within this range there is still a positive linear correlation. This could be due to a

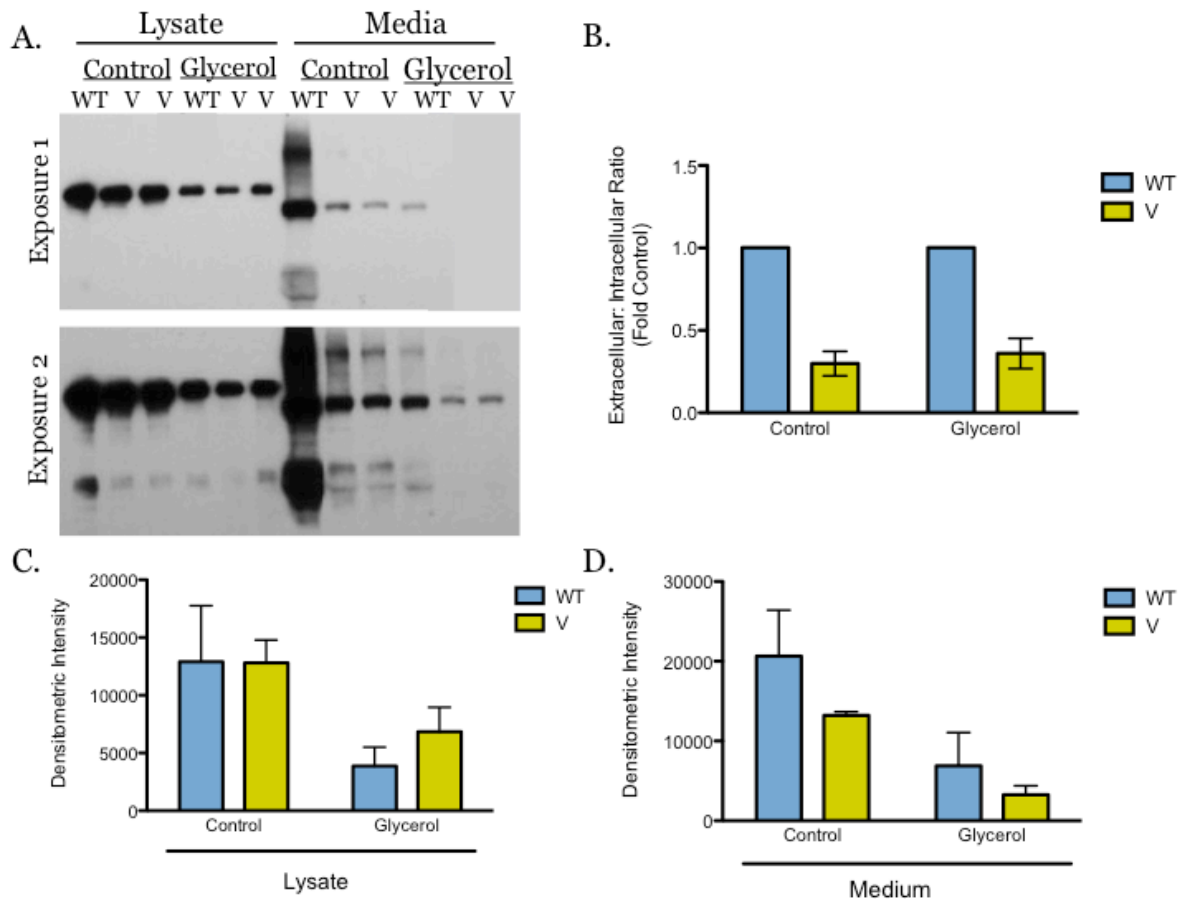


Figure 13. Glycerol Treatment Can Not Rescue KLV Shedding Differences. Shedding was evaluated after treatment of HEK293 cells transfected with KLWT or KL containing the V substitution with 10% glycerol or control media for 24 hours. Shedding was evaluated by western blot as a change in the extracellular: intracellular KL protein ratio. (A) Representative blot showing KL expression in the lysate (intracellular) and media (extracellular) in the presence of 0 or 10% glycerol. (B) Quantification of band intensity was measured across multiple independent experiments. Graph represents the mean fold change of the extracellular:intracellular KL ratio (error bars are \pm SEM; n=3). (C) Graph represents change in densitometric intensity of KL in the lysate (error bars are \pm SEM; n=3). (D) Graph represents change in densitometric intensity of KL in the media (error bars are \pm SEM; n=3). Glycerol treatment did not correct for KLV alterations in shedding, but did lead to a decrease in the amount of KL expression of both KLWT and KLV ($p < 0.05$, 2-way ANOVA).

longer half-life of GLucKL in the media versus half-life of intracellular KL resulting in a buildup of GLucKL over time. Despite lack of plateau, we were able to see increase in KL shedding with treatment of insulin.

1.5.2 IGF-2 Increases KL Shedding

The validated assay was used to determine whether other IGFs could also have an effect on KL shedding. In particular, we examined the ability of IGF2 to increase the shedding of KL. To this end, cells stably expressing GLucKL were treated with 0.1 µg insulin or IGF-2 or with serum free media as a negative control. All treatments were added to GLucKL cells for 15 minutes or 6 hours (Figure 16). Changes in shedding due to insulin could be seen starting at 15 minutes and were amplified at 6 hours as there was a 100% increase in the shedding compared to control between these times (n=3, p<0.05). The IGF-2 increase in shedding was not significant at 15 minutes despite the average percent change being greater than that seen by insulin treatment (194.48% increase with insulin versus 240.05% increase with IGF2), although there was a trend towards significance (n=3, p=0.06). At 6 hours, however, there was a significant increase in shedding compared to control and the magnitude of the change was larger than that seen by insulin treatment (n=3, p<0.05).

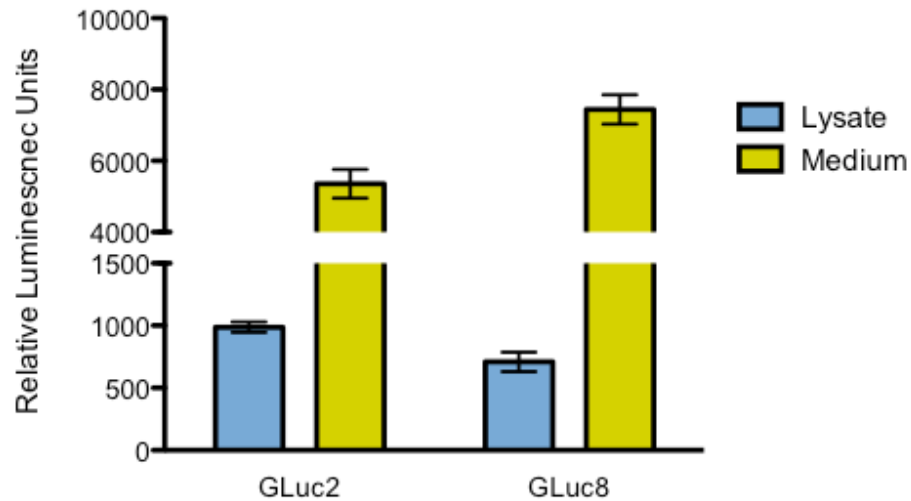


Figure 14. Designing Screen to Discover Modulators of KL Shedding. Shedding of HEK 293 cell lines stably transfected with gaussia luciferase KL (GLucKL) were evaluated by measuring relative luciferase activity in the media and lysate. Bar graph depicts quantification of relative luciferase units measured over independent experiments for expression of the construct (error bars are \pm SEM; n=3). Two cell lines GLucKL 2 and GLucKL8 were chosen for their high expression of GLucKL constructs as evaluated by luciferase activity.

These results suggest that other growth factors are also capable of regulating KL shedding. Compounds found by this screening method could be tested for their ability to increase the shedding of KLV leading to a more normally trafficked protein. This could potentially elucidate the mechanisms of decreased KLV shedding as well as give insight into therapeutic targets that could increase serum levels of KL.

1.6 KLV Expression Results in Decreased Half-life

Altered secretion could be the result of changes in KL processing. To begin investigation of this possibility we assessed variant half-life using biotinylation to measure changes in protein degradation (449). The proteins of HEK 293 cells transfected with KLWT or the variants were biotinylated for 30 minutes. After quenching the reaction cells were allowed to incubate at 37°C before lysates were collected at time points from 30 minutes to 6 hours (Figure 17). Biotinylated proteins were isolated using neutravidin beads and changes in biotinylated KL were measured by western blotting to assess the amount of KL remaining as compared to the amount seen at time zero. There was a decrease in the half-life of the KLV variant ($t^{1/2}=0.6554$, $r^2 = .8245$), compared to KLWT ($t^{1/2}=1.325$, $r^2 = .7065$, $p<0.05$), but no differences in KLS ($t^{1/2}=2.036$, $r^2 = 0.6021$) or KLVS ($t^{1/2}=2.572$, $r^2 = 0.6862$) found by comparing non-linear regression models for one-phase decay through an extra sum-of-squares F test

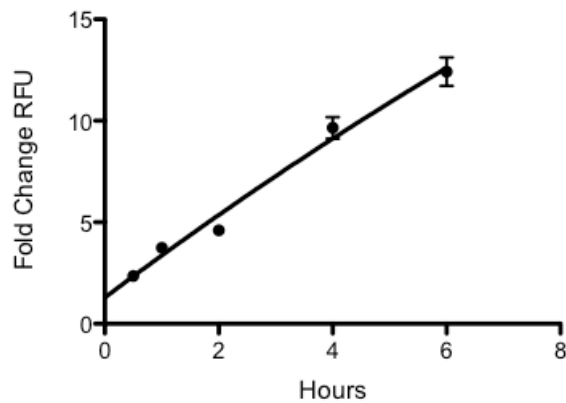


Figure 15. Validating GLucKL as Screening Platform. To evaluate the validity of the GLucKL cell lines as a screening platform for discovering modulators of KL shedding, HEK 293 cell lines stably transfected with GLucKL were treated with 0.2 $\mu\text{g}/\text{mL}$ insulin in serum free media for 0, .5, 1, 2, 4 or 6 hours or with serum free media without insulin for the same time. At each time point, media and lysate were collected and luciferase activity was measured. Graph represents quantitation of relative luciferase units at each time point measured over independent experiments (error bars are \pm SEM; n=3). Line represents line of best fit generated by GraphPad Prism Software.

(Figure 17). These observations coincide with our previous results, further implicating the role of intragenic complementation: while the KLV variant leads to a decrease in half-life, the expression of the KLS variant attenuates the phenotype when expressed together in the KLVS variant.

1.7 Membrane Localization of KL Variants

1.7.1 KL Isoforms Result in Altered Membrane Localization

To determine whether KL trafficking to and from the membrane was affected in the KL variants, we compared the amount of KLWT and KL variant on the membrane with and without endocytosis blocked by the presence of DN. To assess changes in plasma membrane localization, cells co-transfected with either KLWT or variants and pCDNA 3.1 EV or DN were biotinylated at 4°C for 30 minutes with a cell impermeable biotin. The lower temperature slows internalization to ensure that membrane-bound proteins are biotinylated but not internalized. After collection of lysates, biotinylated proteins were isolated and KL levels were measured by western blotting. The amount of KL on the membrane was normalized to total KL expression to correct for differences in construct expression. When co-transfected with EV, the KLV variant shows a decrease in the amount of KL on the membrane compared to KLWT (n=4, $p < 0.05$) (Figure 18A and B).

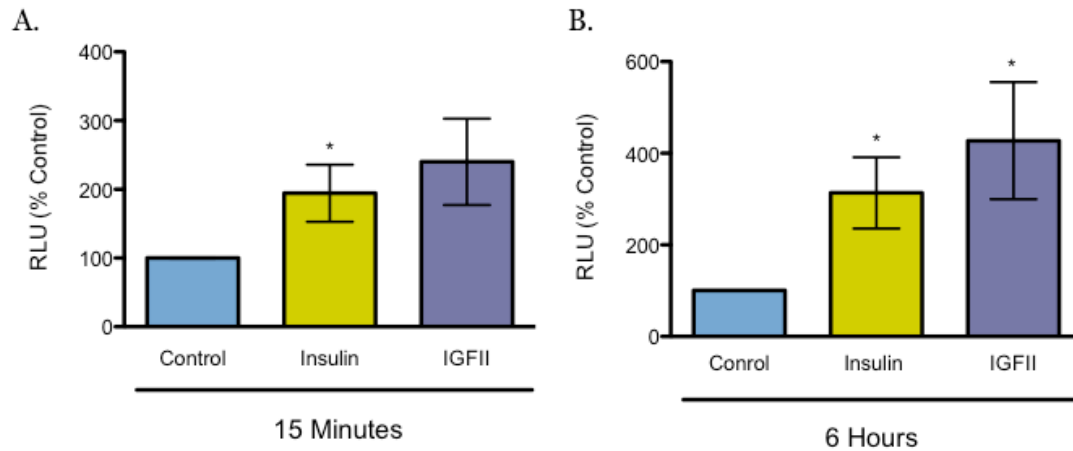


Figure 16. IGF-2 Increases KL Shedding. HEK 293 cells stably expressing GLucKL were treated with 0.1 μ g insulin or IGF-2 or with serum free media as a negative control. All treatments were added to GLucKL cells for 15 minutes or 6 hours after which lysate and media was collected and analyzed for luciferase activity. Differences in shedding were determined by analyzing the extracellular: intracellular ratio of each treatment group and comparing it to the untreated control. (A) Bar graph depicting changes in shedding after 15 minutes of insulin or IGF-2 treatment as compared to control serum free treatment (n=3, *p<0.05, ANOVA). (B) Bar graph showing differences in shedding after 6 hours of insulin or IGF-2 treatment as compared to serum free (error bars are \pm SEM; n=3, *p<0.05, ANOVA). Both insulin and IGF-2 led to increases in KL shedding, although the increase in IGF-2, despite being a larger fold change, was not significant until 6 hours of treatment (p<0.05).

Conversely, KLS shows an increase in the amount on the membrane (n=5, p<0.05) (Figure 10A and B). This difference in membrane localization suggests that trafficking to or from the membrane could explain the altered shedding and half-life of the variants.

1.7.2 KL Variants Alteration of Membrane Localization Attenuated by Inhibition of Endocytosis

Co-transfection with DN allowed us to test whether the changes in membrane localization were due to alterations in trafficking to the membrane without having to account for differences in internalization of the variants. In contrast to the pattern seen when the KL variants are co-transfected with EV, in the presence of DN, there are no significant differences in membrane localization (n=5) (Figure 18C). These findings suggest that changes in secretion and half-life are the result of altered endocytosis and that these changes are normalized when internalization is blocked.

1.8 SORLA Does Not Alter KLVS Processing

If the KLV variant leads to increased endocytosis, it may be possible to rescue its decreased shedding by guiding the endocytosed protein towards recycling endosomes instead of to the late endosomes where it is targeted for degradation by the lysosomes. To test this, HEK 293 cells were co-transfected

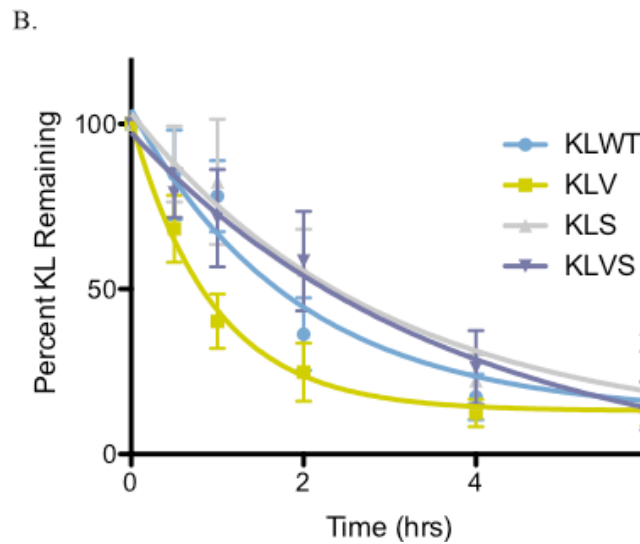
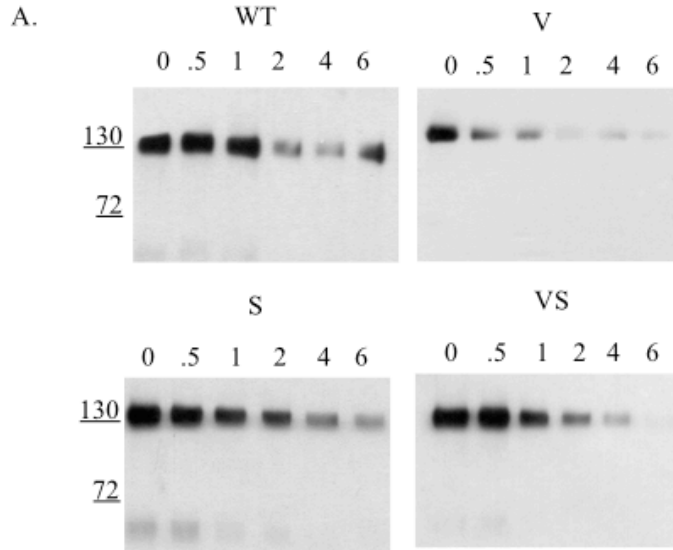


Figure 17. KLV Decreases Protein Half-life. HEK 293T cells transfected with wild-type (WT) KL or KL containing one or two of the amino acid substitutions in KLVS (V, S or VS) were tested for changes in half-life of KL. Cells were biotinylated and half-life was assessed as amount of KL left 0.5, 1, 2, 4 or 6 hours after biotinylation was quenched and cells were placed back at 37°C. (A) Representative western blot of lysates pulled down with neutravidin beads after biotinylation and probed for presence of KL protein. (B) Graph depicts half-life of KL variants represented by percent KL remaining over time (hrs) (error bars are \pm SEM; n=5). Half-life values of variants measured by non-linear regression model of one-phase decay were as follows: KLWT- 1.33 hrs, KLV-0.66 hrs, KLS-2.03 hrs and KLVS-2.53 hrs. The non-linear regression of KLV was found to significantly differ from KLWT by using the extra sum-of-squares F test (n=5, $p < 0.05$).

with KLWT or the variants and SORLA, a sorting receptor that regulates trafficking and processing of proteins sending them towards the recycling endosomes. After transfection, media and lysate were collected to analyze differences in shedding caused by the presence of SORLA. By measuring the fold change in the extracellular: intracellular ratio it was determined that there is no significant change in shedding due to SORLA expression (n=3) (Figure 19). This result suggests that SORLA does not affect KL trafficking, although it does not rule out the possibility that there is altered endocytosis in the KLV variant or that another sorting molecule is involved.

1.9 Altered Subcellular Localization of KL Variants in Endosomes

Immunofluorescence experiments were employed to further elucidate any changes in subcellular localization caused by the KLVS variant. To determine this, HEK 293 cells were transfected with either KLWT-GFP or KL-GFP expressing the variants and were stained for markers of varying subcellular compartments including wheat germ agglutinin (WGA) or cadherin (plasma membrane), GM130 (golgi), calnexin (endoplasmic reticulum), M6P (late endosome) and LAMP1 (lysosome). All of the variants had a similar distribution mainly localized to the ER, golgi and late endosomes (Figure 20). Although there is not a striking difference in the localization of the variants, there is a difference in the intensity of staining in the late endosome. While there is increased

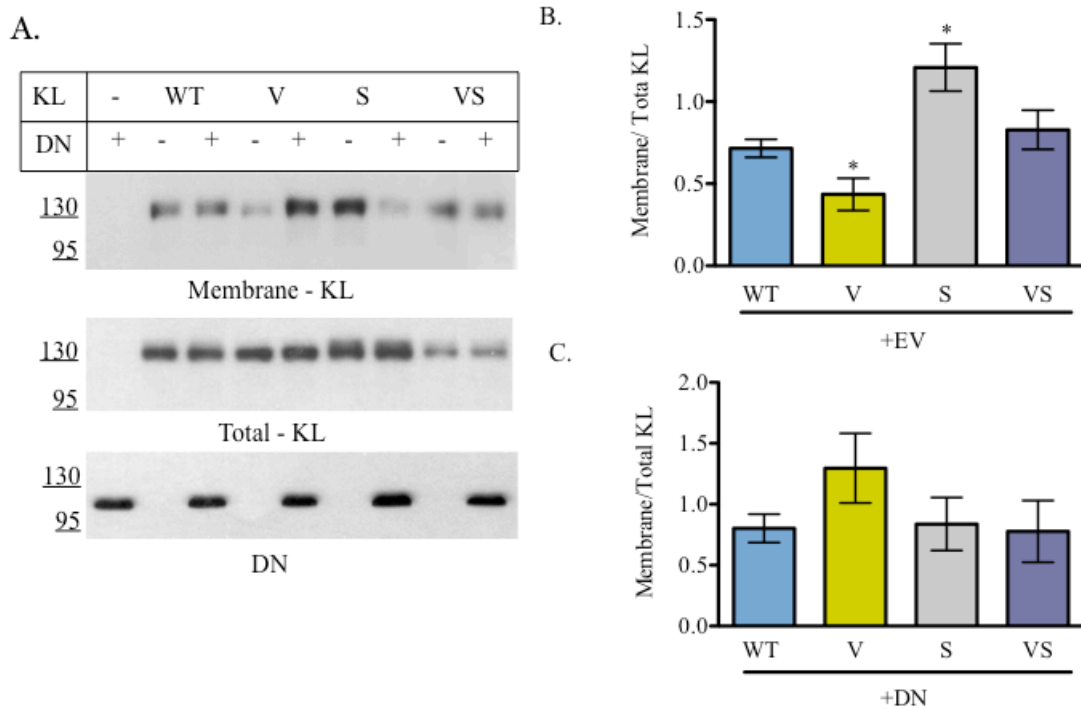


Figure 18. Blocking internalization differentially affects KL V and S variant membrane localization. HEK 293T cells transfected with wild-type (WT) KL or KL containing one or two of the amino acid substitutions in KLVS (V, S or VS) in the presence of an empty vector (EV) or dominant negative dynamin (DN) construct were assessed for differences in membrane localization of KL. Membrane abundance was measured by membrane biotinylation and subsequent pull-down of biotinylated proteins with neutravidin beads. (A) Representative western blot illustrating differences in membrane abundance of the KL variants in the presence of EV or DN compared to expression in total lysate as well as confirming expression of the DN construct. (B) Bar graph showing membrane abundance of KL co-transfected with EV normalized to total KL in cell lysate across multiple experiments (error bars are \pm SEM; $n=4$, $*p<0.05$, ANOVA). (C) Bar graph depicting membrane localization of KL in the presence of DN normalized to amount of total KL in the cell lysate across multiple experiments (error bars are \pm SEM; $n=4$, ANOVA). Changes in membrane localization due to KLV or KLS expression are normalized when dynamin-dependent endocytosis is blocked.

localization of the KLS variant in the late endosome as indicated by the increased yellow staining, the KLV variant has a decreased localization. This could be an indication of altered processing of the protein after endocytosis into the cell. It is known that KL is present in exosomes found in the urine (453) so this increased localization of the KLS variant to the late endosomes could be a sign of an increased amount of KL being packaged in the microvesicles that are formed in the late endosomes. Increased presence of KL in the exosomes would lead to increased amount of KL in the media because these microvesicles are secreted into the media. This could explain the increase in S seen in the media. Although it might be expected that there would be increased KLV in the late endosomes due to its increased endocytosis, its diminished presence could be explained by increased rates of trafficking to lysosomes where the protein would be degraded and not visible.

2. AIM 2: KLVS VARIANT LEADS TO ALTERED KL FUNCTION

2.1 KLVS Variant Alters KL Dimerization

2.1.1 Alteration in Dimerization Assessed by Crosslinking

Dimerization can affect trafficking of proteins (454,455) and can be important in internalization of transmembrane proteins (456,457). Since KL forms dimers (230,231) and dimerization is important for the activity of many β -glycosidases, the effect of the KL variants on dimerization was investigated by two different assays. First, crosslinking was performed with a cell-permeable and

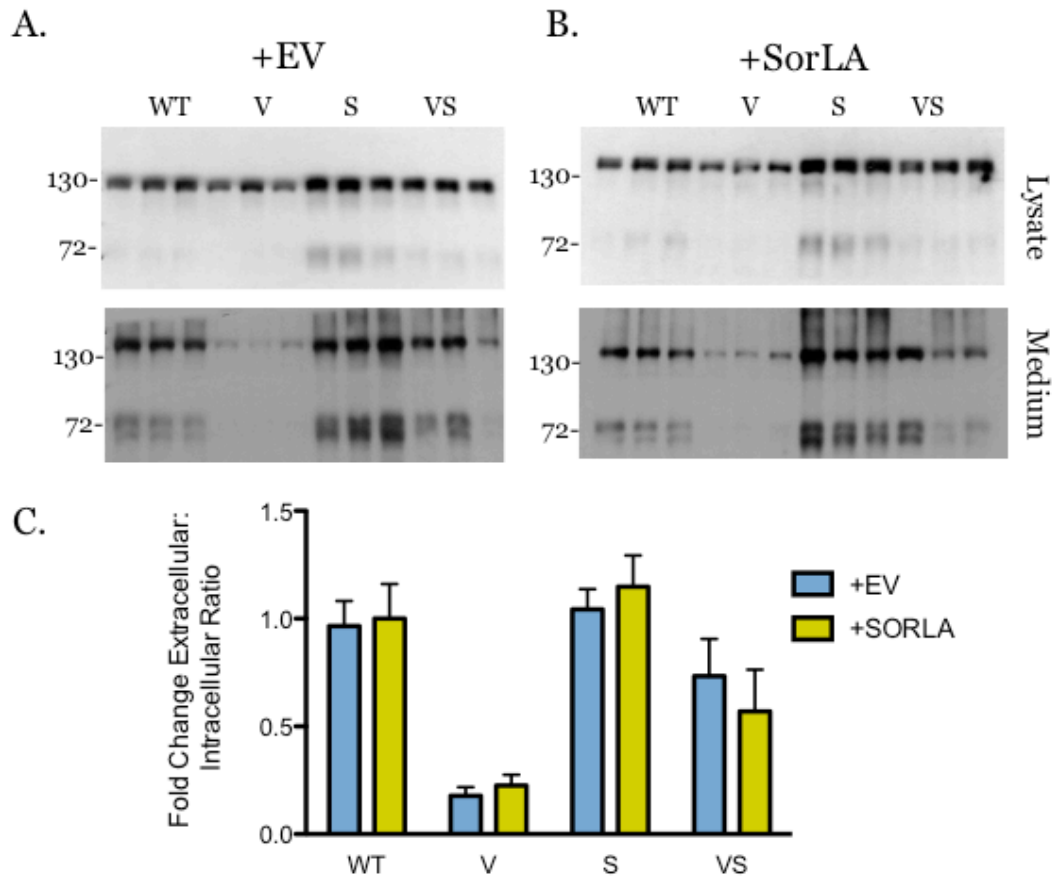


Figure 19. SORLA Does Not Affect KLVS Processing. Shedding was examined by co-transfecting HEK 293 cells with wildtype (WT) KL or KL containing one or both of the amino acid substitutions in KLVS (V, S or VS) and SORLA. Shedding differences were measured by western blot as change in the extracellular:intracellular KL protein ratio. (A) Representative blot showing KL expression in the lysate and media of cells co-transfected with KL and EV. (B) Representative blot showing KL expression in the lysate and media of cells co-transfected with KL and SORLA. (C) Quantification of band intensity was measured across multiple independent experiments. Graph represents the mean fold change of extracellular:intracellular KL in the presence or absence of SORLA (error bars are \pm SEM; n=3).

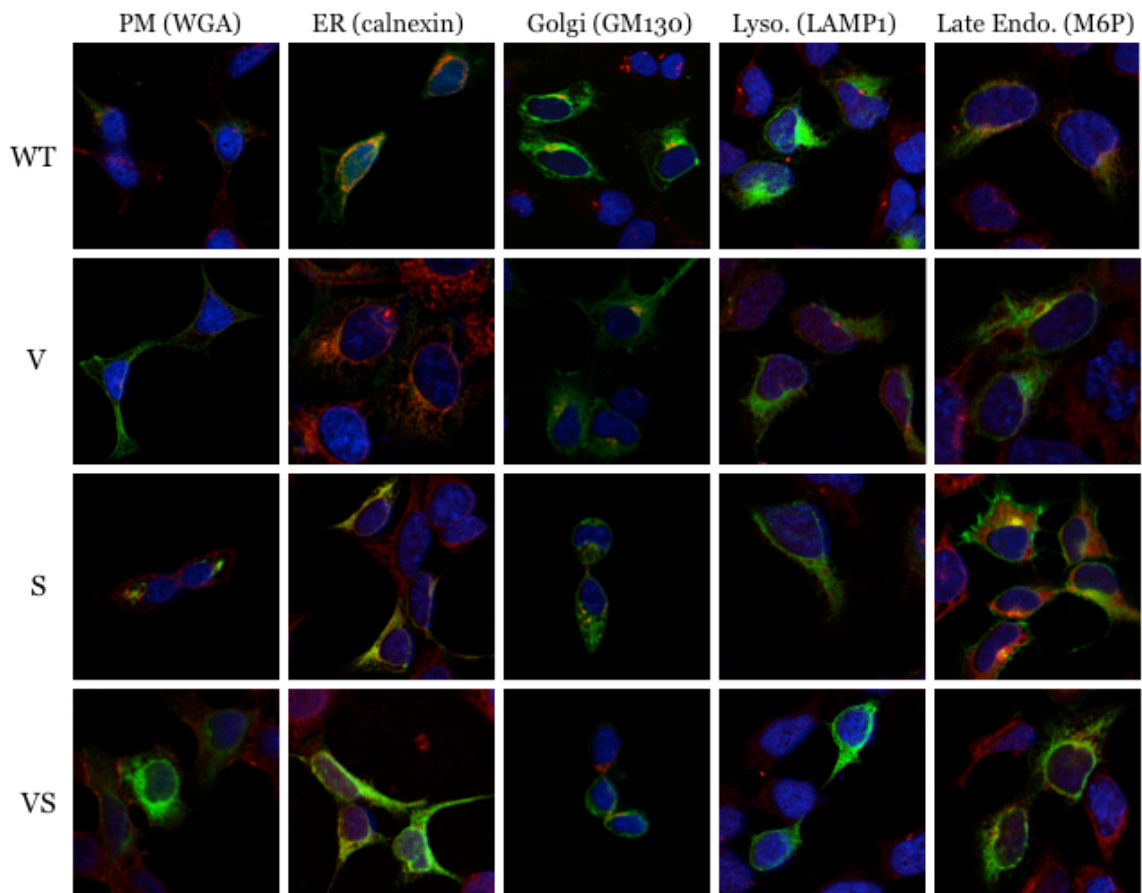


Figure 20. Altered Subcellular Localization of KL Variants in Endosomes. Subcellular localization of KL was determined by transfecting HEK 293 cells with either KLWT-GFP or KL-GFP containing one or both of the amino acid substitutions in KLVS (V, S or VS) after which they were stained for one of the following subcellular compartment markers: wheat germ agglutinin (WGA) for plasma membrane (PM), calnexin for endoplasmic reticulum (ER), gm130 for golgi, LAMP1 for lysosomes (Lyso.) and M6P for late endosomes (Late Endo.). Subcellular compartment markers were visualized with AlexaFluor 633 (red) and the cells were counterstained for the nuclear marker DAPI (blue). KL was visualized using the attached GFP tag (green). Image J was used to make a composite of all three images and any overlap between green and red, indicating co-localization appeared yellow. Overall, there were not many changes in co-localization compared with KLWT with the exception of the late endosomes, where KLS was localized more and KLV localization was decreased.

thiol-cleavable reagent on cells transfected with either KLWT or the variants. Differences in amounts of monomers and dimers were expressed as a ratio of dimers or monomers to total KL protein expressed (Figure 21). Although not all trends were significant, there was a pattern of increased monomers and decreased dimers for both KLS and KLVS ($n=3$, $p<0.05$). In the case of crosslinking, there was no significant change in the KLV variant. Since crosslinking can sometimes create false interactions between proteins, Blue Native-PAGE was also performed to confirm the results found with this method.

2.1.2 Differences in Dimerization as Shown by Blue Native PAGE

Blue Native PAGE allows for a more natural look at alterations in dimerization. For this protocol, cells were transfected with KLWT or the variants and proteins were separated on Blue Native PAGE gels or in denaturing conditions by SDS-PAGE. Running the same samples in denaturing conditions allowed a comparison between the amount of KL found in dimeric and monomeric forms to the total amount of KL in cells, even though constructs may not be expressed equally. While the amount of monomeric KLV was decreased compared to KLWT, the amount of dimerization was increased with KLV ($n=3$, $p<0.05$). On the other hand, expression of both KLS ($n=3$, $p=0.06$) and KLVS ($n=3$, $p<0.05$) showed a decrease in the dimeric form and KLVS, a significant increase in the monomeric form ($n=3$, $p<0.05$) (Figure 22).

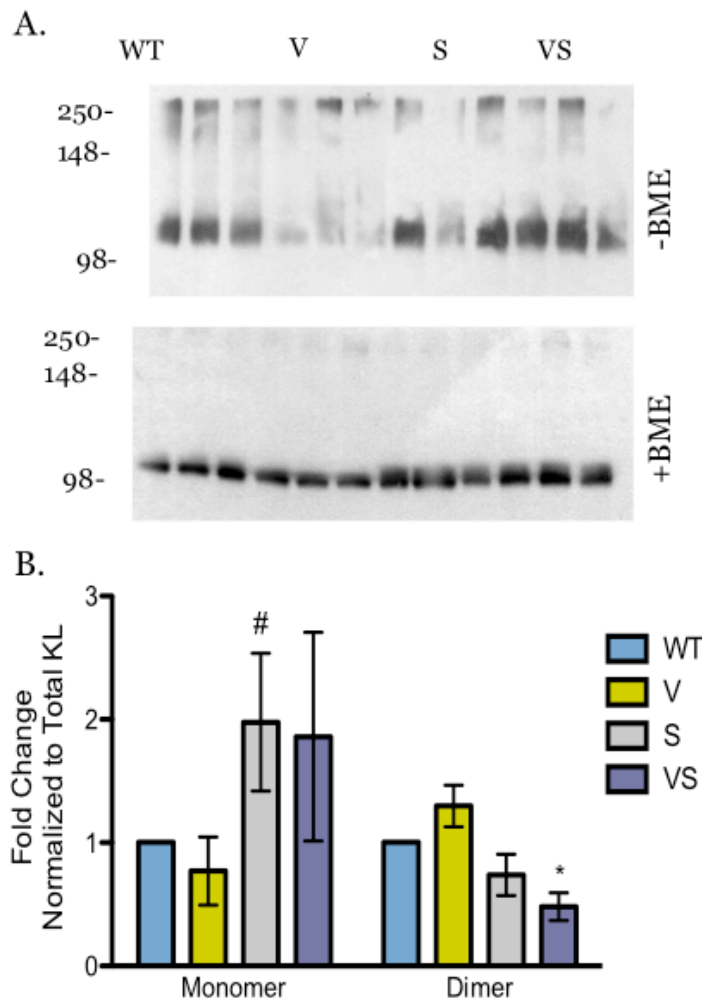


Figure 21. Crosslinking Reveals Differences in KLVS Dimerization. To assess differences in KL dimerization, HEK 293 cells transfected with either KLWT or KL containing one or both amino acid substitutions in KLVS (V, S, or VS) was cross-linked using dithiobis[succinimidyl]propionate. Cross-linked samples were analyzed for differences in dimerization by analyzing the dimer/total KL protein ratio (A) Representative western blots showing expression of KL monomers and dimers in the absence of β -mercaptoethanol (BME) and total KL expression in the presence of BME. (B) Quantification of band intensity was measured across multiple experiments. Bar graph shows fold changes in monomer and dimer expression normalized to total KL expression (error bars are \pm SEM; $n=3$; $\#p=0.06$, $*p<0.05$, ANOVA). Data shows a decrease in KLV monomer and an increase in KLVS monomer as well as a decrease in KLS and KLVS dimers.

Similar to the results seen in previous experiments, the effect of the KLV variant is corrected by co-expression with the KLS variant. However, unlike previous experiments where co-expression resulted in a variant that behaved like KLWT, for the first time we found a distinct effect of the KLVS variant compared to KLWT. When KLVS is overexpressed, more monomeric and less dimeric KL is found.

2.1.3 Homodimerization Decreased by KLVS: Implications for Heterozygote KLVS Carriers

Analysis of KLVS dimers by blue native show there is a decrease in dimerization, but it is not clear whether the complexes measured are homodimers or heterodimers. Further analysis by co-immunoprecipitation was used to determine whether KL's ability to form homodimers was affected. These experiments also allowed us to look at homodimers containing one KLWT and one KLVS allele. Cells were transfected with combinations of KLWT and KLVS containing either a V5 or GFP tag. Lysates were collected and the interaction of KLWT and KLVS were assessed by co-immunoprecipitation. A GFP antibody was used to pull down GFP tagged KL and V5 conjugated beads were used to pull down V5 tagged KL. Alternatively, separate lysates were transfected with GFP or V5 tagged KLWT or KLVS alone and were pulled down with the opposite antibody (KLVS pulled down with GFP antibody and vice versa) in order to rule out

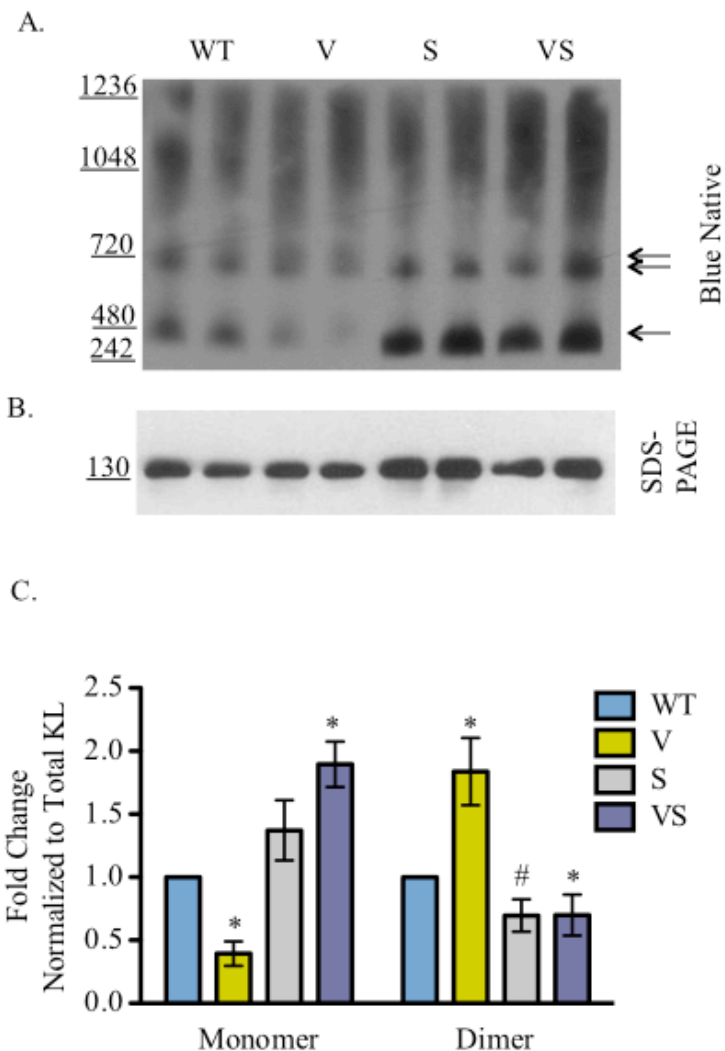


Figure 22. KL Variants Alter the Levels of Both Monomeric and Dimeric KL in Cells. HEK 293T cells transfected with wild-type (WT) KL or variants (V, S or VS) were assessed for differences in dimerization using Blue Native PAGE. (A) Western blot illustrating differences seen in monomers and dimers of KL using the Blue Native-PAGE method. The single arrow points to KL monomers and double arrows indicate KL dimers. (B) Western blot showing total KL amounts in denaturing and reducing conditions. (C) Bar graph depicting fold change across multiple experiments in monomer and dimer formation in the KL variants compared to wild-type normalized to total KL levels (error bars are \pm SEM; $n=3$, # $p=0.06$, * $p<0.05$, ANOVA). KLV leads to increases in dimeric KL and decreases in monomeric KL while both KLS and KLVS show the opposite effect leading to increased monomeric and decreased dimeric KL.

the presence of non-specific immunoprecipitation due to antibody cross-reactivity. After pull-down, samples were separated by SDS-PAGE and the amount of interaction partner was determined by western blot analysis (Figure 23). By this method, we could ascertain differences in levels of dimerization between KLWT and KLVS either to themselves as homodimers or each other as heterodimers. Results show that when compared to levels of KLWT homodimerization, the dimerization of KLWT to KLVS and of KLVS to KLVS are reduced irrespective of the antibody used to pull-down the interaction partners ($n=3$, $p<0.05$) (Figure 24). The reduction in KLVS dimerization to KLVS confirmed the previous results from Figure 22 and suggests that the difference in dimerization seen may be due to the difference in homodimerization. The control immunoprecipitation also shows that these results are not due to pull-down of the opposite tag by non-specific antibody interactions (Figure 25). The results also expand the Blue Native findings to suggest that regardless of binding partner, KLWT or KLVS, KLVS dimerizes less efficiently than KLWT.

2.2 KLVS Increases Heterodimerization with FGFR1c

Changes in KL's ability to homodimerize may impact its function alone or in combination with other binding partners. We used KL's function as a co-receptor with FGFR1c (285,288,291,458) as a model to determine whether the altered dimerization capacity of KLVS could affect protein-protein interactions. For the purpose of the FGF experiments, effect of KLVS was focused on due to its

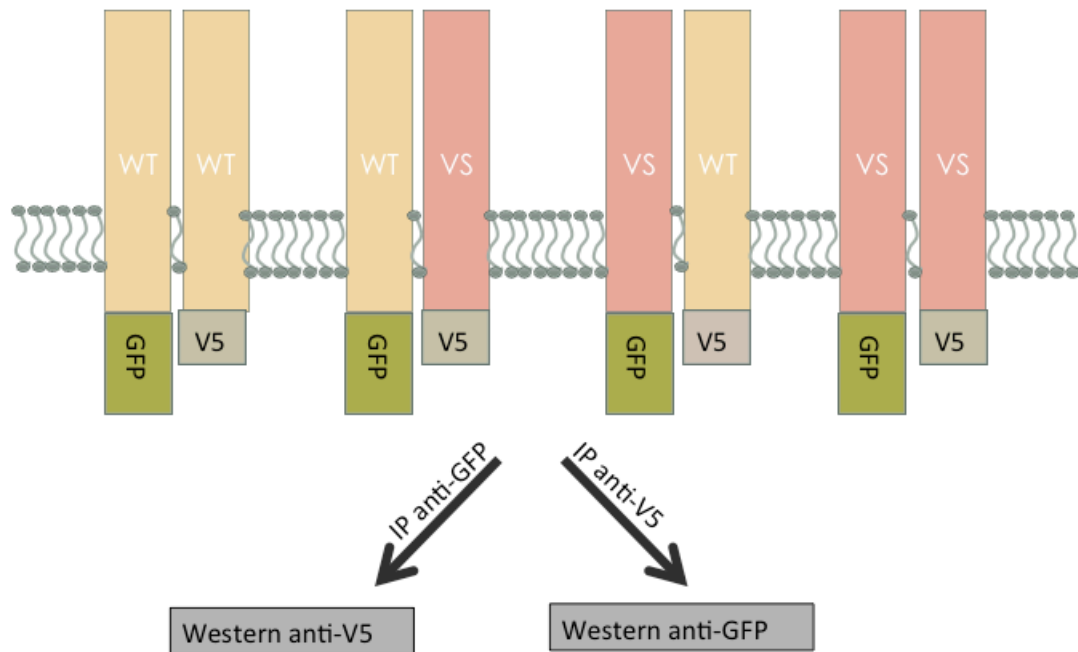


Figure 23. Diagram of KL-KL Co-Immunoprecipitation. Diagram outlining the procedures taken to assess homodimerization of KL by co-immunoprecipitation. Cells co-transfected with combinations of WT and/or VS KL with either a GFP or V5 tag were immunoprecipitated with an antibody to one of the tags (GFP or V5) and then probed by western blotting for presence of the opposite tag. This allowed for the assessment of KL interactions with other KL molecules coding for either WT or VS KL.

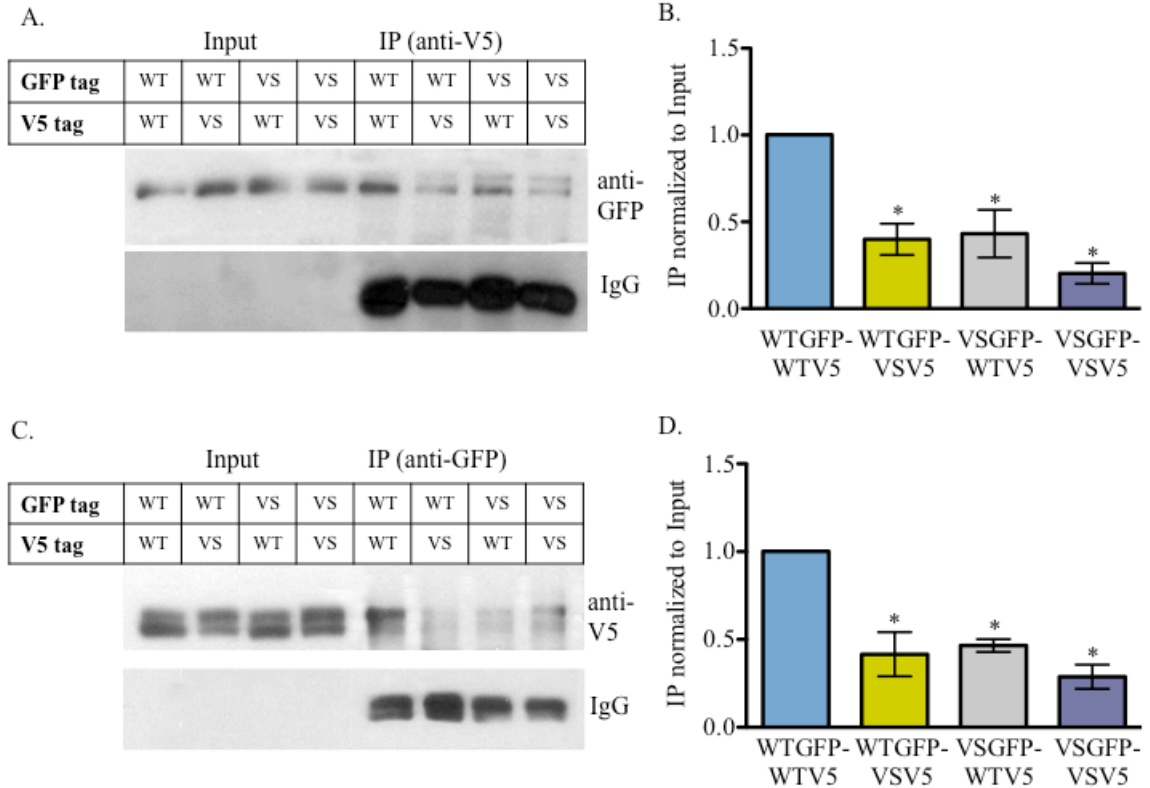


Figure 24. KL homodimerization is altered by the presence of the VS variant. HEK 293T cells were co-transfected with combinations of wild-type and/or VS KL containing either a V5 or GFP tag. Resulting lysates were assessed for differences in dimerization using co-immunoprecipitation (A) Western blot for GFP shows KL_{GFP} pulled down by interactions with co-expressed KL_{V5} variant through use of V5 beads. Non-precipitated lysate is shown as a control. (B) Bar graph depicting fold changes across multiple experiments in amount of GFP tagged KL immunoprecipitated by V5 beads normalized to non-precipitated lysate. (error bars are \pm SEM; $n=3$, $*p<0.05$, ANOVA). (C) Representative western blot showing changes in pull-down of V5 tagged KL when associated with GFP tagged KL. Non-precipitated lysate is shown as a control. (D) Bar graph depicting fold changes in amount of V5 tagged KL immunoprecipitated by GFP beads normalized to non-precipitated lysate. (error bars are \pm SEM; $n=3$, $*p<0.05$, ANOVA). The presence of KL_{VS} in KL dimers leads to an overall decrease in dimerization even if KL_{WT} is present in the dimer.

increased physiological impact compared to KLV and KLS alone. HEK 293 cells were co-transfected with KLWT or KLVS-GFP and EV or FGFR1c-V5. Lysates were collected and the interaction of KL and FGFR1c was assessed by co-immunoprecipitation. A GFP antibody was used to pull down KLWT or KLVS and a V5 antibody was used to pull down FGFR1c. To test for non-specific interactions, samples were also pulled down with mIgG. After pull-down, the amount of co-receptor was assessed by separating the immunoprecipitated proteins on SDS-PAGE followed by western blot analysis for the interaction partner. Amount of interaction was assessed by determining the amount of interaction partner precipitated, compared to the amount of interaction partner in the lysate. Immunoprecipitation with either member of the complex shows an increase in interaction between KLVS and FGFR1c compared to KLWT and FGFR1c (n=3, p<0.05) (Figure 26), which was not due to non-specific antibody interactions (Figure 27). These data taken together suggest that either the VS variant is a better binding partner for FGFR1 and/or that KL must be a monomer to function as a FGFR co-receptor.

2.3 KLVS Increases Downstream Signaling Through FGFR1c

Previous studies have shown that KL is necessary for FGF23 signaling through FGFR (285,291). Since KLVS increases KL's ability to interact with FGFR, it would be predicted to impact FGF23 signal transduction. To assess whether KLVS affects FGF signaling, HEK 293 cells were transfected with KLWT or KLVS.

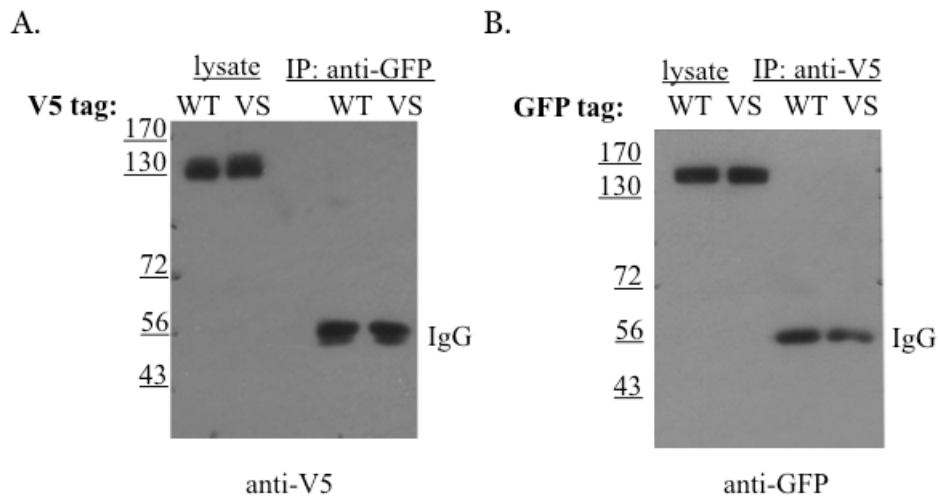


Figure 25. Control Co-immunoprecipitation of KLGFP and KLV5. In order to assess whether there was any non-specific pull-down between the tags used for co-immunoprecipitation, HEK 293 cells were transfected with either KL-GFP or KL-V5. (A) Resulting KL-V5 lysates were pulled down with GFP antibody and then were probed by western blotting for presence of KL-V5 in the pull-down. No non-specific pull-down of KL-V5 was seen. Non-precipitated KL-V5 lysate is shown as control. (B) Resulting KL-GFP antibodies were pulled down with V5 antibody and then were probed by western blotting for presence of KL-GFP in the pull-down. No non-specific pull-down of KL-GFP was seen. Non-precipitated KL-GFP lysate is shown as control.

After transfection, recombinant FGF23 was added to media overlaying cells. The resulting lysates were separated by SDS-PAGE and analyzed for the levels of phosphorylated ERK (pERK) and total ERK (tERK), a kinase that is activated downstream of FGF23 receptor binding. Similar to the increase in binding of KLVS to FGFR1c, there is also a significant increase in ERK activation as measured by pERK/tERK expression (n=3, p<0.01) (Figure 28A and B). Together these data indicate that while trafficking of the VS variant may be distinct from the wild-type, the most profound alteration of this polymorphism would be in enhancing KLVS-FGFR heterodimerization and thus FGF23 signaling.

2.4 KLVS Has Decreased Enzymatic Activity

Arking *et al.* reported that the highly conserved phenylalanine at amino acid 352 is necessary for the glucuronidase activity of a KL paralog (442). Using the fluorescent substrate, 4-methylumbelliferyl- β -D-glucuronide (4Mu-GlcU), as described previously (312), the enzymatic activity of shed KLWT or KLVS concentrated from serum-free media removed from the KL980 stable cells or concentrated serum-free media of HEK293 non-transfected cells (EV) was assessed. Before assaying enzymatic activity, the concentrated media was analyzed by western blot to determine KL protein concentration and the amount used in the assay was corrected based on these results (Figure 29A). To investigate the enzymatic activity of KLWT and KLVS, the concentrated KL was

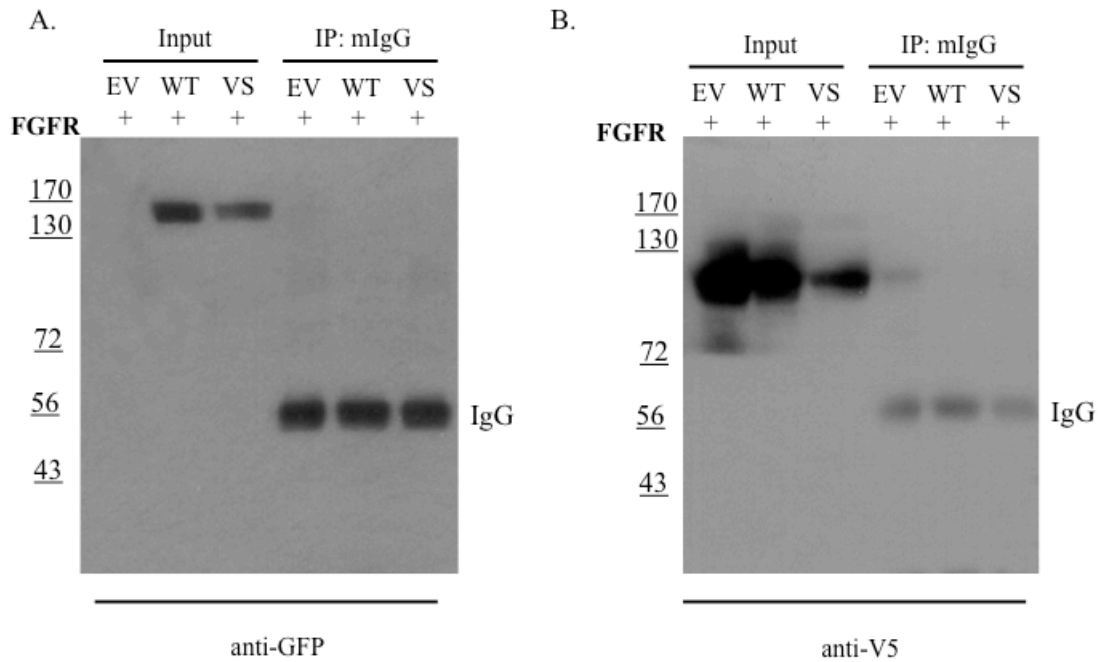
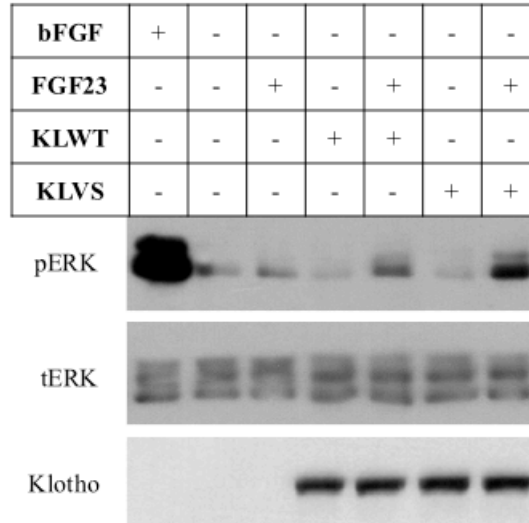


Figure 27. Control Co-immunoprecipitation Shows No Non-Specific Pulldown of FGFR1c/KL Complex. HEK 293T cells were transiently transfected with FGFR1c-V5 (FGFR) and either empty vector (EV), wild type (WT) or mutant (VS) KL-GFP. (A) Western blot showing samples that were pulled down with mIgG and probed for KL-GFP presence. Blot shows no non-specific pulldown of KL-GFP. Non-precipitated lysate samples shown as control. (B) Western blot showing samples that were pulled down with mIgG and probed for FGFR1c-V5 presence. Blot shows no non-specific pulldown of FGFR1c-V5. Non-precipitated lysate samples shown as control.

incubated with a series of 4Mu-GlcU concentrations ranging from 0.1 to 15 μM and fluorescence was measured at 30-minute intervals for 2.5 hours. The concentration of product was plotted over reaction time for EV, KLWT and KLVS and the initial velocity (v_0) of each reaction was determined for every initial substrate concentration ($[\text{S}_0]$) by calculating the slope of the resulting graphs (Figure 29 B and C). $[\text{S}_0]$ and v_0 were then plotted to obtain the Lineweaver-Burke plot for the samples (Figure 18D). From this plot, the K_m and v_{max} were calculated. While the K_m for WT (4.44 ± 0.956) and VS (6.09 ± 1.55) do not significantly differ, the V_{max} of VS (0.227) is significantly lower than KLWT (0.613) ($n=3$, $p < 0.01$) (Figure 29D). This suggests that the VS variant does not change the ability of KL to bind to its substrate, but instead alters the enzyme's capacity to cleave its product.

In order to determine whether the activity seen was due to KL specific enzymatic activity, KL980 samples were concentrated as in the previous experiments, but then were incubated with V5 sepharose beads. Incubation with V5 sepharose beads allowed for immunodepletion of KL from the media due to the V5 tag on the C-terminus of the KL construct. The level of immunodepletion was measured by western blot analysis and was compared to non-immunodepleted samples (Figure 30A). Although depletion of KL was not complete, the depletion was significant. After immunodepletion, the media were analyzed for enzymatic activity as described above. Depletion of KLWT led to a significant decline in enzymatic activity, however, the depletion of KLVS did not

A.



B.

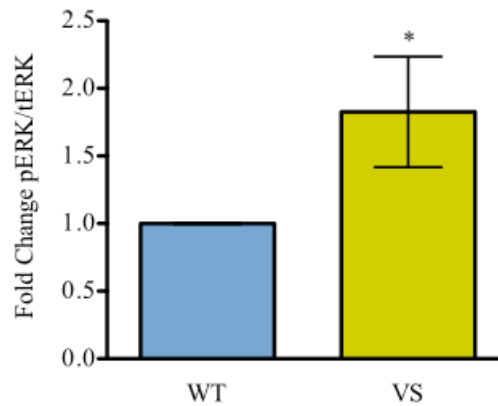


Figure 28. KLVS enhances FGF23 signal transduction. HEK 293T cells transiently transfected with KLWT or KLVS were treated with FGF23 to activate FGFR1c signaling. (A) Representative western blot showing differences in ERK phosphorylation compared to total expression of ERK after transfection of KLWT or KLVS. (B) Bar graph depicting fold change across multiple independent experiments in ERK phosphorylation normalized to total ERK expression (error bars are \pm SEM; $n=3$, $*p<0.05$).

have any effect (n=3) (Figure 30B). Interestingly, immunodepletion increased the background of the experiment as measured by immunodepletion of EV media. The increase background could be due to residual V5 beads in the media, which could give false absorbance readings. Overall, these data show that the activity seen is specific KL enzymatic activity. If the increase in background is taken into consideration, the level of depletion achieved for KLWT in this experiment may be enough to totally abolish the enzymatic activity seen. This finding also confirms that the decreased enzymatic activity of KLVS is due to the specific actions of KL and not another glucuronidase found in the media.

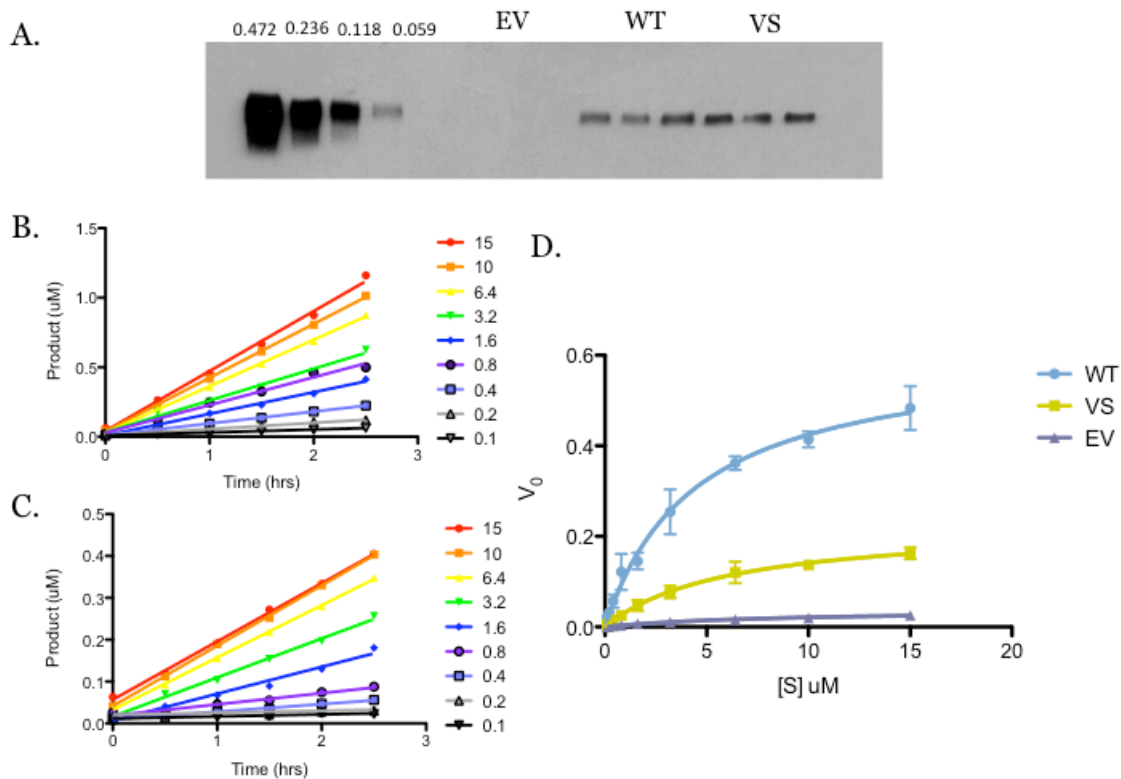


Figure 29. KLVS Leads to Decreased Enzymatic Activity. Media concentrated from HEK2 293 cells stably transfected with KLWT980 or KLVS980 or EV were (A) run on a western blot to determine approximately equal concentration of KL in the two variant samples. KLWT and KLVS were incubated with a series of 4Mu-GlcU concentrations ranging from 0.1 to 15 μ M and fluorescence was measured at 30-minute intervals for 2.5 hours. The concentration of product was plotted over reaction time for EV, (B) KLWT and (C) KLVS. The slope of the resulting graphs were calculated to determine initial velocity (v_0) for each initial substrate concentration ($[S_0]$). (D) The resulting v_0 were plotted against the $[S_0]$ to obtain the Lineweaver-Burke plot. From this plot, the K_m and v_{max} were calculated. While the K_m did not differ between KLWT and KLVS, the v_{max} of KLVS was significantly lower ($n=3$, $p<0.05$).

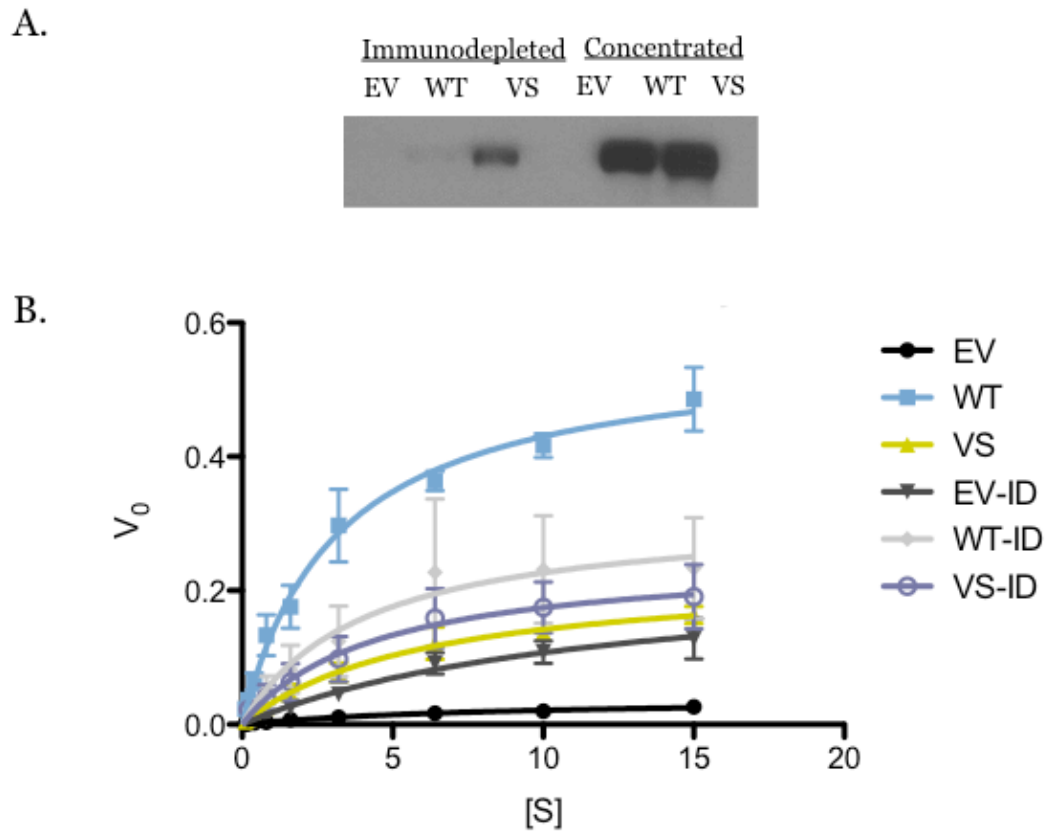


Figure 30. Immunodepletion of KL Eliminates KL Specific Enzymatic Activity. Media concentrated from HEK2 293 cells stably transfected with KLWT980 or KLVS980 or EV were immunodepleted with V5 sepharose beads to remove KL from the media. (A) Resulting samples were run on western blot to determine the efficiency and equality of KL depletion between KLWT and KLVS. Non-immunodepleted samples are shown as a control. (B) The samples were then incubated with a series of 4Mu-GlcU concentrations ranging from 0.1 to 15 μ M and fluorescence was measured at 30-minute intervals for 2.5 hours. After determining v_0 , it was plotted against $[S_0]$ to obtain the Lineweaver-Burke plot. From this plot, the K_m and v_{max} were calculated. There was a significant decrease in the v_{max} of the depleted KLWT compared to the non-depleted control ($n=3$, $p<0.05$). Between the depleted samples, there was no difference in v_{max} suggesting that the specific activity of KL is depleted.

CHAPTER IV: DISCUSSION

1. KLOTHO AND AGING

The KL protein has gathered a lot of interest from members in the aging field since its discovery 16 years ago due to the close approximation of KL deficient mice phenotypes to normal human aging. Not only does lack of KL lead to a shortened lifespan, but the phenotypes leading up to this decrease in lifespan resemble many of the diseases that are prevalent in aging populations. KL's organism-wide aging effects are made more interesting by the systems it has been shown to regulate. Many of the functions attributed to KL overlap with pathways and systems that have been independently associated with theories of aging. As concluded previously, there is not one current theory of aging that completely encompasses the complexities that lead to aging organisms. KL's ability to modulate pathways and systems implicated by multiple theories including oxidative stress, inflammation, senescence, growth factor signaling and pituitary function adds to that argument suggesting that a dysregulation of multiple different pathways leads to aging. How one molecule is able to have such diverse effects is still not sufficiently elucidated, but it is clear that it has an overarching regulatory role in many systems and pathways that are intricately linked with the aging process.

2. KLOTHO POLYMORPHISMS

Analysis of KL polymorphisms gives insight into how the protein that causes such a drastic phenotype in mice may affect human longevity and risk of disease. Many polymorphisms have been identified by Genome Wide Association studies (GWAS) in both coding and non-coding regions of KL that are associated with increased risk of disease. Despite the plethora of polymorphisms, only a handful have been associated with changes in KL function or KL levels.

One polymorphism, the KLVS has not only been associated with increased disease risk, but has also been associated with alterations in longevity. A few studies on this variant have also suggested it may alter the function of KL. Transfection of the KL1 domain into HeLa cells showed that KLV leads to a decrease in secretion while the expression of KLS and the combination of both SNPs led to an increase in secretion (442). This study also showed that the F352V substitution alone led to a decrease in the enzymatic activity of KL1 through measuring the activity of a KL paralog with a corresponding amino acid substitution (442). However, the use of the KL1 domain is non physiologic as KL1 is only detected at the mRNA level and has been identified as a target for nonsense-mediated mRNA decay by the National Center for Biotechnology Information (NCBI) (reference sequence: NM_153683.2) (459). A second study examining KLVS mechanism also showed that the full-length KLV variant did not have the same growth inhibitory effect as KLWT in breast cancer cells (447). While this study again suggests there is a functional difference in the variant, it

only looked at the V substitution alone and not KLVS, which is more physiologically relevant. Both of these studies suggest that KLVS may cause a functional difference in the protein, but are limited by the constructs used. This prompted a more thorough study of the KLVS and resulting substitutions to better understand how they may lead to changes in longevity and disease risk.

3. ALTERATIONS IN PROCESSING OF KLVS ISOFORMS

3.1 KLV Expression Leads to Altered KL Processing

Maintaining proper trafficking and shedding of KL is important for maintenance of function. Many KL functions rely on the far-reaching effects of the extracellular domain that is cleaved from full-length KL at the plasma membrane. Altering the shedding of KL would alter the ability of KL to exert its humoral-like effects. The importance of KL shedding is emphasized by the rescue of age-related phenotypes in KL deficient mice by undergoing parabiosis with wild-type mice (278). Rescue of KL expression limited to brain and testes was also capable of rescuing almost all of the age-related phenotypes in KL deficient mice (198) further emphasizing the importance of KL shedding into blood and CSF (230). The importance of KL shedding coupled with previous studies suggesting KLVS secretion may be altered led to the current studies that sought to further elucidate how KLVS alters processing and the implications it may have for KL function.

In this thesis, investigations of KLVS processing showed that KLV, but not KLVS led to alterations in shedding of full length KL, which seem to be dependent on trans golgi processing due to the attenuation of this phenotype in a construct lacking the intracellular and transmembrane domain. Further analysis of the KLV processing revealed that the decrease in shedding was paired with a decrease in half-life indicating the reason for the decreased amount of shedding could be due to increased rate of degradation of the KLV. It was first hypothesized that the decreased KLV shedding and half-life could be explained by misfolding of the protein. Despite this connection, shedding is only weakly correlated with the changes in half-life, suggesting there may be a second factor that connects these two similar changes (Figure 31). Treatment with two different chemical chaperones that are known to decrease levels of misfolded proteins was not able to rescue the KLV phenotype. Glycerol treatment had no effect on KLV shedding, while treatment with MT-1, a small molecule chemical chaperone, increased shedding equally in both KLWT and KLV suggesting that its effect was not specific to the phenotype seen with KLV. Since alterations in processing could explain both decreased shedding and half-life, we decided to examine whether there are alterations in membrane localization of the variants. These experiments showed that there was a decrease in KLV localization to the membrane that could be blocked by inhibiting dynamin-dependent endocytosis. This result suggests that the decrease in membrane localization is caused by increased endocytosis of the KLV variant.

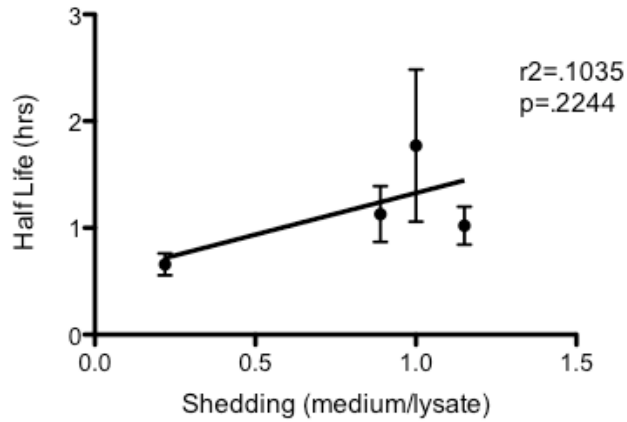


Figure 31. Correlation Between Shedding and Half-life Changes in KL Variants. Mean values from full-length shedding experiments were plotted on the x-axis against half-life values calculated for the same variants. GraphPad Prism was used to calculate the line of best fit and whether the slope of the line significantly differed from 1. There was no significant correlation between shedding and half-life ($r^2=.1035$, $p=.2244$).

Increased endocytosis, which would lead to trafficking to endosomes followed by lysosomal degradation, could explain the decrease in half-life. An increase in KLV endocytosis could also explain the decrease in shed KL because less would be present at the membrane, which is the site of KL sheddases, ADAM10 and ADAM17. Due to the alterations in endocytosis, the variants were co-transfected with SORLA, which is a protein known to aid in trafficking of endocytosed proteins. It was hypothesized that SORLA may be able to direct the endocytosed KLV towards the recycling pathway where it would return to the trans golgi network instead of getting degraded by the lysosomes. If SORLA could accomplish this, it would rescue the phenotype seen in KLV. Unfortunately, SORLA did not lead to a significant difference in KLV shedding suggesting that it was not able to rescue the endocytosed KL. This does not rule out the possibility that the phenotype seen in KLV is due to alterations in endocytosis, but does suggest that KL is not a substrate for SORLA.

Since proper shedding is necessary for functioning KL, a luciferase assay was designed to screen for modulators of KL shedding. This assay was able to confirm the effect of insulin on the shedding of KL and demonstrated that IGF-2 can also increase the amount of KL shed from the plasma membrane. These results could be used to find other modulators of KL shedding, which would not only be useful for targeting diseases in which plasma KL is decreased, but could also lead to a better understanding of the mechanisms of KL shedding regulation. These compounds could also be screened for an ability to correct the KLV

decrease in shedding, which could lead to a better understanding of how the KLV alters the shedding and trafficking of KL.

Although KLV leads to alterations in trafficking, analysis of the subcellular localization of the variants does not show much difference. This could be due to the insensitivity of the assay or because it is only looking at a frozen moment in time so the cell dynamics cannot be seen. While there is no striking difference in subcellular localization there is a small difference in the amount of KL seen in the late endosomes stained with mannose-6-phosphate (M6P). Compared to KLWT expression, KLV co-localizes less to the late endosomes. The decrease of KLV in the endosomes is contrary to what might be expected due to the increase in KLV endocytosis. This may be due to a faster rate of KLV processing into the lysosomes where it is degraded. The increase in KLS could be due to an increased amount being packaged into microvesicles called exosomes that form in the late endosomes. Exosomes in the urine have been shown to contain KL suggesting that KL may be secreted into the extracellular milieu in this fashion (453).

3.2 KLS Alters Endosomal Trafficking

Despite the large differences seen in KLV trafficking, the trafficking of KLS mostly trends with wild type. The one exception is the apparent link to alterations in endosomal trafficking. Not only do immunofluorescence studies suggest that KLS has an increased localization to the late endosomes, but membrane localization studies also show that KLS has an increased presence on

the plasma membrane that is attenuated by inhibition of endocytosis. Together, this data suggests that retromer trafficking of KL from the endosomes back to the plasma membrane via the trans-golgi network is important for proper KL trafficking. It is possible that KLS leads to other alterations in shedding and trafficking that were not detectable with the power of this study.

3.3 Intragenic Complementation of KLV and KLS Result in Like Normal KLVS Processing

The relative lack of KLVS trafficking phenotype suggests that expression of the S substitution in the presence of the V substitution is able to correct for the alterations in trafficking due to intragenic complementation. This could be through increased trafficking of KL containing the S substitution from the endosomes back to the plasma membrane as is suggested from the differences seen in KLS endosomal trafficking. This complementation is similar to cases of mut-o methylmalonic aciduria where one substitution is able to rescue the aberrant phenotype caused by another substitution (460). Despite being able to rescue KLV aberrant trafficking, the association of KLVS with disease risk suggests that there are other functional changes that the KLS is not able to rescue. This led to further investigation in Aim 2 of how the KLVS may lead to alterations in KL function.

4. KLVS VARIANT ALTERS KL FUNCTION

4.1 KLVS Decreases Homodimerization

Despite normal trafficking of the KLVS, expression of the polymorphism in humans leads to increased risk of disease suggesting that KLVS leads to alterations in protein function independent of processing alterations. In an attempt to try to explain why KLVS would change protein function, we first looked at dimerization of the variants. Dimerization is often necessary for proper trafficking and function of transmembrane proteins (454) and KL is known to form dimers or higher oligomers in the blood and CSF of mice and humans as well as in conditioned media from cells overexpressing KL (230,231). This makes it plausible that the KLVS variant may lead to differences in dimerization, which could explain the alterations in function. Further evidence of a possible role of dimerization in the actions of KLVS was provided by analysis of KL using disulfide bond prediction software, DIANNA (461). This software predicted that the cysteine changed to a serine in the S substitution is involved in an intramolecular disulfide bond. Given the fact that cysteines are known to be important for the formation of disulfide bonds, which are critical in proper folding of proteins (462) and can play crucial roles in dimerization (463-466) analysis of KLVS dimers seemed like a good starting point.

Both crosslinking and Blue Native gel analysis of dimerization showed that KLS and KLVS lead to decreases in dimerization, however the increase in KLV dimerization was only seen by Blue Native electrophoresis. Because of the

artificial cross-linking of bonds involved in the first technique, the results gathered from Blue Native separation are deemed to be more accurate representations of KL dimerization. Based on the Blue Native gel results, the increase in KLV dimerization coupled with decreased KLS and KLVS dimerization suggests that KLS attenuates the KLV phenotype by correcting for the increase in dimerization. Although the KLS induced decrease in dimerization corrects the aberrant trafficking caused by the V substitution, the KLVS still has altered dimerization suggesting it has altered function. This is the first difference attributed to the KLVS variant. Previously all results have shown changes in KLV or KLS alone, but when combined they have led to a protein that behaves like KLWT. Since KLVS is the variant found in humans, finding a functional difference in this variant is necessary for understanding how the VS affects risk of disease.

This difference in dimerization could also help explain the alteration in endocytosis that is seen with the KLV. Although it is unlikely that the V substitution in the extracellular N-terminus of the protein has a direct role in controlling endocytosis, it is possible that the KLV increase in dimerization could be a signal for internalization leading to the altered trafficking of KLV. It is known that KL interaction with other proteins, namely the TRPC1-VEGFR2 complex is able to increase the internalization of the complex showing that is capable of regulating endocytosis. This would further explain how the KLS

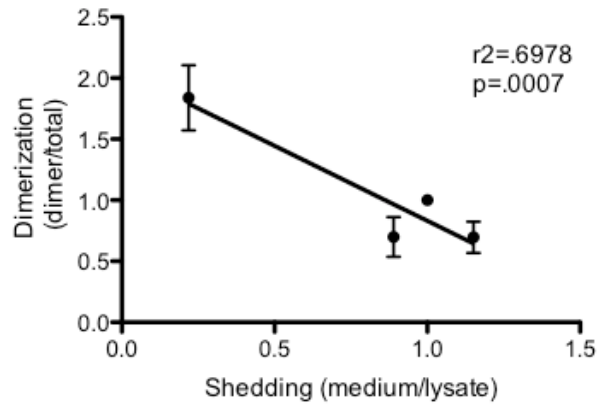


Figure 32. Shedding and Dimerization Changes in KL Variants are Inversely Correlated. Mean values from full-length shedding experiments were plotted on the x-axis against mean values of dimer/total calculated for the same variants. GraphPad Prism was used to calculate the line of best fit and whether the slope of the line significantly differed from 1. There was a significant correlation between shedding and dimerization ($r^2 = .6978$, $p = .0007$).

variant decrease in dimerization could correct for the alteration in KLV shedding by inhibiting the increase in dimerization. It is also possible that homodimerization of KL inhibits retromer trafficking to the plasma membrane, which would explain the opposite effect of KLV and KLS on plasma membrane localization and why both effects are inhibited by expression of dominant negative dynamin. These speculations are bolstered by analysis of associations between dimerization and shedding (Figure 32) and dimerization and half-life (Figure 33). Both analyses suggest that changes in dimerization are inversely correlated with these two measures of changes in KL processing, further implicating a role in dimerization in the control of KL processing.

To confirm the difference in KLVS dimerization with a more quantitative method and to expand upon these findings, co-immunoprecipitation studies were performed. These experiments allowed us to look at the dimerization of KLWT to itself in the presence or absence of the KLVS variant. These results showed that the decrease in KLVS dimerization persists even when there is one molecule of KLWT in the dimer. It is possible that with extra power added to the experiment, the decrease in dimerization seen with VS:VS dimers might be greater than that seen with WT:VS dimers, but in this study there was no significant difference between the two. This suggests that differences in disease risk seen between heterozygous and homozygous expressors of KLVS are not due to variances in dimerization, however it does show a change in function of the KLVS for the first time.

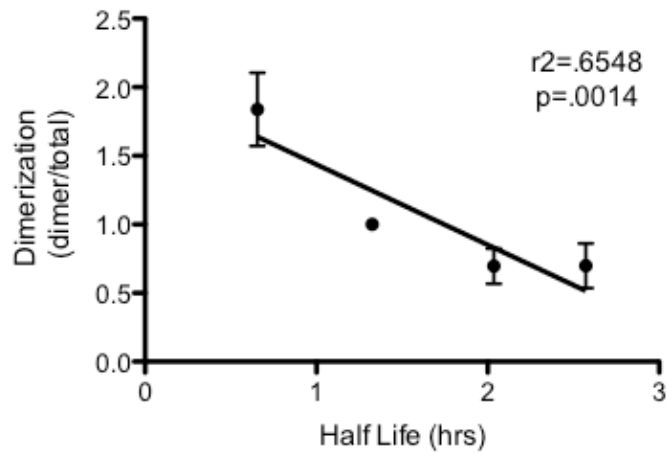


Figure 33. Half-Life and Dimerization Changes in KL Variants are Inversely Correlated. Mean values from half-life experiments were plotted on the x-axis against mean values of dimer/total calculated for the same variants. GraphPad Prism was used to calculate the line of best fit and whether the slope of the line significantly differed from 1. There was a significant correlation between shedding and dimerization ($r^2=.6548$, $p=.0014$).

Overall these findings suggest that despite the correction of trafficking, the KLVS variant may still have altered function through altered protein-protein interactions.

4.2 KLVS Increases Heterodimerization with FGFR1c

KL's interaction with FGFR as a co-receptor for FGF23 binding is the most well studied KL protein-protein interaction and is critical in proper kidney function (467,468). KL activity as an FGF co-receptor is vital to many of the hallmark functions of KL as suggested by the similarities of the KL^{-/-} and FGF23^{-/-} mice (291). Drastic changes in KL's ability to bind to FGFR can also lead to devastating illness (H193R) further emphasizing the significance of KL-FGF function (469). Because of the importance of FGF signaling in KL function, we decided to determine whether KLVS would alter interaction with FGFR and thus affect its subsequent FGF23 signaling cascade. Contrary to the reduced ability of KLVS to bind other KL molecules, the ability of KLVS to bind FGFR1c is increased. This suggests that the increased presence of KL monomers in carriers of KLVS leaves more KL free to bind to FGFR increasing the amount of KL-FGF complexes that form. This implies that α KL may behave much like β KL, which binds with the FGFR signaling complex as a monomer (470). These results indicate that the KLVS variant leads to alterations in KL's ability to act as a co-receptor.

To further elucidate KLVS's effect on the FGFR signaling complex, we tested whether there was a difference in KLVS's ability to activate signaling downstream of the complex. Since previous studies have shown that KL is necessary for downstream ERK activation via FGF23 (285,291), we wanted to determine whether KLVS would alter the amount of downstream ERK phosphorylation. In line with the changes in binding, we found that KLVS also leads to an increase in downstream ERK activation. This significant increase in ERK activation could explain the differences seen between heterozygosity for KLVS versus homozygosity. In heterozygous individuals, the small increase in FGF23 signaling could be beneficial, as seen by the many protective effects of the FGF23-KL axis. This could lead to the advantageous effects of KLVS heterozygosity. While a small increase can be beneficial for heterozygous individuals, homozygous individuals would have a larger increase in FGF23 signaling which could lead to detrimental health effects such as hypophosphatemia, aberrant vitamin D metabolism, impaired growth and rickets/osteomalacia (471). These changes in health could lead to the increased risk of disease found in homozygous individuals. Hypophosphatemia, for example, has been linked to both cardiovascular disease and metabolic syndrome (472), which have both been associated with KLVS homozygosity (435,441,443,444). These findings suggest there is a fine line between a beneficial increase in FGF23 signaling versus a detrimental increase illustrated

by the differences in individuals heterozygous for KLVS versus homozygous individuals

4.3 Effect of KLVS on Enzymatic Activity of KL

Previous studies have shown that a homolog of KLV has reduced glucosidase activity and evidence from other glucosidases show that many enzymes from this family form homo or heterodimers that are necessary for their enzymatic activity (473-475). This led us to conclude that the KLVS may change the ability of KLVS to act as an enzyme due to its changes in dimerization. Analysis of the enzymatic activity of KLVS did show that there was a reduced enzymatic activity of KLVS. Immunodepletion of KL from the media also confirmed that this activity was due to the presence of KL in the concentrated media. These findings are necessary for understanding the effect of KLVS because the enzymatic activity of KL is important for its regulation of ion channel membrane localization. This suggests that KLVS might also lead to alterations in the membrane localization of ion channels regulated by KL, which could affect KL dependent ion homeostasis. Further studies could investigate whether KLVS has a decreased ability to alter localization of ion channels such as TRPV5.

5. SUMMARY

In humans the KLVS polymorphism is the result of a collection of 6 SNPs that always occur together. The current studies focused on the two SNPs that

lead to amino acid changes. We show alterations in shedding and processing but the effects are small overall and intragenic complementation may further minimize these effects *in vivo*. Despite the ability of intragenic complementation to allow proper trafficking of the VS variant, we have identified for the first time, KLVS specific changes in KL function, namely differences in the protein-protein interactions as illustrated by the dimerization and FGFR1c interaction and signaling experiments and alterations in KL enzymatic activity as shown through β -glucuronidase assays. Although we have identified clear KLVS changes, it is likewise possible that the four other SNPs contribute to disease risk in ways we have not appreciated. As the SNPs are near the intron/exon borders, splicing or splicing efficiency could be affected by the SNPs. A small decrease in proper splicing coupled to dimerization or other protein binding changes could compound to create a bigger problem than presently appreciated.

6. FUTURE DIRECTIONS

The research described in this thesis has provided insight into how the KLVS variant affects the function of KL, but much more could be ascertained about how the VS variant leads to the increased risk of disease. More information could be gathered about the role of dimerization in the shed form of KL. As mentioned previously, KL is found as a dimer in the blood and CSF (230) suggesting dimerization may be important for its humoral functions. Studies could investigate whether the reduced dimerization of KLVS has an effect on the

half-life of the shed protein and could investigate whether it has an effect on the other functions that have been ascribed to the shed form of the protein. Further studies could also analyze more in-depth into how the KLVS alters FGF23 signaling. Studies have shown that KL is able to protect against vitamin D induced apoptosis through its regulation of FGF23 signaling (476) so it is possible that KLVS has a higher ability to protect against this. Since this is through KL's inhibition of 1- α -hydroxylase, the enzyme responsible for synthesis of the active form of vitamin D, levels of this enzyme could also be analyzed as well as other downstream proteins regulated by KL such as Egr-1. Evidence from the enzymatic assay performed also suggests that the VS variant may affect KL's ability to regulate membrane localization of ion channels. As mentioned previously, future studies could assess the effect of KLVS on the localization of TRPV5 as well as other ion channels known to be affected by KL.

7. CONCLUDING REMARKS

In this study, the effect of the KLVS variant on trafficking and function of KL has been studied. Through this investigation, we have determined that KLV has altered trafficking leading to a decrease in the amount of KL shed from the membrane as well as a decrease in half life. This aberrant processing caused by the KLV variant can be attenuated by co-expression of the KLS substitution suggesting there is intragenic complementation between the two substitutions. It is possible that the ability of KLS to rescue the KLV phenotype is through its

inhibition of dimerization. When expressed together in the VS variant, the increase in dimerization seen with the V variant is attenuated and in fact reversed. This reversal of dimerization could be the reason for the amelioration of the processing phenotype seen in the KLVS variant suggesting that it is the increase in dimerization that causes the increased endocytosis and other related phenotypes. The alteration in dimerization is also linked to the changes seen in FGFR signaling as well as the decrease in enzymatic activity highlighting the effects of KLVS on dimerization as the key difference leading to the other functional alterations seen (Figure 34). Overall, this research has shown the first effect of the KLVS isoform on KL function giving insight into how this polymorphism may affect risk of age-related disease.

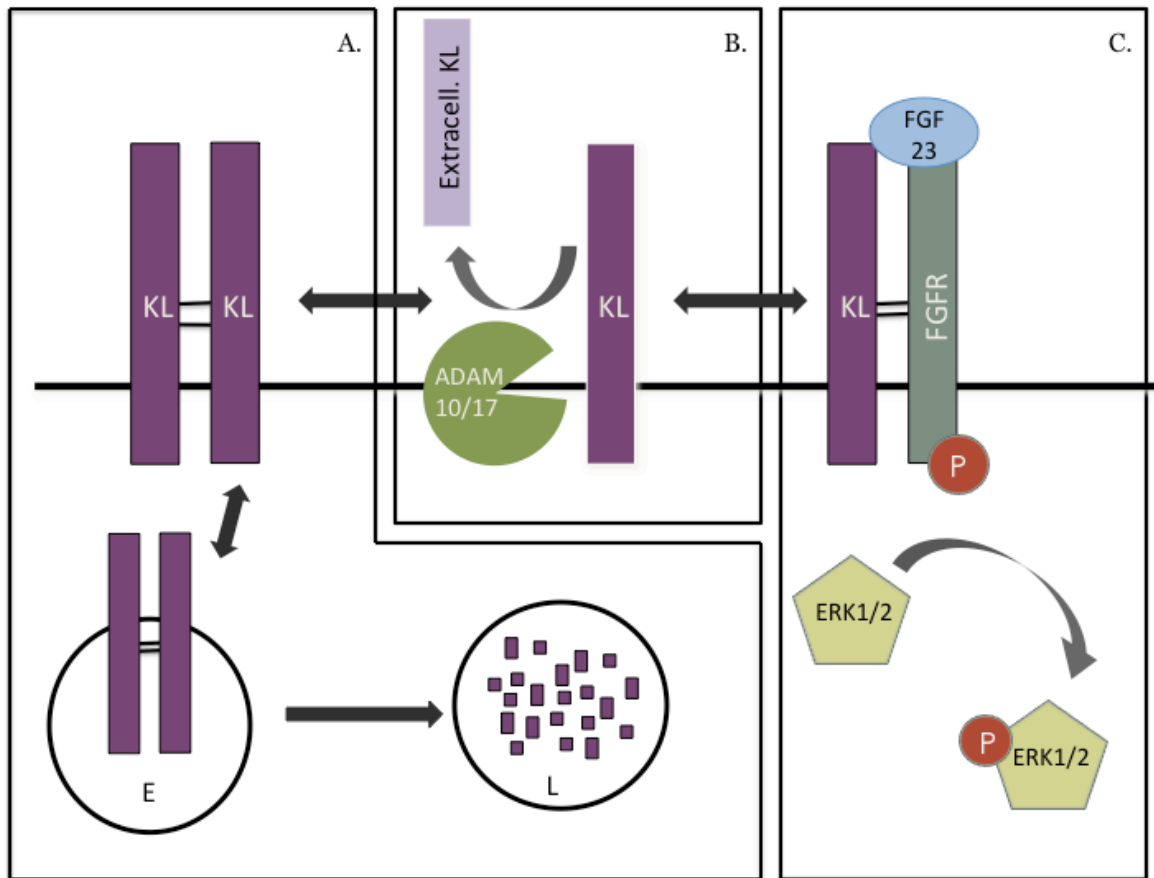


Figure 34. Interactions of Different KL Functions on the Plasma Membrane. Diagram showing the interactions of various KL functions that are altered by the KLVS variant. (A) KL dimerization, which is decreased by the KLVS variant mediates the amount of KL endocytosed and degraded. Increased endocytosis, as seen by KLV, leads to an increase in degradation leading to less KL available on the plasma membrane where it can be (B) cleaved by ADAM10/17 leading to the shedding of the extracellular domain (extracell. KL) into the media. (C) Alternatively, as a monomer, KL can also participate in formation of heterodimers such as the one with FGFR1c. This heterodimer leads to FGF23 binding to FGFR and initiates downstream signaling. Together, this shows how regulation of KL dimerization can lead to regulation of other KL functions.

LIST of JOURNAL ABBREVIATIONS

Acta Neuropathol	Acta Neuropathologica
Acta Otolaryngol	Acta Oto-Laryngologica
Acta Pharmacol Sin	Acta Pharmacologica Sinica
Age (Dordr)	Age (Dordrecht)
Age Ageing	Age and Ageing
Ageing Res Rev	Ageing Research Reviews
Aging Dis	Aging and Disease
Am J Cancer Res	American Journal of Cancer Research
Am J Clin Nutr	The American Journal of Clinical Nutrition
Am J Hum Genet	The American Journal of Human Genetics
Am J Kidney Dis	American Journal of Kidney Diseases
Am J Nephrol	American Journal of Nephrology
Am J Physiol	American Journal of Physiology
Am J Physiol Endocrinol Metab	American Journal of Physiology-Endocrinology and Metabolism
Am J Physiol Lung Cell Mol Physiol	American Journal of Physiology-Lung Cellular and Molecular Physiology
Am J Physiol Renal Physiol	American Journal of Physiology - Renal Physiology
Am J Respir Cell Mol Biol	American Journal of Respiratory

Ann Endocrinol (Paris)	Cell and Molecular Biology
Ann Intern Med	Annales d Endocrinologie (Paris)
Ann Med	Annals of Internal Medicine
Ann N Y Acad Sci	Annals of Medicine
Annu Rev Cell Biol	Annals of the New York Academy of Science
Annu Rev Physiol	Annual Review of Cell Biology
Arch Intern Med	Annual Review of Physiology
Arch Pharm Res	Archives of Internal Medicine
ASN Neuro	Archives of Pharmacal Research
Biochem Biophys Acta	American Society for Neurochemistry Neuro
Biochem Biophys Res Commun	Biochimica et Biophysica Acta
Biochem J	Biochemical and Biophysical Research Communications
Biol Rev Camb Philos Soc	Biochemical Journal
Biophys Res Commun	Biological reviews of the Cambridge Philosophical Society
Blood Cells Mol Dis	Biophysical Research Communications
BMC Nephrol	Blood Cells, Molecules & Diseases
Br J Haematol	BMC Nephrology
Brain Res	British Journal of Haematology
Brain Res Rev	Brain Research
	Brain Research Reviews

Breast Cancer Res Treat	Breast Cancer Research and Treatment
Calcif Issure Int	Calcified Tissue International
Can J Physiol Pharmacol	Canadian Journal of Physiology and Pharmacology
Cancer Biol Ther	Cancer Biology and Therapy
Cancer Cell Int	Cancer Cell International
Cancer Invest	Cancer Investigation
Cancer Sci	Cancer Science
Cell Mol Life Sci	Cellular and Molecular Life Sciences
Cell Physiol Biochem	Cellular Physiology and Biochemistry
Cell Struct Funct	Cells - Structure and Function
Cir Res	Circulation Research
Clin Cancer Res	Clinical Cancer Research
Clin Chim Acta	Clinica Chimica Acta
Clin Endocrinol (Oxf)	Clinical Endocrinology (Oxford)
Clin Exp Immunol	Clinical & Experimental Immunology
Clin J Am Soc Nephrol	Clinical Journal of the American Society of Nephrology
Clin Sci (Lond)	Clinical Science (London)
Contrib Nephrol	Contributions to Nephrology
Curr Biol	Current Biology

Curr Eye Res	Current Eye Research
Curr Med Chem	Current Medicinal Chemistry
Curr Opin Cell Biol	Current Opinion in Cell Biology
Curr Opin Nephrol Hypertens	Current Opinion in Nephrology & Hypertension
Curr Top Microbiol Immunol	Current Topics in Microbiology and Immunology
Curr Top Pathol	Current Topics in Pathology
Dev Dyn	Developmental Dynamics
EMBO J	EMBO Journal
Eur J Biochem	European Journal of Biochemistry
Eur J Cancer	European Journal of Cancer
Eur J Clin Nutr	European Journal of Clinical Nutrition
Eur J Endocrinol	European Journal of Endocrinology
Eur J Hum Genet	European Journal of Human Genetics
Eur J Immunol	European Journal of Immunology
Eur J Neurosci	European Journal of Neuroscience
Exp Aging Res	Experimental Aging Research
Exp Cell Res	Experimental Cell Research
Exp Gerontol	Experimental Gerontology

Exp Neurol	Experimental Neurology
Expert Rev Endocrinol Metab	Expert Review of Endocrinology & Metabolism
FASEB J	FASEB Journal
Free Rad Biol Med	Free Radical Biology and Medicine
Free Radic Res Commun	Free Radical Research Communications
Gen Comp Endocrinol	General and Comparative Endocrinology
Genes Dev	Genes & Development
Genes Immun	Genes and Immunity
Geriatr Gerontol Int	Geriatrics & Gerontology International
Glycoconj J	Glycoconjugate Journal
Hear Res	Hearing Research
Hum Pathol	Human Pathology
Human Genet	Human Genetics
Hypertens Res	Hypertension Research
Immunol Rev	Immunological Reviews
Inflamm Res	Inflammation Research
Int Heart J	International Heart Journal
Int Immunol	International Immunology
Int J Biochem Cell Biol	The International Journal of Biochemistry & Cell Biology

Int J Cardiol	International Journal of Cardiology
Int J Mol Med	International Journal of Molecular Medicine
Invest Ophthalmol Vis Sci	Investigative Ophthalmology & Visual Science
J Am Geriatr Soc	Journal of the American Geriatric Society
J Am Soc Nephrol	Journal of the American Society of Nephrology
J Anat	Journal of Anatomy
J Appl Physiol	Journal of Applied Physiology
J Biol Chem	Journal of Biological Chemistry
J Biomed Biotechnol	Journal of Biomedicine and Biotechnology
J Bone Miner Res	Journal of Bone and Mineral Research
J Cell Physiol	Journal of Cellular Physiology
J Cell Sci	Journal of Cell Science
J Clin Endocrinol Metab	Journal of Clinical Endocrinology and Metabolism
J Clin Epidemiol	Journal of Clinical Epidemiology
J Clin Immunol	Journal of Clinical Immunology
J Clin Invest	Journal of Clinical Investigation
J Endocrinol	Journal of Endocrinology
J Endocrinol Metab	Journal of Endocrinology and Metabolism

J Exp Clin Cancer Res	Journal of Experimental & Clinical Cancer Research
J Gerontol	Journal of Gerontology
J Gerontol A Biol Sci Med Sci	Journals of Gerontology Series A: Biological Sciences and Medical Sciences
J Immunol	Journal of Immunology
J Intern Med	Journal of Internal Medicine
J Invest Dermatol	Journal of Investigative Dermatology
J Investig Med	Journal of Investigative Medicine
J Med Genet	Journal of Medical Genetics
J Mol Med (Berl)	Journal of Molecular Medicine (Berlin)
J Musculoskelet Neuronal Interact	Journal of Musculoskeletal and Neuronal Interactions
J Neurochem	Journal of Neurochemistry
J Neuroinflammation	Journal of Neuroinflammation
J Neurosci Res	Journal of Neuroscience Research
J Nutr	Journal of Nutrition
J Orthop Res	Journal of Orthopaedic Research
J Ren Nutr	Journal of Renal Nutrition
J Theor Biol	Journal of Theoretical Biology
JAMA	Journal of the American Medical Association

Jpn J Pharmacol	Japanese Journal of Pharmacology
Kidney Blood Press Res	Kidney and Blood Pressure Research
Kidney Int	Kidney International
Kobe J Med Sci	Kobe Journal of Medical Sciences
Korean J Intern Med	Korean Journal of Internal Medicine
Lab Invest	Laboratory Investigation
Life Sci	Life Sciences
Mamm Genome	Mammalian Genome
Matrix Biol	Matrix Biology
Mech Ageing Dev	Mechanisms of Ageing and Development
Med Biol Eng Comput	Medical & Biological Engineering & Computing
Med Electron Microsc	Medical Electron Microscopy
Mol Cancer	Molecular Cancer
Mol Carcinog	Molecular Carcinogenesis
Mol Cell	Molecular Cell
Mol Cell Biochem	Molecular and Cellular Biochemistry
Mol Cell Biol	Molecular and Cellular Biology
Mol Endocrinol	Molecular Endocrinology
N Engl J Med	New England Journal of Medicine

Nat Cell Biol	Nature Cell Biology
Nat Commun	Nature Communications
Nat Genet	Nature Genetics
Nat Immun Cell Growth Regul	Natural Immunity and Cell Growth Regulation
Nat Rev Genet	Nature Reviews Genetics
Nephrol Dial Transplant	Nephrology Dialysis Transplantation
Nephron Exp Nephrol	Nephron Experimental Nephrology
Neurobiol Aging	Neurobiology of Aging
Neurosci Lett	Neuroscience Letters
Nucleic Acids Res	Nucleic Acids Research
Oncol Rep	Oncology Reports
Pflugers Arch	Pflugger Archive
Physiol Rev	Physiological Reviews
Proc Natl Acad Sci U S A	Proceedings of the National Academy of Sciences of the United States of America
Proc R. Soc. Lond.	Proceedings of the Royal Society London
Prog Biophys Mol Biol	Progress in Biophysics & Molecular Biology
Prog Brain Res	Progress in Brain Research
Q Rev Biol	Quarterly Review of Biology
Rejuvenation Res	Rejuvenation Research

Trans N Y Acad Sci

Transactions of the New York
Academy of Sciences

Thromb Res

Thrombosis Research

Toxicol Appl Pharmacol

Toxicology and Applied
Pharmacology

Trends Biochem Sci

Trends in Biochemical Sciences

Trends Genet

Trends in Genetics

Tumour Biol

Tumor Biology

Vopr Onkol

Voprosy Onkologii

Z Gerontol Geriatr

Zeitschrift für Gerontologie

REFERENCES

1. Hobbs, F. a. D. B. (1996) Bureau of the Census. Current population reports, Special Studies, P23-190, Sixty-Five Plus in the United States. Government Printing Office, Washington D.C.
2. Murphy, S., Xu, J., and Kochanek, K. (2013) Deaths: Final Data for 2010. in *National Vital Statistics Report*, U.S. Department of Health and Human Services, Center for Disease Control and Prevention, Hyattsville, MD
3. Medvedev, Z. A. (1990) An attempt at a rational classification of theories of ageing. *Biol Rev Camb Philos Soc* **65**, 375-398.
4. Haldane, J. B. S. (1941) *New Paths in Genetics*, Allen & Unwin, London, UK
5. P.B, M. (1952) *An Unsolved Problem of Biology*, H.K. Lewis & Co. LTD, London, UK
6. Williams, G. C. (1957) Pleiotropy, natural selection, and the evolution of senescence. *Evolution* **11**, 398-411.
7. Partridge, L., and Gems, D. (2002) Mechanisms of ageing: public or private? *Nat Rev Genet* **3**, 165-175.
8. Partridge L, P. N. a. P. P. (1999) Another set of responses and correlated responses to selection on age at reproduction in *Drosophila melanogaster*. *Proc. R. Soc. Lond. B* **266**, 255-261.
9. Rose, M., and Charlesworth, B. (1980) A test of evolutionary theories of senescence. *Nature* **287**, 141-142.
10. SN, A. (1993) Retarded senescence in an insular population of opossums. *Journal of Zoology* **229**, 695-708.
11. Kirkwood, T. B. (1977) Evolution of ageing. *Nature* **270**, 301-304.
12. Ackermann, M., Chao, L., Bergstrom, C. T., and Doebeli, M. (2007) On the evolutionary origin of aging. *Aging Cell* **6**, 235-244. 2049046

13. Gerschman, R., Gilbert, D. L., Nye, S. W., Dwyer, P., and Fenn, W. O. (1954) Oxygen poisoning and x-irradiation: a mechanism in common. *Science* **119**, 623-626.
14. Harman, D. (1956) Aging: a theory based on free radical and radiation chemistry. *J Gerontol* **11**, 298-300.
15. Harman, D. (1972) The biologic clock: the mitochondria? *J Am Geriatr Soc* **20**, 145-147.
16. Beckman, K. B., and Ames, B. N. (1998) The free radical theory of aging matures. *Physiol Rev* **78**, 547-581.
17. Chance, B., Sies, H., and Boveris, A. (1979) Hydroperoxide metabolism in mammalian organs. *Physiol Rev* **59**, 527-605.
18. Finkel, T., and Holbrook, N. J. (2000) Oxidants, oxidative stress and the biology of ageing. *Nature* **408**, 239-247.
19. RJ, M. (2003) Oxidants and antioxidants in aging. in *Physiological Basis of Aging and Geriatrics* (PS, T. ed.), 3rd Ed., CRC, Boca Raton, FL. pp 61-83
20. Stadtman, E. R. (1992) Protein oxidation and aging. *Science* **257**, 1220-1224.
21. Carney, J. M., Starke-Reed, P. E., Oliver, C. N., Landum, R. W., Cheng, M. S., Wu, J. F., and Floyd, R. A. (1991) Reversal of age-related increase in brain protein oxidation, decrease in enzyme activity, and loss in temporal and spatial memory by chronic administration of the spin-trapping compound N-tert-butyl-alpha-phenylnitron. *Proc Natl Acad Sci U S A* **88**, 3633-3636. 51506
22. Aksenova, M. V., Aksenov, M. Y., Carney, J. M., and Butterfield, D. A. (1998) Protein oxidation and enzyme activity decline in old brown Norway rats are reduced by dietary restriction. *Mech Ageing Dev* **100**, 157-168.
23. Hamilton, M. L., Van Remmen, H., Drake, J. A., Yang, H., Guo, Z. M., Kewitt, K., Walter, C. A., and Richardson, A. (2001) Does oxidative damage to DNA increase with age? *Proc Natl Acad Sci U S A* **98**, 10469-10474. 56984
24. Ames, B. N. (1989) Endogenous oxidative DNA damage, aging, and cancer. *Free Radic Res Commun* **7**, 121-128.

25. Agarwal, S., and Sohal, R. S. (1994) DNA oxidative damage and life expectancy in houseflies. *Proc Natl Acad Sci U S A* **91**, 12332-12335. 45431
26. Tower, J. (2000) Transgenic methods for increasing *Drosophila* life span. *Mech Ageing Dev* **118**, 1-14.
27. Sohal, R. S., Agarwal, A., Agarwal, S., and Orr, W. C. (1995) Simultaneous overexpression of copper- and zinc-containing superoxide dismutase and catalase retards age-related oxidative damage and increases metabolic potential in *Drosophila melanogaster*. *J Biol Chem* **270**, 15671-15674.
28. Arking, R., Burde, V., Graves, K., Hari, R., Feldman, E., Zeevi, A., Soliman, S., Saraiya, A., Buck, S., Vettraino, J., Sathrasala, K., Wehr, N., and Levine, R. L. (2000) Forward and reverse selection for longevity in *Drosophila* is characterized by alteration of antioxidant gene expression and oxidative damage patterns. *Exp Gerontol* **35**, 167-185.
29. Larsen, P. L. (1993) Aging and resistance to oxidative damage in *Caenorhabditis elegans*. *Proc Natl Acad Sci U S A* **90**, 8905-8909. 47469
30. Melov, S., Ravenscroft, J., Malik, S., Gill, M. S., Walker, D. W., Clayton, P. E., Wallace, D. C., Malfroy, B., Doctrow, S. R., and Lithgow, G. J. (2000) Extension of life-span with superoxide dismutase/catalase mimetics. *Science* **289**, 1567-1569.
31. Huang, T. T., Carlson, E. J., Gillespie, A. M., Shi, Y., and Epstein, C. J. (2000) Ubiquitous overexpression of CuZn superoxide dismutase does not extend life span in mice. *J Gerontol A Biol Sci Med Sci* **55**, B5-9.
32. Yu, B. P. (1999) Approaches to anti-aging intervention: the promises and the uncertainties. *Mech Ageing Dev* **111**, 73-87.
33. Bezlepkin, V. G., Sirota, N. P., and Gaziev, A. I. (1996) The prolongation of survival in mice by dietary antioxidants depends on their age by the start of feeding this diet. *Mech Ageing Dev* **92**, 227-234.
34. Barger, J. L., Kayo, T., Pugh, T. D., Prolla, T. A., and Weindruch, R. (2008) Short-term consumption of a resveratrol-containing nutraceutical mixture mimics gene expression of long-term caloric restriction in mouse heart. *Exp Gerontol* **43**, 859-866.
35. Hagen, T. M., Ingersoll, R. T., Wehr, C. M., Lykkesfeldt, J., Vinarsky, V., Bartholomew, J. C., Song, M. H., and Ames, B. N. (1998) Acetyl-L-

- carnitine fed to old rats partially restores mitochondrial function and ambulatory activity. *Proc Natl Acad Sci U S A* **95**, 9562-9566. 21378
36. Hagen, T. M., Wehr, C. M., and Ames, B. N. (1998) Mitochondrial decay in aging. Reversal through supplementation of acetyl-L-carnitine and N-tert-butyl-alpha-phenyl-nitrone. *Ann N Y Acad Sci* **854**, 214-223.
 37. Tatchum-Talom, R., and Martin, D. S. (2004) Tempol improves vascular function in the mesenteric vascular bed of senescent rats. *Can J Physiol Pharmacol* **82**, 200-207.
 38. Lee, C. K., Pugh, T. D., Klopp, R. G., Edwards, J., Allison, D. B., Weindruch, R., and Prolla, T. A. (2004) The impact of alpha-lipoic acid, coenzyme Q10 and caloric restriction on life span and gene expression patterns in mice. *Free Radic Biol Med* **36**, 1043-1057.
 39. Park, S. K., Kim, K., Page, G. P., Allison, D. B., Weindruch, R., and Prolla, T. A. (2009) Gene expression profiling of aging in multiple mouse strains: identification of aging biomarkers and impact of dietary antioxidants. *Aging Cell* **8**, 484-495. 2733852
 40. Stadtman, E. R. (2004) Role of oxidant species in aging. *Curr Med Chem* **11**, 1105-1112.
 41. Nunnari, J., and Suomalainen, A. (2012) Mitochondria: in sickness and in health. *Cell* **148**, 1145-1159.
 42. Richter, C., Park, J. W., and Ames, B. N. (1988) Normal oxidative damage to mitochondrial and nuclear DNA is extensive. *Proc Natl Acad Sci U S A* **85**, 6465-6467. 281993
 43. Alexeyev, M. F., Ledoux, S. P., and Wilson, G. L. (2004) Mitochondrial DNA and aging. *Clin Sci (Lond)* **107**, 355-364.
 44. Brand, F. N., Kiely, D. K., Kannel, W. B., and Myers, R. H. (1992) Family patterns of coronary heart disease mortality: the Framingham Longevity Study. *J Clin Epidemiol* **45**, 169-174.
 45. De Benedictis, G., Rose, G., Carrieri, G., De Luca, M., Falcone, E., Passarino, G., Bonafe, M., Monti, D., Baggio, G., Bertolini, S., Mari, D., Mattace, R., and Franceschi, C. (1999) Mitochondrial DNA inherited variants are associated with successful aging and longevity in humans. *FASEB J* **13**, 1532-1536.

46. Tanaka, M., Gong, J. S., Zhang, J., Yoneda, M., and Yagi, K. (1998) Mitochondrial genotype associated with longevity. *Lancet* **351**, 185-186.
47. Ivanova, R., Lepage, V., Charron, D., and Schachter, F. (1998) Mitochondrial genotype associated with French Caucasian centenarians. *Gerontology* **44**, 349.
48. Kujoth, G. C., Hiona, A., Pugh, T. D., Someya, S., Panzer, K., Wohlgemuth, S. E., Hofer, T., Seo, A. Y., Sullivan, R., Jobling, W. A., Morrow, J. D., Van Remmen, H., Sedivy, J. M., Yamasoba, T., Tanokura, M., Weindruch, R., Leeuwenburgh, C., and Prolla, T. A. (2005) Mitochondrial DNA mutations, oxidative stress, and apoptosis in mammalian aging. *Science* **309**, 481-484.
49. Trifunovic, A., Hansson, A., Wredenberg, A., Rovio, A. T., Dufour, E., Khvorostov, I., Spelbrink, J. N., Wibom, R., Jacobs, H. T., and Larsson, N. G. (2005) Somatic mtDNA mutations cause aging phenotypes without affecting reactive oxygen species production. *Proc Natl Acad Sci U S A* **102**, 17993-17998. 1312403
50. Trifunovic, A., Wredenberg, A., Falkenberg, M., Spelbrink, J. N., Rovio, A. T., Bruder, C. E., Bohlooly, Y. M., Gidlof, S., Oldfors, A., Wibom, R., Tornell, J., Jacobs, H. T., and Larsson, N. G. (2004) Premature ageing in mice expressing defective mitochondrial DNA polymerase. *Nature* **429**, 417-423.
51. Schriener, S. E., Linford, N. J., Martin, G. M., Treuting, P., Ogburn, C. E., Emond, M., Coskun, P. E., Ladiges, W., Wolf, N., Van Remmen, H., Wallace, D. C., and Rabinovitch, P. S. (2005) Extension of murine life span by overexpression of catalase targeted to mitochondria. *Science* **308**, 1909-1911.
52. Kanungo, M. S. (1975) A model for ageing. *J Theor Biol* **53**, 253-261.
53. Zou, S., Meadows, S., Sharp, L., Jan, L. Y., and Jan, Y. N. (2000) Genome-wide study of aging and oxidative stress response in *Drosophila melanogaster*. *Proc Natl Acad Sci U S A* **97**, 13726-13731. 17643
54. Lee, C. K., Klopp, R. G., Weindruch, R., and Prolla, T. A. (1999) Gene expression profile of aging and its retardation by caloric restriction. *Science* **285**, 1390-1393.
55. McCarroll, S. A., Murphy, C. T., Zou, S., Pletcher, S. D., Chin, C. S., Jan, Y. N., Kenyon, C., Bargmann, C. I., and Li, H. (2004) Comparing genomic

expression patterns across species identifies shared transcriptional profile in aging. *Nat Genet* **36**, 197-204.

56. Welle, S., Brooks, A. I., Delehanty, J. M., Needler, N., and Thornton, C. A. (2003) Gene expression profile of aging in human muscle. *Physiol Genomics* **14**, 149-159.
57. Weindruch, R., Kayo, T., Lee, C. K., and Prolla, T. A. (2002) Gene expression profiling of aging using DNA microarrays. *Mech Ageing Dev* **123**, 177-193.
58. Park, S. K., and Prolla, T. A. (2005) Lessons learned from gene expression profile studies of aging and caloric restriction. *Ageing Res Rev* **4**, 55-65.
59. Pletcher, S. D., Macdonald, S. J., Marguerie, R., Certa, U., Stearns, S. C., Goldstein, D. B., and Partridge, L. (2002) Genome-wide transcript profiles in aging and calorically restricted *Drosophila melanogaster*. *Curr Biol* **12**, 712-723.
60. Duce, J. A., Podvin, S., Hollander, W., Kipling, D., Rosene, D. L., and Abraham, C. R. (2008) Gene profile analysis implicates *Klotho* as an important contributor to aging changes in brain white matter of the rhesus monkey. *Glia* **56**, 106-117. 17963266
61. Kirkwood, T. B. (2002) New science for an old problem. *Trends Genet* **18**, 441-442.
62. Herskind, A. M., McGue, M., Holm, N. V., Sorensen, T. I., Harvald, B., and Vaupel, J. W. (1996) The heritability of human longevity: a population-based study of 2872 Danish twin pairs born 1870-1900. *Hum Genet* **97**, 319-323.
63. Ljungquist, B., Berg, S., Lanke, J., McClearn, G. E., and Pedersen, N. L. (1998) The effect of genetic factors for longevity: a comparison of identical and fraternal twins in the Swedish Twin Registry. *J Gerontol A Biol Sci Med Sci* **53**, M441-446.
64. Gudmundsson, H., Gudbjartsson, D. F., Frigge, M., Gulcher, J. R., and Stefansson, K. (2000) Inheritance of human longevity in Iceland. *Eur J Hum Genet* **8**, 743-749.
65. Heijmans, B. T., Westendorp, R. G., and Slagboom, P. E. (2000) Common gene variants, mortality and extreme longevity in humans. *Exp Gerontol* **35**, 865-877.

66. Perls, T., Shea-Drinkwater, M., Bowen-Flynn, J., Ridge, S. B., Kang, S., Joyce, E., Daly, M., Brewster, S. J., Kunkel, L., and Puca, A. A. (2000) Exceptional familial clustering for extreme longevity in humans. *J Am Geriatr Soc* **48**, 1483-1485.
67. Puca, A. A., Daly, M. J., Brewster, S. J., Matise, T. C., Barrett, J., Shea-Drinkwater, M., Kang, S., Joyce, E., Nicoli, J., Benson, E., Kunkel, L. M., and Perls, T. (2001) A genome-wide scan for linkage to human exceptional longevity identifies a locus on chromosome 4. *Proc Natl Acad Sci U S A* **98**, 10505-10508. 56990
68. Myung, K., Datta, A., Chen, C., and Kolodner, R. D. (2001) SGS1, the *Saccharomyces cerevisiae* homologue of BLM and WRN, suppresses genome instability and homeologous recombination. *Nat Genet* **27**, 113-116.
69. Yamagata, K., Kato, J., Shimamoto, A., Goto, M., Furuichi, Y., and Ikeda, H. (1998) Bloom's and Werner's syndrome genes suppress hyperrecombination in yeast *sgs1* mutant: implication for genomic instability in human diseases. *Proc Natl Acad Sci U S A* **95**, 8733-8738. 21145
70. Vogel, H., Lim, D. S., Karsenty, G., Finegold, M., and Hasty, P. (1999) Deletion of Ku86 causes early onset of senescence in mice. *Proc Natl Acad Sci U S A* **96**, 10770-10775. 17958
71. Difilippantonio, M. J., Zhu, J., Chen, H. T., Meffre, E., Nussenzweig, M. C., Max, E. E., Ried, T., and Nussenzweig, A. (2000) DNA repair protein Ku80 suppresses chromosomal aberrations and malignant transformation. *Nature* **404**, 510-514.
72. Vanyushin, B. F., Tkacheva, S. G., and Belozersky, A. N. (1970) Rare bases in animal DNA. *Nature* **225**, 948-949.
73. Vanyushin, B. F., Nemirovsky, L. E., Klimenko, V. V., Vasiliev, V. K., and Belozersky, A. N. (1973) The 5-methylcytosine in DNA of rats. Tissue and age specificity and the changes induced by hydrocortisone and other agents. *Gerontologia* **19**, 138-152.
74. Wilson, V. L., Smith, R. A., Ma, S., and Cutler, R. G. (1987) Genomic 5-methyldeoxycytidine decreases with age. *J Biol Chem* **262**, 9948-9951.
75. Barbot, W., Dupressoir, A., Lazar, V., and Heidmann, T. (2002) Epigenetic regulation of an IAP retrotransposon in the aging mouse: progressive

- demethylation and de-silencing of the element by its repetitive induction. *Nucleic Acids Res* **30**, 2365-2373. 117196
76. Slagboom, P. E., de Leeuw, W. J., and Vijg, J. (1990) Messenger RNA levels and methylation patterns of GAPDH and beta-actin genes in rat liver, spleen and brain in relation to aging. *Mech Ageing Dev* **53**, 243-257.
 77. Ono, T., Uehara, Y., Kurishita, A., Tawa, R., and Sakurai, H. (1993) Biological significance of DNA methylation in the ageing process. *Age Ageing* **22**, S34-43.
 78. Takatsu, M., Uyeno, S., Komura, J., Watanabe, M., and Ono, T. (1999) Age-dependent alterations in mRNA level and promoter methylation of collagen alpha1(I) gene in human periodontal ligament. *Mech Ageing Dev* **110**, 37-48.
 79. Issa, J. P. (2000) CpG-island methylation in aging and cancer. *Curr Top Microbiol Immunol* **249**, 101-118.
 80. Richardson, B. (2003) Impact of aging on DNA methylation. *Ageing Res Rev* **2**, 245-261.
 81. Kenyon, C., Chang, J., Gensch, E., Rudner, A., and Tabtiang, R. (1993) A *C. elegans* mutant that lives twice as long as wild type. *Nature* **366**, 461-464.
 82. Holzenberger, M., Dupont, J., Ducos, B., Leneuve, P., Geloën, A., Even, P. C., Cervera, P., and Le Bouc, Y. (2003) IGF-1 receptor regulates lifespan and resistance to oxidative stress in mice. *Nature* **421**, 182-187.
 83. Migliaccio, E., Giorgio, M., Mele, S., Pelicci, G., Reboldi, P., Pandolfi, P. P., Lanfrancone, L., and Pelicci, P. G. (1999) The p66shc adaptor protein controls oxidative stress response and life span in mammals. *Nature* **402**, 309-313.
 84. Salih, D. A., and Brunet, A. (2008) FoxO transcription factors in the maintenance of cellular homeostasis during aging. *Curr Opin Cell Biol* **20**, 126-136. 2387118
 85. Nelson, J. F., Strong, R., Bokov, A., Diaz, V., and Ward, W. (2012) Probing the relationship between insulin sensitivity and longevity using genetically modified mice. *J Gerontol A Biol Sci Med Sci* **67**, 1332-1338. 3670160
 86. Hayflick, L., and Moorhead, P. S. (1961) The serial cultivation of human diploid cell strains. *Exp Cell Res* **25**, 585-621.

87. Todaro, G. J., Wolman, S. R., and Green, H. (1963) Rapid Transformation of Human Fibroblasts with Low Growth Potential into Established Cell Lines by Sv40. *J Cell Physiol* **62**, 257-265.
88. Lingner, J., Hughes, T. R., Shevchenko, A., Mann, M., Lundblad, V., and Cech, T. R. (1997) Reverse transcriptase motifs in the catalytic subunit of telomerase. *Science* **276**, 561-567.
89. d'Adda di Fagagna, F., Reaper, P. M., Clay-Farrace, L., Fiegler, H., Carr, P., Von Zglinicki, T., Saretzki, G., Carter, N. P., and Jackson, S. P. (2003) A DNA damage checkpoint response in telomere-initiated senescence. *Nature* **426**, 194-198.
90. Takai, H., Smogorzewska, A., and de Lange, T. (2003) DNA damage foci at dysfunctional telomeres. *Curr Biol* **13**, 1549-1556.
91. Herbig, U., Jobling, W. A., Chen, B. P., Chen, D. J., and Sedivy, J. M. (2004) Telomere shortening triggers senescence of human cells through a pathway involving ATM, p53, and p21(CIP1), but not p16(INK4a). *Mol Cell* **14**, 501-513.
92. Rodier, F., Coppe, J. P., Patil, C. K., Hoeijmakers, W. A., Munoz, D. P., Raza, S. R., Freund, A., Campeau, E., Davalos, A. R., and Campisi, J. (2009) Persistent DNA damage signalling triggers senescence-associated inflammatory cytokine secretion. *Nat Cell Biol* **11**, 973-979. 2743561
93. Rodier, F., Munoz, D. P., Teachenor, R., Chu, V., Le, O., Bhaumik, D., Coppe, J. P., Campeau, E., Beausejour, C. M., Kim, S. H., Davalos, A. R., and Campisi, J. (2011) DNA-SCARS: distinct nuclear structures that sustain damage-induced senescence growth arrest and inflammatory cytokine secretion. *J Cell Sci* **124**, 68-81. 3001408
94. Bodnar, A. G., Ouellette, M., Frolkis, M., Holt, S. E., Chiu, C. P., Morin, G. B., Harley, C. B., Shay, J. W., Lichtsteiner, S., and Wright, W. E. (1998) Extension of life-span by introduction of telomerase into normal human cells. *Science* **279**, 349-352.
95. Vaziri, H., and Benchimol, S. (1998) Reconstitution of telomerase activity in normal human cells leads to elongation of telomeres and extended replicative life span. *Curr Biol* **8**, 279-282.
96. Funk, W. D., Wang, C. K., Shelton, D. N., Harley, C. B., Pagon, G. D., and Hoeffler, W. K. (2000) Telomerase expression restores dermal integrity to

- in vitro-aged fibroblasts in a reconstituted skin model. *Exp Cell Res* **258**, 270-278.
97. Ramirez, R. D., Morales, C. P., Herbert, B. S., Rohde, J. M., Passons, C., Shay, J. W., and Wright, W. E. (2001) Putative telomere-independent mechanisms of replicative aging reflect inadequate growth conditions. *Genes Dev* **15**, 398-403. 312628
 98. Fusenig, N. E., and Boukamp, P. (1998) Multiple stages and genetic alterations in immortalization, malignant transformation, and tumor progression of human skin keratinocytes. *Mol Carcinog* **23**, 144-158.
 99. Yaswen, P., and Stampfer, M. R. (2002) Molecular changes accompanying senescence and immortalization of cultured human mammary epithelial cells. *Int J Biochem Cell Biol* **34**, 1382-1394.
 100. Parrinello, S., Samper, E., Krtolica, A., Goldstein, J., Melov, S., and Campisi, J. (2003) Oxygen sensitivity severely limits the replicative lifespan of murine fibroblasts. *Nat Cell Biol* **5**, 741-747.
 101. von Zglinicki, T. (2002) Oxidative stress shortens telomeres. *Trends Biochem Sci* **27**, 339-344.
 102. Tyner, S. D., Venkatachalam, S., Choi, J., Jones, S., Ghebranious, N., Igelmann, H., Lu, X., Soron, G., Cooper, B., Brayton, C., Park, S. H., Thompson, T., Karsenty, G., Bradley, A., and Donehower, L. A. (2002) p53 mutant mice that display early ageing-associated phenotypes. *Nature* **415**, 45-53.
 103. Maier, B., Gluba, W., Bernier, B., Turner, T., Mohammad, K., Guise, T., Sutherland, A., Thorner, M., and Scrable, H. (2004) Modulation of mammalian life span by the short isoform of p53. *Genes Dev* **18**, 306-319. 338283
 104. Itahana, K., Dimri, G., and Campisi, J. (2001) Regulation of cellular senescence by p53. *Eur J Biochem* **268**, 2784-2791.
 105. Ide, T., Tsuji, Y., Ishibashi, S., and Mitsui, Y. (1983) Reinitiation of host DNA synthesis in senescent human diploid cells by infection with Simian virus 40. *Exp Cell Res* **143**, 343-349.
 106. Shay, J. W., Pereira-Smith, O. M., and Wright, W. E. (1991) A role for both RB and p53 in the regulation of human cellular senescence. *Exp Cell Res* **196**, 33-39.

107. Shay, J. W., Wright, W. E., Brasiskyte, D., and Van der Haegen, B. A. (1993) E6 of human papillomavirus type 16 can overcome the M1 stage of immortalization in human mammary epithelial cells but not in human fibroblasts. *Oncogene* **8**, 1407-1413.
108. Hinkal, G. W., Gatza, C. E., Parikh, N., and Donehower, L. A. (2009) Altered senescence, apoptosis, and DNA damage response in a mutant p53 model of accelerated aging. *Mech Ageing Dev* **130**, 262-271. 2722837
109. Keyes, W. M., Wu, Y., Vogel, H., Guo, X., Lowe, S. W., and Mills, A. A. (2005) p63 deficiency activates a program of cellular senescence and leads to accelerated aging. *Genes Dev* **19**, 1986-1999. 1199570
110. Baker, D. J., Perez-Terzic, C., Jin, F., Pitel, K. S., Niederlander, N. J., Jeganathan, K., Yamada, S., Reyes, S., Rowe, L., Hiddinga, H. J., Eberhardt, N. L., Terzic, A., and van Deursen, J. M. (2008) Opposing roles for p16Ink4a and p19Arf in senescence and ageing caused by BubR1 insufficiency. *Nat Cell Biol* **10**, 825-836. 2594014
111. Varela, I., Cadinanos, J., Pendas, A. M., Gutierrez-Fernandez, A., Folgueras, A. R., Sanchez, L. M., Zhou, Z., Rodriguez, F. J., Stewart, C. L., Vega, J. A., Tryggvason, K., Freije, J. M., and Lopez-Otin, C. (2005) Accelerated ageing in mice deficient in Zmpste24 protease is linked to p53 signalling activation. *Nature* **437**, 564-568.
112. Hayflick, L. (1965) The Limited in Vitro Lifetime of Human Diploid Cell Strains. *Exp Cell Res* **37**, 614-636.
113. Martin, G. M., Sprague, C. A., and Epstein, C. J. (1970) Replicative life-span of cultivated human cells. Effects of donor's age, tissue, and genotype. *Lab Invest* **23**, 86-92.
114. Lipman, R. D., and Taylor, A. (1987) The in vitro replicative potential and cellular morphology of human lens epithelial cells derived from different aged donors. *Curr Eye Res* **6**, 1453-1457.
115. Gilchrist, B. A. (1979) Relationship between actinic damage and chronologic aging in keratinocyte cultures of human skin. *J Invest Dermatol* **72**, 219-223.
116. Blake, D. A., Yu, H., Young, D. L., and Caldwell, D. R. (1997) Matrix stimulates the proliferation of human corneal endothelial cells in culture. *Invest Ophthalmol Vis Sci* **38**, 1119-1129.

117. Perillo, N. L., Walford, R. L., Newman, M. A., and Effros, R. B. (1989) Human T lymphocytes possess a limited in vitro life span. *Exp Gerontol* **24**, 177-187.
118. Faragher, R. G., and Kipling, D. (1998) How might replicative senescence contribute to human ageing? *Bioessays* **20**, 985-991.
119. Loffredo, F. S., Steinhauser, M. L., Jay, S. M., Gannon, J., Pancoast, J. R., Yalamanchi, P., Sinha, M., Dall'Osso, C., Khong, D., Shadrach, J. L., Miller, C. M., Singer, B. S., Stewart, A., Psychogios, N., Gerszten, R. E., Hartigan, A. J., Kim, M. J., Serwold, T., Wagers, A. J., and Lee, R. T. (2013) Growth differentiation factor 11 is a circulating factor that reverses age-related cardiac hypertrophy. *Cell* **153**, 828-839. 3677132
120. Villeda, S. A., Luo, J., Mosher, K. I., Zou, B., Britschgi, M., Bieri, G., Stan, T. M., Fainberg, N., Ding, Z., Eggel, A., Lucin, K. M., Czirr, E., Park, J. S., Couillard-Despres, S., Aigner, L., Li, G., Peskind, E. R., Kaye, J. A., Quinn, J. F., Galasko, D. R., Xie, X. S., Rando, T. A., and Wyss-Coray, T. (2011) The ageing systemic milieu negatively regulates neurogenesis and cognitive function. *Nature* **477**, 90-94. 3170097
121. Jiang, H., Ju, Z., and Rudolph, K. L. (2007) Telomere shortening and ageing. *Z Gerontol Geriatr* **40**, 314-324.
122. Shay, J. W., and Bacchetti, S. (1997) A survey of telomerase activity in human cancer. *Eur J Cancer* **33**, 787-791.
123. Harley, C. B., Futcher, A. B., and Greider, C. W. (1990) Telomeres shorten during ageing of human fibroblasts. *Nature* **345**, 458-460.
124. Dimri, G. P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E. E., Linskens, M., Rubelj, I., Pereira-Smith, O., and et al. (1995) A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc Natl Acad Sci U S A* **92**, 9363-9367. 40985
125. Franceschi, C. (1989) Cell proliferation, cell death and aging. *Aging (Milano)* **1**, 3-15.
126. Kirkwood, T. B., and Franceschi, C. (1992) Is aging as complex as it would appear? New perspectives in aging research. *Ann NY Acad Sci* **663**, 412-417.
127. Franceschi, C., Bonafe, M., Valensin, S., Olivieri, F., De Luca, M., Ottaviani, E., and De Benedictis, G. (2000) Inflamm-aging. An

- evolutionary perspective on immunosenescence. *Ann N Y Acad Sci* **908**, 244-254.
128. Swift, M. E., Burns, A. L., Gray, K. L., and DiPietro, L. A. (2001) Age-related alterations in the inflammatory response to dermal injury. *J Invest Dermatol* **117**, 1027-1035.
 129. Ortega, E., Garcia, J. J., and De la Fuente, M. (2000) Modulation of adherence and chemotaxis of macrophages by norepinephrine. Influence of ageing. *Mol Cell Biochem* **203**, 113-117.
 130. Antonini, J. M., Roberts, J. R., Clarke, R. W., Yang, H. M., Barger, M. W., Ma, J. Y., and Weissman, D. N. (2001) Effect of age on respiratory defense mechanisms: pulmonary bacterial clearance in Fischer 344 rats after intratracheal instillation of *Listeria monocytogenes*. *Chest* **120**, 240-249.
 131. Bradley, S. F., and Kauffman, C. A. (1990) Aging and the response to *Salmonella* infection. *Exp Gerontol* **25**, 75-80.
 132. Seth, A., Nagarkatti, M., Nagarkatti, P. S., Subbarao, B., and Udhayakumar, V. (1990) Macrophages but not B cells from aged mice are defective in stimulating autoreactive T cells in vitro. *Mech Ageing Dev* **52**, 107-124.
 133. Zissel, G., Schlaak, M., and Muller-Quernheim, J. (1999) Age-related decrease in accessory cell function of human alveolar macrophages. *J Investig Med* **47**, 51-56.
 134. Stout, R. D., and Suttles, J. (2005) Immunosenescence and macrophage functional plasticity: dysregulation of macrophage function by age-associated microenvironmental changes. *Immunol Rev* **205**, 60-71. 1201508
 135. Colonna-Romano, G., Aquino, A., Bulati, M., Di Lorenzo, G., Listi, F., Vitello, S., Lio, D., Candore, G., Clesi, G., and Caruso, C. (2006) Memory B cell subpopulations in the aged. *Rejuvenation Res* **9**, 149-152.
 136. Lazuardi, L., Jenewein, B., Wolf, A. M., Pfister, G., Tzankov, A., and Grubeck-Loebenstien, B. (2005) Age-related loss of naive T cells and dysregulation of T-cell/B-cell interactions in human lymph nodes. *Immunology* **114**, 37-43. 1782064
 137. Tsukamoto, H., Clise-Dwyer, K., Huston, G. E., Duso, D. K., Buck, A. L., Johnson, L. L., Haynes, L., and Swain, S. L. (2009) Age-associated

- increase in lifespan of naive CD4 T cells contributes to T-cell homeostasis but facilitates development of functional defects. *Proc Natl Acad Sci U S A* **106**, 18333-18338. 2759371
138. Gregg, R., Smith, C. M., Clark, F. J., Dunnion, D., Khan, N., Chakraverty, R., Nayak, L., and Moss, P. A. (2005) The number of human peripheral blood CD4+ CD25high regulatory T cells increases with age. *Clin Exp Immunol* **140**, 540-546. 1809384
 139. Wayne, S. J., Rhyne, R. L., Garry, P. J., and Goodwin, J. S. (1990) Cell-mediated immunity as a predictor of morbidity and mortality in subjects over 60. *J Gerontol* **45**, M45-48.
 140. Sansoni, P., Cossarizza, A., Brianti, V., Fagnoni, F., Snelli, G., Monti, D., Marcato, A., Passeri, G., Ortolani, C., Forti, E., and et al. (1993) Lymphocyte subsets and natural killer cell activity in healthy old people and centenarians. *Blood* **82**, 2767-2773.
 141. Levy, S. M., Herberman, R. B., Lee, J., Whiteside, T., Beadle, M., Heiden, L., and Simons, A. (1991) Persistently low natural killer cell activity, age, and environmental stress as predictors of infectious morbidity. *Nat Immun Cell Growth Regul* **10**, 289-307.
 142. Giuliani, N., Sansoni, P., Girasole, G., Vescovini, R., Passeri, G., Passeri, M., and Pedrazzoni, M. (2001) Serum interleukin-6, soluble interleukin-6 receptor and soluble gp130 exhibit different patterns of age- and menopause-related changes. *Exp Gerontol* **36**, 547-557.
 143. Sansoni, P., Vescovini, R., Fagnoni, F., Biasini, C., Zanni, F., Zanlari, L., Telera, A., Lucchini, G., Passeri, G., Monti, D., Franceschi, C., and Passeri, M. (2008) The immune system in extreme longevity. *Exp Gerontol* **43**, 61-65.
 144. De la Fuente, M. (2002) Effects of antioxidants on immune system ageing. *Eur J Clin Nutr* **56 Suppl 3**, S5-8.
 145. Libby, P. (2006) Inflammation and cardiovascular disease mechanisms. *Am J Clin Nutr* **83**, 456S-460S.
 146. Coussens, L. M., and Werb, Z. (2002) Inflammation and cancer. *Nature* **420**, 860-867. 2803035
 147. Lee, Y. J., Han, S. B., Nam, S. Y., Oh, K. W., and Hong, J. T. (2010) Inflammation and Alzheimer's disease. *Arch Pharm Res* **33**, 1539-1556.

148. Oberg, B. P., McMenamin, E., Lucas, F. L., McMonagle, E., Morrow, J., Ikizler, T. A., and Himmelfarb, J. (2004) Increased prevalence of oxidant stress and inflammation in patients with moderate to severe chronic kidney disease. *Kidney Int* **65**, 1009-1016.
149. O'Mahony, L., Holland, J., Jackson, J., Feighery, C., Hennessy, T. P., and Mealy, K. (1998) Quantitative intracellular cytokine measurement: age-related changes in proinflammatory cytokine production. *Clin Exp Immunol* **113**, 213-219. 1905038
150. Kiecolt-Glaser, J. K., Preacher, K. J., MacCallum, R. C., Atkinson, C., Malarkey, W. B., and Glaser, R. (2003) Chronic stress and age-related increases in the proinflammatory cytokine IL-6. *Proc Natl Acad Sci U S A* **100**, 9090-9095. 166443
151. Campuzano, O., Castillo-Ruiz, M. M., Acarin, L., Castellano, B., and Gonzalez, B. (2009) Increased levels of proinflammatory cytokines in the aged rat brain attenuate injury-induced cytokine response after excitotoxic damage. *J Neurosci Res* **87**, 2484-2497.
152. Csiszar, A., Ungvari, Z., Koller, A., Edwards, J. G., and Kaley, G. (2003) Aging-induced proinflammatory shift in cytokine expression profile in coronary arteries. *FASEB J* **17**, 1183-1185.
153. Lio, D., Scola, L., Crivello, A., Colonna-Romano, G., Candore, G., Bonafe, M., Cavallone, L., Marchegiani, F., Olivieri, F., Franceschi, C., and Caruso, C. (2003) Inflammation, genetics, and longevity: further studies on the protective effects in men of IL-10 -1082 promoter SNP and its interaction with TNF-alpha -308 promoter SNP. *J Med Genet* **40**, 296-299. 1735442
154. Bonafe, M., Olivieri, F., Cavallone, L., Giovagnetti, S., Mayegiani, F., Cardelli, M., Pieri, C., Marra, M., Antonicelli, R., Lisa, R., Rizzo, M. R., Paolisso, G., Monti, D., and Franceschi, C. (2001) A gender--dependent genetic predisposition to produce high levels of IL-6 is detrimental for longevity. *Eur J Immunol* **31**, 2357-2361.
155. Lio, D., Scola, L., Crivello, A., Bonafe, M., Franceschi, C., Olivieri, F., Colonna-Romano, G., Candore, G., and Caruso, C. (2002) Allele frequencies of +874T-->A single nucleotide polymorphism at the first intron of interferon-gamma gene in a group of Italian centenarians. *Exp Gerontol* **37**, 315-319.
156. Lio, D., Scola, L., Crivello, A., Colonna-Romano, G., Candore, G., Bonafe, M., Cavallone, L., Franceschi, C., and Caruso, C. (2002) Gender-specific

association between -1082 IL-10 promoter polymorphism and longevity. *Genes Immun* **3**, 30-33.

157. Weinert, B. T., and Timiras, P. S. (2003) Invited review: Theories of aging. *J Appl Physiol* **95**, 1706-1716.
158. Everitt, A. V. (1973) The hypothalamic-pituitary control of ageing and age-related pathology. *Exp Gerontol* **8**, 265-277.
159. Samorajski, T. (1977) Central neurotransmitter substances and aging: a review. *J Am Geriatr Soc* **25**, 337-348.
160. Denckla, W. D. (1975) A time to die. *Life Sci* **16**, 31-44.
161. Quay, W. B. (1972) Pineal homeostatic regulation of shifts in the circadian activity rhythm during maturation and aging. *Trans N Y Acad Sci* **34**, 239-254.
162. FM, B. (1974) *The Biology of Aging*, Oxford University Press, Oxford, UK
163. McGeer, P. L., Hattori, T., and McGeer, E. G. (1975) Chemical and autoradiographic analysis of gamma-aminobutyric acid transport in Purkinje cells of the cerebellum. *Exp Neurol* **47**, 26-41.
164. Ordy, J. M., and Schjeide, O. A. (1973) Univariate and multivariate models for evaluating long-term changes in neurobiological development, maturity and aging. *Prog Brain Res* **40**, 25-51.
165. Miller, A. E., Shaar, C. J., and Riegler, G. D. (1976) Aging effects on hypothalamic dopamine and norepinephrine content in the male rat. *Exp Aging Res* **2**, 475-480.
166. Simpkins, J. W., Mueller, G. P., Huang, H. H., and Meites, J. (1977) Evidence for depressed catecholamine and enhanced serotonin metabolism in aging male rats: possible relation to gonadotropin secretion. *Endocrinology* **100**, 1672-1678.
167. Pecile, A., Muller, E., Falconi, G., and Martini, L. (1965) Growth hormone-releasing activity of hypothalamic extracts at different ages. *Endocrinology* **77**, 241-246.
168. Clemens, J. A., and Meites, J. (1971) Neuroendocrine status of old constant-estrous rats. *Neuroendocrinology* **7**, 249-256.

169. Frolkis, V. V., Bezrukov, V. V., Duplenko, Y. K., and Genis, E. D. (1972) The hypothalamus in aging. *Exp Gerontol* **7**, 169-184.
170. Dilman, V. M. (1971) Age-associated elevation of hypothalamic, threshold to feedback control, and its role in development, ageing, and disease. *Lancet* **1**, 1211-1219.
171. Dilman, V. (1976) The hypothalamic control of aging and age-associated pathology. The elevation mechanism of aging. in *Hypothalamus, pituitary and aging* (Burgess, A. E. J. ed.), Thomas, Springfield, Ill. pp 634-667
172. Finch, C. E. (1976) The regulation of physiological changes during mammalian aging. *Q Rev Biol* **51**, 49-83.
173. Riegle, G. D., Meites, J., Miller, A. E., and Wood, S. M. (1977) Effect of aging on hypothalamic LH-releasing and prolactin inhibiting activities and pituitary responsiveness to LHRH in the male laboratory rat. *J Gerontol* **32**, 13-18.
174. Aschheim, P. (1976) Aging in the hypothalamic-hypophyseal ovarian axis in the rat. in *Hypothalamus, pituitary and aging* (JA, E. A. a. B. ed.), Thomas, Springfield, Ill. pp 376-418
175. Rosen, C. J. (2000) Growth hormone and aging. *Endocrine* **12**, 197-201.
176. Mortola, J. F., and Yen, S. S. (1990) The effects of oral dehydroepiandrosterone on endocrine-metabolic parameters in postmenopausal women. *J Clin Endocrinol Metab* **71**, 696-704.
177. Nestler, J. E., Barlascini, C. O., Clore, J. N., and Blackard, W. G. (1988) Dehydroepiandrosterone reduces serum low density lipoprotein levels and body fat but does not alter insulin sensitivity in normal men. *J Clin Endocrinol Metab* **66**, 57-61.
178. Morales, A. J., Nolan, J. J., Nelson, J. C., and Yen, S. S. (1994) Effects of replacement dose of dehydroepiandrosterone in men and women of advancing age. *J Clin Endocrinol Metab* **78**, 1360-1367.
179. Morales, A. J., Haubrich, R. H., Hwang, J. Y., Asakura, H., and Yen, S. S. (1998) The effect of six months treatment with a 100 mg daily dose of dehydroepiandrosterone (DHEA) on circulating sex steroids, body composition and muscle strength in age-advanced men and women. *Clin Endocrinol (Oxf)* **49**, 421-432.

180. Rudman, D., Feller, A. G., Nagraj, H. S., Gergans, G. A., Lalitha, P. Y., Goldberg, A. F., Schlenker, R. A., Cohn, L., Rudman, I. W., and Mattson, D. E. (1990) Effects of human growth hormone in men over 60 years old. *N Engl J Med* **323**, 1-6.
181. Harman, S. M., and Blackman, M. R. (2004) Use of growth hormone for prevention or treatment of effects of aging. *J Gerontol A Biol Sci Med Sci* **59**, 652-658.
182. Anisimov, V. N., Zavarzina, N. Y., Zabezhinski, M. A., Popovich, I. G., Zimina, O. A., Shtylick, A. V., Arutjunyan, A. V., Oparina, T. I., Prokopenko, V. M., Mikhalski, A. I., and Yashin, A. I. (2001) Melatonin increases both life span and tumor incidence in female CBA mice. *J Gerontol A Biol Sci Med Sci* **56**, B311-323.
183. Anisimov, V. N., Zavarzina, N., Zabezhinskii, M. A., Popovich, I. G., Anikin, I. V., Zimina, O. A., Solov'ev, M. V., Shtylik, A. V., Arutiunian, A. V., Oparina, T. I., Prokopenko, V. M., and Khavinson, V. (2000) [The effect of melatonin on the indices of biological age, on longevity and on the development of spontaneous tumors in mice]. *Vopr Onkol* **46**, 311-319.
184. Kawas, C., Resnick, S., Morrison, A., Brookmeyer, R., Corrada, M., Zonderman, A., Bacal, C., Lingle, D. D., and Metter, E. (1997) A prospective study of estrogen replacement therapy and the risk of developing Alzheimer's disease: the Baltimore Longitudinal Study of Aging. *Neurology* **48**, 1517-1521.
185. Ettinger, B., Genant, H. K., and Cann, C. E. (1985) Long-term estrogen replacement therapy prevents bone loss and fractures. *Ann Intern Med* **102**, 319-324.
186. Henderson, B. E., Paganini-Hill, A., and Ross, R. K. (1991) Decreased mortality in users of estrogen replacement therapy. *Arch Intern Med* **151**, 75-78.
187. Steinberg, K. K., Thacker, S. B., Smith, S. J., Stroup, D. F., Zack, M. M., Flanders, W. D., and Berkelman, R. L. (1991) A meta-analysis of the effect of estrogen replacement therapy on the risk of breast cancer. *JAMA* **265**, 1985-1990.
188. Grady, D., Wenger, N. K., Herrington, D., Khan, S., Furberg, C., Hunninghake, D., Vittinghoff, E., and Hulley, S. (2000) Postmenopausal hormone therapy increases risk for venous thromboembolic disease. The

- Heart and Estrogen/progestin Replacement Study. *Ann Intern Med* **132**, 689-696.
189. Lacey, J. V., Jr., Mink, P. J., Lubin, J. H., Sherman, M. E., Troisi, R., Hartge, P., Schatzkin, A., and Schairer, C. (2002) Menopausal hormone replacement therapy and risk of ovarian cancer. *JAMA* **288**, 334-341.
 190. Blalock, J. E. (1989) A molecular basis for bidirectional communication between the immune and neuroendocrine systems. *Physiol Rev* **69**, 1-32.
 191. Turnbull, A. V., and Rivier, C. L. (1999) Regulation of the hypothalamic-pituitary-adrenal axis by cytokines: actions and mechanisms of action. *Physiol Rev* **79**, 1-71.
 192. Besedovsky, H., Sorkin, E., Felix, D., and Haas, H. (1977) Hypothalamic changes during the immune response. *Eur J Immunol* **7**, 323-325.
 193. McEwen, B. S., Biron, C. A., Brunson, K. W., Bulloch, K., Chambers, W. H., Dhabhar, F. S., Goldfarb, R. H., Kitson, R. P., Miller, A. H., Spencer, R. L., and Weiss, J. M. (1997) The role of adrenocorticoids as modulators of immune function in health and disease: neural, endocrine and immune interactions. *Brain Res Brain Res Rev* **23**, 79-133.
 194. Knudsen, P. J., Dinarello, C. A., and Strom, T. B. (1987) Glucocorticoids inhibit transcriptional and post-transcriptional expression of interleukin 1 in U937 cells. *J Immunol* **139**, 4129-4134.
 195. Lew, W., Oppenheim, J. J., and Matsushima, K. (1988) Analysis of the suppression of IL-1 alpha and IL-1 beta production in human peripheral blood mononuclear adherent cells by a glucocorticoid hormone. *J Immunol* **140**, 1895-1902.
 196. Giunta, S. (2008) Exploring the complex relations between inflammation and aging (inflamm-aging): anti-inflamm-aging remodelling of inflamm-aging, from robustness to frailty. *Inflamm Res* **57**, 558-563.
 197. Rowe, J. W., and Kahn, R. L. (1997) Successful aging. *Gerontologist* **37**, 433-440.
 198. Kuro-o, M., Matsumura, Y., Aizawa, H., Kawaguchi, H., Suga, T., Utsugi, T., Ohyama, Y., Kurabayashi, M., Kaname, T., Kume, E., Iwasaki, H., Iida, A., Shiraki-Iida, T., Nishikawa, S., Nagai, R., and Nabeshima, Y. I. (1997) Mutation of the mouse klotho gene leads to a syndrome resembling ageing. *Nature* **390**, 45-51. 9363890

199. Matsumura, Y., Aizawa, H., Shiraki-Iida, T., Nagai, R., Kuro-o, M., and Nabeshima, Y. (1998) Identification of the human klotho gene and its two transcripts encoding membrane and secreted klotho protein. *Biochem Biophys Res Commun* **242**, 626-630.
200. Shiraki-Iida, T., Aizawa, H., Matsumura, Y., Sekine, S., Iida, A., Anazawa, H., Nagai, R., Kuro-o, M., and Nabeshima, Y. (1998) Structure of the mouse klotho gene and its two transcripts encoding membrane and secreted protein. *FEBS Lett* **424**, 6-10.
201. NM_153683.2. in *Nucleotide Database*, National Center for Biotechnology Information
202. Grabnitz, F., Seiss, M., Rucknagel, K. P., and Staudenbauer, W. L. (1991) Structure of the beta-glucosidase gene bglA of *Clostridium thermocellum*. Sequence analysis reveals a superfamily of cellulases and beta-glycosidases including human lactase/phlorizin hydrolase. *Eur J Biochem* **200**, 301-309.
203. Mantei, N., Villa, M., Enzler, T., Wacker, H., Boll, W., James, P., Hunziker, W., and Semenza, G. (1988) Complete primary structure of human and rabbit lactase-phlorizin hydrolase: implications for biosynthesis, membrane anchoring and evolution of the enzyme. *EMBO J* **7**, 2705-2713. 457059
204. Mian, I. S. (1998) Sequence, structural, functional, and phylogenetic analyses of three glycosidase families. *Blood Cells Mol Dis* **24**, 83-100.
205. Li, S. A., Watanabe, M., Yamada, H., Nagai, A., Kinuta, M., and Takei, K. (2004) Immunohistochemical localization of Klotho protein in brain, kidney, and reproductive organs of mice. *Cell Struct Funct* **29**, 91-99.
206. German, D. C., Khobahy, I., Pastor, J., Kuro, O. M., and Liu, X. (2012) Nuclear localization of Klotho in brain: an anti-aging protein. *Neurobiol Aging* **33**, 1483 e1425-1430. 3328593
207. Ben-Dov, I. Z., Galitzer, H., Lavi-Moshayoff, V., Goetz, R., Kuro-o, M., Mohammadi, M., Sirkis, R., Naveh-Many, T., and Silver, J. (2007) The parathyroid is a target organ for FGF23 in rats. *J Clin Invest* **117**, 4003-4008. 2066196
208. Kamemori, M., Ohyama, Y., Kurabayashi, M., Takahashi, K., Nagai, R., and Furuya, N. (2002) Expression of Klotho protein in the inner ear. *Hear Res* **171**, 103-110.

209. Wolf, I., Levanon-Cohen, S., Bose, S., Ligumsky, H., Sredni, B., Kanety, H., Kuro-o, M., Karlan, B., Kaufman, B., Koeffler, H. P., and Rubinek, T. (2008) Klotho: a tumor suppressor and a modulator of the IGF-1 and FGF pathways in human breast cancer. *Oncogene* **27**, 7094-7105. 18762812
210. Donate-Correa, J., Mora-Fernandez, C., Martinez-Sanz, R., Muros-de-Fuentes, M., Perez, H., Meneses-Perez, B., Cazana-Perez, V., and Navarro-Gonzalez, J. F. (2013) Expression of FGF23/KLOTHO system in human vascular tissue. *Int J Cardiol* **165**, 179-183.
211. Pan, J., Zhong, J., Gan, L. H., Chen, S. J., Jin, H. C., Wang, X., and Wang, L. J. (2011) Klotho, an anti-senescence related gene, is frequently inactivated through promoter hypermethylation in colorectal cancer. *Tumour Biol* **32**, 729-735.
212. Azuma, M., Koyama, D., Kikuchi, J., Yoshizawa, H., Thasinas, D., Shiizaki, K., Kuro-o, M., Furukawa, Y., and Kusano, E. (2012) Promoter methylation confers kidney-specific expression of the Klotho gene. *FASEB J* **26**, 4264-4274. 3448772
213. King, G. D., Rosene, D. L., and Abraham, C. R. (2012) Promoter methylation and age-related downregulation of Klotho in rhesus monkey. *Age (Dordr)* **34**, 1405-1419. 21922250. PMC3528360
214. Xie, B., Zhou, J., Yuan, L., Ren, F., Liu, D. C., Li, Q., and Shu, G. (2013) Epigenetic silencing of Klotho expression correlates with poor prognosis of human hepatocellular carcinoma. *Hum Pathol* **44**, 795-801.
215. Zhang, C., Liang, Y., Lei, L., Zhu, G., Chen, X., Jin, T., and Wu, Q. (2013) Hypermethylations of RASAL1 and KLOTHO is associated with renal dysfunction in a Chinese population environmentally exposed to cadmium. *Toxicol Appl Pharmacol*
216. Rubinek, T., Shulman, M., Israeli, S., Bose, S., Avraham, A., Zundeleovich, A., Evron, E., Gal-Yam, E. N., Kaufman, B., and Wolf, I. (2012) Epigenetic silencing of the tumor suppressor klotho in human breast cancer. *Breast Cancer Res Treat* **133**, 649-657.
217. Wang, L., Wang, X., Wang, X., Jie, P., Lu, H., Zhang, S., Lin, X., Lam, E. K., Cui, Y., Yu, J., and Jin, H. (2011) Klotho is silenced through promoter hypermethylation in gastric cancer. *Am J Cancer Res* **1**, 111-119. 3180103
218. Gan, L. H., Pan, J., Chen, S. J., Zhong, J., and Wang, L. J. (2011) [DNA methylation of ZIC1 and KLOTHO gene promoters in colorectal

carcinomas and its clinicopathological significance]. *Zhejiang Da Xue Xue Bao Yi Xue Ban* **40**, 309-314.

219. Lee, J., Jeong, D. J., Kim, J., Lee, S., Park, J. H., Chang, B., Jung, S. I., Yi, L., Han, Y., Yang, Y., Kim, K. I., Lim, J. S., Yang, I., Jeon, S., Bae, D. H., Kim, C. J., and Lee, M. S. (2010) The anti-aging gene KLOTHO is a novel target for epigenetic silencing in human cervical carcinoma. *Mol Cancer* **9**, 109. 2885346
220. Choi, B. H., Kim, C. G., Lim, Y., Lee, Y. H., and Shin, S. Y. (2010) Transcriptional activation of the human Klotho gene by epidermal growth factor in HEK293 cells; role of Egr-1. *Gene* **450**, 121-127.
221. Mitobe, M., Yoshida, T., Sugiura, H., Shirota, S., Tsuchiya, K., and Nihei, H. (2005) Oxidative stress decreases klotho expression in a mouse kidney cell line. *Nephron Exp Nephrol* **101**, e67-74.
222. Moreno, J. A., Izquierdo, M. C., Sanchez-Nino, M. D., Suarez-Alvarez, B., Lopez-Larrea, C., Jakubowski, A., Blanco, J., Ramirez, R., Selgas, R., Ruiz-Ortega, M., Egido, J., Ortiz, A., and Sanz, A. B. (2011) The inflammatory cytokines TWEAK and TNFalpha reduce renal klotho expression through NFKappaB. *J Am Soc Nephrol* **22**, 1315-1325. 3137579
223. Tang, C., Pathare, G., Michael, D., Fajol, A., Eichenmuller, M., and Lang, F. (2011) Downregulation of Klotho expression by dehydration. *Am J Physiol Renal Physiol* **301**, F745-750.
224. Xu, Z. L., Gao, H., Ou-Yang, K. Q., Cai, S. X., and Hu, Y. H. (2004) Establishment of a cell-based assay to screen regulators for Klotho gene promoter. *Acta Pharmacol Sin* **25**, 1165-1170.
225. King, G. D., Chen, C., Huang, M. M., Zeldich, E., Brazee, P. L., Schuman, E. R., Robin, M., Cuny, G. D., Glicksman, M. A., and Abraham, C. R. (2012) Identification of novel small molecules that elevate Klotho expression. *Biochem J* **441**, 453-461. 21939436
226. Yoon, H. E., Lim, S. W., Piao, S. G., Song, J. H., Kim, J., and Yang, C. W. (2012) Statin Upregulates the Expression of Klotho , an Anti-Aging Gene, in Experimental Cyclosporine Nephropathy. *Nephron Exp Nephrol* **120**, e123-e133.
227. Forster, R. E., Jurutka, P. W., Hsieh, J. C., Haussler, C. A., Lowmiller, C. L., Kaneko, I., Haussler, M. R., and Kerr Whitfield, G. (2011) Vitamin D

receptor controls expression of the anti-aging klotho gene in mouse and human renal cells. *Biochem Biophys Res Commun* **414**, 557-562. 3209523

228. Alcendor, R. R., Gao, S., Zhai, P., Zablocki, D., Holle, E., Yu, X., Tian, B., Wagner, T., Vatner, S. F., and Sadoshima, J. (2007) Sirt1 regulates aging and resistance to oxidative stress in the heart. *Circ Res* **100**, 1512-1521.
229. Mehi, S. J., Maltare, A., Abraham, C. R., and King, G. D. (2013) MicroRNA-339 and microRNA-556 regulate Klotho expression in vitro. *Age (Dordr)*
230. Imura, A., Iwano, A., Tohyama, O., Tsuji, Y., Nozaki, K., Hashimoto, N., Fujimori, T., and Nabeshima, Y. (2004) Secreted Klotho protein in sera and CSF: implication for post-translational cleavage in release of Klotho protein from cell membrane. *FEBS Lett* **565**, 143-147. 15135068
231. Chen, C. D., Podvin, S., Gillespie, E., Leeman, S. E., and Abraham, C. R. (2007) Insulin stimulates the cleavage and release of the extracellular domain of Klotho by ADAM10 and ADAM17. *Proc Natl Acad Sci U S A* **104**, 19796-19801. 18056631
232. Bloch, L., Sineshchekova, O., Reichenbach, D., Reiss, K., Saftig, P., Kuro-o, M., and Kaether, C. (2009) Klotho is a substrate for alpha-, beta- and gamma-secretase. *FEBS Lett* **583**, 3221-3224. 2757472
233. Kamitani, A., Yamada, H., Kinuta, M., Watanabe, M., Li, S. A., Matsukawa, T., McNiven, M., Kumon, H., and Takei, K. (2002) Distribution of dynamins in testis and their possible relation to spermatogenesis. *Biochem Biophys Res Commun* **294**, 261-267.
234. Toyama, R., Fujimori, T., Nabeshima, Y., Itoh, Y., Tsuji, Y., Osamura, R. Y., and Nabeshima, Y. (2006) Impaired regulation of gonadotropins leads to the atrophy of the female reproductive system in klotho-deficient mice. *Endocrinology* **147**, 120-129.
235. Min, D., Panoskaltsis-Mortari, A., Kuro, O. M., Hollander, G. A., Blazar, B. R., and Weinberg, K. I. (2007) Sustained thymopoiesis and improvement in functional immunity induced by exogenous KGF administration in murine models of aging. *Blood* **109**, 2529-2537. 1852207
236. Kuro-o, M. (2009) Klotho and aging. *Biochim Biophys Acta* **1790**, 1049-1058. 2743784

237. Steinmann, G. G. (1986) Changes in the human thymus during aging. *Curr Top Pathol* **75**, 43-88.
238. Kendall, M. D., Johnson, H. R., and Singh, J. (1980) The weight of the human thymus gland at necropsy. *J Anat* **131**, 483-497. 1233247
239. Aspinall, R. (1997) Age-associated thymic atrophy in the mouse is due to a deficiency affecting rearrangement of the TCR during intrathymic T cell development. *J Immunol* **158**, 3037-3045.
240. Aspinall, R., and Andrew, D. (2000) Thymic involution in aging. *J Clin Immunol* **20**, 250-256.
241. Tsujikawa, H., Kurotaki, Y., Fujimori, T., Fukuda, K., and Nabeshima, Y. (2003) Klotho, a gene related to a syndrome resembling human premature aging, functions in a negative regulatory circuit of vitamin D endocrine system. *Mol Endocrinol* **17**, 2393-2403. 14528024
242. Yoshida, T., Fujimori, T., and Nabeshima, Y. (2002) Mediation of unusually high concentrations of 1,25-dihydroxyvitamin D in homozygous klotho mutant mice by increased expression of renal 1 α -hydroxylase gene. *Endocrinology* **143**, 683-689.
243. Kawaguchi, H., Manabe, N., Miyaura, C., Chikuda, H., Nakamura, K., and Kuro-o, M. (1999) Independent impairment of osteoblast and osteoclast differentiation in klotho mouse exhibiting low-turnover osteopenia. *J Clin Invest* **104**, 229-237. 408412
244. LJ Melton, B. R. (1988) Clinical Spectrum. in *Osteoperosis: Etiology and Management* (LJ, R. B. a. M. ed.), Raven, New York, NY. pp 155-179
245. Yamashita, T., Nifuji, A., Furuya, K., Nabeshima, Y., and Noda, M. (1998) Elongation of the epiphyseal trabecular bone in transgenic mice carrying a klotho gene locus mutation that leads to a syndrome resembling aging. *J Endocrinol* **159**, 1-8.
246. Yamashita, T., Nabeshima, Y., and Noda, M. (2000) High-resolution micro-computed tomography analyses of the abnormal trabecular bone structures in klotho gene mutant mice. *J Endocrinol* **164**, 239-245.
247. A. Chuttanai, B. G. (1995) Section 11: Aging. in *Handbook of Physiology* (Mesoro, E. ed.), Oxford University Press, Oxford, England. pp 309-324

248. Funada, Y., Nishimura, Y., and Yokoyama, M. (2004) Imbalance of matrix metalloproteinase-9 and tissue inhibitor of matrix metalloproteinase-1 is associated with pulmonary emphysema in Klotho mice. *Kobe J Med Sci* **50**, 59-67.
249. Ishii, M., Yamaguchi, Y., Yamamoto, H., Hanaoka, Y., and Ouchi, Y. (2008) Airspace enlargement with airway cell apoptosis in klotho mice: a model of aging lung. *J Gerontol A Biol Sci Med Sci* **63**, 1289-1298.
250. Suga, T., Kurabayashi, M., Sando, Y., Ohyama, Y., Maeno, T., Maeno, Y., Aizawa, H., Matsumura, Y., Kuwaki, T., Kuro, O. M., Nabeshima, Y., and Nagai, R. (2000) Disruption of the klotho gene causes pulmonary emphysema in mice. Defect in maintenance of pulmonary integrity during postnatal life. *Am J Respir Cell Mol Biol* **22**, 26-33.
251. Sato, A., Hirai, T., Imura, A., Kita, N., Iwano, A., Muro, S., Nabeshima, Y., Suki, B., and Mishima, M. (2007) Morphological mechanism of the development of pulmonary emphysema in klotho mice. *Proc Natl Acad Sci U S A* **104**, 2361-2365. 1892918
252. Nagai, T., Yamada, K., Kim, H. C., Kim, Y. S., Noda, Y., Imura, A., Nabeshima, Y., and Nabeshima, T. (2003) Cognition impairment in the genetic model of aging klotho gene mutant mice: a role of oxidative stress. *FASEB J* **17**, 50-52. 12475907
253. Shiozaki, M., Yoshimura, K., Shibata, M., Koike, M., Matsuura, N., Uchiyama, Y., and Gotow, T. (2008) Morphological and biochemical signs of age-related neurodegenerative changes in klotho mutant mice. *Neuroscience* **152**, 924-941.
254. Anamizu, Y., Kawaguchi, H., Seichi, A., Yamaguchi, S., Kawakami, E., Kanda, N., Matsubara, S., Kuro-o, M., Nabeshima, Y., Nakamura, K., and Oyanagi, K. (2005) Klotho insufficiency causes decrease of ribosomal RNA gene transcription activity, cytoplasmic RNA and rough ER in the spinal anterior horn cells. *Acta Neuropathol* **109**, 457-466.
255. O'Callaghan, J. P., and Miller, D. B. (1991) The concentration of glial fibrillary acidic protein increases with age in the mouse and rat brain. *Neurobiol Aging* **12**, 171-174.
256. David, J. P., Ghozali, F., Fallet-Bianco, C., Watzet, A., Delaine, S., Boniface, B., Di Menza, C., and Delacourte, A. (1997) Glial reaction in the hippocampal formation is highly correlated with aging in human brain. *Neurosci Lett* **235**, 53-56.

257. Sloane, J. A., Hollander, W., Rosene, D. L., Moss, M. B., Kemper, T., and Abraham, C. R. (2000) Astrocytic hypertrophy and altered GFAP degradation with age in subcortical white matter of the rhesus monkey. *Brain Res* **862**, 1-10.
258. Gotow, T., Tanaka, J., and Takeda, M. (1995) The organization of neurofilaments accumulated in perikaryon following aluminum administration: relationship between structure and phosphorylation of neurofilaments. *Neuroscience* **64**, 553-569.
259. Gotow, T., Leterrier, J. F., Ohsawa, Y., Watanabe, T., Isahara, K., Shibata, R., Ikenaka, K., and Uchiyama, Y. (1999) Abnormal expression of neurofilament proteins in dysmyelinating axons located in the central nervous system of jimpy mutant mice. *Eur J Neurosci* **11**, 3893-3903.
260. Gou, J. P., Eyer, J., and Leterrier, J. F. (1995) Progressive hyperphosphorylation of neurofilament heavy subunits with aging: possible involvement in the mechanism of neurofilament accumulation. *Biochem Biophys Res Commun* **215**, 368-376.
261. Gou, J. P., Gotow, T., Janmey, P. A., and Leterrier, J. F. (1998) Regulation of neurofilament interactions in vitro by natural and synthetic polypeptides sharing Lys-Ser-Pro sequences with the heavy neurofilament subunit NF-H: neurofilament crossbridging by antiparallel sidearm overlapping. *Med Biol Eng Comput* **36**, 371-387.
262. Gotow, T. (2000) Neurofilaments in health and disease. *Med Electron Microsc* **33**, 173-199.
263. Uchida, A., Yorifuji, H., Lee, V. M., Kishimoto, T., and Hisanaga, S. (1999) Neurofilaments of aged rats: the strengthened interneurofilament interaction and the reduced amount of NF-M. *J Neurosci Res* **58**, 337-348.
264. Uchida, A., Tashiro, T., Komiya, Y., Yorifuji, H., Kishimoto, T., and Hisanaga, S. (2004) Morphological and biochemical changes of neurofilaments in aged rat sciatic nerve axons. *J Neurochem* **88**, 735-745.
265. Uchida, A., Komiya, Y., Tashiro, T., Yorifuji, H., Kishimoto, T., Nabeshima, Y., and Hisanaga, S. (2001) Neurofilaments of Klotho, the mutant mouse prematurely displaying symptoms resembling human aging. *J Neurosci Res* **64**, 364-370.
266. Chen, C. D., Sloane, J. A., Li, H., Aytan, N., Giannaris, E. L., Zeldich, E., Hinman, J. D., Dedeoglu, A., Rosene, D. L., Bansal, R., Luebke, J. I., Kuro-

- o, M., and Abraham, C. R. (2013) The antiaging protein Klotho enhances oligodendrocyte maturation and myelination of the CNS. *J Neurosci* **33**, 1927-1939.
267. Takumida, M., Ishibashi, T., Hamamoto, T., Hirakawa, K., and Anniko, M. (2009) Age-dependent changes in the expression of klotho protein, TRPV5 and TRPV6 in mouse inner ear. *Acta Otolaryngol* **129**, 1340-1350.
268. Takeshita, K., Fujimori, T., Kurotaki, Y., Honjo, H., Tsujikawa, H., Yasui, K., Lee, J. K., Kamiya, K., Kitaichi, K., Yamamoto, K., Ito, M., Kondo, T., Iino, S., Inden, Y., Hirai, M., Murohara, T., Kodama, I., and Nabeshima, Y. (2004) Sinoatrial node dysfunction and early unexpected death of mice with a defect of klotho gene expression. *Circulation* **109**, 1776-1782.
269. Shimada, T., Takeshita, Y., Murohara, T., Sasaki, K., Egami, K., Shintani, S., Katsuda, Y., Ikeda, H., Nabeshima, Y., and Imaizumi, T. (2004) Angiogenesis and vasculogenesis are impaired in the precocious-aging klotho mouse. *Circulation* **110**, 1148-1155.
270. Utsugi, T., Ohno, T., Ohyama, Y., Uchiyama, T., Saito, Y., Matsumura, Y., Aizawa, H., Itoh, H., Kurabayashi, M., Kawazu, S., Tomono, S., Oka, Y., Suga, T., Kuro-o, M., Nabeshima, Y., and Nagai, R. (2000) Decreased insulin production and increased insulin sensitivity in the klotho mutant mouse, a novel animal model for human aging. *Metabolism* **49**, 1118-1123.
271. Saito, Y., Yamagishi, T., Nakamura, T., Ohyama, Y., Aizawa, H., Suga, T., Matsumura, Y., Masuda, H., Kurabayashi, M., Kuro-o, M., Nabeshima, Y., and Nagai, R. (1998) Klotho protein protects against endothelial dysfunction. *Biochem Biophys Res Commun* **248**, 324-329. 9675134
272. Masuda, H., Chikuda, H., Suga, T., Kawaguchi, H., and Kuro-o, M. (2005) Regulation of multiple ageing-like phenotypes by inducible klotho gene expression in klotho mutant mice. *Mech Ageing Dev* **126**, 1274-1283.
273. Kurosu, H., Yamamoto, M., Clark, J. D., Pastor, J. V., Nandi, A., Gurnani, P., McGuinness, O. P., Chikuda, H., Yamaguchi, M., Kawaguchi, H., Shimomura, I., Takayama, Y., Herz, J., Kahn, C. R., Rosenblatt, K. P., and Kuro-o, M. (2005) Suppression of aging in mice by the hormone Klotho. *Science* **309**, 1829-1833. 16123266. PMC2536606
274. van Heemst, D. (2010) Insulin, IGF-1 and longevity. *Aging Dis* **1**, 147-157. 3295030

275. Yamamoto, M., Clark, J. D., Pastor, J. V., Gurnani, P., Nandi, A., Kurosu, H., Miyoshi, M., Ogawa, Y., Castrillon, D. H., Rosenblatt, K. P., and Kuro-o, M. (2005) Regulation of oxidative stress by the anti-aging hormone klotho. *J Biol Chem* **280**, 38029-38034. 16186101

PMC2515369

276. Xie, J., Cha, S. K., An, S. W., Kuro, O. M., Birnbaumer, L., and Huang, C. L. (2012) Cardioprotection by Klotho through downregulation of TRPC6 channels in the mouse heart. *Nat Commun* **3**, 1238. 3526952
277. Mitani, H., Ishizaka, N., Aizawa, T., Ohno, M., Usui, S., Suzuki, T., Amaki, T., Mori, I., Nakamura, Y., Sato, M., Nangaku, M., Hirata, Y., and Nagai, R. (2002) In vivo klotho gene transfer ameliorates angiotensin II-induced renal damage. *Hypertension* **39**, 838-843.
278. Saito, Y., Nakamura, T., Ohyama, Y., Suzuki, T., Iida, A., Shiraki-Iida, T., Kuro-o, M., Nabeshima, Y., Kurabayashi, M., and Nagai, R. (2000) In vivo klotho gene delivery protects against endothelial dysfunction in multiple risk factor syndrome. *Biochem Biophys Res Commun* **276**, 767-772. 11027545
279. Haruna, Y., Kashihara, N., Satoh, M., Tomita, N., Namikoshi, T., Sasaki, T., Fujimori, T., Xie, P., and Kanwar, Y. S. (2007) Amelioration of progressive renal injury by genetic manipulation of Klotho gene. *Proc Natl Acad Sci U S A* **104**, 2331-2336. 1892974
280. Bektas, A., Schurman, S. H., Sharov, A. A., Carter, M. G., Dietz, H. C., and Francomano, C. A. (2004) Klotho gene variation and expression in 20 inbred mouse strains. *Mamm Genome* **15**, 759-767.
281. Shimada, T., Kakitani, M., Yamazaki, Y., Hasegawa, H., Takeuchi, Y., Fujita, T., Fukumoto, S., Tomizuka, K., and Yamashita, T. (2004) Targeted ablation of Fgf23 demonstrates an essential physiological role of FGF23 in phosphate and vitamin D metabolism. *J Clin Invest* **113**, 561-568. 338262
282. Sitara, D., Razzaque, M. S., Hesse, M., Yoganathan, S., Taguchi, T., Erben, R. G., Juppner, H., and Lanske, B. (2004) Homozygous ablation of fibroblast growth factor-23 results in hyperphosphatemia and impaired skeletogenesis, and reverses hypophosphatemia in PheX-deficient mice. *Matrix Biol* **23**, 421-432. 2894977

283. Liu, S., Zhou, J., Tang, W., Jiang, X., Rowe, D. W., and Quarles, L. D. (2006) Pathogenic role of Fgf23 in Hyp mice. *Am J Physiol Endocrinol Metab* **291**, E38-49.
284. Nakatani, T., Sarraj, B., Ohnishi, M., Densmore, M. J., Taguchi, T., Goetz, R., Mohammadi, M., Lanske, B., and Razzaque, M. S. (2009) In vivo genetic evidence for klotho-dependent, fibroblast growth factor 23 (Fgf23)-mediated regulation of systemic phosphate homeostasis. *FASEB J* **23**, 433-441. 18835926
285. Urakawa, I., Yamazaki, Y., Shimada, T., Iijima, K., Hasegawa, H., Okawa, K., Fujita, T., Fukumoto, S., and Yamashita, T. (2006) Klotho converts canonical FGF receptor into a specific receptor for FGF23. *Nature* **444**, 770-774. 17086194
286. Yamashita, T., Yoshioka, M., and Itoh, N. (2000) Identification of a novel fibroblast growth factor, FGF-23, preferentially expressed in the ventrolateral thalamic nucleus of the brain. *Biochem Biophys Res Commun* **277**, 494-498.
287. Itoh, N., and Ornitz, D. M. (2008) Functional evolutionary history of the mouse Fgf gene family. *Dev Dyn* **237**, 18-27.
288. Goetz, R., Beenken, A., Ibrahimi, O. A., Kalinina, J., Olsen, S. K., Eliseenkova, A. V., Xu, C., Neubert, T. A., Zhang, F., Linhardt, R. J., Yu, X., White, K. E., Inagaki, T., Kliever, S. A., Yamamoto, M., Kurosu, H., Ogawa, Y., Kuro-o, M., Lanske, B., Razzaque, M. S., and Mohammadi, M. (2007) Molecular insights into the klotho-dependent, endocrine mode of action of fibroblast growth factor 19 subfamily members. *Mol Cell Biol* **27**, 3417-3428. 17339340. PMC1899957
289. Harmer, N. J., Pellegrini, L., Chirgadze, D., Fernandez-Recio, J., and Blundell, T. L. (2004) The crystal structure of fibroblast growth factor (FGF) 19 reveals novel features of the FGF family and offers a structural basis for its unusual receptor affinity. *Biochemistry* **43**, 629-640.
290. Schlessinger, J., Plotnikov, A. N., Ibrahimi, O. A., Eliseenkova, A. V., Yeh, B. K., Yayon, A., Linhardt, R. J., and Mohammadi, M. (2000) Crystal structure of a ternary FGF-FGFR-heparin complex reveals a dual role for heparin in FGFR binding and dimerization. *Mol Cell* **6**, 743-750.
291. Kurosu, H., Ogawa, Y., Miyoshi, M., Yamamoto, M., Nandi, A., Rosenblatt, K. P., Baum, M. G., Schiavi, S., Hu, M. C., Moe, O. W., and Kuro-o, M.

- (2006) Regulation of fibroblast growth factor-23 signaling by klotho. *J Biol Chem* **281**, 6120-6123. 16436388. PMC2637204
292. Saito, H., Kusano, K., Kinoshita, M., Ito, H., Hirata, M., Segawa, H., Miyamoto, K., and Fukushima, N. (2003) Human fibroblast growth factor-23 mutants suppress Na⁺-dependent phosphate co-transport activity and 1 α ,25-dihydroxyvitamin D₃ production. *J Biol Chem* **278**, 2206-2211.
293. Segawa, H., Kawakami, E., Kaneko, I., Kuwahata, M., Ito, M., Kusano, K., Saito, H., Fukushima, N., and Miyamoto, K. (2003) Effect of hydrolysis-resistant FGF23-R179Q on dietary phosphate regulation of the renal type-II Na/Pi transporter. *Pflugers Arch* **446**, 585-592.
294. Segawa, H., Yamanaka, S., Ohno, Y., Onitsuka, A., Shiozawa, K., Aranami, F., Furutani, J., Tomoe, Y., Ito, M., Kuwahata, M., Imura, A., Nabeshima, Y., and Miyamoto, K. (2007) Correlation between hyperphosphatemia and type II Na-Pi cotransporter activity in klotho mice. *Am J Physiol Renal Physiol* **292**, F769-779.
295. Shimada, T., Urakawa, I., Yamazaki, Y., Hasegawa, H., Hino, R., Yoneya, T., Takeuchi, Y., Fujita, T., Fukumoto, S., and Yamashita, T. (2004) FGF-23 transgenic mice demonstrate hypophosphatemic rickets with reduced expression of sodium phosphate cotransporter type IIa. *Biochem Biophys Res Commun* **314**, 409-414.
296. Miyamoto, K., Ito, M., Tatsumi, S., Kuwahata, M., and Segawa, H. (2007) New aspect of renal phosphate reabsorption: the type IIc sodium-dependent phosphate transporter. *Am J Nephrol* **27**, 503-515.
297. Shimada, T., Mizutani, S., Muto, T., Yoneya, T., Hino, R., Takeda, S., Takeuchi, Y., Fujita, T., Fukumoto, S., and Yamashita, T. (2001) Cloning and characterization of FGF23 as a causative factor of tumor-induced osteomalacia. *Proc Natl Acad Sci U S A* **98**, 6500-6505. 33497
298. Shimada, T., Hasegawa, H., Yamazaki, Y., Muto, T., Hino, R., Takeuchi, Y., Fujita, T., Nakahara, K., Fukumoto, S., and Yamashita, T. (2004) FGF-23 is a potent regulator of vitamin D metabolism and phosphate homeostasis. *J Bone Miner Res* **19**, 429-435.
299. Williams, K. B., and DeLuca, H. F. (2007) Characterization of intestinal phosphate absorption using a novel in vivo method. *Am J Physiol Endocrinol Metab* **292**, E1917-1921.

300. Krajisnik, T., Bjorklund, P., Marsell, R., Ljunggren, O., Akerstrom, G., Jonsson, K. B., Westin, G., and Larsson, T. E. (2007) Fibroblast growth factor-23 regulates parathyroid hormone and 1alpha-hydroxylase expression in cultured bovine parathyroid cells. *J Endocrinol* **195**, 125-131.
301. Berndt, T., and Kumar, R. (2007) Phosphatonins and the regulation of phosphate homeostasis. *Annu Rev Physiol* **69**, 341-359.
302. Kumar, R. (2006) The phosphatonins and the regulation of phosphate homeostasis. *Ann Endocrinol (Paris)* **67**, 142-146.
303. Dusso, A. S., Brown, A. J., and Slatopolsky, E. (2005) Vitamin D. *Am J Physiol Renal Physiol* **289**, F8-28.
304. Stubbs, J. R., Liu, S., Tang, W., Zhou, J., Wang, Y., Yao, X., and Quarles, L. D. (2007) Role of hyperphosphatemia and 1,25-dihydroxyvitamin D in vascular calcification and mortality in fibroblastic growth factor 23 null mice. *J Am Soc Nephrol* **18**, 2116-2124.
305. Ohnishi, M., Nakatani, T., Lanske, B., and Razzaque, M. S. (2009) Reversal of mineral ion homeostasis and soft-tissue calcification of klotho knockout mice by deletion of vitamin D 1alpha-hydroxylase. *Kidney Int* **75**, 1166-1172. 3143194
306. Razzaque, M. S., Sitara, D., Taguchi, T., St-Arnaud, R., and Lanske, B. (2006) Premature aging-like phenotype in fibroblast growth factor 23 null mice is a vitamin D-mediated process. *FASEB J* **20**, 720-722. 2899884
307. Hesse, M., Frohlich, L. F., Zeitz, U., Lanske, B., and Erben, R. G. (2007) Ablation of vitamin D signaling rescues bone, mineral, and glucose homeostasis in Fgf-23 deficient mice. *Matrix Biol* **26**, 75-84.
308. Morishita, K., Shirai, A., Kubota, M., Katakura, Y., Nabeshima, Y., Takeshige, K., and Kamiya, T. (2001) The progression of aging in klotho mutant mice can be modified by dietary phosphorus and zinc. *J Nutr* **131**, 3182-3188. 11739863
309. Lu, P., Boros, S., Chang, Q., Bindels, R. J., and Hoenderop, J. G. (2008) The beta-glucuronidase klotho exclusively activates the epithelial Ca²⁺ channels TRPV5 and TRPV6. *Nephrol Dial Transplant* **23**, 3397-3402. 18495742

310. Chang, Q., Hoefs, S., van der Kemp, A. W., Topala, C. N., Bindels, R. J., and Hoenderop, J. G. (2005) The beta-glucuronidase klotho hydrolyzes and activates the TRPV5 channel. *Science* **310**, 490-493. 16239475
311. Nijenhuis, T., Hoenderop, J. G., and Bindels, R. J. (2005) TRPV5 and TRPV6 in Ca(2+) (re)absorption: regulating Ca(2+) entry at the gate. *Pflugers Arch* **451**, 181-192.
312. Tohyama, O., Imura, A., Iwano, A., Freund, J. N., Henrissat, B., Fujimori, T., and Nabeshima, Y. (2004) Klotho is a novel beta-glucuronidase capable of hydrolyzing steroid beta-glucuronides. *J Biol Chem* **279**, 9777-9784. 14701853
313. Cha, S. K., Ortega, B., Kurosu, H., Rosenblatt, K. P., Kuro, O. M., and Huang, C. L. (2008) Removal of sialic acid involving Klotho causes cell-surface retention of TRPV5 channel via binding to galectin-1. *Proc Natl Acad Sci U S A* **105**, 9805-9810. 18606998
314. Cha, S. K., Hu, M. C., Kurosu, H., Kuro-o, M., Moe, O., and Huang, C. L. (2009) Regulation of renal outer medullary potassium channel and renal K(+) excretion by Klotho. *Mol Pharmacol* **76**, 38-46. 19349416
315. Hu, M. C., Shi, M., Zhang, J., Pastor, J., Nakatani, T., Lanske, B., Razzaque, M. S., Rosenblatt, K. P., Baum, M. G., Kuro-o, M., and Moe, O. W. (2010) Klotho: a novel phosphaturic substance acting as an autocrine enzyme in the renal proximal tubule. *FASEB J* **24**, 3438-3450. 20466874. PMC2923354
316. Dermaku-Sopjani, M., Sopjani, M., Saxena, A., Shojaiefard, M., Bogatkov, E., Alesutan, I., Eichenmuller, M., and Lang, F. (2011) Downregulation of NaPi-IIa and NaPi-IIb Na-coupled phosphate transporters by coexpression of Klotho. *Cell Physiol Biochem* **28**, 251-258.
317. Huang, C. L. (2011) Regulation of Ion Channels by Secreted Klotho. in *Endocrine FGFs and Klothos* (Kuro-o, M. ed.), Landes Bioscience.
318. Kusaba, T., Okigaki, M., Matui, A., Murakami, M., Ishikawa, K., Kimura, T., Sonomura, K., Adachi, Y., Shibuya, M., Shirayama, T., Tanda, S., Hatta, T., Sasaki, S., Mori, Y., and Matsubara, H. (2010) Klotho is associated with VEGF receptor-2 and the transient receptor potential canonical-1 Ca²⁺ channel to maintain endothelial integrity. *Proc Natl Acad Sci U S A* **107**, 19308-19313. 20966350. PMC2984167

319. Takahashi, M., Tsuda, T., Ikeda, Y., Honke, K., and Taniguchi, N. (2004) Role of N-glycans in growth factor signaling. *Glycoconj J* **20**, 207-212.
320. Salhanick, A. I., and Amatruda, J. M. (1988) Role of sialic acid in insulin action and the insulin resistance of diabetes mellitus. *Am J Physiol* **255**, E173-179.
321. Sasaki, A., Hata, K., Suzuki, S., Sawada, M., Wada, T., Yamaguchi, K., Obinata, M., Tateno, H., Suzuki, H., and Miyagi, T. (2003) Overexpression of plasma membrane-associated sialidase attenuates insulin signaling in transgenic mice. *J Biol Chem* **278**, 27896-27902.
322. Lorenzi, O., Veyrat-Durebex, C., Wollheim, C. B., Villemin, P., Rohner-Jeanrenaud, F., Zanchi, A., and Vischer, U. M. (2010) Evidence against a direct role of klotho in insulin resistance. *Pflugers Arch* **459**, 465-473. 19756714
323. Chateau, M. T., Araiz, C., Descamps, S., and Galas, S. (2010) Klotho interferes with a novel FGF-signalling pathway and insulin/Igf-like signalling to improve longevity and stress resistance in *Caenorhabditis elegans*. *Aging (Albany NY)* **2**, 567-581. 20844315
324. Kenyon, C. (2005) The plasticity of aging: insights from long-lived mutants. *Cell* **120**, 449-460.
325. Tatar, M., Kopelman, A., Epstein, D., Tu, M. P., Yin, C. M., and Garofalo, R. S. (2001) A mutant *Drosophila* insulin receptor homolog that extends life-span and impairs neuroendocrine function. *Science* **292**, 107-110.
326. Bluher, M., Kahn, B. B., and Kahn, C. R. (2003) Extended longevity in mice lacking the insulin receptor in adipose tissue. *Science* **299**, 572-574.
327. Selman, C., Lingard, S., Choudhury, A. I., Batterham, R. L., Claret, M., Clements, M., Ramadani, F., Okkenhaug, K., Schuster, E., Blanc, E., Piper, M. D., Al-Qassab, H., Speakman, J. R., Carmignac, D., Robinson, I. C., Thornton, J. M., Gems, D., Partridge, L., and Withers, D. J. (2008) Evidence for lifespan extension and delayed age-related biomarkers in insulin receptor substrate 1 null mice. *FASEB J* **22**, 807-818.
328. Brown-Borg, H. M., Borg, K. E., Meliska, C. J., and Bartke, A. (1996) Dwarf mice and the ageing process. *Nature* **384**, 33.
329. Liu, H., Fergusson, M. M., Castilho, R. M., Liu, J., Cao, L., Chen, J., Malide, D., Rovira, II, Schimel, D., Kuo, C. J., Gutkind, J. S., Hwang, P. M.,

- and Finkel, T. (2007) Augmented Wnt signaling in a mammalian model of accelerated aging. *Science* **317**, 803-806. 17690294
330. Zhou, L., Li, Y., Zhou, D., Tan, R. J., and Liu, Y. (2013) Loss of Klotho contributes to kidney injury by derepression of Wnt/beta-catenin signaling. *J Am Soc Nephrol* **24**, 771-785. 3636797
331. Anastas, J. N., and Moon, R. T. (2013) WNT signalling pathways as therapeutic targets in cancer. *Nat Rev Cancer* **13**, 11-26.
332. Feigenson, K., Reid, M., See, J., Crenshaw, E. B., 3rd, and Grinspan, J. B. (2009) Wnt signaling is sufficient to perturb oligodendrocyte maturation. *Mol Cell Neurosci* **42**, 255-265.
333. Feigenson, K., Reid, M., See, J., Crenshaw, I. E., and Grinspan, J. B. (2011) Canonical Wnt signalling requires the BMP pathway to inhibit oligodendrocyte maturation. *ASN Neuro* **3**, e00061. 3155198
334. Sugiura, H., Yoshida, T., Shiohira, S., Kohei, J., Mitobe, M., Kurosu, H., Kuro-o, M., Nitta, K., and Tsuchiya, K. (2012) Reduced Klotho expression level in kidney aggravates renal interstitial fibrosis. *Am J Physiol Renal Physiol* **302**, F1252-1264.
335. Doi, S., Zou, Y., Togao, O., Pastor, J. V., John, G. B., Wang, L., Shiizaki, K., Gotschall, R., Schiavi, S., Yorioka, N., Takahashi, M., Boothman, D. A., and Kuro-o, M. (2011) Klotho inhibits transforming growth factor-beta1 (TGF-beta1) signaling and suppresses renal fibrosis and cancer metastasis in mice. *J Biol Chem* **286**, 8655-8665. 3048747
336. Nakamura, T., Saito, Y., Ohyama, Y., Masuda, H., Sumino, H., Kuro-o, M., Nabeshima, Y., Nagai, R., and Kurabayashi, M. (2002) Production of nitric oxide, but not prostacyclin, is reduced in klotho mice. *Jpn J Pharmacol* **89**, 149-156. 12120757
337. Fodde, R., and Brabletz, T. (2007) Wnt/beta-catenin signaling in cancer stemness and malignant behavior. *Curr Opin Cell Biol* **19**, 150-158.
338. Julien-Grille S, M. R., Denat L, et al. (2000-) The Role of Insulin-like Growth Factors in the Epithelial to Mesenchymal Transition. in *Madame Curie Bioscience Database [Internet]*, Landes Bioscience, Austin TX.
339. Bates, R. C., and Mercurio, A. M. (2005) The epithelial-mesenchymal transition (EMT) and colorectal cancer progression. *Cancer Biol Ther* **4**, 365-370.

340. Willis, B. C., and Borok, Z. (2007) TGF-beta-induced EMT: mechanisms and implications for fibrotic lung disease. *Am J Physiol Lung Cell Mol Physiol* **293**, L525-534.
341. Lee, J. M., Dedhar, S., Kalluri, R., and Thompson, E. W. (2006) The epithelial-mesenchymal transition: new insights in signaling, development, and disease. *J Cell Biol* **172**, 973-981. 2063755
342. Matsuzaki, H., Daitoku, H., Hatta, M., Tanaka, K., and Fukamizu, A. (2003) Insulin-induced phosphorylation of FKHR (Foxo1) targets to proteasomal degradation. *Proc Natl Acad Sci U S A* **100**, 11285-11290. 208749
343. Kops, G. J., Dansen, T. B., Polderman, P. E., Saarloos, I., Wirtz, K. W., Coffey, P. J., Huang, T. T., Bos, J. L., Medema, R. H., and Burgering, B. M. (2002) Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress. *Nature* **419**, 316-321.
344. Kobayashi, Y., Furukawa-Hibi, Y., Chen, C., Horio, Y., Isobe, K., Ikeda, K., and Motoyama, N. (2005) SIRT1 is critical regulator of FOXO-mediated transcription in response to oxidative stress. *Int J Mol Med* **16**, 237-243.
345. Inoue, S., Sato, T., Suzuki-Utsunomiya, K., Komori, Y., Hozumi, K., Chiba, T., Yahata, T., Nakai, K., and Inokuchi, S. (2013) Sepsis-induced hypercytokinemia and lymphocyte apoptosis in aging-accelerated Klotho knockout mice. *Shock* **39**, 311-316.
346. Witkowski, J. M., Soroczynska-Cybula, M., Bryl, E., Smolenska, Z., and Jozwik, A. (2007) Klotho--a common link in physiological and rheumatoid arthritis-related aging of human CD4+ lymphocytes. *J Immunol* **178**, 771-777.
347. Okada, S., Yoshida, T., Hong, Z., Ishii, G., Hatano, M., Kuro, O. M., Nabeshima, Y., Nabeshima, Y., and Tokuhi, T. (2000) Impairment of B lymphopoiesis in precocious aging (klotho) mice. *Int Immunol* **12**, 861-871.
348. Maekawa, Y., Ishikawa, K., Yasuda, O., Oguro, R., Hanasaki, H., Kida, I., Takemura, Y., Ohishi, M., Katsuya, T., and Rakugi, H. (2009) Klotho suppresses TNF-alpha-induced expression of adhesion molecules in the endothelium and attenuates NF-kappaB activation. *Endocrine* **35**, 341-346.

349. Zhao, Y., Banerjee, S., Dey, N., LeJeune, W. S., Sarkar, P. S., Brobey, R., Rosenblatt, K. P., Tilton, R. G., and Choudhary, S. (2011) Klotho depletion contributes to increased inflammation in kidney of the db/db mouse model of diabetes via RelA (serine)536 phosphorylation. *Diabetes* **60**, 1907-1916. 3121423
350. Liu, F., Wu, S., Ren, H., and Gu, J. (2011) Klotho suppresses RIG-I-mediated senescence-associated inflammation. *Nat Cell Biol* **13**, 254-262.
351. Ikushima, M., Rakugi, H., Ishikawa, K., Maekawa, Y., Yamamoto, K., Ohta, J., Chihara, Y., Kida, I., and Ogihara, T. (2006) Anti-apoptotic and anti-senescence effects of Klotho on vascular endothelial cells. *Biochem Biophys Res Commun* **339**, 827-832. 16325773
352. Maekawa, Y., Ohishi, M., Ikushima, M., Yamamoto, K., Yasuda, O., Oguro, R., Yamamoto-Hanasaki, H., Tatara, Y., Takeya, Y., and Rakugi, H. (2011) Klotho protein diminishes endothelial apoptosis and senescence via a mitogen-activated kinase pathway. *Geriatr Gerontol Int* **11**, 510-516.
353. Carracedo, J., Buendia, P., Merino, A., Madueno, J. A., Peralbo, E., Ortiz, A., Martin-Malo, A., Aljama, P., Rodriguez, M., and Ramirez, R. (2012) Klotho modulates the stress response in human senescent endothelial cells. *Mech Ageing Dev* **133**, 647-654.
354. de Oliveira, R. M. (2006) Klotho RNAi induces premature senescence of human cells via a p53/p21 dependent pathway. *FEBS Lett* **580**, 5753-5758. 17014852
355. Adijiang, A., Shimizu, H., Higuchi, Y., Nishijima, F., and Niwa, T. (2011) Indoxyl sulfate reduces klotho expression and promotes senescence in the kidneys of hypertensive rats. *J Ren Nutr* **21**, 105-109.
356. Wong, Y. F., and Xu, Q. (2009) Ablation of klotho and premature aging: is 1,25-dihydroxyvitamin D the key middleman? *Kidney Int* **75**, 1137-1139.
357. Ishizaka, N., Mitani, H., and Nagai, R. (2002) [Angiotensin II regulates klotho gene expression]. *Nihon Rinsho* **60**, 1935-1939.
358. Taal, M. W., and Brenner, B. M. (2000) Renoprotective benefits of RAS inhibition: from ACEI to angiotensin II antagonists. *Kidney Int* **57**, 1803-1817.
359. Yoon, H. E., Ghee, J. Y., Piao, S., Song, J. H., Han, D. H., Kim, S., Ohashi, N., Kobori, H., Kuro, O. M., and Yang, C. W. (2010) Angiotensin II

blockade upregulates the expression of Klotho the anti-ageing gene, in an experimental model of chronic cyclosporine nephropathy. *Nephrol Dial Transplant* 20813770

360. Hu, M. C., Shi, M., Zhang, J., Quinones, H., Kuro-o, M., and Moe, O. W. (2010) Klotho deficiency is an early biomarker of renal ischemia-reperfusion injury and its replacement is protective. *Kidney Int* **78**, 1240-1251. 3237296
361. Hu, M. C., Shi, M., Zhang, J., Quinones, H., Griffith, C., Kuro-o, M., and Moe, O. W. (2011) Klotho deficiency causes vascular calcification in chronic kidney disease. *J Am Soc Nephrol* **22**, 124-136. 3014041
362. Sugiura, H., Yoshida, T., Mitobe, M., Yoshida, S., Shiohira, S., Nitta, K., and Tsuchiya, K. (2010) Klotho reduces apoptosis in experimental ischaemic acute kidney injury via HSP-70. *Nephrol Dial Transplant* **25**, 60-68.
363. Yamagishi, T., Saito, Y., Nakamura, T., Takeda, S., Kanai, H., Sumino, H., Kuro-o, M., Nabeshima, Y., Kurabayashi, M., and Nagai, R. (2001) Troglitazone improves endothelial function and augments renal klotho mRNA expression in Otsuka Long-Evans Tokushima Fatty (OLETF) rats with multiple atherogenic risk factors. *Hypertens Res* **24**, 705-709.
364. Koh, N., Fujimori, T., Nishiguchi, S., Tamori, A., Shiomi, S., Nakatani, T., Sugimura, K., Kishimoto, T., Kinoshita, S., Kuroki, T., and Nabeshima, Y. (2001) Severely reduced production of klotho in human chronic renal failure kidney. *Biochem Biophys Res Commun* **280**, 1015-1020.
365. Kim, H. R., Nam, B. Y., Kim, D. W., Kang, M. W., Han, J. H., Lee, M. J., Shin, D. H., Doh, F. M., Koo, H. M., Ko, K. I., Kim, C. H., Oh, H. J., Yoo, T. H., Kang, S. W., Han, D. S., and Han, S. H. (2013) Circulating alpha-Klotho Levels in CKD and Relationship to Progression. *Am J Kidney Dis* **61**, 899-909.
366. Akimoto, T., Yoshizawa, H., Watanabe, Y., Numata, A., Yamazaki, T., Takeshima, E., Iwazu, K., Komada, T., Otani, N., Morishita, Y., Ito, C., Shiizaki, K., Ando, Y., Muto, S., Kuro-o, M., and Kusano, E. (2012) Characteristics of urinary and serum soluble Klotho protein in patients with different degrees of chronic kidney disease. *BMC Nephrol* **13**, 155. 3519712

367. Wang, Y., and Sun, Z. (2009) Klotho gene delivery prevents the progression of spontaneous hypertension and renal damage. *Hypertension* **54**, 810-817. 2814175
368. O'Neill, W. C., Sigrist, M. K., and McIntyre, C. W. (2010) Plasma pyrophosphate and vascular calcification in chronic kidney disease. *Nephrol Dial Transplant* **25**, 187-191.
369. Voormolen, N., Noordzij, M., Grootendorst, D. C., Beetz, I., Sijpkens, Y. W., van Manen, J. G., Boeschoten, E. W., Huisman, R. M., Krediet, R. T., and Dekker, F. W. (2007) High plasma phosphate as a risk factor for decline in renal function and mortality in pre-dialysis patients. *Nephrol Dial Transplant* **22**, 2909-2916.
370. Gordon, P. L., and Frassetto, L. A. (2010) Management of osteoporosis in CKD Stages 3 to 5. *Am J Kidney Dis* **55**, 941-956.
371. Jean, G., Terrat, J. C., Vanel, T., Hurot, J. M., Lorriaux, C., Mayor, B., and Chazot, C. (2009) High levels of serum fibroblast growth factor (FGF)-23 are associated with increased mortality in long haemodialysis patients. *Nephrol Dial Transplant* **24**, 2792-2796.
372. Vervloet, M. G., van Ittersum, F. J., Buttler, R. M., Heijboer, A. C., Blankenstein, M. A., and ter Wee, P. M. (2011) Effects of dietary phosphate and calcium intake on fibroblast growth factor-23. *Clin J Am Soc Nephrol* **6**, 383-389. 3052230
373. Fliser, D., Kollerits, B., Neyer, U., Ankerst, D. P., Lhotta, K., Lingenhel, A., Ritz, E., Kronenberg, F., Kuen, E., Konig, P., Kraatz, G., Mann, J. F., Muller, G. A., Kohler, H., and Riegler, P. (2007) Fibroblast growth factor 23 (FGF23) predicts progression of chronic kidney disease: the Mild to Moderate Kidney Disease (MMKD) Study. *J Am Soc Nephrol* **18**, 2600-2608.
374. Stenvinkel, P., and Larsson, T. E. (2013) Chronic kidney disease: a clinical model of premature aging. *Am J Kidney Dis* **62**, 339-351.
375. Ravizza, T., Balosso, S., and Vezzani, A. (2011) Inflammation and prevention of epileptogenesis. *Neurosci Lett* **497**, 223-230.
376. Foresti, M. L., Arisi, G. M., and Shapiro, L. A. (2011) Role of glia in epilepsy-associated neuropathology, neuroinflammation and neurogenesis. *Brain Res Rev* **66**, 115-122.

377. Li, G., Bauer, S., Nowak, M., Norwood, B., Tackenberg, B., Rosenow, F., Knake, S., Oertel, W. H., and Hamer, H. M. (2011) Cytokines and epilepsy. *Seizure* **20**, 249-256.
378. Vezzani, A., Moneta, D., Richichi, C., Aliprandi, M., Burrows, S. J., Ravizza, T., Perego, C., and De Simoni, M. G. (2002) Functional role of inflammatory cytokines and antiinflammatory molecules in seizures and epileptogenesis. *Epilepsia* **43 Suppl 5**, 30-35.
379. Teocchi, M. A., Ferreira, A. E., da Luz de Oliveira, E. P., Tedeschi, H., and D'Souza-Li, L. (2013) Hippocampal gene expression dysregulation of Klotho, nuclear factor kappa B and tumor necrosis factor in temporal lobe epilepsy patients. *J Neuroinflammation* **10**, 53. 3651326
380. Sata, A., and Ho, K. K. (2007) Growth hormone measurements in the diagnosis and monitoring of acromegaly. *Pituitary* **10**, 165-172.
381. Corvilain, J., Abramow, M., and Bergans, A. (1962) Some effects of human growth hormone on renal hemodynamics and on tubular phosphate transport in man. *J Clin Invest* **41**, 1230-1235. 291036
382. Takamoto, S., Tsuchiya, H., Onishi, T., Morimoto, S., Imanaka, S., Mori, S., Seino, Y., Uozumi, T., and Kumahara, Y. (1985) Changes in calcium homeostasis in acromegaly treated by pituitary adenomectomy. *J Clin Endocrinol Metab* **61**, 7-11.
383. Lund, B., Eskildsen, P. C., Lund, B., Norman, A. W., and Sorensen, O. H. (1981) Calcium and vitamin D metabolism in acromegaly. *Acta Endocrinol (Copenh)* **96**, 444-450.
384. Holdaway, I. M., Bolland, M. J., and Gamble, G. D. (2008) A meta-analysis of the effect of lowering serum levels of GH and IGF-I on mortality in acromegaly. *Eur J Endocrinol* **159**, 89-95.
385. Melmed, S. (2009) Acromegaly pathogenesis and treatment. *J Clin Invest* **119**, 3189-3202. 2769196
386. Sze, L., Bernays, R. L., Zwimpfer, C., Wiesli, P., Brandle, M., and Schmid, C. (2012) Excessively high soluble Klotho in patients with acromegaly. *J Intern Med* **272**, 93-97.
387. Neidert, M. C., Sze, L., Zwimpfer, C., Sarnthein, J., Seifert, B., Frei, K., Leske, H., Rushing, E. J., Schmid, C., and Bernays, R. L. (2013) Soluble

alpha-klotho: a novel serum biomarker for the activity of GH-producing pituitary adenomas. *Eur J Endocrinol* **168**, 575-583.

388. Kohler, S., Tschopp, O., Sze, L., Neidert, M., Bernays, R. L., Spanaus, K. S., Wiesli, P., and Schmid, C. (2013) Monitoring for potential residual disease activity by serum insulin-like growth factor 1 and soluble Klotho in patients with acromegaly after pituitary surgery: Is there an impact of the genomic deletion of exon 3 in the growth hormone receptor (d3-GHR) gene on "safe" GH cut-off values? *Gen Comp Endocrinol* **188**, 282-287.
389. Lu, L., Katsaros, D., Wiley, A., de la Longrais, I. A., Puopolo, M., and Yu, H. (2008) Klotho expression in epithelial ovarian cancer and its association with insulin-like growth factors and disease progression. *Cancer Invest* **26**, 185-192.
390. Chen, B., Wang, X., Zhao, W., and Wu, J. (2010) Klotho inhibits growth and promotes apoptosis in human lung cancer cell line A549. *J Exp Clin Cancer Res* **29**, 99. 2912837
391. Camilli, T. C., Xu, M., O'Connell, M. P., Chien, B., Frank, B. P., Subaran, S., Indig, F. E., Morin, P. J., Hewitt, S. M., and Weeraratna, A. T. (2011) Loss of Klotho during melanoma progression leads to increased filamin cleavage, increased Wnt5A expression, and enhanced melanoma cell motility. *Pigment Cell Melanoma Res* **24**, 175-186. 3021583
392. Usuda, J., Ichinose, S., Ishizumi, T., Ohtani, K., Inoue, T., Saji, H., Kakihana, M., Kajiwara, N., Uchida, O., Nomura, M., Tsutsui, H., Ohira, T., and Ikeda, N. (2011) Klotho is a novel biomarker for good survival in resected large cell neuroendocrine carcinoma of the lung. *Lung Cancer* **72**, 355-359.
393. Wang, X., Chen, B., Xu, W., Liu, S., Zhao, W., and Wu, J. (2011) Combined effects of klotho and soluble CD40 ligand on A549 lung cancer cells. *Oncol Rep* **25**, 1465-1472.
394. Usuda, J., Ichinose, S., Ishizumi, T., Ohtani, K., Inoue, T., Saji, H., Kakihana, M., Kajiwara, N., Uchida, O., Nomura, M., Ohira, T., and Ikeda, N. (2011) Klotho predicts good clinical outcome in patients with limited-disease small cell lung cancer who received surgery. *Lung Cancer* **74**, 332-337.
395. Chang, B., Kim, J., Jeong, D., Jeong, Y., Jeon, S., Jung, S. I., Yang, Y., Kim, K. I., Lim, J. S., Kim, C., and Lee, M. S. (2012) Klotho inhibits the capacity

- of cell migration and invasion in cervical cancer. *Oncol Rep* **28**, 1022-1028.
396. Chen, B., Ma, X., Liu, S., Zhao, W., and Wu, J. (2012) Inhibition of lung cancer cells growth, motility and induction of apoptosis by Klotho, a novel secreted Wnt antagonist, in a dose-dependent manner. *Cancer Biol Ther* **13**, 1221-1228. 3469480
397. Xie, B., Zhou, J., Shu, G., Liu, D. C., Zhou, J., Chen, J., and Yuan, L. (2013) Restoration of klotho gene expression induces apoptosis and autophagy in gastric cancer cells: tumor suppressive role of klotho in gastric cancer. *Cancer Cell Int* **13**, 18. 3599807
398. Zhu, Y., Xu, L., Zhang, J., Xu, W., Liu, Y., Yin, H., Lv, T., An, H., Liu, L., He, H., Zhang, H., Liu, J., Xu, J., and Lin, Z. (2013) Klotho suppresses tumor progression via inhibiting PI3K/Akt/GSK3beta/Snail signaling in renal cell carcinoma. *Cancer Sci* **104**, 663-671.
399. Wang, Y., Chen, L., Huang, G., He, D., He, J., Xu, W., Zou, C., Zong, F., Li, Y., Chen, B., Wu, S., Zhao, W., and Wu, J. (2013) Klotho sensitizes human lung cancer cell line to cisplatin via PI3k/Akt pathway. *PLoS One* **8**, e57391. 3578855
400. Abramovitz, L., Rubinek, T., Ligumsky, H., Bose, S., Barshack, I., Avivi, C., Kaufman, B., and Wolf, I. (2011) KL1 internal repeat mediates klotho tumor suppressor activities and inhibits bFGF and IGF-I signaling in pancreatic cancer. *Clin Cancer Res* **17**, 4254-4266.
401. Nagai, R., Saito, Y., Ohyama, Y., Aizawa, H., Suga, T., Nakamura, T., Kurabayashi, M., and Kuroo, M. (2000) Endothelial dysfunction in the klotho mouse and downregulation of klotho gene expression in various animal models of vascular and metabolic diseases. *Cell Mol Life Sci* **57**, 738-746. 10892340
402. Shroff, R., and Shanahan, C. M. (2011) Klotho: an elixir of youth for the vasculature? *J Am Soc Nephrol* **22**, 5-7.
403. Munzel, T., Sinning, C., Post, F., Warnholtz, A., and Schulz, E. (2008) Pathophysiology, diagnosis and prognostic implications of endothelial dysfunction. *Ann Med* **40**, 180-196.
404. Lim, K., Lu, T. S., Molostvov, G., Lee, C., Lam, F. T., Zehnder, D., and Hsiao, L. L. (2012) Vascular Klotho deficiency potentiates the

development of human artery calcification and mediates resistance to fibroblast growth factor 23. *Circulation* **125**, 2243-2255.

405. Semba, R. D., Cappola, A. R., Sun, K., Bandinelli, S., Dalal, M., Crasto, C., Guralnik, J. M., and Ferrucci, L. (2011) Plasma klotho and cardiovascular disease in adults. *J Am Geriatr Soc* **59**, 1596-1601. 3486641
406. Kitagawa, M., Sugiyama, H., Morinaga, H., Inoue, T., Takiue, K., Ogawa, A., Yamanari, T., Kikumoto, Y., Uchida, H. A., Kitamura, S., Maeshima, Y., Nakamura, K., Ito, H., and Makino, H. (2013) A decreased level of serum soluble Klotho is an independent biomarker associated with arterial stiffness in patients with chronic kidney disease. *PLoS One* **8**, e56695. 3576368
407. Cheng, M. F., Chen, L. J., and Cheng, J. T. (2010) Decrease of Klotho in the kidney of streptozotocin-induced diabetic rats. *J Biomed Biotechnol* **2010**, 513853. 2896693
408. Asai, O., Nakatani, K., Tanaka, T., Sakan, H., Imura, A., Yoshimoto, S., Samejima, K., Yamaguchi, Y., Matsui, M., Akai, Y., Konishi, N., Iwano, M., Nabeshima, Y., and Saito, Y. (2012) Decreased renal alpha-Klotho expression in early diabetic nephropathy in humans and mice and its possible role in urinary calcium excretion. *Kidney Int* **81**, 539-547.
409. Devaraj, S., Syed, B., Chien, A., and Jialal, I. (2012) Validation of an immunoassay for soluble Klotho protein: decreased levels in diabetes and increased levels in chronic kidney disease. *Am J Clin Pathol* **137**, 479-485.
410. Devaraj, S., Dasu, M. R., and Jialal, I. (2010) Diabetes is a proinflammatory state: a translational perspective. *Expert Rev Endocrinol Metab* **5**, 19-28. 2829992
411. Brownstein, C. A., Adler, F., Nelson-Williams, C., Iijima, J., Li, P., Imura, A., Nabeshima, Y., Reyes-Mugica, M., Carpenter, T. O., and Lifton, R. P. (2008) A translocation causing increased alpha-klotho level results in hypophosphatemic rickets and hyperparathyroidism. *Proc Natl Acad Sci U S A* **105**, 3455-3460. 2265125
412. Shimoyama, Y., Taki, K., Mitsuda, Y., Tsuruta, Y., Hamajima, N., and Niwa, T. (2009) KLOTHO gene polymorphisms G-395A and C1818T are associated with low-density lipoprotein cholesterol and uric acid in Japanese hemodialysis patients. *Am J Nephrol* **30**, 383-388.

413. Ichikawa, S., Imel, E. A., Kreiter, M. L., Yu, X., Mackenzie, D. S., Sorenson, A. H., Goetz, R., Mohammadi, M., White, K. E., and Econs, M. J. (2007) A homozygous missense mutation in human KLOTHO causes severe tumoral calcinosis. *J Clin Invest* **117**, 2684-2691. 17710231. PMC1940239
414. Rhee, E. J., Oh, K. W., Yun, E. J., Jung, C. H., Lee, W. Y., Kim, S. W., Baek, K. H., Kang, M. I., and Park, S. W. (2006) Relationship between polymorphisms G395A in promoter and C1818T in exon 4 of the KLOTHO gene with glucose metabolism and cardiovascular risk factors in Korean women. *J Endocrinol Invest* **29**, 613-618.
415. Jo, S. H., Kim, S. G., Choi, Y. J., Joo, N. R., Cho, G. Y., Choi, S. R., Kim, E. J., Kim, H. S., Kim, H. J., and Rhim, C. Y. (2009) KLOTHO gene polymorphism is associated with coronary artery stenosis but not with coronary calcification in a Korean population. *Int Heart J* **50**, 23-32.
416. Wang, H. L., Xu, Q., Wang, Z., Zhang, Y. H., Si, L. Y., Li, X. J., Yang, Q. H., and Xiao, H. (2010) A potential regulatory single nucleotide polymorphism in the promoter of the Klotho gene may be associated with essential hypertension in the Chinese Han population. *Clin Chim Acta* **411**, 386-390.
417. Imamura, A., Okumura, K., Ogawa, Y., Murakami, R., Torigoe, M., Numaguchi, Y., and Murohara, T. (2006) Klotho gene polymorphism may be a genetic risk factor for atherosclerotic coronary artery disease but not for vasospastic angina in Japanese. *Clin Chim Acta* **371**, 66-70. 16579981
418. Kim, Y., Kim, J. H., Nam, Y. J., Kong, M., Kim, Y. J., Yu, K. H., Lee, B. C., and Lee, C. (2006) Klotho is a genetic risk factor for ischemic stroke caused by cardioembolism in Korean females. *Neurosci Lett* **407**, 189-194.
419. Rhee, E. J., Oh, K. W., Lee, W. Y., Kim, S. Y., Jung, C. H., Kim, B. J., Sung, K. C., Kim, B. S., Kang, J. H., Lee, M. H., Kim, S. W., and Park, J. R. (2006) The differential effects of age on the association of KLOTHO gene polymorphisms with coronary artery disease. *Metabolism* **55**, 1344-1351.
420. Ko, G. J., Lee, E. A., Jeon, U. S., Pyo, H. J., Chin, H. J., Chae, D. W., Kim, S., and Kwon, Y. J. (2012) The association of Klotho polymorphism with disease progression and mortality in IgA nephropathy. *Kidney Blood Press Res* **36**, 191-199.
421. Kim, Y., Jeong, S. J., Lee, H. S., Kim, E. J., Song, Y. R., Kim, S. G., Oh, J. E., Lee, Y. K., Seo, J. W., Yoon, J. W., Koo, J. R., Kim, H. J., Noh, J. W., and Park, S. H. (2008) Polymorphism in the promoter region of the klotho

- gene (G-395A) is associated with early dysfunction in vascular access in hemodialysis patients. *Korean J Intern Med* **23**, 201-207. 2687682
422. Yamada, Y., Ando, F., Niino, N., and Shimokata, H. (2005) Association of polymorphisms of the androgen receptor and klotho genes with bone mineral density in Japanese women. *J Mol Med (Berl)* **83**, 50-57.
423. Mullin, B. H., Wilson, S. G., Islam, F. M., Calautti, M., Dick, I. M., Devine, A., and Prince, R. L. (2005) Klotho gene polymorphisms are associated with osteocalcin levels but not bone density of aged postmenopausal women. *Calcif Tissue Int* **77**, 145-151.
424. Kawano, K., Ogata, N., Chiano, M., Molloy, H., Kleyn, P., Spector, T. D., Uchida, M., Hosoi, T., Suzuki, T., Orimo, H., Inoue, S., Nabeshima, Y., Nakamura, K., Kuro-o, M., and Kawaguchi, H. (2002) Klotho gene polymorphisms associated with bone density of aged postmenopausal women. *J Bone Miner Res* **17**, 1744-1751. 12369777
425. Tsezou, A., Furuichi, T., Satra, M., Makrythanasis, P., Ikegawa, S., and Malizos, K. N. (2008) Association of KLOTHO gene polymorphisms with knee osteoarthritis in Greek population. *J Orthop Res* **26**, 1466-1470.
426. Katsoulis C, C. A., Xita N, Gergiou I, Tsatsoulis A. (2009) Klotho gene polymorphism may be a genetic risk factor for metabolic syndrome in men. in *11th European Congress of Endocrinology*, Endocrin Abstracts, Istanbul, Turkey
427. Shimoyama, Y., Nishio, K., Hamajima, N., and Niwa, T. (2009) KLOTHO gene polymorphisms G-395A and C1818T are associated with lipid and glucose metabolism, bone mineral density and systolic blood pressure in Japanese healthy subjects. *Clin Chim Acta* **406**, 134-138.
428. Sotiriou I, K. A., Chazikyriakidou A, Tryfon S, Froudarakis ME, Georgiou I, Bourous D. Klotho Gene Polymorphism -395 G<A IN Patients with Chronic Obstructive Pulmonary Disease (COPD). *PNEUMONIA* **4**, 348-354.
429. Nolan, V. G., Baldwin, C., Ma, Q., Wyszynski, D. F., Amirault, Y., Farrell, J. J., Bisbee, A., Embury, S. H., Farrer, L. A., and Steinberg, M. H. (2005) Association of single nucleotide polymorphisms in klotho with priapism in sickle cell anaemia. *Br J Haematol* **128**, 266-272. 15638863
430. Nolan, V. G., Adewoye, A., Baldwin, C., Wang, L., Ma, Q., Wyszynski, D. F., Farrell, J. J., Sebastiani, P., Farrer, L. A., and Steinberg, M. H. (2006)

- Sickle cell leg ulcers: associations with haemolysis and SNPs in Klotho, TEK and genes of the TGF-beta/BMP pathway. *Br J Haematol* **133**, 570-578. 1679888
431. Telci, D., Dogan, A. U., Ozbek, E., Polat, E. C., Simsek, A., Cakir, S. S., Yeloglu, H. O., and Sahin, F. (2011) KLOTHO gene polymorphism of G395A is associated with kidney stones. *Am J Nephrol* **33**, 337-343.
432. Friedman, D. J., Afkarian, M., Tamez, H., Bhan, I., Isakova, T., Wolf, M., Ankers, E., Ye, J., Tonelli, M., Zoccali, C., Kuro-o, M., Moe, O., Karumanchi, S. A., and Thadhani, R. (2009) Klotho variants and chronic hemodialysis mortality. *J Bone Miner Res* **24**, 1847-1855. 2765930
433. Baldwin, C., Nolan, V. G., Wyszynski, D. F., Ma, Q. L., Sebastiani, P., Embury, S. H., Bisbee, A., Farrell, J., Farrer, L., and Steinberg, M. H. (2005) Association of klotho, bone morphogenic protein 6, and annexin A2 polymorphisms with sickle cell osteonecrosis. *Blood* **106**, 372-375. 1895132
434. Ogata, N., Matsumura, Y., Shiraki, M., Kawano, K., Koshizuka, Y., Hosoi, T., Nakamura, K., Kuro, O. M., and Kawaguchi, H. (2002) Association of klotho gene polymorphism with bone density and spondylosis of the lumbar spine in postmenopausal women. *Bone* **31**, 37-42. 12110410
435. Majumdar, V., and Christopher, R. (2011) Association of exonic variants of Klotho with metabolic syndrome in Asian Indians. *Clin Chim Acta* **412**, 1116-1121. 21376714
436. Majumdar, V., Jose, D., and Christopher, R. (2011) Influence of Klotho genotypes on plasma NO(x) levels in South Indian population. *Thromb Res* **128**, 251-255. 21543107
437. Xu, C., Song, R. J., Yang, J., Jiang, B., Wang, X. L., Wu, W., and Zhang, W. (2013) Klotho gene polymorphism of rs3752472 is associated with the risk of urinary calculi in the population of Han nationality in Eastern China. *Gene*
438. Oguro, R., Kamide, K., Kokubo, Y., Shimaoka, I., Congrains, A., Horio, T., Hanada, H., Ohishi, M., Katsuya, T., Okamura, T., Miyata, T., Kawano, Y., and Rakugi, H. (2010) Association of carotid atherosclerosis with genetic polymorphisms of the klotho gene in patients with hypertension. *Geriatr Gerontol Int* **10**, 311-318.

439. Bostrom, M. A., Hicks, P. J., Lu, L., Langefeld, C. D., Freedman, B. I., and Bowden, D. W. (2010) Association of polymorphisms in the klotho gene with severity of non-diabetic ESRD in African Americans. *Nephrol Dial Transplant* **25**, 3348-3355. 2948839
440. Invidia, L., Salvioli, S., Altilia, S., Pierini, M., Panourgia, M. P., Monti, D., De Rango, F., Passarino, G., and Franceschi, C. (2010) The frequency of Klotho KL-VS polymorphism in a large Italian population, from young subjects to centenarians, suggests the presence of specific time windows for its effect. *Biogerontology* **11**, 67-73. 19421891
441. Arking, D. E., Atzmon, G., Arking, A., Barzilai, N., and Dietz, H. C. (2005) Association between a functional variant of the KLOTHO gene and high-density lipoprotein cholesterol, blood pressure, stroke, and longevity. *Circ Res* **96**, 412-418. 15677572
442. Arking, D. E., Krebsova, A., Macek, M., Sr., Macek, M., Jr., Arking, A., Mian, I. S., Fried, L., Hamosh, A., Dey, S., McIntosh, I., and Dietz, H. C. (2002) Association of human aging with a functional variant of klotho. *Proc Natl Acad Sci U S A* **99**, 856-861. 11792841
443. Arking, D. E., Becker, D. M., Yanek, L. R., Fallin, D., Judge, D. P., Moy, T. F., Becker, L. C., and Dietz, H. C. (2003) KLOTHO allele status and the risk of early-onset occult coronary artery disease. *Am J Hum Genet* **72**, 1154-1161. 12669274
444. Majumdar, V., Nagaraja, D., and Christopher, R. (2010) Association of the functional KL-VS variant of Klotho gene with early-onset ischemic stroke. *Biochem Biophys Res Commun* 21093413
445. Riancho, J. A., Valero, C., Hernandez, J. L., Ortiz, F., Zarrabeitia, A., Alonso, M. A., Pena, N., Pascual, M. A., Gonzalez-Macias, J., and Zarrabeitia, M. T. (2007) Association of the F352V variant of the Klotho gene with bone mineral density. *Biogerontology* **8**, 121-127. 16955217
446. Zarrabeitia, M. T., Hernandez, J. L., Valero, C., Zarrabeitia, A. L., Ortiz, F., Gonzalez-Macias, J., and Riancho, J. A. (2007) Klotho gene polymorphism and male bone mass. *Calcif Tissue Int* **80**, 10-14. 17205327

447. Wolf, I., Laitman, Y., Rubinek, T., Abramovitz, L., Novikov, I., Beerli, R., Kuro, O. M., Koeffler, H. P., Catane, R., Freedman, L. S., Levy-Lahad, E., Karlan, B. Y., Friedman, E., and Kaufman, B. (2010) Functional variant of KLOTHO: a breast cancer risk modifier among BRCA1 mutation carriers of Ashkenazi origin. *Oncogene* **29**, 26-33. 19802015
448. Laitman, Y., Kuchenbaecker, K. B., Rantala, J., Hogervorst, F., Peock, S., Godwin, A. K., Arason, A., Kirchhoff, T., Offit, K., Isaacs, C., Schmutzler, R. K., Wappenschmidt, B., Nevanlinna, H., Chen, X., Chenevix-Trench, G., Healey, S., Couch, F., Peterlongo, P., Radice, P., Nathanson, K. L., Caligo, M. A., Neuhausen, S. L., Ganz, P., Sinilnikova, O. M., McGuffog, L., Easton, D. F., Antoniou, A. C., Wolf, I., and Friedman, E. (2012) The KLVS sequence variant of Klotho and cancer risk in BRCA1 and BRCA2 mutation carriers. *Breast Cancer Res Treat* **132**, 1119-1126. 22212556

PMC3352679

449. Fimbel, S., Dechaud, H., Grenot, C., Tabard, L., Claustrat, F., Bador, R., and Pugeat, M. (1995) Use of non-radioactive labels for half-life measurement of sex hormone-binding globulin in the rabbit. *Steroids* **60**, 686-692. 8539777
450. Chen, A. C., Guo, L. Y., Ostaszewski, B. L., Selkoe, D. J., and LaVoie, M. J. (2010) Aph-1 associates directly with full-length and C-terminal fragments of gamma-secretase substrates. *J Biol Chem* **285**, 11378-11391. 20145246

PMC2857016

451. Engelmann, K., Kinlough, C. L., Muller, S., Razawi, H., Baldus, S. E., Hughey, R. P., and Hanisch, F. G. (2005) Transmembrane and secreted MUC1 probes show trafficking-dependent changes in O-glycan core profiles. *Glycobiology* **15**, 1111-1124.
452. Sato, S., Ward, C. L., Krouse, M. E., Wine, J. J., and Kopito, R. R. (1996) Glycerol reverses the misfolding phenotype of the most common cystic fibrosis mutation. *J Biol Chem* **271**, 635-638.
453. Gonzales, P. A., Pisitkun, T., Hoffert, J. D., Tchapyjnikov, D., Star, R. A., Kleta, R., Wang, N. S., and Knepper, M. A. (2009) Large-scale proteomics and phosphoproteomics of urinary exosomes. *J Am Soc Nephrol* **20**, 363-379. 19056867. PMC2637050
454. Hurtley, S. M., and Helenius, A. (1989) Protein oligomerization in the endoplasmic reticulum. *Annu Rev Cell Biol* **5**, 277-307. 2688707

455. Pelham, H. R. (1989) Control of protein exit from the endoplasmic reticulum. *Annu Rev Cell Biol* **5**, 1-23. 2688704
456. Li, W., and Stanley, E. R. (1991) Role of dimerization and modification of the CSF-1 receptor in its activation and internalization during the CSF-1 response. *EMBO J* **10**, 277-288. 1825054. PMC452644
457. Wang, Q., Villeneuve, G., and Wang, Z. (2005) Control of epidermal growth factor receptor endocytosis by receptor dimerization, rather than receptor kinase activation. *EMBO Rep* **6**, 942-948. 16113650. PMC1369181
458. Wu, X., Lemon, B., Li, X., Gupte, J., Weiszmann, J., Stevens, J., Hawkins, N., Shen, W., Lindberg, R., Chen, J. L., Tian, H., and Li, Y. (2008) C-terminal tail of FGF19 determines its specificity toward Klotho co-receptors. *J Biol Chem* **283**, 33304-33309. 18829467. PMC2662259
459. Database, N. N. NM_153683.2. National Center for Biotechnology Information, U.S. National Library of Medicine 8600 Rockville Pike, Bethesda MD, 20894 USA
460. Qureshi, A. A., Crane, A. M., Matiaszuk, N. V., Rezvani, I., Ledley, F. D., and Rosenblatt, D. S. (1994) Cloning and expression of mutations demonstrating intragenic complementation in muto methylmalonic aciduria. *J Clin Invest* **93**, 1812-1819. 294249
461. Ferre, F., and Clote, P. (2005) DiANNA: a web server for disulfide connectivity prediction. *Nucleic Acids Res* **33**, W230-232. 1160173
462. Creighton, T. E. (1978) Experimental studies of protein folding and unfolding. *Prog Biophys Mol Biol* **33**, 231-297.
463. Banerjee, R. R., and Lazar, M. A. (2001) Dimerization of resistin and resistin-like molecules is determined by a single cysteine. *J Biol Chem* **276**, 25970-25973.
464. Romano, C., Yang, W. L., and O'Malley, K. L. (1996) Metabotropic glutamate receptor 5 is a disulfide-linked dimer. *J Biol Chem* **271**, 28612-28616.
465. Boyson, J. E., Erskine, R., Whitman, M. C., Chiu, M., Lau, J. M., Koopman, L. A., Valter, M. M., Angelisova, P., Horejsi, V., and Strominger, J. L. (2002) Disulfide bond-mediated dimerization of HLA-G on the cell surface. *Proc Natl Acad Sci U S A* **99**, 16180-16185. 138585

466. Scheuermann, S., Hambsch, B., Hesse, L., Stumm, J., Schmidt, C., Beher, D., Bayer, T. A., Beyreuther, K., and Multhaup, G. (2001) Homodimerization of amyloid precursor protein and its implication in the amyloidogenic pathway of Alzheimer's disease. *J Biol Chem* **276**, 33923-33929. 11438549
467. Hu, M. C., Kuro, O. M., and Moe, O. W. (2013) Klotho and chronic kidney disease. *Contrib Nephrol* **180**, 47-63.
468. Olauson, H., and Larsson, T. E. (2013) FGF23 and Klotho in chronic kidney disease. *Curr Opin Nephrol Hypertens*
469. Ichikawa, S., Imel, E. A., Kreiter, M. L., Yu, X., Mackenzie, D. S., Sorenson, A. H., Goetz, R., Mohammadi, M., White, K. E., and Econs, M. J. (2007) A homozygous missense mutation in human KLOTHO causes severe tumoral calcinosis. *J Musculoskelet Neuronal Interact* **7**, 318-319. 18094491
470. Ming, A. Y., Yoo, E., Vorontsov, E. N., Altamentova, S. M., Kilkenny, D. M., and Rocheleau, J. V. (2012) Dynamics and Distribution of Klothobeta (KLB) and fibroblast growth factor receptor-1 (FGFR1) in living cells reveal the fibroblast growth factor-21 (FGF21)-induced receptor complex. *J Biol Chem* **287**, 19997-20006. 3370183
471. Martin, A., David, V., and Quarles, L. D. (2012) Regulation and function of the FGF23/klotho endocrine pathways. *Physiol Rev* **92**, 131-155. 3306265
472. Brunelli, S. M., and Goldfarb, S. (2007) Hypophosphatemia: clinical consequences and management. *J Am Soc Nephrol* **18**, 1999-2003.
473. Pelletier, M. F., Marcil, A., Sevigny, G., Jakob, C. A., Tessier, D. C., Chevet, E., Menard, R., Bergeron, J. J., and Thomas, D. Y. (2000) The heterodimeric structure of glucosidase II is required for its activity, solubility, and localization in vivo. *Glycobiology* **10**, 815-827.
474. Watanabe, T., Sato, T., Yoshioka, S., Koshijima, T., and Kuwahara, M. (1992) Purification and properties of *Aspergillus niger* beta-glucosidase. *Eur J Biochem* **209**, 651-659.
475. Esen, A. (1993) β -Glucosidases. in \square -*Glucosidases*, American Chemical Society. pp 1-14
476. Medici, D., Razaque, M. S., Deluca, S., Rector, T. L., Hou, B., Kang, K., Goetz, R., Mohammadi, M., Kuro, O. M., Olsen, B. R., and Lanske, B.

(2008) FGF-23-Klotho signaling stimulates proliferation and prevents vitamin D-induced apoptosis. *J Cell Biol* **182**, 459-465. 18678710

CURRICULUM VITAE

