DIETARY- AND FASTING-BASED INTERVENTIONS AS NOVEL APPROACHES TO IMPROVE THE EFFICACY OF CANCER TREATMENT

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For my grandparents.

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Abbreviations

·O2-	superoxide radical
40S	ribosomal protein
4E-BP1	eIF4E-binding protein
4T1-HO1	murine breast cancer cells over-expressing heme oxygenase-1
5-FU	5-fluorouracil
60S	ribosomal protein
A1	Bcl-2-related protein A1
AC	adenyl cyclase
acetyl-CoA	acetyl-Co enzyme A
ACL	ATP citrate lyase
ad lib	ad libitum
age-1	homolog of PI3K in C. elegans
AHA	azidohomoalanine, methionine analogue
AIN93G	rodent standard chow
AMPK	adenosine monophosphate-activated protein kinase
AP-1	activator protein-1
Apaf-1	apoptotic protease activating factor-1
ATP	adenosine triphosphate
BACH1	BTB and CNC homology 1, basic leucine zipper transcription factor 1
Bad	BCL2-associated agonist of cell death
Bax	BCL2-associated X protein
Bcl-2	B-cell CLL/lymphoma 2
Bcl-W	BCL2-like 2
Bcl-XL	BCL2-like 1
Bim	BCL2-like 11
BLI	bioluminescence imaging
bp	base-pair
B-Raf	member of the Raf kinase family of serine/threonine-specific protein kinases
BubR1	regulator of chromosomal segregation
C. elegans	*Caenorhabditis elegans*
Ca	calcium
CAM	cell adhesion molecule
cAMP	cyclic adenosine monophosphate
CDDP	Cisplatin
cDNA	complementary DNA
CFU	colony forming units
CO	carbon monoxide
CO_2	carbon dioxide
CP	Cyclophosphamide
CR	calorie restriction
CREB	cyclic adenosine monphosphate response element-binding protein

CTCAE	Common Terminology Criteria for Adverse Events
D. melanogaster	*Drosophila melanogaster*
DAF-16	FOXO transcription factor in *C. elegans*, homolog to FOXO3
daf-2	homologs of insulin/IGF-I receptor in *C. elegans*
DHE	dihydroethidium
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNP	dinitrophenol
DR	dietary restriction
DSR	differential stress resistance
DSS	differential stress sensitization
DXR	Doxorubicin
E2F	transcription factor super-family
ECAR	extra-cellular acidification rate
ECM	extra-cellular matrix
Eef1γ	elongation factor 1 gamma
EGF	epidermal growth factor
eIF2α	elongation factor 2 alpha
ELISA	enzyme-linked immuno-sorbent assay
ERK	extracellular signal-regulated kinase
Eto	etoposide
FAO	fatty acid oxidation
FAS	fatty acid synthase
FBS	fetal bovine serum
FCCP	carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone
FDA	Food and Drug Administration (USA)
FGF	fibroblast growth factor
FMD	fasting mimicking diet
FOXO	forkhead box protein
G418	genecitin
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GBM	glioblastoma multiforme
GH	growth hormone
GHBP	growth hormone binding protein
GHIH	growth hormone-inhibiting hormone
GHR	growth hormone receptor
GHRD	growth hormone receptor deficient
GHRH	growth hormone-releasing hormone
GHR-KO	growth hormone-receptor knock out
GIS1	GIg1-2 Suppressor 3
Glut1	glucose transporter-1
GRB2	growth factor receptor-bound protein 2

GRP78	glucose-regulated protein 78
GSK3β	glycogen synthase kinase 3β
GTP	guanosine triphosphate
gy	gray (unit), SI unit of absorbed radiation
H_2O	water
H_2O_2	hydrogen peroxide
HF	high-fat
HIF-1	hypoxia inducible factor 1
HIF1α	hypoxia-inducible factor 1α
HLA	human leucocyte antigen
HO-1	heme oxygenase-1
HPG	hypothalamic-pituitary-gonadal
HSF	heat shock factor
Hsp32	heat-shock protein 32
Hsp70	heat-shock protein 70
i.p.	intra-peritoneal
i.v.	intra-venous
IACUC	Institutional Animal Care and Use Committee
ICR	intermittent calorie restriction
IF	intermittent fasting
IGF-BP	insulin-like growth factor 1 binding protein
IGF-I	insulin-like growth factor 1
IGF-IR	insulin-like growth factor 1 receptor
Ins	insulin
Irs2	insulin signaling adaptor
ISG15	interferon-stimulated gene 15
JAK	Janus kinase
kDa	kilo Dalton
LCHP	low carbohydrate, high protein
LCT	long-chain triglycerides
LDH	lactate dehydrogenase
LDH-A	lactate dehydrogenase A
LID	liver *Igf1* gene deletion
LKB1	liver kinase B1
LON	ATP-dependent serine peptidase
MAC	mitochondrial apoptosis-induced channel
MAPK	mitogen-activated protein kinase
Mcl-1	myeloid leukemia cell differentiation protein-1
MCT	medium-chain triglyceride
MDH	malate dehydrogenase
ME	malic enzyme
Mg	magnesium
MMP	matrix metalloproteinases

MnSOD	see SOD-2
MSN2	multicopy suppressor of SNF1 mutation 2
MSN4	multicopy suppressor of SNF1 mutation 4
mTOR	mammalian target of rapamycin
MTT	methyl-thiazolyldiphenyl-tetrazolium bromide
Myc	myelocytomatosis viral oncogene
NAD+	nicotinamide adenine dinucleotide (oxidized)
NADH	nicotinamide adenine dinucleotide (reduced)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NCAM	neural cell adhesion molecule
NCCSC	National Cancer Chemotherapy Service Center (USA)
NCI	National Cancer Institute (USA)
NF1	neurofibromin 1
NF-E2	nuclear factor-erythroid 2
NFkB	nuclear factor-kappa B
NH_4OH	ammonium hydroxide
NIA	National Institute on Aging
NIH	National Institutes of Health
O_2	oxygen
OAA	oxaloacetate
OCR	oxygen consumption rates
OD	optical density
p107	retinoblastoma-associated protein homolog
p130	retinoblastoma-associated protein homolog
$p16^{INK4A}$	cyclin-dependent kinase inhibitor 2A
P53	tumor protein 53
$p66^{shc}$	66-kilodalton isoform of the growth factor adapter Shc
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDH	pyruvate dehydrogenase
PDK1	3-phosphoinositide-dependent kinase 1
PI3K	phosphoinositide 3-kinase
PIP_2	phosphatidylinositol 4,5-bisphosphate
PIP_3	phosphatidylinositol (3,4,5) trisphosphate
Pit-1	pituitary-specific transcription factor-1
PKA	protein kinase A
Prlr	prolactin receptor
Prop-1	homeobox protein prophet of *PIT-1*
PTEN	phosphatase and tensin homolog
Puma	P53 upregulated modulator of apoptosis
qRT-PCR	quantitative real-time polymerase chain reaction
RAS	small GTPase protein family member
Ras GAP	Ras GTPase-activating protein

Ras GEF	Ras guanine nucleotide exchange factor
RB	retinoblastoma-associated protein
RelA	transcription factor p65
rHO-1	rat heme oxygenase-1
Rip1Tag2	transgenic mouse model of β-cell carcinogenesis
RNA	ribonucleic acid
RNAi	ribonucleic acid interference
RNS	reactive nitrogen species
ROS	reactive oxygen species
rpm	rounds per minute
RTP	radio-therapy
RT-PCR	reverse transcriptase polymerase chain reaction
s.c.	subcutaneous
S6K	ribosomal protein S6 kinase
SCH9	protein kinase and functional ortholog of mammalian S6 kinase
SDC	synthetic dextra complete
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
siRNA	small interfering ribonucleic acid
Sod-2	mitochondrial superoxide dismutase
STAT	signal transducer and activator of transcription
STS	short-term starvation
TCA	tricarboxylic acid cycle
TGFβ	transforming growth factor beta
TMZ	Temozolomide
TSC	tuberous sclerosis factor
TSP-1	thrombospondin-1
UCLA	University of California, Los Angeles
USC	University of Southern California
UV	ultra-violet
VEGF	vascular endothelial growth factor
VHL	von Hippel–Lindau
WNPRC	Wisconsin National Primate Research Center
WT	wild type
YPD	yeast extract peptone dextrose
ZnPP	zinc protoporphyrin
αIR3	antagonistic IGF-IR antibody

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Abstract

Short-term starvation (STS or fasting) protects normal cells, mice and potentially humans from the harmful side-effects of chemotherapeutic drugs. In this dissertation, I demonstrate that fasting-like cell culture conditions reduce cancer cell survival and sensitize human and murine cancer cell lines to chemotherapy. *In vivo*, cycles of STS were as effective as chemotherapeutics in delaying the progression of specific tumors and increased the effectiveness of these drugs and radiotherapy against melanoma, glioma, and breast cancer cells. In mouse models of neuroblastoma, STS cycles in combination with chemotherapy, but not either treatment alone, resulted in long-term cancer-free survival. In 4T1 breast cancer cells, STS led to increased phosphorylation of the stress-sensitizing AKT and S6 kinases, increased oxidative stress, caspase-3 activation, DNA damage, apoptosis, and reduced expression of the stress resistance transcription factor NFkB; all changes were not observed in normal tissues. Several of these effects are linked to the activity of the stress-responsive enzyme heme oxygenase-1, whose modulation was central in regulating chemotherapy-dependent cell death in breast cancer cells. These studies suggest that multiple cycles of STS promote differential stress sensitization in a wide range of tumors and could potentially replace or augment the efficacy of some toxic chemotherapy drugs in the treatment of various cancers.

In addition, we evaluated the contribution of calorie restricted (CR) diets and defined macronutrient (carbohydrate, protein, fat) ratios for their effects on stress sensitization markers and protection in mice treated with high-dose chemotherapy. Short-term 50% CR, combined with either severe protein-deficient or ketogenic diets, improved chemotoxicity resistance similarly to the standard 50% CR, but did not result in the high protection caused by STS. Notably, a high protein diet reversed the beneficial effects of short-term CR. In a subcutaneous mouse model of glioma, feeding a low protein diet (4% calories from protein *vs.* 18% in the control) for more than 20 days did not delay tumor progression once the tumor became palpable. Also, cycles of short-term (3 days) 50% CR did not augment the chemotherapy efficacy of cisplatin in a murine breast cancer model. These results indicate that the protection from chemotoxicity and retardation of tumor progression achieved with fasting could not be obtained with short-term calorie and/or macronutrient restriction.

Zusammenfassung

Kurzzeitiges Fasten (STS) schützt normale Zellen, Mäuse und potenziell auch Patienten vor den schädlichen Nebenwirkungen einer Chemotherapie. In dieser Dissertation demonstriere ich, dass fasten-ähnliche Zellkulturkonditionen das Überleben von Nagetier- und menschlichen Krebszellen reduziert und diese gleichzeitig gegenüber chemotherapeutischen Interventionen sensitiviert. *In vivo* sind Fastenzyklen für manche Krebszellen ähnlich effektiv wie die verwendete Chemotherapie und reduzieren das Tumorwachstum. In Tierversuchsmodellen für Melanome, Gliome und Brustkrebs konnte die Effektivität der verwendeten Chemotherapeutika und auch Strahlentherapie in Kombination mit Fasten erhöht werden. In Mausmodellen für Neuroblastome resultierte die Chemotherapie zusammen mit Fasten in einem langzeitigen Überleben der Mäuse, während die einzelnen Therapien erfolglos blieben. In dem 4T1 Maus Brustkrebs-Modell resultierte STS in einer erhöhten Phosphorylierung der Stress sensitivierenden Kinasen AKT und S6, erhöhtem oxidativen Zellstress, Spaltung der Caspase-3, DNS-Schäden, Apoptose und der gleichzeitig reduzierten Expression von Transkriptions-Faktoren (z.B. NFkB) die eine Rolle im Widerstand gegen Zellstress spielen. Interessanterweise konnten diese Veränderungen in normalen Zellen nicht aufgefunden werden. Mehrere dieser intrazellulären Effekte stehen mit der Aktivität des Enzyms heme oxygenase-1 in Verbindung, dessen Regulierung eine zentrale Rolle in der durch das Fasten bedingten Sensitivierung von 4T1 Brustkrebs spielt. Unsere Studien deuten darauf hin, das Fasten eine differentielle Stress-Sensitivierung in einer Vielzahl von Tumoren auslösen kann, die Effektivität von Chemotherapeutika erhöhen kann und somit möglicherweise eine Alternative zu Chemotherapie darstellt.

Um Alternativen zum Fasten zu finden, evaluiere ich in dieser Dissertation zusätzlich noch die Effekte von kalorienreduzierten (CR), und Nährstoff (Eiweiß, Fett und Kohlenhydrate) definierten, Nahrungsmitteln hinsichtlich ihrer Effektivität im Schutz gegen akut hohe Chemotherapie und Tumorprogression. Eine kurzzeitige 50% Reduzierung der konsumierten Kalorien, auch in Zusammenhang mit Proteindefiziten oder ketogenen Diäten, erhöhte die Resistenz gegenüber der Chemotherapie, blieb jedoch weniger effektiv als Fasten *per se*. Eine Diät mit hohem

Proteingehalt reversierte die positiven Effekte der CR. In subkutan implantierten Gliomen blieb die Ernährung mit auf niedrigem Eiweiß basierten Kalorien (4%) in der Maus nach mehr als 20 Tagen ohne Effekt auf das bereits etablierte Tumorwachstum. Zyklen in denen die Kalorien auf 50% reduziert wurden hatten, im Gegenteil zu Fasten, keine additive Wirkung auf die Behandlung von Brustkrebszellen mit Cisplatin in der Maus. Die präsentierten Ergebnisse belegen dass Fasten, jedoch nicht kurzeitige CR oder die Modifizierung der konsumierten Nährstoffe, vor der Toxizität von Chemotherapeutika schützt und gleichzeitig Krebszellen zu einer Vielzahl von Chemotherapien sensitiviert.

1 Introduction

1.1 Aging and Senescence

Aging in humans refers to a multidimensional process that includes, but is not limited to, the physiological, psychological, and social changes that accumulate chronologically in the lifetime of an individual. While some dimensions of aging grow and expand with time in the elderly, e.g. wisdom, others, such as physical strength, may drastically decline. Aging plays not only an important part of all humans on a personal level, but its consequences have a large impact on all human societies, as manifested in many cultural and societal conventions to date. Yet, a more biological definition of aging is the physiological deterioration that accompanies increasing age, raising the risk of death form a variety of causes (Finch and Schneider 1985). Alex Comfort defines aging as a progressive increase throughout life, or after a given condition, in the likelihood that an individual will die during the next succeeding unit of time from randomly distributed causes (Comfort 1956). Aging therefore includes a defining element: the increase in mortality (and decrease in fertility) that occurs in older organisms and is based on a gradual decline in the capacity to respond to environmental challenges (Rose 1991), also known as fitness.

Senescence (from Latin: senescere: to grow old) is commonly described as the accumulation of changes that cause alterations in the molecular and cellular structure, thereby affecting the metabolic capacity over time and consequently resulting in deterioration and death. Senescence occurs on the level of individual cells (cellular senescence) and subsequently causes alterations, including the onset of age-related diseases, within the whole of the organism (organismal senescence). There are a numerous hypotheses as to why senescence occurs. For example, some propose senescence occurs due to changes of gene expression (*a cause for failure*), others claim it is the cumulative damage caused by biological processes (*a consequence of failure*). If senescence as a biological process can be slowed down, halted or even reversed is a subject of ongoing scientific research.

Cellular senescence is the phenomenon by which normal cells lose the ability to divide and multiply; after about 50 cell divisions *in vitro*, known as the Hayflick limit in honor of Dr. Leonard Hayflick (Hayflick 1965). Some cells become senescent after

fewer replication cycles as a result of DNA double strand breaks, exposure to toxins or the shortening of telomeres located at the end of eukaryotic chromosomes (a phenomenon generally attributed to "replicative senescence"). In 2009, Elizabeth Blackburn, Carol Greider and Jack Szostak were awarded the Nobel Prize in Physiology or Medicine (the closest Nobel Prize to date related to the field of aging) for their significant contributions of how telomeres are maintained by the specialized reverse transcriptase telomerase. Rapid telomere shortening may indicate cellular hyper-activation, hyper-proliferation and/or hyper-secretory phenotypes that are often associated with cellular senescence, stem cell exhaustion and diseases of aging. In agreement with these possibilities, telomere shortening has been shown to regulate the expression of the interferon-stimulated gene 15 (ISG15). Lou and colleagues demonstrated, for the first time, that an endogenous human gene can be regulated by telomere length prior to the onset of telomere dysfunction and DNA damage signals. Therefore, ISG15 up-regulation in response to telomere shortening may contribute to a chronic inflammation commonly associated with human aging (Lou, Wei et al. 2009). In 2009, several publications by Epel, Blackburn and co-workers provided a new link between telomere length and age-related diseases. As published in the first issue of *Aging*, the rate of telomere shortening in peripheral leukocytes predicts mortality from cardiovascular disease in elderly men (Epel, Merkin et al. 2009). In response to DNA damage or shortened telomeres, cells either “age” or self-destruct through apoptosis, a highly regulated process that involves characteristic changes such as cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation. This “cellular suicide”, the death of one or more cells may benefit the organism as a whole because it depletes compromised or deficient cells from the body; thereby preventing the accumulation of potentially cancerous cells.

1.2 Theories of Aging

Theories of Aging try to explain the various aspects of senescence. Some of the major cellular and functional changes of aging and the potentially underlying theories are shown in **Table 1-1**. Due to the process of senescence being complex, and deriving from a variety of different mechanisms, the closest we can currently get to understand the exact etiology of aging is by accepting that all aging-theories contribute to a holistic understanding of the aging process.

Table 1-1. Major cellular and functional changes of aging by prominent theories and major associated clinical disease outcomes.

Modified from (Cefalu 2011).

Organ System	Major Theories	Cell Level	Structural/Functional Changes of Aging	Disease Outcomes
Integumentary	Oxidative stress; free radical; genetic; autoimmune	Melanocytes, mast, and Langerhans cells	Thinning of stratum corneum and subcutaneous layer	Squamous and basal cell carcinoma; malignant melanoma
Oral	Oxidative Stress; free radical; genetic; autoimmune	Buccal	Increased thickness of tooth dentin, decreased dental pulp; thinning of oral mucosa and receding of gums; decreased sensitivity for smell and taste	Squamous cell carcinoma; tooth decay
Visual	Oxidative stress; free radical; genetic	Rods and cones	Reduced night vision, accommodative ability and increased glare	Macular degeneration; cataracts; diabetic retinopathy
Hearing	Oxidative stress; free radical; genetic	Sensory and neural cells	Stiffening of the inner ear bones	Presbycusis; osteosclerosis
Musculoskeletal	Oxidative stress; genetic; autoimmune	Myocytes	Apoptosis, reduced size of myofibrils, decreased type 2 muscle fibers; decreased hand grip strength with more in the lower extremities	Falls; disuse atrophy; chronic musculoskeletal disorders
Skeletal	Oxidative stress; free radical; neuro endocrine	Osteoblasts and osteoclasts	Change in bone architecture and accumulation of microfractures, disparity in the concentration of deposited minerals, changes in the crystalline properties of mineral deposits and protein content of the matrix; decreased height and thinning of bone	Fractures
Cardiovascular	Oxidative stress; free radical; neuroendocrine; genetic	Myocyte; pacemaker cell	Increase in left ventricular stiffness and decrease in compliance; decreased left ventricular diastolic filling and relaxation, increased stroke volume, reduction in maximal cardiac output and vasodilator response to exercise	Congestive heart failure; cardiomyopathy; heart block
Pulmonary	Oxidative stress; free radical; genetic; autoimmune	Alveolar cells	Chest wall stiffness; decreased arterial oxygenation and impaired carbon dioxide elimination; decrease in vital capacity and forced expiratory volume, increased residual volume and functional residual capacity	Chronic lung disease; carcinoma
Gastrointestinal	Oxidative stress; free radical	Mucosal cell	Decreased elasticity of connective tissue; reduction in phase I metabolism	Carcinoma; increased risk of drug–drug and drug–disease interactions
Renal/urogenital	Oxidative stress; free radical; genetic; neuroendocrine; autoimmune	Renal cell	Diminished proliferative reserve; apoptosis; loss of glomerular and tubular mass; decline in GFR, loss of tubular volume and narrowed homeostatic control of water and electrolyte balance	Carcinoma; chronic renal failure
Neurologic	Oxidative stress; free radical; genetic; neuroendocrine	Neurons; glial cells	Decrease in size of hippocampus and frontal and temporal lobes; decreased number of receptors of all types in the brain with increased sensitivity; decrease in complex visuoconstructive skills and logical analysis skills; decrease in processing speed, decrease in reaction time and decrease ability to shift cognitive sets rapidly; memory distraction and decline in executive function; abnormal reflexes	Neuropathy; neurodegenerative disorders
Hematologic	Autoimmune; genetic; oxidative stress; free radical	Stem cells	Decreased marrow cellularity, increase in bone marrow fat and reduction in cancellous bone	Chronic anemia; myelofibrosis; leukemia
Neuroendocrine	Neuroendocrine; oxidative stress; genetic	Neuroendocrine cells; mitochondria	Decrease or increase in hormone levels; inability to conserve or dissipate heat	Autonomic neuropathy; thyroid disease; adrenal insufficiency; male and female menopause

Existing theories of aging can be divided into the programmed or stochastic theories of aging and are subsequently classified into *evolutionary*, *physiological, structural* and *functional* changes. Physiologic processes included into theories of aging emphasize the role of oxidative stress, immunology, neuro-endocrinology, the metabolism, insulin signaling pathways, and caloric restriction. At the cellular level, structural and functional processes that emphasize the role of intrinsic timing mechanisms and signals (for example through hormone signaling or programmed genetic signals), free radical formation, nuclear or mitochondrial DNA mutations or damage, the accumulation of damaged and abnormal proteins, accidental chance events, cross-linkage, glycation, waste accumulation, general molecular wear and tear, and specific cellular components such as genes, chromosomes or mitochondria, are all included into the aging theories (Cefalu 2011). Theories that include parts of these aspects are presented here after.

Understanding the biological evolution of aging and lifespan is based on observations of the life cycles of biological species. Evolutionary aging theories try to explain the differences in aging rates and longevity across different biological species (e.g. mice and humans) through the interplay of mutation- and selection-processes. For example, observations of the “suicidal” life cycles of species like the pacific salmon have promoted the idea that sexual reproduction may come with a cost for species longevity. Thus, in addition to mutation and selection, the reproductive cost, or, more generally, the trade-offs between different traits of organisms may also contribute to the evolution of species aging and longevity (Gavrilov and Gavrilova 2002). The “disposable soma theory” was proposed by Kirkwood in 1977 and describes that all living organisms have finite energy resources (Kirkwood 1977). The available energy must be balanced between maintenance and reproduction. Reproduction occurs when nutrients are sufficiently available to secure offspring survival. Under conditions of reduced nutrient availability, the reproductive rate is reduced in order to ensure cellular function. It is the compromise of allocating energy between reproduction and repair that causes the body gradually to deteriorate with age.

The logical foundations for most of the modern evolutionary theories of aging were completed relatively late in the 1950s and almost a century after Darwin suggested his theory of biological evolution (Darwin 2003). The biological evolution

of aging was initially studied in a purely theoretical and non-experimental way by Weismann (Programmed death theory, 1882; later adapted), Medawar (Mutation accumulation theory, 1952), Williams (Antagonistic Pleiotropy theory, 1957) and others. Their evolutionary theories of aging were subsequently (although only partially) tested by evolutionary experiments on *D. melanogaster* (Stearns, Ackermann et al. 2000) and on natural populations of *Poecilia reticulata* guppies (Reznick, Buckwalter et al. 2001). In these two studies, selection for later reproduction (artificial selection of late-born progeny) produced, as expected, longer-lived fruit flies while placing guppies in a more dangerous environment with high extrinsic mortality redirected evolution to a shorter lifespan in the following generations. These experiments found that aging and lifespan do evolve in subsequent generations of biological species in a theoretically predicted direction depending on the particular living conditions. Therefore, the early criticism of the evolutionary theory of aging as merely theoretical speculation, with limited and indirect supporting evidence obtained from retrospective and descriptive studies, has been overturned. A detailed overview about evolutionary aging theories is provided by Gavrilov et al. (Gavrilov and Gavrilova 2002).

The evolutionary theories of aging are closely related to the genetics of aging because biological evolution can only be possible through inheritable manifestations of aging. Programmed aging theories propose that senescence is the final destination in a developmental pathway that culminates in death. These theories suggest that aging is under control of “biological clocks” which operate throughout the lifespan of an organism. The regulatory effects of these clocks seem to be depending on changes in the gene expression pattern of systems involved in cellular-maintenance, -repair, and -defense. One such theory is the “Reproductive-Cell Cycle Theory” which reasons that cell growth, development, and death are under the regulation of reproductive hormones derived from the hypothalamic-pituitary-gonadal (HPG) axis. According to this theory, reproductive hormones, such as estrogens, progestagens, androgens and gonadotropins, and their receptors, have an essential function in promoting growth and development of the organism. Normal function in early life is important in order to achieve a maximal reproductive rate. With the onset of hormonal changes (in men around age 30 and in women when they reach menopause, around age 50), the HPG axis becomes unbalanced,

cellular growth and development become deregulated, cellular-dysfunction and cell-death are increasingly more prominent and thus promote senescence (Atwood and Bowen 2011). Supporting evidence for the reproductive-cell cycle theory includes studies that demonstrate that women with menopause occurring later in their life show reduced heart disease, fewer incidences of stroke, are less prone to dementia, and experience less osteoporosis. Conversely, early surgical menopause has been demonstrated to increase the incidence of these diseases (Ossewaarde, Bots et al. 2005). In addition, studies in support of the theory have shown that suppressing growth hormone, regulated by the neuro-secretory nuclei of the hypothalamus, and insulin-like growth factor 1 (IGF-I) signaling (a part of the growth hormone axis), such as caused by caloric restriction or exercise, increases lifespan (Atwood and Bowen 2011). Another programmed theory of aging is related to a similar “fixed” life span model. The developmental–genetic theory of aging proposes that the genetically programmed induction of senescence occurs during adult lifespan, which results in either the activation or suppression of specific “aging” genes. Support for this theory comes from studies that indicate that longevity in humans seems to be hereditable related to the presence of specific genes. One such “aging gene” is the human leucocyte antigen (HLA), which showed significantly high levels in a study of old (over the age of 85) healthy subjects (Ricci, Colombo et al. 1998). However, research that showed that physical fitness improves longevity in humans speaks against the developmental–genetic theory.

Stochastic theories position environmental impacts, which induce cumulative damage at various levels, as the cause of aging. Examples include oxygen radicals (widely known as free radicals and countered by the even more prominent antioxidants) that can damage DNA (e.g. by cross-linking), cells and tissues. The “Chromosomal Alterations Theory” proposes that normal aging is related to alterations in the chromosomal structure: deletions, mutations, translocations, and polyploidy acquired chromosomal instabilities contribute to gene silencing or gene specific expression over time (Cefalu 2011). Another popular theory of aging is the autoimmune theory, which is based on the hypothesis that the human body produces auto-antibodies against its own tissues and/or that deficits, primarily in T-cell function, predispose the elderly to the development of infections, chronic disease, cancer, and autoimmune diseases, e.g. rheumatoid arthritis (Kent 1977).

One of the most commonly accepted aging theories is the “Free Radical Theory of Aging”, first proposed by Harman in the 1950s (Harman 1956). The theory states that highly reactive oxygen-derived substances (free radicals) result in the accumulation of protein-, lipid-, and DNA-damage as a result of normal cellular metabolism. In later years, the theory was extended to include the role of mitochondrial free radical production (Harman 1972). Free radicals are mainly produced inside cellular organelles, in particular the mitochondria, which convert energy for the cell into adenosine triphosphate (ATP). Oxidative phosphorylation, the process in which ATP is produced, involves the shuttled transport of protons (hydrogen ions) through the electron transport chain across the inner mitochondrial membrane. For ATP production, electrons are passed through a series of proteins; each acceptor protein along the chain has a greater reduction potential than the previous one. The last destination for an electron along the electron transport chain is an oxygen molecule which is then reduced to produce H_2O. The reduction of oxygen and the transported electrons, however, is not 100% efficient. Approximately 0.1–2%, oxygen is prematurely and incompletely reduced to produce the superoxide radical (·O2-), mostly documented for Complex I and Complex III (Gomez and Hagen 2012). Reactive oxygen species are therefore a byproduct of normal cellular metabolism. Superoxide is not particularly reactive by itself, but plays a role in hydrogen peroxide formation (H_2O_2), which can leak from the mitochondria into the cell. It is postulated that reactive oxygen may be a signal for aging and its levels in tissues may determine the aging process and lifespan.

However, multiple experiments critically question the free radical theory. First, the over-expression of major antioxidant enzymes (e.g. superoxide dismutase or catalase), does not extend the lifespan of mice (Perez, Van Remmen et al. 2009) and mitochondrial superoxide dismutase (Sod-2) haplo-insufficiency does not accelerate murine aging, even in mice with dysfunctional telomeres (Guachalla, Ju et al. 2009). Even more surprisingly is that the deletion of Sod-2 extends the lifespan in the nematode *Caenorhabditis elegans* (Van Raamsdonk and Hekimi 2009). It was also reported that RNAi of five genes encoding components of mitochondrial respiratory complexes I, III, IV, and V leads to increased life span in flies. Long-lived flies with reduced expression of these electron transport chain genes do not consistently show a reduced assembly of respiratory complexes or reduced ATP

levels. In addition, extended longevity is not consistently correlated with increased resistance to the free-radical generator paraquat (Copeland, Cho et al. 2009). These results are in agreement with previous papers showing that antioxidant over-expression causes minor effects in life span extension in yeast, flies, and mice compared to those caused by mutations in signal transduction genes. It is likely that the increased protection against superoxide must be accompanied by a number of other changes to be effective in life span extension. For instance, LON, a protease located in the mitochondrial matrix, increases stress tolerance, prevents the accumulation of oxidized proteins (Ngo, Pomatto et al. 2011) and increases mitochondrial oxygen consumption, while decreasing oxidative damage of proteins in the fungal aging model *Podospora anserine* (Luce and Osiewacz 2009). In the same model organism, deletion of a gene encoding an O-methyltransferase, which decreases levels of reactive oxygen species, leads to a decreased lifespan (Kunstmann and Osiewacz 2009).

1.3 Aging-related Diseases

In the United States, the geriatric population, individuals 65 years of age or older, has been continuously increasing in size. Between 1989 and 2010, this population rose from 25 to 40 million and is expected to grow to almost 90 million by 2050 (Zoorob, Kihlberg et al. 2011). Aging is by far the leading cause of death: an estimated 100,000 people die of age-related causes per day, about two thirds of the roughly 150,000 people who die each day across the globe. In industrialized nations, the proportion is much higher, reaching 90% (Lopez, Mathers et al. 2006). The majority of these deaths can be attributed to age-associated diseases which include a wide array of illnesses, including cardiovascular disease, cancer, arthritis, cataracts, osteoporosis, type 2 diabetes, hypertension and Alzheimer's disease. Essentially, all of these aging-associated diseases are complications that arise from cellular deterioration. Despite the pronounced interconnection of aging and disease is it important to distinguish age-associated diseases from the aging process itself: all animals will age, yet not all adult animals, as either an individual or as a species, experience age-associated diseases. Mice for example generally do not, unless genetically altered, experience atherosclerosis (Breslow 1996).

In a study conducted by Baker and colleagues in 2011 on mice, senescent $p16^{INK4A}$ -positive cells were deliberately eradicated, leading to greater resistance against aging-associated diseases in the BubR1 progeroid mouse background. Therefore, cellular senescence is causally implicated in generating age-related phenotypes, and the removal of senescent cells can prevent, or delay, tissue dysfunction and extend health span (Baker, Wijshake et al. 2011).

1.4 The Epidemiology of Cancer

Although all the aforementioned aging-related diseases present a particular challenge for ongoing and future research, the data I present in this dissertation will focus mainly on the treatment of malignant neoplasms known as cancer.

In 2008, 12.7 million cases of cancer are estimated to have occurred and 7.6 million people died of cancer worldwide (**Fig. 1-1**). The exact number however can only be estimated because incidence rates and treatment modalities for some parts of the world are not fully established. With the expected increase in the population of the elderly, as well as the adaption to cancer-causing behaviors (e.g. cigarette smoking), the death rate is projected to increase to 13.2 million in 2030 (Brawley 2011). Malignant neoplasms are the major cause of death in economically developed countries and the second leading cause of death in developing countries. Breast cancer in the female population and lung cancer in the male population are the most frequently diagnosed malignancies in both developed- and developing-countries (Jemal, Siegel et al. 2008). Of particular interest is that a large percentage of most cancer deaths seem to be avoidable. In 2005, Danaei and colleagues proposed that about 35% of all cancer deaths worldwide could have been avoided through adjustments to lifestyle- and environmental-factors (Danaei, Vander Hoorn et al. 2005). Based on these (rough) estimations, out of the 7.6 million cancer deaths in 2008, about 2.6 million (or ~7000 deaths/day) could have been avoided through the prevention of exposure to major risk factors such as tobacco use, diet, infections and alcohol use. Tobacco use is of significant importance to these statistics, as it not only drastically increases the risk of lung cancer, but at least 15 additional locations, and is the most preventable cause of cancer-related death. Cancer-deaths attributed to tobacco usage account for 20% (1.6 million) of the total deaths worldwide. A particular contribution to cancer rates in developed countries can be correlated to lifestyle choices such as reduced exercise (and the correlating increase in obesity)

and unhealthy diets. Several cancers, including the most common malignancies in developed countries such as breast, colorectal, stomach, liver, kidney and uterine cancers have been shown to have a clear link to these risk-factors (Watson and Collins 2011; Cabibbo, Maida et al. 2012; Cramer 2012; Li, Zhang et al. 2012; Navai and Wood 2012; Wang, Lehuede et al. 2012).

Due to the impact of dietary choices on cancer, parts of my dissertation are focused on the role of dietary macronutrient compositions and the effects on stress-resistance and tumor progression, as well as potential implications of short-term fasting regimes for the improvement of existing cancer treatments, including chemo- and radio-therapy.

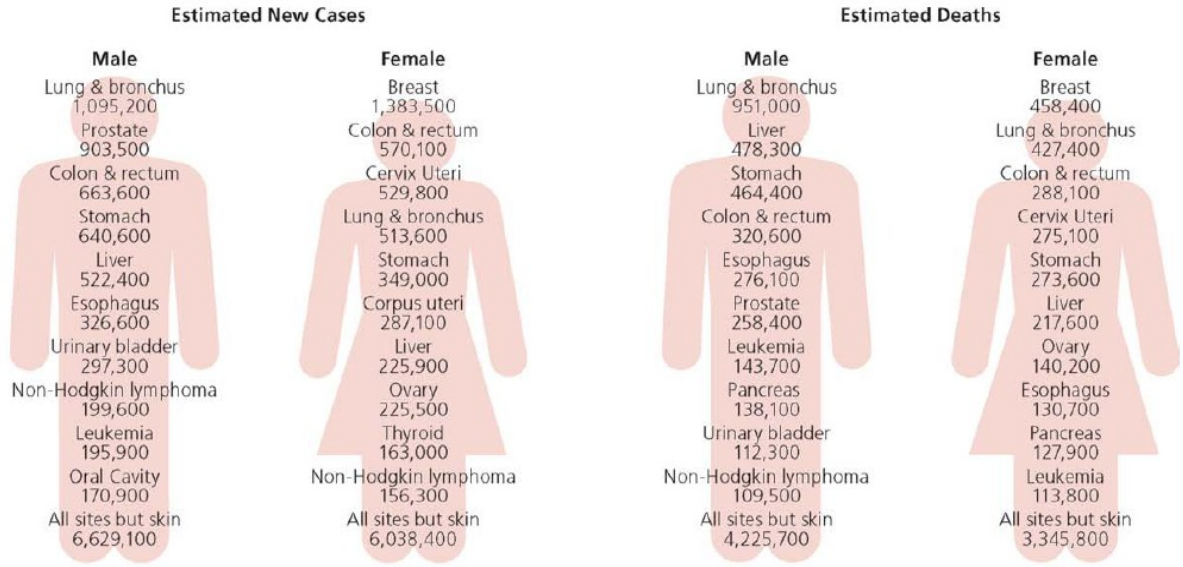

Figure 1-1. Estimation of new cancer cases and deaths worldwide for the leading cancer sites in 2008.

Source: GLOBOCAN 2008. Modified from (Jemal, Siegel et al. 2008).

1.5 Development of Malignant Cells

In 2000, Douglas Hanahan and Robert Weinberg postulated the “hallmarks of cancer” and formulated the hope that cancer-research of the next century will “*become understandable in terms of a small number of underlying principles*” and “*that the teachings of cell biology that virtually all mammalian cells carry a similar molecular machinery regulating their proliferation, differentiation and death*” will be the foundation of cancer research for the new century (Hanahan and Weinberg 2000). Over the last 50 years, research has made progress in at least partly recognizing the underlying mechanisms that govern the transformation from normal into malignant cells.

The genetic alterations of cancer cells, known as the hallmarks of cancer, include the 1) *self-sufficiency in growth signaling* and the 2) *insensitivity to growth-inhibiting signals*, 3) *evasion of apoptosis*, 4) a *limitless replicative potential*, 5) *sustained angiogenesis*, and finally 6) the *capacity to metastasize*. Work of the past decade has made substantial contribution to further our understanding and required the addition of new hallmarks: 7) *genome instability*, 8) *inflammation*, 9) *avoiding of immune destruction* and 10) *reprogramming of energy metabolism* (**Fig. 1-2**) (Hanahan and Weinberg 2000). Based on the variety of cancer types in the human population, with more than 100 distinct types and various subtypes, it is clear that tumor cells have experienced alterations ranging from small point mutations (Rodenhiser, Chakraborty et al. 1996) up to significant changes in their chromosomes (Albertson, Collins et al. 2003). In tissue culture models, the transformation of rodent cells to achieve tumorigenic competence requires the acquisition of at least two genetic alterations; human cells are even more difficult to transform *in vitro* (Hahn, Counter et al. 1999). *In vivo*, transgenic mouse models of tumorigenesis led to the conclusion that multiple steps have to occur before rodent cells can become malignant (Berns, van der Lugt et al. 1994; Wu and Pandolfi 2001). In the human population, a minimum of four stochastic, rate-limiting events with multiple intermediate steps are required to transform normal cells into an invasive cancer cell, thus explaining why the development of cancer is a relatively rare event (when considering the estimated up to 100 trillion cells that form the human body) and why it occurs in an aging-related manner during the average human lifetime (Foulds 1954; Renan 1993). Considering that tumorigenesis is a

multi-step process that requires specific genetic alterations to successfully avoid the regulatory mechanisms that govern normal cell proliferation and homeostasis, it is not surprising that research increasingly focuses on cancer-specific treatment options and personalized medicine.

In the following section, I will describe some of the underlying mechanisms that are essential to cancerous cells. However, a comprehensive review on transformation and malignancy is beyond the scope of this dissertation.

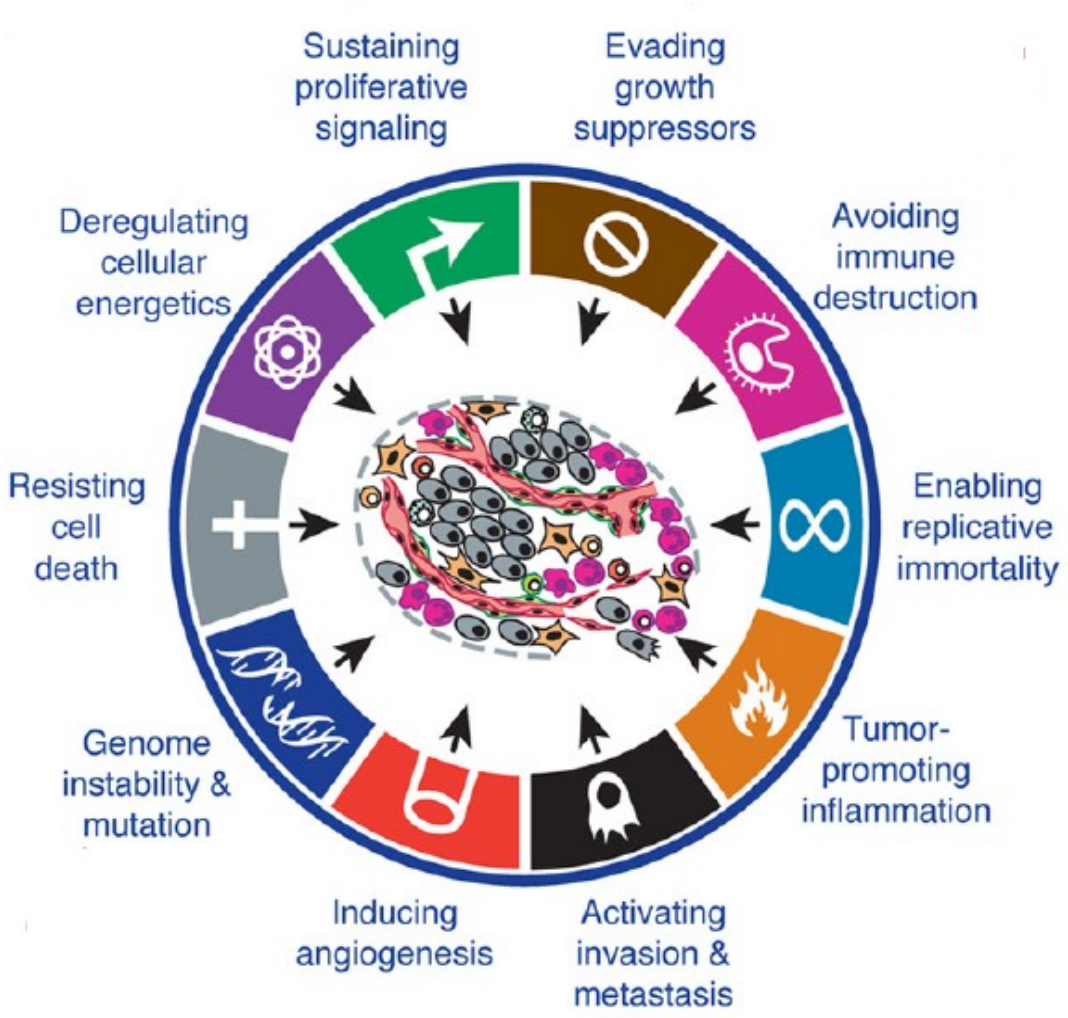

Figure 1-2. The hallmarks of cancer.

The six original hallmarks postulated in 2000 include self-sufficiency in growth signaling and the insensitivity to growth-inhibiting signals, the evasion of apoptosis, a limitless replicative potential, sustained angiogenesis, and metastatic capacity. The addition of the new hallmarks genome instability, inflammation, avoiding of immune destruction, and reprogramming of energy metabolism are added based on recent discoveries in the development of cancerous cells (cartoon in the center of the image). From (Hanahan and Weinberg 2011).

Self-sufficiency in growth signaling was among the first clearly identified capacities of malignant cells due to the defined roles of oncogenes. Normal cells rely on carefully released mitogenic signals (mediated by intercellular communication such as autocrine, paracrine or endocrine signaling pathways) to actively exit a quiescent state and proliferate, to maintain a balanced cell number, and to ensure normal tissue architecture/function. Growth signals are transmitted through a wide variety of transmembrane receptors which can bind diffusible growth factors (e.g. IGF-I or EGF), act as cell-cell adhesion molecules (e.g. cadherins) and can bind extra-cellular matrix components (e.g. integrins). The majorities of transmembrane receptors that mediate growth signals belong to the tyrosine kinase family and transmit signals through downstream signaling cascades to regulate gene expression, protein-modifications, and functions; thereby modulating energy metabolism and survival. Deregulated growth signaling in tumor cells can be due to the increased number of cell surface receptors, which causes cells to be hyper-sensitive to their ligands. Further, hyper-responsiveness of signaling receptors to their ligands can result from structural alterations in the extra-, transmembrane-, and/or intra-cellular structure of the receptor itself; these alterations may even cause ligand independent receptor activation (Lyons, Rao et al. 2008). Malignant cells may also be independent of exogenous signals through oncogenes, located downstream of the transmembrane receptors, that mimic, or even enhance, normal growth signaling; thus disrupting cellular homeostasis.

Mutations downstream of the IGF-I receptor, such as the hyper-activation of phosphoinositide 3-kinase (PI3K), or the mutations in the protein B-Raf (resulting in activation of the mitogen-activated protein kinase (MAPK) pathway) have been detected in multiple tumor types (Jiang and Liu 2009; Davies and Samuels 2010; Sander, Calado et al. 2012). Further, malignant cells are capable of synthesizing growth factors to create a positive feed-back loop with autocrine function (Schlange, Matsuda et al. 2007; Wilson, Lee et al. 2011). Alternatively, cancer cells can signal the surrounding tissue, and normal cells in the tumor-associated stroma, to stimulate growth factor production (Bhowmick, Neilson et al. 2004; Cheng, Chytil et al. 2008).

Sustained proliferative signaling can also result from the disruption of negative feedback-loops. One of the most commonly found proto-oncogenes in human cancer is the Ras proto-oncogene, which activates proteins that ultimately regulate genes

involved in cell growth, differentiation and survival (Downward 2003; Pylayeva-Gupta, Grabocka et al. 2011). Another example of the disruption of negative feedback signaling are loss-of-function mutations in the phosphatase and tensin homolog PTEN. In normal cells, PTEN negatively regulates PI3K signaling by degrading its product phosphatidylinositol (3,4,5) trisphosphate (PIP_3). Therefore loss-of-function mutations in PTEN promote tumorigenesis by sustained PI3K signaling (Song, Salmena et al. 2012). Of further interest, in particular due to the role of integrating the input from upstream pathways, including insulin, growth factors (e.g. IGF-I), and amino acids, is the serine/threonine protein kinase mammalian target of rapamycin (mTOR) (Laplante and Sabatini 2012). The modulation of mTOR activation can, via negative feedback-loops, affect PI3K signaling. The pharmacological inhibition of mTOR, by rapamycin for example, results in an increased activity of PI3K and therefore rapamycin treatment might actually blunt the anti-proliferative effects of mTOR inhibition (O'Reilly, Rojo et al. 2006).

The excessive expression of proto-oncogenes, including RAS and PI3K, and the resulting proliferative signaling can trigger cellular senescence (**Fig. 1-3**) and hence seems to reflect an intrinsic cellular defense against cells that experience an uncontrolled proliferative state. Senescent cells, identified by markers such as the expression of senescence-induced β–galactosidase, enlarged cytoplasm and the absence of markers indicative of proliferation, are prominent in tissues of mouse-models of proto-oncogene deregulation (Collado and Serrano 2010), such as *ras* (Serrano, Lin et al. 1997), but can also be found in human tumor samples (Michaloglou, Vredeveld et al. 2005). The maximal proliferative stimulation in malignant cells must therefore be accompanied by mutations that help to avoid the anti-proliferative response of “induced senescence”.

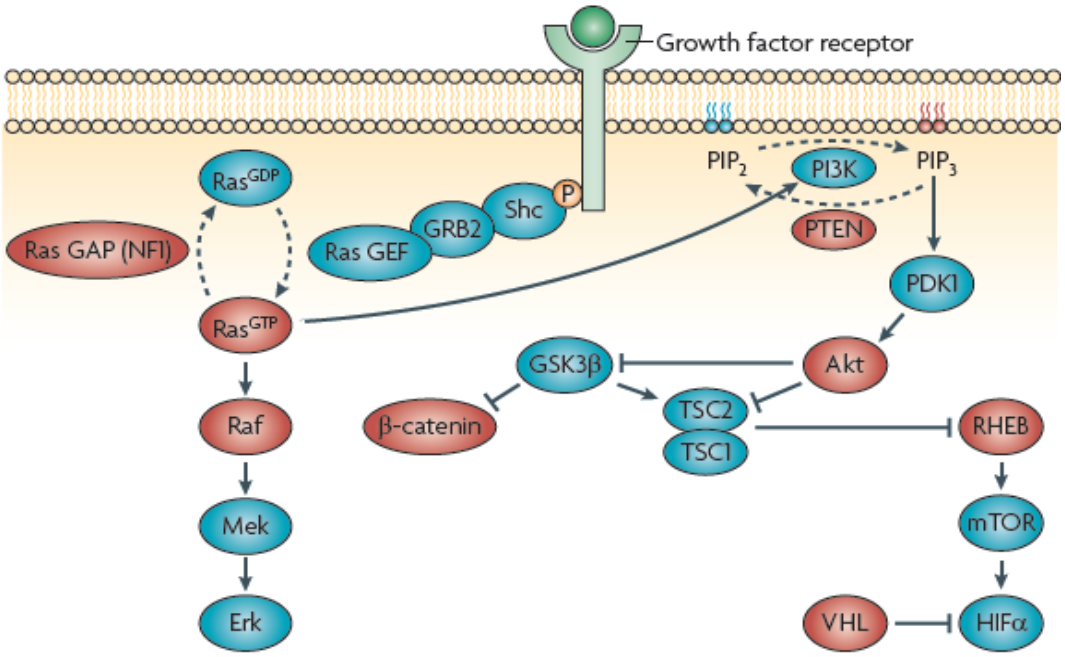

Figure 1-3. Pathways of oncogene-induced senescence.

Signaling pathways that lead to senescence induction *in vivo* when aberrantly activated. Oncogenes and tumor suppressers shown in red correspond to those that, when mutated, have been shown to lead to senescence *in vivo*. (GRB2, growth factor receptor-bound protein 2; GSK3β, glycogen synthase kinase 3β; HIF1α, hypoxia-inducible factor 1α; NF1, neurofibromin 1; P, phosphorylation; PDK1, 3-phosphoinositide-dependent kinase 1; PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; Ras GAP, Ras GTPase-activating protein; Ras GEF, Ras guanine nucleotide exchange factor; TSC, tuberous sclerosis; VHL, von Hippel–Lindau. From (Collado and Serrano 2010)

Evading growth suppression is the second important hallmark. Cancer cells must evolve to evade multiple anti-proliferative signals that regulate cellular quiescence to maintain tissue homeostasis. These anti-growth signals act as tumor-suppressors and include both soluble and immobilized inhibitors (e.g. on the cell surface of neighboring cells or the extracellular matrix), which can interfere with cellular proliferation in two ways: 1) signal cells to enter the quiescent G_0 cell cycle-state or 2) to differentiate into a post-mitotic cell state. The two most well-known tumor-suppressors are the retinoblastoma-associated (RB) and P53 proteins. The RB protein, and its two homologs p107 and p130, govern the transit of the proliferating cell through the G_1-phase of the cell cycle by integrating signals from extra- and intra-cellular sources (Burkhart and Sage 2008). The hypo-

phosphorylation of RB blocks proliferation by inhibiting the E2F transcription factors, which control the expression of genes necessary to progress from G_1 to S phase. Transforming growth factor beta (TGFβ) prevents the phosphorylation of, and thus inactivates, RB and blocks the progression into the S phase of the cell cycle. Cancer cells with disrupted TGFβ and RB pathways thus have active E2F function, resulting in cell proliferation and render cells insensitive to anti-proliferative signaling. RB signaling disruption can result from various alterations, such as the down-regulation of TGFβ-receptors (Meng, Xia et al. 2011) or displaying dysfunctional TGFβ-receptors (Markowitz, Wang et al. 1995).

P53 functions as a sensor that integrates input from stress and abnormalities such as damage to the genome, reduced levels of nucleotides, reduced glucose levels, disrupted growth-signaling and suboptimal levels of oxygenation and halts cell cycle progression until normal cellular conditions have been re-established. If the conditions do not normalize, P53 can induce apoptosis. P53 null (-/-) mice develop tumors at a young age: 75% of p53 -/- mice develop tumors of various types by 6 months of age and all succumb to tumors by 10 months of age (Donehower, Harvey et al. 1992). The p53 heterozygous (p53+/-) mice are also susceptible to tumors, but these neoplasms arise later than those observed in the p53 -/- mice; tumors are rarely observed until nine months of age. By 18 months, roughly 50% have developed cancers, and by 24 months, over 95% of the animals have had tumors or died (Venkatachalam, Shi et al. 1998).

Evading apoptosis is essential for cancer initiation because growth and expansion are not only based on the rate of cellular proliferation, but also on the capacity to maintain the already established pool of cells. Apoptosis serves as a check point to uncontrolled proliferation because apoptosis can be triggered in response to abnormalities such as oncogene-related imbalances (Vaux, Cory et al. 1988) and DNA damage associated with hyper-proliferation (Zindy, Eischen et al. 1998). Many apoptotic signals converge on the mitochondria and in response to pro-apoptotic signals (e.g. cytokines, nitric oxide, temperature, hormones and toxins) cytochrome C is released from the mitochondria through the formation of the mitochondrial apoptosis-induced channel (MAC) in the outer mitochondrial membrane. The MAC formation is regulated by various proteins, such as those

encoded by the mammalian Bcl-2 family with either anti-apoptotic (Bcl-2, Bcl-XL, Bcl-W, Mcl-1, A1) or pro-apoptotic (Bax, Bak, Bid, Bim, Puma) properties. The released cytochrome C binds with the apoptotic protease activating factor-1 (Apaf-1) and ATP, which then bind to pro-caspase-9, creating a protein complex known as the apoptosome. The apoptosome cleaves the pro-caspase to its active form (caspase-9), which in turn activates an intra-cellular cascade of proteases (effector caspases 3, 6 and 7) to execute cell death.

The deregulation of apoptosis is, in part, mediated by the loss of the tumor-suppressor p53 in malignant cells, eliminating this “damage sensor”. Further, tumor cells may avoid the induction of apoptosis by increasing the expression levels of anti-apoptotic regulators such as Bcl-2 and Bcl-XL (Kroemer 1997), or the down-regulation of pro-apoptotic factors such as Bax, Bim or Puma (LeBlanc, Lawrence et al. 2002). The underlying principles to evade apoptosis are based on the structure of the pro- and anti-apoptotic pathways, but also rely on autophagy (which can mediate tumor cell survival, and death, based on genetic and cell-physiological conditions that dictate either fate) or necrosis (which has a pro-inflammatory and tumor-promoting potential). See Favaloro et al. for a review on the role of apoptosis in diseases (Favaloro, Allocati et al. 2012).

Enabling replicative immortality is another hallmark of cancer cells and is important to avoid cell death. The potential for limitless proliferation is a phenomenon generally not attributed to normal cells; stem- and germline-cells are an exception from this rule. This limitation, known as the Hayflick limit (Hayflick 1965), depends on two hurdles: cellular senescence (and the decrease in the replicative potential) as well as a subsequent crisis, resulting in cell death. Telomeres, composed of thousands of 6 base-pair (bp) sequence repeats at the end of the chromosomes are constantly shortened by 50-100 bp during each replication. The eventual loss of telomeres leaves the end of the chromosome unprotected and exposed to end-to-end fusions, resulting in unstable dicentric chromosomes, karyotypic disarray and death. The shortening of telomeres is caused by the incapacity of the DNA polymerase to completely replicate the 3’ ends of the chromosomal DNA. Telomerase, a reverse transcriptase, protects the chromosome by adding hexanucleotide repeats onto the end of the telomeric DNA. Although the above

described acquired capabilities (*self-sufficiency in growth signaling*, *insensitivity to growth-inhibiting signals* and *evasion of apoptosis*) are necessary for the malignant cell to evade organismal regulation, the maintenance of the telomeres is essential to maintain a constant pool of cells. Otherwise, pre-malignant cells eventually succumb to senescence and death based on their own up-regulated proliferative capacities. The role of telomere maintenance in mediating replicative immortality has been demonstrated *in vitro*: the expression of telomerase conveyed unlimited replicative potential to a set of normal, pre-senescent cells (Bodnar, Ouellette et al. 1998; Vaziri and Benchimol 1998), and late passage cells (Counter, Hahn et al. 1998; Halvorsen, Leibowitz et al. 1999). In tumor-suppressor P53 deficient mouse models, engineered to lack telomerase, pre-malignant cells could be pushed towards a senescent state that contributed towards attenuated tumor development (Artandi, Chang et al. 2000).

In contrast to normal cells, virtually all tumor cells have increased telomere maintenance, largely (85-90%) depending on the up-regulation of telomerase expression (Shay and Bacchetti 1997) or through the activation of a recombination-based telomere maintenance known as alternative lengthening of telomeres (Bryan, Englezou et al. 1995; Biessmann and Mason 2003). Mice carrying a homozygous deletion of the negative cell cycle regulator $p16^{INK4A}$ with elevated telomerase activity are particularly prone to develop tumors. However, applying carcinogen treatment to $p16^{INK4A}$–null mice which lack telomerase activity results in reduced tumor incidence and telomere shortening with karyotypic disarray (Greenberg, Chin et al. 1999).

It can be argued that senescence, similar to apoptosis, reflects a protective mechanism that forces deregulated cells into a non-proliferative cell state when DNA damage becomes increasingly abundant. Tumor cells evade these protective means, thus making sustained telomerase function essential to maintain high cell proliferation rates.

Induced and sustained angiogenesis is essential to maintain the progressive growth of solid tumors. Like most normal tissues, cancer cells rely on the sustained availability of O_2 and nutrients. Further, residing close to the vasculature is necessary to remove carbon dioxide and metabolites. It is this dependency that obligates metabolically-active cells in the tissue to maintain in close proximity, no more than 100 µm, to capillary blood vessels. This connection emphasizes why

tumor progression is essentially depending on continued angiogenesis (the sprouting of vessels from the existing vasculature). In the adult tissue, angiogenesis is a rare and highly regulated event with the main physiologic function to ensure wound healing, but further plays a role in the female menstrual cycle and under hypoxic conditions. Tumor cells cannot passively rely on the surrounding vessels but have to induce the sprouting of new vessels from the quiescent vasculature, a step known as the “angiogenic switch”. Mechanisms to induce this switch are based on alterations in balancing the production of angiogenic inducers or inhibitors through changes in the gene expression profile (e.g. increased levels of the vascular endothelial growth factor (VEGF) and the fibroblast growth factor (FGF), reduced levels of thrombospondin-1 and β-interferon). VEGF and FGF both bind to tyrosine receptor kinases presented on endothelial cells and induce sprouting. The prototype of angiogenic inhibitory proteins, thrombospondin-1 (TSP-1), binds to multiple receptors (including CD36, integrins and integrin-associated proteins) and suppresses pro-sprouting stimuli.

Mice lacking genes encoding for angiogenic inhibitors, e.g. TSP-1 null mice (Rodriguez-Manzaneque, Lane et al. 2001) or TSP-1/P53 deficient mice (Lawler, Miao et al. 2001), show enhanced tumor progression. *Vice versa*, the genetic increase of angiogenic inhibitors in mouse models impairs tumor progression; examples of this inhibition include the delayed and decreased papilloma formation in the skin of TSP-1 over-expressing mice in a chemical-induced skin carcinogenesis model (Hawighorst, Oura et al. 2002). Further, anti-VEGF antibodies (Kim, Li et al. 1993) and the dominant interfering VEGF-receptor 2 (Millauer, Shawver et al. 1994) reduced the growth of subcutaneous tumor models, mediated by an impaired angiogenesis.

The mechanisms that cause shifts in the equilibrium of angiogenic regulators towards an increase in neo-vascularization/angiogenesis in malignant cells are still only poorly understood. However, proto-oncogene and tumor-suppressor gene levels are tightly knit to regulate levels of angiogenic regulators. The p53 tumor-suppressor negatively regulates TSP-1 and loss of function mutations in *p53* can result in decreased levels of the angiogenic inhibitor TSP-1 (Dameron, Volpert et al. 1994). The *ras* oncogene, on the other hand, causes the up-regulation of the pro-angiogenic VEGF (Rak, Mitsuhashi et al. 1995).

Activating tissue invasion and metastasis enable cancer cells to leave the primary tumor and invade nutrient- and oxygen-rich tissues, where they begin to form new tumor sites through a multistep process known as the “invasion-metastasis cascade”. This cascade is based on local invasion, the intra-vasation into proximal blood- and lymph-vessels, transition through the lymphatic- and hematologic-system, infiltration of the parenchyma of distant tissues, nodule formation (micro-metastasis) and the subsequent growth of macroscopic tumors (**Fig. 1-4**). Metastases are the cause of up to 90% of cancer-related deaths in humans (Sporn 1996). It is of particular interest that tumor cells can undergo dormancy, leading to recurrence after therapy and long periods of remission also known as “minimal residual disease” (MRD) (Aguirre-Ghiso 2007). For example, 20 to 45% of patients with breast or prostate cancer will relapse years or decades later (Karrison, Ferguson et al. 1999; Weckermann, Muller et al. 2001). The formation of metastases, like the formation of the primary tumor, depends on the previously described hallmarks of cancer (see above). The capacity to metastasize involves alterations in the interaction of tumor cells with the surrounding normal cells/tissue through cell adhesion molecules (CAM), including members of the immunoglobulin family, integrins and cadherins.

E-cadherin is ubiquitous expressed in epithelial cells and bridges adjacent cells, resulting in anti-growth signaling and contact inhibition (Aplin, Howe et al. 1999). In the majority of epithelial cancers, including squamous cell carcinoma, E-cadherin function is drastically reduced and even lost due to mutational inactivation of the E-cadherin or β-catenin (a functional subunit of the cadherin protein complex) genes (Jiang and Mansel 2000), by transcriptional repression (Hajra, Ji et al. 1999) or by proteolysis of the extracellular domain (Bergers and Coussens 2000). Subsequently, exploring the role of E-cadherin in tumor cell lines *in vitro* demonstrated that re-establishing a functional cadherin complex resulted in the reversion from malignant, invasive cancer cells into a more benign epithelial tumor cell phenotype (Frixen, Behrens et al. 1991). Studies exploring the role of E-cadherin in tumor development *in vivo* demonstrated that the expression of this cell adhesion molecule in a transgenic mouse model (Rip1Tag2) of carcinogenesis blocked invasion and metastases (Perl, Wilgenbus et al. 1998).

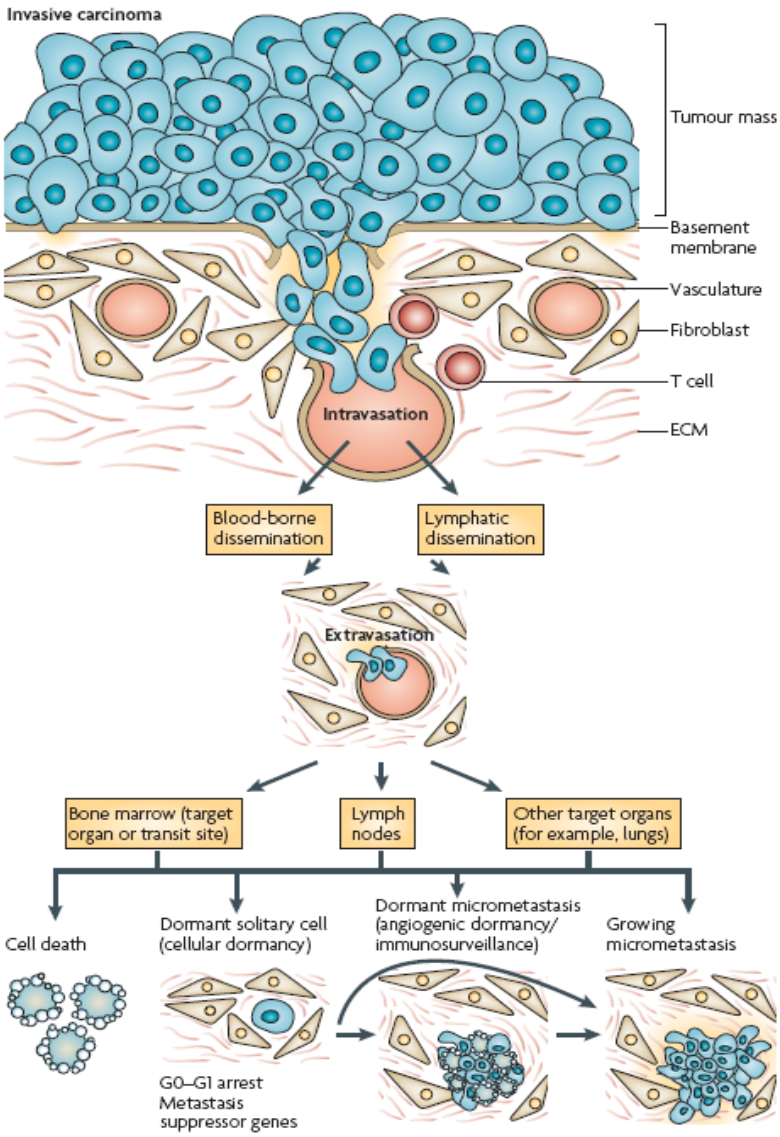

Figure 1-4. The invasion-metastasis cascade of malignant cells.

Tumor cells carrying genetic or epigenetic changes, enabling motile and invasive properties, can invade the underlying stroma and interact with fibroblasts or immune cells and the stromal matrix. Tumor cells (in cooperation with stromal cells) can degrade the extracellular matrix (ECM) and the vascular walls and intra-vasate through either arterial or lymphatic routes. Tumor cells that arrest in the vasculature of the bone marrow can proliferate or remain dormant. Although the bone can be a target organ, it might also serve as a transit site from which cells can again disseminate, through yet unknown mechanisms, to their final destination. Tumor cells can arrest in lymph nodes or in the target organ vasculature, where they can extra-vasate into the organ parenchyma. At this stage, intra- or extra-vascularly lodged tumor cells have four possible fates: 1) they die (the vast majority of cells undergo apoptosis), they can enter a state of quiescence or dormancy, either as 2) a single solitary cell or 3) as a micro-metastatic lesion that underwent a proliferative expansion and cannot recruit a vascular bed, or 4) they can resume proliferation into micro-metastases. Modified from (Aguirre-Ghiso 2007).

In addition to cadherins, immunoglobulins play a critical role in invasion and metastasis. The most prominent member of this super-family is the neural cell adhesion molecule (NCAM). NCAM deregulation in tumor cells can be based on poorly adhesive, even repulsive, isoforms (Rutishauser, Acheson et al. 1988), or on reduced expression (Fogar, Basso et al. 1997). In a transgenic mouse model of β-cell carcinogenesis (Rip1Tag2), the tumor-phenotype correlates with a changed expression from the 120 kDa isoform in normal tissue to the 140/180 kDa isoforms in the tumor. NCAM-deficient Rip1Tag2 mice develop metastases, which has generally not been observed in normal Rip1Tag2 mice. In contrast, over-expression of NCAM 120 in NCAM-deficient Rip1Tag2 mice rescued this phenotype (Perl, Dahl et al. 1999).

Although there are shared and common attributes of tumor cells (the abilities to be self-sufficient in growth signaling, insensitive to growth-inhibiting signals, evade apoptotic signaling, replicate without limits, induce angiogenesis and to metastasize), the underlying mutations in cellular pathways can be highly variable. Even further, mutations in certain proto-oncogenes and tumor-suppressor genes can occur relatively early in development of one tumor or late in others. The subsequent acquisition of any other of the cellular alterations, such as resistance to apoptosis, determines how fast tumor development progresses (**Fig. 1-5**). All these necessary changes that I briefly described above, demonstrate that the development of a single malignant cell relies on the acquisition of a vast variety of genomic alterations to allow sustained growth. Considering how small the chances are to successfully gain all these “cancer hallmarks”, despite the generally very well maintained genome, demonstrates the rarity of the development of a malignant cell. It is therefore that the occurrence of these chance-events is strongly depending on time.

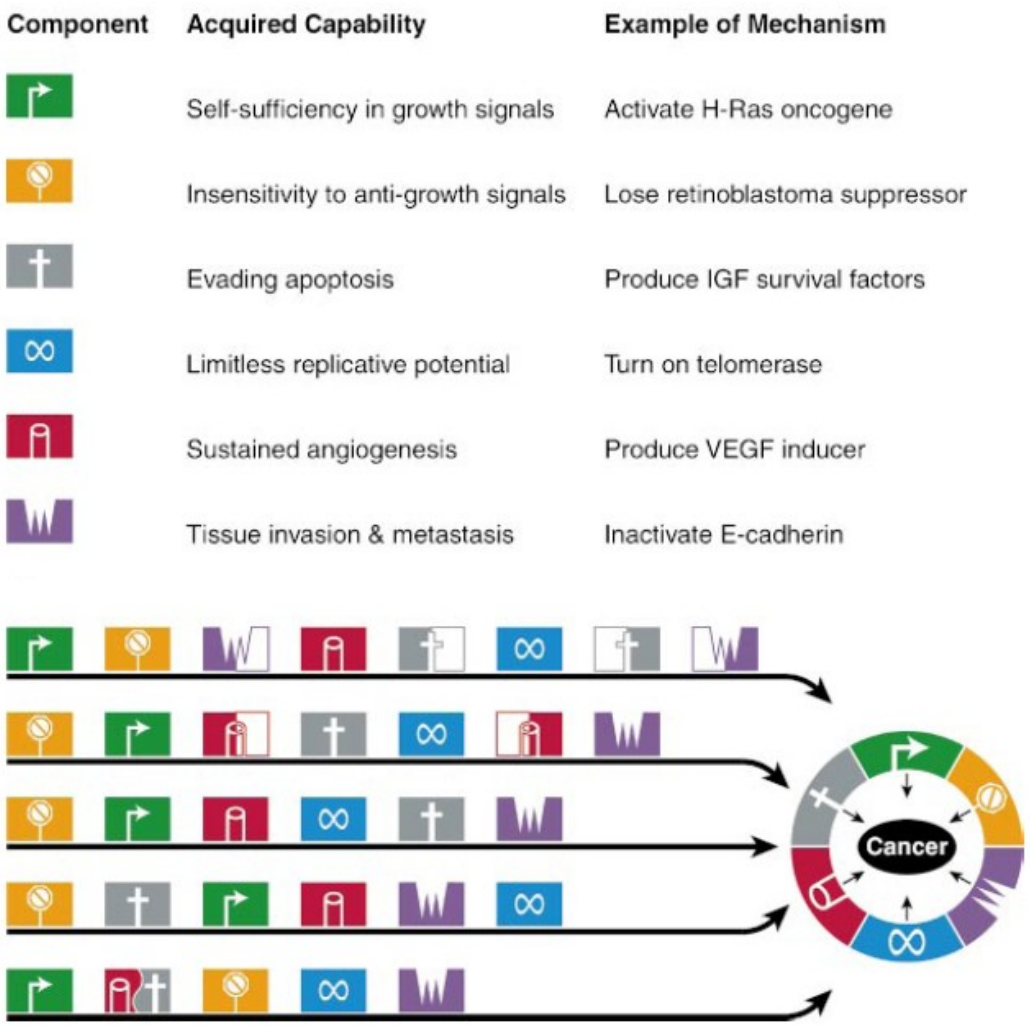

Figure 1-5. Parallel pathways of tumorigenesis

Virtually all cancers must acquire the same six hallmark capabilities (top panel), yet their means of doing so will vary significantly, both mechanistically (see text) and chronologically (bottom panel). The order in which these capabilities are acquired seems likely to be quite variable across the spectrum of cancer types and subtypes. Moreover, in some tumors, a particular genetic lesion may confer several capabilities simultaneously, decreasing the number of distinct mutational steps required to complete tumorigenesis. For example, loss of function of the p53 tumor suppressor can facilitate both angiogenesis and resistance to apoptosis (e.g. in the five-step pathway shown), as well as enabling the characteristics of genomic instability. In other tumors, a capability may only be acquired through the collaboration of two or more distinct genetic changes, thereby increasing the total number necessary for completion of tumor progression. Thus, in the eight-step pathway shown, invasion/ metastasis and resistance to apoptosis are each acquired in two steps. Modified from (Hanahan and Weinberg 2000).

Research over the past decade has expanded our understanding of tumor development and revealed that tumors cannot be simplified as a random assembly of these six cancer hallmarks. Comprehensive studies increasingly reported that it requires more than these hallmarks to successfully form and maintain a cancer cell. In addition, the tumor micro-environment significantly allows malignant cells to survive and thrive. In the normal tissue, deregulated cells are usually detected by the immune system which antagonizes uncontrolled growth. It is thus of particular importance that malignant cells acquire attributes to evade the immune surveillance, e.g. through the secretion of TGF-β which suppresses cytotoxic T lymphocytes and natural killer cells (Yang, Pang et al. 2010). Immune surveillance is not always fully successful, resulting in "edited" tumor cells that have escaped the immune surveillance and remain intact (Ostrand-Rosenberg 2008). Nonetheless, immune processes play a conflicting role: in the majority of all neoplastic lesions tumor-antagonizing and tumor-promoting leukocytes can be found; emphasizing that tumor development relies on the well orchestrated connection of all the contributing factors (**Fig. 1-6**). Immune cells such as certain macrophage subtypes, neutrophils, mast cells, and T- and B-lymphocytes, have all been attributed with tumor-promoting function (Johansson, Denardo et al. 2008); often resembling a chronic inflammatory state (Grivennikov, Greten et al. 2010). The pro-malignant qualities of some immune cells seem to be depending on signaling molecules such as the tumor growth factor EGF, the pro-angiogenic VEGF, chemokines and cytokines that amplify the inflammatory state and pro-invasive matrix degrading metalloproteinases (Egeblad and Werb 2002; Qian and Pollard 2010). *Vice versa*, studies have demonstrated that persistent inflammation is associated with pathologies including fibrosis, aberrant angiogenesis and cancer (Mantovani, Allavena et al. 2008).

In addition, the reprogramming of the cellular metabolism plays an essential role for the sustained growth and proliferation of cancer cells. I will discuss the metabolic requirements for cellular proliferation in **Chapter 1.7**.

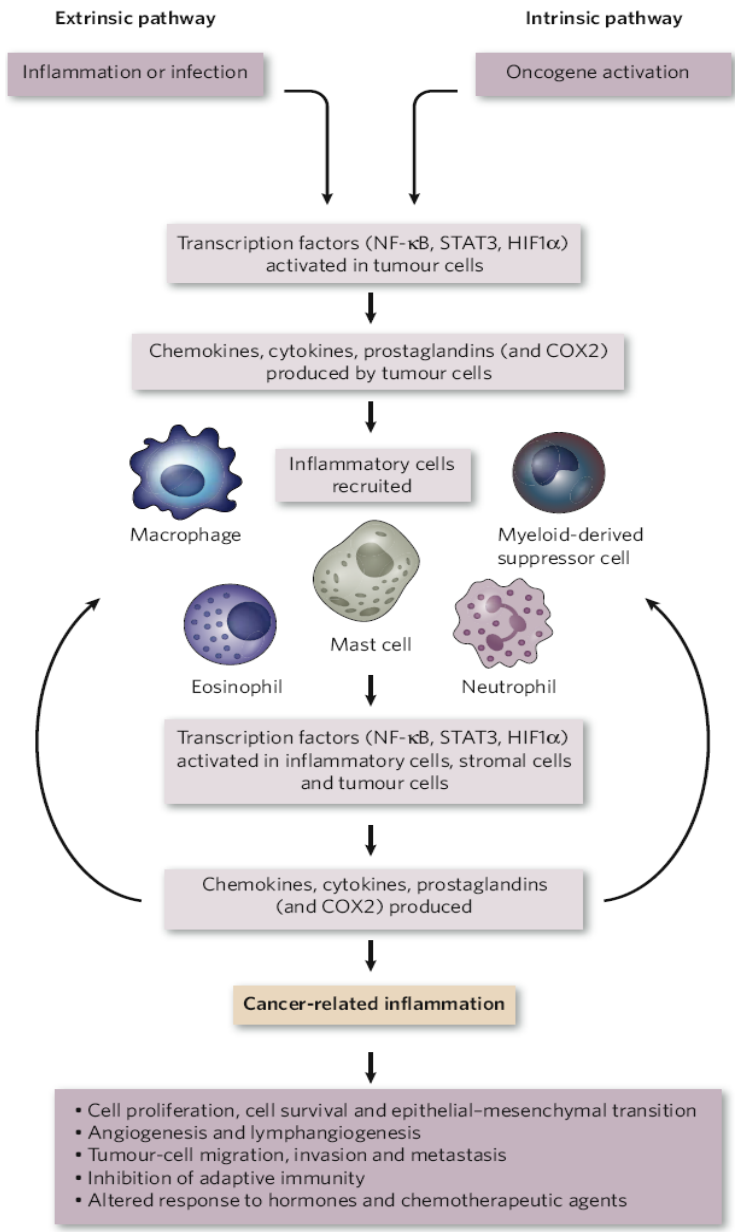

Figure 1-6. Pathways connecting inflammation and cancer.

Cancer and inflammation are connected by two pathways: 1) the intrinsic pathway is activated by genetic events, including the activation of various types of oncogenes, chromosomal rearrangement or amplification, and the inactivation of tumor-suppressor genes. Cells transformed in this manner produce inflammatory mediators and generate an inflammatory environment without any underlying inflammatory condition. 2) In the extrinsic pathway, inflammatory or infectious conditions augment the risk of developing cancer (e.g. in the colon, prostate and pancreas). The two pathways converge, resulting in the activation of transcription factors that coordinate the production of inflammatory mediators, including cytokines and chemokines, and recruit and activate various leukocytes. Cytokines activate the same key transcription factors in inflammatory-, stroma- and tumor-cells, resulting in the production of more inflammatory mediators and a cancer-related inflammatory microenvironment. Modified from (Mantovani, Allavena et al. 2008).

1.6 Overview of Cancer Treatment

Cancer has become a leading global health issue and cause of mortality, only second to heart disease. In the United States, it is predicted that one out of every four deaths will be due to cancer. However, compared to the fast improvements in the treatment of heart disease, cancer treatment has been relatively steady over the past 30 years (Jemal, Siegel et al. 2008). Before the 1950s, tumor therapy remained largely in the hands of the surgeon. Understanding that an effective treatment for cancer-patients requires every organ in the body to be reached, led to the development of drugs that could be systemically administered. With the invention of the linear accelerator, radiation therapy became a valuable tool for the control of local and regional cancer after 1960, but could not eradicate metastatic cancer. As of today, chemotherapy and radiotherapy are still the main treatment choices for many cancers. Although they are effective in the treatment of certain cancers, the clinical potential is greatly limited by their toxic side effects.

Chemotherapy is a major part of current cancer treatment. The first chemotherapy drug was adapted based on the discovery that exposure to the chemical warfare agent mustard nitrogen gas, *bis*-(2-chloroethyl) sulfide, led to the depletion of bone-marrow cells. Frank Adair and Halsey Bagg, researchers in the Douglas Laboratory of the Memorial Hospital in New York, put the idea that this agent could reduce tumor progression to the test. They applied tar to mice to induce skin cancer and treated neoplastic lesions with diluted solutions of mustard gas. The lesions were reduced and disappeared after some scabbing and the mice appeared cured. Encouraged by the results, Adair and Bagg applied their solution to 13 human patients, mainly suffering from some form of skin cancer (Adair and Bagg 1931): "*The patient had a lesion 2.5 by 3 by 1 cm thick. Frequent applications were made to the lesion of small doses of the mustard gas. The tumor has been destroyed gradually and at present, four months after beginning treatment, there is no evidence of the disease and there is good healing over the site of the original tumor"*. In 1942, Goodman and Gilman were commissioned by the United States Department of Defense to examine the potential therapeutic value of a series of toxins developed for chemical warfare. Goodman and Gilman treated a patient with non-Hodgkin's lymphoma with nitrogen mustard (Gilman and Philips 1946). The mediastinal and lymphatic masses of the patient regressed shortly after the treatment. However, the

observed remission lasted only a few weeks and was followed by disease progression. Nonetheless, the principle was established that the systemic administration of drugs could induce tumor regression.

It has been almost 60 years since the establishment of the National Cancer Chemotherapy Service Center (NCCSC) at the National Cancer Institute (NCI), which has significantly contributed to the discovery of various chemotherapeutic drugs (**Fig. 1-7**). The underlying strategy of chemotherapeutic drugs relies on the targeted killing of rapidly proliferating cells, by means of genotoxicity and the production of reactive oxygen species (Look and Musch 1994; Conklin 2004; Kaina, Christmann et al. 2007). The US Food and Drug Administration (FDA) has approved 132 cancer chemotherapy drugs, of which 56 have been reported to cause oxidative stress, including anthracyclines (e.g. cyclophosphamide, cisplatin, doxorubicin, busulfan, fluorouracil, cytarabine, and bleomycin), which lead to collateral damage to normal cells (Chen, Jungsuwadee et al. 2007). Although these drugs were first believed to be quite selective, we now know that normal cells also experience severe toxicity, leading to dose-limiting side effects.

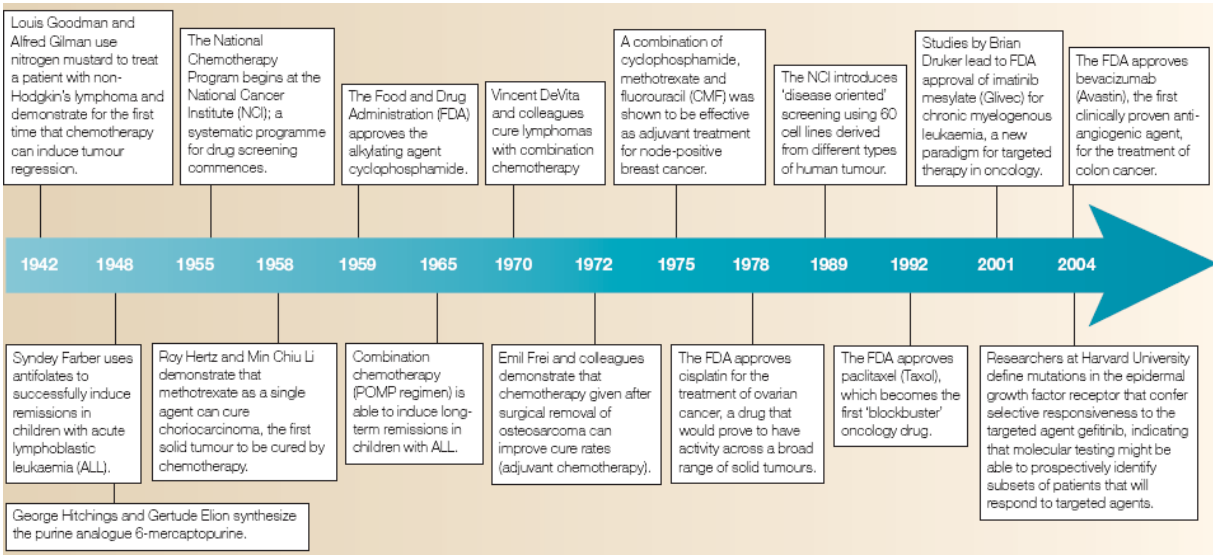

Figure 1-7. The history of chemotherapy.

Modified from (Chabner and Roberts 2005).

The other major arm of modern cancer treatment is radiation-based therapy. Irradiating biological materials leads to a rapid burst of ROS, which is generated primarily from the ionization of water molecules and direct ionization of target molecules (Riley 1994). It is estimated that ~ 60% of the damage is caused by ROS (Barcellos-Hoff, Park et al. 2005). In addition, X-rays and γ-rays cause direct macromolecular damage by energy deposition. Despite its therapeutic benefits, ROS significantly damage normal cells and are responsible for various side-effects (Wilson, Taffe et al. 1993; Bialkowski, Kowara et al. 1996; Olinski, Zastawny et al. 1996; Zastawny, Czerwinska et al. 1996). To make matters worse, it has been reported that even localized small field radiotherapy of head and neck cancers can cause oxidative damage at the organismal level, and may even lead to the development of secondary mutations (Roszkowski, Gackowski et al. 2008). Currently, fractionation strategies are considered as an effective approach to improve tumor control rates without increasing chronic toxicity, yet often causing enhanced acute toxicity (Schulz, Harari et al. 2001). Multiple studies indicated that the administration of antioxidants in combination with radiotherapy reduces the toxic side-effects. For instance, the free-radical scavenger amifostine has been shown to reduce the side-effects in patients with differentiated thyroid cancer (Bohuslavizki, Klutmann et al. 1998); although it will be necessary to critically assessed if this treatment option might result in collateral tumor protection (Andreassen, Grau et al. 2003). In another study, the pharmacological targeting of the toll-like receptor 5 (TLR5) which activates nuclear factor-kappa B (NFκB) signaling and in turn increases superoxide dismutase 2 (SOD2) expression, selectively protected mice and rhesus monkeys against radiation induced damage (Burdelya, Krivokrysenko et al. 2008).

As different as these strategies are, both therapies ultimately converge on cytotoxicity directed against proliferative cells, aiming to preferentially kill malignant cells. However, the selectivity is far from ideal, and collateral damage to normal cells is inevitable and commonly leads to severe side-effects such as emotional distress, myelo-suppression, fatigue, vomiting, diarrhea, and in some cases even death (Love, Leventhal et al. 1989; Partridge, Burstein et al. 2001). Although chemotherapy improves the survival rates of cancer patients, oxidative stress–mediated impairment of normal tissues is a significant side effect and decreases the quality of life of

patients. Today the same fundamentals still apply to modern chemo- and radio-therapy, and its clinical potential is greatly shadowed by its side effects. Despite the focused efforts on drug development designed to target certain cancer markers, cytotoxic drugs will have accompanying side effects unless novel interventions or strategies are adopted. Chemo-protectants such as amifostine, glutathione, mesna, and dexrazoxane are investigated and shown to provide drug-dependent protection to specific tissues, but the use of these compounds has not been shown to increase disease-free or overall survival (Links and Lewis 1999).

Chemotherapy and/or radiotherapy in combination with surgical removal of the tumor mass, by popular belief the single best option for cancer treatment, remains standard care. It is noteworthy that chemotherapy is not the efficacious remedy it promised to be in the 1970s (Devita, Serpick et al. 1970; Einhorn and Donohue 1977) and that early results could not be translated into an efficient treatment for the more common cancers (Kearsley 1986; Tannock 1998). For the majority of cancer patients, cytotoxic chemotherapy is being used as palliative care, with prolongation of survival often remaining the less important outcome (Slater 2001). Despite the development of “new” (and expensive) single and/or combination drugs to increase the response rates, there has been little impact from new regimens and barely any changes in the prescribed regimens over the past 20 years. Examples for this are non-Hodgkin’s lymphoma and ovarian cancer in which the standard choices of care are cyclophosphamide (introduced in 1959, **Fig. 1-7**), doxorubicin and platinum. The median survival benefit that has been achieved by the adjuvant treatment of breast, colon, head and neck cancers is less than 5% (Dube, Heyen et al. 1997; Pignon, Bourhis et al. 2000). For lung cancer, the most frequently diagnosed malignancy in the male population (Jemal, Siegel et al. 2008), the median survival has only increased by two months (Carney and Hansen 2000; Breathnach, Freidlin et al. 2001). In 2004, a literature search for randomized-controlled trials by Morgan et al. came to the conclusion that the overall contribution of curative and adjuvant cytotoxic chemotherapy to 5-year survival in adults (>20 years of age) with cancer was estimated to be 2.1% in the USA (Morgan, Ward et al. 2004). Overall, 13 out of the 22 evaluated malignancies showed an improvement in the 5-year survival, with an improvement bigger than 10% in only 4 out of the 22 cancer sites tested (**Table 1-2**).

Table 1-2. Impact of cytotoxic chemotherapy on 5-year survival in American adults.

*Numbers from the Cancer Statistics Branch NCI. SEER Cancer Incidence Public-use Database 1973-1998. Bethesda: NCI; 2000. † Absolute numbers. ‡ Percent for individual malignancy. Modified from (Morgan, Ward et al. 2004).

Malignancy	Number of cancers in people aged >20 years*	Absolute number of 5-year survivors due to chemotherapy†	Percentage 5-year survivors due to chemotherapy‡
Head and neck	5139	97	1.9
Oesophagus	1521	82	4.9
Stomach	3001	20	0.7
Colon	13936	146	1.0
Rectum	5533	189	3.4
Pancreas	3567	–	–
Lung	20741	410	2.0
Soft tissue sarcoma	858	–	–
Melanoma	8646	–	–
Breast	31133	446	1.4
Uterus	4611	–	–
Cervix	1825	219	12
Ovary	3032	269	8.9
Prostate	23242	–	–
Testis	989	373	37.7
Bladder	6667	–	–
Kidney	3722	–	–
Brain	1824	68	3.7
Unknown primary site	6200	–	–
Non-Hodgkin's lymphoma	6217	653	10.5
Hodgkin's disease	846	341	40.3
Multiple myeloma	1721	–	–
Total	154971	3306	2.1%

As outlined above, the present approach to cancer treatment mimics a "trial and error" or "one size fits all" practice that is inefficient and frequently results in inappropriate therapy and treatment-related toxicity (Duffy and Crown 2008). In contrast, personalized treatment shows the potential to increase efficacy and decrease chemo-toxicity. As of now, personalized treatment for cancer is only beginning, with a small number of validated drug-test products available (Allison 2008). For breast cancer, tests such as ER, uPA/PAI-1, HER-2, Oncotype DX, and MammaPrint are available to personalize the treatment decisions. Although some progress has been made, the evolution of personalized treatment is likely to be slow and costly.

In my dissertation, I will describe a novel approach to cancer treatment that integrates the fields of biogerontology and oncology and is based on well accepted dietary and genetic interventions that extend lifespan and/or enhance stress

resistance. Studies from our lab, including results from yeast, mammalian cells, mice, and preliminary results from humans greatly emphasizes the potential usefulness in the clinical scenario. In this thesis, I will present evidence that fasting specifically sensitizes tumor cells to chemo- and radiotherapy and thus separates normal and malignant cells.

1.7 Metabolic Requirements of Cellular Proliferation

Cells have evolved metabolic control systems to sense the nutrient-level in their periphery, coupling cellular proliferation to sufficient nutrient supply. In order to allow proliferation, cells must increase the rate of their metabolism to generate energy (mostly in form of ATP) and provide lipids, nucleotides and amino acids as building blocks for daughter cells. In most organisms, sufficient nutrients are generally available and thus require a system that regulates nutrient uptake. The uncontrolled uptake is blocked because mammalian cells do not take up nutrients from their proximity unless a stimulatory impulse is triggered by growth-factors. As outlined above, malignant cells do not rely on this growth-factor mediated regulation and can thus utilize this constant nutrient supply to divide. In addition, mutations in regulatory pathways may result in constitutively active uptake, in particular of glucose. In fact, glucose plays a critical role for malignant cells and is displayed in metabolic alterations that increase the rate of glycolysis, e.g. through the up-regulation of the glucose transporter Glut1 (Macheda, Rogers et al. 2005). Otto Warburg noted that cancer cells have a high rate of glycolysis followed by lactic acid fermentation in the cytosol, even in the presence of oxygen (Warburg 1956). This observation, known as the “Warburg Effect”, seems initially odd because our general understanding is that the complete catabolism of glucose through mitochondrial oxidative phosphorylation maximizes energy yield. It is therefore of interest to determine which requirements of cell division are being fulfilled by metabolizing glucose through aerobic glycolysis.

In the presence of oxygen, supplied by the surrounding vasculature of the differentiated tissue, most non-proliferating cells metabolize glucose through glycolytic pyruvate in the mitochondrial TCA- (also known as Krebs-) cycle into reduced nicotinamide adenine dinucleotide (NADH) and carbon dioxide (**Fig. 1-8**). The produced NADH fuels the mitochondrial oxidative phosphorylation to yield energy in the form of ATP. Under normoxic conditions, the production of lactate is

minimal, whereas under hypoxia differentiated cells produce large amounts of lactate. The complete oxidative phosphorylation of one glucose molecule generates up to 36 ATPs, the lactate metabolism only 2 ATPs. The fact that cancer cells rely on aerobic glycolysis, despite the disadvantage in terms of ATP generation, implies that this form of metabolism provides sufficient energy for cellular proliferation. Yet, as recent studies indicated, ATP is not the limiting factor for proliferating cells because the ratios of ATP/ADP and NADH/NAD^+ remain constantly high (Vander Heiden, Cantley et al. 2009). Additional support for this hypothesis is based on the observations that even minor disturbances in the ATP concentration could impair growth and trigger apoptosis (Eguchi, Shimizu et al. 1997; Izyumov, Avetisyan et al. 2004).

Cell division imposes a large requirement for metabolites beyond ATP production, namely nucleotides, amino acids and lipids that are all essential to replicate the cellular content of the dividing cell. For instance, the anabolic synthesis of nucleotides and amino acids requires more carbon and reduced nicotinamide adenine dinucleotide phosphate (NADPH) than actual ATP. An even better example is the synthesis of the cellular membrane lipid palmitate which requires 7 molecules of ATP but an additional 16 carbons (supplied from 8 molecules acetyl-coenzyme A (CoA)) and 28 electrons from 14 NADPH molecules. One glucose molecule can generate 36 ATPs, or two molecules NADH and 30 ATPs from the pentose phosphate shunt, or 6 carbons (Lunt and Vander Heiden 2011). To synthesize palmitate, a 16-carbon fatty acid chain, 7 glucose molecules are needed to generate the necessary NADPH while the required ATP could be supplied by a single glucose molecule alone (Vander Heiden, Cantley et al. 2009). This exemplifies that in order for a cell to divide, converting all of the glucose to CO_2 to maximize ATP production would become a limiting factor for the synthesis of macromolecular precursors, e.g. acetyl-CoA for fatty acids, glycolytic intermediates for (non-essential) amino acids and ribose for nucleotides, which are needed as cellular building blocks. The majority of glucose can therefore not be shunted towards ATP production. Uptake of the amino acid glutamine appears to play an important role for lipid synthesis because it supplies carbon (from mitochondrial oxalacetate) to maintain citrate production in the TCA cycle (**Fig. 1-8**) (DeBerardinis, Mancuso et al. 2007), supporting the production of acetyl-CoA and NADPH together with glucose.

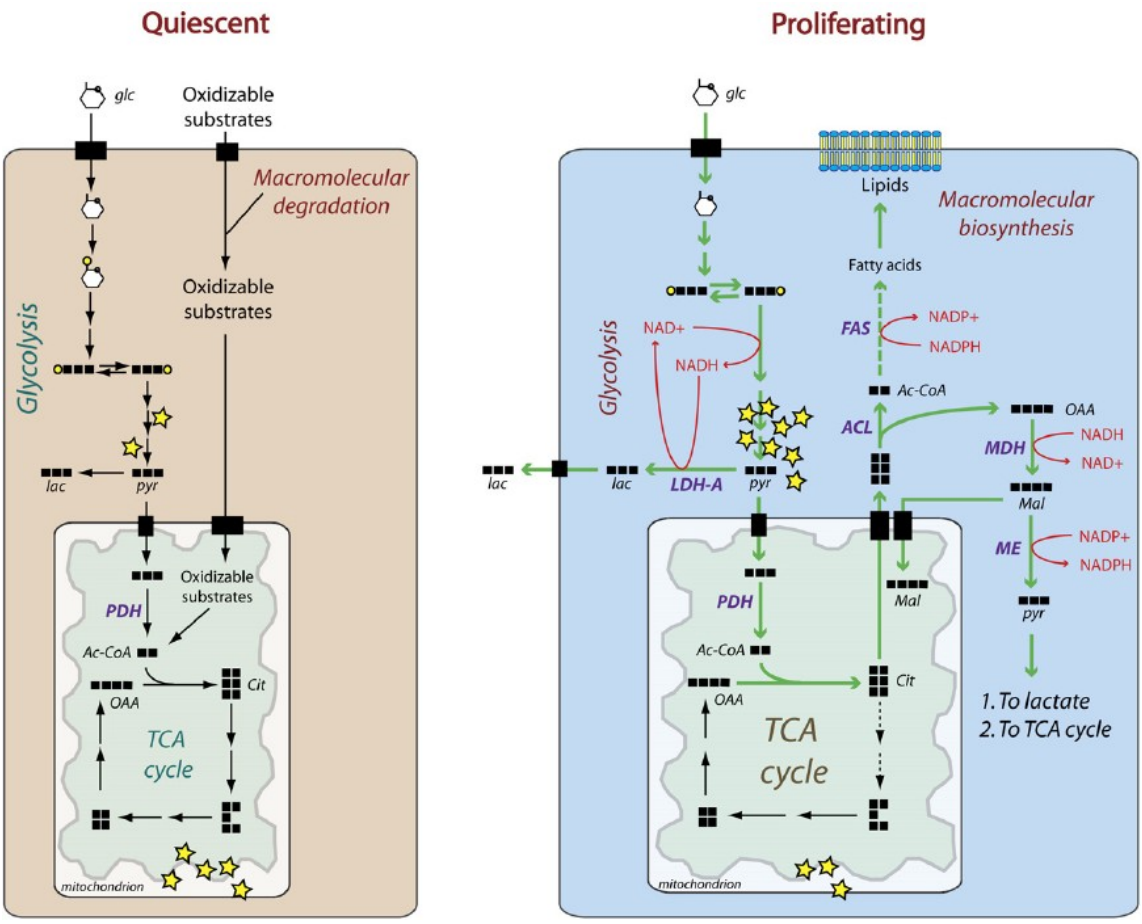

Figure 1-8. Carbon flux differs in quiescent versus proliferating cells.

Quiescent cells have a basal rate of glycolysis, converting glucose (glc) to pyruvate (pyr), which is then oxidized in the TCA cycle. Cells can also oxidize other substrates like amino acids and fatty acids, obtained from either the environment or the degradation of cellular macromolecules. The majority of ATP (yellow stars) is generated by oxidative phosphorylation in the mitochondria. During proliferation, the large increase in glycolytic flux rapidly generates ATP in the cytoplasm. Most of the resulting pyruvate is converted to lactate (lac) by lactate dehydrogenase A (LDH-A), which regenerates NAD^+ from NADH; allowing glycolysis to persist, and the lactate is secreted from the cell. Some of the pyruvate is converted to acetyl-CoA (Ac-CoA) by pyruvate dehydrogenase (PDH) and enters the TCA cycle, where it is converted into intermediates like citrate (cit) that can be used for macromolecular biosynthesis. Citrate is required for the synthesis of fatty acids and cholesterol used to generate lipid membranes for daughter cells. After export to the cytoplasm, citrate is cleaved by the enzyme ATP citrate lyase (ACL). The resulting acetyl-CoA is used by fatty acid synthase (FAS) to synthesize lipids, while the oxaloacetate (OAA) is converted to malate (mal) by malate dehydrogenase (MDH). Malate can either be returned to the mitochondria during citrate-malate antiport or be converted to pyruvate by malic enzyme (ME), generating NADPH to be used in fatty acid synthesis. Modified from (DeBerardinis, Lum et al. 2008)

One could argue that the excess generation of lactate appears to be a very ineffective use of metabolic resources because each lactate that gets excreted from the cell wastes three carbons that might be utilized for ATP production and/or the biosynthesis of macromolecular precursors. One possibility is that the dumping of excess carbon as lactate is effective because it allows for a faster incorporation of carbon into biomass, in turn facilitating rapid cell proliferation (DeBerardinis, Lum et al. 2008; Lunt and Vander Heiden 2011).

The regulation of the glucose metabolism and utilization is under control of the phosphoinositide 3-kinase (PI3K) pathway, which also plays a vital role in sensing amino acids levels and directing them into protein synthesis via the mammalian target of rapamycin (mTOR) (Laplante and Sabatini 2012). Activation of the PI3K/AKT/mTOR regulatory pathway in growth factor dependent- and tumor-cells can regulate the expression of glucose transporters (Taha, Liu et al. 1999), increase the uptake of amino acids (Roos, Jansson et al. 2007), enhance hexokinase and phosphofructose activity (Jones and Thompson 2009) and renders cells dependent on high glucose flux (Buzzai, Bauer et al. 2005). Akt induction increases glycolysis and lactate production and induces the Warburg effect in non-transformed and cancer cells by effecting gene expression and enzyme activity (**Fig. 1-9**) (Plas, Talapatra et al. 2001; Rathmell, Fox et al. 2003). Together, PI3K and Akt stimulate the expression of lipogenic genes, e.g. ATP-citrate lyase and lipid synthesis (Bauer, Hatzivassiliou et al. 2005; Chang, Wang et al. 2005); the inhibition of ATP-citrate synthase, on the other, hand can suppress tumor growth (Hatzivassiliou, Zhao et al. 2005).

Hypoxic conditions stimulate higher glucose uptake rates and lactate production. This response is coordinated by the hypoxia inducible factor 1 (HIF-1) transcription factor complex (Lum, Bui et al. 2007; Denko 2008). Gene targets of HIF-1 include glucose transporters (Mobasheri, Richardson et al. 2005), glycolytic enzymes (O'Rourke, Pugh et al. 1996; Marin-Hernandez, Gallardo-Perez et al. 2009) and lactate dehydrogenase A (LDH-A). Constitutive cellular stabilization of HIF-1 can occur in malignant cells as a result of mutations in the tumor suppressor von Hippel-Lindau (VHL), which usually targets HIF-1 for ubiquitination and degradation (Pugh and Ratcliffe 2003). A more detailed description of the metabolic transformations in cancer cells can be reviewed in Tennant et al. (Tennant, Duran et al. 2009).

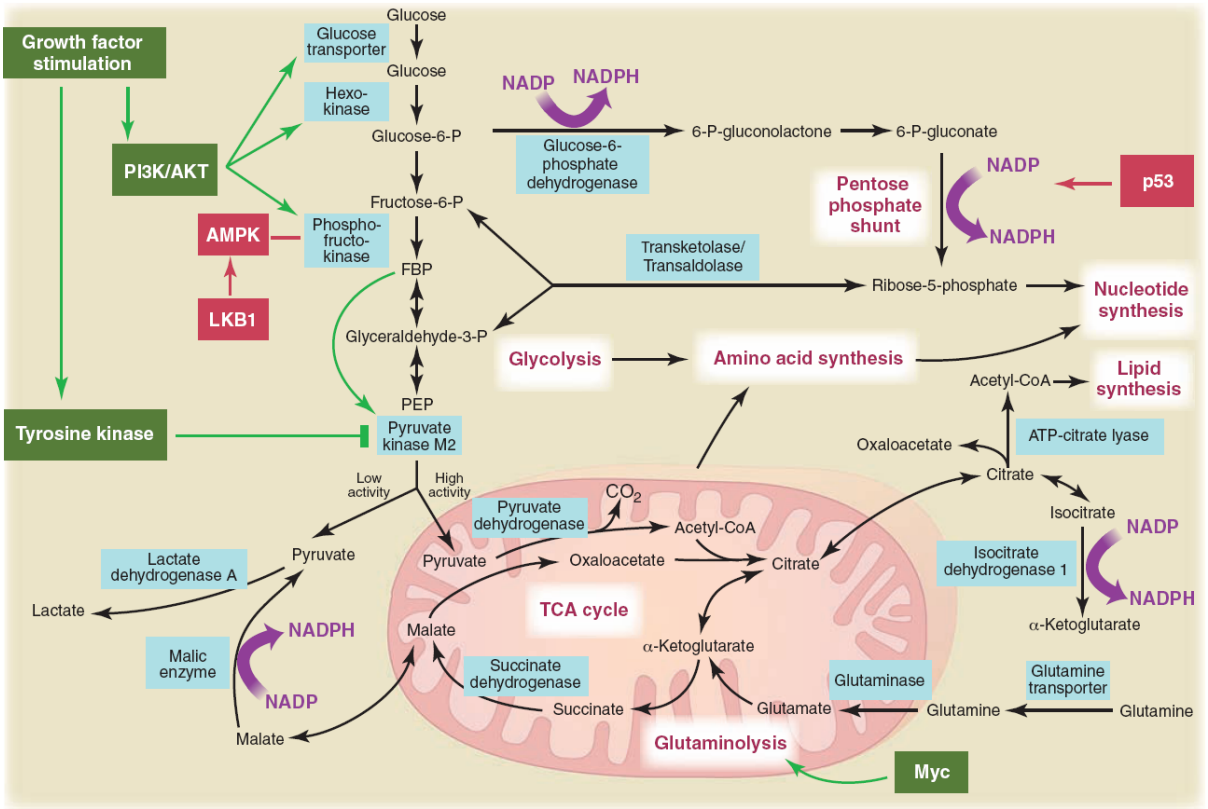

Figure 1-9. Rewiring of metabolic pathways in cancer cells.

Metabolic pathways active in proliferating cells are directly controlled by signaling pathways involving known oncogenes and tumor suppressor genes. The metabolic wiring allows NADPH production and acetyl-CoA flux to the cytosol for lipid synthesis. Key-steps in these metabolic pathways can be influenced by signaling pathways known to be important for cellular proliferation. Activation of growth factor receptors leads to both tyrosine kinase signaling and PI3K activation. Via AKT, PI3K stimulates glucose uptake and flux through the early part of glycolysis. Tyrosine kinase signaling negatively regulates flux through the late steps of glycolysis, making glycolytic intermediates available for macromolecular synthesis as well as supporting NADPH production. Myc drives glutamine metabolism, which also supports NADPH production. LKB1/AMPK signaling and p53 decrease metabolic flux through glycolysis in response to cell stress. Decreased glycolytic flux in response to LKB/AMPK or p53 may be an adaptive response to shut off proliferative metabolism during periods of low energy availability or oxidative stress. Tumor suppressors are shown in red, and oncogenes are in green. Key metabolic pathways are labeled in purple with white boxes, and the enzymes controlling critical steps in these pathways are shown in blue. Some of these enzymes are candidates as novel therapeutic targets in cancer. Malic enzyme refers to $NADP^+$-specific malate dehydrogenase. Modified from (Vander Heiden, Cantley et al. 2009).

1.8 Conserved Regulation of Longevity and Stress Resistance: Dietary and Genetic Interventions

Dietary, genetic, and pharmacological interventions have all contributed greatly to our understanding of lifespan regulation. The most powerful and reproducible dietary intervention that increases life- and health span across species is known as calorie restriction (CR), or dietary restriction (DR; the reduced intake of specific macronutrients). In the majority of animal models, an increase in longevity is often correlated with an increase in the resistance to various stressors, such as heat and toxic agents (e.g. hydrogen peroxide, H_2O_2) (Berg, Breen et al. 1994; Schulz, Zarse et al. 2007). Genetic studies demonstrated that increased lifespan and stress resistance converge on the growth hormone (GH)/IGF-I axis and its homologs (Longo and Fontana 2010). In fact, it is suggested that the GH/IGF-I axis is a major mediator of the beneficial effects of CR (Bonkowski, Dominici et al. 2009). Pharmacological manipulations, such as rapamycin have also shown to increase lifespan (Harrison, Strong et al. 2009; Robida-Stubbs, Glover-Cutter et al. 2012). Rapamycin inhibits the mammalian target of rapamycin (mTOR), which is located downstream of IGF-I and is a major regulator of cellular proliferation, metabolism, and stress (Reiling and Sabatini 2006; Laplante and Sabatini 2012).

CR is the most effective and reproducible intervention to decelerate the rate of aging and increase healthy lifespan, as studied in various model organisms ranging from the simple yeast to worms, flies, mice, rats (**Fig. 1-10**) (Guarente and Kenyon 2000; Kenyon 2001; Fontana, Partridge et al. 2010). CR reduces oxidative stress (Youngman, Park et al. 1992; Sohal and Weindruch 1996) and cell proliferation while enhancing autophagy (Cuervo, Bergamini et al. 2005; Wohlgemuth, Julian et al. 2007) and certain DNA repair processes (Weraarchakul, Strong et al. 1989). CR effectively reduces the levels of plasma insulin, cholesterol, triglycerides, growth factors such as IGF-I (Mahoney, Denny et al. 2006), and inflammatory cytokines (Matsuzaki, Kuwamura et al. 2001). CR also elevates plasma high-density lipoprotein levels (Larson-Meyer, Newcomer et al. 2008), resulting in a reduced risk for developing a metabolic syndrome, e.g. atherosclerosis, diabetes and obesity (Paoletti, Bolego et al. 2006). Additional benefits of CR include reduced blood glucose levels and elevated ketonebodies, which lead to lower oxygen free radical

	Life-span increase		Beneficial health effects	
	Dietary restriction	Mutations/ drugs	Dietary restriction	Mutations/ drugs
 Yeast	3-fold	10-fold (with starvation/ DR)	Extended reproductive period	Extended reproductive period, decreased DNA damage/mutations
 Worms	2- to 3-fold	10-fold	Resistance to misexpressed toxic proteins	Extended motility Resistance to mis-expressed toxic proteins and germ-line cancer
 Flies	2-fold	60–70%	None reported	Resistance to bacterial infection, extended ability to fly
 Mice	30–50%	30–50% (~100% in combination with DR)	Protection against cancer, diabetes, atherosclerosis,cardio-myopathy, autoimmune, kidney, and respiratory diseases; reduced neurodegeneration	Reduced tumor incidence; protection against age-dependent cognitive decline, cardio-myopathy, fatty liver and renal lesions. Extended insulin sensitivity
 Monkeys	Trend noted	Not tested	Prevention of obesity; protection against diabetes, cancer, and cardiovascular disease	Not tested
 Humans	Not determined	Not determined (GHR-deficient subjects reach old age)	Prevention of obesity, diabetes, hypertension Reduced risk factors for cancer and cardiovascular disease	Possible reduction in cancer and diabetes

Figure 1-10. Effects of DR, mutations and drugs on life- and health span.

Experiments on dietary restriction (DR) and genetic or chemical alteration of nutrient-sensing pathways have been performed on a range of model organisms. The results differ widely, and little is known about the long-term effects in humans. Modified from (Fontana, Partridge et al. 2010)

production (Verdery and Walford 1998; Veech 2004). In 1934, Mary Crowell and Clive McCay reported that feeding rats with a calorie restricted diet from the time of weaning resulted in nearly doubled life spans (McCay CM 1935). Following this seminal discovery, Roy Walford and Richard Weindruch from UCLA reported that "adult-initiated" dietary restriction ("undernutrition without malnutrition"), which began at 12 months of age, not only retarded growth and increased life-span, but also reduced spontaneous cancer incidence in two different strains of laboratory mice by more than 50% (Weindruch and Walford 1982). Weindruch et al. further reported that calorie restriction initiated at the time of weaning increased lifespan, reduced tumor incidence, and delayed immunologic aging in laboratory mice (Weindruch, Walford et al. 1986), confirming the CR studies of rats. Further support arose from the recently published 20-year longitudinal adult-onset CR study in rhesus monkeys showing that CR (30%) delayed disease onset and mortality, with a 50% decrease in cancer incidence (Colman, Anderson et al. 2009). However, a CR regimen implemented in young and older age rhesus monkeys at the National Institute on Aging (NIA) has not improved survival outcomes (Mattison, Roth et al. 2012). These findings are in contrast with an ongoing study at the Wisconsin National Primate Research Center (WNPRC), which reported improved survival associated with 30% CR initiated in adult rhesus monkeys (Colman, Anderson et al. 2009) and a preliminary report with a small number of CR monkeys (Bodkin, Alexander et al. 2003).

Although the underlying mechanisms of CR are not yet fully established, energy allocation appears to play a fundamental role. Given that only a certain amount of energy is available at any time, the cellular energetic network must economically balance this finite amount of energy between reproduction/growth and repair/maintenance (Kirkwood 2005). However, under conditions of limited nutrient availability, such as starvation or chronic calorie restriction, the favored cellular survival strategy seems to discourage reproduction/growth and rather to invest the remaining energy to preserve repair/maintenance (Kirkwood, Kapahi et al. 2000). In turn, this approach allows for future reproduction when nutrients become available and would favor the survival of the offspring. Throughout this dissertation, I will refer to this switch of energy allocation as entering a "maintenance mode". Reallocating energy when nutrients are scarce or absent could explain why calorie restricted mice have reduced size and fertility, but increased lifespan and stress resistance (Shanley

and Kirkwood 2000). Notably, mice exposed to chronic calorie restricted diets show parallel characteristics with long-lived growth hormone deficient mice (**Table 1-3**), which are resistant to stress and also have smaller body size (known as “dwarfism”), suppressed fertility, reduced plasma GH/IGF-I, insulin, and glucose (Longo and Finch 2003). Because of this broad ability to promote stress resistance, CR could in theory be applied in the clinic to protect patients from toxic side effects of chemotherapy. However, CR is not feasible for patients already prone to weight loss, from the cancer itself or from the chemotherapy, because, based on animal studies, several months may be necessary for patients undergoing CR to reach a protected state. Thus, in addition to requiring major life-style changes, CR would inevitably also cause chronic weight loss. Also, CR only retards the progression of specific cancers, possibly because of its relatively small effect on glucose and growth factors (Kalaany and Sabatini 2009; Lee and Longo 2011). In humans, CR does not reduce growth-promoting IGF-I unless it is combined with protein restriction (Fontana, Weiss et al. 2008).

Table 1-3. Comparison between CR and GH/IGF-I deficient mice.

Many physiological characteristics of GH/IGF-I deficiency are shared by calorie restriction (CR). Modified from (Longo and Finch 2003).

	CR mice	Dwarf mice
Glucose regulation		
Plasma insulin	Reduced	Reduced
Plasma glucose	Reduced	Reduced
Somatotropic axis		
Plasma GH	Reduced	Absent
Plasma IGF-I	Reduced	Greatly reduced
Body size	Reduced	Reduced
Thyroid function and metabolism		
Plasma thyroid hormones	Reduced	Greatly reduced
Body core temperature	Reduced	Reduced
Reproduction		
Sexual maturation	Delayed	Delayed
Fertility	Reduced	Suppressed
Glucocorticoids and adiposity		
Plasma corticosterone	Elevated	Normal
Percent body fat	Reduced	Elevated (old)

Macronutrient defined diets, with altered proportions of fat, carbohydrates and protein, do not generally alter the lifespan of rodents; but applying caloric restriction to these modified diets does provide the beneficial effects of CR on health span (Ross and Bras 1973; Iwasaki, Gleiser et al. 1988; Masoro 1990). Of note, a severe restriction of dietary protein can extend the lifespan of rodents by up to 20%, independently of the caloric intake (Pamplona and Barja 2006). Reduced levels of serum IGF-I in rats and mice fed with protein-restricted diets might explain the beneficial effects on longevity (Sonntag, Lynch et al. 1999).

Restricting a single essential amino acid in a normal diet can also increase lifespan and stress resistance (Segall 1977; De Marte and Enesco 1986; Pamplona and Barja 2006) and indicates that amino acids are central to CR-dependent lifespan regulation. In flies, adding back essential amino acids to the CR diet decreased lifespan to that of the normally fed group (Grandison, Piper et al. 2009). Laboratory rodents fed a methionine or tryptophan restricted diet showed extended lifespan (Miller, Buehner et al. 2005) with decreased age-dependent diseases and increased resistance to oxidative stress, in part due to increased antioxidant capacity (Richie, Leutzinger et al. 1994). In 1969, Lorincz et al. demonstrated that tumor growth was moderately reduced in mice fed with a tyrosine and phenylalanine restricted diet (Lorincz, Kuttner et al. 1969). Methionine restricted diets can reduce tumor growth in laboratory animals (Breillout, Hadida et al. 1987; Tan, Xu et al. 1996), which seems to rely partially on alterations in the glucose metabolism (Fu, Lin et al. 2010). In patients with advanced cancers, restricting methionine has shown promising results (Epner 2001; Epner, Morrow et al. 2002). The effects of amino acid restriction may be due to the high requirement of methionine in cancer cells in response to elevated protein synthesis and trans-methylation in part owing to epigenetic alterations (Hoffman 1997), whereas normal cells are relatively resistant to methionine restriction (Cellarier, Durando et al. 2003). In addition to its potential protective benefits to the patients, amino acid restriction has been shown to sensitize cancer cells to chemotherapy drugs (Goseki, Yamazaki et al. 1992; Hoshiya, Kubota et al. 1996; Yoshioka, Wada et al. 1998; Kokkinakis, Hoffman et al. 2001). A more detailed overview of CR can be found in (Spindler 2010).

Genetics have also contributed greatly to our understanding of the pathways involved in the regulation of senescence. Studies in *S. cerevisiae*, *C. elegans*, *D. melanogaster* and mice have demonstrated that the insulin and GH/IGF-I axis are major regulators of lifespan and stress resistance and can be modified in response to nutrient and/or growth factor signaling (**Fig. 1-11**) (Longo 1999; Guarente and Kenyon 2000; Longo and Finch 2003; Kenyon 2005; Piper, Selman et al. 2008). Our laboratory has demonstrated in yeast that deleting human homologs of Ras (RAS2) and/or Akt (SCH9/S6K) increased lifespan by more than 200%, while providing increased stress resistance against oxidants, genotoxins, and heat-shock (Fabrizio, Pozza et al. 2001; Wei, Fabrizio et al. 2008). Similarly, in *C. elegans*, mutations in the human homologs of insulin/IGF-I receptor (daf-2) and PI3K (age-1) extended lifespan to 200% and showed increased resistance to thermal and oxidative stress (Kleemann and Murphy 2009; Panowski and Dillin 2009). In the fruit-fly *D. melanogaster*, mutations in the insulin receptor substrate chico lead to a 150% lifespan extension (Clancy, Gems et al. 2001; Giannakou and Partridge 2007). In laboratory mice, mutations in the insulin- GH/IGF-I axis have an increased lifespan of up to 150% (Brown-Borg, Borg et al. 1996; Murakami 2006) and conversely, mice that over-express GH have a shortened lifespan (Bartke, Chandrashekar et al. 2002). In addition, *in vitro* stress resistance studies on cell cultures from long-lived mice with deficiencies in the GH/IGF-I axis have shown to be resistant against oxidative stress (H_2O_2, paraquat), UV, genotoxins (methylmethanesulfonate), heat, and cadmium (Salmon, Murakami et al. 2005; Murakami 2006), suggesting that enhanced stress resistance is partially responsible for longevity, and the possibility to enhance protection by interventions such as CR or down-regulation of the GH/IGF-I axis. In mice the longevity effects of CR appear to involve reduced activity of the GHR/IGF-I pathways because CR does not further extend the life span of GH signaling-deficient mice (Bonkowski, Dominici et al. 2009). These results were further confirmed by studies that applied intermittent fasting (IF, known as every other day feeding) to GH-receptor deficient (GHR-KO) mice (Arum, Bonkowski et al. 2009). Fasting on alternating days increased the survivorship and improved insulin sensitivity of male wild-type mice, but failed to affect either parameter in GHR-KO mice. I will discuss the role of the GH/IGF-I axis on growth and metabolism for mammals in greater detail in the following chapter.

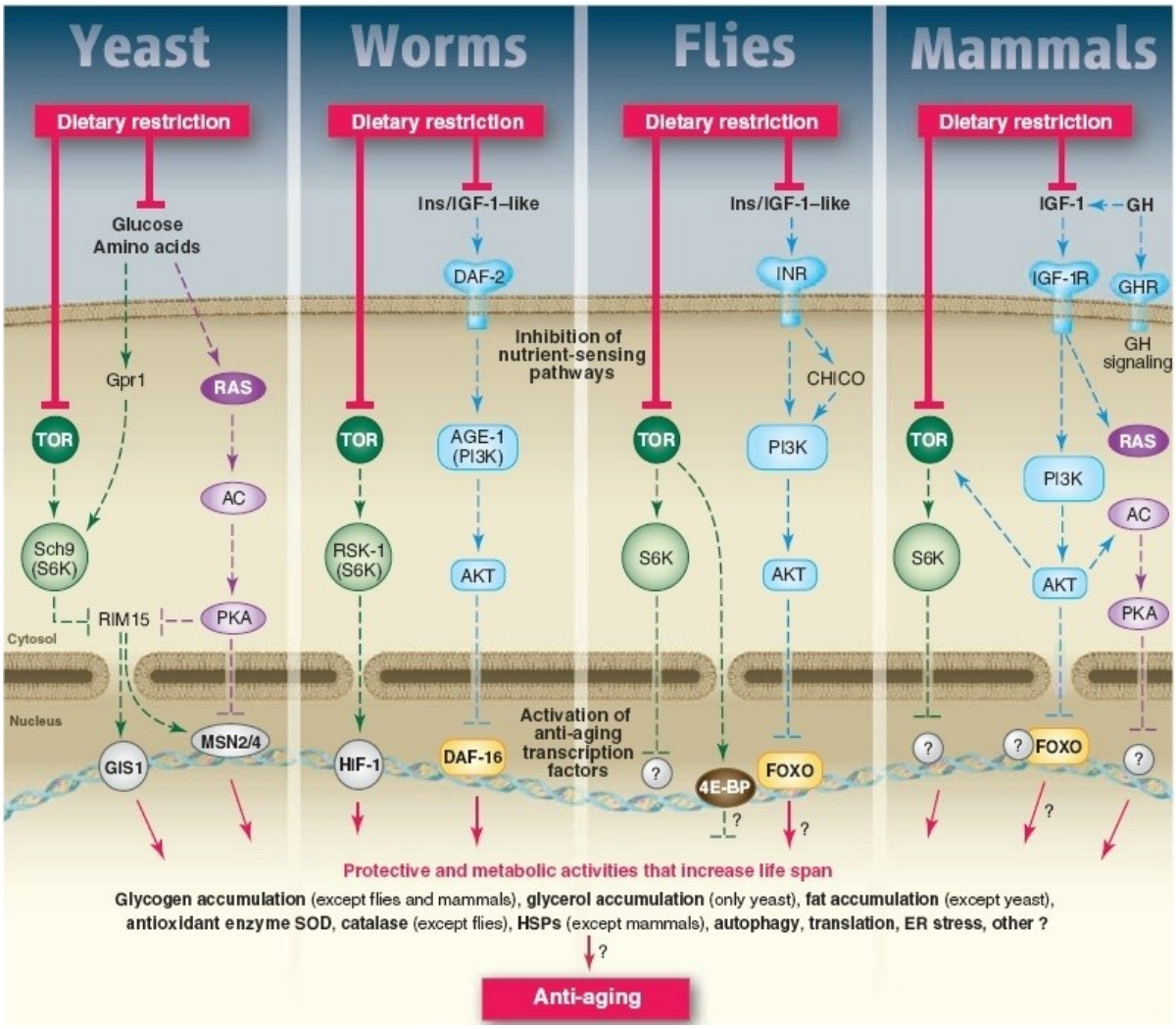

Figure 1-11. Model for conserved nutrient signaling pathways that regulate longevity in various organisms and mammals.

Dietary restriction reduces the activity of various signal transduction pathways either directly (yeast) or indirectly through the reduced levels of growth factors such as IGF-I (worms, flies, mammals). The role of TOR and S6K in promoting aging appears to be conserved in yeast, worms, flies, and mice. The AC/PKA pathways and the TOR/S6K pathway promote aging in yeast and mammals, whereas an insulin/IGF-I like receptor or the upstream growth hormone (mammals) accelerates aging in worms, flies, and mice. Similar transcription factors (GIS1, MSN2/4, DAF-16, FOXO) inactivated by the AC/PKA, IGF-I/AKT, or TOR/S6K pathways affect cellular protection and/or aging in all the major model organisms. Notably, in the multi-cellular worms, flies, and mice, these genes may promote aging within the cells in which they are expressed but also in other cells through the regulation of circulating factors. The mechanisms proposed for the longevity extension caused by inhibition of these nutrient signaling pathways include a decrease in the free radical superoxide (mediated in part by SODs) and of its damage to macromolecules,

protection of proteins by chaperones (Hsp70), decreased translation, the activation of autophagy, and the switch to hypoxia-associated gene expression patterns (in yeast and mice). In yeast, the effects of DR on lifespan extension are also associated with reduced activities of the Tor-Sch9 and Ras-AC-PKA pathways and require the serine-threonine kinase Rim15 and transcription factors Gis1, Msn2, and Msn4. In worms, transcription factors regulated by the TOR-S6K and AGE-1-AKT pathways are implicated in the anti-aging effects of DR. In flies, the reduced activity of both Ins/IGF-I and TOR can protect against shortening of life span by increased food intake. However, in both worms and flies the deletion of DAF-2/FOXO shortens life span, but the animal continues to respond to DR. Modified from (Fontana, Partridge et al. 2010).

1.9 The GH/IGF-I Axis in Growth and Metabolism

The GH/IGF-I axis is of particular interest to aging researchers because, as mentioned above, many genes that regulate lifespan converge on its downstream effectors (Longo and Finch 2003; Yang, Anzo et al. 2005). Physiologically, the GH/IGF-I axis is a major regulator of primarily two important processes: growth and metabolism. GH plays a central role in regulation of organismal growth as indicated in animal studies showing that deficiency in GH production, or the deletion of the GH receptor gene, cause retardation and growth defects (physically characterized by a severely reduced body size) (**Fig. 1-12**).

Figure 1-12. Effects of decreased glucose or Insulin/IGF-I signaling on body size.

Wild-type (left) and long-lived dwarf (right) yeast, flies, and mice. Yeast *sch9* null mutants form smaller colonies. *chico* homozygous mutant female flies are dwarfs and exhibit an increase in life-span of up to 50%. The GHR/BP mice are dwarfs deficient in IGF-I and exhibit a 50% increase in life-span. Modified from (Longo and Finch 2003)

Growth hormone (GH), also known as somatotropin, is a polypeptide that is synthesized, stored, and secreted by somatotropic cells within the anterior pituitary gland (**Fig. 1-13**). Secretion of GH from the pituitary gland is regulated by neuro-secretory nuclei located within the hypothalamus, which release growth hormone-releasing hormone (GHRH) and growth hormone-inhibiting hormone (GHIH). GH release in the pituitary is primarily determined by the balance of GHRH vs. GHIH, which in turn is regulated by physiological stimulators such as exercise, nutrition, sleep and inhibitors (e.g. free fatty acids and hyperglycemia). GH exerts its anabolic function by stimulating IGF-I production primarily in the liver. IGF-1 is released into the periphery and upon binding to its specific tyrosine kinase receptor, the insulin-like growth factor 1 receptor (IGF-R), intracellular signaling is initiated. IGF-I is one of the most potent natural activators of the pro-growth AKT signaling pathway (resulting in cell growth and proliferation) and a potent inhibitor of apoptosis. Approximately 98% of IGF-I is always bound to one of 6 binding proteins (IGF-BPs), which prolong the half-life of IGF and can alter their interaction with cell surface receptors. IGFBP-3 is the most abundant IGF-I binding protein and accounts for 80% of all serum bound IGF-I. GH and IGF-I thus function by translating nutrient availability (among other factors) in cellular growth. It is therefore that GH deficiency causes reduced body size.

There are many mouse models showing the role of GH in growth, including the Snell and Ames dwarf mice, which have point mutations in the pituitary-specific transcription factor-1 *Pit-1* or its upstream gene *Prop-1* (Li, Crenshaw et al. 1990; Andersen, Pearse et al. 1995). Dwarf mice do not produce GH and weigh only a third of their WT littermates during their adulthood, but live about 50% longer than their littermates (Brown-Borg, Borg et al. 1996). In addition, GH receptor/binding protein (GHR/BP) knockout mice, which were initially developed to model the human Laron dwarfism syndrome, have undetectable levels of hepatic GH receptor expression and serum GHBP, leading to a significant decrease in body size and approximately 50% increase in longevity. Due to the lack of the negative feedback inhibition of IGF-I on GH production, these mice have elevated GH levels (but a 90% reduction in IGF-I levels) (Zhou, Xu et al. 1997; Coschigano, Clemmons et al. 2000). Unlike the GHR/BP KO mice, mice that expressed a GH antagonist eventually reached normal body weight and their lifespan remained comparable to their WT littermates

(Coschigano, Holland et al. 2003). A possible explanation for this effect might be based on differences in GH signaling level, i.e. complete deletion of the receptor compared to receptor antagonism; suggesting that the degree of GH signaling inhibition is central for the regulation of growth and lifespan. Anabolic pathways downstream of GH, IGF-I in particular, have been shown to be involved in the effects of lifespan regulation. For example, female heterozygotes of the IGF-I receptor (IGF-1R) display a 33% increase in lifespan, whereas male heterozygotes show a modest 16% increase in lifespan which is not statistically significant. The homozygous deletion of the *IGF-Ir* gene is lethal in mice (Holzenberger, Dupont et al. 2003). Remarkably, the heterozygotes were also better protected against oxidative

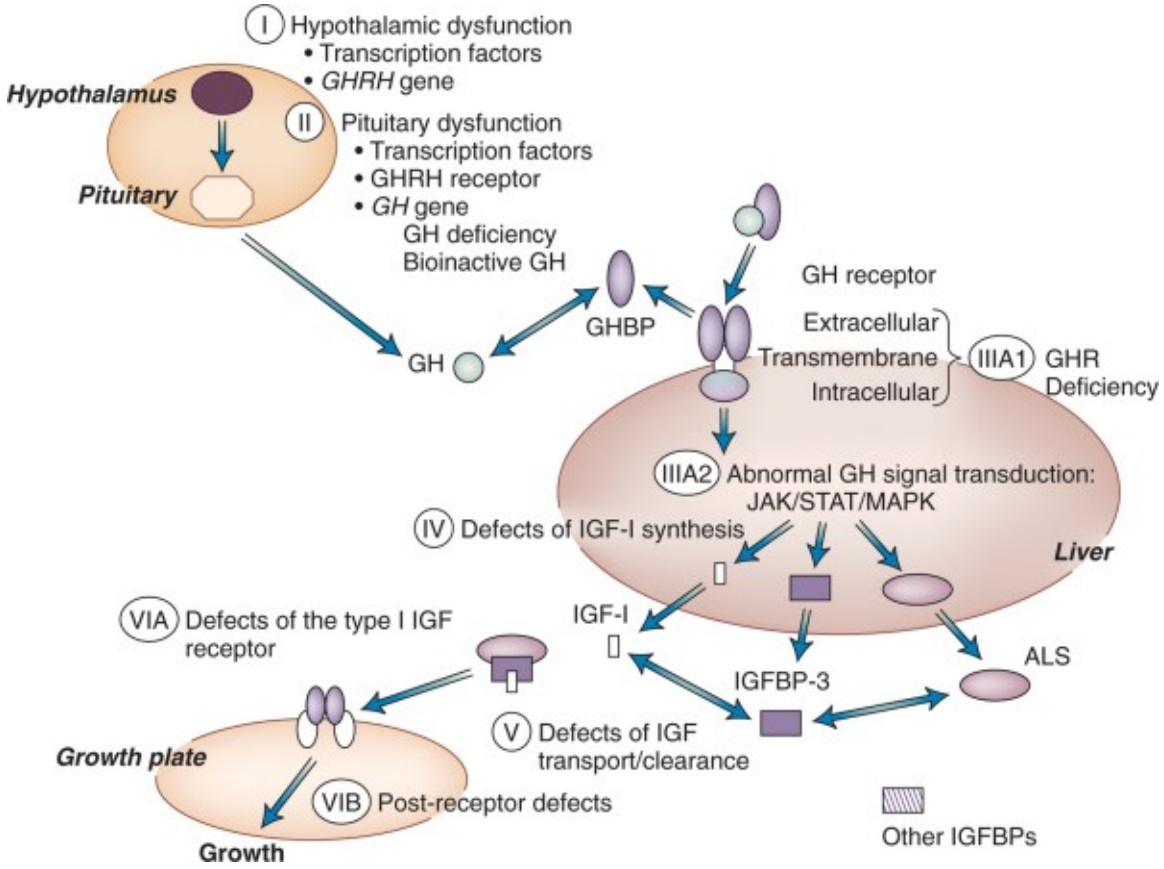

Figure 1-13. The hypothalamic–pituitary–IGF axis with sites of established and hypothetical genetic defects.

Abbreviations: ALS, acid-labile subunit; GH, growth hormone; GHBP, GH binding protein; GHRH, GH-releasing hormone; IGF, insulin-like growth factor; IGF-BP, IGF-binding protein; JAK, Janus kinase; MAPK, mitogen-activated protein kinase; STAT, signal transducer and activator of transcription. Modified from (Lopez-Bermejo, Buckway et al. 2000).

stress, as determined by increased survival following paraquat (an oxidant) treatment. Embryonic fibroblasts obtained from this *IGF-IR +/-* mice showed reduced activation of its major effectors including Akt and $p66^{shc}$, a cytoplasmic signal transducer delivering mitogenic signals from activated receptors to Ras (Ma, Liu et al. 2010), when cultured *in vitro* (Holzenberger, Dupont et al. 2003). Interestingly, $p66^{shc}$ has been correlated with effects on lifespan prior to the discovery of its role in the *IGF-IR +/-* mice: $p66^{shc}$ -/- mice have an increased resistance to oxidative stress and enhanced lifespan (Migliaccio, Giorgio et al. 1999).

Circulating IGF-I, acting synergistically with other hormones, regulates energy metabolism, cell proliferation and differentiation, body size and lifespan in response to calorie and protein availability (Prisco, Romano et al. 1999; Flototto, Djahansouzi et al. 2001; Giovannucci, Pollak et al. 2003; Yu, Shu et al. 2003). As outlined in the previous chapter, GH/IGF-I converges on intracellular nutrient sensing pathways that mediate growth and proliferation. In addition, IGF-I exerts a potent tumorigenic effect on a variety of cancer cells by increasing their proliferative rate and inhibiting apoptosis (Prisco, Romano et al. 1999; Ramsey, Ingram et al. 2002). Studies in mice with deficiencies in the downstream effectors of IGF-R signaling, including mTOR inhibition by rapamycin (Harrison, Strong et al. 2009) and S6K1 (Selman, Tullet et al. 2009), demonstrate the central role of intracellular mitogenic pathways in regulating lifespan and stress resistance while simultaneously reducing tumor growth (Ikeno, Bronson et al. 2003; Pinkston, Garigan et al. 2006; Garcia, Busuttil et al. 2008; Ikeno, Hubbard et al. 2009).

Our laboratory, in collaboration with investigators in Ecuador, has recently published a study in which we monitored cellular responses to stress, and markers of cancer and diabetes in an Ecuadorian cohort with GHR deficiency (GHRD), which results in IGF-I deficiency. We found that human GHRD subjects are protected against age-related pathologies (Guevara-Aguirre, Balasubramanian et al. 2011) (**Fig. 1-14**). Yet, unlike model organisms with similar mutations, human GHRD subjects do not live longer lives. The lack of life-span extension in GHRD subjects may be explained in large part by the high proportion of deaths (70%) caused by convulsive disorders, alcohol toxicity, accidents, liver cirrhosis, and other non–age-related causes.

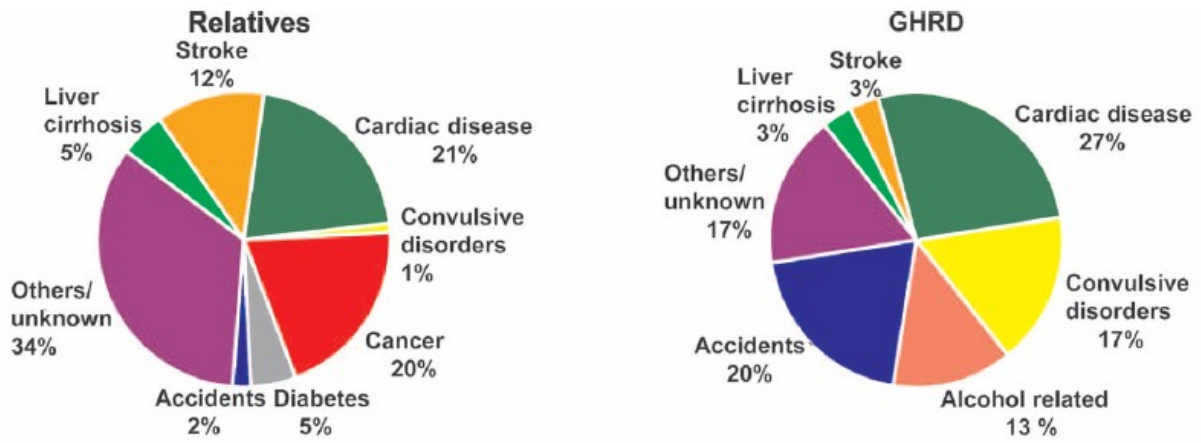

Figure 1-14. Causes of death in the Ecuadorian GHRD cohort.

Causes of death in unaffected relatives (left) and GHRD (right) subjects are shown. Modified from (Guevara-Aguirre, Balasubramanian et al. 2011).

1.10 The Feasibility of Fasting: History, Clinical Application, and Stress Resistance

Mammals undergo three metabolic stages when deprived of food (Wang, Hung et al. 2006). The first stage is a post-absorptive phase, lasting for 10 or more hours following food ingestion. This stage involves the utilization of glycogen as the main stored energy source. 2) Once the liver glycogen storage has been depleted, amino acids serve as the substrate for gluconeogenesis. 3) The remaining glucose is mostly consumed by the brain while glycerol and fatty acids are released from adipose tissue and become the major energy source. As a result, the fat-derived ketonebodies become the main carbon sources within a few days of fasting. During the last phase of prolonged food deprivation, the fat storage is eventually exhausted and rapid muscle degradation occurs for gluconeogenesis. Initially, the weight loss is rapid but later on tapers off as shown in a human study where an average of 0.9 kg/day was lost daily during the first week, subsiding to 0.3 kg/day by the third week of fasting, causing an approximately 20% body weight loss after 30~35 days of fasting (Kerndt, Naughton et al. 1982). Despite this considerable weight loss, it is estimated that a 70 kg person can sustain the basal caloric requirements from fat reserves during two to three months of fasting (Cahill and Owen 1968; Cahill 2006). Within the body, these changes trigger a cellular response including the down-regulation of pathways involved in proliferation, cell growth and the reduced

production of reactive oxygen species, while simultaneously increasing genomic stability and cellular stress resistance (Sohal and Weindruch 1996; Hursting, Slaga et al. 1999; Holzenberger, Dupont et al. 2003; Longo and Fontana 2010; Lee and Longo 2011). GH level in response to fasting are also a critical metabolic switch. GH acts as the primary anabolic hormone during stress and fasting by predominantly stimulating the release and the oxidation of free fatty acids from lipids (lipolysis). This allows the organism to decrease glucose and protein oxidation and consequently preserve lean body mass (Moller and Jorgensen 2009). In fasting humans, GH levels initially increase, whereas IGF-I levels diminish (Ho, Veldhuis et al. 1988), a phenomenon known as “GH resistance”, causing the body to switch fuel sources, to discourage growth, and to enter into a maintenance mode.

Historically, fasting has been performed for a number of reasons including medical, cosmetic, religious, and political purposes (e.g. Gandhi engaging in several hunger strikes to protest the British rule of India) (Kerndt, Naughton et al. 1982; Johnstone 2007). Over the last centuries, much has been learned through either involuntary fasting from the victims of famine and war (Scrimshaw 1987; Kalm and Semba 2005), or voluntary fasting such as the biblical 40 days of food abstinence (Kerndt, Naughton et al. 1982). But beyond its traditional practice, fasting has also been demonstrated to have vast clinical benefits. Notably, clinical studies have shown that water-only fasting for 10-14 days significantly improved hypertension by reducing systolic blood pressure points more than 2-fold compared to that of a combined vegan, low-fat, low salt diet and exercise (Goldhamer, Lisle et al. 2001; Goldhamer 2002). This study is of particular importance as the leading cause of death in the US is heart disease (Jemal, Siegel et al. 2008). Moreover, the safety of consuming a very low calorie diet of 350 kcal/day (an almost fasting-like approach), in patients with chronic disease, has been studied in a large cohort with over 2000 participants (Michalsen, Hoffmann et al. 2005). Fasting was considered safe and appears promising to be incorporated into an integrative medicine ward. The vast majority of the participating patients indicated that the very low calorie diet seemed to be beneficial to counter their chronic disease. Within the last 5 years, fasting has also been proposed in clinics to protect the patient from ischemic reperfusion damage, in which oxidative stress is largely responsible for the damage (Mitchell, Verweij et al. 2009; van Ginhoven, Mitchell et al. 2009).

A prolonged period of fasting is feasible and generally well tolerated, however mild side-effects including headaches, light-headedness, nausea, weakness, edema, anemia, amenorrhea (Bloom 1959; Drenick and Smith 1964), and, in some rare cases, fatal complications such as renal failure, heart failure, and lactic acidosis, may occur (Garnett, Barnard et al. 1969; Runcie and Thomson 1970).

1.11 Short-term Starvation to Improve the Stress Resistance against Chemotherapy

Calorie restriction (CR) is a well-established intervention to increase lifespan and reduce the rate of cancer (Tannenbaum and Silverstone 1949; McCay, Crowell et al. 1989; Tannenbaum 1996). In addition, CR increases the stress resistance in normal cells and organisms (Yu and Chung 2001). The effect of CR on cancer has been largely focused on preventing and reducing tumor growth, whereas its effect on stress resistance has been poorly explored. Since the efficacy of CR as a single treatment modality is limited in curing cancer, and considering that several weeks to months are required to be effective, it would be challenging to incorporate CR into the lifestyle of healthy individuals, let alone cancer patients.

We aimed to determine if short cycles of starvation could achieve stress resistance because both CR and fasting mediate signaling through the GH/IGF-I axis and their downstream signaling pathways. Initial results from our lab in yeast demonstrated that glucose reduction (from 2% to 0.5%) increases protection against oxidative stress, whereas complete starvation (by switching the population of cells to water), promotes not only protection to oxidative insults and heat shock but also resulted in a major life span increase (Wei, Fabrizio et al. 2008). Deleting the transcription factors Msn2/Msn4 and Gis1, located downstream of intracellular nutrient sensing pathways, reversed the protection caused by glucose restriction or starvation conditions, suggesting an important role for genes involved in metabolism, cellular protection and repair. To expand this approach from unicellular yeast to a multi-cellular organism, this starvation mediated protection was applied to mouse models treated with high-dose chemotherapy (Raffaghello, Lee et al. 2008), because most chemotherapy agents cause considerable damage to normal cells. Out of 28 mice, from three genetic backgrounds, which were assigned to short-term starvation (STS) for 48–60 hours before treatment with the chemotherapeutic drug etoposide (Eto), only one mouse died. In contrast, of the 37 control mice treated with etoposide

alone, 20 died of toxicity (**Fig. 1-15**). Of course, utilizing fasting to protect against high-dose chemotherapy would be useless in a clinical scenario if cancer cells are also entering a protected mode. Considering the hallmarks of cancer, and in particular the self-sufficiency in growth signaling, we proposed that malignant cells would not, or to a lesser extent, be protected against high doses of oxidative damage or chemotherapy under STS conditions. Short-term starved *S. cerevisiae* were up to 1,000 times better protected against oxidative stress or chemotherapy drugs than yeast expressing the oncogene homolog $Ras2^{val19}$ (Raffaghello, Lee et al. 2008). Low glucose or low-serum media also protected primary glial cells but not six different rat and human glioma and neuroblastoma cancer cell lines against hydrogen peroxide or the chemotherapy drug/pro-oxidant cyclophosphamide (Raffaghello, Lee et al. 2008). We aimed to determine whether the effects we observed in yeast and mammalian cells with regard to the protection against chemotherapy would also occur *in vivo*. Mice were injected with cancer cells and subsequently treated with etoposide. Short-term starvation provided complete protection to mice, but not to the injected NXS2 neuroblastoma cells, against high dose etoposide (Raffaghello, Lee et al. 2008). These studies describe a starvation-based strategy that enhances the protection of normal, not malignant cells; an effect we termed “differential stress resistance” (DSR).

To evaluate how DSR mediates its effects in differential protection, we analyzed markers of nutrient availability and the connected GH/IGF-I axis. STS and CR both lower circulating IGF-I, which is regulated by GH and nutrition (Clemmons and Underwood 1991; Underwood, Thissen et al. 1994). Under normal conditions, GH directly regulates the production of IGF-I, but during starvation, several changes in the GH/IGF-I axis occur as a result of physiological adaptation to the new environment. In humans, IGF-I levels decrease dramatically in response to a short-term starvation (36-72 hours) despite increased GH secretion (Norrelund 2005; Moller and Jorgensen 2009). The reduction in IGF-I, in turn, down-regulates many of the intracellular growth signals such as AKT, Ras, and up-regulates stress resistance pathways such as FoxO, GRP78 and DNA repair genes (Lee, Raffaghello et al. 2012; Pfaffenbach, Pong et al. 2012).

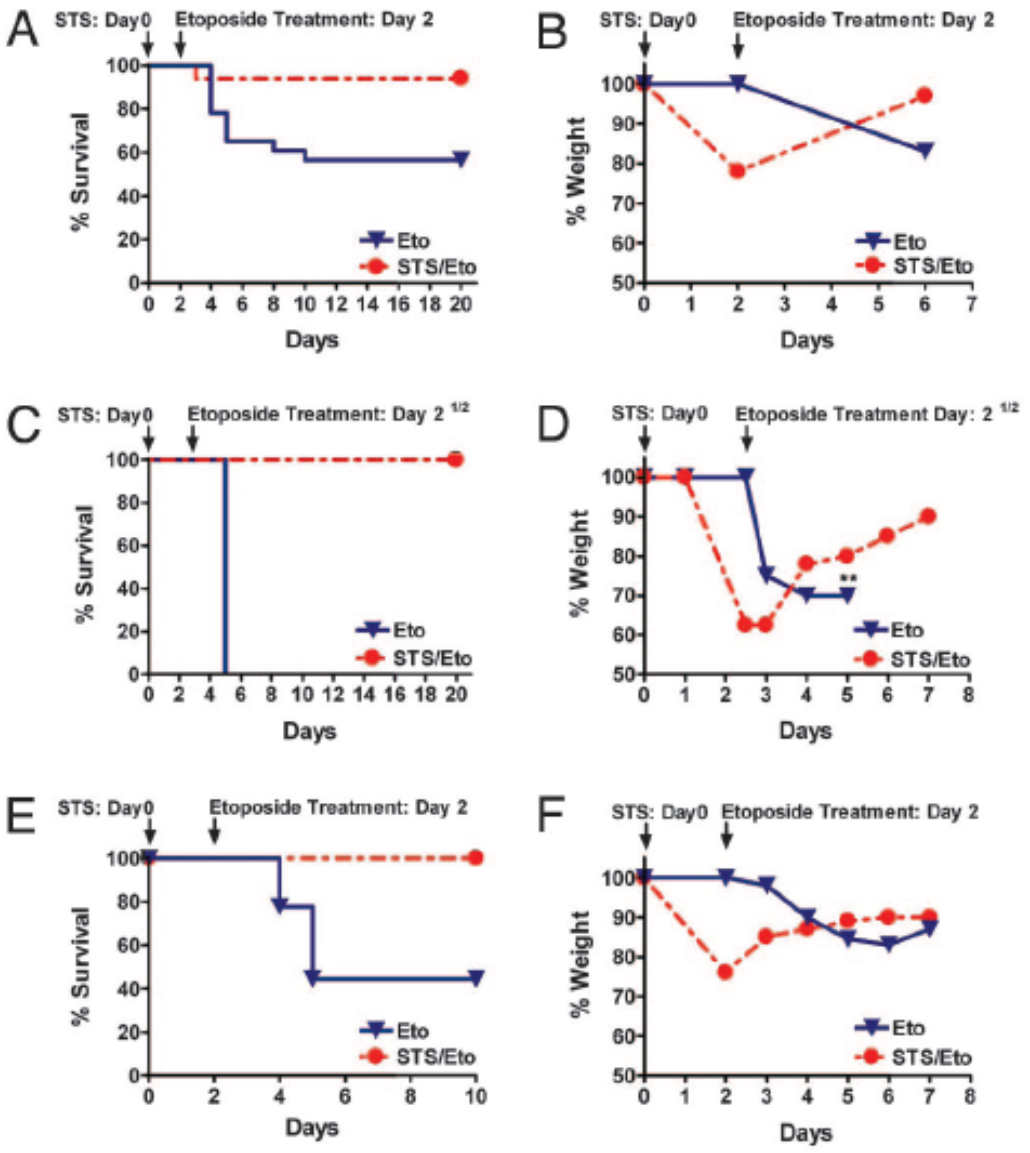

Figure 1-15. Short-term starvation protects against high-dose chemotherapy *in vivo.*

(**A**) A/J mice were treated with 80 mg/kg etoposide with (STS/Eto) or without (Eto) a prior 48 hour starvation (STS). (**B**) Percent weight loss (a measure of toxicity) after etoposide treatment in STS-treated or untreated A/J mice. (**C**) CD-1 mice were treated (i.v.) with 110 mg/kg etoposide with (STS/Eto) or without (Eto) a 60 hour prior starvation. (**D**) Percent weight loss after etoposide treatment in STS-treated (n=5) or untreated CD-1 mice. Asterisks indicate the day at which all mice died of toxicity. (**E**) Athymic (Nude-nu) mice were treated (i.v.) with 100 mg/kg etoposide with (STS/Eto) or without (Eto) a 48 hour prior starvation. (**F**) Percent weight loss after etoposide treatment in the treated (STS/Eto) or untreated (Eto) athymic (Nude-nu) mice. Modified from (Raffaghello, Lee et al. 2008).

In mice and rats, short-term starvation (24-72 hours) decreases IGF-I production (Tannenbaum, Rorstad et al. 1979; Frystyk, Delhanty et al. 1999). In agreement, studies from our lab have obtained similar results when the levels of circulating GH and IGF-I, and its binding proteins IGFBP-1 and IGFBP-3, were measured in mice undergoing 72 hours of STS (Lee, Safdie et al. 2010). The mice lost approximately 20% of their pre-fasted body weight, blood glucose levels were reduced by 40%, GH levels were slightly increased, and serum IGF-I levels decreased by 70%. The bioavailability of IGF-I is regulated by IGF binding proteins. In fasted mice, the level of IGFBP-1, which normally reduces IGF-I signaling, increased by 11.4-fold and decreased IGFBP-3 levels by 40% (**Fig. 1-16**).

To determine whether reducing IGF-I signaling protects from high-dose chemotoxicity, we tested a transgenic mouse model with a conditional liver *Igf1* gene deletion (LID), resulting in a 70% to 80% postnatal reduction of circulating IGF-I (Yakar, Liu et al. 1999; Anzo, Cobb et al. 2008). These results are similar to that of a 72 hour fasted mouse. Therefore, LID mice provide a model for investigating the mechanistic relationship between IGF-I and fasting in chemotherapy resistance. To determine the range of protection by reduced IGF-I, we tested four chemotherapeutic drugs: cyclophosphamide (CP), etoposide (Eto), 5-fluorouracil (5-FU) and doxorubicin (DXR) (**Fig. 1-17**). The LID mice experienced a significant protection from most drugs, although the protection against Eto in the LID model could not be established. These results thus indicate that the compatibility between specific drugs and IGF-I reduction/blockade therapy has to be carefully tested in preclinical studies before being considered as a candidate.

To test if restoring the IGF-I levels during STS reverses the protection against chemotherapy induced toxicity, CD-1 mice underwent a 48 hour STS with IGF-I (200 µg/kg) administration every 12 hours to match levels of *ad libitum* fed mice. Following the STS/IGF-I treatments, mice were intra-venously (i.v.) injected with 16 mg/kg DXR. Restoring IGF-I during STS abolished the protective effect of STS on DXR toxicity, resulting in a 100% vs. 38% survival in the STS and STS/IGF-I groups, respectively (**Fig. 1-18**).

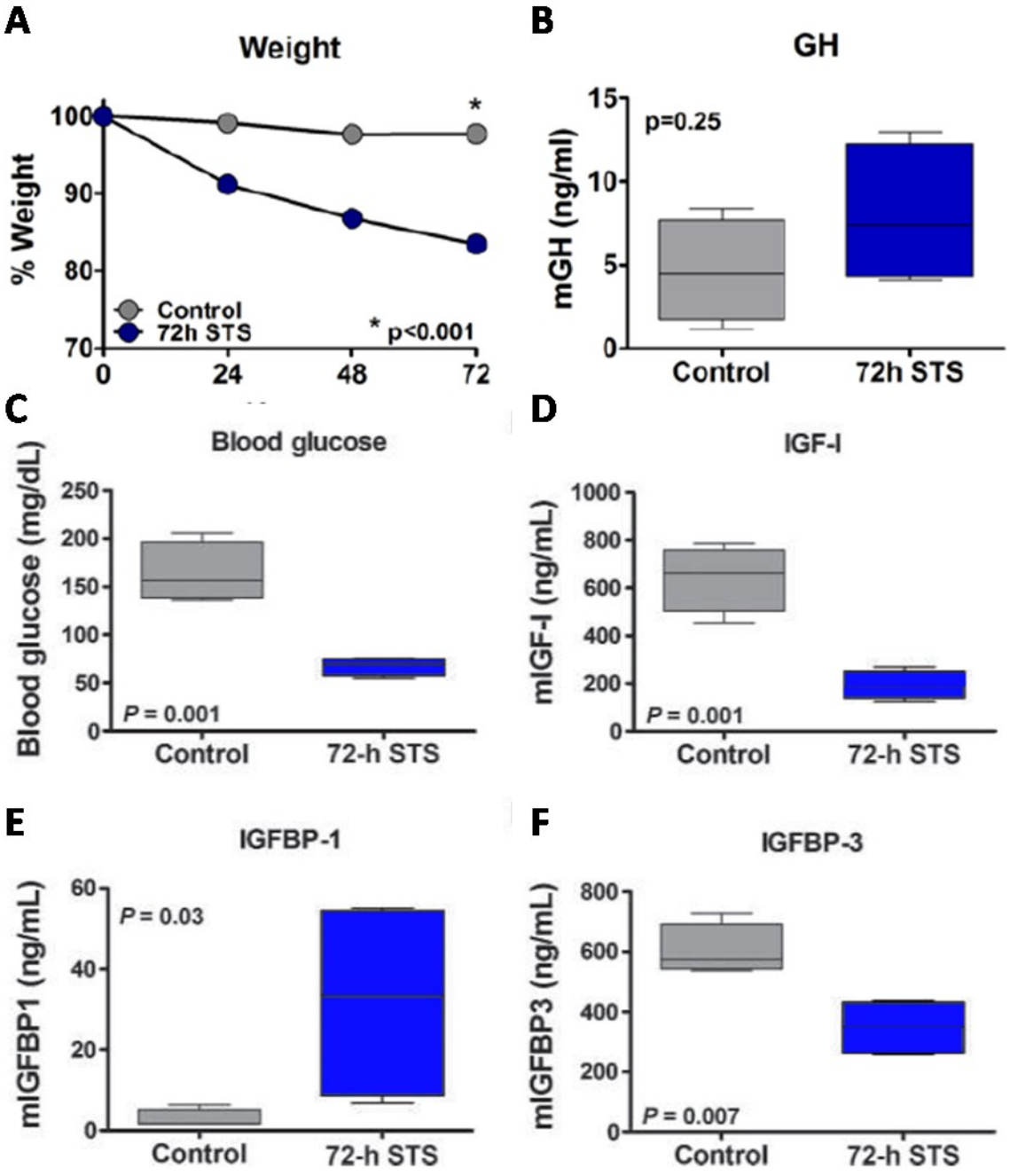

Figure 1-16. The effect of 72 hour fasting on glucose, IGF-I, IGFBP-1 and IGFBP-3 levels.

Thirty week old CD-1 mice were fasted for 72 hours (**A**) and sacrificed. Blood was collected via cardiac puncture under anesthesia, and blood glucose (**C**) was measured immediately. Plasma GH (**B**), IGF-I (**D**) and IGFBP-1/ IGFBP-3 (**E** and **F**) levels were measured by a mouse-specific ELISA (Cohen lab, UCLA). All p-values were calculated by Student's t-test except that for IGFBP-1, which was done by the Mann-Whitney U test. Modified from (Lee, Safdie et al. 2010).

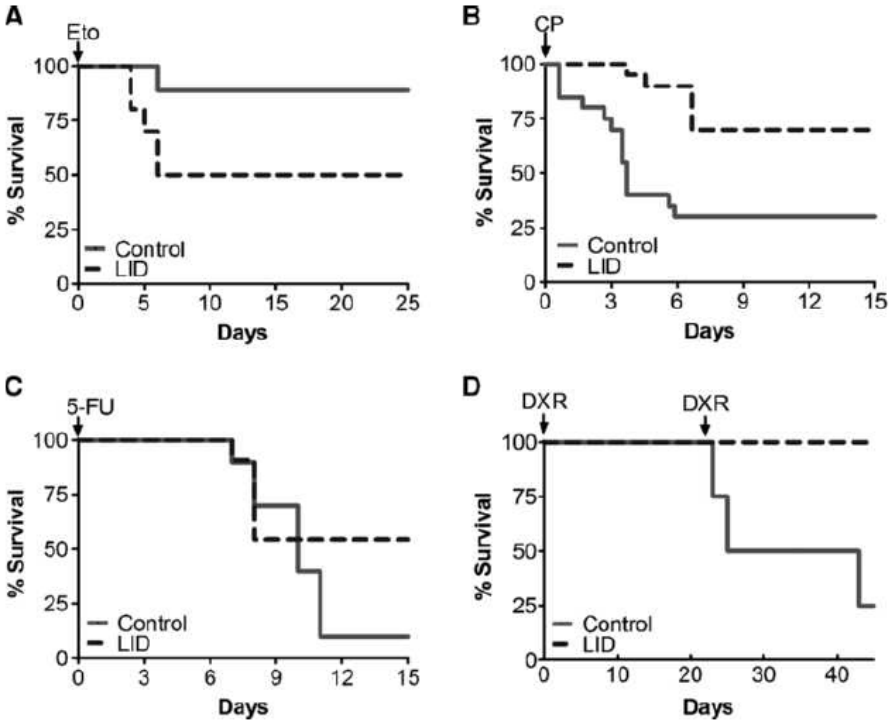

Figure 1-17. Stress resistance testing in a liver *Igf1* gene deletion (LID) mouse model with high-dosed chemotherapeutic drugs.

LID and control mice received (**A**) a single injection of 100 mg/kg etoposide (Eto), (**B**) a single injection of 500 mg/kg cyclophosphamide (CP), (**C**) a single injection of 400 mg/kg 5-fluorouracil (5-FU), or (**D**) two injections of doxorubicin (DXR). The first injection of 20 mg/kg was given on day 0, and the second injection of 28 mg/kg was given on day 22. Toxicity evaluated by percent survival is shown. Modified from (Lee, Safdie et al. 2010).

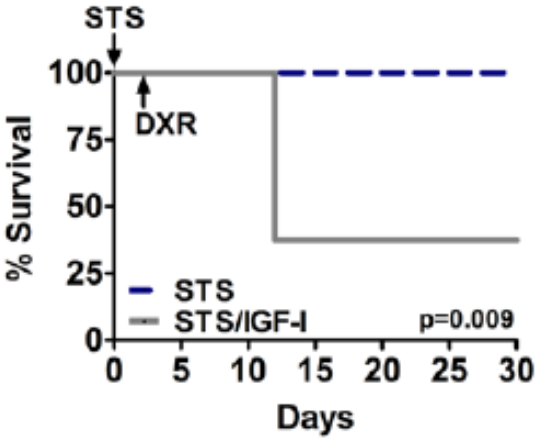

Figure 1-18. IGF-I restoration during STS reverses STS-dependent protection.

16 to 20 week old female CD-1 mice were fasted for 48 hours (STS). During STS, IGF-I (200 µg/kg) was intra-peritoneally injected every 12 hours to restore IGF-I levels. Immediately following STS/IGF-I treatment, all mice were intra-venously injected with 16 mg/kg DXR. Modified from (Lee, Safdie et al. 2010).

Previous human studies have shown that alternate day dietary restriction and short-term fasting (5 days) are well tolerated and safe (Isley, Underwood et al. 1983; Maccario, Aimaretti et al. 2001; Johnson, Summer et al. 2007). In a study published in 2002, children ranging from 6 months to 15 years of age were able to complete 14 to 40 hours of fasting in a clinical study that was carried out by the Children's hospital of Philadelphia (Katz, DeLeon et al. 2002). Following the publication of our pre-clinical work on the effects of DSR, several patients, diagnosed with a wide variety of cancers, elected to undertake fasting prior to chemotherapy and shared their experiences with us. In 2009, our laboratory published a case series report of 10 patients diagnosed with various types of cancer (**Table 1-4**), who have voluntarily fasted prior to and following chemotherapy (Safdie, Dorff et al. 2009). Out of the 10 cancer patients receiving chemotherapy, 7 females and 3 males with a median age of 61 years (range 44-78 yrs), four suffered from breast cancer, two from prostate cancer, and one each from ovarian, uterine, non small cell carcinoma of the lung, and esophageal adenocarcinoma. All patients voluntarily fasted for a total of 48 to 140 hours prior to and/or 5 to 56 hours following chemotherapy administered by their treating oncologists. The obtained results, based on self-assessed health outcomes from a questionnaire and laboratory reports, suggest that fasting is safe and raises the possibility that it can reduce some of the commonly with chemotherapy-associated side effects, e.g. nausea and vomiting (**Fig. 1-19**), although a placebo effect cannot be fully excluded.

In July 2009, the University of Southern California (USC)/Norris Comprehensive Cancer Center initiated a clinical trial (ClinicalTrials.gov Identifier: NCT00936364) to test the efficacy and safety of 24 to 72 hours of fasting in combination with platinum-based chemotherapy treatments in patients diagnosed with bladder cancer. A more recent extension includes patients affected by breast, ovarian and lung cancer (this trial is still ongoing). As of August 2010, the Mayo Clinic Cancer Center (ClinicalTrials.gov Identifier: NCT01175837) started to enroll, and is still recruiting, patients older than 18 years who are affected by lymphomas and leukemia for fasting in combination with chemotherapy. A clinical trial sponsored by the Leiden University Medical Center (ClinicalTrials.gov Identifier: NCT01304251) will study the effects of short-term fasting on tolerance to adjuvant chemotherapy in breast cancer.

Table 1-4. Demographic and clinical information of patients that voluntarily fasted prior to chemotherapeutic treatment.

Modified from (Safdie, Dorff et al. 2009).

	Gender	Age	Primary Neoplasia	Stage at Diagnosis
Case 1	Female	51	Breast	IIA
Case 2	Male	68	Esophagus	IVB
Case 3	Male	74	Prostate	II
Case 4	Female	61	Lung (NSCLC)	IV
Case 5	Female	74	Uterus	IV
Case 6	Female	44	Ovary	IA
Case 7	Male	66	Prostate	IV/DI
Case 8	Female	51	Breast	IIA
Case 9	Female	48	Breast	IIA
Case 10	Female	78	Breast	IIA

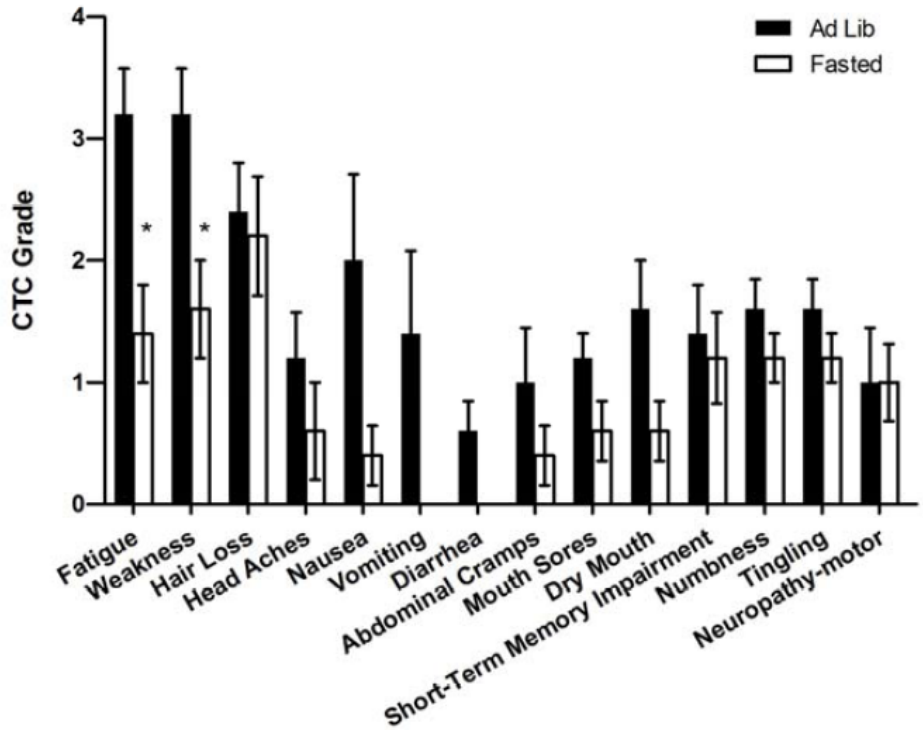

Figure 1-19. Self-reported side-effects after chemotherapy in patients with or without fasting.

Data represent average of Common Terminology Criteria for Adverse Events (CTCAE) grade from matching fasting and non-fasting cycles (*Ad lib*). Six patients received chemotherapy alone, or chemotherapy/ fasting treatments. Side effects from the closest two cycles were compared to one another. Data presented as standard error of the mean (SEM). P-value was calculated with unpaired, two tailed t-test; *, $P<0.05$. Modified from (Safdie, Dorff et al. 2009).

From the introduction above, it becomes clear that normal and cancer cells differ in many ways. In general, normal cells obey the regulatory signals for growth, differentiation, and apoptosis. In contrast, cellular transformation, as a result of mutations, collectively empowers a cell with self sufficiency in growth signaling and insensitivity to growth inhibitory signaling (**Fig. 1-2**), which uncouples cancerous cells from the organism (Hanahan and Weinberg 2008; Luo, Solimini et al. 2009). Even more specific, this self sufficiency in growth signaling is supported by gain-of-function mutations in oncogenes (e.g. *Ras*, Akt, mTor, etc) that enable the constitutive activation of proliferation pathways; regardless if the conditions support cellular growth. On the contrary, insensitivity to growth inhibitory signals is mediated by loss-of-function mutations in tumor-suppressor genes (e.g. *Rb*, *p53*, *PTEN*, etc), enabling cancer cells to disregard anti-proliferation signals (Hanahan and Weinberg 2000). This distinct response to growth regulation between normal and cancer cells is the foundation for the differential stress resistance strategy that we employed to protect mice, and potentially patients, from chemotherapy induced toxicity. The therapeutic potential of fasting may be even greater if it also increases the death of cancer cells.

I will describe in the following sections of this thesis that the self sufficiency in growth signals, insensitivity to growth inhibitory signals, as well as strong dependence on glucose, causes cancer cells to be sensitized to chemotherapy, radiotherapy and other toxins. Malignant cells under normal cellular conditions represent a highly efficient and adapted cell within their proximate niche. However, the same mutations that prone tumor cells to unrestricted growth under optimal conditions, render them also sensitive to alterations in the cellular environment. One such example is the change of the metabolism under fasting conditions. Normal, non-malignant, cells have evolved over thousands of years to cope with the ever changing environment that they are exposed to. Our fasting based sensitization exploits this deficit in cancer cells.

2 Material and Methods

2.1 Yeast Stress Resistance

Yeast strains used in this study are derivatives of the DBY746 (MATα *leu2–3,112, his3Δ1 trp1-289α ura3-52 GAL+*) strain. The over-expressing RAS2^{val19} strain was generated by transforming wild-type DBY746 cells with pMW101 (a pRS416 vector carrying the Cla I–*ras2*^{val19}–Hind III fragment form pMF100; a gift from J. Broach, Princeton). Yeast cells were grown in SDC supplemented with a 4-fold excess of tryptophan, leucine, uracil, and histidine to avoid possible artifacts due to auxotrophic deficiencies of the strains. Medium and growth conditions were as described (Wei, Fabrizio et al. 2008). Briefly, overnight SDC culture was diluted (1:200) in fresh SDC medium to a final volume of 10 ml (with a flask to culture volume of 5:1) and was maintained at 30°C while shaking (200 rpm) to ensure proper aeration. This time point was considered day 0. Every 2 days, aliquots from the culture were properly diluted and plated on to YPD plates. The YPD plates were incubated at 30°C for 2 to 3 days, and viability was accessed by Colony Forming Units (CFUs).

Heat shock resistance was measured by spotting serial dilutions (10-fold dilution started at OD_{600} of 10) of cells removed from SDC cultures onto YPD plates and incubating at either 55°C (heat-shocked) or 30°C (control) for 45 min to 150 min. After the heat shock, plates were transferred to 30°C and incubated for 2 to 3 days. For oxidative stress resistance assays, cells were diluted to an OD_{600} of 1 in K-phosphate buffer (pH 6.0) and treated with 100 mM to 200 mM of hydrogen peroxide for 60 min, or cells were treated with 250 mM of menadione for 30 min in K-phosphate buffer (pH 7.4). Serially diluted (10-fold) control or treated cells were spotted onto YPD plates and incubated at 30°C for 2 to 3 days, and viability was accessed by CFUs.

2.2 Cell Culture of Mammalian Cells

4T1luc murine breast cancer cells were purchased from SibTech. B16fluc murine melanoma cells were provided by N. Craft [University of California, Los Angeles (UCLA)]. GL26luc murine glioma cells, as well as human glioblastoma cell lines U251, LN229, A172, C6 and U87-MG were provided by T. Chen [University of

Southern California (USC)]. PC3 and 22Rv1 human prostate cancer cells were provided by P. Cohen (UCLA). MCF-7 and C42B human breast cancer cells and HeLa human cervical cancer cells were provided by A. Lee (USC). LOVO human colon cancer cells were provided by D. Shibata (USC). NXS2 and Neuro-2a murine neuroblastoma, ACN and SH-SY5Y human neuroblastoma, OVCAR3 human ovarian carcinoma, MZ2-MEL human melanoma, A431 human epidermoid carcinoma, and MDA-MB-231 human breast cancer cells were routinely cultured in the Laboratory of Oncology of Gaslini Institute. Mouse primary mixed glial cells were obtained from the cerebral cortices of 1-3 day old C57BL/6 mouse pups. All cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) and 10% fetal bovine serum (FBS) at 37°C and 5% CO^2.

2.3 *In vitro* Starvation Model

Cellular fasting was done by glucose and/or serum restriction to achieve blood glucose levels typical of fasted and normally fed mice. The lower glucose level was approximated to 0.5 g/liter and the upper level to 2.0 g/liter based on previous results (Raffaghello, Lee et al. 2008; Lee, Safdie et al. 2010). For human cell lines, normal glucose was considered to be 1.0 g/liter. Serum (FBS) was supplemented at 1% for starvation conditions to mimic the reduction in growth factors. Cells were washed twice with phosphate buffered saline (PBS) before changing to fasting medium.

2.4 *In vitro* Proliferation Assays

To determine cellular proliferation, we seeded 50,000 cells in 6-well plates and immediately upon attachment switched medium to starvation (0.5 g/liter, 1% FBS) or control (2.0 g/liter, 10% FBS) conditions. Forty-eight hours later, cell number was assessed by trypan blue exclusion.

2.5 Treatments of Mammalian Cells *in vitro*

Cells were seeded into 96-well microtiter plates at 20,000 cells/well and incubated for 2 days in DMEM (4.5 g/L glucose and 10% FBS) and washed with PBS prior to treatments. Cells were then incubated in glucose free DMEM (Invitrogen) supplemented with either 10% or 1% FBS and glucose (0.5 g/L, for starvation-mimicking condition or 2.0 g/L, for *ad lib* condition) for 24 hours followed by 24 hour treatments with varying concentrations of Temozolomide (TMZ, Schering),

Doxorubicin (DXR, Bedford Laboratories) and Cyclophosphamide (CP, Sigma-Aldrich and Baxter). Optimum drug doses were determined for each individual cell line.

For the treatment with the neutralizing monoclonal antibody α-IR3 (Millipore), cells were incubated in DMEM (2.0 g/L glucose with 1% serum) for 24 hours prior to and during drug treatment with 4 mM cyclophosphamide.

The IGF-I treatment was carried out by incubating cells in DMEM with 1% FBS and rhIGF-I (200 μM, ProSpec-Tany TechnoGene, Rehovot, Israel) for 24 hours prior to and during drug treatment with 16 μM Doxorubicin.

AHA incorporation was measured and normalized against Hoechst 33342 values with the Click-iT AHA Alexa Fluor 488 Protein Synthesis HCS Assay (Invitrogen) following the manufacturer's instructions.

2.6 *In vitro* Cytotoxicity Assays

Cytotoxicity was measured by the ability to reduce methyl-thiazolyldiphenyl-tetrazolium bromide (MTT) or the release of lactate dehydrogenase (LDH).

MTT was prepared at 5 mg/ml in PBS, diluted to a final concentration of 0.5 mg/ml for assays and incubated for 3 to 4 hours at 37°C. Formazan crystals were dissolved overnight (16 hours) at 37°C with 100 μl of lysis buffer [15% (w/v) SDS, 50% (v/v) dimethylformamide, pH 4.7]. Survival was presented as percentage of MTT reduction level of treated cells to control cells. Absorbance was read at 570 nm with the microplate reader SpectraMax 250 (Molecular Devices) and SoftMax Pro 3.0 software (Molecular Devices).

Cell survival with the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega) quantitatively measures the release of lactate dehydrogenase (LDH) upon cell lysis. Released LDH in culture supernatants is measured using a coupled enzymatic assay, resulting in the reduction of a tetrazolium salt (INT) into a red formazan product. The absorbance was read at 490 nm with the microplate reader SpectraMax 250 (Molecular Devices) and SoftMax Pro 3.0 software (Molecular Devices). Survival presented as percentage of untreated control.

2.7 *In vitro* Superoxide Assays

Superoxide levels were estimated by oxidation of the fluorescent dye DHE (Invitrogen). Cells were cultured on slides, treated, and washed twice with PBS

before incubation with DHE (10 mM; in 0.1% dimethyl sulfoxide) for 30 min. Total cell fluorescence was quantified with ImageJ [National Institutes of Health (NIH)]. Corrected fluorescence was calculated with the following equation: integrated density × (area of selected cell × mean fluorescence of background readings).

Mitochondrial superoxide levels were selectively measured with the MitoSOX^{TM} Red flourogenic dye (Molecular Probes). MitoSOX™ Red reagent is live-cell permeant and is rapidly and selectively targeted to the mitochondria. Once in the mitochondria, MitoSOX™ Red reagent is oxidized by superoxide and exhibits red fluorescence. MitoSOX™ Red reagent is readily oxidized by superoxide but not by other ROS, or reactive nitrogen species (RNS), generating systems and oxidation of the probe is prevented by superoxide dismutase. The oxidation product becomes highly fluorescent upon binding to nucleic acids. The experimental design was modified based on the manufacturer's protocol with the following variations: we prepared a 2.5 μM working reagent solution in PBS/Ca/Mg (Cellgro) buffer and incubated for 10 min at 37°C after applying 1 ml for cells grown in 6-well plates (for imaging) or 100 μL per well of a 96-well plate (for quantification). Microscopic imaging was performed with the Nikon Eclipse TE300 system, following the manufacturer's protocol with the excitation and emission at 510 nm and 580 nm, respectively. The 96-well plate quantifications were performed using a fluorescence microplate reader (Labsystems Fluoroskan Ascent FL) with Ex/Em at 510 nm and 580 nm, respectively. Fold-increase presented normalized to untreated control (2 g/L glucose, 10% FBS).

2.8 **Comet Assay Protocol**

Cells were diluted to 10^5/ml in culture medium (DMEM with 10% FBS) and treated with 50 mM DXR for 1 hour at 37°C. Cells were then washed once with ice-cold PBS and subjected to CometAssay (Trevigen) according to the manufacturer's recommended procedure. Comet images were acquired with a Nikon Eclipse TE300 fluorescence microscope and analyzed with the Comet Score software (TriTek Corp., version 1.5). Cells (100 to 300) were scored for each genotype per treatment group.

2.9 Immunoblotting Assay

Cells were rinsed once in ice-cold PBS and harvested in radio-immuno-precipitation assay (RIPA) lysis buffer containing protease inhibitors (Roche) and a cocktail of phosphatase inhibitors (Sigma). Tumor tissues were homogenized in RIPA lysis buffer supplemented with the same protease and phosphatase inhibitors. Proteins from total lysates were resolved by 8 to 12% SDS–polyacrylamide gel electrophoresis and analyzed by immunoblotting with antibodies for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Akt and phospho-Ser473 Akt, p70 S6K and phospho-Thr389 p70 S6K, and eIF2a and phospho-Ser51 eIF2a (1:1000 to 1:2000, Cell Signaling Technology).

2.10 Measurements of Mitochondrial Bioenergetic Function using the XF-24 Extracellular Flux Analyzer

The XF-24 Extracellular Flux Analyzer (Seahorse Biosciences) was used to examine the effects of *ad lib* (2 g/L glucose, 10% FBS) and fasting-mimicking (0.5 g/L glucose, 1% FBS) cell culture conditions on glycolysis and mitochondrial function. Briefly, the mitochondrial function assay is based on inhibitors of the electron transport chain and uncoupling agents to identify alterations in the respiratory function (Yadava and Nicholls 2007). Cells were seeded into special 24-well microtiter plates (Seahorse Biosciences) at 20,000 cells/well and incubated for 2 days in DMEM (4.5 g/L glucose and 10% FBS) and washed with PBS prior to treatments. Cells were then incubated in glucose free DMEM (Invitrogen) supplemented with either 10% or 1% FBS and glucose (0.5 g/L, for starvation-mimicking condition or 2.0 g/L, for *ad lib* condition) for 48 hours. Four basal oxygen consumption rates (OCR) are measured every 8 minutes to obtain baseline OCRs, followed by the measurement of OCR after the sequential injection of 1 μM oligomycin, 1 μM carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) and 0.75 μM rotenone. Glycolysis was assayed through the addition of 75 μM 2,4-dinitrophenol (DNP), fatty acid oxidation was evaluated by adding 200 μM palmitate to the wells. The optimal drug concentration for 4T1 cells was measured prior to the experiments. Owing to the effects of starvation on reduced proliferation and cell death in cancer cells, total cellular protein was measured following each experiment and used to normalize the mitochondrial function rates. To allow comparison

between experiments, data are expressed as the rate of oxygen consumption in pmol/min or the extra-cellular acidification rate (ECAR) in mpH/min. In some experiments, the data are expressed as a percentage of the basal O_2 consumption rate.

2.11 Microarray analysis

Microarray analysis was performed at the NIA/NIH by Alejandro Martin-Montalvo. In brief, RNA from mouse tissues (heart, liver and lung) was isolated according to the procedures described by the manufacturer with the RNeasy kit (Qiagen). Then, RNA was hybridized to BD-202-0202 chips from Illumina Beadchips. Raw data were subjected to Z normalization as described (Cheadle, Vawter et al. 2003). Briefly, for each pathway under each pair of conditions, a Z score was computed as [Z (pathway)= (sm mu)*pow(m,0.5)/delta], where mu= mean Z score of all gene symbols on the microarray, delta = SD of Z scores of all gene symbols on the microarray, sm= mean Z score of gene symbols comprising one pathway present on the microarray, and m= number of gene symbols in a pathway present on the microarray. For each Z (pathway), a P value was also computed in JMP 6.0 to test for the significance of the Z score obtained. These tools are part of DIANE 1.0 (NIH). Parameterized significant analysis is finished according to the SAM protocol (Tusher, Tibshirani et al. 2001) with analysis of variance (ANOVA) filtering (ANOVA $P < 0.05$). Significant genes are selected for each pair-wise comparison. Gene set enrichment was tested with the PAGE method as previously described (Kim and Volsky 2005). Figures were selected on the basis of the names and descriptions provided by Gene Ontology Database and Pathway Data Set (Subramanian, Tamayo et al. 2005). Further gene regulatory relation and canonic pathway analysis is done by the Ingenuity Pathway Analysis System (Ingenuity Systems). All raw data are available in the Gene Expression Omnibus database.

2.12 Modulation of HO-1 Expression

To modulate HO-1 activity, 4T1 cells were treated with 10 μM hemin (Sigma) or 20 μM zinc protoporphyrin (ZnPP; Sigma) for 24 hours prior to and 24 hours during chemotherapy treatment.

The rHO-1 construct, a kind gift from Dr. Claude A. Piantadosi of Duke University, was prepared consisting of pcDNA 3.1/V5-His-rHO-1 containing the entire

protein-coding region of the rat HO-1 gene. 4T1 cells were transfected using the XtremeGENE HP DNA transfection reagent according to the manufacturer's protocol (Roche Applied Science). Selection for successful transfection was carried out with G418 (Genecitin, Sigma). Cells grown from a single clone with the highest HO-1 expression (based on qRT-PCR) were used for experiments. 4T1-HO1 cells were maintained in DMEM (4.5 g/L, 10% FBS with 100 µg/ml G418). 4T1 cells transformed with the empty vector were established as controls following the same protocol.

2.13 **Real time-PCR**

RNA from tissues was isolated according to the procedures described by the manufacturer using the RNeasy kit (Qiagen). cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (AB Applied Biosystems) and RT-PCR was performed using the SYBR Green PCR master mix (AB Applied Biosystems).

The primer sequences for HO-1 are:

forward 5' TGCTCGAATGAACACTCTGG 3'

reverse 5' TCCTCTGTCAGCATCACCTG 3'

The primer sequences for NFkB (p50) are:

forward 5' GGAGGCATGTTCGGTAGTGG 3'

reverse 5' CCCTGCGTTGGATTTCGTG 3'.

Each analyzed treatment was performed with three biological replicates and at least three reactions were used to calculate the expression. The expression ratio was calculated according to the $2^{-\Delta\Delta CP}$ method (Pfaffl 2001).

2.14 **Mouse Cancer Models**

All animal experiments were performed according to procedures approved by USC's Institutional Animal Care and Use Committee (IACUC), the licensing and ethical committee of the National Cancer Research Institute, Genoa, Italy, and the Italian Ministry of Health. All mice were maintained in a pathogen-free environment throughout the experiments.

To establish a subcutaneous cancer mouse model, we injected 12 week old female BALB/c, 12 week old female and male C57BL/6 mice, and 7 week old nude

mice with 4T1 breast cancer cells, B16 melanoma, and GL26 glioma cells, respectively. 5 to 7 week old nude mice were injected with ACN human neuroblastoma cells, MDA-MB-231 human breast cancer cells, or OVCAR3 human ovarian carcinoma cells.

For metastatic mouse models of cancer, 12 week old female BALB/c and 12 week old female and male C57BL/6 mice were injected intravenously via the lateral tail veins with 2×10^5 4T1 or B16 cells, respectively, and 6 week old female A/J mice were injected via lateral tail veins with 2×10^5 NXS2 and 1×10^6 Neuro-2a cells. Before injection, cells in log phase of growth were harvested and suspended in PBS at 2×10^6 cells/ml, and 100 µl (2×10^5 cells per mouse) was injected subcutaneously in the lower back or intra-venously via the lateral tail veins. ACN and Neuro-2a cells were suspended in PBS at a density of 5×10^7 and 1×10^7 cells/ml, and 100 µl (5×10^6 ACN cells per mouse and 1×10^6 Neuro-2a cells per mouse) was injected subcutaneously in the lower back or intra-venously via the lateral tail veins, respectively.

For the intracranial glioma model, mice were fixed in a stereotactic frame, a paramedian incision was made and a 1.5 mm bur hole was drilled in the right frontal lobe of the skull (1 mm anterior and 3 mm lateral relative to the bregma). Using a Hamilton syringe, 1×10^4 GL26luc cells in 5 µL PBS were implanted 5 mm deep into the brain of each mouse. GL26luc cells are genetically engineered to express the firefly luciferase gene. Two animals received a mock surgery without the implantation of tumor cells. The skin incision was then closed with silk thread 3/0. To evaluate tumor progression, luciferin (50 mg/kg body weight) was administered via intra-peritoneal injections and animals were subjected to Bioluminescence Imaging (BLI) with the Xenon IVIS200 system at the USC Small Animal Imaging Center.

Tumor inoculation, drug treatment, radiotherapy and tumor measurements were all performed under inhalant anesthesia, utilizing 2% Isoflurane. All mice were shaved before subcutaneous tumor injection and were gently warmed before intra-venous injections to dilate the veins. Body weights were determined periodically, and tumor size was measured with a digital vernier caliper. The subcutaneous tumor volume was calculated with the following equation: tumor volume (mm^3) = Product (length × width × height)/2, where the length, width, and height are in millimeters.

Animals with a tumor volume bigger than 2500mm^3 and animals showing signs of severe stress and/or deteriorating health status were designated as moribund and euthanized.

2.15 *In vivo* Fasting

For short-term starvation (STS), mice were single caged and maintained in standard shoebox-cages without access to food for 48 to 60 hours. To reduce cannibalism and to avoid coprophragy or feeding on residual chow, cages were changed immediately before STS was initiated. Animals had access to water at all times. Body weight of each individual animal was measured routinely during the STS regime to ensure controlled loss of body weight to no less than 20%; according to the guidelines of our IACUC protocols.

2.16 Cancer Treatments

DXR (Bedford Laboratories), CP (Sigma and Baxter), cisplatin (Teva Parenteral Medicines Inc.) and Temozolomide (Schering) were used. For *in vivo* studies, DXR and TMZ were injected intra-venously via lateral tail veins, cisplatin and CP was injected intra-peritoneally.

Whole body irradiation was performed with Cesium 137 as the radiation source, at a dose of 5 Gy for the first treatment and 2.5 Gy for the second treatment, with or without 48 hour starvation. Mice were monitored daily. Animals showing signs of severe stress, deteriorating health status or excess tumor load (s.c, 2500 mm^3) were designated as moribund and euthanized. The data presented in the graphs showing moribund animals is based on these exclusion criteria.

2.17 *In vivo* Modulation of Heme Oxygenase-1

Hemin (Sigma) was dissolved in 0.7 N ammonium hydroxide (NH_4OH) at 50 mg/ml to obtain a stock solution. This stock solution was dissolved 1:10 in saline and prepared fresh prior to injections. Hemin was delivered by intra-peritoneal injection every 12 hours for a total of 6 injections, at a dose of 15 mg/kg each injection. Control animals were injected with hemin-free dissolvent.

Zinc protoporphyrin (ZnPP, Sigma) was prepared as a 100 mg/ml stock solution dissolved in dimethyl sulfoxide (DMSO). Prior to injections, the stock solution was dissolved 1:10 in DMSO. ZnPP was delivered by intra-peritoneal

injection every 12 hours for a total of 6 injections, at a dose of 20 mg/kg each injection. Control animals were injected with ZnPP-free DMSO.

2.18 Blood Collection for Glucose and IGF-I Measurements

Mice were anesthetized with 2% inhalant isoflurane and blood was collected by left ventricular cardiac puncture. Blood was collected in tubes coated with K^2-EDTA (BD) to process serum. Blood glucose was measured with the Precision Xtra blood glucose monitoring system (Abbott Laboratories) by clipping the tail. IGF-I was measured using a mouse specific ELISA kit following the manufacturer's protocol (R&D Systems).

2.19 Macronutrient Defined Diets

AIN93G standard chow (Harlan) was used as the reference diet and supplied to all mice if not indicated otherwise. Diets modified in the macronutrient composition (fat, protein and carbohydrates) were all based on the AIN93G diet (**Table 2-1**). Diets 20% P-1 (soybean oil as fat source) and 20% P-2 (coconut oil as fat source) had calories from protein sources reduced to 20% compared to the AIN93G formulation; the 0% P diet contained no protein; all these diets were isocaloric to the AIN93G standard chow (**Table 2-2**). The low carbohydrate LCHP diet had calories from carbohydrates reduced to 20% compared to the AIN93G formulation (13% vs. 63.9%) but contained more protein (45.2%) and fat (41.8%). The high fat diet 60% HF was designed to supply 60% of the consumed calories from fat sources, the calories coming from protein and carbohydrates were reduced proportionally. The 90% HF diet is a ketogenic diet which contains 90% of fat with minimal carbohydrates (less than 1%) and half of the protein content (9%). Detailed diet composition and calorie content are summarized in **Table 2-2**. Mice were fed with the AIN93G control diet before the beginning of the experiments and based on their initial bodyweight grouped into the experimental groups (N=5/ group). Mice were acclimated to the test diets one week prior to the experiments. All diets were supplied *ad lib* unless indicated otherwise.

Table 2-1. Detailed composition of macronutrient defined diets.

Modified from Brandhorst et al.; *under review*.

g/kg diet	AIN93G	20% P-1	20% P-2	0% P	LCHP	60% HF	90% HF
Corn Starch	397.486	487.222	487.222	511.636	85.406	179.986	
Dextrinized cornstarch	132.000	161.964	161.964	170.000	28.380	170.000	
Sucrose	100.000	122.700	122.700	128.700	21.500	66.200	
Casein	200.000	40.000	40.000		566.000	133.200	180
Fat	70.000	87.500	87.500	92.050	198.100	350.000	709.37
Cellulose	50.000	50.000	50.000	50.000	50.000	50.000	60
Mineral (AIN-93G-MX)	35.000	35.000	35.000	35.000	35.000	35.000	35.000
Vitamin (AIN-93-VX)	10.000	10.000	10.000	10.000	10.000	10.000	10.000
L-Cystine	3.000	3.000	3.000		3.000	3.000	3
Choline bitartrate	2.500	2.500	2.500	2.500	2.500	2.500	2.500
tert-Butylhydroquinone	0.014	0.014	0.014	0.014	0.014	0.014	0.130
Calcium-Phosphate	0.000	0.000	0.000		0.000	0.000	0.000
Food Color		0.1	0.1	0.1	0.1	0.1	
Total (g)	**1000.00**	**1000.00**	**1000.00**	**1000.000**	**1000.00**	**1000.00**	**1000.000**

Table 2-2. Overview about the macronutrients and calories contained in the experimental diets.

Modified from Brandhorst et al.; *under review*.

	% Calories of Diet from:			
Diet	**Protein**	**Carbohydrates**	**Fat**	**kcal/g**
AIN93G	18.8	63.9	17.2	**3.8**
[a] 20% P-1	3.9	75.7	20.4	**3.9**
[b] 20% P-2	3.9	75.7	20.4	**3.9**
0% P	0	78.8	21.2	**3.9**
LCHP	45.2	13	41.8	**4.4**
60% HF	9	30.9	60	**5.3**
90% HF	9	0.99	90.1	**7.1**

[a] Soybean oil as fat source

[b] Coconut oil as fat source

2.20 Caloric Restriction

For caloric restriction using the AIN93G diet, the standard chow was grounded into a powder and mixed in hydrogel (Clear H_2O) in the necessary amounts to achieve 60%, 50%, 40%, 20%, 10% calorie density of AIN93G (**Table 2-3**). The caloric restricted macronutrient modified diets were prepared the same way (**Table 2-4**). To avoid malnutrition all diets were supplemented with vitamins, minerals, fiber and essential fatty acids matching those in AIN93G. Baseline food intake (3.7 g or 14 kcal/day) was determined with AIN93G feeding prior to the experiments (data not shown). For all CR experiments, mice were single caged in standard shoebox-cages which were refreshed daily to avoid coprophragy or feeding on residual chow. Animals had access to water at all times to ensure sufficient hydration. Bodyweight of each individual animal was measured routinely during the CR regimens.

Table 2-3. Composition of calorie restricted diets.

Modified from Brandhorst et al.; *under review*.

for total diet (kg) 0.2

	40% CR	50% CR	60% CR	80% CR	90%CR	STS
AIN-93G (g)	116.20	95.60	74.80	33.40	12.60	0.00
Essential FA (ml)	0.43	0.43	0.43	0.43	0.43	0.43
Fiber (g)	4.19	5.22	6.26	8.33	9.37	10.00
AIN-93G-MX (g)	2.93	3.65	4.38	5.83	6.56	7.00
AIN-93-VX (g)	0.84	1.04	1.25	1.67	1.87	2.00
Hydrogel (g)	75.44	94.08	112.91	150.37	169.20	180.60

Table 2-4. Composition of calorie restricted macronutrient defined diets.

Modified from Brandhorst et al.; *under review*.

50% CR diets based on:

	AIN93G	0% P	LPHC	60% HF	90% HF
kcal/g	***3.76***	***3.90***	***4.39***	***5.25***	***7.11***
Diet (g)	95.6	92.1	81.9	68.5	50.5
Essential FA (ml)	0.43	0.43	0.43	0.43	0.43
Fiber (g)	5.22	5.22	5.22	5.22	5.22
AIN-93G-MX (g)	3.65	3.65	3.65	3.65	3.65
AIN-93-VX (g)	1.04	1.04	1.04	1.04	1.04
Hydrogel (g)	94.1	97.6	107.8	121.2	139.1
Sum (g)	200	200	200	200	200

2.21 Resistance to High-Dose Chemotherapy

12 to 15 week old female CD-1 mice weighing 25 to 32 g were starved for up to 60 hours (STS) or fed with the macronutrient modified 50% CR diets for 3 days, followed by an intra-venous injection of 24 mg/kg Doxorubicin (DXR, Bedford Laboratories). In all experiments mice were offered AIN93G standard chow after chemo-drug injection and monitored daily. Animals showing signs of severe stress and/or deteriorating health status were designated as moribund and euthanized.

2.22 Statistical Analysis

Statistical analysis was performed using GraphPad Prism v.5. Survival curve comparison was performed with the Log-Rank test. All statistical analyses were two-sided and *P* values <0.05 were considered significant.

3 Results

A prolonged 20 to 40% reduction in calorie intake or dietary restriction (DR) protects a wide variety of organisms against oxidative stress and aging (Weindruch, Walford et al. 1986; Longo and Finch 2003; Longo and Fontana 2010). By contrast, a limited exposure to a severely restricted diet (short-term starvation or fasting) can protect yeast, mammalian cells, mice, and possibly even patients from the toxic effects of oxidative and chemotherapeutic agents without the chronic weight loss generally associated with prolonged CR (Longo, Ellerby et al. 1997; Raffaghello, Lee et al. 2008; Safdie, Dorff et al. 2009; Lee, Safdie et al. 2010; Lee, Raffaghello et al. 2012). We proposed that the fasting mediated protection of normal cells is based on the reallocation of energy towards cellular maintenance pathways from reproduction and growth processes, when nutrients are scarce or absent (Kirkwood 2005). This switch to a protected mode occurs only in normal cells, not cancer cells, because oncogenes prevent the activation of stress resistance pathways in malignant cells. This feature of cancer cells thus provides a way to enhance cancer treatment by selectively increasing protection of normal cells [differential stress resistance (DSR), **Fig. 3-1**] rather than by the more typical strategy of increasing the toxicity of drugs to cancer cells (Raffaghello, Lee et al. 2008; Safdie, Dorff et al. 2009; Lee, Safdie et al. 2010). DSR in mice and mammalian cells is mediated in part by the reduction of extra-cellular glucose and IGF-I concentration/signaling. Potentially harnessing DSR for clinical cancer therapy is attractive because fasting for 2 to 3 days before and 24 hours after chemotherapy is well tolerated by cancer patients receiving a variety of toxic treatments and might even reduce the common side effects caused by chemotherapy (Safdie, Dorff et al. 2009). Further, in mouse models, fasting protects against ischemia-reperfusion injury (Mitchell, Verweij et al. 2009), and deprivation of a single amino acid results in both lower IGF-I levels and protection against renal and hepatic ischemic injury (Peng, Robertson et al. 2011).

The therapeutic potential of fasting would be even greater if it also increased the death of cancer cells. We tested this possibility in collaboration with researchers at the NIA (Baltimore, USA) and the Giannina Gaslini Institute (Genova, Italy) by studying the effect of fasting on cancer cell survival in the presence or absence of chemotherapeutic agents (Lee, Raffaghello et al. 2012; Safdie, Brandhorst et al. 2012).

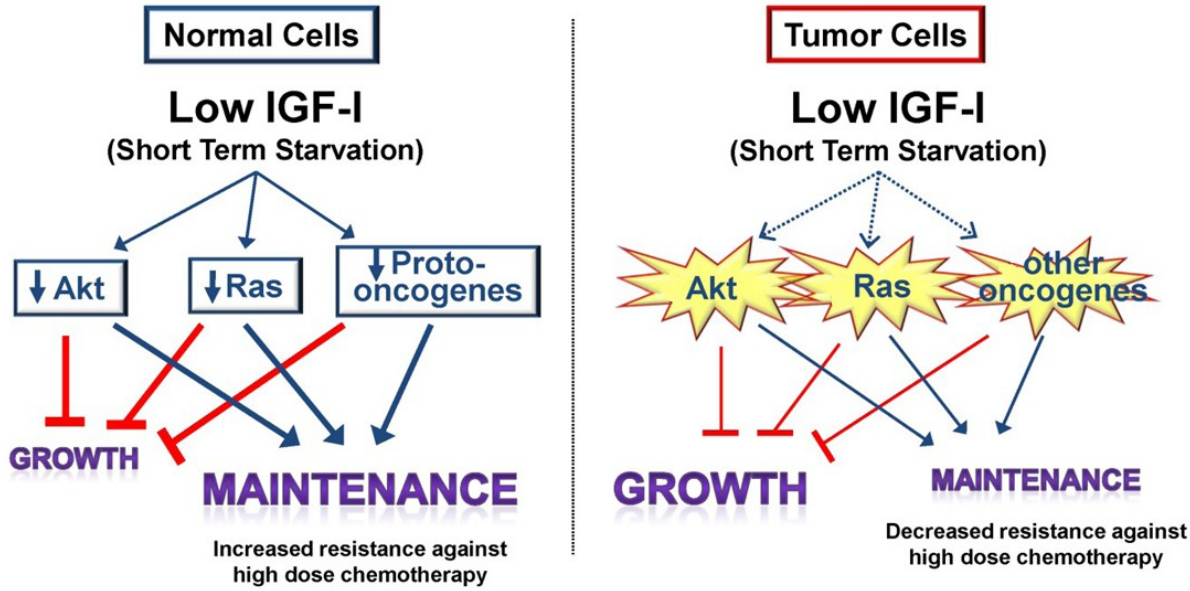

Figure 3-1. Fasting provides a differential stress resistance to chemotherapy.

The investment of the finite energy available in a cell or an organism is efficiently balanced between growth/ reproduction and maintenance/repair. However, challenging conditions, such as fasting and its consequent reduction of IGF-I, withdraws energy from growth/reproduction and reinvests it in maintenance/repair, thereby increasing cellular protection. This switch in cellular metabolic policy is mediated by negatively regulating mitotic pathways such as those major effectors downstream of IGF-I (PI3K/Akt and Ras/ERK). By contrast, oncogenic mutations, which often regulate cellular metabolism and growth, render tumor cells less responsive to fasting because of their independence from external cues. Therefore, cancer cells fail to, or only partially respond to fasting, and continue to promote growth, leaving them vulnerable to chemotherapy drugs. Modified from (Lee and Longo 2011)

3.1 Starvation Sensitizes Yeast and Cancer Cells to Toxins

We have previously shown that, unlike the wild-type DBY746 cells, yeast cells expressing an oncogene-like constitutive active form of Ras ($RAS2^{val19}$) are not protected from oxidative and chemotherapeutic agents by prior starvation (Raffaghello, Lee et al. 2008; Lee, Safdie et al. 2010). We repeated some of the DSR based experiments in yeast (Fabrizio and Longo 2003; Fabrizio, Hoon et al. 2010), by switching normal yeast cells from the standard glucose medium to water for 24 hours, followed by the exposure to various toxic agents and stressors. This starvation-mimicking incubation in water was used as a treatment to increase their

ability to survive in response to heat shock or oxidative stress, induced by hydrogen peroxide or menadione, both of which mimic the oxidation-dependent cytotoxicity of many chemotherapy drugs (**Fig. 3-2**) (Raffaghello, Lee et al. 2008; Lee, Safdie et al. 2010). In contrast, the water-based starvation sensitized yeast cells carrying the $RAS2^{val19}$ mutation and rendered them less able to withstand heat shock or oxidative stress (**Fig. 3-2**). This data suggests that, in contrast to the protection afforded to normal cells, starvation increases the susceptibility of yeast cells expressing an oncogene-like protein to stress.

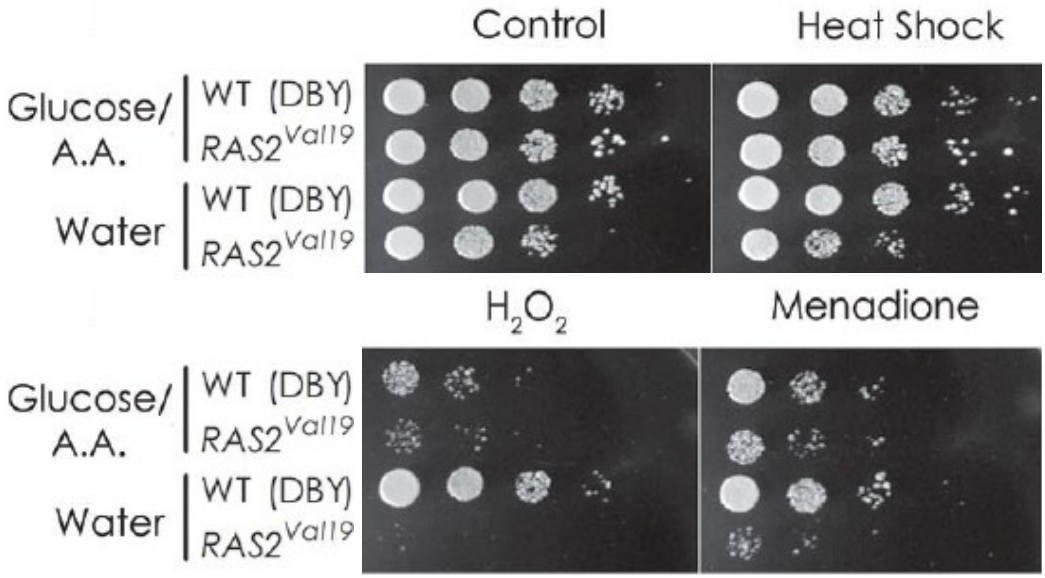

Figure 3-2. Effect of 24 hours of starvation prior to stress treatment in yeast.

Effects of 24 hours of starvation prior to treatment on the survival of wild type (DBY746) and yeast cells expressing constitutively active Ras (*RAS2*^{Val19}). Starvation was modeled by culturing non-dividing yeast cells in water for 24 hours. Ten-fold serial dilutions of cells (from left to right) were spotted on culture plates and incubated at 30°C for 2-3 days. For heat shock resistance, cells were incubated at 55°C for 40 min. For oxidative stress resistance assays, cells were diluted to an OD_{600} in K-phosphate buffer (pH 6) and treated with 100-200 mM of hydrogen peroxide (H_2O_2) for 60 minutes, or cells were treated with 250 mM of menadione for 30 min in K-phosphate buffer (pH 7.4). Collaborative data, modified from (Lee, Raffaghello et al. 2012).

To test whether this sensitization by short-term starvation may also occur in mammalian tumor cells, we incubated murine breast cancer cells (4T1) in cell culture medium containing serum collected from mice that were either fed *ad lib* or fasted for 48 hours. 4T1 cells cultured in the medium supplemented with serum from fasted mice were more susceptible to the chemotherapeutic drugs doxorubicin (DXR) and cyclophosphamide (CP) compared to cells incubated in serum from mice fed *ad lib* (**Fig. 3-3**) and thus extended our fasting-based sensitization approach from yeast into mammalian cells. Based on our previous results that demonstrated that fasting greatly reduces the concentration of serum glucose and growth factors ((Lee, Safdie et al. 2010), see p.74 for reference), we incubated cells in concentrations of glucose mimicking those of normally fed and fasted mice, supplemented with either 10% or 1% serum, respectively, to also model the reduction in IGF-I and other growth factors caused by fasting. Restricting the availability of glucose and growth factors in this way greatly retarded the proliferation of murine 4T1 breast cancer, B16 melanoma and GL26 glioblastoma cell lines by roughly 50%, 35% and 75%, respectively (**Fig. 3-4**).

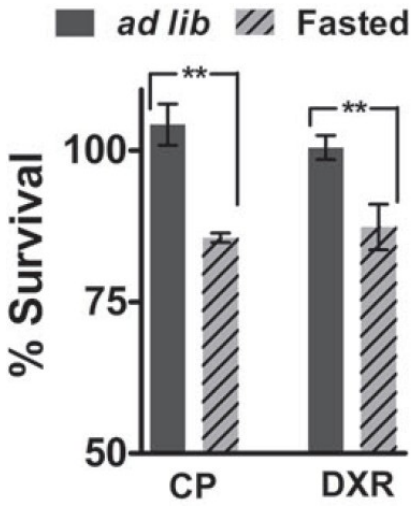

Figure 3-3. Effect of serum from fasted and *ad lib* fed mice on survival of DXR- and CP-treated breast cancer cells.

Murine breast cancer cells (4T1) were cultured in DMEM media supplemented with serum from mice that were either fed *ad lib* or fasted for 48 hours prior to serum collection and treated with 8 mM cyclophosphamide (CP) or doxorubicin (DXR). Survival presented as percentage of MTT reduction of treated cells to untreated control cells. All data presented as mean ± SEM; ** $p < 0.01$, Student's t-test, two-tailed. Collaborative data, modified from (Lee, Raffaghello et al. 2012).

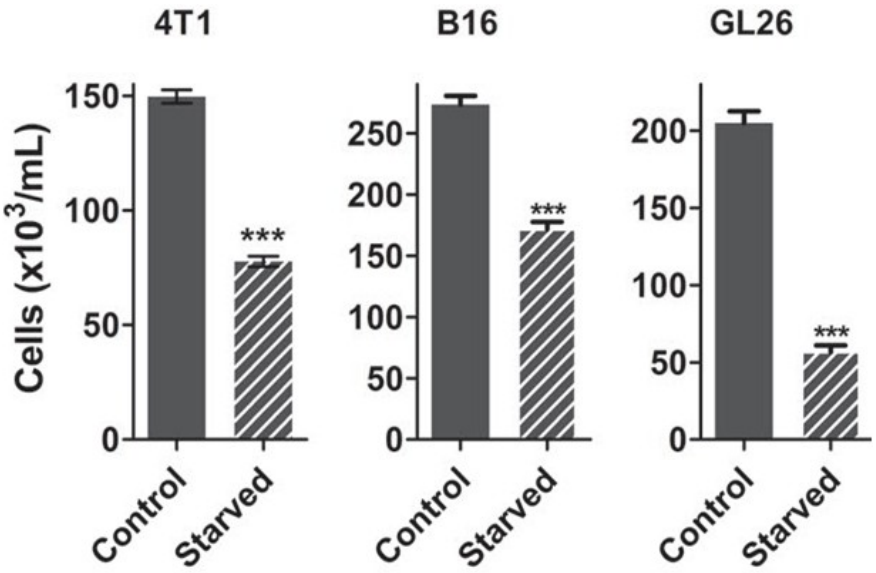

Figure 3-4. Effect of starvation on cellular proliferation *in vitro.*

Murine breast cancer (4T1), melanoma (B16) and glioblastoma (GL26) cell lines were seeded 50,000 cells/well and cultured either in DMEM media supplemented with 2 g/L glucose and 10% FBS (control, mimicking *ad lib* conditions) or 0.5 g/L and 1% FBS (starved, mimicking fasting-like conditions) immediately upon attachment. 48 hours later, cell number was assessed by trypan blue exclusion. All data presented as mean ± SEM; *** $p < 0.001$, Student's t-test, two-tailed. Collaborative data, modified from (Lee, Raffaghello et al. 2012).

Applying this combination of glucose and serum restriction for 24 hours before and during a 24 hour chemotherapy drug treatment in culture, sensitized 15 of 17 cancer cell lines to DXR and/or CP (**Fig. 3-5**, and data not shown). We tested a wide variety of murine cancer (4T1 breast cancer, GL26 glioma, B16 melanoma, NXS2 and Neuro-2a neuroblastoma) and human cancer (U87-MG glioblastoma, PC3 and 22Rv1 prostate cancer, MCF-7 and C42B breast cancer, LOVO human colon cancer, HeLa cervical cancer, ACN and SH-SY5Y neuroblastoma, OVCAR3 ovarian carcinoma, MZ2-MEL melanoma, A431 epidermoid carcinoma and MDA-MB-231 breast cancer) cell lines. Of note is that not all cancer cell lines were sensitized when treated with DXR (e.g. LOVO), although this adverse effect was abolished when CP was used to treat the cells.

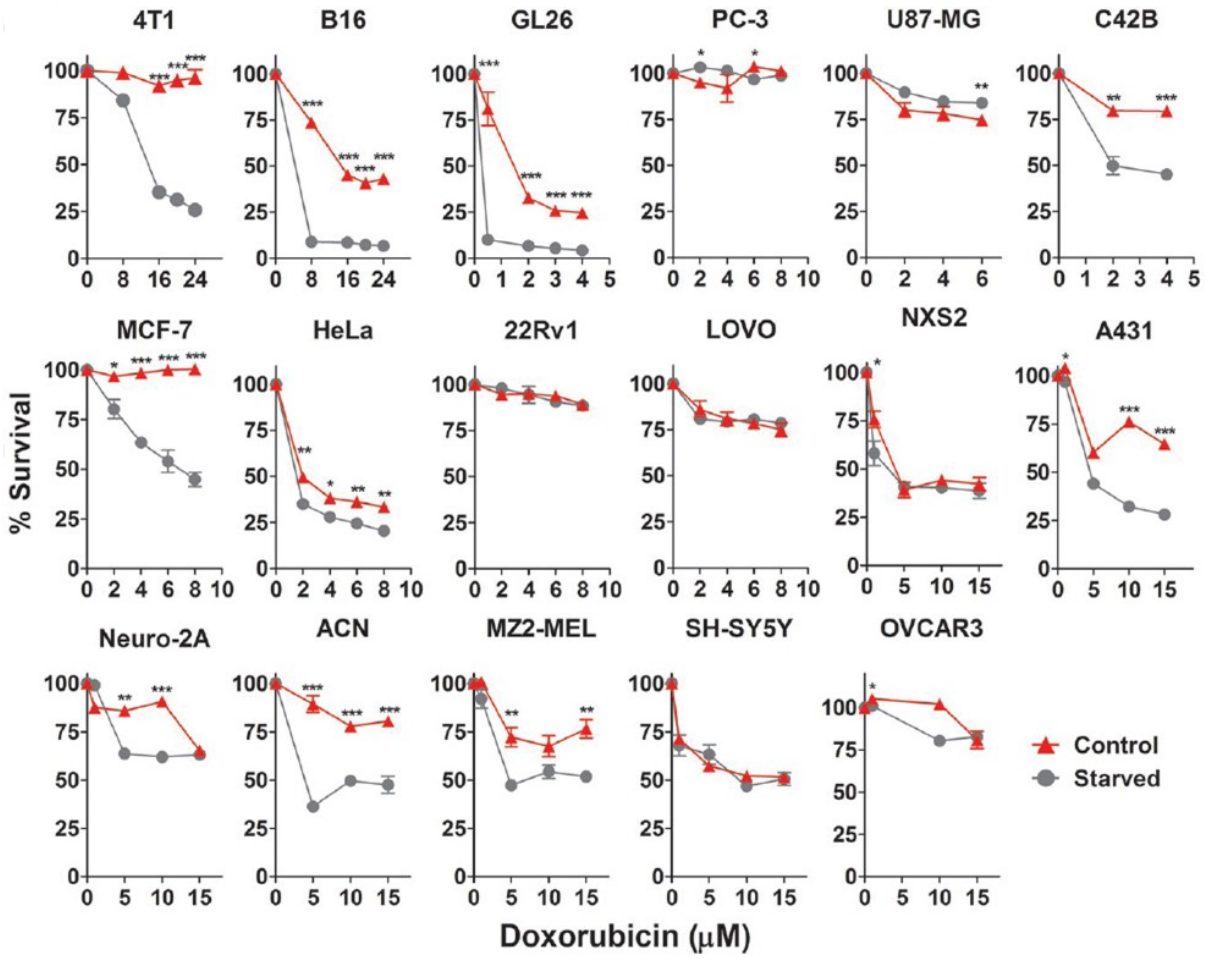

Figure 3-5. Effect of starvation on DXR sensitivity of 17 different cancer cells *in vitro.*

Cells were exposed to starvation-like conditions 24 hours before and 24 hours during DXR treatment. Control groups (red triangle) were cultured in 1.0 g/L and 2.0 g/L glucose, for human and murine cells respectively, supplemented with 10% FBS. Starved cells (grey dots) were cultured in 0.5 g/L glucose supplemented with 1% FBS. Survival was determined by MTT-reduction for murine (4T1 breast cancer, GL26 glioma, B16 melanoma, NXS2 and Neuro-2a neuroblastoma) and human (U87-MG glioblastoma, PC3 and 22Rv1 prostate cancer, MCF-7 and C42B breast cancer, LOVO human colon cancer, HeLa cervical cancer, ACN and SH-SY5Y neuroblastoma, OVCAR3 ovarian carcinoma, MZ2-MEL melanoma, A431 epidermoid carcinoma and MDA-MB-231 breast cancer) cancer cell lines. Survival is presented as percentage of MTT reduction of treated cells to untreated control cells. All data presented as mean ± SEM; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, Student's t-test, two-tailed. Collaborative data, modified from (Lee, Raffaghello et al. 2012).

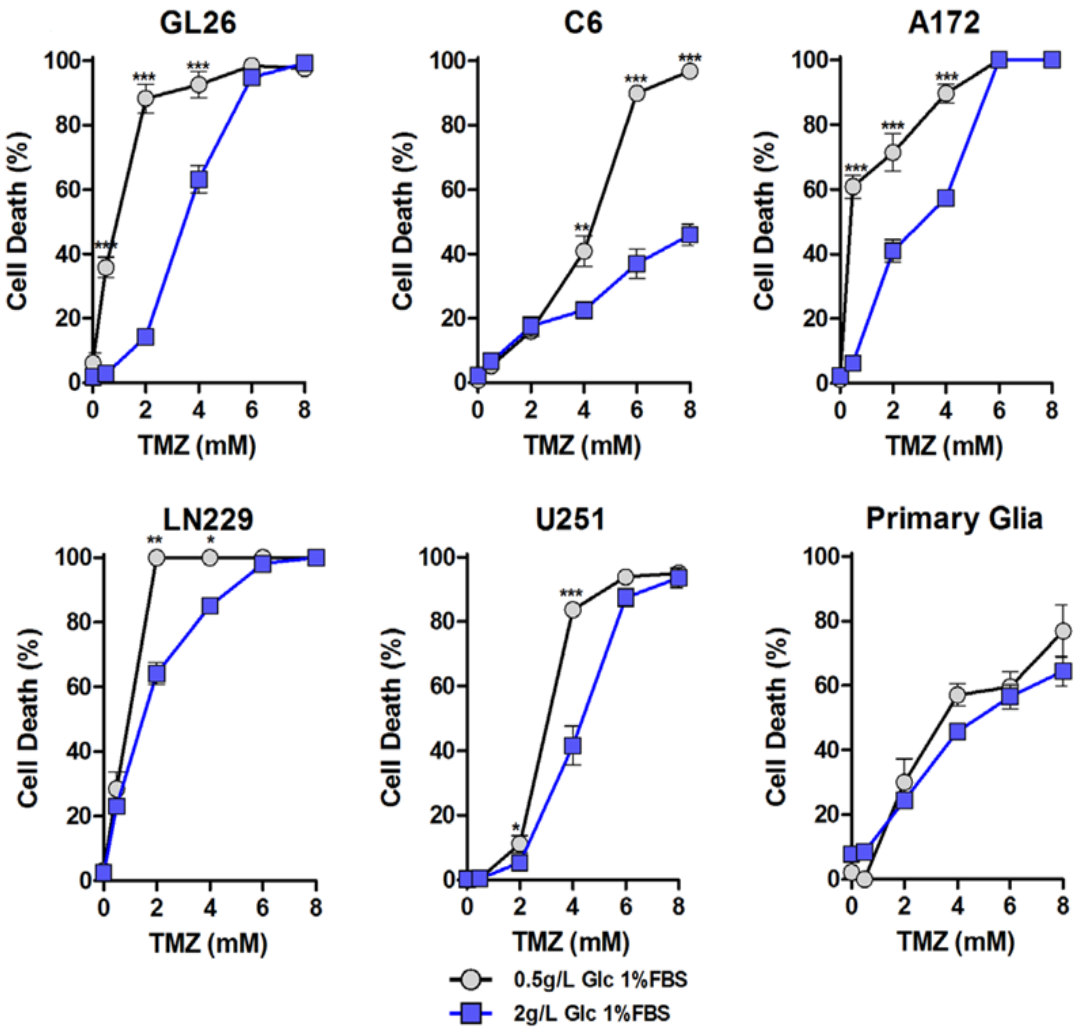

Figure 3-6. Glucose restriction sensitizes glioma cells to Temozolomide treatment *in vitro*.

Glioma cell lines (GL26, C6, LN229, A172 and U251) and murine primary mixed glial cells were tested for glucose-restriction induced sensitization to Temozolomide (TMZ). Cells were incubated in low glucose (0.5 g/L) or normal glucose (2.0 g/L) media, supplemented with 1% FBS for 24 hours. Low glucose modeling STS conditions sensitized murine GL26 glioma cells, rat C6 glioma cells and human A172, LN229, U251 glioma cells to TMZ *in vitro*. Murine primary glial cells were used to represent matching normal cells. Percent cell death was determined based on quantitative measurements of lactate dehydrogenase (LDH) release after 24 hour treatment with 0–8 mM TMZ. All data presented as mean ± SEM; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, Student's t-test, two-tailed. Collaborative data, modified from (Safdie, Brandhorst et al. 2012).

We also tested the effect of glucose restriction alone on cellular stress resistance to Temozolomide (TMZ), the standard chemotherapy drug for glioblastoma multiforme (GBM) treatment (Purow and Schiff 2009). Cells were incubated in either low glucose (0.5 g/L) or normal glucose (2.0 g/L) for 24 hours prior to and during drug treatment to model the serum glucose levels during short-term starvation (STS) and *ad libitum* feeding, respectively (**Fig. 3-6**). Murine GL26 cells, maintained in medium mimicking blood glucose levels of starved mice, displayed elevated cell death after TMZ treatment when compared to cells treated in normal conditions. A 3-fold higher TMZ dose (6 mM vs. 2 mM) was necessary to cause ~90% death in GL26 cells cultured in media containing 2.0 g/L glucose compared to 0.5 g/L glucose in the starvation-mimicking media. Similar effects were observed in the human glioblastoma cell lines LN229, A172, and to a lesser extent, U251 cells, suggesting that sensitization of cancer cells to the TMZ treatment by glucose restriction may apply to human glioma. In contrast to the malignant cells, TMZ toxicity to primary glial cells was not significantly affected by altering the glucose level. Our data suggest that glucose limitation sensitizes glioma cells, but not primary glial cells, to TMZ treatment *in vitro* (**Fig. 3-6**). These results indicate that short-term starvation can not only sensitize murine and human cancer cells, but further sensitize a broad range of different cancer types to chemotherapeutic agents.

Our previous studies suggested that reduced serum levels of IGF-I are important for the protective effects of fasting for normal cells and showed that IGF-I infusion can reverse the fasting-induced protection of mice from the side effects caused by high-dose chemotherapy (Lee, Safdie et al. 2010). To test the role of IGF-I in cancer cell sensitization, we incubated 4T1 cells with the antagonistic IGF-IR antibody (αIR3) for 24 hours prior to and during drug treatment in normal glucose conditions (**Fig. 3-7**). Normal glucose was used to eliminate the role of glucose deprivation on cancer cells as our previous experiment demonstrated that glucose-deprivation plays a partial role during the fasting mediated sensitization. High levels of FBS (e.g. 10%) interfered with the αIR3 treatment, an effect most likely due to competitive binding of IGF-I and the antibody at the receptor, and we therefore tested the antibody in media supplemented with 1% FBS. The addition of αIR3 sensitized murine breast cancer cells to treatment with 4 mM CP and the toxicity was significantly increased in a αIR3 dose dependent manner (**Fig. 3-7**).

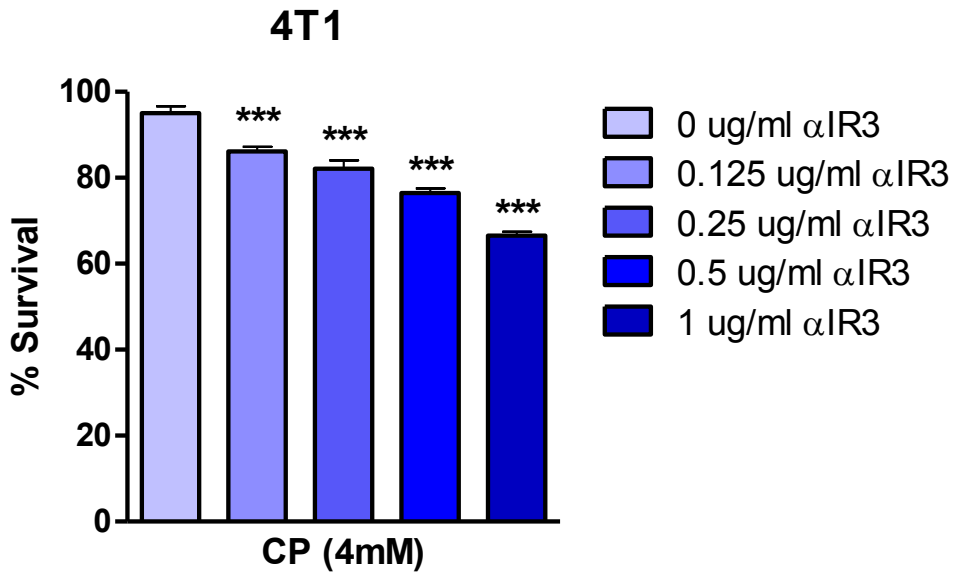

Figure 3-7. Effect of inhibiting IGF-I signaling in the starvation-dependent sensitization of murine breast cancer cells to CP.

4T1 breast cancer cells were incubated in DMEM (2.0 g/L glucose with 1% serum) and various doses of the neutralizing anti–IGF-IR monoclonal antibody α-IR3 for 24 hours prior to and during drug treatment with 4 mM cyclophosphamide (CP). Survival presented as percentage of MTT reduction of treated cells to untreated control cells. All data presented as mean ± SEM; *** p <0.001, ANOVA, Tukey's multiple comparison, compared to untreated control.

Next, we tested whether re-substituting IGF-I, which is well known to inhibit apoptosis, can reverse the effects of starvation on the sensitization of 4T1 and B16 cells. We found that adding 200 μM IGF-I to starvation-treated 4T1 and B16 cells (incubated in 0.5 g/L DMEM) reversed the sensitization (**Fig. 3-8**), suggesting that IGF-I can protect 4T1 breast cancer and B16 melanoma cells from chemotherapy. Thus, this data shows that both glucose and growth factor signaling, in particular the decrease in IGF-I levels caused by fasting, contribute to the sensitization of malignant cells to cytotoxic stress. *In vitro*, this fasting-mimicking model of glucose and growth factor deprivation causes the sensitization of tumor cells in strong similarity to the sensitization of oncogene expressing yeast cells. In contrast, we previously demonstrated that normal cells enter a protected mode when nutrients are scarce or absent. Fasting provides dual action by protecting normal cells and simultaneously sensitizing different cancer cell types of murine and human origin (Lee, Raffaghello et al. 2012).

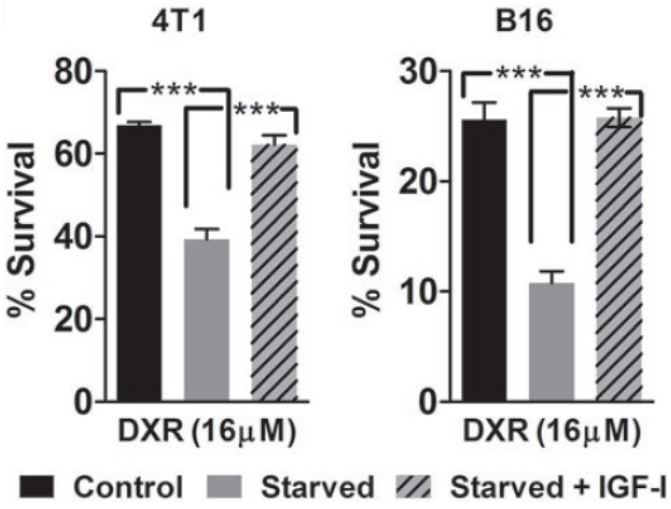

Figure 3-8. Effect of IGF-I on starvation-dependent sensitization of cancer cells to DXR.

4T1 breast cancer and B16 melanoma cell lines s were treated with rhIGF-I (200 μM) during glucose restriction (0.5 g/L vs. 2.0 g/L, under 1% FBS), followed by DXR (16 μM) treatment. All data presented as mean ± SEM; *** p <0.001, Student's t-test, two-tailed (N=3). Collaborative data, modified from (Lee, Raffaghello et al. 2012).

3.2 Short-Term Starvation improved Efficacy of Chemo- and Radiotherapy in Subcutaneous Tumor Models

To explore whether our *in vitro* results apply to mice, we studied subcutaneous allografts of murine glioma (GL26), breast cancer (4T1), melanoma (B16), metastatic neuroblastoma models (NXS2, Neuro-2a), and xenografts of human neuroblastoma (ACN), breast cancer (MDA-MB-231), and ovarian cancer (OVCAR3) cell lines. Fasting was achieved by complete food withdrawal for 48-60 hours while allowing mice access to water.

Malignant glioma, including anaplastic astrocytoma and glioblastoma multiforme (GBM), account for more than 50% of all primary brain tumors, with GBM being the most common malignant brain tumor in adults (Ohgaki and Kleihues 2005). This tumor is highly invasive and angiogenic (Purow and Schiff 2009), resulting in incurability and mortality rates higher than those for any other brain tumor, with the median survival for GBM being ~12 to 15 months (Stupp, Dietrich et al. 2002; Stupp, Mason et al. 2005). The multimodal treatment of GBM includes maximal surgical resection, followed by adjuvant radio- and chemotherapy (Zimmerman 1955; Gupta

and Sarin 2002; Fisher and Buffler 2005). Other than for the introduction of the chemotherapeutic drug Temozolomide (TMZ), the treatment protocol has generally not changed over the past decades. TMZ has a high tolerability and has been shown to prolong patient survival. In consequence, TMZ is currently the standard chemotherapy drug adopted for the treatment of high grade glioma of astroglial origin (Friedman, Kerby et al. 2000). We evaluated the effects of combining STS and TMZ treatments in a subcutaneous (s.c.) model of GL26 glioma (**Fig. 3-9**). GL26 tumors in the untreated control animals progressed rapidly, reaching the endpoint size of 2500 mm^3 within 22 days after tumor implantation. Two cycles of treatment with TMZ (30 mg/kg each cycle) alone led to a deceleration of tumor progression; however the majority of the tumor-bearing animals in the TMZ treated group reached the endpoint volume at day 22 and there was considerable variability in this experimental group. Notably, two cycles of STS for 48 hours, in the absence of chemotherapy, retarded the progression of the glioma as effectively as the TMZ treatment during days 12 to 20 and slowed tumor growth even further during and after the second STS cycle. The greatest effect in decreasing tumor progression was observed when starvation was combined with the TMZ treatment for two consecutive treatment cycles (**Fig. 3-9, A**).

Mice with tumor mass (s.c.) exceeding 2500 mm^3 or showing signs of severe stress and deteriorating health (regardless of tumor load) were described as moribund. Starvation alone did not cause obvious signs of discomfort, but rather improved the animal condition as indicated by body weight and behavior (data not shown). In the STS+TMZ group, 85% of the mice appeared healthy with the tumor-size below the tolerated endpoint volume by day 28. This indicates that the combination of both treatments was well tolerated and improved tumor-bearing survival (**Fig. 3-9, B**). In both the STS and TMZ groups, 40% of the animals survived until day 28 after tumor implantation. Notably, animals in the STS group had to be sacrificed solely based on tumor-size during days 23 to 28 while animals in the TMZ group showed an earlier onset of morbidity starting at day 17, in part caused by symptoms of chemo-toxicity, e.g. reduced food intake, loss of body weight and hypo-activity. On the other hand, survival rates were dramatically lower in the control group (8.3%). 50% of the untreated glioma-bearing mice reached the maximum tumor load by day 20; by day 22 all but one mouse in this group had to be sacrificed.

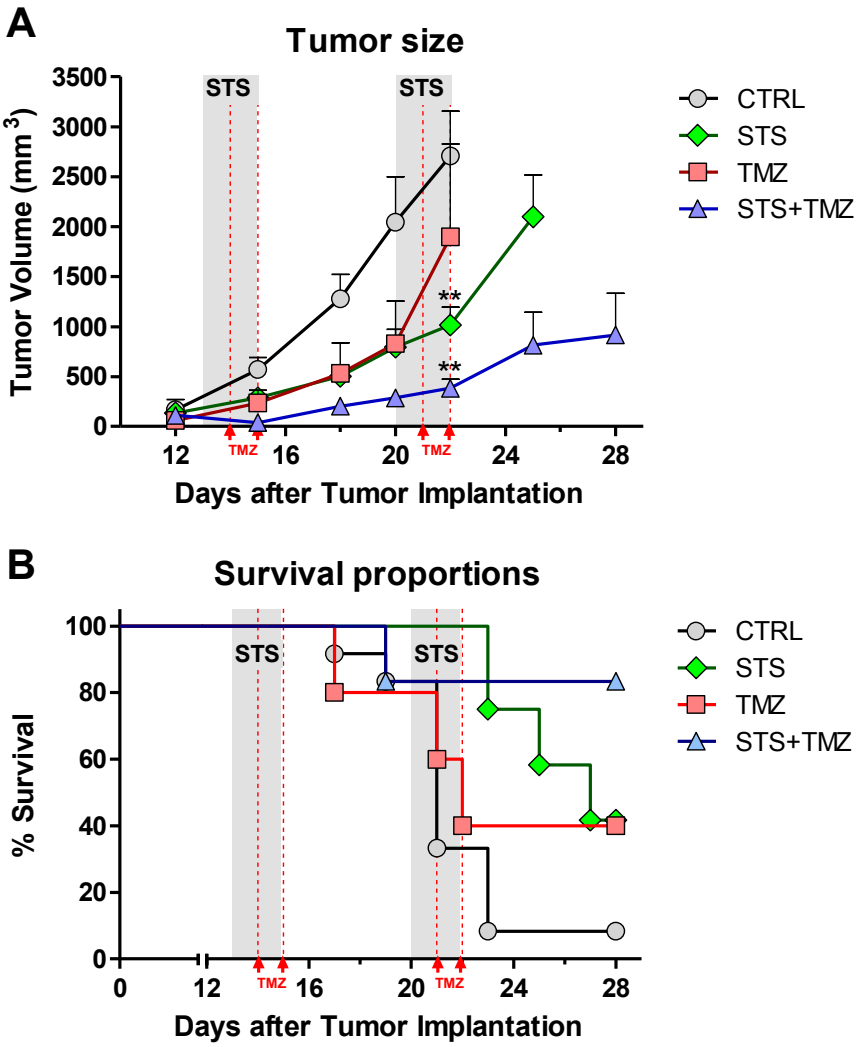

Figure 3-9. Enhanced chemotherapy efficacy by fasting in a murine GL26 glioma model extends onset of morbidity in tumor-bearing animals.

(**A**) Subcutaneous tumor progression of murine GL26 glioma is shown as total tumor volume in mm^3. Tumor measurement was started once the tumor became palpable under the skin at day 12. Control animals (N= 12) received no treatment and the tumor progressed rapidly. STS (N= 12) and STS+TMZ group (N= 6) were deprived of food for two 48 hour cycles (day 13-15; day 20-22, grey area). TMZ animals (N= 5) received 15 mg/kg/day of TMZ (red lines), totaling 30 mg/kg for each treatment cycle. STS+TMZ animals were injected at 24 hours and 48 hours of fasting with 15 mg/kg TMZ per injection. Animals from STS and STS+TMZ groups showed reduced tumor progression and could therefore be monitored for a prolonged period compared to animals from the control and TMZ group. All data presented as mean ± SEM; ** $p<0.01$, ANOVA, Tukey's multiple comparison, compared to control. (**B**) Morbidity of animals inoculated subcutaneous with GL26 glioma. Animals were euthanized once tumor volume exceeded 2500 mm^3 or based on overall appearance and health status. Curve comparison with Log-Rank test (Mantel-Cox, * $p<0.05$). Collaborative data, modified from (Safdie, Brandhorst et al. 2012).

Surgical resection followed by radiotherapy is another standard care for glioma patients. As STS delayed tumor progression and improved survival when combined with chemotherapy, we wanted to determine whether fasting could also be beneficial in combination with radiotherapy. Radiotherapy (RTP) delivered in two cycles of 5 Gy and 2.5 Gy effectively retarded tumor growth when compared to the untreated control group (**Fig. 3-10, A)**. Notably, up to day 22 the tumor progression for the RTP and STS groups was comparable. Once the second cycle of fasting was stopped, a more rapid growth in the STS group was observed compared to that in the RTP group. The most striking effect was noticed in mice receiving two cycles of fasting in combination with RTP, showing a significant reduction in tumor volume when compared to the control, STS and RTP groups (**Fig. 3-10, A)**.

As for the TMZ experiments, animals were considered moribund and were sacrificed once the tumor volume reached 2500 mm^3 or animals showed signs of a deteriorating health status (**Fig. 3-10, B)**. Two cycles of radiotherapy alone were capable of significantly prolonging survival compared to the untreated control group and ~40% of the animals in this group survived until day 31, emphasizing the well-established benefits of radiotherapy for the treatment of GBM. As discussed in the TMZ experiment, animals in the STS group displayed an enhanced survival when compared to the untreated control animals. Despite the fact that the tumor volume in the STS group progressed shortly after the 2^{nd} fasting cycle, animals in this group benefited with delayed tumor progression and showed extended survival. These data suggest that STS augments the efficacy of TMZ and RTP in treating aggressive GBM (**Fig. 3-10, B).**

To the best of our knowledge, we demonstrate for the first time that short-term starvation enhances the efficacy of radiotherapy for the treatment of gliomas (Safdie, Brandhorst et al. 2012). Considering the poor prognosis for patients diagnosed with GBM, the median survival being ~12 to 15 months (Stupp, Dietrich et al. 2002; Stupp, Mason et al. 2005), our results presented here may provide additional means to improve survival after diagnosis.

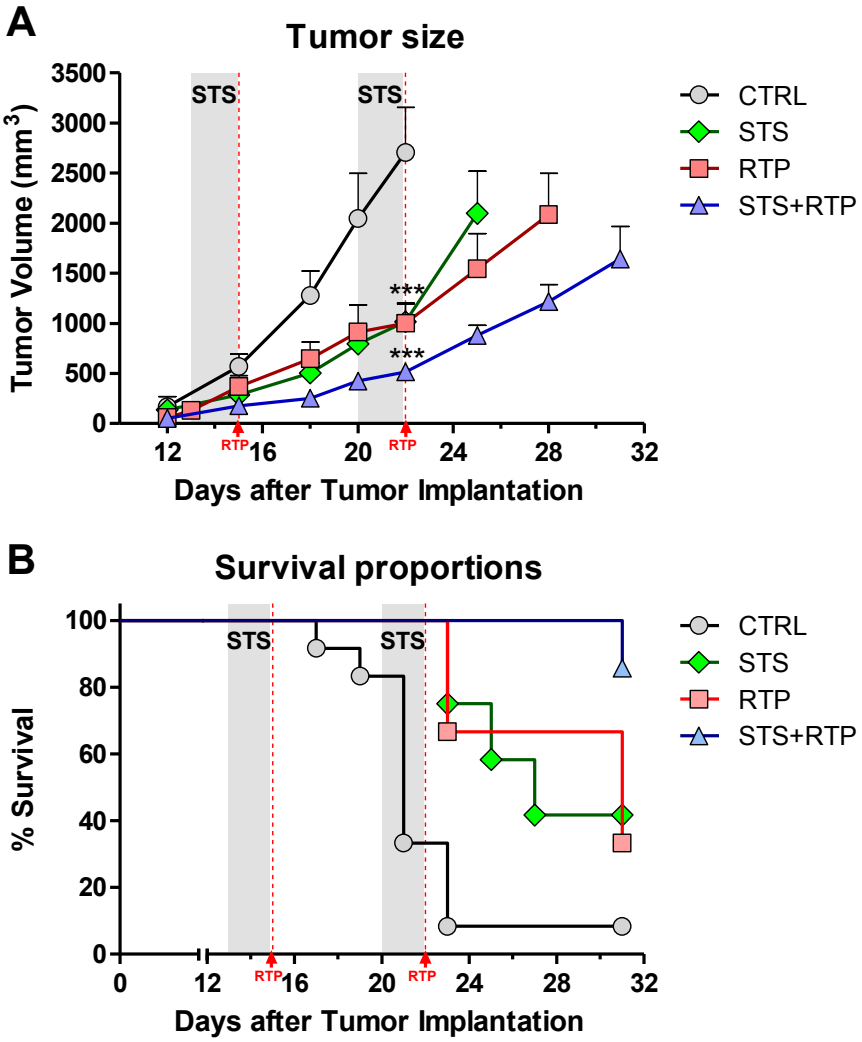

Figure 3-10. Enhanced radiotherapy (RTP) efficacy by fasting for GL26 glioma.

(**A**) Subcutaneous tumor progression of murine GL26 glioma is shown by total tumor volume in mm^3. Tumor measurement was started once the tumor became palpable under the skin at day 12. Control animals (N= 12) received no treatment and tumor progressed rapidly. STS (N = 12) and STS+RTP group were deprived of food for two 48 hour cycles (day 13 to day 15 and day 20 to day 22, grey area). RTP (N= 9) and STS+RTP animals (N= 9) were treated with 5 Gy at day 15 and 2.5 Gy at day 22, totaling 7.5 Gy for the combined radiotherapy treatment; 2^{nd} dose was lowered to 2.5 Gy to reduce radiotoxicity. Animals from STS, RTP and STS+RTP groups showed reduced tumor progression and could therefore be monitored for a prolonged period compared to animals from the control group. All data presented as mean ± SEM; *** $p<0.001$, ANOVA, Tukey's multiple comparison, compared to control at day 22. (**B**) Morbidity of animals inoculated subcutaneously with GL26 glioma and treated with radiotherapy, two 48 hour fasting cycles or combination of both (STS+RTP). Animals were considered moribund once tumor volume exceeded 2500 mm^3 or based on overall appearance and health status. Curve comparison with Log-Rank test (Mantel-Cox; ** $p<0.01$). Collaborative data, modified from (Safdie, Brandhorst et al. 2012).

In mice bearing subcutaneous breast cancer (4T1), two cycles of fasting alone (48 hours each) were as effective as two cycles of CP treatment (**Fig. 3-11, A**), yet the mice were able to regain normal weight within 5 days of re-feeding (**Fig. 3-11, B**). Similar effects were observed in mice bearing subcutaneous melanoma masses (B16 cells) treated with DXR (**Fig. 3-11, C, D**) and also in mice bearing subcutaneous glioma masses (GL26 cells) treated with DXR (**Fig. 3-11, E, F**), although the growth of melanoma cells was not affected by the second cycle of fasting, indicating that cancer cells may in some cases acquire resistance to fasting. The greatest therapeutic effect was observed when fasting was combined with chemotherapy drugs (DXR, 10 mg/kg or CP, 150 mg/kg) (**Fig. 3-11, A, C, E**). For 4T1 breast cancer, two fasting cycles combined with CP maintained tumors size at less than half of that in the CP treatment alone group, even 20 days after the last treatment (**Fig. 3-11, A**). Similar effects were observed in subcutaneous melanoma and glioma models (**Fig. 3-11, C, E**). Pre-fasted mice receiving chemotherapy returned to their normal weight soon after the last fasting cycle (**Fig. 3-11, B, D, F**). The growth of subcutaneous human breast cancer (MDA-MB-231) and ovarian cancer (OVCAR3) xenografts was also retarded by fasting but returned to a size similar to that in animals on the control diet after refeeding (**Fig. 3-11, G, H**). This post-fasting cancer growth may be the result of binge-feeding and weight gain by pre-fasted mice given an *ad lib* diet. However, when fasting was combined with DXR, the progression of the human breast cancer tumors was dramatically retarded and the tumor did not progress (**Fig. 3-11, G, H**).

To test the effect of fasting alone in a model of human cancer, we applied cycles of fasting in immune-compromised nude mice that were subcutaneously injected with human ACN neuroblastoma cells. After 34 days and 5 fasting cycles, the tumor size was less than half of that in normally fed mice (**Fig. 3-12, A**). In agreement with the other tumor mouse models, animals were able to return to the normal weight after each cycle (**Fig. 3-12, B**). These results suggest that cycles of fasting can delay the growth of some cancer cell types, in some cases as effectively as chemotherapy drugs, but that the combination of fasting and chemotherapy cycles provides a more effective, consistent and potent toxic effect on a wide range of tumors.

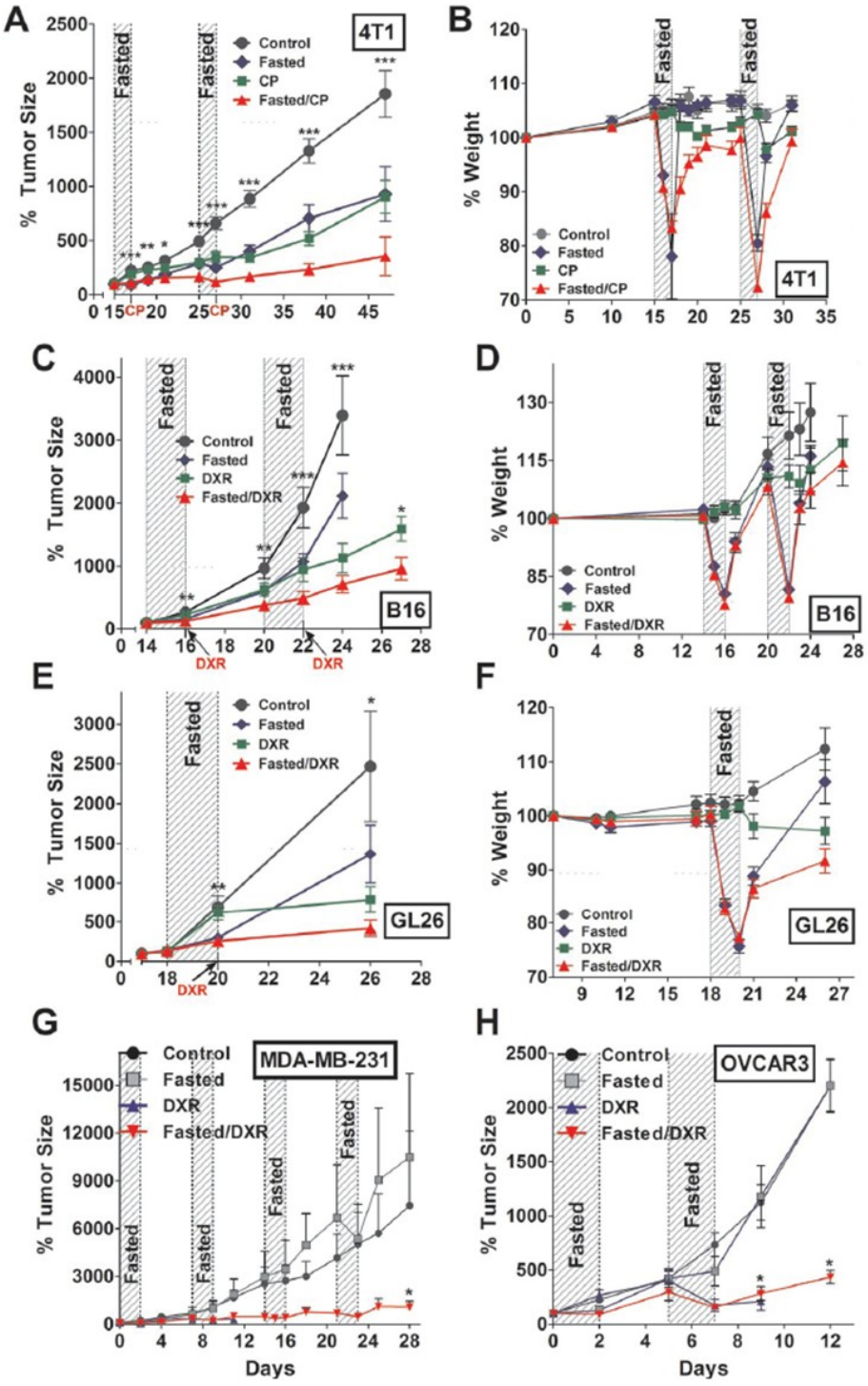

Figure 3-11. The sensitivity of allografted and xenografted tumors to STS and chemotherapeutic agents in mice.

(**A, C, E, G, H**) Tumor progression as percent of initial size; (**B, D, F**) body weight. (**A, B:** 4T1; N=12), (**C, D:** B16; N=11) melanoma, and (**E, F:** GL26; N=8) glioma. Fasting in the glioma model was applied only once due to the rapid tumor growth in the control (*ad lib*, no chemotherapy) group. (**G**) Human breast cancer cells (MDA-MB-231) were subcutaneously xenografted into nude mice. 4 cycles of fasting (48 hours) and/or DXR were performed. Tumor measurements from mice that were fed

ad lib and treated with DXR were terminated at day 11 due to death of all mice from DXR toxicity (N=5). (**H**) Human ovarian cancer cells (OVCAR3) were subcutaneously xenografted into nude mice. 2 cycles of fasting (48 hours) and/or DXR were performed. Tumor measurements from mice that were fed *ad lib* and treated with DXR were terminated at day 9 due to death of all mice from DXR toxicity (N=5). In both xenograft models, fasted mice treated with DXR did not experience death from chemotherapy toxicity. All data presented as mean ± SEM; * p <0.05; ** p <0.01; *** p <0.001, Student's t-test, two-tailed. Collaborative data, modified from (Lee, Raffaghello et al. 2012).

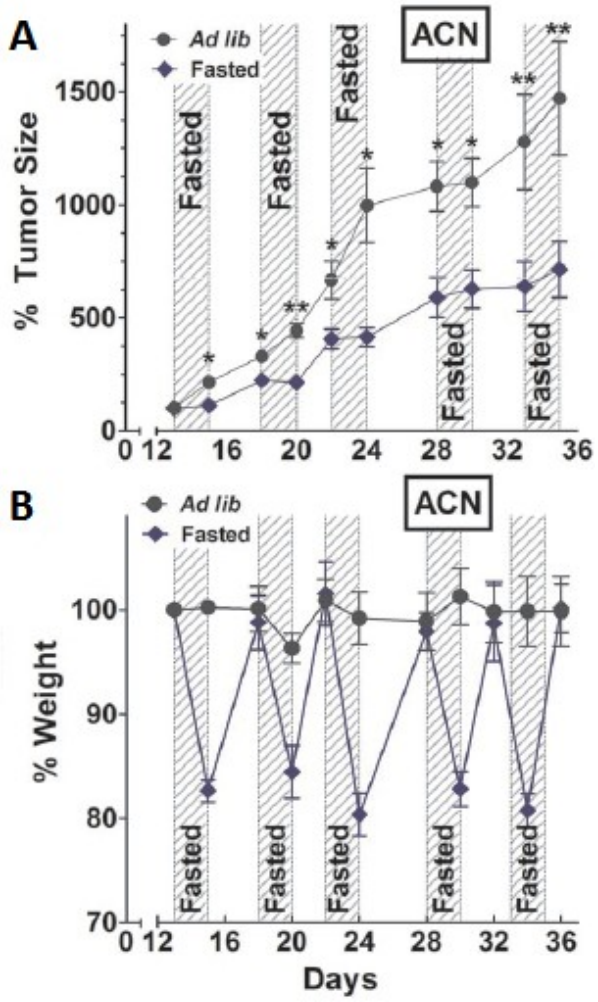

Figure 3-12. Effect of STS on tumor progression of a human neuroblastoma xenograft.

(**A**) Effect of fasting alone (48 hours for 5 cycles) in nude mice on tumor progression of a xenografted human neuroblastoma cell line (ACN; N=7) and on (**B**) body weight. All data presented as mean ± SEM; * p <0.05; ** p <0.01; *** p <0.001, Student's t-test, two-tailed. Collaborative data, modified from (Lee, Raffaghello et al. 2012).

3.3 Short-Term Starvation improves the Efficacy of Chemotherapy in an Intracranial GL26 Tumor Model

In the United States, malignant glioma account for approximately 70% of the 22,500 new cases of malignant primary brain tumors that are diagnosed in adults each year, with a 5 year survival rate that is less than 3% (Stupp, Mason et al. 2005; Wen and Kesari 2008). Due to the high mortality rates and low median survival in brain tumor patients, we were interested in determining if the effect of fasting on an intracranial GBM tumor model would be as effective as that seen in the s.c. model (see p. 103, **Fig. 3-9, A**). GL26 cells, engineered to express the firefly luciferase gene (GL26luc), were stereotactically implanted into C57BL/6 mice in the right frontal lobe. This allows us to monitor tumor progression by live bioluminescence imaging. Within six days of tumor implantation, the luciferase expression by tumor cells could be measured in all mice by bioluminescence imaging (BLI). Based on the bioluminescence intensity, mice were randomized into four experimental groups to achieve an even distribution (**Fig. 3-13**, Day 6).

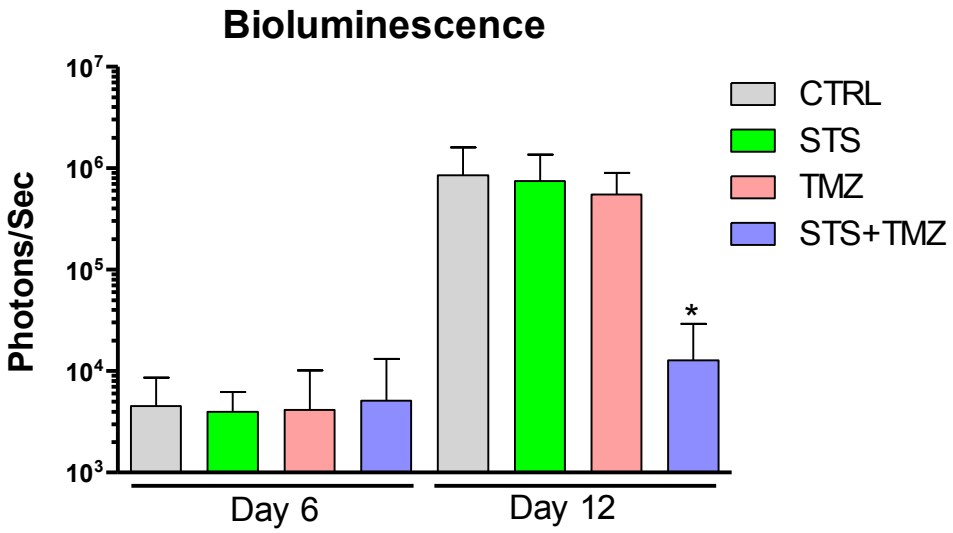

Figure 3-13. Bioluminescence expression in the intracranial GL26 tumor model.

Bioluminescence expression at day 6 *vs.* day 12 after the tumor inoculation. Animals at day 6 were randomized into the four experimental groups (Control, N= 6; TMZ, N= 5; STS, N= 11; or STS+TMZ, N= 12) and treatment was initiated. Tumor progression was monitored at day 12 to determine treatment benefits. Bioluminescence signaling measured as photons/sec. All data presented as mean ± SEM; * $p<0.05$, ANOVA, Tukey's multiple comparison, compared to control at day 12. Collaborative data, modified from (Safdie, Brandhorst et al. 2012).

Temozolomide treatment in human patients is performed as 5 consecutive daily injections per treatment cycle (Villano, Seery et al. 2009). To accommodate the 48 hour STS regimen, TMZ was delivered by two injections of 15 mg/kg into the lateral tail-vein after 24 hours and 48 hours of starvation. On day 12 after the tumor implantation, the tumor progression in all experimental groups was assessed by BLI (**Fig. 3-14**). Bioluminescence in the control group increased within six days from 4500 to 850.000 photons/sec; an almost 200-fold increase (**Fig. 3-13**, Day 12). The STS and TMZ groups displayed a similar trend of increase in bioluminescence at day 12 after the tumor implantation. However, one cycle of the combination of starvation and TMZ treatment reduced the bioluminescence signal of the implanted GL26luc cells significantly ($p < 0.05$, compared to control at day 12) (**Fig. 3-14**).

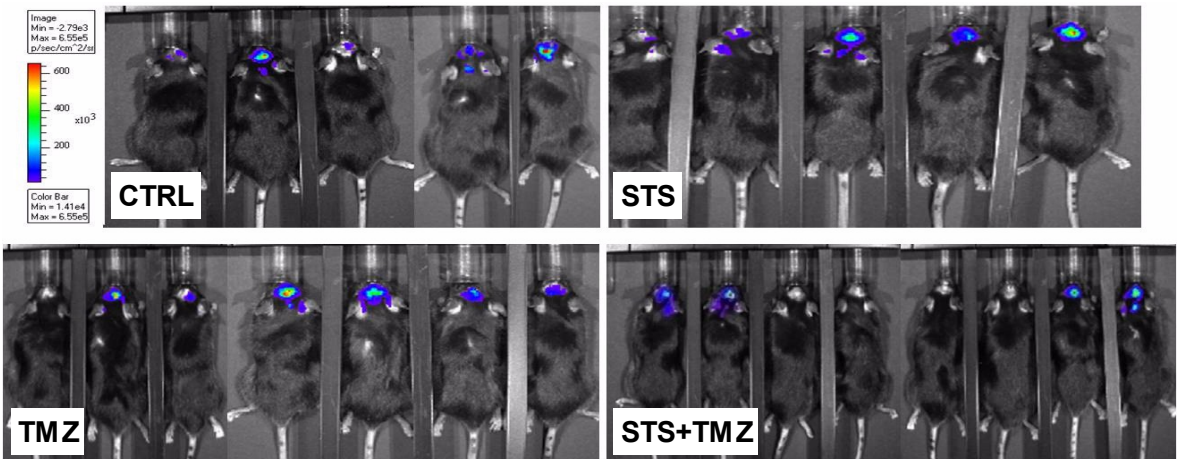

Figure 3-14. Bioluminescence expression in the intracranial GL26luc tumor model at day 12.

Bioluminescence imaging of GL26luc glioma bearing C57BL/6 mice at day 12 after intracranial tumor implantation. Animals are shown according to experimental group. Collaborative data, modified from (Safdie, Brandhorst et al. 2012).

The clinical condition and general health aspect of the mice declined rapidly, likely because of the increase in intracranial pressure caused by tumor proliferation. Amongst the most common manifestations of declining health was continuous weight loss in all groups, notable 6 to 8 days after the tumor implantation (**Fig. 3-15**). Despite the weight loss during the starvation cycle, mice in the STS group regained the bodyweight to that of control animals within one to two days after resuming feeding. Furthermore, we also observed improved activity, exploring of the cage and better grooming in STS+TMZ treated mice when compared to animals from all other groups. These results are consistent with our previous observation that animals treated with chemotherapy after fasting display a better health status compared to their *ad lib* fed counterparts in chemotherapy-treated groups (Raffaghello, Lee et al. 2008).

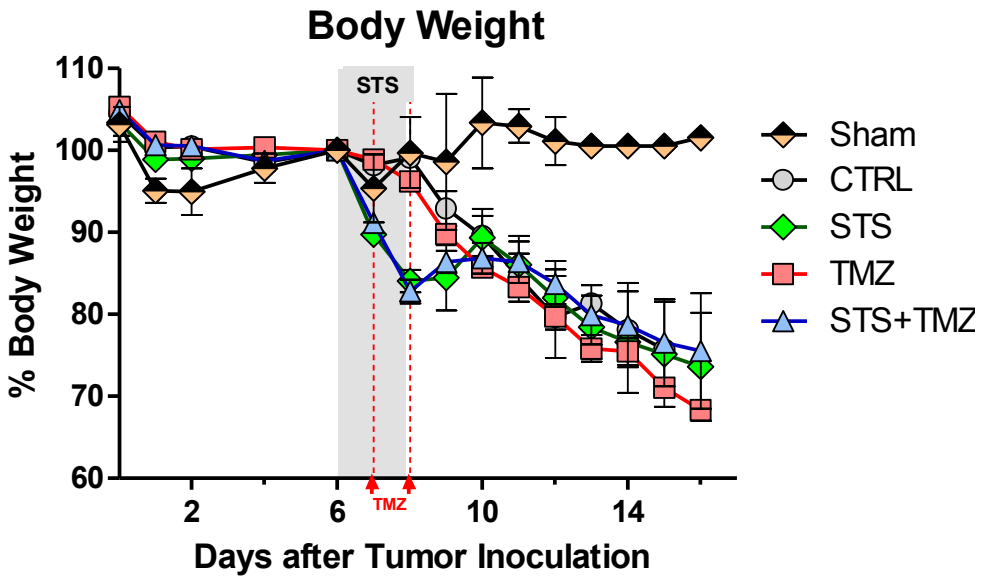

Figure 3-15. Body weight profile for mice inoculated with intracranial GL26luc glioma.

Body weight profile for glioma-bearing animals receiving one cycle of either short-term starvation (STS), TMZ treatment or both. STS and STS+TMZ animals initially reduce body weight during STS cycles (grey area) but regain the weight of untreated control and TMZ-treated animals within 1 to 2 days after re-feeding. TMZ and STS+TMZ animals received i.v. injections of 15 mg/kg Temozolomide on day 7 and day 8 (red lines), totaling 30 mg/kg for the treatment cycle. Tumor progression causes rapid weight loss following treatment in all experimental groups while saline injected sham animals (no tumor) recovered within one to three days from the inoculation procedure. Collaborative data, modified from (Safdie, Brandhorst et al. 2012).

Mice that were starved prior to the administration of TMZ had a significant survival advantage ($p < 0.05$) over the TMZ, STS and control groups in the intracranial GL26 tumor model (**Fig. 3-16**). Animals in the control, STS and TMZ groups showed an onset of morbidity 13 days after tumor implantation. The onset of morbidity caused by tumor burden was delayed by one day in the STS+TMZ group and survival was slightly prolonged in this group; consistent with the reduced bioluminescence signal at day 12. Remarkably, when considering the fast progression of this tumor model, one mouse from the STS+TMZ group was alive for more than 80 days after tumor implantation. These results suggest that fasting in combination with TMZ treatment can decelerate tumor progression in the mouse model and may potentially lead to long-term survival in a small sub-population.

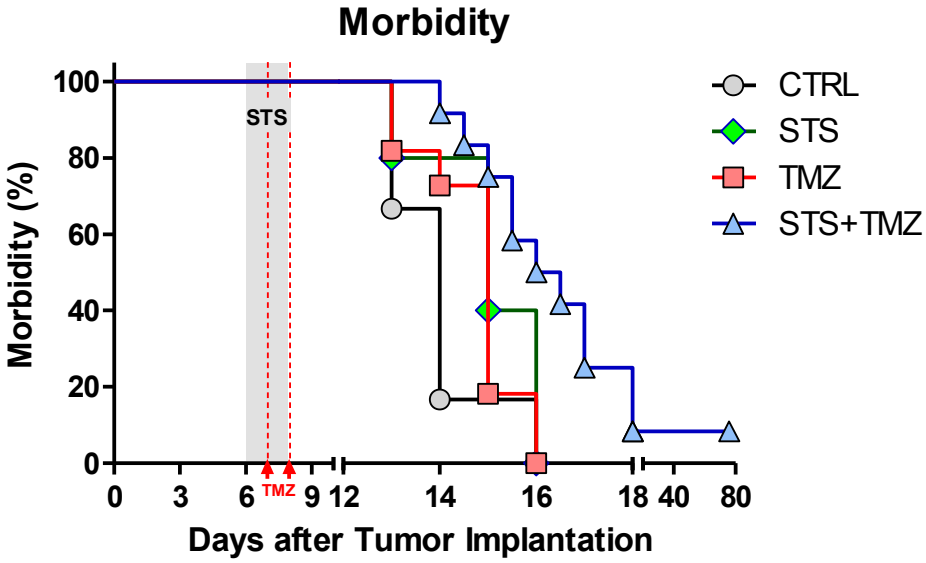

Figure 3-16. Morbidity rate comparison of animals intracranially inoculated with GL26luc glioma cells.

STS and STS+TMZ animals were fasted for 48 hours starting at day 6 (grey area). TMZ and STS+TMZ animals received i.v. injections of 15 mg/kg Temozolomide at day 7 and day 8 (red lines), totaling 30 mg/kg/cycle. Curve comparison with Log-Rank test (Mantel-Cox; * $p<0.05$). Collaborative data, modified from (Safdie, Brandhorst et al. 2012).

To mimic oncologic treatment conditions in human patients, we attempted to extend survival by applying multiple cycles of chemotherapy treatment. As early as 4 days after implantation of the tumor, mice received two injections of TMZ (total 30 mg/kg, TMZ group) or were fasted for 48 hours (STS group). Mice in the STS+TMZ group received the combination of both treatments (**Fig. 3-17, A**). Before starting a second treatment cycle, we allowed animals from both STS treated groups to regain body weight to the values of non-starved groups (**Fig. 3-17, B**). By this time most animals had lost around 15% of bodyweight and appeared weak, making a shorter starvation period (24 hours) and a single dose of 15 mg/kg of TMZ at day 10 the only additional intervention possible. Two cycles of treatment (STS or TMZ) delayed the mortality of the tumor-bearing animals in comparison to the control group (**Fig. 3-17, A**), which showed a survival rate akin to that observed in our previous intracranial experiment (**Fig. 3-16**). Animals that received multiple doses of TMZ showed delayed onset of mortality compared to those in the control and STS groups (day 14 *vs.* day 12 and day 8, respectively). Furthermore, the onset of morbidity was delayed in comparison to the animals that received a single dose of TMZ in the previous experiment (**Fig. 3-16**, day 13). The additional 2nd cycle of STS and TMZ further delayed morbidity in the experimental group receiving the combined treatments. Of note is however, that one mouse died relatively early (day 8) in the STS group which might be due to weight loss and tumor progression, resulting in a scenario that overwhelms the ability of the mouse to cope with these conditions. Nonetheless, the majority of the STS treated mice showed improvement over the untreated control group (**Fig. 3-17, A**).

Despite our ability to delay the onset of mortality in tumor-bearing mice by intervening with treatments such as chemotherapeutic drugs and short-term starvation or the combination of both STS and TMZ, the extremely rapid progression of the intracranial GL26luc glioma model did not allow long-term survival. In a clinical scenario, where the tumor burden is reduced by resection, or where the glioma is less aggressive, STS in combination with adjunctive therapy may offer potentially greater benefits.

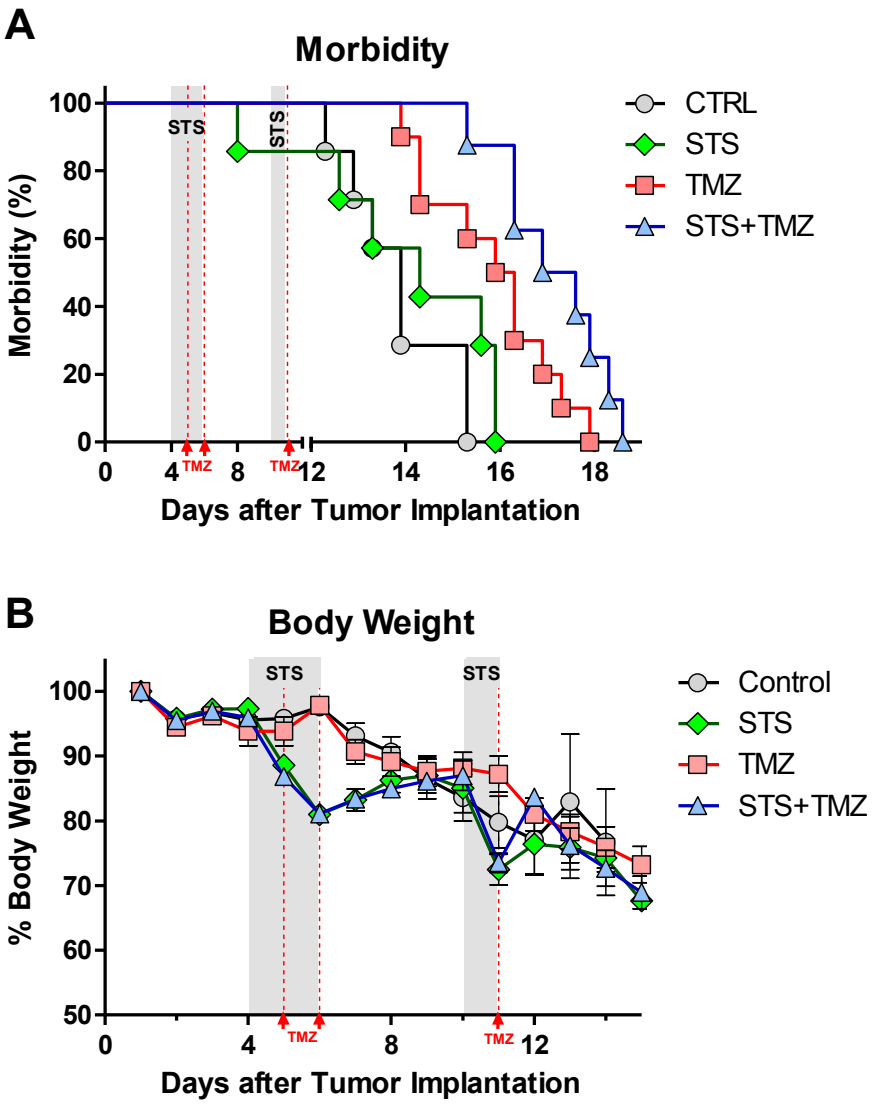

Figure 3-17. Two fasting cycles augment effects of TMZ-chemotherapy in the intracranial GL26 glioma model.

(**A**) Morbidity rate comparison of animals inoculated intracranially with GL26luc glioma cells. Animals at day 4 were randomized into the four experimental groups (Control, N= 7; TMZ, N= 7; STS, N= 10; or STS+TMZ, N= 8). Treatment of all experimental groups was initiated earlier to allow increased response to short-term starvation and/or TMZ. STS and STS+TMZ animals were fasted for 48 hours starting at day 4 followed by a second 24 hour fasting regiment at day 10 (grey areas). TMZ and STS+TMZ animals received i.p. injections of 15 mg/kg Temozolomide at day 5 and day 6 to match the first STS cycle and at day 11 to match the shorter second cycle (red lines), totaling 45 mg/kg. Curve comparison with Log-Rank test (Mantel-Cox; *** p< 0.001). (**B**) Body weight profile for glioma-bearing animals receiving multiple rounds of either short-term starvation, TMZ-treatment or both. STS and STS+TMZ animals initially reduce body weight during STS cycles (grey area) but regain the weight of untreated control and TMZ-treated animals rapidly after refeeding. Collaborative data, modified from (Safdie, Brandhorst et al. 2012).

3.4 Fasting Enhances Cancer-free Survival of Mice with Metastatic Cancer receiving Chemotherapy

Advanced metastatic tumors are extremely difficult to cure once tumor masses have spread to different organs. We therefore studied whether the combination of multiple fasting cycles and high-dose chemotherapy can increase survival in aggressive metastatic models before large tumor masses can be formed. To perform this experiment, we intravenously injected immune-competent mice with murine breast cancer cells (4T1), melanoma cells (B16), or two neuroblastoma cell lines (NXS2 and Neuro-2a). Fasting potentiated the effects of chemotherapy and extended the survival of mice injected with each type of cancer cell (**Fig. 3-18** and **Fig. 3-19**).

In the model of metastatic melanoma, one cycle of DXR treatment alone was not sufficient to extend survival, and only mice that were both fasted and treated with DXR showed extended maximum survival time (**Fig. 3-18, A**). DXR and fasting alone, but to a greater extent fasting combined with DXR, reduced the metastases of B16 melanoma cells to some organs when compared to mice fed the standard diet (**Fig. 3-18, B**). Lung metastases were detected in all mice, as expected from i.v. injections, and served as a control for tumor seeding. Melanoma spleen metastases were found in ~40% of mice treated with DXR, but only in 20% of mice that were fasted for a single cycle and 10% of mice that were fasted and also received DXR. In addition, unlike normally fed mice, metastases were not detected in the ovaries of fasted mice whether they received chemotherapy or not (**Fig. 3-18, B**). Overall, mice fasted and treated with DXR had a 40% reduction in metastases compared to controls (**Fig. 3-18, C**). In agreement with the results for melanoma cells, a single fasting cycle potentiated the effects of chemotherapy and extended survival in mice bearing metastatic breast tumor cells (4T1) (**Fig. 3-18, D**).

To more closely mimic cancer treatment in humans and to determine whether the combination of fasting cycles and chemotherapy has the potential to cure subjects with aggressive metastatic malignancies, we tested the effect of multiple cycles of fasting cycles in combination with chemotherapy drugs on a schedule that more closely reflects the therapy regimen administered to children with neuroblastoma. We monitored the survival of two mouse models of metastatic neuroblastoma. Long-term survival (more than 180 days) was achieved in 42% of

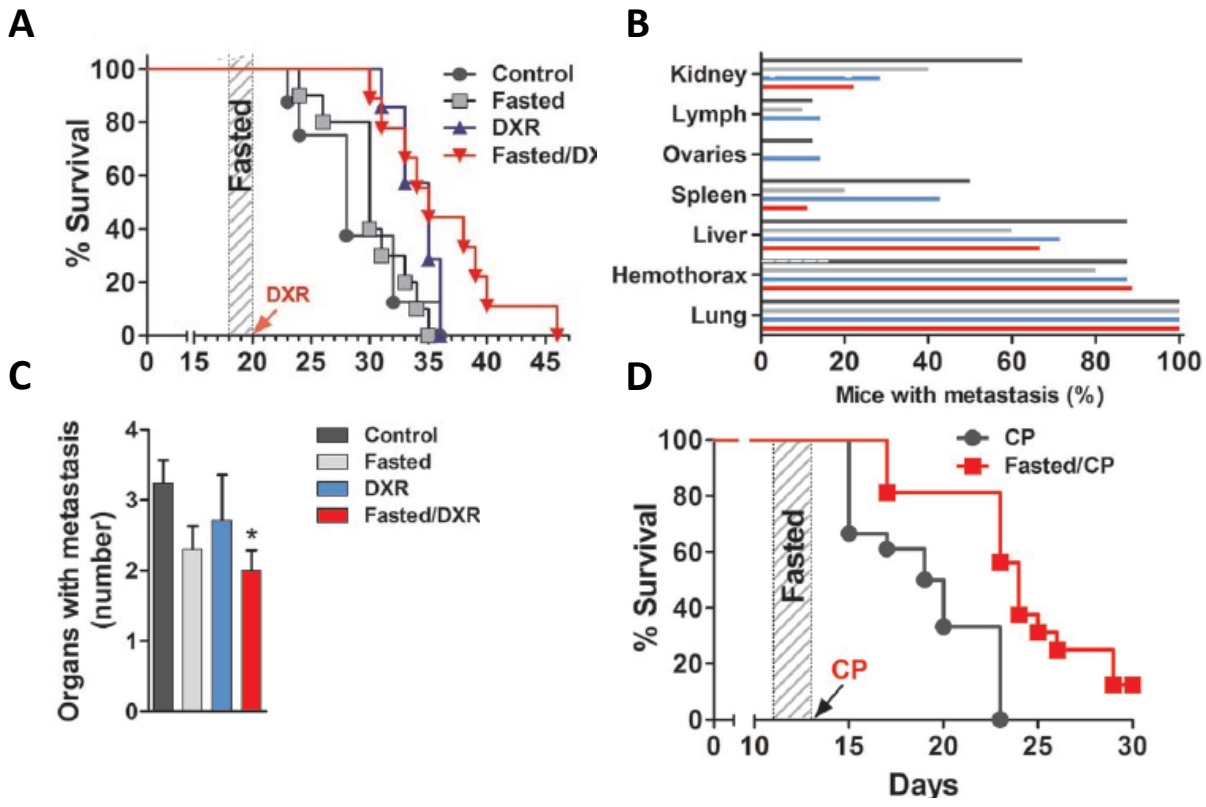

Figure 3-18. Effect of fasting and chemotherapy treatment on survival and tumor load in metastatic melanoma and breast cancer mouse models.

(**A**) Effect of 48 hours of fasting on survival of DXR-treated mice with metastatic murine melanoma (B16; N=9-10; P<0.05) (**B**) and metastasis in various organs. (**C**) The total number of organs with metastasis. All data presented as mean ± SEM; * p <0.05, Student's t-test, two-tailed. (**D**) Effect of 48 hours of fasting on survival of CP-treated mice with metastatic breast cancer (4T1). Collaborative data, modified from (Lee, Raffaghello et al. 2012).

murine neuroblastoma (NXS2)–bearing mice, which underwent two cycles of fasting with high-dose DXR (16 mg/kg) treatment (Fasted/DXR) (**Fig. 3-19, A**), compared to the 100% mortality in the group receiving chemotherapy under a normal diet (DXR). To test the effect of fasting on another model of metastatic neuroblastoma, we combined fasting with a cocktail of two chemotherapy drugs administered only once [DXR (10 mg/kg) + cisplatin (8 mg/kg)]. We injected murine neuroblastoma cells (Neuro-2a) intravenously into mice and allowed the tumor cells to spread for 9 days before initiating chemotherapy (**Fig. 3-19, B**). Whereas all mice treated with the chemotherapy cocktail combined with an *ad lib* diet died by day 75, ~25% of the mice that were fasted, in addition to receiving the chemotherapy cocktail, achieved

long-term survival (**Fig. 3-19, B**) while maintaining a normal weight (data not shown). At 300 days, these mice remained cancer-free. These results suggest that fasting cycles potentially sensitize a wide variety of tumor cell types to several of the most widely used chemotherapy drugs. This fasting-dependent potentiation of chemotherapeutic action significantly extended overall survival time and allowed long-term cancer-free survival, even in mice with a large number of dispersed cancer cells (Lee, Raffaghello et al. 2012).

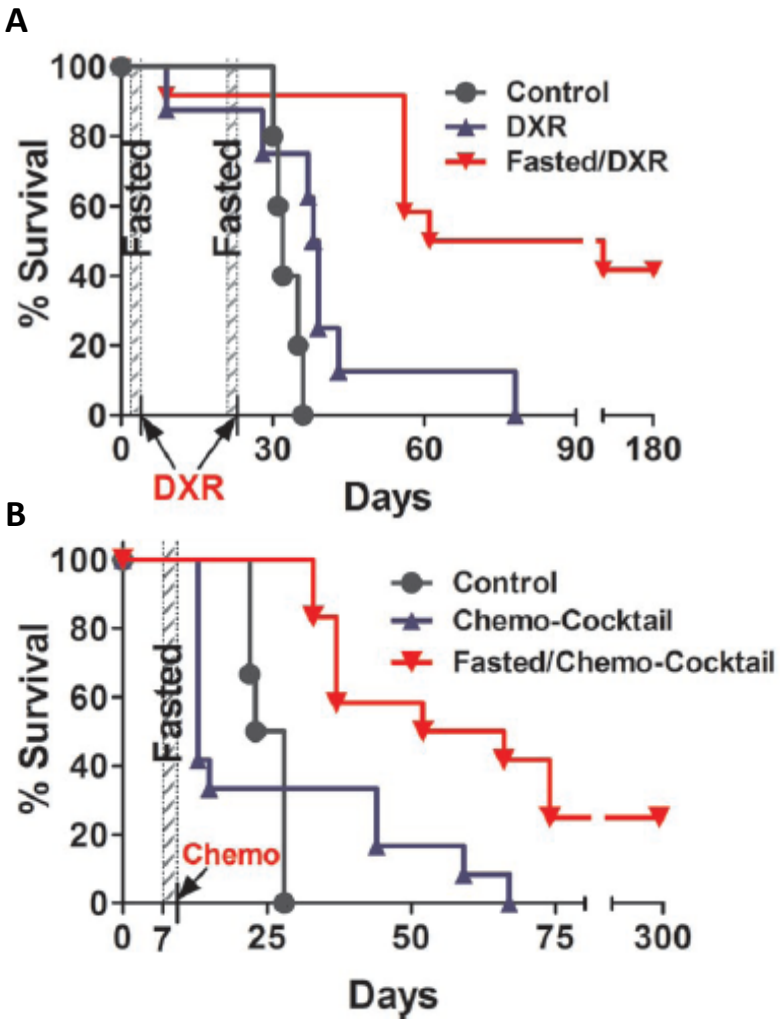

Figure 3-19. Effect of fasting and chemotherapy treatment on survival in metastatic neuroblastoma mouse models.

(**A**) Effect of 48 hours of fasting on survival of mice with murine neuroblastoma NXS2 that were treated twice with high dose DXR (16mg/kg). (**B**) Effect of 48 hours of fasting on survival of chemo-cocktail [DXR (10 mg/kg) + cisplatin (8 mg/kg)] treated mice with Neuro-2a neuroblastoma. Collaborative data, modified from (Lee, Raffaghello et al. 2012).

In summary, the data I presented so far, as well as previously established results (see introduction), all demonstrate that normal cells and cancer cells differ in their response to fasting. In the absence of nutrients, normal cells switch their metabolism towards maintenance pathways, whereas tumor cells are unable to activate this protective response. Our fasting approach is based on differences in metabolism between normal cells and cancer cells that can be used to enhance anticancer therapy by selectively increasing the resistance of normal cells to chemotherapy while simultaneously sensitizing malignant cells (**Fig. 3-20**).

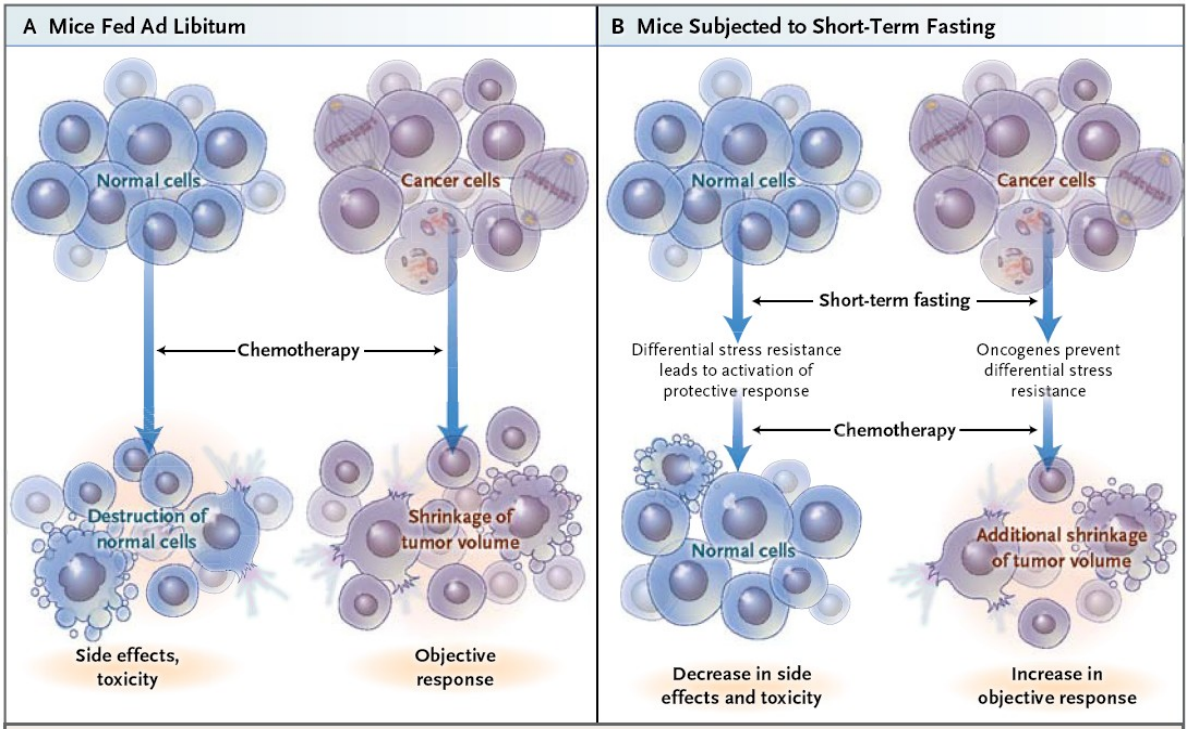

Figure 3-20. Modulating the effects of chemotherapy by means of fasting.

Chemotherapy-induced oxidative stress reduces the rates of both the proliferation and the survival of cancer cells. It yields an objective response that can be quantified on the basis of shrinkage of the tumor volume (**Panel A**). However, chemotherapy also affects normal cells, leading to toxic side effects. Short-term fasting before or after chemotherapy, or at both times, induces differential stress resistance in normal and cancer cells. In normal cells, fasting activates protective metabolic pathways that confer resistance to oxidative stress (**Panel B**). In contrast, yeast cells transformed with an activated oncogene are unable to turn on the protective response and thus remain sensitive to oxidative stress. Additional experiments using mouse models of murine and human cancer showed that fasting specifically augments levels of oxidative stress and sensitivity to oxidative damage (e.g., that inflicted by chemotherapeutic agents) in cancer cells and that these effects are accompanied by DNA damage and apoptosis. Modified from (Laviano and Rossi Fanelli 2012)

3.5 Fasting Differentially Regulates Translation and Proliferation Genes

To identify the gene expression changes that could differentially regulate chemotherapy resistance in normal and cancer cells in response to fasting, we collaborated with Rafael de Cabo and Alejandro Martin-Montalvo at the National Institute on Aging (NIA). Genome-wide microarray analyses were performed on liver, heart, skeletal muscle, and subcutaneous 4T1 breast tumor masses removed from mice that were either fasted for 48 hours or fed an *ad lib* diet. Fasting differentially regulated many genes involved in cellular proliferation such as the insulin signaling adaptor (Irs2) and the mitogenic hormone prolactin receptor (Prlr) in normal and cancer cells (**Fig. 3-21, A**). In many cases, proliferation-associated genes were down-regulated in normal tissues but up-regulated, or unaffected, in cancer cells. Surprisingly, the expression of translation and ribosome biogenesis/assembly genes such as elongation factor 1γ (Eef1g) and components of the 60S and 40S ribosomal proteins were significantly increased in response to fasting in breast cancer allografts (4T1), whereas in normal tissues they were either repressed or minimally affected (**Fig. 3-21, B**). A gene list of protein translation related clusters from the gene ontology analysis can be found in the supplementary material of our publication in Science Translational Medicine (Lee, Raffaghello et al. 2012).

In agreement with these results, 4T1 cells cultured under STS conditions for 48 hours displayed a major increase in cellular protein concentration (**Fig. 3-21, C**) and in translation as estimated by incorporation of the methionine analog azidohomoalanine (AHA) (**Fig. 3-21, D**). In addition, the phosphorylation of the Akt and S6K proteins, which regulate translation and proliferation when phosphorylated, was elevated, and that of eIF2α, which impairs protein synthesis when phosphorylated, was reduced in tumors from fasted mice (**Fig. 3-22, A**). Similar results were observed in 4T1 cells undergoing STS *in vitro*, particularly in combination with CP treatment (**Fig. 3-22, B**). Despite this starvation-dependent activation of translation mechanisms, cancer cell numbers were greatly reduced *in vitro* (**Fig. 3-4**), a finding consistent with the retardation of tumor progression by fasting *in vivo*. In agreement with our previous studies (Raffaghello, Lee et al. 2008; Safdie, Dorff et al. 2009; Lee, Safdie et al. 2010; Safdie, Brandhorst et al. 2012) these results indicate that fasting promotes a differential regulation of pro-growth and pro-translation genes in normal and cancer cells.

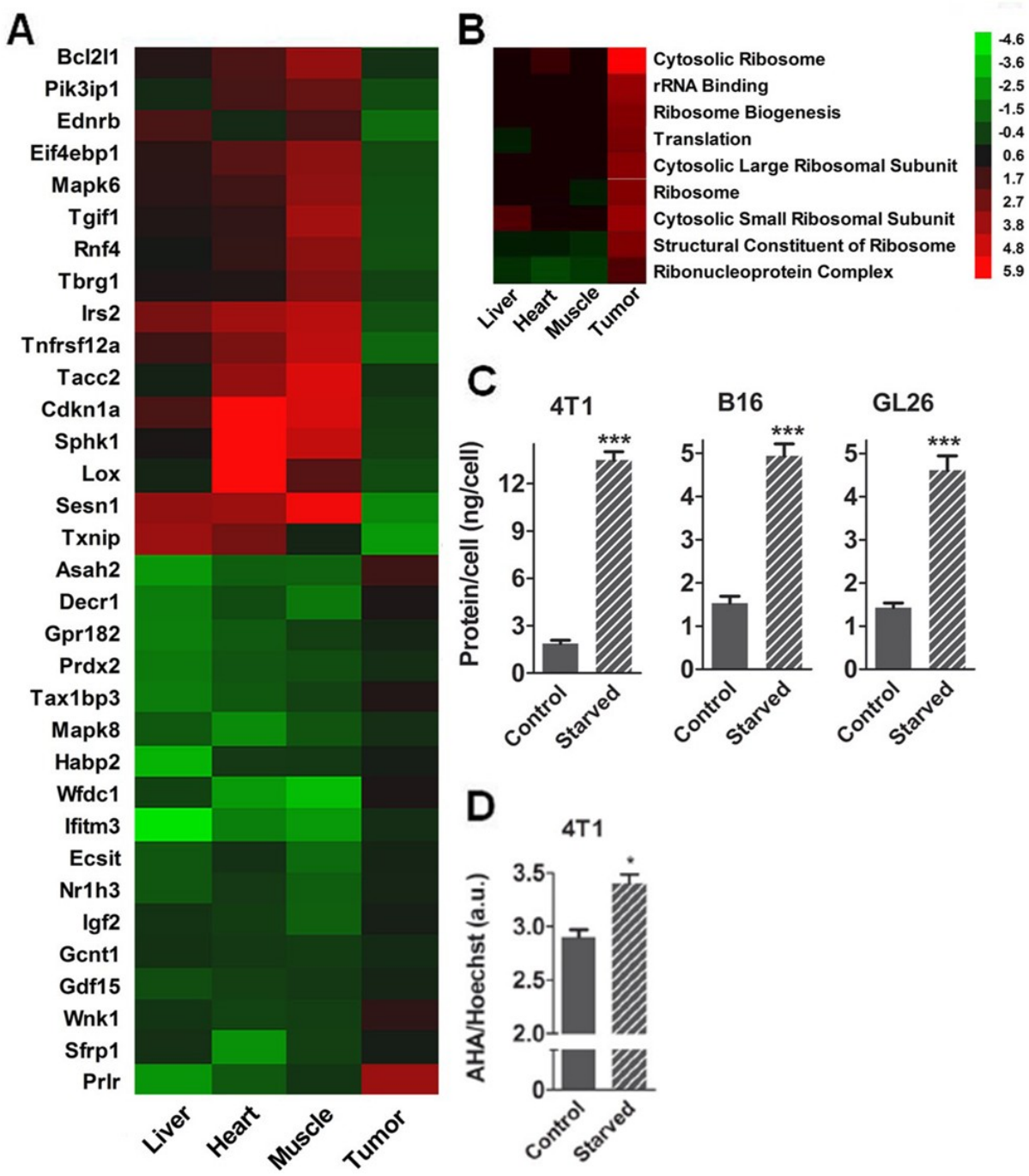

Figure 3-21. Effect of fasting on genes involved in growth and proliferation.

Microarray analysis on the liver, heart, skeletal muscle, and subcutaneous breast tumors (4T1) from normally fed or fasted (48 hours) mice of (**A**) cellular proliferation pathways and (**B**) translational machinery including ribosome assembly/biogenesis. (**C**) Effect of starvation on protein concentration in 4T1, B16, and GL26 cells (N=3). (**D**) Effect of starvation on the rate of AHA (methionine analog) incorporation in 4T1 cells (N=3). All data presented as mean ± SEM; * p <0.05; *** p <0.001, Student's t-test, two-tailed. Collaborative data, modified from (Lee, Raffaghello et al. 2012).

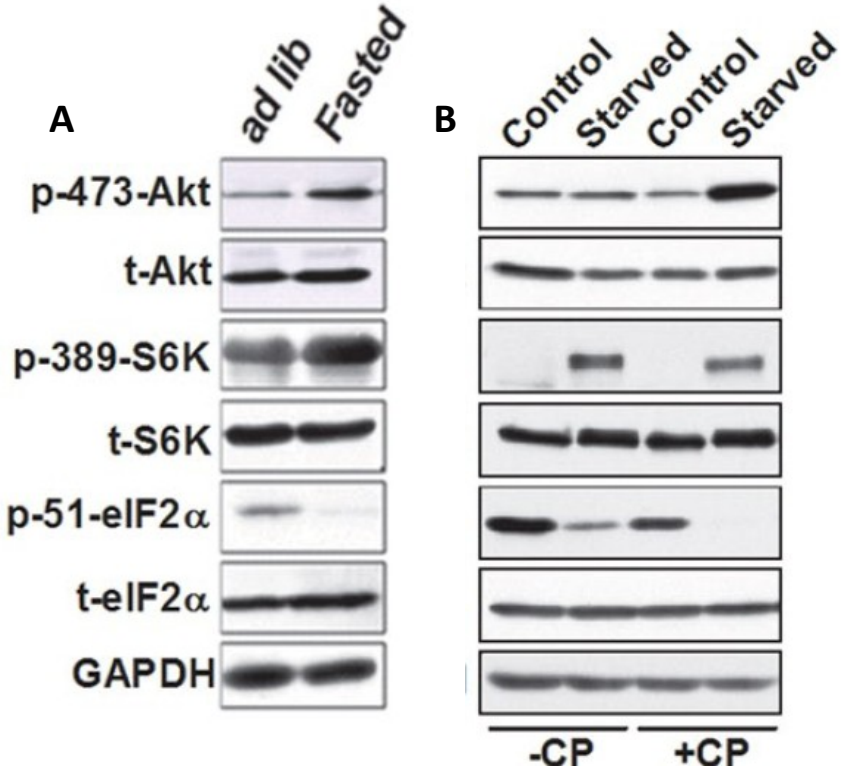

Figure 3-22. Effect of fasting on pro-proliferative pathways in murine breast cancer cells.

(**A**) Effect of fasting on Akt, S6K, and eIF2α phosphorylation in 4T1 murine breast cancer cells *in vivo*. (**B**) Effect of fasting, with our without cyclophosphamide (CP) treatment on Akt, S6K, and eIF2α phosphorylation in 4T1 murine breast cancer cells *in vitro*. Collaborative data, modified from (Lee, Raffaghello et al. 2012).

3.6 Synergistic Effect of STS and Chemotherapy on DNA Damage

To determine the mechanisms by which short-term starvation cycles reduce tumor progression, we tested the hypothesis that lower concentrations of extracellular glucose and growth factors may increase DNA damage in cancer cells. In both cultured 4T1 breast cancer cells (**Fig. 3-23, A**) and B16 melanoma cells (**Fig. 3-23, B**), the reduction of glucose concentrations from those in *ad lib* fed mice (2.0 g/L) to a concentration similar to that reached after fasting (0.5 g/L), in combination with low serum (1% FBS to mimic the fasting-dependent reduction in blood growth factors and proteins), increased DNA damage more than chemotherapy alone. Indeed, the combination of low glucose concentrations and treatment with a

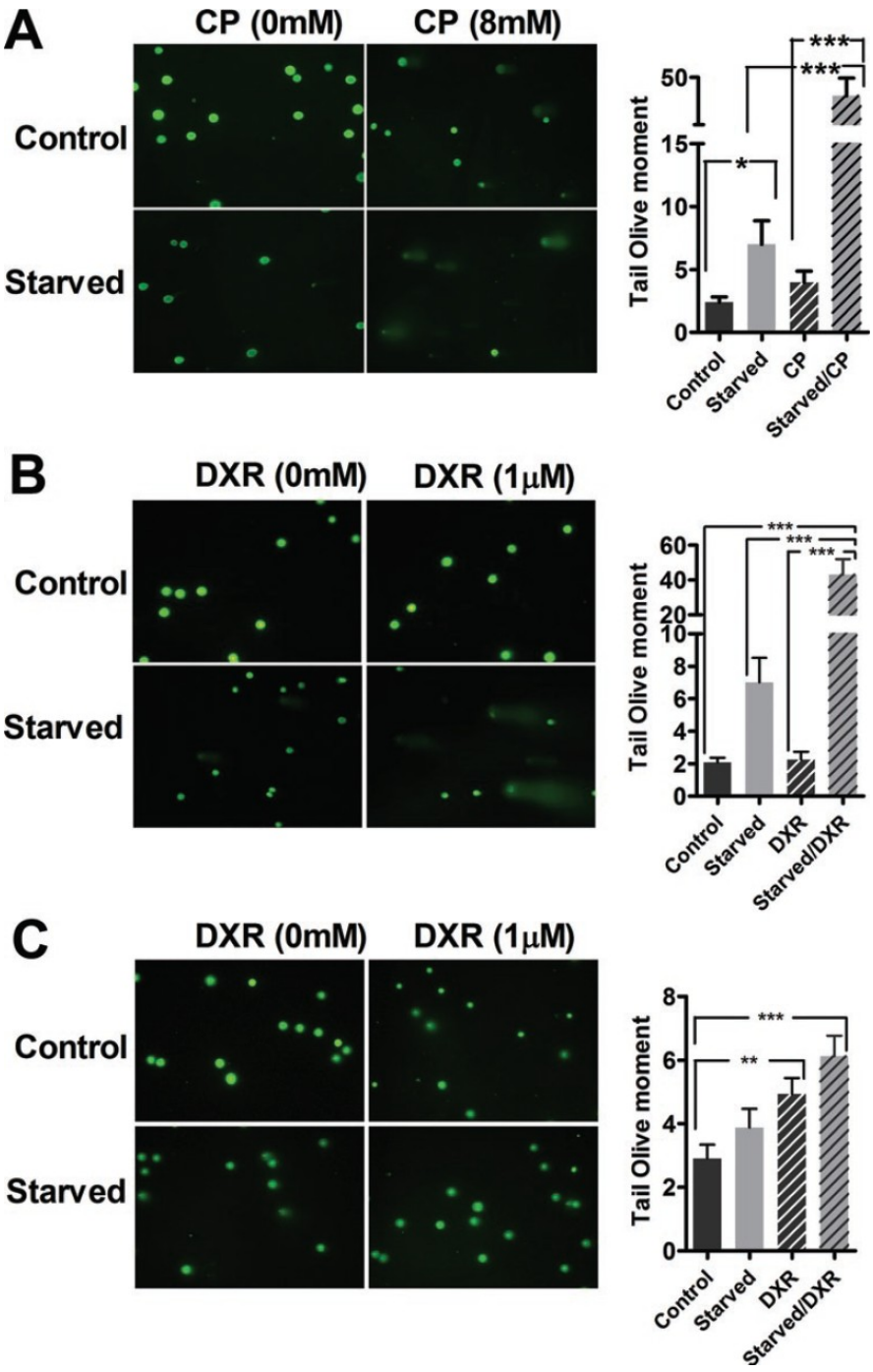

Figure 3-23. Effect of fasting on chemotherapy-induced DNA damage.

(**A**) Effect of starvation alone and when combined with CP in breast cancer (4T1) and (**B**) with DXR in melanoma (B16), and (**C**) with DXR in glioma (GL26) cells as determined by comet assay (N=6). The green signal represents intact and fractured DNA. Cells in both groups were cultured in normal glucose (2.0 g/L) or low glucose (0.5 g/L), respectively, supplemented with 1% FBS. Drugs were selected to match those in Fig. 3-11, A, C and E. Data from at least 3 independent experiments. All data presented as mean ± SEM; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, Student's t-test, two-tailed. Collaborative data, modified from (Lee, Raffaghello et al. 2012).

chemotherapeutic agent promoted a seemingly synergistic, 20-fold increase in DNA damage in both breast cancer and melanoma cells. In GL26 glioma cells, the effect of reduced glucose concentrations on DNA damage was additive with the drug doxorubicin (**Fig. 3-23, C**). These results indicate that starvation conditions and chemotherapy drugs can act in an additive or synergistic manner to promote DNA breaks in cancer cells. These effects may contribute to the chemotherapy-potentiating effects that we observed *in vivo*.

3.7 Fasting-induced Sensitization of 4T1 Cells is Associated with Oxidative Stress and Caspase-3 Activation

Various chemotherapy drugs, including CP, damage DNA and trigger cell death in part by promoting oxidative damage (Berndtsson, Hagg et al. 2007). We therefore measured the level of reactive oxygen species, as indicated by dihydroethidium (DHE) oxidation, in 4T1 cells under standard and starvation conditions after treatment with CP. We detected more DHE oxidation in the cells after starvation, suggesting that starvation conditions promoted oxidative stress and possibly increased superoxide levels (**Fig. 3-24, A** and **B**). CP alone increased DHE oxidation (2-fold), and treatment of the cells with both starvation and CP resulted in even higher DHE oxidation levels (3.2-fold) (**Fig. 3-24, B**) compared to control. This increase in ROS may have contributed to the synergistic DNA damage caused by fasting and chemotherapy (**Fig. 3-23**).

Because oxidative stress is a central promoter of apoptosis, we measured the effect of fasting on the activation of the pro-apoptotic enzyme caspase-3 both *in vivo* and *in vitro*. Cleaved caspase-3 was increased in allografted tumors, but not in normal organs, in response to fasting in mice (**Fig. 3-25, A**) and also in cancer cells undergoing starvation *in vitro*; an effect that was potentiated by treatment with CP (**Fig. 3-25, B**). Because caspase-3 was activated during fasting only in the transplanted tumors (**Fig. 3-25, A** and **B**), and because starvation conditions increased DNA damage, particularly in the presence of CP (**Fig. 3-23**), we conclude that the retarded tumor growth by fasting and the cell death caused by the combination of starvation and chemotherapy drugs may be at least partially attributed to increased apoptosis.

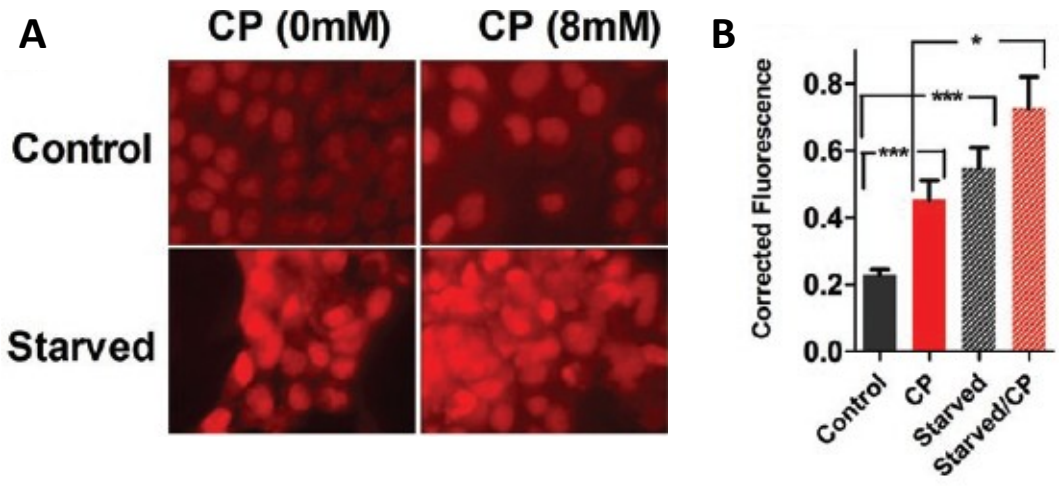

Figure 3-24. Effect of starvation on intracellular oxidative stress as estimated by a superoxide marker (DHE) *in vitro*.

(**A**) Murine breast cancer cells (4T1) were fasted and treated with cyclophosphamide (CP) *in vitro*. Superoxide levels were estimated by dihydroethidium (DHE) staining. 100X magnification. (**B**) Corrected total cell fluorescence was quantified using ImageJ (NIH) and corrected for background fluorescence. All data presented as mean ± SEM; * p <0.05; *** p <0.001, Student's t-test, two-tailed. Collaborative data, modified from (Lee, Raffaghello et al. 2012).

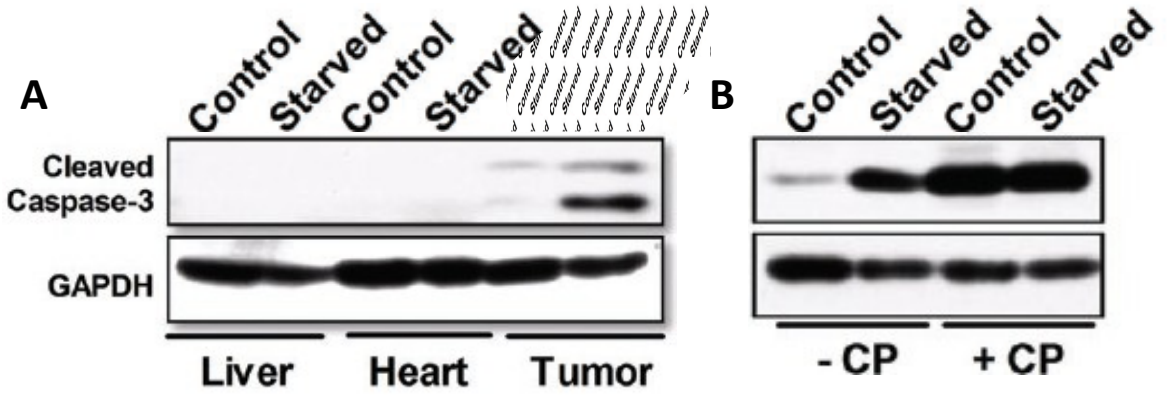

Figure 3-25. STS mediated activation of apoptosis in murine breast cancer cells.

(**A**) Effect of fasting on caspase-3 cleavage in the liver, heart and allografted 4T1 breast tumors *in vivo* and (**B**) in 4T1 breast cancer cells cultured under starvation conditions (restricted glucose (0.5 g/L) and growth factor (1% FBS) concentrations) *in vitro*. Cyclophosphamide (CP) treatment increases cleaved caspase-3 levels. Collaborative data, modified from (Lee, Raffaghello et al. 2012).

3.8 Reduced HO-1 Expression Mediates Part of the Cancer-sensitizing Effect of Fasting

To understand the mechanisms of fasting-induced sensitization, we searched among the stress-responsive genes whose expression is regulated by fasting and CR. Based on our microarray (**Fig. 3-26)**, we selected heme oxygenase-1 (HO-1), also known as heat-shock protein 32 (HSP32), as a promising candidate (Bakken, Thaler et al. 1972; Pearson, Lewis et al. 2008; Verweij, van Ginhoven et al. 2011). HO-1 protects against oxidative damage and apoptosis (Chen, Liu et al. 2004) and inhibits the cleavage of caspase-3 (see **Fig. 3-25**) (Inguaggiato, Gonzalez-Michaca et al. 2001; Liu, Chen et al. 2004). In addition, reduced HO-1 levels increase translation through the regulation of the heme-regulated eIF2α kinase, in agreement with our results on AKT, S6K and eIF2α phosphorylation (**Fig. 3-22**) (Chen 2007; Liu, Suragani et al. 2008). In our microarray, there was a trend for differential HO-1 repression in the transplanted 4T1 tumors and we verified this observation by qRT-PCR. Fasting caused a major increase in HO-1 expression in normal organs, but significantly repressed its expression in the transplanted tumors (**Fig. 3-27, A)**.

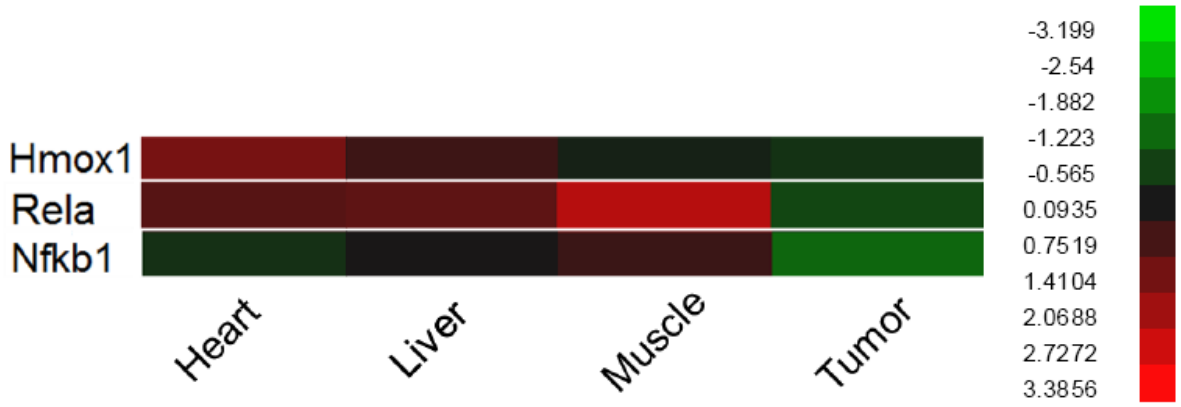

Figure 3-26. Heme oxygenase-1 (HO-1) and NFkB/RelA expression heatmap in normal organs and transplanted 4T1 tumor.

Microarray analysis on the liver, heart, skeletal muscle, and subcutaneous breast tumors (4T1) from normally fed or fasted (48 hours) mice demonstrates a trend for the differential expression of HO-1, NFkB and RelA between normal tissue and transplanted 4T1 tumor. Data in collaboration with the NIA, not published.

In our microarray, transplanted 4T1 tumors showed a trend of differential down-regulation of NFkB (p50) and its partner RelA (p65) (**Fig. 3-26**). Because NFkB reduces oxidative stress via HO-1 and/or MnSOD (Mercurio and Manning 1999; Luo, Kamata et al. 2005; Alam and Cook 2007) and since functional binding sites for NFkB are present in the promoter of HO-1 (Paine, Eiz-Vesper et al. 2010), we tested whether NFkB expression was also reduced in 4T1 cells in response to fasting. qRT-PCR confirmed that fasting reduces the expression of NFkB (p50) in 4T1 tumors (**Fig. 3-27, B**), suggesting that the HO-1 response to fasting may, at least, be partially regulated by NFkB.

The increase in HO-1 expression in normal organs by fasting is consistent with the fact that fasting protects mice against DXR induced toxicity (Lee, Safdie et al. 2010), which can also be achieved by increasing HO-1 *in vivo* and *in vitro* (Suliman, Carraway et al. 2007). In contrast, inhibiting HO-1 expression has been well documented to inhibit tumor progression and increase cancer cell death, as well as sensitize them to toxins; thus positioning HO-1 as a promising target for cancer treatment (Sahoo, Sawa et al. 2002; Fang, Sawa et al. 2004; Marinissen, Tanos et al. 2006; Nowis, Legat et al. 2006; Hirai, Sasahira et al. 2007; Kim, Kim et al. 2008; Mayerhofer, Gleixner et al. 2008; Liu, Liang et al. 2011).

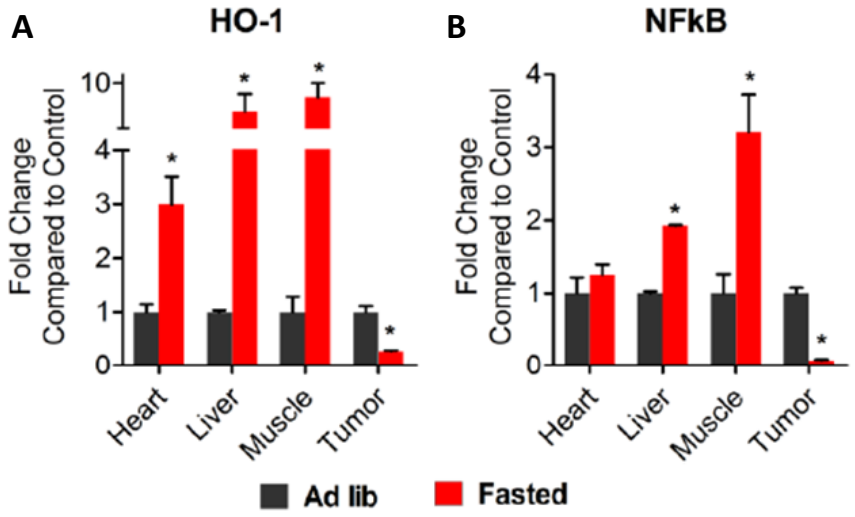

Figure 3-27. Fasting differentially regulates the expression of heme oxygenase-1 (HO-1) and NFkB.

Expression of (**A**) HO-1 and (**B**) NFkB was significantly induced in the heart, liver, and skeletal muscle of mice fasted for 48 hours but significantly repressed in the grafted breast tumor cells. All data presented as mean ± SEM; * $p < 0.05$; Student's t-test, two-tailed. Collaborative data, not published.

To test the role of heme oxygenase-1 in the fasting-dependent sensitization to chemotherapy *in vitro*, we induced HO-1 expression in 4T1 cells using hemin and found that we could partially reverse the sensitization to CP under fasting conditions (**Fig. 3-28, A**). Conversely, the HO-1 inhibitor zinc protoporphyrin (ZnPP) sensitized 4T1 cells to CP under normal, *ad lib* mimicking conditions (**Fig. 3-28, B**) but did not cause additional sensitization under fasting mimicking conditions. To confirm these findings, we over-expressed HO-1 in 4T1 cells by stably transfecting a rHO-1 construct (**Fig. 3-28, C**). Similar to the induction of HO-1 by hemin, the over-expression of HO-1 causes the partial reversion of the fasting induced sensitization in murine breast cancer cells. Cells that were transfected with the empty pcDNA vector do not show these effects and remained sensitized when treated with CP in fasting mimicking media (**Fig. 3-28, D**).

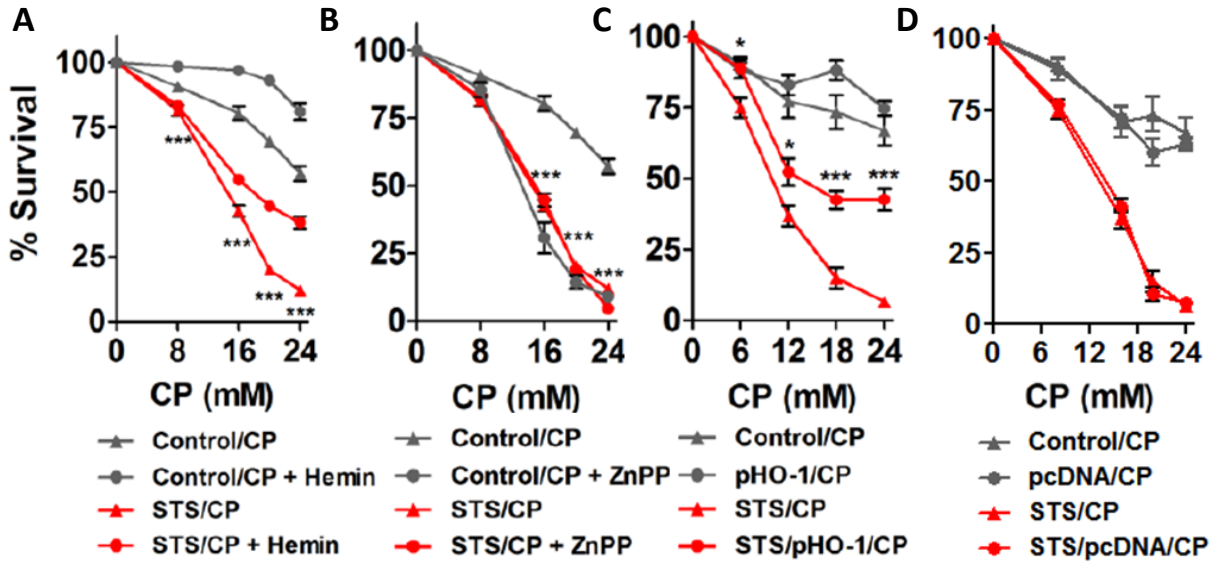

Figure 3-28. Heme oxygenase-1 (HO-1) mediated sensitization of murine breast cancer cells.

Murine breast cancer cells (4T1) were treated with either (**A**) hemin, a commonly used inducer of HO-1 (10 μM), or (**B**) ZnPP (20 μM), a commonly used HO-1 inhibitor, in normal (2.0 g/L glucose, 10% FBS) or fasting mimicking (0.5 g/L glucose, 1% FBS) cell culture conditions for 24 hours, followed by an additional 24 hours with cyclophosphamide (CP). (**C**) Stably transfected 4T1 cells expressing HO-1 were cultured in normal or STS conditions for 24 hours, followed by an additional 24 hours with CP. (**D**) Stably transfected 4T1 cells expressing the empty pcDNA vector were cultured in normal or STS conditions for 24 hours and an additional 24 hours with CP. All data presented as mean ± SEM; * $p < 0.05$; *** $p < 0.001$, Student's t-test, two-tailed. Collaborative data, not published.

Changes in metabolic pathways of malignant cells cause a high dependence on glucose as the main carbon source to maintain high cellular proliferation rates; fasting however deprives malignant cells of this energy/carbon source. We evaluated mitochondrial function of tumor cells grown in either *ad lib*- or fasting-mimicking media conditions because in order to maintain proliferation, malignant cells need cellular energy such as ATP. ATP deficiencies force cells to undergo cell cycle arrest or even apoptosis (Izyumov, Avetisyan et al. 2004; Lum, Bauer et al. 2005). In order to test whether the depletion of nutrients causes functional changes, we measured basal and stimulated mitochondrial functions utilizing the Seahorse Bioscience XF24 analyzer. The Seahorse Bioscience XF24 instrument measures the rate of change of analytes, in particular the amount of dissolved oxygen and pH, in the media immediately surrounding living cells cultured in a 24-well microplate in real-time. Changes in the extracellular media are caused by the consumption or production of analytes by the cells. Therefore, a sensitive measurement of the media flux can be used to determine rates of cellular metabolism with great precision and in a non-invasive, label-free manner.

Our data reveals that cells grown under *ad lib* conditions show a significantly higher basal oxygen consumption rate (OCR) than cells grown under glucose- and serum-restricted conditions, suggesting reduced mitochondrial respiration under fasting-like conditions (**Fig. 3-29, A**). Next, we characterized mitochondrial function by sequential addition of pharmacological inhibitors of the mitochondrial respiratory chain (**Fig. 3-29, B**). To estimate the proportion of the basal OCR linked to ATP synthesis through ATP-synthase, we injected the complex V inhibitor oligomycin. Oligomycin decreases the OCR to the extent to which cells use mitochondria for ATP production (**Fig. 3-29, C**, ATP), while the remaining OCR is attributed to processes other than cytochrome c oxidase (**Fig. 3-29, C**, Non-mito) or proton leak (**Fig. 3-29, C**, Proton) (Brand 1990; Brown, Lakin-Thomas et al. 1990; Brown 1992). To measure the maximal OCR potential that 4T1 tumor cells can undergo in *ad lib* or fasting-mimicking media conditions, the uncoupler FCCP was added (**Fig. 3-29, C**, Max). FCCP, a proton ionophore, renders the inner mitochondrial membrane permeable for protons which cause the electron transfer chain to be uncoupled from the proton gradient (Dranka, Hill et al. 2010). Taken together, the data shown in **Fig. 3-29** indicates that 4T1 tumor cells, grown under fasting-mimicking media conditions

have a sub-maximal capacity when compared to cells that were maintained in a glucose- and serum-rich environment. Reduced mitochondrial integrity has been linked to the intracellular production of reactive oxygen species (Lee and Wei 2007). Future work will have to establish if the reduction in maximal respiratory capacity provides a link to the increased ROS levels we measured in fasted cells.

To determine whether HO-1 plays a role in maintaining mitochondrial respiration under fasting conditions, we exposed 4T1 cells over-expressing heme oxygenase-1 to the same assay conditions. Over-expressing HO-1 had no rescue effects under fasting conditions on baseline oxygen consumption rate (**Fig. 3-29, A**) and the oxygen consumption attributed with mitochondrial ATP production, proton leak and the maximal respiratory capacity (**Fig. 3-29, C**).

Fasting causes an increase in ketone bodies that are produced from acetyl-CoA in the mitochondrial matrix by breaking down fatty acids. The ability to oxidize fatty acids supplies normal cells with energy when other nutrients are scarce or absent. We therefore tested if injections of palmitate, the most common fatty acid in mammals, provide efficient means for malignant cells to produce energy. Murine breast cancer cells grown in media conditions mimicking glucose and growth factor levels of *ad lib* fed mice were able to utilize palmitate for fatty acid oxidation, as indicated by an increased OCR after palmitate injection (**Fig. 3-30**). Surprisingly, in culture-media conditions mimicking glucose and serum levels of fasted mice, 4T1 cells did not respond to the palmitate injection. We detected no increase in oxygen consumption, suggesting that these cells were unable to utilize fatty acids for mitochondrial energy production when glucose is scarce. In stark contrast to normal cells, which can utilize fatty acids, this proves to be a striking disadvantage for fasted malignant cells. As mentioned above, a sufficient supply of nutrients is essential for cellular growth. Fasting and the acute deprivation of glucose, as well as the inability to utilize fatty acids, might provide effective means to intervene with the rapid proliferation of malignant cells. The over-expression of HO-1 reversed this deficiency of murine breast cancer cells, indicating that cellular redox homeostasis and fatty acid utilization in cancer cells are interconnected and plays a crucial role in the fasting-mediated sensitization.

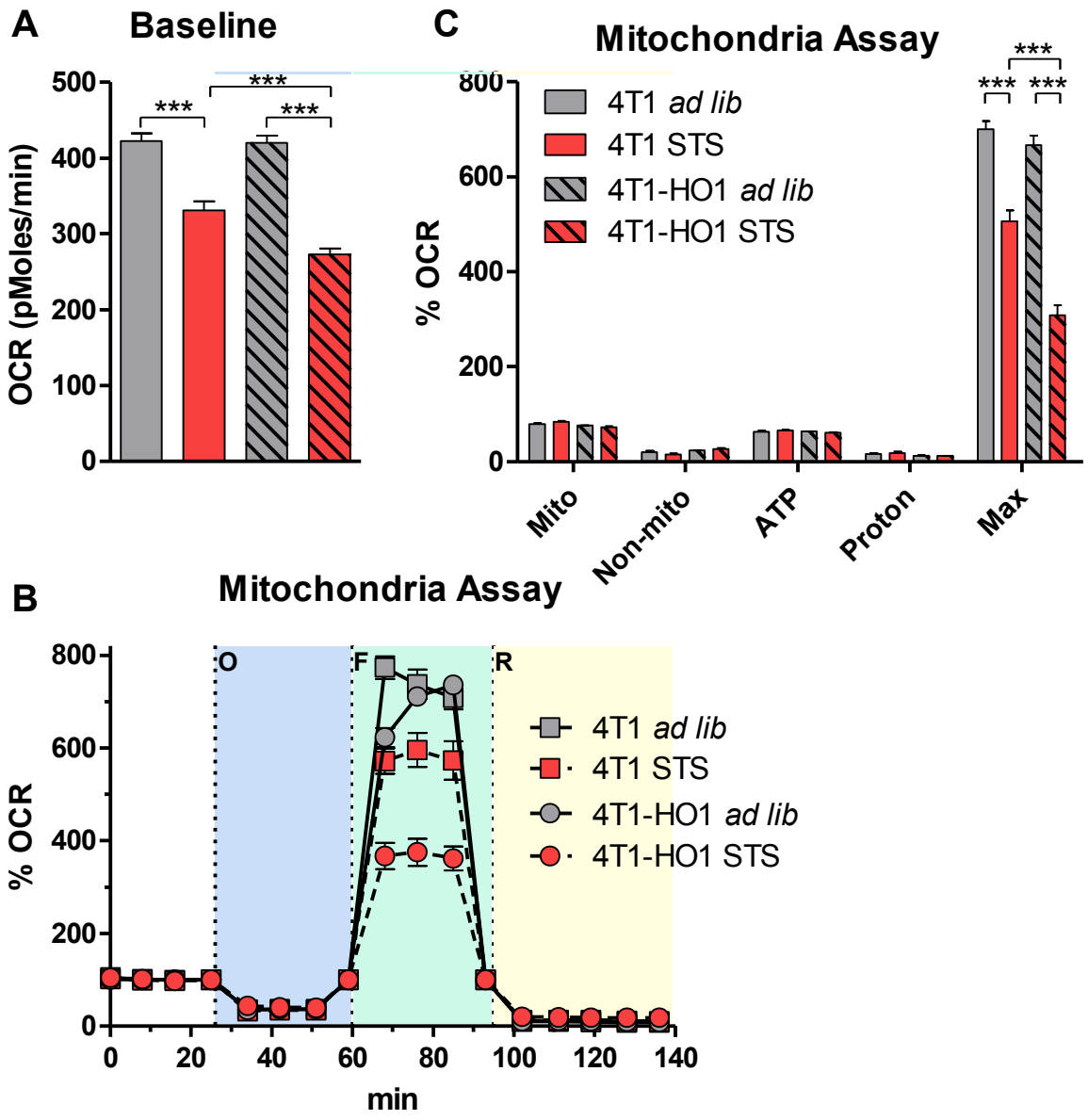

Figure 3-29. Mitochondrial function is altered by cellular starvation in murine breast cancer cells.

(**A**) Baseline oxygen consumption rates (OCR) of normal (4T1) and heme oxygenase-1 over-expressing (4T1-HO1, striped bar) murine breast cancer cells grown for 48 hours in either *ad lib* (grey bar) or STS (red bar) mimicking media. (**B**) Mitochondrial respiration assay for 4T1 cells grown for 48 hours in either *ad lib* (grey bar)- or fasting (red bar)- mimicking media conditions as assayed by mitochondrial oxygen consumption rate. O= Oligomycin, a complex V inhibitor; F= FCCP, a proton-uncoupler; R= Rotenone, a complex I inhibitor. Normalized to basal levels. (**C**) The contribution of the mitochondria, non-respiratory chain, ATP production, proton leakage and maximal capacity to the total oxygen consumption. Normalized to basal levels. All data presented as mean ± SEM; ** $p<0.01$, *** $p<0.01$, ANOVA, Tukey's multiple comparison. Unpublished results.

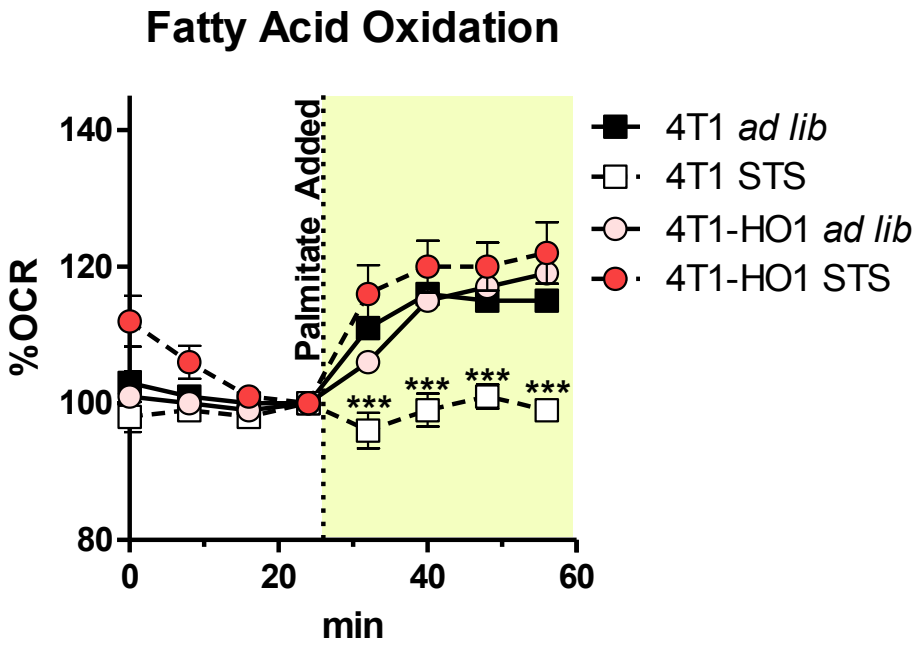

Figure 3-30. Cellular starvation alters fatty acid oxidation in murine breast cancer cells.

Fatty acid oxidation (FAO) of 4T1 cells grown for 48 hours in either *ad lib* (grey circle)- or fasting (red square)-mimicking media. Mitochondrial respiration was assayed through the mitochondrial oxygen consumption rate (OCR). **P< 0.01; ***P< 0.001, Student's t test. Unpublished results.

Because we detected higher levels of DHE oxidation, indicative for higher amounts of reactive oxygen species in 4T1 cells under starvation conditions after treatment with CP *in vitro* (**Fig. 3-24)**, we measured superoxide levels in the mitochondria of 4T1-HO1 cells using the MitoSox Red flourogenic dye. Imaging of stained 4T1 cells revealed that fasting mimicking cell culture conditions increased the fluorescence, which could be partially reversed by over-expressing HO-1 (**Fig. 3-31, A**). We analyzed the relative fluorescence of 4T1 and HO-1 over-expressing cells in a 96-well plate after exposure to fasting mimicking media conditions and in combination with cyclophosphamide (**Fig. 3-31, B**). 4T1 cells treated with 16 mM CP had a measurable increase in their relative super oxide levels (**Fig. 3-31, B**; *, P< 0.05) and fasting had a similar effect (**, P< 0.01). Fasting in combination with chemotherapy resulted in a roughly 30% increase in the measurable superoxide levels and thus confirms prior results (***, P< 0.001). 4T1 cells over-expressing HO-1 showed a trend towards lower superoxide levels in *ad lib*

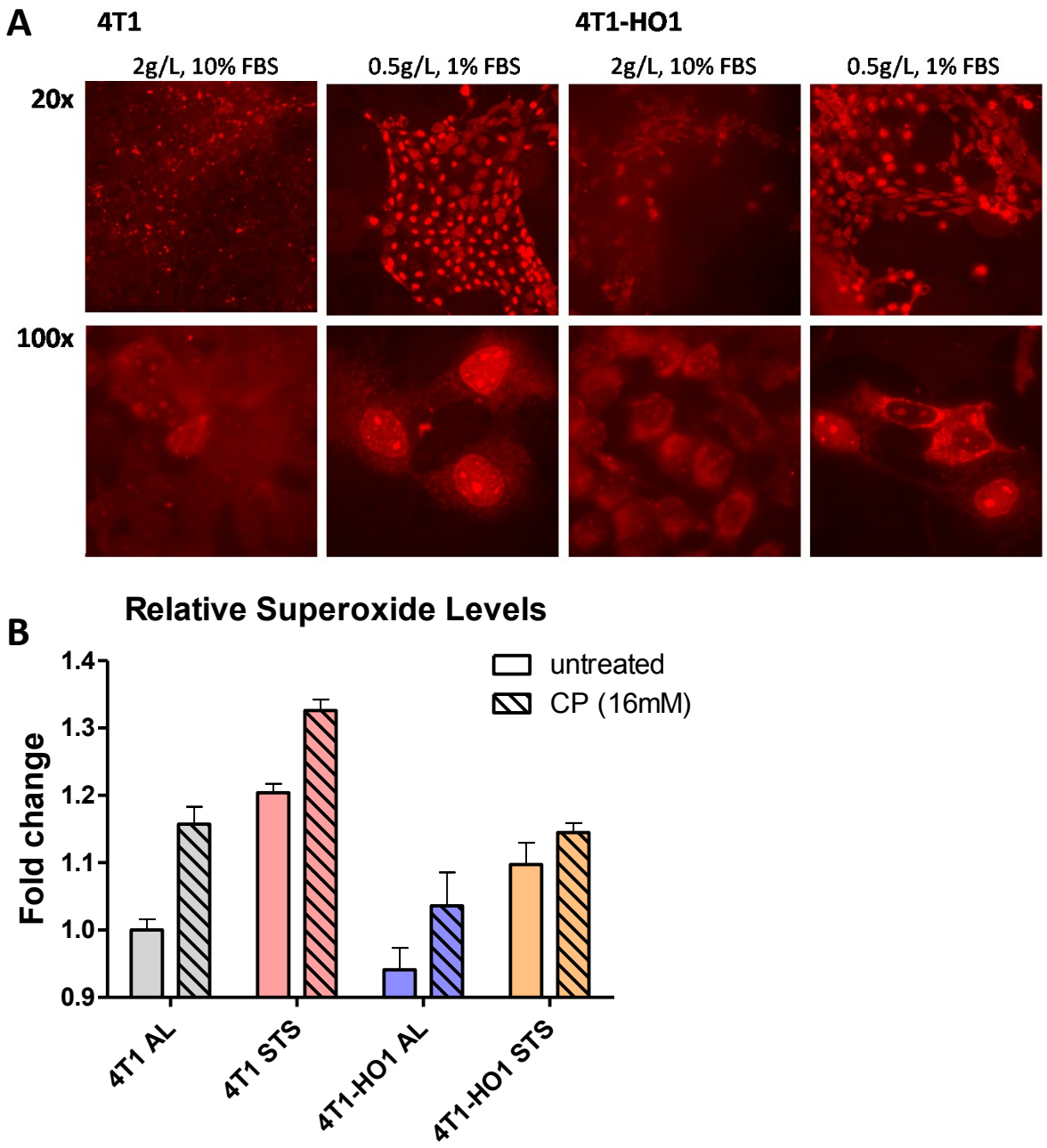

Figure 3-31. Relative superoxide levels are reduced in HO-1 over-expressing murine breast cancer cells.

(**A**) Normal murine breast cancer cells (4T1) and HO-1 over expressing cells (4T1-HO1) were grown in either *ad lib* or fasting mimicking cell culture conditions for 48 hours and incubated with 2.5 μM MitoSOX reagent for 10 min at 37°C. Fluorescent microscopy at 20X (top row) and 100X (bottom row) magnification. (**B**) Relative superoxide levels were measured in either untreated or chemotherapy treated 4T1 and 4T1-HO1 cells grown in *ad lib* (AL) or fasting-mimicking (STS) cell culture conditions for 48 hours. All data presented as mean ± SEM; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, ANOVA, Tukey's multiple comparison. See text for details. Collaborative data, not published.

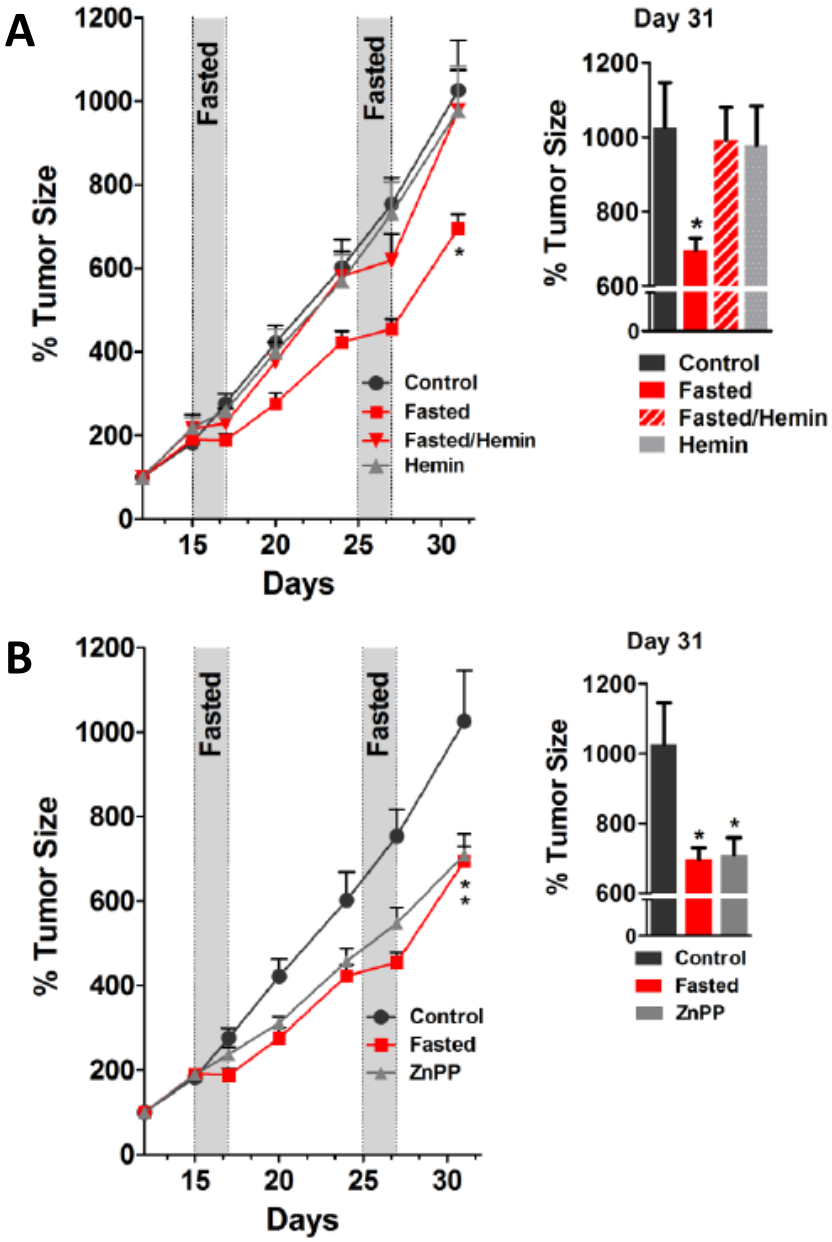

Figure 3-32. Effect of HO-1 modulation on tumor size in fasted mice.

(**A**) Mice bearing subcutaneous 4T1 breast tumors were treated intra-peritoneally with hemin, an inducer of HO-1 (30 mg/kg/day), during each 48 hour fast (N=6-8), or (**B**) ZnPP, an inhibitor of HO-1 (40 mg/kg/day) for 48 hours (D; N=6-8). The tumor size was measured as percent increase compared to the first tumor measurement at day 12 post tumor implantation. The panels on the right side demonstrate the final tumor volume for each treatment group at day 31 post tumor implantation. All data presented as mean ± SEM; * $p<0.05$; Student's *t*-test, Fasted or Control *vs.* Fasted/ Hemin or ZnPP. Collaborative data, not published.

conditions with or without CP treatment but demonstrated significantly less relative superoxide levels when treated with CP in fasting mimicking media conditions (**, P< 0.01). This data thus demonstrates that fasting and cyclophosphamide both, individually or in combination, increase mitochondrial superoxide production and that over-expressing HO-1 in *ad lib* mimicking conditions provides no additive beneficial effects but becomes increasingly important when cellular conditions are causing cell stress. The over-expression of HO-1 thus confers higher stress resistance when cells are exposed to fasting and or chemotherapeutic drugs and makes heme oxygenase-1 central to our hypothesis.

To test whether the stimulation HO-1 expression could reverse the anti-proliferative effects of fasting *in vivo*, we injected mice bearing subcutaneous 4T1 tumors with hemin (30 mg/kg/day; i.p.) during the fasting regimen. Two cycles of 60h fasting caused a significant reduction in tumor size, which was reversed by the concurrent hemin treatment (**Fig. 3-32, A**). Notably, normally fed mice treated with the HO-1 inhibitor ZnPP (40 mg/kg/day; i.p.), displayed a tumor size that was comparable to the tumor volume observed in fasted mice (**Fig. 3-32, B**).

However, these studies only tested the fasting depending reduction of tumor growth and did not consider chemotherapeutic agents. To test the role of HO-1 in the fasting-mediated sensitization of malignant cells to chemotherapeutic drugs, we next used the previously established HO-1 over-expressing 4T1 cell line, as well as cells expressing the empty pcDNA vector, to evaluate the tumor growth without the HO-1 modulation by hemin or ZnPP (**Fig. 3-33**). As expected, fasting significantly reduced the growth of the tumor derived from cells expressing the empty control vector. However, fasting did not retard the growth of grafted 4T1 tumors that over-express HO-1, but instead reversed growth in these tumors to a level that was not significantly different from the untreated tumor. Short-term fasting for two cycles, when combined with CP treatment, significantly reduced the tumor progression compared to the untreated and fasting-alone treated groups and was the most effective treatment option in the normal 4T1 cells. Over-expressing HO-1 on the other hand partially protected 4T1 cells from this fasting depending sensitization (**Fig. 3-33**). The tumor size, at day 32 post tumor implantation, for the 4T1 cells expressing high levels of HO-1 treated with STS and CP was not significantly different from normal 4T1 cells treated with fasting alone.

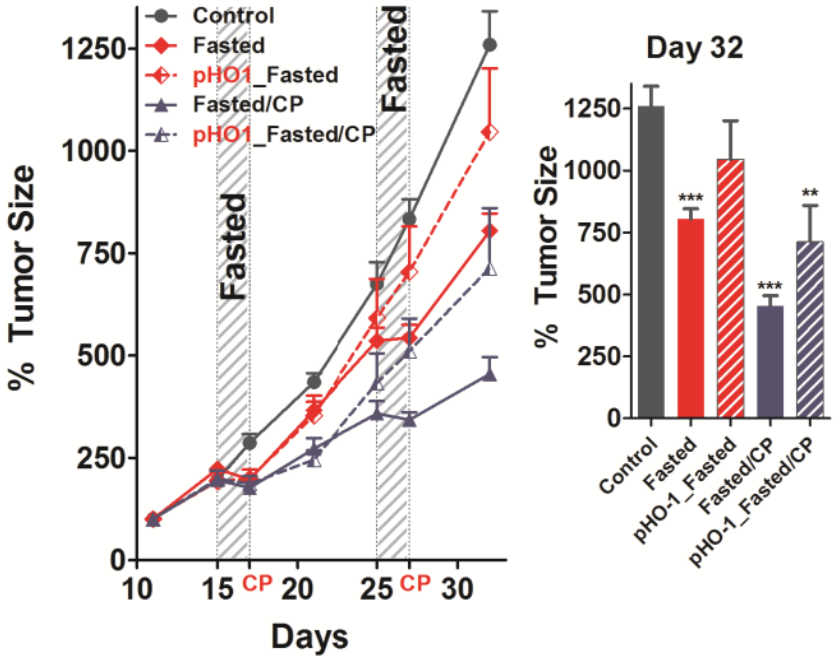

Figure 3-33. Effect of heme oxygenase-1 (HO-1) over-expression on the fasting-dependent sensitization in 4T1 cells.

Effect of fasting on tumor size in mice bearing subcutaneous 4T1 tumors over-expressing HO-1 (pHO-1) and control (empty vector) 4T1 tumors for 48 hours and treated with CP (150 mg/kg; i.p.; N= 8-10). Tumor values on day 32 are shown in the right panel. All data presented as mean ± SEM. Student's *t*-test, ** $P<0.01$, *** $P<0.001$, compared to the control group. Collaborative data, not published.

Together, these studies indicate that reduced HO-1 expression is important for the fasting-dependent sensitization of 4T1 breast cancer cells. Of note is however that "rescuing" HO-1 level, either through the induction with hemin or by over-expressing HO-1, does not fully reverse the fasting-mediated sensitization to chemotherapeutic agents (**Fig. 3-33**). Based on the fact that fasting causes a wide array of intracellular changes, including changes in genes associated with stress protection, as shown here with HO-1 and NFkB, as well as changes in the extracellular surroundings of the tumor (e.g. a lower blood pressure, hormonal changes, reduced availability of glucose and other metabolites necessary to maintain cellular proliferation), it is unlikely that fasting mediates its effect solely through heme oxygenase-1. Therefore, future experiments will need to address additional changes that cause tumor cells to become sensitized to chemotherapeutic agents by short cycles of starvation.

3.9 Dietary Interventions: The Effects of Short-Term Calorie Restriction on Glucose and IGF-I Levels

The studies I described above, including cell culture- and animal-models, indicate that fasting may actually reduce chemotherapy side effects by selectively protecting normal cells, while simultaneously sensitizing tumor cells to chemotherapeutic agents. However, we are aware of the fact that most oncologists will not actively recommend a fasting–based approach to their patients if alternative diet-based strategies are available. Dietary recommendations during cancer treatment are based on the prevention, or reversal, of nutrient deficiencies to preserve lean body mass and minimize nutrition-related side effects such as decreased appetite, nausea and taste- or bowel-changes (Doyle, Kushi et al. 2006). Consequently, for cancer patients who have been weakened by prior chemotherapy cycles or are emaciated, many oncologists could consider a fasting-based strategy to be potentially harmful. We thus aimed to establish if CR and/or diets defined in macronutrient composition (including dietary protein, carbohydrate and fat) can be used as an alternative to our short-term starvation based models of differential stress resistance (DSR) and differential stress sensitization (DSS).

Short-term starvation increases cellular protection against high-dose chemotherapy (Raffaghello, Lee et al. 2008; Lee, Safdie et al. 2010), and sensitizes malignant cells to chemotherapeutic drugs (Lee, Raffaghello et al. 2012; Safdie, Brandhorst et al. 2012). All these effects were linked to reduced serum levels of glucose and IGF-I. STS has the most pronounced effects once animals lose approximately 20% bodyweight, which occurs usually relatively fast after 60 hours of a water-only fasting regimen. Once at the 20% weight loss point, serum IGF-I is reduced by approximately 75%, and glucose by 70%. These are usually the lowest levels we can measure while still maintaining the capacity in mice to recover from the fasting cycle. Thus, we used the 20% weight-loss as a criterion to compare glucose and IGF-I levels of calorie restricted diets to those obtained from a 60 hour STS regimen.

We tested different calorie-restricted diets, ranging from a relatively mild 40% reduction to the very severe 90% reduction, in 12 to 15 week old female CD-1 mice. The 20% weight-loss threshold was reached at 4 days for 90% CR, 6 days for 80%

CR, 9 days for 60% CR, or 13 days for 40% CR (**Fig. 3-34, A**). As expected, the time to achieve 20% weight-loss is strongly dependent on the severity of the calorie restriction (linear fit with r^2= 0.9976; **Fig. 3-34, B**). At 48 hours, the reduction in blood glucose levels showed a trend to correlate with the severity of the calorie restriction (linear fit with r^2= 0.7931; data not shown). The 60 hour fasting regimen (STS) reduced blood glucose levels by 70% compared to that in *ad lib* fed mice (**Fig. 3-34, C**; P< 0.001). The 4 day 90% CR regime reduced blood glucose by approximately 40%, significantly less than STS (*P*< 0.05). In addition, we observed a trend for the effect of CR in lowering blood glucose depending on the length of CR-feeding: the glucose levels in the 13-day 40% CR feeding was significantly (P< 0.05) lower than in the 4 day long 90% CR group. However, no calorie restricted group resulted in blood glucose levels that were lower than in the 60 hour fasting group; and 9 or more days of CR were required to obtain glucose lowering effects in the range of those in the fasted group (**Fig. 3-34, C**). Mice of all experimental CR groups, independent of the severity of the restriction, reached similar serum IGF-I levels once the 20% weight-loss margin was reached and had significantly (P< 0.001) lower IGF-I levels than mice in the *ad lib* control group (**Fig. 3-34, D**).

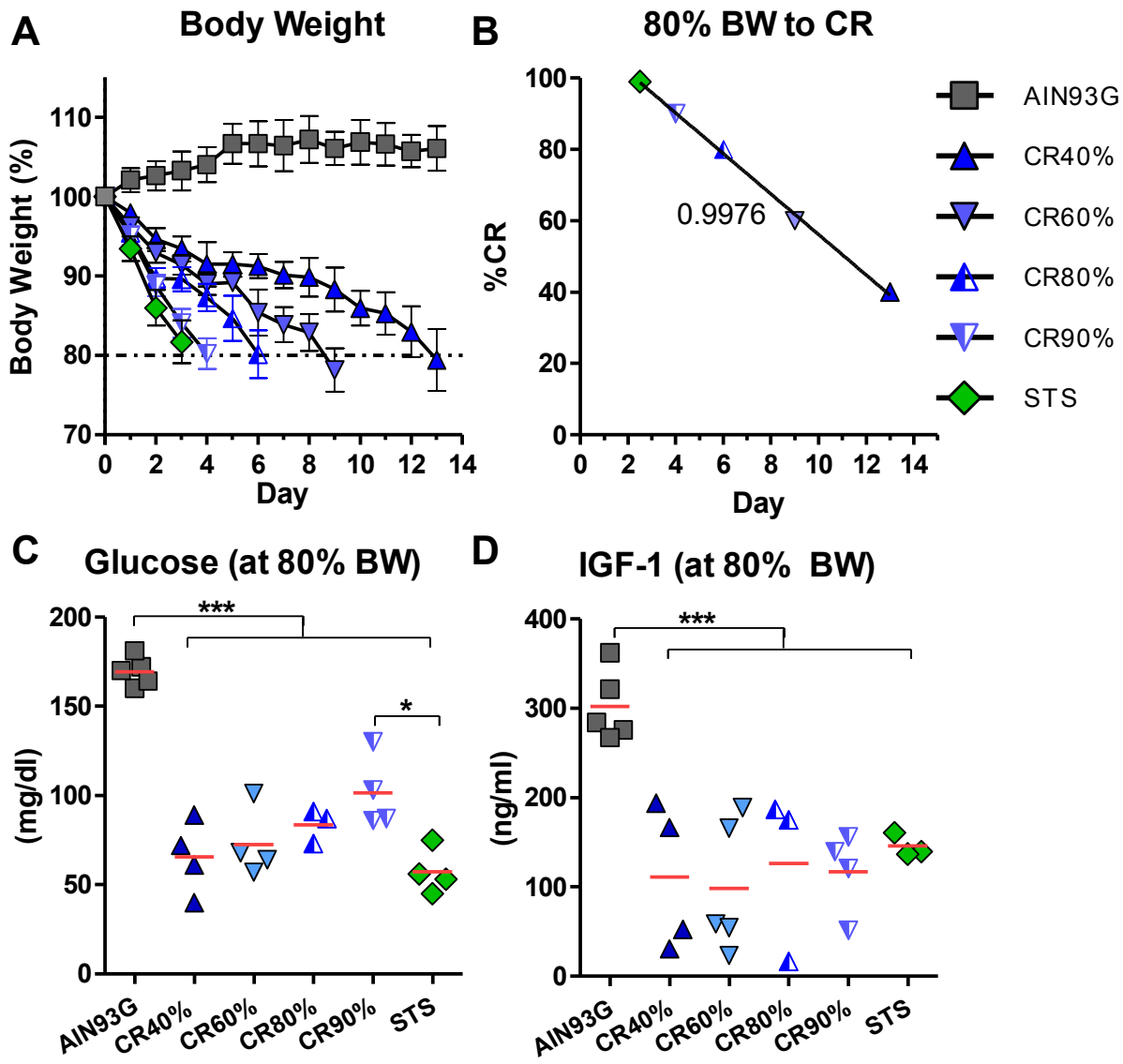

Figure 3-34. Calorie restriction reduces bodyweight, glucose and IGF-I.

(**A**) Female CD-1 mice, age 12-15 weeks were either fed *ad lib* (grey square) with AIN93G rodent standard chow, exposed to 40%, 60%, 80% and 90% calorie restricted (CR) AIN93G diets (triangles) or fasted (STS, green rectangle) until mice lost 20% of their initial bodyweight (dotted line). N= 5 per experimental group. All data presented as mean ± SEM. (**B**) Linear fit for the severity of the CR regimen *vs.* the duration (days) until 80% bodyweight was reached. (**C**) Blood glucose levels for mice once 80% bodyweight was reached. Red line represents mean (**D**) Serum IGF-I levels for mice once 80% bodyweight was reached. Red line represents mean; *** $p<0.001$, ANOVA, Tukey's multiple comparison. Modified from Brandhorst et al.; *under review*.

3.10 The Effect of Macronutrient Defined Diets on Glucose and IGF-I levels

We designed a set of macronutrient-defined diets (**Fig. 3-35**) based on the AIN93G rodent chow to determine whether the restriction of specific dietary constituents could mimic the effects of STS or short-term CR, on blood glucose and/or serum IGF-I. The low protein diets 20% P-1 (soybean oil as fat source) and 20% P-2 (coconut oil as fat source) have calories from protein sources reduced to 20% compared to the original AIN93G formulation while carbohydrates and fat are increased to maintain the diets isocaloric to AIN93G. The 0% P diet contains no protein; carbohydrates as well as fat are increased proportionally to keep the diet isocaloric to the standard chow. The LCHP diet has the calories from carbohydrate sources reduced to 20% compared to the original AIN93G formulation (13% vs. 63.9%) but supplies more protein and fat. The high fat ketogenic diet 60% HF was designed to supply 60% of the consumed calories from fat sources, the calories coming from protein and carbohydrates were reduced proportionally. The 90% HF diet is a ketogenic diet that contains 90% of the calories from fat while supplying only minimal (less than 1%) carbohydrates and has 9% of the calories from protein. Due to the higher fat proportions, the LCHP (4.4 kcal/g), 60% HF (5.3 kcal/g) and 90% HF (7.1 kcal/g) diets have a high caloric-density compared to the AIN93G standard chow (3.8 kcal/g) (**Fig. 3-35**).

Female CD-1 mice were fed *ad lib* with the experimental diets for nine consecutive days to establish bodyweight profiles (**Fig. 3-36, A** and **C**) and to monitor the caloric intake (**Fig. 3-36, B** and **D**). We did not observe any significant food aversion but noticed that mice fed with the diet lacking proteins completely (0% P) reduced food consumption after 6 days (**Fig. 3-36, B**). The reduced calorie intake caused weight-loss for animals in this experimental group (**Fig. 3-36, A**). Mice in the ketogenic high-fat groups (60% HF and 90% HF) consumed more calories during the 9 days of feeding than mice fed with the AIN93G standard chow (**Fig. 3-36, D**) and mice fed *ad lib* with the ketogenic 90% HF diet rapidly gained weight after 4-5 days (**Fig. 3-36, D**). CD-1 mice in the experimental groups fed with diets 20% P and LCHP showed no difference in calorie intake or bodyweight compared to the mice fed with the AIN93G control diet (**Fig. 3-36, A** and **B**).

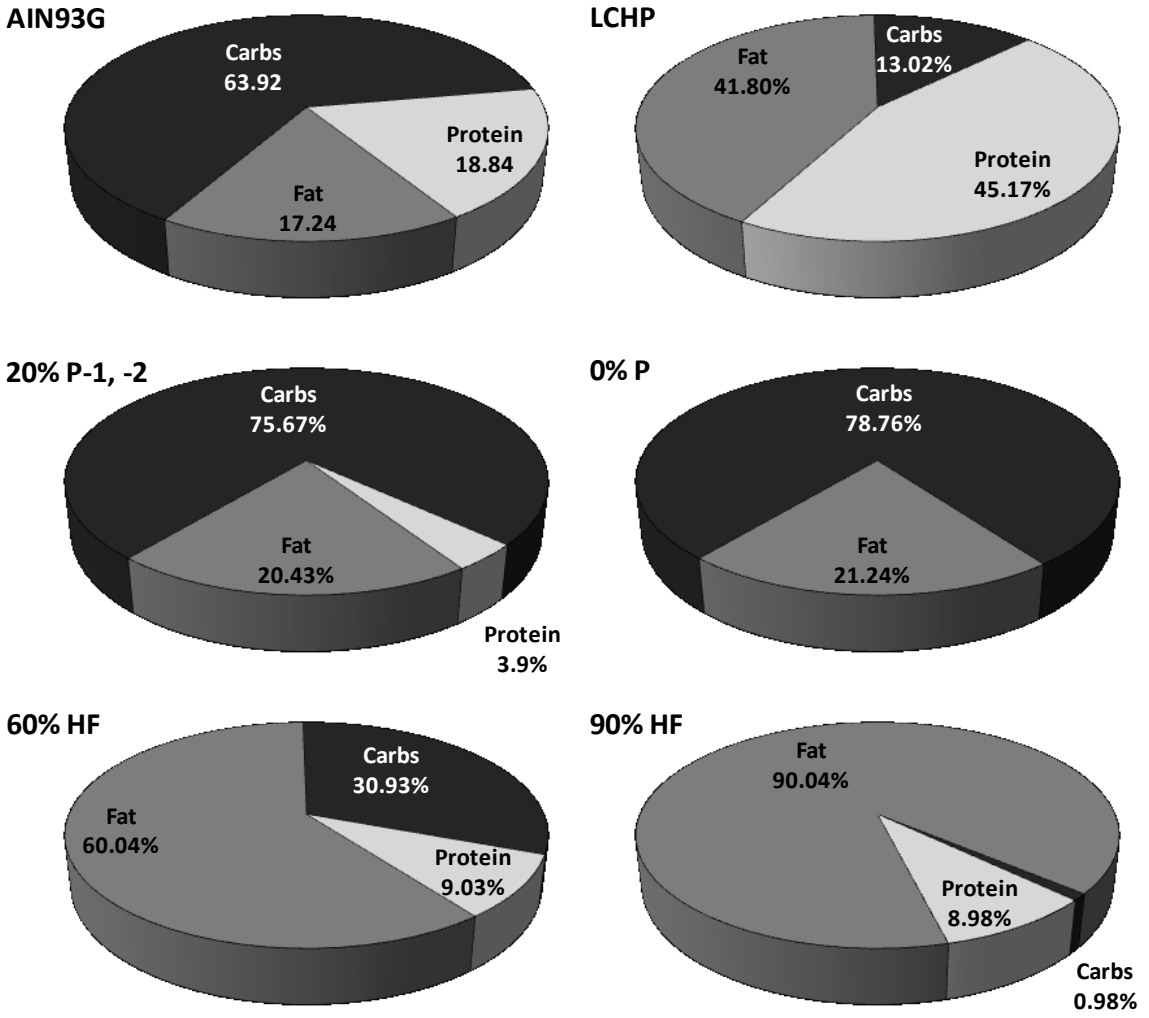

Figure 3-35. Calories supplied by macronutrients of experimental diets in %.

AIN93G standard chow was the reference diet and supplied to all mice. The experimental diets modified in the macronutrient composition (fat, protein and carbohydrates) were all based on this diet. The low carbohydrate LCHP diet had calories from carbohydrates reduced to 20% compared to the AIN93G formulation (13% *vs*. 63.9%) but contained more protein (45.2%) and fat (41.8%). Diets 20% P-1 (soybean oil as fat source) and 20% P-2 (coconut oil as fat source) had calories from protein sources reduced to 20% compared to the AIN93G formulation; the 0% P diet contained no protein; all these diets were isocaloric to the AIN93G standard chow. The ketogenic high fat diet 60% HF was designed to supply 60% of the consumed calories from fat sources. The calories coming from protein and carbohydrates were reduced proportionally. The 90% HF diet was a ketogenic diet which contains 90% of fat while supplying only minimal carbohydrates (less than 1%) and half of the protein content (9%). Modified from Brandhorst et al.; *under review*.

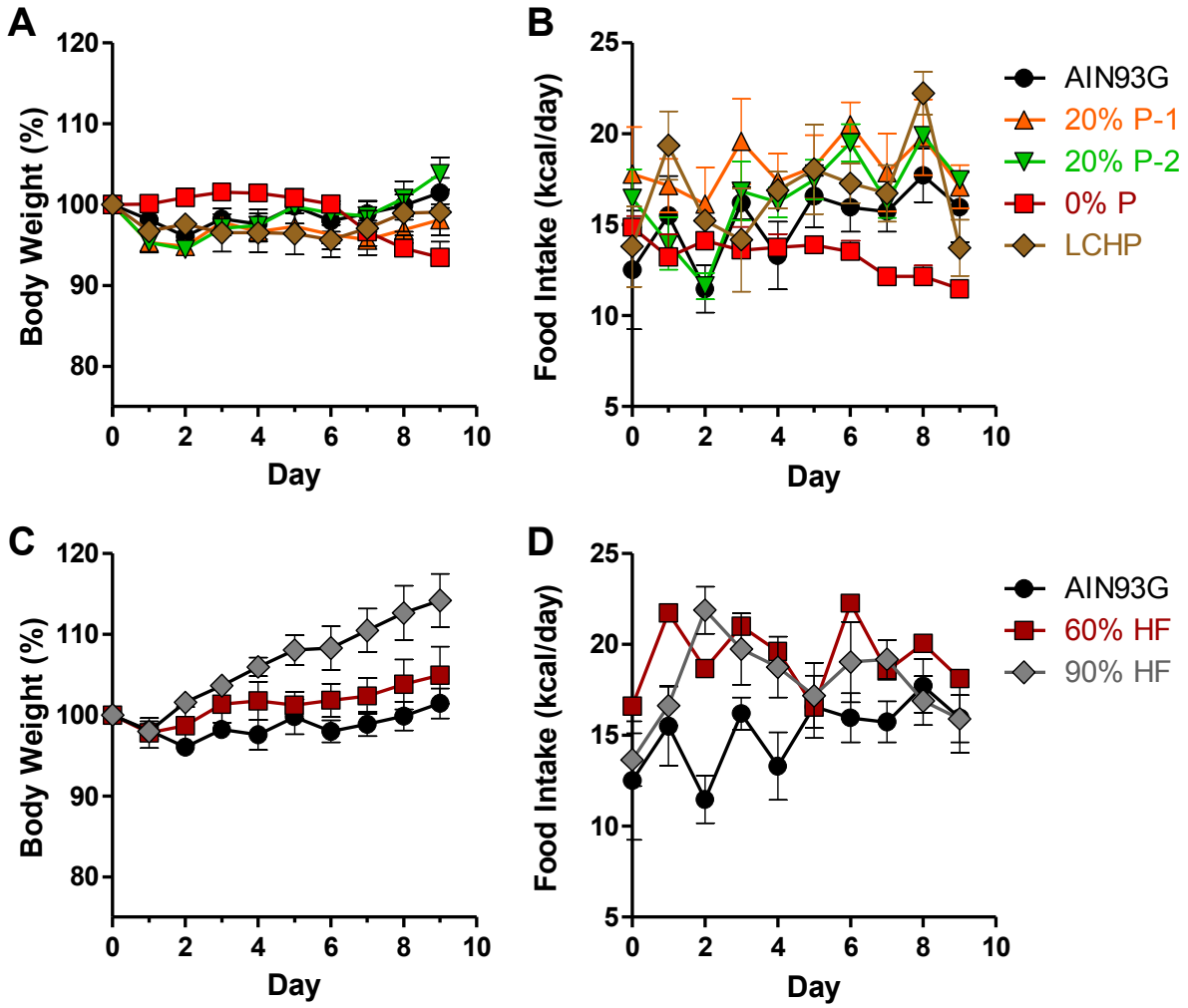

Figure 3-36. Effects of macronutrient defined diets on bodyweight and food intake.

Five female CD-1 mice, age 12-15 weeks were either fed *ad lib* with AIN93G rodent standard chow (black circle) or with (**A**) two different low protein diets (20% P-1 and 20% P-2), a diet low in carbohydrates but high in protein (LCHP), a protein deficient diet (0% P) or (**C**) a high fat diet (60% HF) and ketogenic diet (90% HF). (**B**) Daily *ad lib* calorie intake for diets AIN93G, 20% P-1, 20% P-2, LCHP and 0% P. (**D**) Daily *ad lib* calorie intake for diets 60% HF and 90% HF; AIN93G shown as reference. All data presented as mean ± SEM. Modified from Brandhorst et al.; *under review*.

At days 2, 5 and 9, blood glucose levels from mice on the macronutrient modified diets were not different from those on the standard chow diet (**Fig. 3-37, A** and data not shown). By contrast, serum IGF-I levels were significantly elevated (P< 0.05) in mice on the ketogenic 60% HF diet for 9 days but not for mice fed with the ketogenic 90% HF diet (**Fig. 3-37, B**). Interestingly, not only the macronutrient composition (e.g. the protein content) but also the fatty acid source differentially modulated circulating IGF-I levels: the low protein diet 20% P-1 (containing soybean oil as the only fat source) did not reduce IGF-I levels but the low protein diet 20% P-2 (coconut oil as the only fat source) significantly (P< 0.05) reduced IGF-I levels and there were no differences in these diets other than the fat source. The most noticeable effect on serum IGF-I was in mice fed the protein deficient diet 0% P for 9 days. Circulating IGF-I was reduced to approximately 30% of that in mice on the standard chow (**Fig. 3-37, B**). The protein deficient diet 0% P was the only diet that reduced serum IGF-I levels comparable to the 60 hour short-term starvation.

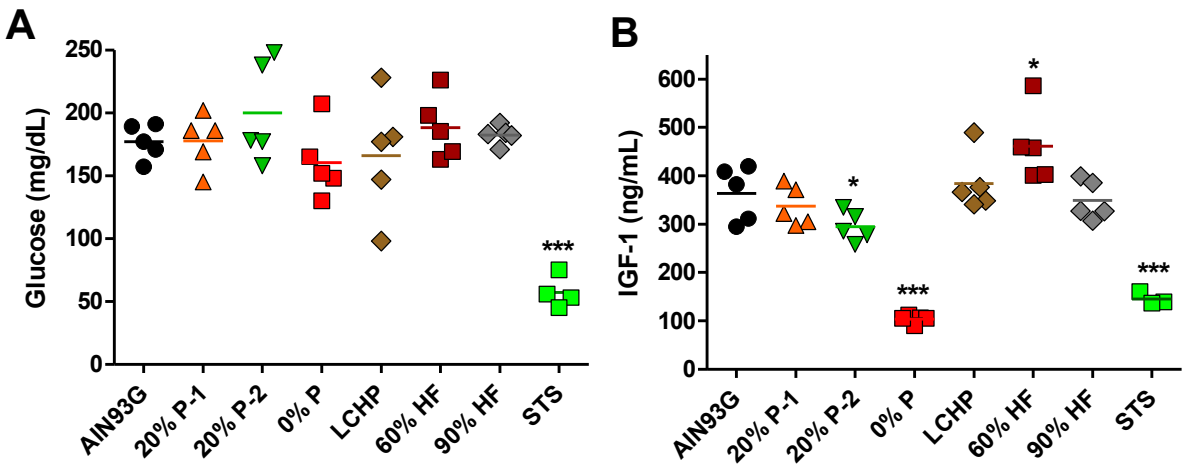

Figure 3-37. Effects of macronutrient defined diets on glucose and serum IGF-I.

Five female CD-1 mice, age 12-15 weeks were either fed *ad lib* with AIN93G rodent standard chow or fed with macronutrient defined diets as described in detail in the text. (**A**) Blood glucose levels after 9 days of *ad lib* feeding. STS (60 hours) as a reference. (**B**) Serum IGF-I levels after 9 days of *ad lib* feeding. STS (60 hours) as a reference. Lines represent mean; *p<0.05,*** p<0.001, ANOVA, Tukey's multiple comparison compared to AIN93G control. Modified from Brandhorst et al.; *under review*.

3.11 Short-term Caloric Restriction and Fasting improve Stress Resistance

In mice, reduced serum IGF-I and blood glucose levels promote the capability to cope with toxicity induced by high-dose chemotherapeutic agents (Lee et al., 2010; Raffaghello et al., 2008). Since short-term calorie restriction, but not the macronutrient defined diets (except for complete protein removal), reduced IGF-I and glucose levels, we used a combined approach to test whether diets with defined macronutrient deficiency fed at 50% of the regular daily calorie intake could result in enhanced chemo toxicity protection. 20% P diets were not included in the stress resistance experiments due to the fact that the 0% P diet showed much more pronounced effects on serum IGF-I.

Stress resistance was tested in CD-1 mice fed either *ad lib* with AIN93G standard chow or with macronutrient defined diets reduced to 50% of the normal calorie intake for three days prior to doxorubicin (DXR, 24 mg/kg, i.v.) treatment (**Fig. 3-38**). In the 50% calorie restricted groups mice lost 12-15% of their initial bodyweight after 3 days, whereas in the STS group mice lost 20% of their weight after 60 hours. Following DXR treatment, AIN93G chow was provided *ad lib* for all animals and the mice regained weight until chemotoxicity-induced weight-loss set in (**Fig. 3-39, A** and **B**). The weight-loss continued in all experimental groups until day 8 post injections, after which many animals slowly recovered. Mice fed the calorie restricted 0% P and LCHP diets never fully recovered their initial weight loss (**Fig. 3-39, A**). Animals started to succumb to chemotoxicity 9-18 days post injection (**Fig. 3-38**), in agreement with the reported onset and nadir days of myelo-suppression after DXR treatment (http://dailymed.nlm.nih.gov). Mice were considered survivors if they remained alive 25 days post DXR injection. Mice fed *ad lib* with the AIN93G diet ,3 days prior to DXR injection, showed the worst outcome with only 16% surviving by day 25 (**Fig. 3-38**). In contrast to the *ad lib* fed mice, the great majority (89%) of fasted (60 hours) mice survived the high-dose chemotherapy. Control mice treated with DXR showed signs of toxicity including reduced mobility, ruffled hair and hunched back posture whereas mice in the STS group showed no visible signs of stress or pain after the treatment (data not shown). Three-day feeding of the combination of 50% CR with macronutrient modification prior to DXR injection improved the stress resistance in mice and resulted in 45-55% survival (**Fig. 3-38**).

There was no indication that fat or carbohydrate content affected the results because all diets achieved a similar protection. The data indicates that short-term CR, not the fat or carbohydrate composition of the diet, confers partial chemo-protection which are not as potent as to those caused by fasting. Mice fed the 50% CR LCHP diet performed worse than all other CR fed groups, presumably because of the effect of the high protein content of this diet on IGF-I (**Fig. 3-35**).

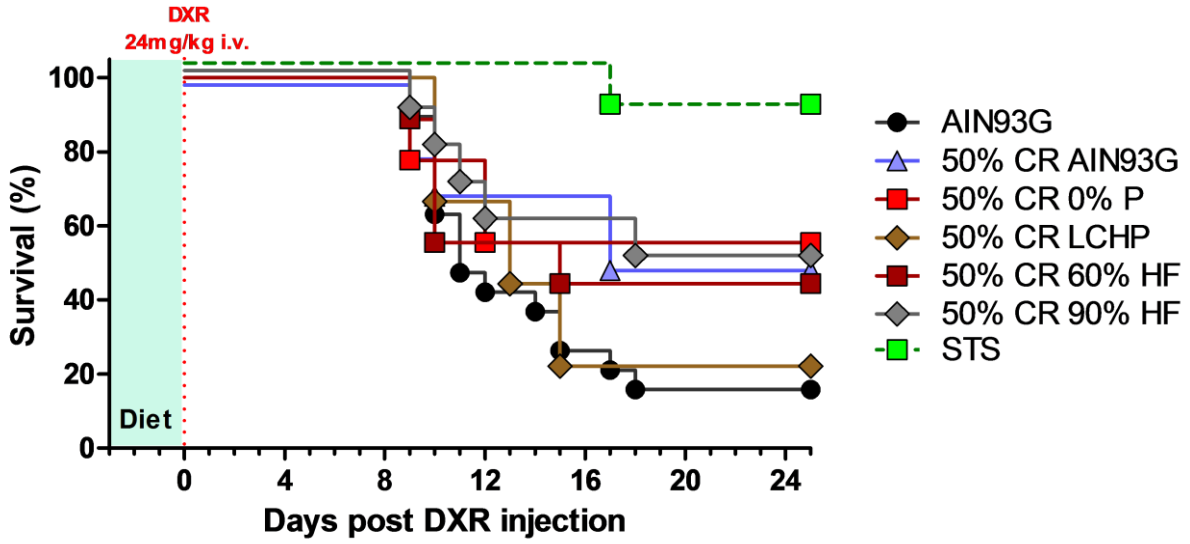

Figure 3-38. Stress resistance test for calorie restricted macronutrient defined diets.

Female CD-1 mice were fed *ad lib* (AIN93G), were fasted for 60 hours (STS) or fed with 50% calorie restricted diets (CR) with defined macronutrient compositions (AIN93G, LCHP, 0% P, 60% HF, 90% HF) for 3 days (green box) prior to an intra-venous injection of doxorubicin (24 mg/kg, red dashed line). Survival was followed for 25 days post injection, after which the remaining animals were considered survivors. Survival data plotted from pair-matched pooled experiments with the statistical software Prism (GraphPad Software). Modified from Brandhorst et al.; *under review*.

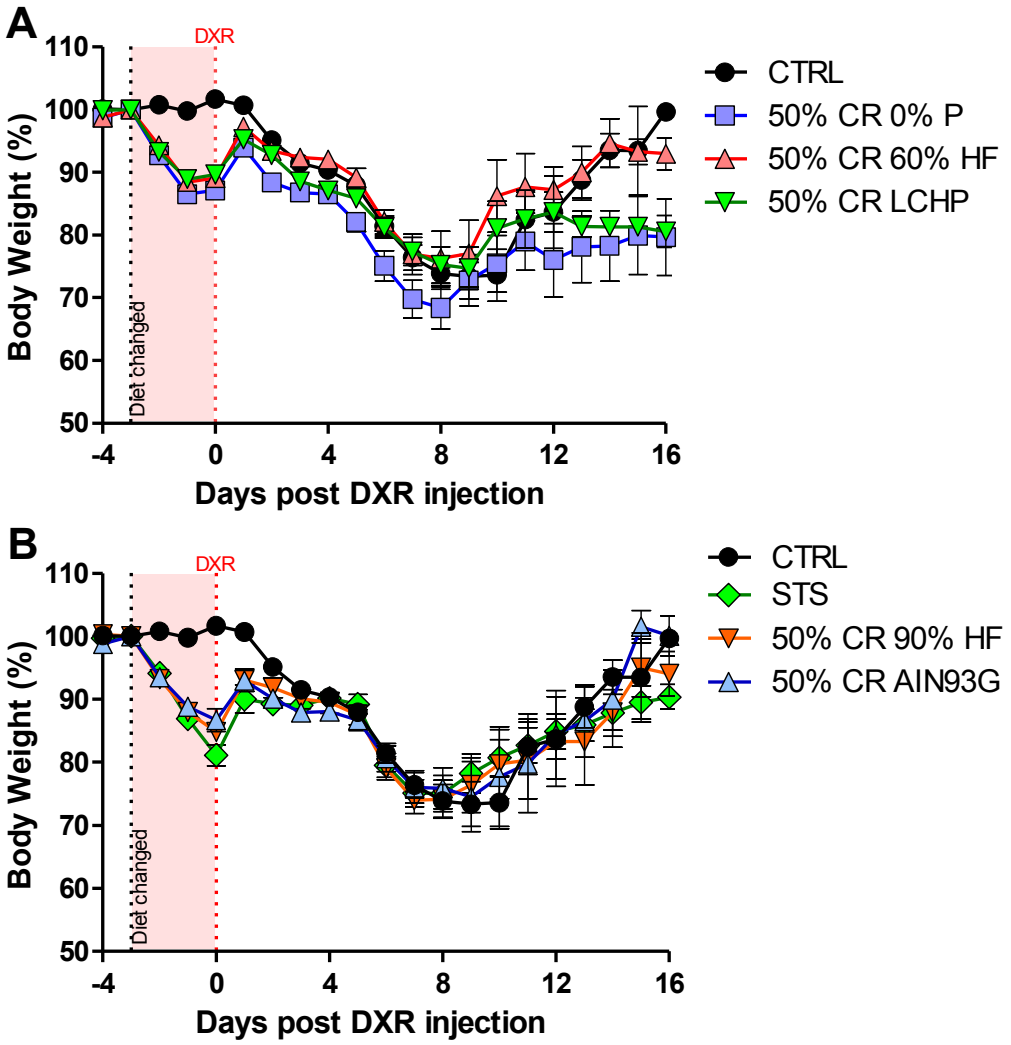

Figure 3-39. Bodyweight profile during the stress resistance test for calorie restricted macronutrient defined diets.

(**A**) Bodyweight profile for mice that were fed *ad lib* (AIN93G) or fed with 50% calorie restricted (CR) diets with defined macronutrient compositions (60% HF, LCHP, 0% P) for 3 days (green box) prior to an intra-venous injection of doxorubicin (24 mg/kg, red dashed line). (**B**) Bodyweight profile for mice that were fed *ad lib* (AIN93G), fed with 50% calorie restricted diets with defined macronutrient compositions (AIN93G, 90% HF) for 3 days or were fasted for 60 hours (red box) prior to an intra-venous injection of doxorubicin (24 mg/kg, red dashed line). Modified from Brandhorst et al.; *under review*.

Blood glucose measurements showed that three-day feeding of the calorie restricted and macronutrient modified diets was not sufficient to significantly reduce glucose levels, with the exception of the 50% CR ketogenic 90% HF diet (**Fig. 3-40**). The reduction in glucose levels in the ketogenic group however was not associated with enhanced stress resistance (**Fig. 3-38**). Mice in the STS group had significantly lower blood glucose levels than all other experimental groups (**Fig. 3-40**).

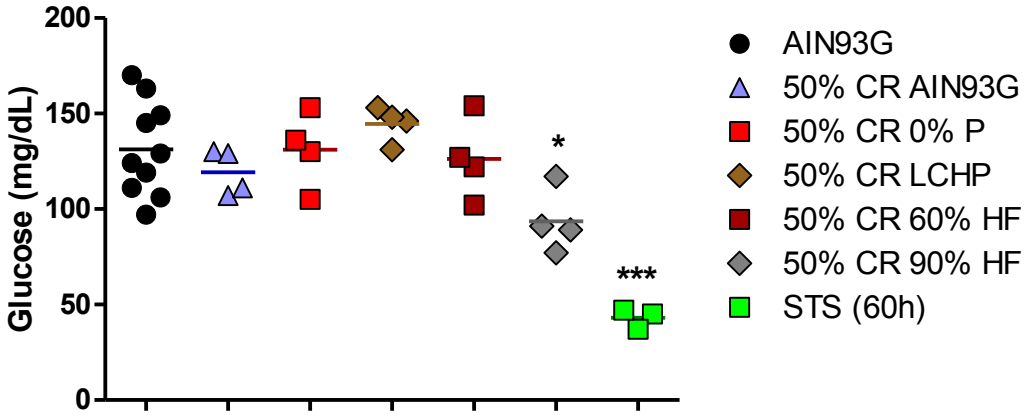

Figure 3-40. Blood glucose for calorie restricted macronutrient defined diets after 72 hours.

Blood glucose levels were measured after 3 days of feeding *ad lib* and CR diets, as well as after 60 hours of STS. Lines represent mean.* $p<0.05$, *** $p<0.001$, ANOVA, Tukey's multiple comparison. Modified from Brandhorst et al.; *under review*.

3.12 A Low Protein Diet does not Delay GL26 Glioma Progression

Diets low in protein have been shown to lower cancer risks (Youngman 1993) while high-calorie and high-protein diets are linked to obesity and promote hormonal, metabolic, and inflammatory alterations that modulate carcinogenesis (Kaaks and Lukanova 2002; Calle and Kaaks 2004). To test the effects of a low protein diet in a subcutaneous glioma model we switched mice from the standard chow (18.8% protein-derived calories, **Fig. 3-35**) to a diet low in protein (20% P-1, 3.9% protein-derived calories) 10 days after the implantation of GL26 cells when the tumor became palpable (**Fig. 3-41**). Low protein diet fed mice displayed tumor progression that was not distinguishable from that in mice fed *ad lib* with the AIN93G diet (**Fig. 3-41**). These results indicate that the tumor progression could not be retarded by protein-restriction once the tumor was established.

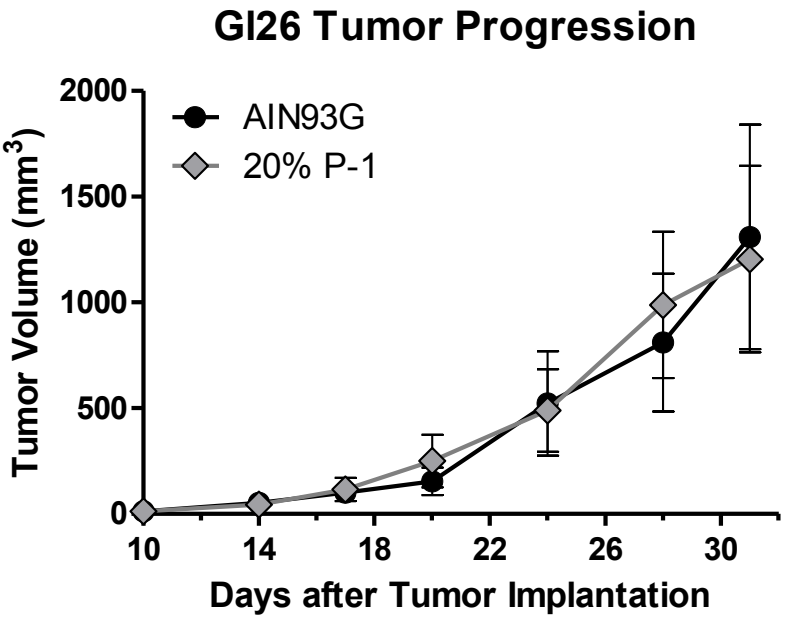

Figure 3-41. The Effect of protein restriction on GL26 tumor progression *in vivo.*

Subcutaneous tumor progression of murine GL26 glioma is shown by total tumor volume in mm^3. Tumor measurements were started once the tumors became palpable under the skin at day 10. Animals were fed *ad lib* with either AIN93G (N= 5) as a control, or with the low protein diet 20% P-1 (N= 6). All data presented as mean ± SEM. Modified from Brandhorst et al.; *under review*.

3.13 Short-term Intermittent Calorie Restriction does not Enhance Efficacy of Chemotherapy against Breast Cancer

The efficacy of STS in augmenting the treatment of various cancers is two-fold: it protects normal cells/tissues against chemotherapy-induced toxicity and sensitizes malignant cells to chemotherapeutic agents (Lee, Safdie et al. 2010; Lee, Raffaghello et al. 2012; Safdie, Brandhorst et al. 2012). Nonetheless, even short cycles of fasting (e.g. 4 days) can be a challenge for the majority of people and thus the “milder” calorie restricted approach could be a more feasible solution. To test whether a short-term intermittent 50% CR (ICR) diet could result in similar beneficial effects as the established fasting protocols, we implanted murine 4T1 breast cancer cells subcutaneously into female BalB/C mice and monitored the tumor progression. Twelve days after tumor implantation, the tumor volume was measured and mice were assigned to either the untreated control group (AIN93G), a group treated with

cisplatin (CDDP) or a group intermittingly fed with 50% CR (ICR) for three days prior to cisplatin treatment. The tumor in the untreated control group progressed rapidly and reached the experimental endpoint 54 days after tumor implantation (**Fig. 3-42**). Three cycles of cisplatin treatment delayed the tumor progression; the tumor volume of these mice was approximately half the size of that in untreated mice (**Fig. 3-42**). In contrast to STS (Lee, Raffaghello et al. 2012), an intermittent 50% calorie restricted AIN93G feeding regimen fed to mice for three days prior to the cisplatin injections did not result in the sensitization of the tumor and did not augment the chemotherapy (**Fig. 3-42**). Tumor volumes in this experimental group did not significantly differ from tumor volumes in mice that were treated with cisplatin alone.

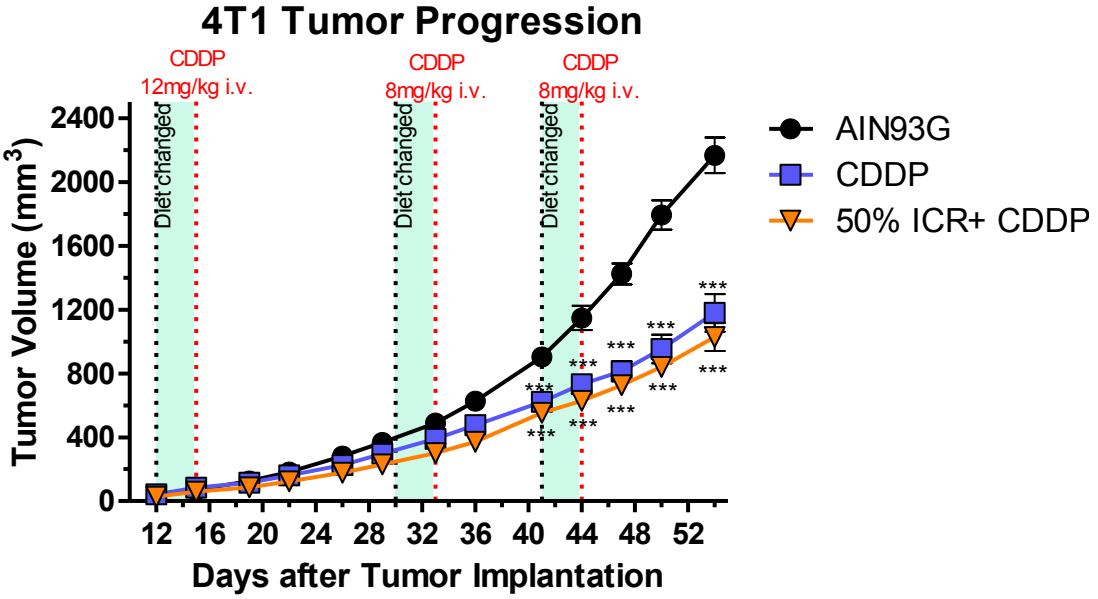

Figure 3-42. The effect of caloric restriction on 4T1 tumor progression *in vivo.*

Subcutaneous tumor progression of murine 4T1 breast cancer is shown by total tumor volume in mm^3. Tumor measurements were started once the tumors became palpable under skin at day 12. Control animals (N= 10) received no treatment, cisplatin (CDDP) animals (N= 9) were injected at days 15, 33 and 44. The first CDDP dose was delivered at 12 mg/kg by intra-venous injection, the two subsquent injection were delivered at 8 mg/kg to avoid chemotoxicity. Mice in the 50% ICR+ CDDP group (N= 9) were fed in intermittent regimens with the AIN93G diet reduced to 50% of the daily calorie intake of the control group for three days (ICR, green box) prior to cisplatin injection. Injection schedule inditical as for the CDDP group. All data presented as mean ± SEM; *** $p<0.001$, ANOVA, Tukey's multiple comparison, compared to control of the respective day. Modified from Brandhorst et al.; *under review*.

4 Discussion

Most cancer drugs are being developed for specific tumor types, and in many cases, they are effective for only a subset of patients with a specific tumor. Furthermore, the development of each of these targeted cancer drugs is extremely costly, and assembling sufficient data for Food and Drug Administration approval requires years of clinical testing (Crown 2001; Garattini and Bertele 2002). Although there are undoubtedly targeted drugs that will be effective in the treatment of specific cancers, there would be advantages to therapies that could be available sooner, at a lower cost, and that could be effective for a wide range of cancers. Here, we have taken advantage of the relative independence of cancer cells from growth signals and their unresponsiveness to anti-growth signals (Hanahan and Weinberg 2011) to show that this inability of cancer cells renders them unable to cope effectively with the markedly altered cellular environments during short cycles of complete starvation (Lee, Raffaghello et al. 2012; Safdie, Brandhorst et al. 2012).

4.1 Fasting vs. Calorie Restriction

Fasting is a simple, cost-effective, and feasible dietary intervention to increase cellular protection. We have previously reported its ability to selectively protect the host, but not malignant cells, to chemotherapy agents (Raffaghello, Lee et al. 2008; Lee, Safdie et al. 2010). Here, I provide evidence for a fasting-dependent sensitization of a wide variety of murine and human cancer cells. Nutrient and growth factor deprivation causes the sensitization to various chemotherapy drugs and thereby increases the chemotherapeutic index for *in vivo* and *in vitro* treatment modalities. However, the beneficial effects of fasting do not reside in the field of cancer treatment alone, but extend even further to renal (Mitchell, Verweij et al. 2010), hepatic (Domenicali, Caraceni et al. 2001; Verweij, van Ginhoven et al. 2011), cerebral (Go, Prenen et al. 1988), and cardiac ischemic injury (Varela, Marina Prendes et al. 2002), which cause considerable cellular damage by glucose restriction, hypoxia, and oxidative stress (Loor and Schumacker 2008). Furthermore, fasting also imparts a potent anti-inflammatory effect *in vivo* (Mitchell, Verweij et al. 2010), whose role in enhancing cancer-therapy and reducing the side effects of the chemo-treatment warrants further investigation (Zitvogel, Kepp et al. 2011).

Adaptation to fasting involves a multiplicity of physiological responses including metabolic changes, e.g. the switch to alternative nutrient sources due to reduced glucose availability and the reduction of growth factors such as IGF-I (Greenspan 2001; Strohman 2002; Lee, Safdie et al. 2010). In this thesis, and in previously published results, we have shown that a major reduction in blood glucose and IGF-I levels is partly responsible for the beneficial effects of DSR and DSS. In mice, a 60 hour short-term fasting regime reduces bodyweight by 20% or more, serum IGF-I by up to 75%, and glucose by up to 70%, increases IGFBP-1 11-fold and allows animals to regain their normal weight rapidly after re-feeding (Lee, Safdie et al. 2010). Under these conditions, animals become highly stress resistant (Raffaghello, Lee et al. 2008; Lee, Safdie et al. 2010), in agreement with results obtained from *S. cerevisiae* (Longo 1999). When we applied 20% weight-loss as an experimental endpoint, various degrees of CR regimens resulted in progressively quicker weight loss and also reduced IGF-I and glucose. However, we also observed that the much shorter (60 hours) STS regimen had more pronounced effects on blood glucose level than most of the CR diets, even when the CR diets were maintained for 9-13 days and caused an equivalent 20% weight loss.

For established murine breast cancer allografts, the combined treatment with short-term intermittent 50% CR and cisplatin did not result in the augmentation of chemotherapy efficacy (in contrast to the combination of STS and chemotherapy). The results I describe suggest that short cycles, up to 72 hours, of a 50% CR did not significantly reduce blood glucose levels and may not cause a sufficient reduction in the carbon sources metabolized by murine breast cancer cells within this regime. Thus, short periods of an intermittent CR regiment, although “milder”, are not feasible to reduce tumor progression and to induce tumor sensitization. Still, this does not exclude that long-term CR regiments might have beneficial effects. Seyfried et al. demonstrated that calorie restriction reduced biomarkers of tumor progression and angiogenesis (Kari, Dunn et al. 1999; Yu and Rohan 2000) and led to a reduction in tumor growth in a CT-2A malignant mouse astrocytoma model. However a 13 day 40% CR resulted only in a 45% reduction in IGF-I levels when compared to *ad lib* fed control animals, and also caused a major and chronic weight loss (Seyfried, Sanderson et al. 2003).

The less pronounced effects of calorie restricted diets, when compared to short-term starvation, might be explained by a distinct physiological response that is unique to conditions under which nutrients are completely absent (Lee and Longo 2011). For example, the decrease in blood glucose caused by short-term fasting in this study was 70% and occurred within 60 hours *vs.* the 40% glucose reduction caused by a 90% CR diet after 96 hours. When deprived of food, mammals generally undergo distinct metabolic stages (Wang, Hung et al. 2006) and these changes trigger the down-regulation of pathways involved in proliferation, cell growth and the reduced production of reactive oxygen species, while simultaneously increasing genomic stability and cellular stress resistance (Sohal and Weindruch 1996; Hursting, Slaga et al. 1999; Holzenberger, Dupont et al. 2003; Longo and Fontana 2010; Lee and Longo 2011).

The effects of prolonged CR have been known for over a century now (Moreschi 1909; McCay CM 1935) and one could argue why calorie restriction as a treatment modality is generally not considered. The problems associated with translating CR into any clinical application is that long-term CR delays, but does not stop, the progression of many malignant diseases (Mukherjee, Abate et al. 2004; Bonorden, Rogozina et al. 2009). CR is also associated with a chronic reduced weight state that might be detrimental for cachectic cancer patients, or patients at risk to become cachectic. In addition, CR might chronically reduce fat and other reserves that may increase frailty, particularly in elderly patients (Seyfried, Sanderson et al. 2003). In fact, prolonged CR can delay wound healing and immune function, which might present an additional hurdle for the great majority of patients receiving chemotherapy or undergoing surgery (Kim and Demetri 1996; Reed, Penn et al. 1996; Kristan 2008; Fontana, Partridge et al. 2010). Furthermore, the 75% reduction in serum IGF-I caused by a 2 to 5 day fast in mice and humans cannot be achieved by a more moderate CR which does not reduce IGF-I levels in humans unless the protein intake is also restricted (Clemmons and Underwood 1991; Fontana, Weiss et al. 2008; Lee, Safdie et al. 2010). Even when combined with protein restriction, chronic CR only causes a 30% reduction of IGF-I in humans (Fontana, Weiss et al. 2008).

By contrast, based on the DSR studies and on clinical data, fasting for a limited period can have a potent protective effect on the host. In a recent report published by our lab, 10 patients with a variety of malignancies, including tumor stages IA to IV/DI, voluntarily fasted for up to 180 hours in combination with chemotherapy, and reported reductions in common chemotherapy-associated side-effects such as vomiting, diarrhea, fatigue and weakness (Safdie, Dorff et al. 2009). Notably, in the cases where cancer progression could be followed, there was no evidence that fasting protected tumors or interfered with chemotherapy efficacy. At the University of Southern California Norris Comprehensive Cancer Center (Los Angeles, CA, USA), the Mayo Clinic Cancer Center (Rochester, MN, USA) and the Leiden University Medical Center (Leiden, Netherlands), there are currently ongoing clinical trials to study the effects of shorter fasting cycles (two to three days) in combination with chemotherapy in cancer patients (ClinicalTrials.gov Identifier: NCT00936364, NCT01175837, NCT01304251), which should begin to reveal the potential of STS in the enhancement of cancer treatment for humans within the next few years. Notably, both in the ongoing clinical trials and in the case studies, patients fasted (water only) for 48-72 hours before and also for 24 hours after chemotherapy. A period of fasting after chemotherapy administration may be of particular importance because chemotherapy drugs can act as carcinogens by causing DNA damage, potentially leading to secondary tumors and other toxicities (Carter 1984; Margison, Santibanez Koref et al. 2002). In fact, the combination of re-feeding after fasting and treatment with potent carcinogens can enhance the growth of aberrant crypt foci in liver, colon and rectum (Laconi, Tessitore et al. 1995; Premoselli, Sesca et al. 1998; Sesca, Premoselli et al. 1998), probably because prolonged fasting can cause cell death and atrophy in organs such as the liver. Re-feeding, which causes the cell proliferation needed to re-establish normal organ size, may lead to DNA damage if high concentrations of toxins are still present. Chemotherapy drugs are generally not potent carcinogens and we showed that toxicity to multiple organs is reduced, not increased, even when re-feeding is initiated immediately after chemotherapy (Raffaghello, Lee et al. 2008; Safdie, Dorff et al. 2009; Lee, Safdie et al. 2010). Still, our recommendation is that the period of fasting after chemotherapy should be based on the half-life of the chemotherapy drug to minimize toxicity to

normal cells when re-feeding begins. In the near future, animal and clinical studies should focus on the investigation of the effect of post-chemotherapy fasting.

4.2 The Induction of Differential Stress Sensitization by STS

Mutations that increase cellular protection and extend longevity (e.g. growth hormone deficiencies) often cause reduced growth and fertility (Kirkwood, Kapahi et al. 2000). Changes in the extracellular environment, such as caused by fasting, can have similar effects: In simple organisms such as *S. cerevisiae*, stress resistance to oxidants and chemotherapeutic drugs can be increased by up to 10-fold in response to fasting and up to 1,000-fold in those cells lacking homologs of Ras, AKT and S6 kinase (Raffaghello, Lee et al. 2008). Nevertheless, this improved resistance to oxidative stress can be completely reversed, and even further decreased below wild type levels, in yeast cells expressing oncogene-like genes such as sch9 (Longo and Finch 2003; Raffaghello, Lee et al. 2008), thereby linking stress resistance to the GH/IGF-1 axis.

The IGF-I pathway has been shown to affect both animal life span and sensitivity to oxidative stress, consistent with the greater resistance to oxidative stress in IGF-I receptor deficient mice (Holzenberger, Dupont et al. 2003). The forkhead box protein O1 (FOXO1), a down-stream target of IGF-I/AKT signaling, can enter the nucleus, in the absence/reduction of IGF-I/AKT signaling, where it can modulate a wide array of genes involved in oxidative stress resistance, longevity, and metabolism (Kim, Kim et al. 2008; Partridge and Bruning 2008; Guevara-Aguirre, Balasubramanian et al. 2011). In consequence, it is a key mechanism involved in protection against age associated stress and disease development. IGF-I exerts a potent tumorigenic effect on a variety of cancer cells by increasing their proliferative rate and inhibiting apoptosis (Prisco, Romano et al. 1999; Ramsey, Ingram et al. 2002). Studies in mice with deficiencies in the downstream effectors of IGF-R signaling, including mTOR inhibition by rapamycin (Harrison, Strong et al. 2009) and S6K1 (Selman, Tullet et al. 2009), demonstrate the central role of intracellular mitogenic pathways downstream of IGF-I in regulating lifespan and stress resistance while simultaneously reducing tumor growth (Pinkston, Garigan et al. 2006; Garcia, Busuttil et al. 2008; Ikeno, Hubbard et al. 2009). In addition, humans with growth hormone receptor deficiency have significantly lower circulating IGF-I levels, and also exhibit drastically reduced incidence of cancer and diabetes,

which are more common among age matched relatives with intact growth hormone receptor (Guevara-Aguirre, Balasubramanian et al. 2011).

Cancer cells are self sufficient in growth signals, with little to no responsiveness to physiological anti-growth signals, and in many cases do not undergo cell cycle arrest due to check point deregulation (Longo, Lieber et al. 2008; Hanahan and Weinberg 2011). In fact, it has been shown that pre-treatment with non-toxic doses of cell cycle arresting drugs (e.g. DXR) or growth factor inhibitors (inhibitors of MEK or EGF receptor) protect normal cells but not cancer cells against chemotherapy (Blagosklonny, Bishop et al. 2000; Blagosklonny, Robey et al. 2000; Demidenko, Halicka et al. 2005). In cancer cells, the acquisition of mutations and epigenetic modifications seems to impose a trade off: the loss of sophisticated responses acquired during evolution to rapidly adapt to a variety of environments, including starvation. Whereas cancer cells are often able to overcome the obstacles generated by anti-cancer therapies, including chemotherapy drugs as well as inhibitors of angiogenesis, signal transduction, and growth factors, they may not be sufficiently heterogeneous or adaptable to overcome wide-acting conditions such as starvation, particularly if combined with cancer therapeutics. One such example is the dependency on glucose to maintain high levels of cellular proliferation (Vander Heiden, Cantley et al. 2009).

In vitro, 19 out of the 21 murine and human cancer cell lines we tested were sensitized under fasting-mimicking conditions. We also noticed that glucose or serum restriction both individually sensitize cancer cells, yet the combination of both of these restrictions has, as can be expected, more potent effects. Glucose restriction alone was sufficient to sensitize various glioma cell lines. Glucose is the major energy source for proliferating cells, such as malignant cells, with elevated blood glucose being associated with increased cancer risk (Rapp, Schroeder et al. 2006; Stocks, Rapp et al. 2009). Many cancer cells have elevated glucose uptake rates and rely on glycolysis followed by lactic acid fermentation even in the presence of oxygen, a phenomenon known as the Warburg effect (Warburg 1956; Oudard, Boitier et al. 1997). In normal cells, the reduction of blood glucose, as well as IGF-I, likely contribute to a differential regulation of the activation of stress resistance transcription factors that are negatively regulated by nutrient sensing pathways (Longo, Lieber et al. 2008; Fontana, Partridge et al. 2010) and cell cycle progression

(Blagosklonny and Pardee 2001). In contrast, for cancer cells low glucose presents a specific and major challenge, particularly in the presence of chemotherapy drugs. Serum starvation alone had similar effects in a multitude of human and murine cell lines. I have also demonstrated that the effects of serum starvation can be imitated by the dose dependent and specific blocking of the IGF-I receptor with the αIR3 antibody in murine breast cancer cells. Even more so, the substitution of IGF-I under low serum conditions reversed the sensitization of 4T1 breast cancer and B16 melanoma cell lines *in vitro*.

Not every cell line responded to each drug we tested (e.g. the human colon cancer cell line LOVO showed no response to DXR but was sensitized when treated with CP) and thus indicates that our fasting-based approach can individually be optimized to the chemotherapeutic agent. We have yet to establish why two of the cancer cell lines did not respond to the starvation-mimicking conditions *in vitro*. One possibility could be that these cancer cell lines acquired mutations that make them irresponsive even to the reduction in glucose and/or IGF-I. Sabatini and colleagues showed that certain human cancer cell lines, when grown as tumor xenografts in mice, are highly sensitive to the anti-growth effects of caloric restriction, whereas others are resistant (Kalaany and Sabatini 2009). Cancer cells that form CR-resistant tumors carry mutations that cause constitutive activation of the phosphatidylinositol-3-kinase (PI3K) pathway and, in culture, proliferate in the absence of insulin or IGF-I. The substitution of an activated mutant allele of PI3K with wild-type PI3K in otherwise isogenic cancer cells, or the restoration of PTEN expression in a PTEN-null cancer cell line, was sufficient to convert a CR-resistant tumor into one that is sensitive to CR (Kalaany and Sabatini 2009). However another, more simplified, explanation for why we did not see sensitizing effects in a couple of cell lines could simply be the usage of chemotherapeutic agents that are not effective for this particular malignancy. For example, one of the cell lines that did not respond to DXR or CP treatment screen was the human astrocytoma cell line U87-MG. The treatment of this malignancy would usually involve Temozolomide (TMZ), the standard chemotherapy drug adopted for the treatment of high grade glioma of astroglial origin (Friedman, Kerby et al. 2000; Villano, Seery et al. 2009). When we tested mouse, rat

and human glioma cell lines under fasting-like conditions, we sensitized these glioma cell lines to TMZ treatment.

To test the efficacy of a fasting-mediated sensitization *in vivo*, we utilized a variety of different tumor models, including subcutaneous, intra-cranial and metastasizing models of tumor allografts and xenografts. We studied the progression of subcutaneous allografts of murine glioma (GL26), breast cancer (4T1) and melanoma (B16) and all of these models showed the most potent effects when short cycles of fasting were combined with chemotherapy. Interestingly, for some allograft models such as breast cancer, two repeated cycles of fasting for only 60 hours were as effective as two cycles of chemotherapy. STS alone may have the potential to be utilized in cases where patients cannot continue, or opt out of, the treatment with chemotherapeutic agents. However, it should be noted that the growth of B16 melanoma cells was not affected by the second cycle of fasting, which might potentially indicate that some cancer cells may have acquired resistance to fasting alone. Future studies should evaluate if repeated cycles of fasting may cause cancer cells to adapt to the challenges induced by starvation conditions. We also tested the growth of subcutaneous human breast cancer (MDA-MB-231) and ovarian cancer (OVCAR3) xenografts. Both cancer cell lines were also retarded by short-term starvation but returned to a size similar to that in animals on the control diet after re-feeding. Of note is that, when mice are re-fed after starvation and chow is provided *ad lib*, mice respond with severe binge eating (personal observation). The food intake in the first 2 to 3 days post fasting can be increased by up to 100-150%. During this period the mice consume significantly more calories and macronutrients, which can essentially “fuel” tumor growth. The effects of starvation alone in the two models might be more potentiated if animals are allowed to consume only control-matched amounts of food. However, this binge eating seems to be a rodent specific effect and human cancer patients are not encouraged to consume high calorie meals after ending their fast, but rather slowly re-adopt the body to normal food. Nonetheless, in the murine xenografts model the progression of the human breast cancer tumors was dramatically retarded and the tumor did not progress when fasting was combined with DXR. We have encountered no situation in which fasting was protective for cancer cells.

We aimed to evaluate the potential benefits of STS for glioma treatment in greater detail due to the severity of this disease. The multimodal treatment of GBM, based on surgical removal of the tumor followed by chemotherapy and radiotherapy, has improved the survival of GBM patients. However, the frequency of recurrence and rapid progression in adults emphasizes the need for a major enhancement of the therapy to achieve long-term survival without relying exclusively on the uncertain and very long and expensive drug development process. In addition, chemotherapy often causes severe toxic side effects and might even fail due to the development of drug resistance as glioma, and GBM in particular, are among the most inherent chemotherapy-resistant tumors (Pyrko, Schonthal et al. 2007; Kreisl 2009). The aim of our study was to investigate whether fasting, which could be rapidly, inexpensively and widely integrated into existing cancer treatments by clinicians, can improve the efficacy of chemo-and radiotherapies in treating mouse models of aggressive GBM. The intracranial inoculation of C57BL/6 mice with GL26luc cells showed a fast tumor progression that led to severe signs of illness such as back-hunching, reduced grooming (ruffled coat) and hypo-activity, possibly due to increasing intracranial pressure. One cycle of TMZ treatment at day 7 and 8 after tumor inoculation, or a 48 hour STS alone, extended the median survival from 14 to 15 days, although this effect was not statistically significant. By contrast, the combination of STS and TMZ delayed the onset of mortality and significantly extended median survival to 16 days. Remarkably, one animal in the STS+TMZ group achieved long-term survival. These results indicate that the combination of starvation with TMZ, the standard chemotherapy drug for the treatment of malignant glioma, has the potential to extend survival, at least in a portion of the subjects treated. When we expanded the treatment to two cycles, we limited the 2nd treatment to 24 hours of starvation because of a loss of body weight prior to STS, presumably due to tumor burden. Despite the shortened starvation regimen and the reduced TMZ dose, the STS+TMZ group showed an extended survival compared to that of the single cycle treated group. Although this may be seen as a limited improvement for the treatment of advanced stage glioma, the fasting-based sensitization has the potential to achieve a better health outcome in the treatment of a residual disease after the tumor resection.

The longer survival in the subcutaneous glioma model compared to the intracranial one allowed us to analyze the beneficial effects of STS in combination with TMZ- or radiotherapy. Both treatments, which are standard care in glioma treatment, significantly delayed tumor progression when combined with fasting. Notably, STS alone was capable of retarding tumor progression in the subcutaneous model to a degree comparable to that caused by radiotherapy during the earlier stages of tumor development. Consistent with our STS-based approach, which results in blood glucose reduction, others have shown that the reduction in blood glucose availability, e.g. by 2-deoxy-D-glucose (a glucose analog and glycolytic inhibitor), can selectively enhance radiation-induced damage in tumor cells while protecting normal cells (Singh, Singh et al. 1990; Mohanti, Rath et al. 1996). However, fasting, which affects the levels of many growth factors and metabolites, is likely to be much more effective against a wider range of tumors than interventions that affect the levels of specific metabolites, such as glucose, alone. Thus, this dietary intervention may represent an alternative to GBM patients who are unable to receive or opt out of the conventional treatments.

Advanced metastatic tumors are extremely difficult to cure once tumor masses have spread to different organs. We therefore we aimed to investigate whether the combination of multiple fasting cycles and high-dose chemotherapy can increase survival in aggressive metastatic cancer models. In models of intravenously injection of murine breast cancer cells, melanoma cells and two neuroblastoma cell lines (NXS2 and Neuro-2a) in immune-competent mice, the combination of STS and chemotherapy significantly extended survival and reduced the spreading of tumor cells into distant organs. As outlined in the introduction, cancer metastasis is a multistep process driven by the complex molecular interactions that occur between the disseminating cancer cell and its changing microenvironment. The rate limiting steps regulating this process are tumor-induced angiogenesis, extracellular matrix (ECM) degradation, cell movement through tissue barriers, including entry into and exit from blood vessels, and survival/proliferation. Previous studies demonstrate that manipulation of IGF-I levels, e.g. by dietary intervention such as CR, influences tumor growth and metastasis. Up-regulation of the IGF-I pathway stimulates tumor proliferation, progression and metastasis and conversely, the down-regulation of this

pathway results in anti-tumorigenic activity *in vivo* (Baserga, Peruzzi et al. 2003). The functional disruption of the IGF-I receptor decreases breast cancer metastasis in immune deficient nude mice by suppressing cellular adhesion, invasion, and metastasis of breast cancer cells to the lung, lymph nodes, and lymph vessels (Kari, Dunn et al. 1999). Of particular interest for the reduced metastasis are molecules that enable motile and invasive properties, thus allowing malignant cell to invade the underlying stroma. Such molecules include the matrix metalloproteinases (MMPs). These zinc-dependent collagenases have been implicated in ECM degradation and are associated with tumor-dependent angiogenesis and invasion (Samani, Yakar et al. 2007). The IGF system plays a role in the regulation of several of the MMPs and can thereby promote tumor invasion. Several labs identified the IGF-I receptor as a promoter of MMP(-2) synthesis and tumor invasion, as measured *in vitro* (Yoon and Hurta 2001; Grzmil, Hemmerlein et al. 2004; Zhang, Bar-Eli et al. 2004). Further, the down- or up-regulation of IGF-IR expression altered MMP-2 expression levels, invasion, and metastasis in a Lewis lung carcinoma model of liver metastasis (Long, Navab et al. 1998). We have not yet established if STS indeed modulates MMP expression. I propose that the pronounced effects of STS on IGF-I might result in decreased MMP expression and thus might be involved in the reduced metastasis in our models.

Gene expression profiling by genome-wide microarray analyses on the liver, heart, skeletal muscle, and subcutaneous 4T1 breast tumor mass from mice, fasted for either 48 hours or fed an *ad lib* diet, was performed to investigate genes that are differentially regulated in normal and cancer cells in response to fasting. The microarray analysis clearly indicates that fasting differentially regulates genes involved in cellular proliferation and found that the expression of translation and ribosome biogenesis/assembly genes was significantly increased in response to fasting in the autografted breast cancer (4T1). In normal tissues these genes were either repressed or minimally affected. In agreement with this increase in the expression of translation genes, tumor cells cultured under STS for 48 hours showed increased cellular protein concentration, had increased levels of phosphorylation of Akt and S6K proteins, and reduced phosphorylation of eIF2α in autografted tumors of fasted mice. It thus appears as if 4T1 tumor cells, when exposed to starvation,

attempt to compensate for the lack of nutrients and growth factors by increasing translation, although counter-intuitive. As a result, cancer cells appear to consume even more energy, eventually promoting oxidative stress and cell death. In fact, translation is closely coupled with cell cycle progression and cell growth (Meric and Hunt 2002), and is a costly process that can consume 50 to 75% of the cellular energy in rapidly dividing cells (Rolfe and Brown 1997; Princiotta, Finzi et al. 2003). The regulation of translation in mammalian cells is tightly coupled to nutrient status through the mTOR pathway and the modulation of translation initiation by altering the phosphorylation status of the eIF4E-binding protein (4E-BP1) and S6K. S6K and 4E-BP1 are also partly regulated by PI3K and its downstream protein kinase Akt and thus the phosphorylation of S6K is stimulated by several growth factors, such as platelet-derived growth factor and IGF-I (Graves, Bornfeldt et al. 1995). PTEN is a phosphatase that negatively regulates PI3K signaling. $PTEN^{-/-}$ cells have constitutively active Akt, causing increased S6K activity and S6 phosphorylation (Neshat, Mellinghoff et al. 2001). Consequently, S6K activity is inhibited both by the PI3K inhibitors wortmannin and LY294002 or by the mTOR inhibitor rapamycin (Thomas and Hall 1997).

In accordance with the hypothesis that the promotion of cellular proliferation is accompanied by a decrease in cellular maintenance (Kirkwood, Kapahi et al. 2000), we observed increased DNA damage and the induction of apoptosis in cancer cells. The reduction in cellular maintenance might be responsible for the effect of fasting on the sensitization of 4T1 cells. We noted a considerable increase in the ROS-dependent staining in malignant cells with DHE and MitoSox Red under fasting-mimicking cell culture conditions. Under these conditions, cancer cells responded with the effort to increase pro-proliferative signaling. The hyper-activation of the Akt and Tor-S6K pathways may be the underlying cause for the increase in reactive oxygen species (Nogueira, Park et al. 2008). In fact, in both yeast and higher eukaryotes, the activation of Akt and Tor-S6K pathways sensitizes cells to oxidative damage, in part by inactivating stress resistance transcription factors (Fabrizio, Pozza et al. 2001; Wei, Fabrizio et al. 2008). In addition, data suggest that high levels of intracellular ROS contribute to PTEN inactivation by oxidation with a subsequent PI3K/Akt pathway hyper-activation (Silva, Yunes et al. 2008). These pro-

oxidizing conditions caused by starvation may in turn promote the observed increase in DNA damage and apoptosis.

4.3 HO-1 is a Mediator of Differential Stress Sensitization in Malignant Cells.

The ability to communicate with the environment to respond to changes, in particular to adverse events, is a critical component to maintain cellular function and enables survival even in stressful conditions. Therefore, most cells have developed a cellular stress response that allows adjusting gene expression profiles in response to these stimuli, particularly through the establishment of proteins involved in cellular defense mechanisms. On the other hand, the continuous, or rather, exaggerated translational activity in fasted tumor cells may consume the limited cellular energy at the cost of inducible stress-resistance factors such as NFkB and HO-1, when nutrients are scarce or absent. This fasting-induced decrease in NFkB and HO-1 expression in transplanted 4T1 tumors may explain at least parts of the sensitization effects observed in these cells.

Heme oxygenase-1 is a microsomal membrane protein that catalyzes the oxidative cleavage of heme molecules to generate biliverdin, free heme iron and carbon monoxide (CO). HO-1 is critical in at least two physiological pathways: a) the recycling of iron molecules from senescent erythrocytes (Kovtunovych, Eckhaus et al. 2010) and b) the maintenance of cellular homeostasis under stressful conditions (Otterbein, Soares et al. 2003; Gozzelino, Jeney et al. 2010). Previous research demonstrated that the maintenance of cellular homeostasis by HO-1 is promoted by potent anti-inflammatory, anti-proliferative and anti-oxidant activities of CO and bile pigments. In turn, this may be of particular importance in human pathologies including inflammation (e.g. sepsis and asthma), cardiovascular diseases (e.g. myocardial infarct), atherosclerosis, hypertension, transplant graft rejection and cancer (Liu, Chen et al. 2004; Ryter and Choi 2009).

In normal cells, the expression of HO-1 is triggered by heme, as well as a diversity of stressors including ultraviolet radiation, hypoxia, inflammation, heavy metals, hydrogen peroxide and nitric oxide (Prawan, Kundu et al. 2005). In addition, we show here that STS induces HO-1 expression in the liver, heart and muscle. Owing to this vast variety of stressors, HO-1 is regulated by a multitude of transcription factors to fine-tune the response to specific conditions. Among these factors are members of the heat shock factor (HSF), nuclear factor-κB (NFkB),

nuclear factor-erythroid 2 (NF-E2 and more particular Nrf2) and activator protein-1 (AP-1) families as well as the hypoxia inducible factor-1 (HIF-1) and cAMP response element-binding protein (CREB) (Alam, Stewart et al. 1999; Prawan, Kundu et al. 2005; Alam and Cook 2007). Although heme itself is proposed to enhance *hmox-1* gene transcription by binding BACH1 (Igarashi and Sun 2006), most of the HO-1 induction is regulated by transcription factors that are themselves regulated by signaling cascades that integrate protein phosphorylation status and redox reactions. As such, a substantial amount of data emphasizes the role of mitogen activated protein kinases (MAPK), the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling cascade and the PI3K/AKT in the regulation of HO-1 (Kyriakis and Avruch 2001; Yang, Sharrocks et al. 2003).

HO-1 levels are usually elevated in cancer cells, including lymphosarcomas, prostate cancer, brain tumors, adenocarcinomas, hepatomas and melanomas (Was, Dulak et al. 2010), compared to the surrounding tissue. However, during the fasting-induced sensitization, HO-1 was significantly reduced. Due to the protective role of HO-1 in protecting cancer cells from oxidative damage and reduction of apoptosis (Baranano, Rao et al. 2002; Gozzelino, Jeney et al. 2010), we hypothesized that restoring HO-1 levels, via induction with hemin or over-expression with a rHO-1 construct, could partially reverse the STS induced sensitization to chemotoxic agents. *Vice versa*, the reduction of HO-1 by ZnPP treatment caused sensitizing effects under *ad lib* conditions. To test whether the depletion of nutrients by STS causes functional changes, we measured basal and stimulated mitochondrial functions. When we aimed to analyze the changes of mitochondrial respiration caused by STS, we noticed that cells grown under *ad lib* conditions show a significantly higher basal oxygen consumption rate (OCR) than cells grown under glucose- and serum-restricted conditions. These findings suggest reduced mitochondrial respiration under fasting-like conditions. The over-expression of HO-1 had no effects on baseline oxygen consumption rate and the oxygen consumption attributed with mitochondrial ATP production, proton leak and the maximal respiratory capacity; indicating that HO-1 does not alter the mitochondrial respiration.

Under normal conditions, differentiated cells rely primarily on mitochondrial respiration to produce ATP and utilize glucose to maintain energy production. Fasting conditions result in the fast depletion of glucose while increasing

ketonebodies in the blood. Fatty acids become the main source of cellular energy and reducing equivalents such as NADPH. We therefore tested if palmitate provides sufficient energy for malignant cells when glucose is depleted. Murine breast cancer cells, grown in media conditions mimicking glucose and growth factor levels of *ad lib* fed mice, were able to oxidize fatty acids while 4T1 cells, grown in culture-media conditions mimicking glucose and serum levels of fasted mice, suggested that these cells cannot utilize fatty acids. The over-expression of HO-1 reversed this deficiency of murine breast cancer cells, indicating that cellular redox homeostasis and fatty acid utilization in cancer cells are interconnected and plays a crucial role in the fasting-mediated sensitization. This hypothesis is supported by a recent report showing that the inhibition of fatty acid oxidation by etomoxir (a fatty acid transport inhibitor), reduced cell viability and elevated intracellular ROS levels (Pike, Smift et al. 2011). In our study, under STS conditions, we measured increased DHE and MitoSox Red staining, both indicative for increased levels of reactive oxygen species production. Elevated ROS levels may significantly contribute to the STS-mediated sensitization of cancer cells. Pike et al. noted that the inhibition of fatty acid oxidation resulted in decreased levels of reduced glutathione and NADPH (Pike, Smift et al. 2011). The induction of HO-1, via hemin or the over-expression of a rHO-1 construct might reverse this sensitization and restore fatty acid oxidation by maintaining cellular redox homeostasis. We will aim future studies to address in greater detail if STS indeed depletes the intracellular pool of ATP, as well as NADPH and other reducing systems, and how HO-1 mediates its essential role in reversing the observed effects on fatty acid oxidation. In addition, experiments to block fatty acid oxidation, such as the utilization of etomoxir, might provide means to improve the sensitization of cancer cells.

The experiments on HO-1, mitochondrial respiration and fatty acid oxidation were all performed in one murine cell line. Yet, matching the above reported observation on the central role of HO-1 in 4T1 cells is that the proliferative action of HO-1 can be down-regulated by siRNA, resulting in the inhibition of pancreatic tumor growth (Berberat, Dambrauskas et al. 2005), while in murine and human melanomas the over-expression of HO-1 increased cellular proliferation (Was, Cichon et al. 2006). Still, the observed reduction in HO-1 levels may only be of minor significance for other cell lines, since different oncogenes and tumor-specific combinations of

mutations may affect the response to fasting. We will therefore test more human and murine cancer cells to establish the role of HO-1 and to determine additional mediators (e.g. FOXO) of the STS-induced cancer sensitization.

The data I present in this dissertation revealed that the beneficial effect of STS in cancer treatment can be twofold. On the one hand, STS dampens the levels of nutrients and growth factor signaling, resulting in the cellular protection of normal cells, but not cancer cells because oncogenic mutations negate this adaptive stress resistance response. On the other hand, STS causes changes in the blood that promote cancer cell death and further sensitize cancer cells to cytotoxic stimuli, such as chemotherapy drugs and radiation (**Fig. 4-1**). However, the twofold contribution of the STS-induced DSR may be unique to the cancer cell types.

4.4 Macronutrient Deficient Diets do not Mimic STS Effects

Above, I outlined that fasting can differentially protect mice but not the tumor allograft to chemotherapeutic agents while protecting the host from chemotherapy induced toxicities. Fasting itself acts growth-static, i.e., inhibited tumor progression, which was particularly noticeable during the fasting cycles where we could usually observe only very limited tumor growth. Despite the fact that fasting is safe, economical, feasible, and effective in reducing chemotherapy-related side-effects in cancer patients at advanced stages (Safdie, Dorff et al. 2009), prolonged and/or repeated fasting cycles still impose a challenge to even the healthiest individuals. Therefore, understanding the major dietary components that mediate the beneficial effects of fasting may introduce the use of substitution diets to selectively protect the host against chemotherapy toxicity.

Dietary protein, and the resulting amino acid-content, seems to affect longevity and healthy aging (Ross and Bras 1973; Pamplona and Barja 2006). Restricting protein intake shares some of the physiological effects of CR, including a decreased metabolic rate, reduced oxidative damage, enhanced hepatic resistance to toxins and oncogenic insults, decreased preneoplastic lesions and tumors (Maeda, Gleiser et al. 1985; Rodrigues, Sanchez-Negrette et al. 1991; Youngman, Park et al. 1992; Ayala, Naudi et al. 2007). Furthermore, both CR and protein restriction reduce serum IGF-I levels (Spindler 2010), which might be one of the contributors to longevity extension as the IGF-I-like signaling pathways regulate

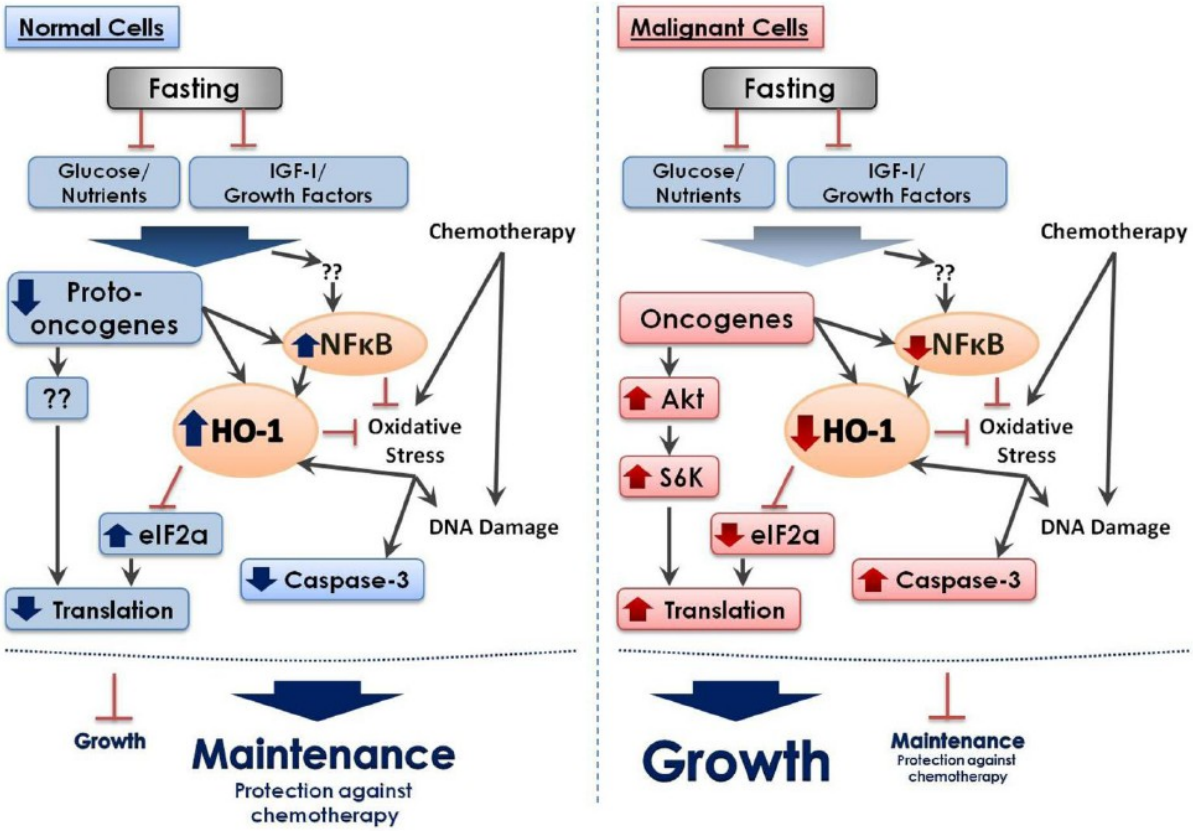

Figure 4-1. A model for fasting-dependent sensitization of tumor cells to chemotherapy.

In response to fasting, glucose and IGF-I, as well as other pro-growth proteins/factors (oncogenes) are reduced. Malignant cells respond to this reduction by activating AKT/S6K and eIF2α in an attempt to increase translation. This comes at the price of reducing the expression of stress resistance proteins NFkB and HO-1. Consequently, in contrast to normal cells, the finite amount of cellular energy would have to be allocated favorably to growth over maintenance in malignant cells. These changes lead to the increase in oxidative stress, DNA damage, activation of caspase-3, and eventually cell death.

lifespan in various model organisms such as *C. elegans*, *D. melanogaster* and mice (Guarente and Kenyon 2000; Kenyon 2005; Russell and Kahn 2007; Bartke 2008; Fontana, Partridge et al. 2010). Despite the well-established effects of protein deficient diets in reducing IGF-I and inducing cellular stress resistance, we did not detect any benefits in short-term protein restricted mice (0% P and 20% P, 72 hours to allow a comparison to STS) with regard to stress resistance and tumor progression. In a subcutaneous glioma model, *ad lib* feeding of a diet low in protein did not significantly reduce tumor progression once the tumor was palpable. This may be partially due to this diet not reducing glucose and/or IGF-I levels during the feeding regimen. However, glioma is a particularly aggressive tumor, so many additional cancer models should be tested in combination with protein restricted or a combination of CR and protein restricted diets to establish whether these interventions can be effective in delaying cancer progression. Mice in the group fed with a calorie restricted low carbohydrate diet (LCHP) had the worst survival of all CR groups, comparable to that of mice in the control group. The fact that mice in this group consumed similar, or higher amounts of fat-derived calories (20.9% in 50% CR LCHP vs. 17.2% *ad lib* AIN93G) and more importantly protein-derived calories (22.6% in 50% CR LCHP vs. 18.8% *ad lib* AIN93G) during three days of feeding, might explain this lack of protection. Of note is that the results presented on the induction of stress resistance are based on relatively short (72 hour) feeding periods, thus we cannot exclude that longer CR regimen with either altered calorie and/or macronutrient restrictions could result in an improved stress resistance.

Ketogenic diets are used extensively in the treatment of refractory epilepsy in children, but have also been studied in cancer treatment (Seyfried, Sanderson et al. 2003). To determine how this approach would compare with our stress resistance and potentially tumor sensitization experiments, we designed two ketogenic diets: our 90% HF diet (% calorie ratio of fat: carbohydrates: protein of 90%: 1%: 9%; Fig. 1) is nearly identical (± 0.5% variation) to the classic ketogenic diet with a ratio of fat: carbohydrates: protein of 90%: 1.4%: 8.6% respectively. The high-fat diet 60% HF (% calorie ratio of fat: carbohydrates: protein of 60%: 31%: 9%) contains fat ratios similar to the fat ratio used in the modified Atkins diet (% calorie ratio of fat: carbohydrates: protein of 60%: 5%: 35%)(Kossoff and Dorward 2008), but we

reduced the protein content because previous work has established that protein, and not carbohydrates, regulate IGF-I levels in human (Cahill 1970; Fontana, Weiss et al. 2008). The results we describe here, demonstrate that neither glucose nor IGF-I levels were significantly reduced after feeding both ketogenic diets for 9 consecutive days.

To evaluate the effects of saturated *vs.* unsatured fatty acids, as well as medium- *vs.* long-chain fatty acids in cancer treatment, we designed two diets that were isocaloric to the control diet with soybean oil or coconut oil as a fat source but had low protein content. Long-chain unsaturated fatty acids are found in most commonly used dietary fats and vegetable oils such as soybean oil, while short- and medium-chain saturated fatty acids (e.g. lauric acid and myristic acid) are found in relatively high abundance in palm kernel oil and coconut oil. The medium-chain triglycerides (MCT) can easily by hydrolyzed in the gastro-intestinal tract and can be transported through the portal venous system towards the hepatocytes, while most of the long-chain fatty acids are transported as chylomicrons in the lymphatic system and packaged into triglycerides in the liver. MCTs can easily be fed into the mitochondrial β-oxidation (Aoyama, Nosaka et al. 2007), while long-chain triglycerides (LCT) rely on transporters, such as carnitine, to enter the mitochondrial matrix in hepatocytes (Calabrese, Myer et al. 1999). Data from human studies has indicated that consumption of MCTs or diets with higher unsaturated to saturated fatty acid ratio are associated with decreasing blood glucose, improving lipid profile, and reducing obesity (Ghosh 2007; Xue, Liu et al. 2009). In a study of biochemical and anthropometric profiles in women with abdominal obesity, dietary supplementation with coconut oil promoted a reduction in abdominal obesity (Assuncao, Ferreira et al. 2009).

In agreement with the partial effects on blood glucose and IGF-I, the results I presented above indicate that 72 hours of 50% CR, but also of diets restricted in carbohydrates or proteins, have only partial effects on stress resistance. None of the 50% dietary restricted and macronutrient defined diets fed for 3 days, except for the ketogenic 90% HF diet, lowered blood glucose levels, which has been shown to promote host-protection and tumor sensitization. Interestingly, a 50% reduction in the calories consumed on a ketogenic diet leads to a 30% reduction in blood glucose levels after 3 days of feeding, an effect presumably due to the very low carbohydrate

content (less than 1%) of this diet. However, our stress resistance experiment indicates that this reduction did not improve survival. It is noteworthy, that no mice from any of the CR diets, neither the control diet nor the macronutrient defined diets, achieved protection equivalent to that caused by 60 hours of fasting. Thus it appears that the limited changes in blood glucose and IGF-I, caused by specific carbohydrates or protein-restricted diets, will most likely not improve, or have limited effects on, stress-resistance and/or reduce tumor progression. However, additional studies with extended feeding regimes and larger experimental group size will be necessary to understand whether specific diets may be sufficient to achieve DSR and DSS effects that are close to those caused by fasting cycles. Future studies could also evaluate the effects of various macronutrient-defined and CR diets on ROS production, tumor progression and stress resistance.

In summary, the previously described fasting-dependent differential protection of normal and cancer cells (DSR) (Raffaghello, Lee et al. 2008; Safdie, Dorff et al. 2009; Lee, Safdie et al. 2010) and the tumor cell-specific sensitization presented here (Differential Stress Sensitization, DSS) indicate that short-term starvation conditions, or modified diets that promote similar changes, have the potential to enhance a wide variety of standard cancer therapies. Owing to the consistent effects on glucose and IGF-I, and consequent effects on protection of normal and sensitization of cancer cells without the chronic under-weight, periodic fasting cycles appear to have the highest potential to protect patients treated with a variety of chemotherapy drugs, while simultaneously augmenting treatment-efficacy. If confirmed in the currently still ongoing clinical trials, repeated fasting cycles could also provide an alternative to chemotherapeutic treatment for early stage patients that may not be considered sufficiently at risk to receive chemotherapy, or for patients with more advanced malignancies who receive chemotherapy and are at high risk for recurrence. In addition, fasting cycles in combination with chemotherapy, could extend the survival of advanced stage cancer patients by retarding the tumor progression and reducing the side effects.

5 Perspectives

According to the American Cancer Society, the general dietary recommendation for cancer patients that receive chemotherapeutic agents is to maintain or even to increase their caloric intake (Doyle, Kushi et al. 2006). This seems to be common wisdom and generally well accepted and practiced, presumably because it appears as if feeding the body during times of sickness implies a better health status. Notably, circulating IGF-I is regulated by protein intake and total energy (Clemmons and Underwood 1991; Underwood, Thissen et al. 1994). Low-protein and low-calorie diets have a strong effect on circulating concentrations of several pro-growth factors, including IGF-I, and clinical biomarkers (Fontana 2006; Fontana, Weiss et al. 2008). Considering the fact that the average American consumes more protein than needed (Fulgoni 2008), which has been shown to be a major risk factor for tumor progression (Metges and Barth 2000), there is an urgent need to re-evaluate the current dietary recommendation for cancer patients. The studies I presented in this dissertation suggest that a shift in the dietary recommendations for cancer patients might allow for an improvement of the treatment efficacy.

Our data demonstrates that short cycles of fasting are able to selectively protect mice but not tumor cells from chemotherapy. In addition, the effect of short-term starvation is extended beyond selectively protecting normal cells to sensitizing murine and human cancer cells to chemo- and radio-therapy. Furthermore, mimicking starvation-like conditions reveals that IGF-I signaling and glucose deprivation, without the use of chemotherapeutic agents, reduces tumor progression. This is important since it provides a basis to study mechanisms of stress resistance through metabolic manipulations, with the potential of discovering drug targets that can weaken the cellular defense of malignant cells. Our studies showed that fasting causes a differential expression of stress regulating transcription factors and indicate that reduced heme oxygenase-1 level, under STS conditions, plays a central role in mediating cancer cell sensitization. This understanding of DSR also reveals another approach to mimic fasting: targeting specific signal transduction pathways by pharmacological interventions. The data I presented in this dissertation suggests multiple candidate targets including IGF-I (extracellular) and its receptor,

downstream effectors such as the PI3K/Akt and Ras/ERK pathways (intracellular) and transcription factors that mediate the fasting induced cancer sensitization, e.g. NFkB and HO-1. Nonetheless, metabolic reprogramming during fasting is proposed to impact various pathways within the cell and it appears unlikely that the targeted disruption of one of these pathways in cancer cells will have as a broad impact as starvation.

Despite its beneficial effects, even short cycles of fasting remain a challenge to most patients, especially for those patients prone to weight loss. Ongoing research from Dr. Longo's lab at the University of Southern California focuses on developing substitution diets that mimic the beneficial effects of fasting, while providing sufficient supply of minerals, vitamins, etc. In various communications with patients enrolled in the clinical trials at the Keck School of Medicine that focus on fasting, the majority of patients (~70%) indicated they would rather participate in a dietary substitution study. A product designed in our lab, described here only as "fasting mimicking diet" (FMD) due to pending patents, was distributed to patients that volunteered in helping us to adopt the taste and to test the effects on blood parameters. One volunteer, a male patient diagnosed with multiple myeloma in April 2006, started a fasting-related dietary regimen in June 2011 after he became unresponsive to his treatment regiment (Revlimid, Velcade and Dexamethasone). After the patient tested multiple rounds of fasting or fasting mimicking diets, his marker protein stabilized. The patient donated serum to us that was collected at the end of one FMD-regime; as well as during a period of normal food intake. I used this serum for preliminary measures to determine if changes occurred that have sensitizing effects on a human cancer cell line (**Fig. 5-1**). In strong similarity to the STS *in vitro* results I presented before, serum collected at the end of an FMD regime significantly reduced cell survival of a human prostate cancer cell line (PC3) by 40% when compared to cells grown in *ad lib* serum.

I propose that in the near future, fasting and fasting-mimicking diets will be applied to the clinical scenario where they will improve the treatment efficacy for a wide range of diseases, not limited to cancer alone.

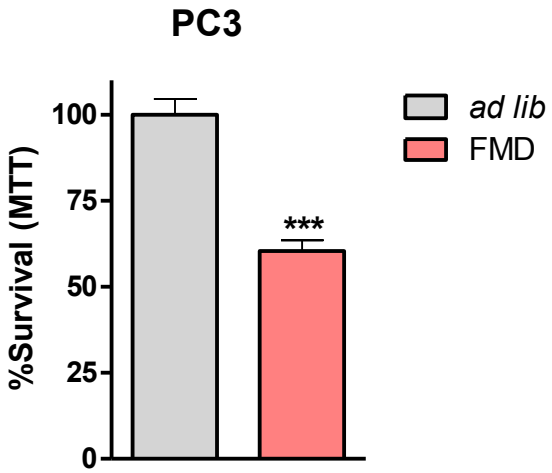

Figure 5-1. A fasting-mimicking diet reduces survival of a human prostate cancer cell line *in vitro*.

Human prostate cancer cells (PC3) were incubated for 48 hours in human serum that was collected after normal food intake (*ad lib*) or at the end of a fasting mimicking diet (FMD) regime. All cells were grown in 10% of each serum; glucose was matched to the patients glucose levels at the time of collection (1.25 g/L *ad lib*, 0.9 g/L FMD). Survival presented as percentage of MTT reduction of *ad lib* to FMD. All data presented as mean ± SEM; *** $p < 0.001$, Student's t-test, two-tailed.

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Curriculum vitae

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Erklärungen

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. (2) f) der Promotionsordnung der Falultäten für Biologie, Chemie und Mathematik zur Erlangung der Dr. rer. nat., dass ich das Arbeitgebiet, dem das Thema "*Dietary- and Fasting-based Interventions as Novel Approaches to Improve the Efficacy of Cancer Treatment"* zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von *Sebastian Brandhorst* befürworte und die Betreuung auch im Falle eines Weggangs, wenn nicht wichtige Gründe dem entgegenstehen, weiterführen werde.

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Hiermit erkläre ich, gem. § 6 Abs. (2) d) + f) der Promotionsordnung der Falultäten für Biologie, Chemie und Mathematik zur Erlangung des Dr. rer. nat., dass ich keine anderen Promotionen bzw. Promotionsversuche in der Vergangenheit durchgeführt habe und dass diese Arbeit von keiner andern Fakultät/Fachbereich abgelehnt worden ist.

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