Characterizing Longevity-Related Insulin-like Growth Factor-1 Pathways.

A. Overview and Aims

The insulin-like growth factor (IGF) axis has been strongly implicated in the negative control of lifespan longevity for a large number of model systems including the fruitfly, nematode and mouse. Consequently, much research has been invested in understanding the IGF pathways involved in controlling longevity. We are interested in exploring two currently unanswered problems regarding IGF related longevity control mechanisms in the mouse model.

Broad Aim 1: "Dissociating" the activities of the growth hormone receptor (GHr) and insulin-like growth factor receptor.
Specific Aim 1.1: Create mouse knockouts for (1) growth hormone receptor (2) insulin like growth factor-1r.
Specific Aim 1.2: Characterize the differential gene expression profiles for GHr and IGF-1r knockouts by microarray analysis.

Broad Aim 2: Testing whether IGF's longevity regulation pathways are modulated by a neuroendocrine system.
Specific Aim 2.1: Creating the brain-specific IGF-1r knockouts.
Specific Aim 2.2: Comparing longevity phenotypes of the three mouse lines: (1) the non-specific 1GF-1r mouse knockout, (2) the brain-specific IGF-1r mouse knockout and (3) wild type mice.
Specific Aim 2.3: Comparing oxidative stress responses, metabolic activity and IGF-1 related activity of the three mice lines: (1) the non-specific 1GF-1r mouse knockout (2) the brain-specific IGF-1r mouse knockout and (3) wild type mice.

Acronyms
GH – Growth Hormone; GHr – Growth Hormone receptor; IGF-1 – Insulin-like Growth Factor-1; IGF-1r – Insulin-like Growth Factor-1 receptor; ES cell – Embryonic Stem cell.

B. Background and Significance

The first mammalian longevity studies began more than seventy years ago with the famous establishment of the inverse relationship between caloric intake and lifespan in mice (McCay *et al*., 1935). The story of the first *genetic* studies, however, began more recently with worms. The allowance for high-throughput gene screening within C elegans models resulted in the cataloging of hundreds of genes that affected worm life-span longevity (see Braungart *et al*., 2004; Jones *et al*., 2005; Springer *et al*., 2005). A handful of those genes (especially daf-2) proved to be extraordinarily interesting due to homologies to mouse genes belonging to the *IGF signaling cascade* (Kenyon *et al*., 1993; Guarente and Kenyon, 2000; Kenyon, 2001). Now, after many knockouts in many systems, it is believed that the IGF axis plays an important and evolutionarily conserved role in regulating lifespan longevity within yeasts, (Fabrizio *et al*., 2001), fruit flies (Clancy, *et al*., 2001), the nematodes (Kenyon at al., 1993), and more recently and famously, mice (Holzenberger *et al*., 2003). Furthermore, epidemiological studies have shown that the IGF longevity pathway is also present in humans (Barbieri *et al*., 2003; Bonafè et al., 2003; review: Longo and Finch, 2003). All of the above studies showed the following result: reduction of IGF-1r activity (through knockouts or RNAi) resulted in increased adult longevity (sometimes to up to 300% as shown in yeast) i.e. the IGF axis negatively regulates longevity.

In mammals, the insulin like growth factor "axis" (IGF axis) includes IGF-1, IGF-2, their respective receptors (IGF-1r and 2r), the IGF binding proteins (IGFBP) and the IGFBP proteases. It is currently believed that all of the signaling pathways mediated by the IGF axis is mediated by the binding of ligands (either IGF-1 or IGF-2) to the IGF-1 receptor (IGF-1r). Apart from the recently discovered longevity control, components within IGF axis have been shown to be tightly linked to cancer proliferation (Kanter-Lewensohn *et al*., 1998; Jiang *et al*., 1999; Werner and LeRoith, 1996; Baserga *et al*., 2003), brain growth and development (D'Ercole *et al*., 1996; Werther et al., 1998), neuronal regeneration (Narasimhan, 2006), apoptosis (Chrysis *et al*., 2001; D'Ercole *et al*., 1996), cell cycle reentry (Hodge et al., 2004) and other mitogenic pathways.

In aims of understanding the pathways controlling longevity in mammals, we will focus on longevity control by the IGF axis, in particular, the IGF-1r pathway because it is (1) the key player in most IGF-related signaling pathways and (2) ubiquitous within most tissue types (Chrysis *et al*., 2001).

This paragraph briefly describes the study of longevity control in mice. Following cues from nematode studies, IGF-1r heterozygous knockout mice showed a statistically significant (26%) average increase in lifespan in some experimental cohorts without major changes in physiological attributes (Holzenberger *et al*., 2003). From a followup study (Holzenberger, 2004), the longevity was shown to be due to (in large part) increased resistance to oxidative stress. The manner in which IGF-1 regulates these anti aging mechanisms (oxidative stress) is still unknown (Holzenberger, 2004).

(B.1) A need to resolve the IGF/GH Holzenberger paradox. As mentioned above, the suppression of IGF-1r activity results in greater organism longevity. It is also known that the growth hormone (GH) upregulates the expression of IGF-1r through various autocrine/endocrine mechanisms (review: Daughaday, 1989). Therefore, it would be expected that the upregulation of GH will result in reduced longevity. So, it is puzzling that the topical application of GH results in visible youngness of skin (example of this line of study: Ehrlich *et al*., 2006). Scattered reports of these rejuvenative results may, at most, me intriguing and not troublesome; however, these reports have triggered a boom in GH related rejuvenating facial creams (such as VesPro Life Sciences' Regeniskin, Somatoprofen S33 transdermal creams, Boca Raton's HGH creams, etc.). By conventional knowledge, the use of GH should result in overactivity of the IGF-1r pathway and therefore should result in adverse (not rejuvenative) effects on aging. The reason for the rejuvenative effects of GH has

eluded scientists so far, and this has been a source of concern. As opined by Holzenberger:

> *"We conclude that more in-depth studies on the possible dissociation of GH and IGF actions are urgent, and that we need to understand, in more detail, how, on the one hand, the administration of GH can show beneficial effects on ageing and, on the other hand, how lowering IGF signalling can increase longevity."* (Holzenberger, 2004)

Work done by Kenyon and colleagues on DNA-microarrays on the IGF-1r homolog in C. elegans shed light on the network of genes that were regulated downstream of the IGF-1r activity (Murphy *et al*., 2003). In section (C.1), we propose to help "dissociate" the functions and pathways of GH and IGF-1r using similar microarray methods. This will be done by comparing DNA microarray profiles of IGF-1r knockout mice to that of GHr knockout mice using wildtype mice as the baseline. We believe that analysis of this sort may uncover key components to the GH/IGF pathways that will help in resolving what we will henceforth called the *Holzenberger paradox* (for the sake of brevity).

(B.2) IGF's control on longevity in mice may be modulated by a neuroendocrine system. The IGF related longevity in C elegans was recently shown to be regulated in a non-autonomous manner (Apfeld and Kenyon, 1998) i.e. the longevity of the entire organism may be dictated by signaling from a small subset of cells (endocrine signaling). It was further evidenced in C elegans that the nervous system plays a key role in IGF related longevity (Wolkow *et al*., 2000), indicating that a neuroendocrine system regulates longevity. Due to the large number of similarities between IGF pathways in the worm, fly and mouse, we believe that longevity regulation is also moderated via similar neuroendocrine mechanisms in mice. The implications of non-autonomous longevity regulation are great – for only a small (and select) fraction of IGF-1r receptors within the body will need to be targeted in order to affect the aging process. To test whether mice moderate aging via neuroendocrine mechanisms, in section (C.2) we will propose to create a brain-specific IGF-1r mouse knockout and compare the longevity differences between the wild type mouse and the longer living *non-tissue-specific* IGF mouse knockout.

(B.3) The possible consolidation of various findings. The mechanism with which IGF-1r negatively regulates longevity, along with the mechanism of GH/IGF signaling is yet unknown. However, a commonly proposed mechanism involves IGF-1 upregulation by the GH/GHr binding event (Daughaday, 1989; Scanes and Daughaday, 1995), which then results in the negative regulation of longevity. A simplified schematic of this conventional model is shown at the *left side* of *Figure 1*. Although where (liver, all tissues etc) and by what means (endocrine, autocrine etc) the upregulation of IGF happens is not known, this general schematic has not yet been proven wrong.

Complexities add on. It is well understood that the conventional model pathway is highly simplified. This is strikingly evidenced by the presence of the *Holzenberger paradox* (described in *B.1*). Here, we modify the conventional model by introducing a negative-feedback loop that associates IGF-1r with the negative regulation of GH. This single assumption resolves the Holzenberger paradox, as the downregulation of GHr and IGF-1r *and* the upregulation of GH will result in the same longevity effects. This model is further supported by the fact that GH has been shown to have direct effect on target tissues (see Green *et al*., 1985; Lindahl *et al*., 1987). If this model stands true, our microarray studies proposed in (C.1) should show high GH gene expression as a result of the low GHr and IGF-1r expression in both the knockout mice. In this manner, while the aims in (C.1) and (C.2) will be specifically tailored towards (1) dissociating pathways and (2) testing the neuroendocrine model, the presence of the negative feedback loop will be probed in both sections as an auxiliary test.

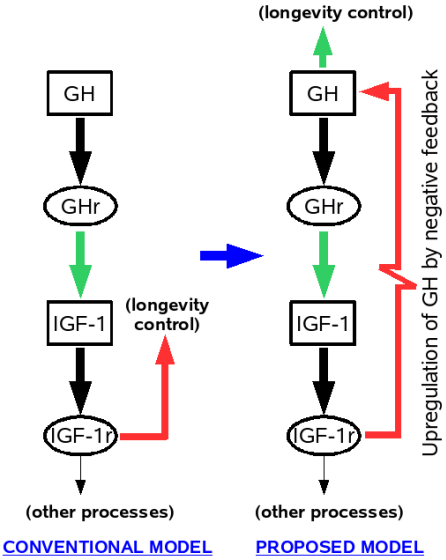

Figure 1. The conventional signaling model (*left*) of IGF-1r expression and the proposed modification to that schematic (key: red arrows indicate negative regulation and green arrows indicate positive or up-regulation).

Note that, If it exists, this negative feedback loop is not necessarily localized, and may be carried out in a (neuro)endocrine fashion as discussed in (B.3); the control of GH production by the pitituary gland (see Coschigano, 2006) is a tantalizing, in that it eludes to a possible mechanism by which the IGF-1 mediated negative loop is mediated.

C. Experimental Design and Methods

(C.1) Broad Aim 1: "Dissociating" the activities of the growth hormone receptor (GHr) and insulin-like growth factor receptor (IGF-1r).
Here, we will study gene expression levels modulated by GH and IGF-1r in hopes of finding genes that are differentially controlled by GHr and IGF-1r. The differences in expression levels will be studied by cDNA microarray analysis.

(C.1.1) Specific Aim **1.1:** Create mouse knockouts for (1) growth hormone receptor (2) insulin like growth factor-1r.
The production of a conditional knockout (where the a Cre mediated knockout event is initiated by a ligand) is believed to be variably expressed in different tissues and is least expressed in the brain (8%) and is therefore not employed in our knockout preparations (see Müller, 1999). Therefore, we followed the regular Cre-loxP method involving embryonic stem (ES) cells in a manner similar to that described by Müller (1999). The method is outlined as follows:
1. Obtain transgenic mice possessing Cre recombinase that is upstream of a tissue nonspecific promoter.
2. Obtain transgenic β-galactosidase-indicator mice possessing loxP sites flanking the gene to be deleted (GH, GHr, IGF-1, and IGF-1r), a stop codon and a lacZ gene. This is our target mouse strain. If unavailable, one may create this strain by homologous recombination in ES; the employment of ES libraries is quicker and more efficient than oocyte modification (reviewed in: Evans *et al*., 1997) which makes the creation of four knockout strains manageable.
3. Upon crossing of the two strains, Cre mediated recombination is expected to happen in all tissues where the promoter is recognized. A convenient indication of Cre recombinase expression is that the tissue will turn blue upon X-gal staining. With the completion of these steps, our two knockouts will be ready for experiments. The knockouts produced will be of heterozygous type, which can be converted to homozygous form by mating.

Possible problems in heterozygous mouse knockouts. For a molecule as important as those being discussed (GHr and IGH-1r), homozygous knockouts may result in adverse effects in a number of unanticipated pathways. The worst of these effects will result death of the knockout mouse (possibly prenatally). If this is the case, one may be able to study heterozygous knockouts, where only one of the two alleles have been inactivated. Previous studies have shown that heterozygous knockouts of key players in the IGF-1r pathway remain relatively normal, while still showing marked effects on the longevity pathways (Holzenberger *et al*., 2003). So, it is our belief that, with the failure of the homozygous models, the heterozygous models will be a reasonable replacement.

(C.1.2) Specific Aim **1.2:** Characterize the differential gene expression profiles for IG, IGr, IGF-1 and IGF-1r knockouts by microarray analysis.
The method used is schematically described in the *Figure 2*. The description of experiments follows:

Brain, liver and muscle tissue total RNA will be extracted from each mouse (the knockout mutants described in *C.1.1* and the wild type mouse) by TRIzol extractions (Life Technologies, Inc.). We chose to analyze the brain vs. the liver and muscle because: (1) the liver is known to be higher in GH/IGF-1 activity, (2) the brain is expected to be the starting point in the neuroendocrine pathway, and (3) the muscle is a "spectator" tissue which should show changes due to neuroendocrine stimulation.
This total RNA will be converted to fluorescently labeled cDNA by SuperScript II reverse transcriptase (Life Technologies, Inc.) following industry protocol (the labels to be used are Cy-3 and Cy-5 for each of the two knockout

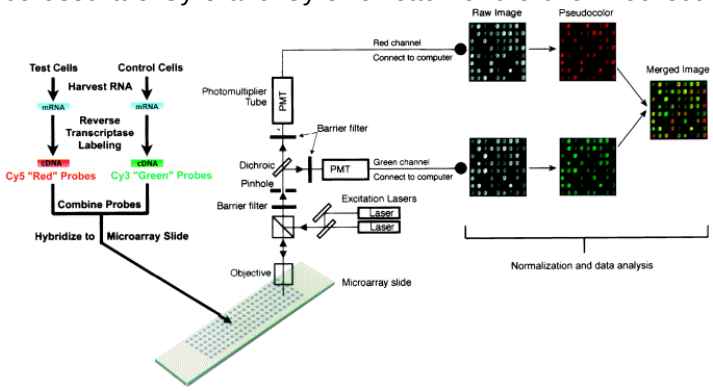

Figure 2. The schematic of cDNA probe preparation, hybridization, scanning and image analysis (take from the review: Khan *et al*., 1999)

mutants and Cy-2 for the wild type mouse). The resulting cDNA libraries are hybridized to a microarray, which will then be scanned using a (core facility) confocal laser scanning microscope. Both of the mutants may be analyzed on the same chip ("two-color fluorescence") because the dyes used are spectrally distinct (Khan *et al*., 1999; Lippincott-Schwartz *et al., online text*). Ratio measurements will be used to discover genes that are differentially expressed (and therefore differentially hybridized) between the different knockouts. The threshold of two will be used to decide whether a gene is to be marked as a candidate gene or not; two is a regularly used ratio threshold for this purpose (Feng *et al*., 2000); These candidate genes will be further analyzed by verifying expression differences using polymerase chain reaction with reverse transcriptase (RT-PCR; as done by Dupont *et al*., 2001; Baur *et al*., 2006). The differences in expression are expected to be most evident in neuronal development, muscular development, mitogenic pathways, and stress response pathways. However, other pathways that are currently unknown are also expected to show up.

Possible problems in total RNA collection. There are many difficulties associated with the collection of total RNA from animal tissues (Khan et al., 1999). Most of them are related to the fact that an animal is a heterogeneous medium which is controlled by both genetic *and* environmental cues. This leads to heterogeneity in the RNA sample resulting in unnecessary noise. One may counter this problem by doing each experiment in triplicate and only counting results that tally in all three readings. In extreme cases, one may create mouse fibroblast cultures from from mouse tissues that may then be kept under tight environmental and biochemical control.

(C.2) Broad Aim 2: *Testing whether IGF's longevity regulation pathways are modulated by a neuroendocrine system.*
Here, we will (1) create a brain-specific IGF-1r knockout and compare longevity characteristics (longevity, oxidative stress resistance, metabolic activity and IGF axis activity) against the already well studied wildtype mice and tissue non-specific IGF-1r mouse knockouts.

A NOTE (small print!): It has been indicated, from a discussion by Holzenberger et al., (2003) that work on brain-specific mouse models for the purpose of studying

non-autonomous age-related IGF signaling pathways has been commenced in their lab. In that, our studies and theirs will coincide. However, except for a single-line comment on the usage of Cre-Lox for brain-specific studies, the authors do not elaborate on specific methods or specific aims. Further, even after three years, no reports of their studies have been published in public record (BioMed). Thus, we believe that our investigations on this model are ethical and still warranted.

(C.2.1) Specific Aim **2.1:** *Creating the brain-specific IGF-1r knockouts.*

The method used to produce the brain specific IGF-1r knockouts was obtained from (Müller, 1999), and is outlined as follows:

1. Obtain transgenic mice possessing Cre recombinase that is upstream of a brain-specific promoter.
2. Obtain transgenic β-galactosidase-indicator mice possessing loxP sites flanking the gene to be deleted (IGF-1r), a stop codon and a lacZ gene. This is our target mouse strain. (if unavailable, one may create this strain by homologous recombination in embryonic cells).
3. Upon crossing of the two strains, Cre mediated recombination is expected to happen only in the brain.

Tissue specific knockout issues. In addition to problems associated with homogeneous knockout mice addressed in (C.1.1), it is of serious importance to make sure that the knockouts are localized to exactly those tissues that are needed. If not, the results of the experiments will be misinterpretations. Accordingly, spatial expression will be checked within founding lines to find one that expresses Cre recombinase exclusively within the brain (brain, muscle and liver tissues will be looked at). A convenient indication of Cre recombinase expression is that the tissue will turn blue upon X-gal staining. With the verification of the specificity using this method, our brain-specific IGF-1r$^{-/-}$ knockouts will be ready for experiments.

The other two mice strains, the non-specific IGF-1r knockout and the wildtype mice will be obtained from the Holzenberger lab (also obtainable from *http://www.emma.rm.cnr.it*). With the three mice strains at hand (the non-specific IGF knockout, brain-specific IGF knockout, and wt mouse), we can now ask the question: do the specific and non-specific knockout mice behave similarly with respect to age related phenotypes (longevity, systemic 1GF-1r quantities, etc.)? The mice will be grouped into two cohorts (each identical in constitution, containing all three mouse types) and each cohort may be further divided into male and female groups. The first cohort will be used in studying longevity of the three mice lines. The second cohort will be used for the testing of various age related and metabolic activities, while the third cohort will be used to study responses to oxidative stress.

If the neuroendocrine system is used to relay age-related regulation, added longevity (and its associated phenotypes such as stress resistance, etc) will be seen in both non-specific and brain specific knockouts (in contrast to the wild-type phenotype). Although the essential aim of this section is to test this hypothesis, it will be interesting to see how different all three mouse lines are with respect to biochemistry. For example, if the proposed model pathway described in (B.3) is true, then even if both brain-specific and nonspecific IGF-1r knockouts show similar age-related phenotypes, the levels of serum GH and IGF-1 may be different (GH may be the same IGF-1 will probably be much lower in brain-specific knockouts, as IGF-1 will only need to pervade that brain to be effective in the pathway).

(C.2.2) Specific Aim **2.2:** *Comparing longevity of the three mice lines: (1) the non-specific 1GF-1r mouse knockout, (2) the brain-specific IGF-1r mouse knockout and (3) wild type mice.*

The procedure used will be similar to the one used by (Holzenberger, 2003). Each mouse within the first cohort will be allowed to live under normal feeding conditions with standard settings (temperature, etc.). The female mice will be placed in individual cages while the male mice will be given the company of a wt female mouse. The weight of the mice will be checked and recorded every week. The cages will be otherwise checked daily until the advent of natural death (termimations will be performed on mice whose deaths appear painful and violent), upon which, necropsy will be performed to ascertain the type of death (including tumor immunohistochemistry). The ages, causes of death, and weights and longevity (reflected by Kaplan-Meier survival curves) will be compared between each mouse type (and male/female group).

(C.2.3) Specific Aim **2.3:** *Comparing IGF related activities of the three mice lines: (1) the non-specific 1GF-1r mouse knockout, (2) the brain-specific IGF-1r mouse knockout and (3) the wild type mice.*

A battery of tests will be performed to characterise the IGF-1r related activities within the second *and* third cohort in a manner similar to previous work (Holzenberger, 2003).

Second cohort studies. The tests performed on the second cohort will include:

IGF-1 assay: As described in (Holzenberger, 2004), IGF-1 levels would be measured using radioimmunoassay-based assays that may be obtained from DSL (Webster, Texas, USA) following industry protocol.

GH assay: GH levels in the serum will be assayed using a polyclonal immunometric assay (AutoDELFIA, Wallac).

Fertility studies: Cohort males and females will be crossed with their respective wildtype partners and the female will then be analyzed for the expected gestation period (30 days). Littersize will be used as a metric for female fertility. The ability to sire a litter will be used as a metric for male fertility.

Third cohort studies: Finally, the third cohort will be used to analyze glucose tolerance, energy expenditure and oxidative stress responses. The methods will be briefly described below:

Glucose tolerance: Glucose tolerance *will be measured by a handheld electronic device (Gluocotouch, Lifescan, Johnson & Johnson, Milpitas, CA, USA) as described previously (Holzenberger, 2004)*

Energy expenditure: The metabolic rate will be analyzed using indirect calirometry and O_2 and CO_2 flow as described previously (Oudart *et al*., 2000).

Oxidative stress respons analysis (Holzenberger, 2003): A herbicide that is known to induce the formation of reactive oxygen species (paraquat) will be

administered by injection, upon which stress resistance reflected by the fraction alive with respect to time will be noted.

E. References

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