Progress Report
"Development of a platform to screen for insulin signaling pathway components"
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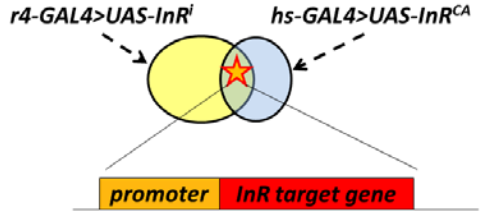
The goal of this project is to generate a rapid and convenient way to measure insulin sensitivity *in vivo*. We have focused on the *Drosophila* fat body, a liver- and adipose-like endocrine organ that exhibits insulin resistance and lipid accumulation in the presence of caloric excess. Fat body-specific loss of the insulin receptor (InR) resulted in tissue-specific lipid accumulation and insulin resistance as well as hyperglycemia in high calorie-fed *Drosophila*, a model for type 2 diabetes. Therefore, we used the fat body as a platform to identify genes that act as markers of insulin signaling.

Figure 1. Insulin pathway target gene identification strategy. RNA-seq and differential expression analysis were used to identify genes that might act as reporters of insulin pathway activity. (A) Differentially expressed mRNA from insulin receptor knockdown fat bodies (yellow) were compared with mRNA changes from constitutively active InR-expressing fat bodies (blue).



First, we characterized downstream markers of insulin signaling in the fat body by both loss- and gain- of insulin pathway activity. To identify gene expression changes resulting from increased insulin signaling, we transiently expressed a constitutively active InR (InR^{CA}) and measured transcript abundance in the fat body. To identify gene expression changes resulting from chronic reduction of insulin signaling, complementary gene expression data from chronic loss of InR was generated using InR RNA^{i} (InR^{i}) fat bodies. RNA-seq and differential expression analysis were used to identify genes that were regulated by insulin signaling in the fat body (Figure 1).

We identified 220 genes that were insulin-dependent in the fat body using both strategies (see DCC site for raw data). The presence of a gene in both datasets was considered an indicator that the gene was insulin-responsive over both short- and long-term perturbations of the pathway. Most (128/220) genes were regulated in opposing directions under the two conditions tested, as expected (Table 1). Many genes, however, were regulated in the same direction. These changes in gene expression may represent an attempt to compensate for low levels of insulin signaling, rather than direct targets of the insulin pathway. Only genes regulated in opposite directions during loss- and gain-of-function were considered likely to serve as effective short- and long-term insulin pathway sensors.

Table 1: Putative insulin pathway target genes. Positive genes are those upregulated by constitutively active InR and downregulated by loss of InR. Negative genes seem to be expressed in contrast to the degree of insulin signaling.

Positive					
ATPCL	CG14567	CG30463	CG42847	CR34335	Obp56d
Buffy	CG14855	CG31496	CG5656	CR40621	sage
CG11912	CG15186	CG32137	CG7763	Cyp6t1	Sgs4
CG12310	CG15210	CG32335	CG8180	Fbp2	Sgs7
CG12715	CG15772	CG33232	CG8664	Lcp3	Sgs8
CG13731	CG17105	CG3394	CG9486	Lcp4	
CG13947	CG18557	CG42798	comm3	Lcp9	
CG14332	CG1969	CG42834	Cpr11A	Mal-A6	
Negative					
aay	CG1702	CG5321	CG8389	GNBP1	P58IPK
Acox57D-p	CG18031	CG5789	CR40963	GRHR	Pdk
aralar1	CG18067	CG5966	Cyp4d14	Idgf1	Pepck
AttA	CG30118	CG6428	Cyp4g1	IM3	Plod
AttB	CG32626	CG6912	Cyp4p1	IP3K2	RpL4
CG10433	CG33459	CG7224	Cyp6a22	kel	Spn28D
CG10814	CG33460	CG7442	Cyp9b1	lectin-24A	Spn43Ab
CG11652	CG33462	CG8026	daw	melt	stv
CG12428	CG34136	CG8051	Dif	Men	svp
CG13654	CG3835	CG8129	DptB	mrt	TotC
CG15293	CG43085	CG8160	Ect3	niki	Trc8
CG15879	CG43153	CG8249	Ero1L	Nplp2	Ugt58Fa
CG1667	CG4335	CG8317	Fuca	Odc2	Victoria
CG16712	CG4757	CG8353	GIIIspla2	Ork1	

Gene ontology analysis highlighted both known and novel pathways that appear to be controlled by insulin signaling (Table 2). We used GOStat (1) and AmiGO (2) to analyze the group of 45 genes that are positively regulated by insulin signaling and the group of 83 genes that are negatively regulated by insulin signaling. GOStat and AmiGO gave roughly the same results and both identified the significantly enriched categories shown (Table 2). Several cuticle synthesis genes in the Sgs and Lcp classes were positively regulated by the insulin signaling pathway, which is surprising due to the opposing actions of insulin and EcR, which also positively regulates these genes (3). InR activity negatively regulated gamma-butyrobetaine dioxygenases, which promote β-oxidation of free fatty acids (4). In this context, insulin probably reduces lipid catabolism in order to increase lipid storage. This particular step in the β-oxidation pathway is sometimes considered rate-limiting although the select trans-regulation of the pathway is unexpected. The insulin-pathway-associated increase in the expression of ATP-citrate lyase (ATP-CL), which promotes the synthesis of fatty acids, also supports a positive role for insulin in lipogenesis. This role for insulin is expected and is supported by the literature (5).

Table 2: Gene ontology categories. *Enriched categories identified in a list of co-expressed genes that were positively or negatively regulated by insulin signaling.*

positive	p-value	negative	p-value
cuticle	7 e-5	gamma-butyrobetaine dioxygenase	<0.001
		defense response to bacterium	<0.001
		oxidoreductase	<0.05

Interestingly, insulin signaling seems to attenuate the immune response, as antimicrobial peptide expression is reduced ("defense response to bacterium"). This result could stem from a reduced bacterial load in healthy animals; compromised immunity in the insulin-resistant fat body might lead to upregulation of these infection response genes. It is important to note that some of these genes respond to the transcription factor NF-κB, which can be activated by ER stress, a hallmark of insulin resistance. Finally, the cytochrome p450 class of oxidoreductases is overrepresented in the list of genes that are negatively regulated by insulin signaling. These enzymes metabolize a broad range of substrates from steroid hormones to environmental toxins, and so further study will be required to gain insight into the reasons why insulin might regulate them. Future experiments will use loss-of-function genetics to understand the roles of these insulin target genes in the pathophysiology of insulin resistance.

From the genes in these categories, we will undertake promoter comparison analysis to identify conserved motifs that would act as insulin-response elements. This strategy is based on the assumption that related genes are likely to be co-regulated by the same factors. After identifying promoters that can be used to express reporter genes, we will synthesize artificial promoters upstream of reporter genes to generate "insulin sensor" flies. An alternate strategy we have recently considered is the use of the target genes themselves as reporters, for example by using multiplex qPCR. While this approach will be more costly, it allows the simultaneous quantification of several promoters' activity and simplified the genetics required. Exogenous insulin will be used to test the validity of insulin reporters, using culture conditions we have benchmarked (6). These reporter strategies will serve as a platform for identifying proteins that modify insulin signaling.

Sharing Plan

Gene expression data will be deposited at the NIH's Gene Expression Omnibus site and at the DCC website.

References

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